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Melanoma

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Editors

Melanoma

With 160 Figures and 55 Tables

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To the memory of our son Samuel Fisher (1991–2016)
—David E. Fisher

To my children Lennart, Ingmar, Stella, and Juliet
—Boris C. Bastian

Preface

The scientific and clinical melanoma research communities have made tremendous progress over the last two decades, which produced a dramatic improvement in outcome for patients with advanced disease as well as remarkable insights into the biology of the disease. These advances were catalyzed by a quantum leap in techniques to interrogate the cancer genomes and in breakthrough discoveries into ways through which cancers evade eradication by immune cells. Massive parallel sequencing enabled the large-scale cataloging of genetic alterations and development of matching drugs that set the stage for therapies that target specific mutations in the tumor of the individual patient. Functional analyses of the interaction of immune cells with each other and with cancer cells have informed ways to block key mechanisms that tumor cells hijack to paralyze the immune system.

The field continues to move forward at high speed after this phenomenally productive period in research that is akin to the Cambrian explosion in the evolution of life on Earth. This book represents a systematic effort of many of the thought leaders in the field to comprehensively portrait the current understanding of key aspects related to melanoma. It is written with a broad audience in mind that we hope will include students and professionals from basic and translational research as well as the community of clinical caregivers. One of the most impactful aspects of this "melanoma revolution" has been the rapid deployment of similar tools and therapeutic strategies to other highly challenging human cancers. It is our hope that the revolution will continue, both for patients with melanoma who still require great advances due to unmet needs and for the broader cancer community which may continue to benefit from ongoing progress in melanoma science and clinical discovery.

July, 2019

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David E. Fisher, M.D., Ph.D., is chief of the Department of Dermatology at Massachusetts General Hospital (MGH). He also serves as director of Mass General's Cutaneous Biology Research Center, director of the Melanoma Center, and chair of the MGH Executive Committee on Research. A professor of dermatology and of pediatrics at Harvard Medical School, Dr. Fisher came to Mass General from the Dana-Farber Cancer Institute, where he previously directed the melanoma program. Dr. Fisher's research has focused on understanding the molecular and genetic events which underlie formation of melanoma as well as skin pigmentation. As a clinician, he has worked to translate these understandings into advances in diagnosis, treatment, and prevention of human diseases related to the skin and associated disorders.

A graduate of Swarthmore College with a degree in biology and chemistry, Dr. Fisher is also an accomplished concert cellist and graduated from the Curtis Institute of Music in Philadelphia. He received his doctorate under Nobel Laureate Gunter Blobel and Henry Kunkel at Rockefeller University and his medical degree at Cornell University Medical College. Dr. Fisher's specialty training in Medicine, Pediatrics, and Oncology were carried out at Dana-Farber Cancer Institute, Boston Children's Hospital, and Brigham and Women's Hospital, Harvard Medical School. His research contributions

include elucidation of the pathway through which UV radiation induces pigmentation, identification of MITF as master transcriptional regulator in melanocytes and melanoma oncogene, novel approaches to melanoma treatment and prevention, and discovery of the link between ultraviolet radiation and addictive behaviors.

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Boris C. Bastian, M.D., received his medical degree from the University of Munich, Germany. He completed his residency in dermatology at the University of Würzburg, Germany. He did a postdoctoral fellowship at the Cancer Genetics Program at the University of California, San Francisco, and joined the UCSF faculty in 1999 with appointments in dermatology and pathology. He served as the chair of the Pathology Department at Memorial Sloan-Kettering Cancer Center and was president of the Society of Melanoma Research from 2010 to 2013. In 2011 he returned to UCSF, where he now leads a research laboratory focused on the genetic underpinnings of skin cancer with the goal of improving disease classification and diagnosis, therapy, and prevention.

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Part I

Introductory Concepts

Developmental Biology of Melanocytes

1

Lukas Sommer

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Abstract

Apart from embryonic stem cells (ESCs) in the blastocyst, neural crest stem cells (NCSCs) in vertebrate embryos represent the stem cell population in our body with the broadest developmental potential, generating most of the neurons and glia of the peripheral nervous system (PNS) as well as various nonneural cell types, such as smooth muscle cells in the outflow tract of the heart, craniofacial bone, and cartilage and, in particular, melanocytes in the skin. It is assumed that a third of all congenital birth defects are due to failures in neural crest development, illustrating the significance of this stem cell population. Moreover, processes underlying melanocyte development appear to be recapitulated, at least partially, during formation of melanoma, the most aggressive skin tumor. For instance, it has recently been shown that an embryonic NCSC gene expression signature is reactivated upon tumor initiation in a zebrafish model of melanoma, suggesting a functional involvement of a NCSC program in tumors originating from neural crest derivatives. Thus, to gain insights into melanoma biology, it is important to understand the mechanisms regulating NCSC and melanocyte development, as outlined in this chapter.

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Neural crest · Neural crest stem cell · Melanocyte · Embryonic development · Lineage specification · Tumor cell of origin

Introduction: Neural Crest Stem Cells, the Embryonic Progenitors of Melanocytes

Melanocytes not only provide the pigmentation to our skin but are also found in the inner ear, the eye, and some internal organs such as the heart. In the skin, melanocytes transfer the pigment melanin to keratinocytes and the growing hair in order to protect skin tissue from damage caused by UV light of the sun. In the inner ear, melanocytes control development and function of a structure termed the stria vascularis, which is required for proper hearing. In the eye, melanocytes are found both in the iris (which, among others, adjusts the amount of light entering the eye) and the choroidea (a thin epithelial structure behind the retina shielding deeper tissues from excessive light). Thus, defects in melanocyte formation as found, for instance, in Waardenburg syndrome in humans are associated with abnormal pigmentation of the skin and the eyes as well as varying degrees of hearing loss.

During embryonic development of vertebrates, melanocytes are produced from the so-called neural crest, a structure unique to vertebrates. The neural crest emerges during neurulation in the dorsal neural tube between the anlage of the future central nervous system and the surface ectoderm. Neural crest cells in the neural tube undergo an epithelial to mesenchymal transition before migrating to various structures in the embryo and producing, apart from melanocytes, a wealth of other cell types as diverse as neurons and glia of the peripheral nervous system, craniofacial chondrocytes and bone cells, smooth muscle cells in the heart outflow tract, chromaffin cells of the adrenal medulla, and others.

While melanocytes as well as sensory neurons and peripheral glia are produced from all axial levels in the embryo, other neural crest derivatives – such as craniofacial cartilage and bone, cardiovascular smooth muscle cells, and the enteric nervous system innervating the gastrointestinal system – only emerge from discrete axial levels of the neural tube. This indicates that the fate of neural crest cells is influenced by positional cues along the neuraxis (Le Douarin et al. 2008). In principle, such fate-determining factors could act selectively to favor the development of specific predetermined precursor cells (e.g., neuroblasts) over precursor cells of other cell lineages (e.g., melanoblasts); in this case, neural crest cells would represent a heterogeneous population composed of several lineage-restricted precursors (Fig. 1). Alternatively, local fate specification could be achieved by factors that act instructively on multipotent cells. According to this second model, the neural crest would constitute a homogenous population of stem cells, each with the potential to produce distinct cell types in response to instructive factors (Fig. 1).

Pioneering studies by Nicole Le Douarin and others, involving in vivo transplantation of neural crest cells in avian embryos, have shown that on the population level neural crest cells of virtually all axial levels exhibit similar potentials (Le Douarin and Dupin 2003). For instance, when cranial neural crest cells of a donor embryo were transplanted into the neural tube at the trunk level of a host embryo, the cells from the donor adapted the fates normally acquired by the host cells in that area. Thus, environmental factors present in the host trunk influenced the development of the transplanted donor cells. Similarly, neural crest cells from the trunk, when put into cell culture, were able to produce bone and cartilage (i.e., derivatives that normally only arise from the cranial neural crest), when exposed to appropriate growth factors (Graham et al. 1996; Shah et al. 1996). Likewise, although in quail neural tube explant culture cells biased to the melanocyte lineage were preferentially found in late emigrating neural crest cells (Henion and Weston 1997), formation of melanocytes can readily be induced by appropriate growth factor combinations in early migratory neural crest cells (Shakhova and Sommer 2015).

However, the ultimate demonstration that neural crest cells are multipotent had to be provided on the single cell level in so-called clonal analyses, in which prospectively identified,

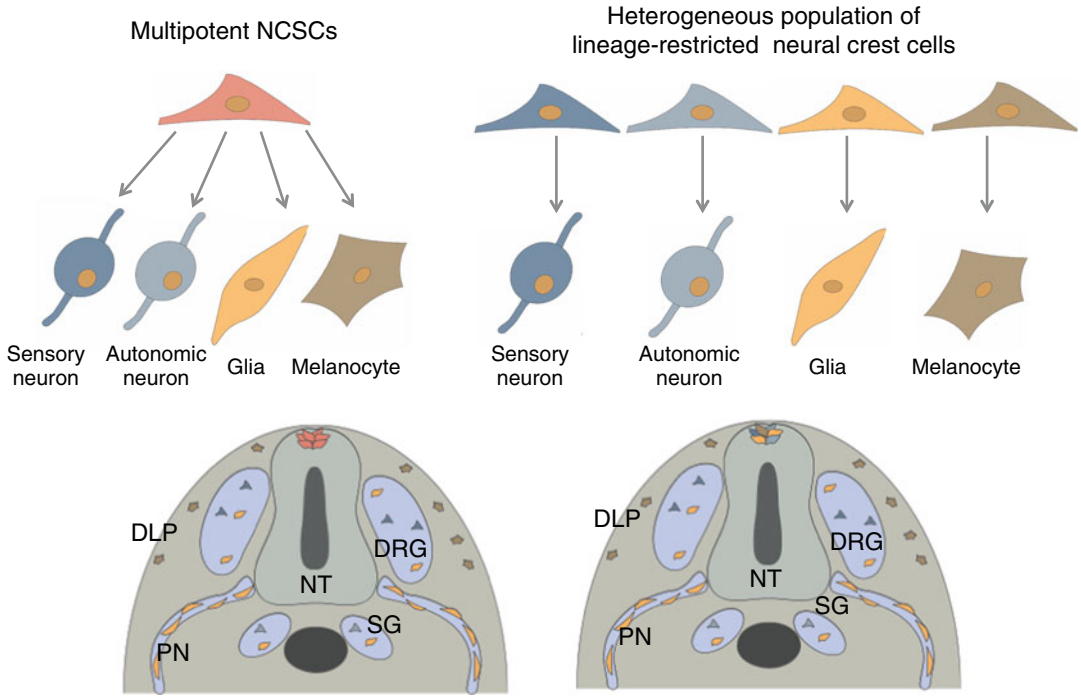

Fig. 1 During embryonic development of vertebrates, the neural crest emerges in the dorsal-most part of the neural tube (NT). Subsequently, neural crest cells delaminate from the NT to migrate either along a dorsolateral pathway (DLP) to produce melanocytes or along a ventral pathway to produce, for instance, sensory neurons and satellite glia in dorsal root ganglia (DRG), autonomic neurons and satellite glia in sympathetic ganglia (SG), or Schwann cells along peripheral nerves (PN). In principle, two models can explain the generation of these various derivatives. According to the model on the left, premigratory neural crest cells consist of a homogeneous population of stem cells (*red*) that all have the capacity to give rise to multiple cell types (see, e.g., Bronner 2015). Melanocytes, in this case, would derive from multipotent NCSCs. According to the model on the right, the neural crest consists of distinct lineage-restricted progenitors that each generate a specific derivative (see, e.g., Krispin et al. 2010a). In this case, melanocytes would originate from progenitors that normally do not give rise to other cell types. Recently, it has been reported that melanoma-initiating cells acquire properties of their embryonic counterpart (Kaufman et al. 2016). The molecular nature of a melanoma-initiating cell, and ultimately mechanisms of melanoma initiation, might thus depend on the mode of melanocyte generation during embryonic development (Figure by courtesy of Dr. Arianna Baggiolini, University of Zurich)

undifferentiated neural crest cells were individually traced, while they proliferated and differentiated to form a clone of multiple neural crest derivatives (Dupin and Sommer 2012). Such experiments were done, among others, with early migratory neural crest cells isolated from neural tube explant cultures that can be derived from avian, rodent, and even human embryos (Etchevers 2011). In avian and rodent systems, neural crest cells from explant cultures were replated at clonal density to demonstrate the existence of multipotent neural crest cells in cell culture (Baroffio et al. 1988; Cohen and Konigsberg 1975; Sieber-Blum and Cohen 1980; Stemple and Anderson 1992). In particular, studies performed with cranial neural crest from chicken embryo revealed the presence of neural crest cells with a very broad potential that had the capacity as single cells to give rise to peripheral neurons and glia, chondrocytes, osteoblasts, smooth muscle cells, as well as melanocytes (Calloni et al. 2009). In addition, neural tube explant cultures were also used to demonstrate by serial subcloning that a considerable fraction of neural crest cells are not only multipotent but also display the capacity for self-renewal (Stemple and Anderson 1992). Thus, at least in culture, some neural crest cells appear to be functional neural crest stem cells (NCSCs).

In other studies, however, in which individually labeled neural crest cells were followed in

high-density neural tube explant cultures rather than in low-density cultures after replating, most cells appeared to be fate-restricted rather than multipotent (Henion and Weston 1997). Although neural crest cell fates may be influenced in high-density cultures by fate-restricting community effects acting between the cells (Hagedorn et al. 1999), these studies raised a debate of whether multipotency of neural crest cells might be the consequence of a cell culture artifact, dependent on the culture condition. Indeed, cells with NCSC features could also be derived from various post-migratory neural crest derivatives, including dorsal root ganglia (DRG), the gut, sciatic nerves, and the skin (Delfino-Machin et al. 2007). For instance, neural crest cells isolated from the epidermis of quail embryos were able to produce neurons and glia in culture, although these cells supposedly generate only melanocytes in normal development (Richardson and Sieber-Blum 1993). Even some adult neural crest-derived cells, when exposed to a new microenvironment as, for instance, presented by certain cell culture conditions, maintain the capacity to self-renew and to generate a plethora of cell types typically produced from embryonic neural crest cells (Shakhova and Sommer 2010). In particular, prospective identification and direct isolation of neural crest-derived cells marked by genetic in vivo fate mapping indicated the presence of NCSC-like cells in peripheral nerves of the skin and in the bulge of adult hair follicles (Wong et al. 2006), which comprises melanocyte stem cells (MSCs) (Nishimura et al. 2002). Possibly, adult neural crest-derived structures such as peripheral nerves or hair follicles contain a small reservoir of multipotent adult NCSC-like cells that may normally be quiescent and only activated in a pathophysiological context. Alternatively, isolation or other processes disturbing the physiological 3D situation may promote reprogramming in differentiated cells, such as Schwann cells or melanocytes, resulting in dedifferentiation and acquisition of stem cell features. In support of this idea, pigmented melanocytes from chicken could be induced to dedifferentiate in culture, to self-renew, and to produce neural crest lineages other than melanocytes (Dupin et al. 2000). Similarly, melanoblasts from postnatal murine skin were shown to exhibit "stemness" properties upon exposure to stromal cells (Motohashi et al. 2009).

The combined data demonstrate that cells with stem cell features can be isolated from the neural crest and neural crest-derived tissues, from early embryonic stages up to postnatal and even adult stages. Such NCSCs can be propagated in vitro and are able to generate virtually all cell types that are normally generated in the embryo from the neural crest. In animal models, researchers have already made use of this potential to repair myelination and bone defects, respectively, by transplantation of cells exhibiting NCSC properties (Lavoie et al. 2009; McKenzie et al. 2006). Similarly, the potential of NCSCs to produce melanocytes might be applied in the future to treat pigmentation defects, for instance, in skin substitutes grafted onto burn patients.

Generation of the Melanocyte Lineage from the Neural Crest In Vivo

From a scientific perspective, however, a question to be addressed is whether neural crest-derived multipotent stem cells indeed reside in the living organism or whether NCSCs solely represent "in vitro stem cells" (Smith 2006), which comparable to embryonic stem (ES) cells isolated from the blastocyst reveal their potential only upon expansion in culture or exposure to other, hitherto unknown stimuli. Over the years, various laboratories aimed to tackle this issue by clonal analyses in vivo, with sometimes contradicting results (Bronner 2015). Although clonal assays have not yet been performed in postnatal neural crest derivatives, single neural crest cells have been traced in embryos of various species both at premigratory and migratory stages. In avian embryos, for instance, single neural crest cells in or emerging from the dorsal neural tube were labeled by dye labeling or by low-titer infection with marker-expressing virus (Bronner-Fraser and Fraser

1988, 1989; Frank and Sanes 1991). Likewise, labeling of single neural tube cells by dye injection was performed in whole mouse embryos (Serbedzija et al. 1994). In these early studies, many neural crest cells generated clones composed of multiple derivatives, including peripheral neurons and glia, smooth muscle cells, as well as melanocytes (Fig. 1). Although the experiments did not exclude the presence of lineage-restricted neural crest cells, these findings revealed the existence of multipotent neural crest cells giving rise to the melanocytic and other lineages.

Others suggested, however, that melanocytes arise from precursor cells that are either already fate-restricted in the neural tube or segregate from other neural crest lineages at early developmental stages while emigrating from the neural tube. Unlike neural progenitors, which migrate ventrally, the first melanoblasts (as defined by neural crest-derived cells expressing melanocyte lineage markers) are detectable in the so-called migration staging area close to the neural tube, before they continue their journey on a dorsolateral pathway underneath the ectoderm (Weston 1991). Ventral migration occurs before dorsolateral migration in avian and zebrafish embryos, and pigment cells express first signs of lineage specification later in development than, e.g., peripheral neuroblasts (Erickson et al. 1992; Hari et al. 2012; Raible et al. 1992), consistent with a temporal pattern of lineage segregation. More recent work also pointed to a temporal switch from neural to melanocyte specification in the avian neural crest and reported lineage segregation of neural and melanocyte progenitors already in the premigratory neural crest, before emigration from the neural tube (Krispin et al. 2010b). This study repeated dye labeling experiments of single neural crest cells similar to those done before (Bronner-Fraser and Fraser 1988) but – unlike the earlier studies – found that migratory neural crest cells are lineage-committed and sequentially localize first to sympathetic ganglia, then to the DRG, and finally to the skin to generate melanocytes. According to these findings, the premigratory neural crest represents a heterogeneous cell population, consisting of discrete fate-restricted cell lineages (Krispin et al. 2010a) (Fig. 1).

It is difficult to reconcile this work with those previous studies that identified multipotent neural crest cells in embryos of chicken and other species (Bronner 2015). With the goal to potentially solve this controversy, a recent study sought to follow the development of single cells and their progeny without invasive manipulation in order to avoid potential experimental influences on cell fate decisions. This was achieved by means of clonal analyses in mouse embryos in vivo using a genetic cell tracking system that allows fate mapping of single cells (Baggiolini et al. 2015; Snippert et al. 2010) (Fig. 2). In this system, Cre-mediated recombination of a multicolor Cre-reporter allele termed *R26R-Confetti* leads to expression of various colors in a stochastic manner (nuclear green, cytoplasmic yellow, cytoplasmic red, membrane-bound blue, or combinations thereof in the case of homozygous *R26R-Confetti*) (Snippert et al. 2010). When combined with a tamoxifen-inducible form of Cre recombinase, the extent of Cre-mediated fluorescent marker expression can be restricted to few cells, the clonal progeny of which can be distinguished by expression of different colors. Using Cre lines that activate *R26R-Confetti* either in the premigratory crest or in neural crest cells having just emigrated from the neural tube, it was demonstrated that the vast majority of neural crest cells are multipotent, with only very few clones being restricted to single derivatives (Baggiolini et al. 2015). Intriguingly, multipotency was even maintained in neural crest cells after their emigration from the neural tube. Of note, all clones containing melanocytes were founded by multipotent cells, speaking against early segregation and lineage restriction of the melanocytic lineage (Fig. 2). The data do not exclude that melanocyte-restricted progenitors exist at stages later than those analyzed. However, given that unlike in avian embryos, dorsolateral migration in mouse embryos already takes place as soon as neural crest cells start to emigrate from the neural tube, i.e., concomitantly with ventral migration (Serbedzija et al. 1990), it is conceivable that at least in mammals a

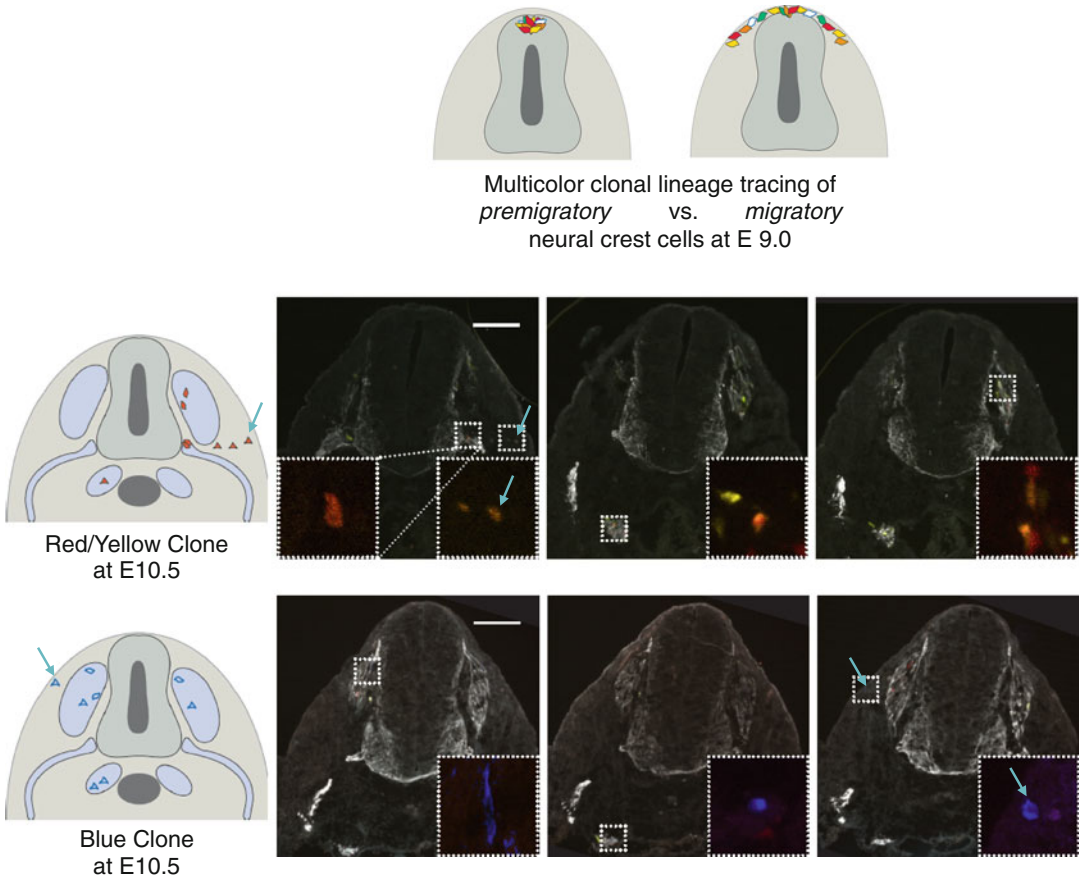

Fig. 2 Clonal in vivo tracing of premigratory vs. migratory neural crest cells using a multicolor Cre reporter allele *(R26R-Confetti)* induced at embryonic day (E)9.0 by *Wnt1-CreERT* or by *Sox10-CreERT2*. Analysis of the embryos at E10.5 revealed that the vast majority of neural crest cells are multipotent, both at premigratory and at migratory stages (Baggiolini et al. 2015). Two examples of clones are shown that produced multiple neural cell types in different structures of the PNS and also contributed to the melanocytic lineage (arrows). Scale Bars, 50 μm. Of note, all melanocytes analyzed in this study derived from multipotent NCSCs rather than from committed neural crest progenitor cells (Figure by courtesy of Dr. Arianna Baggiolini, University of Zurich)

considerable fraction of melanocytes derive from multipotent neural crest cells.

Generation of Melanocytes by an Alternative Path: From Nerves to Pigment Cells

Apart from melanocytes arising from dorsolaterally migrating neural crest cells, some melanocytes appear to originate from ventrally migrating neural crest cells that usually give rise to the neural cells of the PNS (Sommer 2011). For instance, cells expressing pigment cell markers were detectable on both the dorsolateral and the ventral pathway in zebrafish (Camp and Lardelli 2001), consistent with the presence of ventrally migrating bipotent glial-melanocytic progenitors in zebrafish in vivo (Raible and Eisen 1994). Extending on these findings, in vivo imaging of pigment cell precursors traced by green fluorescent protein (GFP) expression showed that, at postembryonic stages, peripheral nerves serve as reservoir for specific subsets of adult pigment cells in zebrafish (Budi et al. 2011). Similarly, Ernfors and colleagues reported that in chicken and mice some ventrally migrating neural crest-derived cells are fated to become melanocytes (Adameyko et al. 2009; Ernfors 2010). According

to these studies, cells associated with nerves innervating the skin start to upregulate melanocyte lineage markers after detachment from the nerves, once these have reached their target structure (Adameyko et al. 2009, 2012). This alternative way of melanocyte cell production was detected in chicken by electroporation of neural tube cells with GFP, allowing tracing of GFP-positive melanocytic cells in nerve endings in the skin. Likewise, in mice, inducible Cre recombinase-mediated cell fate mapping was used to demonstrate that cells traced during a restricted time period (around embryonic day 11) were later found to detach from peripheral nerves and to express early melanocyte markers. However, it is difficult to estimate how many melanocytes in the adult skin originate from nerve-associated cells, because the Cre line used in these studies appears to display expression at least in some multipotent neural crest cells as well as in melanoblasts in the skin (Hari et al. 2012; Leone et al. 2003). Nonetheless, the finding that a fraction of melanocytes derives from peripheral nerves is intriguing and raises the question of whether this potential is restricted to a particular embryonic stage or whether it can also be realized at later stages, including after birth. Indeed, under the influence of appropriate culture conditions, Schwann cells isolated form peripheral nerves were able to generate pigmented melanocytes in vitro (Dupin et al. 2003; Sherman et al. 1993). Apparently, such a fate-switch can even be triggered in vivo upon injury of the sciatic nerve, which leads to appearance of pigmented areas at the nerve stump (Rizvi et al. 2002). However, it remains to be shown whether melanocytes derived from nerves are produced from residual multipotent stem cells potentially present in peripheral nerves, from bipotent glial-melanocytic progenitor cells, or from Schwann cells dedifferentiating, e.g., upon nerve injury.

Transcription Factors Regulating NCSCs and Melanocyte Specification

During neurulation, the neural crest becomes specified in the dorsal neural tube by the combined activity of so-called neural crest specifier genes that include transcription factors such as FoxD3, Ets1, Snai1/2, and SoxE (Prasad et al. 2012; Simoes-Costa and Bronner 2015). These factors are part of a complex gene regulatory network that, besides neural crest specification, also regulates delamination and emigration of neural crest cells from the neural tube. Other transcription factors, such as Pax3 and Zic1, act upstream of these neural crest specifier genes and also modulate signaling pathways involved in early neural crest development, such as canonical Wnt (Plouhinec et al. 2014). As demonstrated by the *R26R-Confetti*-based lineage-tracing analysis described above (Baggiolini et al. 2015), neural crest cells delaminate from the neural tube as multipotent cells. However, it is only poorly understood how multipotency of migratory neural crest cells is controlled. Recently, it has been reported that in *Xenopus* embryos, neural crest cells retain a gene expression program active in the blastula stage and controlling pluripotency (Buitrago-Delgado et al. 2015). Among the factors identified was FoxD3, which is not only a neural crest specifier but has also been shown by loss-of-function experiments to regulate neural crest cell survival and, hence, proper development of multiple neural crest derivatives (Lister et al. 2006; Teng et al. 2008). Likewise, the SoxE family factor Sox10 is involved in early neural crest specification and also required for proper development of neural crest derivatives (Cheung et al. 2005; Taylor and Labonne 2005). In particular, overexpression of Sox10 in NCSCs in culture conferred multipotency while suppressing neuronal differentiation (Bondurand et al. 2006; Kim et al. 2003), whereas depletion of *Sox10* affected the generation of PNS structures and melanocytes, but not of other nonneural derivatives of the NC (Britsch et al. 2001).

Intriguingly, key factors involved in neural crest specification and NCSC maintenance, such as FoxD3, Pax3, and Sox10, also play a role in melanocyte development from NCSCs. These factors, together with the β-catenin effector proteins Lef1/Tcf and the basic-leucine zipper protein Creb, are part of a transcriptional network that controls in a positive or negative manner the expression of the microphthalmia-associated

transcription factor Mitf (Mort et al. 2015). The so-called M-Mitf isoform of this basic helix-loop-helix leucine zipper transcription factor marks melanoblasts at an early developmental stage, is central to melanocyte specification, and directly controls several pigmentation genes (Cooper and Raible 2009; Levy et al. 2006; Mort et al. 2015). In human, mice, and other species, mutations in *Mitf* are associated with pigmentation defects, consistent with roles of Mitf in melanocyte development and survival (Hornyak et al. 2001; Opdecamp et al. 1997). In contrast, overexpression of Mitf or Mitf orthologues promotes melanocyte marker expression in mouse fibroblasts (Tachibana et al. 1996), ectopic pigmentation in zebrafish in vivo (Lister et al. 1999), and melanocyte formation in Medaka blastula cells (Bejar et al. 2003).

Although these findings clearly underline a role of Mitf in the specification of melanocytes from neural crest cells, it is likely that other factors are implicated in this process as well. Indeed, although lacking Mitf, cells expressing the early melanocyte lineage marker Dopachrome tautomerase (Dct) are initially present at early stages of development in *Mitf*-mutant embryos (Hornyak et al. 2001; Opdecamp et al. 1997). Moreover, Mitf is apparently dispensable for melanoblasts to engage in dorsolateral migration (Thomas and Erickson 2008). Thus, other factors in addition to (and likely in conjunction with) Mitf support melanoblast development from the neural crest.

In NCSCs and neural progenitors, *Mitf* expression is repressed by FoxD3, presumably by direct binding to *Mitf* promoter sequences (Curran et al. 2009). Accordingly, FoxD3 expression is normally downregulated in melanocytic cells (Kos et al. 2001), which at least in zebrafish is mediated by histone deacetylase1 (hdac1) (Ignatius et al. 2008). When overexpressed in neural crest cells, FoxD3 prevented *Mitf* transcription, promoting generation of glia at the expense of melanocytes in culture (Thomas and Erickson 2009) and counteracting dorsolateral cell migration and melanocyte formation in avian embryos (Kos et al. 2001). In contrast, inactivation of *FoxD3* in neural crest cells promoted dorsolateral migration in avian embryos and led to increased and expanded *Mitf* expression in zebrafish (Curran et al. 2009; Ignatius et al. 2008; Kos et al. 2001). Of note, however, *FoxD3* inactivation on its own is not sufficient to promote the formation of pigmented cells from neural crest cells, indicating that this transcription factor represents only one of the players in the gene regulatory network that controls melanocyte specification in neural crest cells.

In zebrafish, FoxD3 is involved in additional steps of pigment cell development, which are associated with the production of differentially colored cells typical for this species. The majority of pigment cells in zebrafish are directly generated from the neural crest. However, a subpopulation of these cells originate from a bipotent Mitf-positive progenitor either producing iridophores or melanophores. This fate decision is controlled by FoxD3. When reexpressed in progenitors, FoxD3 represses *Mitf*, thus promoting iridophore production; conversely, FoxD3-negative/Mitf-positive progenitors give rise to melanophores (Curran et al. 2010).

In contrast to FoxD3, the NCSC regulator Sox10 is involved in activation of *Mitf* expression. Sox10, in synergy with Pax3, binds to the *Mitf* promoter to activate *Mitf* expression in cell culture (Bondurand et al. 2000; Potterf et al. 2000). However, Pax3 is apparently dispensable for melanocyte specification and rather controls melanoblast numbers. Indeed, in mice homozygous for a *Pax3* loss-of-function mutation, dorsolaterally migrating melanoblasts were detectable (although drastically reduced in numbers), speaking against a role of this factor in melanocyte lineage specification (Hornyak et al. 2001). This is in contrast to mouse embryos lacking *Sox10*, in which melanoblasts are virtually absent (Bondurand et al. 2000; Britsch et al. 2001). Consistent with its crucial role in melanocyte development, Sox10 not only regulates the expression of melanocytic genes such as *Mitf* and *Dct* but also controls survival and expansion of the lineage. Moreover, the function of Sox10 is gene dosage- and context-dependent (Paratore et al. 2001). In the embryo, *Sox10* haploinsufficiency results in diminished melanoblast numbers (Britsch et al. 2001) and,

in adult mice and human patients, causes Waardenburg-Hirschsprung disease associated with defects in the enteric nervous system and in pigmentation (Bondurand et al. 1999; Kuhlbrodt et al. 1998; Pingault et al. 1998). Such pigmentation defects are significantly increased in mice heterozygous for both *Sox10* and *Mitf* mutations, demonstrating genetic interactions between *Sox10* and *Mitf* in vivo (Potterf et al. 2000). Other genes interact with *Sox10* in an antagonistic rather than synergistic manner during melanocyte development. Deletion of *Sox5*, encoding a member of the SoxD subfamily of Sox transcription factors, does not affect the formation of melanocytes (Stolt et al. 2008). However, loss of *Sox5* partially rescues the pigmentation defects associated with *Sox10* heterozygosity, revealing that Sox5 is able to modulate the activity of Sox10.

Consistent with the above-described loss-of-function data, overexpression of Sox10 in zebrafish and *Xenopus* embryos resulted in expansion of Mitf expression and pigment cell formation, respectively (Aoki et al. 2003; Dutton et al. 2001; Elworthy et al. 2003). However, there appear to be species-specific differences with respect to the role of Sox10 in pigment cell specification. Indeed, Sox10 overexpression in cultured mouse neural crest cells did not induce Mitf expression but rather increased the cells' neural potential (Kim et al. 2003). In addition, overexpression of Mitf was able to rescue pigmentation defects in *Sox10*-mutant zebrafish but not in *Sox10*-mutant mice, pointing to activities of Sox10 in mice that go beyond transcriptional regulation of *Mitf* (Elworthy et al. 2003; Hou et al. 2006). Unlike in zebrafish, Sox10 protein is detectable at later stages of pigment cell development in mice and human skin, including in differentiated melanocytes (Dutton et al. 2001; Shakhova et al. 2015). Conditional ablation of *Sox10* in the melanocytic lineage in adult mice led to depletion of both the MSC pool and differentiated melanocytes, showing a requirement for *Sox10* at multiple developmental stages of the lineage (Harris et al. 2013). Intriguingly, overexpression of Sox10 also led to loss of MSCs and hair graying by promoting premature differentiation, indicating that proper levels of Sox10 are crucial for melanocyte development and maintenance.

Growth Factors Regulating NCSCs and Melanocyte Specification

It is well established that the cell-intrinsic gene regulatory networks described above are engaged in an interplay with signaling pathways activated by extracellular growth factors. As mentioned before, such signaling pathways elicit either selective effects during neural crest development by promoting survival and proliferation of specific lineage-restricted progenitor cells or instructive effects by inducing a particular fate in multipotent cells at the expense of other possible fates (Dupin and Sommer 2012). However, it was so far not possible to identify specific growth factors stimulating melanocyte production in NCSCs in an instructive manner. Nonetheless, melanocytes can be efficiently obtained in neural tube explant cultures by treatment with a cocktail of factors, including Edn3 (endothelin-3), SCF (stem cell factor), α-MSH (alpha-melanocyte-stimulating hormone), and TPA (12-O-tetradecanoylphorbol-13-acetate) (Shakhova and Sommer 2015). Best studied among these cues with respect to melanocyte development is Edn3 (Saldana-Caboverde and Kos 2010), which rather acts selectively than instructively on NCSCs. Indeed, Edn3 appears to foster formation of glial and melanocytic unipotent and glial/melanocytic bipotent progenitor clones in quail neural crest cells and, surprisingly, also when added to differentiated melanocytes or Schwann cells (Dupin et al. 2000, 2003; Lahav et al. 1998). Thus, at least in these neural crest derivatives in culture, Edn3 can trigger a de- or even transdifferentiation program. However, in mouse neural crest cell cultures, treatment with Edn3 supported melanoblast proliferation and differentiation, but not their specification from undifferentiated neural crest cells (Opdecamp et al. 1998). The combined data indicate that Edn3 signaling can promote the expansion of progenitor cells with melanocytic potential but does not instruct NCSCs to exclusively produce melanoblasts.

In addition, Edn3 plays a role in the melanocytic lineage at later stages of development. Mutations in genes coding for ligands or receptors of the endothelin signal transduction pathway result in pigmentation defects (Saldana-Caboverde and Kos 2010). However, inactivation of the Edn3 receptor *Ednrb* in murine neural crest cells interfered with pigment cell differentiation without affecting melanoblast generation in culture (Hou et al. 2004). Likewise, pigmentation defects in *Ednrb* mutant mice could be rescued when *Ednrb* overexpression was induced at a developmental stage after melanoblast specification (Shin et al. 1999). Apart from melanocyte differentiation, Edn signaling also appears to regulate guidance of migratory melanoblasts to the dorsolateral pathway. For instance, overexpression of an avian-specific Edn receptor in mouse ES cells was sufficient to steer these cells preferentially toward the dorsolateral pathway upon in ovo transplantation (Pla et al. 2005). Similarly, neural crest cells overexpressing an Edn receptor remain undifferentiated but prefer the dorsolateral migratory pathway over the medioventral pathway normally chosen by most neural crest cells (Harris et al. 2008; Krispin et al. 2010b), again confirming a role of the Edn signaling pathway in melanocyte development independent of lineage specification.

Comparable to Edn, sonic hedgehog (Shh) has also been reported to sustain neural crest progenitor cells with melanocytic potential without influencing melanocyte specification (Dupin and Sommer 2012). In particular, in cultures of cranial neural crest cells, Shh treatment elevated the frequency of clones with a very broad developmental potential that apart from neural and osteochondrogenic potentials also maintained the capacity to generate melanocytes (Calloni et al. 2009; Dupin et al. 2010). The in vivo relevance of these findings remains to be elucidated. In contrast, signaling mediated by the tyrosine kinase receptor Kit or its ligand Kitl controls melanocyte development in vitro as well as in vivo. Indeed, mutations in this signaling pathway result in pigmentation defects due to impaired melanoblast survival, proliferation, migration along the dorsolateral pathway, and possibly differentiation (Mackenzie et al. 1997; Wehrle-Haller and Weston 1995; Hou et al. 2000, 2004). However, melanocyte specification from NCSCs and expression of early lineage markers such as Mitf and Dct is Kit signaling independent (Hou et al. 2000; Mackenzie et al. 1997; Wehrle-Haller and Weston 1995).

A central signal transduction pathway in melanocyte development is canonical Wnt signaling, which via activation of the intracellular signaling component β-catenin and of downstream transcription factors of the Tcf/Lef family directly controls expression of Mitf (Dorsky et al. 2000; Takeda et al. 2000; Widlund et al. 2002). However, Mitf expression as a Wnt signaling readout seems to be context dependent: Indeed, addition of a canonical Wnt ligand or ectopic expression of a constitutively active, stabilized form of β-catenin did not promote Mitf expression and melanocyte lineage specification in NCSCs of murine neural tube explant cultures but rather fostered sensory neurogenesis (Lee et al. 2004) (Fig. 3). In accordance with these data, conditional expression of stabilized β-catenin in premigratory neural crest cells in mice induced sensory neuron formation in vivo, while actually suppressing generation of melanocytes and other neural crest lineages.

In striking contrast to these findings, expression of constitutively active β-catenin in cranial neural crest cells in zebrafish induced pigment cell formation, while repressing neural differentiation (Dorsky et al. 1998). Similarly, melanocyte numbers in cultured quail neural crest cells were increased upon Wnt signal activation without overt changes in proliferation, consistent with a melanocyte lineage-inducing activity of Wnt (Jin et al. 2001). In support of studies claiming a role of Wnt/β-catenin in melanocyte formation in vivo, inactivation of canonical Wnt signaling either by injection of a dominant-negative Wnt ligand or of a mutant form of Tcf3 in zebrafish, or by conditional deletion of *β-catenin* in neural crest cells in mouse embryos, interfered with Dct and Mitf expression and pigment cell production (Dorsky et al. 1998; Hari et al. 2002). Unlike in zebrafish, however, loss of Wnt/β-catenin signaling in the murine neural crest not only affected the

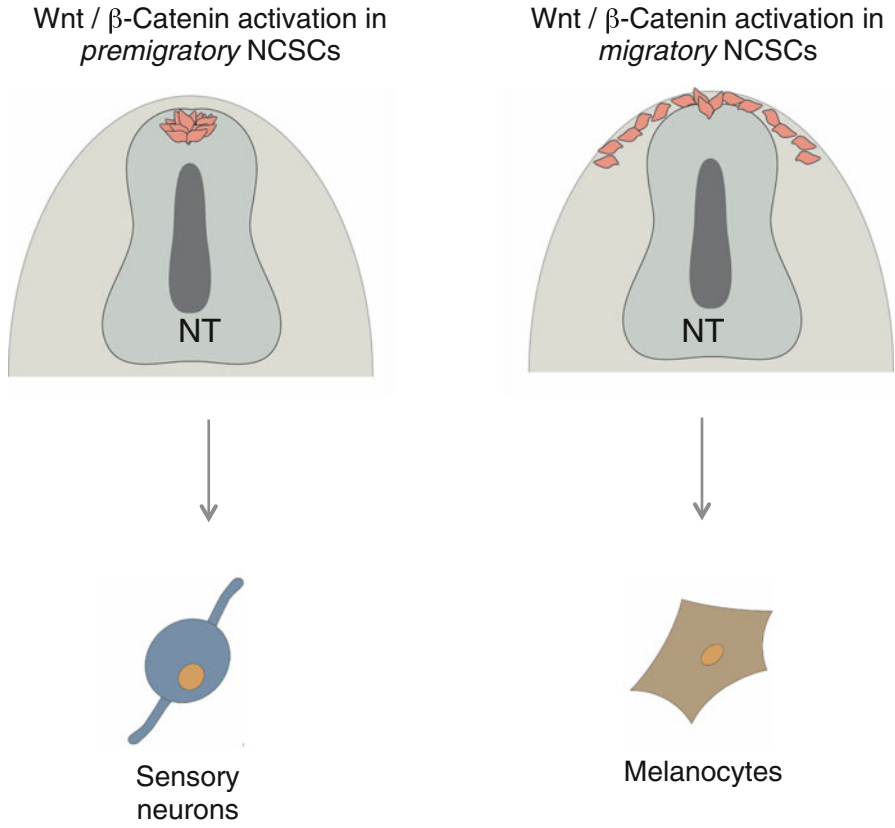

Fig. 3 The readout of Wnt/β-Catenin signal activation in NCSCs is stage dependent: When activated in premigratory NCSCs, canonical Wnt signaling promotes sensory neurogenesis; when activated in migratory NCSCs, the same pathway promotes melanocyte formation (Hari et al. 2012; Lee et al. 2004). Thus, induction of a melanocytic program is dependent on the progenitor cell's intrinsic molecular configuration. This finding is likely relevant for melanoma formation as well: Assuming that melanoma initiation involves oncogene-induced reprogramming (Kaufman et al. 2016; Van Keymeulen et al. 2015; Wong et al. 2008), the extent of cellular dedifferentiation might determine whether a cell is responsive to further cues promoting a melanocytic/melanoma program (Figure by courtesy of Dr. Arianna Baggiolini, University of Zurich)

melanocyte but also the sensory neuronal lineage (Hari et al. 2002), which is again consistent with the abovementioned finding that gain-of–βcatenin induces sensory neurogenesis (Lee et al. 2004).

The combined data, although controversial at first sight, speak for a context- and, in particular, stage-dependent role of canonical Wnt signaling in neural crest cells. From cell culture experiments, it is known that Wnt activity can be modulated by other signaling pathways (Kleber and Sommer 2004). For instance, when added together with BMP, Wnt is unable to induce sensory neurogenesis in mouse neural crest cells (Kleber et al. 2005) and melanocyte formation in quail neural crest cells (Jin et al. 2001). Similarly, in melanoblasts, Wnt promotes proliferation and pigmentation, but only when applied concomitantly with Edn (Dunn et al. 2000). Thus, whether or not Wnt/β-catenin signal activation affects melanocyte generation appears to rely on the presence or absence of factors modulating its activity. This, in turn, conceivably depends on spatiotemporal parameters. To test this idea, Hari and colleagues (Hari et al. 2012) used an inducible form of Cre recombinase to conditionally overexpress constitutively active β-catenin at different stages of neural crest development (Fig. 3). When expression was induced in premigratory neural

crest cells, sensory neurons rather than melanocytes were predominantly generated, as described before (Lee et al. 2004). Intriguingly, when the active form of β-catenin was induced in migratory neural crest cells, i.e., only after their delamination from the neural tube, the cells gave rise predominantly to melanocytes (Fig. 3), which moreover migrated extensively throughout the embryo including at ectopic places usually not harboring neural crest-derived structures (Hari et al. 2012). These data are in line with the previous report that conditional *β-catenin* ablation in the neural crest results in loss of both sensory neurons as well as melanocytes (Hari et al. 2002). Of note, the capacity of activated Wnt/β-catenin to promote melanocyte formation was restricted to migratory NCSCs present during a narrow time window of development, and activation at a later time point failed to induce melanocytes (Hari et al. 2012). In sum, a melanocytic program can apparently be activated only within a very specific and finely tuned context.

Conclusion: A Link Between NCSCs, Melanocyte Development, and Melanoma Formation

It is believed that the biology of a given tumor is dependent on its ontogeny and that tumor cells share properties with normal cells of the tissue, from which the tumor arises. In particular, initiation of tumors has been functionally associated with reactivation of embryonic or organ-specific stem cell programs (Van Keymeulen et al. 2015; Wong et al. 2008). As melanoma derives from melanocytic cells originating during embryonic development from NCSCs, it is conceivable that the broad developmental potential and migratory capacity of NCSCs is causally linked with the aggressiveness of melanoma. As discussed above, melanocytes emerge during development from multipotent migratory NCSCs (Baggiolini et al. 2015) as well as from peripheral nerve-associated cells (Adameyko et al. 2009), which could be residual stem cells located along the nerve, restricted progenitors, or even differentiated Schwann cells. On the other hand, cells with NCSC features can be induced from differentiated pigment cells (Dupin et al. 2000; Motohashi et al. 2009). Whether such dedifferentiation processes ever occur under normal physiological circumstances is unknown. But the question whether during development and even more so in the adult melanocytes arise from multipotent stem cells, from restricted progenitors, or also upon reprogramming from differentiated cell types is not just relevant for basic developmental and stem cell biology but also for tumor biology: If differentiated cells, such as Schwann cells or melanocytes, can under certain circumstances acquire the potential for self-renewal and, hence, extensive proliferation, could tumorigenic events also lead to dedifferentiation and foster self-renewal in these cells? Moreover, if dedifferentiation is accompanied by acquisition of multipotency, could, for instance, Schwann cells be promoted to dedifferentiate and to respond to a melanocytic program in the context of tumorigenesis? This would imply that melanoma could potentially have multiple origins, presumably dependent on the cell-intrinsic and extrinsic oncogenic events driving tumor formation. According to this view, melanoma could derive from MSCs as well as from differentiated melanocytes and even from Schwann cells along peripheral nerves.

It has to be noted, however, that the cell of origin in human melanoma is currently unknown. The most compelling evidence for a dedifferentiation event being at the origin of melanoma formation has recently been provided by live-cell tracing in a zebrafish model of melanoma (Kaufman et al. 2016). In this study, the tumor-initiating cell emerged in a field of tumor-prone melanocytes, which apparently involved a process of in vivo reprogramming and expression of a gene normally only active in the developing neural crest. In mouse models, both MSCs and melanocytes might be involved in melanoma initiation, depending on the genetic driver of tumorigenesis. However, the lack of genetic tools exclusively marking stem cells as opposed to differentiated melanocytes in vivo has so far precluded researchers from answering this issue by genetic cell fate mapping. Moreover, to what extent findings in animal models are applicable to the situation in human patients is unclear given the species-specific histology (with, for instance,

human skin harboring interfollicular melanocytes unlike most areas of mouse skin).

Independent of the question of whether a stem cell rather than a differentiated cell is at the origin of melanoma formation, there is increasing evidence for stem cell features being implicated in melanomagenesis. Emergence or upregulation of particular NCSC markers in melanoma has been functionally associated with tumorigenesis in genetic mouse models of spontaneous melanoma formation and in human melanoma cells. For instance, expression of the NCSC marker CD271 correlates with increased metastasis formation in human melanoma patients (Boiko et al. 2010; Civenni et al. 2011). Likewise, the NCSC transcription factor Sox10 is upregulated by oncogenic signaling in melanoma and required at high levels for tumor initiation and growth in vivo (Shakhova et al. 2012). The RhoGTPase Rac1 regulates self-renewal of NCSCs at a particular developmental stage (Fuchs et al. 2009), and gain-of-function mutations in *RAC1* have been implicated in driving human melanoma (Krauthammer et al. 2012). Finally, the roles of both Mitf and canonical Wnt signaling in melanoma are well established (Damsky et al. 2011; Delmas et al. 2007; Mort et al. 2015; Murakami et al. 2001; Zuidervaart et al. 2007). In sum, it seems that initiation and progression of melanoma involves the reuse of an embryonic NCSC regulatory program. Thus, targeting mechanisms regulating NCSCs might represent a promising treatment strategy.

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The Biology of Pigmentation

2

Allison S. Dobry and David E. Fisher

Contents

Abstract

Having roots in the earliest Mendelian experiments, the scientific examination of pigmentation offers the unique opportunity to better understand the contributions of genetics, signaling pathways, hormones, and the external environment on the phenotype of our body's largest organ system: the skin. Epidermal pigmentation is a product of the genetically determined melanin content, the cellular response to external stimuli, and the individual capacity for tanning. These processes are dependent upon a functional pigmentation pathway, which requires proper melanocyte migration, adequate melanogenic enzyme activity, and correct packaging and transfer of melanin to neighboring cells. Disruption of any of these processes leads to alterations in pigmentation. Although cutaneous pigmentation is most heavily focused upon, information about pigment patterning can also be gleaned from other pigmented tissues, including the hair and eyes. Numerous molecular signaling pathways and hormone systems converge to modulate pigment at the cellular level, which further contribute to the overall phenotype. These systems acquire greater importance when considered in

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the context of melanoma development, as these pathways are frequently found to be dysregulated.

Keywords
Pigmentation · Melanocyte · MC1R · MITF · Melanocyte stem cells · Tanning · Melanin · Ultraviolet

Introduction

Evolution is perhaps the greatest artist of all time. The natural world is ripe with masterpieces: from the iridescent blue and green plumage of the peacock to the blotchy dark spots scattered along the cream-colored fur of the cheetah, these pigmentary patterns all add to the beauty of the natural world. External coloration is an important evolutionary trait in the animal world – it confers a survival advantage via thermoregulation, camouflage, protection from solar radiation, and the facilitation of reproductive and social behavior. Most of our understanding of human pigmentation – the exterior coloration that absorbs or reflects light – is derived from studies on our animal counterparts.

Epidermal pigmentation is not a shared trait among all mammals. While many mammals don pigmented coats, their underlying skin is often surprisingly lacking in color. For these species, their pelage is an adequate form of defense against solar radiation. The appearance of cutaneous pigmentation in humans is thought to have coincided with the loss of body hair on the majority of the skin surface area. This evolutionary event was believed to take place when the *Homo erectus* population migrated to the African savannah (Maresca et al. 2015). Over countless generations, our ancestors developed an increased number of sweat glands and a reduced amount of body hair. Concurrently, hair became more heavily concentrated on top of the head. These changes were thought to develop in response to the hot environment, which created physiological stress and, via selective pressure over several generations, favored survival of those with improved thermoregulatory capacity. Hypotheses for the gradual pigmentation of skin are more variable. Some researchers argue that pigmentation developed as a protective mechanism against ultraviolet (UV)-mediated skin damage, whereas others argue that it developed because pigmented skin operates as a more effective barrier against the harsh environment. Over time, migration and dominion of new lands led to variations in skin phenotype. Those that migrated to Europe and Asia, where solar radiation is less intense, escaped the functional constraint on the gene(s) promoting dark pigmentation of the skin and developed more varied and lighter shades of pigmentation.

The scientific examination of pigmentation is not new. In the early 1900s, French biologist Lucien Cuénot performed one of the earliest mammalian genetic experiments, in which he crossed normally pigmented mice with unpigmented albino mice to demonstrate Mendelism applied to animals as well as plants. Although Cuénot demonstrated the important role of genetics in skin/fur phenotype, pigmentation is truly a result of a complex interplay between genetics, molecular signaling pathways, hormones, and the external environment. This chapter will cover the biology of pigmentation in depth and will touch upon the molecules and cells that act as building blocks of pigmentary units, the underlying signaling pathways that mediate pigmentation, and finally the relationship between pigmentation, solar radiation, and melanoma.

Part 1: Creating the Pigmentary Phenotype

Skin color results from reflected and absorbed light of unpigmented skin in combination with its constitutive pigments. The primary molecules that contribute to the coloring of human skin are melanin, hemoglobin, and carotenoids, although melanin is the greatest contributor to the resulting phenotype. When light hits the surface of the skin, it may be reflected back, scattered, or absorbed by molecules called chromophores, within the skin. The epidermal layers scatter light, while epidermal melanin granules absorb light and create brownish-black or reddish-yellow colors depending

on melanin type. Some light also reaches the dermis, where part of it is scattered and part of it is absorbed by collagen, creating a yellow color. Oxygenated hemoglobin produces a reddish tint, whereas reduced hemoglobin generates a bluish tint.

Skin pigmentation is primarily a trait of epithelial cells, in which most of the epidermal melanin resides after being synthesized and transferred from melanocytes. Epidermal pigmentation can be divided into two categories: constitutive and facultative. Constitutive pigmentation is the baseline color of the skin and is a function of the genetically determined melanin content (i.e., amount, type, and distribution). Facultative pigmentation is the result of the interplay between UV exposure, hormones, and the capacity for tanning. For instance, UV exposure in individuals with good tanning capability leads to an increase in the amount and type of melanin produced by melanocytes.

In 1975, Harvard dermatologist Thomas Fitzpatrick devised a numerical skin phototype classification system for use in clinical practice (see Table 1). The thereafter named Fitzpatrick scale ranges from phototypes I to VI and classifies persons by skin complexion and tolerance of sunlight. At the extremes, persons with phototype I (red-haired individuals) have light skin and eyes, always burn, and do not tan. Persons with phototype VI have dark-brown or black skin, never burn, and tan darkly. Although this scale is imperfect, it is a widely used metric both in research and in the clinic. Dermatologists most frequently utilize this tool to assess a patient's cancer risk.

Melanin, Melanocytes, and Melanosomes

Melanin

Melanins are a class of polymorphous biopolymers derived from the amino acid tyrosine. Two kinds predominate in the skin, hair, and eyes: eumelanin and pheomelanin (Thody et al. 1991). They differ both in their chemical composition and physical properties and thus respond disparately to light. Eumelanins are dark brown/black and highly polymerized, whereas the sulfur-containing pheomelanins are blond/red and less polymerized. Eumelanin is found in abundance in individuals with darker hair and skin. In contrast, pheomelanin predominates in individuals of skin types I and II but is also found together with eumelanin in darker phototype individuals. Hair concentrations of eumelanin and pheomelanin are higher than those in the skin.

As seen in Fig. 1, both eumelanin and pheomelanin share the first step of biosynthesis, in which tyrosinase catalyzes the conversion of tyrosine to dihydroxyphenylalanine (DOPA), which is then further oxidized by tyrosinase to dopaquinone. At this point, the pathways diverge. If available, cysteine or glutathione will rapidly conjugate with dopaquinone to form cysteinyldopa or glutathionyldopa before ultimately forming

Table 1 Fitzpatrick scale. The Fitzpatrick scale classifies persons to skin phototype according to both constitutive and facultative pigmentations. This chart demonstrates characteristics associated with each phototype, including sunburn and/or tanning history and minimal erythema dose (MED) (Table adapted from Astner and Anderson 2004)

Phototype	Constitutive color	Sunburn and tanning history	UVA-MED (mJ/cm^2)	UVB-MED (mJ/cm^2)
I	Ivory white	Burns easily, never tans	20–35	15–30
II	White	Burns easily, tans minimally with difficulty	30–45	25–40
III	White	Burns moderately, tans moderately and uniformly	40–55	30–50
IV	Beige-olive	Burns minimally, tans moderately and easily	50–80	40–60
V	Moderate brown	Rarely burns, tans profusely	70–100	60–90
VI	Dark brown-black	Never burns, tans profusely	100	90–150

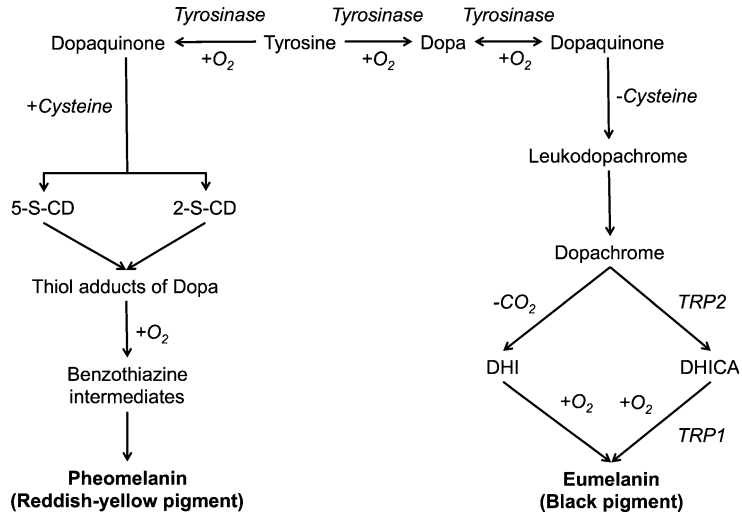

Fig. 1 Melanin synthesis. There are two separate biosynthetic pathways for eumelanin and pheomelanin synthesis (Image from Seo et al. 2007) (Adapted from Lamoreux et al. 2001)

pheomelanin. Otherwise, dopaquinone is transformed to leukodopachrome followed by a series of oxidoreduction reactions that result in the intermediates dihydroxyindole (DHI) and DHI carboxylic acid (DHICA), which become oxidized and polymerize to form eumelanin. Individual melanocytes can synthesize both eumelanin and pheomelanin, but only one pathway can remain active at a time since it is determined by the presence or absence of reduced thiols. The ratio of eumelanin to pheomelanin within the cell is a product of tyrosinase activity, as well as availability of tyrosine and sulfhydryl-containing reducing agents in melanosomes (the organelles responsible for pigment production, storage, and transport).

Several enzymes play a key role in producing pigment and are thus named melanogenic enzymes. These include tyrosinase, the tyrosinase-related proteins TRP1 and TRP2 (also commonly known as DCT), melan-A (MLNA), the P protein, and premelanosome protein (PMEL) (Hirobe 2011). Tyrosinase is a copper-containing enzyme that catalyzes three reactions in the pigment-producing pathway: (1) the hydroxylation of tyrosine to DOPA, (2) the oxidation of DOPA to dopaquinone, and (3) the oxidation of DHI to indolequinone. TRP1 and TRP2 share approximately 40% homology with tyrosinase (Lin and Fisher 2007). Both TRP1 and TRP2 are thought to stabilize the enzymatic activity of tyrosinase. Mutations in these enzymes can result in oculocutaneous albinism (OCA1-4), in which melanocytes are intact but have altered ability to produce pigment (see Table 2). Specifically, defects in the *TYR* gene, which encodes tyrosinase, lead to tyrosinase-negative OCA1. OCA2 results from mutation of the *OCA2* gene encoding P protein, OCA3 is due to mutation of *TRP1*, and OCA4 is a product of mutation of the gene encoding membrane-associated transport protein (*SLC45A2*).

In humans, eumelanin is the primary determinant of dark eyes, hair, and skin color. Additionally, eumelanin has been established as a potent photoprotective agent due to its broadband absorption spectrum. Eumelanin may partially shield organisms from intense sunlight, through dissipating a percentage of UV energy as heat in a nanosecond or less. Despite the ubiquity of melanins in nature, the underlying physical structure has been surprisingly elusive to researchers. The structure of melanin is difficult to study outside of living organisms, as, once it is isolated, it loses its structure and may transform into an amorphous mass. More than 100 variations of eumelanin composition have been noted to exist. Interestingly, a group of researchers has recently uncovered a unique optical property of eumelanin in that eumelanin has geometric disorder in addition to previously recognized chemical disorder (Chen et al. 2014a). Geometric disorder

Table 2 Genetics of oculocutaneous albinism. The TYR, OCA2, TRP1, and MATP genes are all implicated in oculocutaneous albinism (Image adapted from Grønskov et al. 2007, available under a CC BY 2.0 license. URL: http://ojrd.biomedcentral.com/articles/10.1186/1750-1172-2-43. © Grønskov et al.; licensee BioMed Central Ltd. 2007)

Gene	Chr. localization	Size	Disease name	Prevalence
TYR	11q14.3	65 kb (529aa)	OCA1	1:40,000
			OCA1A	
			OCA1B (yellow alb.)	
OCA2 (p gene)	15q11.2–q12	345 kb (838aa)	OCA2 (brown OCA in Africans)	1:36,000 (white Europeans), 1:3,900 (Africans)
TRP1	qp23	17 kb (536aa)	OCA3 (Rufous OCA)	Rare (white Europeans, Asians), 1:8,500 (Africans)
MATP	5p13.3	40 kb (530aa)	OCA4	Rare (white Europeans), 1:85,000 (Japanese)

is a result of randomly oriented and randomly sized molecules forming the aggregate structures. The interplay of geometric order and disorder of eumelanin aggregate structures generates random excitonic couplings among the molecules. These couplings broaden the absorption spectrum.

Eumelanin and pheomelanin respond differently to UV radiation (UVR). Eumelanin, as a heterogeneous polymer of DHI, DHICA, and their derivatives, acts as a UV filter and also scavenges for UV-induced free radicals. Additionally, the melanin precursor DHICA has been shown to inhibit lipid peroxidation as well as stimulate the antioxidant defense systems and differentiation of keratinocytes. Pheomelanin is a poor UV filter and has also been found to increase formation of reactive oxygen species (ROS). Within melanosomes containing dark/eumelanin pigment, it has been noted that the initial (deepest) melanins are pheomelanin, but these become overlaid or "caged" by layers of eumelanin which may protect the cell from ROS production by the underlying pheomelanin. Absorption of radiation by various melanin species, and particularly pheomelanin, can generate radicals that are strong oxidants. Furthermore, melanin synthesis involves a series of highly reactive quinone intermediates that promote ROS and oxidative DNA damage.

Melanocytes

Melanin is the most important molecule in producing skin pigmentation, and proper functioning of melanocytes is vital for appropriate melanin production. As seen in Fig. 2, melanocytes are found within the human skin dispersed along the dermal/epidermal border. There, they can interact with the underlying fibroblasts of the dermis and the surrounding keratinocytes in the dermis. However, melanocytes are not merely pigment producers. They release a diverse array of signaling peptides (e.g., melanocortin peptides, catecholamines, serotonin, eicosanoids, and nitric oxide) that tightly connects them with both the neural and immune systems. Because of this, melanocytes are thought to play an important role in general epidermal homeostasis.

Cutaneous melanocytes are derived from pluripotent neural crest cells. Neural crest cells also give rise to neurons, glial cells, the adrenal medulla, cardiac cells, and craniofacial tissue. Upregulation of microphthalmia-associated transcription factor (MITF) by paired-box 3 (PAX3), the Wingless-type (WNT) signaling pathway, and sex-determining region Y (SRY)-box 10 (SOX10), in combination with downregulation of forkhead box D3 (FOXD3) and SRY-box 2 (SOX2), is important in creating committed melanocyte lineage cells. Melanoblasts, the melanocyte precursors, proliferate and migrate dorsolaterally from the dorsal portion of the neural tube to populate the basal epidermis and hair follicles. An additional pathway of melanocyte development from Schwann cell precursors has recently been found to be a significant contributor to cutaneous pigment cell formation (Mort et al. 2015). Human melanocytes can be detected in the

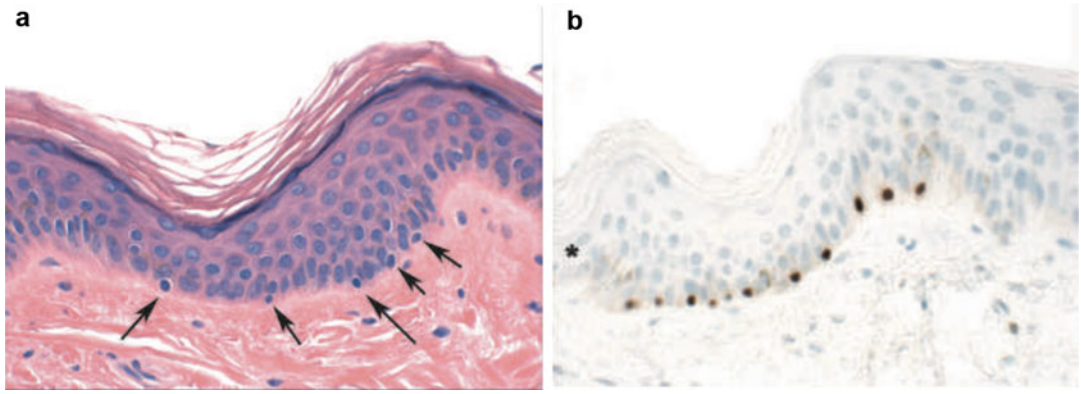

Fig. 2 Skin histology. (**a**) Hematoxylin and eosin staining of normal human skin tissue. Hematoxylin stains nuclei purple and eosin stains cytoplasm and collagen pink. The arrows point to melanocytes within the basal layer of the epidermis. (**b**) Immunohistochemistry of the same tissue. The melanocytes are stained brown by the antibody D5 (antimicrophthalmia-associated transcription factor) (Image from Lin and Fisher (2007) with permission from S. R. Granter)

dermis and epidermis by 7 weeks of estimated gestational age. Defects in melanoblast migration result in unpigmented patches of the skin.

Many signaling pathways and transcription factors provide input for proper melanocyte migration and proliferation. Integration of spatial and temporal signals from these pathways allows for precise control of melanocytes. Signals important in homing of melanocytes to the skin include the KIT proto-oncogene receptor tyrosine kinase (KIT) and its cognate ligand KITLG, as well as endothelin and its receptor B (EDNRB). Furthermore, the appropriate migration of melanoblasts and melanocytes is dependent upon integrins, cadherins, and extracellular matrix molecules. For example, mouse studies show that at embryological day 11.5 in mice, most dermal melanoblasts are E- and P-cadherin negative, but over the next 48 h during migration from the dermis to the epidermis, the majority of melanoblasts become E-cadherin high/P-cadherin low (Mort et al. 2015).

Overall, absolute melanocyte numbers are not the main drivers in pigmentary differences between races. Rather, it is the overall cellular activity, the type of melanin produced, and the size, number, and packaging of melanosomes that determine pigmentation. Variation in melanosome size can be seen between different skin types. Individuals with dark skin types have larger melanosomes that are packaged as single units, which limit their degradation in keratinocytes and provide greater visible pigmentation. Lighter-skinned individuals have smaller melanosomes that are packaged in groups, making them more vulnerable to degradation.

There is high variability in melanocyte population densities both within individuals and between individuals. For instance, there are twice as many melanocytes in the head and forearm skin compared to elsewhere on the body. The density of melanocytes within the skin is a function of UVR and stimulatory factors secreted by neighboring cells. Studies have suggested that after reaching 30 years of age, a person will lose approximately 10–20% epidermal melanocytes per decade. However, it is also possible that a reduction in pigmentation may be due to a reduction in melanogenic enzyme activity rather than complete cell loss. Aging additionally causes changes in melanocyte morphology and proliferative capacity, as well as a reduction in melanogenic enzyme activity. Terminally differentiated melanocytes are characterized by an accumulation of cyclin-dependent kinase inhibitors (e.g., $p16^{INK4A}$), hypophosphorylation of retinoblastoma protein (pRB), and decreased levels of cyclin D1. After time, terminally differentiated melanocytes also suffer from high levels of ROS due to a reduction in catalase activity and downregulation of anti-apoptotic factor BCL2.

Reduced activity of the mitogen-activated protein kinase (MAPK) pathway also leads to a reduction in melanocyte proliferation.

Melanosomes

Within melanocytes, there are large, specialized pigment-producing organelles (measuring up to 500 nm in diameter) called melanosomes, which are responsible for melanin synthesis, storage, and transport. Melanosomes are a type of lysosome-related organelle and protect the rest of the melanocyte from the toxic by-products of melanin synthesis. Melanosomes are synthesized in the perinuclear region of the melanocyte. Melanosome development occurs in four morphologically distinct stages; the two earliest stages are associated with little to no pigment, whereas melanosomes are pigmented by the later stages. Stage I premelanosomes are formed by an outpouching of a smooth membrane from the rough endoplasmic reticulum. At stage II, PMEL is sorted into intraluminal vesicles and undergoes proteolytic cleavage to form the fibrillar matrix. The melanogenic enzymes tyrosinase and TRP1 are delivered to the organelle, inducing pigment synthesis. In stage III, melanin pigment is deposited onto the fibrillar matrix. In stage IV, the melanosome matures and is fully melanized (Slominski et al. 2004). This stepwise process can become dysregulated under pathological conditions like melanoma. In such states, tyrosinase can become activated by stage I of melanosomes synthesis, and melanin can be deposited without an underlying matrix.

For melanosomes to be transferred to surrounding epithelial cells, they must travel from the perinuclear region to the tips of dendrites within the melanocyte. Transport to the melanocyte dendrite is mediated by microtubules and microtubule-associated motor proteins (kinesins and cytoplasmic dyneins). Once at the tip, melanosomes are captured for migration to nearby keratinocytes. Myosin VA (MYO5A) is involved in the capture of melanosomes at the tip. Ras-related protein Rab-27a (RAB27A) assists in phosphatidylserine addition to synaptotagmin-like protein2-a (SLP2A), which docks melanosomes to the protein membrane.

Pigment transfer from melanocytes to keratinocytes has been extensively studied but remains incompletely understood. There are currently four non-mutually exclusive models for melanin transfer: (1) phagocytosis of melanocytic dendrites by keratinocytes, (2) melanosome transport by membrane nanotubules, (3) melanosome exocytosis of a polymerized melanin extracellularly followed by keratinocyte internalization, and (4) shedding by melanocytes of plasma membrane-enclosed melanosome-rich packages that are then phagocytosed by keratinocytes (Wu and Hammer 2014). The third and fourth models have gained considerable traction due to supporting evidence from experimental studies (Tarafder et al. 2014). More studies are required for the underlying mechanism to be fully elucidated. Once transferred to keratinocytes, the melanosomes are positioned over the superficial/top (sun-exposed) side of the nucleus, potentially shielding DNA from the deleterious effects of UVR.

Melanosomal disorders include Chédiak-Higashi syndrome (CHS), Hermansky-Pudlak syndrome (HPS), and Griscelli syndrome (GS). These disorders involve aberrant melanosome biogenesis. They are often accompanied by immunodeficiency and neurological dysfunction due to parallel roles of their genetically encoded mediators, which regulate melanosome-like vesicular structures in other cellular compartments. Other disorders include macromelanosomes and autophagic giant melanosomes, which can be seen in complexes of nevocellular nevi, lentigo simplex, and malignant melanoma.

Pigmented Lesions

Pigmented lesions arise on the skin from alterations in melanocyte cellular activity or increases in melanocyte number. One of the most focused upon pigmented lesions in the dermatology clinic is the melanocytic nevus, more commonly known as a mole. Striking in appearance, most people have a few dozen melanocytic nevi on their body, with many fair-skinned people having more. Nevi may either be congenital, developing in utero, or acquired during a person's lifetime, which is the more common presentation.

Typically found in sun-exposed sites, the common acquired melanocytic nevus can appear within the first 6 months of life, reaching its largest diameter in young adulthood and regressing with advancing age. Dermatology patients are provided with the mnemonic ABCDE (asymmetry, border irregularity, color variation, diameter, and evolutionary change) to assist in at-home monitoring of their nevi. Nevi meeting any one of these criteria should be pointed out to a dermatologist for appropriate clinical evaluation, which may include a biopsy and histopathologic analysis.

A melanocytic nevus is defined as a local proliferation of melanocytes in contact with each other, forming nests. Melanocytic nevi can be further subclassified into junctional, compound, or intradermal nevi based upon the histologic location of melanocytic nests. Nevi can progress from junctional to compound to intradermal locations as they migrate deeper into the skin, evolving from a flat macule to a raised papule.

Although benign, melanocytic nevi are formed by an activating mutation in an oncogene, causing proliferation until the onset of senescence which is thought to limit further growth. Although higher numbers of melanocytic nevi are associated with an increased risk of melanoma formation, only about one in four melanomas is derived from an apparent preexisting melanocytic nevus. Congenital and acquired melanocytic nevi vary in their oncogenic drivers. Congenital melanocytic nevi are thought to form from an error in neuroectodermal development and migration. Mutations in both neuroblastoma RAS viral oncogene (*NRAS*) and B-raf proto-oncogene (*BRAF*) have been found in CMN, with *NRAS* mutations being the primary driver in giant congenital melanocytic nevi. Acquired melanocytic nevi are thought to be a result of UV exposure and carry *BRAF* mutations in 50–70% of cases.

Other pigmented lesions that are commonly seen are ephelides (also known as freckles), lentigines (also known as age spots), and café au lait spots. Ephelides are small brown macules that arise on sun-exposed skin. They are seen most frequently in fair-skinned people, especially those with red hair. After UV exposure, increased melanin production occurs within melanocytes, which then transfer the pigment to neighboring keratinocytes. Accumulation of melanin in a localized group of keratinocytes creates the brown macular appearance of an ephelis. Typically, ephelides will fade during the winter months. Lentigines are brown macules that commonly arise in middle age, often due to sun damage. They are most frequently found on the face and hands and are generally larger and more defined than ephelides. They persist for a long time and do not fade in the winter months like ephelides. They are a localized proliferation of melanocytes and can be distinguished from melanocytic nevi by the absence of melanocyte nests. Café au lait spots are hyperpigmented lesions caused by an increase in melanin content in combination with giant melanosomes. They often develop in isolation, but a large number of them are suggestive of neurofibromatosis type 1 (NF1). Interestingly, café au lait spots of NF1 patients have increased melanocyte density and higher levels of KITLG than in those of individuals without NF1.

Pigment-Recipient Phenotype + Pigment Patterning

The Pigmentary Unit

Although melanocytes are the primary focus of pigment biology, epithelial cells play an equally important role. Indeed, the skin and hair are made up almost entirely of keratinocytes, while melanocytes are much fewer in number. Melanocytes may be the pigment generators, but proper skin color patterning ultimately relies on a finely controlled communication network between melanocytes and the surrounding epithelial cells. The underlying process by which this coloration happens has been named the "pigment-recipient phenotype." In this model, melanocytes act as the pigment donors, and dedicated epithelial cells behave as pigment recipients. Together they comprise what is called a "pigmentary unit," in which the melanocyte makes dendritic connections to a defined group of epithelial cells (typically there is one melanocyte interacting with 30–40 keratinocytes). Four major classes of pigment recipients have been identified in humans:

(1) keratinocytes of the basal layer of the epidermis, (2) keratinocytes of the first suprabasal layer of the epidermis, (3) progenitors of the hair cortex, and (4) precursors of medulla follicular cells.

In the epidermis, melanocytes sit above the basement membrane. From there, they communicate with basal and first suprabasal layers of epithelial cells. Via this arrangement, melanocytes are able to deliver pigment to the least differentiated keratinocytes with the greatest proliferative capacity. After melanin acquisition, epidermal keratinocytes concentrate the pigment on the apical side of the nucleus, forming a nuclear cap that functions as a parasol to shield the nucleus from sunlight.

Clues that keratinocytes may influence melanocyte behavior arose in early in vitro experiments. When isolated human melanocytes are placed into a culture dish with epidermal keratinocytes, the melanocytes localize to the basal layer, just as they do in the human epidermis. Thus, it appears that the epidermal cells provide vectorial signals to the melanocytes that assist in their positioning. Keratinocytes can also influence melanocyte survival, proliferation, melanogenesis, and dendricity by production of paracrine growth factors and cell adhesion molecules. Factors secreted by the keratinocyte include α-melanocyte-stimulating hormone (α-MSH), endothelin 1, KITLG, basic fibroblast growth factor (FGF2), nerve growth factor (NGF), prostaglandins, and granulocyte macrophage colony-stimulating factor (GM-CSF).

Epithelial Cell Targeting

As the skin develops, some epithelial cells become pigmented, while others do not. What drives this difference? Central to this question is whether epithelial cells are simply passive recipients of melanin or are self-driven to actively recruit melanin from melanocytes. Several studies have suggested the latter may be the case, and the major molecules thought to activate the pigment-recipient phenotype will be covered here. Two molecules that have been heavily studied in the interactions between epithelial cells and melanocytes are the transcription factor Forkhead box protein N1 (FOXN1) and its target FGF2. Both of these molecules are expressed in epithelial cells that are pigment recipients.

In humans, FOXN1 is present in the differentiating hair cortex, first suprabasal layer of the epidermis, and small portions of the basal layer of the epidermis. *FOXN1* is most highly expressed in these areas during cellular transitions from proliferation to differentiation. Humans with a nonsense mutation in *FOXN1* suffer from T cell immunodeficiency, congenital alopecia, and nail dystrophy. Mutation of *Foxn1* in mice, a gene that is 86% identical to the human gene, results in a nude phenotype. Mouse studies by Weiner and colleagues have suggested that *Foxn1* appears to have a role in pigmentation directioning (Weiner et al. 2007). FOXN1 is thought to cause keratinocytes to release FGF2, which is detected by melanocytes and allows them to recognize FOXN1-positive epithelial cells as pigment targets. Thus, through FOXN1 and the release of FGF2, epithelial cells appear to engineer their own pigmentation. This means that pigment recipients, like pigment donors, are also specialized cells dedicated to a pigmentary function. However, humans do not have FOXN1-mutation associated abnormalities, and thus, more research must be done to clarify its role in human pigmentation.

KITLG plays a clear and important role in melanocyte development. Mutations in KITLG (or KIT receptor itself) result in piebaldism: patches of unpigmented hair. KITLG exists both in diffusible and cell-bound forms, which are produced by alternative splicing of the same RNA transcript. The diffusible form is thought to mediate chemotaxis, whereas the cell-bound form is thought to direct cell positioning and promote proliferation and survival. KITLG activates the MAP kinase pathway in melanocytes through the KIT receptor, which is a receptor tyrosine kinase. KITLG may help identify or activate pigment recipients, either in conjunction with FOXN1 or in place of it. Other factors that may play a role in the recipient phenotype are noggin (NOG), epidermal growth factor receptor (EGFR), F2R like trypsin receptor 1 (F2RL1), and derivatives of proopiomelanocortin (POMC).

This division of activities – one cell producing pigment, another cell using it – is specific to the skin and carries unique advantages: specificity in pigmentary interactions and the creation of a finely mapped template for pigmentation.

Pigmentation in Other Tissues

Hair Pigmentation

The hair is one of the defining characteristics of mammals. In contrast to most mammals, humans grow long, thick hair on the scalp, with relatively short, thin hairs on the remainder of the body. The biological and evolutionary significance of this is uncertain, but purported theories range from UV protection on the scalp to serving as a method of expelling built-up toxic chemicals via melanin binding (Tobin and Paus 2001). A hair shaft is comprised of compact terminally differentiated keratinocytes known as trichocytes. Hair shafts grow from a follicle at a rough rate of 1 cm per month. Humans have different types of hair, including terminal, vellus, and androgenic hairs, which serve different biological purposes. Vellus hair is generally more lightly pigmented, fine, and short in length, whereas terminal and androgenic hair are thicker, darker, and longer.

The color of the hair is a function of the ratio of eumelanin to pheomelanin. Black hair follicles have melanocytes with a large number of eumelanosomes with a fibrillar matrix. Brown hair is associated with smaller bulb melanocytes. Blonde hair has poorly melanized melanosomes, such that only the melanosomal matrix is visible. Red hair is associated with pheomelanosomes that contain a vesicular matrix and irregularly deposited melanin. Most of the human population has dark hair. This was thought to arise evolutionarily as a selective advantage in tropical UV-intense climates. It is also believed that persons with a loss-of-function melanocortin 1 receptor (*MC1R*) mutation, which gives rise to the red hair phenotype, might have escaped this evolutionary pressure, explaining the wider variety of hair colors within Northern European populations. Interestingly, hair and skin pigmentations do not always perfectly correlate. Melanin is processed differentially by recipient hair cortical keratinocytes and epidermal keratinocytes. Melanin transferred to epidermal keratinocytes is partially digested. In contrast, melanin processing in hair keratinocytes is minimal. These variable degrees of melanin processing are thought to account for seemingly unrelated hair and skin phenotypes, such as dark hair and light skin.

The hair follicle is considered to be a mini-organ. It is tightly connected to many cell types, including keratinocytes, fibroblasts, nerves, and immune cells, all of which influence the hair growth cycle. The hair follicle is made up of concentric cylindrical layers of cells: the innermost layers make up the hair shaft, and the intermediate layers make up the inner root sheath. Stem cells at the hair bulb, which forms the base of the follicle, generate the cells forming the hair shaft and inner root sheath. These segments are displaced up and through the surrounding outer root sheath. Another important structure is the bulge region of the outer root sheath, which is the site of arrector pili muscle attachment.

The hair follicle pigmentary unit is tightly coupled to the hair growth cycle, whereas the epidermal pigmentary unit experiences continuous melanogenesis. It is comprised of follicular melanocytes, matrix keratinocytes, and dermal papilla fibroblasts. Hair becomes actively pigmented by proliferating melanocytes during anagen, the first phase of the hair cycle (see Fig. 3). During the subsequent two phases, catagen and telogen, melanogenic enzymes are downregulated before melanocytes ultimately undergo apoptosis. Melanogenesis and pigmentation then begin anew during the next cycle (Schneider et al. 2009).

Hair pigmentation develops via a coordinated sequence of events: the melanogenic activity of follicular melanocytes increases followed by transfer of melanin granules into cortical and medullary keratinocytes, ultimately producing a pigmented hair shaft. Melanocytes achieve this specific targeting of pigment transfer by extending their dendrites upward along the columns of cortical and medullary cells. Within the hair follicle, melanocytes exist in a 1:5 ratio with keratinocytes, which is more balanced than the

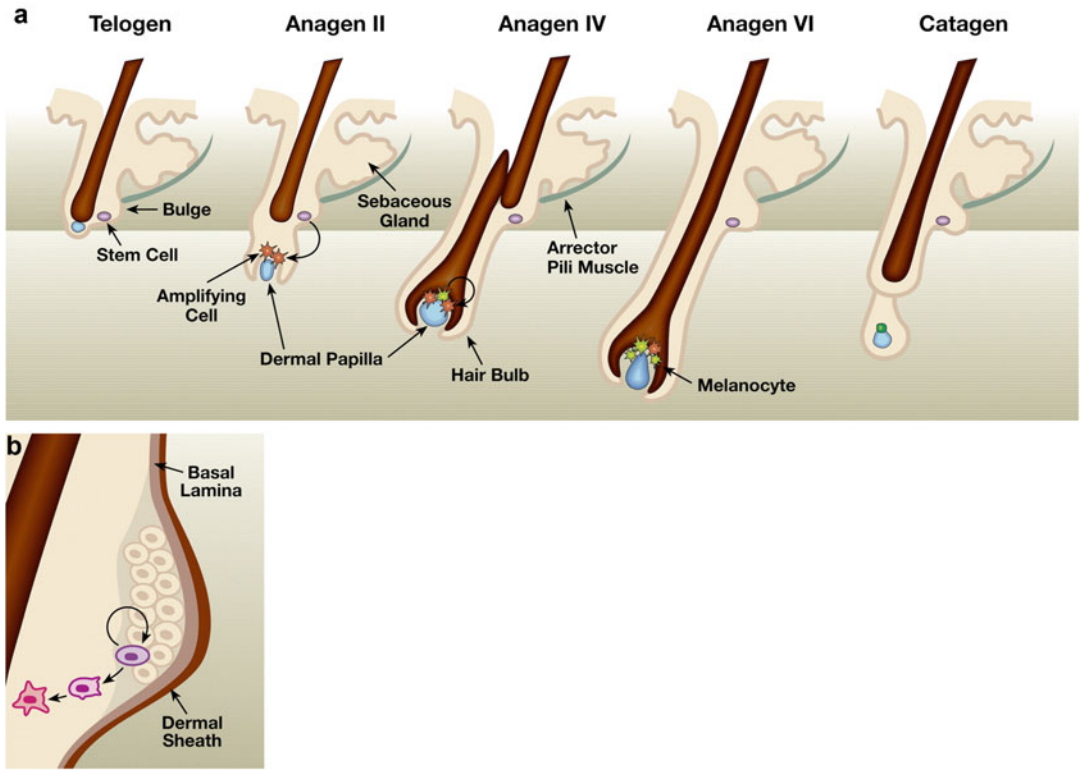

Fig. 3 Melanocyte stem cells and the hair cycle. (**a**) Shown here are the different stages of the hair cycle, including telogen, anagen, and catagen. At the beginning of anagen, melanocyte stem cells (purple) in the hair bulge give rise to amplifying cells (red) that migrate to the dermal papilla. Once there, they differentiate into melanocytes (green). (**b**) Melanocyte stem cells give rise to two types of daughter cells: those that self-renew (purple) and those that differentiate (red) (Image from Steingrímsson et al. 2005) (Adapted from Nishimura et al. 2002)

ratio within the epidermis. Melanin production within the anagen hair bulb is thought to mediate cellular stress caused by production of ROS through the free radical scavenger properties of melanin.

Like the rest of cutaneous melanocytes, hair follicle melanocytes are derived from neural crest cells. Homing to the follicular pigmentary unit during embryogenesis is thought to be directed by two receptor-ligand pairs that have been previously discussed: (1) receptor tyrosine kinase KIT and its ligand KITLG and (2) endothelin-3 and its receptor EDNRB. Melanoblasts expressing KIT migrate into the KITLG-positive hair follicle epithelium. Once there, differentiated KIT-positive melanocytes travel to the bulb when it is KITLG-positive. KIT-negative melanoblasts migrate to the outer root sheath and bulge region within the hair follicle. After homing is complete, active melanocytes are found within the basal layer of the infundibulum, the upper dermal papilla, and the basal layer of the sebaceous gland.

Melanocyte Reservoirs and Aging

Observations of vitiligo patients led to the hypothesis that collections of melanocyte stem cells might exist. Vitiligo is a skin condition characterized by loss of groups of melanocytes mirrored by patchy loss of skin pigmentation. Repigmentation in patients with autoimmune vitiligo following immunosuppressive UV therapy manifests as tiny islands of color centered upon hair follicles. This was suggestive of a stem cell reservoir within hair follicles. Early scientific studies supported this idea, as the presence of DOPA-negative amelanotic melanocytes was observed within the

outer root sheath and bulge areas of the hair follicle. Under normal conditions, these melanocytes did not produce pigment. However, after stimulation by UVR or epidermal wounding, these melanocytes could be induced to produce pigment. These observations led researchers to believe that this pool of amelanotic melanocytes might represent a reservoir of melanocyte stem cells (Steingrímsson et al. 2005).

Confirmation that this population was actually a pool of melanocyte stem cells was achieved through mouse studies. Addition of anti-KIT antibodies to mice was observed to induce hair graying via depletion of KIT-dependent melanocytes. However, later hair cycles produced normally pigmented hair, suggesting that the hair bulb melanocytes were replenished by a KIT-independent melanocyte source. In another experiment, when an anti-KIT antibody was given to deplete replicating melanoblasts from neonatal *Dct-LacZ* transgenic mice (historically used to monitor early melanoblast development), LacZ-positive cells were observed predominantly in the bulge area of hair follicles (Nishimura et al. 2002). This, combined with the observation that mice injected with anti-KIT antibody produced gray hair during the first hair cycle before again growing normally pigmented hair, was strongly suggestive of the presence of a resting melanocyte precursor population in the bulge region.

The proposed mechanism of melanocyte stem cell involvement in the hair cycle is that once the hair cycle moves from telogen to anagen, both the expression of *TRP2* and cell size increase in melanocyte stem cells, leading to cell division (see Fig. 3). One cell remains in the bulge region, while the other migrates to the hair matrix, divides further, and differentiates into a pigment-producing melanocyte. It was later shown through transgenic mouse models that the bulge melanocyte stem cells could act a source of melanocytes in the epidermis (under certain conditions) by melanoblasts traveling from the hair bulge to the surrounding epidermis. Maintenance and regulation of the melanocyte stem cell pool within hair follicles depend on the transcription factors PAX3 and MITF.

Improved knowledge of follicular melanocyte stem cells paved the way for better understanding of hair graying. Most humans begin to show gray hair at around 35 years of age. It was long thought that hair graying was a result of long-term toxicity from melanin biosynthesis, causing eventual degradation of melanocytes. However, it was later shown that hair graying is instead a product of melanocyte stem cell reservoir depletion (Nishimura et al. 2005). Two proteins play a particularly active role in the maintenance of hair pigmentation. Specifically, BCL2 protects melanocytes from apoptosis. Mice with a null mutation for *Bcl2* can only produce pigmented hair for a single hair cycle before the hair turns gray. Between postnatal days 6 and 8 (early–mid anagen cycle), a sudden loss of all bulge melanocytes occurs despite a normal number of differentiated melanocytes within the hair bulb. MITF also plays an important role in maintenance of the melanocyte stem cell niche. Premature graying is seen in mice with the $Mitf^{vit}$ mutation. This mutation caused melanocytes to differentiate prematurely (or become "aberrantly pigmented") within the bulge region, causing them to permanently lose their stem cell properties and as a result preventing proper migration to the bulb. Thus, MITF is thought to be important for self-renewal.

Eye Pigmentation

Eye color is a function of both iris pigmentation as well as the scattering of light by the stromal medium. The iris is comprised of five layers: the anterior border layer, stroma, muscular layer, anterior pigment epithelium, and posterior pigment epithelium. Iris pigmentation is dependent upon the concentration of melanin within both the iris pigment epithelium (IPE) and the stroma and the cellular density of the stroma. In the brown iris, there is a large quantity of brownish-black melanin within the anterior border layer and stroma that effectively absorbs light. In contrast, there is very little melanin in the blue iris. Interestingly, there is also no blue pigment present.

Rather, the blue color is a result of optics: as light traverses through the melanin-free layers, longer wavelengths are transmitted, while the shorter blue wavelengths are reflected via scattering by collagen fibrils. This is known as the Tyndall effect. Thus, the blue iris represents structural color. Patients with severe albinism lack pigment in the back of the iris, allowing light from inside the eye to escape through the iris to the front. The only color seen in eyes from these individuals is from hemoglobin in the capillaries, resulting in a reddish-pink eye.

There are three major classes of eye color: brown, blue, and green hazel. The number of melanocytes does not differ between eye colors. Darker eyes have a greater ratio of eumelanin to pheomelanin, whereas lighter eyes have more pheomelanin. Lighter iris colors are found almost exclusively in persons of European descent. The majority of babies of European descent have light-color eyes when they are born, which may later change to darker colors by the age of 1. This is due to an increase in melanin production driven by sympathetic neuronal stimulation. Eye color has also been noted to change in later stages of life, such as during puberty and after trauma. Also of note, half the adult population has iris nevi, which can appear on the surface of the anterior border layer when a group of melanocytes increases their melanin production.

Although eye color is a complex polygenic trait, it has been estimated that 74% of variation in human eye color can be attributed to a portion of the genome containing the *OCA2* gene (Sturm and Larsson 2009). Regulation of *OCA2* gene expression via epigenetic pathways is thought to determine blue-brown eye color in European populations. A single base change, rs12913832 T/C within intron 86 of the upstream *HERC2*, appears to play a role in this process. This SNP has been suggested to be a target site for the SWI/SNF family member helicase-like transcription factor (HLTF). In the proposed mechanism, as shown in Fig. 4, the *HERC2-OCA2* locus initially exists in a closed heterochromatin packaging state. When the rs12913832*T allele is active, HLTF recognition can occur, causing chromatin unwinding and exposure of the regulatory sequences recognized by MITF and lymphoid enhancer binding factor 1 (LEF1), permitting the *OCA2* promoter to be available for transcription factor regulation. Expression of the *OCA2* gene results in brown eye color. In contrast, the rs12913832*C allele prevents HLTF binding, keeping chromatin in a closed state that prevents transcription of the *OCA2* locus. This results in blue eye color due to the inability to form mature/eumelanotic melanosomes.

Extracutaneous Melanin

In addition to the epidermis, hair follicles, and eyes, melanocytes can be found in less suspected areas. They may be found in small numbers throughout the dermis and subcutis, blood vessel walls, and even within the muscle, nerves, and sebaceous glands. Melanocytes have even been detected within lymph nodes in a benign form. It has been suggested that these melanocyte collections are instead due to an error in embryological migration, and they may be seen in association with large congenital melanocytic nevi and blue nevi. Melanocytes present within lymph nodes can sometimes be troubling, as they can be mistaken for metastases.

Melanocytes may additionally be found within the heart valves and septa. Their function there is unclear, but they may contribute to atrioventricular valve function, as well as regulation of calcium and ROS levels. Melanocytes are also located within the stria vascularis of the cochlea and play a critical role in hearing due to maintenance of extracellular potassium in the endolymph. Indeed, the Waardenburg type II (WS2) phenotype resulting from an *MITF* mutation presents with sensorineural deafness that can range from mild to severe. Melanocytes are also present in the brain within the meninges overlying the medulla oblongata and upper cervical spinal cord. Notably, the dark melanin produced by dopaminergic neurons in the substantia nigra is neuromelanin and is believed to be an autooxidative product of dopamine synthesis rather than melanocytic in origin.

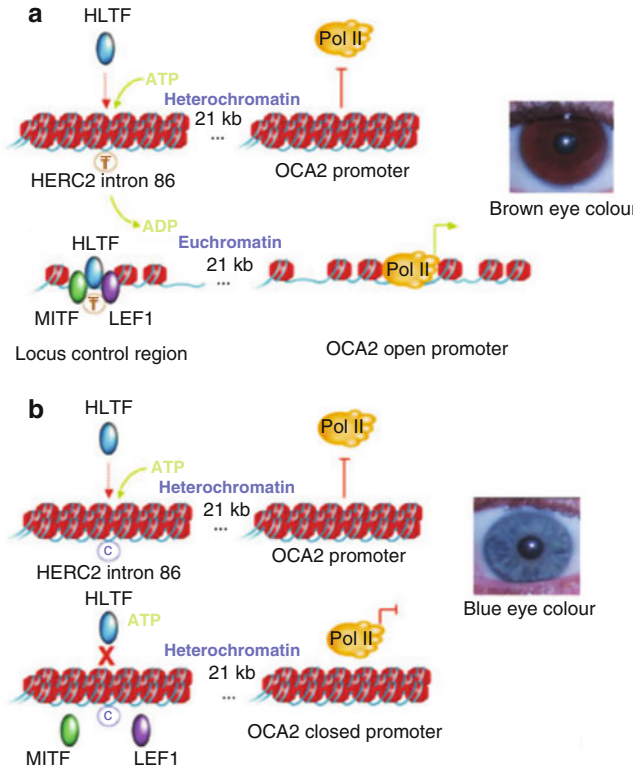

Fig. 4 A model of how regulation of *OCA2* gene expression determines blue-brown eye color. (**a**) Helicase-like transcription factor (*HLTF*), a member of the SWI-SNF family, is able to regulate genes by altering chromatin structure. Here, HLTF recognizes the evolutionary conserved element containing the SNP rs12913832*T within the HERC2 intron 86 region of a DNA molecule (DNA is represented as the blue coil, and nucleosomes are represented as the red spheres). This causes DNA to transform into a more relaxed state, which permits the transcription factors MITF and LEF1 to bind to the locus control region. As a result, RNA polymerase II (Pol II) can transcribe the *OCA2* gene, which stimulates eumelanogenesis and a resulting brown eye color. (**b**) If the SNP rs12913832*C is instead present, HLTF is unable to interact with the heterochromatin, which prevents MITF and LEF1 binding, as well as *OCA2* transcription. A resulting lack of melanin production leads to blue eye color (Image from Sturm and Larsson 2009)

Part 2: Molecular Control of Pigmentation

Multiple factors are involved in the control of pigmentation. More than 150 alleles spread over 90 loci are involved in the regulation of pigmentation, and they encode protein products such as enzymes, structural proteins, transcriptional regulators, transporters, receptors, and growth factors (Slominski et al. 2005). Keratinocytes and fibroblasts release POMC, growth factors, cytokines, and ROS. Hormones like corticosteroids and estrogens can also influence pigmentation. One of the most central interactions in the pigmentation is that between α-MSH and MC1R, which initiates cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling.

MC1R and the Pigment-Type Switching System

Melanocortin 1 Receptor

Much of the attention has been focused on establishing the link between pigmentation phenotype and genetic polymorphisms involving the

MC1R locus. So far, *MC1R* is the only gene identified that can account for the large phenotypic variation in pigmentation. MC1R acts as a regulator of both constitutive and facultative pigmentation. The *MC1R* gene encodes a seven-transmembrane G-protein-coupled receptor (GPCR), which upon activation leads to production of eumelanin within melanocytes. Mutations at this locus can alter the ratio of eumelanin/pheomelanin production within melanocytes and lead to widely variable coloring in the human eyes, skin, and hair.

More than 100 gene polymorphisms have been reported for *MC1R*. *MC1R* variants contain changes in ligand binding, receptor function, or a complete loss of function. In particular, loss-of-function polymorphisms result in the red hair, freckling, and fair skin phenotype, in which individuals have a decreased ability to tan. *MC1R* gene sequence variants are present in over 80% of individuals with red hair or poor-tanning skin (Roider and Fisher 2016). In contrast, variants are found in fewer than 20% of individuals with brown or black hair and in less than 4% of individuals with good tanning ability. Loss-of-function *MC1R* variants are linked to an increased risk of developing both melanoma and non-melanoma skin cancer.

The MC1R receptor is activated by α-MSH, a peptidic hormone derived from POMC in the pituitary and skin, and is inhibited by agouti signaling protein (ASIP). Thus, α-MSH is thought to promote eumelanin production, whereas ASIP induces pheomelanin synthesis. Once activated by α-MSH, MC1R signals through the G-protein α-subunit (Gαs) to increase intracellular levels of the second messenger cAMP via adenylate cyclase, leading to phosphorylation of cAMP responsive element-binding protein (CREB) and transcription of MITF. This leads to generation of eumelanin via transcription of genes for melanogenesis (*TYR*, *TRP1*, *TRP2*), melanosome biogenesis (*PMEL*), and melanosome transport (*RAB27A*). Additionally, α-MSH stimulation of MC1R leads to the creation of even more MC1R proteins. When MC1R is inhibited, cAMP levels remain low, and pheomelanin production is preferred. Adrenocorticotropic hormone (ACTH) can also induce pigmentation through interactions with MC1R.

The Pigment-Type Switching System

Melanocytes possess a pigment-type switching mechanism in which they can individually alternate between eumelanogenic and pheomelanogenic states. Upon binding to MC1R, α-MSH activates the eumelanogenic pathway, whereas ASIP provides an inhibitory signal and promotes pheomelanin synthesis (Walker and Gunn 2010). ASIP is a soluble protein secreted by dermal papilla cells of the hair bulb that competitively antagonizes α-MSH at MC1R and actively suppresses MC1R activity. This inhibits melanogenic enzymes and promotes pheomelanogenesis. Additionally, ASIP can exert effects even in the absence of α-MSH, presumably by down-regulating a degree of ligand-independent MC1R signaling activity.

Mice exhibit one of the clearest examples of pigment switching. In the wild-type "agouti" mice, there is a transient switch between eumelanin and pheomelanin production in the hair follicle, before once again reverting to eumelanogenesis. This manifests as a subapical yellow band on a background of dark hair (see Fig. 5). Agouti banding is a common phenomenon seen in many mammalian species, although it is not observed in humans. Pigment-type switching is absent in certain coat-color mutants. Mice with a null mutation for *ASP* (the murine orthologue to *ASIP*) have a completely black coat color with no yellow banding, indicative of continuous MC1R activity (this is the origin of the "black" designation in the C57Black6 mouse strain). Similarly, mice bred with a continuously active *somber* allele ($MC1r^{E\text{-}So}$) also have completely black coats. In contrast, mice engineered to express *ASP* continuously via a heterozygous lethal yellow allele (A^{Y}) demonstrate a completely yellow coat. This is reflective of ASP inhibition of MC1R. An inactivating mutation in the receptor ($MC1r^{e}$) also produces a similar yellow phenotype. Furthermore, mice with loss-of-function mutations for both *MC1R* and *ASP* exhibit completely yellow coat fur, suggesting that ASP signaling is dependent upon MC1R functionality. This

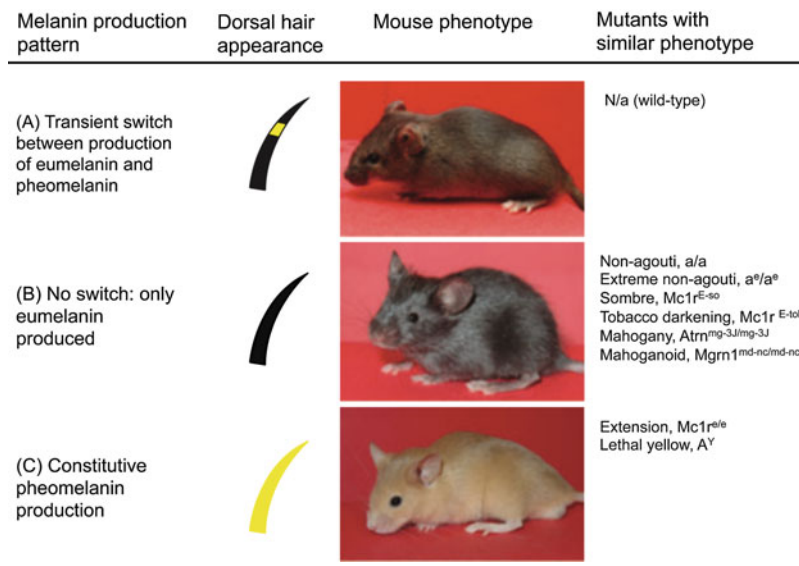

Fig. 5 Pigment-type switching in mice. There are three basic colors of melanin pigmentation in mice: agouti, black/brown, and red. (**a**) Agouti (wild-type mice) is a result of a transient switch to pheomelanin production during a baseline eumelanin-producing state. This creates a yellow band on a black background. (**b**) Black/brown is a result of continuous eumelanin production. This is a result of gain-of-function (*GOF*) *Mc1r* mutations or loss-of-function (*LOF*) mutations in agouti, Atrn, or Mgrn1. (**c**) Yellow fur is a result of continuous pheomelanin production. This is a result of LOF in Mc1r or GOF in agouti (Image from Walker and Gunn 2010)

strengthens the understanding of ASP as a ligand for MC1R, as MC1R is epistatic to ASP. An important caveat is that while increased ASP signaling results in greater pheomelanin production, defective MC1R signaling does not necessarily increase the amount of pheomelanin. Thus, ASP may be necessary to induce pheomelanogenesis. ASIP has two accessory proteins: attractin (ATRN), thought to be an obligatory accessory receptor for ASIP, and mahogunin (MGHN1), an E3 ubiquitin ligase. In vitro studies have suggested that ASIP may signal through MC1R to activate these proteins via a cAMP-independent pathway (Hida et al. 2009). Mutations in these proteins affect the functionality of ASIP. In humans, the larger variety of *ASIP* genetic variations makes understanding its role more difficult.

Interestingly, a third ligand involved in the pigment-type switching system has been identified: β-defensin 103. This ligand was discovered in canine coat-color gene mapping experiments performed by Candille and colleagues to better understand what alleles determined the dominant black coat color in dogs (Candille et al. 2007). Additionally, mice that transgenically express the black canine allele have black coats with small areas of agouti-banded hairs. β-defensin 103 competes against α-MSH binding at MC1R but does not activate the cAMP pathway. Rather, it seems the resultant black coat phenotype is a result of either inhibition of ASP binding to MC1R or interference with an ASP co-receptor by β-defensin 103.

MITF

MITF is widely known as the master regulator of melanocytes, as it has a central role in directing melanocyte development, function, and survival. MITF is a basic helix-loop-helix leucine zipper (bHLHZip) transcription factor encoded by the *MITF* locus that is active in lineage-specific pathway regulation of melanocytes, osteoclasts, and mast cells. Mutation in *MITF* leads to defects in these cell types. MITF is a member of the MIT family and can heterodimerize with related bHLHZip transcription factors, including the transcription factors E3, EB, and EC (TFE3, TFEB,

TFEC). Together with these transcription factors, MITF binds to DNA as a dimer at the E-box motif (CANNTG).

As shown in Fig. 6, the *MITF* gene has at least nine different promoter-exon units for each of the MITF isoforms (Levy et al. 2006). These isoforms differ primarily in their first exon, which encodes the transcriptional activation domain, thus giving amino terminus specificity. All isoforms share the carboxy-terminus, encoded by eight downstream exons, which contains the bHLHZip structure that is used for dimerization and DNA recognition. The melanocyte-specific exon 1 (exon1M) is transcribed exclusively in melanocytes and gives rise to the MITF-M isoform. This exclusive expression is due to its unique melanocyte-restricted promoter enhancer. *MITF-M* transcription is upregulated by several transcription factors that can bind to its promoter region, including SOX10, CREB, PAX3, LEF1, and MITF itself (see Fig. 7). The specificity of the MITF-M isoform to melanocytes is enhanced in part due to the obligate cooperativity between cAMP and SOX10, which is only expressed in cells of neural crest origin. WNT and α-MSH activate pathways responsible for driving activity of the *MITF*-M promoter. For example, in the WNT pathway, WNT proteins bind to Frizzled receptors, initiating interaction between β-catenin and the LEF1 transcription factor, and ultimately induction of the *MITF*-M promoter. Thus, multiple signals converge to induce expression of the *MITF*-M promoter.

MITF also undergoes numerous posttranslational modifications. MITF is phosphorylated by several kinases, including MAPK ribosomal s6 kinase (RSK) and glycogen synthase kinase 3β (GSK3β). Posttranslational modification often leads to MITF repression or degradation. For instance, KIT activation in melanocytes triggers the phosphorylation of two serines on MITF: Ser73 by extracellular signal-regulated kinase 2 (ERK2) and Ser408 by p90 RSK (p90RSK). Phosphorylation at Ser73 targets MITF for ubiquitin-mediated proteolysis. Another form by which MITF undergoes posttranscriptional modification is sumoylation. MITF can be sumoylated at two residues, one of which (E318K) is disrupted by a recurrent mutation in certain individuals with familial melanoma, thereby constitutively increasing MITF's activity.

Severe loss-of-function mutations in *MITF* cause serious autosomal dominant auditory-pigmentary disorders, including the autosomal dominant condition WS2a and Tietz syndrome

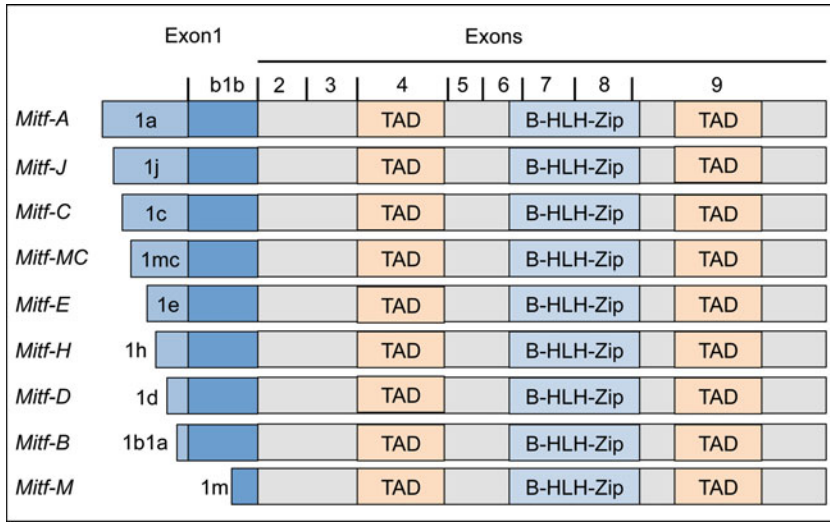

Fig. 6 MITF isoforms. Thus far, nine different promoters have been identified for MITF. MITF isoforms vary within the first exon but share exons 2–9, which contain common functional domains. The *Mitf-M* promoter is expressed only in melanocytes and gives rise to the isoform M (Image adapted from Levy et al. 2006)

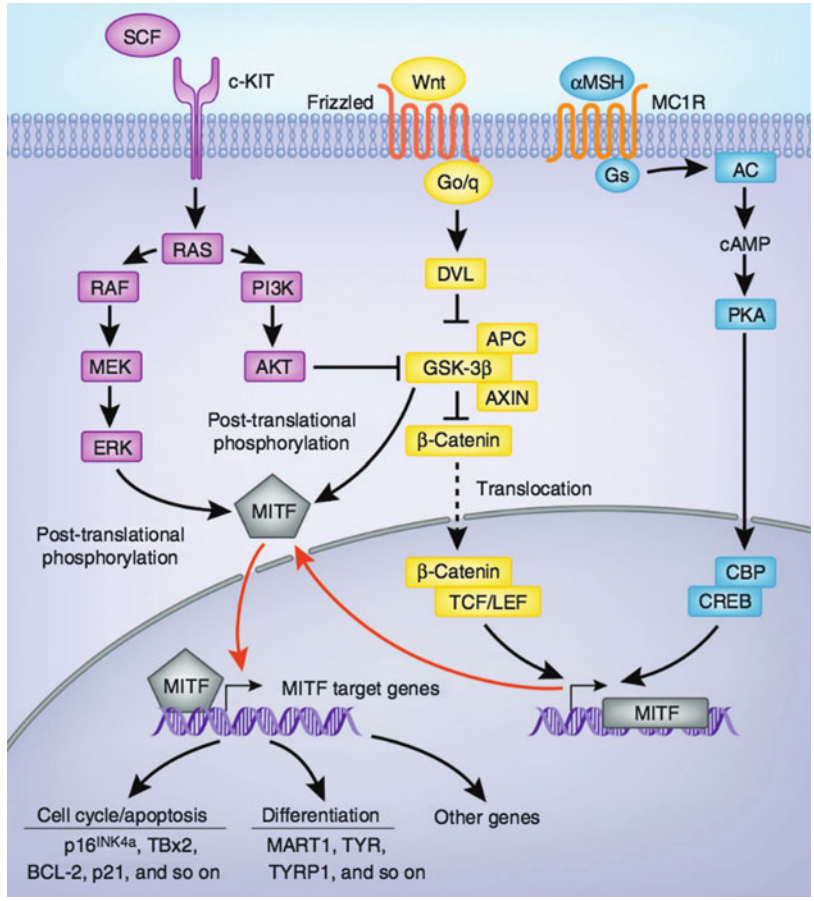

Fig. 7 MITF regulation and its target genes. MITF receives input from several upstream pathways, including c-Kit (purple), Wnt/β-Catenin (yellow), and α-MSH (blue). Activation of c-KIT by SCF activates RAS and the downstream MAPK and PI3K pathways, which influence MITF activity. In the Wnt/β-Catenin pathway, Wnt activates Disheveled (DVL), which inhibits degradation of β-catenin. β-catenin and LEF stimulate MITF expression. Binding of α-MSH to MC1R leads to PKA activation, the phosphorylation of CREB, and the recruitment of CREB-binding protein (CBP) to aid in activation of the MITF promoter (Image from Hocker et al. 2008)

(TS). More than 35 gene mutations have been identified in WS2a, and they range from producing a truncated MITF protein to altering the helix-loop-helix or leucine-zipper motif. These mutations result in disruption of dimer formation. Heterozygous individuals with WS2a are found to have a reduction of melanocytes that results in varying degrees of sensorineural deafness and a patchy distribution of cutaneous hypopigmentation. Mouse models of *Mitf* null mutations are similarly found to lack melanocytes and have white fur, microphthalmia, and deafness. The other types of WS provide additional insight into MITF regulation. WS is divided into four types depending on the presence or absence of additional symptoms. Types I and III are caused by *PAX3* mutations. These types are characterized by additional abnormalities in the facial musculoskeletal system. WS type IV is caused by an array of gene mutations, including those that encode the proteins SOX10, endothelin, and EDNRB.

TS is another manifestation of MITF mutation, in which a single amino acid within the basic domain is altered, resulting in a dominant negative protein as dimers with a wild-type MITF partner are unable to be transported to the cell nucleus to appropriately bind with DNA. The phenotype is one of complete penetrance and is characterized by deafness with light hair and skin color. In contrast, WS2 has a more variable appearance. TS may be considered a more severe form of WS2. Thus far, two *MITF* gene mutations in the basic motif region have been identified in people with TS. Few functional studies have been performed to understand the alterations in MITF signaling in these rare diseases. Depending on the location of the *MITF* mutation in

WS2-associated MITF, varying degrees of alterations in protein activity, DNA-binding ability, and cellular localization may be observed in vitro (Zhang et al. 2013). Mutations in the nuclear localization signal are believed to cause a more dramatic effect. Of note, the observed phenotypic effects in TS are thought to be due to haploinsufficiency rather than a dominant negative effect. Surprisingly, in one study, TS-associated MITF showed comparable in vitro activity to WT MITF, despite the severity of the TS phenotype.

MITF is involved in the expression of genes promoting melanocyte survival (*CDK2*, *p16INK4a*, *TBX2*, *CDKN1A*), motility (*MET*), differentiation and apoptosis (*BCL2* and *HIF1A*), and melanosome production (*TYR*, *TRP1*, *TRP2*, *SLC45A2*, *PMEL*, *RAB27A*). MITF also plays a central role in melanogenesis. Melanogenesis is regulated mainly through the α-MSH/MC1R interaction leading to activation of the cAMP/PKA signaling cascade to induce expression of *MITF*. Transcriptional targets of MITF include the melanogenic genes TYR, TRP1, and TRP2 and the matrix protein PMEL. MITF additionally promotes pigmentation by upregulating EDNRB. EDNRB activation by endothelins 1 and 3 activates MAPK, which phosphorylates MITF, stimulating MITF expression. Expression of DICER, a regulator of miRNA maturation, is also upregulated by MITF during melanocyte differentiation. DICER expression causes posttranscriptional processing of miRNA-17, causing downregulation of BCL2-interacting mediator of cell death (BIM) and thus promoting melanocyte survival.

MITF gene amplification or recurrent E318K mutations have been identified in melanoma. *MITF* amplification is more prevalent in metastatic disease. *BRAF* mutation and *p16* inactivation were found to co-occur with MITF amplification in melanoma cell lines. It was also found that ectopic MITF expression in the presence of a $Braf^{V600}$ mutation transformed human melanocytes into melanoma, establishing MITF's role as a melanoma oncogene (Garraway et al. 2005). Thus, targeting MITF in combination therapies may confer a greater survival benefit. MITF can thus be representative of a "lineage addiction" oncogene.

The Effect of pH on Melanogenesis

It has been noted that melanosomes of melanocytes derived from lighter human skin have lower tyrosinase activity and are more acidic compared to melanocytes from darker human skin, which have higher tyrosinase activity. Based on these observations, a link between pH and melanogenic activity has been suspected, but the mechanism is poorly understood. One of the molecules believed to be involved in pH regulation of melanogenesis is vacuolar (V)-ATPase (Kondo and Hearing 2011). cAMP upregulates the expression of V-ATPase subunits as well as acidification of melanosomes. Ion transporter proteins also regulate the pH of melanosomes and can affect pigmentation. For instance, SLC45A2 (MATP/AIM1) is a transporter protein localized to melanosomes that mediates melanin synthesis. Its mutation leads to OCA4. The P protein, a sodium/sulfate transporter that mediates melanosomal pH neutralization, similarly causes OCA2 when mutated (Ancans et al. 2001). Despite knowledge of these enzymes and membrane proteins, more research has to be done to understand the precise effect pH has on melanogenesis.

Part 3: UVR, Skin Phototype, and the Link to Melanoma

An Overview of UV, Tanning, and Sunburns

Properties of UVR

UVR has a wide array of effects on the skin, including tanning, photoaging, immune suppression, phototoxicity, and carcinogenesis (Armstrong and Kricker 2001). The human skin buffers this damage by thickening of the epidermis, DNA repair mechanisms, antioxidant enzymes, and apoptosis. UV light is an electromagnetic radiation with a wavelength shorter than that of visible light but longer than that of X-rays. Although UVR is invisible to humans, it can have a profound effect on our biology

and health. Common physical manifestations of UVR exposure include suntan, freckling, and sunburn. Long-term effects include melanoma and non-melanoma skin cancer. UVR also provides unseen protective health benefits such as the conversion of vitamin D into a usable form.

There are three regions of the UV spectrum: UVA (400–320 nm), UVB (320–280 nm), and UVC (280–100 nm) All three have been linked to an increased risk of skin cancer. The ozone layer differentially filters the different types of UVR. Sunlight is composed of approximately 94% UVA and 6% UVB. UVC is completely filtered by the ozone layer. Although UVA makes up a greater proportion of solar radiation, UVB delivers more intense dose response. UVA has the longest wavelength and can penetrate into the dermis. It is responsible for generating an immediate tan, premature skin aging, and wrinkles. Indoor tanning equipment typically emits UVA with a smaller proportion of UVB. UVB rays have shorter wavelengths. UVB penetrates the epidermis and is responsible for a delayed tan, sunburns, most skin cancers, and cataracts. UVB is also responsible for the photolysis step involved in vitamin D biosynthesis within the skin. Factors affecting UV delivery to the Earth include time of day, season, latitude, altitude, cloud cover, and reflection off surfaces.

Facultative Pigmentation and Photoproducts

Tanning is the most common form of acquired skin pigmentation and is believed to be a form of environmental adaptation in humans. UVR increases skin pigmentation by increasing active epidermal melanocytes, melanogenic enzymes, and melanocyte dendricity. Soon after UVR exposure, keratinocytes release pro-inflammatory cytokines. UVR induces DNA damage in keratinocytes, which stabilizes the *p53* tumor suppressor gene and activates transcription of POMC (see Fig. 8). POMC is enzymatically cleaved to produce α-MSH, which is then released by keratinocytes and binds to MC1R on melanocytes, ultimately leading to transcription of MITF via CREB.

Generation of ROS followed by depletion of cellular antioxidants also occurs. ROS produced include hydrogen peroxide, hydroxyl radical, singlet oxygen, and peroxyl radicals. The ROS go on to damage lipids, proteins, and DNA. Antioxidant enzymes in the skin (superoxide dismutatase, glutathione peroxidase, and catalase) actively neutralize ROS.

The skin contains several photosensitive molecules called chromophores that, when receiving photons from UVR, are raised to a higher energy state. After absorbing a photon, the chromophores can pass on the excited energy state to other molecules, causing a chain reaction. DNA and RNA contain strongly absorbing chromophores for UVB, as aromatic heterocyclic nitrogen bases absorb wavelengths at 260–265 nm. Although UV targets many epidermal cellular components, including nucleic acids, proteins, lipids, and other macromolecules, its effect on DNA is probably the most profound (Chen et al. 2014b). The premutagenic photoproducts cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (64PP) are commonly generated. These lesions alter the structure of DNA, inhibiting polymerases and arresting replication. CPDs consist of a four-membered ring arising from the coupling of carbon-carbon double bonds of pyrimidines. This structure interferes with base pairing during DNA replication and increases the rate of mutations. 64PP occur at only one-third of the frequency of CPDs but they are more mutagenic. Dimers can be repaired by photoreactivation or nucleotide excision repair. If left unrepaired, they can lead to highly specific mutations known as UVR fingerprint mutations (CC➔TT double-base substitutions and C➔T substitutions) at dypyrimidine sites. Xeroderma pigmentosum, a genetic disorder of nucleotide excision repair in which these types of mutations are left unrepaired, causing a fourfold risk of childhood melanoma.

Other Effects of UVR

Sunburns are understood as one of the greatest risk factors for cutaneous melanoma development. Although it is frequently cited that a history of severe sunburns during childhood is the greatest risk factor for cutaneous melanoma

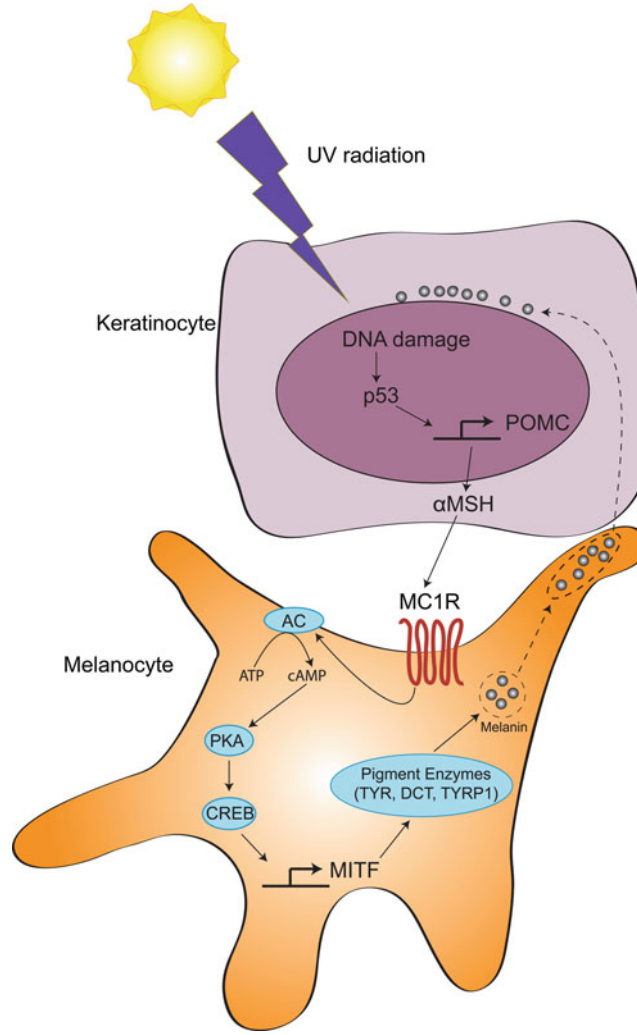

Fig. 8 UV-mediated tanning pathway. UVR causes DNA damage, which activates p53. The p53 protein promotes production of proopiomelanocortin (POMC), which can be processed into either adrenocorticotrophic hormone (ACTH), β-endorphin (β-end), or α-melanocyte-stimulating hormone (α-MSH). α-MSH binds to melanocortin 1 receptor (MC1R) on adjacent melanocytes and promotes melanogenesis and storage of melanin within melanosomes. Melanin is transported to keratinocytes and forms a cap over the nucleus to protect the cell's DNA from UVR (Image from Hsiao and Fisher 2014)

development, a recent meta-analysis taking into account dose-response effects has indicated that melanoma risk rises with increasing number of sunburns during all life periods, whether it be childhood, adolescence, or adulthood (Dennis et al. 2008).

UVB is known to more potently induce sunburns than UVA by about 1,000-fold. UVB-induced erythema can be detected within several hours after UVB exposure and can fade within a day. However, in fair-skinned individuals, the erythema may last significantly longer. At the cellular level, erythema is classically associated with the presence of apoptotic keratinocytes, known as sunburn cells.

As stated above, UVB induces vitamin D production in the skin. Vitamin D plays an important role in calcium metabolism and likely multiple other signaling/regulatory pathways. Use of UV as the sole source of vitamin D synthesis is unlikely to offer stable maintenance of healthy vitamin D-related metabolism, given that UV is an unpredictable source of vitamin D synthesis. Its dose response is dependent upon numerous variables, including latitude, time of year, time of day, phototype of the person, amount of skin exposed, and duration of exposure. Of course, UV as a source of vitamin D also brings concurrent carcinogenic risk. For these reasons it is strongly recommended that individuals define their

vitamin D blood levels using routine blood testing and obtain oral supplementation (which is readily and inexpensively available) to obtain stable, predictable circulating vitamin D.

UVR also causes immunosuppression both in the skin microenvironment and systemically. In particular, Langerhans cells are depleted from the skin following UV exposure. The evolutionary function of this response is unclear but has been suggested that it is a means of limiting immune reactions following DNA damage from UVR. Evidence shows that UV-induced immunosuppression may be a mechanistic contributor to UV-induced tumor development.

Redefining the Roles of UVR and Melanin in Melanomagenesis

Melanoma incidence rates are continually rising and have increased over 30-fold within the last century. Stratospheric ozone depletion will only worsen these numbers, as decreased ozone layer protection results in greater UV delivery to the Earth. Intense intermittent, rather than chronic, UVR exposure is known as the major risk factor for melanoma. Much is still being learned about the relationship between UV, melanin, DNA damage, and cancer risk. Although UVA and UVB have an established role in their contribution to non-melanoma skin cancer, their roles in melanomagenesis have been less clear. However, recent research has begun to unravel the precise contributions of both types of UV to the onset of melanoma formation.

UVR and Melanomagenesis

UVA and UVB cause distinct alterations to the genome and, as a result, on skin pigmentation. Traditionally, UVA has been known to cause oxidative damage, which generates ROS that can damage DNA and increase photoaging of the skin. UVB causes direct DNA damage in the form of CPDs and 64PP almost instantaneously. Although UVA was once thought to primarily induce skin aging, research over the past decade is now implicating it as a causal factor in skin cancer in addition to UVB. Thus, there are believed to be at least two separate UV wavelength-dependent pathways for the induction of melanomagenesis.

Clues that UVA and UVB induce melanoma via separate pathways arose through the observation that UVB irradiation led to similar rates of melanoma in both black and albino hepatocyte growth factor (HGF) transgenic mice (engineered to express melanocytes in the epidermis and dermis). This suggested that initiation of melanoma development by UVB is pigment independent. In contrast, black HGF transgenic mice developed melanoma following UVA irradiation, whereas matched albino strains did not (Noonan et al. 2012). This is supportive of two wavelength-dependent mechanisms for UV-induced melanoma: (1) a pigment-independent pathway that is initiated by UVB and (2) a pigment-dependent pathway caused by UVA.

These authors performed several studies to better characterize these two pathways. CPD and 64PP were detectable in both black and albino transgenic HGF mice following UVB irradiation, but not UVA irradiation. UVA irradiation led to only low levels of TT-CPD lesions and no 64PP in both strains. Thus, it was thought that UVA-induced CPD formation could not explain the pigment-dependent mechanism of UVA-induced melanoma. However, one of the photooxidative products of UVA, 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), was found only in pigmented UVA-irradiated HGF transgenic mice, but not in their UVA-irradiated albino counterparts. In contrast, UVB is not effective at producing 8-oxodG. Thus, it appears that 8-oxodGuo development requires both UVA and melanin.

Additional information about UVA, melanin, and melanomagenesis came from the discovery of "dark CPDs." UVA has been traditionally considered to be inefficient at making CPDs. However, it was found that melanin-containing murine melanocytes generated CPDs for at least 3 h after UVA exposure (Premi et al. 2015). This effect was not seen in melanocytes derived from albino mice, implicating melanin as an active player in DNA damage. Dark CPDs were shown to constitute half of all CPDs. The presence of melanin itself, rather

than its synthesis following UVR, is thought to contribute to CPD formation as CPDs were particularly increased in keratinocytes at the 2-h time point. Pheomelanin is even more potent in the production of dark CPDs as both initial and dark CPDs are twice as frequent in *Mc1r*$^{e/e}$ mice compared to black mice. Thus, pheomelanin appears to be both an inferior shield and a more potent producer of dark CPDs. Ultimately, it was found that irradiating melanin-containing cells with UV induced both superoxide and nitric oxide formation and caused a spike in levels of their product and strong pro-oxidant peroxynitrite. Peroxynitrite leads to melanin degradation and the development of melanin-like granules in the nucleus. Peroxynitrite is one of the few molecules in the body that can excite electrons to a triplet state. Thus, up to several hours after the initial UV exposure, peroxynitrite also continues to excite melanin derivatives to a triplet state that has the high energy of a UV photon. These electronically excited melanin fragments can then transfer their energy to DNA, generating dark CPDs. Thus, melanin has a dual nature, in that it is both carcinogenic and protective.

Based on these results, it appears that people with fair skin are at particularly high risk of instantaneous DNA damage from UVB due to poor melanin shielding and are still vulnerable to the pigment-dependent pathway of UVA-induced melanoma.

Pigmentation and Melanomagenesis

Although the protective benefits of melanin, and eumelanin in particular, have long been lauded, its role as a protective agent has become more nuanced with the aforementioned discoveries. Despite this, there is a wealth of evidence that supports the fact that individuals with darker skin are more protected against skin cancer. Individuals with the lightest skin types are at approximately a 70-fold greater risk of developing skin cancer than individuals with the darkest skin types. It has been shown that the epidermis of the lightest skin types allows 55% of UVA and 24% of UVB to penetrate the skin, whereas the epidermis of the darkest skin types permits only 17.5% of UVA and 7.4% of UVB (Brenner and Hearing 2008). Differences in melanosome processing are also present in different skin types. Melanosomes in dark skin are resistant to degradation by lysosomal enzymes and are able to form supranuclear caps in keratinocytes. In contrast, melanosomes in light skin are degraded to "melanin dust" within the suprabasal layers, which is less effective in providing UV protection.

The increased melanoma risk within individuals with red hair and light skin has historically been attributed to inadequate UV protection, but research in recent years has revealed a UVR-independent pheomelanin-dependent mechanism of melanoma development. Mitra and colleagues developed a preclinical model of melanomagenesis in redheads by engineering pheomelanin-expressing *Mc1r* loss-of-function mice possessing a *Braf*^{V600E} mutation selectively expressed within melanocytes (Mitra et al. 2012). These red-haired mice were observed to develop spontaneous melanomas in the absence of UVR at a tenfold higher rate than mice without pheomelanin. Additionally, in the absence of UV exposure, the red-haired mice were found to have significantly elevated levels of lipid peroxidation and oxidative DNA damage within the skin compared to genetically matched albino mice. This strongly suggested that pheomelanin, and in particular the oxidative stress resulting from its presence, plays an independent role as a driver of melanoma formation (Morgan et al. 2013).

Wendt and colleagues sought to study this phenomenon in humans. Specifically, they generated a large case-control study to examine the effects of *MC1R* variants on melanoma incidence among 991 melanoma patients and 800 controls (Wendt et al. 2016). To isolate UV-independent effects, the researchers adjusted the analysis for age, sex, and variables related to sun exposure (e.g., history of sunburns in childhood and adolescence and visible signs of actinic sun damage) and discovered there was a 1.5-fold (95% CI, 1.01–2.21; $P = 0.04$) to 2.63-fold (95% CI, 1.82–3.81; $P < 0.001$) melanoma risk increase. Thus, humans with *MC1R* variants also appear to suffer from the same pheomelanin-driven and UV-independent risk of melanoma development.

These findings further confirm the significant genetic contribution to melanomagenesis and highlight the need for an improved understanding of the mechanism behind pheomelanin-mediated melanoma formation. It is not clear how pheomelanin increases the amount of oxidative stress in the skin. It has been hypothesized that one of two mechanisms may occur: (1) pheomelanin generates ROS that cause oxidative DNA damage, or (2) pheomelanin synthesis consumes antioxidants that leave the cell susceptible to ROS-mediated damage. Pheomelanin has also been shown to affect the cellular redox system itself, as Panzella and colleagues demonstrated that pheomelanin significantly lowered levels of both reduced glutathione (GSH) and nicotinamide adenine dinucleotide (NADH) (Panzella et al. 2014). Although individuals with light skin and red hair are most strongly affected by the negative effects of pheomelanin, it is possible that a dose response occurs in which individuals with lower pheomelanin levels may have tempered but still significant negative effects from oxidative stress in the skin. It is unclear whether specific antioxidants might be able to combat this newly recognized UV-independent mechanism of carcinogenesis, but it is highly likely that this effect is significantly amplified by UV exposure.

The p53 Protein and Melanomagenesis

It is understood that UV exposure of the skin causes DNA damage and that the cumulative effect of repeated damage is a contributor to skin cancer development. However, the precise mechanism whereby UVR initiates melanomagenesis is poorly understood. In an effort to answer this, the role of DNA damage in the tanning pathway as well as carcinogenesis has been closely examined. Melanization after UVR is enhanced by DNA repair. Furthermore, topical application of small dipyrimidine DNA fragments, which imitate photodamaged pyrimidine dinucleotides excised during DNA repair, upregulates tyrosinase and increases pigmentation. Alternative DNA-damaging processes, such as X-ray irradiation and exposure to chemotherapeutic agents, can also elicit a tanning response likely through overlapping pathways. Studies have established evidence of the ability of UVR to generate tumor-initiating DNA mutations in melanocytes and exome sequencing studies of melanoma have clearly demonstrated a major contribution of UV signature mutations.

The tumor p53 protein (p53), which is encoded by the *TP53* gene, has been nicknamed the "guardian of the genome" because of its role in preventing genome mutation. The p53 protein has a central role in the skin's response to UVR. p53 is a transcription factor whose stability is rapidly increased following DNA damage. p53 is involved in several signaling pathways that become activated after stressors such as DNA damage, oxidative stress, and membrane compromise. Depending on the degree of damage, p53 may activate genes involved in DNA repair or may initiate apoptosis. The actions of p53 are mediated via control of cell cycle checkpoint activity and regulation of DNA repair machinery.

UVR has been linked to p53 expression. Immunohistochemistry of UV-irradiated human skin demonstrates an increase in p53 within suprabasal cells peaking at 4 h after exposure, as well as in basal cells peaking 48 h after exposure (Pontén et al. 1995). Topical sunscreen and more darkly pigmented skin eliminated UV-induced expression of p53. Following p53 upregulation, p53 simultaneously stimulates expression of POMC in keratinocytes, leading to transcription of tyrosinase and TRP1 genes within underlying melanocytes (Cui et al. 2007).

Two common polymorphisms in p53 are an arginine and proline at position 72 (Arg72 and Pro72). The Arg72 form has a greater tendency toward promoting apoptosis, whereas the Pro72 form confers elevated transcriptional activity (Miller and Tsao 2010). The effects of the Pro72 polymorphism are nuanced, as it is more commonly found in individuals living closer to the equator (likely due to the improved tanning ability conferred by this polymorphism) but has also been discovered to be associated with a greater risk for melanoma and basal cell carcinoma (Han et al. 2006). In particular, the Pro72 allele may be especially detrimental in individuals with loss of function in MC1R, as damaged cells would have a lower tendency to undergo apoptosis after DNA

damage. Indeed, Nan and colleagues found the highest risk of melanoma in women with light pigmentation along with MC1R variants (Nan et al. 2008).

Sunscreen

Sunscreen is a topical product containing UV filters that absorb or reflect a fraction of solar UVR and thus protects against sunburn. Sunscreens can be divided into inorganic UV filters (those that reflect sunlight) and organic UV filters (those that absorb UV light). Inorganic filters include titanium dioxide and zinc oxide, and organic UV filters include avobenzone, oxybenzone, octinoxate, octocrylene, and padimate O.

Sunscreens are categorized according to SPF (sun protection factor), which is measured by calculating the minimal dose of UVR necessary to cause confluent erythema at 24 h after exposure on the protected skin of a certain phototype, compared to the unprotected skin. SPF, as a measure of erythema, is primarily based on UVB protection rather than UVA, because erythema is induced by UVB irradiation. "Broad-spectrum" solar protection would extend into the UVA range, but the United States currently lacks a federally regulated system for quantitatively rating UVA protection. In Europe there are several different rating systems to measure effectiveness in blocking UVA rays, such as the immediate pigment darkening assay (IPD) and the persistent pigment darkening assay (PPD).

Fewer UVA-protecting sunscreen ingredients are available in the United States compared to other countries. However, in 2006, the Food and Drug Administration (FDA) approved an efficacious UVA-protecting compound, ecamsule. Despite this increased availability of UVA-protecting UV filters, it was discovered that half of all sunscreens marketed as having broad-spectrum protection within the United States provided only low to medium protection against UVA. This may be due in part to the observation that the UVA filter avobenzone is degraded in the presence of the UVB filter octinoxate following UV exposure (Sayre et al. 2005).

Despite the widespread and continued recommended use of UV filters (including by these authors), there have been some uncertainties about the safety details of these compounds. It has been suggested that the aromatic organic compounds might penetrate through either the stratum corneum or hair follicles into the epidermis. Researchers have also voiced concerns about systemic absorption, especially after noting discernable levels of UV filters within both breast milk and urine samples following topical sunscreen application. Furthermore, both organic and inorganic UV filters have been observed to induce ROS following UVR within the epidermis.

Even though numerous epidemiologic studies have demonstrated both an increased incidence and risk of melanoma with increased ambient solar radiation and cutaneous sun sensitivity, the protective effect of sunscreen against melanoma has been surprisingly difficult to demonstrate. Mouse experiments have shown that sunscreen can delay the onset of melanomagenesis. However, findings from case-control and cohort studies on sunscreen use in humans have been largely uninformative and generally fail to achieve statistical significance. Unfortunately, many past studies were based upon antiquated sunscreen formulas, which make results difficult to extrapolate to current day usage. Studies were also often based on indirect measures of melanoma risk, such as nevi quantification. One commonly cited study performed by Gallagher and colleagues on Canadian children revealed a small reduction in new melanocytic nevi following regular sunscreen use (Gallagher et al. 2000). It is difficult to interpret how exactly these observations contribute to melanomagenesis, particularly when current thinking is that only a minority of melanomas arise from preexisting nevi.

A key study published by Green et al. examined the incidence of melanoma in 1,621 patients in Australia in a randomized controlled trial of daily sunscreen application and beta-carotene supplementation over a 5-year period with a 10-year follow-up (Green et al. 2011). Participants assigned to the sunscreen intervention were asked to apply on a daily basis a broad-spectrum sunscreen containing the chemical filters 2-ethylhexyl-*p*-methoxycinnamate and 4-*tert*-butyl-4′ methoxy-4-dibenzoylmethane,

with an overall SPF of 16. Control participants were asked to continue their normal behavior of sun protection, which ranged from sporadic sunscreen use to no sunscreen use. Also of note, half of study participants were randomly assigned to supplementation with 30 mg of the antioxidant beta-carotene that has been hypothesized to counteract UV-induced oxidative DNA damage, whereas the other half was provided a placebo supplement. The results demonstrated a 50% reduction in invasive melanoma among those who used sunscreen compared to those who did not, and this trial provides the strongest evidence to date of reduction in the incidence of invasive melanoma after regular application of sunscreen in adults. Thus, the evidence suggests that sunscreen affords partial UV protection, and it is suggested that sunscreen be utilized in combination with sun avoidance strategies.

There are several potential explanations for the modest or even conflicting results from large studies analyzing melanoma prevention by sunscreen use. These include old formulations, inadequate application or reapplication of the formulations, and insufficient follow-up intervals. It is also possible that ROS produced by chemical sunscreens within the skin may antagonize the UV-protective benefits. Other confounding factors include selection bias of study participants (in which higher-risk individuals use sunscreen more often) and inadequate education of proper sunscreen use. However, there are promising alternatives that may soon be coming our way. Deng and colleagues recently devised a method of encapsulating UV filters within bioadhesive nanoparticles, which have the advantages of being adherent to the stratum corneum without penetrating deeper into the epidermis (Deng et al. 2015). Broad-spectrum protection against UVA as well as UVB is hopefully shortly on the horizon in the United States and may provide significantly enhanced protection.

The role of topical antioxidants in the prevention of melanoma is also debatable. Within this study, there was no observed effect of the beta-carotene intervention on either increasing or decreasing the risk of melanoma incidence. One of the concerns of antioxidant supplementation is that if applied to an area of the skin with islands of UV-induced mutations, the presence of antioxidants may in fact stabilize cells containing these mutations and allow their continued survival via anti-apoptotic mechanisms.

Therapeutic Pigmenting Agents

Several therapeutic pigmenting agents have been researched to harness the protective properties of eumelanization. A UV-independent agent that hyperstimulates pigment synthesis may be valuable, as pigmentary protection gained by tanning cannot otherwise be achieved without the detrimental side effects from UVR. In particular, researchers have attempted to perturb the UV signaling pathway at various points to modulate the activity of MC1R, adenylate cyclase, cAMP, and MITF.

Piperine, a compound extracted from black peppers, has been marketed as a natural supplement to aid in tanning. Experimental studies have suggested that piperine can induce melanocyte proliferation and dendrite formation in combination with UV exposure in vitro (Soumyanath et al. 2006), as well as repigmentation in a sparsely pigmented vitiligo mouse model (Faas et al. 2008). However, human studies are lacking. Additionally, the combination of UV exposure to maximize tanning is obviously not ideal as it apparently requires UV and its effects on UV carcinogenesis are unknown.

In an effort to sidestep the DNA damage pathway, a chemically modified superpotent α-MSH analogue, [Nle^4-D-Phe^7]-α-MSH, has been tested via subcutaneous injection in human subjects. Researchers demonstrated that supplementation with an α-MSH analogue led to a melanin increase of 41% in subjects with low-MED skin type and a melanin increase of 12% in those with high-MED skin type (Barnetson et al. 2006). Additionally, they observed that formation of epidermal sunburn cells and thymidine dimer formation was halved in subjects with low-MED skin type following UV exposure.

Forskolin, a cAMP activator, is a small molecule that has been able to activate the pigmentation pathway downstream of Mc1r, thus achieving pigmentation even in nonfunctional

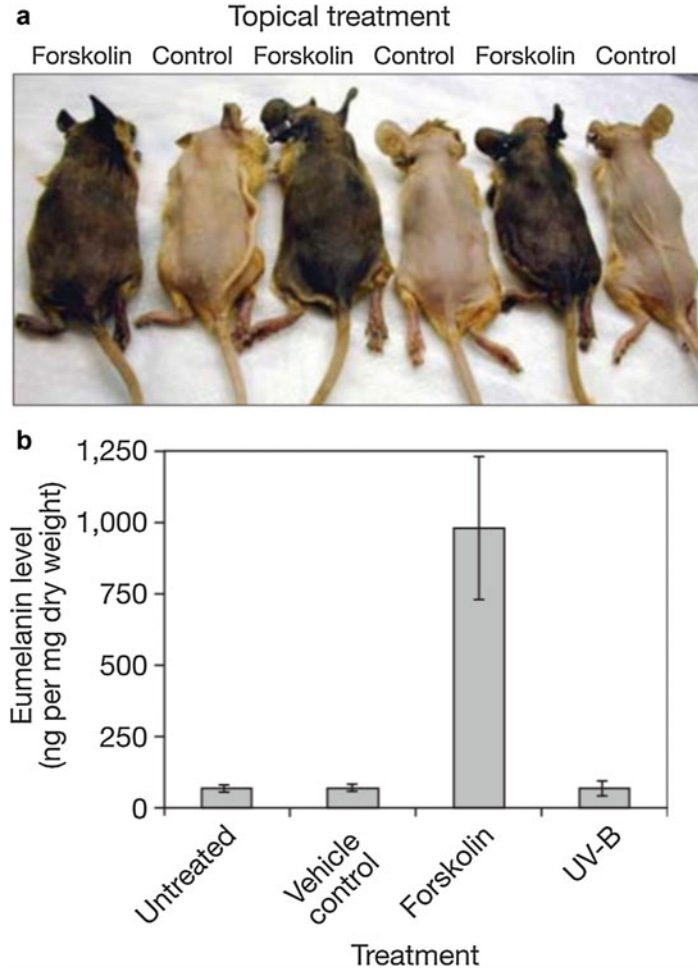

Fig. 9 Pigmentation "rescue" by topical forskolin in "redhaired/fairskinned" mice (D'Orazio et al 2006). The adenylate cyclase agonist forskolin was topically applied to the skin of redhaired (Mc1r(e/e) mice containing the K14-SCF transgene that causes retention of epidermal melanocytes. (a) Forskolin treatment induced significant skin darkening, as compared to the control vehicle treatments. (b) Quantification of eumelanin pigment demonstrated significant induction of eumelanin synthesis by forskolin. In contrast, UV-B treatment did not induce eumelanin synthesis, consistent with the observation that redhaired individuals do not tan after UV

Mc1r mutant mice (see Fig. 9). Regular topical forskolin application in mouse models leads to dark pigmentation, protection against both UVR-mediated damage and carcinogenesis, and decreased levels of both CPDs and 64PP in keratinocytes (D'Orazio et al. 2006). Unfortunately, forskolin does not have good topical penetration in the human skin.

Therapeutic pigmenting agents may not be without risk. It has been reported that use of these agents can result in atypical nevi and melanoma. Specifically, case reports exist of melanoma developing in an extremely short time period after heavy Melanotan 2 (an α-MSH analogue) usage. However, a recent controlled clinical study did not reveal evidence of increased melanoma risk (Langendonk et al. 2015).

Conclusion

Pigmentation is a multistep process. It requires adequate melanocyte development, proper homing to epidermal and follicular locations, the formation of appropriate dendritic connections with recipient epithelial cells, melanin production by melanosomes, and precise transfer of this pigment to the surrounding epithelial network. These pathways are further controlled precisely at the molecular level by enzymes, structural proteins, transcriptional regulators, transporters, receptors, and growth factors. Alterations in any of these pathways or factors can cause variations or defects in pigmentation. In addition to its social significance, pigmentation plays an important role in the

development of skin cancers including melanoma. Over the past years, there has been an explosion of knowledge about the interplay between melanin, UVR, and melanoma progression. Additionally, attempts have been made to increase pigmentation therapeutically as well as target mediators of pigmentation in melanoma. Although pigmentation is extremely complex, improved knowledge of the intricate pathways involved in the engineering of pigmentation will allow us to both better understand evolutionary conserved processes and improve health outcomes in a vast array of dermatological disorders and diseases.

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Ultraviolet Radiation and Melanoma

3

Thomas M. Rünger

Contents

Abstract

There is overwhelming epidemiologic, animal model, and molecular evidence that ultraviolet radiation (UVR) is a major pathogenic factor in the development of the most common subtypes of melanoma. Both UVB and UVA induce pyrimidine dimer DNA photoproducts, possibly through different mechanisms, induce mutations (mostly the C → T UV-signature mutation), and contribute to melanoma formation. There is no conclusive evidence that oxidative DNA base modifications play a significant role in melanomagenesis. Sun seeking and artificial tanning are most likely to blame for the steady increases of melanoma incidence over the past decades. Photoprotection can reduce melanoma risk. Education of the public about the dangers of UVR exposure from natural and artificial sources and about effective measures of photoprotection with the goal to modify behavior

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and to reduce UVR exposure is the best strategy to reverse the increases in melanoma incidence.

Keywords
Ultraviolet radiation (UVR) · UVA · UVB · C → T mutation · UV-signature mutation · DNA photoproduct

Introduction

Exposure to sunlight has long been recognized as an important risk factor for the development of melanoma in sun-exposed skin, and the doubling of melanoma incidence in the past 25 years (Linos et al. 2009) has been attributed to increased ultraviolet radiation (UVR) exposures through sun seeking and artificial tanning. This chapter first reviews the epidemiologic and clinical evidence that exposures to sunlight and to artificial sources of UVR cause melanoma and which wavelengths of UVR contribute to this effect. Today, there is also strong molecular evidence for the role of UVR in the pathogenesis of melanoma and these newer insights, which are reviewed in the subsequent paragraphs, also shed light on the wavelength question.

Epidemiologic and Clinical Evidence that Exposure to Sunlight Causes Melanoma

Several lines of clinical and epidemiological evidence implicate sunlight exposure in the pathogenesis of melanoma. These are that activities related to sun seeking, a history of sunburns, a high number of melanocytic nevi (which is a marker of past sun exposures), and pigmentary characteristics with fair skin, blond hair, and blue eyes with higher sunburn susceptibility and lower ability to tan (lower Fitzpatrick skin phototypes) all significantly increase melanoma risk (reviewed by Gandini et al. 2011).

Location of residence studies show that living in sunny areas increases melanoma risk (Weinstock et al. 1989; Osterlind et al. 1988). In addition, migrant studies show an increased risk in individuals who have spent childhood and adolescence in sunny geographical areas (Holman and Armstrong 1984; English and Armstrong 1988; Autier et al. 1997).

Moreover, the observation that photoprotection with use of sunscreens prevents melanoma (Green et al. 2011) also supports the link between solar UVR exposure and melanoma.

It is important to recognize, however, that melanoma is not a monolithic entity. Different melanoma subtypes are not only genetically distinct, but also differ with regard to pathogenic factors, including UVR (Whiteman et al. 2011). For example, acral and mucosal melanomas are not thought to be induced by UVR. Within the group of UVR-induced melanomas, the mechanisms of how UVR induces malignant transformation also appear be different. Melanomas arising in chronically sun-damaged skin (high-CSD melanomas), which are most commonly found on the face in the elderly, but can also occur in other heavily photodamaged areas of the skin, are associated with high cumulative sun exposure and often arise from in situ melanomas termed lentigo maligna. These high-CSD melanomas show an extremely high mutation burden (Bastian 2014; Hodis et al. 2012). Desmoplastic melanomas have a similarly high mutation burden (Shain et al. 2015) and a similar age and anatomic site distribution as high-CSD melanomas.

Melanomas arising in sun-exposed skin without signs of pronounced chronic photodamage (low-CSD melanomas) are most commonly found on the lower legs in women and on the back in men (Gordon et al. 2015). At least in Western cultures, these areas tend to be photoprotected for long periods of time, e.g., during winter, and are then suddenly exposed to high doses of UVB (Bodekær et al. 2016). Chronically UVR-exposed skin is characterized by an upregulation of anti-mutagenic mechanisms. The absence of such protective states in photoprotected skin, in combination with a relative inability of melanocytes to undergo apoptosis upon high-dose UVR exposures has been suggested as an explanation for the induction of melanoma by intermittent UVR exposures

(Gilchrest et al. 1999). Low-CSD melanomas often arise from melanocytic nevi acquired during childhood, which are themselves also associated with sun exposure (Satagopan et al. 2016). Low-CSD melanomas differ from high-CSD melanomas also genetically. They have a lower mutation burden and are characterized by a different pattern of BRAF mutations (Bastian 2014; Menzies et al. 2012; Shain et al. 2015; Alexandrov et al. 2013). The BRAF V600E mutation is common in low-CSD melanomas, but not in high-CSD melanomas, which tend to either not carry BRAF mutations at all, or have other types of BRAF mutations, such as BRAF 600 K. While low-CSD melanomas still have a very high mutation burden, it is lower than in high-CSD melanomas.

Epidemiologic and Clinical Evidence that Exposure to Artificial Sources of UVR Causes Melanoma

Natural sunlight is not the only source of UVR. There are also exposures to artificial UVR from industrial sources in occupational settings and from devices used in tanning parlors. Many studies have shown a significantly increased risk of melanoma subsequent to sunbed/sunlamp use (Boniol et al. 2012; Gallagher et al. 2005), including a large prospective cohort study in 106,379 women in Sweden and Norway, showing a relative melanoma risk of 1.42–2.58 with use of tanning devices (Veierød et al. 2003). A melanoma epidemic in Iceland with increasing rates of melanoma between 1990 and 2006, mainly in young women, has also been associated with increased tanning bed use (Autier et al. 2011). Melanomas at usually covered sites, e.g., skin in the sacral and pubic areas, may also be attributable to UVR exposure from sunbed use (Higgins and Du Vivier 1992).

Various types of artificial UVR sources are also used for phototherapy of inflammatory skin disorders. An increased risk of melanoma has never been reported following phototherapy with broadband or narrow-band UVB. This may be due to the fact that the UVR doses used for phototherapy are much lower than those from a lifetime of sun exposures and that phototherapy dosing regimens usually start with low doses and allow for careful slow adaptation with measured dose increments. However, extensive longitudinal studies on the risk of UVB phototherapy have not been done. This is in contrast to photochemotherapy with UVA in combination with the photosensitizer 8-methoxy-psoralen (PUVA), where a large cohort of the first patients treated since the inception of PUVA in the 1970s has been followed carefully. These patients do not only have an increased risk of cutaneous squamous cell carcinomas with high numbers of treatments and high cumulative doses, but also for melanoma (Stern and PUVA Follow Up Study 2001). PUVA treatment involves formation of DNA-psoralen adducts with inter- and intrastrand crosslinks, which is quite different in its DNA damaging and mutagenic properties than UVR without psoralen. Nevertheless, the increased melanoma risk in patients who have received PUVA treatment is a reminder that phototherapy with UVR carries risks and should, if possible, be avoided in patients with an increased melanoma risk.

Which Wavelengths of UVR Cause Melanoma – UVB or UVA? Evidence from Epidemiologic Studies and Animal Models

The strong evidence that UVR causes melanoma raises the question as to which wavelengths of the UVR spectrum this effect can be attributed. When a few decades ago it was increasingly recognized that UVB (280–315 nm) had skin damaging effects, but UVA (315–400 nm) was still considered relatively safe, the tanning industry reduced the UVB output in their devices and claimed that a UVA-induced tan would be safe. It has then been postulated that the increased risk of melanoma with the use of tanning devices may be attributed to high amount of UVA emitted by these devices. However, tanning machines differ widely in their spectral output (Facta et al. 2013; Gerber et al. 2002). With the increasing recognition that UVA is not safe (e.g., UVA has been

classified as a complete carcinogen by the WHO; El Ghissassi et al. 2009), more and more UVB has been added back into tanning devices. With this information in mind, the increased melanoma risk with sunbed use cannot be clearly attributed to the high fraction of UVA, but may also be due to the UVB that is still emitted by tanning devices.

Another way to answer the wavelengths question of melanoma is the use of animal models. Ley (1997) observed the induction of melanoma precursor lesions in the opossum with UVA, more than with UVB. However, no infiltrative melanomas could be induced. Setlow et al. (1993) used swordfish and observed that UVA induces melanomas more effectively than UVB. However, this observation could not be reproduced and the opposite result was reported by Mitchell et al. (2010). De Fabo et al. (2004) used HGF/SF transgenic mice and observed melanoma induction only with UVB, but not with UVA. Later, the same group (Noonan et al. 2012) observed that UVA does induce melanoma, but only in pigmented mice, not in the previously studied nonpigmented mice. With that, it appears that, at least in this mouse model, the presence of melanin is required for the induction of melanoma with UVA, but not with UVB, and that both wavelengths, UVA and UVB, can induce melanoma, but possibly via a different mechanism. Today, we may be able to provide a molecular explanation for this difference (see below).

Molecular Evidence that UVR Causes Melanoma

Upon absorption by cellular chromophores and their subsequent excitation, UVR induces photochemical reactions, either in the excited chromophore itself or indirectly through a reaction of the chromophore with another cellular molecule (photosensitized reaction). Nucleic acids, DNA and RNA, have an absorption maximum at 260 nm, which is within the UVC range (100–280 nm). UVC does not penetrate the earth's atmosphere. Even if it did, it would not penetrate deep enough into the skin to reach the basal layer of the epidermis, the most important compartment of the skin for the development of nonmelanoma skin cancer and melanoma, and is therefore not thought to play a major role in photocarcinogenesis. However, UVB (280–320 nm) does reach the earth's surface and is still able to strongly excite nucleic acids. It also penetrates deeper into the epidermis than UVC and induces photochemical reactions in the basal keratinocytes and in melanocytes. A critical consequence of UVR-induced excitation of DNA is the formation of covalent chemical bindings between two adjacent pyrimidine bases, thymine and cytosine (Fig. 1, left column: classic pathway). There are two main types of such DNA photoproducts formed by UVR, cis-syn cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts (6–4 PPs). Both types of dimers can form between any combination of thymine and cytosine: T:T, T:C, C:T, or C:C.

These pyrimidine dimer photoproducts impair DNA replication and can result in mispairing if not removed prior to cells entering the S-phase of the cell cycle for DNA replication. A mispairing results in the change of the DNA sequence, a mutation. Several types of such mutations are observed after UV exposure, but the most common ones are C → T base exchange mutations at sites of two adjacent pyrimidines (dipyrimidine sites). Without UV exposure, e.g., in noncutaneous tissues or tumors, such mutations are observed much less frequently (Alexandrov et al. 2013), and the C → T and the C:C → T:T tandem mutations at dipyrimidine sites have therefore been named UV-signature mutations (Brash 2015). They are caused by misincorporation of adenine(s) opposite a cytosine-containing pyrimidine dimer. Once these mutations are formed, they remain for the life span of the affected cells and are also propagated during cell division. They can be considered the memory of a cell's lifetime exposure to UVR. Some authors use the term UVB signature mutations. However, this term is misleading, as they are also formed by UVA (Brash 2015; Rünger 2008) and should be avoided.

Most mutations can be generated by different mutagens and mechanisms. While UV-signature mutations appear to be relatively specific for

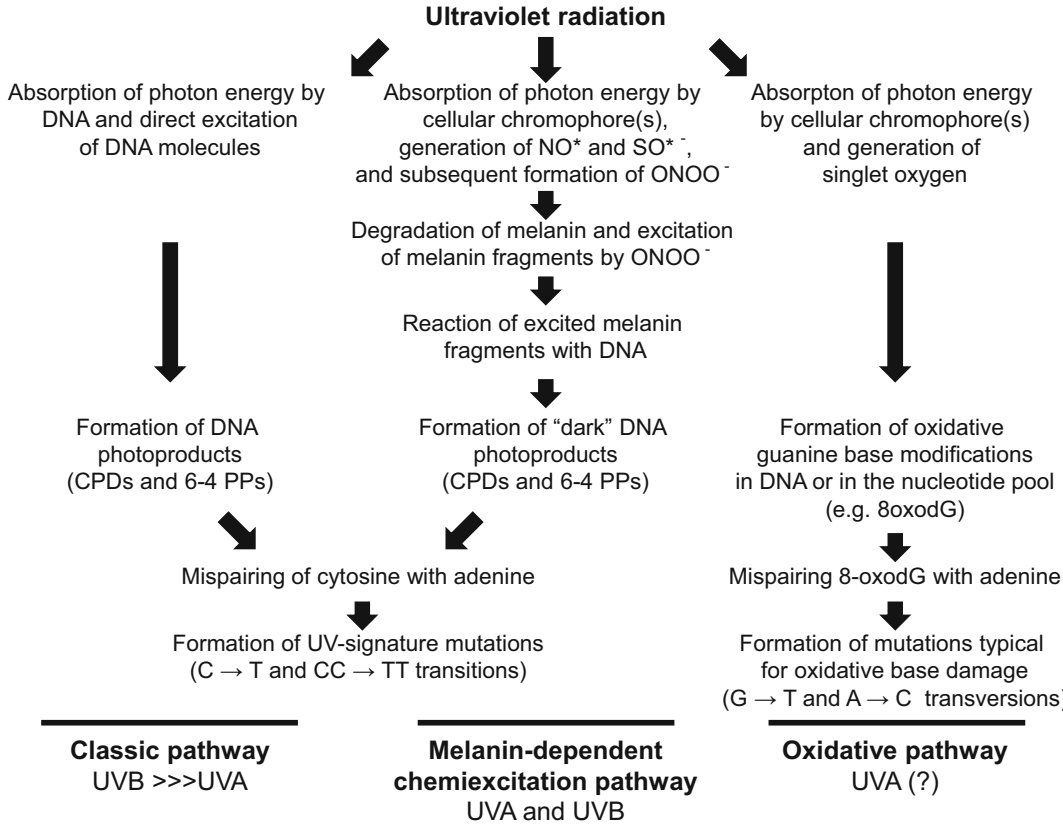

Fig. 1 Mechanisms of UVR-induced mutation formation in melanocytes. In the classic pathway (left panel), adjacent pyrimidines react with each other and dimerize to form DNA photoproducts after direct excitation of DNA molecules by photons. The C → T and CC → TT UV-signature mutations may form at sites of DNA photoproducts by mispairing of cytosine(s) with adenine. Although 260 nm is the absorption maximum of DNA, UVB still strongly excites DNA and generates mutations through this pathway. UVA's ability to excite DNA is much weaker, but may still generate mutations via this mechanism. CPDs, cis-syn cyclobutane pyrimidine dimers; 6–4 PPs, pyrimidine (6–4) pyrimidone photoproducts.
The melanin-dependent chemiexcitation pathway (middle panel) was recently described by Brash (2015). It describes the formation of DNA photoproducts not by direct excitation of DNA, as in the classic pathway, but by energy transfer from excited melanin fragments to DNA, which involves nitric oxide (NO*), superoxide (SO^{*-}), and peroxynitrite ($ONOO^{-}$). This mechanism generates UV-signature mutations both after irradiation with UVA and with UVB.
The oxidative pathway (right panel) involves a photosensitized reaction with formation of singlet oxygen after excitation of a cellular chromophore other than DNA. Singlet oxygen then reacts with guanine either in DNA or in the nucleotide pool. A common oxidative base lesion is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). By mispairing with adenine, G → T and A → C transversions can form. UVA has long been thought to contribute to UVR-induced mutation load through this mechanism. However, molecular evidence, in particular the low frequency of such transversions in melanomas, does not support a major role in the formation of melanoma

UVR, a single mutation, even a C → T mutation at a dipyrimidine site, can never be ascribed to only one possible cause. Nevertheless, a relative increase of these transitions compared to other base pair changes in melanomas and other cutaneous malignancies clearly point to UVR as their causative agent.

Pleasance et al. (2010) were the first to report the full genomic sequence of a melanoma as compared to the normal sequence of a lymphoblast cell line from the same patient. Out of 33,345 base substitution mutations observed in the melanoma, more than 70% were C → T UV-signature mutations. Although not 100% of these mutations

may have been caused by pyrimidine dimers following UV exposure, the predominance of this type of mutation that is otherwise rare in non-UV-exposed tumors is an impressive molecular account of a lifetime of sun exposure of a single melanocyte that has ultimately given rise to this melanoma. This data have since been confirmed in large series of hundreds of melanomas, showing that more than 75% of all mutations in melanoma are UV-signature mutations (The Cancer Genome Atlas Network 2015; Hodis et al. 2012; Krauthammer et al. 2012). These data also show that the mean mutation rate in melanomas from sun-exposed sites along with mismatch repair deficient cancers is the highest of all cancers investigated in this way. This high burden of mutations with mostly C → T transitions, both in high-high-CSD and low-CSD melanomas, again emphasizes the role of UVR's high mutagenic properties and its role in the pathogenesis of cutaneous melanoma. In contrast, melanomas in sun-protected skin, including, e.g., acral and mucosal melanomas, have a much lower mutation burden and do not show the high rate of UV-signature mutations.

UV-signature mutations are also found in many of the mutated genes that play a particular role in the development of melanoma. For example, 62% of CDKN2A, 52% of PTEN, 69% of TP53 (Hocker and Tsao 2007), and 33–85% of TERT promotor mutations are UV-signature mutations (Horn et al. 2013; Huang et al. 2013). UV-signature mutations in the TERT promoter have also been reported in few internal tumors that have never been exposed to UVR (Vinagre et al. 2013). These may have been caused by another, unidentified carcinogen or may be due to locus-specific genetic instability at these sites.

BRAF mutations, however, which are very common in melanocytic nevi (approximately 80%) and in melanomas (approximately 50%; Tsao et al. 2012) are not UV-signature mutations. The most common BRAF mutation, V600E, is a T → A base substitution transversion mutation. The observation that this mutation is not or only rarely observed in melanomas from UVR-protected areas indicates that it is indeed induced by UVR. Thomas et al. (2006) proposed that it could be induced by a DNA photoproduct in the vicinity of this locus. While CPDs and 6–4 PPs at dipyrimidine sites are the most common UV-induced DNA lesions, UVR also generates several other, yet rare types of DNA damage, and one of those may give rise to this otherwise uncommon type of mutation in BRAF. It is, however, not a mutation that is commonly caused by oxidative DNA damage. The elucidation of the mechanism as to how UVR generates this T → A transversion in BRAF V600E mutations or the GT → AA tandem mutations in BRAF V600 K mutations remains an enigma. These BRAF mutations have also been described in internal malignancies, suggesting non-UVR-related mechanisms of formation and that the ability of UVR to generate these mutations may be indirect, rather than through UVR-induced DNA damage directly. It is also important to recognize that BRAF is an oncogene that is activated by a gain-of-function mutation. Because gain-of-function mutations can only occur for certain amino acid changes, the DNA base change likely indicates a constraint on the amino acid sequence rather than the identity of the mutagen. The same is true for NRAS mutations in melanoma, which are also not UV-signature mutations. Instead, they are most commonly G → T transversions, base exchange mutations typically induced by oxidative base modifications.

Only a small fraction of UV-induced DNA photoproducts results in mutation formation, because the majority of DNA lesions are removed by cellular DNA repair mechanisms before they can give rise to mutations. Pyrimidine dimer photoproducts are removed by a multienzyme pathway called nucleotide excision repair. This repair is faster in transcribed genes (transcription-coupled vs. global genome nucleotide excision repair), resulting in a lower frequency of mutations in transcribed genes. As expected, this has also been confirmed in the full genome sequencing studies of melanomas, where transcribed genes in melanoma have a lower mutation frequency than nontranscribed genes (Pleasance et al. 2010).

Cells from patients with the autosomal recessive condition xeroderma pigmentosum are characterized by a deficient nucleotide excision repair.

Consequently, they not only have a highly increased risk of nonmelanoma skin cancer in UVR-exposed skin, but also of melanoma (high-CSD melanomas). As expected, many of the mutations in melanomas of xeroderma pigmentosum patient are also UV-signature mutations (Wang et al. 2009). Interestingly, polymorphisms in DNA repair genes of xeroderma pigmentosum have also been reported to increase the melanoma risk and decrease melanoma survival in otherwise normal individuals (Li et al. 2013).

DNA repair is not the only cellular defense against mutation formation at sites of DNA damage. In response to DNA damage, cells arrest in the G1 phase of the cell cycle and do not enter the S-phase. This prevents replication of damaged DNA and misincorporation of DNA bases at sites of DNA damage before repair has taken place and with that prevents mutation formation. This is of critical importance in rapidly proliferating cells, e.g., keratinocytes, but probably not as important for the prevention of mutation formation in the slowly proliferating melanocytes. Apoptosis in response to UV-induced DNA damage is also a protective mechanism against photocarcinogenesis, as it removes heavily damaged cells from the pool of cells that may undergo malignant transformation. However, while apoptotic keratinocytes (sunburn cells) are commonly observed in sunburned skin, melanocytes are relatively resistant to apoptosis following exposure to UVR (Bowen et al. 2003; Zhai et al. 1996). It has been proposed that this is due to high expression levels of antiapoptotic proteins in melanocytes, such as BCL2 (Gilchrest et al. 1999). With this, high-dose intermittent UVR exposures may be particularly mutagenic for melanocytes and may explain why low-CSD melanomas occur in intermittently UVR-exposed skin.

Which Wavelengths of UVR Cause Melanoma – UVB or UVA? Evidence from Molecular Studies

As described above, there is some epidemiologic, clinical, and animal model evidence that exposure to UVA may be a particular risk factor for melanoma. UVR represents a range of different wavelengths. In general, the photophysical and photobiologic properties of UVA are different from those of UVB. With increasing wavelengths from UVB to UVA, the ability to excite the DNA molecule directly and to so generate pyrimidine dimer photoproducts declines exponentially. This is only partially offset by the 50- to 100-fold higher abundance of UVA in natural sunlight. Conversely, the ability to generate oxidative stress, e.g., via formation of singlet oxygen, increases. This has led to the hypothesis that oxidative DNA lesions mediate mutation formation in UVR-induced melanoma, in particular when induced by UVA (Rünger 1999). The most common nucleic acid damaged by singlet oxygen is guanine, and the most common type of singlet oxygen induced oxidative DNA lesion is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). By mispairing of this lesion with adenine during replication, 8-oxodG gives rise to G → T transversions. If guanosine in the nucleotide pool is oxidized, A → C transversions can arise by misincorporation of 8-oxodG opposite adenine during DNA replication (Fig. 1, right panel: oxidative pathway). Unlike pyrimidine dimers, which are repaired by the nucleotide excision repair pathway, the single base modifications of oxidized guanosine are repaired by the simpler process of base excision repair. When contemplating oxidative base damage as a possible mechanism how UVA may generate the mutations that lead to the formation of melanoma, it is important to recognize that at least in fibroblasts and keratinocytes, UVA has been shown to produce more pyrimidine dimers than 8-oxodG (Courdavault 2004). However, unlike with UVB, which generates all combinations of pyrimidine dimers – T:T, T:C, C:T, and C:C – UVA generates mostly T:T CPDs and no 6–4 PPs. Courdavault et al. (2004) hypothesized that UVA-induced dimers are generated via a photosensitized triplet energy transfer, rather than by direct photoexcitation, as with UVB. Unlike early reports of a separate UVA signature mutation observed in transformed rodent cells (Drobetsky et al. 1995), the majority of UVA-induced mutations has later been shown to be C → T transitions at

dipyrimidine sites, without a particular signal for a separate UVA signature mutation or a high rate of mutations typical for oxidative base modifications, both in vitro and in vivo (Ikehata et al. 2013; Rünger and Kappes 2008).

An attractive candidate for generation of oxidative stress upon exposure to UVR in melanocytes is melanin. Melanin has been described to have some photosensitizing properties, in particular the more reddish-colored pheomelanin (Kollias et al. 1991; Micillo et al. 2016). Wang et al. (Wang et al. 2010; see also commentary by Rünger 2011) reported that upon irradiation with UVA, melanocytes generate more oxidative DNA damage, have less efficient repair of oxidative DNA damage, and produce more mutations. They proposed that oxidative DNA damage is a major driver in melanomagenesis. However, they did not sequence the UVA-induced mutations and with that did not provide ultimate proof that the UVA-induced mutations are indeed the G → T or A → C transversions that are typical for oxidative DNA damage. In addition, the abovementioned mutation data from melanomas show only a small percentage of such mutations typical for oxidative DNA damage (Pleasance et al. 2010; The Cancer Genome Atlas Network 2015; Hodis et al. 2012; Krauthammer et al. 2012). Taken together, there is no compelling evidence that oxidative DNA base lesions play a major role in UVR-induced mutation formation that drives melanoma.

Recently, Premi et al. (Premi et al. 2015, see also Brash 2016) reported formation of DNA photoproducts up to 3 h after UV exposure and showed that the formation of these so-called "dark CPDs" is mediated by photoexcitation of melanin, in particular of pheomelanin, through formation of peroxynitrite from UV-induced nitric oxide and superoxide (Fig. 1, middle panel: melanin-dependent chemiexcitation pathway). With this, it appears that melanin is indeed not only a photoprotector, but may also cause cancer. This observation also provides an attractive explanation why UVA induces melanoma only in pigmented mice, but not albino mice (Noonan et al. 2012).

The bystander effect describes the phenomenon that nonexposed cells in the vicinity of irradiated cells also undergo similar stress responses and changes. UVA in particular has been shown to exert such bystander effects in melanocytes, and long-lived UVR-induced radicals have been proposed to mediate it (Nishiura et al. 2012; Redmond et al. 2014). It is tempting to speculate that the peroxynitrite described by Premi et al. could possibly not only generate "dark CPDs" in irradiated cells, but also in unirradiated bystander cells.

Role of the Effects of UVR on the Immune System in the Pathogenesis of Melanoma

Exposure of the skin to UVR has profound consequences on the immune system, not only locally in the exposed skin, but also systemically. Pro-inflammatory responses, e.g., those seen in the acute sunburn reaction, are mediated by innate immunity, whereas immunosuppressive effects are mediated by adaptive immunity. One example of a systemic immunosuppressive effect of UVR is the induction of tolerance to antigens applied to UVR-exposed skin. This may include UVR-induced melanoma antigens, as melanoma is well recognized to be an immune-controlled tumor. Immunosuppression does increase the risk for melanoma, e.g., in kidney transplant patients, and it is therefore likely that the UVR-induced immunosuppression also contributes to melanomagenesis. As immune-escape is a hallmark of melanoma progression, these immune-effects probably not only contribute to initiation of melanomas, but also to their progression. On the other hand, UVR-induced inflammation from innate immunity has been reported to promote angiogenesis and, in turn, melanoma metastasis (Bald et al. 2014).

Photoprotection Is Melanoma Prevention

From the evidence discussed above, no doubt remains that exposure to UVR is a major risk factor for the development of melanoma and that good photoprotection reduces melanoma risk. Consequently, the use of sunscreens as one mode

of photoprotection (in addition to sun and tanning parlor avoidance, shade seeking, and protective clothing) has been advocated to reduce melanoma risk. However, for many years, the efficacy of sunscreen use for melanoma prevention had been questioned, as epidemiologic studies yielded conflicting results. This is likely due to difficulties of retrospectively assessing UVR exposures and sunscreen use and the complexity of human behavior with an interplay between personal sun sensitivity/tendency to burn (skin phototype), sun exposure, and sunscreen use (Klug et al. 2010). With more recent animal data (Klug et al. 2010; Viros et al. 2014) and a large prospective clinical trial (Green et al. 2011) showing reduction in melanoma incidence with sunscreen use, however, sunscreens are now well established as an effective means of melanoma prevention. This requires, however, that sunscreens are not used to prolong sun exposure.

Given that both UVB und UVA contribute to UVR-induced DNA damage and mutation formation in melanocytes, melanoma prevention is likely most effective when photoprotection includes protection against both, e.g., in the form of modern broad-spectrum sunscreens. The large UVR-induced mutational burden is observed not only in spontaneous melanomas, but also in cases of familial malignant melanoma and in patients with genetically determined increased melanoma risk, such as xeroderma pigmentosum. This underscores the point that very good photoprotection is of even higher importance for those patients who already have a higher melanoma risk.

The abovementioned immunosuppressive effects of UVR and their role in facilitating melanoma progression also emphasize that photoprotection is not only important for the primary prevention of melanoma, but also for secondary prevention even after melanoma initiation. For this, there is also some molecular evidence: mutations of TP53 are rare in early stage melanoma, but more common in advanced melanoma (Hocker and Tsao 2007). These late mutations, also harbor C → T mutations (Hocker and Tsao 2007; Hodis et al. 2012), suggesting that the mutagenic properties of UVR also contribute to melanoma progression.

And finally, photoprotection is also important to prevent secondary melanomas. Melanoma patients already have a high UVR-induced mutational burden in their skin, and further UV exposures will only add to that burden and further increase the chance that the tens of thousands of random mutations will affect a sufficient number of genes critical for melanoma development and with that entail the malignant transformation of another melanocyte.

When thinking about public awareness campaigns, it is important to recognize that sun seeking and tanning device seeking behavior is, at least in some individuals, associated with a pleasurable central nervous effect (Aubert et al. 2016; Fell et al. 2014). Such features of addiction to UVR exposure are reminiscent of the addictive nature of nicotine which has made smoking cessation programs for lung cancer prevention difficult. The fact that antismoking campaigns have been able to overcome nicotine addiction and have resulted in a decline in smoking and consequently in lung cancer incidence is encouraging for efforts to reduce melanoma rates by addressing addictive tanning and sun seeking.

Conclusion

Environmental insults by UVR play a pivotal role in the pathogenesis of the most common types of cutaneous melanoma and interplay with genetically determined predisposition in some patients. Fortunately, these insults can be addressed. A large fraction of melanomas should be preventable by measures of sun protection and avoidance of tanning beds.

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Key Signaling Pathways in Normal and Neoplastic Melanocytes

4

Andrew E. Aplin and Ashani T. Weeraratna

Contents

Abstract

Signal transduction pathways regulate the proliferation, differentiation, migration, and survival of melanocytes. These signaling pathways are dysregulated during the transformation of melanocytes, often due to somatic mutation of genes within the pathway. One major signaling pathway that highlights this paradigm is the mitogen-activated protein (MAP) kinase pathway. Growth factor signaling via the MAP kinase pathway is required for melanocyte proliferation and survival. MAP kinase signaling is activated in the majority of melanomas through somatic mutations in NRAS, BRAF, and MEK1/2. Regulation of proliferation and survival is also controlled by phosphatidyl-inositol 3′-kinase (PI3K) signaling. PI3K is a major regulator of melanocyte biology and is commonly activated through the mutation/loss of expression of negative not

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pathway regulators such as PTEN. Alterations in cyclin-dependent kinase signaling are also frequent in melanoma and promote aberrant cell cycle progression. Other pathways such as Gαq, Wnt (canonical and noncanonical), Hippo, Notch, and signaling downstream of Rho family GTPases also play important roles in the aforementioned biological processes, and in some cases are altered in selective subsets of melanoma. The high mutation burden within genes in signaling pathways, the important role of these pathways in melanocytic neoplasms, and the knowledge that melanomas adapt their signaling mechanisms in response to targeted inhibitors make it essential to have a thorough understanding of the key signaling pathways in melanocytes and melanomas.

Keywords
RAS · BRAF · ERK1/2 · PI3K · PTEN · AKT · GNAQ · GNA11 · Rac · Rho · PREX · CDK4/6 · Wnt · β-catenin · YAP · TAZ · Notch

Introduction

Regulation of melanocyte proliferation and differentiation is modulated by a diverse set of cues that cells receive from their extracellular milieu. Stimuli range from soluble peptide growth factors to interaction via cell-cell adhesion molecules and generate a range of signal transduction pathways that tightly control processes in normal melanocytes and are aberrantly regulated during melanoma initiation and progression. Pathways often de-regulated in melanoma range from protein kinase and lipid kinase transduction events to heterotrimeric and monomeric GTPase signaling. In this chapter, we review the key signaling pathways that regulate melanocyte proliferation, growth, and migration/invasion. Furthermore, we illustrate how these pathways become deregulated during the transformation of melanocytes to melanoma.

Mitogen-Activated Protein (MAP) Kinase Pathway

The mitogen-activated protein (MAP) kinase cascade is one of the ubiquitously important signaling cascades in different cell types. In normal melanocytes, as in other normal cells, MAP kinase cascade activation is triggered by soluble peptide growth factors binding to their cognate receptor tyrosine kinases (RTKs), which are expressed on the cell surface. Growth factor binding leads to dimerization and autophosphorylation of receptors. Subsequent transphosphorylation of additional residues within the intracellular C-termini of RTKs which serve as binding sites to recruit Src homology 2 (SH2)-domain and phosphotyrosine binding (PTB) domain-containing adaptor proteins. These adaptor proteins then link through to signaling networks. One example of an adaptor protein is Grb2, which binds to phosphorylated tyrosine residues of RTKs and localizes SOS, a guanine nucleotide exchange factor, to the plasma membrane. In turn, SOS catalyzes GDP to GTP nucleotide exchange in not of RAS GTPases.

A key step between RTKs and the MAP kinase pathway is the activation of RAS. There are three main forms of RAS: N-RAS, H-RAS, and K-RAS, which act as molecular switches being active in their GTP-bound state. RAS binds multiple effectors but relevant to the MAP kinases ERK1 and 2 are the RAF (*R*apidly *A*ccelerated *F*ibrosarcoma) serine-threonine kinases ARAF, BRAF, and CRAF. RAS recruits RAFs to the plasma membrane where they are activated and initiate a phospho-relay cascade. RAFs phosphorylate and activate the dual specificity kinases MEKs at key serine residues, S218 and S222. RAFs act as dimers, with BRAF-CRAF heterodimers eliciting higher activity than homo-dimers (Rushworth et al. 2006). C-terminal 14-3-3 binding sites in RAF (serine 621 in CRAF; serine 729 in BRAF) also play an important role in RAF dimerization (Weber et al. 2001). Activated MEKs phosphorylate and activate ERK1/2. ERK1/2 phosphorylate a variety of cytoplasmic

targets (e.g., focal adhesion kinase, p90RSK, Bim) as well as translocate to the nucleus to control transcriptional events (Balmanno and Cook 2009).

The ERK1/2 pathway is fine-tuned through multiple adaptor complexes, scaffolding molecules, and feedback pathways. As noted above, 14-3-3 proteins are highly conserved adapters that bind phosphorylated residues in RAFs creating an inhibitory intra-molecular bridge, which is relieved by activated RAS. Kinase suppressor of RAS (KSR) is a pseudokinase that translocates to the plasma membrane and provides a scaffold function by binding and co-localizing RAFs, MEKs, and ERK1/2 (McKay et al. 2011). Negative control of the pathway is mediated by negative feedback loops including direct phosphorylation of SOS and C-terminal sites on BRAFs by ERK1/2 (Brummer et al. 2003), upregulation of Sprouty proteins that inhibit RAF activity (Pratilas et al. 2009), and regulation of dual-specificity phosphatases (DUSP) 4 and 6 that de-phosphorylate the activation sites on ERK1/2 (Lito et al. 2012).

Regulation of signaling through the ERK1/2 pathway is critical for melanocyte proliferation. Basic FGF that is secreted from keratinocytes promotes proliferation and activates ERK1/2 signaling in primary human melanocytes. Additional growth factors such as stem cell factor and endothelin 1 also activate the pathway. ERK1/2 signaling is key to G1-S cell cycle progression in normal human melanocytes through control of cyclin D1 levels as well as downregulation of the cyclin-dependent kinase (CDK) inhibitor, p27Kip1 (Bhatt et al. 2005). ERK1/2 signaling also impacts survival mechanisms by controlling the levels of the pro-apoptotic proteins, Bim EL and Bmf. In addition to RTK stimulation, MAP kinase signaling is also triggered downstream of G-protein-coupled receptors (GPCRs). For example, melanocortin 1 receptor (MC1R), expressed in melanocytes, is required for melanocyte proliferation and is a major determinant of skin pigmentation. Binding of the ligand, alpha melanocyte stimulating hormone, to MC1R activates adenylate cyclase-mediated production of cyclic adenosine monophosphate (cAMP) via the Gs alpha subunit, as well as ERK1/2 signaling (Busca et al. 2000). The role of cAMP in ERK1/2 activation may differ between model systems.

Cutaneous melanomas have a high burden of somatic mutations. The advent of next generation sequencing technologies has led to the identification of multiple recurrent mutations within components of the RAS-RAF-MEK-ERK1/2 pathway (Fig. 1). The first oncogene identified as being mutated in melanoma was NRAS. Most NRAS mutations occur at the Q61 locus and less frequently at the G12 or G13 loci. Other forms of RAS are mutated infrequently in melanoma; mutation rates in HRAS and KRAS are both ~1%. NRAS mutations at the Q61 locus impair the GTP hydrolysis activity resulting in a GTP-bound state for NRAS and constitutive activation of downstream effector pathways.

Approximately 50% of cutaneous melanomas harbor an activating mutation within the BRAF gene (Davies et al. 2002; TCGA 2015). The most frequent BRAF mutation is a thymine (T) to adenine (A) transversion that results in a valine to glutamic acid substitution at codon 600. This V600E alteration introduces a phosphomimetic change within the activation loop of BRAF and leads to constitutive, RAS-independent activation of BRAF and downstream MEK-ERK1/2 signaling. Furthermore, BRAF V600E mainly exists as a monomer and is refractory to negative feedback mechanisms mediated by Sprouty proteins (Brady et al. 2009). Additional non-V600 mutations have been identified; most are activating but a subset of mutations actually inactivate BRAF kinase activity. Nevertheless, these mutations still activate MEK, as the kinase-defective BRAF protomer can dimerize with CRAF and lead to an activated heterodimer (Wan et al. 2004). Other forms of RAF are mutated at very low frequencies; <2% for ARAF and 1.1–3.6% for CRAF according to the dataset in cBioPortal for Cancer Genomics. More recently, BRAF fusions have been identified, in which the intact kinase domain of BRAF is fused to a broad variety of different 5′ partner

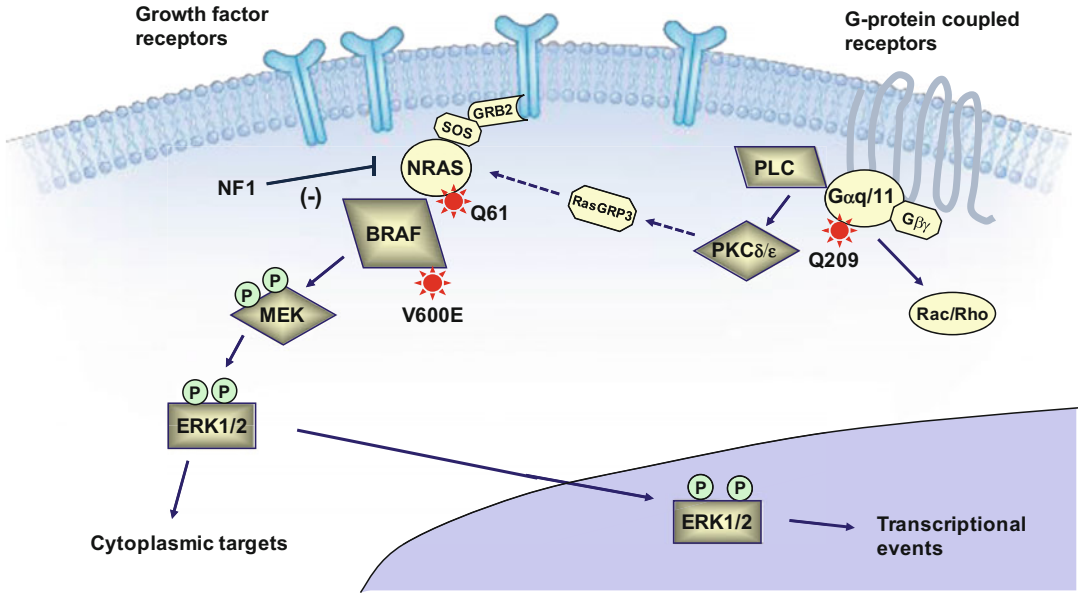

Fig. 1 Mutations in the RAF-MEK-ERK1/2 signaling pathway associated with cutaneous and uveal melanoma. The MAP kinase signaling is activated in the majority of cutaneous melanomas through somatic mutations in NRAS, BRAF, and MEK1/2. This pathway is activated in uveal melanoma by mutations in GNAQ and GNA11 and downstream signaling via PKC and RasGRP3

genes (Hutchinson et al. 2013; Botton et al. 2013). The 5′ partners often promote dimerization of fusion kinases, resulting in constitutive activation.

A third frequent alteration in the MAP kinase pathway affects the *NF1* gene. NF1 encodes a GTPase activating protein (GAP) that upregulates the GTPase activity of RAS proteins. NF1 is mutated frequently in melanomas on chronically sun-damaged skin and desmoplastic melanoma (Krauthammer et al. 2015; Shain et al. 2015). Although all the mutations have not been functionally analyzed, many are damaging alterations in both alleles and therefore likely result in loss of NF1 function. NF1 mutations can co-occur with NRAS mutations but are anticorrelated with BRAF V600 mutations, which arise in a specific melanoma subtype. Less frequent mutations have also been detected within MEK1 and -2 (Nikolaev et al. 2012).

As noted above, the ERK1/2 pathway is regulated by scaffold proteins including kinase suppressor of RAS (KSR), IQGAP, and MEK partner 1 (MP1). However, as exemplified by studies in KSR, while a role for scaffold proteins in modulating the ERK1/2 pathway has been shown in developmental biology models and mouse embryo fibroblasts, there have been few studies in melanocytes and melanoma cells.

BRAF V600E signaling via the MEK-ERK pathway contributes to several malignant traits in melanoma. BRAF V600E signaling promotes cell cycle progression via upregulation of cyclin D1 and downregulation of p27Kip1 (Bhatt et al. 2007). Resistance to apoptotic cell death is mediated by pathway signaling leading to downregulation of two BH3-only proteins, Bim-EL (BCL2-like 11) and Bmf (BCL2-modifying factor) (Brocklin et al. 2009; Boisvert-Adamo and Aplin 2008; Cartlidge et al. 2008; Shao and Aplin 2012). Additionally, phosphorylation of dynamin-related protein 1, a protein involved in mitochondrial division, may also contribute to transformation mediated by MEK-ERK signaling. Migratory and invasive properties of melanoma cells are enhanced by BRAF V600E-MEK-ERK upregulation of the Rho family GTPase, Rnd3 (Klein et al. 2008), and the EMT transcription factor Twist1 (Weiss et al. 2012) and downregulation of the cGMP-selective

phosphodiesterase, PDE5A (Arozarena et al. 2011a). MAP kinase signaling also cross-talks to metabolic pathways via ERK1/2 and RSK phosphorylating LKB1, which suppresses LKB1 function and promotes BRAF V600E-driven tumorigenesis (Zheng et al. 2009). By contrast, expression of BRAF V600E in normal melanocytes leads to senescence (Michaloglou et al. 2005; Gray-Schopfer et al. 2006; Dankort et al. 2009) and the formation of nevus-like structures in mice (Dankort et al. 2009). These data are consistent with the notion of oncogene-induced senescence and the presence of BRAF mutations in the majority of human nevi (moles) (Pollock et al. 2003).

The MAP kinase cascade is also activated via mutations in NRAS, which occur in a mutually exclusive manner from BRAF mutations and are more frequent in older patients (Jakob et al. 2012). Mutations in NRAS at Q61, G12, and G13 lead to a persistent GTP-bound state of NRAS and constitutive activation of downstream effector pathways. Furthermore, inactivation of NF1 occurs frequently and is associated with a high level of active RAS, although additional mechanisms of RAS activity regulation appear to be important in some NF1-null tumors (Krauthammer et al. 2015). In contrast to their BRAF-mutant counterpart, NRAS mutant melanomas signal predominantly via CRAF, which is associated with a disruption in cAMP metabolism (Dumaz et al. 2006). Other major effectors downstream of activated RAS are phosphatidyl-inositol 3′-kinase (PI3K) and Ral GEF. PI3K signaling will be discussed in section "Phosphatidyl-Inositol 3′-Kinase (PI3K) Pathway."

Suppression of NRAS expression in human melanoma cell lines harboring NRAS mutations can induce apoptosis (Eskandarpour et al. 2005); however, the effects are likely variable across a larger panel of lines. More compelling data for a driver role for mutant NRAS comes from mouse models of melanoma. Expression of NRas Q61K in the melanocytic lineage (under the control of tyrosinase-regulatory sequences) leads to skin hyperpigmentation and increased phosphorylation of ERK1/2 (Ackermann et al. 2005). Tyr: NRas Q61K mice crossed onto a p16Ink4a null background develop primary melanoma with high penetrance and short latency, a subset of which metastasize to the liver and lung (Ackermann et al. 2005). Expression of a mutant form of CDK4 that is resistant to inactivation by p16INK4a in combination with NRAS Q61K in melanocytes leads to dysplastic nevus-like lesions and metastatic melanoma, although the time to onset and multiplicity is dependent on the mouse strain utilized (Ferguson et al. 2015). By contrast, tyrosinase-driven HRas G12V expression in p16INK4a null mice gives rise to amelanotic melanomas but few/no metastases (Chin et al. 1997).

Due to the varied mechanisms of activation and multiple opportunities for therapeutic targeting, considerable efforts have been focused on targeting the ERK1/2 pathway in melanoma. However, the dependency of melanoma cell lines on the ERK1/2 pathway varies with BRAF V600E/K mutant cell lines being highly sensitive to MEK inhibitors, inhibiting G1-S progression and proliferation in vitro and in vivo (Solit et al. 2006). BRAF dependency of BRAF V600E/K lines has also been demonstrated with selective inhibition by vemurafenib/PLX4032 (Joseph et al. 2010). These studies have formed the initial basis for therapeutic blockade of the BRAF-MEK-ERK1/2 signaling pathway in BRAF V600 melanomas. Other factors also influence the pathway and provide therapeutic strategies. For example, copper influx binding to MEK1 enhances MEK1 phosphorylation of ERK1/2, and disrupting this binding inhibits BRAF V600E signaling through MEK-ERK1/2 and tumorigenesis (Eskandarpour et al. 2005). This study forms the basis of copper chelation strategies to enhance the effects of BRAF-MEK-ERK1/2 pathway inhibitors in mutant BRAF melanoma patients.

Phosphatidyl-Inositol 3′-Kinase (PI3K) Pathway

A second pathway strongly implicated in melanoma initiation and metastasis is the phosphatidyl-inositol 3′-kinase (PI3K) signaling cascade. Class I PI3Ks consist of a regulatory p85 subunit and a catalytic p110 subunit. Typically, growth

factor signaling leads to PI3K activation via the phosphorylation of cytoplasmic adaptor proteins containing Y-x-x-M motifs that serve as strong docking sites for the p85 subunit. However, direct binding to a receptor may also occur. For example, neuregulin binding to ERBB3 leads to receptor heterodimerization with a co-receptor (typically EGFR, ERBB2 or EBB4) and phosphorylation of Y-x-x-M motifs in the cytoplasmic domain of ERBB3. As with adaptor proteins, phosphorylation of Y-x-x-M motifs leads to PI3K binding and activation. The p110 catalytic subunit phosphorylates phospholipid phosphatidylinositol -4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3) to trigger downstream signaling. PIP3 binds to pleckstrin-homology domains within several proteins to transmit signals. These targets include phosphoinositide-dependent protein kinase-1 (PDK1), AKT1-3, and serum/glucocorticoid-regulated kinase (SGK) 1-3. The PI3K signaling pathway is dampened by the action of phosphatases. Phosphatase and tensin homologue (PTEN) dephosphorylates PIP3 at the 3′ position and inositol polyphosphate 4-phosphatase type II (INPP4B) dephosphorylates PI(3,4)P2 to PI(3)P. In normal melanocytes, PI3K is activated in vitro by growth factors such as stem cell factor and provides a pro-survival signal (Larribere et al. 2004). Furthermore, PTEN deletion in melanocytes in mice leads to increased numbers of melanocytes in the dermis and retention of coat color by resisting hair greying (Inoue-Narita et al. 2008). Inpp4b-/- mice are viable (Li Chew et al. 2015), but the effect of loss of Inpp4b on melanocytes in vivo is yet to be described. INPP4B loss has been reported in 21% of melanoma (Gewinner et al. 2009); however, genetic alterations are less frequent in more recent next-generation sequencing analyses of melanoma. Further studies are warranted to understand the influence of INPP4B in melanoma initiation and progression.

PI3K signaling plays a key role in growth, survival, and metabolism; thus, it is unsurprising that PI3K signaling has been linked to melanoma progression. Importantly, use of patient samples has linked PI3K signaling to melanoma brain metastasis (Anastas et al. 2014), providing a strategy to limit a severe complication of melanoma that is currently an unmet clinical need. Activating mutations are frequent in the catalytic PIK3CA subunit are prevalent in breast cancer, colorectal cancer, and uterine corpus endometrial carcinoma. By contrast, PIK3CA mutations are they are infrequent (~3%) and in cutaneous melanoma and their contribution to the malignant phenotype is poorly characterized. A frequent alteration leading to activation of PI3K signaling in melanoma is the loss of PTEN (Fig. 1). Alterations in PTEN occur through frameshift mutations leading to premature truncations, intragenic microdeletions and epigenetic silencing (Xing et al. 2012). Loss of PTEN has been strongly linked to progression of melanoma, often occurring in concert with BRAF mutations and in wild-type BRAF/NRAS tumors. PTEN loss is rare in mutant NRAS melanomas. Mice with the loss of Pten in melanocytes do not develop spontaneous melanomas unless induced with a carcinogen (Inoue-Narita et al. 2008). Additionally, while expression of BRAF V600E leads to the formation of nevus-like structures in mice, concurrent loss of PTEN leads to the formation of invasive tumors with 100% penetrance and metastasis to the lymph and lungs (Dankort et al. 2009). In this model, melanoma growth was prevented by inhibitors of mTorc1 (Dankort et al. 2009). There is also growing evidence of PTEN modulating the response to targeted therapy and immune checkpoint inhibitors (Paraiso et al. 2011). Overall, these data strongly implicate PTEN loss in the malignant progression of melanomas.

In BRAF V600E/PTEN null tumors, combinations of BRAF inhibitors and PI3K inhibitors elicit more potent effects in vivo than either alone (Marsh Durban et al. 2013; Deuker et al. 2015); thus, it is important to understand the signaling downstream of PI3K. PDK1 plays an active role since its depletion or pharmacological inhibition impairs tumor growth in BRAFV600E CDKN2A -/- mice (Scortegagna et al. 2014). PDK1 phosphorylates AKT at threonine 308 within the activation loop but is not frequently altered in melanomas. E17K alterations in the pleckstrin homology domains in AKT isoforms 1 and 3 have been identified at low

frequencies in melanoma tumors and cell lines (Davies et al. 2008). However, AKT3 is frequently (20–40%) deregulated in melanomas through copy number increases and loss of PTEN expression (Stahl et al. 2004). AKT signaling contributes to malignant properties of melanomas through control of mTOR signaling, phosphorylation of PRAS40 (proline-rich Akt substrate of 40 kDa) (Madhunapantula et al. 2007), and regulation of Notch1 (Bedogni et al. 2008). Importantly, in mouse models, expression of a constitutively active (myristoylated) form of Akt1 promotes melanoma formation and metastasis to the lung and brain in the setting of BRaf V600E and silencing of p16INK4A and p19ARF. PI3K and PDK1-dependent and AKT-independent signaling may also contribute to malignant traits. AKT inhibitors demonstrate only modest effects on growth in BRAF V600E/PTEN null tumors (Marsh Durban et al. 2013), and SGK3 has been implicated as a major target downstream of PDK1 that contributes to the growth of mutant BRAF melanoma (Scortegagna et al. 2015). PTEN may also signal independent of its role in the PI3K-AKT pathway, for example, utilizing protein phosphatase activity, to contribute to malignant behavior. PTEN represses β-catenin nuclear localization and transcriptional activity via a caveolin-1 regulated mechanism (Conde-Perez et al. 2015). PTEN inhibits the activity of the Rac-selective GTP exchange factor PREX2 (Mense et al. 2015). PREX2 mutations have been found in ~14% of melanomas (see below), and expression of mutant PREX may promote the transformation of TERT-immortalized mutant NRAS human melanocytes (Berger et al. 2012). However, the exact role for PREX2 requires further examination (Horrigan et al. 2017).

Gαq Signaling

In contrast to the high frequency of *BRAF* and *NRAS* mutations found in cutaneous melanoma, alterations in are absent in nevi, or melanocytomas of the central nervous system. Rather, activating mutations in GNAQ and GNA11 genes (typically Q209 in exon 5 but also R183 in exon 4) are found in 80–90% of uveal melanomas. GNAQ mutations have also been identified at a high frequency in blue nevi (Van Raamsdonk et al. 2009, 2010) and melanocytomas (Koelsche et al. 2015). Alterations in GNAQ and GNA11 occur early in disease progression; however, additional mutations are required, often including inactivating BAP1 mutations on chromosome 3. BAP1 encodes a deubiquitylating enzyme and loss of function mutations in this gene are found in 32–50% of primary uveal melanomas and are associated with aggressive disease and higher likelihood of metastasis (Harbour et al. 2010).

GNAQ and GNA11 encode Gqα and G11α, the alpha subunits of heterotrimeric G-proteins signaling downstream of G-protein coupled receptors (GPCR). Normally, Gα is in a complex with Gβ and Gγ subunits, but following receptor stimulation the α subunit switches from a GDP-bound form to a GTP-bound form and dissociates from the β and γ subunits. Of note, Gqα has been shown to be downstream of the endothelin receptor, which is required for melanoblast migration during neural crest development (Shin et al. 1999). A major effector of Gqα and G11α is phospholipase Cβ, which when activated hydrolyzes membrane PIP2 to release diacylglyerol (DAG) and inositol triphosphate (IP3). These second messengers ultimately lead to activation of members of the protein kinase C (PKC) family and the RAS GEF, RasGRP3 (Chen et al. 2014, 2017). The Q209 residue in Gqα and G11α lies within the RAS-like domain and its mutation disrupts GTP-hydrolysis, leading to constitutive activation of the aforementioned pathways and transformation of immortalized melanocytes (Van Raamsdonk et al. 2009). Downstream of PKC and RasGRP3 signaling, the MEK-ERK1/2 pathway is activated in mutant GNAQ/11 uveal cell lines (Fig. 1). These findings have contributed to the basis for phase II and phase III clinical trials to target MEK in uveal melanoma patients. To date, these trials have met with limited success (Carvajal et al. 2014).

Gqα and G11α may also signal independently of phospholipase Cβ. Several guanine nucleotide

exchange factors (GEFs) for Rho family GTPases are also effectors of Gqα and G11α. Specifically, LARG, p115 RhoGEF, PDZ Rho GEF, lbc Rho-GEF, and p63 Rho-GEF have all been reported to interact with Gqα and/or G11α. A distinct Rho/Rac GEF, Trio, was identified through a genome-wide RNA interference screen to signal downstream of Gqα and is required for the growth of mutant GNAQ uveal melanoma cells. Trio promotes Rho and Rac GTPases activity, actin stress fiber formation, and YAP1 (Yes associated protein 1) nuclear translocation and YAP-dependent transcription. YAP is a transcriptional co-activator within the Hippo signaling pathway. Phosphorylation at serine 127 leads to the cytoplasmic sequestration of YAP and inhibition of its transcriptional activity. The Notch ligand, Jag-1, and the Notch target gene, Hes-1, are possible downstream targets of YAP signaling in uveal melanoma cells (Liu et al. 2015). Uveal melanoma display high levels of nuclear YAP and depletion of YAP impairs the growth of uveal melanoma lines in vitro and in vivo. Thus, selectively targeting YAP represents a therapeutic strategy for advanced-stage uveal melanoma patients. A recent advance toward testing such strategies is the generation of a transgenic mouse model of uveal melanoma. Mice with MITF-Cre regulated expression of GNAQ Q209L develop melanocytic neoplasms with a 100% penetrance after 3 months with evidence of invasive lesions and lung metastasis (Huang et al. 2015).

The Rho/Rac Signaling Pathway

The Ras superfamily is comprised of multiple members. Of these, the Rho/Rac subfamily is, in itself, comprised of 26 members, in varying isoforms of Rho, Rac, Cdc42, Rnd, and the atypical RhoBTB and RhoT/Miro GTPases. In this chapter, we will focus on Rho/Rac GTPases specifically. Rho/Rac GTPases control motility, and other processes critical to melanocyte function, and melanoma progression. Rho/Rac GTPases, when bound to guanosine di-phosphate (GDP), are inactive. When GDP are exchanged for guanosine tri-phosphate (GTP), with the help of guanosine exchange factors (GEFs), Rho/Rac proteins are activated. Specificity of Rho/Rac activities is determined by (1) lipid modifications, which determine their subcellular localization, (2) the GTPase effector proteins present in those locales, and (3) phosphorylation by molecules such as PKC and PKA.

Rho and Rac signaling have both collaborative and divergent functions in cytoskeletal remodeling. Rho proteins are involved in the formation of stress fibers, and Rac proteins dictate the formation of lamellipodia and dendrite extension. In response to UV irradiation, melanocytes form dendrites, which allow them to pass melanosomes to neighboring keratinocytes, to protect them. This process of dendrite formation is dictated by Rho/Rac signaling and is very similar to that observed in the formation of dendrites from neurons, perhaps reflecting the neural crest origins of melanocytes. After exposure to UV, the hormone alpha-MSH is increased, which results in stimulation of cAMP. cAMP signals to activate PKA, which in turn signals to deactivate Rho, and increase Rac signaling (Scott et al. 1997). Activation of Rac, therefore, is a major contributor to cell motility.

In melanoma cells, Rac can be activated in multiple ways. One mechanism is via the mutation of the NRAS oncogene. When NRAS is mutated, Rac is activated (Li et al. 2012). While this does not affect the motility of melanoblasts, it does promote their survival. Inhibiting Rac1 suppresses tumor growth and progression. In addition to upstream mutations, it has recently been discovered that Rac1 itself is recurrently mutated in melanoma. Unlike BRAF V600E, NRAS, or GNAQ/11, Rac1 P29S is not thought to be an initiating mutation, as it is not present in nevi. The mutation is likely environmentally induced as is formed through a C to T transition, at a dipyrimidine, as typical for UV-induced mutations. This mutation renders Rac1 constitutively active, allowing it to maintain binding with molecules such as PAK1, driving transformation and a metastatic program (Watson et al. 2014). Data showing that inhibiting Rac in Rac1 mutant melanocytes inhibits malignant transformation suggest that this might make Rac1 P29S a good target for therapy.

Finally, mutations in one additional family of Rac exchange factors have been identified in melanoma. The PIP3-dependent Rac GEFs PREX1 and -2 have recently been shown to play a role in melanoma (Welch 2015). These proteins are large >180 kDa proteins that encompass pleckstrin homology domains, a Dbl homology domain, DEP domains, and PDZ domains among others. The most important interaction in melanoma is likely the DH-PH domains, as this both acts as a Rac GEF and allows for the binding of PREX to PTEN. This inhibits PTEN activity, resulting in the activation of AKT and therefore cell survival. Knockout of PREX1 in mice causes the development of white bellies, indicative of a melanoblast migration defect, and crossing these mice to melanoma-prone mice results in suppression of tumor growth and metastasis (Lindsay et al. 2011, 2015).

PREX2 binds and inactivates PTEN. In turn, loss of PTEN can increase PREX2 activity (Mense et al. 2015). PTEN is commonly deleted in melanoma, and this has been shown to have multiple implications for both melanoma progression and therapy resistance (Dankort et al. 2009). Intriguingly, melanoma cells that develop resistance to BRAF inhibitors are largely PTEN deficient. In addition to this, it has been shown that the metabolism of these cells is altered as well (Baenke et al. 2016) and vemurafenib resistant cells tend to rely more on oxidative phosphorylation and less on glucose (Zhang et al. 2016). Since PREX2 knockout mice have been shown to be insulin resistant due to defects in the metabolism and uptake of glucose, it may be that PREX2 can also contribute to therapy resistance in melanoma. Overall, melanoma cells appear to find multiple ways to maintain and promote Rac1 activity, resulting in increases in both tumor progression and therapy resistance.

CDK4/6-RB1 Pathway

Abnormal cell cycle progression is one of the hallmark features of cancer. Notably the progression through G1 phase, through the restriction point, and into the DNA synthesis (S) phase is a tightly regulated process. The molecular machinery controlling the G1-S progression comprises a series of cyclins and cyclin-dependent kinases (CDKs). Within the early phase of G1, D-type cyclins (D1, D2 and D3) form a complex with two closely related CDKs, CDK4 and CDK6. Later in G1, cyclins E and A bind to CDK2 and CDK1 to promote G1-S and M phase progression. In general, cyclin levels fluctuate across the cell cycle, whereas CDK levels are more constant. A major function for cyclin-CDK complexes is to phosphorylate and inactivate the pocket protein, retinoblastoma (RB), and its related proteins, p107 and p130. Hyper-phosphorylation of RB releases E2F transcription factors that can activate transcription of genes involved in further cell cycle progression and division. Opposing the kinase activities of cyclin-CDK complexes are two families of cyclin-dependent kinase inhibitors (CDKI). The Cip/Kip family of CDKIs includes p21Cip1, p27Kip1, p57Kip2, which primarily inhibit cyclin E and cyclin A-bound CDK complexes by forming ternary complexes. By contrast, the INK4 family, which includes p16 INK4A, p15 INK4B, p18 INK4C, and p19 INK4D/ARF, more selectively inhibits D-type binding CDK4 and CDK6 complexes through an allosteric mechanism.

Multiple mechanisms drive aberrant G1-S cell cycle progression in melanoma and lead to aberrant proliferation. D-type cyclins act as mitogenic sensors within G1. The RAF-MEK-ERK1/2 pathway, which is constitutively activated by mutations in NRAS (20–30% frequency) and BRAF (~50% of melanomas), transcriptionally upregulates cyclin D1 and cyclin D3, as well as downregulates p21Cip1 and p27Kip1 (Bhatt et al. 2005). Cyclin D1 is also regulated by ubiquitin-proteasomal systems. In melanoma, cyclin D1 may be posttranscriptionally upregulated through mutation of the E3 ubiquitin ligase, FBXO4, and thus impairs ubiquitylation and degradation of cyclin D1 (Lee et al. 2013). However, the frequency of these mutations in the melanoma TGCA dataset seems very low at approximately 1%. Cyclin D1 is recurrently amplified, in particular in melanomas on acral and mucosal sites (Sauter et al. 2002). Inactivation of RB1 may

also occur through mutations in CDK4 and loss of CDKI expression and, to a lesser degree, loss of RB1 itself. Somatic and germline R24C/H mutations in CDK4 are detected in cutaneous melanoma (Wolfel et al. 1995), and CDK4 amplifications occur in a mutually exclusive pattern with CDKN2A deletions (Curtin et al. 2005). The CDK4 mutations occur within the p16INK4a domain and render CDK4 less susceptible to inhibition by INK family proteins. CDK4 R24C knock-in mice are susceptible to melanoma induced by chemical carcinogenesis (Sotillo et al. 2001). Loss of the Ink4a/Arf tumor suppressor locus is a more frequent mechanism leading to RB-deregulation in melanoma. In mouse models, deletion of p16Inka in combination with expression of mutant RAS promotes aggressive melanoma formation (Monahan et al. 2010), and loss of p19Arf in combination with tyrosinase-driven mutant HRAS promotes melanomagenesis, an effect that is exacerbated following UV irradiation (Kannan et al. 2003). Deficiency in both Ink4a and Arf promotes UV-induced melanomagenesis in a HGF transgenic mouse model (Ha et al. 2007). Thirdly, the RB1 gene is mutated albeit infrequently (3–5% in cBioportal databases) in melanoma. RB1 loss may occur either through focal intragenic homozygous deletion or through truncation mutations (Xing et al. 2012).

CDKs are targetable with small molecule inhibitors; however, early generation CDK inhibitors were relatively nonselective and elicited limited therapeutic effects in melanoma patients. Parke-Davies developed PD0332991/palbociclib/IBRNACE, an orally available, highly selective inhibitor of CDK4/6. A very similar compound LEE011/ribociclib from Novartis and a CDK4/6/9 inhibitor, LY2835219/abemaciclib from Eli Lilly have also been developed. The FDA-approval of palbociclib in postmenopausal estrogen receptor (ER)-positive/HER2-negative breast cancer has re-ignited interest in targeting cell cycle progression in cancer. In melanoma, palbociclib inhibits the hyper-phosphorylation of RB and blocks the proliferation of RB-proficient melanoma cell lines. Also, the combination of palbociclib with inhibition of MEK leads to apoptosis in vitro and tumor shrinkage in vivo (Kwong et al. 2012). These preclinical findings have translated into a clinical trial of ribociclib and the MEK inhibitor, MEK162/binimetinib, in late-stage, mutant NRAS melanoma patients. In summary, the CDK4/6 pathway is activated across most, if not all melanoma subtypes, and provides an attractive option for therapeutic intervention in combination with other targeted agents.

The Wnt Signaling Pathway

The Wnt signaling pathway involves 19 known Wnt ligands, which signal via any one of 10 frizzled (fzd) receptors to activate G-protein signaling downstream of ligand-binding. Wnt signaling can be divided into canonical and non-canonical signaling (Fig. 2), where canonical Wnt signaling involves the activation of the DIX-domain protein, Disheveled (Dsh), ultimately activating β-catenin (Webster et al. 2015a). β-Catenin plays important roles both at the cell surface, where it regulates adhesion, and in the nucleus where it activates the transcription of genes involved in cell proliferation. β-Catenin activity is regulated by the GSK3β/APC/Axin destruction complex. In this complex, Axin acts as a docking protein, as it has a GSK3β docking motif as well as a β-catenin docking motif, bringing together the two proteins in close proximity. This is further enhanced by APC, a protein with multiple β-catenin docking sites. When β-catenin is recruited to this complex, which is what occurs in the absence of Wnt ligand, it is phosphorylated and targeted for ubiquitination and degradation. Once Wnt ligand binds its cognate receptor, Dsh is activated. Dsh inhibits the ability of GSK3β to phosphorylate β-catenin to target it for destruction, increasing the levels and activity of β-catenin and enhancing cellular proliferation. Canonical Wnt ligands mostly include Wnt1, 3A, 7, 8, and 8b, and the canonical Fzd receptors that most commonly mediate this signaling include Fzd 1 and 7 (Webster et al. 2015a).

In noncanonical Wnt signaling, β-catenin is not a key component and in fact, as discussed below, may even be targeted for degradation in a GSK3-β-independent manner. There are two key non-canonical pathways, the Wnt/Ca^{2+} pathway, and the planar cell polarity (PCP) pathway (Fig. 2).

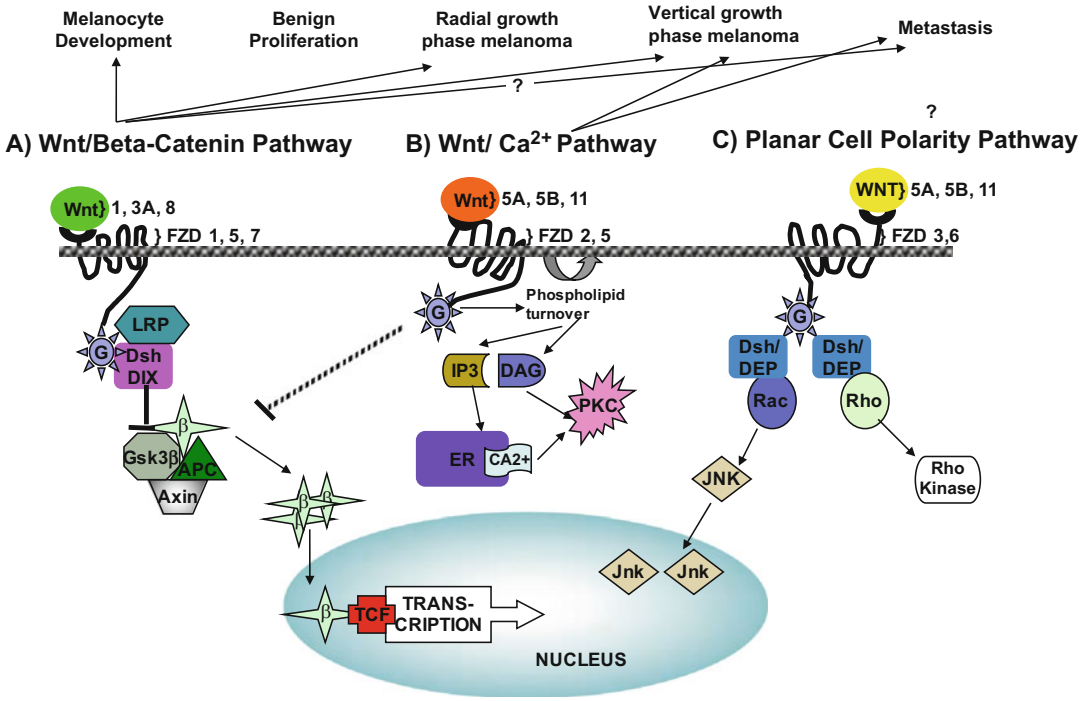

Fig. 2 The Wnt signaling pathway. The three main Wnt signaling pathways (Wnt/β-catenin; Wnt/Ca^{2+} and planar cell polarity) and their probable roles in melanocyte development, and melanoma progression

The planar cell polarity pathway results in the activation of Rho kinases. First, Wnt binds to its receptor, activating Dsh, which signals to activate the disheveled associated activator of morphogenesis (Daam1). Daam1 activates Rho signaling via the activation of GEFs. In the PCP pathway, Dsh can also complex with Rac, activating Jun kinases (JNK). Both the activation of Rho and Rac can result in cytoskeletal remodeling, thereby promoting migration. In the Wnt/Ca^{2+} pathway, Wnt binding to its receptor triggers the direct activation of Gαq. This results in the activation of PLCγ, lipid turnover in the membrane, and the generation of the second messengers, diacylglycerol (DAG) and inositol triphosphate (IP3). DAG can signal to activate PKC, and IP3 translocates to the endoplasmic reticulum where it releases calcium from its intracellular stores. PKC has multiple downstream signaling effects, and calcium release results in the activation of CAMKII and calcineurin, which also signal to activate and inhibit multiple different pathways. Noncanonical Wnt signaling is most commonly mediated by Wnts 4, 5, 5B and 11, and Fzd 2 and 5 (recently reviewed in (Webster et al. 2015a)).

In addition to the Fzd receptors, Wnt signals require co-receptors for transduction. These co-receptors help to determine through which pathway the signals are transduced, as the Wnt/Fzd combination can otherwise be rather promiscuous. Low-density lipoprotein receptors (LRPs), specifically LRP5 and 6, are single transmembrane receptors that complex with Fzd to mediate canonical Wnt signaling. Orphan receptor tyrosine kinases ROR1 and ROR2 are also single transmembrane receptors, but have intracellular tyrosine and serine-threonine kinase domains, as well as extracellular immunoglobulin-like, cysteine-rich domains. These receptors seem to play antagonizing roles to each other in melanoma, and most commonly mediate noncanonical Wnt signaling (O'Connell et al. 2013). In addition, there are other co-receptors that are not well described in humans, including Ryk/Derailed and FRL1/Crypto. Given the enormous amount of possible ligand/receptor/

co-receptor interactions, and the multiple downstream amplification steps in this cascade, Wnt signaling plays multiple, varied roles in the development and pathogenesis of cancer.

In melanoma, canonical and noncanonical Wnt signaling (specifically, the Wnt/Ca^{2+} pathway) play important and opposing roles. First, Wnt signaling directs the migration of melanocytes from the neural crest. Then, during the early stages of melanomagenesis, β-catenin is critical for the immortalization of melanocytes and the bypassing of melanocyte senescence (Delmas et al. 2007; Larue et al. 2009). Activation of the POU-domain transcription factor, Brn2, by β-catenin activates the melanocyte transcription factor MITF, leading to increased proliferation of melanocytes. As melanoma cells become more aggressive, they switch from a canonical Wnt signaling state, where they are very proliferative, to a noncanonical Wnt signaling state, where they now become invasive (O'Connell and Weeraratna 2009). This delineation of proliferation versus invasion in melanoma is known as phenotype switching. When melanoma cells switch to an invasive phenotype, they express higher levels of Wnt5A. Wnt5A has been shown to play multiple roles in melanoma metastasis from modulating the cytoskeleton, to promoting an EMT-like switch, to activating a series of metalloproteinases and tumor homing antigens such as CD44 (Weeraratna et al. 2002; Dissanayake et al. 2008). Many of these effects require the presence and activity of ROR2, but not ROR1, which is not present in metastatic melanoma.

The role of β-catenin in melanoma metastasis is controversial. While some studies have shown that β-catenin is inhibitory to melanoma invasion (Arozarena et al. 2011b), others have shown that β-catenin promotes invasion (Damsky et al. 2011). Studies on human samples have shown a loss of β-catenin during the progression from nevi to metastatic melanoma, and high levels of β-catenin correlate with better prognosis for melanoma patients in multiple studies (Hoek et al. 2006; Chien et al. 2009; Bachmann et al. 2005). Recently, a new study also showed that an inhibitor of β-catenin signaling, sFRP2, is increased in the aged microenvironment (specifically fibroblasts) and shuts off β-catenin signaling in melanoma cells, contributing to age-related increases in melanoma metastasis in both human samples, and in vivo in mice (Kaur et al. 2016). Additionally, in mice, overexpression of the canonical Wnt ligand, Wnt3A, inhibited tumorigenesis. However, as mentioned, other mouse models show that stabilizing β-catenin promotes metastasis (Damsky et al. 2011). The difference between these studies could be due to whether or not β-catenin is mutated (constitutively stabilized). Therefore, studies that look at genetically altered β-catenin tend to show associations with invasion, but studies that look at endogenous β-catenin may show the opposite.

The canonical WNT pathway is constitutively activated in a subset of melanocytic neoplasms called deep penetrating nevi (DPN) (Yeh et al. 2017). DPN are deeply invasive, highly pigmented lesions that are considered benign, but which can occasionally metastasize. DPNs are characterized genetically by a combination of a MAPK pathway activating mutation at the level of BRAF or MEK1 and an activating mutation of β-catenin. It has been shown that WNT signaling in benign nevi with only a BRAF V600E mutation decreases gradually with increasing distance of nevus cells from epithelial structures such as the epidermis and hair follicles and coincides in a decrease in cell size and pigmentation. The latter phenomenon is called "maturation" by pathologists and considered as an important criterion for benignancy. DPN do not show maturation and show constitutive activation of the WNT pathway, as the mutations activating β-catenin override the dependency on secreted WNTs, allowing the tumor cells to invade deeply.

As well as effects on metastasis, the dichotomy between β-catenin and Wnt5A also dictates therapy resistance in melanoma. Activation of β-catenin in cells with a loss of Axin1 has been shown to sensitize melanoma cells to vemurafenib-induced apoptosis, and overexpression of Wnt3A does the same (Biechele et al. 2012). Inhibiting β-catenin induced resistance to PLX4720, and this was confirmed in a later study, where Wnt5A was shown to increase resistance to PLX4720, in part via the hypoxia/

Siah2-induced degradation of β-catenin (O'Connell et al. 2013) and in part via the induction of a pseudo-senescent state (Webster et al. 2015b). Additionally, in aged mice, β-catenin loss correlated to increased resistance to BRAF inhibitor. MEK inhibitor (AZD6244) treatment of NRAS mutant tumors showed a similar dependence on β-catenin for effective apoptosis, suggesting that this might be a universal response to inhibition of the RAS/RAF/MEK/ERK pathway (Yeh et al. 2017). Intriguingly, however, continued, long-term treatment of melanoma patients with vemurafenib did not show the same association between nuclear β-catenin and response, suggesting that this pathway may get reactivated after long-term BRAF inhibitor treatment (Chien et al. 2014).

In addition to targeted therapy, immune therapy plays a large role in the current clinical landscape of melanoma. Wnt5A plays critical roles in hematopoietic stem cell self-renewal, suppressing β-catenin to maintain stem cell quiescence (Nemeth et al. 2007). In melanoma, we have shown that Wnt5A signals to decrease MITF and shuts off melanocytic antigens such as GP100 and MART1, thereby rendering melanoma cells less recognizable to the immune microenvironment (Dissanayake et al. 2008). β-catenin, especially mutant β-catenin, has been considered a melanoma antigen for some time and indeed was used to design some of the earlier tumor-infiltrating lymphocyte therapies (Robbins et al. 1996). A new study suggests that mutant β-catenin may actually do the opposite and decrease immune infiltration, but this has yet to be confirmed in other studies (Spranger et al. 2015). Overall, Wnt signaling plays critical roles in the development and pathogenesis of melanoma. The incredible complexity of this signaling pathway leads to its roles in multiple aspects of melanoma, from metastasis to therapy resistance.

Hippo Signaling

The Hippo signaling pathway (known as MST1/2 in humans) is important in mediating the interplay between contact inhibition and mitogenic signaling. Thus, it is the key pathway that regulates organ size during development and as such is highly conserved. MST1/2 are serine/threonine kinases that phosphorylate LATS1/2, which is activated and signals to phosphorylate and inhibit the activities of the Yes-associated protein (YAP) and the transcriptional coactivator with PDZ-binding motif (TAZ). Once YAP/TAZ signaling is inhibited they are sequestered in the cytoplasm via binding to 14-3-3 proteins. By contrast, unphosphorylated YAP is able to translocate to the nucleus and activate the transcription of a number of genes involved in mitosis and proliferation, such as cyclin A and cyclin E, as well as the TEA family of transcription factors. In addition to MST1/2 (Hippo), this signaling cascade can also be initiated by other MAP kinases, including MAP4K4/6/7 and MAP4K1/2/3/5 (Sanchez and Aplin 2014).

In cancer, the aberrant activation of YAP/TAZ leads to a loss of contact inhibition and subsequently, uncontrolled proliferation of tumor cells. This can be regulated in part by molecules involved in cell-cell adhesion, such as E-cadherin. As cells transition towards a more metastatic phenotype, they lose E-cadherin, allowing for increased activation of YAP/TAZ. YAP/TAZ are expressed in both melanocytes and melanoma cells, but are increased in metastatic disease. In one study, staining of YAP/TAZ in nevi and early stage melanoma revealed a mixed cytoplasmic/nuclear pattern with increased TAZ levels in more invasive lesions. Knockdown of YAP/TAZ led to a loss of tumorigenic and metastatic properties, while overexpressing YAP in melanoma cells increased their ability to metastasize to distant sites (Nallet-Staub et al. 2014).

The observed increase in invasion in YAP-overexpressing cells was attributed to the activation of TEA domain transcription factors, which recognize TEA/ATTS elements as their DNA binding domains (Lamar et al. 2012). YAP activation of TEADs was shown to increase transcription of the connective tissue growth factor, which is also overexpressed in metastatic melanoma, and thought to contribute to melanoma invasion. Transcriptome analysis of melanoma indicated that TEAD was a key regulator of the

invasive phenotype in melanoma (Lamar et al. 2012). In keeping with this, TEAD knockdown has been shown to decrease invasion and increase sensitivity of melanoma cells to targeted therapy. This suggests then that inhibition of the Hippo/YAP/TEAD pathway might be of interest for melanoma therapy. Indeed, the small molecule verteporfin, which inhibits the YAP-TEAD interaction, has been shown to decrease metastatic outgrowth of YAP overexpressing melanoma cells. Importantly, this drug may be effective not only in cutaneous melanoma but also in uveal melanoma, as YAP/TAZ signaling can be activated by mutant GNAQ/11, the predominant mutation in uveal melanoma. Verteporfin has been shown to inhibit the growth/invasion of uveal melanomas bearing GNAQ/11 mutations (Feng et al. 2014). This pathway holds great potential for the discovery of therapies that might affect multiple subtypes of melanoma.

The Notch Signaling Pathway

Like the Wnt pathway, the Notch pathway has been extensively studied in melanoma and can be divided into canonical and noncanonical pathways. Canonical Notch signaling is made up of the four transmembrane Notch receptors (Notch 1-4) and the membrane-bound ligands Jag 1 and 2 and delta-like (dll) 1-3. Signals transduced from these ligands, with the exception of dll3, are very similar, resulting in cleavage of notch by γ-secretase, thereby releasing the Notch intracellular domain (NICD). NICD interacts with MAML and other transcription factors in the nucleus, as part of a DNA binding complex known as the CSL (CBF1/Suppressor of Hairless/LAG-1) complex. One intriguing feature of this pathway is that there are no amplification steps in the signaling cascade, i.e., no phosphorylation of a number of different proteins. This, together with the fact that the receptor is cleaved in order to be activated such that one NICD is generated per activated receptor, means that any genetic alterations that change gene dosage can have dramatic effects (Ranganathan et al. 2011).

Unlike canonical Notch signaling, noncanonical Notch signaling occurs independently of CSL. The mediators of noncanonical Notch signaling are largely unknown, but the output is the activation of canonical Wnt signaling (β-catenin). This may have to do with the fact that Dsh, a key component of the Wnt signaling pathway, can bind to the NICD. NICD can also bind to components of the β-catenin destruction complex such as Axin and GSK3β, thereby affecting the stability of β-catenin. In turn, Wnt signaling can also regulate Notch signaling by upregulating ligands such as jagged1 and Dll4, as well as Notch itself (Borggrefe et al. 2016). Given the importance of Wnt signaling in melanoma, investigators explored the relevance of Notch in this disease. In melanocytes, deletion of Notch results in defects in melanoblast formation and the elimination of melanocyte stem cells. In addition to this, the localization of melanoblasts and melanocyte stem cells is also controlled by Notch signaling. Despite this, Notch1 is not expressed at high levels in melanocytes or benign melanocytic lesions, and forced overexpression of active Notch (Notch-NIC) can transform melanocytes both in the skin and in the retinoid pigmented epithelium (Bedogni 2014).

Notch signaling may also play roles in the metastatic progression of melanoma (Bedogni 2014). Notch 3 and Notch 4 have been implicated in angiogenesis and vasculogenic mimicry. Notch 1 was demonstrated to activate NFκB in pancreatic cancer. In melanoma, it has also been shown that NFκB can increase Notch expression via the PI3K/AKT pathway. Depletion of Notch in pancreatic cancer cells led not only to decreases in NFκB, but also in MMP-9, VEGF, survivin, and COX2. Since all of these molecules have also been implicated in metastatic dissemination of melanoma, it is reasonable to assume that a similar signaling cascade exists in melanoma. Indeed, treating melanoma cells with a γ-secretase inhibitor overcomes survivin expression and leads to apoptosis. In addition, forced activation of Notch in primary melanoma cells led to their progression to a metastatic state via the Wnt pathway, which as described earlier can also signal to increase Notch. Finally, Notch signaling can be activated in

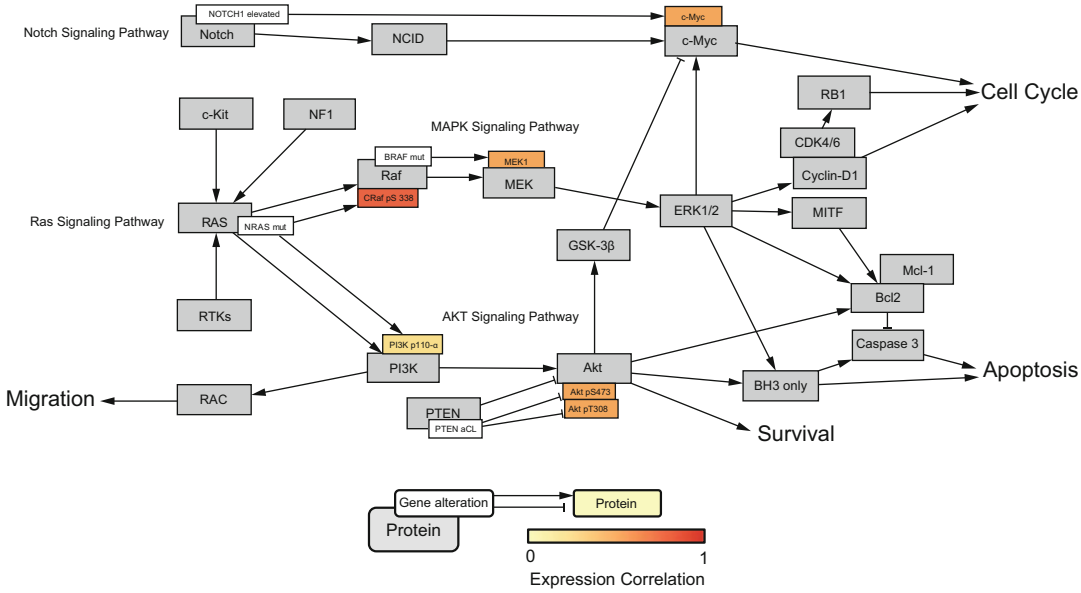

Fig. 3 A network map showing interactions between proteins in cutaneous melanoma signaling pathways. Individual proteins and phosphoproteins are colored according to the spearman correlation results between either mRNA or protein levels and the downstream protein levels for TCGA's cutaneous melanoma samples harboring a gene with a mutation (mut), copy loss (CL), or elevated expression level (elevated). The mutation profile, RNA Sequencing and protein data were generated by the TCGA Research Network: http://cancergenome.nih.gov/. Calculations were performed using Matlab® (v2015b). We acknowledge the help from Tim Purwin (Thomas Jefferson University) for the generation of this figure

situations known to promote metastatic progression such as hypoxia. HIF-1α can regulate Notch1, increasing its expression. This allows for the AKT-dependent transformation of melanocytes to a malignant state, and in the absence of Notch1 this transformation cannot occur (Bedogni et al. 2008).

In addition to hypoxia, other microenvironmental factors can regulate Notch expression in melanoma. It has been shown that fibroblasts transfected with active Notch1 increase their levels of the Wnt-inhibitory secreted protein WISP1. When this happens, Wnt signaling in adjacent melanocytes is inhibited, and their transformation to a malignant state does not occur (Shao et al. 2011). Finally, Notch is also an important player in the immune system as it is critical for hematopoietic stem cell self-renewal and lineage decisions (Bigas et al. 2013). It is not yet known how Notch in infiltrating immune cells may affect the survival, growth, and metastatic progression of melanoma. Understanding the interplay between Notch in the microenvironment and Notch in the tumor cell will be critical before therapies targeting this pathway can be conceived.

Conclusion

The heterogeneity and plasticity of melanoma is reflected in the vast complexity of the signaling pathways described herein (Fig. 3) and indeed in many other pathways considered of minor relevance that were not covered in this review. Studies to understand these signaling pathways mechanistically have provided vital insights into the acquisition of the malignant traits of the melanoma cell. Additionally, they have highlighted strategies to target oncogene addiction, which have translated into the development of novel therapies. The role of these signaling pathways is currently being interrogated for alterations in immune cell infiltration/activation in the melanoma tumor microenvironment.

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Epigenetic Regulation in Melanoma

5

Michelle Dang and Leonard Zon

Contents

Abstract

Cancer is the accumulation of genetic and epigenetic events that lead to the disruption of normal cellular development and homeostasis. Skin cancer originates from transformed melanocytes and is one of the few cancers with a significantly increasing incidence. Melanoma, the most aggressive form of skin cancer, accounts for the majority of skin cancer-related deaths, and the 5-year survival rate for late-stage melanoma is under 40%. While having some initial success in the clinic, targeted therapies and immunomodulators have a varied rate of response in patients and typically result in the development of drug resistance. The genomic landscape, including the identification of both driver and passenger mutations, in melanoma is well characterized. The epigenetic events that drive melanoma development and metastasis is an area of active research. The recent appreciation of epigenetic contributions to tumorigenesis focuses on DNA methylation, histone modifications, and noncoding

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RNAs. Here, we highlight key epigenetic mechanisms and how these epigenetic states can be exploited in (pre)clinical applications leading to novel therapeutic avenues.

Keywords
Melanoma · Epigenetics · DNA methylation · Histone modification · Noncoding RNAs

Introduction

Cancer is a complex disease of involving genetic, epigenetic, immune, and environmental factors. Melanoma, originating from transformed melanocytes, is the most aggressive form of skin cancer with an increase in incidence and an overall 40% 5-year survival rate in late-stage metastatic patients (Siegel et al. 2018). Localized melanomas detected early can be treated surgically, while unresectable melanomas are significantly more difficult to treat due to varying efficacies immunotherapy and targeted therapy. Immune checkpoint blockade is currently the first-line therapy for metastatic melanoma. Immunotherapies utilizing anti-CTLA-4 and anti-PD-1 antibodies have dramatically altered the clinical landscape of melanoma. Immunotherapy has become a frontline treatment for metastatic melanoma, but only 20–30% of patients have long-term benefit from the treatment (Hodi et al. 2010; Wolchok et al. 2017; Gide et al. 2017). Targeted therapies against oncogenic $BRAF^{V600E}$ and MEK can be used and resulted in unprecedented response rates in patients with metastatic melanoma, but these targeted therapies are ineffective long-term treatments due to the inevitable emergence of drug resistance (Robert et al. 2015; Konieczkowski et al. 2018). These challenges in melanoma therapy highlight the critical need to develop better combinations of therapies. A critical issue for the field is that resistance occurs for both drug classes, and methods to restore sensitivity have not been developed.

Genetic alterations to key signaling pathways can drive tumorigenesis by affecting cell cycle, proliferation, survival, and apoptosis. Extensive genomic sequencing studies such as The Cancer Genome Atlas (TCGA) and the Cancer Genome Project revealed melanoma as one of the most heavily mutated cancers per megabase and identified somatic and recurrent mutations (Network et al. 2015). The canonical RAS-RAF-MEK-ERK signaling cascade, also known as the mitogen-activated protein kinase (MAPK) pathway, promotes cellular proliferation and growth via a series of phosphorylation events and is the most frequently mutated pathways in cutaneous melanoma. BRAF, a serine/threonine kinase, is a critical regulator of the MAPK pathway, and approximately 50% of diagnosed cutaneous melanomas are due to an activating mutation on the BRAF oncogene ($BRAF^{V600E}$) (Davies et al. 2002). The second most common genetic driver of melanoma is the NRAS oncogene, where an activating glutamine to arginine (Q61R) mutation occurs in upward to 25% of cutaneous melanoma (Ball et al. 1994). These oncogenic mutations are also observed in benign nevi, suggesting that additional alterations including the loss of tumor suppressor genes are required for tumorigenesis. Sequencing studies identified recurrent mutations in tumor suppressor genes such as CDKN2A, PTEN, and TP53, and the loss of these are critical in melanomagenesis (Hodis et al. 2012; Network et al. 2015). Over the past decade, extensive sequencing studies of the cancer genome have provided significant insight into genetic alterations driving melanoma as well as the identification of passenger mutations.

While genetics has driven the understanding of human development and diseases, it cannot completely explain the complexities of these biological processes. Recent characterization of epigenetic mechanisms has solidified its role in the regulation of normal developmental programs and has been increasingly linked to tumorigenesis. Epigenetics, a concept first introduced in 1939, is defined as heritable and reversible changes that do not alter the DNA sequence. In fact, epigenetic events can dynamically regulate gene expression through alterations to chromatin via DNA methylation, covalent modifications to histones such as acetylation or methylation, and regulation of mRNA translation via noncoding RNAs. This higher-order level of regulation can promote

tumorigenesis, including initiation, maintenance, metastasis, and drug resistance. Understanding the role epigenetics plays and characterizing the melanoma epigenome will help in the development of novel biomarkers and therapeutic targets. This chapter aims to highlight the foundations of epigenetics in melanoma and how these epigenetic states can be exploited in (pre)clinical applications leading to novel therapeutic avenues.

DNA Methylation

DNA methylation, a powerful epigenetic process to regulate gene expression, is defined as the addition of a methyl group to the 5-carbon position of cytosine to generate 5-methylcytosine (5-mC) and is mediated by the DNA methyltransferase (DNMT) enzyme. DNMT1 functions to maintain methylation states by copying DNA methylation during cell division, while DNMT3A/B functions as de novo methyltransferases. This covalent modification typically occurs on regions enriched with repeats of cytosine guanine dinucleotides (CpG islands), and these CpG islands occur in the promoter region of 40% of mammalian genes (Fatemi et al. 2005). Global DNA methylation patterns in human cancers were first characterized in 1983 (Feinberg and Vogelstein 1983). A study in 1989 identified focal hypermethylation in the promoter of the retinoblastoma (RB) gene, which is normally involved in suppressing cellular growth, in patients and provided the first link between DNA methylation and cancer (Greger et al. 1989). It is widely accepted that DNA hypermethylation at the promoter silences gene expression via transcriptional repression (Fig. 1).

Epigenetic Silencing of Tumor Suppressor Genes

Epigenetic silencing is a major driver of tumorigenesis, and promoter hypermethylation of key tumor suppressor genes has been demonstrated in melanoma through targeted and genome-wide studies. The major pathways that are impacted by aberrant methylation include MAPK, phosphatidylinositol 3-kinase (PI3K), cell cycle, DNA repair, apoptosis, and metastasis. While there is increasing appreciation for the role epigenetics, specifically DNA methylation, plays in tumorigenesis, a shortcoming of the field is the mechanistic and functional understanding of these modifications. Major questions that remain include how DNA methylation is regulated in cancer cells and which transcriptionally repressed genes are required for melanoma initiation and maintenance.

Early efforts in identifying the methylation status at specific loci utilized a sodium bisulfite-dependent PCR assay that converted non-methylated cytosine bases to uracil, and the regions of interest would be PCR amplified and sequenced. One of the first tumor suppressors in melanoma identified to be hypermethylated is cyclin-dependent kinase inhibitor 2A (CDKN2A), which encodes both the $p16^{INK4A}$ (p16) and $p14^{ARF}$ (p14) proteins. p16 is a negative regulator of the cell cycle through CDK4 and CDK6 inhibition, and p16 promoter methylation was reported in 25% of analyzed cutaneous melanoma samples (Gonzalgo et al. 1997). p14 is responsible for inhibiting MDM2-mediated p53 ubiquitination and proteasomal degradation, and the p14 promoter is hypermethylated in 57% analyzed cutaneous melanoma samples (Freedberg et al. 2008). Promoter hypermethylation of CDKN2A was observed in cutaneous melanoma of the vertical growth phase (Straume et al. 2002), but not reported in nevi (Conway et al. 2011). Taken together, it suggests that tumor suppressor CDKN2A is expressed in nevi and silenced during melanoma development.

Phosphatase and tensin homologue (PTEN) is another major tumor suppressor gene that has been heavily linked to the development of melanoma. PTEN is a phosphatase that negativity regulates the PI3K signaling pathway and is significantly lost in melanoma. Methylation-specific PCR on human primary and metastatic melanomas identified approximately 60% of promoter methylation of PTEN. Upon treatment with a demethylation agent, PTEN expression levels normalized, and the aberrant PI3K activity ceased (Mirmohammadsadegh et al. 2006). Interestingly,

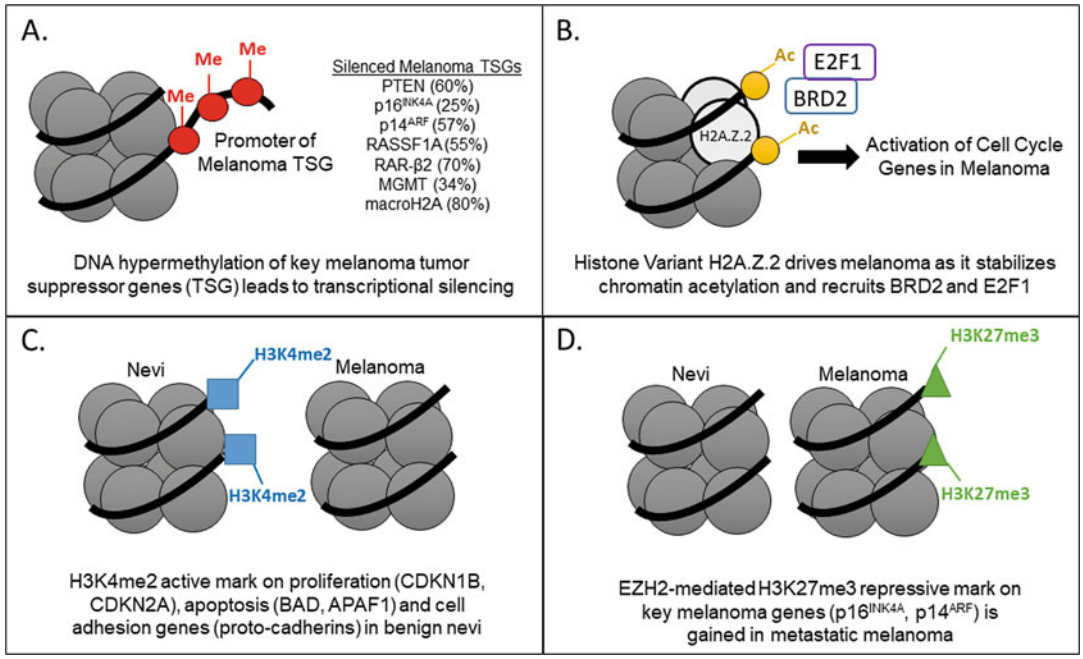

Fig. 1 Epigenetic modifications to regulate transcription. (**a**) DNA promoter hypermethylation of key melanoma tumor suppressor genes results in transcriptional silencing. (**b**) Histone variant H2A.Z.2 drives melanoma as it stabilizes chromatin acetylation and recruits BRD2 and E2F1 to activate cell cycle genes. (**c**) The H3K4me2 active mark is present on key proliferation, apoptosis, and cell adhesion genes in benign nevi but is lost upon tumorigenesis. (**d**) The H3K27me3 repressive mark on key melanoma regulatory genes is gained during melanoma development

the loss of PTEN from promoter methylation is significantly correlated to poor survival (Lahtz et al. 2010).

Ras-associated domain family protein 1A (RASSF1A) is involved in cell cycle regulation and apoptosis and found to be frequently silenced via promoter hypermethylation in several cancers (Hesson et al. 2007). Methylation-specific PCR analyzed 11 human melanoma cell lines and 44 metastatic melanomas and confirmed RASSF1A as a tumor suppressor gene in melanoma. The RASSF1A promoter was hypermethylated in 55% melanoma tumors and correlated with loss of RASSF1A expression. Interestingly, serial biopsies were conducted in a single patient with stage 3 metastatic melanoma. The early biopsies revealed no RASSF1A promoter methylation, but a later biopsy showed hypermethylation at the RASSF1A promoter (Spugnardi et al. 2003). While this was observed in only one patient, it suggests that RASSF1 promoter hypermethylation may not be required for melanoma initiation but more involved in melanoma maintenance and/or metastasis.

Methylation-specific PCR was used on 15 human melanoma cell lines and 130 cutaneous mixed-stage melanomas to assess the tumor suppressive role of candidate factors that had been previously studied in carcinomas (Esteller et al. 2001). The screen identified three hypermethylated tumor suppressor genes in melanoma: retinoic acid receptor-β2 (RAR-β2), RASSF1A, and O^6-methylguanine DNA methyltransferase (MGMT). RAR-β2 was hypermethylated in 70% of the melanoma samples and was consistent across both primary and metastatic samples, suggesting RAR-β2 may be important in melanoma development and maintenance. Additional studies on normal melanocytes and benign nevi can provide more insight in RAR-β2's role in melanoma initiation. The second most hypermethylated gene identified in this study was RASSF1, which was previously reported in an independent study. RASSF1 was

hypermethylated in 57% of melanomas and was more closely associated with metastatic melanomas compared to earlier staged samples, which suggests that RASSF1 is progressively lost via DNA methylation during the metastasis stage in melanoma. Finally, MGMT was silenced in 34% of melanomas and is involved in DNA repair and maintaining genomic stability (Hoon et al. 2004). This study is the first to assess the methylation status of known tumor suppressor genes in the context of melanoma. These findings open up avenues to mechanistically study the methylated tumor suppressor genes, how they are functionally involved in melanoma development and metastasis, and whether they can be specifically targeted with hypomethylating agents.

Genome-Wide Methylation Analysis in Melanoma

The characterization and differential analysis of the methylation landscape of normal human melanocytes, benign nevi, noninvasive melanomas, and metastatic melanomas can further the molecular understanding of tumor initiation, progression, and metastasis. Furthermore, extensive studies of these methylation patterns can be correlated with stages of the disease and consequently be used as biomarkers for disease progression. The first genome-wide methylation study was conducted in 2003 using methylated DNA immunoprecipitation (MeDIP) and identified 68 hypermethylated and 8 hypomethylated genes using early passage human melanoma cell lines (Koga et al. 2009). While methylation patterns identified in the cell lines were independently validated using methylation-specific PCR, it is unclear whether these aberrantly methylated genes are a consequence of in vitro passaging or if they are consistent with primary samples.

Another genome-wide approach is called methylated-CpG island recovery assay (MIRA)-seq. It is a high-throughput method to identify the DNA methylation landscape by using protein complexes (MBD2B and MBD3L1) with high affinity for methylated CpG islands and next-generation sequencing (NGS) (Rauch and Pfeifer 2010). A MIRA-seq study conducted on metastatic melanoma samples from 27 donor patients sought to characterize the DNA methylation landscape of metastatic melanoma and identified 179 melanoma-specific methylation events that were present in all 27 donor samples and absent in the normal tissues assayed. Over 3000 methylated regions were identified in over 40% of the melanoma samples assayed, and a closer look into these methylated genes revealed an enrichment for melanoma differentiation factors such as SOX10, KIT, and PAX3 (Jin et al. 2016). This discovery-oriented study demonstrates the potential in identifying melanoma-specific biomarkers as well as identifying epigenetically modulated genes involved in tumorigenesis. Limitations of this study include the quantity of donor samples queried and the use of normal melanocytes as the baseline control. Since the normal melanocyte originated from three donors, it is likely that methylation patterns in the normal melanocytes are significantly varied and patient specific. Future studies may utilize donor-matched samples comparing benign nevi to normal melanocytes, primary melanoma to nevi, and metastatic to primary melanoma results in more stage-specific methylation patterns.

The dynamics of DNA methylation have been implicated in driving cancer metastasis, yet the specific mechanism is poorly understood. Using bead capture-based technology called Illumina's Infinium HumanMethylation450 (450 K BeadChip), the DNA methylation status of over 450,000 CpG islands were characterized for primary tumors, brain metastasis, and lymph node metastasis derived from melanoma patients. Interestingly, promoter hypermethylation of HOX family members, specifically HOXD9, was observed to be significantly higher in metastatic samples compared to noninvasive samples. Patients with observed hypermethylation HOXD9 correlated with poor survival and could potentially serve as a prognostic factor (Marzese et al. 2014). While the misregulation of HOX transcription factors has been demonstrated in human melanoma compared to benign nevi, the mechanism is poorly understood.

It is known that the melanocyte-specific transcription factor microphthalmia-associated

transcription factor (MITF) plays a critical role in melanocyte development and differentiation (Steingrimsson et al. 2004). Amplification of oncogenic MITF is found in 10% of melanomas and correlated with poor survival (Garraway et al. 2005). It is clear that MITF plays a crucial role in melanocyte biology, as well as melanomagenesis, but the mechanisms of how MITF levels are regulated are incompletely understood and likely involve epigenetic perturbations. The methylation landscapes of melanoma tumors compared to cultured melanocytes revealed global hypomethylation in gene bodies and hypermethylation enriched in the promoter of developmental target genes. MITF and its target genes were shown to be hypermethylated in the melanoma samples. Demethylation of MITF using 5′-aza-2-′-deoxycytidine (5-Aza) was sufficient to restore MITF protein levels (Lauss et al. 2015). This study suggests that MITF expression is regulated by DNA methylation during melanoma development.

Undeniably, the characterization of methylation profiles in matched normal melanocytes, benign nevi, malignant primary tumors, and metastatic tumors in patients has been monumental in understanding the epigenetic contribution to melanomagenesis. The genome-wide studies have been critical in discovering differentially methylated genes, but a major current limitation is understanding which of the aberrantly methylated genes are functionally relevant in driving melanoma initiation, maintenance, and metastasis. Understanding when the changes in the methylation pattern occurred can help define if the modulation is driving an oncogenic phenotype or merely a consequence of dysregulation. Due to the immense heterogeneity of cancers, more patient-matched samples from the various oncogenic stages must be studied before developing a reliable panel of biomarkers for disease progression and response to therapy.

DNA Demethylation

Since epigenetic modifications are dynamic and reversible, DNA demethylation contributes as an important regulatory mechanism in both normal cellular development and cancer (Szyt et al. 2004). DNA demethylation could promote tumorigenesis by reactivating an oncogene that is normally silenced. For example, melanoma-associated antigen 1 (MAGEA1) is a cancer/testis antigen that is typically expressed in the testis but is shown to be upregulated in melanoma tumors. Metastatic melanomas from 56 patients were subjected to methylation-specific PCR to determine the methylation status of MAGE family members and demonstrated gene reactivation of MAGEA1 via DNA promoter hypomethylation. The reactivation of the MAGEA1 in melanoma promotes cellular proliferation (Sigalotti et al. 2004).

DNA demethylation of 5-mC is catalyzed by a family of hydroxylases called ten eleven translocation (TET) proteins into 5-hydroxymethylcytosine (5-hmC) (Tahiliani et al. 2009). TET activity is dependent on alpha-ketoglutarate (α-KG), and α-KG production is controlled by isocitrate dehydrogenases (IDH1 and IDH2) (Xu et al. 2011). Mutations in these IDH1 and IDH2 enzymes have been shown to disrupt TET activity and lead to hypermethylated DNA in acute myeloid leukemia (Figueroa et al. 2010; Ward et al. 2010). In melanoma, TET proteins are mutated in over 20% of cases (Song et al. 2014). From TCGA studies, IDH1 is found to be recurrently mutated in ~6% melanoma, and IDH1-mutated samples exhibited high CpG hypermethylation (Network et al. 2015). To address the demethylation patterns in melanoma, levels of 5-hmC were detected and quantified using immunofluorescent (IF) and immunohistochemical (IHC) staining in over 50 cases of benign nevi, primary melanomas, and metastatic melanomas. The study revealed a global loss of 5-hmC, and this trended with melanoma progression, as the primary and metastatic samples had a significant loss of 5-hmC staining compared to the benign nevi samples. The loss of 5-hmC staining correlated with downregulated TET protein and IDH2 expression levels. Interestingly, human melanoma cell lines overexpressing either TET2 or IDH2 were sufficient to restore 5-hmC levels, and the xenograft mice exhibited reduced proliferation (Lian et al. 2012). This study suggests that

the loss of TET2 or IDH2 is linked with melanoma progression and identified global loss of 5-hmC as a potential diagnostic marker for melanoma.

DNA Methyltransferase (DNMT) Inhibitors

A major class of epigenetic drugs are hypomethylating agents, such as DNMT inhibitors. Essentially, DNMT inhibitors can activate genes that were silenced by DNA methylation, and the re-expression of these genes can potentially reverse the tumorigenic effect. The most popular DNMT inhibitors are 5-azacitidine and decitabine and were approved for the treatment of myelodysplastic syndrome (Raj and Mufti 2006; Saba 2007). While these demethylating agents have shown efficacy in blood malignancies, their role in the context of solid tumors is challenging. Recent clinical trials investigating epigenetic drugs are summarized in Table 1.

A phase I trial in melanoma patients sought to determine the toxicity and antitumor effect of a hypomethylating agent combined with a FDA-approved intervention for metastatic melanoma. Escalating dosing of decitabine and two cycles of high-dose interleukin-2 (IL-2) was administrated to patients and demonstrated a 31% objective response in melanoma patients with minimal toxicity (Gollob et al. 2006). This is the first study to support the tolerance of combination DNMT and IL-2 treatments in melanoma patients.

The clinical landscape for the treatment of metastatic melanoma has been evolving quickly with the FDA approval for dual BRAF/MEK combination therapy, as well as anti-CTLA4 and anti-PD1 immunomodulators. Accordingly, more recent DNMT inhibitor clinical trials aim to assess the safety and efficacy in combination with these BRAF/MEK inhibitors and immunotherapies. For example, a phase Ib trial sought to determine the toxicity and antitumor effect of a DNMT inhibitor (decitabine) in combination with a $BRAF^{V600E}$ inhibitor (vemurafenib). Patients with mutant BRAF melanoma were administered two cycles of escalating doses of decitabine in combination with the normal dosing of vemurafenib. Cohorts that received the lowest dose of decitabine exhibited minimal toxicity and the largest partial response (Zakharia et al. 2017). This study demonstrates the safety of combining low-dose DNMT inhibitors to existing targeted therapies. Additional studies with larger patient cohorts must be conducted to address whether this addition of low-dose decitabine has the potential to prevent or delay drug resistance to vemurafenib.

While DNMTs have shown efficacy in blood malignancies, challenges in dosing and specificity have proven to be a major challenge in solid tumors including melanoma. Low-dose and long-term administration of DNMT seems to have potential, but long-term effects of hypomethylation are undocumented. Additional studies in patients must be conducted to identify the therapeutic window, as there is a delicate balance between toxicity and efficacy. In addition to dosing, an even larger limitation to decitabine and other DNMT inhibitors is the broad-spectrum effect. The lack of specificity of these DNMT inhibitors results in global hypomethylation. These drugs will nonselectively inhibit DNA methylation and consequently have the potential to unintentionally activate both oncogenes and tumor suppressor genes.

Histone Modifications

In addition to DNA methylation, a major epigenetic mechanism to regulate transcription is post-translational modifications on the chromatin. DNA is wound around highly alkaline histone protein octomers (H2A, H2B, H3, H4) to form nucleosomes which are then compacted to form chromatin. It has been known for decades that posttranslational modification on the chromatin can regulate DNA transcription. The type of modification and the location of the mark yield specific consequences, ranging from impacting DNA accessibility to the transcriptional machinery to recruiting transcriptionally repressive or activating protein complexes.

Table 1 Overview of clinical trials of epigenetic drugs in melanoma

Agents	Indication	Phase	Status	Clinical trial ID
DNMT inhibitor (decitabine)	Stage III/IV melanoma	I	Completed	NCT00002980
Decitabine, temozolomide	Stage III/IV melanoma	I/II	Completed	NCT00715793
Decitabine, vemurafenib	BRAF-positive metastatic melanoma	I	Completed	NCT01876641
DNMT inhibitor (azacitidine), pembrolizumab	Stage III/IV melanoma	II	Recruiting	NCT02816021
Azacitidine, recombinant interferon alfa-2b	Stage III/IV melanoma	I	Completed	NCT00217542
Pan-HDAC inhibitor (vorinostat)	BRAF-positive metastatic melanoma, progressed on BRAF and MEK inhibitors	I/II	Recruiting	NCT02836548
Pan-HDAC inhibitor (panobinostat)	Melanoma	I	Completed	NCT01065467
Panobinostat, ipilimumab	Stage III/IV melanoma	I	Active	NCT02032810
Selective HDAC6 inhibitor (ACY-241), ipilimumab, nivolumab	Stage III/IV melanoma	I	Completed	NCT02935790
HDAC1/2/3/11 inhibitor (mocetinostat), ipilimumab, nivolumab	Stage III/IV melanoma	Ib	Recruiting	NCT03565406
HDAC inhibitor (MS-275)	Progressed on immunotherapy or chemotherapy	II	Completed	NCT00185302
HDAC1/3 inhibitor (entinostat), pembrolizumab	Progressed on PD-1 or PD-L1	I/II	Recruiting	NCT02437136
Vorinostat	Uveal melanoma	I	Recruiting	NCT03022565
Entinostat, pembrolizumab	Uveal melanoma	II	Recruiting	NCT02697630

The best studied histone modifications are acetylation and methylation, but other modifications include phosphorylation, ubiquitylation, SUMOylation, and deimination. The acetylation on lysine residues is facilitated by histone acetyltransferases (HAT) and histone deacetylases (HDAC) and tends to occur on residues on the H3 and H4 histones. This acetylation is associated with open chromatin and tends to promote positive gene expression by allowing transcription factor binding. For example, acetylation of lysine 9 on histone 3 (H3K9ac) and acetylation of lysine 27 on histone 3 (H3K27ac) mark active enhancers and are associated with open chromatin and active gene transcription.

The other major histone modification is H3 and H4 methylation on lysine residues and is demonstrated to either activate or repress transcription depending on the specific residue and the number of methyl groups present. The methylation is mediated by histone methyltransferases (HMT) and histone demethylase. Specific histone methylation marks are associated with transcriptional states. For example, tri-methylation of lysine 4 on histone 3 (H3K4me3) occurs at the gene promoter and is associated with active transcription. Monomethylation of lysine 4 on histone 3 (H3K4me1) occurs at enhancer sites and is also indicative of active transcription. Histone modifications can also indicate transcriptionally repressive states, such as tri-methylation of lysine 27 on histone 3 (H3K27me3) and tri-methylation of lysine 9 on histone 3 (H3K9me3).

The dysregulation of histone methyltransferases have been linked to cancers, but their exact role in tumorigenesis is unclear (Albert and Helin 2010). The histone methyltransferase SETDB1 was identified while assessing the oncogenic potential of genes in a recurrently amplified region in melanoma. SETDB1 acts on H3K9me, and its overexpression was sufficient to accelerate melanoma onset in a $BRAF^{V600E}$ zebrafish model.

Microarray analysis of melanomas overexpressing SETDB1 tumors exhibited transcriptional dysregulation of HOX genes, and tissue microarrays on normal melanocytes, benign nevi, and melanomas revealed a strong correlation between SETDB1 expression and melanoma progression (Ceol et al. 2011) This study identified an oncogenic histone methyltransferase important in the progression of melanoma.

Another histone methyltransferase is enhancer of zeste homologue 2 (EZH2), and it is responsible for the repressive H3K27me3 mark. EZH2 expression was characterized via immunostaining on benign nevi, melanoma in situs, and metastatic melanomas. EZH2 expression was significantly higher in metastatic melanomas compared to the nevi, suggesting a link between EZH2 and melanoma progression. Knockdown of EZH2 in cell lines resulted in a global decrease of H3K27me3 repressive mark and an increase in the active H3K14ac mark on p21/CDKN1A. EZH2 drives tumorigenesis by repressing senescence through histone acetylation on the p21/CDKN1A locus (Fan et al. 2011). The potential role of EZH2 inhibitors in melanoma is being investigated.

Characterization of known epigenetic marks in non-transformed human melanocytes and tumorigenic melanomas revealed specific chromatin states linked with tumorigenesis. Comparing the distinct tissues revealed that the nevi harbor histone acetylation and H3K4 methylation on cancer regulatory genes. Most interestingly, these marks are lost upon melanoma transformation. The administration of histone deacetylase inhibitors prevented proliferation of melanoma cells, which suggests the histone deacetylation is functionally important in driving tumorigenesis in melanoma (Fiziev et al. 2017).

Increasing evidence has implicated the role of histone modifiers in melanoma progression and response to therapy. A CRISPR-Cas screen was conducted to screen for chromatin regulators important in driving resistance to MAPK inhibitors and identified histone deacetylase SIRT6. Partial loss of SIRT6 allowed for melanoma cell lines to proliferate in the presence of MAPK inhibitors. Loss of the histone deacetylase resulted in increased chromatin accessibility via H3K65 acetylation at the IGFBP2 locus. The acetylation mark resulted in downstream IGF-1R activation and AKT signaling. The SIRT6-mediated resistance phenotype was abrogated with an IGF-1R inhibitor in combination with $BRAF^{V600E}$ inhibition. Interestingly, high expression of IGFBP2 correlated with drug resistance in matched melanoma samples from patients receiving combination dabrafenib and trametinib therapy (Strub et al. 2018). This screen not only identified a histone deacetylase implicated in melanoma resistance but also showed the potential of IGF-1R inhibitor as a therapeutic strategy.

Bromodomain (BRD)-containing proteins are a major class of histone readers that facilitate the chromatin remodeling by binding acetylated histones. BRD4 is a histone reader of the bromodomain and extra terminal domain (BET) family and is significantly upregulated in primary and metastatic melanoma compared to nevi, suggesting BRD4 could act as an oncogenic contributor to melanoma progression. Treatment of metastatic melanoma cell lines with a small molecule inhibitor of BET proteins inhibited proliferation. Also, administration of the BET inhibitors led to a fivefold decrease of tumor growth and reduced numbers of lung metastasis in a xenograft mouse model. Withdrawal of the BET inhibitors in both the cell line and xenograft model resulted in a restoration of pro-proliferation phenotypes. Specific knockdown of BRD4 recapitulated the chemical studies and demonstrated that BRD4 plays a role in melanoma progression. Interestingly, the oncogenic effects of BRD4 upregulation and the efficacy of BET inhibitors were consistent in cutaneous melanomas driven by $BRAF^{V600E}$, NRAS, or neither (Segura et al. 2013). The identification of upregulated BRD4 as a pro-oncogenic factor in melanoma development and progression could represent a novel therapeutic strategy that is independent of BRAF or NRAS mutational status.

Histone Deacetylase (HDAC) Inhibitors

The involvement of histone modifications in cancer progression has made small molecule

inhibitors targeting HDACs therapeutically promising. HDACs represent the largest group of epigenetic drugs and are thought to exert antitumor effects by promoting cell cycle arrest and apoptosis. Ongoing clinical trials are evaluating the effect of HDAC inhibitors as single agents in treatment solid tumors, as well as the safety and efficacy of HDAC inhibitors in combination with FDA-approved targeted inhibitors and immunotherapies.

Suberoylanilide hydroxamic acid (SAHA) also known as vorinostat was approved for the treatment of T-cell lymphoma in 2006, and this fueled the investigation into the use of HDAC inhibitors for solid tumors (Mann et al. 2007). Patients with solid tumors were administered escalating doses of vorinostat for 6 months in a phase I clinical trial. Of the six melanoma patients in this cohort, two exhibited stable disease for over 8 months upon vorinostat treatment, suggesting that HDAC inhibition could be therapeutically beneficial (Munster et al. 2009). Subsequently, a phase II clinical trial of vorinostat in 32 patients with metastatic melanoma aimed to assess the clinical benefit of HDAC inhibition. Vorinostat was administered for 28 consecutive days per cycle and mostly resulted in stable disease or a partial response with an average 5 months of progression-free survival. Overall, the treatment exhibited moderate toxicity in over half the patients and an unimpressive efficacy in the cohort (Haas et al. 2014). The poor efficacy in the phase II clinical trial highlights the current challenges of demonstrating clinical efficacy of epigenetic drugs. More extensive characterization in preclinical models may provide insight into what melanoma patient subtypes would be most beneficial to the therapy in question. Using in vitro and in vivo preclinical models, melanomas can be categorized by anatomical subtype (cutaneous, acral, mucosal, ocular), mutational status ($BRAF^{V600E}$, NRAS, "wild type"), and disease stage (nonmetastatic, lymph node metastasis). Furthermore, the correlation of each of these categories with response to HDAC inhibitors can better inform patient eligibility for future clinical trials.

Investigation of HDAC inhibitors in combination with targeted therapies and immunotherapies may be more promising. Resistance to BRAF and MEK inhibition is a major clinical challenge in the treatment of melanoma. The major resistance mechanisms include reactivation of the MAPK signaling pathway and activation of the PI3K/AKT survival pathway. To determine if HDAC inhibition could impact drug resistance, patient-derived melanoma cell lines were co-treated with an HDAC inhibitor (panobinostat) and $BRAF^{V600E}$ inhibitor (encorafenib). Panobinostat is a pan-HDAC inhibitor that targets class I, II, and IV HDACs. In human melanoma cell lines and xenograft models that stopped responding to BRAF inhibition, panobinostat was sufficient to restore encorafenib sensitivity. The combination was found to increase caspase-dependent apoptosis by altering PI3K signaling. Western blot analysis revealed that the response correlated to an upregulation of pro-apoptotic BIM or NOXA with reduced expression of anti-apoptotic proteins Bcl-XL (Gallagher et al. 2018). This preclinical study provides a rationale for HDAC inhibition in treatment-resistant melanomas.

In a phase I clinical trial to assess the safety and dosing of panobinostat, patients with late-stage melanoma were dosed with 30 mg 3 days a week. Due to severe toxicity, including thrombocytopenia, the treatment scheme was redesigned for 30 mg panobinostat for 3 days a week every other week. Patients were treated for a median of 1.9 months, and all developed progressive disease, demonstrating that panobinostat is an ineffective single agent for the treatment of late-stage melanoma (Ibrahim et al. 2016). Future clinical trials could assess the combination of HDAC inhibitors in combination with BRAF/MEK inhibitors. The severe toxicity in patients dosed with 30 mg panobinostat suggests that the pan-HDAC inhibitor has too many off-target effects for clinical use. The use of selective HDAC inhibitors may minimize toxicity. For example, entinostat is a less toxic selective HDAC I and IV inhibitor and is currently in a phase Ib/II clinical trial (NCT02437136) for melanoma patients who are progressing on anti-PD1 immunotherapy. Future

studies could focus on the development of better epigenetic drugs and more stringent preclinical models, as the lack of specificity and efficacy in these HDAC inhibitors poses as a major clinical challenge.

Histone Variants

Traditional histones H2A, H2B, and H3 can be replaced with histone variants, and this switch potentially changes the posttranslational modifications to provide a higher degree of regulation. This physical change of the chromatin landscape is thought to play a key role in transcriptional regulation, and there are two major histone variants that have been implicated in melanoma: macroH2A (mH2A) and H2A.Z.2. The first histone variant identified as a tumor suppressor in melanoma is mH2A. Global expression analysis in human melanoma cell lines and melanoma mouse models identified a striking decrease in mH2A expression, resulting in enhanced invasion in cell lines and increased metastasis in mice. The loss of mH2A is due to DNA hypermethylation at the mH2A promoter. Moreover, expression of mH2A was significantly decreased in human melanoma tissues and was lost in over 80% of metastatic melanoma patients. Interestingly, this decrease in mH2A corresponded to an increase in CDK8 expression. The characterization of mH2A in melanoma revealed its tumor suppressive role and demonstrated that methylation silencing of this histone variant mediated CDK8-driven proliferation and invasion (Kapoor et al. 2010). The identification of CDK8 as a mH2A target could support a potential therapeutic avenue. Additionally, the histone variant H2A.Z.2 is highly expressed in metastatic melanoma, and patients with high expression of this histone variant demonstrated lower survival rates. Knockdown studies resulted in cell cycle arrest and a decrease in proliferation. Also, microarray analysis of melanoma cell lines identified that H2A.Z.2-regulated genes were transcriptional targets of E2F1 and E2F4, both of which have been implicated in melanoma (Vardabasso et al. 2015).

Chromatin-Interacting Protein Complexes

Protein complexes, such as the SWI/SNF and the polycomb repressive complexes, have the ability to dynamically modulate transcriptional activity by interacting with the chromatin architecture. Aberrant expression and dysregulation of these proteins can alter normal transcription and promote tumorigenesis.

The most studied chromatin remodeler is the ATP-dependent Brg/Brahma-associated factor (BAF) complex, also referred to as the SWI/SNF complex. SWI/SNF complex regulates transcription by directly binding to the DNA and nucleosomes to modulate positioning and accessibility. SWI/SNF complex consists of 15 subunits and can exist in two forms depending on their core subunits. While most of the subunits are shared, the BAF complex exclusively contains BRM, ARID1A, and ARID1B. Meanwhile polybromo-BAF (pBAF) exclusively contains PBRM1, ARID2, and BRD7. It is hypothesized that the various combinations of subunits that constitute the SWI/SNF complex allow for a higher degree of specificity and regulation. The subunits of the SWI/SNF complex are mutated in approximately 20% of human cancers, including but not limited to breast, lung, colon, and skin cancer. The various mutations on each of the SWI/SNF subunits appear to be cancer-specific and tend to illicit tumor suppressive effects (Kadoch et al. 2013). The complex is mutated in over 30% of melanomas, and ARID2 mutations are found in 12% of melanomas (Hodis et al. 2012). BRG1/SMARCA4 mutated in 5–10% of melanomas. The BRG1 subunit of the complex is tumor suppressors that are repressed in melanoma. BRG1 binds p16, suggesting RB1- and E2F-dependent mechanisms for cell cycle regulation. SMARCB1 is downregulated in melanoma and evades apoptosis. SMARCB1-positive protein expression correlated with better overall and disease-free survival time (Kadoch et al. 2013).

The polycomb repressive complex (PRC) recognizes histone modifications and serves as transcriptional repressors in both normal development and tumorigenesis. Both PRC1 and PCR2

transcriptionally repress by binding and tri-methylating the H3K27 histone. The major subunit of the PRC1 is BMI-1, which is a regulator of p16/INK4a and p14/ARF. Immunohistochemistry on benign and malignant melanocytes revealed that BMI-1 is highly expressed in benign nevi, and it is lost during melanoma development, and it correlated with poor survival (Bachmann et al. 2008). The catalytically active subunit of the PRC2 is EZH2, a methyltransferase responsible for the transcriptionally repressive H3K27me mark. In contrast to BMI-1, high expression of EZH2 is correlated with metastatic diseases, and high expression correlates with poor survivals. H3K27 histone demethylases, such as Jumonji domain-containing 3 (JMJD3), have the ability to reverse EZH2-mediated repression (Barradas et al. 2009).

Noncoding RNA

Approximately 90% of the human genome is transcribed into noncoding RNAs (ncRNAs), and they represent a diverse group of posttranscriptional regulators. There are two major classes of ncRNAs based on their nucleotide length: microRNAs (miRNAs) and long noncoding RNAs (lncRNAs). miRNAs are 22-nucleotides long and function as regulators of gene expression by controlling mRNA stability and degradation. These pre-miRNA hairpins are processed by Dicer into mature miRNAs and subsequently loaded onto the RNA-induced silencing complex (RISC). The loaded miRNA targets complementary sequences on the 3′UTR to of transcripts, resulting in degradation (Bartel 2004).

Dysregulation of these miRNAs has been observed in cancer progression and resistance to therapy. Importantly, while there are over 2,500 annotated miRNAs in the human genome, the specific expression and activity of each miRNAs are tissue and disease-stage specific (Croce 2009). Understanding the miRNA landscape across normal tissue and various stages of cancer can serve as powerful biomarkers for disease staging and prognosis. The advancements of sequencing technologies have spearheaded the generation of global miRNA analysis through microarray and RNA-seq studies and have identified hundreds of miRNAs linked to cancer. Interestingly, miRNAs can harbor either oncogenic or tumor suppressive roles and may drive oncogenesis through a myriad of mechanisms including sustained proliferation, evasion of cell death, and invasion or metastasis.

In melanoma cell lines, overexpression of miR-221/222 has been shown to silence the expression of tumor suppressor gene p27 to positively regulate cell cycle and suppress apoptosis (Felicetti et al. 2008). Subsequent serum analysis in 72 cutaneous melanoma patients identified serum levels of miR-221 were significantly higher compared to serum extracted from 54 healthy subjects. The high serum levels of miR-221 correlated with advanced clinical stage, metastasis, and poor overall 5-year survival rate (Li et al. 2014). The identification of miR-221 overexpression in melanoma samples and the correlation of miR-221 serum levels to prognosis serve as an early example of how miRNAs can serve as biomarkers for melanoma prognosis.

MITF, the master regulator of the melanocyte lineage, plays an undisputed role in normal melanocyte development and melanomagenesis. While it is accepted that expression levels of MITF are variable across melanoma cell lines, stages of primary melanoma, and even in drug-resistant melanoma, the mechanisms that regulate MITF are incompletely understood. miRNAs represent a class of regulators that could mechanistically control expression levels of MITF. One group utilized computational methods to identify all miRNAs located within a genomic region that was previously associated with melanocyte biology. Their efforts identified a miR-137 in the target region, and subsequent gene expression studies found miR-137 to be significantly upregulated in melanoma compared to benign nevi. Further analysis revealed that miR-137 downregulates the oncogenic transcription factor MITF (Bemis et al. 2008). A similar systematic method to identify functionally relevant miRNAs focuses on genomic regions that are lost or gained in melanoma. This method revealed that miR-182 is frequently upregulated in melanoma cell lines,

and high miR-182 levels trend with the transition from noninvasive to metastatic melanomas. miR-182 is found to act as a negative regulator of transcription factors MITF and FOX03, leading to promote survival and metastasis in melanoma (Segura et al. 2009).

A miRNA microarray performed on benign nevi, primary melanomas, and metastatic melanomas sought to identify differentially expressed miRNA involved in melanoma initiation and metastasis. This study identified a significant downregulation of miR-205 in melanoma samples compared to the nevi, suggesting that miR-205 could act as a potential tumor suppressor. Subsequent in vitro studies demonstrated that miR-205 directly suppressed E2F1 and results in the negative regulation of the AKT survival pathway. miR-205 also directly targets E2F5, which is linked to regulating tumor suppressors p130 and p107 (Dar et al. 2011).

In addition to miRNAs, lncRNA plays a critical role in regulating gene expression, and their misexpression or dysregulation has been recently implicated in cancer progression and metastasis (Huarte 2015). lncRNA exploits several varied mechanisms, with the ability to interact with DNA, RNA, and proteins, in order to transcriptionally regulate gene expression. For example, lncRNA has been shown to recruit histone-modifying complexes to activate or silence target genes, interact with regulatory proteins to inhibit transcription, and even interact with miRNAs.

With the growing evidence supporting the role of lncRNAs in tumor development, studies sought to identify lncRNAs involved in the transition from benign nevi to malignant melanoma. Comparing the lncRNA microarrays for normal melanocyte to transformed melanoma cell lines identified a SPRY4 transcript (SPRY4-IT4) as one of the first upregulated lncRNA in melanoma. In vitro knockdown studies of SPRY4-IT4 showed decreased cell growth and enhanced apoptosis, suggesting an oncogenic phenotype of SPRY4-IT4. Interestingly, expression levels of SPRY4-IT4 were assessed in 30 melanoma patient samples and revealed that higher expression of the lncRNA correlated with metastatic samples (Khaitan et al. 2011).

HOX transcript antisense RNA (HOTAIR), an lncRNA from the HOXC locus, was first identified as an oncogenic lncRNA in breast cancer, and its significant upregulation was tightly linked with metastasis and poor prognosis (Gupta et al. 2010). HOTAIR interacts with the polycomb repressive complex 2 (PRC2) and silences critical metastatic suppressor genes via H3K27 trimethylation. The specific mechanisms are still unclear, but it is postulated in breast cancer cell line studies that HOTAIR inhibits HOXD and miR-7 expression. Consequently, downregulation of miR-7 results in increased expression of a histone methyltransferase SETDB1 and activation of transcription factor STAT3 (Zhang et al. 2014). Similarly to other cancers, HOTAIR was found to be consistently upregulated in lymph node metastasis compared to matched primary melanoma tumors (Tang et al. 2013).

Taken together, recent understanding of both miRNAs and lncRNAs has shown that the misexpression or dysregulation of these ncRNAs can promote oncogenic or tumor suppressive effects contributing to melanomagenesis. Several early examples of ncRNAs have been documented to serve as potential, noninvasive biomarkers for metastasis. In general, the field of ncRNA in melanoma is still in the discovery phase working to identify of strong ncRNAs that play a functional role in cancer initiation, maintenance, metastasis, or drug response. Finally, extensive metadata analysis of gene expression datasets, such as RNA-seq, from patient-derived primary and metastatic biopsies will be critical in developing a robust and reliable biomarker for metastasis or prognostic factor for survival.

Conclusion

Melanoma is a complex disease originating from both genetics and epigenetic alterations. The genetic landscape of cutaneous melanoma is well characterized from sequencing studies, but the melanoma epigenome is complex and dynamic. Fueled by a deeper molecular understanding of epigenetic events and the advancements in genome-wide technologies, researchers are

continuing to discover epigenetic events important in normal melanocyte biology, oncogenic transformation of a benign nevi, metastasis of primary melanomas to distant sites, and even response of melanomas to various drug treatments. The epigenome is dynamic, and understanding which differentially regulated gene or histone mark is required for melanoma development or metastasis is critical. Functional characterization of these implicated genes in preclinical melanoma models will be instrumental in determining if the alteration is meaningful.

A major challenge in identifying epigenetic drugs with clinical efficacy is the lack of specificity. The therapeutic window is narrow since DNMT inhibitors result in global hypomethylation, and HDAC inhibitors exhibit non-specific acetylation. The risk of off-target effects and general toxicity is high. While these non-specific inhibitors have shown efficacy in cell lines and in vivo xenograft models, the results do not typically translate in melanoma patients. Extensive characterization in preclinical models may provide insight into what melanoma patient subtypes would be most beneficial to the therapy in question. Using in vitro and in vivo preclinical models, melanomas can be categorized by anatomical subtype (cutaneous, acral, mucosal, ocular), mutational status ($BRAF^{V600E}$, NRAS, "wild type"), and disease stage (nonmetastatic, lymph node metastasis). Furthermore, the correlation of each of these distinctions with response to epigenetic drugs can better inform patient eligibility for future clinical trials.

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Melanoma Metabolism

6

Franziska Baenke, Nathalie Dhomen, Eyal Gottlieb, and Richard Marais

Contents

Abstract

The rewiring of metabolic pathways is one of the hallmarks of cancer and essential for tumorigenesis. It stems from the need of cancer cells to adapt their biosynthetic and bioenergetic demands in order to allow unrestricted proliferation and growth. Melanoma and other cancer cells exhibit a remarkable flexibility within a complex network of metabolic pathways, allowing them to use their often limited resources and direct them to the processes best placed to maximize their growth and survival. For example, during melanomagenesis, cells will often switch their energy production from mitochondrial metabolism to glycolysis, because the products of glycolysis can easily be shifted to essential pathways for macromolecule biosynthesis. Many of these metabolic alterations are initiated in response to signaling cues from genetic alterations that initiated melanomagenesis in the first place, which suggests that the events required to transform cells to malignancy must allow cells to meet their biosynthetic and bioenergetic needs. During progression,

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melanomas appear to display metabolic heterogeneity as vital nutrient sources become scarce and the tumor seeks to overcome these restrictions. As further insights into the metabolic rewiring that occurs during melanoma development and progression are gained, opportunities to target these vulnerabilities for therapeutic benefit may be exploited.

This book chapter provides a brief overview of important metabolic pathways and how these become reprogrammed by known oncogenes that are frequently altered in human melanomas. How these metabolic vulnerabilities are being targeted for clinical use will also be highlighted.

Keywords

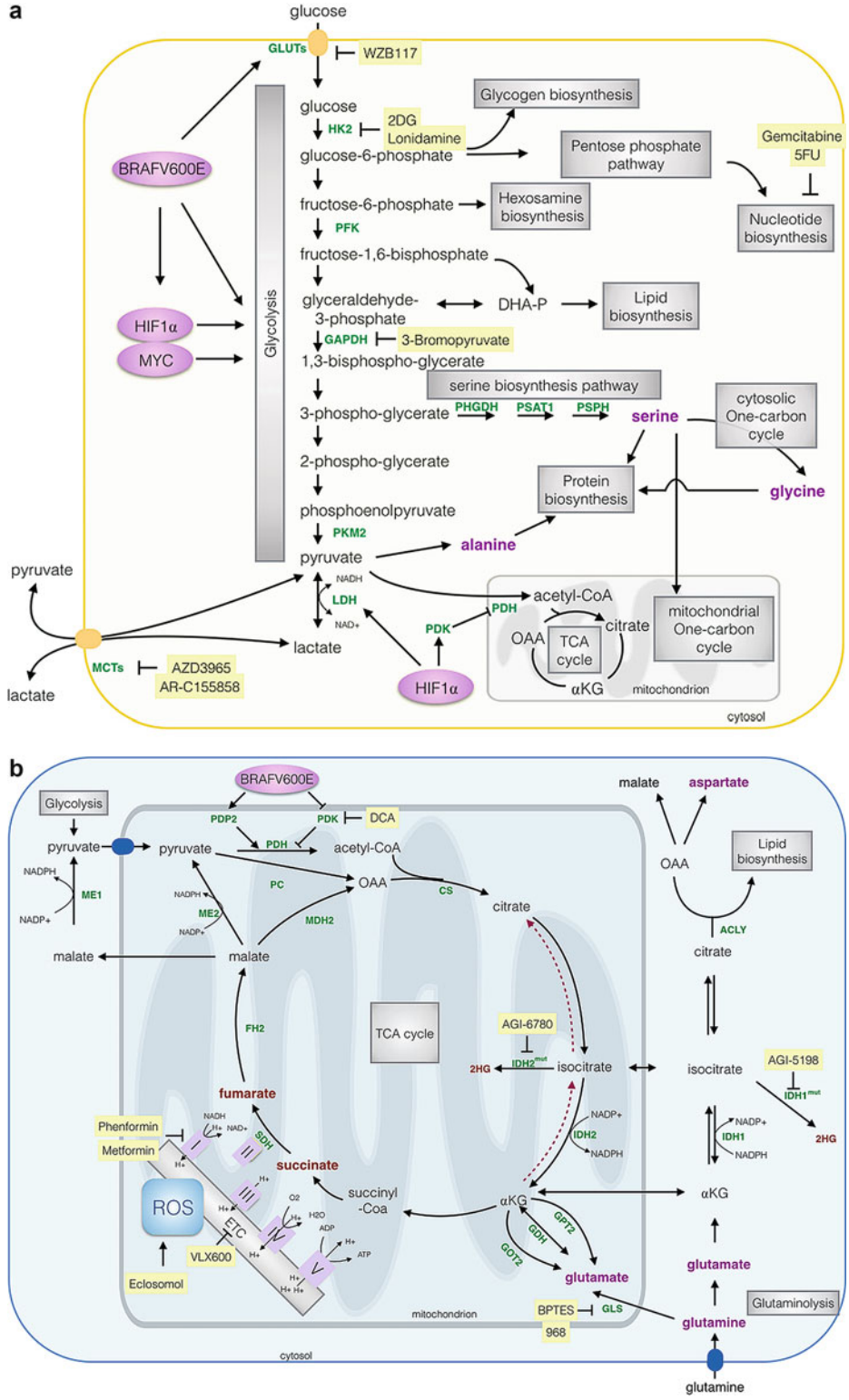
Melanocytes · Melanoma · Metabolism · Signaling · Nutrients · Hypoxia · Autophagy · Microenvironment

Introduction

Metabolism is defined as the sum of all chemical reactions in living cells that produce energy for vital processes and new biomaterial for growth. The process of cell proliferation exemplifies the spectrum of these metabolic reactions, as it involves cell growth and division and the fine-tuning of energy sourcing. Proliferating cells need to acquire and coordinate biomass through the biosynthesis of macromolecules such as proteins, lipids, nucleotides, and carbohydrates, a process that is called anabolism and which requires energy from the breakdown of adenosine-5′-triphosphate (ATP). Critically, the processes of anabolism also require reducing equivalents such as nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), which not only act as cofactors in anabolic reactions but are also essential for mopping up their toxic by-products. Conversely, the degradation of macromolecules to generate ATP is called catabolism. The enzymes that catalyze metabolic reactions are proteins or catalytically active RNA; and require additional molecules such as cofactors, coenzymes, and ions to function. Products of these chemical reactions are called metabolites and can influence cell signaling and gene expression by providing intermediates that are used for pre-transcriptional (epigenetics) and posttranslational modifications (i.e., acetylation, methylation). The direction and rate of a chemical reaction are determined by various factors such as substrate availability, the concentration of emerging products, and substrate specificity and affinity of the enzyme that catalyzes the reaction. Together, these factors dictate the turnover (metabolic flux) of metabolites within these pathways, and any given cell accommodates a variety of metabolic reactions in the cytosol and nucleus, as well as within specialized organelles such as the mitochondria or the endoplasmic reticulum (ER). These interlinked processes coordinate the phenotype of a given cell, whether they are quiescent or proliferative, and are linked to the supply of available nutrients.

The most abundant carbohydrate utilized by cells is glucose, which serves as the primary carbon source for the generation of pyruvate in the glycolysis pathway (Fig. 1a). Glycolysis occurs through a series of enzymatic reactions in the cytosol that catabolize one molecule of glucose to produce two molecules of pyruvate, two molecules of ATP, and two molecules of NADH. Under low oxygen conditions (hypoxia), lactate dehydrogenase (LDH) reduces glucose-derived pyruvate to lactate, regenerating two molecules of NAD^+. Lactate is then secreted from the cells. However, when oxygen is present (normoxia), glucose-derived pyruvate enters the mitochondria and is oxidized to H_2O and CO_2 by the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OxPhos) via the electron transport chain (ETC) to produce another 34 molecules of ATP from each original glucose molecule (Fig. 1a, b).

Critically, in addition to contributing to energy production, intermediates from both glycolysis and the TCA cycle are directed into macromolecule synthesis during cell growth and proliferation. For example, glycolytic intermediates can be used for nucleotide biosynthesis through the pentose phosphate pathway (PPP), for amino acid and fatty acid biosynthesis, and for glycosylation of lipids and proteins through the hexosamine



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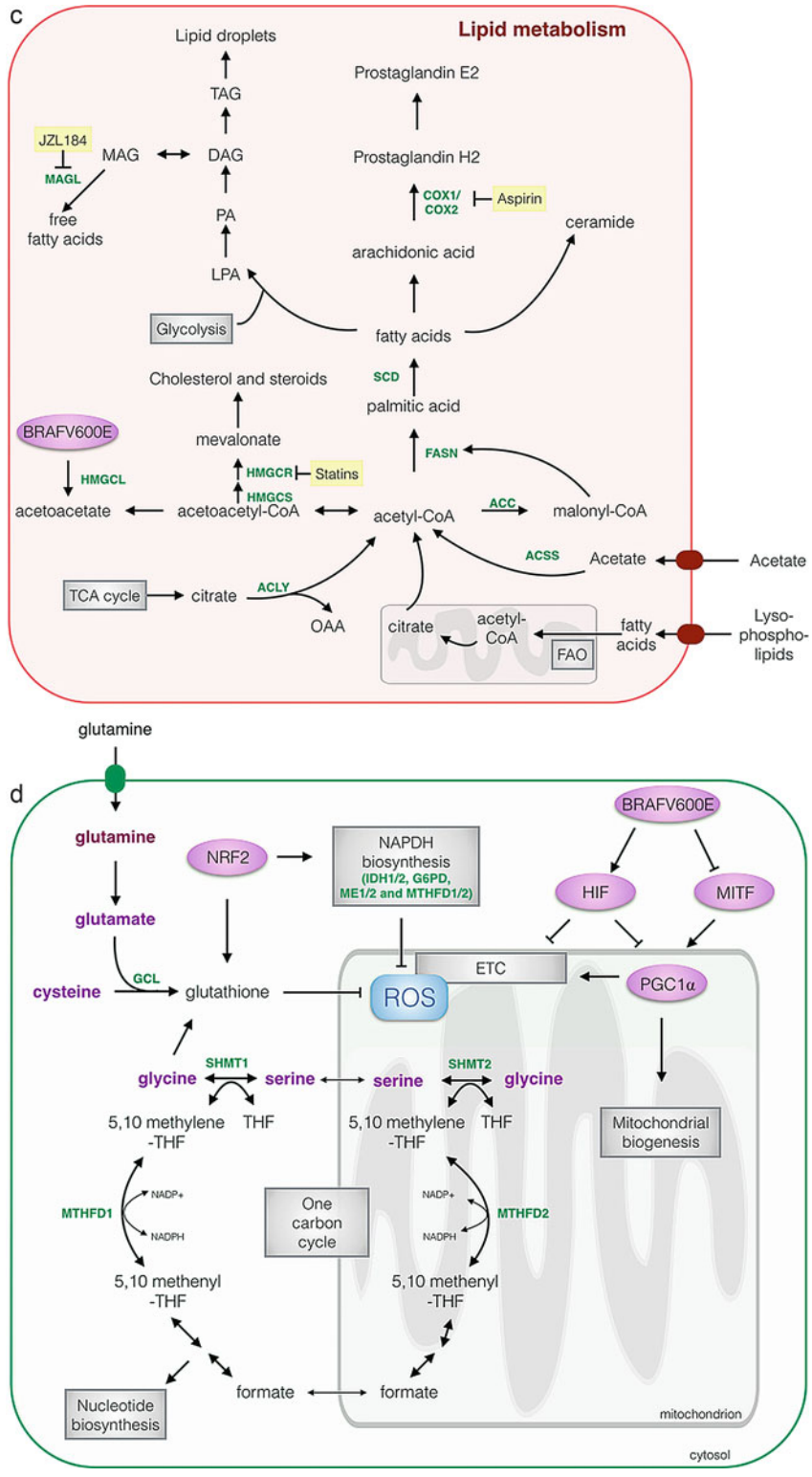

Fig. 1 (continued)

pathway (Fig. 1a). Similarly, oxaloacetate (OAA) and α-ketoglutarate (αKG), key TCA cycle intermediates, can be used for *de novo* synthesis of the amino acids aspartate and glutamate, respectively, and subsequently for synthesis of other amino acids and nucleotides (Fig. 1b). Citrate from the TCA cycle can also be exported to the cytosol for conversion to cytosolic acetyl-CoA, the sole carbon source for lipid synthesis in the cytosol and ER (Fig. 1b, c). As these processes consume vital metabolic intermediates, they must be replenished, or otherwise glycolysis and the TCA cycle will grind to a halt, so the cell resolves this shortfall by consuming other vital nutrients, i.e., amino acids such as glutamine, the most abundant amino acid in the blood. Glutamine is taken up by specific transporters and gets converted by the enzyme glutaminase (GLS) to glutamate, which is then directed into the TCA cycle as αKG after oxidative deamination (Fig. 1b). Glutamate can also donate its amino group to keto acids such as αKG (transamination) to generate amino acids such as aspartate, important for the urea cycle, or serine and glycine via

Fig. 1 Schematic overview of core metabolic processes in melanoma. (**a**) **Carbohydrate metabolism.** Metabolic reprogramming caused by BRAF expression. Glycolysis links multiple molecular pathways (*grey boxes*) associated with the edited metabolic phenotype. (**b**) **Mitochondrial metabolism and amino acid metabolism.** Glucose-derived carbons contribute to the TCA cycle intermediates and to produce energy in the form of ATP by the ETC. The by-products of oxidative metabolism are ROS. Glutamine is another carbon source important for the replenishment of the TCA cycle. Glutamine can either be metabolized by oxidative (in mitochondria) or reductive (in cytosol) carboxylation to produce citrate that is subsequently used for lipid production. Important enzymes of carboxylation are IDH1/2, however they can become mutated and generate 2HG. 2HG, fumarate and succinate can act as oncometabolites (shown here in *red*) that can drive tumorigenesis. (**c**) **Lipid metabolism.** Pathways involved in the synthesis of fatty acids (FAs), cholesterol, phosphoglycerides, eicosanoids and ceramides. The enzymes involved in catalyzing individual reactions are highlighted in green. Glucose- or glutamine-derived citrate is metabolized to acetyl-CoA by ACLY. For the biosynthesis of FAs, acetyl-CoA is converted into malonyl-CoA and repeated condensation of acetyl-CoA and malonyl-CoA by FASN results in palmitic acid. Elongation and desaturation of palmitic acid generates different FAs with distinct saturation levels. Saturated and unsaturated FAs are combined with glycerol-3-phosphate (produced by glycolysis) to generate arachidonic acid, a long-chain polyunsaturated FA that is the source for the synthesis of eicosanoids. Ceramide and sphingolipids can also be generated by fusing acyl chains and polar head groups derived from serine, phosphocholine or phosphoethanolamine with FAs. Cholesterol and steroid biosynthesis is initiated by the conversion of acetyl-CoA to acetoacetyl-CoA, which is converted by subsequent reactions of HMGCS and HMGCR to mevalonate. Acetoacetyl-CoA can also be converted to acetoacetate by HMGCL, which is downstream of oncogenic BRAF. (**d**) **Other important metabolic pathways.** The one-carbon cycle that occurs in both the cytosol and mitochondria metabolizes serine to glycine, which is coupled to folate metabolism. This contributes to NADPH generation in addition to glutathione production, which are both important to counteract ROS. Another important pathway in melanoma is the MITF/PGC1 transcriptional cascade that drives the oxidative phenotype by inducing mitochondrial biogenesis and increasing mitochondrial function such as oxygen consumption. These processes are only able to function if vital nutrients and oxygen are present. Enzymes are shown in green and amino acids are shown in magenta. Genetic drivers and transcription factors that influence these pathways are shown in purple. Compounds targeting metabolic pathways are shown here in yellow. Enzyme abbreviations: *ACC* acetyl-CoA carboxylase, *ACLY* ATP citrate lyase, *ACSS* acyl-CoA synthetase short-chain family member, *COX1/2* prostaglandin-endoperoxide synthase (PTGS), *ELOVL* fatty acid elongase, *FASN* fatty acid synthase, *FH* fumarate hydratase, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *GCL* glutamate-cysteine ligase, *GDH* glutamate dehydrogenase, *GLS* glutaminase, *GLUTs* glucose transporters, *GOT2* aspartate aminotransferase 2, *HK* hexokinase, *HMGCL* 3-hydroxymethyl-3-methylglutaryl-CoA lyase, *HMGCR* 3-hydroxy-3-methylglutaryl-CoA reductase, *HMGCS* 3-hydroxy-3-methylglutaryl-CoA synthase, *LDH* lactate dehydrogenase, *IDH1* isocitrate dehydrogenase 1, *IDH2* isocitrate dehydrogenase 2, *MAGL* monoacylglycerol lipase, *MCT* monocarboxylate transporter, *ME1* malic enzyme 1, *ME2* malic enzyme 2, *MTHFD1/2* methylenetetrahydrofolate dehydrogenase 1/2, *PC* pyruvate carboxylase, *PDH* pyruvate dehydrogenase, *PDK* pyruvate dehydrogenase kinase, *PDP2* pyruvate dehydrogenase phosphatase catalytic subunit 2, *PFK* phosphofructokinase, *PHGDH* phosphoglycerate dehydrogenase, *PKM2* pyruvate kinase M2, *PSAT1* phosphoserine aminotransferase, *PSPH* phosphoserine phosphatase, *SCD* stearoyl-CoA desaturase, *SDH* succinate dehydrogenase, *SHMT1/2* serine hydroxymethyltransferase 1/2. Metabolite abbreviations: 2HG, 2-hydroxy-glutarate; α-KG, α-ketoglutarate, *DAG* diacylglycerol, *FA* fatty acid, *LPA* lysophosphatidic acid, *MAG* monoacylglyceride, *TAG* triacylglyceride, *THF* tetrahydrofolate

the serine biosynthesis pathway (Fig. 1a, b). When the TCA cycle is blocked by environmental (hypoxia) or genetic causes, glutamine/glutamate-derived carbons can sustain citrate through the reductive carboxylation of αKG to isocitrate (Fig. 1b).

While these metabolic processes support energy production and sustain cell growth, a toxic by-product of oxygen metabolism is the generation of partially reduced oxygen derivatives in the form of free radicals or so-called reactive oxygen species (ROS) (Fig. 1b, d). Left unchecked, these will damage the cell, and thus ROS homeostasis requires a balance between ROS-producing and ROS-scavenging systems. In most cells, the major ROS producers are the mitochondria, peroxisomes, and ER, while ROS scavengers include antioxidant enzymes such as catalases, glutathione peroxidases, and peroxiredoxins, as well as the reducing equivalents NADH and NADPH. In particular, NADPH occupies a central role in metabolic pathways, both as an intermediate in biosynthetic processes and a suppressor of ROS, and its importance is reflected by the fact that no less than four metabolic processes contribute to the NADPH pool of a cell: the PPP, via the enzymes glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) (Fig. 1a); the TCA cycle via the isocitrate dehydrogenases IDH1 and IDH2, TCA cycle enzymes which convert isocitrate to αKG (Fig. 1b); the process of pyruvate and malate interconversion, via malic enzymes ME1 and ME3 (Fig. 1b); and the one-carbon pathway, via specific enzymes MTHFD1 and MTHFD2 (Fig. 1d).

Ultimately the activity of all these metabolic processes depends on the state of the cell. Quiescent cells regulate nutrient uptake only to maintain housekeeping functions, whereas proliferating cells need to grow and divide, raising their anabolic need to generate biomass. This can be achieved by increasing nutrient uptake for catabolism to supply carbon, nitrogen, ATP, and the reducing equivalents NADH, $FADH_2$, and NADPH. Many metabolic processes are controlled by intrinsic (cell signaling) and extrinsic (growth factors, nutrients, oxygen) mechanisms that change when cells become malignant, both because of aberrant signaling induced by oncogene activation or loss of tumor suppressors and because of the increased metabolic demand placed on cells that are proliferating independently of external stimuli. This chapter describes the principles of metabolism including glycolysis, amino acid metabolism (glutaminolysis), mitochondrial metabolism, fatty acid synthesis and oxidation, and nucleotide biosynthesis in melanocytes and melanoma. In particular, how oncogenes such as $BRAF^{V600E}$ alter metabolic processes to sustain melanoma growth, progression, and survival and how metabolism mediates resistance to drugs that target this pathway will be highlighted.

Melanocyte Metabolism

Under normal physiological conditions, melanocytes have low proliferative and self-renewal activity and exhibit a quiescent-like phenotype (Fig. 2). They are nevertheless metabolically active and consume nutrients to generate energy and reducing equivalents for use in ion and ROS homeostasis and to maintain housekeeping functions. It is presumed that sufficient nutrients and oxygen are available for these normal functions and that the signaling pathways tightly regulate nutrient uptake and macromolecule synthesis. However, the physiological oxygen concentration in the skin varies between 0.5% and 10% O_2, suggesting that melanocytes may face a range of nutrient and oxygen availability (Bedogni and Powell 2009). Notably, melanocytes are able to grow in hypoxia, and although they present similar metabolite profiles under normoxia and hypoxia, fewer glutamine-derived carbons are incorporated into TCA cycle intermediates under hypoxic conditions, suggesting glutamine use decreases significantly under hypoxia (Scott et al. 2011). Moreover, under normoxic conditions, melanocytes rely heavily on mitochondrial respiration, oxidizing glucose for maximal energy production, whereas under hypoxia they display increased lactate production due to fermentation of glucose-derived pyruvate (Scott et al. 2011).

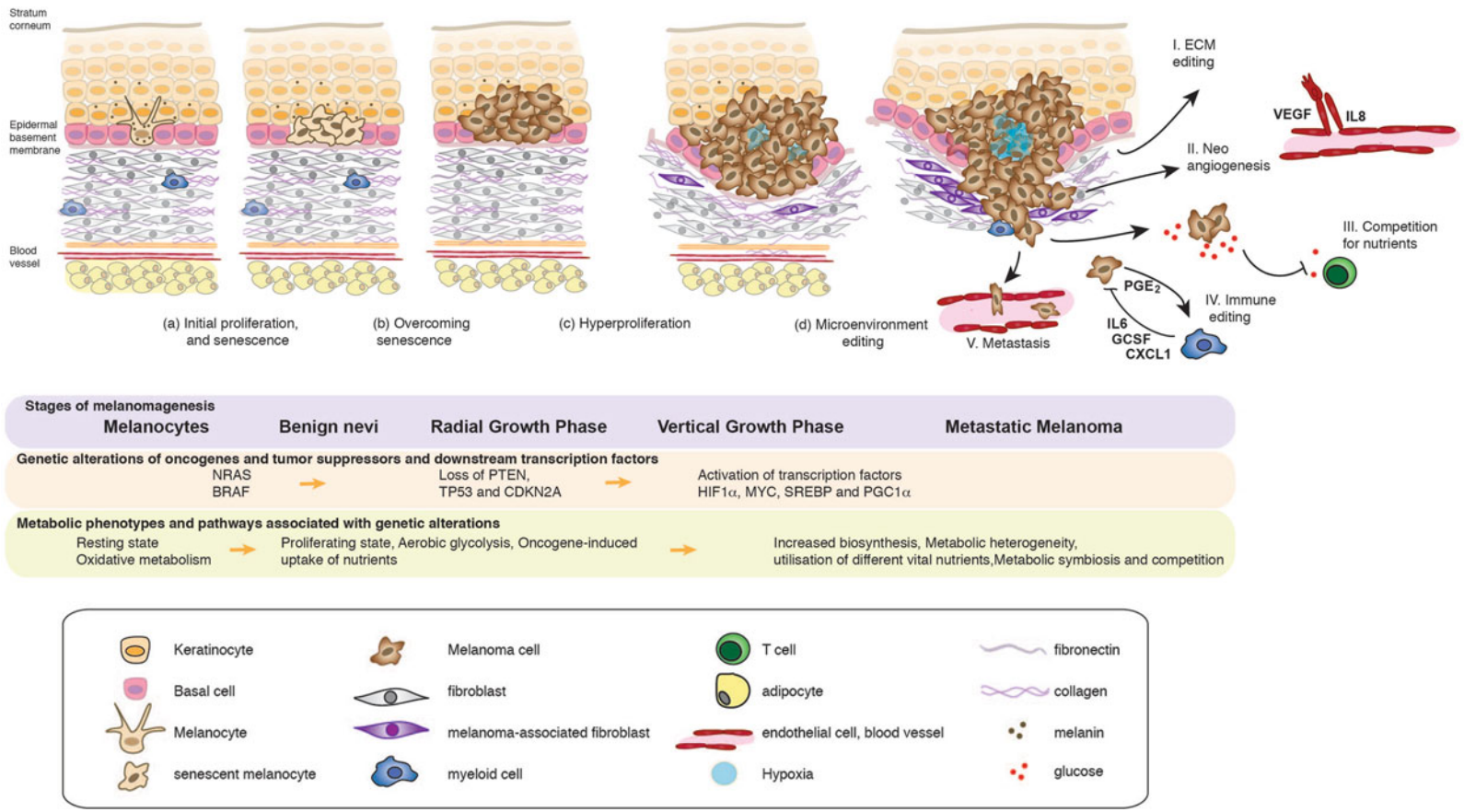

Fig. 2 Genetic alterations and metabolic rewiring during melanomagenesis. Stages of melanomagenesis and the metabolic rewiring associated with the different stage-associated phenotypes. Acquiring mutations in oncogenes such as *BRAF* and *NRAS* results in oncogene-induced senescence (OIS) in melanocytes (**a**). Overcoming OIS occurs upon acquisition of other genetic alterations such as loss of tumor suppressors that drive metabolic rewiring (**b**). This enables unlimited proliferation of melanoma cells (**c**). The metabolic phenotype initiated by genetic events leads to melanoma-induced secretion of metabolites or ions, which exert a multitude of effects on cells such as immune cells, fibroblasts and endothelial cells as well as extracellular matrix components. Reciprocally the microenvironment reacts to these stimuli and influences the cancer cells themselves (**d**)

Thus, melanocytes appear to be able to sustain their metabolic demands and essential cellular functions irrespective of oxygen concentration.

Overall, melanocytes *in vitro* generally rely on mitochondrial metabolism and so are sensitive to mitochondrial poisons and inhibitors of respiration. One of the key regulators of oxidative metabolism in melanocytes is microphthalmia-associated transcription factor (MITF), which controls mitochondrial biogenesis and function through its modulation of the transcriptional peroxisome proliferator-activated gamma co-activator 1 alpha (PGC1α) (Fig. 1d, see section "Mitochondrial Metabolism and ROS"). Additionally, MITF is an important regulator of pigmentation, as it governs the expression of several proteins involved in a series of oxidoreductions that allow melanocytes to convert the nonessential amino acid L-tyrosine to the pigment melanin. Modulation of pigment production, in turn, can have significant consequences for melanocyte metabolism. For example, the amount of melanin generated by melanocytes is correlated to the expression of an important metabolic modulator, the transcription factor hypoxia-inducible factor 1α (HIF1α), and upregulation of glycolytic genes such as *GLUT1*, *LDHA*, and *ALDOA*, which are downstream targets of HIF1α. Melanin, in turn, protects cells from ultraviolet radiation (UVR)-induced DNA damage and oxidative stress (Slominski et al. 2014), not only in the melanocytes but also the surrounding keratinocytes. Thus melanocyte metabolic activity, largely dependent on mitochondrial metabolism, allows them to fulfill one of their main functions and synthesize melanin in response to UVR.

Signaling and Senescence

Over the last decade, enormous insight has been gained into the genomic landscape of melanoma and this has led to a deeper understanding of melanoma biology. One of the earliest events is commonly the acquisition of a mutation that activates the RAS/RAF/MEK/ERK pathway promoting melanocyte proliferation (Fig. 2a). However, after an initial phase of expansion, growth arrest is often observed due to oncogene-induced senescence (OIS), leading to the development of a nevus and allowing the transformed cells to lie dormant for up to several decades (Kuilman et al. 2010, Fig. 2b). OIS, driven by cell cycle checkpoint regulators such as $p16^{INK4A}$ (encoded by *CDKN2A*) or tumor suppressors such as tumor protein 53 (TP53) and phosphatase and tensin homolog (PTEN), explains why the majority of benign and dysplastic nevi carry $BRAF^{V600E}$ mutations but do not progress to melanoma (Pollock et al. 2003). To overcome OIS, melanocytes need to activate other oncogenes and signaling pathways or lose the tumor suppressors that drive this process (Fig. 2c). It has become increasingly clear that these alterations not only induce proliferative or survival signaling cues but must necessarily affect a range of metabolic functions and processes to support the unimpeded proliferation of melanoma cells. For example, genetic alterations in the PI3K/AKT/mTOR pathway are frequently observed in *BRAF*- and *RAS*-driven melanomas, and the tumor suppressor PTEN, which negatively regulates this pathway, is mutated or lost in approximately 15% of cutaneous melanomas. Loss of PTEN has been shown to allow transformed melanocytes to bypass $BRAF^{V600E}$-induced senescence (Vredeveld et al. 2012), as it results in unsuppressed AKT activation and subsequent signaling through mTOR and downstream effectors to support cell growth, proliferation, and survival. Notably, a genetically engineered mouse model (GEMM) overexpressing *PTEN* displayed diminished PI3K/AKT signaling, which resulted in decreased animal size and weight due to higher levels of mitochondrial respiration and increased fatty acid oxidation. This is because, alongside its well-studied signaling role, AKT supports glycolysis by inducing expression of glucose transporters and glycolytic enzymes and also stimulates fatty acid metabolism via the metabolic enzyme ATP citrate lyase (ACLY), and thus mice in which this pathway is blocked exhibited a suppressive metabolic phenotype (Garcia-Cao et al. 2012).

In addition, by using GEMMS, Damsky and colleagues showed that activation of both

mTORC1 signaling (through loss of the tumor suppressor *LKB1*) and mTORC2/AKT signaling (through loss of the tumor suppressor *CDKN2A*) was required to overcome oncogenic *BRAF*-induced OIS, allowing transformed melanocytes to progress to melanoma (Damsky et al. 2015). Loss of either *CDKN2A* or *LKB1* alone was insufficient to drive melanomagenesis, demonstrating the importance of both the mTORC1 and mTORC2 components of the mammalian target of rapamycin (mTOR) signaling pathway in melanomagenesis. These experiments exemplify the role of mTOR as a master regulator of cell growth and proliferation, not only by transducing growth and survival signals but also by controlling protein and lipid biosynthesis (Laplante and Sabatini 2012). Moreover, LKB1 functions as an energy sensor and becomes activated when nutrients and energy levels are low. It then phosphorylates AMP-activated protein kinase (AMPK), which is a key regulator of the catabolic/anabolic machinery that balances the cellular processes involved in energy metabolism and macromolecule biosynthesis. In response to diminished ATP, nutrient and/or oxygen availability, AMPK can inhibit the mTOR signaling pathway to limit energy consumption for protein and lipid synthesis. Moreover, AMPK can induce a cellular state called autophagy that allows tightly controlled degradation and recycling of cellular components, such as proteins or even organelles like mitochondria to maximize energy and macromolecule use (White 2012).

In addition to its well-characterized role as a tumor suppressor, TP53, another important mediator of melanocyte senescence, fulfills regulatory functions at different nodes of the metabolic network. *TP53* encodes a transcription factor that can become activated in response to cellular stresses such as DNA damage and hypoxia. Under normal conditions, TP53 is expressed at low levels; however, under stress conditions such as nutrient deprivation, TP53 becomes stabilized and regulates genes that affect the balance between glycolytic and mitochondrial metabolism. TP53 drives the expression of TP53-induced glycolysis and apoptosis regulator (TIGAR), which results in the inhibition of phosphofructokinase 1 (PFK1), a key regulator of glycolysis (Bensaad et al. 2006). This slows the rate of glycolysis and simultaneously redirects glucose-derived carbons into the PPP. In this manner, cells can generate NADPH and synthesize nucleotides for DNA repair.

TP53 has also been shown to regulate mitochondrial function by stimulating the expression of the SCO2 cytochrome C oxidase assembly protein, a copper transporter, which is important in the assembly of complex IV/cytochrome C oxidase of the mitochondrial ETC, the site of OxPhos. Additionally, TP53 regulates glutamine metabolism by upregulating expression of glutaminase isoform 2 (GLS2) (Suzuki et al. 2010) and dampens the expression of malic enzyme (ME), which coordinates the conversion of malate to pyruvate, by binding the promoters of *ME1* and *ME2*. Abrogation of TP53 by RNAi increases ME protein expression and activity, which positively contributes to the NADPH pool. Conversely, when ME expression is inhibited, a TP53-mediated growth arrest is induced via activation of AMPK, suggesting that ME regulates TP53. This TP53-ME feedback loop is exemplary of how coordinated cell signaling and cell metabolism decide the metabolic fate of transformed cells (Jiang et al. 2013). Thus, alterations in tumor suppressor genes are a common mechanism to circumvent oncogene-induced senescence in melanocytes, leading to melanomagenesis.

OIS is not mediated solely by the action of tumor suppressors. The metabolic rewiring that accompanies oncogenic transformation plays an important role, for example, by altering the cells' use of such carbon sources as pyruvate. $BRAF^{V600E}$ expression in human diploid fibroblasts results in upregulation of pyruvate dehydrogenase phosphatase 2 (PDP2), which positively regulates pyruvate dehydrogenase (PDH) and inhibition of pyruvate dehydrogenase kinase 1 (PDK1), a negative regulator of PDH (Fig. 1b). The net effect of this is reduced pyruvate secretion and increased mitochondrial metabolism, with no overall change in glucose uptake, leading to growth arrest. Notably, subsequent overexpression of PDK1 allows cells to escape senescence and form tumors. Despite exhibiting

similar changes in mitochondrial metabolites and metabolism to those observed in $BRAF^{V600E}$-expressing fibroblasts, fibroblasts expressing $KRAS^{G12V}$ do not display a similar regulation of PDK1 and PDP2 expression, suggesting there are differences in the metabolic rewiring exerted by oncogenic *RAS* and *RAF* (Kaplon et al. 2013).

Despite the high homology observed between RAS isoforms, the exact mechanisms by which they mediate OIS are isoform-specific. Oncogenic *HRAS*, which is commonly amplified or mutated in Spitz nevi, does not mediate growth arrest via alterations in genes more commonly associated with senescence, such as *CDKN2A* or *TP53*, but via ER stress and the unfolded protein response (UPR). Constitutively active HRAS stimulates protein synthesis in transformed melanocytes, resulting in increased protein translocation to the ER and increased ER stress. This leads to activation of the UPR, to counterbalance protein misfolding, and consequently halts a significant number of cellular processes, leading to senescence (Denoyelle et al. 2006). Neither $NRAS^{Q61R}$- nor $BRAF^{V600E}$-expressing cells display the same phenotype of increased ER activity, suggesting that the different oncogenes in the MAPK signaling pathway trigger distinct responses leading to a senescent phenotype. Another way in which RAS^{G12*} expression in fibroblasts has been shown to promote senescence is by causing an increase in ROS levels, particularly mitochondrial-derived ROS. This phenotype was disrupted when fibroblasts were exposed to hypoxic conditions which dampen the production of ROS and cells were able to proliferate (Lee et al. 1999).

Taken together these studies demonstrate how transformed melanocytes/benign nevi precisely orchestrate their metabolic processes to prevent the uncontrolled hyperproliferation that can result from oncogenic transformation. However, when further genetic, metabolic, and environmental alterations allow transformed melanocytes to overcome senescence and become malignant, the resulting melanomas exhibit an extensive rewiring of their metabolic systems to support their increased demand for metabolic intermediates.

Metabolic Rewiring in Melanoma

In order to proliferate, grow and react to stress signaling, melanoma cells need to undergo significant metabolic reprogramming of their energetic and biosynthetic processes. Due to the accelerated and uncontrolled cell growth of most solid tumors, nutrients and oxygen that are normally abundant become scarce as the increasing demand from the tumor cells exceeds that which is supplied by the vasculature, creating a potentially unfavorable microenvironment for cancer cells (Figs. 2d and 3). To sustain proliferation and survival, the tumor cells need to optimize nutrient consumption and maintain flexibility within the metabolic machinery. Most metabolic pathways are centered around glucose and glutamine, as these carbon sources are abundant; however, cancer cells use other amino acids, lipids, and alternative carbon sources to withstand and adapt to these challenging conditions. In addition, many tumor areas may have to respond to fluctuations in oxygen levels. These extracellular influences also dictate tumor heterogeneity, as they influence not only the cancer cells themselves but stromal cells within their microenvironment. Another layer of complexity arises from systemic effects of metabolism-altering conditions such as diabetes and obesity, which can also affect melanoma metabolism and progression. This section describes alterations in the core metabolic processes of melanoma cells and provides a brief overview of how cancer-associated metabolism affects cell signaling and the microenvironment.

Sugar Metabolism

In the 1920s, Otto Warburg discovered that tumor slices from the liver consumed more glucose than healthy liver tissues and also secreted more lactate, even in an oxygen-rich environment (Warburg 1924). This phenomenon is now known as aerobic glycolysis and is exploited for diagnostic purposes in ^{18}F fluoro-2-deoxyglucose PET (^{18}FDG-PET) imaging. The preferential switch to aerobic glycolysis by cancer cells appears puzzling, because its energy output is very inefficient

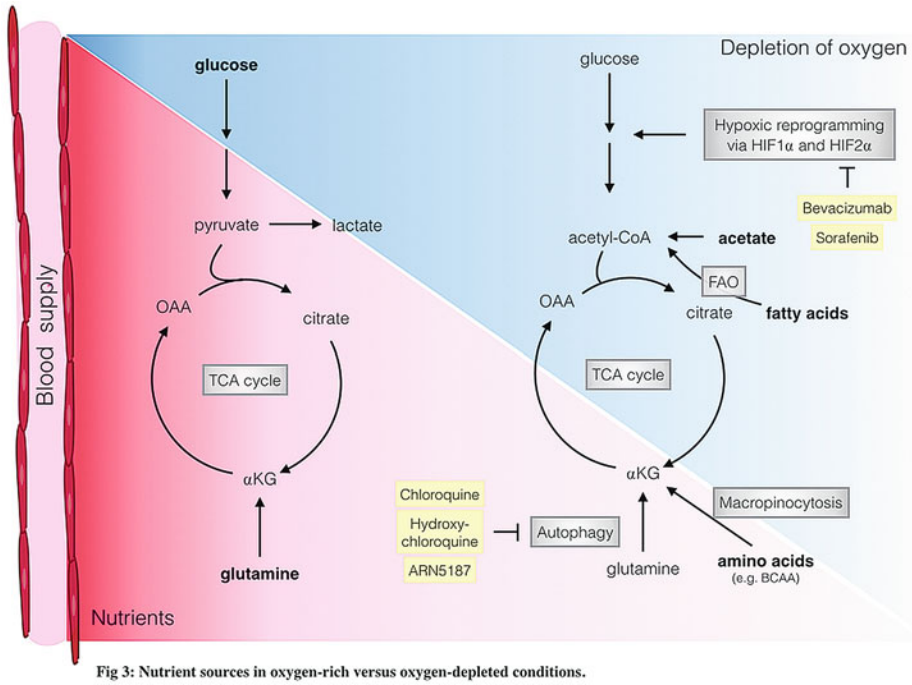

Fig 3: Nutrient sources in oxygen-rich versus oxygen-depleted conditions.

Fig. 3 Nutrient sources in oxygen-rich versus oxygen-depleted conditions. The availability of nutrients depends on the proximity to the blood supply. Glucose and glutamine are the most commonly utilized nutrients in a well-vascularized environment. However, in a nutrient- and oxygen-scarce environment, melanoma cells will utilize alternative carbon sources such as acetate, fatty acids and amino acids, including branched chain amino acids (BCAA). These are processed by such pathways as fatty acid oxidation (FAO), autophagy and macropinocytosis in order to replenish important metabolic processes (i.e. TCA cycle) and generate the macromolecules they require

when compared to mitochondrial metabolism. However in recent years, it has become clear that the glycolytic pathway not only supports energy production, albeit inefficiently, but also provides the glycolytic intermediates that are needed for synthesis of fatty acids, amino acids, nucleotides, and reducing equivalents to support the rapid cell growth and division of cancer cells.

Metabolic profiling reveals significant metabolic heterogeneity, and not all melanoma cells are highly glycolytic (Scott et al. 2011). However, most melanoma cells do show increased dependency on glycolysis, and inhibitors of glycolysis impair their cell growth and survival while having little effect on melanocytes (Scott et al. 2011). The oncogenes that activate the MAPK pathway increase nutrient uptake through processes such as upregulation of glucose transporters to maximize glucose use (Flier et al. 1987; Parmenter et al. 2014). Constitutive RAS signaling also increases expression and activation of the bifunctional glycolytic enzyme PFKFB3, which controls both synthesis and degradation of fructose-2,6-bisphosphate (F-2,6-BP) and is also an allosteric activator of the key glycolytic regulator PFK1 to increase glycolysis (Telang et al. 2006). Furthermore, deregulated MAPK signaling increases transcription of the glycolytic enzymes HK2 and PKM2, and in cancer cells, PKM2 slows glycolytic flux, allowing key intermediates to be siphoned off to macromolecule biosynthesis (Israelsen and Vander Heiden 2015).

Critically, inhibition of cell signaling impedes oncogene-mediated glucose reprogramming. For example, MEK inhibitors suppress levels of intracellular and secreted lactate in melanoma cells (Falck Miniotis et al. 2013), and prolonged genetic or pharmacological inhibition of $BRAF^{V600E}$ causes downregulation of glycolytic transporters such as GLUT1 and enzymes such as

HK2, resulting in reduced availability of glycolytic intermediates (Baenke et al. 2016; Parmenter et al. 2014) (Fig. 1a). Conversely, NRAS-driven resistance to BRAF inhibitors (BRAFi) restores the glycolytic phenotype, and it has been shown that BRAFi-resistant cells become less dependent on glucose metabolism and exhibit increased oxidative phosphorylation (Baenke et al. 2016). Accordingly, combined treatment of BRAF mutant melanoma cells with a BRAFi and dichloroacetate (DCA), a PDK1 inhibitor that stimulates oxidative metabolism (Fig. 1b), suppresses cell proliferation and increases apoptosis through downregulation of a transcription factor axis involving HIF1α, MYC, and MLXIP (MONDOA) (Parmenter et al. 2014).

Glucose metabolism is closely linked to nucleotide and amino acid biosynthesis by pyruvate kinase, a family of enzymes that perform the last step of glycolysis and regulate glycolytic flux, with the PKM2 isoform in particular favoring anabolic reactions (Fig. 1a). Notably, melanoma cell lines exhibit increased secretion of adenosine and inosine compared to other cancer cell types, suggesting that purine synthesis is altered in melanoma (Jain et al. 2012). Nucleotide biosynthesis can be classified into purine and pyrimidine metabolism, and the enzymes associated with purine biosynthesis, particularly those that generate guanosine monophosphate (GMP), are overexpressed in many cancers including melanoma. Inhibition of GMP synthase (GMPS), which catalyzes the conversion of inosine monophosphate (IMP) to GMP, attenuates cell invasion and reduces tumor progression. In contrast, increased levels of GMP release a negative feedback loop via GMP reductase (GMPR), thereby converting GMP back to IMP, which in turn can be used for the generation of AMP. Overexpression of GMPR in melanoma cell lines suppresses invasion *in vitro* and *in vivo* (Wawrzyniak et al. 2013).

The dependency of melanoma cells on glycolysis suggests this may be an avenue for therapeutic intervention. For example, the glucose analog 2-deoxyglucose (2-DG) inhibits glucose metabolism, depleting cellular ATP and causing cell death, and inhibitors of glycolytic enzymes such as 3-bromopyruvate are being tested in clinical trials (Fig. 1a). Another approach is to target lactate metabolism by inhibiting lactate transporters or lactate dehydrogenase (Fig. 1a), both of which reduce cancer cell viability (Vander Heiden et al. 2011).

Amino Acid Metabolism

Amino acids are the precursors of proteins and other biomolecules such as fatty acids, glucose, and nucleotides, but they also influence gene expression, as in the example of methionine, which provides the methyl groups needed for the histone and DNA modifications that are at the core of epigenetic gene regulation. Twenty amino acids contribute to protein synthesis, and nine of these (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) are essential amino acids, as they cannot be synthesized and so must be obtained from the diet. Notably, several amino acids are important in metabolic rewiring of cancer cells.

The most studied nonessential amino acid (NEAA) in cancer is glutamine, due to its high abundance in the blood. It is an important source for cellular carbon and nitrogen and is required for processes involved in amino acid, nucleotide, amine, and carbamoyl phosphate biosynthesis. Metabolic flux analyses show that melanoma cells exhibit increased aerobic glycolysis and glutaminolysis (Scott et al. 2011), likely creating a balance between the reduced flux of glucose-derived pyruvate into the TCA cycle and glutamine-derived anapleurosis (Fig. 1b) (Filipp et al. 2012a). In melanoma, glutamine metabolism plays a key role in overcoming the stresses induced by therapeutic intervention and in the development of resistance to these approaches. Flux analyses with isotope-labeled glucose and glutamine showed that cancer cells with defective mitochondria exhibit very limited incorporation of glucose-derived carbons into citrate, in turn suggesting minimal contribution of glucose-derived carbons into the TCA cycle or oxidative metabolism. Glutamine labeling showed that glutamine refuels the TCA cycle metabolites (Fig. 1b). This mechanism is a general response

in cancer cells that experience mitochondrial dysfunction due to hypoxia, inhibitors of the ETC, or mutations in mitochondria-associated genes, and these observations emphasize the importance of the mitochondria in tumorigenesis. Moreover, under hypoxic conditions, glutamine becomes essential for maintaining *de novo* lipogenesis in cancer cells as glutamine can be metabolized to cytosolic citrate via reductive carboxylation of αKG in the cytosol or the reversed TCA cycle in the mitochondria, requiring IDH1 and IDH2, respectively (Mullen et al. 2012; Filipp et al. 2012b) (Fig. 1b). Low-frequency mutations (6%) in these enzymes have been shown to generate 2-hydroxyglutarate (2-HG), a derivate of αKG that can act as an oncometabolite (Nowicki and Gottlieb 2015).

Cancer cells exposed to BRAFi immediately decrease glucose uptake and glycolysis, leading to metabolic stress. Short-term glucose deprivation causes mild metabolic stress that negatively regulates mTORC1 signaling and simultaneously activates the ER stress response pathway, in part due to increased uptake of most amino acids to compensate for the loss of glycolytic intermediates and the glycosylation process. However, cancer cells cannot cope with prolonged glucose deprivation and die through a pathway driven by ERK2 and the transcription factor ATF4 (Shin et al. 2015). The uptake of amino acids, in particular glutamine, prevents glucose-deprived cells from undergoing cell death, and so if glutaminolysis is also inhibited, the cells die. This is in line with the observation that melanoma cell lines, independent of BRAF status, display higher expression of glutamine transporters compared to melanocytes or benign nevi. The inhibition of glutamine transporters (such as ACST2) suppresses melanoma cell proliferation and spheroid growth *in vitro*, and this may open new therapeutic options for combination with BRAF and MEK inhibitors (Fig. 1b) (Wang et al. 2014). Moreover, prolonged inhibition of BRAF signaling in melanoma cells causes a subset of cells to depend on glutamine metabolism, and depletion of glutamine or inhibition of the enzyme GLS can induce cell death in melanoma cell lines with acquired resistance to BRAFi (Baenke et al. 2016).

Another key player in metabolic rewiring in melanoma is the NEAA serine. Serine biosynthesis is initiated by the conversion of the glycolytic intermediate 3-phosphoglycerate to phosphohydroxypyruvate by the enzyme phosphoglycerate dehydrogenase (PHGDH) (Fig. 1a), and in a subset of melanomas, PHGDH is upregulated and serine synthesis increased (Locasale et al. 2011). Serine is a carbon source for the one-carbon pathway (Fig. 1d), which plays a crucial role in cancer cell survival. In this pathway, serine is metabolized to glycine by serine hydroxymethyltransferase (cytosolic SHMT1/mitochondrial SHMT2), which concomitantly interconverts 5,10-methylenetetrahydrofolate and tetrahydrofolate (THF). 5,10-Methylenetetrahydrofolate is then further metabolized to 10-formyl-THF by MTHFD1 (cytosolic) or MTHFD2 (mitochondrial), which is a crucial contributor to nucleotide biosynthesis. The one carbon pathway is also linked to the generation of cysteine via various trans-sulfuration steps. Cysteine together with glycine and glutamate is required for the synthesis of glutathione, an important antioxidant (Fig. 1d). Studies have shown that withdrawal of extracellular serine or glycine or attenuation of serine and glycine production, for example, by attenuation of PHGDH, diminishes tumor cell growth *in vitro* and *in vivo* (Maddocks et al. 2013; Locasale et al. 2011).

Several other amino acids are important for tumorigenesis. Asparagine, which is derived from glutamine, protects cancer cells from stress and cell death due to glutamine depletion by suppressing the ER stress response (Zhang et al. 2014). Glucose-labeling studies show that besides serine and glycine, alanine and proline are increased in melanoma cells compared to melanocytes (Scott et al. 2011), and it has been suggested that increased secretion of alanine is a mechanism to dispose of excessive nitrogen, although this has yet to be confirmed in melanoma. Similarly, genetic suppression of pyrroline-5-carboxylate synthase (P5CS), an enzyme involved in proline synthesis, suppresses melanoma cell growth *in vitro* and *in vivo*, but this can be partially rescued by exogenous proline. Moreover, when nutrients are scarce, proteins can be catabolized back into amino acids through the processes of autophagy or proteasomal degradation,

allowing cancer cells to redirect their building blocks into the key molecules that will keep them alive (Fig. 3). To carefully regulate these catabolic and anabolic processes, accurate nutrient sensing is required. As all amino acids are structurally and functionally unique and cannot compensate for each other, the cell needs to sense which amino acids are depleted (Efeyan et al. 2015), and one of the key amino acid sensors is general control nonderepressible 2 (GCN2), the activation of which inhibits global protein synthesis and sensitizes cancer cells to cell death (Kardos et al. 2015).

Another as yet unidentified amino acid sensor appears to directly influence the activity of mTOR, the main regulator of protein synthesis (Efeyan et al. 2015). Classically mTOR is activated by growth factors, but it also senses intracellular amino acids and is inhibited by their withdrawal. Not all amino acids regulate mTOR to the same extent, but one of the strongest mTOR activators is leucine, and curiously, melanoma cells with an activated MAPK pathway are highly sensitive to this amino acid. When the essential amino acid leucine is depleted from the media, melanoma cells are unable to induce autophagy and thereby undergo mitochondrial-dependent apoptosis *in vitro*. The combination of an autophagy inhibitor with a leucine-free diet recapitulated this *in vitro* finding by reducing melanoma growth *in vivo* (Sheen et al. 2011).

Thus, a wealth of studies have demonstrated that cancer cells can modulate both the utilization and production of amino acids and other metabolic intermediates to meet their nutrient demands and liabilities and respond to environmental stresses. This remarkable flexibility appears to be both a response to and a result of the variety of genetic alterations that occur in cancer cells. However further study within the field of melanoma is required to distinguish which amino acids are important for tumor initiation, progression, and metastasis and which are essential in a given genetic background.

Lipid Metabolism

Among the most prominent metabolic reprogramming of many cancer cells is an increased rate of lipid synthesis. While nonmalignant cells obtain the majority of their fatty acids from the extracellular environment, the high lipid demand needed for rapid growth and proliferation (e.g., to fuel membrane production) means *de novo* lipogenesis is a common feature of many cancers, including melanoma. Lipogenesis, the process by which nutrient-derived carbons get converted to fatty acids and sterols, occurs in the cytosol but relies mainly on the availability of acetyl-CoA. This is primarily derived from citrate (Baenke et al. 2013), 95% of which is supplied by oxidative metabolism of glucose and glutamine in the mitochondria under normal conditions (Kamphorst et al. 2013). For citrate to be accessible for lipid synthesis, it needs to be transported via a pyruvate/citrate shuttle into the cytosol. Here, citrate is metabolized to acetyl-CoA by ATP citrate lyase (ACLY), which is the starting point of lipid synthesis. Acetyl-CoA is converted to malonyl-CoA via acetyl-CoA carboxylase (ACAC), and both acetyl-CoA and malonyl-CoA serve as substrates for fatty acid synthase (FASN), which in turn generates palmitic acid, the basis for a diverse spectrum of saturated and unsaturated fatty acids including eicosanoids, sphingolipids, and triacylglycerides (Baenke et al. 2013) (Fig. 1c). Chemical inhibition or RNA interference (RNAi) of multiple enzymes within the lipid biosynthesis pathway including FASN has been shown to interfere with cancer cell growth. For example, the occurrence and number of lung metastases in a murine model of melanoma are significantly reduced after treatment with a FASN inhibitor (Seguin et al. 2012) (Fig. 1c). Similarly, statins, cholesterol-reducing drugs, exhibit antitumor effects against cancer stem cells and various cancer cell lines. In particular, atorvastatin, which inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGCR), a key enzyme of the mevalonate pathway required to convert acetyl-CoA to the cholesterol precursor mevalonate, causes a decrease in cholesterol levels and concurrent growth inhibition in some melanoma lines (Fig. 1c) (Warita et al. 2014).

Lipids store energy and act as structural components of cell membranes, but they also play important roles in cell signaling. Eicosanoids, particularly the prostaglandins, are signaling

molecules made by oxidation of 20-carbon fatty acids and exert control over a variety of cell and bodily functions. Prostaglandin H_2 is derived from arachidonic acid by cyclooxygenases (COX1 and COX2). Prostaglandin H_2 is then further metabolized to prostaglandin E_2 (PGE_2) via the rate-limiting enzyme mPGES-1 (Baenke et al. 2013). PGE_2 is reported to activate the RAS/RAF pathway and induce cell proliferation in an autocrine manner, and melanoma cells have been shown to change the behavior of their microenvironment by secreting PGE_2. PGE_2 suppresses the release of type I interferons in myeloid cells, which is known to activate a T-cell-mediated response. Moreover, not only does PGE_2 inhibit T-cell activation, it also modulates the release of other cytokines and growth factors such as IL6, CXCL1, and GCSF in the myeloid cells (Fig. 2d). Intriguingly, these factors have also been implicated in modulating tumor immune escape (Zelenay et al. 2015).

During nutrient starvation, cancer cells limit their anabolic processes, including lipogenesis, but still need to meet their metabolic demands. Cells expressing $BRAF^{V600E}$, but not wild-type *BRAF* or oncogenic *RAS*, can break down lipids to ketone bodies, and $BRAF^{V600E}$ induces expression of the ketogenic enzyme 3-hydroxy-3-methylglutaryl-CoA ligase (HMGCL) leading to the accumulation of acetoacetate (Fig. 1c), which in turn stimulates the interaction of $BRAF^{V600E}$ with MEK1 to drive RAF/MEK/ERK signaling (Kang et al. 2015).

Direct uptake of intracellular acetate, normally thought to be a feature in bacterial metabolism, has been shown to provide an additional source of cytosolic acetyl-CoA under nutrient starvation (Lakhter et al. 2016) or hypoxic conditions (Schug et al. 2015) (Figs. 1c and 3). Flux analysis of labeled acetate showed that this contributes to both the cytosolic and mitochondrial acetyl-CoA pool, suggesting that acetate is an important nutrient for lipid and cholesterol synthesis and ATP production under scarce nutrient and oxygen conditions (Fig. 3). The enzyme acyl-CoA synthetase short-chain family member 2 (ACSS2) mediates the conversion of the acquired acetate to acetyl-CoA, and is regulated by environmentally responsive transcription factors HIF1α and SREBP1. Their induction results in an increased contribution of acetate into lipogenesis and upregulation of ACSS2 in tumor hypoxic areas. Moreover melanoma brain metastases exhibit increased dependency on acetate (Mashimo et al. 2014). Clearly, ACSS2 and its other family members (ACSS1 and ACSS3) bear further investigation in melanoma.

While *de novo* lipogenesis is frequently observed in cancer cells, this does not preclude the utilization of diet-derived lipids under certain conditions. Highly aggressive cancers including melanoma display a distinct lipid signature that is associated with the increased expression of monoacylglycerol lipase (MAGL). MAGL breaks down free fatty acids and monoacylglycerols from triacylglycerides (Fig. 1c), and abrogation of this enzyme negatively affects cell migration and cancer cell survival, as well as growth of tumor xenografts in mice, but the reduction in tumor size due to abrogation of MAGL is rescued by a high-fat diet (Nomura et al. 2010). Hypoxia or *RAS* mutations can also prime cancer cells to consume fatty acids such as lysophospholipids from the microenvironment (Kamphorst et al. 2013), allowing RAS-driven cancer cells to degrade lysophospholipids via fatty acid oxidation (FAO or β-oxidation) to meet their energetic demands. Moreover, fatty acid degradation allows cancer cells to meet their energy demands and also to replenish the TCA cycle as fatty acid-derived carbons are important for the amino acid aspartate, which is involved in purine and pyrimidine biosynthesis (Figs. 1b and 3).

Given that cancer cells depend on *de novo* lipogenesis, inhibiting this process may open up novel therapeutic avenues. Disturbing the lipid synthesis pathway in cancer cells would be expected to have far-reaching consequences for membrane composition, cell migration, angiogenesis, response to therapeutic agents, and interactions with the microenvironment. Drugs that target the enzymes of the fatty acid synthesis pathway are currently under clinical testing, with the cholesterol-reducing statins and the NSAID aspirin having been shown to be effective in preclinical models (Vander Heiden 2011; Rohrig and Schulze 2016).

Mitochondrial Metabolism and ROS

The mitochondria are the powerhouse of the cell and are where the bulk of energy production, in the form of ATP, takes place. It is therefore not surprising that upon discovering that cancer cells tended to prefer the far less efficient process of glycolysis to mitochondrial OxPhos to produce ATP, Otto Warburg erroneously concluded that mitochondrial function must be impaired in cancer cells. However, it has become increasingly apparent that mitochondria in cancer cells not only are intact and functional but play a significant role in cancer cell adaptation and survival. For example, a subset of melanomas exhibit increased dependency on mitochondrial function in response to BRAF inhibition (Haq et al. 2013; Baenke et al. 2016), which is indicative of the adaptive metabolic rewiring required to respond to neoplastic transformation by oncogenes such as $BRAF^{V600E}$, nutrient and oxygen stress, and therapeutic interventions such as BRAF inhibition. Similarly, levels of the mitochondrial enzyme ME2, which converts malate to pyruvate via oxidative decarboxylation, are increased during melanomagenesis, and abrogating this increased expression reduces the NADH and pyruvate levels required to maintain OxPhos and the TCA cycle, respectively, thereby negatively impacting cell survival (Chang et al. 2015b).

Thus, despite melanomas being characteristically highly glycolytic and less oxidative, oxidative metabolism remains important to melanoma cell function and is controlled by one of the key orchestrators of mitochondrial biogenesis and function, the transcription factor PGC1α, encoded by *PPARGC1A*. *PPARGC1A* is overexpressed in a subset of melanomas as well as specifically upregulated in BRAFi-treated BRAF-driven melanomas via a cascade in which MITF is released from oncogenic BRAF-induced repression and stimulates expression of *PGC1α*. Increased expression of PGC1α results in elevated mitochondrial function (specifically increased mitochondrial mass and oxygen consumption) (Baenke et al. 2016) and mediates resistance to ROS and associated oxidative stress (Haq et al. 2013; LeBleu et al. 2014; Shoag et al. 2012; Vazquez et al. 2013). If this high PGC1α expression is subsequently abrogated, the cells display a non-oxidative metabolism phenotype accompanied by reduced cell proliferation and survival. The process comes full circle when loss of PGC1α results in increased ROS production, leading to HIF1α stabilization and increased glycolytic activity (Lim et al. 2014).

Metabolic editing also plays a role in melanoma cell switching from noninvasive to invasive phenotypes. Melanoma cells exposed to the metastasis-promoting protein S100A4 downregulate *MITF* and *TYR* as they become more invasive, resulting in a concomitant decrease in PGC1α expression and a switch from oxidative metabolism to glycolysis (Bettum et al. 2015). Conversely, when the metastasis suppressor *KISS1* is expressed in melanoma cells, glycolysis is downregulated, and mitochondrial activity is induced (Liu et al. 2014), suggesting the balance between glycolysis and oxidative metabolism influences metastasis formation.

ROS production and oxidative stress are inevitable by-products of mitochondrial oxidative metabolism, and thus, the means and extent to which cancer cells respond to or by counteract this oxidative stress have an enormous influence on their survival, growth, and migration (Gorrini et al. 2013). Levels of oxidative stress can markedly influence the behavior of cancer cells: low to moderate levels of ROS can support cell proliferation and survival by acting as signaling molecules, whereas high levels of ROS can induce DNA damage and cell death. As a consequence, cancer cells must maintain and rewire complex systems of overlapping antioxidants such as glutathione and NADPH, produced via various metabolic reactions or obtained from the diet, such as vitamins A, C, and E.

Glutathione is the most abundant antioxidant in a cell and is synthesized via glutamate cysteine ligase (GCL) (Fig. 1d). During tumor initiation, glutathione is required to counteract ROS generated by anabolic processes, and inhibition of glutathione in GEM models of different cancers delayed tumorigenesis. However, once a tumor is established, the necessity for glutathione becomes less urgent as the thioredoxin pathway

can compensate for low glutathione levels. At this stage, only combined inhibition of the glutathione and thioredoxin pathways results in cancer cell death (Harris et al. 2015). Glutathione levels, in turn, are regulated by NFE2-related factor 2 (NRF2), the master regulator of the antioxidant response (Fig. 1d). Under non-stressed conditions, NRF2 is inactive due to its binding to the inhibitor Kelch-like ECH-associated protein1 (KEAP1) which signals the proteasomal degradation of NRF2. Under stress, NRF2 activates a transcriptional program that involves more than 100 genes including antioxidant genes such as catalase and genes that stimulate glutathione production. The role that NRF2 plays in cancer progression is currently unclear, but NRF2 expression promotes increased susceptibility to tumorigenesis in lung and pancreatic cancers (DeNicola et al. 2011), and NRF2 activators commonly found in the diet, such as resveratrol and sulforaphane, are cytoprotective, and sulforaphane can delay tumorigenesis in many cancer types (Sporn and Liby 2012).

Nevertheless, NRF2 appears broadly to promote cell survival under stress conditions, supporting a role as a potential oncogene. Gain-of-function mutations in NRF2 have been found in chemotherapy-refractive skin and lung cancer cells, and the activation of a ROS scavenger program is important in human *KRAS*-driven pancreatic tumors, which show marked upregulation of the NRF2 target gene *NQ1* (DeNicola et al. 2011). Aside from its role in ROS detoxification, NRF2 is also involved in anabolic processes. Metabolomic profiling revealed that NRF2 increases the PPP activity to generate nucleotides as well as glutamine metabolism, important for gluthathione synthesis in a PI3K/AKT-dependent manner. Moreover, NRF2 also regulates the key enzymes of the serine biosynthesis pathway PHGDH, PSAT1, and SHMT2 via ATF4 to support glutathione and nucleotide production in a lung cancer model (DeNicola et al. 2015).

Dietary antioxidants are commonly thought to be cancer protective and are widely used as supplements for cancer patients or as part of a healthy diet. However, clinical trials have shown that the impact of antioxidants on cancer development and progression is far from clear. Studies in mouse models of *BRAF*- and *KRAS*-driven lung cancers have shown that supplementation of antioxidants during tumor progression accelerates tumor progression and burden (Sayin et al. 2014). Similarly, recent studies assessing the impact of antioxidants such as vitamin C, vitamin E, and n-acetylcysteine (NAC) on melanoma progression have found them to be more harmful than protective. Vitamin C has been shown to be toxic for melanoma cell lines, while trolox, a soluble analog of vitamin E, and NAC, a precursor of cysteine and GSH, positively influence the migratory and invasive properties in a murine melanoma model without affecting proliferation. Addition of NAC to the mouse diet increased the number of lymph node and lung metastases in an inducible $BRAF^{V600E}/PTEN^{-/-}$ mouse melanoma model. The antioxidants create a reduced intracellular environment by increasing the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), which in turn induced activation of the pro-migratory RHO GTPase RHOA. This was more pronounced in the metastatic lesions than the primary tumor and could be reversed with a GSH inhibitor, resulting in diminished RHOA-dependent migration and invasion (Le Gal et al. 2015).

Moreover, a study found that the cancer cells' ability to metastasize to distant sites is dependent on the way in which these cells cope with oxidative stress in mice. Metabolomic comparisons of patient-derived cancer cells from metastatic and nonmetastatic tumors implanted into mice revealed that circulating cells and cells within the metastatic lesions displayed higher ROS levels and consequently these cells created a reduced intracellular environment via increased levels of NADPH and glutathione. These adaptations were reversible, as ablation of genes in the one carbon pathway or inhibition with methotrexate reduced the numbers of metastases (Piskounova et al. 2015). This suggests that cancer cells with increased antioxidant reserves are more likely to succeed during the metastatic process.

Thus, the mitochondria are not only the powerhouse of the cell but also important signaling organelles. As some cancers depend on mitochondrial

metabolism, it is of great interest to identify compounds that can specifically target the mitochondrial machinery of cancer cells. Metformin, an approved drug used as therapeutic intervention for diabetes, has been shown to reduce tumor incidence. This is mediated by inhibition of complex I of the respiratory chain (Fig. 1b) and reduction of the glucose levels in the blood via activation of AMPK. *In vitro* and *in vivo* studies have shown how metformin can diminish melanoma survival, but it has been noted that metformin can only act when a specific transporter called OCT is expressed in cancer cells. Phenformin, another member of the biguanide family, is also effective at killing BRAFi-resistant melanoma cells (Yuan et al. 2013), but as this agent can induce lactic acidosis, it is considered a less safe option. Notably however, DCA, which induces a shift from the glycolytic to the mitochondrial phenotype by inhibiting PDK1 (Fig. 1b), a downstream target of HIF1α, has been used to treat lactic acidosis in patients with defects of mitochondrial metabolism. The increased oxidative phenotype in treated cells may support increased oxidative stress in cancer cells and thereby result in cell death as the ROS threshold of the cells is reached. Clinical trials evaluating the prooxidant drug elesclomol (Fig. 1b) are currently running to evaluate its impact in anticancer therapies.

Melanoma Microenvironment

While many studies have focused on intrinsic oncogene-driven metabolic alterations in cancer cells, tumor cells both impact and are influenced by their microenvironment. An inevitable result of unrestrained cancer cell proliferation is the disruption of cellular homeostasis and frequently limited access to the blood supply as tumors expand. Consequently, a subset of tumor cells will experience a reduction in their partial oxygen pressure. Many cancers, including melanoma, therefore need to initiate a molecular response to be able to cope with this oxygen stress (Fig. 3). This involves the upregulation of hypoxia-inducible factors (HIFs), which are transcription factors that consist of heterodimeric complexes containing an oxygen-sensitive α subunit and an oxygen-insensitive constitutively expressed β subunit (LaGory and Giaccia 2016). Hypoxic tumor regions exhibit high levels of HIF1α and HIF2α expression, which in melanoma are associated with disease progression and poor therapeutic outcome.

HIF1α and HIF2α have been shown to drive aerobic glycolysis (Keith et al. 2012) and limit the entry of pyruvate into the mitochondria via PDK. Inhibition of HIF1α signaling, either chemically or genetically, abrogates the glycolytic phenotype and promotes mitochondrial respiration. A decrease in activity of the HIF1α target PDK3 results in PDH stimulation and consequently increased OxPhos and associated ROS production. As such, PDK3 inhibition augments the impact of the oxidative stress inducer elesclomol in melanoma cells (Kluza et al. 2012) (Fig. 1b). HIF activation in cancer cells also stimulates the expression and secretion of VEGF and IL8, which promote neovascularization, providing cancer cells with new blood supply and essential nutrients. Like oxygen, nutrient availability becomes compromised in fast-growing tumors. Although HIF-induced VEGF and IL8 secretion might promote angiogenesis, tumor-induced vasculature has been shown to produce leaky and unstable blood vessels (LaGory and Giaccia 2016) (Fig. 2d). Thus, tumor cells necessarily maintain a high degree of metabolic flexibility (Fig. 3) in order to use the available nutrients efficiently and appear to display a level of symbiosis across different tumor areas to allow maintenance of the whole population, and disruptions of these dependencies will suppress tumor growth (Allen et al. 2016; Pisarsky et al. 2016). For instance, tumor cells in poorly vascularized areas of the tumor preferentially use glucose as a carbon and energy source, while cells within well-vascularized tumor areas will utilize alternatives available to them, such as glutamine (Boroughs and DeBerardinis 2015) (Fig. 3).

An imbalance caused by a paucity of nutrients may also evoke the process of autophagy in cancer cells, a naturally occurring destructive mechanism that disassembles, through a regulated process, unnecessary or dysfunctional cellular components. Ordinarily occurring under stress

conditions, autophagy has been shown to be both pro- and anti-tumorigenic (White 2012), depending on context, and is commonly observed in and thought to be essential to melanoma. In a $BRAF^{V600E}/PTEN^{-/-}$ GEM model of melanoma, inhibition of ATG7, an essential autophagy gene, results in reduced tumor growth, with tumors exhibiting an accumulation of damaged mitochondria. Metabolic profiling of *BRAF*-driven melanoma cell lines after suppression of autophagy revealed a decrease in metabolite levels of the TCA cycle (Xie et al. 2015). These studies suggest that, during *BRAF*-driven tumorigenesis, autophagy is necessary to overcome shortages in metabolites essential for cell growth and proliferation and that autophagy activity is imperative for tumor formation.

A well-known autophagy inhibitor, chloroquine (Fig. 3), has also been shown to reduce melanoma growth and metastasis, although its efficacy appears to stem from both autophagy-dependent and autophagy-independent effects. In addition to reducing autophagic flux and consequently reducing tumor cell proliferation, chloroquine is able to improve the overall fitness and function of tumor vessels, which is not observed in tumor models in which the autophagy gene ATG5 is silenced (Maes et al. 2014). While the observed vessel normalization resulted in decreased tumor necrosis, it counteracted metastatic seeding through improvement of endothelial cell barriers and resulted in the improved delivery and efficacy of chemotherapy. Chloroquine's autophagy-independent effect on tumor vessels is mediated by NOTCH1 signaling in endothelial cells (Maes et al. 2014), and its potential as a therapeutic agent in melanoma is currently being investigated in clinical trials. Targeting autophagy in drug-resistant melanoma is also being explored, following the observation that patients with acquired resistance to BRAFi display increased levels of autophagosomes and this correlates to reduced progression-free survival (Ma et al. 2014).

Outside of the cancer cells themselves, different cell types such as fibroblasts, keratinocytes, and immune cells, extracellular matrix components such as collagen and fibronectins, and small molecules such as ions, nutrients, and oxygen impact tumorigenesis. For example, activation and expansion of latent immune cells into effector cells require a hyperproliferation program, which results in T cells competing with tumor cells for glucose (Fig. 2d). It has been suggested that tumor cells that display increased glucose uptake in a harsh environment may not be recognized by the immune system, as the T cells do not have enough nutrients available to proliferate. Conversely, tumor regression might be due to activated T cells that starve cancer cells of glucose followed by proper recognition of cancer cells as abnormal (Chang et al. 2015a).

Moreover, reciprocal signaling of the tumor itself to the microenvironment influences its structure and functionality. Metabolic rewiring of melanomas results in increased acid production (e.g., lactate), and because cancer cells need to maintain a consistent pH so that their metabolic processes run efficiently, these acids are secreted into the tumor microenvironment. This is mediated by upregulation of proton-coupled transporters such as monocarboxylate transporters (MCTs), and advanced melanomas exhibit increased expression of MCT1 and MCT4, which in turn is associated with poor prognosis (Pinheiro et al. 2016). The increased efflux of acids to the microenvironment contributes to acidosis and the remodeling of the extracellular matrix. The acidic environment induces the expression of degrading enzymes such as matrix metalloproteinases (MMPs) and cathepsins, which have been shown to support angiogenesis, cell invasion, and metastasis (Bohme and Bosserhoff 2016). Tumor-secreted molecules also affect the stiffness of the surrounding extracellular matrix and impede drug efficacy. Culturing melanoma cell lines on stiff matrices also make them more resistant to BRAF inhibition. Nonmalignant cells in the tumor microenvironment are influenced by these secretomes, and they can then impact melanoma cell responses to therapies, as shown by the observation that patient-derived melanoma-associated fibroblasts can induce melanoma cell resistance to BRAFi (Hirata et al. 2015).

Melanoma cells can also orchestrate immunoediting, allowing BRAF-driven melanomas

to escape immune cell-mediated destruction. The eicosanoid PGE2, secreted by the tumor, affects cytokine production in myeloid cells. Rather than releasing type I interferons to activate T cells, myeloid cells edit their cytokine pool and release factors that enable the tumor to evade recognition by T cells (Zelenay et al. 2015). These observations underline the crucial interplay between tumor cell and microenvironment during melanoma progression and metastasis, and there is great interest in exploiting these relationships for therapeutic benefit.

Due to the strong impact of hypoxia and HIF on metabolic processes, cancer cells can become dependent on HIF signaling for survival, and therefore numerous drugs and compounds targeting the HIF axis are currently being tested. Agents blocking VEGFA, a key regulator in neo-angiogenesis, including bevacizumab (a monoclonal antibody targeting VEGFA, Fig. 3) and sorafenib (an inhibitor of VEGFA receptor tyrosine kinase), have shown limited promise as a monotherapy in only a subset of cancers; however, they may prove powerful tools in combination with chemotherapeutics or targeted therapies. The role of autophagy in melanoma needs to be further elucidated, but clinical trials evaluating the combination of the autophagy inhibitor chloroquine (Fig. 3) with known anticancer therapeutics for melanoma and other cancers are underway. Preliminary results suggest that they improve median survival and that chloroquine may prove useful as an adjuvant therapy.

Conclusion

It is clear that metabolic rewiring is essential for melanoma initiation and progression and its consequences are far-reaching. Melanocyte transformation requires a change from quiescence to a highly proliferative and invasive state, often in a poorly oxygenated and nutrient-depleted setting. Thus, melanoma cells need to adapt to a hostile environment, optimally utilize scarce resources, and even support neighboring cells if they are to survive and thrive in such conditions. It follows that the events leading to transformation, particularly those that activate the RAS/RAF/MEK/ERK signaling pathway, must initiate and/or support the metabolic rewiring that accompanies melanomagenesis. The ability of cancer cells to break down alternative carbon sources, to reprogram the metabolic pathways available to them to generate the macromolecules and energy they require, and also to maintain their redox potential in the presence of ROS may mirror underlying molecular changes accompanying melanoma initiation and progression. Therefore a better understanding of the oncogene- and non-oncogene-induced metabolic dependencies of melanocytic lesions is urgently required. To date studies interrogating the metabolic consequences of the molecular changes underlying melanomagenesis have focused primarily on oncogenic BRAF activation and signaling. However, there are likely many more genetic events that drive metabolic reprogramming, independently of whether the RAS/RAF/MEK/ERK pathway has been activated, and these may differ across the melanoma subtypes. The high level of molecular heterogeneity observed in melanoma may well reflect a significant metabolic heterogeneity, and studies are currently focused on revealing and targeting metabolic vulnerabilities across the spectrum of melanoma types. Moreover, following recent advances in the use of immune checkpoint inhibitors for the clinical management of melanoma, it will be important to learn how these agents influence the metabolic liabilities of cancer cells and whether it is possible to identify common metabolic phenotypes in melanoma patients.

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Molecular Genetics of Melanocytic Neoplasia

7

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Abstract

The pathogenesis of melanoma depends on the occurrence of specific genetic changes that drive the neoplastic process. Much research has therefore focused on elucidating these changes with twin goals of comprehending the genetic basis of how a melanocyte becomes a melanoma and identifying vulnerabilities ripe for clinical intervention. One of the defining features of the melanoma genome is an extraordinarily high prevalence of C > T point mutations; in this regard, ultraviolet light represents the predominant force shaping the melanocyte genome. Several subtypes of melanoma and melanocytic neoplasms exist, each of which may harbor specific genetic changes.

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Recent genetic and genomic studies have revealed an overarching framework for the mechanisms that transform melanocytes into benign melanocytic neoplasms and eventually melanomas. Thus, critical aspects of melanoma pathogenesis may be inferred from patterns of genetic alteration recurrent across subtypes of melanoma and melanocytic neoplasms.

Keywords

Melanoma · Melanocytic neoplasia · Genetics of melanoma · Somatic mutations of melanoma · Chromosomal copy number alterations in melanoma · Melanoma genomics · Melanoma genetics · Genetics of nevi

Introduction

Melanoma, like all cancers, is a genetic disease. Cancer-causing mutations subvert the usual checks and balances governing the participation of a single melanocyte in the multicellular human body. The result is unrelenting cellular proliferation.

No single mutation (considered in the broadest sense to mean any change to the genome) suffices to transform a melanocyte into a melanoma. Thus, most of the mutations acquired by melanocytes in their cellular lifetimes bear no neoplastic potential. However, certain mutations can coax a melanocyte to grow into a benign neoplasm, also known as a nevus, or mole. A nevus, visible or microscopic, may sometimes be the first step on the evolutionary path to melanoma. While most nevi do not progress to melanoma, the subsequent acquisition of other specific mutations can cause their progression to an intermediate stage of neoplasia. From here, only a few additional mutations may be needed to pass the threshold into a malignant melanocytic neoplasm, the definition of a melanoma. Thereafter, additional mutations may amplify disease progression or severity.

Mutational Processes

As a cellular evolutionary process, carcinogenesis depends on two main forces: variation and selection. Mutation generates the genetic variation upon which selection may then act. Mutations that confer a relative fitness advantage tend to increase in frequency across the cellular population – such mutations have been defined as driver mutations (Stratton et al. 2009).

The predominant type of mutation affecting the melanocyte genome is a C > T (predominantly TpC > TpT) substitution indirectly caused by exposure to the ultraviolet (UV) spectrum of light. However, various mutagenic processes additionally mold the melanocyte genome, resulting in all manner of substitutions, small insertions and deletions, and structural alterations.

Melanocytic neoplasms can be categorized in part by the primary mutagenic processes that have influenced their evolution. Some types of neoplasm primarily demonstrate mutations attributable to UV light mutagenesis, while other types are mostly characterized by focal chromosomal amplifications and deletions or gene fusions.

Point Mutations

Ultraviolet Light Photoproducts

Epidermal melanocytes reside on the basement membrane of the epidermis, at the epidermal-dermal junction. Although the primary function of these melanocytes is to produce melanin to protect the genomes of neighboring keratinocytes from the UV component of sunlight, the melanocytes themselves are vulnerable to the mutagenic properties of UV light. Nonepidermal melanocytes tend to receive less UV light exposure, specifically those cells located in the dermis, within the eye, or in association with internal organs.

UV light stimulates the creation of promutagenic DNA lesions (Fig. 1a, b). The energy from a UV photon can covalently join two neighboring pyrimidine bases, resulting in either a cyclobutane pyrimidine dimer or a (6-4) photoproduct. UVB

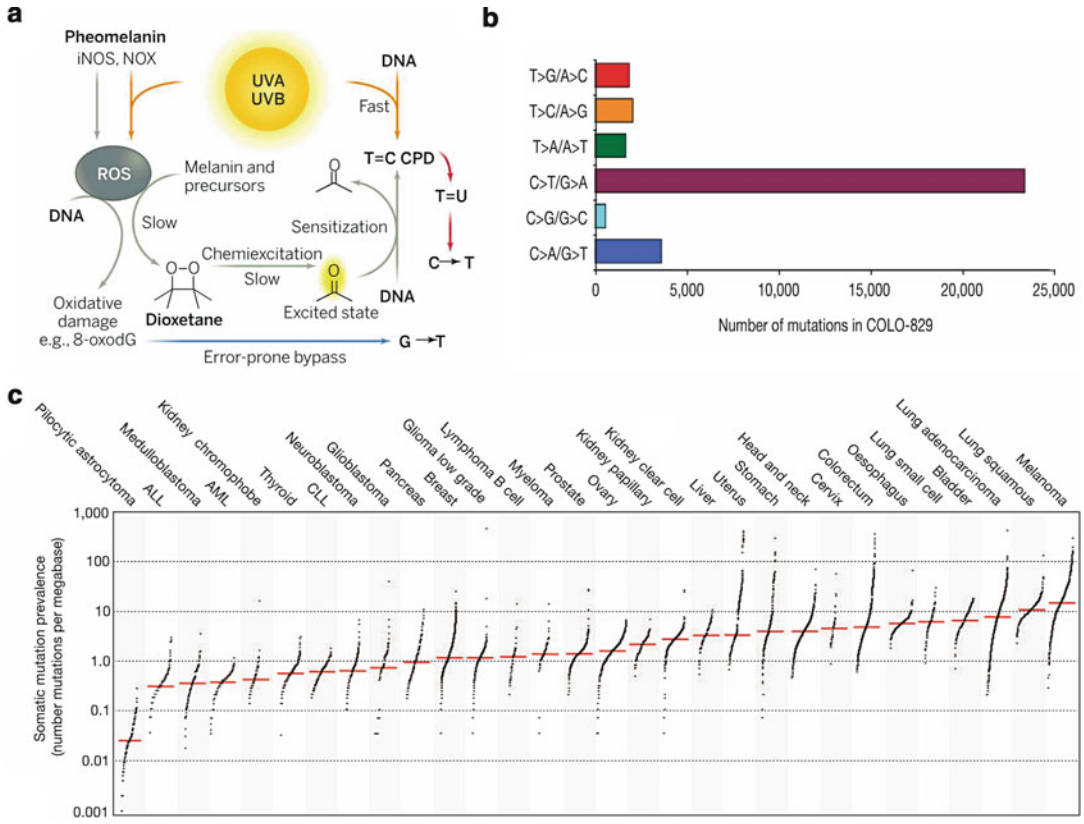

Fig. 1 Somatic point mutations in melanoma. (**a**) Schematic of common pathways leading to somatic point mutations in the melanocyte genome. (**b**) Prevalence of different point mutations in a melanoma genome, the first melanoma genome sequenced. Notice the relative abundance of both C > T/G > A (associated with UV radiation) and C > A/G > T (associated with oxidative damage) mutations, with the former being much more common. (**c**) Number of mutations per one million base pairs (mega base pairs, Mbp) in 30 different cancer types, sorted from left to right by increasing mean number of mutations per Mbp (indicated by red horizontal lines) (Figures reprinted with permission from (**a**) Taylor, Science 2015. https://doi.org/10.1126/science.aaa6578, (**b**) Pleasance et al., Nature 2010. https://doi.org/10.1038/nature08658, and (**c**) Alexandrov et al., Nature 2013. https://doi.org/10.1038/nature12477)

light (280–320 nm) directly generates such lesions, while UVA light (320–400 nm) does so indirectly, in a manner dependent on melanin and reactive oxygen species (Noonan et al. 2012; Premi et al. 2015).

Most cellular polymerases are unable to use pyrimidine dimers as a template, and standard replication or transcription comes to a halt at such lesions. Humans lack the enzyme photolyase, and thus cannot directly reverse pyrimidine dimers. Instead, nucleotide excision repair (NER) proteins are recruited to the lesion to remove and replace the damaged DNA. NER enzymes make two nicks in the DNA strand containing the lesion, one nick upstream and one downstream of the lesion. A single stranded stretch of 24–32 nucleotides containing the lesion is so removed. Using the alternate DNA strand as template, a polymerase fills in the missing 24–32 nucleotides and a ligase seals the remaining nick. The result is perfect repair of the pyrimidine dimer with no change to the DNA sequence. Elucidation of the molecular mechanism of this crucial repair pathway earned Aziz Sancar one third of the 2015 Nobel

Prize in Chemistry (Sancar et al. 2004). Genetic defects in NER are the cause of xeroderma pigmentosum, a disease associated with sensitivity to sunlight and increased risk of skin cancers, including melanoma.

Though NER can correctly repair pyrimidine dimers induced by UV light, such dimers can and do indirectly cause mutagenesis (Fig. 1a).

If a pyrimidine dimer (affecting TpT, CpC, TpC, or CpT) escapes detection and repair and the cell proceeds into S phase, a DNA polymerase capable of translesion synthesis must be recruited opposite the lesion. Specialized translesion polymerases tend to synthesize the correct, matching bases opposite the photoproduct dimer, but they err orders of magnitude more frequently than standard replication polymerases. This elevates the odds of mutagenesis opposite a pyrimidine dimer. When the dimer is eventually repaired by NER through excision and DNA synthesis, the mistake introduced by the translesion polymerase is propagated to the opposing strand.

However, the primary mechanism by which pyrimidine dimers lead to mutagenesis appears to depend on cytosine deamination (Taylor 2005). Cytosine bases in a pyrimidine dimer experience an increased rate of deamination as compared to native cytosine bases. Deamination of cytosine yields uracil, and deamination of 5-methylcytosine yields thymine. If now again the deaminated pyrimidine dimer escapes repair and the cell proceeds to DNA replication, a translesion polymerase will pair an A opposite the deaminated C (U or T) in the dimer, resulting in a mutation. When NER eventually repairs the pyrimidine dimer, the introduced mutation on the opposite strand will propagate to the strand that originally sustained the UV light damage, forming the canonical C > T (most commonly TpC > TpT) UV light–induced mutation (Fig. 1b). Canonical UV light–induced CpC > TpT mutations can similarly result if the pyrimidine dimer comprises two cytosines and both undergo deamination.

Excessive UV light exposure, especially early in life, is associated with a higher risk of melanoma (Rhodes et al. 1987). In addition, C > T substitutions are responsible for a number of melanoma driver mutations, providing strong support for a role of UV light mutagenesis in melanocytic neoplasia. Accordingly, the most recurrent pair of mutations in melanoma are C > T mutations in the promoter of the *TERT* gene, encoding telomerase reverse transcriptase (Horn et al. 2013; Huang et al. 2013). Numerous additional C > T driver mutations in both oncogenes (causing recurrent missense mutations) and tumor suppressors (causing nonsense or damaging missense or splice site mutations) can likely be ascribed to UV light mutagenesis (Hodis et al. 2012).

Other Forms of Mutagenesis

While photoproducts of UV light dominate the mutagenic landscape of sun-exposed melanocytic neoplasms, other mutagenic forces contribute as well (Alexandrov et al. 2013).

Reactive oxygen species (ROS) can be formed by UVA light or during oxidative cellular metabolism (Garibyan and Fisher 2010). Biosynthesis of the red/yellow melanin pigment pheomelanin may also generate ROS (Mitra et al. 2012). ROS cause oxidative DNA damage, especially at uanine bases. Oxidation of guanine produces 7,8-dihydro-8-oxoguanine (8-oxoG). When unrepaired, 8-oxoG pairs with an adenine base during replication, ultimately leading to a G > T mutation upon eventual base excision repair of the oxidized base (Fig. 1a, b).

Point Mutation Probability Across the Genome

Melanomas are notorious for having a high prevalence of point mutations. In a comparison of 30 cancer types, melanoma had the highest median number of point mutations per megabase pair (one million base pairs; Mbp) of genome sequence (Fig. 1c) (Alexandrov et al. 2013). Large-scale sequencing studies of cutaneous melanoma have reported an average point mutation prevalence of roughly 15 mutations per Mbp (TCGA Network 2015; Hodis et al. 2012), and this number varies with the measure of sun exposure experienced by each tumor. The burden of point mutations becomes evident in benign melanocytic neoplasia and steadily increases with evolution to malignancy (Shain et al. 2015a). Roughly 70–80% of an average

sun-exposed melanoma's point mutations are C > T substitutions, the canonical mutation induced by UV light (Fig. 1a, b) (Hodis et al. 2012; Krauthammer et al. 2012).

Early sequencing studies revealed that point mutations are not evenly distributed across the melanoma genome (Pleasance et al. 2010). While this phenomenon is appreciated in all cancer genomes, it is accentuated in melanoma genomes by the malignancy's exceptional point mutation burden.

Genic regions tend to harbor a lower number of mutations per base pair as compared to intergenic regions. And a negative correlation between gene expression level and mutation prevalence was among the first observations made in the melanoma genome, together with a predisposition for C > T and G > T mutations in the untranscribed strand (Pleasance et al. 2010). A reduced prevalence of mutations in the transcribed strand has been attributed to the protective effect of transcription-coupled repair.

Chromatin features have emerged as the strongest predictors of mutation prevalence across the genome to date, capable of explaining 75% of the observed variation (Polak et al. 2015). Epigenetic modifications associated with an open chromatin conformation correlate with lower mutation prevalence and the opposite is true for modifications linked to closed chromatin conformations (Fig. 2a). Similarly, genomic regions accessible to DNaseI digestion (generally interpreted as regions of open chromatin) tend to show a relative decrease in mutation abundance (Fig. 2b).

Epigenetic modifications and regions of closed/open chromatin associate with different genomic loci in different tissue and cell types. Intriguingly, melanoma mutation prevalence correlates most closely with the epigenetic modifications found in human melanocytes, of all 106 tested cell types, including a melanoma cell line (Polak et al. 2015). Beyond suggesting a possible mechanistic link between chromatin state and probability of mutagenesis, these observations may imply that the majority of mutations in a melanoma happen before neoplastic evolution leads to any significant epigenetic restructuring.

Variation in mutation prevalence across the genome has implications for driver mutation discovery. Inference of candidate driver mutations depends on identification of genes or loci that have more mutations than would otherwise be expected by chance. Thus, correct estimation of the mutation abundance expected by chance (that is, under neutral selection) is required, and this estimate should vary across the genome in order to reflect reality. Specialized statistical methods have been developed for driver mutation discovery in melanoma (Hodis et al. 2012).

Point Mutation Burden Associated with Clinical Benefit from Immunotherapy

Melanoma's increased prevalence of point mutations, due largely to DNA damage induced by UV radiation, may be clinically exploitable. Biologics that counteract the immune checkpoint blockade, specifically CTLA4, PD-1, and PD-L1 antibodies, have demonstrated clinical benefit, with a large percentage of patients responding to therapy. Research into determinates of response and benefit in patients receiving immunotherapies has revealed an association between mutation burden and clinical benefit (Van Allen et al. 2015; Hugo et al. 2016; Snyder et al. 2014), as well as between neoantigen load and clinical benefit (Van Allen et al. 2015). Thus it seems that though a high point mutation burden often underlies melanoma evolution, it may auger well in the context of drug-induced immune system reactivation.

Structural Alterations

While the canonical mutation profile associated with melanoma is the C > T UV light induced signature, melanoma genomes also feature significant structural alterations such as DNA copy number changes and rearrangements. Copy number changes are rare in benign melanocytic neoplasms (Bastian et al. 2003) and emerge during progression to malignancy (Shain et al. 2015a). Structural alterations can generate key driver mutations, illustrated most obviously in

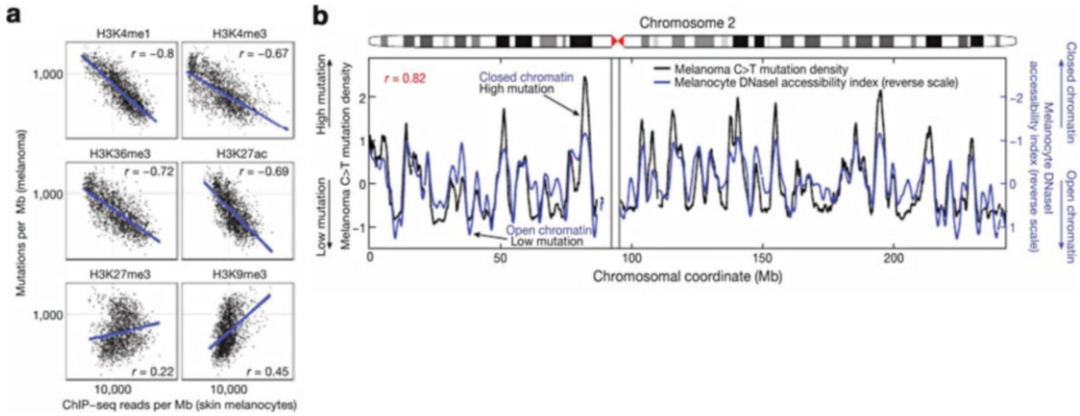

Fig. 2 Somatic point mutation prevalence varies across the melanoma genome. (**a**) Association between several epigenetic histone modifications in the melanocyte genome (as measured by chromatin immunoprecipitation) and average somatic mutation prevalence in the melanoma genome. Pearson's correlation coefficient, *r*, is displayed for each comparison. (**b**) Melanoma somatic mutation density is associated with (reversed) chromatin accessibility. For chromosome 2, C > T mutation density in melanoma overlaid on a plot of melanocyte DNA accessibility (DNaseI; high values correspond to both closed chromatin and high mutation density). Pearson's correlation coefficient, *r*, is displayed in red in the upper left corner (Figures reprinted with permission from Polak et al., Nature 2015. https://doi.org/10.1038/nature14221)

melanocytic neoplasms arising on non-sun-exposed areas of the body and Spitz tumors.

Copy Number Alterations

The majority of cancer types display characteristic patterns of aneuploidy, and melanoma is no exception. Each subtype of melanoma demonstrates its own recurring pattern of gain and loss of entire chromosomes, chromosome arms, and focal regions (Fig. 3a).

The result of extra or lost copies of DNA segments in melanoma is generally interpreted through a gene-centric lens: which genes are recurrently deleted (likely tumor suppressors) or amplified (likely oncogenes)? Such an interpretation may be permissible when one small tract of DNA, centered on one gene, is gained or lost in multiple tumors. However, often times the altered region is large and encompasses many genes, or even a chromosome arm or an entire chromosome. Attributing these larger alterations to a single gene becomes more difficult, and it may be the case that the simultaneous loss or gain of several genes is what provides a selective advantage.

The exact causes of genomic instability in melanoma and cancer in general are not clear, but likely involve errors of replication and recombination (Hastings et al. 2009). Mitotic missegregation can cause gains and losses of entire chromosomes. Replication fork stalling, perhaps due to DNA damage (including UV-induced pyrimidine dimers), can lead to template switching and amplification or deletion of nearby genomic loci. Unequal crossing over, perhaps mediated by low complexity regions, can similarly result in amplifications and deletions. Telomere loss is known to cause a crisis characterized by rampant chromosomal instability. For example, in the absence of telomeres, exposed chromosome ends can fuse by nonhomologous end joining to form a dicentric chromosome that then undergoes breakage-fusion-bridge cycles, leading to multiple fold-back inversions of one locus.

Copy-Neutral Rearrangements

Copy-neutral rearrangements are also commonplace in melanoma genomes. A copy-neutral rearrangement represents the linear joining of two chromosomal regions that were not previously contiguous, without duplication or deletion of the majority of the involved sequences. When such rearrangement affects two genes, the end

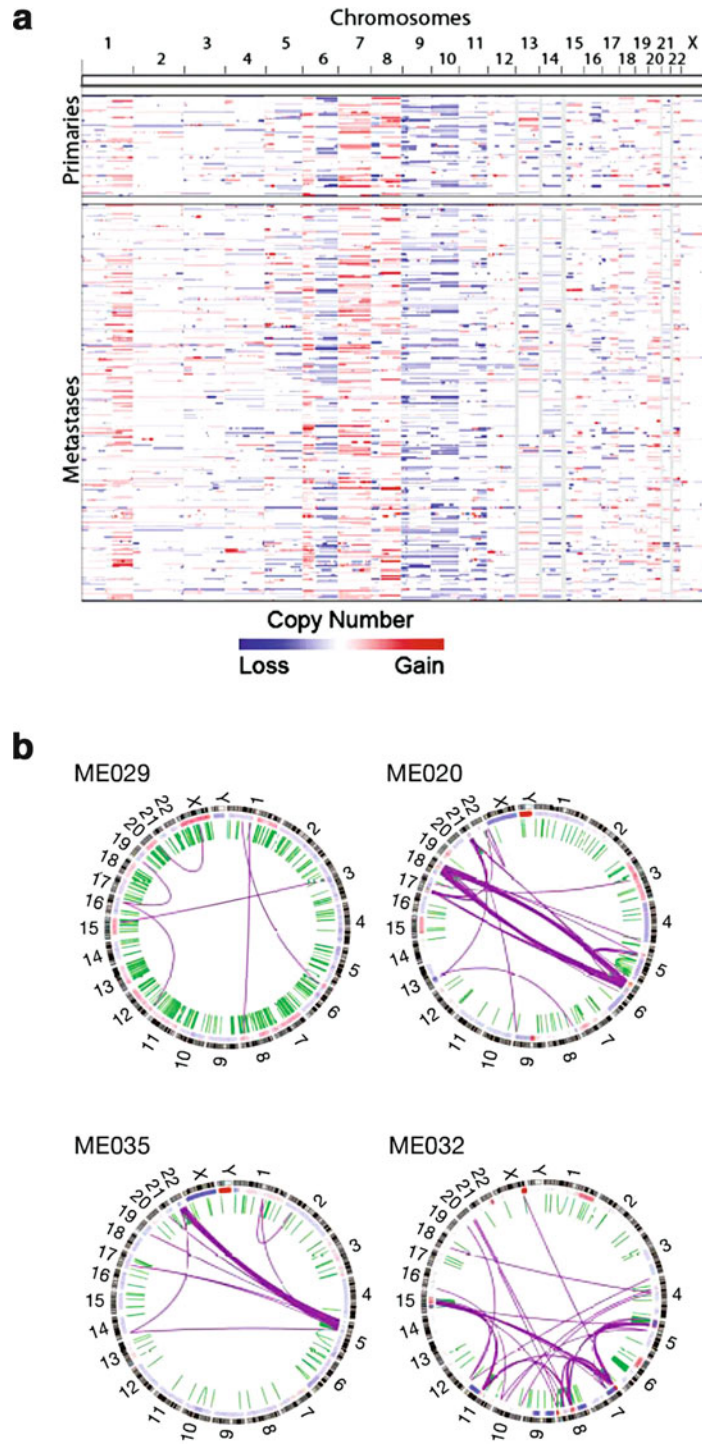

Fig. 3 Somatic structural alterations in melanoma. (**a**) Somatic copy number alterations across many melanomas. Each sampled melanoma tumor is a row in the heatmap and each column represents a chromosomal location. Primary melanomas are grouped as the top rows and metastases as the bottom rows. Frequently gained or lost chromosomal loci are apparent as mostly red or mostly blue columns. (**b**) Circos plots of somatic rearrangements in four melanoma genomes. Purple lines represent interchromosomal rearrangements and green lines represent intrachromosomal rearrangements. Chromothripsis is evidenced by numerous rearrangements affecting only a small set of chromosomes (Figures reprinted with permission from (**a**) TCGA Network, Cell 2015. https://doi.org/10.1016/j.cell.2015.05.044 and (**b**) Berger et al., Nature 2012. https://doi.org/10.1038/nature11071)

product can be a chimeric gene fusion. In Spitz tumors, it is common to find driver gene fusions encoding constitutively active protein products, caused by genomic rearrangements. Evidence of localized, simultaneous, massive-scale rearrangement, termed chromothripsis, has been also observed in some melanoma genomes (Fig. 3b) (Berger et al. 2012), although this phenomenon's

contribution to neoplastic progression remains to be determined.

Structural Alteration Probability Across the Genome

In comparison to what is known about point mutations, much less is understood about how the probability of structural alteration varies across the genome. A more accurate model of the odds of a structural alteration forming by chance at a given locus in the genome would improve statistical discovery of driver structural alterations and better distinguish between fragile sites and true driver events.

Somatic Mutations

Melanocytic neoplasms, benign and malignant, can be classified according to site of origin, driver mutation profile, age of common occurrence, degree of UV-light exposure, and histopathology, among other features. A taxonomic framework of melanocytic neoplasms has been formalized recently by Bastian (Fig. 4) (Bastian 2014).

Melanocytic neoplasms are first divided into two groups based on whether the cell-of-origin is epithelial or nonepithelial in anatomic location. Within the epithelial melanocytic neoplasms, a division is then made between nonglabrous (hair-bearing), glabrous (non-hair-bearing), and mucosal site-of-origin. The nonglabrous group is further divided based on degree of sun exposure. Within the nonepithelial melanocytic neoplasms a division is made based on tissue-of-origin: skin, eye, or internal organ.

Neoplasms Arising from Epithelial Melanocytes

Nonglabrous

Nonglabrous melanocytic neoplasms originate from melanocytes residing in hair-bearing skin. Melanoma of this subtype is the most common of all melanomas and disproportionately affects Caucasians.

Lower-UV

The "Lower-UV" category encompasses melanocytic neoplasms on nonglabrous skin that have suffered comparatively less UV-light mediated damage than their counterparts in the "Higher-UV" category. This distinction is a relative one; exposure to UV radiation is a feature of both categories (Fig. 5).

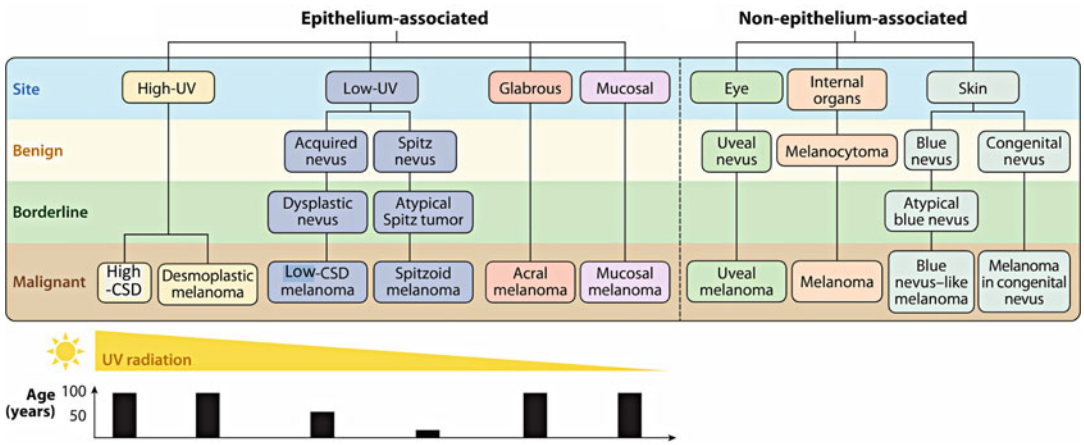

Fig. 4 Classification framework of melanocytic neoplasia. A taxonomy for classification of different subtypes and stages of melanocytic neoplasia (Figure reprinted with (pending) permission from Bastian, Annu Rev Pathol 2014. https://doi.org/10.1146/annurev-pathol-012513-104,658. Figure adapted to reflect WHO nomenclature (high-CSD/low-CSD rather than CSD/no-CSD))

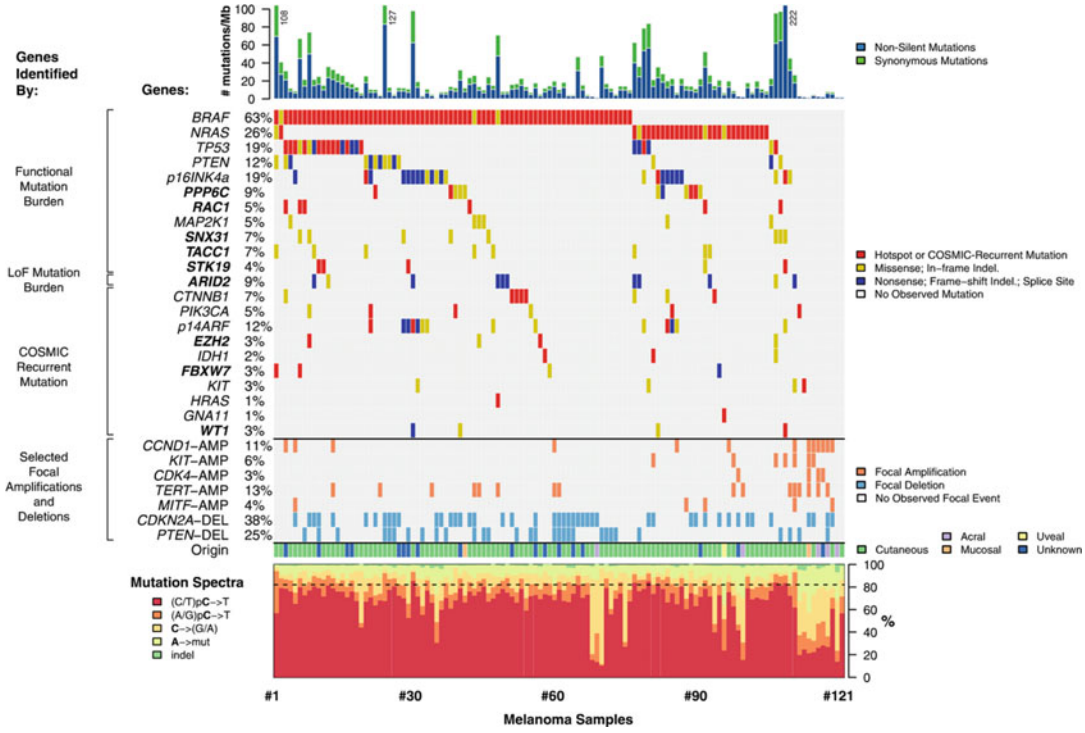

Fig. 5 Landscape of cutaneous melanoma driver mutations. Co-occurrence plot of candidate driver mutations and copy number alterations in cutaneous melanoma. Melanoma tumor samples are represented as columns, with mutation burden of each tumor as the top plot, mutation or copy number state of candidate melanoma genes in the central plot on a gray background, then tissue-of-origin depicted as a single row, and finally the bottom plot shows the relative frequency of various mutation types (e.g., C > T, C > A) in each melanoma sample (Figure reprinted with permission from Hodis et al., Cell 2012. https://doi.org/10.1016/j.cell.2012.06.024)

Cutaneous with Low Cumulative Sun Damage (Low-CSD)

Low cumulative-sun-damage (low-CSD) cutaneous melanomas are the most common subtype of melanoma. Neoplasms of this subtype are so named to distinguish them from melanocytic neoplasms that too arise in nonglabrous epidermis but show signs of extensive sun damage (high-CSD). Low-CSD tumors tend to arise on body regions that suffer only intermittent sun exposure, like the trunk, back, arms, and legs. These tumors lack local signs of cumulative sun-induced damage (e.g., solar elastosis). Other associations with low-CSD melanocytic neoplasms include a younger age of incidence (<55 years old) and an increased number of acquired nevi (Lachiewicz et al. 2008; Whiteman et al. 2003). Melanomas of this low-CSD subtype often (30–90% of the time) display a preexisting nevus component (Bevona et al. 2003; Shitara et al. 2014).

The initiating mutation of a low-CSD melanocytic neoplasm overwhelmingly seems to be a thymidine to adenine substitution yielding *BRAF* V600E (Davies et al. 2002). *BRAF* is a kinase in the mitogen-activated protein (MAP) kinase signaling pathway whose activity stimulates cellular proliferation. V600E mutation of *BRAF* yields a constitutively active kinase that can function as a monomer, whereas wildtype BRAF requires homo- or heterodimerization with other RAF proteins for activity. On its own, V600E mutation of *BRAF* is sufficient to produce an acquired nevus (Pollock et al. 2003; Shain et al. 2015a).

Over 80% of acquired nevi, predominantly arising in the first two decades of life, harbor

BRAF V600E as their sole driver mutation (Pollock et al. 2003). Other early acquired melanocytic neoplasms instead harbor oncogenic mutations in *NRAS* usually affecting Q61 (Shain et al. 2015a). More rarely, mutations in other genes activate the MAPK signaling pathway in lieu of *BRAF* and *NRAS* mutations: including *NF1* loss and oncogenic mutation of *HRAS* or *KRAS* (Hodis et al. 2012). Secondary activating mutations in *MAP2K1* might serve to augment the effects of other MAPK pathway activating mutations.

Progression from nevus to an intermediate evolutionary state appears to depend on expression of *TERT* (Shain et al. 2015a). *TERT* encodes the catalytic protein subunit of telomerase, a ribonucleoprotein polymerase responsible for telomere maintenance. Roughly 70% of melanomas have either one of two C > T mutations in the *TERT* promoter that are thought to induce *TERT* expression by forming an Ets family transcription factor binding site (Horn et al. 2013; Huang et al. 2013). *TERT* amplifications occur as well, but they are less frequent in this subtype of melanoma.

Transition from this intermediate state to unequivocal melanoma most commonly occurs in conjunction with biallelic loss of *CDKN2A*, through a combination of mutations and deletions (Shain et al. 2015a). *CDKN2A* codes for two protein products, p16 (INK4A) and p14 (ARF). The p16 protein inhibits CDK4 and CDK6 which normally phosphorylate the RB protein to mediate progression from G1 to S phase in the cell cycle. The p14 protein prevents MDM2 from degrading p53, a key regulator of apoptosis and a sensor of DNA damage. Thus through loss of both p16 and p14, *CDKN2A* loss leads to dysregulation of pathways controlling the G1/S transition (RB pathway) and apoptosis (p53 pathway). Rare loss-of-function mutations of *RB1* or activating mutations of *CDK4* (R24C) are likely capable of substituting for loss of p16. It has so far been more difficult to determine whether loss-of-function mutations in *TP53* (10–20% of melanomas) substitute for loss of p14.

Mutations in chromatin remodelers such as *ARID2*, *ARID1A*, and *ARID1B* have been noted in the transition to invasive melanoma as well (Shain et al. 2015a), though their functional role is less clear. Of these, only mutations in *ARID2* (~10% prevalence, commonly loss-of-function) have so far shown definitive statistical evidence of positive selection (TCGA Network 2015; Hodis et al. 2012).

Mutations in *PPP6C*, affecting 5–10% of melanomas and associating with *BRAF* and *NRAS* mutations (Fig. 5) (Hodis et al. 2012; Krauthammer et al. 2012), may too occur at this intermediate stage. The functional consequences of both recurrent (mainly R264C) and loss-of-function mutations in *PPP6C* remain to be determined but may play a role in cell cycle progression.

Tertiary mutations become more common once the melanocytic neoplasm has become an unequivocal melanoma. *PTEN* mutations and deletions of its locus on chromosome 10 are particularly frequent in low-CSD melanomas (Tsao et al. 1998). *PTEN* loss is seen in roughly 40% of low-CSD melanomas with *BRAF* V600E mutation, driving the Akt signaling pathway. It is thought that *PTEN* loss does not tend to occur in melanomas with *NRAS* mutations because oncogenic *NRAS* can already drive Akt signaling (Tsao et al. 2000). Mutations of *TP53* affect 10–20% of melanomas (Fig. 5), and current evidence suggests they occur as tertiary mutations (Hodis et al. 2012; Shain et al. 2015a).

Many additional driver mutations contribute to the evolution of this subtype of melanoma, likely as tertiary mutations with single digit percentage prevalence. These mutations include amplification of *MITF*; activating mutations of *RAC1*, *IDH1*, and *EZH2*; recurrent 5′UTR mutations in *RPS27*; and inactivating mutations in *DDX3X*, among others (Hodis et al. 2012; TCGA Network 2015; Dutton-Regester et al. 2014; Garraway et al. 2005; Krauthammer et al. 2012). Mutations with a sub-one-percent prevalence can also act as drivers, although such rare events are difficult to statistically nominate as drivers with current sequencing study sample sizes (TCGA Network 2015; Hodis et al. 2012; Krauthammer et al. 2012, 2015).

UV light is the predominant mutational mechanism in low-CSD melanocytic neoplasms. A C > T signature of UV light mutagenesis peppers

the genomes of these neoplasms, with roughly 5–6 mutations per Mb (Mar et al. 2013). The point mutation burden increases during progression from nevus to melanoma. At the melanoma stage, copy number alterations become apparent as well, with common amplifications affecting chromosomes 1q, 6p, 7, 8q, 17q, and 20q, and deletions of 6q, 8p, 9p, and 10 (Krauthammer et al. 2012).

Spitz Tumors

Spitz nevi, atypical Spitz tumors, and spitzoid melanomas comprise a spectrum of melanocytic neoplasms that predominantly arise in children and young adults. These neoplasms tend to show spindled or epithelioid cells and are usually intradermal. In the Spitz spectrum, atypical Spitz tumors represent borderline neoplasms somewhere between a nevus and a melanoma.

Spitzoid neoplasms can be split into three groups based on mutually exclusive oncogenic driver mutations: (1) 60% harbor kinase fusions (reported to affect *ROS1*, *NTRK1*, *ALK*, *BRAF*, *RET*, and *MET*) (Wiesner et al. 2014; Yeh et al. 2015), (2) 25% have *BRAF* V600E mutations on a background of *BAP1* loss (Wiesner et al. 2012), and (3) 15% possess amplification and/or oncogenic mutation of *HRAS* (Bastian et al. 2000a; Wiesner et al. 2014).

The kinase fusions appear in 55% of Spitz nevi, 56% of atypical Spitz tumors, and 39% of spitzoid melanomas, suggesting such a fusion may act as an initiating oncogenic event (Wiesner et al. 2014). Biallelic *CDKN2A* loss may act as a progression event from Spitz nevus to atypical Spitz tumors, and *TERT* promoter mutations (only observed in patients >10 years old) have been associated with aggressive spitzoid melanomas (Lee et al. 2015).

Loss of *BAP1* in cells of an acquired (non-Spitz) nevus harboring *BRAF* V600E (or more rarely *NRAS* Q61R) can beget transition to an epithelioid morphology and progression to an atypical Spitz tumor (Wiesner et al. 2011). Mutations or isolated amplifications (11p) of *HRAS* have only been seen in Spitz nevi, not in spitzoid melanoma to date (van Engen-van Grunsven et al. 2010).

Higher-UV

Cutaneous with High Cumulative Sun-Induced Damage (High-CSD)

Melanomas arising on nonglabrous skin that shows signs of high cumulative sun-induced damage (high-CSD) constitute a melanoma subtype distinct from nonglabrous melanomas that do not show signs of cumulative sun damage (low-CSD, discussed in earlier section). Signs of cumulative sun damage include solar elastosis and local occurrence of nonmelanoma skin cancers or their precursors. High-CSD melanomas tend to arise on the face, neck, and ears – anatomic sites of continuous sun exposure. Several lines of evidence support a distinction between high-CSD and low-CSD melanomas of nonglabrous skin origin.

Of most direct relevance to the topic of this chapter, specific driver mutations occur at differing frequencies in high-CSD melanomas as compared to low-CSD melanomas. *BRAF* V600E mutations, common in low-CSD melanomas, appear more rarely in high-CSD melanomas (Maldonado et al. 2003). Instead, MAPK pathway-activating mutations in low-CSD melanoma take the form of loss-of-function mutations in *NF1* (30% of cases), oncogenic mutations of *KIT* (10%), *CCND1* amplifications (20%), and *BRAF* V600K mutations (Curtin et al. 2005, 2006; Glatz-Krieger et al. 2006; Krauthammer et al. 2012; Menzies et al. 2012). In contrast with *BRAF* mutation frequency, the frequency of *NRAS* mutations (20%) is comparable between low-CSD and high-CSD melanomas. *ARID2* and *TP53* mutation frequencies are elevated in the high-CSD subtype (Krauthammer et al. 2012), perhaps due in part to the bounty of C > T substitutions that derive from chronic sun exposure. To date, large-scale sequencing studies have analyzed low-CSD and high-CSD melanomas as one group; however, their distinct mutational profiles suggest that separate analyses may be more informative.

Not surprisingly, the mutational landscape of high-CSD melanomas shows a marked increase in C > T substitutions compared to that of low-CSD melanomas, where C > T mutations are already quite common. An average high-CSD melanoma

displays a point mutation rate of approximately 20 mutations per Mb of DNA (Mar et al. 2013). Such an elevated mutation burden is on the high end of all melanoma subtypes and is exceeded only by the desmoplastic subtype, discussed in the following section. As immunotherapy advances in clinical use, the high mutational load of high-CSD melanoma may hopefully render it particularly foreign to a reinvigorated immune system.

In further contrast with low-CSD melanomas, high-CSD melanomas are not associated with nevi (Whiteman et al. 2003). This observation goes hand-in-hand with the *BRAF* V600E mutation, the primary genetic driver of acquired nevi, occurring less commonly in high-CSD melanomas. Instead, high-CSD melanomas are associated with a lentiginous growth pattern where melanocytes are distributed as a single cell along the basement membrane of the epidermis. Such a cellular distribution may be driven by the non-*BRAF*-V600E MAPK pathway mutations common in high-CSD melanoma.

Age of incidence additionally underscores a distinction between low-CSD and high-CSD melanomas. While the proportion of melanomas arising on the trunk of the body (intermittently exposed to the sun) peaks around 55 years of age, the proportion of melanomas arising on the chronically sun exposed face and ears peaks around 75 years (Lachiewicz et al. 2008). Incidence of melanomas on the face and ears continues to rise past 70 years of age, while incidence of melanomas on the trunk has already plateaued by then. It remains a paradox why melanocytes sustaining higher levels of mutagenic UV radiation should develop more slowly into melanomas than their low-CSD counterparts.

Desmoplastic

Like high-CSD melanomas, desmoplastic melanomas arise later in life on areas of high sun exposure, such as the face and the neck. The cells of this melanoma subtype are located primarily in the dermis. However, a high mutation prevalence (62 mutations/Mb on average) with a C > T UV radiation signature intimates an epidermally located cell-of-origin (Shain et al. 2015b).

BRAF V600E and *NRAS* Q61 mutations are not seen in desmoplastic melanomas, nor are *KIT* mutations. The most common mutations in desmoplastic melanoma are *TERT* promoter mutations in 85% of tumors, *NF1* mutations in 52–93%, and *TP53* mutations in 48–60% (Fig. 6a) (Shain et al. 2015b; Wiesner et al. 2015). There are also recurrent mutations, often homozygous, in the promoter of *NFKBIE* in 14.5% of cases. Such mutations likely lead to increased expression of the IKBE protein, an inhibitor of the NF-KB signaling cascade.

Many other genes are mutated in desmoplastic melanoma, but the exceptionally high mutation prevalence makes identifying driver mutations a challenge, especially as few tumors have been sequenced to date. Genes with a relatively high fraction of loss-of-function mutations, likely to serve as drivers, include *CDKN2A*, *ARID2*, *CBL FBXW7*, and *RB1*, among others mutated in 10–30% of cases (Fig. 6a). Several mutations seen recurrently in other melanoma subtypes have also been observed at low levels in desmoplastic melanoma (around 3%), specifically *MAP2K1* P124 mutations, *PPP6C* R264C, and *RAC1* P29S (Shain et al. 2015b).

Copy number alterations occur less often in desmoplastic melanoma than in other melanoma subtypes. Deletion of *CDKN2A* has been noted in 18% of examined tumors and *NF1* in 6.5% (Shain et al. 2015b).

Glabrous

Glabrous, or non-hair-bearing, skin can be found covering the palms of the hands, soles of the feet, and in the nail beds. Melanomas that arise in glabrous skin are termed acral melanomas. Acral melanomas are not associated with acral nevi and tend to grow around eccrine glands during early stages of in situ acral melanoma.

By virtue of both their location and a thick stratum corneum or nail plate, acral melanocytes and their neoplastic outgrowths are not exposed

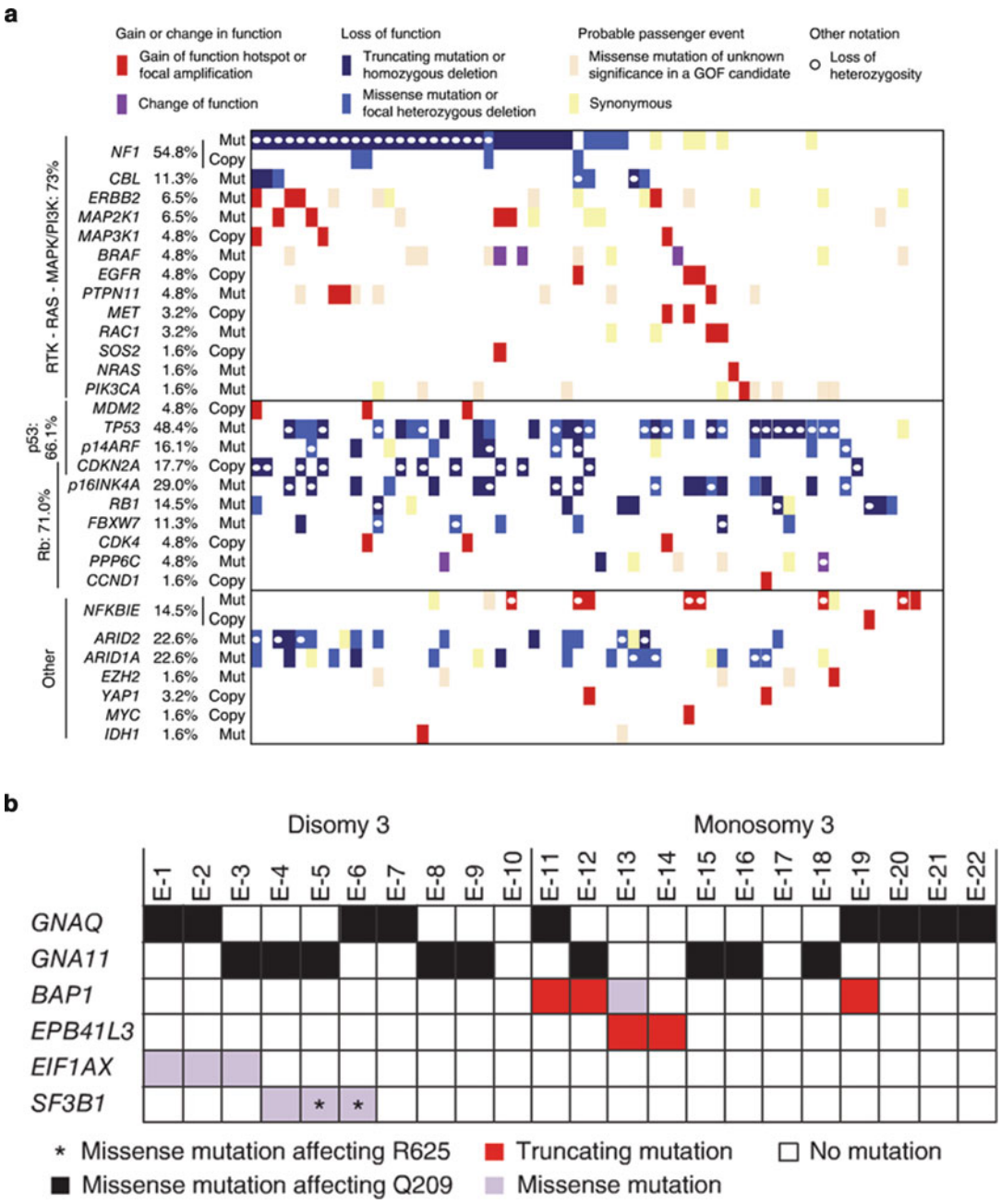

Fig. 6 Driver mutation landscape of desmoplastic and uveal melanoma. (**a**) Co-occurrence plot of putative driver mutations and copy number alterations in desmoplastic melanoma. Genes are represented as rows and individual desmoplastic melanoma samples as columns. (**b**) Co-occurrence plot of mutations in GNAQ, GNA11, BAP1, EPB41L3, EIF1AX, and SF3B1 in uveal melanoma, grouped by disomy/monosomy 3 status. Individual uveal melanoma tumors are represented as columns and genes as rows (Figures reprinted with permission from (**a**) Shain et al., Nat Genet 2015. https://doi.org/10.1038/ng.3382 and (**b**) Martin et al., Nat Genet 2013. https://doi.org/10.1038/ng.2674)

to high levels of UV radiation. As a result, the genomes of acral melanomas do not tend to show a signature of UV light–induced mutagenesis and on average carry only a modest burden of point mutations of around 2–3 per Mbp (Berger et al. 2012; Furney et al. 2014; Hodis et al. 2012; Krauthammer et al. 2012). Instead numerous amplifications and deletions, arising early in neoplastic progression and distinct in pattern from those of cutaneous melanomas, are more characteristic of the mutational landscape of this subtype of melanoma (Bastian et al. 2000b; Curtin et al. 2005).

The most frequent amplifications increase the number of copies of *CCND1*, *TERT*, *CDK4*, and *KIT* (Curtin et al. 2005, 2006). Amplifications of *CCND1* and *CDK4* occur in a mutually exclusive pattern. Frequent deletions also overlap the *CDKN2A* gene locus, specifically in tumors without *CDK4* amplifications.

Mutations commonly affect *KIT* (13.5% of tumors), *BRAF* (16.2%), and *NRAS* (11.4%). Any given acral melanoma tends to be mutant in only one of these three genes, if any (Curtin et al. 2005, 2006; de Vazquez et al. 2016). *TERT* promoter mutations (8.4%) have also been reported in acral melanoma.

Mucosal

Mucosal (Nonocular Mucosal)

Outside of the skin, melanocytes also inhabit the epithelial mucosa that lines the respiratory, intestinal, and urogenital tracts. Neoplasia of these mucosal melanocytes is most common in the anogenital mucosa and in the sinonasal cavity and paranasal sinuses.

Mucosal melanomas tend to have a high frequency of focal amplifications and deletions (Curtin et al. 2005) and a low frequency of point mutations, in comparison with cutaneous melanomas (Furney et al. 2013; Hodis et al. 2012; Krauthammer et al. 2012). Mucosal melanocytes are generally inaccessible to the rays of the sun; correspondingly, a signature of UV light–induced C > T mutations is not a feature of mucosal melanoma.

Focal amplifications and mutations (often activating) affect *KIT* in 30–40% of mucosal melanomas (Beadling et al. 2008; Curtin et al. 2006). *BRAF* or *NRAS* mutations are only seen in 3–11% and 5% of tumors, respectively (Curtin et al. 2005, 2006).

Focal amplifications of *CDK4* and deletions of *CDKN2A* are common and occur in a mutually exclusive fashion (Curtin et al. 2005). Focal deletions and mutations also frequently affect the tumor suppressor gene *PTEN* (Curtin et al. 2005; Furney et al. 2013).

Conjunctival (Ocular Mucosal)

The conjunctiva of the eye is a mucosal epithelium and contains resident melanocytes. Conjunctival melanoma can develop de novo, from conjunctival nevi, or from primary acquired melanosis. Generally, melanocytes in the bulbar conjunctiva receive sun exposure, and malignant neoplasms of these cells have a high point mutation burden (~30/Mb) with a C > T mutational signature of UV light (Rivolta et al. 2015).

BRAF mutations have been identified in 50% of conjunctival nevi and 29–40% of conjunctival melanomas with mutations being more frequent on lesions involving the bulbar conjunctiva, but 0% of primary acquired melanosis (Goldenberg-Cohen et al. 2005; Griewank et al. 2013), suggesting multiple possible neoplastic trajectories. *NRAS* mutations are reported to occur in 18% of conjunctival melanomas, in the absence of *BRAF* mutations, and copy number gains including the *KIT* locus affect 17% of these tumors.

TERT promoter mutations exist in 8% of primary acquired melanosis and 41% of conjunctival melanomas, but 0% of conjunctival nevi (Koopmans et al. 2014).

The copy number alterations observed in conjunctival melanomas parallel those seen in cutaneous rather than uveal melanomas. Loss of the *PTEN* 10q23 locus is common in the context of *BRAF* mutation (Griewank et al. 2013).

Conjunctival melanocytic neoplasms, in particular those arising from the bulbar, sun-exposed conjunctiva, seem to be more similar in terms of driver mutation patterns to cutaneous melanocytic neoplasms (specifically the low-CSD subtype) than to mucosal melanocytic neoplasms.

Melanomas arising from the tarsal conjunctiva that arise from primary acquired melanosis may be more closely related to mucosal melanomas, but additional studies are needed to catalog their genetic alterations.

Congenital

Congenital nevi are melanocytic nevi that arise in utero. Clinically, this definition is not held strict, and nevi acquired shortly after birth are also sometimes called congenital nevi. Congenital nevi are commonly bigger in diameter than acquired nevi, can cover large swathes of the body, and may be associated with multiple satellite congenital nevi.

Over 80% of congenital nevi harbor Q61 activating mutations in *NRAS* (Bauer et al. 2007) as the sole detectable mutation. Because these mutations happen in utero, UV light necessarily cannot play a role.

Less commonly, *BRAF* gene fusions have been reported in congenital nevi with wildtype *NRAS* (Dessars et al. 2007). Whether V600E mutations in *BRAF* exist in congenital nevi is a more contentious question (Charbel et al. 2013), and reports of such may be confounded by the difficulty in ascertaining whether the nevi in question indeed arose in utero.

Although congenital nevi do not appear to arise in association with an epithelium, their driver mutation spectrum strongly suggests they be grouped with other epithelium-associated melanocytic neoplasms. The high prevalence of *NRAS* mutations and corresponding lack of *GNAQ* or *GNA11* mutations (see next section for discussion of these mutations) suggest a melanocyte cell-of-origin fated for epithelial residence.

Within congenital nevi, benign proliferative nodules can develop, which tend not to progress to malignancy, and even regress. Whole chromosome aneuploidy is common in these nodules, most frequently loss of chromosome 7 in 30% of tumors examined (Bastian et al. 2002).

Melanomas may develop within congenital nevi, an especially common event in large or giant congenital nevi. The driving mutations of this neoplastic progression await discovery.

Neoplasms Arising from Nonepithelial Melanocytes

Skin

Blue nevi are benign neoplasms of nonepithelial melanocytes. The melanocytes of these neoplasms primarily populate the dermis.

Acquired blue nevi overwhelmingly display mutation of Q209 in either *GNAQ* or *GNA11*, in roughly 65–80% and 7% of cases, respectively (Raamsdonk et al. 2008, 2010). R183 mutations are significantly less common.

Congenital blue nevi include nevi of Ota, nevi of Ito, and Mongolian spots. Mongolian spots affect the lower back. Nevi of Ota involve the trigeminal nerve, periorbital skin, and the conjunctiva of the eye. Nevi of Ota are a risk factor for uveal melanoma; around 20% have a *GNAQ or GNA11* mutation. The same mutations are also seen in nevi of Ito, which involve the cervical nerve and shoulder (Tse et al. 2016).

Like acquired nevi, blue nevi rarely become malignant, but there is some evidence that loss-of-function mutations of *BAP1* may be a driver of blue nevus-like melanoma (Dai et al. 2016; Yeh et al. 2014).

Eye

Melanocytes also inhabit the uveal tract of the eye: the choroid, ciliary body, and iris. Melanocytic neoplasia in the uveal tract produces uveal nevi and melanomas. Uveal melanoma accounts for 5% of all melanomas and is the most common primary cancer of the eye.

Uveal melanoma risk has not been definitively associated with UV light exposure by epidemiological studies. Additionally, sequencing studies did not reveal a preponderance of C > T mutations, the canonical mutation induced by UV light (Johansson et al. 2016). Instead, uveal melanoma is characterized by low point mutation prevalence (average of 10.6 nonsilent coding mutations (Johansson et al. 2016)) and recurrent chromosomal copy number alterations.

A missense mutation in either *GNA11* or *GNAQ*, encoding homologous G-protein α subunits, appears to be the common initiating event in uveal melanocytic neoplasia. Over 80% of uveal

melanomas have a mutation of Q209 (more common) or R183 in either *GNA11* or *GNAQ* (Raamsdonk et al. 2008, 2010). Both mutations interfere with GTPase activity (Q209 completely abolishes it, R183 only partially) of the Gαq proteins, leading to constitutive activity. GNA11 and GNAQ appear to signal through the protein kinase C pathway, the MAP kinase pathway, and the Hippo pathway, promoting melanocyte proliferation (Feng et al. 2014; Yu et al. 2014). In cases without *GNA11* or *GNAQ* mutations, D630Y mutations in the downstream effector *PLCB4* have been noted (4% of cases) (Johansson et al. 2016) as well as L129Q mutations in the upstream receptor *CYSLTR2* (3%) (Moore et al. 2016). Both mutations are likely oncogenic.

Chromosomal copy number alterations are frequent in uveal melanoma, most often manifesting as monosomy 3, trisomy 8, deletions in chromosome 1, and alterations in chromosome 6 (Chattopadhyay et al. 2016; Horsman and White 1993).

Whereas the presence of a *GNA11* or *GNAQ* mutation is a ubiquitous, early feature of uveal melanomas, monosomy 3 is a later event that splits the malignancy into two groups of roughly equal prevalence. Monosomy 3 is strongly associated with metastasis, primarily to the liver. The target of monosomy 3 appears to be *BAP1* (BRCA1-associated protein 1), since over 80% of the 50–60% of uveal melanomas with monosomy 3 also have deleterious mutations in *BAP1* (Harbour et al. 2010). *BAP1* encodes a nuclear deubiquitinase, possibly involved in chromatin remodeling.

Uveal melanomas disomic in chromosome 3 rarely metastasize and tend to be wildtype in *BAP1*. In place of *BAP1* mutations, mutually exclusive mutations in either *SF3B1* (splicing factor 3b subunit 1) or *EIF1AX* (eukaryotic translation initiation factor 1A, X-linked) are common (Fig. 6b) (Harbour et al. 2013; Martin et al. 2013). *SF3B1* missense mutations in codon 625 occur in roughly 20% of disomy 3 uveal melanomas, while *EIF1AX* N-terminal missense mutations occur in about 45%, together accounting for approximately 65% of disomy 3 uveal melanomas.

Internal Organs

Melanocytoma of the Central Nervous System

Autochthonous melanocytes reside in the leptomeninges of the central nervous system (CNS). Neoplasia of these nonepithelial melanocytes gives rise to blue nevi called melanocytomas of the CNS. Malignancy at this site of origin is termed a primary melanoma of the CNS, and an intermediate grade of melanocytoma is also recognized.

Melanocytomas of the CNS almost universally possess an activating mutation in Q209 of either *GNAQ* or *GNA11* (Küsters-Vandevelde et al. 2010; Murali et al. 2012). Gains of chromosome 6p and losses of 3 or 3q have also been observed in 33% and 17%, respectively, of tumors examined (Koelsche et al. 2015).

Commonalities

The catalog of the driver mutations of each subtype of melanocytic neoplasia reveals the unique inner workings of each subtype (Table 1). But the catalog may also be studied in toto to detect patterns across subtypes, illuminating the basic genetic requirements for the development of melanoma.

There appear to be at least three main requirements for a melanocyte to transform into a melanoma. The first is activation of either the MAPK or the Gαq signaling pathway. The second requirement is disruption of the p16/cyclin/CDK/Rb pathway. The third is activation of telomerase.

The first two requirements have been recognized for some time. The existence of the third was made unimpeachable by the recent discovery of recurrent mutations in the promoter of *TERT* in roughly 70% of nonglabrous melanomas (Horn et al. 2013; Huang et al. 2013).

Should additional requirements exist, the continuing expansion of the catalog of driver mutations in melanocytic neoplasia will undoubtedly aid in their deciphering.

Table 1 Common driver mutations of melanocytic neoplasia. Frequent mutations associated with each subtype of melanoma are listed. Mutations are classified either as primary, initiating events, or events associated with neoplastic progression

Melanoma subtype	Site of origin	Primary (MAPK/Gαq)	Progression
Low-CSD	Skin epithelium	*BRAF* (V600E), *NRAS*, *NF1*	*TERT*, *CDKN2A*, *PTEN*, *TP53*, *ARID2*, *PPP6C*, *RAC1*, *IDH1*, *RPS27*, *MITF*, *MAP2K1*, *DDX3X*
Spitzoid	Skin epithelium	Kinase fusions (*ROS1*, *NTRK1*, *ALK*, *BRAF*, *RET*, *MET*), *BRAF* (V600E), *HRAS*	*TERT*, *CDKN2A*
High-CSD	Skin epithelium	*NF1*, *NRAS*, *BRAF* (V600K), *KIT*, *CCND1*	*TERT*, *TP53*, *ARID2*
Desmoplastic	Skin epithelium	*NF1*	*TERT*, *TP53*, *NFKBIE*, *CDKN2A*, *ARID2*, *CBL*, *FBXW7*, *RB1*, *MAP2K1*, *PPP6C*, *RAC1*
Acral	Skin epithelium	*BRAF*, *KIT*, *NRAS*	*TERT*, *CCND1*, *CDK4*, *CDKN2A*
Mucosal	Mucosal epithelium	*KIT*, *BRAF*, *NRAS*	*CDK4*, *CDKN2A*, *PTEN*
Conjunctival	Mucosal epithelium	*BRAF*, *NRAS*, *KIT*	*TERT*, *PTEN*
Congenital	Develops in utero	*NRAS*	
Blue	Dermal	*GNAQ*, *GNA11*	*BAP1*
Uveal	Uveal tract of eye	*GNAQ*, *GNA11*, *PLCB4*, *CYSLTR2*	*BAP1*, *EIF1AX*, *SF3B1*
Leptomeningeal	Central nervous system	*GNAQ*, *GNA11, or NRAS (when congenital nevus associated)*	

Activation of MAPK or Gαq Signaling

In epithelial melanomas and melanocytic neoplasms, activating mutations in the MAPK signaling pathway are nearly ubiquitous. Lending further credence to the importance of MAPK pathway activation is the observation that there tends to be only one mutated gene in the pathway per tumor. In each subtype, the MAPK pathway mutations are early events that usually suffice to form a nevus.

The classic subtype for making these observations is melanoma on nonglabrous skin, where sequencing studies have noted that mutations in *KIT*, *BRAF*, *NRAS*, *NF1*, *MAP2K1*, *HRAS*, and *KRAS* account for roughly 90% of tumors (Fig. 7) (TCGA Network 2015; Hodis et al. 2012). However, these observations generalize across subtypes: every single subtype of epithelial melanocytic neoplasm harbors driver mutations in the MAPK pathway in a mutually exclusive pattern. Spitzoid melanocytic neoplasms harbor *BRAF* fusions, amplifications or mutations in *HRAS*, or receptor tyrosine kinase fusions of relevance to the MAPK pathway. Desmoplastic melanomas do not have *BRAF* or *NRAS* mutations but instead commonly mutate or lose *NF1*. Glabrous and acral melanomas are often driven by mutations and amplifications of *KIT*, but also have driver mutations in *BRAF* and *NRAS* some of the time. Conjunctival melanomas display mutually exclusive mutations in *BRAF*, *NRAS*, and *KIT*. And finally, congenital nevi are driven by oncogenic *NRAS* mutations, while acquired nevi are mostly driven by *BRAF* mutations. Future sequencing studies are likely to reveal the existence of yet unappreciated MAPK pathway mutations that contribute to melanocytic neoplasia.

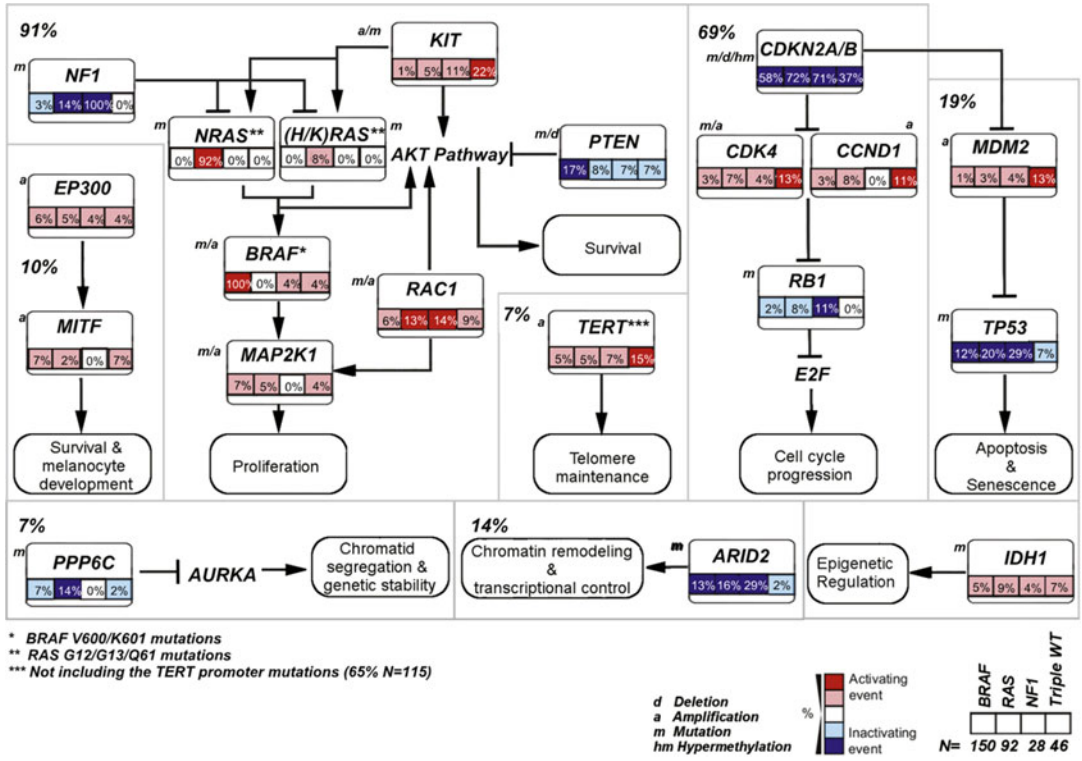

Fig. 7 Pathway-based summary of genetic drivers in cutaneous melanoma. Frequencies of candidate driver events in cutaneous melanoma overlaid on a schematic of the affected biological pathways. Aggregate frequency of alteration of a given pathway is given by the large, bold black percentage in each pathway-box. Frequency of alteration of each gene is displayed as four distinct numbers, each representing the frequency of the gene being altered in a specific melanoma subtype. The four subtypes are *BRAF*-mutant, *NRAS*-mutant, *NF1*-mutant, and triple-wildtype (wildtype in *BRAF*, *NRAS*, and *NF1*) melanoma (Figure reprinted with permission from TCGA Network, Cell 2015. https://doi.org/10.1016/j.cell.2015.05.044)

The clinical successes of small molecule inhibitors of BRAF and MEK in *BRAF* V600E melanomas underscore the importance of the MAPK signaling pathway in epithelial melanomas (TCGA Network 2015; Chapman et al. 2011; Flaherty et al. 2012). Not only is the pathway important in melanoma formation but also in tumor maintenance. Resistance to RAF and MEK inhibitors can come in the form of mutation-based reactivation of the MAPK pathway, further betraying the dependence of melanomas on MAPK pathway activity (Van Allen et al. 2014; Wagle et al. 2014).

In nonepithelial melanomas, activating mutations in the Gαq pathway take the place of mutations in the MAPK pathway. This distinction may reflect a difference in developmental origin between melanocytes residing in epithelia versus those located in nonepithelial tissues, or it may represent a difference in tissue environment. The former seems more likely given the diversity of tissue environments inhabited by both nonepithelial and epithelial melanocytes.

Gαq pathway mutations are nearly ubiquitous in nonepithelial melanomas and melanocytic neoplasms, and only one such mutation tends to exist in each tumor. Again, these mutations are early, initiating events, apparently sufficient for nevus formation. Blue nevi, melanocytomas, uveal nevi, and uveal melanomas are driven by mutation of either *GNAQ* or *GNA11* in more than 80% of cases. In uveal melanomas without *GNAQ* or *GNA11* mutations, mutations in *PLCB4* or *CYSLTR2* often occur instead. Here too it seems reasonable that future studies will reveal novel

Gαq pathway mutations in tumors without recognized mutations in the pathway.

If initial success in targeting the MAPK pathway in cutaneous melanoma serves as any example, attempts to target the Gαq pathway in uveal melanoma deserve prioritization.

Disruption of the p16/Cyclin/CDK/Rb Pathway

One of the earliest hints as to the importance of the Rb pathway in melanoma came with the discovery that *CDKN2A* mutations contribute frequently to both hereditary and sporadic melanomas (Hussussian et al. 1994; Kamb et al. 1994). While questions soon arose as to whether the p16 or the p14 protein product of the *CDKN2A* locus was the main culprit, subsequent sequencing studies credentialed the p16/cyclin/CDK/Rb pathway as more often mutated in melanoma, in comparison with the (still important) p14/MDM2/p53 pathway.

Mutations in the p16/cyclin/CDK/Rb pathway affect roughly 70% of nonglabrous skin melanomas (Fig. 7) (TCGA Network 2015). Only one of the various components of this pathway appears to be altered in any given tumor. Most commonly, p16 is lost through deletions or loss-of-function mutations in *CDKN2A*. In cases where wildtype p16 is present, *RB1* may be lost, *CDK4* may harbor an activating R24C mutation, or *CCND1* may be amplified.

Glabrous skin melanomas show frequent amplifications of *CCND1* or *CDK4* in a mutually exclusive pattern. With the same sort of pattern, mucosal melanomas harbor focal amplifications of *CDK4* or deletions of *CDKN2A*. Biallelic *CDKN2A* loss is seen in atypical Spitz tumors. And, roughly 70% of desmoplastic melanomas harbor mutations in the Rb pathway, including frequent mutations of either *CDKN2A* or *RB1* and amplifications of *CDK4* and *CCND1*.

The high frequency (around 70% in certain subtypes of melanoma) at which one and only one gene member of the p16/cyclin/CDK/RB pathway undergoes mutation in melanoma suggests that derangement of this pathway is compulsory, or nearly so, for melanoma formation.

In melanomas arising in nonepithelial tissues (blue, uveal, internal organ), mutations in the p16/cyclin/CDK/Rb pathway are generally not observed. If a parallel requirement exists in nonepithelial melanoma, loss of *BAP1* appears an attractive candidate for further investigation given its common loss in blue and uveal melanocytic neoplasms that have progressed beyond the nevus state.

The requirement for p16/cyclin/CDK/Rb pathway dysregulation, at least in epithelial melanomas, offers another target for future therapies. Reversing loss of the p16 tumor suppressor is not currently possible, but inhibition of downstream kinases CDK4/6 appears a reasonable strategy. As CDK4/6 inhibitors begin to show promise in clinical trials in other cancers, it will be worth considering simultaneous targeting of both the MAPK pathway and the p16/cyclin/CDK/Rb pathway in melanoma. Co-targeting two required and nearly orthogonal pathways should reduce the odds of acquired resistance to therapy.

Telomerase Activity

Over 90% of melanomas have detectable telomerase activity (Glaessl et al. 1999). Telomerase is a ribonucleoprotein enzyme responsible for replicating the protective repeat sequences found at the end of chromosomes, known as telomeres. Without telomerase, telomeres shorten with each round of cell division, eventually exposing chromosomal ends, which then fuse by nonhomologous end joining leading to di- and multicentric chromosomes and genomic instability. Telomere attrition thus provides a limit on the total number of cell cycles a cell may undergo in the absence of telomerase activity ("the Hayflick limit"). Somatic cells apart from stem cells do not express *TERT*, the catalytic protein subunit of telomerase, so as a result telomerase remains inactive in these cells. Cancers, on the other hand, must find a way to activate telomerase in order to maintain their telomeres in pursuit of replicative immortality.

Telomerase activation often comes by way of turning on expression of *TERT*; the remaining subunit of telomerase, encoded by *TERC*, is usually already expressed in somatic cells.

The majority of melanomas appear to initiate expression of *TERT* through mutating the *TERT* promoter. Recurrent mutations affecting either one of two base pairs in the *TERT* promoter are found in approximately 70% of nonglabrous melanomas, 85% of desmoplastic melanomas, and 41% of conjunctival melanomas, as noted earlier in this chapter. Both promoter mutations are expected to activate expression from the *TERT* locus through creation of an Ets family transcription factor binding site proximal to the start site of transcription. *TERT* amplifications are also found in melanoma, and glabrous melanomas display focal *TERT* amplifications, as well as occasional *TERT* promoter mutations.

Melanomas of the extra-epithelial lineage generally do not harbor *TERT* promoter mutations or *TERT* amplifications, yet have active telomerase (Heine et al. 2000). The common mechanisms by which telomerase is activated in these melanomas remain to be elucidated.

As nearly all melanomas seem to require telomerase activity, at least at some point in pathogenesis, efforts to investigate telomerase as a therapeutic target in melanoma should be revived. Telomerase inhibition offers the benefit of targeting a melanoma requirement that is independent from either the requirement for MAPK (or Gαq) signaling or the requirement for dysregulation of the p16/cyclin/CDK/Rb pathway, and so may present a third orthogonal vulnerability for concurrent targeting.

Conclusion

Based on knowledge of genetic alterations common across subtypes of melanoma, at least three requirements for melanoma pathogenesis may be deduced. The first is activation of either the MAPK or the Gαq signaling pathway, the second is disruption of the p16/cyclin/CDK/Rb pathway, and the third is telomerase activity. The latter two requirements do not yet seem to translate directly to melanomas arising in nonepithelial tissues and may benefit from future redefinition as more is learned about such melanomas. In addition, each subtype of melanoma and melanocytic neoplasia demonstrates its own recurrent cast of mutated genes, some of which may be shared with just one or two other subtypes. UV radiation often fuels the dominant mutagenic process; however, genomic instability too molds the melanoma genome. The genetics of melanocytic neoplasia defines the evolutionary paths to melanoma genesis and progression and provides the ultimate lens through which the disease must be viewed if it is to be understood.

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Disclosure of Potential Conflicts of Interest
E.H. is a consultant for GV. L.A.G. reports receiving a commercial research grant from Novartis, has ownership interest (including patents) in Foundation Medicine, and is a consultant/advisory board member for Warp Drive, Novartis, Boehringer Ingelheim, and Foundation Medicine. L.A.G. is an employee of Eli Lilly and Company.

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Biology of Melanoma Metastasis

8

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Contents

Abstract

Metastasis is the major cause of death in patients suffering from malignant melanoma, one of the most aggressive types of cancer. Local recurrence is rare in melanoma, but regional and distant metastasis, particularly in the brain, bone, lung and liver, may arise even many years after primary tumor resection, probably due to reactivation of dormant melanoma cells. Dormancy of dispersed melanoma cells is regulated by tumor cell intrinsic as well as extrinsic factors, including angiogenesis, immunosurveillance, and other stroma-dependent processes. Melanoma metastasis correlates with local invasion and the vertical growth phase at the primary site. During this phase, cancer cells invade deep into the dermis and interact with various stromal cells to gain access to blood and lymphatic vessels. Subsequently, melanoma cells migrate along the local lymphatic vasculature, sometimes

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resulting in locoregional lesions such as in-transit metastases, before reaching the draining lymph node and spreading systemically. Both lymphatic and blood vessels play a critical role in the dispersion of melanomas. Besides providing a simple transport route for cancer cells, endothelial cells are emerging as important and active players in melanoma metastasis. Conditioned by factors derived from the tumor microenvironment, the vasculature undergoes morphological and functional changes, which facilitate the metastatic process in multiple ways. Consequently, therapeutic manipulation of lymphatic and blood vessels may represent a new way to combat melanoma metastasis.

Keywords
Lymphangiogenesis · Angiogenesis · VEGF · Lymphatic vessels · Dormancy · Local invasion

Introduction

Malignant melanoma is one of the most aggressive cancer types. Whereas the primary tumor is usually completely removed by surgery, metastases are the predominant cause of mortality among melanoma patients. The 5-year overall survival for patients without regional or distant metastasis (Stage I) in a European cohort has been reported to be as high as 95–100%, whereas for patients with distant organ metastases (Stage IV), it was only 28–44% (Svedman et al. 2016). Only around 4% of the patients newly diagnosed with melanoma present with clinically apparent distant metastasis at the initial diagnosis. However, approximately one third of patients experience recurrence after resection of the primary tumor, usually not manifesting as local recurrence at the site of the original tumor, but as distant metastases. This distant metastatic recurrence in melanoma patients can occur within a relative short period of time, but in about 40% patients, it can take up to 5 years or more (Damsky et al. 2014).

Melanoma metastases can be classified based on their location into locoregional, regional, and distant metastases. Locoregional metastases include satellite lesions (in direct proximity to the primary tumor) and in-transit metastases, which develop from metastatic cells that have entered the lymphatic vascular system and grow to form a metastatic nodule at a distance of >2 cm from the primary tumor but proximal to the draining lymph node. The term "regional metastases" denotes lesions forming in the draining lymph node(s). Distant organ metastasis is common in malignant melanoma, and predominantly develops in the brain, bone, lung, and liver in the case of cutaneous melanomas, whereas uveal melanomas metastasize preferentially to the liver (Damsky et al. 2014). In addition to basic parameters such as primary tumor depth and ulceration, the metastatic pattern has an important influence on the prognosis and has consequently been included in the current staging system for cutaneous melanoma, established in 2003 by the Melanoma Staging Committee of the American Joint Committee on Cancer, which has subsequently been adopted by several health-care organizations around the world (Balch et al. 2004). Stage I and Stage II are comprised of patients without regional or distant metastases. Stage III patients have metastases either in the regional lymph nodes and/or regional skin or in-transit metastases, whereas Stage IV is defined by the presence of distant metastasis.

On a cellular level, the metastatic process can be divided into distinct steps, each of which represents a hurdle that cancer cells need to overcome in order to form a metastatic lesion. These stages include (a) local invasion into the dermis, (b) entry into the vasculature, (c) survival in the circulation, (d) extravasation at a distant organ site, and (e) survival and growth to form a metastasis. Consequently, the vascular system plays an important role in metastasis by providing a route for dissemination. In line with this model, it has traditionally been assumed that melanoma cells undergo a stepwise transformation process, acquiring the mutations that enable them to form distant metastasis only at late phases of tumor progression. This view is supported by the good clinical correlation between the depth of the primary tumor (representing the capability for local invasion) and the occurrence of distant metastasis. However, there is evidence that spread of tumor cells can occur very early during primary tumor

formation as well. For example, metastatic spread of uveal melanoma cells has been detected up to 5 years before diagnosis of a primary tumor. In fact, 4–12% of patients with metastatic melanoma never presented with any detectable primary tumor. Thus, metastatic spread of malignant or even pre-malignant melanocytes may occur very early during tumor progression, at least in some cases. In line with this, nonmalignant melanocytes are found within the dermis and even in lymph nodes from non-melanoma-bearing patients, demonstrating an inherent capacity of melanocytes to migrate and disseminate, even before malignant transformation (Damsky et al. 2014).

The sometimes very long lag phase between primary tumor resection and recurrence, called the "dormancy period," indicates that disseminated melanoma cells can survive for extended periods of time in a dormant stage without growing into clinically manifest metastases. How dormancy of cancer cells and their "awakening" is regulated is still not fully understood, but several models have been proposed based both on clinical experience and experimental data.

This chapter summarizes our current knowledge about the regulation of dormancy, local invasion, and distant spread of metastatic melanoma cells through the blood and lymphatic vascular systems.

Dormancy in Metastatic Melanoma

Metastatic dormancy is defined clinically as the disease-free period between removal of the primary tumor and subsequent local or distant recurrence. In melanoma, local recurrence is rather rare, due to the advanced surgery procedures and safety margins. However, depending on the stage of the tumor at the time of resection, distant metastasis is much more common. Similar to, for example, prostate and breast cancer, melanomas often have a very long period of dormancy which may last up to many years after resection of the primary tumor, during which the patient remains clinically tumor-free. Localized melanoma can recur after disease-free intervals of 10 years or more, and a small subset of melanomas have been reported to have ultra-long dormancy with recurrence more than 20 years later (Damsky et al. 2011).

Clinical experience and experimental work has resulted in the proposal of several mechanisms that maintain melanoma cells in a dormant state and which trigger their release from dormancy and outgrowth to form a clinically detectable metastasis, respectively.

Quiescence of Disseminated Tumor Cells

Disseminated, single melanoma cells which are negative for the proliferation marker Ki67 and which show no signs of apoptosis are frequently detected in autopsy material. This phenomenon of solitary dormant tumor cells suggests that disseminated tumor cells (DTCs) can enter a state of reversible cell cycle arrest, or quiescence, during which they can remain viable over long periods of time without undergoing proliferation. Quiescence may result from a "mismatch" between the tumor cell and its host tissue but may also be actively maintained, either by tumor cell intrinsic factors or by signals derived from the microenvironment (Senft and Ronai 2016; Damsky et al. 2014). Signaling pathways associated with tumor dormancy appear to regulate growth, metabolism, and survival of cells in response to microenvironmental changes, such as p21 and p27 which control cell cycle, mTOR signaling, and the PI3K/AKT signaling pathway (Ossowski and Aguirre-Ghiso 2010; Senft and Ronai 2016).

As recurrence at distant sites can occur several years even after removal of low-grade tumors that showed no signs of invasion, it is conceivable that tumor cells or even pre-malignant melanocytes disseminate very early during disease development. These cells therefore may lack some of the mutations which mediate malignant transformation in the primary tumor, which results in their growth arrest. Only upon further genetic or epigenetic alterations, these cells would gain the ability to re-enter the cell cycle and form an overt metastasis. This model of "parallel progression" is supported by genetic analyses that indicated

considerable heterogeneity between primary and matched metastatic tumors (Damsky et al. 2014).

There is significant heterogeneity among the cells within the primary tumor mass. In some cancer types, a relatively small population of cancer cells have been identified that possess stemlike properties such as self-renewal, reminiscent of normal tissue stem cells. These cells have been denoted as "cancer stem cells" (CSCs) or tumor-initiating cells. CSCs are thought to proliferate at a very low rate or remain in a quiescent state for long periods of time, making them resistant to conventional therapies such as chemotherapy and radiation therapy, which specifically target proliferating cells. The self-renewal capacity of CSCs allows them to maintain and drive tumorigenesis, while the differentiation capability contributes to cellular heterogeneity of the tumor. In the case of melanoma, the existence of a CSC pool has remained hypothetical. However, partial dedifferentiation of melanoma cells with a concomitant gain of certain stem-cell-like properties has been observed. For example, inflammatory stimuli were found to result in "phenotypic switching" of melanoma cells between a differentiated and dedifferentiated, stemlike state (Landsberg et al. 2012). Dedifferentiated melanoma cells disseminated to distant sites may account for some of the observed dormant DTCs and could become reactivated upon certain stimulating signals. Dormant cancer cells at distant sites are often found in close proximity to blood vessels, which have been suggested to provide a stemness mediating niche, similar to the niche in which normal body stem cells reside (Ossowski and Aguirre-Ghiso 2010). Somewhat paradoxically, hypoxia has also been implicated in the regulation of cellular dormancy and dedifferentiation of melanoma cells (Senft and Ronai 2016). Melanoma cells in hypoxic tumor regions are often amelanotic, and in vitro studies indicate that hypoxia can downregulate microphthalmia-associated transcription factor (MITF), a key transcription factor for melanocyte differentiation. However, it remains currently unclear to what extent dedifferentiation of melanoma cells in fact contributes to the status of dormancy.

Lack of Blood Vessel Supply and the Angiogenic Switch

Rapid expansion of a tumor mass requires increased supply of oxygen and nutrients. However, angiogenesis is strictly regulated by pro-angiogenic factors such as vascular endothelial factors (VEGFs) and by endogenously expressed angiogenesis inhibitors, such as thrombospondin 1 (TSP-1) (Detmar 2000). Consequently, angiogenesis does not occur under physiologic conditions at distant sites, and small clusters of disseminated tumor cells may fail to recruit sufficient vascular supply due to the lack of expression of angiogenic factors and/or high expression of angiogenic inhibitors in the surrounding tissue. Under such conditions, tumor cell proliferation is counterbalanced by cell death caused by oxygen and nutrient deprivation, so that the metastasis remains very small and nonprogressive. Metastatic outgrowth will only occur upon a break in the balance of pro- and anti-angiogenic factors, termed as "angiogenic switch." This may occur, for example, when a subset of tumor cells adapt to produce elevated levels of angiogenic factors as a consequence of mutations or in response to environmental stress, in particular to inflammation that may be promoted by bone marrow-derived myeloid cells. Tumor-associated fibroblasts and other immune cells recruited to a metastasis are also thought to provide angiogenic factors that may induce the switch to a pro-angiogenic phenotype.

It is difficult to generate direct evidence whether this mechanism is actually relevant for tumor cell dormancy in melanoma patients, since dormant cells cannot be detected until they grow out to form a metastasis. However, it has been reported that human melanoma micrometastases, which proliferate at a low rate, are poorly vascularized, whereas macro-metastases, which proliferate at a high rate, are significantly better vascularized. Also, some experimental evidence suggests that anti-angiogenic therapies induce tumor growth inhibition and inhibit recurrence in animal melanoma models and in human melanoma patients (Ossowski and Aguirre-Ghiso 2010). However, whether the absence of

angiogenesis is responsible for the prolonged quiescence of disseminated melanoma is still unknown. In fact, the observation that dormant tumor cells often reside in close proximity to pre-existing blood vessels speaks against a lack of blood supply as a determinant for the maintenance of dormancy, at least at the cellular level. Nevertheless, the angiogenic switch may still be involved in the transition from micro- to macrometastases.

Immunosurveillance

Organ transplantation between donors carrying dormant melanoma cells and immunocompromised recipients frequently results in rapid growth of metastases. This observation has led to the hypothesis that constant immunosurveillance at distant sites contributes to dormancy in melanoma. Melanoma is considered to be a rather immunogenic tumor type, due to its relatively higher frequency of mutations with effects on the protein sequence, as compared to most other human cancers, and to the expression of highly melanocyte-specific antigens such as Melan-A/MART-1 that can be recognized by cytotoxic T lymphocytes (CTL) in association with MHC class I. Consequently, CTL-mediated killing and/or cytostatic activity of immune cytokines such as IFN-γ can restrict the growth of metastatic lesions (Ossowski and Aguirre-Ghiso 2010). Dormant cells likely evade immunosurveillance, for example, due to partial dedifferentiation and thus downregulation of melanocyte antigens or by the expression of immune-inhibitory signals. Furthermore, constant deletion of immunogenic tumor cells arising from dormant lesions results in the selection of poorly immunogenic and/or immunosuppressive cancer cell clones over time, a process termed "cancer immunoediting" (Vesely and Schreiber 2013). Such clones can then initiate proliferation and generate distant metastasis.

Taken together, the proposed tumor dormancy mechanisms can be divided into two major categories: dormancy of single disseminated tumor cells (quiescence) and dormancy of clusters of disseminated cancer cells at the micrometastatic state (angiogenic suppression and immunosurveillance). As of now, it is not entirely clear which of those mechanisms is the most important one in the case of melanoma, but it is conceivable that these mechanisms function in parallel to maintain the sometimes very long dormancy phases observed in melanoma patients.

Local Invasion

According to the classic "Clark Model" of melanoma growth (Elder 2016), primary melanoma develops from benign nevi or de novo, and progresses through distinct, histologically defined stages, before giving rise to fully malignant, metastatic disease. Tumors evolve from the radial growth phase (RGP), in which tumor growth is confined to epidermal layers, to a vertical growth phase (VGP), in which the tumor cells gain the ability to invade the dermis. This step in the tumor progression enables tumor cells to get in direct contact with blood and lymphatic vessels present in the dermis and to invade them, resulting in systemic dissemination. Molecular changes related to invasiveness, such as upregulation of growth factors, cytokines, angiogenic and fibrogenic factors, chemotactic and motility factors, as well as immune response-related molecules and adhesion molecules, have been identified in the VGP. Thus, angiogenesis and lymphangiogenesis are often induced during the VGP (Fig. 1).

The Molecular Basis of Invasive Growth

The transition from RGP to VGP is associated with changes in the gene expression pattern that help melanocytes to invade and proliferate in the dermal microenvironment. For example, decreased E-cadherin levels have been reported to reduce the interaction with keratinocytes that control the behavior of normal melanocytes; increased levels of N-cadherin on the other hand enable melanoma cells to interact with fibroblasts, facilitating survival outside of the epidermis. This is reminiscent of the epithelial-to-mesenchymal

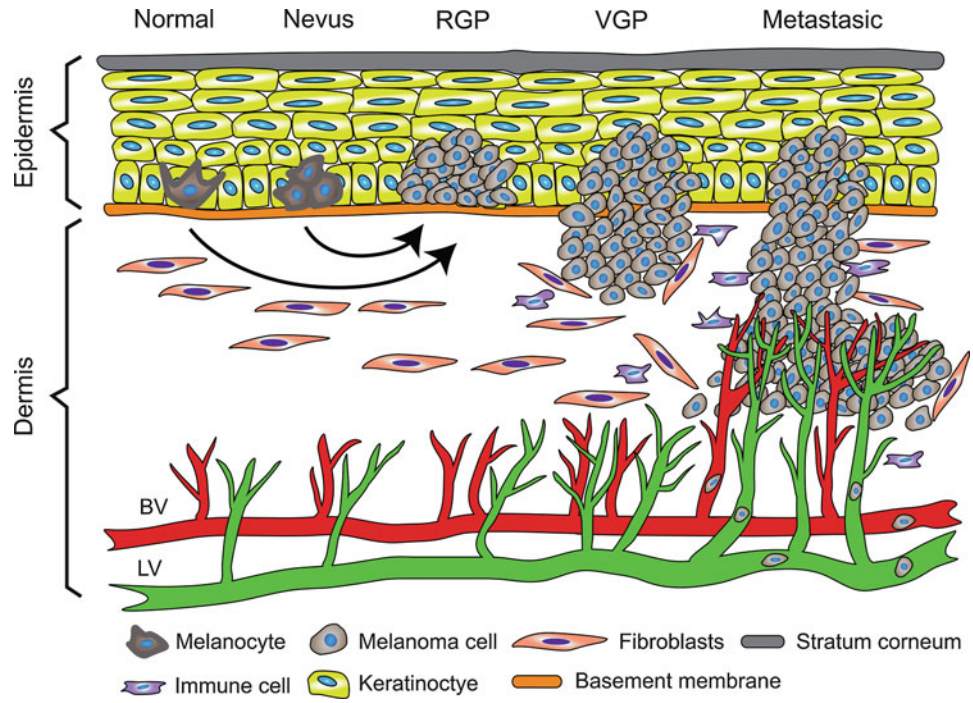

Fig. 1 Development of metastatic melanoma. Primary melanomas develop from benign nevi or de novo (indicated by black arrows) and progress through the radial growth phase (*RGP*) to the vertical growth phase (*VGP*) and finally toward metastatic disease. The transition from RGP to VGP is associated with the acquisition of invasive behavior and metastatic potential by regulating gene expression related to survival, cell adhesion, cell mobility, and (lymph)angiogenesis. Interactions between melanoma cells and stromal cells, including fibroblasts and immune cells, also facilitate the malignant progression. *BV* blood vessel, *LV* lymphatic vessel

transition (EMT) process described in carcinomas, and indeed, transforming growth factor-beta (TGF-β)-induced upregulation of snail family transcription factors has been reported to induce a mesenchymal pattern of gene expression that promotes melanoma cell mobility and invasion. Also, expression changes in regulators of the cell cycle and apoptosis are considered crucial for tumor cell survival in the dermis. For example, combined gain-of-function mutations of the Ras gene and loss of TP53 can drive invasion by increasing survival of invasive cells. Similarly, B-raf signaling, which is often hyper-activated in melanoma due to mutations in the *BRAF* gene, is capable of promoting the expression of fibronectin and its receptor component integrin beta 3. Furthermore, growth factors and cytokines released by melanoma cells, including hepatocyte growth factor (HGF), TGF-β, and basic fibroblast growth factor (bFGF), may stimulate invasive growth in an autocrine manner (Gaggioli and Sahai 2007; Leong et al. 2012). Metalloproteinases (MMPs) from melanoma cells, such as MMP-2 and MMP-13, degrade the extracellular matrix and promote invasion (Damsky et al. 2011; Leong et al. 2012).

Crosstalk between stromal cells in the dermis and melanoma cells facilitates invasion. Growth factors and cytokines produced by melanoma cells have been found to alter the behavior of adjacent fibroblasts, immune cells, and endothelial cells, stimulating production of other growth factors such as platelet-derived growth factor (PDGF) and HGF. VEGFs, in particular VEGF-A and VEGF-C, produced by melanoma cells and some stromal cells, induce (lymph)angiogenesis and vasculature dilation, preparing the routes for metastatic spread. As the invasiveness increases, melanoma cells overexpress the cell

adhesion molecule MCAM (melanoma cell adhesion molecule), which can promote the interaction with endothelial cells and facilitate intravasation (Gaggioli and Sahai 2007; Leong et al. 2012).

Satellite Lesions

In 1981, the term "microscopic satellites" was first used to define nests of melanoma cells separated from the main body of the tumor mass by a layer of collagen or subcutaneous fat. Nowadays, the term is used to denote lesions >0.05 mm in diameter that are separated from the main tumor mass by at least 0.3 mm (Balch 2009). Satellite lesions might represent an early step in the development of intralymphatic metastases and can be regarded as a manifestation of the invasive potential of the melanoma cells. Clinical studies revealed that the presence of satellite lesions is intimately related to other markers of melanoma aggressiveness. For example, presence of microsatellites appears to predict regional lymph node metastases but did not correlate with distant metastasis or overall survival (Shaikh et al. 2005), suggesting that local invasiveness and distant metastasis might not necessarily depend on the same processes.

Lymphatic and Angiogenic Spread

As in most tumor types, angiogenesis and lymphangiogenesis are critical steps in the progression of melanoma. Angiogenesis is induced by the growing tumor as a result of a relative lack of oxygen and nutrient supply in the malignant tissue. The expanding vasculature thus improves the growth conditions for the tumor cells. At the same time, expanding blood and lymphatic vessels also facilitate the distant metastasis of malignant primary melanoma cells. In the following section, the morphological and functional changes of lymphatic vessels and blood vessels leading to the dissemination of metastatic cells, as well as their clinical and pathologic implications, will be discussed.

Structure and Function of the Vascular System

In vertebrates, there are two vascular systems: the blood vascular system and the lymphatic system. These two systems differ from each other structurally and functionally. The blood vascular system is circulatory, driven by the heart, which acts as a central pump. Blood vessels function to transport oxygen and nutrients to the peripheral tissues and carry waste products away for excretion. Arteries carry oxygen-rich blood from the heart into the periphery, where they branch into smaller vessels, arterioles, and capillaries, where the exchange of molecules with the surrounding tissues occurs. Capillaries, now carrying oxygen-poor blood, then converge to venules and veins, returning the blood to the heart and the lungs. All blood vessels are lined by a monolayer of endothelial cells that are surrounded by a basement membrane and different types of perivascular supportive cells. Despite some differences in the microanatomy of blood vessels depending on the organ, the junctions between adjacent endothelial cells are usually very tight and only allow diffusion of small molecules and some proteins, but not of, for example, entire cells.

The lymphatic vascular system is a one-way, blind-ended network that initiates in peripheral tissues and that joins the blood circulation via the junction of the thoracic duct and the subclavian vein. Its principal function is the drainage of interstitial fluid and solutes, which are constantly leaking from the blood vasculature into peripheral tissues, and their transport back to the venous circulation, thereby maintaining fluid homeostasis. Lymphatic vessels also function to transport immune cells and soluble antigens from the periphery to the lymph nodes, where immune responses are triggered. Lymphatic capillaries are composed of a single layer of lymphatic endothelial cells (LECs), whereas larger collecting vessels are covered by a layer of supporting pericytes and smooth muscle cells. Capillary LECs form specialized "button-like" junctions and possess a discontinuous basement membrane, which facilitates entrance of interstitial fluid and cells into the vessel lumen. The capillaries drain to

pre-collecting vessels and thereafter to collecting lymphatic vessels. Larger pre-collecting vessels display tight, "zipper-like" junctions between adjacent LECs, similar to blood vessels. Collecting vessels have a continuous perivascular sheath of smooth muscle cells and are divided into distinct vascular units, called lymphangions, by bileaflet lymphatic valves. Contractions of smooth muscle cells surrounding the lymphangions serve to push the fluid forward, whereas the valves prevent backflow. On their way back to the thoracic duct, lymphatic collectors pass through lymph nodes. On the afferent side of the lymph nodes, the vessels open into a large sinus that lies directly under the lymph node capsule. A ramified network of smaller lymphatic sinuses ensures a close contact between the lymph fluid and immune cells present in the lymph nodes. Finally, the fluid that passed the node is collected in a large vessel that leaves the node on the efferent side (Stacker et al. 2014; Dieterich and Detmar 2016).

The skin is particularly rich in blood and lymphatic vessels. The cutaneous blood microvasculature is organized into a subpapillary and a cutaneous horizontal plexus. Similarly, the lymphatic vasculature in the skin can be divided into two plexuses. Below the epidermis resides a dense capillary network of thin vessels without valves. These are connected to a deep lymphatic plexus, consisting of larger lymphatic vessels containing numerous valves, which is located below the cutaneous arterial plexus in the lower dermis and the superficial zone of the subcutaneous tissue (Skobe and Detmar 2000).

Lymphatic Remodeling and Metastasis in Melanoma

Under pathological conditions, blood vessels and lymphatic vessels undergo dramatic morphological and functional changes. During inflammation, blood vessels become leaky, enlarged, and activated with high expression of several adhesion molecules, leading to extravasation of inflammatory cells and fluid into the inflamed tissue. Lymphangiogenesis and lymphatic enlargement are also induced in inflammation, increasing the transport of fluid, extravasated leukocytes, and antigen-presenting cells and supporting the initiation of antigen-specific immune responses in the draining lymph nodes (Zgraggen et al. 2013).

In contrast to their beneficial role in inflammatory conditions, lymphatic vessels play a harmful role in many tumor types, including melanoma. Metastasis to tumor-draining lymph nodes is commonly seen in melanoma and correlates with distant metastasis and poor disease-free and overall survival (Streit and Detmar 2003). The density of lymphatic vessels within and surrounding the primary tumor correlates with sentinel lymph node metastasis and a poor outcome (Dadras et al. 2003). These clinical observations, together with the occurrence of in-transit metastases and the early detection of metastasis in melanoma draining lymph nodes, strongly indicate an important role of lymphatic metastasis in the progression of melanoma (Stacker et al. 2014; Dieterich and Detmar 2016). However, whether sentinel lymph node metastasis is the first step on the road toward distant metastasis, or whether it merely reflects the high aggressiveness of a tumor, is still incompletely understood. Genetic analyses performed in prostate cancer patients demonstrated that metastasis can spread from organ to organ, including lymph nodes, in complex patterns (Gundem et al. 2015). However, it is currently unclear to what extent this might happen in melanoma patients as well.

During tumor progression, the lymphatic system undergoes remodeling both at the site of the primary tumor and in the periphery, along the primary drainage route from the tumor. This remodeling includes the sprouting and growth of new lymphatic capillaries (lymphangiogenesis), vessel dilation, rerouting, and remodeling of the draining lymph nodes. All of these changes are considered to facilitate the dissemination of tumor cells through the lymphatic system (Fig. 2).

Lymphatic Remodeling at the Primary Tumor Site

In a significant percentage of primary melanomas, the density of lymphatic vessels is increased, both within the tumor mass and in the tumor

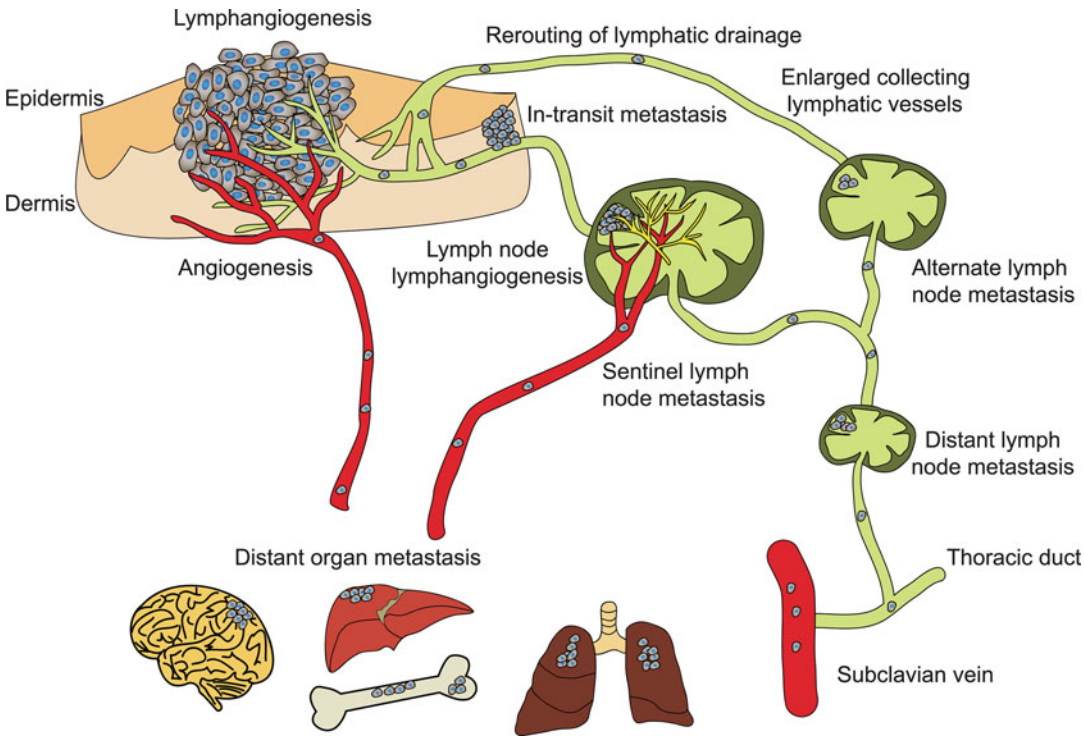

Fig. 2 The metastasis routes of melanoma. At the site of the primary tumor, the blood and lymphatic vascular networks are expanded due to tumor-induced angiogenesis and lymphangiogenesis. Lymph node lymphangiogenesis is induced by lymphangiogenic factors drained from the primary site and may occur prior to arrival of tumor cells. In-transit metastases develop along the lymphatic vessels from the primary site to the sentinel nodes. Increased lymphatic flow and enlargement of collecting vessels contribute to sentinel lymph node metastasis. Growth of metastases in the sentinel lymph nodes may lead to the blockade of the principal drainage route and subsequent rerouting of flow and metastasis toward alternate lymph nodes. Lymphatic vessels join the blood circulation at the subclavian vein, from where tumor cells can reach distant organs. Alternatively, tumor cells may gain access to the blood circulation already within the primary tumor or in tumor-colonized lymph nodes

periphery (Dadras et al. 2003, 2005). This is due to lymphangiogenesis induced by lymphangiogenic factors released by tumor cells and stromal cells, in particular macrophages. The proliferation and migration of LECs result in sprouting and formation of new lymphatic capillaries, as well as enlargement of pre-existing lymphatic vessels in the tumor proximity. Tumor lymphangiogenesis and lymphatic dilation increase the lymphatic surface area and thus the potential contact interface with tumor cells, facilitating their entry and spread through the lymphatic system (Stacker et al. 2014; Dieterich and Detmar 2016).

Increased lymphatic vessel density in primary melanomas correlates with poor disease-free survival and poor overall survival of melanoma patients (Dadras et al. 2003). The extent of lymphangiogenesis in primary cutaneous melanoma can also serve as a novel prognostic indicator to predict the presence of sentinel lymph node metastases at the time of surgery, exceeding the significance of tumor thickness (Dadras et al. 2005). Whereas in most carcinomas the major expansion of lymphatic vessels occurs at the tumor-stroma interface, enhancing lymphatic tumor seeding, in malignant cutaneous melanomas, intratumoral lymphatic vessel density also significantly correlates with sentinel lymph node metastases. The presence of intratumoral lymphatic vessels is significantly correlated with poorer disease-free survival, suggesting that

these vessels do play a functional role in melanoma progression (Dadras et al. 2003).

In addition to lymphatic vessels within or surrounding the primary tumor, lymphatic collectors, which drain away from the tumor and toward the sentinel lymph node, can undergo substantial remodeling during tumor progression. Soluble factors drained from the primary tumor induce the proliferation of LECs and the dilation of collecting lymphatic vessels, resulting in an increased flow rate in the lymphatic vessels contributing to the dissemination of tumor cells and the formation of lymph node metastasis (Karaman and Detmar 2014).

Lymphangiogenesis in Tumor-Draining Lymph Nodes

Extensive remodeling of the lymphatic vasculature in the sentinel lymph node occurs early during tumor progression, even before the arrival of metastatic cells, probably in response to soluble factors drained from the primary tumor (Hirakawa et al. 2005, 2007). This has led to the hypothesis of the "pre-metastatic" niche, which facilitates the later colonization of the node by disseminated tumor cells. After the establishment of a metastatic focus, lymphangiogenic factors secreted by metastatic cells or other lymph node resident cells such as macrophages or B cells provide a constant source of stimulation for LECs in the lymph node. Similar observations of pre-metastatic tissue changes have been made at distant organ sites as well, in the case of melanoma, for example, in the lung. Here, recruitment of VEGFR1-positive bone marrow-derived hematopoietic progenitor cells was found to precede the arrival of tumor cells and to promote the growth of lung metastasis (Kaplan et al. 2005).

In addition to soluble factors, melanoma-derived exosomes might play an important role in establishing the pre-metastatic niche. Exosomes are small extracellular vesicles (around 100 nm in diameter) that are produced by virtually all cells and present in all body fluids. Exosome release from tumor cells is often elevated, and there is increasing evidence that these exosomes serve as long-distance information carriers from tumor cells to host-derived cells in the periphery, transmitting molecular and genetic messages and modulating cell motility, angiogenesis, and immune responses (Whiteside 2016). Exosomes released from melanoma cells at the site of primary tumors are drained to sentinel lymph nodes, where they prepare the pre-metastatic niche for the colonization of the lymph node by arriving metastatic cells (Hood et al. 2011). Systemic effects of melanoma-derived exosomes have also been described, for example, the priming of cells in the bone marrow to migrate to the lung, where they take part in the niche formation as described above (Peinado et al. 2012).

Re-routing of Lymphatic Flow

Lymph node metastasis, when grown to a significant size, can lead to the obstruction of the nodal sinuses and thus of the path of lymphatic flow. In a preclinical study of melanoma metastasis, this has been found to result in a re-routing of the lymph flow, via newly formed lateral lymphatic vessels, which then drain to different lymph nodes (Proulx et al. 2013). Of note, such re-routing events may have severe clinical implications, as the metastasis-bearing lymph node may not be correctly identified by the surgeon during the sentinel lymph node dissection, which might result in a falsely negative diagnosis (Karaman and Detmar 2014). After surgical removal of tumor-draining lymph nodes, similar re-routing events have been observed as well (Blum et al. 2013).

Mechanisms of Tumor Cell Entry into Lymphatic Vessels

Lymphatic invasion (LI) is a term used to indicate the presence of tumor cells within lymphatic vessels in histological samples. Whereas lymphatic vessels are often difficult to distinguish in routinely stained histological sections, immunostaining for the lymphatic marker podoplanin (D2-40 antibody) makes detection of LI on histological sections much more sensitive than routine histology alone. In cutaneous malignant melanoma, LI occurs more frequently than blood vessel invasion and is strongly associated with sentinel lymph node metastasis, independently from tumor thickness (Doeden et al. 2009).

Several mechanisms have been identified to explain how tumor cells gain access to the lymphatic system. Due to the increased leakage of tumor-associated blood vessels, the tumor mass generally has a high interstitial tissue pressure, leading to an increased interstitial flow toward lymphatic vessels. Tumor cells may simply follow the flow and be "swept" into lymphatic vessel passively. At the same time, lymphatic vessel endothelium expresses chemokines such as CXCL12 and CCL21, which act as guidance molecules for cancer cells expressing the corresponding receptors, CXCR4 or CCR7. Once reaching a lymphatic capillary, tumor cells may transmigrate across the endothelium via the relatively loose "button" junctions. This process is analogous to the way recirculating leukocytes "sense" lymphatic vessels and enter them. In addition, tumor cells might physically damage and penetrate the lymphatic vessels. This process has been reported to be facilitated by secretion of the enzyme 15-lipoxygenase-1, inducing the formation of holes in LECs (Kerjaschki et al. 2011).

In-Transit Metastasis

In-transit metastasis is defined as any dermal or subcutaneous metastasis localized more than 2 cm from the primary lesion but proximal to the draining regional lymph nodes. The phenomenon of in-transit metastasis is almost unique to melanoma. In-transit metastases are regarded to arise from metastatic cells that entered the lymphatic system but became trapped in lymphatic vessels before reaching the draining lymph node. The mechanisms responsible for the development of these lesions are not completely understood, but likely LEC-derived chemokines provide a microenvironment that supports the stemness of melanoma cells. Patients who develop in-transit disease have an increased risk to develop additional locoregional and distant disease. A clinical study with 11,614 patients revealed that the in-transit metastasis rate for sentinel lymph node positive patients was fivefold higher than for sentinel lymph node negative patients (Read et al. 2015). However, whether the occurrence of in-transit metastasis has a prognostic value for patient survival has remained unclear.

Hematogenous Metastasis

Tumor cells may directly enter tumor-associated blood vessels or reach the blood circulation after a transit through the lymphatic system. In either case, hematogenous metastasis is a prerequisite for the colonization of distant organs.

Angiogenesis is defined as the growth of new blood vessels from a pre-existing vasculature and is thought to be required for continuous tumor growth. As oxygen from the blood stream can only diffuse for a few hundred micrometers into the skin, tumor cells at a distance larger than this become hypoxic, which leads to phenotypic changes, induction of angiogenic growth factors, or cell death. Tumor-associated blood vessels differ structurally and functionally from normal blood vessels. They have a less regular structure with a partial or complete loss of the normal vessel hierarchy, deficient endothelial cell lining, and a relative lack of vessel-supporting pericytes. Additionally, the basal lamina is thinner than in normal vessels and discontinuous, which makes these vessels highly permeable. In consequence, tumor-associated blood vessels are less functional in terms of oxygen and nutrient transport, which further increases tumor hypoxia and the release of angiogenic factors. At the same time, tumor-associated blood vessels are more permissive for the invasion by tumor cells.

The clinical and prognostic significance of tumor angiogenesis for melanoma progression and metastasis has remained controversial. Some clinical studies indicated that increased vascular density was correlated with melanoma progression, and tumor vascularity was the most important determinant of overall survival, surpassing tumor thickness. By contrast, several other investigators failed to detect any correlation between melanoma vascularization and prognosis. Thus, the potential prognostic value of tumor vascularization in human cutaneous melanomas remains unsolved (Streit and Detmar 2003).

Tumor cell vasculogenic mimicry, also known as vascular mimicry, represents an alternative mechanism by which tumors can obtain blood supply and direct access to the circulation. Vascular mimicry refers to the phenomenon of

tumor cells themselves forming tubelike structures, which lack an endothelial layer but nevertheless contain blood and are connected to the blood vascular system. Thus, tumor cells reside in direct contact to the blood stream, which conceivably facilitates their dissemination. First introduced as a novel paradigm for tumor perfusion in melanoma in 1999 (Maniotis et al. 1999), evidence for vascular mimicry has by now been reported in several malignant tumors, including breast cancer, prostate cancer, bladder cancer, colorectal cancer, and lung cancer, and has been linked to poor prognosis of cancer patients (Cao et al. 2013). The precise mechanisms and molecular pathways regulating vascular mimicry are incompletely understood, but factors related to cancer stemness and plasticity, such as the Notch pathway, have been suggested to play a role. VEGF-A has been reported to be involved in vascular mimicry in melanoma, by activating VEGFR-1 expressed on melanoma cells, which promoted tumor cell invasion, migration, and plasticity. Consequently, these signaling pathways might represent potential therapeutic targets and diagnostic indicators of vascular mimicry (Hendrix et al. 2016).

Molecular Mediators of Angiogenesis and Lymphangiogenesis in Melanoma

Members of the vascular endothelial growth factor (VEGF) family are the most important factors in tumor-induced (lymph)angiogenesis and vessel remodeling. Several VEGFs have been discovered, which preferentially act on blood or lymphatic endothelial cells, respectively (Fig. 3).

The major angiogenesis factor VEGF-A signals via its receptor tyrosine kinases VEGFR-1 and VEGFR-2, as well as their co-receptors, neuropilin (NRP)-1 and NRP-2. VEGFR-1 is expressed in blood vessels, whereas VEGFR-2 is expressed on both blood and lymphatic vessels. Consequently, VEGF-A can induce both angiogenesis and lymphangiogenesis. Although VEGFR-1 binds to VEGF-A with a higher affinity than VEGFR-2, activation of VEGFR-1 has only minor effects on endothelial cells, and the relevance of VEGFR-1 downstream signaling for angiogenesis remains largely unexplored (Simons et al. 2016). The main activity of VEGF-A is transmitted by VEGFR-2, directly regulating endothelial cell proliferation, migration, and permeability, which are key steps for tumor (lymph)angiogenesis (Alitalo and Detmar 2012). Expression of VEGF-A is induced by hypoxia, due to stabilization of the transcription factor HIF (hypoxia-inducible factor), a mechanism which is probably responsible for the high VEGF-A expression in most tumors (Krock et al. 2011). VEGF-A expression is also regulated by inflammatory cytokines such as TNF-α (Zgraggen et al. 2013). VEGF-A-induced recruitment of bone marrow-derived inflammatory cells and endothelial progenitors has been suggested to contribute to the growth of tumor-associated blood (and lymphatic) vessels in an indirect way.

Another member of the VEGF family, placenta growth factor (PlGF), binds specifically to VEGFR-1 and is expressed in a large number of human melanoma cell lines, suggesting that PlGF plays a role in melanoma growth, angiogenesis, or progression (Streit and Detmar 2003).

Lymphangiogenesis in cutaneous melanoma is mainly induced by VEGF-C and VEGF-D. Both VEGF-C and VEGF-D exert their lymphangiogenic activity through their cognate receptor VEGFR-3 and the co-receptor NRP-2, which are predominantly expressed on lymphatic endothelial cells. VEGF-C- and VEGF-D-induced VEGFR-3 signaling can lead to proliferation and enlargement of peritumoral and intratumoral lymphatic vessels in melanoma. Fully mature VEGF-C and VEGF-D additionally gain affinity for VEGFR-2, expressed on both blood and lymphatic vessel endothelial cells. In addition, VEGFR-3 expression was reported on some tumor-associated blood vessels, indicating that under certain conditions VEGF-C and VEGF-D may also affect angiogenesis and blood vessel permeability (Dieterich and Detmar 2016).

In clinical studies, increased VEGF-C expression levels significantly correlated with lymphatic vessel density in primary melanomas (Dadras et al. 2005) and with lymph node metastasis (Dadras et al. 2005; Schietroma et al. 2003).

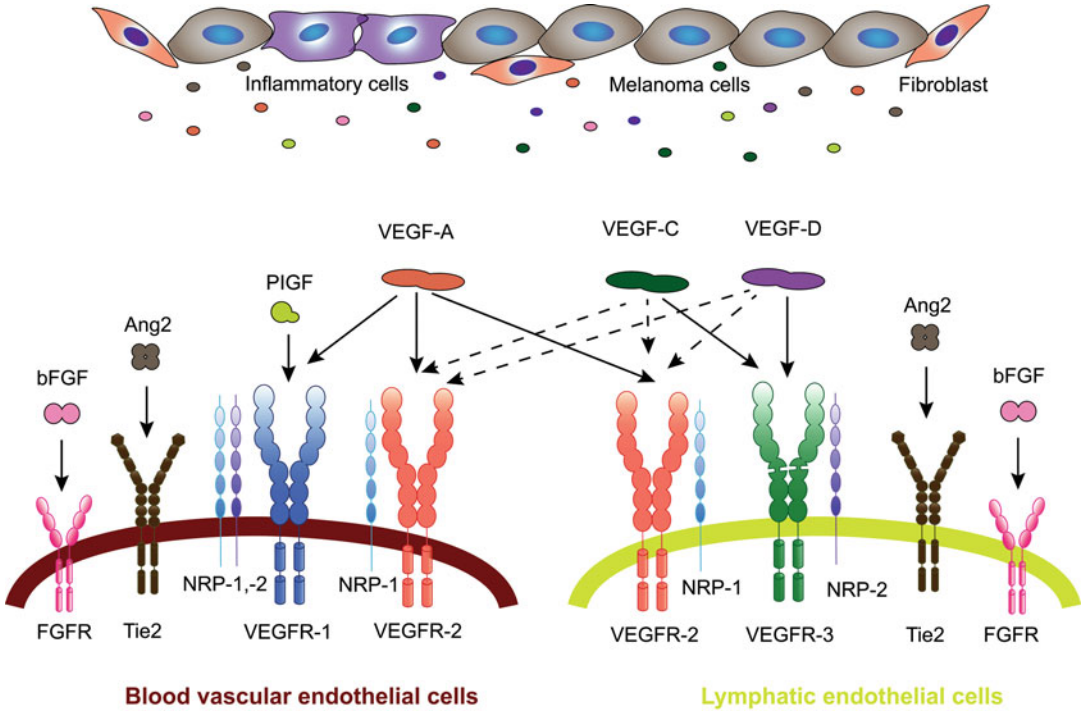

Fig. 3 Mediators of angiogenesis and lymphangiogenesis in melanoma. VEGF-A is the main mediator of angiogenesis. VEGF-A binds to VEGFR-1 and VEGFR-2, as well as its co-receptors NRP-1 and NRP-2, and can induce both angiogenesis and lymphangiogenesis. VEGF-C and VEGF-D, which mainly bind to VEGFR-3, are the main lymphangiogenic mediators. Ang2 and bFGF have also been identified as (lymph)angiogenic factors in melanoma. *VEGF* vascular endothelial growth factor, *PIGF* placenta growth factor, *bFGF* basic fibroblast growth factor, *Ang2* angiopoietin 2, *NRP* neuropilin

VEGF-C expression in melanoma cells was prognostic of a shorter overall and disease-free survival. However, VEGF-C expression in melanoma cells was not associated with Breslow thickness, Clark level, or ulceration of the primary cutaneous melanoma. Primary melanomas in the vertical growth phase were reported to express more VEGF-C than those in the radial growth phase (Rinderknecht and Detmar 2008). Besides melanoma cells, tumor-associated macrophages (TAMs) also represent major sources of VEGF-C in melanoma. The percentage of VEGF-C positive TAMs was higher in primary tumors of patients with sentinel lymph node metastasis compared to patients without. On the other hand, VEGF-C expression in TAMs was not associated with pathological characteristics of the primary tumor such as Breslow thickness, Clark level, or ulceration nor with disease-free or overall survival (Dadras et al. 2005). In contrast to VEGF-C, no correlation was found between the expression levels of VEGF-D and the incidence of lymph node metastasis, although VEGF-D has been detected in melanomas and has been associated with melanoma angiogenesis (Rinderknecht and Detmar 2008).

Another important family of angiogenic factors in melanoma are the fibroblast growth factors (FGFs). In particular, basic FGF (bFGF) expression has been found in melanoma cells but not in normal melanocytes. Expression of bFGF has paracrine effects, through the stimulation of local angiogenesis, and also autocrine effects, directly increasing tumor cell proliferation through activation of FGF receptors expressed on the melanoma cells themselves (Streit and Detmar 2003).

Finally, angiopoietin 2 (Ang2), which is released by endothelial cells themselves, is believed to contribute to angiogenesis and lymphangiogenesis by

destabilizing endothelial junctions and "priming" of endothelial beds for the induction of (lymph) angiogenesis. Ang2 mediates its effects by binding to the receptor Tie2, expressed by all endothelial cells. Circulating levels of Ang2 correlated with tumor progression in malignant melanoma patients, indicating that this pathway is active in melanoma (Helfrich et al. 2009).

Angiotropism, a Special Form of Vascular Metastasis

Angiotropism denotes the histological finding of tumor cells closely associated with abluminal vascular surfaces, without intravasation into the vessel lumen. Angiotropic cells are generally detected close to the advancing front of a tumor mass and may involve both capillaries and larger vessels. The presence of angiotropic melanoma cells has been interpreted as a marker of "extravascular migratory metastasis" (EVMM), a mechanism of infiltrative tumor cell migration along the abluminal face of vessels, representing an alternative mechanism of melanoma spread. Angiotropism has been reported as an independent prognostic marker, significantly associated with locoregional metastases, such as in-transit metastasis and microsatellites, and with distant metastases, as well as with ulceration and Breslow thickness (Landsberg et al. 2016). Angiotropic melanoma cells have been found to sometimes "replace" pericytes, a phenomenon termed "pericytic mimicry." Pericytic mimicry by angiotropic melanoma cells and interaction between melanoma cells and the abluminal vascular surface may induce the expression of genes linked to cancer migration, embryonic/stem cell properties, and inflammation (Lugassy et al. 2014).

Additional Roles of Lymphatic and Blood Vessels in Tumor Progression

The Perivascular Stem Cell Niche

Stemlike properties of dedifferentiated cancer cells are at least partially maintained by signals from their microenvironment, which may be composed of various types of stromal cells. For example, tumor-associated blood vessels are thought to provide multiple signals, including cell-cell contact-dependent signaling, for the maintenance of stemlike properties, which is referred to as the "perivascular stem cell niche." Similarly, lymphatic vessels might provide a "lymphovascular niche" to maintain metastatic melanoma cells in a dedifferentiated state, which might contribute to the occurrence of in-transit metastases and to the persistence of metastatic cells in the lymph node subcapsular sinus. Thus, melanoma cells trapped in the lymphatic vessels could be kept quiescent in the lymphovascular niche for extended time periods. A recent study indicated that expression of CCR7, a receptor for the lymphatic chemokine CCL21, in a breast cancer model contributed to the maintenance of a pool of stemlike cells and promoted tumor progression (Boyle et al. 2016). Furthermore, at sites of distant metastasis, including lung and lymph nodes, CXCR4 expressing $CD133^+$ melanoma cells with stemlike properties were found to be attracted by CXCL12 from tumor-activated LECs and were localized in the vicinity of lymphatic vessels (Kim et al. 2010). However, it is currently unclear whether factors derived from lymphatic endothelium actively regulate the differentiation status of adjacent melanoma cells.

Immune Regulation

Endothelial cells of both blood and lymphatic vessels have been directly implicated in the regulation of immune responses, which may affect tumor growth, particularly of immunogenic tumor types such as melanoma. Endothelial cells act as non-professional antigen-presenting cells and can modulate the activation state of immune effector cells by expression of stimulatory or inhibitory surface molecules and cytokines.

Under the influence of tumor-derived factors such as VEGFs, blood vascular endothelial cells can directly inhibit T-cell activation by upregulating inhibitory molecules such as PD-L1 and IL-10. On the other hand, tumor endothelial cells can also express FasL, which leads to apoptosis of Fas-expressing T cells. Furthermore, through the

downregulation of intracellular adhesion molecule 1 (ICAM1) and vasculature cell adhesion molecule 1 (VCAM1), which are required for extravasation, tumor-associated blood vessels may efficiently prevent effector T cells from infiltrating into tumor tissues (Lanitis et al. 2015).

The lymphatic system has traditionally been thought to passively transport lymph and immune cells and thus to affect the immune system rather indirectly. Surprisingly, recent studies revealed that LECs are actively involved in the regulation of T-cell immunity and the tumor microenvironment. Lymph node LECs are involved in the maintenance of peripheral tolerance by presenting self-antigens and concomitantly expressing T-cell inhibitory signals such as PD-L1. As tumor-derived antigens are transported with the lymph to the draining lymph nodes, where they are taken up and presented by LEC, these cells may actively contribute to the disruption of tumor-specific $CD8^+$ T-cell responses as well. Indeed, in a melanoma mouse model, LECs in draining lymph nodes were found to present a tumor-derived peptide and to inhibit responses of antigen-specific $CD8^+$ T cells toward the tumor (Rouhani et al. 2014). On the other hand, in a mouse model that lacked dermal lymphatic vessels, implanted melanomas grew robustly but exhibited drastically reduced cytokine expression and leukocyte infiltration compared with those implanted in control animals (Lund et al. 2016). This indicates that the lymphatic system plays an important role in shaping immune responses in melanoma.

Conclusion

Malignant melanoma is a tumor type with an inherently high capacity to metastasize. Metastases can develop locoregionally, in tumor-draining lymph nodes, and in distant organs, up to many years after surgical resection of the primary tumor, implying that melanoma cells disseminate during tumor progression and remain dormant in the body periphery over extended periods of time. In order to disseminate, melanoma cells first need to invade locally into the dermis, and subsequently enter the blood or the lymphatic vascular system, which allow them to spread throughout the body. Consequently, lymphatic and blood vessels play important roles in the metastatic process of melanoma, and the expression of lymphangiogenic factors and the density of lymphatic vessels at the primary tumor site strongly correlate with the occurrence of lymph node metastasis and a poor prognosis. Thus, therapeutic manipulation of lymphatic and blood vessels might represent a promising approach to inhibit metastasis in melanoma.

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Principles of Targeted Therapy

9

Gideon Bollag and Keith T. Flaherty

Contents

Abstract

With the increasing volume of genetic melanoma profiling, key oncogenic driver mutations have attracted substantial attention as therapeutic targets. Chief among these are BRAF and KIT. Since BRAF mutations occur in about half of all melanomas, BRAF pathway inhibition has attracted the bulk of therapeutic attention. The discovery of BRAF mutations in cutaneous melanomas led to considerable research into the role of BRAF/MEK/ERK signaling and its role in melanomagenesis. In parallel, drug discovery efforts targeting BRAF, MEK, and ERK led to promising therapeutic candidates. Single-agent BRAF inhibitors showed strong efficacy in metastatic melanoma patients, effectively transforming the treatment for BRAF-mutant cutaneous melanoma patients. MEK inhibitors also showed efficacy as single agents, but the combination of BRAF and MEK inhibitors was clearly superior to either single-agent treatment. ERK inhibitors are currently undergoing clinical development. KIT mutations are

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primarily found in acral and mucosal melanomas, and several KIT inhibitors have been tested in KIT-mutant melanoma clinical trials; so far none have been approved by regulatory agencies. The challenge for KIT inhibitors may lie in the rarity and diversity of KIT genetic mutations. This chapter explores the biology of BRAF- and KIT-mutant melanoma cells and describes discovery of therapeutic candidates and reviews their role in clinical care.

Keywords
BRAF · KIT · MEK · ERK · Resistance · RAF inhibitor paradox

Introduction

In order to understand the role of oncogenes as driver mutations in melanoma, it is important to review the genesis of melanoma. This is discussed in other chapters (e.g., Sommer, Ma et al.). One of the seminal observations is that melanoma cells are more closely related to neural stem precursor cells than to melanocytes (Boiko et al. 2010). This is consistent with the general notion that cancer represents a dedifferentiated state of the tissue of origin (Hanahan and Weinberg 2011). Importantly, unlike chemotherapies and immunotherapies, targeted therapies generally drive differentiation of the tumor cells (Boni et al. 2010). In some cases, this differentiation results in re-expression of antigens that are recognized and neutralized by the immune system, and in some cases, this results in cell-intrinsic dysfunction that causes necrosis or apoptosis. However, unless the consequence of targeted therapy is death of the tumor cell, the redifferentiated tumor cell mass is only temporarily benign: almost inevitably, reignition of the tumorigenic and metastatic properties prevail over time (and often very quickly) (Solit and Rosen 2014).

Melanoma as a class bears one of the highest somatic mutation frequencies among all tumor types (see ▶ Chap. 7, "Molecular Genetics of Melanocytic Neoplasia" by Hodis and Garraway). Among the somatic mutations, BRAF is by far the most commonly mutated gene, found in 63% of cutaneous melanomas. Many additional gene mutations have been discovered, but few have been druggable. While occasional melanomas bear mutations in genes for which there are targetable proteins such as KIT, PIK3CA, NTRK1, and ALK, these are generally quite rare. This chapter will focus primarily on BRAF and KIT, since they have garnered the most therapeutic efforts.

Signaling Pathways

BRAF

Since the discovery of the BRAF oncogene in 2002 (Davies et al. 2002), considerable laboratory research has focused on understanding its role in melanomagenesis. BRAF is one of three genes that encode the RAF family of protein kinases (also including the ARAF and CRAF genes) (Holderfield et al. 2014). These protein kinases have one predominant substrate: the kinase MEK. MEK in turn also has one predominant substrate: the kinase ERK. ERK, in turn, phosphorylates many target genes, including nuclear transcription factors. Indeed, nuclear translocation of the ERK kinase is an important occurrence during activation of the RAF pathway.

BRAF mutations are likely initiating events, as they are frequently found in nevi, which are benign collections of locally proliferating melanocytes, often characterized as moles (Pollock et al. 2003). Whether malignant melanomas progress from these nevi or initiate independently is still under investigation, but the discovery that BRAF mutations are found even at the earliest stages indicates an acute dependence on this event. As indicated by their characterization as oncogenes, BRAF mutations are gain-of-function lesions even though some of the mutational effects actually impede kinase activity (Wan et al. 2004); these will be described in more detail in a subsequent section. In addition to the RAF genes, there are also two closely related genes called KSR (Neilsen et al. 2017). During normal signaling, KSR proteins play an important role in assembling RAF/MEK complexes and in

mediating transphosphorylation of BRAF and MEK. However, to date the role of KSR proteins in melanoma has been largely ignored. It is possible that KSR plays a much larger role in normal signaling, in which case inhibition of KSR function would be unwanted in a drug.

While there are three RAF genes in the human genome, there are only two MEK genes (MEK1, MEK2) and two ERK genes (ERK1, ERK2) (Caunt et al. 2015). The relative roles of the two MEKs and the two ERKs are still in early stages of elaboration by research teams. Regarding therapeutic targeting, current efforts favor pan-MEK and pan-ERK approaches.

In the two decades preceding the discovery of the BRAF oncogene, copious literature cemented the role of RAF kinases as important effectors of the RAS proteins (Holderfield et al. 2014). RAS is the most frequent oncogene in human cancers, so understanding the RAS pathway has been a major focus of scientific investigation (Simanshu et al. 2017). RAF kinase was the first described effector of RAS, and subsequent work has identified quite a few additional effectors including phosphatidylinositol 3′kinase (PI3K). PI3K is an enzyme that converts phosphatidyl inositol 4,5-bisphosphate to phosphatidyl inositol 2,4,5-triphosphate, an important second messenger that induces multiple cellular events such as calcium release from intracellular stores. Other effectors of RAS include, for example, RAL GDS, an enzyme that catalyzes replacement of GDP by GTP on the RAL small GTPase. The GTP-bound state of RAL is an activated effector for other enzymes such as phospholipase D. While important in other cancers, and likely in bypassing targeted agents for melanoma, these other pathways have considerably less signaling importance in melanoma compared to the BRAF/MEK/ERK pathway.

RAS

The next most frequently found mutations in cutaneous melanomas are gain-of-function RAS (primarily NRAS) oncogenes, or loss-of-function mutations in the NF1 gene (► Chap. 7, "Molecular Genetics of Melanocytic Neoplasia" by Hodis and Garraway). RAS and BRAF mutations are almost always mutually exclusive, except in a subset of BRAF mutations (see class 3 BRAF mutations below) in which RAS activation plays an obligate role. RAS is a small protein that binds to GTP and GDP (Simanshu et al. 2017). Just like RAL, RAS forms a molecular switch: when bound to GTP, it binds to effectors and drives the cognate pathway. When bound to GDP, RAS is unable to bind to effectors and likely plays a purely passive role. Regulation of the amount of GTP bound to RAS is thus the critical determinant of pathway output. An intricate post-translational processing sequence is critical to RAS biology (Cox et al. 2015). Efforts to silence RAS with small molecule drugs have been attempted at countless pharmaceutical companies for well over 30 years, yet no drugs are currently available. RAS becomes prenylated primarily by farnesyl groups; unfortunately for drug discoverers, NRAS and KRAS can readily substitute geranylgeranyl groups in order to bypass the effects of farnesyl transferase inhibitors. Subsequent to prenylation at a cysteine residue four residues from the carboxy-terminus, RAS proteins become proteolyzed by an endoprotease and then carboxymethylated at the newly exposed cysteine carboxylate by an enzyme called isoprenyl cysteine methyltransferase. The net effect of all these events is localization of RAS proteins to the plasma membrane where RAS signal transduction is most critical.

In cutaneous melanomas, BRAF and NRAS mutations are found in about 80% of the tumors (see ► Chap. 7, "Molecular Genetics of Melanocytic Neoplasia" by Hodis and Garraway). As mentioned above, RAF is only one of the effectors of RAS. However, the biology of melanoma suggests that RAF/MEK/ERK signaling is likely the most important effector pathway for NRAS mutations. Still, the efficacy of MEK inhibitors is limited to a relatively small subset of NRAS-mutant melanomas, suggesting that other RAS effector pathways do play an important, yet poorly understood, role.

NF1 is a gene that encodes the very large protein neurofibromin (Philpott et al. 2017).

While neurofibromin is over 3000 residues in length, the primary determinant of NF1 biology is found in a 300-residue segment near the middle of the NF1 gene: this is the GAP domain. GAPs are *G*TPase-*a*ctivating *p*roteins that were first described for RAS back in 1987: they bind to RAS proteins and stimulate the GTPase activity. By stimulating the GTPase activity, these enzymes turn RAS off (Fig. 1). Therefore, loss-of-function NF1 mutations result in activation of the RAS pathway. The degree of RAS pathway activation is highly critical: too much activity can lead to catastrophic cell death, so tumors calibrate the level of RAS activation by the types of RAS mutations. It is generally believed that the loss of NF1 yields modest activation of the RAS pathway. In fact, the loss of additional negative regulators of RAS (such as RASA2) appears frequently along with NF1 inactivating mutation in melanoma (Arafeh et al. 2015). Just as mentioned above, it is believed that the primary (but not exclusive) role of NF1 mutation in melanomas is activation of the RAF/MEK/ERK pathway (Nissan et al. 2014). Therefore, RAF, MEK, or ERK inhibitors deserve to be tested as therapeutics for melanomas with NRAS oncogenic mutations or loss-of-function NF1 mutations. As described in ▶ Chap. 32, "Targeted Therapy in Advanced Melanoma" by Johnson and Sosman, these agents have activity as single agents, but likely insufficient to have truly meaningful impact on the disease.

During typical RAS signaling in undiseased tissues, extracellular growth factors engage their cognate receptors which subsequently recruit and activate guanine nucleotide "exchange factors" for RAS (Vigil et al. 2010)."Exchange factors" have a variety of names in historical literature, including GEFs (Fig. 1) GDS (guanine nucleotide dissociating factors), GRFs (guanine nucleotide releasing factors), and GNRPs (guanine nucleotide releasing proteins). Regardless of the name, the net result of exchange factors is to cause dissociation of GDP from RAS. Due to the large excess of GTP to GDP in a typical cell, this dissociation results in rebinding of GTP and hence an active RAS-GTP form. RAS-GTP at the plasma membrane recruits RAF dimers which in turn phosphorylate and activate MEK, which in turn phosphorylate and activate ERK (Rajakulendran et al. 2009). This is the typical means for propagating a RAS/RAF-dependent signal in normal cells. Note that RAF dimerization is a key event in this process and RAF monomers are typically inactive.

BRAF Alleles

By far the most common BRAF oncogenic allele is BRAF-V600E: substitution of glutamate for valine at codon 600 of the BRAF gene (Heinzerling et al. 2013). Other codon 600 substitutions are also found, with lysine (V600K), aspartate (V600D), and arginine (V600R) being the most common (see ▶ Chap. 7, "Molecular Genetics of Melanocytic Neoplasia" by Hodis and Garraway). As a group these codon 600 lesions have been called class 1 mutations. Current understanding of the biochemistry of these alleles is that they function independently of RAS and likely function as monomers. Approximately 80% of all BRAF mutations in any cancer are class 1 mutations, and in cutaneous melanoma, they comprise about 90% of BRAF mutations. V600E versus V600K mutations are strongly associated with younger age. And, V600E mutations are more commonly associated with overall low mutation burden. Conversely, V600K mutations are more commonly associated with co-occurring genetic alterations in tumor suppressor genes such as PTEN, CDKN2A, and p53. These findings have ramifications for differential therapeutic vulnerability by BRAF mutation type and even within the V600 population.

Nonetheless, it is important to understand the other classes of BRAF mutations since they shed considerable light on BRAF biology and will likely be important in resistance to targeted therapies.

Class 2 mutations are exemplified by mutations in codons 464, 469, 597, and 601 (Yao et al. 2015). These mutations cause constitutive dimerization of RAF, resulting in enhanced kinase activity. MEK and ERK inhibitors are likely to be active against class 2 mutations but may be

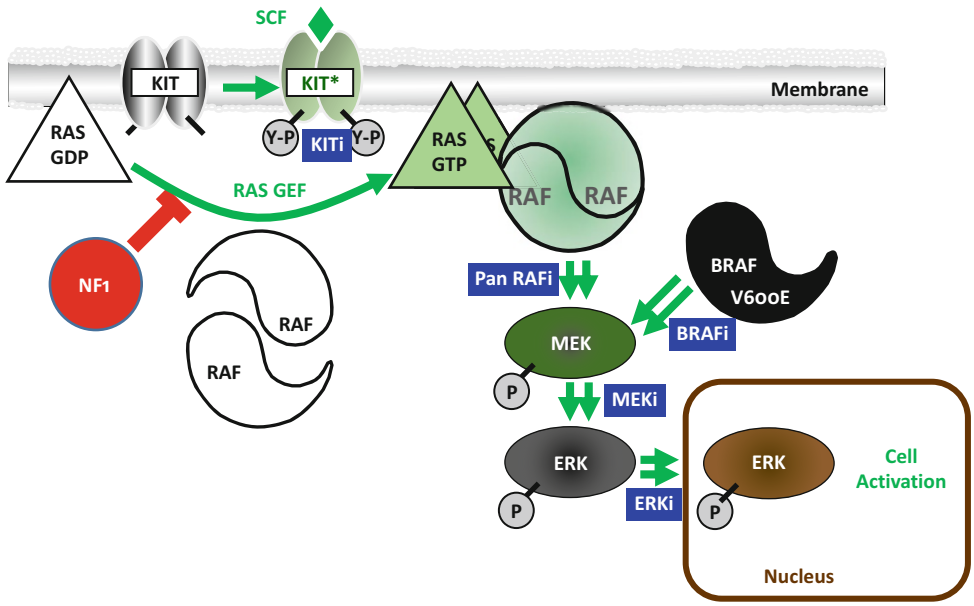

Fig. 1 Cartoon of melanoma signaling pathways, highlighting RAF/MEK/ERK and KIT signaling. Normal KIT signaling happens when stem cell factor (SCF) engages the dimeric KIT receptor. In melanoma, KIT activation can occur through KIT mutations (indicated by an asterisk). KIT (and many other) receptors signal through RAS, effecting exchange of GDP (inactive) for GTP (active) via guanine nucleotide exchange factors (RAS GEFs). This activation can be countered by GTPase-activating proteins (GAPs), including NF1; the loss of NF1 in melanomas results in elevated RAS-GTP levels and signaling through the RAF/MEK/ERK pathway (and other pathways not shown here, such as the PI3K pathway). RAS-GTP (perhaps as dimers) cause dimeric RAF to translocate to the plasma membrane resulting in activation of RAF kinase activity. In melanomas, RAF activation can frequently occur through mutation in BRAF – typically the V600E mutation that signals independently of RAF. RAF phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. Activated ERK translocates to the nucleus where phosphorylation of multiple substrates including transcription factors leads to widespread changes in gene expression that result in melanoma cell growth. Avenues to intervene through targeted therapies that are discussed in this chapter are indicated in blue

limited by therapeutic window. BRAF inhibitors could be active if they could block dimeric RAF isoforms. With increased sequencing of melanoma samples, fusions of dimerization domains to RAF kinase domains (usually but not always BRAF) are becoming more widely recognized as melanoma oncogenes; these are also class 2 mutations.

Class 3 mutations are exemplified by mutations in codons 466, 581, and 594 (Yao et al. 2017). These mutations typically cause reduced kinase activity, a phenomenon that was initially confusing (Wan et al. 2004). Subsequent analysis revealed that these mutations co-occur with RAS mutations or other events that cause upstream activation of the pathway. Therefore, these class 3 mutations stabilize a dimeric RAF enzyme in the presence of RAS-GTP. Inhibition of class 3 mutations with RAF inhibitors will be challenging due to the co-occurrence of RAS pathway activation.

RAF Inhibitors

After the discovery that RAF kinase is a direct effector of RAS-GTP, multiple drug discovery efforts led to CRAF kinase inhibitors. Several examples include sorafenib (BAY 43-9006, Nexavar), ZM 336372, L-779,450, and GW 5074 (Bollag et al. 2003). Of these compounds, only sorafenib made it to clinical development (Wilhelm et al. 2006). Sorafenib underwent clinical trials with the strategy to interfere with the RAS pathway and non-intuitively showed clinical

efficacy in renal cell carcinoma, hepatocellular carcinoma, and thyroid cancer. Subsequent mechanistic studies suggest that clinical activity in these diseases is primarily due to inhibition of VEGF receptors (VEGFRs). A close analog of sorafenib, known as regorafenib (Stivarga), subsequently showed activity in colorectal cancer, hepatocellular carcinoma, and gastrointestinal stromal tumors, presumably due to activity against VEGFR and KIT kinase (the receptor for stem cell factor, Fig. 1) (Pelosof et al. 2018). However, despite initial optimism, sorafenib was tested in large phase III clinical trials for BRAF-mutated melanoma and failed to show efficacy in second or third lines (Bollag et al. 2012).

Postmortem analysis suggests that sorafenib, while binding to BRAF, binds to the inactive form and therefore is a poor inhibitor of the oncogenic, hyperactive kinase activity presented by the BRAF-V600E allele that predominates in melanoma tumors. Structural characterization of kinase inhibitors has identified at least two different binding modes: type I and type II. Type I inhibitors bind to the activated form of kinases (also known as DFG-in), while type II inhibitors bind to inactive (DFG-out) forms that block activation but not so much constitutively active kinases (Fig. 2). Sorafenib is a type II inhibitor, and its co-crystal structure with BRAF was a landmark development that catalyzed the discovery of subsequent, type I inhibitors (Wan et al. 2004).

A subsequent elaboration of sorafenib identified a compound with improved specificity for the RAF proteins, known as RAF265. RAF265, also a type II inhibitor, displayed some efficacy against BRAF-mutant patients in phase I clinical trials but was insufficiently improved to proceed in clinical trials after the success of type I inhibitors was documented (Izar et al. 2017).

Despite the costly failure of sorafenib in melanoma clinical trials, drug discovery efforts targeting BRAF kinase activity ramped up after the discovery of the oncogene in 2002. Specifically, the drug discovery efforts focused on type I kinase inhibitors. Multiple compounds were discovered by diverse pharmaceutical chemistry efforts, and three compounds have achieved striking clinical efficacy in melanoma: vemurafenib, dabrafenib, and encorafenib. Description of the drug discovery projects is detailed below.

Vemurafenib was the first type I kinase inhibitor to enter clinical trials (Bollag et al. 2012). The compound was discovered at Plexxikon through structure-guided drug identification and optimization. Characterization of a sister compound, PLX4720, was detailed in 2008. Initial screening identified a 5-substituted azaindole as a reproducible binder to multiple kinase active sites, including PIM and FGFR kinases. Subsequent lead optimization focused on dual substitution at both 3- and 5-positions of the azaindole scaffold. Perhaps the most important empirical discovery entailed elaboration of a phenyl-sulfonamide at the 3-position of the azaindole. Fluorine substitution of the phenyl group was key in order to impart acidity to the sulfonamide nitrogen: this makes key interactions with a newly revealed specificity sub-pocket. After identification of PLX4720, the 5-chloro azaindole substituent was replaced by a 5-p-chloro-phenyl moiety yielding vemurafenib (PLX4032, RG7420, Zelboraf). Clinical development of vemurafenib is summarized in ▶ Chap. 32, "Targeted Therapy in Advanced Melanoma" by Johnson and Sosman.

In order to improve on PLX4032, additional chemistry efforts identified an analog known as PLX3603 (RG7526). While PLX3603 possessed certain improvements on vemurafenib, the pharmacokinetic profile was insufficient to pursue the compound beyond a phase I clinical trial. Nonetheless, the clinical efficacy of PLX3603 was similar to other BRAF inhibitors, and several patients achieved several years duration of clinical response.

A related drug discovery project led to the identification of dabrafenib (GSK2118436, Tafinlar). Dabrafenib has a different scaffold to bind to the hinge region of BRAF but again utilizes the fluoro-phenyl sulfonamide to engage the specificity sub-pocket (King et al. 2013). This compound is also a potent type I kinase inhibitor, and its clinical results are also summarized in ▶ Chap. 32, "Targeted Therapy in Advanced Melanoma" by Johnson and Sosman.

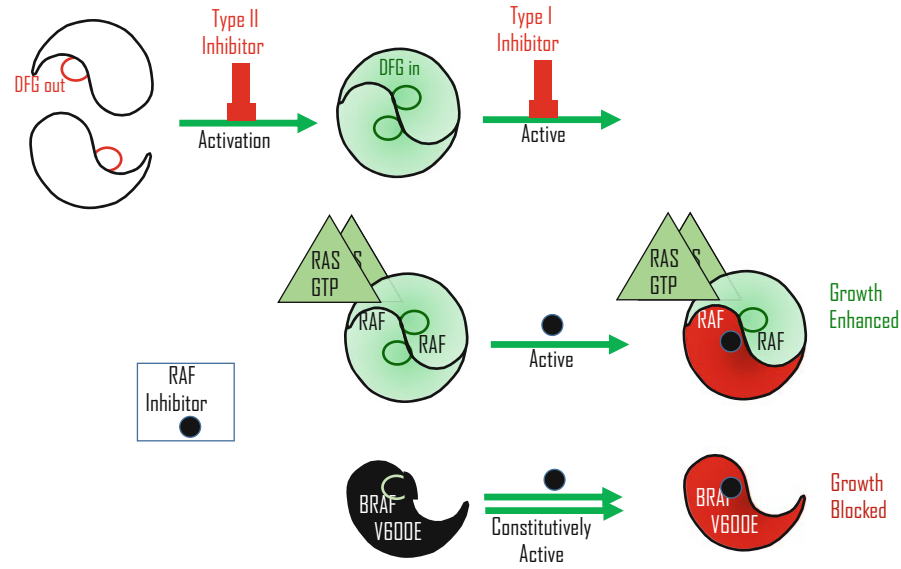

Fig. 2 Cartoon of biochemical mechanisms that can be altered through targeted therapies. The top row illustrates a typical activation mechanism for protein kinases. Unactivated kinase domains possess an activation loop bearing amino acids Asp-Phe-Gly (DFG) in an "out" conformation that sterically blocks ATP binding. Activation – for example, through ligand binding to a receptor kinase or phosphorylation by an upstream kinase – causes the DFG loop to switch to the "in" conformation that enables ATP binding. Type II inhibitors bind to the DFG-out conformation and block kinase activation. Type I inhibitors bind to the DFG-in conformation, thereby blocking kinase activity directly. The middle row shows an idiosyncrasy of the RAF kinases, namely, paradoxical activation by RAF inhibitors. This occurs since RAS induces formation of an asymmetric RAF dimer; binding of the inhibitor to one protomer of the dimer prevents ATP binding to that protomer but causes an allosteric shift that enhances ATP binding to the neighboring protomer resulting in an active RAF enzyme. The bottom row illustrates that BRAF-V600E, found in half of cutaneous melanomas, signals as an active monomer which is effectively blocked by type I RAF inhibitors

An additional type I inhibitor has been added to the clinical repertoire and is now known as encorafenib (LGX818). This compound is remarkable in having a very slow off-rate from the BRAF enzyme and has subsequently been used to elucidate differential binding affinities for each protomer of asymmetric BRAF dimers (Yao et al. 2015). Since standard of care for metastatic melanoma has advanced to BRAF/MEK inhibitor combinations, the focus of encorafenib development has been in combination with the MEK inhibitor binimetinib. Results of this development path are encouraging (see ▶ Chap. 32, "Targeted Therapy in Advanced Melanoma" by Johnson and Sosman) (Flaherty 2017).

Efficacy of BRAF inhibitors in BRAF-V600-mutant metastatic melanoma patients was evident early in the clinical development of the compounds (Bollag et al. 2012; Luke et al. 2017). Vemurafenib showed a high tumor regression (response) rate and progression-free survival in phase I trials, which was confirmed in phase II trials. A phase III trial comparing vemurafenib to the chemotherapeutic agent dacarbazine also showed improved overall survival. One notable side effect was increased incidence of cutaneous squamous cell carcinoma (cSCC) and the related malignancy keratoacanthoma (Su et al. 2012). Even though this treatment-emergent cancer was relatively easily controlled by routine dermatological methods, the prevalence was troubling. Careful review of the literature revealed that similar findings had been seen in sorafenib-treated patients, although incidence was at a lower rate. This led to intense scientific scrutiny, and the resulting experimental analyses led to the identification of a surprising etiology: RAF inhibition could lead to paradoxical activation of the

RAF/MEK/ERK pathway. This phenomenon was termed the RAF inhibitor paradox, and the putative mechanism is discussed below. An important consequence of this mechanism is that RAF/MEK/ERK signaling is likely stimulated or unaffected in normal tissues, as opposed to inhibition achieved in BRAF-V600-mutant cancer cells, with a resultant wider therapeutic window.

A number of "pan-RAF" inhibitors have entered the clinic as well (Yao et al. 2015). These compounds appear to have broader activity on the RAF isoforms, in part through blocking dimeric RAF proteins (Kortum and Morrison 2015). Clinical compounds in this class include LXH254, TAK-632, MLN2480, LY3009120, CCT196969, BGB-283, and CEP-32496 (RXDX-105). These compounds are all relatively early in clinical development, and further data on efficacy and safety are eagerly awaited. If these agents inhibit RAF/MEK/ERK signaling in normal tissues, this may produce a therapeutic window that is more like MEK and ERK inhibitors than paradoxical activating BRAF inhibitors.

MEK and ERK Inhibitors

MEK inhibitors have been the subject of drug discovery efforts as long as RAF inhibitors and for the same reason: since the RAF/MEK/ERK is directly downstream of the RAS oncogene (Sebolt-Leopold and Herrera 2004). The MEK inhibitor U0126 was identified in the late 1990s from a primary drug screen at DuPont. This compound has poor pharmaceutical properties but was initially used as a tool compound to block MEK activity in cellular assays. In parallel efforts, the Parke-Davis drug PD98059 was identified as a more selective MEK inhibitor but also possessed poor pharmaceutical properties. Nonetheless, many publications have used PD98059 as a potent and selective MEK inhibitor to probe the RAF/MEK/ERK pathway in cells. PD98059 binds in an allosteric site of MEK, providing exceptional selectivity.

Subsequent optimization of an unrelated lead at Parke-Davis identified the compound PD184352 (CI1040) as a potent and selective MEK inhibitor with much improved pharmaceutical properties. The crystal structure of an analog of CI1040 as a ternary complex with ATP and MEK1 or MEK2 provided the structural basis for further drug elaboration (Ohren et al. 2004). However, clinical results with this compound revealed suboptimal bioavailability, and this compound was also discontinued. An improved version of this same pharmacophore yielded the compound PD325901, a compound with much improved human bioavailability. Safety issues have delayed development of this compound, yet it remains a clinical compound at this time.

The ternary binding mode has spawned multiple lead optimization efforts at several pharmaceutical companies, and at this writing, cobimetinib is an approved drug, while the compounds selumetinib and binimetinib are in late stages of clinical development.

A completely different scaffold that also binds allosterically to MEK was discovered through a phenotypic screen for inhibition of cell cycle in cancer cells. This effort identified the compound trametinib (JTP-74057, GSK1120212). This compound has favorable bioavailability in humans and was rapidly developed as a single agent and in combination with dabrafenib in melanoma patients (see ► Chap. 32, "Targeted Therapy in Advanced Melanoma" by Johnson and Sosman).

Additional MEK inhibitors currently in clinical development include pimasertib, LNP3794, RO4987655 (CH4987655), RO5126766, and GDC-0623 (Cheng and Tian 2017). Time will tell if these newer compounds supersede any of the currently approved agents.

Subsequently, the MEK inhibitor cobimetinib (XL518, GDC-0973) showed promising results when combined with vemurafenib in BRAF-mutant melanoma patients. A phase III trial showed clear superiority of the cobimetinib/vemurafenib combination versus single-agent vemurafenib. Recent results for the combination of encorafenib and binimetinib are also very promising, so these agents may also become available for melanoma patients with BRAF mutations. With the clinical success of combined BRAF/MEK inhibition, this combination is

currently the targeted therapy of choice for BRAF-mutant melanoma.

Therapeutic targeting of ERK has lagged behind BRAF and MEK, perhaps because of concerns about the many substrates and roles of ERK1 and ERK2 (Samatar and Poulikakos 2014). Nonetheless, several ERK inhibitors have now advanced to clinical study. The most advanced compound is ulixertinib (BVD-532, VTX11e), which recently reported phase 1/2 trial results (Sullivan et al. 2018). At least two other compounds, MK-8353 (SCH900353) and GDC0994 (RG7842), are currently in phase 1 clinical development. MK-8353 is mechanistically differentiated from ulixertinib (an ATP-competitive inhibitor) since in addition to blocking ATP-binding MK-8353, it binds deeper in the active site and blocks MEK-dependent phosphorylation. A broader set of BRAF- and NRAS-mutant melanoma cell lines were sensitive to this agent than to MEK inhibition, for mechanistic reasons that remain poorly understood. Given the success of dual BRAF/MEK inhibition, but also the limited durability, it is hoped that ERK inhibitors may have activity in tumors that have become resistant to BRAF/MEK inhibition; data so far point to clear, but limited, activity. For BRAF V600-mutant melanomas, the optimal application of ERK inhibitors may ultimately be in combination with BRAF inhibitors, in place of MEK inhibitors.

The RAF Inhibitor Paradox

A key discovery that was made early in RAF inhibitor clinical development was the finding of cSCC/keratoacanthomas in a significant number of patients (Su et al. 2012). These lesions often arise within a few weeks, so de novo development is highly unlikely. Much more likely is a pronounced acceleration of tumor development in response to RAF inhibition. A further clue to the mechanism was found from genetic analyses of the lesions: a disproportionate number of lesions had been initiated by RAS mutation. Further investigations in many labs strongly linked these lesions to the RAF inhibitor paradox.

Empirically, the RAF inhibitor paradox can be readily demonstrated in vitro: tumor cells with RAS mutations or alternative upstream activation of the RAS pathway suffer MAP kinase pathway activation in response to RAF inhibitors. Treatment of these cells with RAF inhibitors of diverse chemotypes has relatively modest effects on growth properties, but when growth is affected, increased proliferation is observed. Consistently, treatment of these RAS-driven cells with RAF inhibitors results in pronounced increase in downstream signaling. Hence, the paradox: an inhibitor of the RAF enzyme results in stimulation of the downstream pathway. This was particularly puzzling at the time since such dramatic tumor regressions were observed in patients with BRAF-mutant melanomas.

The mechanism of the RAF inhibitor paradox was known to be intrinsic to RAF binding (rather than due to off-target effects) since such a wide variety of chemotypes (including both type I and type II binding modes) demonstrated paradoxical activation (Cox and Der 2012). It was shown that dimerization was important, since all of the offending compounds caused increased RAF dimerization. While specific details are still under investigation, the hypothesis is that binding of a RAF inhibitor to one protomer of a RAF dimer allosterically induces enhanced ATP binding to the neighboring protomer. This is consistent with the role of RAS in stimulating RAF dimer formation at the membrane. The efficacy in BRAF-V600-mutant melanomas is thus due to the fact that type I dimer inhibitors bind effectively to BRAF monomers to stymie signaling. BRAF-mutant melanoma is "addicted" to MAP kinase pathway signaling, so blocking at the BRAF monomer level is an effective antitumor modality. In class 2 and class 3 BRAF-mutant tumors, the first-generation RAF inhibitors are generally ineffective; these BRAF mutations are rare in melanomas. One compound has been described in the literature that has substantially reduced liability for the RAF inhibitor paradox (Zhang et al. 2015). PLX8394 binds to the active site of BRAF, but instead of allosterically stimulating the neighboring protomer, this compound

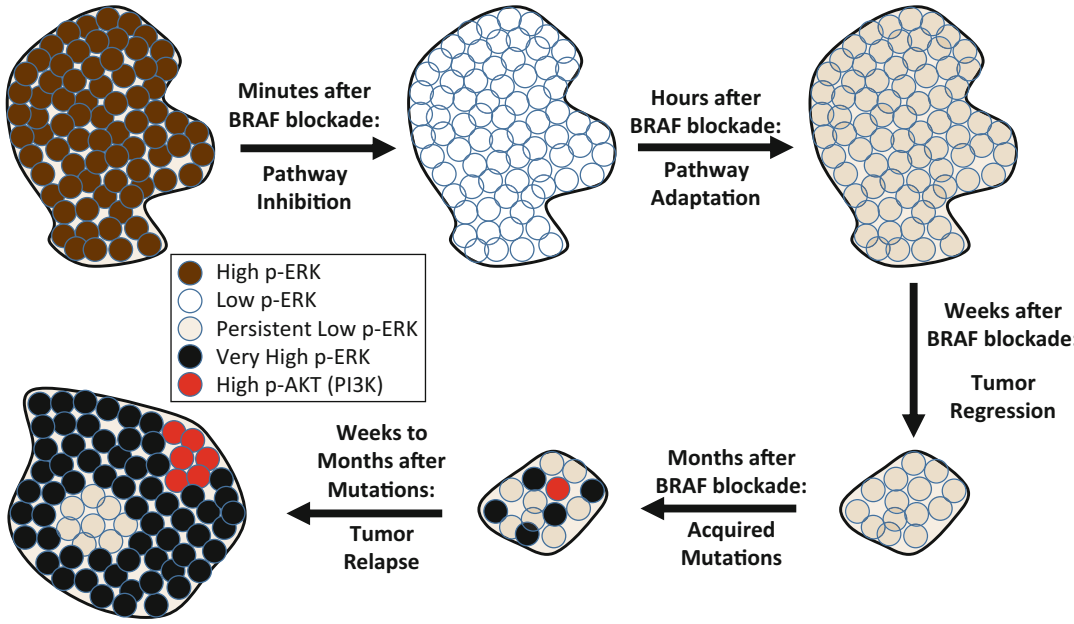

Fig. 3 Cartoon illustrating a typical tumor response to targeted BRAF pathway inhibition. Initially, BRAF-mutant tumors have strong pathway activation, indicated by brown phospho-ERK (p-ERK) staining. Within minutes, BRAF inhibitors effectively silence the pathway resulting in low p-ERK levels (white). Within hours, feedback adaptation occurs to cause a reset of pathway output (since p-ERK normally self-limits pathway output and this pathway control is abrogated by the inhibitor). This steady state (light brown) persists, likely for months, while epigenetic reprogramming enhances persistence of the tumor (not through direct effects on p-ERK). During this time, tumor regression occurs, likely through a combination of necrosis, apoptosis, and immune cell activity. Most frequently, though, residual tumor persists, supported by epigenetic and stromal retaliation dictated by the tumor cell. Within the persisting tumor mass, selection for mutations that overcome the targeted therapy results in resistant clones. Most commonly, resistant mutations cause reignition of the RAF/MEK ERK pathway (black staining). However, relapsed tumors are often polyclonal, and alternative pathways can be triggered; in this cartoon example, the PI3K pathway is stimulated (e.g., through loss of PTEN), and phospho-AKT (p-AKT) levels increase (red). Over the following weeks to months, these resistant clones proliferate and predominate, reestablishing a progressively growing tumor mass

actually destabilizes the dimer. This compound is currently in early clinical development.

Resistance to RAF/MEK/ERK Inhibitors

Response to MAP kinase pathway inhibition is usually followed by some form of adaptation or true resistance. Three chronologically distinct phases can be defined: (1) within a day, "rewiring" of cellular signaling leads to a new homeostasis; (2) within a month, epigenetic, immuno-, and microenvironmental adaptation leads to tolerance; and (3) after months (to years), genetic mutations result in outgrowth of resistant clones (Fig. 3).

When BRAF-mutant cancer cells are treated with BRAF inhibitors, ERK-dependent feedback inhibition is abrogated (Pratilas and Solit 2010). This occurs through a variety of pathways, including DUSP and Sprouty proteins whose upregulation in response to ERK activation normally leads to pathway attenuation either by dephosphorylating ERK directly or through blocking receptor signaling. A new "steady state" is achieved, as the tumor cells adapt to the tonic presence of the inhibitor.

Over time, epigenetic and microenvironmental factors induce tolerance to pathway inhibition. Thus, a subset of "tolerant" cells persist and can seed recurrence of the tumor (Sharma et al. 2010; Konieczkowski et al. 2014). Furthermore, stromal

cells can support melanoma growth, and one of the most common factors that mediates this event is HGF, hepatocyte growth factor (Straussman et al. 2012; Wilson et al. 2012). The adaptation events sometimes lead to a state in which the tumor is "addicted" to the presence of the targeted inhibitor. Indeed, intermittent removal of the inhibitor can lead to improved efficacy (Das Thakur et al. 2013).

Substantial efforts have tried to address the question about acquired resistance to BRAF pathway inhibitors (Solit and Rosen 2011). Single-agent BRAF inhibitors typically have a relatively short progression-free survival (PFS) of ~6 months (▶ Chap. 32, "Targeted Therapy in Advanced Melanoma" by Sosman and Johnson). Clearly, resistance to BRAF inhibition is a frequent and rapid event. Dual inhibition of BRAF and MEK increases durability resulting in a PFS of ~12 months. Still, resistance is a major problem, and dual BRAF/MEK inhibition has only modest activity once BRAF inhibitor resistance transpires. Note, however, that the occurrence of cSCC/keratoacanthomas decreases significantly in BRAF/MEK dual inhibitor trials compared to single-agent BRAF inhibitors. This adds credence to the proposed mechanism of the cSCC/keratoacanthomas: the RAF inhibitor paradox can predictably be antagonized by MEK inhibition.

After much research, the mechanisms known to cause resistance to BRAF/MEK inhibition are numerous (Johnson et al. 2015). Unfortunately, this dampens hope that a single add-on therapy could be used in all patients. Among the mechanisms of resistance are the following: RAS mutation (primarily NRAS), BRAF amplification, BRAF splicing, growth factor receptor activation, stromal growth factors, activation of the phosphatidylinositol 3′-kinase pathway, and other less frequent events. Analysis of tumor samples from resistant patients has yielded much of this information, although about 40% of the samples did not reveal an identifiable mechanism. In samples with identifiable mechanisms, it appears that most (perhaps 75%) of the resistant tumors rely on the reactivation of the RAF/MEK/ERK pathway. To make follow-up therapy even more challenging, analysis of multiple metastases from the same patient yields multiple resistance mechanisms (Shi et al. 2014).

Interplay between Immuno-Oncology and Targeted Therapy

Immuno-oncology research has burgeoned, seeking to take advantage of the patient's innate immune potential to antagonize the tumor directly (Luke et al. 2017). This approach makes strategic sense, since tumors develop intricate mechanisms to hide from innate immune cells. Immune evasion and immune suppression make up one of the hallmarks of cancer, "avoiding immune destruction." Furthermore, "tumor-promoting inflammation" is another hallmark that is mediated by the tumor microenvironment.

In the case of BRAF-mutant melanoma cells, two important phenotypes include dedifferentiation such that antigenic epitopes are not displayed on the cell surface and expression of immune inhibitors such as PD-L1. BRAF mutation thus serves to protect melanoma cells from the innate immune response. BRAF pathway inhibition, therefore, not only blocks proliferation and causes cell death but also impedes immunosuppressive mechanisms that are key to melanoma cell survival. While BRAF inhibition can lead to increased expression of the immunosuppressive ligand PD-L1, this is not a universal feature (Cooper et al. 2015). In any case, targeted therapies overlap with immuno-oncology therapies to block melanomas. Therefore, combination of targeted therapies with immune checkpoint inhibitors is a promising development in the treatment landscape (Luke et al. 2017).

KIT Inhibitors

Most of this chapter has been devoted to targeting the BRAF pathway, since that approach has led to successful therapies. A small subset of tumors express another targetable oncogene, namely, the mutated or amplified KIT receptor (Postow and Carvajal 2012). KIT is the receptor for stem cell factor, which is important for the development of

key myeloid cells, and in particular for mast cell function. KIT also functions in melanocytes, for example, in the pathway of melanin biosynthesis. Mutations in KIT are primarily found in acral, mucosal, and chronic sun damage (CSD) surface forms of melanoma (▶ Chap. 7, "Molecular Genetics of Melanocytic Neoplasia" by Hodis and Garraway).

Since KIT mutations are found at much higher frequency in gastrointestinal stromal tumors (GIST), the targeted therapy approach has been explored in great detail in those tumors (Maki et al. 2015). Current GIST treatment consists of multiple lines of KIT inhibitor treatment. Imatinib, an inhibitor of several kinases including KIT and ABL, is the first line of therapy for GIST patients and is typically effective for about 2 years. Subsequent KIT inhibitors include sunitinib and regorafenib, which have diminishing durability due to the accumulation of KIT mutations during the development of resistance.

Imatinib has also been tested in a number of trials for KIT-mutant melanoma (▶ Chap. 32, "Targeted Therapy in Advanced Melanoma" by Sosman and Johnson). Clear tumor responses are seen in a subset of patients (15–25%), with most activity in KIT-mutant tumors and minimal activity in KIT-amplified tumors. Nilotinib, which inhibits KIT and ABL, and sunitinib, which inhibits KIT and multiple other kinases, have also been trialed in KIT-mutant melanoma patients, and response rates to date have been lower than those for imatinib. As with GIST tumors, KIT mutation heterogeneity presents a substantial challenge in the treatment of KIT-mutant melanomas with KIT inhibitors. The diversity of KIT mutations, the heterogeneity of mutations within patients, and the scarcity of patients with KIT mutations have made the development in KIT-mutant melanoma particularly difficult, and no inhibitors have yet been approved.

Other Targetable Drivers of Melanoma

While BRAF and KIT have attracted the most clinical attention, additional oncogenic drivers with associated targeted therapies have been described. For example, in ▶ Chap. 7, "Molecular Genetics of Melanocytic Neoplasia" by Hodis and Garraway, mutations in genes such as MEK, EZH2, and IDH1 reveal likely genetic drivers that may be addressed by the current therapeutic armamentarium. In ▶ Chap. 18, "Spitz Tumors" by Yeh and Bastian, a variety of targetable kinase gene fusions are noted in spitzoid melanoma, including ROS1, NTRK1, NTRK3, ALK,RET, and MET. Since approved or experimental drugs exist for each of these fusion-activated kinases, it is likely that future clinical care of these genetically identifiable oncogenes with targeted agents will become standard practice. In the meantime, anecdotal reports are emerging, such as response in a ROS1 fusion-driven melanoma to the kinase inhibitor entrectinib (Drilon et al. 2017).

RAS pathway activation occurs frequently in melanomas, most frequently through NRAS or KRAS mutations, loss of NF1, or activation of GNAQ and GNA11. While RAS inhibitors have been a holy grail of targeted therapy discovery efforts for 30 years, no drugs are currently available. MEK inhibitors have been tested in NRAS-mutant melanoma with modest activity (Dummer et al. 2017). However, further analysis of these patients revealed no survival advantage (▶ Chap. 32, "Targeted Therapy in Advanced Melanoma" by Johnson and Sosman). MEK inhibitors have also been tested in uveal melanoma patients which mostly have GNAQ/GNA11 mutations. Again, initial results were promising (Carvajal et al. 2014), but late-stage studies failed to show a significant progression-free survival effect or any overall survival advantage (Komatsubara et al. 2016). Reinvigorated efforts to target RAS in different ways will hopefully provide additional targeted therapeutic options for melanoma patients (Simanshu et al. 2017).

Conclusion

While immunotherapies have taken center stage in recent years, targeted therapies will remain important options in the melanoma patient's journey. As future scientific discoveries reveal the vulnerabilities of available therapeutic options, subsequent drug discoveries will address these

vulnerabilities. The genetics and biochemistry of resistance will inform target identification along with the proper constituents and scheduling of therapeutic combinations. The resulting treatment paradigms will enable long-term melanoma abatement, perhaps even cures.

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Melanomics: Comprehensive Molecular Analysis of Normal and Neoplastic Melanocytes

10

Xuan Tien Steven Nguyen and Ian R. Watson

Contents

Abstract

Cutaneous melanoma is the deadliest form of skin cancer. An immense understanding of this disease has emerged through the molecular characterization of melanocytes and melanomas, which has resulted in the development of therapies that have impacted patient outcomes. This chapter will review studies that have characterized the melanoma genome, transcriptome, as well as the epigenome of non-acral, cutaneous, and uveal melanoma. Specifically, first-generation sequencing findings that discovered many of the frequently mutated oncogenes and tumor suppressors in melanoma will be described. Subsequently, next-generation sequencing studies that revealed novel UV-induced driver mutations and frequent noncoding mutations will be covered. Finally, integrative analyses of melanoma across multiple data platforms that have led to the discovery of new biomarkers and increased our understanding of the molecular basis of this disease will be reviewed.

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Introduction and Etiology

Found most abundantly in the basal layer of the epidermis, melanocytes are derived from the neural crest lineage and are distributed to various anatomical sites. These cells function to produce melanin, which provides pigmentation for the skin, eyes, and hair. Melanocytes can produce different subsets of neoplasms that differ in clinical features, histopathological appearance, and biological behavior (reviewed in Bastian 2014). Melanocytic neoplasms are most frequently found in the skin; however, they can arise from melanocytes in the eye, the central nervous system, and numerous internal organs. Nevi and melanoma are the terms used to describe the benign and malignant melanocytic neoplasms, respectively.

This chapter focuses on studies that have performed analyses of melanocytic neoplasms with the following data platforms: first-generation sequencing, whole exome sequencing (WES), whole genome sequencing (WGS), copy number variation (CNV) analysis, mRNA/miRNA profiling, genome-wide epigenetic characterization, and integrative analysis across multiple data platforms. First, genome-wide and integrative analyses of cutaneous melanoma from skin with either marked (high-CSD) or intermediate or little (low-CSD) signs of chronic sun-induced damage will be reviewed. (These melanoma subtypes were originally termed chronic sun damaged (CSD) and non-CSD, respectively. The 2018 WHO Classification of Skin Tumors uses the terms high- and low-CSD instead, and this nomenclature is used in this chapter for consistency.) The second part of this chapter will cover integrative analyses from melanomas originating from the uveal tract of the eye (uveal melanoma). The publications reviewed herein will be limited to treatment-naïve melanomas. As large-scale integrative analyses for the rarer forms of melanomas have not yet been performed, this chapter will focus primarily on non-acral cutaneous melanoma and uveal melanoma. Where appropriate, major genomic findings for the less well-studied forms of melanomas will be briefly described. The latter types include melanomas from glabrous (non-hair-bearing) skin (acral melanoma) and melanomas originating from mucosal membranes (mucosal melanoma), as well as desmoplastic and Spitz melanomas.

Next-Generation Sequencing

Studies of familial melanoma using array-based comparative hybridization and first-generation sequencing technologies that include PCR amplification and Sanger-based capillary sequencing methodologies have been instrumental in identifying genes involved in melanoma progression. However, from 2010 to 2016, two next-generation sequencing (NGS) approaches have led to major genomic discoveries in melanoma. First, whole exome sequencing (WES), where exons are captured using biotinylated RNA "baits" and undergo massive parallel sequencing, has allowed investigators to identify indels and SNVs in the coding portion of melanoma genomes. Second, whole genome sequencing (WGS) has enabled the identification of single nucleotide variants (SNVs) in both coding and noncoding portions of the genome, as well as structural variants. These approaches facilitated the discovery of driver mutations that were not detected by first-generation sequencing methods.

Throughout this chapter, the following terms will be used. A ***mutation*** is a change of the normal structure of a gene caused by alterations of single base units in DNA (SNVs), deletions, insertions or amplifications, as well as rearrangement of genes or chromosomes that can lead to expressed gene fusions. ***Driver mutations*** are somatic mutations in a gene that provide a selective advantage for cells, with statistical evidence for positive selection. A ***hotspot mutation*** is a recurrent mutation that leads to the same nucleotide (for noncoding) or amino acid substitution (for coding mutations),

which generally signifies positive selection. In contrast, ***passenger mutations*** do not provide a selective advantage to cancer cells, reflected by the lack of statistical evidence for positive selection (reviewed in Watson et al. 2013). Herein, genomic studies and comprehensive molecular analyses of normal and neoplastic melanocytes that have provided insight into the etiology of this disease will be reviewed.

Cutaneous Melanoma

Detected in its earliest stages (stage I, II, and resectable stage III), melanoma is generally curable with early detection and surgery. Unfortunately, patient prognosis significantly decreases for patients with late stage metastatic disease (unresectable stage III and stage IV), where lesions spread to distant sites (Balch et al. 2009). The American Cancer Society estimates that approximately 87,110 new melanomas will be diagnosed in 2017 in the United States, of which nearly 10,000 are expected to die from this disease (Siegel et al. 2017). Unlike the majority of cancers, melanoma rates have been increasing significantly in both men and women (Linos et al. 2009). Ultraviolet (UV) radiation is a major environmental risk factor for cutaneous melanoma. Melanomas from the sun-exposed skin are commonly categorized into two groups: originating from severely sun-damaged (high-CSD) or skin with little or intermediate sun-induced damage (low-CSD) (Bastian 2014). High-CSD melanomas are generally found in older patients (>55 years old) on the head, neck, and dorsal surfaces of extremities. They show a high degree of solar elastosis histologically, as the defining criterion. Melanomas found on the trunk and proximal extremities of younger patients typically show low or intermediate degrees of solar elastosis and therefore are considered low-CSD melanomas (Whiteman et al. 2003). First-generation sequencing analyses identified driver mutations in frequently mutated melanoma oncogenes and tumor suppressors, but interestingly, few possessed characteristic UV signature mutations.

Genomics of Cutaneous Melanoma

First-Generation Sequencing: Cutaneous melanoma is characterized by mutually exclusive hotspot mutations in the mitogen-activated protein kinase (MAPK) regulators, *BRAF* (p.V600) and *NRAS* (p.G12, G13, and Q61L), found in approximately 50% and 20% of patients, respectively. These hot spot mutations in *BRAF* and *NRAS* were discovered using PCR amplification and Sanger sequencing (Albino et al. 1989; Davies et al. 2002; Sekiya et al. 1984; van 't Veer et al. 1989). The vast majority of hotspot mutations in *BRAF* and *NRAS* are not caused by characteristic UV-induced SNVs in the form of C > T transitions found at dipyrimidines (described in more detail below). These findings led to the development of BRAF inhibitors (e.g., vemurafenib and dabrafenib) and MEK kinase inhibitors (e.g., trametinib) for the treatment of melanoma, which has elicited dramatic antitumor responses in the clinic, although drug resistance remains problematic (reviewed in Girotti et al. 2014).

Prior to the era of next-generation sequencing (NGS), studies employing various approaches discovered mutations in genes that encode for regulators of canonical cancer pathways in addition to the MAPK pathway. These include the INK4A-CDK4/6-RB, ARF-MDM2-P53, and PI(3)K-AKT signaling pathways (reviewed in Tsao et al. 2012). Many of these melanoma genes were discovered from studying melanoma-prone families, such as the initial discovery of mutations in *CDKN2A* that co-segregated with melanoma susceptibility (Hussussian et al. 1994; Kamb et al. 1994). *CDKN2A* encodes for both p16INK4A and p14ARF that regulate the INK4A-CDK4/6-RB cell cycle and the ARF-MDM2-TP53 apoptotic pathways, respectively. Other germline mutations that predispose individuals to melanoma include variants in *CDK4*. CDK4 phosphorylates and inhibits the RB1 tumor suppressor to promote cell cycle progression. The most common mutation in *CDK4* encodes for the p.R24C amino acid change that renders CDK4 insensitive to p16INK4A inhibition (Wolfel et al. 1995; Zuo et al. 1996). Furthermore, retinoblastoma patients

that possess germline *RB1* mutations have a higher incidence of melanoma (Draper et al. 1986). Although portions of *CDKN2A* that encode for p16INK4A are more frequently mutated in melanoma, alterations affecting p14ARF have been reported in some types of familial melanoma (Randerson-Moor et al. 2001). ARF positively regulates TP53 function by inhibiting its negative regulator, the E3 ubiquitin ligase MDM2, which promotes TP53 degradation (reviewed in Tsao et al. 2012). TP53 is a tumor suppressor frequently mutated in many cancers that promotes apoptosis in response to DNA damage. *MDM2* amplifications were also found in approximately 3% of melanomas (Muthusamy et al. 2006). Another important pathway involved in the regulation of melanoma cell growth and survival is the PI(3)K-AKT pathway, where *AKT3* amplifications, *PTEN* mutations and deletions were discovered as frequent events (Guldberg et al. 1997; Teng et al. 1997). Notably, *PTEN* mutations/deletions were found to occur more frequently in *BRAF* mutant melanomas (Stahl et al. 2004).

Copy Number Variations (CNV): Copy number approaches that include the use of high-density single-nucleotide polymorphism (SNP) and comparative genomic hybridization (CGH) arrays played important roles in identifying oncogenes and tumor suppressors in melanoma. Some of the earliest CGH studies discovered losses of chromosomes 6q, 8p, and 10 as well as gains in chromosomes 1q, 6p, 7, and 8 in primary melanomas (Balázs et al. 2001; Bastian et al. 1998, 2000a, 2003). SNP array analysis from NCI60 human tumor cell lines revealed amplifications of the lineage-specific master regulator of melanocytes, microphthalmia-associated transcription factor (MITF), as one of the first lineage-specific oncogenes (Garraway et al. 2005). Integration of array CGH analysis combined with clinical information and focused sequencing of known driver mutations in *BRAF* and *NRAS* revealed insight into differences among clinical and histologic subtypes of melanoma (Curtin et al. 2005). These analyses demonstrated distinct patterns of genetic alterations in four groups of primary melanomas: acral (minimal or no sun exposure), mucosal (protected from sun), and skin melanomas with CSD and non-CSD (Fig. 1). Melanomas on the skin with intermitted sun exposure (low-CSD) had higher frequencies of *BRAF* (p.V600) mutations and chromosome 10 loss, which encompasses the *PTEN* tumor suppressor (Curtin et al. 2005). In contrast, high-CSD melanomas had infrequent *BRAF* (p.V600) mutations, but possessed more frequent *CCND1* amplifications. Acral melanomas, defined as melanomas originating from the glabrous (non-hair-bearing) skin of the palms and soles or the nail apparatus, as well as mucosal melanomas that are protected from direct UV light, had a much higher frequency of focal amplifications and deletions. For example, amplifications of *CDK4* and *CCND1* were found more frequently in both acral and mucosal melanomas. By contrast, copy number increases of chromosome 7 were more common in low-CSD melanomas and preferentially affected the chromosome encompassing the mutant *BRAF* allele. Hotspot mutations in *BRAF* and *NRAS* mutations were mutually exclusive (Davies et al. 2002; Maldonado et al. 2003; Pollock et al. 2003) (Fig. 1). Large-scale SNP array studies coupled with statistical tools that include GISTIC and CONTRA improved the genomic resolution of minimal common regions (MCR) encompassing amplified or deleted genes (Hodis et al. 2012; Krauthammer et al. 2012; Lin et al. 2008; Cancer Genome Atlas Network 2015) (Figs. 2 and 3). Such studies revealed 14 major significant regions of amplifications and 13 regions of deletion in over 100 melanoma short-term cultures and cell lines (Lin et al. 2008). Lin et al. reported that the most statistically significant MCRs of amplification included the genomic regions of 7q32.3, 7q34 that spans *BRAF*, 20q13.2, 7p21.2, and 3p13 that encompasses *MITF*. The most significant MCRs of deletions were 9p21.3 that spans *CDKN2A*, 10q23.31 that includes *PTEN*, 4q34.3, and 6q26 covering *PACRG* and *PARK2* (Lin et al. 2008). Similar results were observed by The Cancer Genome Atlas (TCGA) (described in more detail below) (Figs. 2 and 3).

Whole Exome and Whole Genome Sequencing: Epidemiological and animal model studies have linked UV exposure to melanoma risk.

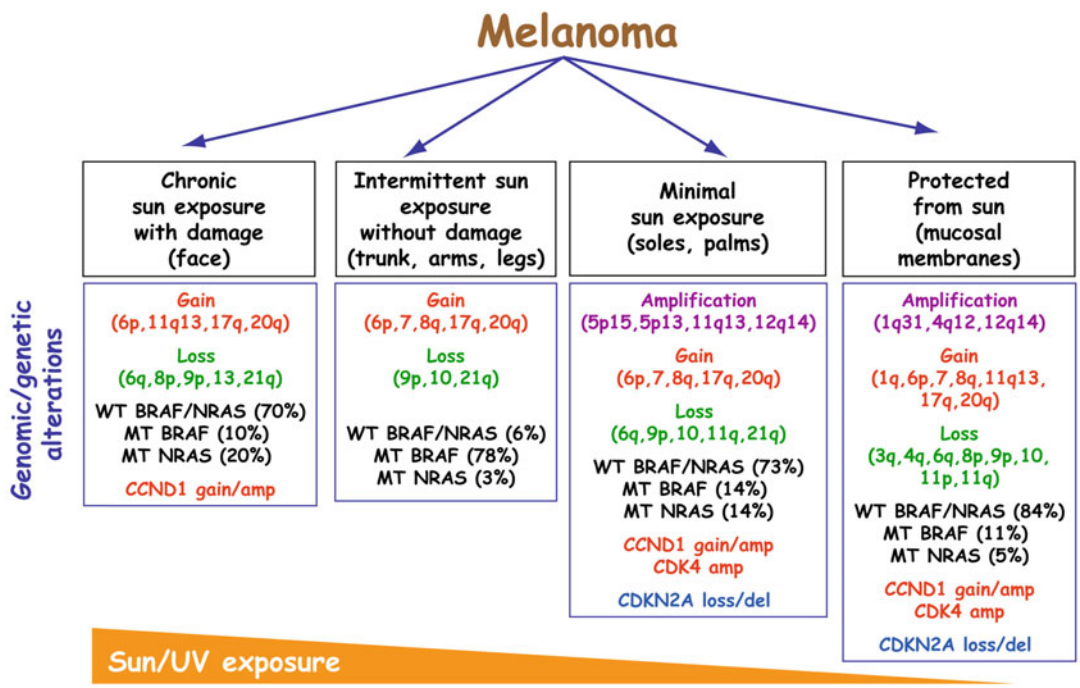


Fig. 1 Genetic alterations in melanoma patients affected by varying degrees of sun exposure (From Curtin et al. 2005). Shown in the four panels are genetic alterations of melanomas with chronic sun damage, intermittent sun/UV exposure without sun damage, minimal sun/UV exposure, and protected from sun/UV exposure. The genetic alterations listed include chromosomal gains/losses and *BRAF/NRAS* mutation status that differ in frequency in melanomas with varying degrees of sun/UV exposure. For example, melanomas that occurred with minimal or no sun/UV exposure were reported to possess *CDK4/CCND1* amplifications and *CDKN2A* loss (Reprinted from *Cancer Cell*, Vol 8/edition number 6, Omar Kabbarah and Lynda Chin, Revealing the Genomic Heterogeneity of Melanoma, Pages 439–441, Copyright (2005), with permission from Elsevier)

Driver mutations caused by UV signature mutations were not well known prior to NGS studies of melanoma (Hodis et al. 2012; Krauthammer et al. 2012). However, NGS technology provided the capability to catalogue SNVs and indels from the melanoma genome. The first whole genome sequencing (WGS) analysis of melanoma was performed on one melanoma cell line and a lymphoblastoid cell line derived from the same patient, revealing the presence of UV signature mutations (Pleasance et al. 2010). Subsequent larger scale studies revealed that cutaneous melanoma has one of the highest mutation burdens of cancers sequenced to date, with the latest estimate being ~17 mutations/Mb (Berger et al. 2012; Hodis et al. 2012; Krauthammer et al. 2012; Cancer Genome Atlas Network 2015). As a comparison, childhood cancers and leukemias possess mutation burdens of ~1 mutation/Mb (Lawrence et al. 2013).

The high mutation burden in cutaneous melanoma is attributed to UV radiation, as evidenced by the elevated number of UV signature mutations in melanoma genomes. UV radiation is composed of three components that differ in wavelength: UVA (320–400 nm), UVB (290–320 nm), and UVC (200–290 nm) (reviewed in Garibyan and Fisher 2010). The two wavelengths that humans are exposed to are UVA and UVB, as 95% and 10% of these wavelengths reach the Earth's surface, respectively (UVC gets absorbed by the atmosphere and ozone layer). The higher the wavelength of UV radiation, the deeper it can penetrate the skin. As a result, the most cutaneous damage has been attributed to UVB, which induces DNA damage in the form of cyclobutane

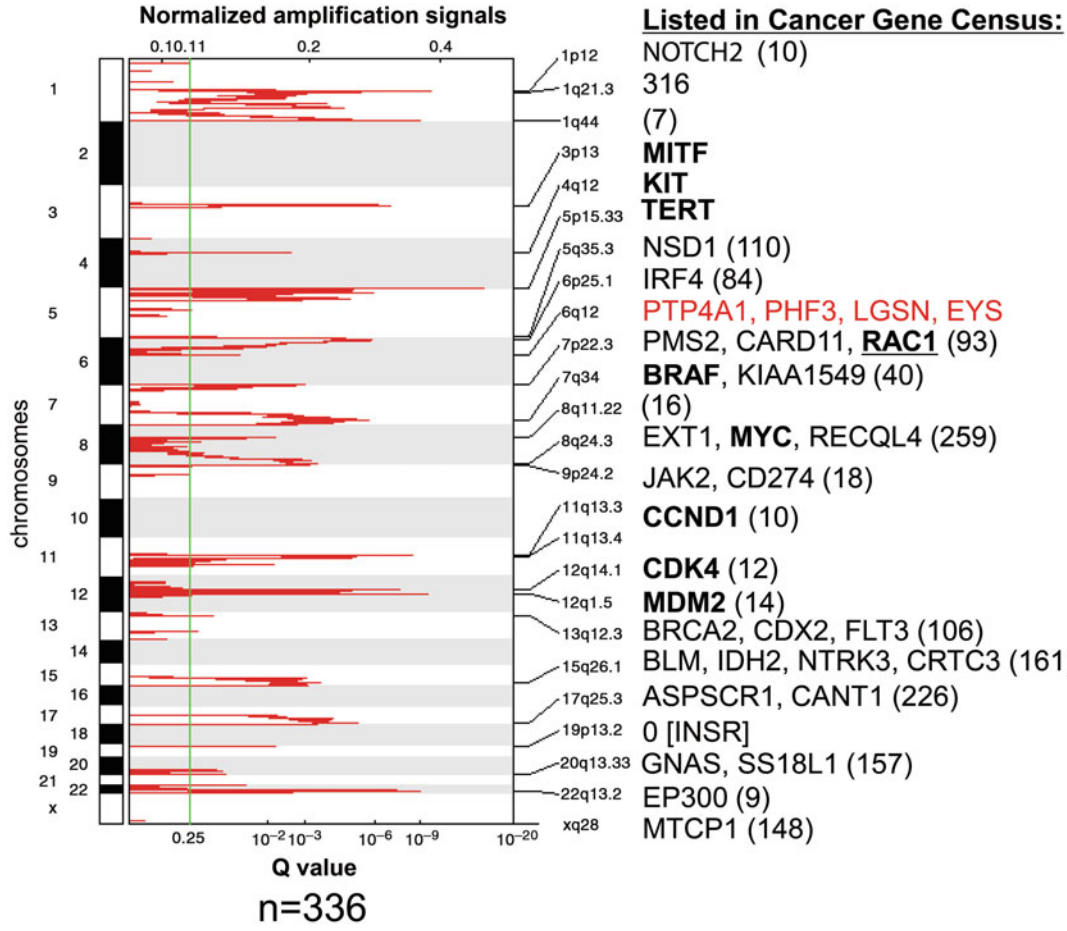


Fig. 2 Significantly amplified regions in cutaneous melanoma from the TCGA dataset. GISTIC2 analysis from the Melanoma TCGA data of 336 samples indicating significance of minimal common regions (MCRs) (right side) from chromosomal regions (left side) by Q-values. The threshold for significance was set at a Q-value of 0.25 (green line). Numbers of genes found in MCRs are indicated in brackets. Genes listed are found in the Cancer Gene Census List from the Wellcome Trust Institute (black) and/or have been previously linked to melanoma (bold and/or underlined), or are genes with unclear roles in cancer (red) (Unpublished work from the TCGA and the Watson lab)

pyrimidine dimers (CPDs) and pyrimidine 6–4 pyrimidone photoproducts (Garibyan and Fisher 2010). The best understood mutational mechanism that leads to UV signature mutations is that cytosine or 5-methyl-cytosine deaminates in the CPDs to uracils or thymidines that are subsequently replicated in an error-free process, thus generating C > T transitions at dipyrimidine sites (Taylor 2015). Meta-analyses from experimental sequencing data with defined UV exposure established that having C > T transitions at dipyrimidines in more than 60% of the total mutational burden or CC > TT mutations in more than 5% defines the presence of a UV signature (Brash 2015). Experimental systems have demonstrated that UVA-mediated oxidative DNA damage induces G > T and T > G transversions (Drobetsky et al. 1995; Palmer et al. 1997). More recent studies have identified mechanisms in which UVA can also induce CPDs through a reactive oxidative species (ROS)-dependent mechanism involving a reaction of the phototoxic pheomelanin as well as the photoprotective eumelanin leading to the generation of C > T UV

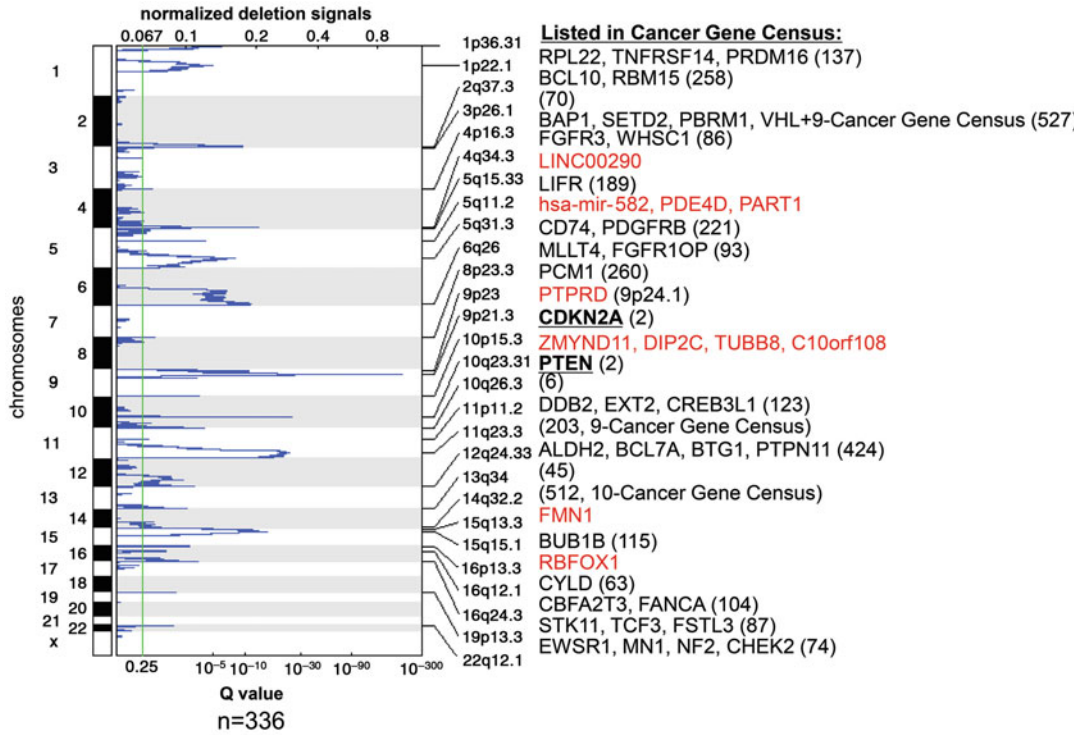


Fig. 3 Significantly deleted regions in cutaneous melanoma from the TCGA dataset. GISTIC2 analysis from the Melanoma TCGA data of 336 samples indicating significance of minimal common regions (MCRs) (right side) from chromosomal regions (left side) by Q-values. The threshold for significance was set at a Q-value of 0.25 (green line). Numbers of genes found in MCRs are indicated in brackets. Genes listed are found in the Cancer Gene Census List from the Wellcome Trust Institute (black) and/or have been previously linked to melanoma (bold and/or underlined), or are genes with unclear roles in cancer (red) (Unpublished work from the TCGA and the Watson lab)

signature mutations (Mitra et al. 2012). Pheomelanin is thought to be phototoxic through its innate ability to generate ROS, which has been shown to induce melanoma in mice in the absence of UV exposure (Mitra et al. 2012).

Early WES studies of cutaneous melanoma produced mutation data in a limited number of samples (Wei et al. 2011). Such studies identified recurrent mutations in additional members of the MAPK pathway, such as *MAP 2 K1* (p.P124S) (Emery et al. 2009; Nikolaev et al. 2012). However, due to the high and heterogeneous mutation burden in melanoma, identifying driver mutations remains a challenge even to date. This is especially true in the case of tumor suppressors and infrequently mutated oncogenes. This problem was first raised in analyses of melanoma exomes in 2012. To identify novel significantly mutated genes (SMGs) in melanoma, research groups needed to sequence large sample cohorts (>100 samples), and develop novel algorithms to identify SMGs (Hodis et al. 2012; Krauthammer et al. 2012). To address this challenge, Hodis et al. developed InVEx, which is a statistical tool that uses sequencing data from intronic and untranslated regions to infer a gene-specific mutation burden that allows for the identification of SMGs. By employing this tool, well-established melanoma tumor suppressors and oncogenes were found to be significantly mutated, including *BRAF, NRAS*, *MAP 2 K1*, *PTEN,* and *CDKN2A*, as well as novel SMGs (Hodis et al. 2012) (Fig. 4). These novel SMGs possessed hotspot and loss of function (LoF) mutations caused by UV signature mutations. Hotspot mutations were identified in the Rho GTPase *RAC1* (p.P29S), in the catalytic

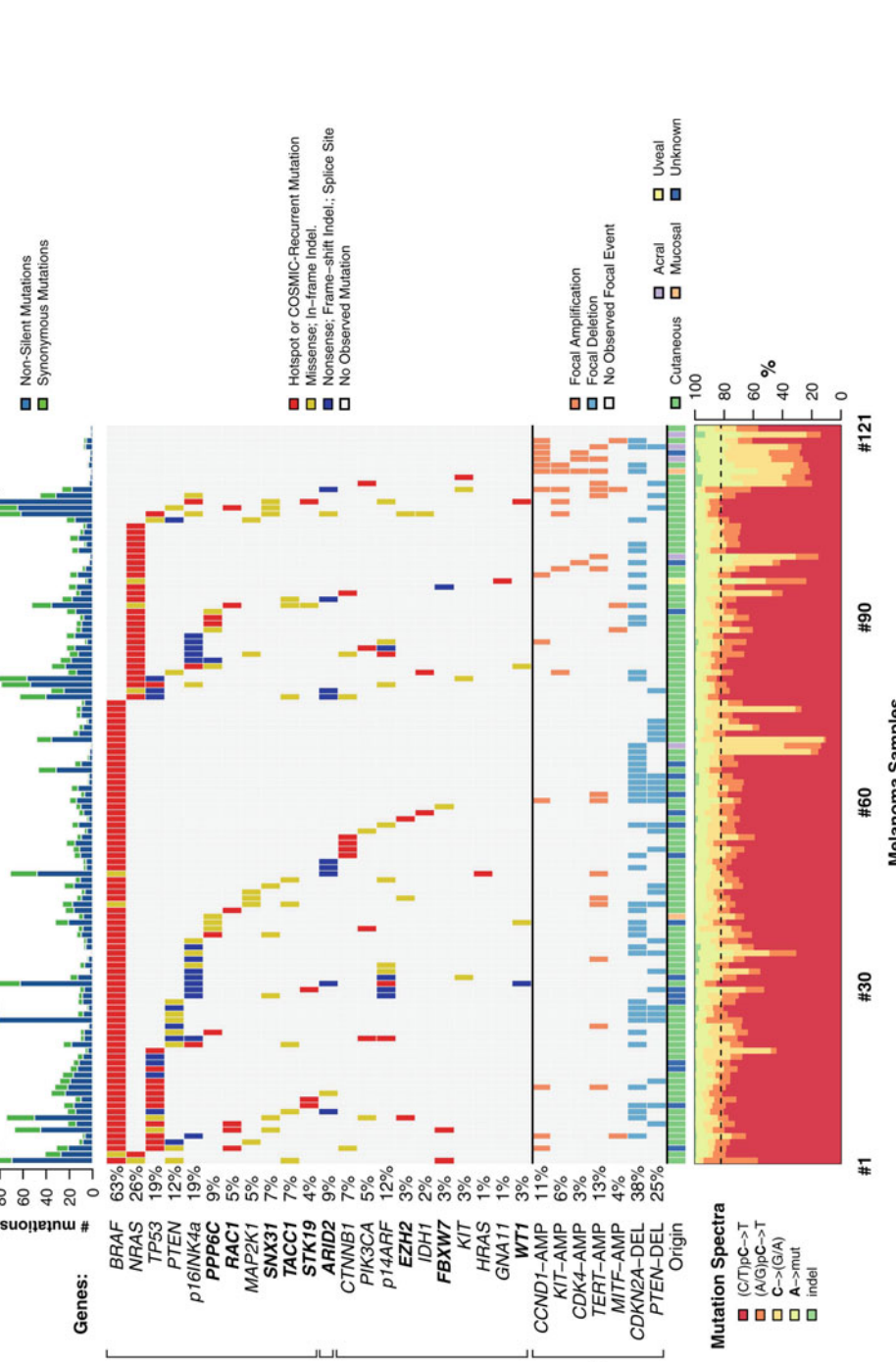


Fig. 4 The landscape of driver mutations in melanoma. Significantly mutated genes identified by the statistical tool InVEx that possessed significant mutational burden or loss of function (LoF) burden are indicated according to prevalence. Mutations found frequently in the COSMIC database that have been reported to be driver mutations in other cancers are shown. Selected significant focal amplifications and deletions determined by GISTIC 2 and linked to melanoma are also displayed. The color-coded matrix indicates type of

subunit of the heterotrimeric PP6 protein phosphatase complex *PPP6C* (p.R301C), and in the serine threonine kinase *STK19* (p.D89N). LoF mutations were also found in *ARID2*, a component of the SWI/SNF chromatin-remodeling complex (Hodis et al. 2012; Krauthammer et al. 2012) (Fig. 4). These SMGs were concurrently discovered by Krauthammer et al. in an exome sequencing study of 147 cutaneous melanoma samples that used an alternate method that took into account gene expression in order to identify SMGs (Krauthammer et al. 2012). These findings provided a missing mechanistic link between melanoma and driver UVB-induced signature mutations with the identification of driver mutations caused by C > T transitions in new melanoma oncogenes and in tumor suppressors.

Mutations Within the Noncoding Regions: Some of the most significant discoveries in melanoma genomics from NGS technologies have come from the discoveries of noncoding mutations in melanoma. For instance, telomerase activity is upregulated in almost all cancers (Kim et al. 1994). However, the mechanisms that mediate these processes were not entirely clear. TERT encodes the catalytic subunit of the telomerase enzyme, which together with the telomerase RNA component (TERC) lengthen telomeres (reviewed in Armanios and Blackburn 2012). In melanoma, overexpression of TERT is one of three factors commonly used to immortalize melanocytes, and DNA copy number studies have shown significant *TERT* amplifications in ~5–15% of melanomas (Garraway et al. 2005; Hodis et al. 2012; Krauthammer et al. 2012; Cancer Genome Atlas Network 2015). In 2013, two concurrent studies discovered two mutually exclusive recurrent *TERT* promoter mutations in a large fraction of cutaneous melanoma samples from the investigation of familial melanoma patients and analysis of WGS data (Horn et al. 2013; Huang et al. 2013). In the familial study, a disease-segregating germline mutation in the promoter of *TERT* was found by linkage analysis and high-throughput sequencing of a melanoma-prone family (Horn et al. 2013). This same *TERT* promoter mutation was not identified in subsequent sequencing of sporadic melanomas. Instead, Horn et al. found that 33% of primary melanomas, 74% of metastatic melanoma cell lines, and 85% of metastatic tissue possessed *TERT* promoter mutations predominantly at two positions, chr 5: 1,295,228 C > T (C228T) and chr 5: 1,295,250 (C250T), in a mutually exclusive manner (Table 1). These hotspot mutations create new binding sites for E-twenty-six (ETS) transcription factor (Horn et al. 2013; Huang et al. 2013), which specifically recruits the multimeric GA-binding protein (GABP) transcription factor to the mutant promoter (Bell et al. 2015).

A number of additional hotspot noncoding mutations were discovered through WES and WGS analyses from 2014 to 2016. A somatic C > T hotspot mutation in the 5′ UTR of a component of the 40S eukaryotic small ribosomal subunit, *RPS27*, was discovered in ~10% of cutaneous melanomas (Dutton-Regester et al. 2014) (Table 1). This mutation is thought to increase RPS27 expression by expanding the 5′ terminal oligopyrimidine tract (5′ TOP), which is a sequence that controls translation and is regulated by the PI(3)K/AKT and mTOR pathways (Dutton-Regester et al. 2014). RPS27 aberrant expression has been found in a number of cancers, including melanomas (Santa Cruz et al. 1997). Other noncoding mutations, such as the bidirectional promoter mutations in *DPH3* and *OXNAD1,* have been shown to positively regulate their expression (Denisova et al. 2015; Fredriksson et al. 2014) (Table 1). To date, the

Fig. 4 (continued) mutation (hotspot, missense, and nonsense) or focal amplifications and deletions (rows) found in patient samples (columns). The top panel indicates mutation burden in mutations per Mb, and the bottom panel displays the mutation spectrum. Hodis et al. primarily focused on non-acral cutaneous melanoma; however, a few acral, uveal, mucosal, and melanomas of unknown origin were analyzed (Reprinted from *Cell*, Vol 150/edition number 2, Eran Hodis, Ian R. Watson, Gregory V. Kryukov, Stefan T. Arold, Marcin Imielinski, Jean-Philippe Theurillat, Elizabeth Nickerson, Daniel Auclair, Liren Li, Chelsea Place, Daniel DiCara, Alex H. Ramos, Michael S. Lawrence, Kristian Cibulskis, Andrey Sivachenko et al., A Landscape of Driver Mutations in Melanoma Pages No., 251–263 Copyright (2012), with permission from Elsevier)

role of *DPH3* and *OXNAD1* in melanoma remains unclear.

Hotspot noncoding mutations that have been shown to result in a decreased expression of the targeted gene include *SDHD* and *NDUFB9* (Poulos et al. 2015; Weinhold et al. 2014). *SDHD* is a tumor suppressor and a component of the succinate dehydrogenase (SDH) enzyme that plays roles in both the citric acid cycle and oxidative phosphorylation energy conversion pathways (reviewed in Bardella et al. 2011) (Table 1). The reported *SDHD* promoter mutation leads to its decreased expression, which is consistent with its tumor suppressor function. *NDUFB9* is a subunit of the NADH dehydrogenase (ubiquinone) 1, another important component of the oxidative phosphorylation pathway, and its promoter mutation is thought to decrease its expression (Poulos et al. 2015). Finally, a 5′ UTR mutation in *MRPS31* that encodes for a ribosomal protein required for protein synthesis in the mitochondria was reported in ~6% of melanoma samples (Cancer Genome Atlas Network 2015) (Table 1).

Hotspot promoter mutations have also been found in desmoplastic melanoma, a rare form of cutaneous melanoma with sarcomatous histology, which mostly occurs in chronically sun-exposed skin of elderly patients. Shain et al. performed WES of 20 desmoplastic melanoma samples and discovered recurrent promoter mutations in *NFKBIE* in ~15% of cases (Shain et al. 2015a) (Table 1). This mutation alters the binding motif for several transcription factors, such as GABPA and ELF1. *NFKBIE* encodes for IκBε, which functions to inhibit NF-Kβ signaling pathway by sequestering the NF-Kβ transcription factors in the cytoplasm. Consistent with a proposed gain of function role for promoter *NFKBIE* mutations, *NFKBIE*-mutant cell lines were shown to lack NF-Kβ nuclear localization (Shain et al. 2015a). For the majority of these hotspot noncoding mutations, their biological role in melanoma remains unclear and requires further functional and mechanistic studies.

Recurrent Synonymous Mutations in Melanoma: Synonymous or silent SNVs are generally considered passenger mutations, as the altered codon sequence of a gene does not result in an amino acid change for the encoding protein. Nevertheless, some groups have identified recurrent silent mutations that they propose to be genetic drivers through mutational mechanisms that include: changes in splicing/exon regulation; alterations in transcript stability through mRNA secondary structure folding and RNA-binding protein affinity; modulation of translational rates; and abolishment of miRNA binding sites (reviewed in Gotea et al. 2015). Gartner et al. performed WES and WGS studies of 29 melanomas identifying 16 recurrent synonymous mutations (Gartner et al. 2013). The authors provided evidence that a recurrent mutation in *BCL2L12* (p.F17F) resulted in an increase in its transcript and expression levels, leading to enhanced inhibition of TP53-mediated UV-induced apoptosis (Gartner et al. 2013). In silico prediction identified *ha-miR671-5p* miRNA as a regulator of wild-type *BCL2L12*, but not its mutant form (Gartner et al. 2013). Melanomas possess an abundance of silent passenger mutations due to the role UV plays in this disease. As a result, a combination of sophisticated statistical tools, in silico miRNA/mRNA prediction programs, and functional studies will be required to determine how many silent mutations are in fact driver events.

Comprehensive Integrative Analyses of Cutaneous Melanoma Across Multiple Data Platforms

Genomic Classification of Cutaneous Melanomas: As depicted above, single high-throughput data-platform analysis of large-scale sample sets (e.g., WES) played an important role in revealing new insights into the etiology of melanoma. However, multiplatform integrative analyses had only been performed on relatively small sample cohorts up until 2015. TCGA is a collaboration between the National Cancer Institute (NCI) and National Human Genome Research Institute (NHGRI) whose goal is to characterize approximately 40 cancer types across multiple data platforms. In 2015, the TCGA published the largest integrative multiplatform analysis through systematic characterization at the DNA, RNA, and protein levels in a relatively large sample cohort of melanomas (>300 samples). The TCGA project

Table 1 Recurrent noncoding mutations in cutaneous melanoma. Genes with recurrent noncoding mutations (genomic coordinates indicated) found in exome and genome sequencing studies that occur in greater than 5% of cutaneous melanomas are shown. Where reported, effects of noncoding mutations on mRNA expression levels are listed. Of note, recurrent *NFKBIE* noncoding mutations were found in desmoplastic melanoma subtype. (SNV = single nucleotide variant) (This table was first published by Shivshankari Rajkumar and Ian R Watson in *British Journal of Cancer*. 2016 Jun 23. https://doi.org/10.1038/bjc.2016.195

Gene name	Gene symbol	Reported genomic coordinates for predominant SNV	Frequency (cutaneous melanoma)	mRNA expression
Telomerase reverse transcriptase	***TERT***	chr5: 1,295,228 C>T chr5: 1,295,250 C>T	~70%	Increase
Ribosomal protein S27	***RPS27***	chr1: 153,963,239 C>T	~10%	Increase
Succinate dehydrogenase complex, subunit D, integral membrane protein	***SDHD***	chr11: 111,957,523 C>T chr11: 111,957,541 C>T	~5–10%	Decrease
Mitochondrial ribosomal protein S31	***MRPS31***	chr13: 41,345,346 C>T	~5%	Unknown
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9	***NDUFB9***	chr8: 125,551,344 C>T	~5%	Decrease
Diphthamide biosynthesis 3	***DPH3***	chr3: 16,306,504 C>T chr3: 16,306,505 C>T/A	~10%	Increase
Oxidoreductase NAD-binding domain containing 1	***OXNAD1***	chr3: 16,306,504 C>T chr3: 16,306,505 C>T/A	~10%	Increase
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	***NFKBIE***	chr6: 44,233,400 C>T (clustered C>T from chr6: 44,233,379 – 44,233,439)	~15%	Unknown (proposed GoF)

included samples ($n = 333$) that consisted of 67 (20%) primary cutaneous melanomas (all originating from nonglabrous skin) and 266 (80%) metastases. Primary melanomas are generally small at initial diagnosis compared with most other solid tumors (thicknesses average ≤ 1 mm and ≤ 4 mm for $>60\%$ and $>90\%$, respectively) (Baade et al. 2012; Criscione and Weinstock 2010). In routine clinical practice, most or all of the primary tumor tissues is generally used for diagnostic evaluation and are not available for multiplatform molecular analyses. Therefore, melanoma samples analyzed by the TCGA were mainly metastases, with the majority obtained from regional lymph nodes, skin, or soft tissue (i.e., first site of metastasis). WES analysis identified 228,987 mutations that included both SNV and indels. Seventy-six percent of primary samples and 84% of metastatic samples had a UV mutational signature (Cancer Genome Atlas Network 2015). To identify SMGs in the context of this high mutational burden, the TCGA analysis working group employed InVEx, as well as MutSigCV. MutSig is one of the earliest and most commonly used statistical tools to determine SMGs that had been modified to take into consideration covariates (CV) of mutational burden variation, such as patient-specific mutation frequency and spectra, mRNA expression, as well as gene-specific DNA replication times (Lawrence et al. 2014; Cancer Genome Atlas Network 2015). MutSigCV found 42 SMGs that were expressed ($Q < 0.1$), and InVEx identified 13 SMGs (Bonferroni $p < 0.05$, or 20 SMGs at $Q < 0.1$) (Cancer Genome Atlas Network 2015). InVEx ascertained most of the well-established melanoma oncogenes and tumor suppressors as being significantly mutated (*BRAF*, *NRAS*, *PTEN,* and *CDKN2A)*, recently identified SMGs (*RAC1*, *PPP6C*, *MAP 2 K1*, *ARID2*) and genes previously linked to melanoma, but found for the first time to be significantly mutated (*NF1*, *RB1*, and *IDH1*). In addition, *DDX3X*, which functions as a putative RNA helicase, was discovered as a novel SMG. MutSigCV also identified three SMGs with 5′UTR or promoter mutations including *RPS27*,

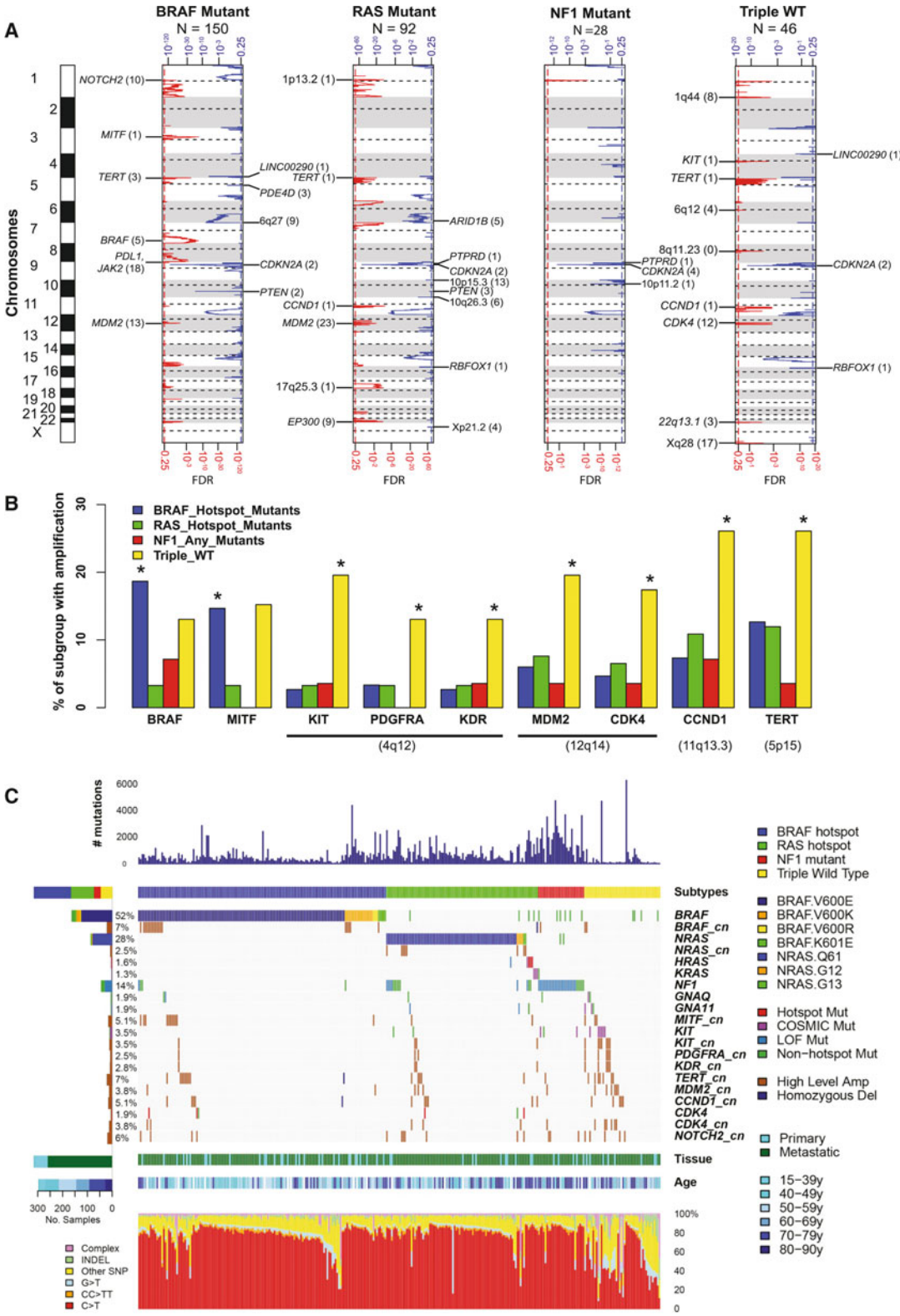
NDUFB9, and *MRPS31* (Cancer Genome Atlas Network 2015) (Table 1).

A major knowledge-gap in the melanoma genomic field pertains to the genetic driver events in *BRAF/NRAS* wild-type cutaneous melanomas. The TCGA analysis working group observed that 40–50% of *BRAF/NRAS* wild-type melanomas possessed loss of function mutations in *NF1*, which was concurrently reported by Krauthammer et al. (2015; Cancer Genome Atlas Network 2015). While *BRAF* and *NRAS* hotspot mutations are known to be mutually exclusive, significant *NF1* LoF mutations were shown to be anticorrelated with hotspot *BRAF* (p.V600E/K/R and p.K601E) mutations. Based on these mutational relationships, the TCGA and Krauthammer et al. proposed that cutaneous melanoma from nonglabrous skin can be categorized into four genomic subgroups: mutant *BRAF* (~50%), mutant *RAS* (~25%), mutant *NF1* (lacking BRAF/RAS driver mutations) (~10%), and *triple wild-type (WT)* melanomas (Krauthammer et al. 2012; Cancer Genome Atlas Network 2015) (Fig. 5).

A number of molecular characteristics were associated with each genomic subgroup. *BRAF* hotspot mutant patients were generally younger (mean average age of ~50 years old at diagnosis) consistent with previous studies (low-CSD subtype), while patients from the *NF1* subgroup were significantly older (mean average age ~67 years old at diagnosis) (Cancer Genome Atlas Network 2015). *NF1* subgroup patients also had the highest mutational burden possessing a mean of ~40 mutations/Mb compared to 9–17 mutations/Mb for the other three subgroups, suggesting they were from areas of chronic sun exposure (high-CSD subtype). Krauthammer et al. also observed significant cooccurrence of somatically mutated RASopathy genes that were enriched in the *NF1* subgroup, which included *RASA1*, *RASA2*, *SOS1*, *PTPN11, SPRY, SPRED1,* and *RAF1*, suggesting that *NF1* mutant melanomas require additional cooperating factors for robust MAPK activation (Krauthammer et al. 2015). RASopathies are a group of developmental syndromes caused by germline mutations in genes regulating RAS family and MAPK regulators, with the most common being Noonan syndrome and Neurofibromatosis type 1. Functional studies demonstrated that loss of *RASA2* increased RAS activation and melanoma cell line growth (Arafeh et al. 2015). Notably, Shain et al. elucidated that over 50% of desmoplastic melanomas had LoF and/or deletions in *NF1* and, together with the TCGA and Krauthammer et al. studies, demonstrated that *NF1* mutations occur in cutaneous melanomas with CSD (Shain et al. 2015a).

Although the genomic subgroups of cutaneous melanoma were defined by driver mutations in the MAPK pathway, reverse phase protein array (RPPA) analysis revealed some of the differential signaling between *BRAF*, *RAS*, *NF1,* and *Triple WT* subgroups. RPPA is a high-throughput antibody-based technique that evaluates phosphoprotein as well as total protein levels for over 200 antibodies per sample across large cohorts. The TCGA analysis working group noted that while phospho-S217/S221 MAP 2 K1/MAP 2 K2 (MEK1/2) levels were elevated in both *BRAF* and *RAS* mutant subgroups, only *RAS* hotspot mutations possessed significant higher levels of phospho-T202/Y204 MAPK1/MAPK3 (ERK1/2) (Cancer Genome Atlas Network 2015). Total CRAF levels were highest in the *NF1* subgroup, demonstrating how different signaling components vary between the MAPK subtypes. Furthermore, *BRAF* mutant melanomas possessed higher levels of IGFBP2, which is a regulator in the insulin signaling pathway. The *Triple WT* melanomas had higher levels of antiapoptotic protein, BCL-2 and KIT (Cancer Genome Atlas Network 2015).

Triple WT melanomas were defined by an absence of hotspot *BRAF*, *RAS* as well as loss of function *NF1* mutations, and were heterogeneous in putative driving events. Only ~30% of *Triple WT* samples had a UV signature, compared to over 90% in the other genomic subgroups (Cancer Genome Atlas Network 2015). To identify low-frequency driver mutations that may not have reached mutational significance based on a lack of power, Hodis et al. and the TCGA analysis working group cross-referenced the Catalogue of Somatic Mutations in Cancer (COSMIC) database that compiles mutation data from all NGS studies to identify recurrent/driver mutations found in other cancers that may be present in


A
BRAF Mutant
N = 150
RAS Mutant
N = 92
NF1 Mutant
N =28
Triple WT
N = 46
Chromosomes
NOTCH2 (10)
MITF (1)
TERT (3)
BRAF (5)
PDL1, JAK2 (18)
MDM2 (13)
LINC00290 (1)
PDE4D (3)
6q27 (9)
CDKN2A (2)
PTEN (2)
1p13.2 (1)
TERT (1)
CCND1 (1)
MDM2 (23)
17q25.3 (1)
EP300 (9)
ARID1B (5)
PTPRD (1)
CDKN2A (2)
10p15.3 (13)
PTEN (3)
10q26.3 (6)
RBFOX1 (1)
Xp21.2 (4)
PTPRD (1)
CDKN2A (4)
10p11.2 (1)
1q44 (8)
KIT (1)
TERT (1)
6q12 (4)
8q11.23 (0)
CCND1 (1)
CDK4 (12)
22q13.1 (3)
Xq28 (17)
LINC00290 (1)
CDKN2A (2)
RBFOX1 (1)
FDR
B
% of subgroup with amplification
BRAF_Hotspot_Mutants
RAS_Hotspot_Mutants
NF1_Any_Mutants
Triple_WT
BRAF
MITF
KIT
PDGFRA
KDR
MDM2
CDK4
CCND1
TERT
(4q12)
(12q14)
(11q13.3)
(5p15)
C
mutations
Subtypes
BRAF
BRAF_cn
NRAS
NRAS_cn
HRAS
KRAS
NF1
GNAQ
GNA11
MITF_cn
KIT
KIT_cn
PDGFRA_cn
KDR_cn
TERT_cn
MDM2_cn
CCND1_cn
CDK4
CDK4_cn
NOTCH2_cn
Tissue
Age
No. Samples
Complex
INDEL
Other SNP
G>T
CC>TT
C>T
BRAF hotspot
RAS hotspot
NF1 mutant
Triple Wild Type
BRAF.V600E
BRAF.V600K
BRAF.V600R
BRAF.K601E
NRAS.Q61
NRAS.G12
NRAS.G13
Hotspot Mut
COSMIC Mut
LOF Mut
Non−hotspot Mut
High Level Amp
Homozygous Del
Primary
Metastatic
15−39y
40−49y
50−59y
60−69y
70−79y
80−90y

melanoma (Hodis et al. 2012; Cancer Genome Atlas Network 2015). This analysis revealed low frequency driver mutations in *EZH2* ($n = 1$), *KIT* ($n = 6$), *CTNNB1* ($n = 3$), as well as *GNAQ* ($n = 1$) and *GNA11* ($n = 2$) found in 46 *Triple WT* melanomas (Cancer Genome Atlas Network 2015) (Fig. 5). Notably, *TERT* promoter mutations were rare in *Triple WT* melanomas (6.7%) compared to over 70% in the other MAPK driving subtypes.

To identify driving event in *Triple WT* melanomas, the TCGA analysis working group performed GISTIC 2 copy number analysis of the four genomic subgroups, revealing that *Triple WT* melanomas possess significant amplifications in *KIT*, *TERT*, *CDK4*, *MDM2,* and *CCND1* genes as well as 1q44, 6q12, 8q11.23, 22q13.1, and Xq28 chromosome regions that were significantly enriched in comparison to *BRAF*, *RAS,* and *NF1* mutant melanomas, consistent with other studies (Curtin et al. 2005, 2006; Cancer Genome Atlas Network 2015) (Fig. 5). The *KIT* amplicon of 4q12, which also contains additional receptor tyrosine kinases (RTK), *PDGFRA* and *KDR* (also known as *VEGFR2*), were significantly coamplified solely in *Triple WT* melanomas compared to the other genomic subgroups (Fig. 5). It is possible that these genetic findings indicate that some melanoma subtypes, such as acral melanoma or mucosal melanoma (*KIT* mutant, CCND1 amplification, low mutation burden) and blue nevus-like melanoma (with *GNAQ* or *GNA11* mutations discussed later), may have been included in the TCGA analysis.

Other subtype-specific alterations included significant *BRAF and MITF* amplifications found solely in *BRAF* mutant melanomas that were consistent with earlier findings, as well as novel significant *CD274* (PD-L1) and *JAK2* amplifications (Garraway et al. 2005; Cancer Genome Atlas Network 2015) (Fig. 5). PD-L1 amplifications are notable in light of the clinical success of anti-PD-1 immune checkpoint therapies in melanoma.

Integrative Pathway Analysis: Early melanoma studies clearly demonstrated that the RAS-MAPK-AKT, INK4A-CDK4/6-RB, and ARF-MDM2-P53 pathways are important in melanoma, which was confirmed by the TCGA study to be deregulated in 91%, 69%, and 19%, respectively, via genetic aberrations in the form of SNVs, copy number alterations, and hypermethylation changes (Fig. 6). The more recent NGS studies have underscored the importance of the telomerase pathway in melanoma with the identification of highly recurrent *TERT* promoter mutations (Horn et al. 2013; Huang et al. 2013). One aspect highlighted by the TCGA study relates to the trends by which these pathways are differentially deregulated in the various genomic subgroups. For example, *TP53* mutations were more commonly found in the three MAPK subgroups (*BRAF*, *RAS*, *NF1*), whereas the TP53 pathway is deregulated more often in *Triple WT* melanomas via significant amplifications of *MDM2* (Figs. 5 and 6). *CDKN2A* SNVs, homozygous deletions, and hypermethylation occur more frequently in the three MAPK subtypes compared to *Triple WT* melanomas (range of 58–72% compared to 37%); however, *CDK4* and *CCND1* are significantly enriched in the *Triple WT* subgroup (Figs. 5 and 6). The PI3-AKT pathway is affected more frequently in *BRAF* mutant melanomas by significant *PTEN* deletions and mutations, while AKT3 amplification and overexpression are enriched in the *RAS*, *NF1,* and *Triple WT* subgroups (Fig. 6).

Fig. 5 Copy number analysis of cutaneous melanoma mutation subgroups from the TCGA dataset. (**a**) GISTIC 2 analysis of significant amplifications and deletions across the four genomic subgroups reported by the TCGA. Certain minimal common regions were only found to be significant in a subset of genomic subgroups, such as *BRAF*, *JAK2*, and *PDL1* amplifications found in *BRAF* mutant melanomas. (**b** and **c**) Amplifications of *KIT*, *PDGFRA*, *KDR*, *MDM2*, *CDK4*, *CCND1*, and *TERT* were found to be significantly enriched in *Triple WT* melanomas. The *Triple WT* subtype also possessed *KIT*, *GNAQ,* and *GNA11* mutations (Reprinted from *Cell*, Vol 161/edition number, The Cancer Genome Atlas Network, Genomic Classification of Cutaneous Melanoma, Pages No. 1681–1696, Copyright (2015), with permission from Elsevier)

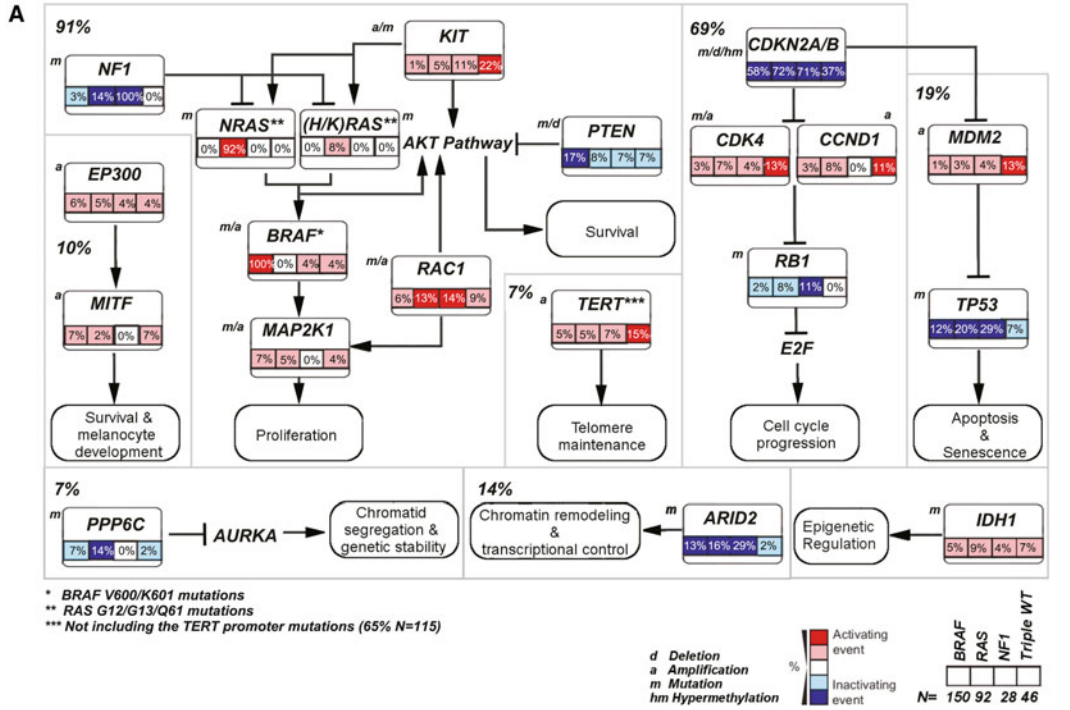


Fig. 6 Pathways altered in melanomas across genomic subgroups from the TCGA dataset. (**a**) Percentage of genetic alterations of canonical cancer pathways for MAPK/PI(3)K (% case altered = 91%), CDKN2A/RB1 pathway (69%), and MDM2/TP53 pathways (19%) are indicated. Furthermore, where data was available, the telomerase pathway was reported to be altered in 65% of cutaneous melanomas by promoter mutations and in 7% by amplifications. TCGA demonstrated that although these canonical pathways are frequently altered in melanoma, there are trends regarding how these pathways are deregulated at the gene level within subtypes. (**b**) As an example, the PI(3)K/mTOR pathway is affected in *BRAF* mutant melanomas through significantly more alterations in *PTEN*, whereas *RAS*, *NF1*, and *Triple WT* melanomas have more frequent alterations in AKT3 (Reprinted from *Cell*, Vol 161/edition number, The Cancer Genome Atlas Network, Genomic Classification of Cutaneous Melanoma, Pages No. 1681–1696, Copyright (2015), with permission from Elsevier)

Furthermore, *TERT* promoter mutations are rare in *Triple WT* melanomas and instead possess significantly more TERT amplifications (Figs. 5 and 6). Conversely, *TERT* promoter mutations are found in the range of 72–83% in the *BRAF*, *RAS,* and *NF1* subgroups. Thus, although large fractions of melanomas possess alterations in key canonical cancer pathways, there are trends of which component of these pathways are affected at the genetic level in the four subtypes. Finally, the more recent NGS studies have clearly demonstrated additional pathways that are frequently altered, but their role in melanoma is much less clear. These include genetic alterations in epigenetic regulators (*ARID2*, *IDH1,* and *EZH2*), the *RAC1* pathway, and significantly mutated genes

with poorly understood functions (*STK19*, *PPP6C,* and *DDX3X*).

Structural Aberrations in Melanoma: In the TCGA study, integrative analysis between copy-number, RNA-seq, and WGS data (low-pass and deep sequencing) identified 224 candidate fusion drivers in 333 samples. A number of low frequency in-frame fusion events involving melanoma-associated genes fused to various gene partners were discovered, such as *BRAF* ($n = 2$), *RAF1* ($n = 3$), *AKT3* ($n = 4$), *MITF* ($n = 3$), and *HMGA2* ($n = 3$) (Cancer Genome Atlas Network 2015). However, only one recurrent fusion was identified (*GRM8-CNTNAP2*). *Triple WT* melanomas were enriched for candidate fusion drivers and had significantly more complex structural rearrangements (Cancer Genome Atlas Network 2015).

Hutchinson et al. previously reported *BRAF* fusions in approximately 4–8% of pan-negative melanomas without discernable driver mutations, and that cell lines that possess *BRAF* fusions are sensitive to MEK inhibitor treatment (Hutchinson et al. 2013). In 2014, Wiesner et al. discovered that 60% of Spitz tumors possess a kinase fusion as a driver event, involving either *ROS1* (17%; $n = 24$), *NTRK1* (16%; $n = 23$), *ALK* (10%; $n = 14$), *BRAF* (5%; $n = 7$), or *RET* (3%; $n = 4$) (Wiesner et al. 2014) (Fig. 7). The following year, fusions in *c-MET* were discovered in a number of cancers, including melanomas with spitzoid morphology (Yeh et al. 2015). Spitz tumors are a subtype of melanocytic neoplasms of the skin that are composed of spindled as well as epithelioid melanocytes with enlarged nuclei (Bastian 2014). They range from benign (Spitz nevi) to intermediate (atypical Spitz tumor) to malignant (malignant Spitz tumor). The gene fusion products of RTKs (*ROS1*, *NTRK1*, *ALK*, *RET,* and *c-MET*) activate growth signaling pathways and are likely constitutively active (Wiesner et al. 2014; Yeh et al. 2015). In light of these studies, Hutchinson et al. reviewed the pathology of melanomas possessing *BRAF* fusions, and reported that these melanomas possessed spitzoid morphology (Hutchinson et al. 2014). The term spitzoid melanoma has also been used for melanoma with some features of Spitz tumors, but genetically these mostly share genetic alterations of low-CSD melanomas (Lazova et al. 2017). By contrast, the term malignant Spitz tumor is intended to designate the malignant end of the Spitz spectrum, which is genetically characterized by the above mentioned kinase fusions or activating mutations of *HRAS* (Bastian et al. 2000b).

Temporal Acquisition of Mutations and Tumor Heterogeneity: Numerous NGS studies described previously helped define the landscape of driver mutations in melanoma. Nonetheless, a key question remained regarding the temporal acquisition of genetic driving events. To address this, Shain et al. performed focused sequencing of over 290 cancer-related genes in 150 areas of 37 primary melanomas and their adjacent precursor lesions (Shain et al. 2015b). A team of eight dermatopathologists scored the various sequenced areas of melanoma by stage of progression that included benign, intermediate, and melanoma in situ. Integrating this information, the authors observed that precursor lesions were initiated by mutations in the MAPK pathway, and provided strong evidence that benign lesions possess *BRAF* (p.V600E) mutations exclusively, consistent with previous studies (Pollock et al. 2003; Shain et al. 2015b). Conversely, intermediate lesions, often with histopathological characteristics of dysplastic nevi, were enriched with *NRAS* and additional driver mutations. *TERT* promoter mutations were found in 77% of intermediate lesions and in situ melanomas. The observation of a high fraction of *TERT* promoter mutations in intermediate lesions indicated that they occurred earlier in disease progression than the authors expected. Based on the estimated number of cells in intermediate lesions and the amount of cell divisions of founder cells, the authors suspect activation of TERT becomes a selective advantage before malignant transformation occurs (reviewed in Shain and Bastian 2016). This suggests that some premalignant lesions are more proliferative than the senescence model of permanent cell cycle arrest of nevi predicts. In addition, Shain et al. found that biallelic inactivation of *CDKN2A* coincided with the transition to invasive melanomas, whereas *TP53* and *PTEN* mutations were found solely in advanced invasive melanomas with considerable tumor thickness (Shain et al. 2015b). Both point-mutation burden

Fig. 7 Landscape of driver fusions and other genetic aberrations in spitzoid melanoma. (**a**) Wiesner et al. discovered spitzoid melanomas possessed frequent fusions involving kinases that included *ROS1*, *ALK*, *NTRK1*, *RET,* and *BRAF*. In addition, frequent hotspot *HRAS* mutations were discovered in this melanoma subtype. Notably,

and copy number changes were found to increase from benign to intermediate lesions and melanoma in situ. A UV signature was detected at all evolutionary stages.

Several WES studies of multiple matched primary and metastatic samples from the same patient have revealed important insights into the mechanisms of metastatic spread in melanoma. The traditional view of metastatic progression implies metastatic dissemination from the regional lymph nodes and from there to distant sites. Sanborn et al. reported that primary tumors exhibit more complex metastatic patterns with parallel dissemination of melanoma cells to regional and distant sites (Sanborn et al. 2015). Moreover, identical subclones can be found at different sites indicating that metastases and possibly primary tumors can be reseeded. Single cell RNA sequencing analysis provided additional resolution of tumor heterogeneity at the transcription level (Tirosh et al. 2016). Tirosh et al. observed that bulk melanoma tumors can possess individual melanoma cells that have different transcription states that confer either sensitivity or resistance to targeted therapies.

Transcription Factors: A number of transcription factors that include MITF, SOX10, PAX3, and FOXD3 regulate the differentiation and development of melanocytes from the neural crest (Sommer 2011). Furthermore, Yang et al. demonstrated that human and mouse fibroblasts can be converted into functional melanocytes through overexpression of SOX10, PAX3, and MITF (Yang et al. 2014). Many of the transcription factors that control melanocyte differentiation also play roles in melanoma progression. As discussed previously, *MITF* was discovered by SNP array-based assays to be an oncogene amplified in melanoma and was correlated with poor patient survival (Garraway et al. 2005). Normal MITF transcriptional targets encompass several genes that are involved in cell cycle regulation (e.g., *CDK2*, *CDKN2A*, *TBX2*, and *CDKN1A*), differentiation/pigmentation (e.g., *TYR*, *TYRP1*, *DCT*, *MLANA*, *AIM1,* and *PMEL*), RTKs (e.g., *MET*), and transcriptional regulation (e.g., *HIF1A*) (reviewed in Cheli et al. 2010; Levy et al. 2006). While increased MITF expression activates differentiation genes, several studies have shown MITF-low melanomas exhibit a more invasive and stem-like behavior (Cheli et al. 2011; Rambow et al. 2015). Thus, levels of MITF play an important role in determining tumor subpopulation identity. The *MITF*-M promoter region is positively regulated by transcription factors such as PAX3, CREB, SOX10, LEF1, ONECUT-2, and even MITF itself, and negatively by transcription factors that include ATF2, BRN2, and FOXD3 (Levy et al. 2006; Shah et al. 2010). Transcriptionally active ATF2 is thought to act early in melanoma progression by fine-tuning MITF expression (Shah et al. 2010). Shah et al. demonstrated that MITF is downregulated by the ATF2 transcription factor, and that a high ATF2 to MITF ratio is associated with metastasis and poor prognosis (Shah et al. 2010). Furthermore, functional studies from zebrafish demonstrated that melanocytes that bypass oncogene-induced senescence reactivate a neural crest expression progenitor state that is recapitulated by overexpression of SOX10 (Kaufman et al. 2016). A number of

Fig. 7 (continued) HRAS mutations and driver fusions were found to be mutually exclusive. In the mutamatrix, patient numbers (columns) and type of genetic alterations (rows) are indicated by color. (Brown = gene fusions, red = point mutations and indels, green = amplifications, purple = truncating mutations). (**b**) Illustration of fusions and predicted breakpoints discovered in spitzoid melanomas. (Grey bars = exons of genes, vertical blue line = breakpoint of the gene, green-shaded regions = kinase domain, blue-shaded region = coil-coil domain of the fusion gene) (Reprinted by permission from Macmillan Publishers Ltd.: Nature Communications, Wiesner T, He J, Yelensky R, Esteve-Puig R, Botton T, Yeh I, Lipson D, Otto G, Brennan K, Murali R, Garrido M, Miller VA, Ross JS, Berger MF, Sparatta A, Palmedo G, Cerroni L, Busam KJ, Kutzner H, Cronin MT, Stephens PJ, Bastian BC. Kinase Fusions Are Frequent in Spitz Tumors and Spitzoid Melanomas. Nat Commun. 2014;5:3116. https://doi.org/10.1038/ncomms4116. Copyright (2014))

earlier studies have reported SOX10 overexpression in giant congenital nevi and desmoplastic melanoma (Mohamed et al. 2013; Shakhova et al. 2012). Thus, transcription factors that control melanocyte fate play a central role in modulating melanoma phenotypic plasticity.

Melanomas do express certain epithelial-mesenchymal-transition transcription factors (EMT-TFs) (Hoek et al. 2004; Shields et al. 2007). While melanocytes do not come from the epithelial lineage and EMT is not normally emphasized as an important process in melanomagenesis, varying degrees of expression changes of EMT markers have been implicated in melanoma progression. For example, comparative analysis of melanocytes and melanomas has revealed differential regulation and function of EMT-TFs. Melanomas have been reported to possess increased *TWIST1* and *ZEB1* expression, whereas normal melanocytes express *SNAIL2* and *ZEB2* transcription factors (Caramel et al. 2013). *SNAIL2* and *ZEB2* behave like tumor suppressors and activate MITF-dependent melanocyte differentiation. Functional studies demonstrated upon BRAF/NRAS activation that ZEB1 and TWIST1 levels increase resulting in enhanced cell invasion and E-cadherin loss (Caramel et al. 2013).

Other transcription factors implicated in melanoma development and progression include AP1, AP2alpha, CREB, ETS-1, HMGB1, LEF-1, TCF-4, STAT1/3, SKI, ETV1, MYC, and NF-κB (reviewed in Poser and Bosserhoff 2004). NF-κB is involved in pro-inflammatory responses and induces transcription of several antiapoptotic genes: *BCL2L1*, *TRAF1/2* (tumor necrosis factor receptor-associated factor 1/2), and *BIRC2/3* (inhibitors-of-apoptosis 1/2) (reviewed in Madonna et al. 2012). The NF-κB family of transcription factors consists of p50, p52, RelA/p65, c-rel, or RelB, although NF-κB1/p105 and NF-κB2/p100 are the inactive precursors of p50 and p52, respectively. These transcription factors dimerize once activated and colocalize to the nucleus. They are normally sequestered in the cytoplasm by the inhibitors of NF-κB (IκBs) family (e.g., IκBα, IκBβ, and IκBε). Dissociation between the transcription factors and the IκB proteins depends on the inhibitors of IκB (IKKs) complex (IKKα, IKKβ, IKKγ/NEMO), which phosphorylate the IκBs, marking them for proteasomal degradation (Madonna et al. 2012). In dysplastic nevi and melanoma cells, overexpression of p50 and p65 transcription factors has been observed in the nucleus compared to normal nevi and melanocytes (McNulty et al. 2004). Furthermore, expression of IκBα was shown to be significantly lower in metastatic melanomas than in intradermal nevi (McNulty et al. 2004). Recent exome sequencing analysis by Shain et al. detected recurrent hotspot promoter mutations predicted to alter transcription of *NFKBIE*, the gene encoding IκBε, in ~15% of desmoplastic melanoma (Shain et al. 2015a). Other transcription factors implicated in melanoma progression include *MYC* (Chr.8q24) and *ETV1* (Chr.7p), which are frequently amplified and overexpressed in melanoma (Jané-Valbuena et al. 2010; Moore et al. 2008).

In addition to transcription factors, mRNA-binding proteins such as 4E-BP1 that stabilize mRNAs have been reported to be upregulated in melanoma. Both the PI(3)K and the MAPK pathways are known to play roles in regulating phosphorylation and deactivation of 4E-BP1 resulting in the dissociation from translation initiation factors (e.g., eIF4E) (O'Reilly et al. 2009). O'Reilly et al. demonstrated that 4E-BP1 hyperphosphorylation occurs in melanoma cell lines with concurrent *BRAF* and *PTEN* mutations (O'Reilly et al. 2009). Other translation initiation factors, such as eIF-4A1 and eIF2α, are overexpressed in melanoma cell lines (Eberle et al. 1997; Rosenwald et al. 2003). 4E-BP1 hyperphosphorylation has been linked to poor survival, which raises the possibility of targeting mRNA translation and inhibiting 4E-BP1 as a melanoma therapeutic strategy.

mRNA Expression Profiling in Melanoma: To the authors' knowledge, the first study to employ microarray technology in order to classify melanoma based on gene expression was performed by Bittner et al. in 2000. In this study, mRNA expression profiles were assessed for 31 melanoma biopsies or cell lines and seven controls using a microarray platform containing probes representing 6971 unique genes (Bittner et al. 2000). The authors discovered a gene

expression subtype of highly aggressive melanomas characterized by dysregulation of the WNT signaling pathway, affecting both cell motility and invasion (Bittner et al. 2000; Weeraratna et al. 2002). Subsequent studies using microarrays for ~14,500 transcripts identified novel pathways from genes differentially expressed between melanocytes and melanoma cell lines, which included: (1) activation of the NOTCH pathway; (2) increased TWIST expression and deregulated expression of transcription factors of EMT; (3) activation of cancer testes antigens; (4) downregulation of immune modulatory genes, including the IFN pathway; (5) decreased expression of membrane trafficking genes; and (6) downregulation of growth suppressors, including *NECDIN (NDN)* (Hoek et al. 2004). Integrating focused sequencing mutation data, Shields et al. performed complete human genome microarray analysis to identify downstream expression changes mediated by BRAF and NRAS activation of the ERK kinase, as well as gene expression signatures in *BRAF*/*NRAS* wild-type melanomas. Their analysis revealed over 80 genes associated with ERK activation, which included *TWIST1*, *HIF1α,* and *IL-8* (Shields et al. 2007). *BRAF*/*NRAS* wild-type melanomas demonstrated lower ERK activation at similar levels seen in human melanocytes. This subtype was also characterized by p53 inactivation, decreased FGF2 expression, and increased MITF expression as well as epithelial markers (e.g., increased keratin 8/18, E-cadherin, P-cadherin, and CD24 levels; and decreased MCAM, N-cadherin, and TWIST1 levels). A number of early microarray studies also observed distinct gene expression signatures between nevi, primary melanomas, and melanoma metastases, as well as differences comparing laser-captured microdissected radial phase spreading melanomas (encompassing minimally invasive tumors) and vertical growth phase tumors (where melanomas gain the ability to metastasize) (Haqq et al. 2005; Jaeger et al. 2007; Riker et al. 2008; Talantov et al. 2005).

These early microarray studies described above shed light on pathways deregulated in melanoma that were evident through clustering analysis or by comparing human melanocytes to melanoma during various phases of progression. Subsequent genome-wide mRNA studies would incorporate clinical data to identify gene expression signatures associated with clinical outcome to both understand mechanisms of melanoma progression and to develop prognostic assays for the clinic. In one of the first studies of this nature, Winnepenninckx et al. performed gene expression profiling on 83 primary melanomas that were systematically collected since the early 1980s with available long-term follow-up information (Winnepenninckx et al. 2006). Their analysis identified ~250 genes associated with distant metastasis-free survival, which included genes involved in the activation of DNA replication origins (Winnepenninckx et al. 2006). Of the ~250 genes, 23 were validated at the protein level through immunohistochemistry in an independent melanoma cohort, and five were significantly associated with survival in the validation process (*MCM3*, *MCM4*, *MCM6*, *KPNA2,* and *GMNN*) (Winnepenninckx et al. 2006). KPNA2 regulates nuclear import of proteins, and GMNN plays a role in controlling genomic stability by ensuring DNA is replicated only once per cell cycle. GMNN orchestrates the recruitment of minichromosome maintenance proteins (MCM) to the replication origins that are necessary for DNA replication (Luo and Kessel 2004). Overexpression of MCM proteins was previously found in other cancer types, but was found in melanoma for the first time to be associated with survival (Winnepenninckx et al. 2006). In a related study, Wardwell-Ozgo et al. identified a *HOXA1* transcriptional signature, which encodes for homeobox transcription factor A1, as a pro-invasion oncogene that promotes melanoma progression (Wardwell-Ozgo et al. 2014). Utilizing data from the Winnepennickx et al. study, Wardwell-Ozgo et al. demonstrated that patients who exhibited poor 4-year distant metastasis-free survival also had a high *HOXA1* signature (Wardwell-Ozgo et al. 2014).

A number of other gene expression signatures have been identified with prognostic significance. For example, Brunner et al. performed expression profiling on primary cutaneous melanoma between short-term (overall survival $\leq$4 years;

$n = 20$) and long-term (overall survival ≥5 years; $n = 21$) survivors and determined 92 differentially expressed genes (Brunner et al. 2008). A follow-up study assessing these differentially expressed genes pinpointed a 9-gene signature significantly associated with overall survival in a 91-patient cohort (Brunner et al. 2013). This gene signature included *KRT9*, *KBTBD10*, *DCD*, *ECRG2*, *PIP*, *SCGB1D2*, *SCGB2A2*, *COL6A6*, *HES6*. Furthermore, gene expression analysis of primary melanomas that had not yet metastasized ($n = 116$) compared to melanomas that had already spread since initial diagnosis ($n = 72$) reported expression of *CD24* and *EVL* to be strong metastatic predictors (Gschaider et al. 2012). Using a cancer-gene-focused approach, Conway et al. identified high levels of a secreted phosphoprotein, SPP1, that was associated with reduced recurrence-free survival following profiling of over 500 cancer genes on >350 formalin-fixed, paraffin-embedded (FFPE) cutaneous primary melanomas (Conway et al. 2009). These studies elucidate a variety of genes and pathways linked with metastatic risk and patient survival outcome in primary melanomas. However, few genes were found to overlap with the numerous gene signature identified in these studies, which is likely due to the degree of contaminating normal tissue, diverse methodologies used, and cohorts analyzed (reviewed in Weiss et al. 2015). Whether one could develop an expression-based assay to inform on clinical outcome with high specificity and sensitivity into a commercial assay remained unclear, although recent progress has been made.

The American Joint Committee on Cancer (AJCC) TNM (primary tumor; regional lymph node; distant metastases) has defined cutaneous melanoma into stages 0–IV (Balch et al. 2009). While stage I and stage II melanomas exhibit low risk of metastatic recurrence, up to 20% patients will develop metastatic disease and die within 4 years of initial diagnosis (Gerami et al. 2015b). Prognosis between clinical stages II and III is also highly variable, with 5-year survival rates of 53–82% and 22–68%, respectively (Gerami et al. 2015b). One group has proposed and commercialized a gene signature that predicts the risk of distant metastasis at 5 years for patients with stage I–III melanoma. Through the analysis of previously published mRNA biomarker studies in addition to the incorporation of prognostic genes identified in earlier studies, Gerami et al. tested a gene signature of approximately 30 genes predictive of metastatic risk (Gerami et al. 2015b). In the first study, this group tested their gene expression signature in a training cohort of 164 melanomas and a validation cohort of 104 samples (Gerami et al. 2015b). The authors used radial base machine (RBM) modeling to stratify patients into two classes of melanomas predicted to have either a class 1 (low risk) or class 2 (high risk) of developing metastatic disease. The authors reported that metastatic risk was predicted with high accuracy in both the training set (receiving operating characteristic (ROC) of 0.93) and validation cohort (ROC = 0.91). In a follow-up study, they restricted their analysis to patients that had undergone a sentinel lymph node biopsy (SLNB), which is a minimum invasive procedure used for regional melanoma staging (Gerami et al. 2015a). This expression signature was reported to be a better predictor of disease-free, distant metastasis-free, and overall survival in univariate and multivariate analyses than SLNB (Gerami et al. 2015a). Genes included in this assay are: *BAP1*, *MGP*, *SPP1*, *CXCL14*, *CLCA2*, *S100A8*, *BTG1*, *SAP130*, *ARG1*, *KRT6B*, *GJA1*, *ID2*, *EIF1B*, *S100A9*, *CRABP2*, *KRT14*, *ROBO1*, *RBM23*, *TACSTD2*, *DSC1*, *SPRR1B*, *TRIM29*, *AQP3*, *TYRP1*, *PPL*, *LTA4H*, and *CST6* (Gerami et al. 2015b). The majority of these genes had decreased expression in early-phase melanomas in the Class 2 category, except for *SPP1*, *KRT6B,* and *EIF1B*, which are frequently upregulated (Gerami et al. 2015b). This expression signature has been commercialized by Castle Biosciences (Friendswood, Texas) into a diagnostic test called DecisionDx-Melanoma as an approach to predict distant metastatic risk of class 1 (reported low risk of a 3% chance of metastasis within 5 years) and class 2 (reported high risk of a 69% chance of developing metastasis within 5 years) for patients with stage I to III cutaneous melanoma. However, this test is not currently recommended by any standard treatment guidelines, and requires further assessment in prospective analyses (Weiss et al. 2015).

Integrative Analysis of mRNA Expression Signatures: The vast majority of early mRNA biomarker studies focused solely on one data platform in relatively large patient cohorts. The relationships between mRNA expression signatures and other genetic driving events and epigenetic changes were poorly understood. The first cutaneous melanoma TCGA marker study provided some insight into the relationships of various melanoma molecular and genomic subgroups. The genomic melanoma subgroups of *BRAF, NRAS, NF1,* and *Triple WT* proposed by the TCGA and Krauthammer et al. were not associated with patient outcome (Krauthammer et al. 2012; Cancer Genome Atlas Network 2015). In contrast, hierarchical clustering of the top 1500 differentially expressed genes from 329 melanoma samples revealed three transcriptomic subgroups with similarities to previously described mRNA expression groups that were associated with survival. The three mRNA expression subgroups were characterized by the skin/neuronal/organ-specific embryonic development genes ("keratin" subclass) ($n = 102$; 31%), low melanocytic lineage specific transcription factor (MITF) expression ("MITF-low" subclass) ($n = 59$; 18%), and immune function ("immune" subclass) ($n = 168$; 51%) (Cancer Genome Atlas Network 2015) (Fig. 8).

The keratin subclass was similar to an expression signature found by Shields et al. of a molecularly distinct subset of melanomas with high expression of keratins, pigmentation regulators, and genes involved in epithelium as well as neural- or organ-specific embryologic development (Cancer Genome Atlas Network 2015; Shields et al. 2007). Regional metastatic melanomas that had high keratin expression levels had worse outcomes when compared to the other two transcriptomic subclasses (Cancer Genome Atlas Network 2015) (Fig. 8). The "MITF-low" subgroup was characterized by low expression of genes associated with pigmentation and epithelial expression, most of which were MITF target genes (Cancer Genome Atlas Network 2015). These genes were involved in cell adhesion, cell migration, and modeling of the extracellular matrix. Many of the significantly enriched genes were preferentially expressed in the nervous system and/or were associated with neuronal or other organ-specific embryologic development (Cancer Genome Atlas Network 2015) (Fig. 8). This subclass was similar to mRNA expression subgroup described in previous publications that possessed low MITF expression and exhibited invasive and stem-like behavior driven by neuronal transcription factors (Cheli et al. 2011). Tumors classified in "MITF-low" had a significant higher percentage of *BRAF* hotspot mutations compared to the other transcriptomic subclasses (Cancer Genome Atlas Network 2015). Furthermore, the MITF-low expression group was associated with tumors that possessed genome-wide hypomethylation (Fig. 9).

The "immune" subclass overexpressed genes associated with immune cells (T cell, B cell, NK cells, Mast cells), co-inhibitory/co-stimulatory immune checkpoint proteins, cytokines, and immune-related receptors. High expression levels of these immune-related genes and the presence of tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment was associated with improved post-accession survival in patients, consistent with previous studies (Azimi et al. 2012; Clemente et al. 1996; Cancer Genome Atlas Network 2015) (Fig. 8). Histological and Reverse Phase Protein Array (RPPA) analysis demonstrated high protein levels of LCK (lymphocyte-specific protein tyrosine kinase) expression and high lymphocytic infiltration statistically correlated with mRNA levels of the immune subclass genes and conferred improved patient outcome compared to the keratin and MITF-low subgroups (Fig. 8). This immune mRNA subgroup was found equally distributed across all four melanoma genomic subgroups (*BRAF*/*NRAS*/*NF1*/*Triple WT*) (Cancer Genome Atlas Network 2015). PD-1 and PD-L1 expression levels were significantly elevated in the "immune" cluster compared to the other two groups. PD-1 is a co-inhibitory T-cell receptor that inhibits T-cell activation. PD-L1 is the ligand for PD-1 expressed on many cell types, including tumor cells (Sharma and Allison 2015). Both are targets of monoclonal antibodies that activate the immune system, and together with another antibody targeting the T-cell

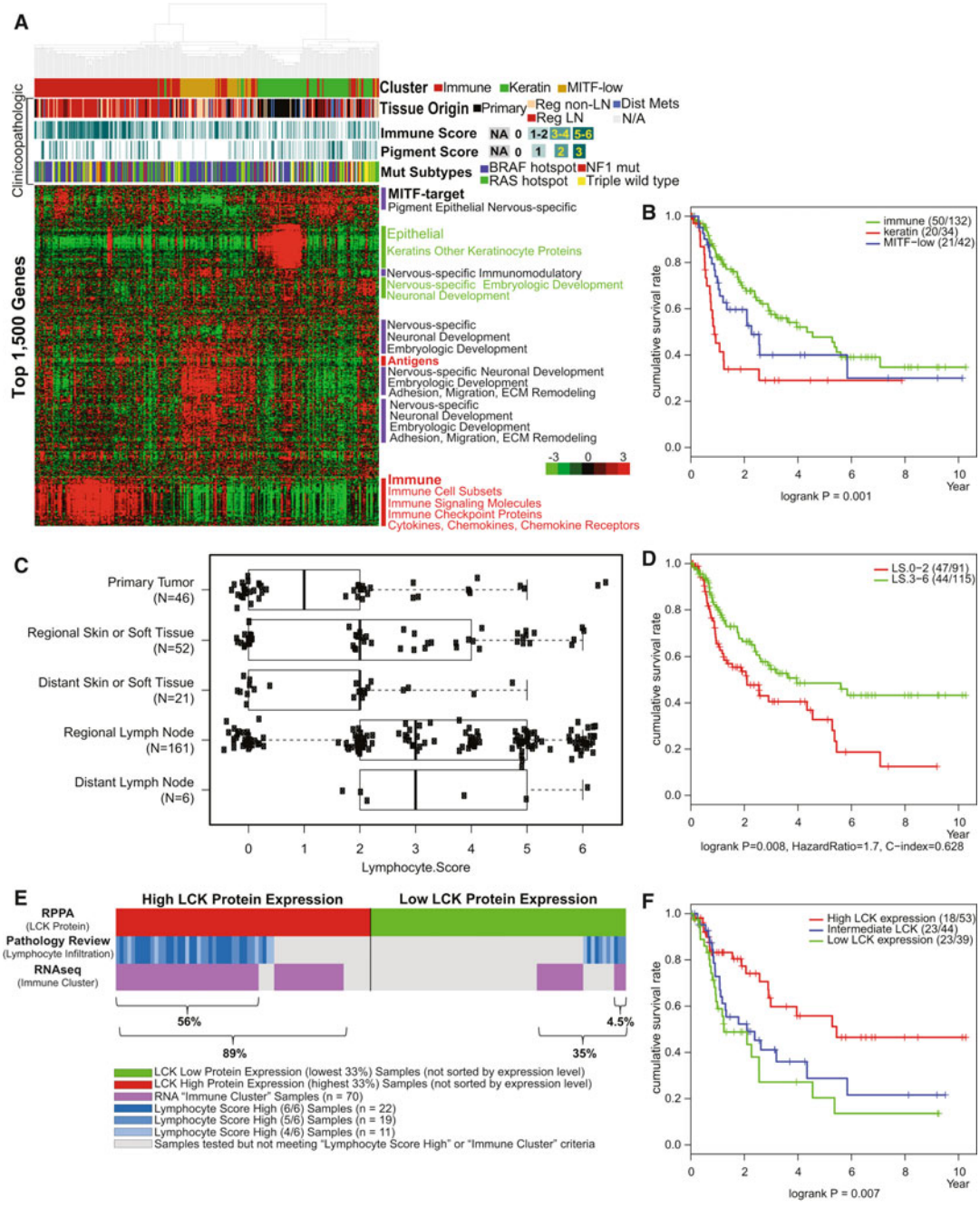


Fig. 8 mRNA subgroups of cutaneous melanomas reported by the TCGA. (**a**) Unsupervised clustering of 329 samples from the top 1500 differentially expressed genes from RNA sequencing data performed by the TCGA. This analysis identified three clusters separating patients into categories defined by mRNA signatures characterized by high immune-related genes (immune), high keratin-expressing genes (keratin), and low MITF mRNA levels and MITF-target genes (MITF-low). (**b**) Patient that had the immune signature had improved post-accession survival (survival interval from when the melanoma was removed to death or last follow-up). Patients with the keratin expression signature had the worst post-accession survival. (**c**) Histopathology analysis of infiltrating lymphocytes determined by a lymphocyte score (taking into consideration both distribution and density) observed high lymphocytic infiltration in regional metastases (**d**) and was associated with improved survival. (**e**) Clustering of

checkpoint inhibitor, CTLA-4, have shown impressive clinical results. These immune checkpoint inhibitor antibodies, anti-PD-1 and anti-CTLA-4, have produced durable responses and increased overall survival in a subset of melanoma patients (reviewed in Ott et al. 2013). Approximately 25–40% of patients respond to immune checkpoint inhibitors, and the TCGA study raised the possibility that patients with melanomas possessing an immune signature make up the patient population responding to immune therapies.

It should be noted that studies published prior to the TCGA marker publication have linked immune expression signatures with survival outcome in melanoma patients. For example, Harlin et al. performed microarray analysis of melanomas associated with CD8+ T-cell recruitment and elucidated a subset of six chemokines preferentially expressed in tumors that more effectively recruited CD8+ effector T cells (Harlin et al. 2009). Messina et al. identified a 12-chemokine gene expression signature (*CCL2*, *CCL3*, *CCL4*, *CCL5*, *CCL8*, *CCL18*, *CCL19*, *CCL21*, *CXCL9*, *CXCL10*, *CXCL11*, and *CXCL13*) that can predict intratumoral immune reaction in stage IV metastatic melanoma (Messina et al. 2012). This gene expression signature has been reported to predict the presence of unique, lymph nodal structures that contain CD20+ B cells, CD3+ T cells, and CD83+ cells. Upregulation of this 12-chemokine signature correlates with the presence of the lymph node-like structures as well as overall survival of melanoma patients (Messina et al. 2012). In addition, Sivendran et al. identified a 53-immune-gene panel that was predictive of disease-specific survival and recurrence-free survival by using the NanoString focused mRNA quantification technology (Sivendran et al. 2014). Currently, a number of studies are focused on identifying biomarkers of immune therapy response by performing integrative analysis of melanomas from pre-, on-, and post-treatment specimens for patients treated with immune checkpoint inhibitors (reviewed in Rajkumar and Watson 2016).

Integrative Epigenetic Analysis of Cutaneous Melanoma: When performing clustering analysis of the top 1% of the most variable CPG methylated loci the TCGA analysis working group identified four subgroups that were defined by CpG-island methylator phenotype (CIMP), hypermethylation, hypomethylation, and normal-like methylation patterns. The CIMP subgroup of melanomas had a higher frequency of *NRAS* hotspot mutations, a lower frequency of *BRAF* hotspot mutations, a strong association with *IDH1* and *ARID2* mutations, and a high overlap with the keratin expression subgroup (Cancer Genome Atlas Network 2015) (Fig. 9). *ARID2* is part of the SWI/SNF chromatin-remodeling complex, which facilitates ligand binding onto DNA for transcriptional activation through nucleosome positioning alterations. *ARID2* is mutated in approximately 15% of melanomas with the majority being nonsense LoF mutations (Hodis et al. 2012; Cancer Genome Atlas Network 2015). The *ARID2* mutations observed in melanoma are similar to those discovered in hepatitis-C-virus-associated hepatocellular carcinomas, which encode for truncated ARID2 variants that lack the C2H2 Zn-finger motifs needed for DNA binding (Li et al. 2011). The *IDH1* mutations identified in melanomas encoded primarily the p.R132C or p.R132H amino acid substitutions in approximately 5% of melanomas (Cancer Genome Atlas

Fig. 8 (continued) reverse phase protein array (RPPA) data revealed a subgroup of patients characterized by high LCK protein expression. LCK is a member of SRC family of protein tyrosine kinases that plays a key role in selection and maturation of developing T-cells. TCGA analysis working group observed a high overlap in patients with the mRNA immune signature, high lymphocytic infiltration by histopathology review, and high LCK protein expression. (**f**) Patients with regional metastases with high LCK protein expression were found to have improved survival (Reprinted from *Cell*, Vol 161/edition number, The Cancer Genome Atlas Network, Genomic Classification of Cutaneous Melanoma, Pages No. 1681–1696, Copyright (2015), with permission from Elsevier)

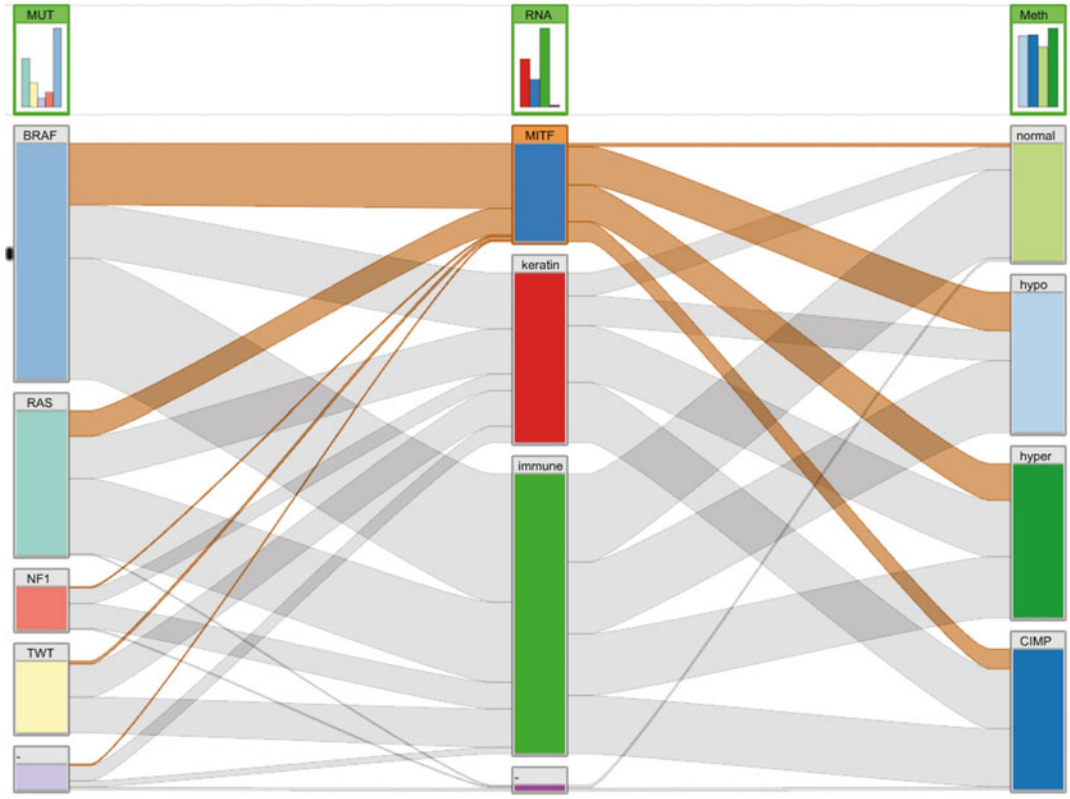


Fig. 9 Relationships and overlap of various melanoma subgroups from multiple data platforms from the cutaneous melanoma TCGA analysis. Shown above is a StratomeX plot illustrating the relationships indicated by connecting bars showing overlap between the various subgroups identified in each specific data platform analysis. The genomic subgroups shown by mutation (MUT) include *BRAF*, *RAS*, *NF1*, and *Triple WT*, by mRNA sequencing (RNA) MITF, keratin and immune, and methylation (meth) normal, hypo-, and hypermethylation as well as the CIMP signature. Highlighted in the brown bars is the relationship of the MITF-low RNA expression subgroup that overlaps more frequently with *BRAF* mutant melanomas and rarely expresses a normal-like methylation pattern. In contrast, the majority of melanoma samples with a normal-like methylation pattern also possess an immune expression signature (Reprinted from *Cell*, Vol 161/edition number, The Cancer Genome Atlas Network, Genomic Classification of Cutaneous Melanoma, Pages No. 1681–1696, Copyright (2015), with permission from Elsevier)

Network 2015; Shibata et al. 2011). Genome-wide gene expression profiling studies demonstrated that *IDH1* upregulates growth-related transcription factors (*JUN*, *MYCN,* and *ATF3*) and reduces *RASSF1*, *DHRS1,* and *ADH5* expression in melanoma cells (Shibata et al. 2011). *IDH1* hotspot mutations were originally discovered in gliomas (Parsons et al. 2008). While normally involved in glucose metabolism, mutant *IDH1* acquires the ability to reduce α-ketoglutarate (αKG) to 2-hydroxyglutarate (2HG), where excess levels contribute to disease progression (Dang et al. 2009). Melanomas that possessed normal-like methylation profiles most often possess an immune mRNA expression signature and low copy number alterations consistent with lymphocytic infiltration (Fig. 9).

Genes targeted by aberrant hypermethylation in melanoma include the *CDKN2A* tumor suppressor promoter region, and melanocyte differentiation factors *KIT*, *PAX3,* and *SOX10* (Dahl et al. 2015; Jin et al. 2015; Lauss et al. 2015; Cancer Genome Atlas Network 2015). *CDKN2A* has been reported to be hypermethylated in ~20%

of vertical growth phase melanomas leading to lower levels of p16INK4A levels, increased tumor cell proliferation, and significantly reduced patient survival (Straume et al. 2002). Using MIRA sequencing to characterize metastatic melanoma-specific hypermethylation, Jin et al. discovered 179 tumor-specific methylation peaks present in all samples analyzed and 150 upstream of transcription start sites for genes regulating melanocyte differentiation *KIT*, *PAX3,* and *SOX10*, marked by high levels of methylation on H3K27 (H3K27me3) (Jin et al. 2015). Another 22 genes have been reported to be differentially methylated during melanoma progression. By comparing DNA methylation signatures between nevi and melanomas using high-throughput DNA-methylation array-based profiling followed by unsupervised hierarchal clustering, Conway et al. identified 26 CpG sites in 22 genes with significantly different methylation levels (Conway et al. 2011). Hypermethylated genes included *KCNK4*, *GSTM2*, *TRIP6* (two CpG sites), *FRZB*, *COL1A2*, and *NPR2*. Genes found to be hypomethylated were *CARD15*/*NOD2*, *KLK10*, *MPO*, *EVI2A*, *EMR3* (two sites), *HLA-DPA1*, *PTHR1*, *IL2*, *TNFSF8*, *LAT*, *PSCA*, *IFNG*, *PTHLH*, *RUNX3* (three CpG sites), *ITK*, and *CD2* (Conway et al. 2011). Other comparative studies have reported hypermethylation of tumor suppressor genes in progression from primary to metastatic melanomas that include *MGMT*, *RASSF1A,* and *DAPK* (Hoon et al. 2004). Further analysis of melanoma patient plasma revealed circulating hypermethylated *MGMT*, *RAR*-β2, and *RASSF1A* DNA in ~30% of patients indicating that hypermethylation increases over the course of melanoma progression (Hoon et al. 2004). In contrast, loss of 5-hydroxymethylcytosine (5hmC) has been reported in the transition of nevi to melanoma, which is thought to be regulated by TET family and IDH2 (isocitrate dehydrogenase 2) (Lian et al. 2012).

A number of groups have studied changes in acetylation and other epigenetic marks revealing mechanisms of melanoma progression. For example, histone hypoacetylation mediated by histone deacetylases (HDAC) is thought to play an important role in melanoma progression affecting similar pathways normally associated with CpG island hypermethylation. Utilizing the HDAC inhibitor, trichostatin A (TSA), Florenes et al. revealed that melanomas exhibit loss of tumor suppressor genes, such as *CDKN1A* and p21*,* through reversible deacetylation of lysine residues in local histones (Flørenes et al. 2004). Other genes downregulated by histone hypoacetylation encode for the proapoptotic factors that include APAF-1, BAX, BAK, BID, BIM, caspase-3, caspase-8, TNFRSF10A, and TNFRSF10B, raising the possible clinical utility of HDAC inhibitors for treatment of cutaneous melanoma (Facchetti et al. 2004; Zhang et al. 2003, 2004). Other studies have utilized immunohistochemistry techniques to identify specific chromatin mark changes during melanoma progression. Immunohistochemistry profiling of 89 melanoma lesions demonstrated lower levels of H3K4me2 and H3K27me3 in metastatic samples compared to primary melanoma cases (Kampilafkos et al. 2015). Furthermore, protein levels of a known chromatin remodeling gene, EZH2, which is a polycomb-group (PcG) protein that transcriptionally represses gene expression via histone methylation, was shown to be elevated in melanoma cells compared to nevi (Fan et al. 2011; Kampilafkos et al. 2015). Functional studies and mouse models have recently demonstrated that mutant EZH2 promotes melanoma progression by inactivating tumor suppressors and immune response genes (Fan et al. 2011; Souroullas et al. 2016; Tiffen et al. 2015; Zingg et al. 2015). Targeting EZH2 through conditional ablation in mice or through pharmacological inhibition leads to reactivation of tumor suppressors, such as *ATF3* and *CDKN1A*, that halt tumor growth demonstrating the clinical utility of targeting EZH2 signaling in melanoma.

In addition to genetic analysis of human melanomas, animal model studies have discovered epigenetic factors that cooperate with oncogenes to induce melanoma. Zebrafish melanoma models engineered with the *BRAF* p.V600E mutation revealed that the histone methyltransferase SET Domain, Bifurcated 1 (*SETDB1*) is upregulated and accelerates melanoma progression (Ceol et al. 2011). SETDB1 promotes trimethylation of

histone H3K9 and gene repression. Human SETDB1 protein levels are only elevated in melanomas, but not in benign nevi or normal melanocytes, suggesting that *SETDB1* acts as an oncogene and plays a role in chromatin dysregulation to promote tumorigenesis (Ceol et al. 2011).

Noncoding RNA in Melanoma Development: Recent studies have shed light on the dysregulation of microRNA (miRNA) and long noncoding RNA (lncRNA). For example, high-throughput miRNA screening of blood samples from melanoma patients as well as normal healthy individuals showed that there are over 50 differentially regulated miRNAs, with ~20 being downregulated and ~30 being upregulated, in comparison to controls (Leidinger et al. 2010). Microarray-based miRNA profiling of melanocytes and melanoma cell lines derived from primary and metastatic melanomas revealed that certain miRNA expression patterns are associated with melanoma initiation, progression, and metastasis (Mueller et al. 2009). When comparing primary melanoma cell lines to normal human epidermal melanocytes, 77 miRNAs were upregulated (49 of which were highly upregulated during early progression) and 14 were downregulated (Mueller et al. 2009). Validation studies with metastatic samples revealed 11 miRNAs were confirmed to be upregulated and two downregulated. Notably, the miR-17-92 cluster, which had been previously reported to promote tumor progression by cooperating with MYC and blocking apoptosis, was found to be upregulated in primary tumor cell lines compared to melanocytes. The miR-106-363 cluster that promotes cell attachment, mobility, and proliferation was increased in both primary and metastatic melanoma cells (Mueller et al. 2009). Mirzaei et al. identified aberrant activation of additional miRNAs in melanoma, which included *let-7a/b*, *miR-148*, *miR-155*, *miR-182*, *miR-200c*, *miR-211*, *miR-214*, *miR-221,* and *miR-222* (Mirzaei et al. 2016). These miRNAs have been recognized as regulators of a number of melanoma-associated genes, such as *NRAS*, *MITF*, *KIT,* and *ATF2* (Mirzaei et al. 2016).

Various other processes are regulated by miRNAs in melanoma, such as the regulation of epigenetics (e.g., *miR-18b*, *miR-29c*); apoptosis (e.g., *miR-18b*, *miR-155*, *miR-26a*); immune response (e.g., *miR-210*, *miR-34a/c*, *miR-30b/d*); uncontrolled replication (e.g., *miR-205*, *miR-203*); metastasis (e.g., *miR-214*, *let-7a*); and cell signaling (e.g., *miR-137*, *miR-221*) (reviewed in Aftab et al. 2014; Bennett et al. 2013; Sarkar et al. 2015). Several studies demonstrated clear tumor suppressor functions of miRNAs that are either suppressed or deleted in melanoma, which include the *miR-100* family and *miR-31* that negatively regulate oncogenes EZH2, SRC, MET, NIK, and RAB27A (Asangani et al. 2012; Varambally et al. 2008). CpG island methylation upstream of regulatory regions has been shown to modulate *miR-375* and *miR-34b* expression in stage II to IV melanomas, demonstrating stage-specific methylation patterns of miRNA in melanoma (Aftab et al. 2014). Recently, a mouse model study elucidated a more complex mechanism by which miRNAs regulate melanomagenesis. Damsky et al. discovered that BRAF p.V600E-induced upregulation of *miR-99/100* caused senescence by downregulating mTOR and IGF1R signaling as a mechanism of oncogene-induced senescence in melanoma (Damsky et al. 2015).

Given the roles of miRNA in various aspects of melanoma biology, a number of groups have searched for miRNA biomarkers with diagnostic potential. In particular, three studies have identified miRNAs prognostic signatures. Segura et al. revealed an 18 miRNA signature from analysis of metastatic tissue that demonstrated significant overexpression in patients with longer survival, which was subsequently narrowed to a six miRNA signature (*miR-150*, *miR-342-3p*, *miR-455-3p*, *miR-145*, *miR-155*, and *miR-497*) that could predict post-recurrence survival with approximately 80% accuracy (Segura et al. 2010). Tembe et al. observed that *miR-150-5p*, *miR-142-3p,* and *miR-142-5p* were associated with poor prognosis in 45 metastatic melanoma samples from Stage III patients (Tembe et al. 2015). Finally, Jayawardana et al. identified a 12-miRNA signature from a re-analysis of publicly available datasets, and a 15-miRNA signature that predicted longer survival from TCGA data (Jayawardana et al. 2016). Jayawardana

et al. also performed a cross-validation analysis and elucidated that five miRNAs (*miR-142-5p*, *miR-150-5p*, *miR-342-3p*, *miR-155-5p*, and *miR-146b-5p*) were reproducibly associated with patient outcomes suggesting they should be prioritized in future biomarker studies, functional work, and drug discovery (Jayawardana et al. 2016).

Long noncoding RNAs (lncRNAs) have a number of functions including regulation of transcription via the interaction of regulatory proteins, mRNA splicing by influencing ribosomal RNA modification, and epigenetic regulation through the recruitment of histone modifying complexes in either a *trans* or *cis* manner (reviewed in Sarkar et al. 2015). Since the first functional lncRNA, *XIST*, was reported in 1992 (Brown et al. 1992), progressively more studies are beginning to unravel the roles of lncRNA in melanoma. For example, in patients with melanoma-neural system tumor syndrome found in families that develop both cutaneous melanoma and neural system tumors, Pasmant et al. identified *ANRIL* within the germ-line deletion that included the entire gene cluster *p15/CDKN2B-p16/CDKN2A-p14/ARF* (Pasmant et al. 2007). Subsequent genome-wide association studies revealed *ANRIL* as an unexpected major target for various cancer types that coregulates CDKN2A/B expression through a *cis*-acting mechanism (Pasmant et al. 2011).

Noncoding RNA microarray studies revealed 77 lncRNAs that are differentially expressed between melanocytes and melanoma cell lines, of which four were aberrantly expressed in patient samples (Khaitan et al. 2011). Follow-up functional studies demonstrated that one of the overexpressed lncRNAs, *SPRY4-IT,* found in an intron of the gene *SPRY4,* plays an oncogenic role leading to increased cell growth and migration upon its overexpression (Khaitan et al. 2011; Mazar et al. 2014). The lncRNA, *HOTAIR*, was also found to be overexpressed in lymph node metastases tumors when compared to primary melanomas (Tang et al. 2013). Decreasing *HOTAIR* expression led to reduced motility and invasion in human melanoma cell lines, suggesting its role in promoting metastasis (Tang et al. 2013). Aberrant expression of lncRNAs also correlated with increased RNA-binding ability of polypyrimidine tract-binding (PTB) protein associated splicing factor (PSF) in both mouse and human tumors (Wu et al. 2013). Through RNA-SELEX affinity chromatography, Wu et al. elucidated *Llme23* as the lncRNA that binds specifically to PSF in melanoma cell lines (Wu et al. 2013). Furthermore, this interaction is exclusively detected in human melanoma cell lines, and knockdown of *Llme23* suppressed its oncogenic function and prevented the expression of the proto-oncogene, *Rab23* (Wu et al. 2013).

lncRNAs have also shown to play important roles in oncogenic BRAF signaling. Flockhart et al. identified 39 differentially expressed lncRNAs in *BRAF* p.V600E melanoma cells, and found *BANCR* as the most recurrently overexpressed lncRNA that was regulated by BRAF in malignant melanoma (Flockhart et al. 2012). As the annotation of lncRNAs increases, future studies will undoubtedly reveal additional roles for lncRNAs in melanoma. For example, a recent study discovered the lncRNA, *SAMMSON*, which is found in the 3p13–3p14 region and coamplified with MITF, regulates targeted therapy response and mitochondrial function in melanoma (Leucci et al. 2016). Interestingly, silencing *SAMMSON* in cell lines and patients derived xenografts sensitized melanomas to targeted therapy. These various studies have uncovered the new roles of lncRNAs in melanoma, as well as their potential as biomarkers and drug targets.

Perspective on Future Studies in Cutaneous Melanoma

Recent power calculations have estimated that up to 5300 samples are needed to be sequenced in order to identify driving mutations in genes at a frequency of 2% (Lawrence et al. 2014). The largest sequencing studies to date have analyzed in the range of 300–500 samples (Arafeh et al. 2015; Hodis et al. 2012; Cancer Genome Atlas Network 2015). Thus, not all SNV-mediated driving events in melanoma have been identified. Future studies will certainly elucidate new

driving mutations in tumor suppressors and low frequency oncogenic mutations. With the advancement of statistical tools to identify significantly mutated noncoding regions of the genome, forthcoming work will likely reveal additional noncoding driving mutations.

As illustrated by a number of recent studies, the discovery of amplified and deleted regions that contain noncoding RNAs that play roles in melanoma initiation, disease progression, and metastasis should be elucidated in the coming years. In addition to identifying new genetic alterations, the functional role and the signaling pathways modulated by many of the newly identified significantly mutated genes possessing coding and noncoding mutations remain an important area of investigation. In order to identify potential new therapeutic strategies to treat melanomas, generation of new pre-clinical human and mouse melanoma model systems and detailed mechanistic investigation will be required for the characterization of these new melanoma-associated genes. Finally, as this chapter has focused on treatment-naïve melanomas, future "omic" studies should continue to incorporate how genetic driving events influence and evolve in response to the latest melanoma treatment modalities. To date, a number of studies have already shed important insight into mechanisms of resistance to targeted therapies and biomarkers linked to immune therapy response (reviewed in Carlino et al. 2015; Rajkumar and Watson 2016). As the treatment paradigms begin to evolve in melanoma with new therapeutic strategies, it will be important to continue to link detailed clinical history and treatment response to the molecular characterization of melanomas.

Uveal Melanoma

Uveal melanoma is a distinct subtype of melanoma that originates from melanocytes in the choroidal plexus of the eye, the ciliary body, or the iris. It is the most common primary cancer of the eye and accounts for ~5% of melanoma cases in North America, with 1200–1500 new cases each year (reviewed in Harbour 2012). Uveal melanoma metastasizes to several sites; however, the overwhelming majority spread to the liver. The median survival rate for patients with metastatic uveal melanoma is approximately 9 months (Kath et al. 1993). Uveal melanoma has one of the lowest mutation burden of any cancer sequenced to date and has distinct driver mutations, and there is no evidence of a UV signature. Characterization of the genetics and expression profiling of uveal melanoma have aided significantly in understanding the etiology of this disease, as well as in the identification of different subgroups with prognostic significance.

Early Genetic Studies of Uveal Melanoma

First-Generation Sequencing: As illustrated above, the landscape of driver mutations differs significantly in the various melanoma subtypes. Notably, early clues about the identity of possible driver oncogenes in melanoma originated from forward genetic screening of mice. For example, Van Raamsdonk et al. noted that hypermorphic mutations in *Gna11* or *Gnaq* caused hyperpigmentation in mice attributed to an increase in intradermal but not epidermal melanocytes (Van Raamsdonk et al. 2004). The melanocytic hyperplasia in these mice was reminiscent of blue nevi in humans, which prompted sequencing of *GNAQ* and *GNA11* in blue nevi. In the first analysis, only GNAQ mutations were identified in blue nevi (Van Raamsdonk et al. 2009). Blue nevi are dermal spindled and dendritic melanocyte proliferations without significant epithelial involvement that can progress to blue nevus-like melanoma (Bastian 2014). Nevus of Ota is a specific variant of blue nevus which affects the facial skin corresponding to the innervation site of the first branch of the trigeminus nerve. It is frequently accompanied by an increased number of melanocytes in the uvea and conjunctival hyperpigmentation. In Caucasians, nevus of Ota is a risk factor for uveal melanoma, which prompted sequencing of uveal melanoma to determine the mutation status for *GNAQ* and later for *GNA11*. Strikingly, these two G-protein subunits of the Gαq family were mutated in a substantially large

fraction of blue nevi and uveal melanoma. Analogous to *BRAF* and *NRAS* SNVs, hotspot *GNA11* and *GNAQ* mutations resulting in an amino acid change in either p.Q209 or p.R183 point mutations are found in over 80% of uveal melanoma in a mutually exclusive manner (Van Raamsdonk et al. 2010). p.Q209 mutations in *GNAQ* were found in 55% of blue nevi, 45% of primary uveal tumors, and 22% of uveal melanoma metastasis, while mutations affecting p.Q209 in *GNA11* were found in 7% of blue nevi, 32% of primary uveal tumors, and 50% of uveal melanoma metastasis (Van Raamsdonk et al. 2009, 2010). *GNAQ* and *GNA11* encode for the α-subunit of the heterotrimeric G proteins, which are composed of α, β, and γ subunits. The α-subunit functions as a switch for the G-protein and possesses specific arginine and glutamine residues that are of critical importance for its intrinsic GTPase activity when interacting with GTP. In *GNA11,* the critical glutamine at position 209 was found to be mutated to either a leucine in 97% or a proline in ~3% of uveal melanomas, which is homologous to amino acid p.Q61 in RAS (Van Raamsdonk et al. 2010). While less frequent, mutations in p.R183 in GNAQ and GNA11 were found in 4.8% of primary uveal melanomas, the majority of which encoded for p.R183C in the case of *GNA11*. Uveal melanomas do not possess many of the commonly mutated genes in cutaneous melanoma including *BRAF* and *NRAS*, and alternatively activate the MAPK pathway through *GNAQ* and *GNA11* mutations (Van Raamsdonk et al. 2009).

Copy Number Variations (CNVs): Cytogenetic studies of samples from patients with uveal melanoma have identified many genetic alterations and chromosomal aberrations in this subtype. Recurring nonrandom chromosomal aberrations in uveal melanoma were initially identified through standard karyotypic analyses, which include loss of chromosomes 1p, 3, 6q, 8p, and 9p as well as gains of 1q, 6p, and 8q, which were later confirmed with additional approaches (Harbour 2012). Although many of these genetic alterations have been associated with uveal melanoma outcomes, loss of a copy of chromosome 3 (monosomy 3) that occurs in ~50% of cases is by far the most significant prognostic chromosomal marker (Prescher et al. 1990). Prescher et al. studied over 50 patients with monosomy 3 and found that this chromosomal aberration is a significant predictor of both poor relapse-free and overall survival outcomes in uveal melanoma (Prescher et al. 1996).

Loss of chromosome 1p is observed in ~25% of uveal melanomas, and generally cooccurs with monosomy 3 (Hausler et al. 2005; Hoglund et al. 2004). Candidate tumor suppressor and oncogenes proposed to be targeted by chromosomal losses include *HES2*, *HES5,* and *TP73* (Kilic et al. 2008). Gains of 6p and loss of 6q were found respectively in ~30% and ~40% of uveal melanomas, and 6q loss were present in ~50% of tumors that also harbored 6p gain (Hoglund et al. 2004). This suggested that this event occurs by isochromosome formation, which is produced by the transverse splitting of the centromeres resulting in the simultaneous duplication and deletion of genetic material in which the arms of the chromosomes are the mirror images of each other (Aalto et al. 2001). Moreover, 6p gain frequently occurs in the absence of monosomy 3 (Ehlers et al. 2008; Parrella et al. 1999). Loss of chromosome 8p and gain of 8q occur in 25% and 40% of uveal melanoma cases, respectively (Hoglund et al. 2004). Reports indicate that 8q gain is significantly associated with uveal melanoma metastasis, and candidate oncogenes in the minimal common region of 8q amplification are *MYC*, *DDEF1,* and *NBS1* (Ehlers and Harbour 2005; Ehlers et al. 2005; Parrella et al. 2001). *LZTS1* is a candidate tumor suppressor on 8p, which was found to be both silenced by hypermethylation and deleted on chromosome 8p12–22, where functional data supports a role for this gene in preventing metastasis (Onken et al. 2008). Finally, loss of chromosome 9p is found in ~25% of uveal melanomas. This region contains the *CDKN2A* locus whose promoter region is also commonly silenced in many uveal melanoma cell lines (Hoglund et al. 2004; Merbs and Sidransky 1999; van der Velden et al. 2001).

Next-Generation Sequencing and Comprehensive Integrative Analyses of Uveal Melanoma Across Multiple Data Platforms

mRNA/miRNA Expression Profiling: Monosomy 3 is observed in ~50% of melanoma patients and is significantly associated with metastatic spread. Approximately 70% of patients with monosomy 3 will die within 4 years after initial diagnosis, while patients with disomy 3 do not develop metastatic disease (Tschentscher et al. 2003). Various groups have performed gene expression profiling of primary uveal melanomas to gain insight into its biology. Early studies identified differentially expressed genes between normal melanocytes and uveal cell lines as well as monosomy 3 versus disomy 3 tumors (Tschentscher et al. 2003; Zuidervaart et al. 2003). Further studies performed unsupervised clustering analysis to identify expression signatures that classified uveal melanomas into two prognostically significant groups independent of cytogenetic status that outperformed other prognostic indicators, including clinical, pathological, and cytogenetic variables, including monosomy 3 (Onken et al. 2004, 2012) (Fig. 10). This expression classification places uveal melanoma into two discrete molecular groups: class 1 (low-risk) and class 2 (high-risk) groups. These early analyses used high-density oligonucleotide arrays that identified 3075 significant genes employing stringent statistical cut-offs. Principal component analysis revealed two distinct clusters where follow-up filter processing identified 62 discriminating genes (Onken et al. 2004). This original signature included genes that were downregulated on chromosome 3 and up-regulated on 8q. Pathway analysis revealed that the top 26 discriminate genes of the 62 gene signature had roles in cell communication, development, cell growth, motility, and cell death (Onken et al. 2004). Class 2 tumors exhibited epithelial features characterized by polygonal cell morphology, acinar clustering, and increased cell adhesion, with concomitant upregulation of E-cadherin that colocalized with β-catenin at the plasma membrane (Onken et al. 2006). Onken et al. sought to identify the most highly discriminate genes and invariant control genes to develop a clinical PCR-based assay that could be used for small samples obtained by fine needle aspiration biopsy (FNAB) and archival FFPE tissues (Onken et al. 2010). Their work led to the development of a PCR-based 15-gene assay composed of 12 discriminating genes (*CDH1*, *ECM1*, *E1F1B*, *FXR1*, *HTR2B*, *ID2*, *LMCD1*, *LTA4H*, *MTUS1*, *RAB31*, *ROBO1*, and *SATB1*) and three endogenous control genes (*MRPS21*, *RBM23*, and *SAP130*) that are expressed equally in both classes (Harbour and Chen 2013). A prospective multicenter study confirmed the assay's prognostic accuracy showing it to be superior to monosomy 3 testing. This assay, DecisionDx-UM, is now commercially available from Castle Biosciences Inc. In many ocular oncology centers, this assay has become the standard of care for prognostic testing (reviewed in Decatur et al. 2016).

Although not developed and characterized to the same degree as mRNA expression profiles, a few groups have identified miRNA that are prognostic. For example, miRNA clustering analysis revealed subgroups with prognostic significance where *let-7b* and *miR-199a* were the most significant discriminators (Worley et al. 2008). Furthermore, functional studies have shown that *miR-34a* can downregulate c-Met, leading to decreased uveal melanoma cell line proliferation (Yan et al. 2009). *miR-137* also has tumor suppressor activity by downregulating MITF and CDK6 (Chen et al. 2011). Conversely, *miR-454* has been shown to be upregulated in uveal melanoma tissue and has oncogenic functions by reducing *PTEN* expression (Sun et al. 2015).

Next-Generation Sequencing, Epigenetics, and Integrative Analysis: Numerous studies have attempted to identify the tumor suppressor (s) located on chromosome 3 that are crucial for uveal melanoma progression. However, it was not until WES was utilized in the analysis of uveal melanoma that Harbour et al. identified *BRCA1-Associated Protein-1 (BAP1),* located on chromosome 3 (3p21.1), as the key tumor suppressor (Harbour et al. 2010). *BAP1* encodes for a

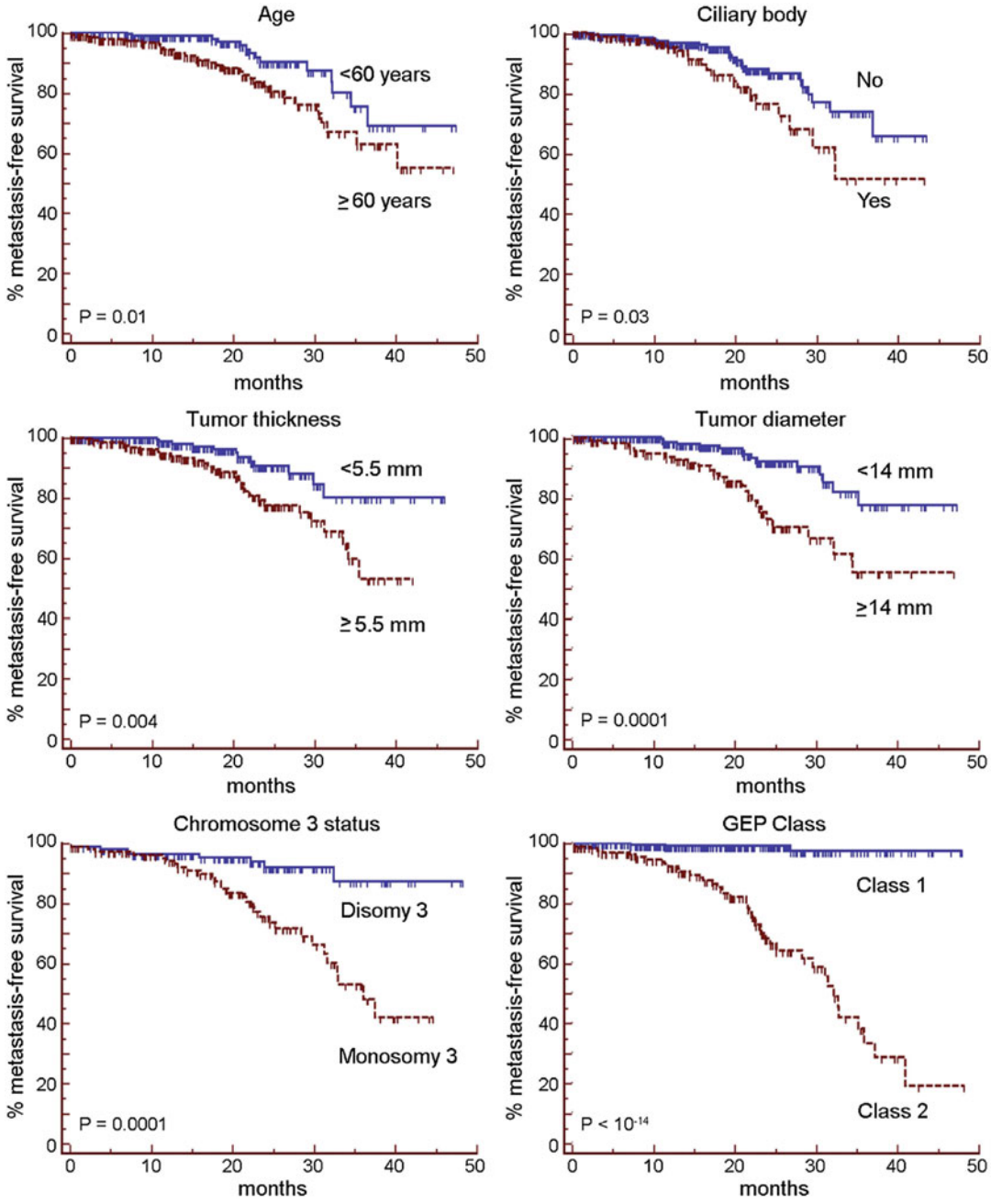


Fig. 10 Demonstration of the prognostic value of metastatic risk of the gene expression profiling (GEP) classification by Onken et al. (2012). Kaplan-Meier plots for the indicated prognostic factors (age, ciliary body, tumor thickness, tumor diameter, chromosome 3 status) and GEP classes are shown with P-values determined by log-rank method displayed in the bottom left portion of the graphs. This study demonstrated that GEP assay was the most accurate prognostic marker among all factors tested (Reprinted from *Ophthalmology*, Vol 119/edition number 8, Michael D. Onken, Lori A. Worley, Devron H. Char, James J. Augsburger, Zelia M. Correa, Eric Nudleman, Thomas M. Aaberg, Michael M. Altaweel, David S. Bardenstein, Paul T. Finger, Brenda L. Gallie, George J. Harocopos, Peter G. Hovland, Hugh D. McGowan et al., Collaborative Ocular Oncology Group Report Number 1: Prospective Validation of a Multi-Gene Prognostic Assay in Uveal Melanoma, Pages No. 1596–1603., Copyright (2012), with permission from Elsevier)

nuclear deubiquitinating enzyme of the ubiquitin carboxy-terminal hydrolase class that was originally identified as a BRCA1 binding protein (Jensen et al. 1998). WES analysis revealed *BAP1* was the only gene possessing SNVs on chromosome 3, and was mutated in ~85% of class 2 metastasizing uveal tumors (Harbour et al. 2010). The vast majority of *BAP1* mutations were inactivating events, with 15/26 mutations identified resulting in premature protein termination and 5/26 affecting the ubiquitin hydrolase domain. All 17 *BAP1*-mutant class 2 tumors where cytogenetic data was available had one copy of chromosome 3 missing. Functional data demonstrated that knockdown of BAP1 developed a more epithelial morphology and a shift towards a class 2 gene expression signature. Thus, this study identified inactivation of *BAP1* as the key event in the acquisition of metastatic competence of uveal melanomas. Although somatic mutations in *BAP1* are infrequent in cutaneous melanoma, rare germline *BAP1* variants were discovered in families with an autosomal dominant syndrome characterized by multiple melanocytic tumors, including cutaneous and uveal melanomas. Affected family members had distinctive melanocytic neoplasms that show cytologic features of Spitz tumors, but lack other features characteristic of Spitz nevi, such as epidermal hyperplasia. These lesions are now termed *BAP1*-inactivated spitzoid nevi, and arise from *BRAF*- (or less commonly *NRAS*-) mutant nevi through biallelic inactivation of *BAP1* (Wiesner et al. 2011).

To date, a number of studies have characterized the tumor suppressor function of *BAP1*. In 2010, Scheuermann et al. demonstrated that the Drosophila homolog of *BAP1, calypso*, physically interacted with Polycomb group protein *ASX* to form the Polycomb repressive deubiquitinase complex (PR-DUB), which removes mono-ubiquitin moieties from H2A histones, thus preventing transcription of important Hox genes (Scheuermann et al. 2010). *BAP1* was also shown to interact and form a stable complex with *ASXL1*, the human homolog of *ASX*, to deubiquitinate H2Aub1 in nucleosomes. This study demonstrated an important role for BAP1 in transcriptional and epigenetic regulation during Drosophila development (Scheuermann et al. 2010). BAP1 was also found to interact with HCF1, which is a chromatin-associated protein that regulates gene expression by maintaining complex formation between chromatin-modifying enzymes and transcription factors (Machida et al. 2009). These studies provided evidence for the role of *BAP1*'s deubiquitinating activity in epigenetic regulation. Furthermore, gene set enrichment analysis (GSEA) comparing class 1 and class 2 tumors determined that genes upregulated by HDAC inhibitors (e.g., SAHA and depsipeptide) were deemed the most significant. Thus, Landreville et al. tested whether histone deacetylase (HDAC) inhibitors can serve as a therapeutic strategy to treat uveal melanomas with *BAP1* inactivating mutations (Landreville et al. 2012). They observed that loss of BAP1 sensitized uveal melanoma cell lines to HDAC inhibitors, which decreased cell proliferation and tumor growth in vivo. Currently, HDAC inhibitors, Vorinostat (NCT01587352), are in clinical trials for treatment of patients with metastatic uveal melanoma.

To elucidate the landscape of driver mutations in uveal melanoma, a number of groups performed larger sequencing studies that identified previously unreported driver events. Martin et al. performed WES on uveal melanomas to determine the genetic differences between patients that had monosomy 3 compared to those that had disomy 3, which seldom metastasize (Martin et al. 2013). Recurrent somatic mutations in either *EIF1AX* (15/31; 48%) or *SF3B1* (9/31; 29%) were identified, both specifically occurring in uveal melanomas with disomy 3. *EIF1AX* and *SF3B1* mutations were found in patient samples with partial monosomy 3, where only portions of chromosome 3 were lost. However, they were rarely mutated in uveal melanomas with monosomy 3 (Martin et al. 2013). *EIF1AX* encodes for the eukaryotic translation initiation factor 1A X-linked, and all of its mutations were missense mutations that affected the N-terminus of the protein. *SF3BP1* mutations were anticorrelated with *EIF1AX* mutations in disomy 3 uveal melanomas and WES detected heterozygous p.R625C or p.R625H hotspot mutations in low-grade uveal melanomas with good prognosis (Harbour et al.

2013; Martin et al. 2013). Mutations in *SF3BP1*, which encodes for the subunit 1 of the splicing factor 3b complex, were mutually exclusive with *BAP1* mutations (Harbour et al. 2013; Martin et al. 2013). *SF3BP1* is a component of the major U2-like and minor U12-like spliceosome. To determine the effects of mutant *SF3B1* on uveal melanoma transcripts, Furney et al. utilized the Affymetrix Human Transcriptome arrays (HTA2) that contains both exon and exon-exon junction probes in the analysis of mRNA from *SF3B1* mutant and wild-type uveal melanomas (Furney et al. 2013a). Recurrent *SF3B1* mutations were associated with differential alternative splicing of several protein coding genes. The genes that had the strongest SF3B1-mediated aberrant splicing and were validated by secondary PCR methods were *GUSBP11, UQCC, ANKHD1, GAS8, F8, ADAM12,* and *ABCC5* as well as the lncRNA *CRNDE* (Furney et al. 2013a). However, the role these alternative splicing transcripts play in uveal melanoma biology is still poorly understood.

Johansson et al. carried out deep sequencing of 28 uveal melanoma samples (either tumors or primary cell lines), and discovered recurrent mutations in *PLCB4* (c.G1888 T, p.D630Y), which encodes for phospholipase C β4, a canonical downstream effector of the Gαq signaling pathway (Johansson et al. 2016) (Fig. 11). *PLCB4* gain-of-function mutations were mutually exclusive with *GNAQ* and *GNA11*, suggesting that this novel mutation activates the same pathway to promote uveal melanoma development. Moore et al. also identified recurrent mutations *CYSLTR2*, which encodes for cysteinyl leukotriene receptor 2, another gene of the Gαq signaling pathway (Moore et al. 2016) (Fig. 11). Furthermore, the p.L129Q constitutively activating mutation in *CYSLTR2* was found in four out of nine uveal melanoma samples that lacked mutations in *GNAQ*, *GNA11,* and *PLCB4*. Mutant forms of CYSLTR2 promotes phorbol ester-independent growth in vitro and tumorigenesis in vivo (Moore et al. 2016). These account for the missing oncogenes in uveal melanomas without mutations in *GNAQ* or *GNA11*.

Due to the high frequency of *TERT* promoter mutations in cutaneous melanomas, groups have performed focused analysis of the TERT locus. Gene expression, copy number, and focused sequencing analyses of 50 primary uveal melanomas revealed that hotspot *TERT* promoter mutations are extremely rare in uveal melanoma (1/50) (Dono et al. 2014). However, the C228T *TERT* promoter mutation did lead to higher TERT expression in the samples where it was discovered.

Perspective on Future Studies in Uveal Melanoma

In contrast to many of the melanoma subtypes, the landscape of driver mutations in uveal melanoma has been more clearly defined compared to other melanoma subtypes. This accomplishment has been aided in part by the lack of UV-induced passenger mutations and low mutation burden that necessitated sequencing fewer samples to clearly define driver mutations. Furthermore, the genetic driving events found in primary melanomas that metastasize (monosomy 3 and *BAP1* mutations), or are alternatively found in patients with better prognosis (*S3FB1* and *EIF1AX* mutations), have been clearly defined. Clinically amenable prognostic expression assays that are able to distinguish good and poor outcomes in patients are also currently available. While the driver events in this melanoma subtype are now well-defined, the mechanism of action and signaling changes caused by uveal melanoma oncogenes and tumor suppressors are still not well-understood. Unquestionably, this knowledge gap will be addressed in future studies.

In contrast to cutaneous melanoma, where immune and targeted therapies are producing long-term survival benefits in a subset of patients, few effective therapies are available for uveal melanoma patients with metastatic disease. Recent studies identified PKC and YAP signaling as key downstream meditators of mutant GNAQ/GNA11 signaling (Chen et al. 2014; Feng et al. 2014;

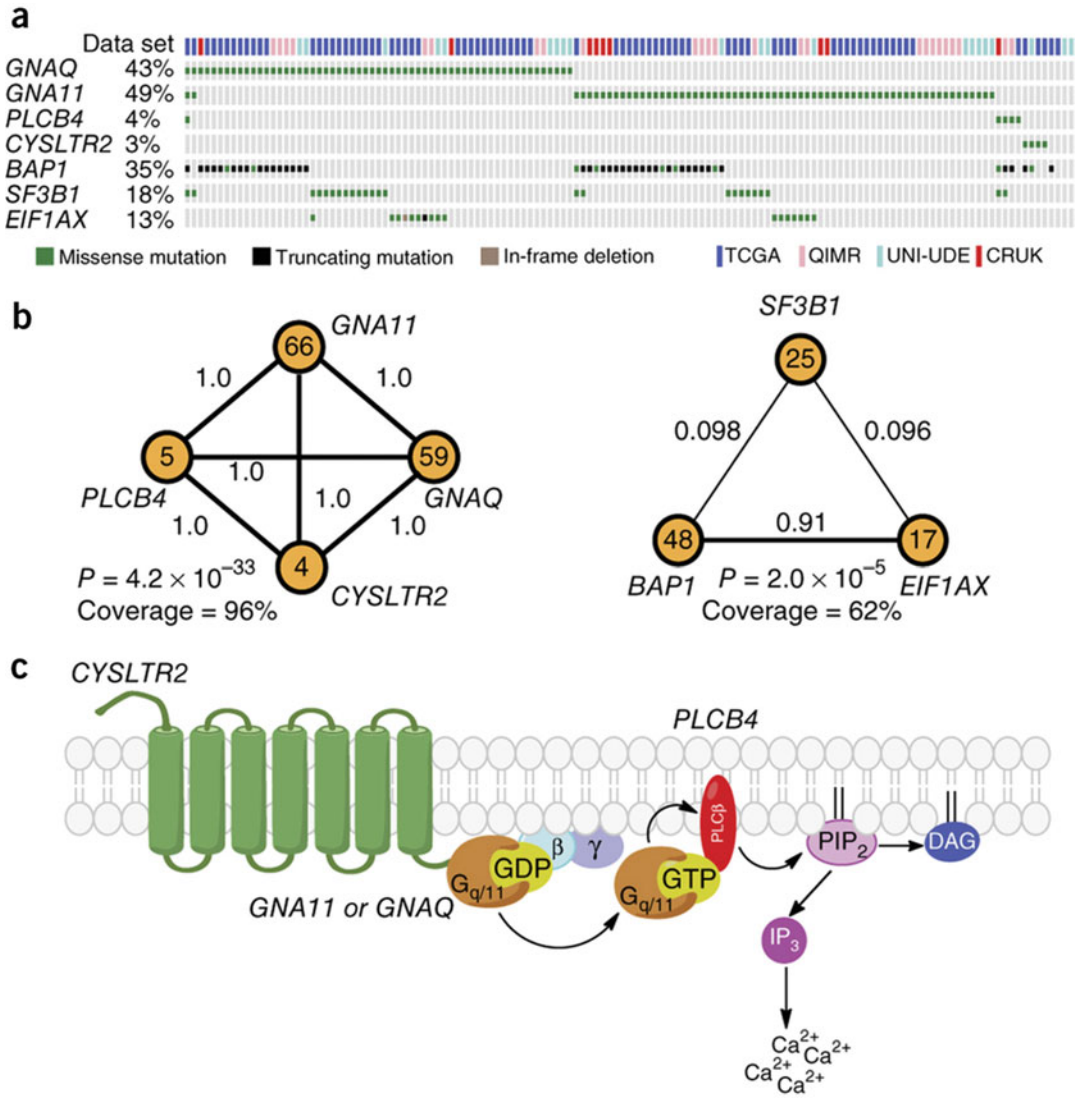


Fig. 11 Landscape of driving mutations in uveal melanoma (From Moore et al. 2016). (**a**) Mutamatrix of significantly mutated genes in uveal melanoma identified in exome datasets from The Cancer Genome Atlas (TCGA), Cancer Research UK (CRUK), QIMR Berghofer Medical Research Institute (QIMR), and University of Duisburg-Essen (UNI-UDE). (**b**) Statistical analysis with P-values indicate mutual exclusivity between *GNA11*, *GNAQ*, *PLCB4*, and *CYSLTR2* mutated in 96% of uveal melanoma samples (left panel) and *SF3B1*, *BAP1*, *EIF1AX* mutated in 62% of samples. (**c**) Illustration of signaling pathway affected by mutations in uveal melanoma is shown. In summary, the G protein receptor (GPCR), *CYSLTR2*, promotes the exchange of GDP for GTP binding of GNAQ/11 resulting in increased interaction with PLCB4 that promotes the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce diacylglycerol (DAG) and inositol triphosphate (IP_3), leading to calcium release and activation of PKC (Reprinted by permission from Macmillan Publishers Ltd., Nature Genetics, Amanda R Moore, Emilie Ceraudo, Jessica J Sher, Youxin Guan, Alexander N Shoushtari, Matthew T Chang, Jenny Q Zhang, Edward G Walczak, Manija A Kazmi, Barry S Taylor, Thomas Huber, Ping Chi, Thomas P Sakmar & Yu Chen. Nat Genet. 2016 Jun;48(6):675–80. https://doi.org/10.1038/ng.3549. Copyright (2016))

Yu et al. 2014). Combination PKC and MEK inhibitors are currently in clinical trials to treat metastatic uveal melanoma patients, although preliminary results from the PKC monotherapy trials have not been impressive, emphasizing the need for further studies (Chen et al. 2014).

Although HDAC clinical trials are underway to treat metastatic uveal melanoma, the mechanisms of action of BAP1 loss are still not entirely clear. Addressing these knowledge gaps will be better addressed with improved human and mouse models of uveal melanoma. For instance, there are few human uveal melanoma cell lines with monosomy 3 available to the research community. In addition, currently, there is no genetically engineered mouse model (GEM) for metastatic uveal melanoma. For example, the *Bap1* knockout mouse does not develop uveal melanoma (Dey et al. 2012). The transgenic GNAQ p.Q209L mice develop uveal melanoma, but do not develop liver metastases (Huang et al. 2015). Improved GEM models with appropriate gene targeting of mutations found in uveal melanoma will likely lead to better mouse models recapitulating the phenotypes observed in the human disease. With the identification of the current landscape of driver mutations in uveal melanoma along with the development of better model systems for functional studies, the melanoma community is poised to accelerate the understanding of uveal melanoma biology. Hopefully, such work will lead to improved therapeutic options for patients.

Conclusions

A number of studies have identified causative germline variants in familial melanoma that were not covered in this chapter. However, an understanding of how the "ground state" normal genetic variation in patients influences the landscape of somatic mutations and melanoma biology remains unclear, and is an important avenue of investigation. Future studies will also need to functionalize the melanoma genome through mechanistic studies and the development of appropriate model systems. Such work will hopefully lead to new therapeutic strategies. Given the recent clinical success of immune checkpoint inhibitors, understanding the molecular changes associated with response and resistance mechanisms to immune therapies should also lead to more effective management of melanoma patients.

Molecular characterization of non-acral cutaneous and uveal melanoma has led to the development of new therapies and the discovery of biomarkers that predict patient outcome. However, rarer forms of melanoma require similar study to both gain an understanding of the molecular causes of the disease and to discover new drug targets and biomarkers. In particular, the landscape of driving mutations in acral and mucosal melanoma are not well-understood (Furney et al. 2012, 2013b). To date, molecular characterization of these subtypes across multiple data platforms is still lacking and represents an important area of study. Other rare forms of melanoma with relatively limited genomic characterization have revealed important insights into the disease, such as work done in desmoplastic and spitzoid melanomas, as illustrated above (Shain et al. 2015a; Wiesner et al. 2016). Undoubtedly, larger scale multidimensional characterization of melanoma will emerge in the near future that will reveal new insights into the etiology and biology of the disease.

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Inherited Contributions to Melanoma Risk

11

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Abstract

While exposure to ultraviolet radiation (UVR) is a significant factor contributing to the risk of developing cutaneous malignant melanoma (CMM), genetics plays a significant role in both the context of high-risk melanoma families as well as the general population. While two high-risk melanoma susceptibility genes, *CDKN2A* and *CDK4*, have been known for decades, technological advances in recent years have enabled the recent identification of numerous genes and/or genetic loci mediating risk. A review of this progress, including rare high-penetrance gene mutations involved in familial melanoma as well as more common population variants of intermediate to low effect, is summarized in this chapter.

Keywords

Melanoma genetics · Melanoma susceptibility genes · Exome sequencing · Genome sequencing · *CDKN2A* · *CDK4* · *MC1R* · Heritability · *TERT* · *BAP1* · *POT1* · *ACD* · *TERF2IP* · *PARK2* · Shelterin Complex Mutations · GWAS · Pigmentation genetics · Nevus genetics · DNA repair · Telomeres · *MITF*

Introduction: Heritability and Phenotypes

Melanoma risk is determined by a combination of genetic and environmental factors. Historically, melanomas have been separated into "familial" and "sporadic" forms. The important role of genetic factors in susceptibility is clear in "familial" melanoma, where there is a large aggregation of melanoma cases in a particular family due to the segregation of a high-risk mutation. The proportion of melanoma cases that classify as "sporadic" varies across world populations. For example in Australia, where melanoma is more common, the occurrence of two close relatives with melanoma is not uncommon and would be less likely to be denoted "familial" than say in a similar family in Europe. In practice, the vast majority (>90%) of melanomas worldwide are of the "sporadic" form.

The term "sporadic" or (nonfamilial) implies that melanoma cases occur randomly, without respect to shared genetic factors. In fact, there is a substantial germline contribution to cutaneous melanoma risk in general. This contribution has been estimated in three ways: firstly from family studies; secondly from traditional twin studies; and thirdly from more recent approaches which use large-scale genome-wide genetic data from genotyping microarrays (see the end of this chapter for details). Family studies show that melanoma occurs in the first-degree relatives of probands at approximately 2–3 times the rate it occurs in general population samples; this is consistent with a genetic contribution to melanoma risk, although it is difficult in such studies to disentangle the contributions of shared environment and genetic factors. Recognizing this limitation, traditionally in genetic epidemiology twin studies have been used as a better way of establishing the relative importance of genes and environment. Monozygotic (MZ) twins share all their DNA while dizygotic (fraternal) twins share only half (on average). By making an assumption that the different zygosity types have similar environments, it is possible to estimate the relative contributions of genes and environment by estimating concordance rates in MZ and DZ twins. If a condition has a heritable genetic component then the MZ concordance will be proportionally higher than the DZ concordance. Under a statistical model which partitions the observed trait variability into a component due to genes and a component due to environment, one can define heritability as the proportion of variance due to genetic variation among individuals. Over the last 20 years, Scandinavian twin studies have enabled

large-scale twin studies of melanoma risk. These studies have established that germline genetic factors explain a substantial proportion of variation in (sporadic) melanoma risk. The most recent Scandinavian twin study (Mucci et al. 2016) examined over 200,000 twins and estimated a heritability of 58% (95% confidence interval 43–73%) for melanoma (a small subset of these would have been familial cases since the study included all melanomas). This work confirms that although environmental factors (particularly UVR) play a major role in melanoma risk, within countries, genes are very important in explaining variation in melanoma risk.

Like the majority of complex traits including cancer, the penetrance of individual variants ranges considerably; highly penetrant *CDKN2A* and equivalent mutations are primarily found in familial melanoma (discussed below), while the genetic risk for "sporadic" melanoma mainly derives from the combination of multiple genetic variants that vary in penetrance and population frequency. The authors will discuss these different classes of variation in this chapter.

Genetics of Familial Melanoma

CDKN2A

CDKN2A was the first highly penetrant gene found to mediate melanoma risk in melanoma-prone families. It was discovered as a result of a positional cloning effort that relied on a combination of the detection of loss of heterozygosity (LOH) (Dracopoli et al. 1987), cytogenetically detectable rearrangements at 9p21-p22 in melanoma cell lines and uncultured tumors (Cowan et al. 1988; Cowan and Francke 1991), a case study of an individual with multiple melanomas, atypical moles and rearrangement between the p arms of chromosomes 5 and 9 leading to the deletion of 9p21 (Petty et al. 1993a, b), and multiple studies showing significant evidence of genetic linkage to chromosome bands 9p21–22 in high-risk melanoma families (Cannon-Albright et al. 1992; Gruis et al. 1993; Nancarrow et al. 1993). These efforts culminated in the eventual identification of germline melanoma-predisposing mutations in the *CDKN2A* gene (Hussussian et al. 1994; Kamb et al. 1994).

The *CDKN2A* gene is unusual in the human genome in that it encodes two distinct proteins, p16 (INK4A) and p14 (ARF; alternative reading frame), each derived in part from common second and third exon sequence read in alternate reading frames. p16 and p14 are transcribed from two different promoters and utilize unique first exons, respectively. The first exon of each is spliced to the same splice acceptor sites of exon 2, but each is translated in different reading frames and thus encode fully distinct proteins. Both the p16 and p14 proteins are tumor suppressor proteins. p16 functions as a negative regulator of the cell cycle, acting to inhibit the activity of cyclin-dependent kinases CDK4 and CDK6. These kinases phosphorylate the retinoblastoma protein resulting in the release of E2F transcription factors that transactivate genes required for entry into S phase (Serrano et al. 1993; Serrano et al. 1995). By contrast, p14 antagonizes the ability of MDM2 to induce degradation of the p53 tumor suppressor (Zuo et al. 1996; Pomerantz et al. 1998).

CDKN2A mutations are observed in approximately 40% of melanoma families worldwide (Goldstein et al. 2006). Most familial *CDKN2A* mutations are loss of function mutations that are spread out over the length of the gene in the form of missense, deletion, insertion, duplication, nonsense, and splicing mutations, with a number of population-specific founder mutations reported. In addition, rare pathogenic mutations in nonprotein coding sequence at this locus have been identified, including variants in the 5′-UTR and deep within an intron (Liu et al. 1999; Harland et al. 2001). More than 95% of *CDKN2A* mutations alter the p16 protein, while more than half also alter p14. Roughly 5% of *CDKN2A* mutations (2% of melanoma families overall), however, harbor mutations in the form of large deletions or splicing mutations, which are predicted to only affect the p14 protein, suggesting that p14 may act as an independent melanoma susceptibility gene within the *CDKN2A* locus in some instances. In addition to predisposing to melanoma, there is

considerable evidence that families with *CDKN2A* mutations have a higher incidence of pancreatic cancer, and probably nervous system tumors (NSTs) in families harboring mutations that alter p14 but not p16 (Goldstein et al. 2006).

CDK4

Based on the identification of *CDKN2A* as a melanoma risk gene, candidate gene screens of the p16-interacting gene cyclin-dependent kinase 4 (*CDK4*) in high risk melanoma families resulted in the identification of two melanoma-predisposing mutations to a single *CDK4* codon, R24C (Zuo et al. 1996) and R24H (Soufir et al. 1998). The affected arginine plays a key role in the binding of CDK4 to p16, and alteration of this amino acid results in loss of the ability for p16 to bind and inhibit CDK4 activity. Perhaps not surprisingly *CDK4* families, similar to families harboring *CDKN2A* mutations, have early onset melanomas, often in multiplicity, and have clinically atypical nevi (Puntervoll et al. 2013). *CDK4* mutations are found in 2–3% of melanoma families (Goldstein et al. 2006).

Linkage Studies: Other Potential Susceptibility Loci

Beyond the discovery of these two genes and the characterization of their role in melanoma predisposition, progress toward identifying additional susceptibility genes had been limited until quite recently. Several linkage analyses had been published, including those identifying loci for melanoma risk on chromosome bands 1p36 (Bale et al. 1989; Goldstein et al. 1993) and 1p22 (Gillanders et al. 2003), as well as a locus for both uveal and cutaneous susceptibility on chromosome band 9q21 (Jonsson et al. 2005; Cannon-Albright et al. 2013). To date, no specific melanoma-predisposing mutations at these loci have been identified. This may be due to significant heterogeneity and/or polygenic inheritance (i.e., familial clustering is due to aggregated risk from multiple, low penetrance risk genes; see below) in melanoma families without *CDKN2A* and *CDK4* mutations (Gillanders et al. 2003).

BAP1

The BRCA1-associated protein 1 (*BAP1*) gene was initially implicated in melanoma via whole-exome sequencing of uveal melanomas, which found the gene to be somatically mutated in more than 80% of uveal melanomas that became metastatic. Wiesner and colleagues evaluated two families with multiple unpigmented to reddish brown melanocytic tumors with Spitzoid features that segregated in an autosomal dominant pattern, with some family members also developing uveal or cutaneous melanomas (Wiesner et al. 2011). Comparative genomic hybridization of multiple tumors from members of one of these families showed a common region of chromosomal loss on chromosome 3p21, and analysis of germline DNA from this family showed evidence of linkage to the same region. Targeted capture of this region followed by parallel sequencing identified inactivating mutations in *BAP1* segregating in both families, with loss of the wild-type allele in the melanocytic tumors with Spitzoid features, uveal and cutaneous melanomas. Soon thereafter, multiple studies reported *BAP1* mutations in additional families with cutaneous or uveal melanoma and other cancers (reviewed in Aoude et al. 2015). It is now established that *BAP1* mutations cause a multi-cancer predisposition syndrome, with carriers at an increased risk of a number of different cancers including uveal and cutaneous melanoma, mesothelioma, and clear cell renal cell cancer. Uveal and cutaneous melanomas are found in only approximately 30% and 13% of *BAP1* mutation carriers reported to date (Rai et al. 2016), and account for only a small proportion of melanoma families.

Familial Mutations Associated with Telomere Biology

The end replication problem of DNA leads to the progressive loss of a small portion of the

telomeric sequences of all chromosomes each time cells divide. Telomeres consist of repetitive DNA sequences that cap and protect the end of chromosomes. Once cells have exhausted their replicative lifespans, a DNA damage signal ensues and results in senescence or apoptosis. Studies of both melanoma families, as well as sporadic melanoma cases (discussed later in this chapter), have begun to implicate alterations in multiple genes involved in the maintenance of telomeres in mediating susceptibility to melanoma.

TERT

The first evidence that mutations in genes associated with telomere maintenance may play an important role in familial melanoma was the discovery of a mutation to the promoter of the gene encoding telomerase reverse-transcriptase (*TERT*) by Horn and colleagues (Horn et al. 2013). Targeted high-throughput sequencing of a large melanoma family showing linkage to chromosome 5p identified a cosegregating sequence variant at −57 base pairs from the ATG translation start site of *TERT*. The mutation creates a new binding motif for ETS transcription factors and ternary complex factors (TCFs), and resulted in increased transcriptional activity in reporter assays. A subsequent screen of 675 multicase melanoma families suggested that melanoma-predisposing mutations in the promoter of *TERT* are exceedingly rare, identifying only one additional family harboring the same −57 germline mutation (Harland et al. 2016). Subsequent sequencing of melanoma cell lines and primary melanomas and metastases by Horn and colleagues, as well as a second group (Huang et al. 2013), further identified recurrent somatic *TERT* promoter mutations at positions −124 and −146, with a higher frequency in metastases. Both of these somatic mutations also create novel ETS/TCF motifs utilized specifically by the GA-binding protein transcription factor (GABP) (Bell et al. 2015; Makowski et al. 2016). Recent studies have identified similar *TERT* promoter mutations in preneoplastic melanocytic lesions (Shain NEJM 2015), an unexpected finding that suggests that these lesions, which include dysplastic nevi, have a history of more cell divisions than their number of cells would predict, pointing to a role in telomere length in constraining the proliferation of preneoplastic lesions. These data suggest that familial *TERT* promoter mutations are likely associated with increased *TERT* expression and align with findings from population studies (Iles et al. 2014) that more broadly implicate telomere maintenance genes in melanoma risk. Most likely the increased *TERT* expression contributes to melanoma risk by extending the replicative lifespan of preneoplastic lesions.

Shelterin Complex Mutations: *POT1*, *ACD*, *TERF2IP*

Consistent with the hypothesis that telomere maintenance may play a key role in melanoma predisposition, several recent whole-genome and -exome sequencing studies of high-risk melanoma families have implicated additional telomere maintenance proteins, specifically members of the shelterin complex. The shelterin complex is composed of six components and protects telomere ends, preventing their degradation and processing of telomere ends by DNA damage response pathways, as well as regulating telomere interaction with the telomerase complex (Palm and de Lange 2008). Telomeric TTAGGG repeat sequences are recognized by three members of the shelterin complex. TERF1 and TERF2 bind to double-stranded telomere repeats, whereas protection of telomeres 1 (POT1) recognizes single-stranded telomeric repeats at 3′ overhangs or in alternative loop structures. The shelterin complex subunit and telomerase recruitment factor ACD has been found to increase the affinity of POT1 for telomeric ssDNA (Wang et al. 2007), and in a complex with POT1, mediates interactions between the shelterin complex and TERT; inhibition of the POT1/ACD subunit facilitates the elongation of telomeres by telomerase (Xin et al. 2007). POT1, together with ACD, facilitates the formation of t-loop structures, which protect the 3′

overhangs by promoting the annealing within duplex telomeric repeat sequences.

Two parallel studies initially identified melanoma-predisposing mutations within the *POT1* gene. Robles-Espinoza and colleagues performed whole-exome or whole-genome sequencing of 184 melanoma cases drawn from high-risk pedigrees collected in the UK, Netherlands, and Australia that lacked *CDKN2A*, *CDK4*, or *BAP1* mutations (Robles-Espinoza et al. 2014). Four pedigrees were found to harbor potentially deleterious *POT1* mutations. Three of these mutations were to highly conserved residues of the two POT1 oligonucleotide/oligosaccharide-binding (OB) fold domains (Y89C, Q94E, and R273L), while a fourth was in the splice acceptor between exons 17 and 18, compromising splicing (g.124465412C > T). Importantly, the variants in three of these families cosegregated with melanoma (the fourth family was unassessable). Concurrently, Shi and colleagues sequenced exomes of 101 cases from 56 unrelated Italian families and identified a novel missense variant (S270N) in the second POT1 OB domain, which cosegregated with the melanoma phenotype in five families (Shi et al. 2014) along with two other missense variants. Consistent with a role in susceptibility, both of these studies noted significant enrichment for rare or novel *POT1* variants in familial (Robles-Espinoza et al. 2014) or population-based (Shi et al. 2014) melanoma cases relative to controls. Recognition of single-stranded telomeric repeats by POT1 are mediated by the OB domains; modeling of POT1 protein structure suggests that several of the amino acid residues found to be altered in these families are in close proximity to DNA binding sites, suggesting that the observed *POT1* mutations are likely to weaken or abolish POT1-ssDNA binding. Consistent with this notion, *in vitro* translated POT1 harboring the Y89C, Q94E, and R273L failed to bind to TTAGGG sequences in electromobility shift assays, and both studies found the telomeres of carriers of these *POT1* mutations to be longer than those of non-carriers.

Based on these findings, a subsequent study screened all six genes encoding shelterin complex members (*POT1*, *ACD*, *TERF1*, *TERF2*, *TERF2IP*, and *TINF2*) in a considerably larger number of melanoma families (Aoude et al. 2014). From 510 melanoma families with unknown genetic etiology screened using a combination of whole-genome, whole-exome, and targeted gene sequencing, five novel *ACD* mutations were found in six families, including a cosegregating nonsense mutation (Q320X). Of note, the novel variants found in melanoma families were found to be nonrandomly clustered in the POT1 binding domain relative to those found in population controls, suggesting that this domain and the interaction between ACD and POT1 may play a key role in telomere maintenance and melanoma susceptibility. Similarly, novel *TERF2IP* mutations were found in four families, including a segregating nonsense mutation; like *ACD*, novel and rare variants in *TERF2IP* were found to be significantly enriched in melanoma families relative to population-based controls, supporting a potential role for variation in the *TERF2IP* gene in mediating melanoma risk. Family members harboring *TERT*, *POT1*, *ACD*, or *TERF2IP* mutations were found to have developed a number of other cancer types, suggesting that melanoma-predisposing mutations in these genes may confer susceptibility to a broader range of cancers (Bainbridge et al. 2015).

Undetected Familial Melanoma Genes

While considerable progress has been made in the identification of high-penetrance melanoma risk genes, nearly half of high-risk families nonetheless cannot be explained by mutations in known genes. The failure to identify high-penetrance mutations in these additional families could be explained by a number of possibilities. Firstly, a proportion of these additional families may harbor deleterious variants altering the function of predisposition genes that are not readily detected via exome sequencing. Specifically, the identification of rare promoter mutations in *TERT* (Horn et al. 2013), as well as the identification larger melanoma-predisposing structural aberrations in *CDKN2A* (Petty et al. 1993a, b), both highlight the possibility that noncoding gene-regulatory

mutations and/or deleterious structural variants, including gene deletions, may play a wider role in melanoma predisposition than currently appreciated. Additionally, some families could harbor rare deleterious gene mutations that remain unrecognized as predisposing due to limited sample size, and/or imperfect familial cosegregation due to the phenocopies in sequenced families. Still, a proportion of melanoma families may not harbor mutations in high-penetrance genes at all, but instead may be explained by shared environment, random chance, or the presence of multiple intermediate- to low-penetrance melanoma risk alleles segregating within individual families.

Intermediate Risk, Candidate Gene Studies, and the Dawn of GWAS

Early candidate gene studies found *MC1R* missense variants that induce red hair and pale skin convey an intermediate melanoma risk (OR 1.4–2.4) whereas variants in *SLC45A2* that are associated with darker skin reduce susceptibility. However as is often the case with candidate studies the results were inconsistent across other genes, likely due to limited power and low single nucleotide polymorphism (SNP) coverage. Identification of additional intermediate or low-risk genetic variant for melanoma would require very large sample sizes and genome-wide coverage.

Genome-wide association studies (GWAS) simultaneously test genetic variation genome-wide for association with the trait of interest. Briefly, high-density arrays are used to genotype hundreds of thousand, or millions, of SNPs with high accuracy. These SNPs are generally chosen to leverage patterns of correlation (linkage disequilibrium, LD) across the genome in order to provide maximal coverage of common genetic variation. SNPs which are directly genotyped on the high-density arrays are in LD with nearby genetic variants that are not on the arrays (LD decays as a function of number of generations/recombinations, physical distance, and relative allele frequency). This means that given a detailed map of the LD between all genome-wide genetic variation in an appropriate

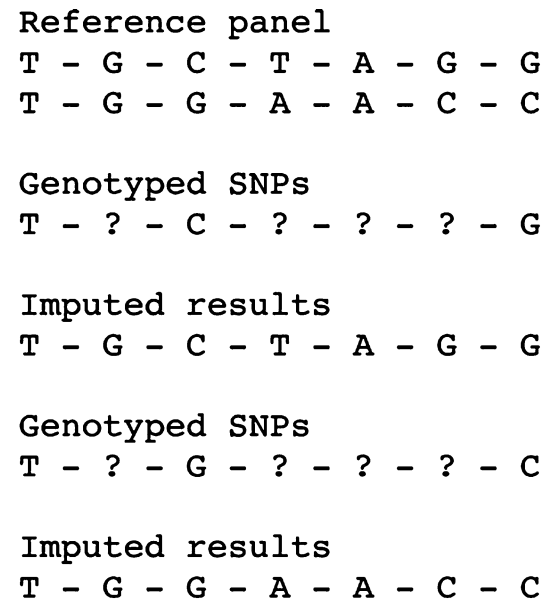

Fig. 1 Imputation example. In this simplistic example, there are only two possible sets of SNPs in LD (two haplotypes). In reality, there are multiple haplotypes formed from recombination of various sets of SNPs, and the imputation may assign probabilities of certain genotypes rather than absolutes

population, as provided by the HapMap and the 1000 Genomes Project, the directly genotyped SNPs can be used to infer (impute) nearby genetic variation with high accuracy. Figure 1 shows a simplified example where the genotyped SNPs are in perfect LD with those not genotyped.

Thus high-density SNP genotyping arrays can be leveraged to impute the majority of human genetic variation down to ~1% minor allele frequency, and a large proportion of rarer SNPs. In addition to giving greater coverage when testing for a disease association, imputation assists meta-analysis of datasets by increasing the number of SNPs that overlap between studies. The main advantage of GWASs is that they are unbiased, making no assumptions about which parts of the genome influence the trait of interest. However, the high number of loci tested comes at the cost of an increased false discovery rate, requiring appropriate statistical corrections. Various methods have been used to determine the effective number of independent tests, generally converging on a million tests for all MAF $>$ 5% SNPs, leading

to the accepted practice of setting genome-wide significance at $p < 5 \times 10^{-8}$. The number of tests may further increase as less and less common SNPs are included in GWAS, potentially requiring (slightly) more stringent significance thresholds.

Which classes of variation (rare vs. common, high vs. low penetrance) can be detected by GWAS is a function of power, which is primarily driven by sample size. Generally, initial GWAS detects a mix of lower frequency, moderate penetrance variants, or high frequency, lower penetrance variants (the so-called "low-hanging fruit"). As sample sizes increase, usually by meta-analyses of multiple GWAS, further risk variants are detected with lower allele frequencies and/or effect size. For ease of discussion, the known melanoma GWAS loci have been grouped by putative risk pathway (pigmentation, nevus count, DNA repair/telomeres) where possible (Table 1).

While extremely powerful, GWAS provides only evidence for association between a genetic region and the trait of interest; further work is often required to fully characterize how genetic variation in that region influences trait biology. The majority of GWAS-identified SNPs do not include coding variants (with prominent exceptions for melanoma discussed below), or even span genes. In many situations, the most-associated SNP resides in an intergenic region or gene desert. Instead, many of the genetic variants identified by GWAS influence gene expression levels. These expression quantitative trait loci (eQTLs) can modify the activity of gene enhancers and promoters, and thereby regulate the expression of genes up to distances of a megabase or more.

Pigmentation

MC1R red hair alleles are a number of independent non-synonymous variants in the *MC1R* gene that impact the receptor's ability to activate the cAMP pathway and mediate a protective response to UVR DNA damage. When melanocytes or keratinocytes detect UVR-induced DNA damage they upregulate the expression of melanocyte-stimulating hormone (MSH), which via binding to MC1R induces the production of melanin, which is subsequently transported to neighboring keratinocytes (Fig. 2). In addition, MC1R signaling induces DNA repair and antioxidants (Mitra et al. 2012). The first GWAS of melanoma, published in 2008 and 2009 (Brown et al. 2008; Bishop et al. 2009), confirmed the importance of red hair alleles in *MC1R* for melanoma risk, and additionally identified common variants in other pigmentation genes. The SNP near *MC1R* most strongly associated with melanoma in the recent meta-analysis, rs75570604, is in LD $r^2 = 0.8$ with the red hair missense variant rs1805007 (R151C), which is itself melanoma associated (Meta-analysis $p = 5 \times 10^{-83}$) (Law et al. 2015). A second red hair allele, rs1805008 R160W, is also melanoma associated (6×10^{-25}), as is rs11648879 ($p = 2.0 \times 10^{-9}$), which is in LD r^2 0.6 with a third red hair allele, rs2228479 V92M (Law et al. 2015).

MC1R activity is negatively regulated by the MC1R antagonist agouti signaling protein (Fig. 2), which is encoded by the *ASIP* gene. SNPs near *ASIP* have also been found to be associated with melanoma by GWAS (Brown et al. 2008). These same variants are also associated with human pigmentation and are strongly associated with *ASIP* expression in the skin (Grundberg et al. 2012). The major allele rs1885120 is an eQTL associated with reduced *ASIP* expression, high pigmentation, and decreased melanoma susceptibility, presumably as a consequence of increased *MC1R* signaling. While *ASIP* is a very plausible candidate in the chromosome 20 melanoma locus it is worth noting that additional genes in the region, including *GSS* which is involved in melanogenesis, may also contribute to skin coloration and thus modulate melanoma susceptibility (Liu et al. 2015).

Activation of MC1R promotes melanin synthesis, and *Tyrosinase* (*TYR*) is a rate-limiting enzyme in the conversion of tyrosine into

Table 1 Summary of melanoma-associated loci (Law et al. 2015). For each locus, the peak SNPs is tabulated; detailed results for all SNPs with a *p*-value $<1 \times 10^{-7}$ can be found in the supplementary material (Law et al. 2015)

CHR	Suggested or nearest gene	Peak SNP	Melanoma *p*-value[1]	Nevus *p-value*[2]	Pigmentation *p*-value[3]
1	*ARNT, SETDB1*	rs12410869	5.2×10^{-13}	n.s	n.s
1	***PARP1***	**rs1858550**	**1.7×10^{13}**	**n.s**	**n.s**
2	*CYP1B1*	rs6750047	2.9×10^{-7}*	n.s	n.s
2	*CASP8*	rs7582362	8.9×10^{-9}	0.1	n.s
5	***TERT***	**rs380286**	**1.7×10^{-17}**	**0.05**	**n.s**
5	*SLC45A2*	rs250417	2.3×10^{-12}	n.s	0.07
6	*CDKAL1*	rs6914598	2.6×10^{-8}	n.s	n.s
7	*AGR3*	rs1636744	1.8×10^{-9}	0.09	n.s
9	*MTAP, CDKN2A*	rs7852450	4.7×10^{-32}	0.09	n.s
9	*TMEM38B*	rs10739221	9.6×10^{-9}	n.s	n.s
10	***OBFC1***	**rs2995264**	**8.5×10^{-7}***	**n.s**	**n.s**
11	***CCND1***	**rs498136**	**1.0×10^{-10}**	**n.s**	**n.s**
11	*TYR*	rs1393350	2.5×10^{-25}	n.s	2.6×10^{-8}
11	***ATM***	**rs73008229**	**1.4×10^{-12}**	**n.s**	**n.s**
15	*OCA2*	rs4778138	3.1×10^{-9}	n.s	2.2×10^{-6}
16	*FTO*	rs12596638	1.8×10^{-9}	0.05	n.s
16	*MC1R*	rs75570604	6.2×10^{-92}	n.s	4.7×10^{-39}
20	*ASIP*	rs6059655	5.4×10^{-29}	n.s	2.9×10^{-6}
21	*MX2*	rs408825	3.2×10^{-15}	n.s	n.s
22	*PLA2G6*	rs2092180	2.1×10^{-11}	0.002	0.04s

[a]Meta-analysis *p* fixed or random if $I^2 > 0.31$ (see Law et al. 2015 for details)
[b]*p*-Value for association with nevus count in the Leeds cohort of the melanoma-meta-analysis (Law et al. 2015). n.s.: not significant ($p > 0.1$)
[c]*p*-Value for association with pigmentation in the Leeds cohort of the melanoma-meta-analysis (Law et al. 2015). Dark shading indicates likely role in pigmentation; light shading indicates a likely role in nevus count based on existing literature or the results within the Leeds cohort. Bold text indicates likely role in senescence, DNA repair, or telomere function
[d]Genome-wide significance in replication sets (Law et al. 2015)

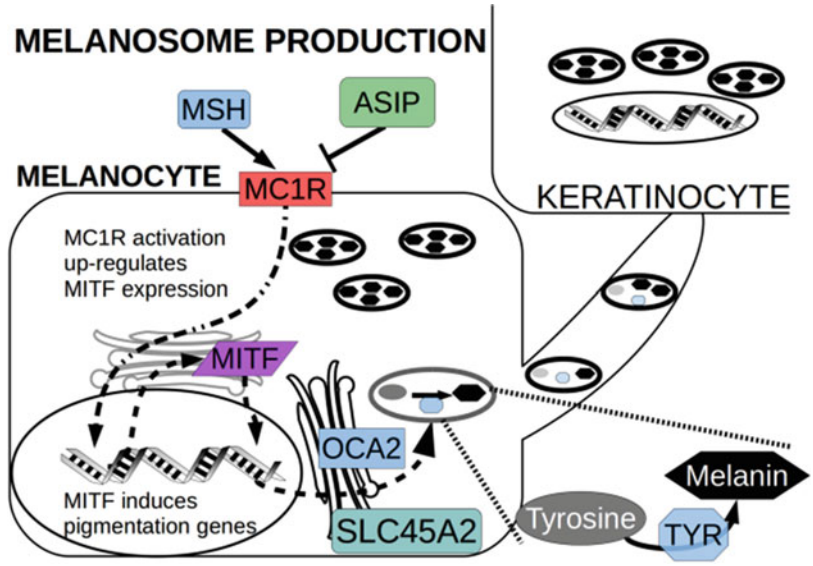

Fig. 2 Melanogenesis. MSH signaling via MC1R (which can be repressed by ASIP) leads to *MITF* expression, and upregulations of genes required to make functional melanosome, including *OCA2* and *TYR*. In the melanosomes, the TYR protein takes part in the conversion of tyrosine to either eumelanin or pheomelanin

eumelanin (Fig. 2). SNPs near *TYR* are also strongly associated with melanoma risk (Bishop et al. 2009), tanning (Nan et al. 2009), and to a lesser extent eye and skin pigmentation. While the peak melanoma SNPs have not been reported as *TYR* eQTLs, they include the nonsynonymous *TYR* rs1126809 R402Q variant. The 402Gln allele has also been associated with sunburns, tanning, and pigmentation, and results in reduced enzymatic activity at physiological temperatures due to protein misfolding. An impaired ability to produce eumelanin, rather than a complete absence, may explain why 402Gln is more associated with sunburn and tanning than overall pigmentation. While other nonsynonymous mutations in *TYR* also are associated with pigmentation/tanning traits (e.g., rs1042602), they are not as strongly associated with melanoma susceptibility ($p > 1 \times 10^{-7}$, Law et al. 2015).

Melanin and melanin-producing enzymes are packaged as melanosomes in melanocytes prior to their distribution to keratinocytes, a process which requires the function of SLC45A2 and OCA2 proteins. GWAS has identified an SNP near *SLC45A2* that conveys strong susceptibility to melanoma (Table 1; Barrett et al. 2011), and this SNP rs250417 is in LD ($r^2 = 0.51$) with the nonsynonymous L374F SNP rs16891982. The L374F variant is associated with reduced pigmentation and may impair the incorporation of tyrosinase into melanosomes (Fig. 2). 374L is ancestral and common in most human populations, whereas 374F has been under selection in European populations and has neared fixation.

Lastly, as with *SLC45A2,* SNPs near *OCA2* have also been associated with melanoma by GWAS (Table 1 Amos et al. 2011). A SNP at this locus, rs12913832, functionally modulates *OCA2* expression (Fig. 2) and is a major determinant of blue/brown eye color and influences skin pigmentation (Liu et al. 2015). The peak melanoma associated SNP at the 15q13.1 locus, rs4778138, is in LD ($r^2 = 0.12$) with rs12913832 in Europeans, so it is possible both SNPs contribute to the melanoma association (Law et al. 2015). rs4778138 has also independently been associated with variation in human eye color, and likely also modulates *OCA2*.

Nevus Number

In addition to rare familial variants at *CDKN2A* associated with high nevus counts and greatly increased risk of melanoma, common SNPs in the 9p21.3 have been associated with both melanoma and nevus count through GWAS (Bishop

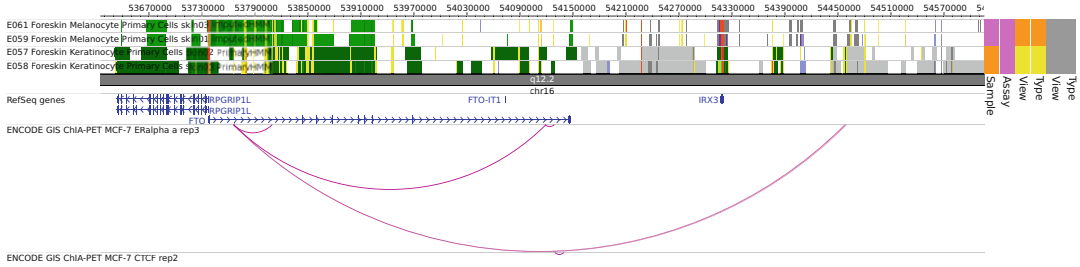

Fig. 3 Melanoma-associated SNPs, including the initially reported SNP rs16953002, in an enhancer that interacts with the promoter of the *FTO* gene in melanocytes. Plot generated using the WashU Epigenome Browser (http://epigenomegateway.wustl.edu/browser/)

et al. 2009; Falchi et al. 2009). Given the critical role of the p16 protein in cell cycle checkpoint control, common and rare variants at this locus may contribute to both melanoma and nevus count through altered cell division propensity, leading to clonal expansion of melanocytes. However, *CDKN2A* may not be the only important gene at this locus. Melanoma-associated SNPs at 9p21.3, including rs935055 and rs10811629 (European LD $r^2 = 0.45$) are eQTLs for the adjacent methylthioadenosine phosphorylase (*MTAP*) gene in a range of tissues including skeletal muscle, skin, and adipose tissue (Grundberg et al. 2012; Consortium 2015), with the protective allele (Barrett et al. 2015) associated with decreased *MTAP* expression across tissues. *MTAP* expression is required for the salvage of methiothine. This gene is somatically silenced in melanoma and the resultant accumulation of 5′-methylthioadenosine may lead to the induction of pro-cancer pathways (Limm et al. 2014). This may suggest a paradox – the protective allele is associated with reduced *MTAP* expression yet absence of MTAP function promotes melanoma – but it is worth noting that *PARP1* alleles associated with reduced risk for melanoma have also been associated with worse survival (Davies et al. 2014). While there is less evidence addressing the potential role for MTAP in nevus development, it is likely that genetic variants affecting *CDKN2A* could promote the clonal expansion of melanocytes in a nevus, thus affecting nevus size and number.

SNPs spanning the *AGR3* gene at 7p21.1 are associated with *AGR3* expression in lung and thyroid tissues (GTEx gene expression dataset Consortium 2015), and *AGR3* has been implicated in ovarian, breast, and other cancers. The peak *AGR3* SNP in the melanoma meta-analysis, rs1636744, showed a trend toward association with nevus count (Law et al. 2015), which may point to a role in this risk phenotype. As the function of this gene is poorly understood, it is not yet clear how *AGR3* might influence nevus development or melanoma.

Variants in or near the *FTO* gene on chromosome 16 are associated with nevus count (Law et al. 2015) and melanoma (Iles et al. 2013). Functional analysis of the FTO protein suggests it is an RNA demethylase involved in modulating mRNA translation relative to amino acids availability. Prior studies have revealed a strong association with body mass index (BMI), however the linked SNPs appear to influence the function of distal *IRX3* and *IRX5* genes rather than *FTO* (Smemo et al. 2014). By contrast, the melanoma-associated SNPs are independent of the BMI-associated SNPs (Iles et al. 2013), and it is not immediately obvious how *FTO* may influence melanoma biology or the development of nevi. The melanoma risk allele rs12596638-A (Table 1) is strongly associated with *FTO* expression in whole blood (Westra et al. 2013). This SNP is in LD ($r^2 = 1.0$) with the initial reported SNP, rs16953002 (Iles et al. 2013), which is situated in an intronic enhancer in *FTO* that interacts with the promotor of *FTO* (Fig. 3).

A SNP on chromosome 22, rs2092180, is also associated with melanoma risk and nevus count and to a lesser extent pigmentation (Law et al. 2015),

and overlapping SNPs in this region have also been identified in nevus count GWAS (Falchi et al. 2009). The LD r^2 between top melanoma SNP rs2092180 and the top nevus count SNP rs2284063 is 0.64 in Europeans. These linked loci at this chromosome 22 harbour numerous genes, and eQTLs associated with the melanoma risk variant have been established for *TMEM184B, MAFF*, *CSNK1E,* and *PLA2G6* genes in a range of tissues and databases (Westra et al. 2013; Consortium 2015) and *BAIAP2L2* in solely sun-exposed skin (Consortium 2015). The protein product of *BAIAP2L2* plays a role in stabilizing plasma membrane structures, while TMEM184B can promote migration and metastasis of oral squamous cell carcinoma cells via cytoskeletal modification (Fukumoto et al. 2015). *MAFF* heterodimerizes with a range of transcription factors and regulates gene expression in response to a range of cellular stressors including oxidative damage, and *MAFF* expression is downregulated in tumors; *CSNK1E* is a component of the circadian rhythm system, which impacts melanogenesis as well as response to sunburn (Gaddameedhi et al. 2015); *PLA2G6* encodes a phospholipase A2, an enzyme that cleaves fatty acids from membrane phospholipids, and as a result is involved in a variety of cellular processes. Consequently, any of these genes may be plausibly involved in melanocytic neoplasia directly or indirectly but evidence implicating specific genes at this locus is currently lacking.

Telomeres, Senescence, and DNA Repair

In addition to the rare familial variants discussed above, common variants in genes involved in telomere function, DNA repair, and senescence are associated with melanoma. A polygenic risk score based on the top seven SNPs previously found to be associated with longer leukocyte telomere length has been found to be associated with higher risk of melanoma (Iles et al. 2014). rs2995264, a SNP on chromosome 10 located in an intron of the telomere maintenance gene *OBFC1,* is strongly associated with both mean telomere length (Codd et al. 2013) and melanoma risk (Law et al. 2015), suggesting that an increased risk for melanoma from longer telomeres is not simply due to confounding (e.g., UVR damaging telomeres in parallel to inducing somatic oncogenic mutations in melanocytes). It is plausible that delayed senescence due to an extended replicative lifespan increases the pool of partially transformed melanocytes (e.g., as present in nevi) and thereby the probability for the accumulation of additional pathogenic mutations and progress toward melanoma.

However, the influence of telomere genes on melanoma may not solely be through effects on telomere length. For example, GWAS and fine-mapping efforts suggest there are a number of independent common SNPs associated with cancer at the telomerase reverse transcriptase gene (*TERT*), with many cancer types associated with a different but overlapping subset of these SNPs (Wang et al. 2014; Barrett et al. 2015). These include rs2736100, which has been found to be associated with both cancer and telomere length (Codd et al. 2013), with rs2736100-G associated with risk of myeloproliferative neoplasms, glioma, acute myeloid leukemia, lung, thyroid, and bladder cancers while being protective for colorectal, pancreatic, and testicular cancer (Turnbull et al. 2010; Campa et al. 2015; Li et al. 2017). rs2736100-G, however, is only modestly associated with risk of melanoma ($p = 0.02$) (Iles et al. 2014). Melanoma associations at *TERT* are strongest at rs401681 and rs380286 (LD $r^2 = 1$ with each other; Table 1) (Law et al. 2015), which are not in LD with rs2736100 (European LD $r^2 = 0.003$). In contrast to rs2736100, rs401681-G is protective for melanoma, pancreatic, and testicular cancer but associated with an increased risk for basal cell carcinoma, as well as lung, bladder, prostate, and cervix cancers (Rafnar et al. 2009; Stacey et al. 2009; Turnbull et al. 2010; Campa et al. 2015). These data suggest that there may be multiple pathways to melanoma from telomere genes, with some loci influencing telomere length while others presumably mediate other telomere-related processes. Notably, melanoma-associated SNPs at the *TERT* locus have also been associated with nevus count in some studies (Law et al. 2015)

but not others (Bodelon et al. 2012), perhaps suggesting a role in senescence.

In additional to telemere genes, other risk genes highlight the importance of senescence and /or DNA repair in melanoma. The association signal at 1q42 is localized to the Poly(ADP-ribose) Polymerase 1 (*PARP1*) gene (Macgregor et al. 2011), a critical mediator of DNA repair and genomic stability. Among its many roles in transcriptional regulation and apoptosis, *PARP1* binds to damaged DNA, activating its poly (ADP-ribose) polymerase (PARP) function, which signals and enables the binding of additional cofactors to initiate DNA repair. The peak melanoma associated SNPs (e.g., rs1858550) are eQTLs for *PARP1* expression in melanoma cell lines and tumors. These SNPs tag a 6 bp insertion/deletion variant, rs144361550, that alters a *PARP1* enhancer, suggesting the functional gene at 1q42 in melanoma is *PARP1* (Choi et al. 2017). Indeed, increased expression of *PARP1* promotes proliferation of primary melanocytes, rescues melanocytes from $BRAF^{V600E}$-induced senescence, promotes malignant transformation of immortalized melanocytes independently of PARP1 catalytic activity, and promotes expression of the melanocyte-lineage survival oncogene *MITF* (Choi et al. 2017). The missense variant D1853N in *ATM* (rs1801516) was associated with melanoma in a GWAS (Barrett et al. 2011) and confirmed in a meta-analysis (Law et al. 2015). *ATM* is an attractive candidate given its importance in DNA damage response, cell cycle progression, and telomere maintenance (for review see Di Domenico et al. 2014). Rare, *ATM* variants with medium penetrance are associated with familial breast cancer and potentially other cancers (Renwick et al. 2006; Fletcher et al. 2010; Helgason et al. 2015). These variants appear to be different from those associated with melanoma.

Other Risk Loci

For the remaining loci containing common melanoma risk genes (Table 1), a unifying pathway is more difficult to discern, though eQTL datasets may allow for some inference. The peak SNP of the broad association signal at 1q21, rs12410869, is an eQTL for a number of the nearby genes including *ARNT*, *HORMAD1*, *GOLPH3L*, *CTSS*, and *CTSK* (Law et al. 2012) in a range of tissues in GTEx (Table 1; Consortium 2015). The strong, long-range LD in this region limits the ability to fine-map the association with melanoma (Macgregor et al. 2011; Barrett et al. 2015). Prominently, this local region also encompasses *SETDB1*, which has been shown to accelerate melanoma formation in a zebrafish model (Ceol et al. 2011). While GTEx reports rs12410869 is an eQTL for *SETDB1,* its association with melanoma is not significant, after genome-wide correction.

rs7582362 at 2q33.1 is an eQTL for both *CASP8* and *ALS2CR12* (Consortium 2015); of these two, *CASP8* is a stronger *a priori* candidate given its role in cell survival and/or apoptosis (for review see Shalini et al. 2015). rs7582362 is in LD (r^2 0.97) with rs1830298, the lead SNP for a breast cancer GWAS hit at this locus. These and other linked SNPs at this locus are eQTLs for *CASP8* and may represent a broader cancer risk locus.

rs498136, the strongest melanoma association at 11q13.3, is upstream of the *CCND1* promoter and is an eQTL for *CCND1* in monocytes (eQTL $p = 1 \times 10^{-13}$) (Westra et al. 2013); however, the melanoma risk rs498136 allele is associated with reduced expression, which is counterintuitive, considering the role cyclin D1, the gene product of CCND1. Cyclin D1 is the coenzyme of *CDK4,* which itself is a high penetrance melanoma risk gene. Another familial risk gene, *CDKN2A* ($p14^{ARF}$), blocks cell cycle progression in response to UVR DNA damage via p53-mediated inhibition of CCND1. While the observation of an eQTL in monocytes may not be relevant for melanoma, this suggests a potential indirect involvement with DNA repair as discussed above. *CCND1* is frequently amplified in melanomas located at acral sites and sites of chronic sun damage (Sauter et al. 2002; Curtin et al. 2005), and this amplification is associated with ulceration, thicker tumors, and worse survival (Vizkeleti et al. 2012).

The melanoma association signal on chromosome 21 spans the 5′ end of *FAM3B* and the promotor of *MX2* (Barrett et al. 2011), and includes SNPs that are strong eQTLs for *MX2* in testis and fibroblasts (Consortium 2015). In whole blood, the melanoma-associated rs431563 is an eQTL for *MX1*; while not passing the threshold for gene-wide significance the same SNP is an eQTL for *FAM3B* and *MX2* ($p < 5 \times 10^{-4}$ Westra et al. 2013). Likewise, while not gene-wide significant ($p = 0.0012$), rs431563 is an eQTL for *MX2* in adipocyte tissue (Grundberg et al. 2012). MX1 and MX2 appear to act as inhibitors of viral infection by limiting the ability of viruses to enter the nucleus. MX1 has also been associated with alopecia areata in a small case control study, which may suggest a role for this gene in modulating the immune response in the skin.

For the remaining loci, a clear pathway to melanoma remains elusive. As discussed in Law et al. (2015) rs6750047 on chromosome 2 is an eQTL for *CYP1B1*; however that gene is better known for its role in hormonal cancers. Likewise, while the melanoma-associated SNPs at 6p22.3 are within an intron of *CDKAL1*, searches of bioinformatics or functional databases could not identify the gene(s) functionally affected by these SNPs (Law et al. 2015). Melanoma-associated SNPs at 9q31.2 are near *RAD23B*, a gene involved in nucleotide excision repair; however, there is as yet no direct functional evidence linking these SNPs to any nearby genes, including *RAD23B*.

"Missing" Heritability and Rare Melanoma Risk Variants

Heritability estimates derived from GWAS for melanoma (Lu et al. 2014) have been calculated in Australian (30%, 95% CI 10–50%) and American samples (19%, 95% CI 1–37%), providing a guide to the extent to which large GWAS may explain the total genetic contribution to melanoma at the current time. There is much debate over what may fill the gap between estimates of melanoma heritability previously derived from twin studies (~50%) and these "GWAS heritability" estimates. Two possible contributions to this gap are the effects of gene-gene interactions (where the genetic effects of two independent loci are larger than the effects of either regarded separately) and of gene-environment interactions. There are, however, two more likely contributors to this gap: melanoma risk due to common variants with effect sizes too small to accurately measure without much larger sample sizes, and the presence of rarer melanoma risk variants not assessable via GWAS (e.g., variants exhibiting insufficient LD with SNPs on GWAS arrays).

In terms of common variants with small effect sizes, it is worth noting that the genome-wide significant loci described in this chapter explain only about a third of the 30% "GWAS" heritability for melanoma, indicating there are likely many more common variants conferring small effects on melanoma risk that do not yet reach genome-wide significance. Powering a genome-scale study to identify such variants for a role in melanoma risk while correcting for multiple testing presents significant technical, monetary, and sample-size challenges, but is nonetheless possible. For some complex traits where much larger sample sizes are already available (height and body mass index, for example), large sample size combined with dense imputation have yielded array heritability estimates which are only 20% lower than the "twin" heritability (Yang et al. 2015), suggesting that better-powered melanoma GWAS may at least partially explain this "missing" heritability.

Further, while the latter set of rare variants not assessable via GWAS includes rare high-penetrance melanoma risk variants found in high-risk melanoma families, it also includes rare variants of medium to low effect for which family studies and current population studies are not well-powered to comprehensively find. Identifying such rare trait-associated variants requires alternative approaches that maximize power by limiting multiple testing. It is hypothesized that many melanoma families without mutations in known high-penetrance risk genes may instead be enriched for medium-penetrance risk variants. Thus, a viable study design is one that utilizes both family-based sequencing to identify imperfectly segregating but rare variants, followed by

genotyping a limited number of them in case-control studies. Alternatively, focusing on strong *a priori* gene candidates can also further maximize the probability of success. Notably, the use of both of these approaches resulted in the identification of the first medium-penetrance melanoma risk variant in the melanoma-lineage-specific oncogene microphthalmia-associated transcription factor (*MITF*; E318K) (Bertolotto et al. 2011; Yokoyama et al. 2011).

MITF E318K

Whole genome-sequencing of probands from high-risk melanoma families resulted in the identification of a rare, missense mutation (E318K, rs149617956, MAF = 0.002 in 1000 Genomes EUR population) that cosegregated imperfectly with melanoma. The variant, however, was found in an additional 31 families, and linkage analysis showed evidence that this variant may represent a medium-penetrance melanoma risk allele, and genotyping in case-control studies from Australia and the UK confirmed the significant association with melanoma risk (OR 2.19, 95% CI 1.41–3.45). E318K was also associated with an increased number of melanocytic nevi and nonblue eye color. However, the association with melanoma risk remained essentially the same when accounting for these phenotypes (OR 1.82, 95% CI 0.85–3.92), suggesting that this variant influences risk at least in part independently of the nevus and pigmentation phenotypes. In sequencing a set of 62 patients with both melanoma and renal cancer, Bertolotto and colleagues found this same *MITF* variant to be highly enriched in this set (5/62 patients; MAF = 4%, OR 14.46, 95% CI 3.74–48.04). Subsequent analysis of this variant in case-control studies demonstrated significant associations with both melanoma (OR 4.78, 95% CI 2.05–11.75) and renal cancer alone (OR 5.19, 95% CI 1.37–16.87) (Bertolotto et al. 2011). This variant alters a consensus site on the MITF protein that is subject to SUMOylation, resulting in altered chromatin occupancy and transcriptional activity of MITF (Bertolotto et al. 2011; Yokoyama et al. 2011).

PARK2

Based on the observation that melanoma incidence is higher in patients with Parkinson's disease (PD), and vice versa, and that several studies have suggested *PARK2* may be a tumor suppressor gene, Hu and colleagues tested whether germline variants inactivating *PARK2* also conferred increased risk of developing melanoma (Hu et al. 2016). Sequencing and assessment of *PARK2* gene dosage in more than 500 French cases and controls, respectively, revealed that melanoma cases more frequently have *PARK2* copy number variants or splicing and missense mutations than controls (*PARK2* CNVs only, OR = 5.11, 95% CI 1.18–14.97; all *PARK2* alterations, OR = 3.95, 95% CI 1.34–15.75). While replication of these findings is needed, this observation is intriguing, as PARK2 acts as a ubiquitin ligase for cyclin D and cyclin E (Gong et al. 2014), providing a plausible link to a signaling pathway activated in melanoma. Notably, the expression of wild-type *PARK2* in melanoma cell lines was found to reduce colony formation, suggesting that PARK2 may act as a tumor suppressor in melanoma (Hu et al. 2016).

Conclusions

While the number of known high-penetrance melanoma risk genes has expanded considerably in recent years, these genes still only account for <50% of high-density melanoma families. *CDKN2A* remains by far the most mutated risk gene in case-dense families, while no other gene has been found to account for more than 2% of families. Other high-penetrance mutations that affect specific genes likely exist and will have to be discovered by detailed studies of individual families. It is also to be expected that additional nongenic alterations affecting the promoters and other regulatory sequences, untranslated regions, and introns will be found. This hypothesis remains largely unexplored; gene promoters, UTR sequences, and intronic sequence have been covered to a variable degree by current exome-

capture platforms, and larger structural changes are poorly detected by exome sequencing platforms. Some proportion of melanoma families could also be explained by a preponderance of medium- to low-penetrance risk alleles (having a high polygenic load), but some may be explained by shared environmental exposures alone.

A similar challenge remains in terms of identifying variants playing a role in sporadic melanoma cases, where less than half of the contribution of genetics to risk is explained by known, genome-wide significant risk loci. As hinted at by the finding that multiple variants associated with telomere length, most of which are not melanoma risk loci at a genome-wide significant level, are predictive of melanoma risk (Iles et al. 2014), it is likely there are many additional common melanoma risk loci remaining to be discovered by larger meta-analyses of melanoma GWAS. Across a range of complex diseases, each doubling of the GWAS sample size has doubled the number of loci identified; it is hence likely that future melanoma GWAS will identify many more loci contributing to risk. However, the search for rare population variants such as *MITF* E318K remains challenging. Additional whole-genome and -exome sequencing at both the family and population levels, improved genome-wide coverage by better imputation panels, as well as pathway-based studies are poised to make a further impact in characterizing such variants.

Lastly, while there has been much progress in the past several years identifying novel melanoma risk genes and loci, there is considerable work to be done to extend these findings into a better biological understanding of the processes influencing melanoma development. While, for example, common themes appear to have emerged from GWAS studies, functional characterization of which genes mediate risk at many of these loci, how risk-associated genetic variants modulate the function of these risk genes, and how these genes influence phenotypes associated with melanoma development remain challenging. This challenge represents a bottleneck in fully utilizing the wealth of genetic data on melanoma susceptibility generated to date, and in the future. Still, while there is much work to be done in terms of identifying genes mediating melanoma risk, the recent advances outlined in this chapter have highlighted important clues as to pathways whose dysregulation play important roles in melanoma risk and development. Rare, high penetrance mutations that contribute to familial melanoma reveal the role of critical cell-cycle pathway genes. Telomere maintenance genes are impacted by both rare, high penetrance mutations and more common, lower effect size variants. Common and low-frequency variation in genes required for human pigmentation reinforce the role of sun exposure in melanoma development, and genes that alter nevus development and modulate senescence increase the potential for melanocytes to become cancerous.

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Epidemiology of Melanocytic Neoplasia

12

Margaret Anne Tucker

Contents

Abstract

Cutaneous melanoma has increased rapidly worldwide over the past 60 years, moving from a rare cancer to one of the most common in the USA. This epidemic has led to epidemiologic and clinical studies to elucidate risk factors for cutaneous melanoma. The risk of melanoma varies by race and ethnicity. Lower socioeconomic status is associated with more advanced melanoma at the time of diagnosis. Identified risk factors for cutaneous melanoma include ultraviolet light exposure (sun and artificial) and host susceptibility factors such as family history of melanoma, dysplastic nevi,

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increased number of nevi, light pigmentation (skin, hair, and eyes), and immunosuppression. Comprehensive sun/ultraviolet radiation (UV) protection measures, such as those implemented in Australia since the 1990s, are now showing an impact with decreasing incidence of cutaneous melanoma. Mucosal melanoma is distinct from cutaneous melanoma and does not appear to be UV-related. No known risk factors have been identified except for race and gender. Ocular melanoma is also a distinct entity with less evidence of UV exposure as a risk factor than for cutaneous. Host factors include light pigmentation and choroidal/iris nevi. Reciprocal increases in ocular and cutaneous melanoma suggest some common risk factors.

Keywords
Incidence · Mortality · Survival · Risk factors · Nevi · Cutaneous melanoma · Mucosal melanoma · Ocular melanoma

Introduction

Before the early 1970s, cutaneous melanoma was considered a rare cancer; most health-care providers in the USA had never seen one. In Connecticut 1950–1954, the incidence of melanoma was 1.9 per 100,000 men and 2.6 per 100,000 women (Geller et al. 2013). The majority of diagnosed primary melanomas were bulky, advanced tumors, and a substantial proportion of melanomas were diagnosed as metastatic disease. In 2015, melanomas were the fifth most frequent cancer diagnosed in men and seventh in women in the USA, many of which are thin lesions. Disentangling the components of this dramatic change in patterns of disease over a relatively short period of time has been a complicated, global, multidisciplinary effort, as evidenced throughout this book. Melanoma rates vary widely across populations and geographic areas (in part because of differing ultraviolet exposures); no one population or geographic area has the whole answer to this puzzle. Pathologists have been crucial in defining subtypes of melanoma, precursor lesions, and prognostic features. Epidemiologists have defined population patterns of risk and risk factors for melanoma. Clinicians have refined both clinical criteria for melanoma and diagnostic accuracy in detecting early melanoma and have developed targeted therapy for advanced disease. Geneticists have identified high- and low-risk germline susceptibility genes within families and populations and somatic drivers of melanocytic neoplasia. Laboratory scientists have elucidated the molecular mechanisms of melanoma development. Psychologists have explored the societal and behavioral components of melanoma risk. A synthetic classification of melanocytic neoplasia integrating many of these observations has been proposed (Bastian 2014). It has yet to be validated in population studies and is not used currently in tumor registries in which most of the population data are collected. Retrospective classification using this system is not feasible for data collected in the past decades but the system should be informative for future studies including whole genome sequencing of melanocytic neoplasia, where additional somatic driver mutations will likely be identified.

This chapter will primarily focus on cutaneous (skin) melanoma in light-skinned populations where the greatest risk occurs and most research has been conducted but will also explore population differences and other primary sites of melanoma: ocular and mucosal. There are few population-based data on the epidemiology of benign nevi, but they are important risk markers for and precursors of melanoma.

Population Patterns of Cutaneous Melanoma Incidence and Mortality

International Variation

Across the globe, incidence (the rate of newly diagnosed melanomas within a defined population) and mortality (the rate of death due to melanoma within a defined population) vary widely, both by extent of skin pigmentation in the population and by differential exposure to ultraviolet radiation.

Table 1 Estimated age-standardized (to world population) 2012 incidence and mortality rates of melanoma by geographic area (Globocan)

	Incidence		Mortality	
Area	Number	ASR (W)	Number	ASR(W)
World: male	120,649	3.3	31,390	0.9
Female	111,481	2.8	24,098	0.6
Europe: male	47,290	8.6	12,058	1.2
Female	53,152	8.9	10,153	1.3
USA: male	40,078	16.8	6735	2.7
Female	29,031	12.6	3489	1.2
Asia: male	11,639	0.5	6377	0.3
Female	10,191	0.4	5366	0.2
Australia/New Zealand: male	8499	40.3	1406	6.0
Female	6239	30.5	613	2.4
South America: male	5766	2.9	1993	1.0
Female	5250	2.2	1526	0.6
WHO Africa region (AFRO): male	2621	1.2	1424	0.7
Female	3461	1.4	1894	0.8

Table 1 shows the number of melanomas diagnosed by geographic area and gender in five continents and the USA in 2012. The incidence and mortality rates shown are age standardized to the world population which allows comparisons between populations with different age structures. The highest rates of melanoma incidence and mortality are in Australia and New Zealand, and the lowest rates of incidence and mortality are in Africa. Table 1 also demonstrates that both incidence and mortality rates are higher in men than women, except in Europe and Africa, where the rates are slightly higher in women than men (Globocan) (Table 2).

The numbers of skin melanomas diagnosed, and population-specific incidences in varying geographic locations, have changed over time with differing patterns. These differing patterns among populations have complicated the interpretation of data with regard to melanoma etiology. Melanoma incidence has been steadily increasing in populations worldwide over the last several decades, particularly in light-skinned groups. The International Agency for Research on Cancer (IARC) estimates that 250,178 melanomas occurred worldwide in 2015 (Globocan). Of these, 130,800 occurred in men and 119,378 in women. Approximately 30% of global melanomas occurred in the USA overall, with gender (33% of global melanoma in men and 26% in women) and age (28% of global melanoma under age 65 and 32% age 65 and older) differences. Evaluating the patterns of melanoma within the USA, however, is instructive because of the diversity and large size of the population, the number of melanomas diagnosed every year, and the geographic size and latitudes of the country, with comparable data being collected in one reporting system. Complications of using US data are that the medical care system is not unified and the population is quite mobile, making tracking of individuals over time more difficult.

US Population-Based Data

In the USA, cancer rates are tracked by the Surveillance, Epidemiology, and End Results (SEER) Program, initiated in 1973 in the National Cancer Institute (Howlader et al. 2015). SEER started as a population-based registry in nine geographic areas. Since then, SEER has expanded to include a representative 28% of the US population. SEER tracks both new cancers that occur in the population within the SEER registry areas and deaths from cancer in total in the USA. Similar to most nationwide tumor registries, although criteria for diagnoses are used and common coding of the

Table 2 Distribution of primary invasive melanomas by 5-year calendar periods in SEER, 1989–2008 (amended from Shaikh et al. 2013)

Thickness in mm	1989–1993	1994–1998	1999–2003	2004–2008
0.01–1.00	9609 (52.4%)	15,624 (56.8%)	35,881 (60.8%)	49,628 (64.2%)
1.01–2.00	2448 (13.3%)	3918 (14.2%)	7696 (13.0%)	10,345 (13.4%)
2.01–4.00	1430 (7.8%)	2138 (7.8%)	4406 (7.5%)	6042 (7.8%)
>4.00	693 (3.8%)	1197 (4.3%)	2534 (4.3%)	4266 (5.5%)
Unknown	4175 (22.7%)	4631 (16.8%)	8472 (14.4%)	7054 (9.1%)

diagnoses occurs, there is no independent systematic histologic review of diagnoses, and no genetic information (either germline or somatic) is available. SEER sites focus primarily on invasive cancers; data presented here are for invasive melanoma only. The majority of cancer diagnoses collected by SEER are obtained from hospital records, but melanoma diagnoses have required additional steps, since a growing number of melanomas are removed as outpatient surgical procedures, rather than occurring in a hospital setting. SEER enhances retrieval of pathology reports from private dermatopathology laboratories within SEER catchment areas, but there is a lag of several years in obtaining these data (Clegg et al. 2002). This delay can result in an apparent downturn in melanoma incidence in the most recent years of reporting, which is adjusted as new data are obtained. In some SEER reports, delay-adjusted analyses are also provided.

Thickness of melanomas is available in the SEER registry only after 1988, and histologic attributes recommended for standard melanoma pathology reports are inconsistently recorded in the original local pathology reports. In a review of 182,184 cases of invasive melanoma records in SEER from 1989 to 2008, thickness was missing in 13% (Shaikh et al. 2013).

The proportion of unknown thickness decreased significantly with time ($P_{trend} < 0.001$) from 23% in 1989–1993 to 9% in 2004–2008. The analyses focused on the attributes of the melanomas with unknown thickness. Unknown thickness cases were twice as likely to be young (0–14), 40% more likely to be Asian/Pacific Islanders, and 20% more likely to be Hispanics, with poor prognosis attributes and threefold increased risk of death due to melanoma. Survival also appeared to be most similar to melanomas 2.01–4.00 mm thick. Shaikh and colleagues used multiple imputation to estimate tumor thickness, which was associated with approximately 30% increased melanoma survival and was also associated with Clark level (85% increased), but the magnitude of these associations was lower than among the melanomas with measured thickness. The authors raised the appropriate concern that not including the melanomas with unknown thickness in population analyses could bias results since they tend to represent poorer prognosis cases.

Melanoma rates can be separated also by the anatomic site where the primary melanoma arose. Site data are probably least reliable for mucosal sites, which even when known can be challenging to code. Data on deaths are also collected by SEER from death certificates. Incidence rates presented in this chapter are the number of new invasive melanomas per 100,000 people in specified groups (for instance, defined by age, gender, geographic location, location of melanoma, etc.) over specified calendar periods. For rare cancers, such as mucosal or ocular melanomas, incidence may be expressed as new cases per million individuals. Mortality rates are similar to incidence rates, but deaths from melanoma are counted instead of new melanomas. Join-point analyses show areas of inflection in curves when rates are significantly changing and are indicated by the lines in Fig. 1.

Variation in US Melanoma Incidence and Mortality Over Time by Race and Ethnicity

Figure 1 shows the incidence of and mortality from cutaneous melanoma in the USA from 1975 to 2012 for Whites and Blacks and from

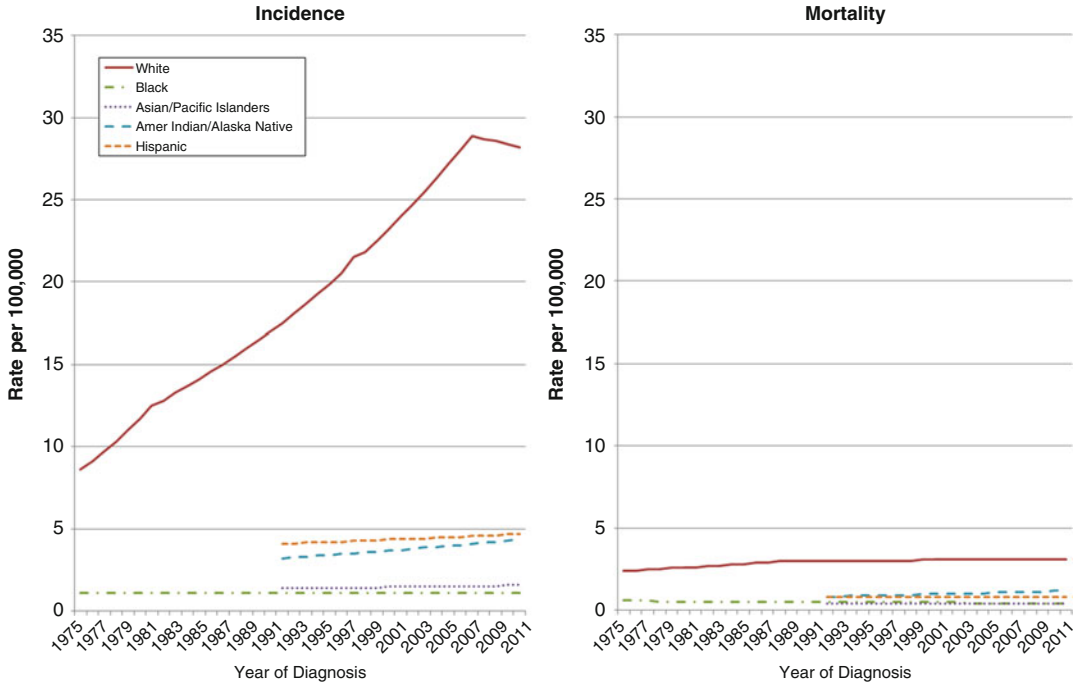

Fig. 1 Join-point analyses of cutaneous melanoma incidence and mortality by race/ethnicity in the US Surveillance, Epidemiology, and End Results Program 1975–2012. Data points are not included for clarity of presentation but may be found at http://seer.cancer.gov/csr/1975_2012/sections.html Fig. 16.2

1992 to 2012 for Asian/Pacific Islanders, American Indians/Alaska Natives, and Hispanics, the racial and ethnic groups captured in SEER data. Hispanic is not mutually exclusive from Whites, Blacks, Asian/Pacific Islanders, and American Indians/Alaska Natives. Melanoma incidence has steadily increased among the White population (with the least pigmentation) over the calendar period. Mortality in Whites has also risen slightly, but the increase is much less than that in incidence. In contrast, the incidence and mortality rates among African-Americans, Native Americans/Alaskans, and Asian/Pacific Islanders have been relatively stable and quite similar. Of note, mortality is proportionately higher in these groups than in Whites. The types and anatomic sites for melanoma differ substantially among lighter- (mostly Whites) and darker-pigmented (Blacks, Asian/Pacific Islanders, and American Indians/Alaska Natives) individuals. The most common site among the lightly pigmented individuals is the trunk, followed by the limbs, and then the head and neck. The sites and subtypes vary by age and gender. Among more deeply pigmented individuals, the most common site of melanomas is on the sole of the foot or palm of the hand or subungual (underneath the nail, usually the great toe or thumb). These acral lentiginous melanomas usually occur at older ages and are frequently more advanced lesions with a poorer prognosis. The incidence rates of acral lentiginous melanomas are similar in all races and ethnicities, but they account for differing percentiles of total melanomas in different groups. They also tend to have a different pattern of somatic mutations than the melanomas arising in sun-exposed skin of less pigmented individuals (see ▶ Chap. 7, "Molecular Genetics of Melanocytic Neoplasia"). Given the lack of UV exposure in the acral sites, and the stability of the rates of these melanomas across differing skin pigmentation, latitudes, and ambient UV flux, it is unlikely that these melanomas develop in response to UV exposure. More deeply pigmented individuals may also develop any of the other melanoma subtypes, in sun-exposed or protected sites, but at lower rates than Whites. The

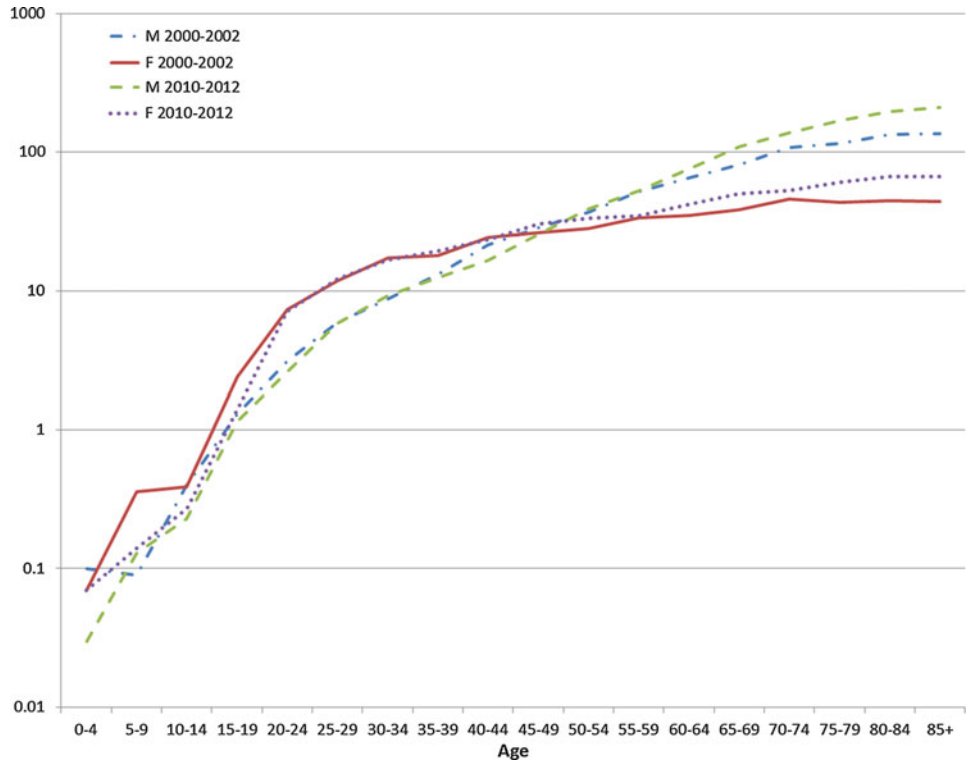

Fig. 2 Age-specific incidence per 100,000 Whites, by gender, of cutaneous melanoma in the US Surveillance, Epidemiology, and End Results Program, 2000–2002 and 2010–2012

relative site and stage distributions of melanomas differ, however. Melanomas are more frequent in the lower limbs in more highly pigmented men than in White men (likely due to the higher percentage of acral lentiginous melanomas). More deeply pigmented women have higher stage disease at diagnosis than white Women (Park et al. 2012). In the USA, non-Hispanic whites tend to be in higher socioeconomic status and to have thinner superficial spreading melanomas, but Hispanics are more likely to have lower socioeconomic status and have melanomas thicker than 2 mm, often acral lentiginous or nodular (Pollitt et al. 2011). One problem contributing to the advanced stage of disease detection among more pigmented individuals is that they may not realize they are at risk and can develop melanoma, in part because of past public messaging about melanoma. Another problem for early detection of melanomas in the USA in the past has been the lack of medical care coverage for many individuals (Amini et al. 2016).

Variation in US Melanoma Age-Specific Incidence by Gender and Calendar Period

Figure 2 shows the age-specific incidence of melanoma in US white males and females in two time periods, 2001–2002 and 2011–2012 (Howlader et al. 2015). In early childhood (before age 10), there is little separation of the incidence between genders in the USA, Germany, Great Britain, or Australia (Howlader et al. 2015; Brecht et al. 2015, Wallingford et al. 2015). These rare, early melanomas may arise in large congenital nevi (often identified by multiple overlapping sites). Melanomas in early childhood may also arise from Spitz nevi, but it is difficult in population-based cancer registries to identify systematically melanomas arising from Spitz nevi without conducting an expert pathology review. Limited data suggest that less than 20% of pediatric melanomas are

Spitzoid melanomas (Brecht et al. 2015). Data from a Spitzoid tumor registry suggest that the mean age at diagnosis of Spitzoid melanomas is 55 and of Spitz nevus is 22 (Lott et al. 2014). Spitz nevi have characteristic somatic genetic alterations (see ▶ Chap. 18, "Spitz Tumors"). In contrast, based on limited numbers of tumors analyzed, Spitzoid melanomas defined by histology show similar somatic genetic changes as unselected melanomas (Lazova et al. 2017).

From age 10 on, the rates of melanoma increase more rapidly in young females than males in the USA, Germany, Denmark, Great Britain, and Australia (Campbell et al. 2015; Brecht et al. 2015; Helvind et al. 2015; Wallingford et al. 2015). In these countries, the distribution of subtypes and anatomic sites of melanoma after age 10 is similar to adult melanomas. In Germany and in England, the trunk is the most frequent site of melanoma in males and legs in females in teenage years (Brecht et al. 2015; Wallingford et al. 2015). After age 10 in the USA, the incidence rates in females into young adulthood have risen steadily over time. Although in the past, similar to adult women, legs were the most frequent anatomic site among teenage girls, currently among teenagers and young adult women (under age 40), truncal melanomas are increasing more rapidly than any other site or age group (Bradford et al. 2010). This change, with the trunk increasing more rapidly than other sites, may be related to patterns of UV exposure and tanning practices in these specific birth cohorts. A similar pattern of female excess in melanoma through young adulthood, with the most common site changing from the lower extremity to trunk, is also seen in Denmark (Helvind et al. 2015). Among Australian teenagers, the trunk was the most common site for both sexes (Wallingford et al. 2015). Australia has had an active public health awareness program and extensive public education about UV exposure and sun-protective practices since the early 1980s. Since the early 1990s, incidence rates of melanoma have decreased substantially in both younger children and adolescents in Australia but have continued to increase in England. These trends suggest the important role of comprehensive UV protection programs, especially at early ages, in preventing melanoma (Wallingford et al. 2015).

Limited data from SEER suggest similar patterns in the USA in those under 20 years old from 2000 to 2010. For boys 15–19, the annual decrease was approximately 8% per year from 2000 to 2010. For girls 15–19, from 2000 to 2003, there was a roughly 17% increase per year; from 2004 to 2010, this trend changed to an 11% decrease per year. The authors suggest that the change in incidence compared to earlier years might reflect the impact of sun protection education since the 1990s or perhaps decreased outdoor activities (Campbell et al. 2015). If the delay-adjusted incidence in the most recent years does not substantively increase (with additional melanomas from private histopathology labs reported to SEER), this would be an encouraging trend.

As seen in Fig. 2, the female predominance in the US Whites lasts until the late 40's in age, after which the incidence in men exceeds the incidence in women. Previously, the rate of increase slowed substantially in women in this age range, accentuating the gender difference as the rates in men rapidly increased. In 2011, the rate of increase in women was similar over the older age range (cumulative doubling of the incidence from age 50–54 to 85+), but the rates in men increased substantially (cumulative 5.5-fold increase from 50–54 to 85+) (Guy et al. 2015). In Denmark, the female incidence rates remain higher than male rates until age 60 and older (Helvind et al. 2015). The male excess at older ages has been of particular concern because the lesions tend to be thicker melanomas, often of the nodular subtype, associated with increased mortality. The rate of ulceration, a poor prognostic feature, is also highest in older men with thicker lesions (over 2.01 mm) and is approximately double that of older women with the same thickness lesions (Richardson et al. 2014). The melanoma mortality in the USA for 50–54 year old women is 2.0, increasing to 14.6 per 100,000 for those over age 85. For men, the melanoma mortality for 50–54 year olds is 3.6, increasing to 40.7 per 100,000 for those over 85 (Guy et al. 2015).

Variation in Melanoma Survival

Survival also differs between the sexes, with women having better survival than men after adjustment for all clinical and demographic risk factors. In both Australia and the USA, men are older at the time of diagnosis, have thicker or ulcerated lesions, and are less likely to have melanomas on the arms or legs. Even after thin melanomas, however, survival is better in women than in men. The gender differences by age groups are broadly consistent with ages of hormonal differences in women, suggesting that this hypothesis could be explored further (Khosrotehrani et al. 2015).

Melanoma is one of the most common cancers occurring during pregnancy, a time of altered immune status. Byrom et al. (2015) and colleagues conducted a systematic review and meta-analysis of published data from cohort studies of women of childbearing age with confirmed diagnoses of melanoma. Eleven studies compared risk of melanoma death among women diagnosed during pregnancy (and up to 1 year postpartum) to those diagnosed when not pregnant. In five of six studies including only primary melanomas without known metastases, there were no significant differences in melanoma mortality. In the five including all stages of melanoma, three also showed no differences in melanoma mortality, accounting for stage at diagnosis. Quantitative mortality estimates were reported in four studies; the pooled hazard ratio was 1.56 (1.23–1.99). Although this hazard ratio is of concern, it is based on relatively limited data.

Survival also varies by calendar period, driven in large part by the decreasing thickness of melanoma over time. Melanoma thickness has been incorporated into the SEER data since 1988. Thickness, however, is missing for a varying proportion of reported melanomas particularly in the early years. A concern that has arisen is that the increase in incidence rates and the improving survival are due to overdiagnosis of very thin lesions. Incorporating imputed data for thickness, incidence overall and within each thickness category (T stage) increased significantly over a 20-year period. Five-year survival also increased for each thickness group, and the median thickness of each group decreased. Since melanoma is by far most common in Whites, these results largely reflect Whites. Other racial and ethnic groups did not show the same benefits (Shaikh et al. 2016). These data, including increases in thicker as well as thinner lesions, suggest that the increasing incidence of melanoma is not due solely to overdiagnosis of very thin lesions.

The concern about overdiagnosis of nevi or in situ melanomas as thin invasive melanomas is based in large part on the dramatic increase of melanoma diagnoses disproportionate to increases in mortality (in the prior absence of effective treatment for metastatic disease) (see Fig. 1). For example, in 1950–1954 in Connecticut, the overall melanoma incidence in men was 1.9 (per 100,000) and in women 2.6 (per 100,000); by 2007, these rates had increased to 33.5 for men and 25.3 for women. Over the same calendar period, the mortality has increased from 1.6 to 4.9 (per 100,000) for men and from 1.3 to 2.6 (per 100,000) for women (Geller et al. 2013). Similar patterns of increases in other geographic areas have led some to postulate overdiagnosis of thin invasive melanomas. In the USA, this could be a valid concern, since misdiagnosis of melanoma (usually missing the diagnosis rather than overcalling thin melanomas) is one of the most frequent reasons for malpractice suits. In a recent survey, one-third of dermatopathologists reported that they had had past malpractice experiences. Most respondents felt that malpractice concerns increased the likelihood of doing additional sections and specialized stains and seeking a second opinion to ensure best care for the patients, independent of whether they had previous experience with a malpractice suit (Carney et al. 2016). Clinically, the differences in excision margins and outcome of a severely dysplastic nevus versus in situ melanoma or an in situ melanoma versus very thin melanoma are relatively small. Previously, however, in the USA, those differences in diagnosis could have major implications for obtaining insurance, before the Affordable Care Act. The differences in risk of progression to metastatic disease between these lesions are also small, and an individual who has

developed a severely dysplastic nevus needs similar surveillance to that of an individual who has had an early invasive melanoma so that the differences in clinical management are minimal (Pomerantz et al. 2015).

Thin invasive melanomas, however, may not be a trivial diagnosis. Even though the risk of progression and metastases is quite low with thin invasive melanomas, in the population with the highest incidence rate of melanoma, Queensland and Australia, more individuals die of melanoma after a diagnosis of thin (≤1 mm) melanomas than thicker (4+ mm) (Whiteman et al. 2015). This is largely due to the majority of melanomas being diagnosed early. The Australian population should be the group in which the phenomenon of more deaths from thin melanomas than from thicker would be recognized sooner than in other populations because of the comprehensive implementation of education about UV protection and early detection of melanoma since the early 1980s. Since then, the percentage of individuals diagnosed by metastases only with no known primary and those with unknown thickness decreased in both incidence and mortality. The percentage of thin melanomas to which death was attributed, however, increased from 14% in 1990–1994 to 23% in 2005–2009. It is also important to note that the latency between diagnosis of thin melanoma and death has increased from median of 5 years (25% died by 3 years; 75% by 6 years) in 1990–1994 to 7 years (25% died by 4 years; 75% by 13 years) in 2005–2009 (Whiteman et al. 2015). The later data may reflect more complete capture of delayed recurrences. In their accompanying commentary, Geller and colleagues appropriately point out that prognostic attributes for recurrence in thick melanomas also pertain to thin melanomas. They questioned whether these attributes were present in those who died and whether their lesions were, in fact, closer to 1 mm than much thinner (Geller et al. 2015). In addition, it will be important to assess if specific somatic alterations also predict risk of recurrence, but this is logistically difficult with the small size of the tumors and the challenges of working with formalin fixed tissues with melanin.

Projections of Future US Melanoma Incidence and Mortality

Using US melanoma data from 1982 through 2011, Guy (2015) projected US melanoma incidence and mortality through 2030 and estimated costs of treatment and potential cost savings, if a nationwide UV protection program were implemented, similar to SunSmart in Australia. As shown in Fig. 1, the incidence of melanoma is highest in non-Hispanic Whites and in Fig. 2, higher in men than women and increases with age. Mortality also increases with age for both genders. Incidence rates are projected to increase for White males and females though 2019, but mortality rates are projected to be stable. Using population data and gender-specific melanoma treatment costs, Guy projected treatment costs for melanoma with and without a reduction in melanoma from implementing a UV protection program, with a 5-year lag period between implementation of the program and reduction of melanoma incidence. Without new UV interventions, 112,000 new melanoma cases are projected for 2030. A comprehensive skin cancer prevention program could decrease melanoma incidence by 20%, for a total reduction of 230,000 new melanomas between 2020 and 2030. Medical costs of treating newly diagnosed melanomas are estimated to increase >250% from 2011 to 2030 ($457 million to $1.6 billion). The reduction in new cases from the UV protection program would save approximately $250 million a year from 2020 to 2030 for a total of $2.7 billion (Guy et al. 2015). Guy was using estimates of treatment cost from 2011, before the new expensive targeted therapies for metastatic melanoma were generally used; these are, therefore, very conservative cost savings estimates if widespread sun/UV protective practices were to be implemented.

Risk Factors for Cutaneous Melanoma

As with other common adult onset cancers, melanoma develops as a result of complex interactions of environmental exposures and host susceptibility factors, many of which have strong genetic

components. The major genetic determinants of melanoma susceptibility will only be very briefly mentioned in this chapter but are comprehensively covered in ▶ Chaps. 7, "Molecular Genetics of Melanocytic Neoplasia" and ▶ 11, "Inherited Contributions to Melanoma Risk" and others.

Solar and Artificial UV Radiation

The recognized epidemic of cutaneous melanoma has led to multiple etiologic studies worldwide, starting in Australia, which has the highest rates of melanoma. Almost all have demonstrated that the major environmental risk factor for melanoma is ultraviolet radiation exposure, whether natural from the sun or artificial from tanning beds or sunlamps. The first studies to convincingly implicate ambient UV levels as a risk factor for melanoma were the studies beginning in the 1950s of the migrants to Australia (Whiteman et al. 2011). At the population level, residential latitude and measures of ambient UV from the sun, along with various measures of time spent outdoors have been used to estimate exposure. The majority of the individual epidemiologic data has been reconstruction of retrospective dose collected from questionnaire data. Lifetime residential data has been used to estimate exposure to measured ambient UV. Recently initiated studies, however, have started to employ wearable UV monitors to sample daily activities over specified times (Petersen et al. 2015). In high sun areas, the major exposures are often ambient UV and time outdoors, modified by extent and type of sunscreen use and sun-protective measures, time of day of exposure, clothing worn including types of hats, recreational activities, and occupational activities. Recall of these routine types of activities is often imprecise and subject to "averaging" over a lifetime. Even in high sun areas, however, tanning bed use is an important risk factor for melanoma among users (Cust et al. 2011). For those in low ambient UV areas, such as Northern Europe, the major UV exposures often occur with vacations in sunny areas (which are often more distinctly remembered) or tanning bed use (Nielsen et al. 2012). Early on in studies of the etiology of melanoma, these variations in types of exposure in different populations were the source of energized discussions about whether intermittent or chronic exposure was the dominant cause of melanoma.

Table 3 shows the results of meta-analyses of both sun exposure and use of tanning devices. Over time, it has become clear that both chronic exposure and intermittent exposure are important for melanoma risk with differences in measurements and in populations (Gandini et al. 2005) and lead to different sites and types of melanoma (Whiteman et al. 2011). Occupational exposure has been complicated to use as a measure, partially due to inherent bias in the selection of working outdoors; individuals who burn easily are less likely to choose high sun exposure employment without protection in a high ambient UV area. Despite the plethora of approaches to assessing UV exposure, the associations are relatively consistent. Lentigo maligna melanoma seems to be the subtype most strongly associated with chronic exposure, typically occurring on the face, neck, hands, and lower arms and legs and frequently related to occupational exposures in the past or extensive recreational time outdoors without sun protection. These lesions often occur in older individuals with badly sun-damaged skin (cumulative sun-induced damage) who often have a previous history of non-melanoma skin cancers (Bastian 2014). The most frequent subtype of melanoma, superficial spreading melanoma, tends to occur on intermittently exposed areas of the body, such as the trunk, upper arms, and legs, often arising from a precursor nevus.

Other measures of UV exposure (and UV sensitivity) that have been used to assess melanoma risk include number of sunburns and age at sunburns (Gandini et al. 2005). Sunburn is a complicated exposure measure, however, because it reflects not only intensity and possible duration of UV exposure but also the individual susceptibility to sunburn, largely a result of genetic factors including pigmentation and in rare cases (such as individuals with Xeroderma pigmentosum), DNA repair mechanisms (see ▶ Chap. 11, "Inherited Contributions to Melanoma Risk"). As shown in Table 3, sunburns are associated with a higher

Table 3 Meta-analysis of relative risks (RR) of melanoma by different types of UV exposure

Exposure	Years of studies	# Studies included	RR (95% CI)	Citation
Sun exposure	1969–2002	57		Gandini et al. (2005)
Total sun	1969–2002	15	1.43 (1.02–1.77)	
Intermittent	1982–2002	33	1.61 (1.31–1.99)	
Chronic sun	1981–2002	41	0.95 (0.87–1.04) (latitude dependent)	
Sunburns	1982–2002	33	2.03 (1.73–2.37)	
Indoor tanning	<2013	31		Colantonia et al. (2014)
Ever/never	1981–2012	31	1.16 (1.05–1.28)	
Before 2000	1981–2008	21	1.12 (1.00–1.26)	
After 2000	2005–2012	10	1.22 (1.03–1.45)	
>10 sessions	1994–2012	10	1.34 (1.05–1.71)	
By continent				
North America	1986–2012	8	1.23 (1.03–1.47)	
Europe	1981–2012	21	1.10 (0.98–1.24)	
Oceania	1986–2011	2	1.33 (0.99–1.78)	
First use <25 years	1998–2012	6	1.35 (0.99–1.84)	
First use ≥25 years	1998–2012	6	1.11 (0.86–1.42)	

risk of melanoma than the various other measures of UV exposure, likely due to sunburns occurring in more susceptible individuals. Freckling has also been assessed as a risk factor for melanoma, and this, too, is a complex measure of both UV exposure and host susceptibility (particularly variations in *MC1R* and other pigmentation genes) (see ▶ Chap. 11, "Inherited Contributions to Melanoma Risk"). Seasonal variation in the diagnosis of melanoma has also been noted in multiple populations, with higher rates of melanoma diagnosis (particularly superficial spreading melanomas) in summer than in winter (Walter et al. 2015), even in tropical climates, perhaps related to evolution of pigmented lesions to invasive melanoma.

The most recent UV exposure that has been extensively evaluated is the use of sunlamps/tanning beds for tanning and maintaining a tan. This, too, has been a complex exposure to disentangle from other exposures, because individuals who tan usually seek both outdoor and indoor UV exposure. In 2009, IARC conducted an expert review of the data regarding the carcinogenicity of tanning beds and considered them carcinogens (El Ghissassi et al. 2009). Meta-analyses of tanning bed exposure have been conducted, but they do not usually account for the environmental UV exposure and tanning (Colantonio et al. 2014) (Table 3). In contrast, Cust et al. (2011) found in young women who used sunbeds multiple times had approximately doubled melanoma risks after adjustment for host factors and sun exposure. In the Swedish cohort of women aged 25–39 at enrollment, both sunny vacation and sunbed use were risk factors for melanoma (Nielsen et al. 2012). In Sweden, UVB in sunbeds was restricted by law in 1982 so that the tanning bed exposure was predominantly UVA. Among those under the age 40, the risk of using a tanning bed more than ten times a year was more than doubled. In an earlier study of adults in Minnesota where the ambient UV is relatively low, tanning beds were the participants' dominant source of UV exposure, approximately doubling their risk of melanoma Lazovich et al. (2010). Risk of melanoma increased with more years of use, total hours of use, and longer sessions at a time. UVB-enhanced devices tripled risk of melanoma, and primarily UVA devices quadrupled risk.

Since 2009, multiple countries have restricted access to tanning beds. Similar to their much earlier adoption of UV protection, Australia was early in control of commercial tanning establishments. In 2015, Australia banned commercial tanning. Several of the provinces of Canada have banned access to commercial tanning establishments to individuals under 18. In December 2015, the US Food and Drug Administration proposed rule changes for access to sunlamp/sunbed products. Only individuals aged 18 and older would be able to use commercial sunlamp/sunbed products. Before their first tanning session and every 6 months afterward, users would have to sign a risk acknowledgement certification before being allowed access to a tanning device. Additional safety improvements in the tanning devices would also be required (FDA 2015).

A relatively new insight into tanning behavior has occurred in the past several years. There is a growing body of evidence that tanning, either outdoor or indoor, is likely an addictive substance abuse-related disorder, as measured by either DSM IV TR (Diagnostic and Statistical Manual of Mental Disorders, 4th edition) criteria or by CAGE (cut down, annoyed, guilty, eye-opener) criteria (Warthan et al. 2005). After UV exposure, skin keratinocytes synthesize the prohormone peptide proopiomelanocortin that is metabolized to melanocyte stimulating hormone, which induces tanning (Fell et al. 2014). Fell and colleagues developed an elegant mouse model to demonstrate that in mice, following UV exposure, skin keratinocytes also release β-endorphin, another peptide derived from proopiomelanocortin. After chronic UV exposure, opioid blockade induces withdrawal signs. The withdrawal effects were sufficient to condition the mice to avoid withdrawal. These findings have substantial implications for approaches to try to modify tanning behavior in humans.

Host Susceptibility Factors

The major host risk factors conferring high melanoma susceptibility are largely genetic, covered in ▶ Chap. 11, "Inherited Contributions to Melanoma Risk". Family history of melanoma is an important risk factor but is not frequent. When melanoma was still a rare cancer, two or more relatives with melanoma were sufficient to identify high-risk families. As melanoma has become more common, current criteria usually require three or more relatives, except in geographic locations where melanoma is still not a frequent cancer. Several high-risk susceptibility genes have been identified in melanoma-prone kindreds over the last two decades. More recently, multiple genes identified through genome-wide association studies (using germline DNA) and candidate gene studies account for an unknown percentage of familial melanoma. Prominent among the genome-wide association findings are pigmentation genes, variants in which may be associated with freckling on sun exposure. These variants explain at least part of the associations with fair skin, light hair and eyes, and freckling, phenotypes that are well-described risk factors for cutaneous melanoma, conferring risks generally in the two- to fourfold increased level in multiple populations. In addition, cell cycle control genes have also been identified by the genome-wide studies. The germline genetic alterations associated with nevi, the major risk markers for, and precursors of melanoma have been more challenging to identify, but some progress has occurred. Part of the difficulty in discovering the genetics of nevi is the heterogeneity in classification and identification of nevi over time in the melanoma studies where germline biospecimens have been systematically collected.

Dysplastic nevi are distinctive lesions first described in the context of familial melanoma, but it was soon apparent that they occurred outside of the familial melanoma setting. The clinical criteria for dysplastic nevi include at least 1 diameter $\geq$5 mm, a flat component, and at least two of the following attributes: variable pigmentation; irregular, asymmetric outline; and indistinct borders. All families participating in the NCI familial melanoma study have dysplastic nevi. Within these families, dysplastic nevi are dynamic lesions. They tend to become clinically evident in adolescence and to disappear/involute with increasing age. With increased UV exposure,

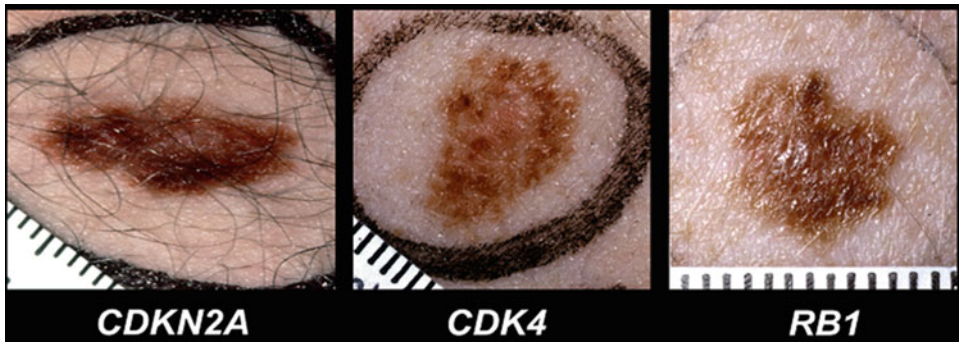

Fig. 3 Dysplastic nevi in individuals with high-risk mutations in *CDKN2A*, *CDK4*, and *RB1* and previous melanoma. All lesions meet the clinical criteria for dysplastic nevi, with at least 1 diameter ≥5 mm, a flat area, irregular, asymmetric outline, indistinct borders, and variable pigmentation

new nevi appear and existing nevi morphologically change. With age, prolonged UV protection, or systemic treatment of advanced melanoma, dysplastic (and other) nevi involute over time (Tucker et al. 2002). Figure 3 shows clinically diagnosed dysplastic nevi from individuals who have had melanoma who also carry high-penetrance melanoma susceptibility genes: *CDKN2A*, *CDK4*, and *RB1*. All of these nevi meet the above criteria.

After the identification of nevi as important risk factors for melanoma, the epidemiology of nevi has been more rigorously investigated. There are few, if any, representative general population assessments of nevi, in large part because they are not reportable or registerable conditions. Nevi have been enumerated in epidemiologic studies by multiple mechanisms from self-reports either by counting moles on an arm or by comparing the pattern of moles on the back to a schematic chart or rarely by full-body nevus counts and classification by trained professionals. Advantages of self-reported nevus counts are that they are more readily accomplished and much less expensive to obtain and for these reasons may possibly be more representative of the general population. Advantages of counts by trained professionals are much less misclassification of lesions and better discrimination between types of nevi. Virtually all studies that have tried to enumerate nevi have found that increased number of nevi is an important risk marker for melanoma. Meta-analyses of nevi as risk factors for melanoma are subject to misclassification by differing ascertainment of nevus counts and types. Few studies have separated the risks associated with common acquired nevi, small and large, and dysplastic nevi. Table 4 shows the mutually adjusted risks of melanoma associated with different types of nevi characterized by expert examiners classifying the nevi and conducting the counts (Tucker et al. 1997).

As shown in Table 4, clinically identified dysplastic nevi confer much higher risks of melanoma than increased number of small or large common acquired nevi. Approximately half of the melanoma cases had documented dysplastic nevi, and approximately one-tenth of controls had clinically confirmed dysplastic nevi. These controls had agreed to a full-body skin exam, photography, and possible nevus biopsy, which they might have been more willing to do if they had pigmented lesions of concern to them. This estimate of dysplastic nevi in controls, therefore, may be somewhat inflated in comparison to a random sample of the White population. Virtually all of the individuals over age 50 with clinical dysplastic nevi were cases (Table 5).

Among the individuals without dysplastic nevi, risks increased with increasing numbers of small and large nevi to about a fivefold maximum increase in risk among those with large numbers (≥50) of intermediate nevi (2–4 mm) and more than five large nevi (5 + mm). No increased risk of melanoma was associated with the presence of congenital nevi in this study.

Table 4 Adjusted relative risks of melanoma by nevus type and number (Tucker et al. 1997)

Number of nevi by type	Number of cases	Number of controls	Adjusted RR (95% CI)[a]
Nevi >2 mm and <5 mm			
0–24	258	658	1.0
25–49	163	190	1.8 (1.3–2.5)
50–99	169	107	3.0 (2.1–4.4)
≥100	123	43	3.4 (2.0–5.7)
Nondysplastic nevi ≥5 mm			
0	239	507	1.0
1	135	224	0.9 (0.7–1.3)
2–4	188	195	1.3 (1.0–1.8)
5–9	86	51	1.7 (1.0–2.7)
≥ 10	65	21	2.3 (1.2–4.3)
Congenital nevi			
0	605	881	1.0
1	74	85	1.1 (0.7–1.6)
≥2	34	32	1.3 (0.7–2.5)
Dysplastic nevi			
0	301	778	1.0
Indeterminate	72	127	1.0 (0.7–1.6)
1	64	50	2.3 (1.4–3.6)
2–4	121	33	7.3 (4.6–12)
5–9	45	15	4.9 (2.5–9.8)
≥10	55	6	12 (4.4–31)

[a]Mutually adjusted and adjusted for age, sex, center, referral pattern, morphologic dysplastic nevi<5 mm, sunburns, freckles, solar damage, scars, nevus excisions, and family history of melanoma

Table 5 Relative risk of melanoma according to nevus categories among study subjects without evidence of dysplastic nevi or clinically atypical nevi of any size, adjusted for age and freckling (Tucker et al. 1997)

Number of large nevi	Number of small nevi	Number of cases	Number of controls	RR (95% CI)
0	<25	68	285	1.0
	25–49	22	40	2.5 (1.4–4.5)
	≥50	11	25	2.1 (1.0–4.6)
1	<25	31	120	1.1 (0.7–1.8)
	25–49	7	28	1.1 (0.5–2.8)
	≥50	7	15	2.1 (0.8–5.6)
2–4	<25	28	77	1.4 (0.8–2.3)
	25–49	14	31	1.9 (0.9–3.7)
	≥50	19	20	3.9 (2.0–8.0)
≥5	<25	9	9	3.8 (1.4–10.0)
	25–49	9	11	3.2 (1.3–8.4)
	≥50	18	17	4.6 (2.2–9.6)

The advent of dermoscopy to characterize and follow nevi over time has changed clinical practice of skin surveillance and indications for biopsy of lesions (see ▶ Chap. 13, "Prevention of Cutaneous Melanoma"). The natural history of and risk factors for increased numbers of nevi have also been studied, with the ultimate goal to decrease total number of nevi and risk of melanoma. Childhood midday sun exposure without UV protection is positively associated with number of nevi (English et al. 2005). Overall nevus number rises with age, although the process is

dynamic. Prospective studies with active follow-up and photographic surveillance have documented development of new nevi, involution of existing nevi, and evolution of nevi, even in low ambient UV areas (Scope et al. 2011). Nevus number changes (both increases and decreases) were more frequent in those with higher numbers of nevi. Children who had had multiple sunburns at the beginning of the study were also more likely to develop pattern changes in their nevi.

Most investigations of risk factors for melanoma have been conducted in White populations, in which the majority of melanomas occur (Fig. 1). Few investigations have evaluated risk factors for melanoma among Asian/Pacific Islanders, Hispanics, and mixed-race individuals. In comparison to Whites, the incidence rates of melanoma are much lower, and melanoma histology, anatomic distribution, thickness, and stage vary in both sexes in high sun areas (Park et al. 2012).

For the risk factor evaluations, acral lentiginous melanomas were not included because they are not likely to be UV related ($n = 2$ in whites; $n = 10$ in nonwhite/multiracial individuals). In the nonwhite/multiracial group, age at entry into the cohort, male gender, higher education, natural hair color, eye color, sunburn phenotype score, and history of non-melanoma skin cancer were all significantly associated with melanoma risk. Information on nevi was not available (Park et al. 2012).

Risk Prediction Models for Melanoma

The identification of multiple risk factors for melanoma has led to the formulation of several risk prediction models which in large part have been developed to identify those individuals who might benefit from intervention, closer surveillance, and/or screening. Vuong et al. (2014) conducted a systematic review of 19 studies reporting 28 risk prediction models. The most common elements included were nevi, skin type, freckle density, age, hair color, and sunburn history. They found large differences among the studies in model development and performance; few studies assessed internal or external validity of the models developed or their use in clinical or population studies. They concluded that the risk prediction tool developed by Fears and colleagues for use by primary care providers in the US population appeared to be the most clinically useful and could potentially assist in identifying high-risk groups for melanoma prevention strategies. Given the large variation in melanoma incidence across populations, it is likely that successful risk models will need to incorporate population- and geography-specific data.

Medical Conditions Associated with an Increased Risk of Melanoma

Subsequent melanoma has been associated with multiple medical conditions, particularly those affecting the immune system. Prominent among these are organ transplantation, lymphoproliferative cancers, treatment with immunosuppressive therapy for autoimmune disorders, and HIV infection. A recent study of melanoma following organ transplantation evaluated melanoma risk and survival in the USA (Robbins et al. 2015). Melanoma incidence among non-Hispanic Whites was doubled after organ transplantation, and regional metastatic disease was quadrupled. Although primary melanoma rates were stable over time after transplantation, regional metastatic disease varied by time since transplantation and by type of immunosuppression. Melanoma mortality was fivefold increased for thin melanomas excised posttransplantation and doubled for thicker melanomas. A common thread in the increased risk of melanoma in immune-deficient conditions is the poor outcome after more advanced disease, likely due to immunosuppression of varying degrees. The high risks and increased mortality among those developing melanoma after organ transplantation argue for vigorous surveillance and screening in this group.

Significantly increased risk of cutaneous melanoma is also observed in individuals with previous cutaneous melanoma; the risk of a second primary melanoma overall in both sexes is 8.80, ranging from 15.41 in the first year to 6.00 at

20+ years (Curtis et al. 2006). Risk of cutaneous melanoma is also significantly increased after ocular melanoma in both sexes (overall risk 3.99), suggesting that they may share some common risk factors (e.g., pigmentation, possibly UV exposures, and susceptibility genes). Risk for developing a primary melanoma is also significantly increased after multiple other cancers in SEER, including the small intestine (almost doubled) usually after small intestinal sarcoma, breast (20% increased), prostate (14% increased), kidney (30% increased), soft tissues (80% increased), brain (about 50% increased), Hodgkin lymphoma (60% increased), non-Hodgkin lymphoma (60% increased), chronic lymphocytic leukemia (more than doubled) with higher risks among those diagnosed at age <50 (more than tripled), and childhood cancer (more than quadrupled overall, with 40% occurring after previous melanoma and two after other soft tissue sarcomas [12-fold increased]). After cutaneous melanoma, significantly increased risks of salivary gland (70% increased), small intestine (60% increased), female breast (about 10% increased), prostate (17% increased), kidney (about 30% increased), soft tissue (more than doubled), thyroid (doubled), and non-Hodgkin lymphoma (25% increased) are observed. The cancers with reciprocal risks of similar magnitude suggest some common etiologic factors. Similar patterns are seen in other populations.

Mucosal Melanoma

Mucosal melanomas may arise from any mucosal surface that contains melanocytes. McLaughlin et al. (2005) evaluated epidemiologic characteristics of mucosal melanomas in data collected from 1996 to 2000 by the North American Association of Central Cancer Registries covering 62% of the US population. Mucosal melanomas comprise approximately 1.4% of melanomas in the USA, with incidence rates in women (2.8 per million) 86.7% higher than those in men (1.5 per million); the male to female rate ratio is 0.54 (95% CI 0.50–0.61). Incidence increases with age (Fig. 4).

The most frequent site in men is the head and neck, including the nasal cavity, accessory sinuses, and oral cavity, all of which have incidence less than 0.5 per million. In women, the most frequent site is the genital tract with 1.6 per million incidence, predominantly the vulva and vagina. Head and neck mucosal melanomas are the second most common site in women, and the genital tract is the second most common site in men, predominantly the penis. Anorectal melanoma comprises 16.5% of mucosal melanoma with minimally higher incidence in women than men but both below 0.5 per million. Incidence of mucosal melanomas varies by race; the incidence in White men is approximately doubled compared to Black men. The incidence in White women is also almost double that of Black women (McLaughlin et al. 2005).

In both races, mucosal melanomas are diagnosed at more advanced stages than cutaneous melanomas. For the genital sites, only about two-thirds are diagnosed with localized disease, about one quarter with regional, and about one-tenth with metastatic disease. The other sites are even more advanced, with only 41.3% diagnosed with localized disease, 33% with regional, and 25.7% with metastatic disease (McLaughlin et al. 2005).

Between 1992 and 2011 in SEER, the incidence rates of anorectal melanoma increased 80-fold between ages 25 and 84 (Callahan et al. 2016). The incidence was minimally higher in women than in men; 64% of the cases were women. Incidence also varied by race and ethnicity. Hispanic Whites had slightly higher incidence rates than non-Hispanic Whites; the highest rates were among elderly Hispanic women. Incidence for Blacks and American Indian/Alaska natives was about half that of Whites; for Asian/Pacific Islanders, the incidence was about the same as Whites.

Specific risk factors (other than gender, race/ethnicity, and age) or precursor lesions for mucosal melanomas have not been identified to date. Conducting case control or cohort studies to identify exposures or other risk factors is challenging, with so few melanomas, most of which are

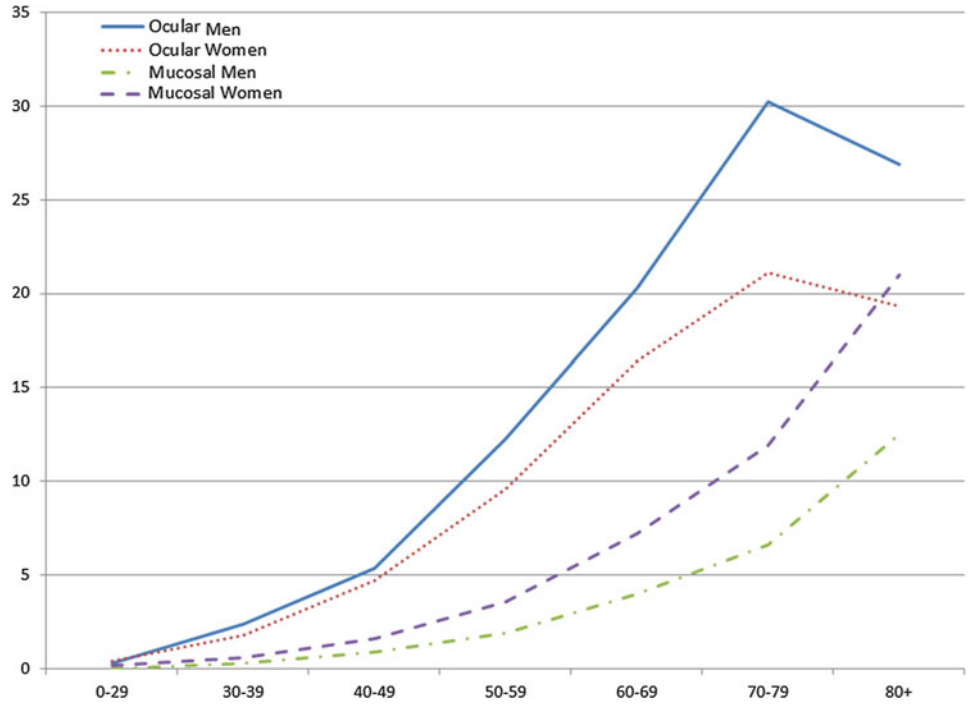

Fig. 4 Age-specific incidence of mucosal and ocular melanoma per million by gender in the USA, standardized to 2000 population, all races (McLaughlin et al. 2005)

relatively advanced stage, occurring in older individuals.

Ocular Melanoma

Ocular melanoma represents approximately 4% of melanomas in the USA (McLaughlin et al. 2005). The incidence increases with age (see Fig. 4) and overall is about six per million. The more common ocular melanomas arise in the uveal tract (82.5%), including the choroid (71.2%) and iris/ciliary body (11.3%). Other sites include the conjunctiva (6.6%) and retina/overlapping or unspecified sites (10.9%). The incidence of ocular melanoma is about 30% higher in men than women (Fig. 4). All subsets of the uveal tract demonstrate similar male predominance. The incidence of ocular melanoma also differs by race. Among White males, the incidence overall is approximately eightfold higher than among Black males. Among White females the incidence is tenfold higher than among Black females (McLaughlin et al. 2005). In the US, the majority of uveal melanomas occur among Whites (96.9%), with many fewer Asian/Pacific Islanders (0.7%), Blacks (0.5%), American Indian/Alaskan Natives (0.2%), and unknown race (1.7%) (Andreoli et al. 2015). In SEER data, race did not significantly affect overall or disease-specific survival.

Ultraviolet light exposure is the major suspected environmental exposure related to the development of ocular melanoma, but the epidemiologic data are not as compelling as for cutaneous melanoma. The lack of UV signature mutations in uveal melanoma renders UV radiation unlikely for this melanoma subtypes (see ▶ Chap. 16, "Primary Cutaneous Melanocytic Neoplasms"). It is also difficult to invoke UV exposure in the posterior eye after early childhood because of UV filtering by the lens. That is the rationale for using birth or early childhood residence as a surrogate for early UV exposure. Another hypothesis for intermittent intense UV exposure has been occupational exposure for

welders after epidemiologic studies showed an increase of ocular melanoma in them. In a meta-analysis of 12 studies with data on UV exposure (total of 14 risk factors; 9 considered intermittent and 5 chronic) and uveal melanoma, Shah et al. (2005) were able to evaluate 4 measures of UV exposure that were available in at least 4 of the studies: welding, outdoor leisure activities, occupational sunlight exposure, and latitude of birth. The first two were considered intermittent and the second two chronic. In this analysis, the only statistically significant UV exposure was welding which conferred a twofold increased risk. Outdoor leisure activity, occupational sunlight exposure, and birth latitude did not reach statistical significance.

The pattern of anatomic distribution of uveal tract melanomas, however, is somewhat suggestive of UV exposure with choroidal melanomas frequently arising in the posterior central choroid and iris melanomas most frequently arising in the inferior and lateral quadrants of the iris that receive most UV exposure. McLaughlin et al. (2005) also evaluated north-south and coastal-not coastal differences in residence at the time of diagnosis as a surrogate for UV exposure. For choroidal melanomas, the south/north comparison did not show an increased risk. For iris/ciliary body melanomas, however, the rate ratio of south to north was 40% increased and reached statistical significance. For iris/ciliary body, the rate ratio of coastal to non-coastal was also 30% significantly increased, suggesting a possible role for UV exposure.

Similar to cutaneous melanoma, host factors are important risk markers for ocular melanoma. There are families with ocular melanoma and other cancers (including cutaneous melanomas) that have *BAP1* germline mutations (see ▶ Chap. 11, "Inherited Contributions to Melanoma Risk"). Other host factors also predispose to developing uveal melanoma, including light eye color (75% increased risk), fair skin color (80% increased risk), and ability to tan (64% increased risk) (Weis et al. 2006). In this meta-analysis, light hair color did not reach statistical significance.

Choroidal nevi are not very common and rarely transform to melanoma (estimated risk 1/8845) (Qiu and Shields 2015). Among individuals from the general population over age 40 in the USA, 4.7% had a choroidal nevus on retinal imaging. The prevalence increased with age, with a 60% increase by age 80+. The prevalence varied slightly by gender with a 14% predominance in men. After adjustment for age and race, Whites were tenfold more likely to have a choroidal nevus than Blacks; Hispanics were fivefold more likely than Blacks; others were fourfold more likely than Blacks; and non-Hispanic Whites were twofold more likely than Hispanics.

For many years, oculodermal melanocytosis (also known as nevus of Ota), a congenital anomaly, has been recognized as a risk factor for, and potential precursor lesion of, ocular melanoma (Shields et al. 2013). It is estimated that 1 in 400 White individuals with oculodermal melanocytosis will develop uveal melanoma, a much higher rate than in the general population. It is estimated that at most, approximately 3% of individuals with uveal tract melanoma have oculodermal melanocytosis. The sites of melanocytosis include the sclera (92%), iris (17%), choroid (12%), eyelid (8%), and temporal fossa (1%). Limited data suggest that those with oculodermal melanocytosis and ocular melanoma were approximately twice as likely to develop metastatic disease. The risks varied by thickness of the tumor and somewhat by site of the melanocytosis with the iris conferring 2.8-fold increased risk; choroid slightly less, 2.6-fold; and sclera 1.9-fold.

Similar to cutaneous melanoma, ocular melanoma occurs at an increased frequency following specific cancers. After ovarian cancer, the risk of ocular melanoma is significantly increased (more than threefold increased), especially in women diagnosed with ovarian cancer under age 70. The risk of ocular melanoma after cutaneous melanoma reaches statistical significance in women (threefold increased) but not men, which might reflect both the age of diagnosis and survival differences between women and men with cutaneous melanoma. After ocular melanoma, renal parenchymal cancers are increased (2.5-fold increased), mostly in males, soft tissue (fourfold increased) predominantly in women, and

cutaneous melanoma (fourfold increase) in both sexes (Curtis et al. 2006).

Conclusion

The melanoma epidemic has led to characterization of the patterns of melanoma in different populations and investigation of risk factors for melanoma. In the past decades, the population-specific differences have been investigated and integrated for an improved understanding of the etiology and risk factors for melanoma, both cutaneous and ocular. Mucosal melanomas present the greatest challenge, since most are diagnosed as relatively advanced lesions in older individuals, making epidemiologic and clinical studies difficult.

For cutaneous melanoma, Australia has set the standard for sun protection programs. Their efforts are now bearing fruit with the decrease in melanoma incidence in the cohort of adolescents and young adults who have been brought up in the era of systematic sun protection. The possible decreases in the incidence seen in Fig. 1 and in adolescents in the USA, if sustained with future data, are also promising, but there is still much to be done. With the recognition of the dangers of tanning beds and increasing regulation regarding access to tanning beds by adolescents, hopefully the excess exposure (and addiction) will be diminished in the next decades.

The clinical and epidemiologic risk factors identified for cutaneous melanoma are important for targeting individuals at increased risk of melanoma for surveillance and prevention measures. Individuals with a strong family history of melanoma and those with multiple dysplastic nevi are at particularly increased risk. Widespread sun-protective measures, similar to those in Australia, could lead to fewer melanomas and markedly decreased medical costs associated with the treatment in the USA. In time, the incidence could decrease so that melanoma returns to a rare cancer instead of one of the most common. Sun protection could also potentially have an effect on the incidence of ocular melanoma, particularly iris/ciliary body melanomas, which may be more UV- or sun-related than choroidal. Mucosal melanomas will continue to be a difficult clinical problem, since visualization and early detection are not likely to be feasible in the near future.

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Prevention of Cutaneous Melanoma

13

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Contents

Abstract

The disease burden associated with melanoma continues to be a significant public health problem. The main modifiable risk factor for cutaneous melanoma is exposure to ultraviolet radiation, whether from the sun or artificial sources such as tanning sunbeds. Here we discuss the evidence for primary prevention and early detection of melanoma, commencing with an historical account of early skin cancer prevention programs and tools such as the Solar UV Index.

At a population level, there is sufficient evidence to support multicomponent, community-wide skin cancer prevention interventions (such as mass media campaigns,

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environmental and legislative interventions) and those that target certain settings. Legislative controls that restrict access to artificial tanning sunbeds can be effective in reducing the number of visitations by young people, thus reducing their risk of melanoma. Considering behavioral strategies, there is a lack of high-quality evidence for the effectiveness of recommendations to seek shade, cover exposed skin with clothing, wear a hat and sunglasses, and to regularly apply sunscreen with a SPF of 15 or higher when outdoors in the sun; however, this is largely due to limitations of the available epidemiological data. There is also lack of evidence that mass screening programs reduce mortality and medical costs due to melanoma, which are important principles of any population-based screening program. Globally, most skin cancer detection guidelines encourage regular self-examination, together with education regarding sun protection and whole body skin examination by a clinician in the presence of any change or concern.

In summary, there is reasonable evidence to suggest that reducing melanoma incidence through evidence-based prevention and early detection interventions is not only likely to be effective in reducing the risk of melanoma at a population level but will also be cost-effective. In Australia, where skin cancer prevention programs were established in the 1980s, melanoma incidence is now declining among younger adults, which most likely reflects changing patterns of sun exposure and the success of primary prevention efforts in recent decades. At a time when the cost of treating melanoma is likely to increase significantly in coming years, now is the time to make the necessary investments to reduce the growing and significant human and financial burden of melanoma into the future.

Keywords
Melanoma · Skin cancer prevention · Sunscreen · Sunbeds · Early detection · Melanoma screening · Ultraviolet radiation · Health promotion

Introduction

The disease burden associated with cutaneous malignant melanoma (*melanoma*) continues to be a significant public health problem. Globally, there were an estimated 232,130 incident cases of melanoma and 55,488 deaths in 2012 (Ferlay et al. 2013). As with other skin cancers, the disease burden (adjusted for population size) is disproportionately carried by fair-skinned populations, especially those living in locations experiencing high levels of sunlight (Lucas et al. 2008).

The main modifiable risk factor for melanoma is exposure to ultraviolet radiation, whether from the sun or artificial sources such as tanning sunbeds. Intermittent sun exposure and sunburn history are risk factors for melanoma, as is exposure to high levels of UV in childhood (Gandini et al. 2005; Whiteman et al. 2001). It is estimated that 65% of melanomas that occur worldwide are due to exposure to ultraviolet radiation; however, this figure could be as high as 95% in countries like Australia where UV levels are extreme for long periods and people spend a significant proportion of their time outdoors and a considerable portion of the population is Caucasian (Armstrong and Kricker 1993). This makes the primary prevention and early detection of melanoma an issue of high public health importance.

In much of Europe and North America, incidence rates of melanoma have increased in recent decades (with significant geographic variations), possibly due to increased sun-seeking behavior among fair-skinned Caucasians, including leisure travel to sunny destinations (Erdmann et al. 2013; Agredano et al. 2006). In other countries such as Australia, rates are starting to decline, particularly in younger age groups, most likely as a result of long-term prevention efforts (Whiteman et al. 2016; Iannacone et al. 2015; Thursfield and Farrugia 2015).

Providing the effort is sustained over the long term, improving sun-protective behaviors at a population level, reducing access to artificial tanning sunbeds, and implementing setting-based sun protection policies and practices, have the potential to reduce the substantial human and financial burden of melanoma.

History

The relationship between UV exposure and skin cancer was known for much of the twentieth century; however, it wasn't until the 1970s, when there was rising concern about a possible diffusion of the stratospheric ozone layer, that global efforts to deliver primary prevention programs increased (de Gruijl 1999).

One of the first efforts to demonstrate the benefits of skin cancer primary prevention was an early detection of melanoma project that started in Queensland (a state of northern Australia) in the 1960s. The project demonstrated that education for health professionals, combined with campaigns for the broader public, resulted in earlier patient presentation and better 5-year survival compared with states and territories that had not implemented this approach (Smith 1979). The early health education campaigns in Queensland were significant in informing and shaping what became the better known Australian 'Slip! Slop! Slap!' (*Slip on a shirt, Slop on some sunscreen, and Slap on a hat*) campaign that was started by the Cancer Council Victoria (then Anti-Cancer Council of Victoria) in the 1980s. Some years later (in 1988), the Victorian SunSmart program was funded by a hypothecated tax on tobacco products. This was Australia's, and indeed the world's, first population-wide multicomponent social marketing campaign that had a significant paid media presence to reduce the burden of skin cancer.

In the United Kingdom, Norway, and Sweden, skin cancer prevention campaigns started much later in the 1990s with generally limited resources (Whiteman et al. 2016). In the United States, multiple agencies have promoted sun protection for several decades but again with only very modest investments.

While community-wide efforts at a population level are being delivered to prevent skin cancer in many other countries (e.g., United Kingdom, New Zealand, Canada, USA, Spain, France, Sweden, Germany), the breadth, length, and magnitude of the intervention relative to the population has not yet been replicated in any other country outside of Australia.

Given the level and breadth of investment and effort in skin cancer prevention that has been sustained in Australia for over 30 years, efforts in this country provide some of the strongest evidence yet of the impact of prevention campaigns to reduce the rates of melanoma at a population level.

The Global Solar UV Index

The Solar UV Index (UV Index) was first developed by Canadian scientists in 1992 and was then adapted to become the Global Solar UV Index in 1995 in conjunction with the International Commission on Non-Ionising Radiation Protection, United Nations Environment Program, World Meteorological Organization, and the World Health Organization (Fioletov et al. 2004). The UV Index is a measure of biologically effective UV radiation intensity at the Earth's surface and provides an indication of potential for skin damage. The UV Index was launched globally in 1995 and was updated in 2002 (World Health Organization 2002).

Importantly, the UV Index provides a universal measure that is useful to guide public health efforts to alert the general population of current (or forecast) UV levels so that they can take appropriate sun safety precautions. UV Index values are either measured in situ or are predicted and adjusted for cloud cover. The UV Index levels vary according to solar elevation (which is related to the time of day, time of year, and latitude of the country where measurement is taken), cloud cover, stratospheric ozone, and the presence of aerosols (particles and pollution in the air) (Repacholi 2000). The UV Index usually reaches its peak annual levels around the middle of the day in summer (Table 1).

Communicated primarily through media channels and smartphone applications, the UV Index provides guidance as to when sun protection is required and what behavioral measures should be taken, according to the UV level. While not considered at the time of its development, the UV Index has also proved to be a useful tool to provide guidance to the general population as to

Table 1 UV levels of selected cities[a]

Location	Latitude	Average UV summer peak	Average UV winter low
Sydney, Australia	34°S	9	2
Tokyo, Japan	36°S	10	2
New York, USA	41°N	9	1
Los Angeles, USA	34°N	10	2
Berlin, Germany	52°N	7	1
Cape Town, South Africa	34°S	10	2
Rio de Janeiro, Brazil	23°S	12	5
Singapore	1°N	13	10
Paris, France	49°N	7	1
Vancouver, Canada	49°N	7	1

[a]Source: World Health Organization. http://www.who.int/uv/intersunprogramme/activities/uv_index/en/index3.html

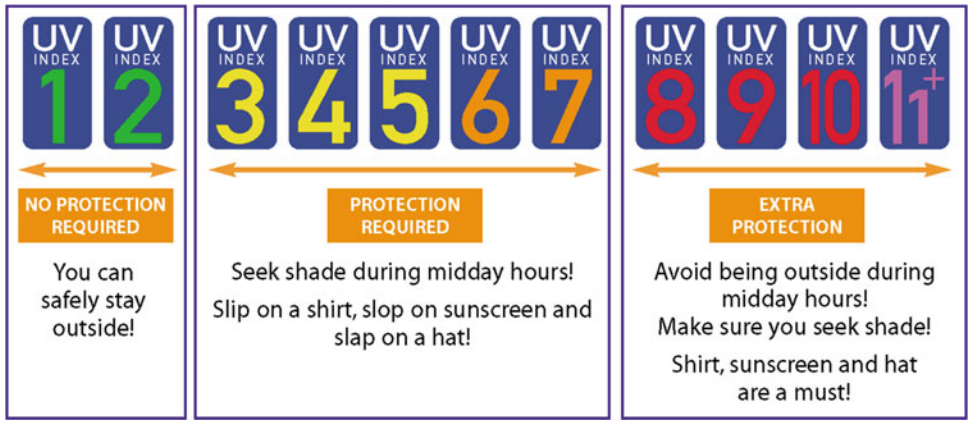

Fig. 1 The UV Index, with sun protection recommendations and simple "sound bite" messages

when sun protection is not recommended in order to enable some unprotected sun exposure in winter months. Encouraging people to not use sun protection (when UV Index <3) assists with the human body being able to maintain sufficient vitamin D levels, important for sustaining healthy levels of calcium in the bloodstream.

While the UV Index has not shown a significant effect in motivating behavior change, it has provided valuable guidance to those working in public health responsible for delivering skin cancer prevention efforts (Italia and Rehfuess 2012) (Fig. 1).

Worldwide, the UV Index remains the most commonly used tool to communicate messages about risk of harmful UV exposure. While the value of the UV Index may be limited on its own, when combined with broader efforts and delivered to individuals in a way that takes into account their location and ambient UV levels, the potential benefit of the UV Index for the purposes of providing sun protection advice is likely to be significant at a population level.

Evidence of Effectiveness

Evidence of Effectiveness of Population Health Interventions

The time lag of up to three to four decades between population-wide changes in UV exposure and changes in melanoma incidence creates challenges for evaluating the effectiveness of melanoma prevention programs. Because of this, cross-sectional snapshots of the target population

at particular points in time are often used to assess the more immediate effects of a prevention intervention. Such interim indicators include sunburn rates and sun-protective behaviors and/or attitudes.

In a recent systematic review of research on skin cancer prevention programs, the Community Preventative Service Task Force in USA found sufficient evidence to support (U.S. Department of Health and Human Services 2014):

- Multicomponent, community-wide interventions, which use a combination of integrated strategies to influence UV protective behaviors. These strategies may include mass media campaigns, environmental interventions (such as shade structures) and policy changes, implemented across multiple settings within the community.
- Interventions that target certain settings, specifically, child care centers, primary and middle schools, outdoor recreational and tourism settings, and outdoor occupational settings.

Studies in USA by the National Cancer Institute show that since the year 2000 there has been inconsequential changes in the US population in the use of shade, sunscreen, or shirts with long sleeves along with no significant changes in sunburn rates (Lazovich et al. 2012). Similarly in the United Kingdom, studies by Cancer Research UK have shown little improvement in sun-protective behavior between 2003 and 2011 despite modest investments in public awareness.

Case Study: Australian SunSmart Program

As an example of a multicomponent intervention, the Australian SunSmart program has had the capacity to deliver paid and unpaid media (social, TV, radio, outdoor, print) campaigns while at the same time engage in key settings such as schools, recreation settings, and workplaces to influence local policies and practices. The success of the SunSmart program has been measured by long-term improvements in attitudes and behaviors, as evident through population-based tracking surveys. Its proven track record has led to other Australian State governments investing in similar multicomponent social marketing campaigns under the SunSmart brand. Within the Australian context, multicomponent SunSmart campaign efforts have led to increased preference for no tan, reductions in sunburn, increased sunscreen use, and overall reduced mean percentage of skin exposed to the sun over successive summer weekends in the adult population (Dobbinson et al. 2015; Volkov et al. 2013).

By 2012, 77% of Australian primary schools had a written sun protection policy, nearly all of which required students to wear hats during summer months. The presence of a school policy signified better sun protection practices than a school with no sun protection policy (Dono et al. 2014).

The key factors that have contributed to changing the behavioral norms in relation to sun protection within an Australian context has been a long-term funding commitment to a population-wide, multicomponent intervention combined with the integration of research and evaluation into program planning and implementation (Sinclair and Foley 2009). However, the experience has also demonstrated the need to repeat and reinforce messages, as sun-protection behaviors appear to fluctuate according to funding levels and corresponding presence of media campaigns (Sinclair and Foley 2009; Dobbinson et al. 2008).

Effectiveness of Prevention Recommendations

Comprehensive sun protection programs include recommendations to seek shade, cover exposed skin with clothing, wear a hat and sunglasses, and to regularly apply sunscreen with a SPF of 15 or higher when outdoors in the sun. These primary prevention strategies target not only melanoma but also the keratinocyte cancers (basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)). While the following section reviews the evidence of effectiveness of these recommendations in reducing the incidence of melanoma, any investment in primary prevention is likely to result in benefits in the form of reduced incidence of BCC and SCC as well.

(a) **Sunscreen**

Sunscreens protect the skin from the damaging effects of UV radiation by reducing the transmission of high-energy photons to the skin cells, thus reducing damage to key skin components including cellular DNA, collagen, elastin, and lipids (Tanner 2006). The efficacy of sunscreens is measured by the sun protection factor (SPF), which is the ratio of the time taken to cause barely perceptible reddening (one minimal erythema dose [MED]) on skin that has been protected by sunscreen compared to the time taken to elicit the same response in unprotected skin (Nash 2006).

Sunscreens are designed, therefore, when applied appropriately, to prevent sunburn, and sunburn is a strong risk factor of melanoma at all latitudes (Chang et al. 2009). Two quantitative reviews on the effect of sunscreen on melanoma risk both reported a nonsignificant association (Dennis et al. 2003; Huncharek and Kupelnick 2002); both meta-analyses were based on case-control studies only (the review by Dennis et al. included 18 case-control studies and the earlier review by Huncharek and Kupelnick included 11 case-control studies). In a 2011 systematic review to update the recommendations from the 2003 US Preventive Services Task Force, the Centre for Disease Control (CDC) assessed the evidence as to the effectiveness of sunscreen in preventing all skin cancer outcomes (Lin et al. 2011). This review was limited to observational studies and Randomized Control Trials (RCTs) that met strict inclusion criteria and predefined quality ratings. The authors concluded that sunscreen use may prevent squamous cell carcinoma but not melanoma, and that the evidence was inconclusive for BCC.

Of all observational studies reporting on the association between sunscreen use and melanoma to date, only 13 were population-based: two cohort studies (the Nurses' Health Study and the Norwegian Woman and Cancer Study) (Cho et al. 2005; Ghiasvand et al. 2016) and 11 case-control studies (Lazovich et al. 2011; Youl et al. 2002; Westerdahl et al. 1995, 2000; Whiteman et al. 1997; Autier et al. 1995; Holly et al. 1995; Herzfeld et al. 1993; Beitner et al. 1990; Osterlind et al. 1988; Holman et al. 1986) (Table 1). One cohort study reported no effect (Cho et al. 2005) and the other a significantly protective effect (Ghiasvand et al. 2016). Of the 11 case-control studies, 2 reported a significant protective effect (Lazovich et al. 2011; Holly et al. 1995), 4 reported a significant increased risk (Westerdahl et al. 2000; Autier et al. 1995; Herzfeld et al. 1993; Beitner et al. 1990), and the remaining 5 studies reported nonsignificant negative or positive associations. The definition of sunscreen use varied across studies.

The inconsistencies in the epidemiologic literature on the association between sunscreen use and melanoma reflect the challenges of examining the relationship with observational study designs. Such studies are unable to disentangle the effect because the main determinants of sunscreen use overlap with causal factors for melanoma (i.e., sun sensitive phenotype and sun exposure). This is known as "confounding by indication" and is almost impossible to control through analytic techniques. Case-control studies can also be affected by recall bias (i.e., whereby cases are prompted to recall past exposures solely because of their diagnosis) and misclassification bias, if cases are more likely to accurately recall their past use of sunscreen than controls. Thus, only well-conducted RCTs with high-quality measurements and rigorous control of confounders can adequately assess the association. Only one RCT examining the effect of daily sunscreen use on skin cancer has been conducted. That trial, which took place in Queensland, Australia, found that adults aged 40–69 years randomized to apply daily sunscreen had a 50% lower incidence of melanoma (Green et al. 2011) than those randomized to discretionary sunscreen use; the protective effect was of borderline statistical significance. Melanoma was not a prespecified outcome of the trial and it was limited by a small sample size

and low number of events. Moreover, the protective effect was seen on both sunscreen-protected and nonprotected body sites. Nonetheless, the trial is unlikely to ever be repeated and thus is likely to remain the highest level of evidence for a protective effect of sunscreen in the development of melanoma.

Because of the limitations of the available epidemiological data, there is a need to examine indirect evidence of a role for sunscreen in protecting against the damaging effects of UV which might initiate melanoma development, including effects on genetic and immune-suppression pathways. Such evidence is emerging from experimental studies on intact human skin. For example, one study has shown that sunscreen protects against UV-induced DNA damage in melanocytes (Hacker et al. 2013). Melanomas have a higher mutation load than other cancers (Hill et al. 2013; Alexandrov et al. 2013) and UV-signature mutations account for most of this elevated mutational burden (Hodis et al. 2012). The findings of Hacker and colleagues (Hacker et al. 2013) support the use of sunscreen to prevent the genetic changes important in melanomagenesis.

(b) **Hats/clothing/shade**

Clothing (including hats) and shade offer varying degrees of protection from exposure to UV radiation, and while there are data to show that people who use these forms of protection are less likely to report sunburns, e.g., Branstrom et al. (2010), direct evidence of a protective effect for melanoma is scant. One population-based case-control study reported lower risks of melanoma among both "inconsistent" and "optimum" users of these sun protection methods (in aggregate) when compared with never users (Lazovich et al. 2011).

Research on clothing and melanoma prevention is limited. A large US cohort (Weinstock et al. 1991) and a population-based Australian case-control study (Holman et al. 1986) both reported an increased risk of trunk melanoma in women who wore bikinis compared to those who wore one piece, high backline swimsuits. The later study also reported a higher risk of site-specific melanoma among outdoor workers if the site was "sometimes exposed" rather than "usually covered" while working outdoors.

While shade is a potentially valuable means of protection from the damaging effects of UV radiation, direct evidence for a protective effect for melanoma is lacking.

Economics of Prevention

Cost-effectiveness studies aim to provide information to decision-makers on whether an intervention of interest provides value for money by systematically comparing the costs and effects of the intervention with alternative strategies. Gordon and Rowell (2015) systematically reviewed the cost-effectiveness of skin cancer prevention. Of seven identified cost-effectiveness studies, three were concerned with melanoma as the outcome of interest (three others focused on all skin cancer and one on the keratinocyte cancers only). Two studies reported incremental cost-effectiveness ratios of US $400 per life-year saved for an early detection/education campaign (versus a "do nothing" approach) (Cristofolini et al. 1993) and US$3357 per life-year saved for an educational campaign (versus a "do nothing" approach) (Garattini et al. 1996) using data from 1977–1985 to 1993, respectively. The third study used a lifetime model (and 2010 prices) and reported AU$40,890 per QALY (~ US$41,851 in 2010) for daily sunscreen use (versus routine use) (Hirst et al. 2012). In the countries where these studies were conducted (Italy and Australia), the interventions were highly cost-effective according to acceptable cost per QALY/life-years saved. Four other studies identified by the review evaluated the cost-effectiveness of various initiatives (solarium regulation, sunscreen use, a school-based program, and a multicomponent intervention) to prevent either "all skin cancer" or the keratinocyte skin cancers and

all reported cost savings and a return on investment to governments.

A recent study investigated the benefits and costs of three skin cancer campaigns implemented in New South Wales, Australia, between 2006 and 2013 (Doran et al. 2016). For melanoma, the study found that during this period, an estimated 885 cases and 109 deaths were averted, with direct and indirect cost savings totalling AU$37.69 million (equivalent to US$34.5 million over the same timeframe, i.e., 2006–2013). The benefit-cost ratio for all skin cancers (melanoma plus non-melanoma skin cancer) was estimated at 3.85, suggesting that for every AU$1 invested in prevention campaigns, AU$3.85 was returned.

The cost-effectiveness of interventions to prevent melanoma is likely to increase substantially with the advent of new beneficial but costly therapies for advanced melanoma. Melanoma treatment costs will rise (on average) and are likely to further exceed the costs of preventive measures, highlighting the continued cost effectiveness of skin cancer prevention.

Evidence of Effectiveness of Interventions on Melanoma Rates

Incidence of cutaneous melanoma increased steadily in the second half of the twentieth century worldwide, but there are encouraging signs that the incidence among young people has plateaued or even declined in some high-incidence populations. In an analysis of incidence trends from 39 population-based cancer registries over the period 1953–2008, incidence rates of melanoma have continued to rise in most European countries, whereas in Australia, New Zealand, North America, Israel, and Norway, rates appear to be stabilizing in adults under 50 (Erdmann et al. 2013). A detailed analysis of melanoma incidence trends and projections of rates to 2031 in six populations based on long-term registry data (Whiteman et al. 2016) showed that age-specific incidence in Australia and New Zealand for those <60 years peaked around 2002–2006 and then declined, while they are not projected to stabilize until 2021 in US, and until 2026 in the UK, Sweden, and Norway. In Queensland, Australia, incidence rates among adolescents and young adults (15–24 years) have declined since the mid-to-late 1990s (Iannacone et al. 2015). A comparison of incidence trends in young people aged under 25 years in Australia and the UK reported declines in Australia from 1996 to 1997 (Wallingford et al. 2015), while rates have steadily increased in the United Kingdom for both males and females in this age group. The declines seen in Australian children and young adults most likely reflect changing patterns of sun exposure and the success of primary prevention efforts in recent decades. While it is possible that other secular trends might be influencing recent trends in melanoma incidence among younger age groups, including overall migration-dilution effects and more "screen time," these factors are unlikely to explain all of the downturns observed (Baade et al. 2015) (Fig. 2).

Sunbeds

(a) **Evidence of association**

Exposure to artificial ultraviolet radiation from indoor tanning is a cause of melanoma. A meta-analysis conducted by the International Agency for Research on Cancer (IARC) working group on artificial ultraviolet light and skin cancer (IARC 2007), using data from 19 studies, reported a modest increase in the risk of melanoma for "ever" compared with "never" exposure to indoor tanning equipment. It also found a higher pooled estimate if first exposure occurred before age 35 years. These findings prompted the World Health Organization to classify tanning beds as a group I carcinogen (El Ghissassi et al. 2009). A subsequent review confirmed these findings (Boniol et al. 2012), and a significant dose-response relationship was further observed, with a 1.8% (95% CI 0–3.8%) increase in risk of melanoma for each additional session of sunbed use per year. Higher risks have been observed among sunbed users who have never experienced a burn from indoor tanning or outdoor sun exposure

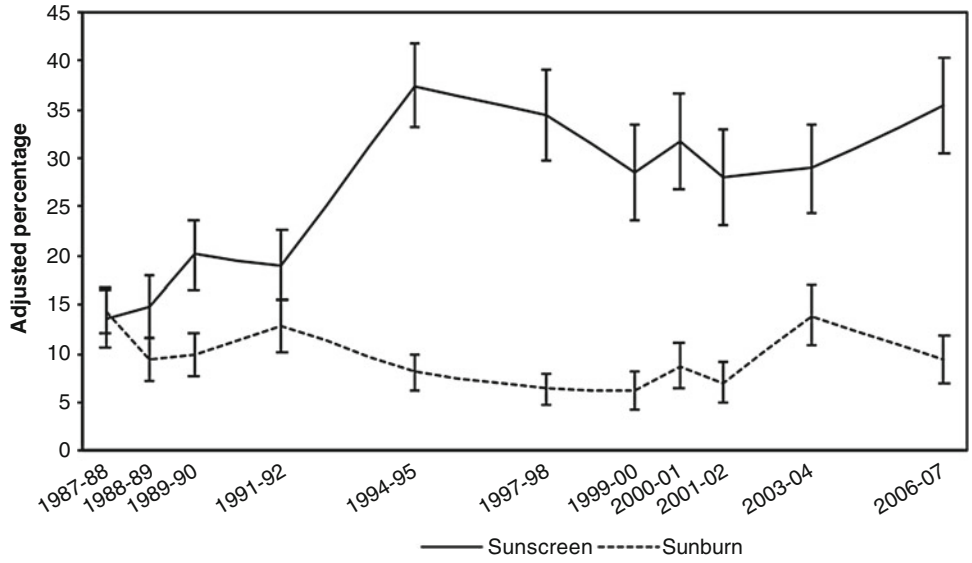

Fig. 2 Trends in sunscreen use and sunburn in Melbourne, Victoria, from 1987–1988 to 2006–2007 (Makin et al. 2013)

(Vogel et al. 2014), that is, in people with a low propensity for sunburn.

Using prevalence estimates of sunbed use from 18 European countries, Boniol and colleagues estimated that 5.4% of melanoma cases (3438 cases each year) in Western Europe could potentially be prevented by avoiding exposure to indoor tanning (Boniol et al. 2012). Data from an Australian case-control study on early-onset melanoma estimated that 76% of melanomas diagnosed between the age of 18–29 years were attributable to sunbed use and 13% of those diagnosed in 30–39-year-olds (Cust et al. 2011), reinforcing the importance of prevention in young people. An ecological study conducted in Iceland reported a marked increase in the incidence of melanoma in women younger than 50 years following the widespread introduction of sunbeds in the early 1990s (Hery et al. 2010). Although inferences about causality cannot be made from ecological studies, the sequence of events in Iceland was highly suggestive and was invaluable in alerting public health authorities to the problematic trend.

(b) **Current sunbed regulations globally**

It has been demonstrated that legislative controls that restrict access to artificial tanning sunbeds can be effective in reducing the number of visitations by young people (Guy et al. 2014; Makin and Dobbinson 2009; Hester et al. 2005). In 1977, France became the first country to introduce age restrictions on indoor tanning for youth 18 years or younger (Pawlak et al. 2012). Since then, there has been a rapid increase in the implementation of controls to restrict under age access in other jurisdictions; with 12 USA states, several Canadian provinces, Israel, and 12 European countries also restricting under 18 access (Sinclair and Makin 2013; Guy et al. 2014).

Besides restrictions on under age use, common legislative restrictions include limits on the intensity of UV lamps (e.g., European Union), warning labels (e.g., USA, Canada), no claim of health benefit (e.g., France), supervision by trained personnel (e.g., Ireland, Northern Ireland, Wales), and the compulsory wearing of protective goggles (e.g., Canada, Israel). While the increase in legislative restrictions has been positive, compliance checking against the laws is often inadequate (Hester et al. 2005; Mayer et al. 2008).

The most significant shift in legislative controls has been the complete ban of

commercial artificial tanning sunbeds in Brazil in 2009 and in all Australian states in 2016. In the Australian context of implementing an outright ban, compliance by commercial operations with the ban was very high. Strong public support for an outright ban combined with comprehensive and strong enforcement practices by regulatory agencies were the main contributing factors to this compliance. Importantly, there was also very little evidence to suggest that there was a significant shift in consumer demand to the domestic sunbed market following the outright ban (Sinclair et al. 2016). An incremental increase in controls to reduce health risks in commercial sunbed operations, combined with educational efforts to warn of the risks of sunbed use, were key factors to the successful implementation of the outright ban in Australia (Sinclair et al. 2016).

Early Detection

Whether identified by the patient, clinician, or as part of a mass screening program, the aim of early detection initiatives are to diagnose melanomas in their earliest stages, at which point lesions are thinner and survival rates are higher.

About half of all melanomas are self-detected (Collins et al. 2014). Self-examination of the skin involves checking all areas of the body (including those that are not exposed to the sun) for changes in the shape, size, or color of existing lesions, or for the emergence of new lesions.

Globally, most skin cancer detection guidelines encourage regular self-examination, together with education regarding sun protection and whole body skin examination by a clinician in the presence of any change or concern (Watts et al. 2015). Further, for "extreme risk" patients, such as those with a personal or familial history of melanoma, multiple (more than five) atypical naevi and genetic mutation of CDKN2A, most international guidelines advise annual surveillance by a dermatologist (Watts et al. 2015).

There are a number of clinical tools that may improve diagnostic accuracy by clinicians. From their systematic review of clinical guidelines, Watts and colleagues found a high level of evidence to support the use of dermoscopy, which may be complemented by sequential digital dermoscopy imaging (SDDI) to record any changes over time, and a moderate level of evidence for the use of total body photography in high risk patient groups (Watts et al. 2015). The review acknowledges that specialized training, including for primary care physicians, is essential to the effectiveness of these tools to improve diagnostic accuracy.

However, not all population groups are equally likely to notice changes in lesions or to seek early medical advice; this is one rationale for selective or mass population screening. Mass screening for melanoma involves examination of whole population groups. In principle, mass screening should reduce the number of deaths due to melanoma, as well as the medical costs due to treatment (Wilson and Jungner 1968); however, there is a lack of evidence to support these principles. As such, population-wide organized screening is not delivered in any country with the exception of Germany, including those countries with the highest rates of melanoma.

(a) **The German Screening Program**

In 1999, Germany introduced a systematic approach to skin cancer screening. This commenced with a pilot program called Skin Cancer Research to Provide Evidence for Effectiveness of Screening in Northern Germany (SCREEN), which provided total body skin examinations for residents of Schleswig-Holstein who were 20 years or older and held statutory health insurance policies. A mass media campaign was used to inform residents about the program. During the 1-year screening period (2003–2004), trained practice-based physicians screened 360,288 residents, or 27% of eligible women and 10% of eligible men in Schleswig-Holstein (Waldmann et al. 2012). In total, 585 of those screened were diagnosed with melanoma, of which close to 90% were classified as thin melanoma (<1 mm) (Breitbart et al. 2012). The screening period was associated with a spike in

melanoma incidence, as well as for non-melanoma incidence, which was not observed in neighboring regions (Eisemann et al. 2014; Waldmann et al. 2012). By 2008, melanoma mortality rates had declined by 47% among men and 49% among women in Schleswig-Holstein (Katalinic et al. 2012).

The program participation rate, its successful integration within the existing health care system, and the apparent positive trends all contributed to conclusions that population-based skin cancer screening was possible and feasible in Germany. This led to a nation-wide population-based screening program, which commenced in Germany in 2008. Under the national program, total body skin examinations were provided by health insurance providers for those aged 35 years and older. An ecological study was conducted to evaluate the impact of the program on the German population, which analyzed trends in the age-standardized incidence and mortality for melanoma in Germany from 1980 to 2012 (Boniol et al. 2015). Although the launch of the program coincided with a 29% increase in melanoma incidence, from 14.5 cases per 100,000 person-years in 2006 to 18.0 cases per 100,000 person-years in 2010, melanoma mortality increased by 2.6% in men and 0.02% in the 5-year period that followed. These trends did not differ from the age-standardized trends observed for surrounding countries that did not have screening programs. Furthermore, melanoma mortality in Schleswig-Holstein doubled from 2009 to 2010, thus returning to rates close to that observed before the pilot project (Boniol et al. 2015).

The failure to achieve the projected decline in melanoma mortality in Germany could not be explained by poor rates of participation in the national screening program, which exceeded those in Schleswig-Holstein. It is possible that a longer period of follow-up was necessary in order to measure any population benefit, although this was not the experience in Schleswig-Holstein. It has been suggested that biases introduced to the reporting of deaths due to melanoma might have contributed to an apparent reduction in melanoma mortality in Schleswig-Holstein, as many doctors who performed screening as part of the trial were also responsible for reporting deaths due to melanoma (Boniol et al. 2015). This highlights the challenge of evaluating the impact of public health policies such as melanoma screening where trial data are not available.

(b) **Effectiveness of Targeted Screening Programs**

Mass screening is indiscriminate. The result is that cost savings associated with detecting early stage cancer may be forfeited to the expense of screening those who are at very low risk of melanoma (Collins et al. 2014). Further, screening low-risk patients can increase the rates of biopsy of benign lesions, and detection and treatment of basal and squamous cell carcinomas, which are less likely to be life-threatening.

Terminology used to define "high risk" differs throughout clinical practice guidelines but considers naevi, phenotypic features, UV exposure, and other factors such as family history and genetics (Watts et al. 2015). Guidelines to identify high-risk individuals are informed by risk prediction models, which quantify an individual's risk of developing melanoma. In a systematic review of the melanoma risk prediction literature, Vuong et al. (2014) found that naevi, skin type, density of freckles, age, hair color, and sunburn history were common to many of the 19 models that were considered' however, there was some variation in how effectively models discriminated between individuals with and without melanoma. Research by Olsen et al. (2015) has demonstrated that risk prediction models need to be calibrated specifically for the target population, using cut-off scores that are suitable for what is considered to be high risk in that setting. Thus, although it is beneficial to use standardized, repeatable measures to identify high-risk individuals, there is no global tool for melanoma risk prediction. The derivation of risk

prediction models using a collaborative approach pooling multiple independent studies may have advantages in comparison with those developed from single studies. For example, pooled studies offer greater precision and increased generalizability, with the ability to incorporate variables that describe differences in risk across populations globally including ethnicity and latitude of residence.

There is some evidence that screening programs that target high-risk individuals may improve melanoma survival rates, particularly if these programs reach those who are less likely to present to a clinician (Gordon and Rowell 2015; Collins et al. 2014). Targeted surveillance programs may need to be accompanied by public campaigns in order to reach high-risk groups, particularly older men, who are less likely to participate in selective or population-based screening (Collins et al. 2014). In France, the Self-Assessment of Melanoma Risk Score (SAMScore) was reported to reduce the size of the population to be screened by a factor of 11 (compared with mass screening) and to identify a subgroup in which melanoma incidence was 25 times higher than in the general population (Quereux et al. 2012; Rat et al. 2015). Population benefits and cost-effectiveness of the French screening program is yet to be established.

A systematic review by Gordon and Rowell (2015) identified four studies on the economic value of melanoma screening in the decade to 2007, three of which were conducted in America and one in Australia. Although the studies are now somewhat dated, the review found evidence from two studies that screening high-risk individuals such as older men and those with a family history of melanoma could be cost-effective. However, cost savings were offset by the removal of squamous and basal cell carcinomas and other skin lesions that were also detected by the screening program (Gordon and Rowell 2015).

Conclusion

After 40 years of investment to reduce the impact of melanoma at a population level, there is reasonable evidence to suggest that reducing melanoma incidence through high-impact, evidence-based prevention, and early detection interventions is not only likely to be effective in reducing the risk of melanoma at a population level but will also be cost-effective.

In many respects, the evidence is clear in terms of what needs to be done and there are a multitude of governmental reports including from USA (U.S. Department of Health and Human Services 2014; Saraiya et al. 2004), UK (National Institute for Health and Clinical Excellence 2011), and Australia (Department of Health 2012) that supports this view by providing guidance towards evidence-based best-practice in skin cancer prevention.

At a time when the cost of treating melanoma, particularly advanced melanoma, is likely to increase significantly in coming years, and with the knowledge currently available to prevent melanoma, now is the time to make the necessary investments to reduce the growing and significant human and financial burden of melanoma into the future.

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ICGC Breast Cancer Consortium, ICGC MMML-Seq Consortium, ICGC PedBrain, Zucman-Rossi J, Futreal PA, McDermott U, Lichter P, Meyerson M, Grimmond SM, Siebert R, Campo E, Shibata T, Pfister SM, Campbell PJ, Stratton MR (2013) Signatures of mutational processes in human cancer. Nature 500(7463):415–421

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Microbiome and Melanoma

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Reetakshi Arora, Amanda Hermann, and Jennifer A. Wargo

Contents

Abstract

Significant advances have been made in the past decade across the melanoma care continuum, with approved systemic therapy for patients with advanced disease as well as in the adjuvant setting. We are gaining an appreciation of the factors that drive response and resistance to these therapies, and there is novel evidence that the microbiome (which refers to the microbes that inhabit our bodies along with their collective genomes) may shape overall immunity and may even impact therapeutic responses (e.g., immune checkpoint blockade). This has profound implications and calls to question if the microbiome could be used as a biomarker or therapeutic target in patients going onto treatment with immune checkpoint blockade (and potentially onto other forms of therapy). Insights are also being gained into the potential influence of the microbiota on melanoma development at the level of the skin and of the gut, though there is a tremendous knowledge yet to be gained. Each of these aspects

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will be discussed herein, as will strategies to target and factors that influence the microbiome.

Keywords
Melanoma · Microbiome · Checkpoint blockade · Immunity

Introduction

The human microbiome is a complex aggregate of microorganisms (including bacteria, archaea, viruses, and fungi) as well as their associated genomes. Though once known for their pathogenic properties, these microbes are now implicated in helping to regulate the delicate balance of health and disease (Sekirov et al. 2010). Many of the initial insights into this role of the microbiome focused on gut microbes and metabolism (Turnbaugh and Gordon 2009); however microbes throughout the body are now implicated regulating a host of physicologic properties including local and systemic immunity (Lloyd-Price et al. 2016). The increased recognition of the role of the microbiome came about in part due to advances in sequencing techniques – which allowed the identification and characterization of these microbes without the need to culture them. Since initial approaches were described (Staley and Konopka 1985), there are now numerous means through which microbiota may be characterized – and may yield insight into their function as well as their phylogeny (Wade 2002; Duncan et al. 2007; Eckburg et al. 2005; Shendure and Ji 2008; Pace 1997; Venter et al. 2004). Such approaches have now been used to characterize the human microbiome in participants worldwide – through efforts such as the Human Microbiome Project (HMP) and American Gut (Human Microbiome Project Consortium 2012a, b; McDonald et al. 2018).

In addition to their role in normal physiology and maintenance of overall health, these microbes may impact disease states, particularly when imbalances of these microbes may exist in a particular body site (termed "dysbiosis") (Frosali et al. 2015). A classic example of this is in the context of *Clostridium difficile* infection in the gut, which is associated with a massive dysbiosis (Khanna et al. 2016). However more subtle disruptions in the microbiota in the gut and at other sites have been associated with diseases and conditions throughout the body – ranging from autism and heart disease to cancer (Strati et al. 2017; Tang et al. 2017; Sheflin et al. 2014; Zitvogel et al. 2015; Zou et al. 2018). Disruptions of the skin microbiome have been associated with conditions such as eczema and psoriasis (Trivedi 2012; Grice 2014), among other conditions.

The link between microbiota and cancer has now been described at multiple levels – with the earliest reports focusing on the contribution of microbes to carcinogenesis (such as in the case of hepatitis viruses and hepatocellular cancer and *Helicobacter pylori* in gastric cancer). However there is now a growing appreciation of the complexity of the potential contribution of these microbes to carcinogenesis and also to response to therapy (Tsilimigras et al. 2017), both at the site of disease and at distant sites. This is poignantly illustrated in the recent observations that microbes may be found within human tumors and that they may either facilitate (Miller et al. 2018) or inhibit (Geller et al. 2017) therapeutic responses. Perhaps even more profound is the recent data supporting the impact of gut microbiota on responses to immunotherapy (specifically immune checkpoint blockade) in patients with melanoma and other cancers (Matson et al. 2018; Gopalakrishnan et al. 2018a; Routy et al. 2018a; Yi et al. 2018; Kroemer and Zitvogel 2018; Bhatt et al. 2017; Chaput et al. 2017).

Together, these findings have profound implications for patients with melanoma and other cancers – as the microbiome could potentially serve as a biomarker and could even be therapeutically targeted (using fecal microbiota transplant among other strategies). Each of these will be discussed herein, with the goal of providing the basis for an understanding of the role and potential of the microbiome in patients with melanoma.

The Microbiome in Health and Disease

Gut Microbiome

As noted previously, the microbiome contributes to numerous critical functions within the host. Perhaps one of the most impactful contributions is the influence of the gut microbiota on systemic immunity – which could potentially alter immune function and immunosurveillance for cancer and can also influence responses to immunotherapy for melanoma as shown in recent studies (Matson et al. 2018; Gopalakrishnan et al. 2018a; Routy et al. 2018a; Chaput et al. 2017; Frankel et al. 2017). Certainly, there is extensive interaction between microbes in the lumen of the gut and immune cells in the lamina propria along its vast length and surface area, and there are also more distantly interactions at the level of the mesenteric lymph node. These host-microbial interactions are paramount to overall health, and there is a delicate balance through which host immune cells recognize and eliminate pathogenic microbes while remaining tolerant to critical commensal microbes as well as food antigens. However despite these immune cells being tolerant to commensals, there is now clear evidence that the overall immunity is in part shaped by interactions with these gut microbes (Honda and Littman 2016), including from mouse models where germ-free mice demonstrate markedly altered immune function (Johansson et al. 2015; Spiljar et al. 2017) but also from studies in human cohorts.

Microbes interact with immune constituents (including T cells, B cells, dendritic cells, neutrophils, and others) scattered along the lamina propria of the gut as well as in the organized structures of the gut-associated lymphoid tissue (GALT) (Fig. 1a). The means through which they interact are numerous and include local interactions such as engagement of pathogen-associated molecular patterns (PAMPs) (such as lipopolysaccharide (LPS) and flagellin) with Toll-like receptors present on innate immune cells and interstitial epithelial cells (IECs). These PAMPs can also induce maturation of dendritic cells in the area, which can then traffic to mesenteric lymph nodes where they may stimulate CD4+ and CD8+ T lymphocytes (Lathrop et al. 2011). These lymphocytes and other immune cells can then act locally to secrete cytokines such as interleukin-10 and interleukin 17 or may traffic directly into the bloodstream where they can mediate distant effects. CD4+ regulatory T cells (Tregs) play an important role in promoting immunologic tolerance to commensal microbes (Furusawa et al. 2013), limiting inflammation. Microbes may also influence immunity via production of metabolites such as short-chain fatty acids (SCFAs) (Reichardt et al. 2014).

Though we do not yet have a deep understanding of the ideal constituents of a "healthy" microbiome, there is evidence that disruptions of the gut microbiome (dysbiosis) may lead to pathologic conditions including autoimmune and inflammatory diseases such as inflammatory bowel disease (IBD), type I diabetes, rheumatoid arthritis (RA), and multiple sclerosis (Tsilimigras et al. 2017; Kim et al. 2017; Mima et al. 2017; Garcia-Castillo et al. 2016) and have also been associated with cancer (Sears and Garrett 2014; Yang and Jobin 2017).

Skin Microbiome

In addition to the gut microbiota, microbiota at other sites (such as the skin) may also influence immunity and overall health (Byrd et al. 2018), with disruption potentially leading to disease states (Grice and Segre 2011). The skin harbors a lower microbial biomass compared to gut, owing to different physical and chemical properties (Chen et al. 2018). The microbial ecology of human skin is complex, and microbiota analysis from healthy donors has identified *Staphylococcus*, *Micrococcus*, *Corynebacterium*, *Brevibacterium*, *Propionibacterium*, and *Acinetobacter* species as normal residents in skin (Gao et al. 2007). The most common fungal species present on normal human skin are *Malassezia* (Sanford and Gallo 2013). Both environmental and host factors can have direct

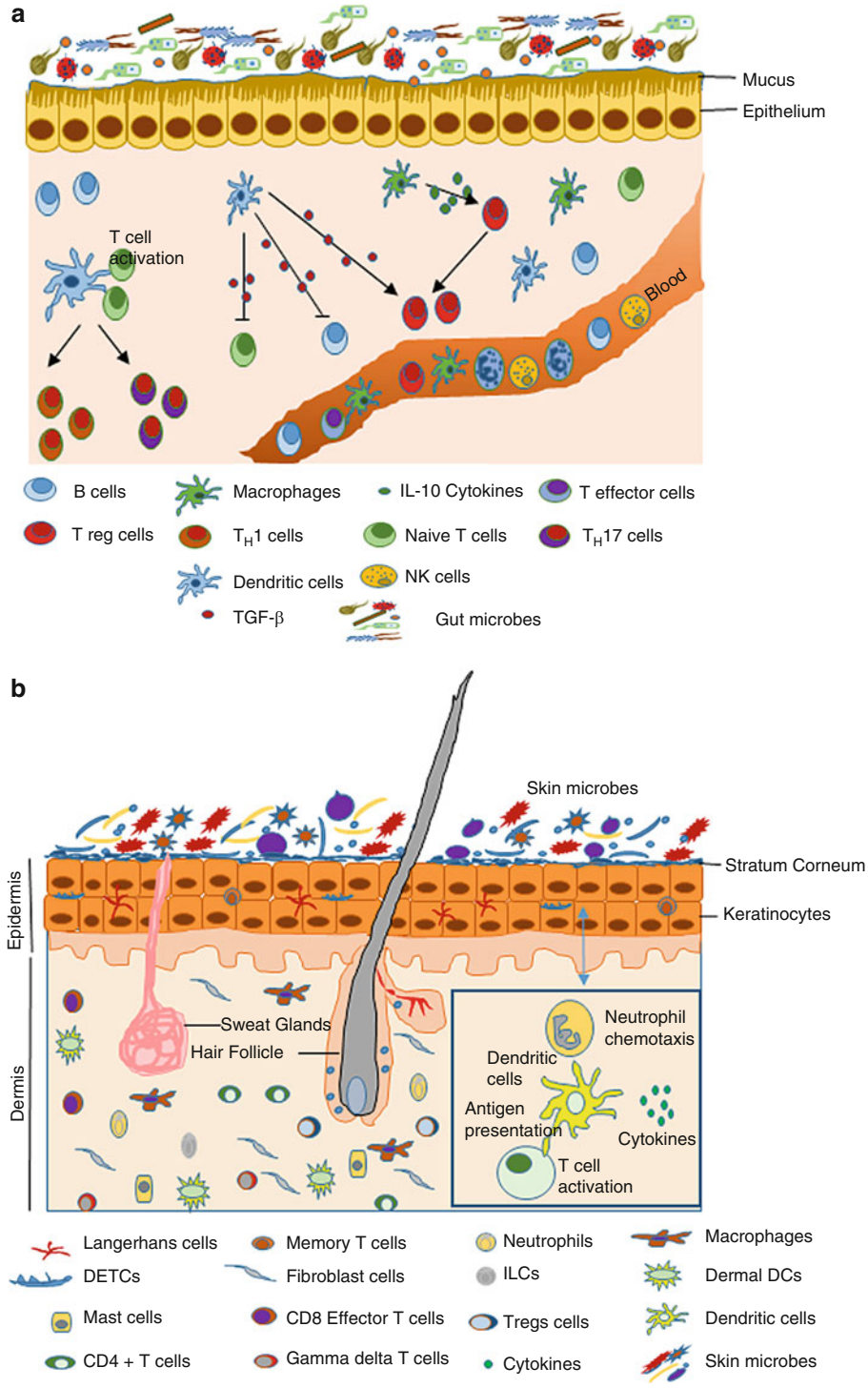

Fig. 1 Microbiota can cross-talk with immune cells: (a) Gut microbiota and immune cells. Gut microbes within the lumen of the gut interact with immune cells to elicit immune response. Microbes or microbial metabolites can activate dendritic cells (DCs) which migrate to the draining lymph node to activate naïve T cells to effector cells. These effector cells then enter into systemic circulation. These microbes and microbial by-products alter DCs

effect on skin microbiome such as physiology, external environment, immune system, lifestyle, body location, age, gender, and underlying medical conditions (Grice and Segre 2011).

The stratum corneum layer of the skin epidermis and epidermal tight junctions are two of the main elements in the barrier function of the skin (De Benedetto et al. 2012). Components of the skin microbiome may influence immunity and other host functions via a number of different mechanisms (Belkaid and Segre 2014) (Fig. 1b). This includes their ability to metabolize host proteins and lipids into bioactive molecules such as free fatty acids (Belkaid and Segre 2014) that in turn can stimulate keratinocyte-derived immune mediators (complement, and IL-1) and other immune cells within the dermis. Work from Work from Dr. Yasmine group showed how skin microbiota plays a key role in promoting protective immunity to dermal infections. Their group demonstrated that skin commensals can induce T cell responses restricted to MHC class I molecules and these commensal-specific T cells express immunoregulatory and tissue repair signature genes promoting accelerated skin wound closure. Therefore, suggesting the capacity of skin microbiota to induce immune responses that couples antimicrobial function with tissue repair (Linehan et al. 2018).

Disruptions of the skin microbiota are associated with a number of disease conditions – including psoriasis, atopic dermatitis, and acne vulgaris (Trivedi 2012; Grice 2014). Psoriasis is a chronic multifactorial autoimmune disorder affecting the skin, characterized by raised, scaly, well-demarcated, erythematous oval plaques (Nestle et al. 2009), and can be provoked or exacerbated by specific pathogens including bacteria (*S. aureus* and *Streptococcus pyogenes*), viruses (human papillomavirus and endogenous retroviruses), and fungi (*Malassezia* and *Candida albicans*) (Fry and Baker 2007). Fahlén et al. found *Streptococcus* as the most common genus in both normal and psoriasis skin, whereas *Staphylococcus* and *Propionibacterium* were significantly lower in psoriasis compared with control limb skin (Fahlen et al. 2012), while Alekseyenko et al. showed significant increase in abundances of *Corynebacterium*, *Propionibacterium*, *Staphylococcus*, and *Streptococcus* in psoriatic plaques (Alekseyenko et al. 2013). In contrast Gao et al. revealed *Propionibacterium* species being less abundant in psoriasis than in normal controls (Gao et al. 2008). In another study, a reduction in *Firmicutes* and an increase in *Proteobacteria* were reported in psoriatic patients (Drago et al. 2016), while Liew et al. reported that *Firmicutes* were significantly overrepresented and *Actinobacteria* and *Propionibacterium* were significantly underrepresented in psoriatic lesions. Although this confirms that psoriasis exhibits a distinct microbiota from healthy unaffected skin, however, conflicting reports warrant a thorough characterization of the abundance of microbes in psoriatic patients. Of note, M proteins, found on Group A, C, and G β-hemolytic streptococci, are associated with worsening of chronic plaque psoriasis by mimicking keratin determinants with subsequent psoriatic T-cell activation (McFadden et al. 1991; Valdimarsson et al. 2009). This theory is validated by the fact that the interaction between type IV collagen and αα integrin found exclusively on epidermal psoriatic T cells results in the expansion of this subset of cells and the manifestation of psoriasis (Conrad et al. 2007). T-cell activation in

Fig. 1 (continued) which can skew the T-cell phenotype – T helper-1 (T_H1), T helper-17 (T_H17), or T regulatory cells (Tregs). (**b**) **Skin microbiota and immune cells**. Commensal organism over the skin surface and associated structures can metabolize the host proteins and lipids into bioactive products that can inhibit the invasion of pathogens. Immune cell types are found within the skin, including Langerhans cells, dendritic epidermal γδ T cells (DETCs), and memory αβ T cells in the epidermis, and subsets of dendritic cells, macrophages, neutrophils, mast cells, γδ T cells, innate lymphoid cells (ILCs), CD4+ and CD8+ T effector cells, and T regulatory cells (Treg) are found in dermis. Skin commensals can also induce T-cell responses, and these commensal-specific T cells express effector genes with immunoregulatory signatures

psoriasis is also shown to be predisposed by antigens such as streptococcal pyogenic toxin A and B as well as peptidoglycan (Boyman et al. 2007; Davison et al. 2001; Baker et al. 2006). The role of the skin microbiome in the development and progression of melanoma and other cancers is incompletely understood at this point, though active investigations are currently underway.

Profiling the Microbiome

As previously mentioned, advances in techniques to characterize the microbiome have resulted in a marked increase in our understanding of these microbes in the setting of health and disease. Several different techniques exist to profile the microbiome, and these each have unique advantages and disadvantages (Lagier et al. 2018; Cogdill et al. 2018) (Table 1).

This includes 16 s sequencing which involves next-generation sequencing techniques to characterize the 16 s subunit of the ribosome (which is unique to prokaryotes). Regions of the 16 s subunit vary between bacterial species – thus allowing use of this technique to determine relative abundances of differential bacterial species within a given sample (Zhang et al. 2018). Additionally, one can use this approach to assess alpha diversity – which is a measure of the differences in abundance of certain bacterial taxa between samples and/or groups. Beta diversity may also be derived using this technique, which refers to the similarity/dissimilarity between groups of samples (Caporaso et al. 2010). An advantage of this technique is the relatively low cost and speed of analysis using this approach; however limitations exist as species-level determination may not be feasible nor are other components of the microbiota assessed using this approach (such as viruses, fungi, and protozoa).

Another technique that can be used to profile the microbiome is whole metagenomic sequencing – or WMS. This approach involves sequencing of the entire genomic content; thus it allows characterization of microbes beyond bacteria and allows better resolution with the ability to characterize down to the species level (and even to specific strains). Thus this approach has many advantages over 16 s sequencing; however it is currently more costly and also requires more advanced bioinformatics approaches for data analysis.

Additional approaches include culturomics and PCR-based approaches targeting specific bacterial taxa. Culturomics is appealing, in that it allows isolation and characterization of specific microbes associated with the specific phenotype of interest. Though somewhat labor intensive, this approach is gaining momentum to overcome some of the limitations of pure sequencing approaches (Seng et al. 2009). PCR-based approaches may also be used to interrogate for single or limited taxa of interest, and the cost and turnaround time of such analyses provide advantages. On top of this, metabolomic profiling and transcriptomic profiling performed in parallel may be quite useful as it may yield additional information regarding functional status of the microbiome (Lagier et al. 2012).

Role of the Microbiome in Melanoma and Other Cancers

Influence of Tumor and Gut Microbiome on Carcinogenesis and Response to Cancer Therapy

The notion that microbes could contribute to carcinogenesis and response to cancer therapy originated many years ago (Littman et al. 2004; Welton et al. 1979; Nagy et al. 1998), though the full impact of this is only now being appreciated with additional insights clearly to be gained. This is perhaps best studied in the case of luminal gastrointestinal malignancies such as gastric cancer and colorectal cancer, where bacteria have a demonstrated link to carcinogenesis (with *Helicobacter pylori* in the case of gastric cancer, and *Fusobacterium nucleatum* in the case of colorectal cancer) (Peek and Blaser 2002; Mima et al. 2015).

Beyond these examples, there is now extensive evidence linking microbes (including bacteria, viruses, protozoa, and others) to cancer – with therapeutic strategies ranging from eradication of these pathogens to facilitate cancer treatment

Table 1 Methods to profile microbiome

Microbiome profiling method	Profiling description	Advantages	Disadvantages
16S rRNA sequencing	Processing relevant samples to DNA PCR amplification of hypervariable region(s) of 16S gene Sequencing and comparison with reference databases	Quantify ecology metrics: alpha- and beta-diversity Characterize differential abundance of bacteria taxa or operational taxonomic units (OTUs) Fairly rapidly performed Low cost of analysis	Reduced accuracy of taxonomic identification due to copy number variations and PCR primer and amplification bias Cannot inform the functional biological capacity of a given microbial community Only accounts for bacteria; cannot quantify viruses, fungi, and protozoa in the sample
Whole metagenomic sequencing (WMS)	Non-targeted sequencing process Involves sequencing of the entire genome of all microbes in a given sample Annotates assembled or unassembled reads against a protein database	Allows sequencing of viruses, fungi, protozoa, and archaea Assesses functional potential of microbial communities Deeper resolution to characterize down to the species level Yields relative abundances of orthologous gene families or pathways	Significantly higher cost in terms of time and money Less tolerant of low biomass or contaminated samples Requires more complex computational analytic approaches
Metatranscriptomic	High throughput sequencing of RNA isolated from complex microbial populations -mRNA/cDNA sequencing for high-resolution gene expression profiling	Help identify the subset of genes within a microbial community expressed in sample High throughput and sensitivity Characterization of known and unknown transcripts	Detection of microbial genes is technique-sensitive due to limited stability of RNA and low proportions of mRNA in stool samples Involve multiple purification steps Computationally intense as they require normalization of transcripts to DNA copy numbers
Metabolomics	Non-sequencing-based, culture-independent approaches to molecular profiling of the human microbiome	Can perform on low amount of sample Time efficient Identify secreted and intracellular microbial products Quantification of small-molecule metabolites generated by microorganisms Yield functional information of the microbiome Study the impact of microorganism in health and disease	Lack accuracy in differentiating between host-derived and microbial-derived molecules Many unknown metabolites in databases Strict identification of compound labor intensive
Metaproteomics	Proteins/peptides are analyzed Protein monitoring and profiling	Quantification of protein or peptide levels that can provide a high-resolution snapshot of bacteria-host interaction and metabolites generated by microorganisms Identify differential microbial proteins production under various physiological/environmental conditions	Heterogeneous stability Difficult to analyze all the metabolites present in the sample Many unknown proteins in databases

(*continued*)

Table 1 (continued)

Microbiome profiling method	Profiling description	Advantages	Disadvantages
Culturomics	High throughput culture method to complement taxonomic identification by metagenomics by advances in mass spectroscopy techniques (MALDI-TOF)	Identification of bacteria that have been considered to be difficult to culture Allows characterization of specific microbes Rapidly and accurately identify large number of colonies Characterizing the viability of detected microorganisms	Labor intensive and time consuming
Targeted/specific PCR	Target-specific microbial taxa	Detection of very small quantities of bacteria that often remain undetected by 16S profiling More accurate bacterial species and strain identification than traditional qPCR Allows detection of archaeal, fungal, and viral communities Accurate species identification	Require harmonization of the extraction and PCR conditions between the studies Cannot inform the biological function of a given microbial community

(Rosenberg et al. 2008; Uribe-Herranz et al. 2018) to prevention of these infections through. Microbes in tumors have also been shown to impact therapeutic responses to systemic therapy such as immune checkpoint blockade, with virally driven tumors exhibiting enhanced responses to therapy likely owing to recognition of "foreign" antigens (Smola 2017; Rieckmann et al. 2013; Tashiro and Brenner 2017).

In addition to microbes at the level of the tumor impacting carcinogenesis and response to cancer therapy, microbes at the level of the gut can do this as well through their impact on immunity and potentially on immunosurveillance of cancer (Routy et al. 2018b; Zitvogel et al. 2017). There is evidence to support the concept that generalized dysbiosis of gut microbiota may contribute to carcinogenesis (Tsilimigras et al. 2017; Garrett 2015), as repeated use of antibiotics has been associated with the development of both gastrointestinal (GI) tract and non-GI tract tumors in large case-control studies (Boursi et al. 2015). Various mechanisms have been proposed by which dysbiosis might affect tumorigenesis and tumor growth, however a comprehensive understanding of the complex mechanisms through which these commensal microbes impact immunity and carinogenesis is critical and work analyzing this is currently underway.

One mechanism through which gut dysbiosis may have an impact is through the induction of an inflammatory state that can promote carcinogenesis via pro-inflammatory toxins (such as produced by *Bacteroides fragilis* (Purcell et al. 2017; Wu et al. 2009)), increased reactive oxygen species (Mangerich et al. 2012), and alterations in signaling pathways (*Fusobacterium nucleatum*) (Kostic et al. 2013). Alternatively, bacterial products/metabolites (Dalmasso et al. 2014; He et al. 2018) may also contribute to carcinogenesis. For example, components of *F. nucleatum* including the FadA adhesion (FadAc) can activate β-catenin/Wnt signaling pathways resulting in oncogenic transcriptional changes (Sears and Garrett 2014; Rubinstein et al. 2013). *F. nucleatum* has been demonstrated to play a role in the development and progression of colon adenomas and colon cancer (Castellarin et al. 2012; McCoy et al. 2013; Warren et al. 2013) and has also been identified in nodal and distant metastasis (Yu et al. 2016; Bullman et al. 2017). Another well-explored example of gut microbiota-associated malignancy is hepatocellular carcinoma (HCC) where microbial modification of primary bile acids produced by the liver to secondary bile acids such as deoxycholic acid (DCA) can cause DNA damage,

hepatotoxicity, and carcinogenesis (Yoshimoto et al. 2013). The gut microbiota is also associated with the response to infectious hepatitis, obesity, and the development of nonalcoholic steatohepatitis (NASH) as well as other forms of cirrhosis, all of which are key risk factors for the development of HCC (Mima et al. 2017).

Although many other studies demonstrate a direct association of dysbiosis and other malignancies, additional preclinical, clinical, and epidemiological studies will certainly strengthen the relationship between dysbiosis and cancer. Furthermore, the harmonization of characterization techniques/pipelines is necessary to bring the parity between the studies to be able to conclude healthy vs tumorigenic microbes. With the current studies, it is undeniably conceivable that strategies to modulate the microbiota may be used to improve cancer immunosurveillance (Zitvogel et al. 2018) and it would be productive to explore gut microbiota and/or their metabolic products as potential biomarkers of cancer development.

Influence of the Gut and Tumor Microbiome on Response to Melanoma Therapy

Though the role of microbes in influencing cancer development has been studied for many years, their role in melanoma was not elucidated until recently. However seminal work by Dr. Gajewski and others has now put melanoma in the spotlight – with a clear and significant demonstrable contribution of the microbiome in response to melanoma therapy.

The earliest of this work was published in 2015, where preclinical studies demonstrated that mice with different gut microbiota demonstrated differential responses to melanoma therapy – specifically to immune checkpoint blockade (Sivan et al. 2015). Specifically, Gajewski's group showed that identical strains of mice (C57BL6) purchased from two different vendors (Taconic Farms vs. Jackson Laboratories) had distinct gut microbiomes, and this was associated with differential response to treatment with immune checkpoint blockade (targeting the programmed death receptor 1 – PD-1) to treat established melanoma tumors (B16). Strikingly, they also found that by modulating the gut microbiota they could enhance responses to therapy in these mice (either through co-housing, as mice are naturally coprophagic, or by transfer of specific bacterial strains). Furthermore, the group provided insight into the mechanism through which these gut microbiota were enhancing antitumor immunity – demonstrating that mice with a "favorable" gut microbiome had more functional antigen-presenting cells (APCs) such as dendritic cells capable of priming antigen-specific T-cell responses (Sivan et al. 2015). Similar work was published in the same issue of *Science* by Zitvogel and colleagues, demonstrating a reliance on gut microbiota to treatment with immune checkpoint blockade in preclinical models (specifically to CTLA-4 blockade) across several cancer types (using sarcoma, melanoma, and colon cancer tumor models) (Vetizou et al. 2015).

These studies sparked excitement in the field though some skepticism given that findings were only demonstrated in preclinical models. This excitement turned to action when several groups then turned to human cohorts to test the relevance of these findings, and this work has now shown an association between gut microbiota in response (as well as toxicity) to immune checkpoint blockade in melanoma in numerous published studies (Matson et al. 2018; Gopalakrishnan et al. 2018a; Chaput et al. 2017) (Table 2). Several of these studies were published together in *Science* in 2018 strengthening the link between gut microbiota and response to immune checkpoint blockade in melanoma as well as in other cancer types (Gopalakrishnan et al. 2018a; Routy et al. 2018a; Chaput et al. 2017; Frankel et al. 2017). In these studies, distinct bacterial "signatures" were noted in the gut microbiota of responders versus non-responders to anti-PD-1 therapy in patients with melanoma (Matson et al. 2018; Gopalakrishnan et al. 2018a; Frankel et al. 2017) and in non-small cell lung cancer and renal cell carcinoma (Routy et al. 2018a) – with higher diversity and enrichment of specific bacterial taxa (such as *Bifidobacterium*, *Ruminococcus*, *Faecalibacterium*, and *Akkermansia*) in

Table 2 Human studies demonstrating modulatory function of gut microbiome on response to immune checkpoint blockade therapy for melanoma

	Influence of gut microbiome in ICB therapy	Outcome	Bacteria	References
1	Enhanced efficacy of PD-1 blockade therapy	Higher abundance in responders Elevating levels of effector T cells in peripheral blood and TILs Increasing densities of CD8+ T cells in tumor microenvironment	*Ruminococcaceae*	Gopalakrishnan et al. (2018a)
2	Enhanced efficacy of PD-1 blockade therapy	Higher abundance in responders	*Veillonella parvula*	Matson et al. (2018)
3	Enhanced efficacy of PD-1 blockade therapy	Higher abundance in responders Decreasing peripherally derived Tregs	*Bifidobacterium adolescents*	Matson et al. (2018)
4	Enhanced efficacy of PD-1 blockade therapy	Higher abundance in responders	*Bifidobacterium longum*	Matson et al. (2018)
5	Reduced efficacy of PD-1 blockade therapy	Higher abundance in non-responders	*Ruminococcus obeum*	Matson et al. (2018)
6	Enhanced CTLA-4 blockade efficacy therapy	Higher abundance in responders Inducing activation of Treg Promoting development of tolerogenic macrophages and dendritic cells Prolonging progression-free survival/ overall survival	*Butyrate-producing bacterium*	Chaput et al. (2017)
7	Enhanced efficacy of PD-1 blockade therapy	Decreasing peripherally derived Tregs	*Collinsella aerofaciens*	Matson et al. (2018)
8	Enhanced efficacy of PD-1 blockade therapy	Decreasing peripherally derived Tregs	*Enterococcus faecium*	Matson et al. (2018)
9	Enhanced CTLA-4 blockade efficacy therapy	Prolonging overall survival Elevating colitis risk	*Faecalibacterium prausnitzii*	Chaput et al. (2017)
10	Enhanced efficacy of PD-1 blockade therapy	Higher abundance in nonresponders	*Klebsiella pneumonia*	Matson et al. (2018)
11	Enhanced efficacy of PD-1 blockade therapy	Decreasing peripherally derived Tregs	*Parabacteroides merdae*	Matson et al. (2018)
12	Enhanced efficacy of CTLA-4 blockade therapy	Prolonging progression-free survival/ overall survival Elevating colitis risk	*Gemmiger formicilis*	Chaput et al. (2017)
13	Reduced efficacy of PD-1 blockade therapy	Higher abundance in nonresponders	*Roseburia intestinalis*	Matson et al. (2018)

responders to therapy. Although only modest overlap has been noted in specific bacterial taxa associated with response across these cohorts, phylogenetic commonalities do exist and functional status (what these microbes are doing to immunity) may be much more important than the names of these bacterial taxa.

Several of these manuscripts demonstrated that these phenotypes could be recapitulated by fecal microbiota transplant (FMT) from responding and nonresponding patients into germ-free mice, with subsequent tumor implantation and treatment with immune checkpoint blockade (Matson et al. 2018; Gopalakrishnan et al. 2018a; Routy et al. 2018a). Modulation of the gut microbiota was shown to enhance therapeutic response in several of these preclinical models. Additionally, there was evidence in *human* cohorts that negative modulation of the gut microbiome could impact therapeutic response, as treatment of patients with antibiotics around the time of first administration of checkpoint blockade was associated with impaired survival on anti-PD-1-based therapy (Routy et al. 2017), which has now been validated in subsequent cohorts (Derosa et al. 2018). Based

on findings from these studies, efforts are currently underway to positively modulate the gut microbiota in patients with melanoma going onto immune checkpoint blockade (NCT03643289, NCT03595683, NCT03341143, NCT03772899).

Importantly, the tumor microbiome may be relevant in patients with melanoma – as microbes have been identified in tumors across several different histologies including lung cancer, breast cancer, colon cancer, gastric cancer, pancreatic cancer, cholangiocarcinoma, ovarian cancer, and prostate cancer. Though the specific mechanism through which these microbes gain access to tumors is incompletely understood, systemic seeding from infection or bacterial translocation from the GI tract may occur and has even been shown to occur in healthy individuals with normal gut mucosal integrity. These microbes have been shown to influence response to chemotherapy (Geller et al. 2017) and immunotherapy (Miller et al. 2018) in other cancer types, and investigations regarding the role of intra-tumoral microbes in melanoma are currently underway.

Targeting the Microbiome to Treat Disease

Though the concept of targeting the microbiome to treat cancer is somewhat novel, this approach has been used in noncancer indications such as *Clostridium difficile* colitis and other conditions for years with proven efficacy in some cases (van Nood et al. 2013; Juul et al. 2018) – particularly with regard to targeting the gut microbiota. The gut microbiome may be targeted using several different approaches (Fig. 2), including through the use of fecal microbiota transplant (FMT), the administration of bacterial consortia (Hibberd et al. 2017), dietary intervention (Ramirez-Farias et al. 2009; Turnbaugh et al. 2007), and targeted approaches against specific taxa using antibiotics or phage (Wong and Santiago 2017; Pranjol and Hajitou 2015; Abedon et al. 2017; Budynek et al. 2010).

The first published report of the use of fecal microbiota transplant (FMT) was in 1958 when Eisenman reported successful treatment of *C. difficile* colitis using this approach in several patients (Eiseman et al. 1958). However the concept has been around for centuries, with efforts to manipulate the composition of the gut microbiota used over 1700 years ago in China for the treatment of diarrhea. Since the approach was first published and reported, it is now being widely evaluated in the treatment of numerous conditions ranging from inflammatory bowel disease and multiple sclerosis (Paramsothy et al. 2017) and now in the treatment of cancer (NCT03353402, NCT03341143, NCT03678493, NCT02928523). FMT can be administered via a number of different routes, including colonoscopy and also via oral administration (Gough et al. 2011), and great consideration needs to be taken into appropriate donor selection to minimize the risk of transmissible infectious diseases and undesirable traits such as obesity (Rao and Young 2015). Thus far, healthy donor FMT has focused primarily on these issues, though the choice of donors is likely to be more complicated when considering treatment for conditions such as cancer – as the "optimal" gut microbiota composition to facilitate antitumor immune responses is incompletely understood (Cogdill et al. 2018; Gopalakrishnan et al. 2018b). However evidence from published studies suggests that responders to immune checkpoint blockade do have distinct signatures in the gut microbiome (Gopalakrishnan et al. 2018a); thus it may be prudent to screen potential donors for this signature in addition to the routine screening tests. To date, most of the planned and ongoing trials to modulate the gut microbiota in patients with melanoma on immune checkpoint blockade incorporate FMT from complete responders to therapy (NCT03353402, NCT03341143). Certainly, use of FMT in such trials is a logical and likely necessary first step in a rational approach to target the gut microbiome in patients with cancer.

Based on published studies, there is also an ongoing effort to target the gut microbiota using a mixture of several (or even single) bacterial strains that have been associated with therapeutic response to immune checkpoint blockade (NCT03595683, NCT03637803). This approach

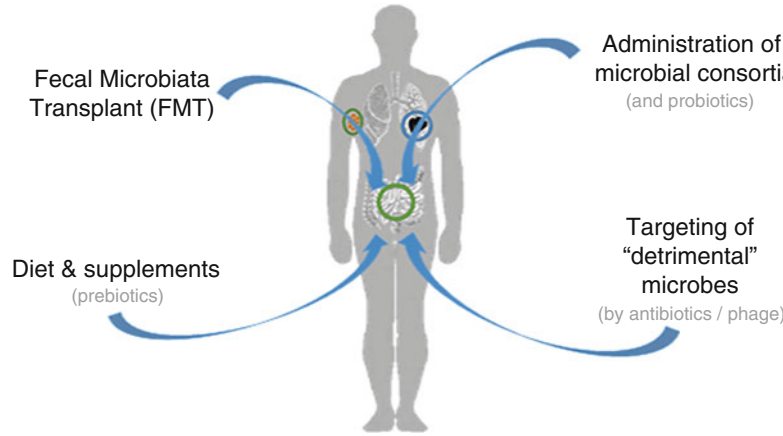

Fig. 2 Methods of microbiome modulation: The gut microbiome may be targeted using several different approaches, including through the use of fecal microbiota transplant (FMT), the administration of bacterial consortia, dietary intervention, and targeted approaches against specific taxa using antibiotics or phage

has some potential advantages over FMT including ease of manufacturing and scalability; however, as noted we do not have a clear understanding of which bacterial taxa and strains may be beneficial when comparing across published cohorts. Additionally the number of composition of an "optimal" consortia of bacteria to enhance therapeutic responses is unknown; thus this approach is likely to be iterative and informed by early trials and studies in larger cohorts of patients. Moreover, there has been interest in testing probiotic preparations in combination with treatment with checkpoint blockade; however substantial limitations exist with this approach as published studies show that there is tremendous variability in the ability of commercial probiotic supplements to engraft in the gastrointestinal tract and these preparations are less well-regulated than other formulations, with recent evidence that such formulations may actually impair engraftment of healthy commensal bacteria (Zmora et al. 2018; Suez et al. 2018).

Approaches to specifically target detrimental microbes are also being used, either with targeted antibiotic approaches or with use of viruses that target specific bacteria (bacteriophages). The phages have the potential to infect bacteria in the gut (Lusiak-Szelachowska et al. 2017) and have been shown to contribute to the efficacy of approaches such as fecal microbiota transplant for noncancer indications (Zuo et al. 2018). However these components of the microbiota are less well-studied, and additional investigation is needed to better understand their role in melanoma and other cancers.

Another means to modulate the gut microbiota is via dietary intervention, though this has not been thoroughly investigated in the setting of treatment of cancer, and incorporation of such analyses is critically needed. However some insights may be gained from studies performed in noncancer populations where such studies have been done. Such studies have focused on diets that have been recommended and have been associated with a lower risk of developing or dying from cancer (such as the Mediterranean Diet and Healthy Eating Index); however the influence of these diets on gut microbiota has not been well-studied. Nonetheless such diets are associated with enhanced immune function and reduced levels of systemic inflammation (Oude Griep et al. 2013), and more formal studies of dietary intervention are currently underway.

Conclusions and Future Directions

Significant advances have been made in the treatment of melanoma, and there is increasing evidence that environmental and host factors may impact melanomagenesis and response to melanoma therapy. This includes the tumor and gut microbiota; however the full impact of these variables is incompletely understood. As we move forward as a field, it will be important to

take these factors into consideration and to use insights gained to derive strategies to improve responses to melanoma therapy and ultimately to prevent melanoma altogether.

Author Contributions
Conception: Hermann and Wargo
Writing: Arora and Wargo
Creation of figures: Arora and Wargo
Critical review and revision of the manuscript: All authors

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Animal Models of Melanoma

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Zackie Aktary, Martin McMahon, and Lionel Larue

Contents

Abstract

Melanoma is a highly aggressive cancer with a significant incidence in western countries and a high mortality rate. Recently developed pathway-targeted or immunotherapies are, at least in part, the fruit of gains of knowledge in chemistry, immunology, genetics, cell signaling, and cell biology. The translation of knowledge from the bench to the bedside was possible because of advanced technologies and techniques but also in vitro and in vivo models that are more refined and relevant for the study of human melanoma. This chapter reviews the different in vivo models that are used to study melanoma, including xenografts of melanoma cell lines, patient-derived xenografts, and genetically engineered animal models.

Keywords

Mouse · Zebrafish · NRAS · BRAF · Proliferation · Senescence

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Introduction

Melanoma is an aggressive cancer and is the leading cause of skin cancer deaths worldwide. Melanoma originates from melanocytes, which are neural crest-derived cells responsible for producing the pigment melanin. Melanocytes are present mainly in the skin, inner ear, meninges, hair follicles, and uveal tract. Epidermal melanocytes make extensive contacts with neighboring keratinocytes, to which they transfer their melanin. During a multi-step process known as melanomagenesis, skin melanocytes are transformed into melanoma. The first steps often involve benign proliferation of melanocytes to form a nevus, or a benign skin lesion, within which the melanocytes are clustered and lose their characteristic contacts with keratinocytes. Eventually, the melanocytes in the nevus cease proliferation and become senescent. As melanomagenesis continues, the melanocytes in the nevus are able to bypass senescence and enter the radial growth phase (RGP), where they typically proliferate superficially toward the epidermal basement membrane. These primary steps constitute melanoma "initiation." Next, during the vertical growth phase (VGP), melanoma cells continue to proliferate actively, and acquire migratory and invasive properties, which enables them to cross the basement membrane and invade the dermis. Eventually the cells progress to acquire metastatic characteristics, as they enter the bloodstream and/or lymphatic vessels and eventually colonize different tissues and organs (Larue and Beermann 2007). These latter steps can be thought of as the "progression" of the disease.

Melanomagenesis is associated with modifications of numerous cellular (proliferation, immortalization, epithelial-mesenchymal transition, and migration) and molecular (signaling pathways, cell cycle, and cell adhesion) processes. At the molecular level, the abovementioned cellular processes are modified primarily in a cell-autonomous manner. For example, the activation of different tyrosine kinase receptors (e.g., KIT, MET, and RET) by the ligands (SCF, HGF, and GDNF, respectively) leads to the induction of a number of signaling pathways (e.g., ERK1/2 MAP kinase, PI3-lipid/PTEN-AKT, and WNT/β-catenin signaling), all of which have been implicated in melanomagenesis both in vivo and in vitro (Easty et al. 2011; Paluncic et al. 2016). The modification of these signaling pathways may act to alter the cell cycle and to promote growth, migration, and invasion of the melanoma cells.

In melanoma, a number of abnormalities of gene activity have been detected, which include genetic and epigenetic lesions and high and low levels of gene expression. In cutaneous melanoma (melanoma that occurs on the skin, which is the most common type of melanoma), abnormalities often involve the activation of oncogenes, the inactivation of tumor suppressor genes, the inhibition of apoptosis, the modification of DNA repair enzyme activities, and the alterations in cell morphology and migration capacity (Larue and Beermann 2007).

Historically, successful therapies to treat melanoma have proven elusive. However in 2011, vemurafenib, an inhibitor of mutationally activated BRAF (V600), was FDA approved for the treatment of advanced melanoma in the USA (Kuzu et al. 2015). While patients initially showed robust responses to this inhibitor, resistance to the drug was almost always observed and patient relapse was frequent. Immunotherapy is another treatment strategy with some clinical success in improving overall melanoma patient survival; however both low patient response rates and relapse have been reported (Kuzu et al. 2015; Zaretsky et al. 2016). Therefore, it is essential to continue to gain a better understanding of the molecular mechanisms regulating melanomagenesis (at both the initiation and progression stages) and to be able to test potential therapeutic agents in the most relevant way possible. The most effective way to accomplish this is to use animal models of melanomagenesis, which are a vital tool in understanding and combating the disease. Since cancer cells exist within a complex tumor microenvironment composed of neighboring cells, blood vessels, host immune cells, and an extracellular matrix, the different animal models must also recapitulate these features and must also allow for the natural proliferation, bypass of

senescence, invasion, and metastasis typically observed during melanomagenesis in humans.

This chapter discusses the various animal models (e.g., mouse, canine, equine, porcine, and zebrafish) that have been used in melanoma research and describes how these different models have contributed to the understanding of melanomagenesis.

Mouse Melanoma Models

To date, the mouse is the most commonly used organism for studying melanomagenesis in vivo. The majority of the mouse models of melanoma are focused on investigating cutaneous melanoma. Mice are advantageous for melanoma researchers as they are relatively easy to genetically manipulate and are, primarily, readily available for use. In addition, the mouse and human genomes are similar, with many noncoding sequences conserved between the two. In addition, due to the large body of knowledge that exists regarding mouse genetics, extensive analyses can be done using this model. Furthermore, mice and humans have comparable organs and physiology. However, mouse models of melanoma have some acknowledged limitations. First, the localization of melanocytes in human versus mouse skin is different with human melanocytes primarily located in the epidermis, whereas mouse melanocytes are primarily located in hair follicles. Second, mice are not prone to spontaneously develop melanoma in response to ultraviolet light, the most likely carcinogen that promotes melanomagenesis in humans (Zaidi et al. 2011; Noonan et al. 2012).

The mouse has been used to study melanomagenesis through the use of engrafted human melanoma cell lines and melanoma biopsies from patients (patient-derived xenografts, hereafter referred to as PDX) and genetically engineered mice. Xenograft models involve the culture and engraftment of either human melanoma cell lines or patient-derived melanomas into immunocompromised mice. In contrast, genetically engineered mouse models make use of sophisticated genetic manipulations that allow for altered expression of known or suspected melanoma "oncogenes" (e.g., *Nras*, *Braf*, *Rac1*) or "tumor suppressors" (*Cdkn2a*, *Nf1*, *Pten*) with temporal and spatial control of melanocyte-specific genetic alterations.

Mouse Xenograft Models

Cell Line Xenografts

Researchers have established a large number of human melanoma cell lines with genetic alterations that are broadly representative of genes implicated both in the initiation and progression of melanoma. These cell lines are useful for basic manipulations and identification of potential genes important in melanoma progression and maintenance. Melanoma cell line xenografts involve the subcutaneous implantation of these melanoma cell lines into immunocompromised/deficient mice that will not reject the cells (e.g., nude athymic (nu/nu) mice or severe combined immunodeficient (SCID/SCID) mice). In doing so, the implanted cells are able to adhere, to grow, to induce angiogenesis, and to interact directly with the blood and lymphatic vessels, allowing for the in vivo assessment of tumor growth as well as response to various therapeutic interventions (Kuzu et al. 2015). This type of model is simple to use, as numerous melanoma cell lines are readily available for manipulation and implantation. However, many such melanoma cell lines have been cultured for several years under nonphysiological conditions (e.g., growth in 2D, on plastic, presence of calf serum, etc.), and cells that were established and propagated may not accurately reflect the initial tumor from which the cell lines were derived. As a result, these xenograft models have lower frequency of correctly predicting clinical outcomes and drugs that are successful using these models often fail during clinical trials (Kuzu et al. 2015).

Patient-Derived Xenografts (PDX)

In this model, tumors from human melanoma patients are surgically removed, cut into small specimens, and subcutaneously implanted into nude mice. PDX have several advantages over

cell line xenografts. For example, tumors that form in these mouse models retain similarities to the original tumors (compared to cell line xenografts) and thus may more accurately reflect the diversity of human melanoma. In several studies, it was observed that PDX have similar histological, transcriptomic, and polymorphic/copy number features to the tumors from which they were derived (Tentler et al. 2012). These PDX are also more useful in accurately predicting therapeutic drug efficiency. This model also allows for the potential of creating a large number of xenografted mice from a single patient tumor, as the tumors can be serially biopsied and injected into different mice. Due to the heterogeneity of the tumors, a variety of different clones, each with different characteristics (including a different potential for therapeutic drug resistance), can be generated and subsequently analyzed (Einarsdottir et al. 2014; Kemper et al. 2015, 2016; Krepler et al. 2016). More recently, genetic screens using PDX have been done (Bossi et al. 2016). In this case, surgically resected melanoma tumors were subcutaneously injected into immunodeficient mice, and the resulting tumors were cultured. Next, the cultured cells were transduced with an shRNA epigenetic library and then re-transplanted into immunodeficient mice. The specific ability of each shRNA-transduced PDX to form tumors would allow for the identification of those genes involved in melanoma tumor formation. PDX also have potential uses in drug screens, since they would provide a more accurate representation of specific responses to specific drug treatments in comparison to cell line xenografts. Treatment of PDX from individual patients with specific drugs may prove useful in identifying specific therapeutic drugs that are useful for individual patients (Kuzu et al. 2015).

Despite these promising features, these models also have limitations. The time for tumors to develop in immunocompromised mice typically ranges from 3 to 9 months, and often, tumors do not develop following implantation. Furthermore, implantation into immunocompromised mice does not accurately reflect the natural, physiological tumor microenvironment. In addition, the PDX are difficult to manipulate genetically in comparison to melanoma cell lines, since traditional gene manipulation protocols are inefficient at inducing a change in gene expression in these tumor xenografts (Kuzu et al. 2015). A major limitation of using PDX models to explore melanoma therapy is that they are largely incompatible with testing various manipulations of the immune system since they are propagated in immunocompromised mice until more cost-effective humanized mice can be produced.

Genetically Engineered Mouse Models

Genome-Editing Tools to Generate Melanoma Mouse Models

Despite the fact that spontaneous melanoma formation in mice is rare, melanomagenesis can be readily initiated in mice that have been suitably genetically manipulated leading to the expression of mutant gene products that promote human melanoma or that alter (by either increasing or decreasing) the expression of genes that are implicated in melanomagenesis. In this regard, the mouse genome is exceptionally tractable for sophisticated and extensive genetic manipulation. First, the genome can be modified by random integration of ectopic transgenes into the genome. This is normally achieved by introducing a transgene into fertilized oocytes, after which the DNA will randomly integrate into the genome, and more often than not, the insertion site will not encode for an endogenous gene. Secondly, homologous recombination can be used to alter the genome by replacing or modifying a particular endogenous gene of interest, which is generally achieved using mouse embryonic stem cells (ES). Next, the modified ES cells are injected into mouse blastocysts, eventually generating mice with either the removal (knockout), replacement (knock-in), or particular modification (conditional allele) of a target gene of interest (GOI). As molecular biology techniques have become more refined and sophisticated, the spatiotemporal control of the expression of specific GOIs has become possible and commonly utilized. Finally, CRISPR/CAS9 is likely to provide a fast and

efficient approach to generate novel mouse models for the melanoma field (Singh et al. 2015).

Genetically engineered mouse models of melanoma have been generated employing a basic principle: a specific GOI is placed under the control of a particular promoter, which allows for a specific pattern of expression of that GOI. The expression of the GOI alone may be enough to cause melanomagenesis, or it may need to be expressed simultaneously in the same mouse with other melanoma-associated genes in order to promote melanomagenesis. Alternatively, chemical (e.g., DMBA) or environmental agents/factors (e.g., UVB light) may be applied to mice to promote melanomagenesis (Zaidi et al. 2011; Viros et al. 2014).

To study the function of a particular GOI in the melanocyte lineage, genetically engineered mice have been generated that express the specific gene under the control of a gene promoter expressed solely in the melanocyte lineage. For the most part, genetically engineered mouse (GEM) models with melanocyte-specific transgene expression have been developed using a transgene that is under the control of the tyrosinase (*Tyr*) gene promoter. Tyrosinase is an enzyme involved in melanin synthesis, a process specific to melanocytes. Thus, genes under the control of this promoter will be expressed in the melanocyte lineage. In addition to the promoter, transgenes that contain GOIs under the control of both the tyrosinase enhancer and promoter also exist, adding further specificity to the regulation of gene expression. Other promoters that have been employed to confer melanocyte-specific expression of different GOIs are from the dopachrome tautomerase (*Dct*), melanoma antigen recognized by T cells (*Mart1*), or microphthalmia transcription factor (*Mitf*) genes (MacKenzie et al. 1997; Alizadeh et al. 2008; Aydin and Beermann 2011).

While the majority of genetically engineered melanoma mouse models employ one of the aforementioned promoters, some studies have made use of the metallothionein gene (*Mt*) promoter to drive gene expression. In these mice, since the metallothionein gene is expressed in all tissues, the GOI is also expressed in all tissues (Iwamoto et al. 1991). In this case, melanoma formation usually results from treatment of the mice with a chemical tumor promoter, which seemingly affects the genetically modified melanocytes either alone or in addition to other cell types, which may themselves, become cancerous. For melanoma researchers, two main chemical agents have been used to induce tumor formation in mice. The first of these agents is 7,12-dimethylbenz[a]anthracene (DMBA), which suppresses the immune system and causes organ-specific carcinogenesis after being metabolized in the body and binding to DNA at adenine and guanine nucleotides (Miyata et al. 2001). The second of these carcinogenic agents is 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which binds to and activates protein kinase C (PKC), leading to various outcomes, including tumor formation in mouse skin (Abel et al. 2009).

The reversible induction of specific GOIs from exogenous promoters has also been made possible through the use of the tetracycline/doxycycline inducible system, i.e., the Tet-On and Tet-Off systems (Zaidi et al. 2011; Bockamp et al. 2008). In the Tet-Off system, the tetracycline transactivator protein (tTA), whose expression is under the control of a melanocyte-specific promoter (e.g., Tyr::tTA), is capable of binding to tetO sequences, which are part of a tetracycline response element (TRE) controlling the expression of a target gene of interest (Zaidi et al. 2011). The binding of the tTA to the TRE results in expression of the target gene of interest. When tetracycline is present, it binds to the tTA and does not allow it to bind to the TRE; therefore target gene expression is silenced. In the Tet-On system, the tTA is modified and is actually a reverse tTA (rtTA), which can only bind to the TRE in the presence of tetracycline. This system has been valuable in allowing researchers to look at the inducible and reversible effects of a certain GOI in a particular lineage, including the melanocyte lineage (Chin et al. 1999).

While these technologies have been useful in identifying the role of various genes in melanomagenesis, one important consideration to be made is that an artificially introduced promoter, and not the endogenous promoter, controls

expression of these genes. The consequences are that the temporal and level of expression of the GOI are modified, which may lead to physiological artifacts. To address this concern, genetically engineered mouse models that allow expression of melanoma-relevant genes under the control of their own endogenous promoter have been generated and are now widely used. In this case, the endogenous GOI is modified using homologous recombination in ES cells, which results in a genetically altered ES cell that is then injected into early embryos to generate allophenic (chimeric) mice. Several founder mice containing the altered gene have to be generated and characterized. Gene alteration can be constitutive or conditional. The conditional gene alteration can be performed using the Flp/Frt or Cre/LoxP system (Larue and Beermann 2007). These two systems are naturally present either in yeast or bacteriophage and are based on a recombinase (Flp or Cre) and sequences that are both specifically recognized and recombined (Frt or LoxP). Cre is an enzyme derived from the P1 bacteriophage that is able to homologously recombine internally DNA between two specific DNA sequences (known as LoxP sites) of 34 nucleotides: 5′-ATAACTTCGTATA ATGTATGC TATACGAAGTTAT-3′, with 2 inverted repeats of 13 nucleotides and a spacer of 8 nucleotides (Nagy 2000). The development of this enzyme as a tool for genetic manipulation for genetically engineered mice has been of immense importance and enabled researchers to perform experiments that were not previously possible. In general, Cre is used in the following way to induce the melanocyte-specific expression of a target GOI:

1. The endogenous gene of interest has been modified using homologous recombination in ES cells, and transgenic mice containing the modified allele have been generated. It should be noted that the modified allele (or "floxed" allele), which is present in all cell types, does not affect these mice, since it's mutant/altered form is not present unless Cre-mediated recombination occurs.
2. The Cre enzyme is under the control of a melanocyte-specific promoter such as Mitf or Mart1 (Alizadeh et al. 2008; Aydin and Beermann 2011). However, in most cases the Tyr promoter is used. These Tyr::Cre mice are characterized and, of course, the Tyr::Cre transgene has no effect on melanomagenesis by itself (Delmas et al. 2003).
3. The crosses of Tyr::Cre and floxed mice generate pups that contain the mutant/altered allele of the GOI (also called the defloxed allele) in the melanocyte lineage specifically with intact/floxed germ cells. Importantly, since the *Tyr* gene is expressed at approximately E9.5 during embryonic development, conditional genetic alterations (defloxing) of the gene of interest occur at approximately this time.

This technology has made it relatively simple to study the effects of alterations in a particular GOI in the melanocyte lineage from its endogenous promoter (Aoki et al. 2015; Mort et al. 2014; Wavre-Shapton et al. 2013; Li et al. 2011; Selfridge et al. 2010; Dhomen et al. 2010; Schouwey et al. 2007; Levy et al. 2010; Pshenichnaya et al. 2012). However, as mentioned, since *Tyr* is expressed during embryonic development, the mutant GOI is also expressed at this time, which may not completely reflect melanomagenesis in humans, as mutation primarily occurs after birth. This issue was addressed by the generation of two independent transgenic mouse lines in which a hormone-dependent form of Cre recombinase ($CreER^{T2}$) is expressed under the control of the tyrosinase promoter/enhancer sequences that will be called $Tyr::CreER^{T2(L)}$ and $Tyr::CreER^{T2(B)}$ (Yajima et al. 2006; Bosenberg et al. 2006). $CreER^{T2}$ is a fusion protein comprising Cre recombinase fused to a modified form of the hormone-binding domain of the human estrogen receptor that is activated by 4-hydroxytamoxifen (4-OHT) but not by endogenous estrogens (Feil et al. 1997). Hence, in the absence of 4-OHT, $CreER^{T2}$ is inactive, but in the presence of 4-OHT, the $CreER^{T2}$ protein is activated to perform its enzymatic activity in the nucleus. Consequently, the use of both $Tyr::CreER^{T2}$ mouse models has allowed for the spatial and temporal control of the expression of the altered GOIs in mouse melanocytes.

More recently, as the development of new technologies has advanced, a new technique known as "Replication-competent avian sarcoma-leukosis virus long terminal repeat with splice acceptor/tumor virus A" has emerged. This system, also known as RCAS/TVA, makes use of an RCAS vector to induce efficient and stable delivery of specific genes of interest in a targeted manner (Loftus et al. 2001; von Werder et al. 2012). When the suite of RCAS vectors is used in conjunction with mice carrying a melanocyte-specific *Dct::TVA* transgene (tva800 or tva950), this allows for genetic manipulation of gene expression in mouse melanocytes. In other words, targeted cells of interest are genetically modified to express the proteins tva800 or tva950 (*Dct::TVA* transgene), which are not expressed in mammalian cells. Once these proteins are expressed in the cells of interest, they can be infected with the RCAS vector and will express the gene of interest in the cells of interest.

Finally, recent advances in genome editing have been made possible by the application and refinement of the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system, which has significantly advanced how researchers can edit their desired genome of interest. This system has the potential to generate cell lines with identical genetic backgrounds that only differ in the loss of the CRISPR-targeted GOI. As such, the effects of losing expression of the GOI can be accurately assessed between the parental cell line and the CRISPR cell lines. In this system, the bacterial CAS9 protein, which cleaves DNA to introduce double-stranded breaks, is expressed in conjunction with a specific guide RNA (sgRNA), which is designed to target a specific GOI. This specific RNA sequence is complementary to a genomic region in the GOI that is found adjacent to a protospacer adjacent motif (PAM), which has a particular sequence and is commonly found within the genome. When the sgRNA aligns with the genomic DNA, CAS9 cleaves both strands of the DNA three nucleotides upstream of the PAM resulting in a double-stranded DNA break. When this break is repaired by host DNA repair mechanisms, the end result is often the disruption (knockout) of the targeted gene of interest. In addition, double-stranded break formation may be followed by the insertion of a specific sequence of interest, provided that an exogenous artificial repair template (e.g., a DNA fragment on which the GOI-targeting sequences flank the sequence to be inserted) is provided (Agrotis and Ketteler 2015). Importantly, since PAM sequences are found throughout the genome, CRISPR/CAS9 technology can be useful in manipulating a large number of genes and DNA sequences to assess their effects on cellular processes including melanomagenesis. To date, genetically engineered mouse lines using the CRISPR/CAS9 system for studying melanomagenesis have not been generated, but their potential is enormous. However, several studies have used this system in human melanoma cell lines (Krachulec et al. 2016; Shalem et al. 2014; Benamar et al. 2016; Kim et al. 2016). One of these studies used this system in a human melanoma cell line to identify genes involved in resistance to vemurafenib, one of the few treatments available for melanoma patients, and identified a number of genes that could potentially be involved in this resistance (Shalem et al. 2014). Future studies will be needed to verify the roles of these proteins in resistance associated with vemurafenib.

Genetically Engineered Mouse Line Tools

In the above section, the different mechanisms that have been used to generate melanocyte-specific expression of particular genes/mutants of interest were described. These mechanisms can also be used to generate transgenic mice that can be useful to help visualize and follow the particular expression or pattern of expression of genes/alleles that have been genetically manipulated (defloxed) following Cre-mediated recombination. This section discusses four such genetically engineered mouse lines that have been very useful in identifying which cells have been genetically altered following recombination events.

Dct::LacZ Mice

The *LacZ* gene encodes β-galactosidase, an enzyme involved in lactose metabolism in

bacteria. Specifically, this enzyme cleaves the disaccharide sugar lactose into the monosaccharides glucose and galactose. In the case of melanoma research, Dct::LacZ mice were generated, can be molecularly tested for homozygosity, and are still very useful because they can be used in X-gal staining experiments to identify Dct-positive cells including melanoblasts, melanocyte stem cells, melanocytes, and melanomas (MacKenzie et al. 1997; Takemoto et al. 2006; Nishimura et al. 2002). When X-gal, which is an organic molecule that contains galactose linked to an indole group, is added to the tissue section or embryo of interest, LacZ-expressing (i.e., Dct-expressing) cells will cleave the X-gal, liberating the lactose moiety and a 5-bromo-4-chloro-3-hydroxyindole moiety. The latter then forms a homodimer and is oxidized to produce a blue color. Other β-galactosidase substrates can be used for other purposes as well. This tool can be very useful in serving as a control for the identification of melanocyte-lineage cells.

Z/EG Mice

Another genetically engineered mouse line that is a useful imaging tool is the Z/EG mouse line, which contains the β-galactosidase gene flanked between two LoxP sites. Downstream of the second LoxP site is the gene encoding the green fluorescent protein, EGFP (Novak et al. 2000). Z/EG mice can be genotyped for homozygosity (Colombo et al. 2010) and express β-galactosidase starting from embryonic development. However, when these mice are crossed with Cre mice, Cre-mediated recombination results in the removal of the β-galactosidase gene and allows the expression of EGFP. As such, cells in which Cre has been active appear green under the microscope. The fluorescence can also be viewed in real time on live cells, providing researchers with the unique ability to visualize the kinetics of Cre-mediated recombination as it occurs within the cells. Moreover, as required, EGFP-expressing cells can be isolated using flow cytometry.

mT/mG Mice

The mT/mG mouse line is another tool for visualization of the spatial and temporal activity of Cre recombinase as well as for lineage tracing and cell morphology studies. In these mice, the gene encoding an N-terminal membrane-tagged tdTomato protein is flanked by LoxP sites, whereas the gene encoding a similarly tagged EGFP is downstream of the second LoxP site. This cassette is encoded within the Rosa26 locus; therefore it is expressed in all cells (Muzumdar et al. 2007). Prior to Cre-mediated recombination, all cells in the mouse express tdTomato and are red fluorescent. Following Cre-mediated recombination, the tdTomato expression is silenced and the cells express EGFP and are green fluorescent. As such, this mouse line is an excellent tool to provide contrast between cells that have or have been subject to Cre-mediated recombination.

Confetti Mice

The Confetti or Brainbow mouse line consists of a series of genes encoding fluorescent proteins (XFPs) back-to-back and separated by LoxP sites (Muzumdar et al. 2007). The genes encoding the XFPs are downstream of a "road block" cassette, which does not allow the expression of the XFPs prior to Cre-mediated recombination. However, it may contain a mutant XFP (e.g., YFP) that does not fluoresce but that can be detected by immunostaining, which would give an indication of the number of cells containing the transgene. Cre-mediated recombination results in the random removal of XFP genes, resulting in a single characteristic fluorescence for each individual cell. Monitoring of these individual cells is useful for addressing cell of origin, for lineage tracing, and for assessing the clonality of tumor formation.

The abovementioned tools make it possible to study the effects of melanocyte-specific mutations in GOIs. In the following section, the different genetically engineered mouse models of melanoma will be summarized. A complete listing of these mouse models is provided in Table 1.

Specific Genomic Alterations in Genetically Engineered Mice

Various signaling pathways including the RAS-activated ERK1/2 MAP kinase (MAPK), PI3-kinase, and WNT/β-catenin are involved in melanoma initiation and progression, as are proteins involved in the cell division cycle, such

Table 1 Summary of genetically engineered melanoma mouse models

Mouse model	Carcinogen	Melanoma	Met	Reference
Cell autonomous: monogenic				
Tyr::CreER$^{T2(B)/\circ}$; Braf$^{CA/+}$	None	No	No	Dankort et al. (2009)
Tyr::CreER$^{T2(L)/\circ}$; Braf$^{LoxP\text{-}V600E/+}$	None	Yes	No	Dhomen et al. (2009)
Tyr::CreER$^{T2(B)/\circ}$; Braf$^{LoxP\text{-}V618E/+}$	None	Yes	No	Perna et al. (2015)
Tyr::Braf$^{V600E/\circ}$	None	No	No	Goel et al. (2009)
Tyr::HRAS^{G12V}	None	No	No	Powell et al. (1995)
Tyr::HRAS^{G12V}	None	No	No	Chin et al. (1997)
Tyr::CreER$^{T2(L)/\circ}$; β-actin::Kras$^{LoxP\text{-}G12V/LoxP\text{-}G12V}$	None	Yes	No	Milagre et al. (2010)
Tyr::NRAS$^{Q61K/\circ}$	None	Yes	Yes	Ackermann et al. (2005)
Tyr::CreER$^{T2(B)/\circ}$; Nras$^{LoxP\text{-}G12D/LoxP\text{-}G12D}$	None	No	No	Pedersen et al. (2013)
Tyr::Cre/°; Nras$^{LoxP\text{-}G12D/LoxP\text{-}G12D}$	None	Yes	No	Pedersen et al. (2013)
Tyr::CreER$^{T2(B)/\circ}$; Nras$^{LoxP\text{-}Q61R/LoxP\text{-}Q61R}$	None	No	No	Burd et al. (2014)
Mt::Hgf	None	Yes	Yes	Takayama et al. (1997)
Mt::Ret	None	Yes	Yes	Iwamoto et al. (1991)
				Kato et al. (1998)
Tyr::CreER$^{T2(B)/\circ}$; Nf1$^{LoxP/LoxP}$	None	No	No	Maertens et al. (2013)
Tyr::CreER$^{T2(B)/\circ}$; Pten$^{LoxP/LoxP}$	None	No	No	Dankort et al. (2009)
Tyr::Cre/°; Pten$^{LoxP/+}$	None	No	No	Puig et al. (2009)
				Conde-Perez et al. (2015)
Ink4a$^{-/-}$	None	No	No	Serrano et al. (1996)
p16$^{Ink4a-/-}$; p19$^{Arf+/-}$	None	Yes	No	Sharpless et al. (2001)
Cdk4$^{R24C/R24C}$	None	No	No	Sotillo et al. (2001)
Tyr::bcat*$^{/\circ}$	None	No	No	Delmas et al. (2007)
Tyr::Cre/°; β-catΔex2-6$^{LoxP/LoxP}$	None	No	No	Luciani et al. (2011)
Tyr::Cre/°; β-catΔex3$^{LoxP/+}$	None	No	No	Yajima et al. (2013)
Tyr::Cre/°; Rosa26::Mdm4$^{LoxP/+}$	None	No	No	Gembarska et al. (2012)
Tyr::SV40Tag	None	Yes	Yes	Bradl et al. (1991)
				Klein-Szanto et al. (1991)
Dct::Grm1	None	Yes	No	Pollock (2003)
Tyr::HRAS^{G12V}	DMBA	Yes	Yes	Gause et al. 1997
p16$^{Ink4a-/-}$	DMBA	Yes	Yes	Krimpenfort et al. (2001)
Cdk4$^{R24C/R24C}$	DMBA/ TPA	Yes	No	Sotillo et al. (2001)
Mt::Hgf	UV	Yes	No	Noonan et al. (2001)
Mt::Hgf	UVB	Yes	Yes	De Fabo et al. (2004)
Cell autonomous: multigenic				
Tyr::CreER$^{T2(L)/\circ}$; Braf$^{LoxP\text{-}V600E/+}$; p16$^{Ink4a-/-}$	None	Yes	Yes	Dhomen et al. (2009)
Tyr::Braf$^{V600E/\circ}$; Cdkn2a$^{+/-}$	None	Yes	Yes	Goel et al. (2009)
Tyr::Braf$^{V600E/\circ}$; p53$^{-/-}$	None	Yes	Yes	Goel et al. (2009)
Tyr::CreER$^{T2(B)/\circ}$; Braf$^{CA/+}$; Pten$^{LoxP/LoxP}$	None	Yes	Yes	Dankort et al. (2009)
Tyr::CreER$^{T2(B)/\circ}$; Braf$^{CA/+}$; Pten$^{LoxP/LoxP}$; β-catΔex2-6$^{LoxP/LoxP}$	None	No	No	Damsky et al. (2011)
Tyr::CreER$^{T2(B)/\circ}$; Braf$^{CA/+}$; Pten$^{LoxP/LoxP}$; β-catΔex3$^{LoxP/LoxP}$	None	Yes	Yes	Damsky et al. (2011)

(*continued*)

Table 1 (continued)

Mouse model	Carcinogen	Melanoma	Met	Reference
Tyr::CreER$^{T2(B)/\circ}$; Braf$^{CA/+}$; Nf1$^{LoxP/LoxP}$	None	Yes	No	Maertens et al. (2013)
Tyr::NRAS$^{Q61K/\circ}$; Cdkn2a$^{-/-}$	None	Yes	Yes	Ackermann et al. (2005)
Tyr::CreER$^{T2(B)/\circ}$; Nras$^{LoxP\text{-}Q61R/LoxP\text{-}Q61R}$; p16$^{LoxP/LoxP}$	None	Yes	No	Burd et al. (2014)
Tyr::NRAS$^{Q61K/\circ}$; Tyr::Cre/$^{\circ}$; Pten$^{LoxP/+}$	None	Yes	Yes	Conde-Perez et al. (2015)
Tyr::NRAS$^{Q61K/\circ}$; Tyr::bcat*$^{/\circ}$	None	Yes	Yes	Delmas et al. (2007)
Tyr::NRAS$^{Q61K/\circ}$; Tyr::Cre/$^{\circ}$; Rosa26::Mdm4$^{LoxP/\circ}$	None	Yes	No	Gembarska et al. (2012)
Tyr::CreER$^{T2(B)/\circ}$; Kras$^{LoxP\text{-}G12D/+}$; p16$^{LoxP/LoxP}$	None	Yes	No	Monahan et al. (2010)
Dct::TVA; Cdkn2a$^{-/-}$; RCAS (NRAS^{Q61R} + Cre)	None	Yes	No	VanBrocklin (2010)
Dct::TVA; Cdkn2a$^{-/-}$; RCAS (NRAS^{G12V} + Cre)	None	No	No	VanBrocklin (2010)
Tyr::CreER$^{T2(B)/\circ}$; p16$^{LoxP/LoxP}$; p53$^{LoxP/LoxP}$	None	No	No	Monahan et al. (2010)
Tyr::HRAS$^{G12V/\circ}$; Cdkn2a$^{-/-}$	None	Yes	No	Chin et al. (1997)
Tyr::HRAS$^{G12V/\circ}$; Cdkn2a$^{+/-}$	None	Yes	No	Chin et al. (1997)
tetO::HRAS^{G12V}; Tyr::rtTA	None	Yes	No	Chin et al. (1999)
Tyr::iRasP1A; Cdkn2a$^{LoxP/LoxP}$	None	Yes	No	Huijbers (2006)
Tyr::HRAS^{G12V}; Cdk4$^{R24C/R24C}$	None	Yes	Yes	Hacker et al. (2006)
Mt::Ret; EdnrB$^{+/-}$	None	Yes	Yes	Kumasaka et al. (2010)
Mt::Ret; Il6$^{-/-}$	None	Yes	No	Von Felbert (2005)
Cdkn2a$^{-/-}$; Pten$^{-/-}$	None	Yes	No	You (2002)
Dct::rtTA; tetHA-Gnaq^{Q209L}; Cdkn2a$^{-/-}$	None	Yes	No	Feng (2014)
Tyr::HRAS^{G12V}; Cdk4$^{R24C/R24C}$	UV	Yes	Yes	Hacker et al. (2006)
Tyr::Mip2; Cdkn2a$^{+/-}$	DMBA	Yes	No	Yang (2001)
Non-cell autonomous				
K14-CreER$^{T2/\circ}$; RXRα$^{LoxP/LoxP}$	DMBA/TPA	Yes	No	Indra et al. (2007)
K14-CreER$^{T2/\circ}$; Taf4$^{LoxP/LoxP}$	DMBA/TPA	Yes	No	Fadloun (2007)

For simplification, the two Tyr::CreERt2 transgenic mouse lines are designated as B and L for the "Bosenberg" and "Larue" lines, respectively. *Met* metastasis

as INK4A-CDK4-RB and ARF-MDM2/4-TP53 signaling. However, it appears that key components of the ERK1/2 MAPK pathway play a crucial role in the early proliferation of initiated melanocytes followed by senescence. By contrast, components of other pathways are more closely associated with the bypass of senescence of initiated melanocytes to melanoma. One current linear model suggests that expression of mutationally activated NRAS or BRAF promotes melanocyte proliferation, resulting in benign melanocytic nevus formation, which ultimately cease proliferation and display features of senescence. Further steps to melanoma therefore require additional genetic/epigenetic events in pathways that promote bypass of senescence leading to melanomagenesis. However, melanoma progression (invasion and metastasis formation) is complex, since it involves multiple cellular mechanisms such as loss of melanocyte-keratinocyte adhesion, loss of melanocyte-basal adhesion, degradation of the basement membrane, migration, invasion, intravasation in blood/lymph vessels, resistance to anoïkis, extravasation, implantation, and angiogenesis. Of course, during the process, melanoma cells must be resistant to apoptosis and the immune system and must also be able to adapt to their environment through their high

molecular and cellular plasticity (phenotypic switch).

The following sections describe different classes of GEM models, which can be thought of as either cell-autonomous or cell non-autonomous (see Table 1). A cell-autonomous melanoma model is defined by the presence of germinal/somatic mutations in the same melanocytes and can be subdivided into three types: (ia) monogenic mouse models associated with proliferation which may or may not form melanoma, (ib) monogenic mouse models associated with immortalization and bypass of senescence, and (ic) multigenic mouse models associated with melanoma formation. A cell non-autonomous melanoma model is defined by at least one modification arising from (iia) the microenvironment (surrounding cells [keratinocytes, fibroblasts, adipocytes] or modification of the amount of nutrients or oxygen) or (iib) the environment (physical irradiation [such as UV] or chemical exposure [such as DMBA and/or TPA]). Here, we will refer to genes/RNA in italics, to human in capital letters, and to mice in lower cases with the first letter capitalized, proteins in non-italicized upper case for human or in lower cases with the first letter capitalized for mouse.

Cell-Autonomous Models

Monogenic Mouse Models Associated with Proliferation Which May or May Not Form Melanoma

Activated BRAF

The RAF protein family consists of three serine-threonine protein kinases, namely, ARAF, BRAF, and CRAF, that function downstream of GTP-bound RAS, which regulate signaling through the MEK1/2 to ERK1/2 MAP kinase signaling pathway. Mutations in the *ARAF* and *CRAF* genes are rare in human melanoma; hence no melanoma models have been generated for either of these two genes. By contrast, mutational activation of *BRAF* (primarily the $BRAF^{T1799A}$ transversion, resulting in the $BRAF^{V600E}$ oncoprotein) is detected in approximately ~70% of human sun-induced benign nevi and in ~50% of human melanomas (Davies et al. 2002; Pollock et al. 2003a). As such, mutated, oncogenic BRAF induces melanocyte proliferation first and senescence after several cell cycles (Michaloglou et al. 2005).

To study the role of the $BRAF^{V600E}$ oncoprotein kinase in melanomagenesis, four different *Braf* mouse models have been generated (Fig. 1). Due to differences between mouse and human *BRAF*, the human $BRAF^{T1799A}$ mutation is equivalent to $Braf^{T1910A}$ in the mouse. Correspondingly, human $BRAF^{V600E}$ is equivalent to $BRAF^{V637E}$ in the mouse. For convenience, we shall use the human numbering throughout. Two of these models employ a conditionally mutated *Braf* gene (encoding $BRAF^{V600E}$ protein) expressed following Cre-mediated recombination of the endogenous *Braf* gene. Even though Tyr::$CreER^{T2}$-mediated recombination results in the expression of the $BRAF^{V600E}$ oncoprotein in the melanocyte lineage, these two melanoma models display some differences. On one hand, these differences are due to different insertion sites of the transgene (Tyr::$CreER^{T2}$) and to a different sequence of the tyrosinase promoter (Yajima et al. 2006; Bosenberg et al. 2006). On the other hand, the *Braf* knock-in is slightly different as well (Mercer et al. 2005; Dankort et al. 2007). The consequence is that these two crosses, "Marais-Larue" and "McMahon-Bosenberg," led to hyperpigmentation of the skin, tails, ears, and paws and nevi formation. However, "Marais-Larue" mice formed melanoma without additional manipulations, whereas the "McMahon-Bosenberg" model did not (Dhomen et al. 2009; Dankort et al. 2009). Besides the intrinsic molecular differences of the transgenes of these two models, the presence/absence of melanoma may be due to the genetic background of the mice and/or a variety of other factors including the intrinsic quality of the animal colonies. The third Braf allele was slightly different, as the LoxP sequences were located in intron 3, flanking a polyA signal, with a mutated exon 15. These mice also produced melanoma after Cre-mediated recombination displaying hyperpigmentation of the skin, tails, ears, and paws and nevi formation (Perna et al. 2015). In the fourth mouse model

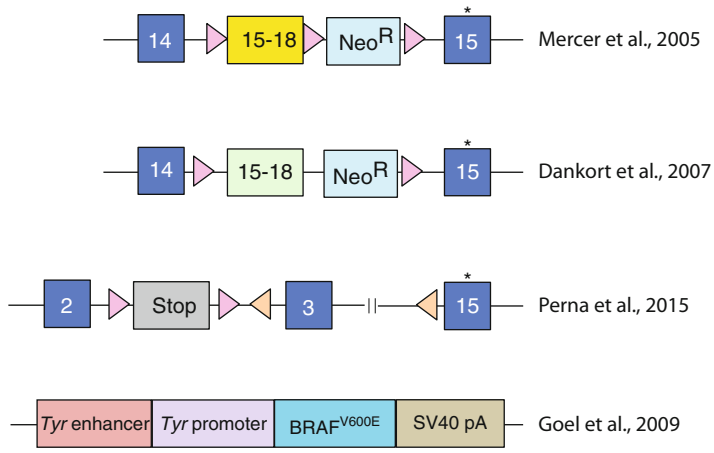

Fig. 1 Schematic summary of the four different Braf^{V600E} alleles. Endogenous exons are shown numbered in dark blue boxes. The star indicates that Exon 15 contains the mutation encoding for the V600E mutant protein. The pink triangles denote LoxP sites and the orange triangles denote FRT sites. Note that the mini-gene from Mercer et al. is of mouse origin and from Dankort et al. is of human origin. Note that exon 15–18 are identical at the protein level in human and mouse except at the C-terminal end. Human BRAF has an alanine at the position 762, whereas the mouse equivalent is a glycine. The BRAF V600E cDNA from Goel et al. is of human origin and obtained A375M melanoma cell line

(*Tyr::BRAF^{V600E}*), the BRAF^{V600E} oncoprotein is constitutively expressed under the control of the tyrosinase promoter (Goel et al. 2009). These mice displayed hyperpigmentation of the skin, tails, ears, and paws, but did not form melanoma. The efficiency to produce these transgenic mice was low, and the level of BRAF^{V600E} expression was also low. The fact that *Tyr::Cre*-mediated, embryonic expression of BRAF^{V600E} is lethal may explain the properties of the *Tyr::BRAF^{V600E}* transgenic mice (Goel et al. 2009; Dhomen et al. 2010).

Activated RAS

RAS proteins are GTPases that regulate intracellular signaling pathways and thereby serve to propagate extracellular mitogenic signals into appropriate biochemical and biological responses. The most cancer-relevant members of this family are NRAS, HRAS, and KRAS (neuroblastoma, Harvey rat sarcoma, and Kirsten rat sarcoma viral oncogene homologues, respectively). In humans, 20–25% of melanomas express mutationally activated NRAS. While HRAS and KRAS are also mutated in melanoma, the frequency of mutation is significantly lower (1% and 2%, respectively (Fernandez-Medarde and Santos 2011)).

Activated HRAS

Transgenic mice expressing constitutively active HRAS^{G12V} under the control of the tyrosinase promoter (i.e., Tyr::HRAS^{G12V}, also referred to as TPras) displayed melanocytic hyperplasia (characteristic of nevi formation) with intense skin pigmentation, but did not form melanoma (Powell et al. 1995). In another mouse model that contained the HRAS^{G12V} oncogene downstream of both the tyrosinase proximal promoter and upstream enhancer element, HRAS^{G12V} did not promote melanoma (Chin et al. 1997). One should note that in both cases human HRAS was used.

Activated KRAS

A genetically engineered mouse melanoma model for activated KRAS has also been developed (Milagre et al. 2010), in which a constitutively active KRAS^{G12V} oncoprotein is expressed under the control of the β-actin promoter (i.e., *β-actin::Kras$^{LoxP\text{-}G12V/LoxP\text{-}G12V}$*). Melanocyte-specific expression of KRAS^{G12V}, elicited with Tyr::CreER^{T2}, led to hyperpigmentation of the back, tail, and ear skin due to the emergence of various melanocytic lesions. The most common of these lesions was similar to human blue nevi.

Furthermore, mice with melanocyte-specific $KRAS^{G12V}$ expression also developed melanoma tumors in all cases, although they appeared not to metastasize throughout the body.

Activated NRAS

Since *NRAS* mutations are observed at a high frequency in melanoma (20–25%), genetically engineered mice expressing different constitutively active NRAS mutants ($NRAS^{Q61K}$, $NRAS^{G12D}$, and $NRAS^{Q61R}$) have been generated. In human melanomas, 84% and 7% of these mutations localize to codon 61 and 12, respectively. The presence of NRAS mutations in humans induces melanocytic proliferation followed by senescence as shown for giant nevus (Charbel et al. 2014).

The first genetically engineered transgenic *NRAS* mouse line, Tyr::$NRAS^{Q61K/\circ}$, was made using melanocyte-specific expression of human constitutively active $NRAS^{Q61K}$ under the control of both the distal regulatory element (DRE) and the promoter of the mouse tyrosinase gene (Ackermann et al. 2005). These mice displayed hyperpigmentation of the skin, ears, paws, and tails. More significantly, in these mice, melanocytes proliferated at ectopic sites of the skin and in some cases developed cutaneous melanoma with metastases in the lung, liver, and brain.

In the next genetically engineered mouse model, oncogenic $Nras^{G12D}$ was also expressed downstream of the endogenous *Nras* promoter (Pedersen et al. 2013) and was expressed in the melanocyte lineage following the activity of the $CreER^{T2}$ recombinase (*Tyr::CreER*$^{T2/\circ}$*; Nras*$^{LoxP\text{-}G12D/LoxP\text{-}G12D}$). In these mice, melanocyte-specific $Nras^{G12D}$ expression resulted in skin hyperpigmentation and nevi formation, but no tumors developed. When the $Nras^{G12D}$ oncogene was expressed in the melanocyte lineage during development (*Tyr::Cre/*$^{\circ}$*; Nras*$^{LoxP\text{-}G12D/LoxP\text{-}G12D}$), the mice had darker skin, tails, paws, and snouts (compared to controls) and also developed benign lesions similar to human blue nevi, but they did not form cutaneous melanoma. Interestingly, these mice developed neurological symptoms typical of motor dysfunction, which was concurrent with significant darkening of the arachnoid mater and pia mater, the two thinnest membranes surrounding the brain and spinal cord. The melanocytes in these meninges eventually progressed to primary central nervous system (CNS) melanomas, which were both aggressive and invasive.

Activated RTKs

Many different signaling pathways are implicated in melanocyte development and in melanomagenesis, including receptor tyrosine kinases (RTKs) such as KIT, MET, and RET (Easty et al. 2011; Paluncic et al. 2016). KIT plays an essential role in melanocyte development, proliferation, survival, migration, and differentiation and is overexpressed and/or mutated (V559A) in melanoma (Walker et al. 2011; Stankov et al. 2014). However, no mouse melanoma models with activating mutations in the Kit have been generated. Genetically engineered mouse models studying the Met and Ret RTKs have been generated as described below.

HGF-MET Signaling

The hepatocyte growth factor (HGF) promotes melanocyte proliferation through its cognate receptor tyrosine kinase MET. HGF-MET-mediated activation of the ERK1/2 MAPK and PI3K pathways most likely promotes melanocyte proliferation leading to melanomagenesis (Hirobe et al. 2004; Li et al. 2001). In a genetically engineered mouse model in which mouse Hgf is expressed under the control of the metallothionein promoter (*Mt::Hgf*), melanomas developed, as did mammary gland tumors and rhabdomyosarcomas (Takayama et al. 1997). Furthermore, it appeared that melanoma formation was driven by an autocrine loop in which the tumors displayed elevated levels of both the Hgf ligand and its receptor Met (Otsuka et al. 1998). Interestingly, while melanocytes in wild-type mice are normally located in the hair follicles, the melanocytes in these transgenic mice were found in the epidermis, in the dermal-epidermal junction, and in the dermis. Thus, since human melanocytes are primarily found in the epidermis, this transgenic mouse model could more accurately reflect the composition of human skin.

GDNF-RET Signaling

The RTK RET is involved in a wide range of biological processes, including neural crest cell migration, and establishment and maintenance of neurons in the central and peripheral nervous systems (Mulligan 2014). The ligands for RET are the glial cell line-derived neurotrophic factor (GDNF) family of proteins. While mutations in RET have been observed in melanoma, their significance remains uncertain (Mulligan 2014). To better understand the role of RET in tumorigenesis, genetically engineered mice were made that express Ret downstream of the mouse metallothionein 1 promoter-enhancer, which resulted in the ubiquitous expression of oncogenic Ret (Iwamoto et al. 1991; Kato et al. 1998). In these transgenic mice, melanoma tumors spontaneously formed primarily in the dermis of the face around the nose (Iwamoto et al. 1991). These tumors were slow growing and did not metastasize on a mixed strain background (C57BL/6 × BALB/c). However, on a pure C57BL/6 background, these tumors progressed to malignancy and metastasized to multiple sites, including the lymph nodes, lungs, and brain (Kato et al. 1998).

G-Protein-Coupled-Receptor

G-protein-coupled-receptor has been shown to be involved in melanomagenesis. Metabotropic glutamate receptor 1 (GRM1) is upregulated in some human melanoma and was sufficient to induce melanoma initiation in mice (*Dct::Grm1*) after inducing proliferation and inhibiting apoptosis (Pollock et al. 2003b).

SV40 Large T-Antigen

The SV40 large T-antigen is an oncoprotein that is derived from the polyoma SV40 virus, which is capable of transforming a wide variety of cell types (for review see An et al. 2012). As the oncogenic activity of the large T-antigen is mediated primarily by its ability to repress the tumor suppressors P53 and RB (An et al. 2012), transgenic mouse models with its expression may display similar phenotypes to those models without expression of both P53 and RB. GEM models with melanocyte-specific expression of this oncoprotein were some of the first mouse models produced. Transgenic mice with expression of the large T-antigen under the control of the *Tyr* promoter (*Tyr::SV40Tag*) spontaneously developed eye and skin melanoma (Bradl et al. 1991; Klein-Szanto et al. 1991; Silvers and Mintz 1998). Moreover, these *Tyr::SV40Tag* melanocytes were prone to form melanoma after UVB irradiation (Larue et al. 1992).

Monogenic Mouse Models Associated with Immortalization and Bypass of Senescence

Loss of NF1

Neurofibromin 1 (*NF1*) encodes a GTPase-activating protein (GAP) that has tumor suppressor activity through its activation of the GTPase activity of RAS proteins. Recently *NF1* mutations have emerged as a frequent event in melanomagenesis being mutated in approximately 10–15% of human melanomas, which now constitutes one of the four major subtypes (*NRAS*, *BRAF*, *NF1*, and triple wild-type) used to classify melanomas, based on their mutational profiles (Cancer Genome Atlas Network and Electronic address IMO, Cancer Genome Atlas Network 2015). As a relatively new mediator of melanomagenesis, studies on mice with melanocyte-specific silencing of Nf1 are limited. In a GEM model, the loss of *Nf1* in the melanocyte lineage ($Tyr::CreER^{T2/\circ}$; $Nf1^{LoxP/LoxP}$) resulted in increased ear, tail, and paw pigmentation; however, it did not induce melanoma when induced after birth (Maertens et al. 2013). This finding was surprising, since loss of NF1 should lead to elevated RAS.GTP, which might reasonably be expected to have promoted melanocyte proliferation. This result suggests that NF1 silencing alone is unable to promote sufficient accumulation of RAS.GTP to promote melanocyte proliferation (Maertens et al. 2013; Posch et al. 2016).

Loss of PTEN

PTEN is a lipid phosphatase that negatively regulates the PI3K signaling pathway in cells and plays an important role in the suppression of melanomagenesis. Indeed, PTEN is mutated or silenced in ~20% of human melanomas (Wu et al. 2003; Whiteman et al. 2002; Zhou et al. 2000; Conde-Perez et al. 2015). However,

GEM models indicate that melanocyte-specific silencing of Pten (*Tyr::CreER*$^{T2/o}$*; Pten*$^{LoxP/LoxP}$ or *Tyr::Cre/*^{o}*; Pten*$^{LoxP/+}$) has little or no phenotypic effect on melanocytes and is not sufficient to promote melanomagenesis (Dankort et al. 2009; Conde-Perez et al. 2015; Puig et al. 2009).

Loss of CDKN2A, Encompassing INK4A and ARF

The *CDKN2A* gene, which is mutated in at least half of all human melanomas, comprises two genes (*INK4A* and *ARF*) that encode two melanoma suppressor proteins, P16^{INK4A} and P14^{ARF} (p19^{Arf} for the mouse) (Bennett 2016). P16^{INK4A} is a stoichiometric inhibitor of D-type cyclin-dependent kinases (CDK) 4 and 6. Expression of P16^{INK4A} inhibits the CDK4/6-mediated phosphorylation of RB and its close homologues p107 and p130, leading to arrest of the cell division cycle prior in G1. By contrast, P14^{ARF} (p19^{Arf} in mice) inhibits MDM2/MDM4 thereby leading to stabilization and activation of TP53 (Bennett 2016). The initial studies looking at the role of the *Cdkn2a* gene (also referred to as Ink4a) in tumorigenesis showed that while Ink4a$^{-/-}$ transgenic mice developed various malignancies, including fibrosarcomas and lymphomas, they did not form melanomas (Serrano et al. 1996). DMBA and/or UV treatment decreased tumor latency, demonstrating that the loss of the *Cdkn2a* gene resulted in increased sensitivity to carcinogenic agents. However, transgenic mice lacking p16^{Ink4a} but containing one copy of p19^{Arf} developed melanoma, although they developed soft tissue sarcoma and lymphoma more frequently (Sharpless et al. 2001). Mice lacking p16^{Ink4a} (p16$^{Ink4a-/-}$) were also more prone to form tumors (including melanoma) following DMBA treatment than those with one functional copy of p16^{Ink4a} (p16$^{Ink4a+/-}$).

Gain of CDK4

CDK4 is implicated in melanomagenesis since a mutationally altered form of the gene, encoding CDK4^{R24C}, was identified as a FAMM family gene (Wolfel et al. 1995). CDK4 regulates early events in the cell division cycle through the phosphorylation of the RB family tumor suppressors. Furthermore, activating mutations in CDK4 (i.e., CDK4$^{R24C/H}$) have been observed in melanoma (Zuo et al. 1996; Puntervoll et al. 2013). However, studies using genetically modified mice expressing the mutant CDK4^{R24C} in the place of the normal protein showed that the mice displayed a wide range of tumors, but not melanomas unless the mice were treated with DMBA/TPA (Sotillo et al. 2001).

Altered β-Catenin Levels

β-Catenin (*CTNNB1*) is an armadillo repeat-containing protein that is both a mediator of cell-cell adhesion through cadherins and a transcriptional co-regulator that regulates gene expression (Aktary et al. 2016). Following WNT-mediated inhibition of the APC destruction complex (among other pathways), the abundance of β-catenin is increased allowing it to translocate into the nucleus where it interacts with transcription factors (including LEF/TCF) to regulate mRNA production. While mutations in the *CTNNB1* gene itself are rare in melanoma, β-catenin cytoplasmic or nuclear localization has been observed in approximately 30% of human melanoma patients, suggesting that its transcriptional activity may be increased (Rimm et al. 1999). To date, three different genetically engineered mouse models have been developed that can be used to look at the role of β-catenin in melanomagenesis. In the first model (*Ctnnb1*$^{\Delta ex2\text{-}6LoxP}$), Cre-mediated recombination results in a truncated and inactive β-catenin (Brault et al. 2001). Two other mouse models allow for expression of a stabilized and activated form of β-catenin. *Tyr::*βcat* mice express a stabilized form of β-catenin (S33A/S37A/T41A/S45A) under the control of the mouse tyrosinase promoter. Importantly, serines/threonine, which are encoded in exon 3 of the *CTNNB1* gene, are essential for regulating the stability (degradation) of β-catenin (Delmas et al. 2007). In *Ctnnb1*$^{\Delta ex3LoxP}$ mice, exon 3 has been flanked by loxP sites such that Cre-mediated recombination results in expression of a truncated but stabilized and constitutively active form of β-catenin (Harada et al. 1999).

Different studies have shown that melanocyte-specific overexpression or loss of β-catenin by

itself is insufficient to elicit melanoma in genetically manipulated mice (Delmas et al. 2007; Luciani et al. 2011; Yajima et al. 2013). However, these studies have shown that any alterations in β-catenin levels appear to have deleterious effects on melanocyte proliferation and pigmentation in mice. *Tyr::*βcat* mice display a gray coat color and a white belly spot similar to mice with hypomorphic allele of $Mitf^{Wh/+}$ (Delmas et al. 2007; Gallagher et al. 2013). By contrast, mice with melanocyte-specific silencing of β-catenin (*Tyr::Cre/°; Ctnnb1*$^{\Delta ex2\text{-}6LoxP/\Delta ex2\text{-}6LoxP}$) displayed a white coat color with a dramatic reduction of the number of melanocytes due a reduction of melanoblast proliferation (Luciani et al. 2011).

Gain of MDM4

The transcription factor TP53 is activated in response to various forms of cellular stress (including DNA damage) and plays an important role in maintaining genome integrity by regulating the expression of genes involved in DNA repair, cell cycle arrest, and apoptosis (Eischen 2016). The stability of TP53 and its transcriptional activity are regulated by a number of its interacting partners, including MDM4 (MDMX), a negative regulator of TP53 function (Eischen 2016). Consistent with TP53 being commonly mutated in many types of cancer, ~15% of human melanomas display alterations in *TP53.* Moreover, recent mouse studies have indicated that TP53 serves as a potent suppressor of melanoma progression in mice with melanocyte-specific expression of oncogenic NRAS or BRAF. Moreover, to study the role of the MDM4 in melanomagenesis, a GEM model was generated in which the Mdm4 was conditionally expressed from the Rosa26 locus (Gembarska et al. 2012). However, no tumors were observed in mice with melanocyte-specific overexpression of Mdm4 (*Tyr::Cre/°; Mdm4*$^{LoxP/°}$).

Multigenetic Mouse Melanoma Associated with Melanoma Formation

While the genetically engineered mouse models described above have been useful in identifying the role of individual genes in melanoma initiation, the majority of GEM models have demonstrated that a single mutated gene is insufficient for the formation of advanced melanomas with the ability to metastasize. However, when a number of these genetically modified alleles are combined, the rate of melanoma initiation and progression (invasion with metastatic dissemination) is very frequently increased.

Multigenic GEM Models of BRAF-Mutated Melanoma

Melanocyte-specific expression of oncogenic $BRAF^{V600E}$ leads to formation of benign nevus-like lesions (Dhomen et al. 2009; Dankort et al. 2009). However, $BRAF^{V600E}$ expression in combination with $p16^{Ink4a}$ silencing (*Tyr::CreER*$^{T2/°}$; *Braf*$^{LoxP\text{-}V600E/+}$; *p16*$^{Ink4a-/-}$) decreased the latency of melanoma initiation and increased the penetrance, number, and metastatic dissemination of melanomas in the mice (Dhomen et al. 2009). Consistent with these results, silencing of Ink4a/Arf or Tp53 promoted melanoma progression in two other $Braf^{V600E}$-driven melanoma models (*Tyr::Braf*$^{V600E/°}$; *Cdkn2a*$^{-/-}$ & *Tyr::Braf*$^{V600E/°}$; *Trp53*$^{-/-}$) (Goel et al. 2009).

Mutational silencing of Pten also strongly potentiates progression of Braf-mutated melanoma. In this case, mice with melanocyte-specific expression of $Braf^{V600E}$ combined with Pten silencing (*Tyr::CreER*$^{T2/°}$; *Braf*$^{CA/+}$; *Pten*$^{LoxP/LoxP}$) displayed rapid onset, fully penetrant primary melanomagenesis with evidence of micrometastases in numerous organs including the lungs and lymph nodes (Dankort et al. 2009). Consistent with its ability to regulate PI3′-lipid signaling in melanocytes, $Braf^{V600E}$ also cooperated with mutationally activated Pik3CA, encoding the catalytic subunit of PI3′-kinase-a (Vredeveld et al. 2012; Marsh Durban et al. 2013; Deuker et al. 2015).

An important role for β-catenin has been discerned in the $Braf^{V600E}$/$Pten^{null}$ GEM model of melanoma. Silencing of β-catenin (*Tyr::CreER*$^{T2/°}$; *Braf*$^{CA/+}$; *Pten*$^{LoxP/LoxP}$; *Ctnnb1*$^{\Delta ex2\text{-}6LoxP/LoxP}$) delayed melanoma formation and inhibited the appearance of lymph node metastases. This result may be explained by previous results showing that melanocyte-specific loss of β-catenin in mice (*Tyr::Cre/; Ctnnb1*$^{\Delta ex2\text{-}6LoxP/\Delta ex2\text{-}6LoxP}$) resulted in a white coat color that is due to an inhibition of proliferation of melanocytes and

in consequence a decreased number of melanocytes in the skin of these mice (Luciani et al. 2011). Furthermore, activation of β-catenin in the $Braf^{V600E}$/$Pten^{null}$ melanocytes (*Tyr::CreER*$^{T2/+}$; *Braf*$^{CA/+}$; *Pten*$^{LoxP/LoxP}$; *Ctnnb1*$^{\Delta ex3LoxP/\Delta ex3LoxP}$) resulted in a significant increase in melanoma growth and metastasis (Damsky et al. 2011).

Melanocyte-specific silencing of Nf1 combined with $Braf^{V600E}$ expression (*Tyr::CreER*$^{T2/+}$; *Braf*$^{CA/+}$; *Nf1*$^{LoxP/LoxP}$) resulted in increased melanoma compared with $Braf^{V600E}$ alone (*Tyr::CreER*$^{T2/+}$; *Braf*$^{CA/+}$) alone (Maertens et al. 2013). Taken together, these results suggest that oncoproteins such as $Braf^{V600E}$ that promote initial melanocyte proliferation can cooperate with genetic alterations pathways that promote melanoma initiation and which may be involved in the bypass of the senescence-like arrest that restrains the continuous proliferation of $Braf^{V600E}$-driven benign nevus cells.

Finally, the sleeping beauty transposon-mediated mutagenesis has also been used in a $Braf^{V600E}$ context (Mann et al. 2015). This study made use of the sleeping beauty transposase, an enzyme that is capable of excising a transposon from DNA (either plasmid or genomic) and then inserting it into another DNA site with a specific sequence (Dupuy et al. 2009). Transposon insertion would then result in the loss or altered expression of a number of different genes, which could potentially affect tumor formation. Analysis of the different tumors formed in each mouse would potentially identify a different gene implicated in tumor formation. In this study, Cre-mediated activation of the sleeping beauty transposase resulted in the melanocyte-specific disruption in the expression of different genes and allowed for the identification of 1,232 candidate melanoma genes. Specifically, it was shown that CEP350, a protein thought to be involved in the organization, binding and anchoring of microtubules at the centrosome, acts as a tumor suppressor (Mann et al. 2015).

Multigenic Mouse Models on a Mutant NRAS Background

Mice with melanocyte-specific expression of $NRAS^{Q61K}$ develop melanomas with evidence of metastases, although the time to tumor formation was approximately 1 year (Ackermann et al. 2005). However, expression of $NRAS^{Q61K}$ in combination with Ink4a-Arf silencing (*Tyr::NRAS*$^{Q61K/+}$; *Cdkn2a*$^{-/-}$) resulted in reduced latency and increased melanoma formation and metastases. Similarly, melanocyte-specific silencing of $p16^{Ink4a}$ combined with $Nras^{Q61R}$ expression (*Tyr::CreER*$^{T2/+}$; *Nras*$^{LoxP\text{-}Q61R/LoxP\text{-}Q61R}$; *Ink4a*$^{LoxP/LoxP}$) also resulted in melanoma, but these tumors did not metastasize (Burd et al. 2014). In the most rigorous analysis of the effects of oncogenic NRAS on melanomagenesis to date, mice with melanocyte-specific expression of either $Nras^{G12V}$ (*Nras*$^{LSL\text{-}G12V}$) or $Nras^{Q61R}$ (*Nras*$^{LSL\text{-}Q61R}$) were compared with an $Ink4a^{Null}$ background. Remarkably, whereas $Nras^{Q61R}$/$Ink4a^{Null}$ melanocytes progressed to melanoma, $Nras^{G12V}$/$Ink4a^{Null}$ melanocytes did not. This is perhaps the clearest example of mutation-specific effects of RAS genes on tumorigenesis to date and may also explain the preponderance of $NRAS^{Q61X}$ versus $NRAS^{G12X}$ alterations in human melanoma (Burd et al. 2014).

In addition to Ink4a and/or Arf silencing, mutational inactivation of Pten expression also contributed to melanomagenesis on an NRAS mutant background (Conde-Perez et al. 2015). Mice with both melanocyte-specific expression of $NRAS^{Q61K}$ combined with reduced Pten expression (*Tyr::NRAS*^{Q61K}; *Tyr::Cre/+*; *Pten*$^{LoxP/+}$) showed that diminished Pten expression accelerated melanomagenesis in conjunction with oncogenic NRAS. Furthermore, when melanocytes with one functional copy of Pten (i.e., Tyr::Cre/+; $Pten^{LoxP/+}$) were isolated, they displayed low efficiency (~25%) of establishment of immortalized cell lines. However, Pten-deficient melanocytes were completely established in culture as immortalized cell lines very efficiently, thereby suggesting that Pten silencing was a contributing factor in the bypass of senescence required for the immortalization of melanocytes in cell culture.

β-catenin has also been shown to promote melanomagenesis in the context of oncogenic NRAS. While *Tyr::βcat** mice alone did not form tumors, mice with combined expression of $NRAS^{Q61K}$ and activated β-catenin (*Tyr::NRAS*$^{Q61K/+}$; *Tyr::βcat**/+) developed melanomas with shorter latency and higher penetrance than mice with $NRAS^{Q61K}$ expression alone.

Mechanistically, β-catenin repressed *Ink4a* transcription in these tumors, which resulted in the bypass of senescence. The presence of the oncogenic form of β-catenin also promoted the formation of lung metastases. These results revealed the association of induction of proliferation ($NRAS^{Q61K}$) with the bypass of senescence in these mice (Delmas et al. 2007).

Finally, melanocyte-specific overexpression of Mdm4 in mice with melanocyte-specific expression of oncogenic $NRAS^{Q61K}$ (Tyr::$NRAS^{Q61K/\circ}$; Tyr::Cre/°; Mdm4 $^{LoxP/\circ}$) resulted in increased melanoma tumor formation in comparison to mice without the overexpressed Mdm4 (Tyr::$NRAS^{Q61K/\circ}$; Tyr::Cre/°). This study also showed that MDM4 protein levels were increased in human melanomas compared to normal melanocytes and benign nevi, which would suggest that while the *TP53* gene may be mutated in melanoma at low frequency, the TP53 pathway may still be inactivated by other means during melanomagenesis.

Constitutive expression of an oncogenic form of Nras in mouse melanocytes represents only partially the situation in humans. Several mouse models were generated including the iNras melanoma model based on the Tet-On system in which Nras activated form is produced in melanocytes after doxycycline induction on a Cdkn2a-null background. iNras mice produce melanoma in 17 weeks with a 50% penetrance (Kwong et al. 2012).

Other Multigenic Mouse Models

While most multigenic mouse models of melanoma are built on a platform of mutationally activated Nras or Braf, a number of studies have been performed using other oncoprotein drivers of melanocyte proliferation. For example, melanocyte-specific silencing of $p16^{Ink4a}$ cooperated with expression of $Kras^{G12D}$ (*Tyr::CreER*$^{T2/\circ}$*; Kras*$^{LoxP\text{-}G12D/+}$*; Ink4a*$^{LoxP/LoxP}$) to promote melanomagenesis, although no metastases were observed in this model (Monahan et al. 2010). In this study, the importance of the oncogenic Kras in promoting melanomagenesis was demonstrated by the fact that mice with melanocyte-specific silencing of $p16^{Ink4a}$ and TP53 (*Tyr::CreER*$^{T2/\circ}$*; p16*$^{LoxP/LoxP}$*; p53*$^{LoxP/LoxP}$) did not form melanoma. When both alleles of the *Cdkn2a* gene were deleted in *Tyr::HRAS*^{G12V} mice (*Tyr::HRAS*$^{G12V/\circ}$*; Cdkn2a*$^{-/-}$), melanomagenesis, but not metastasis, was significantly increased compared to mice with an intact *Cdkn2a* locus (Tyr::$HRAS^{G12V/\circ}$; $Cdkn2a^{+/-}$ Chin et al. 1997). The importance of oncogenic HRAS in the context of *Cdkn2a* null mice was further demonstrated using a doxycycline-inducible $HRAS^{G12V}$ mouse model (i.e., Tyr/Tet-RAS; Chin et al. 1999). In these mice, induced expression of $HRAS^{G12V}$ combined with *Cdkn2a* deletion resulted in melanomagenesis. Furthermore, doxycycline withdrawal from $HRAS^{G12V}$/Ink4a-Arf^{null} melanoma-bearing mice resulted in dramatic regression of pre-existing melanomas. Furthermore, re-administration of doxycycline resulted in prompt melanoma recurrence at the original primary sites.

In another study, mice with melanocyte-specific $HRAS^{G12V}$ expression combined with ubiquitous expression of $Cdk4^{R24C}$ (*Tyr::HRAS*^{G12V}*; Cdk4*$^{R24C/R24C}$) developed melanoma more frequently than $Cdk4^{R24C/R24C}$ mice alone (Hacker et al. 2006). At this point, the cooperation was not studied at the cellular level.

Finally, the Ret melanoma model has been used to show that the endothelin receptor B (EdnrB), which plays an important role in the development of neural crest cells (including melanocytes), can also contribute to melanomagenesis (Kumasaka et al. 2010). Specifically, when melanomas emerged in the *Mt::Ret* mice, it was observed that the expression of EdnrB was decreased in malignant compared to benign tumors. To examine the role of EdnrB in tumor progression in the *Mt::Ret* model more carefully, mice were engineered to express RET but with reduced EdnrB expression (*Mt::Ret; EdnrB*$^{+/-}$). These mice directly developed metastatic melanoma without displaying an evolution from a nevus-like phenotype. Moreover, these mice displayed evidence of lung metastases. While this model might be useful for studying de novo melanomagenesis, the lack of increased RET

signaling in human melanoma may reduce the clinical relevance of this model.

Cell Non-autonomous Models

Cell Non-autonomous Models Associated with the Microenvironment

While studying melanocyte-specific gene mutations and their effect on melanomagenesis is of paramount importance, it must be remembered that, in vivo, melanocytes in the epidermis make contacts with neighboring keratinocytes. In this regard, it may be possible that alterations (e.g., mutations and/or alterations in expression of various genes) within keratinocytes may, in certain contexts, promote melanomagenesis. The first indication of this was from work in mice with keratinocyte-specific silencing of the retinoic acid receptor Rxrα as well as the Taf4 subunit of TFIID, a general transcription factor. In these mice, DMBA and TPA treatment led not only to papilloma formation but also to the formation of nevi and invasive dermal melanoma tumors (Indra et al. 2007). This cell non-autonomous melanoma formation, which occurred in response to genetic changes in the neighboring keratinocytes, reinforces the fact that environmental cues from keratinocytes (e.g., α-MSH) can trigger melanocyte hyperproliferation and/or oncogenic transformation.

Cell Non-autonomous Models Associated with the Environment

Different treatments have been used with different genetically engineered mouse models to induce melanomagenesis: including treatment with DMBA with or without TPA and UV irradiation.

Administration of DMBA to mice with melanocyte-specific oncogenic $HRAS^{G12V}$ resulted in malignant melanoma development (Powell et al. 1995; Gause et al. 1997), which then metastasized to the lungs and the lymph nodes (Gause et al. 1997). Mice lacking $p16^{Ink4a}$ expression are not melanoma-prone; however treatment of these mice with DMBA induced melanoma with evidence of metastases (Krimpenfort et al. 2001). Furthermore, combination treatment of mice with ubiquitous expression of $Cdk4^{R24C}$ with DMBA and TPA induced nevus formation, which eventually progressed to melanoma (Sotillo et al. 2001).

The *Mt::Hgf m*odel has been particularly useful for determining the role of UV irradiation in melanoma. In one study, a single dose of UV radiation of neonates was both necessary and sufficient to induce melanoma (Noonan et al. 2001; Wolnicka-Glubisz and Noonan 2006). This model was also used to demonstrate that it is UVB irradiation, and not UVA, that promotes melanoma initiation in this model (De Fabo et al. 2004). UV irradiation of $Tyr::HRAS^{G12V}$; $Cdk4^{R24C/R24C}$ mice increased tumor development (compared to non-irradiated mice) and resulted in lymph node metastases (Hacker et al. 2006).

The Braf mouse melanoma model was used to evaluate that the single dose of UVR that mimicked mild sunburn in humans induced clonal expansion of the melanocytes, and repeated doses of UVR increased melanoma burden. A large proportion of UVR tumors exhibited Trp53 mutations, and mutant Trp53 accelerated melanoma initiation on a $Braf^{V600E}$ background (Viros et al. 2014).

Other Mammalian Melanoma Models

Canine Melanoma Models

Dogs are now viewed as potentially useful models of human melanoma and can be used in treatment studies. Unlike in mice, malignant melanoma occurs spontaneously in domestic dogs and is relatively common, compared to other animals. In this regard, canine models of melanoma are useful for studying the human disease, since they are heterogeneous and since both tumor formation and metastasis occur spontaneously in immunocompetent animals. In dogs, the most frequent type of melanoma is of mucosal origin, which typically originates in the oral cavity. This type of canine melanoma is highly aggressive and metastasizes rapidly to numerous sites including the lungs and the lymph nodes (for review see van

der Weyden et al. 2016). Other types of melanomas, including cutaneous (occurring in the hairy skin), acral (occurring in the footpad and nails), and uveal (occurring in the eye), also occur in dogs but are less frequent. Importantly, it must be noted that canine cutaneous melanomas are usually benign, which is in contrast to human cutaneous melanomas, which are invariably malignant.

Following the completion of the canine genome project, it was noted that human and dog nucleic acid and protein sequences are more similar to one another than are mouse and human sequences, further supporting the utility of the dog melanoma model in studying the human disease (Lindblad-Toh et al. 2005). However, some differences exist between the human and dog diseases. As mentioned above, the primary disease subtype in humans is cutaneous melanoma, whereas in dogs, it is mucosal melanoma. As such, exposure to UV is not a risk factor in mucosal melanoma in dogs, whereas it is a factor in cutaneous melanoma in humans. This observation appears intuitive since dogs are protected against UV by their fur. As with human melanomas, mutational activation of genes encoding BRAF, NRAS, PTEN, and KIT have all been observed in canine melanomas, albeit to differing extents than the human disease (van der Weyden et al. 2016). Nonetheless, as with human melanoma, multiple signaling pathways (e.g., MAPK, PI3K, WNT) are involved in and responsible for canine mucosal and cutaneous melanomas.

One study looking at over 2,000 dogs with melanoma showed that certain dog breeds, including Labradors, Rottweilers, and Dobermans, developed melanoma more frequently than other breeds. Furthermore, melanomas were more frequent among dogs with black coats, compared to those with white coats (Gillard et al. 2014). This finding is somewhat in disagreement with the occurrence of melanoma in humans, where light-skinned individuals are more likely to develop melanoma than those who are dark-skinned, although this is a comparison made between mucosal and cutaneous melanomas. This may suggest that the genetic predisposition for these two types of melanoma is different.

Typical treatments for mucosal melanoma in dogs include surgical resection and ionizing radiation of the primary tumor. In addition, other therapies such as chemotherapy (e.g., carboplatin or cisplatin) and immunotherapy (e.g., allogeneic cancer vaccines expressing interleukin-2) have also been employed (van der Weyden et al. 2016). Similar to humans, melanoma metastases in dogs are difficult to treat. While immunotherapy has been tested as a potential therapeutic avenue, these trials have shown limited success (van der Weyden et al. 2016). Clinical trials in canines provide the ability for researchers and veterinarians to assess the effects of a particular therapy in a shorter time frame (due to the shorter lifespan of dogs) compared to humans, while assessing the effects on a complex and heterogeneous animal population, which spontaneously forms melanoma and metastases.

Equine Melanoma Models

As in dogs, spontaneous melanomas also occur in horses. There are five types of melanocytic lesions that have been characterized in horses, which can be considered as cutaneous melanomas:

(i) Melanocytoma, melanocytic nevi sometimes resembling human nevi that occur primarily on the legs, body, and neck of horses of any coat color.
(ii) Dermal melanoma, which typically occur in gray horses, are characterized as discrete tumors/nodules with a low propensity to metastasize that typically develop in the anal, perianal, and genital regions as well as in the perineum, lips, and eyelids and under the tail root.
(iii) Dermal melanomatoses are usually characterized as multifocal dermal lesions, which are typically found in the genital or perianal regions. These tumors arise in white and gray horses, and they can eventually become malignant.
(iv) Anaplastic malignant melanoma occurs in all horses, but the risk is higher for non-gray horses.

(v) Besides cutaneous and mucosal melanomas, ocular melanomas may occur in horses with a very low frequency (Valentine 1995).

Unlike humans, exposure to UV irradiation is not considered to be a risk factor for melanoma in horses. The disease is however associated with the age-related development of a gray hair coat color, which is caused by a germline intronic duplication in the *STX17* gene, which encodes syntaxin 17. This mutation leads to the constitutive activation of the ERK1/2 MAPK pathway in the melanocytes of the gray horses. As such, this observation further emphasizes the importance of the ERK1/2 MAPK pathway in melanoma, regardless of the species. The bypass of senescence would be favored with age with an unknown mechanism that could be associated with sFRP2 and β-catenin (Delmas et al. 2007; Kaur et al. 2016).

Horses with mutation in the agouti signaling protein gene (*ASIP*) have increased propensity to develop melanoma, thereby pointing to a role for the melanocortin-1 receptor pathway in the development of equine melanoma (Rosengren Pielberg et al. 2008). Horse melanoma cell lines were established from primary and metastatic tumors, and all of them lacked TP53 expression. However equine melanoma cell lines established from metastases lacked both $P16^{INK4A}$ and PTEN expression (Seltenhammer et al. 2014). Horse melanomas present molecular characteristics similar to humans; therefore a better understanding of their genetics and epigenetics may be useful to discover novel genes and pathways involved in horse melanomagenesis with potential implications for the treatment of the human disease.

Swine Melanoma Models

In pigs, cutaneous melanoma occurs spontaneously around birth but frequently regresses (Baco et al. 2014). Such regression occurs in humans with melanoma and was thought to reflect the patient's immune system gaining the upper hand over the melanoma and therefore spurred research in melanoma immunotherapy. Comparative genomic analyses have demonstrated that the pig and human genomes are very similar. In addition, the skin of humans and pigs is similar with their melanocytes mainly located in the basal layer of the epidermis. The postnatal onset, the lack of contribution of an obvious mutagen, and the practicalities of research in porcine models aside, such similarities could be exploited to discover novel molecular players and therapies (Rambow et al. 2008).

Three breeds of pigs develop melanoma spontaneously and have been used to study melanoma: Sinclair, Munich Troll, and MeLiM (melanoblastoma-bearing Libechov Minipig). The phylogenetics of these three breeds remains unknown, but it does not mean that these breeds are unrelated. More than any other animal model, porcine melanomas have been essential for gaining a better understanding of the natural history of the spontaneous melanoma regression with a complete tumor regression rate of 90%. Regression of the primary melanoma is characterized by the flattening, drying, and loss of pigmentation of the tumors (Vincent-Naulleau et al. 2004). Large-scale analyses of these various cases may be informative to decipher the mechanism(s) responsible for these phenomena.

Nonmammalian Melanoma Models

While all of the abovementioned melanoma animal models involve mammals, nonmammalian models also exist and have played important roles in the understanding of the disease. More specifically, various species of fish have been used to study melanomagenesis and have been successful in identifying important factors that regulate disease initiation and progression.

Work using the swordtail fish *Xiphophorus* has shown that these fish can develop melanoma. More specifically, when different *Xiphophorus* species (*Xiphophorus maculatus*, which is a pigmented platyfish, and *Xiphophorus hellerii*, which is a nonpigmented swordtail fish) were mated, the resulting hybrid offspring developed melanoma. These melanomas were shown to result from the aberrant expression of Xmrk, which is the *Xiphophorus* ortholog of EGF

receptor (Wittbrodt et al. 1989). This tyrosine kinase receptor was able to promote melanophore proliferation, protect against apoptosis, and induce migration (Wellbrock et al. 2002). Reintroduction of the Xmrk gene into medaka (*Oryzias latipes*), another fish species, resulted in the formation of melanoma, confirming the role of Xmrk in the initiation of melanomagenesis in fish (Winnemoeller et al. 2005).

Genetically engineered zebrafish have provided an excellent tool for researchers to perform in vivo imaging experiments as well as large-scale chemical screens and genetic analyses to identify important molecular players and potential therapeutic targets for treatment of melanoma. Overall, the zebrafish and human genomes show approximately 70% similarity, and orthologs of an estimated 80% of human disease-associated genes have been identified in zebrafish (Howe et al. 2013).

Despite the emphasis on GEM models of melanoma, the first model of $BRAF^{V600E}$-driven melanoma was developed in zebrafish (Patton et al. 2005). In this study, zebrafish expressing oncogenic $BRAF^{V600E}$ under the control of the MITF promoter formed nevi. In addition, $BRAF^{V600E}$ expression was combined with TP53 silencing (*Mitf::BRAF^{V600E}; Trp53$^{-/-}$*); melanoma formation was increased compared to $BRAF^{V600E}$ expressing fish alone. The involvement of somatic gain-of-function mutations in *BRAF* has since been confirmed in mice and observed in dogs, thus demonstrating the utility and relevance of the zebrafish model for the better understanding of human melanoma.

Genetically engineered zebrafish have been useful in identifying other genes that are potentially involved in promoting melanomagenesis. More specifically, in one study, a list of several genes that were overexpressed in a set of human melanoma cell lines and tumor cultures was compiled, and each gene was co-expressed in the *Mitf::BRAF^{V600E}; Trp53$^{-/-}$* fish. In doing so, a number of genes that accelerate melanoma progression were identified (Ceol et al. 2011), many of which are also implicated or upregulated in human melanoma.

In addition to mutant $BRAF^{V600E}$, zebrafish models expressing mutant oncogenic $NRAS^{Q61K}$ have also been generated, which by itself resulted in hyperpigmentation of the fish (Dovey et al. 2009). When $NRAS^{Q61K}$ was expressed in $TP53^{null}$ zebrafish, melanomagenesis was again increased. These melanomas were invasive and could be transplanted into other zebrafish that were previously irradiated. Importantly, these tumors overexpressed a number of genes that are typically upregulated in human melanoma (Subramanian et al. 2005).

Genetically modified zebrafish have also been useful in demonstrating the role of the transcription factor MITF in melanomagenesis. MITF is a master regulator transcription factor in the melanocyte lineage and is responsible for the regulated expression of many genes essential for appropriate melanocyte development, migration, and function, including those genes that are involved in the production of melanin. In one study using a transgenic zebrafish model containing a temperature-sensitive MITF allele ($mitfa^{vc7}$), it was shown that while *Mitf::BRAF^{V600E}; mitfa^{vc7}* zebrafish did not form melanoma at the non-permissive temperature (due to a loss of MITF activity resulting from a splicing defect and lack of melanocytes), the same fish formed melanomas at the permissive temperature. These tumors appeared to be less differentiated than tumors from *Mitf::BRAF^{V600E}; Trp53$^{-/-}$* fish, as they had lower levels of the melanocyte markers DCT and TYR but higher levels of the oncogenic signaling protein c-MET. This result showed that mutated MITF, together with oncogenic $BRAF^{V600E}$, were sufficient for melanomagenesis in zebrafish. More impressively, when the fish at the permissive temperature were shifted to the non-permissive temperature, there was a regression of the melanoma tumors. Finally, the melanoma tumors recurred when the fish were shifted back to the permissive temperature (Lister et al. 2014). Thus, this study clearly demonstrated the necessity of MITF in maintaining melanoma tumors, at least in the context of $BRAF^{V600E}$ mutations.

Zebrafish can also be used for transplantation experiments, where human melanoma cells can be transplanted into either the early embryos, the larvae, or the adult animals. Melanoma cell lines

transplanted into the early embryos prior to gastrulation have been useful in identifying important signaling pathways, since the transplanted cells may alter the development of the embryos. Transplantation into the larvae can result in melanoma lesions within several days. Since the larvae are transparent, these types of experiments would allow for the visualization, under the microscope, of tumor-induced vascularization and metastatic spread. Coupling these types of experiments with zebrafish that contain fluorescently tagged vasculature would allow for the live visualization of angiogenesis and/or lymphoangiogenesis. Melanoma cells themselves that are fluorescently labeled can also be visualized in the embryos and larvae. This type of live visualization may be useful in discovering how different tumor cells behave and interact with one another in vivo during angiogenesis or invasion. Transplantation of melanoma cells in adult zebrafish is also useful; however, the issue of immune suppression must be addressed (potentially by gamma irradiation prior to transplantation). These transplantation experiments are useful for examining the tumorigenic and metastatic potential of various cells of interest (van der Weyden et al. 2016).

Conclusion

While experiments done in cell culture and in vitro provide valuable information regarding the processes that regulate melanomagenesis, they are limited in their applicability to human melanoma due to their artificial nature. Therefore, animal models are essential in helping to gain a more relevant biological understanding of the molecular alterations that affect the different biological processes that lead to melanomagenesis. All of the animal models listed in this chapter have their advantages and disadvantages regarding the ease and availability of use and their relevance to human melanoma. Collectively, these models have helped in identifying and confirming a number of genes and proteins that are implicated in the initiation and progression of melanoma (e.g. BRAF, NRAS, $P16^{INK4A}$, β-catenin, PTEN, and TP53). It is this collectivity that is essential for the work on understanding and treating this disease. Each model makes important contributions and the findings from each help to advance the field as a whole.

As molecular biological techniques continue to improve and more options become available for use in animal models, our understanding of the molecular and physiological events that contribute to melanomagenesis will only increase. This will also allow for a more comprehensive strategy for the design and melanoma-specific targeting of various therapeutic compounds/agents, with the eventual goal of more effective treatments for melanoma patients.

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Part II

Types of Melanocytic Neoplasms

Primary Cutaneous Melanocytic Neoplasms

16

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Contents

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Abstract

This chapter on primary cutaneous melanocytic neoplasms of the skin will discuss three major categories of benign melanocytic neoplasms: common acquired, congenital, and blue nevi. The predominant initiating genomic event in common acquired nevi is a mutation in BRAF, while a significantly smaller percentage have a mutation in NRAS. Mutations in NRAS occur far more frequently in congenital nevi; the ratio of NRAS to BRAF mutations in congenital nevi varies depending on the size of the congenital nevus. Giant congenital nevi are almost exclusively NRAS mutated. Blue nevi commonly have mutations in GNAQ and GNA11 and likely have a distinct melanocytic precursor cell compared to many, but not all, common acquired nevi. This chapter will highlight how specific mutations and melanocytic precursor cell types impact morphology of benign melanocytic nevi and how these factors can be integrated into a more reproducible classification system. The author also discusses two major subtypes of melanoma: those occurring on non-chronically sun-damaged skin, which have frequent BRAF mutations, and melanomas occurring in chronically sun-damaged skin, which have less frequent BRAF and NRAS mutations but have occasional mutations in c-Kit or NF1. Likewise, the author discusses how the mutation and cell of origin in these melanomas relate to morphology and ultimately can be used for a more robust classification system.

Keywords

Nevi · Common acquired · Congenital · Blue nevi · Melanoma · Genomics

Introduction

While melanocytic neoplasms can arise in a variety of organs, including in the epithelium of the gastrointestinal system and other mucosal sites, the eye, and the central nervous system, the skin is the site of origin to the majority of benign and malignant melanocytic neoplasms. It has long been recognized that melanocytes originating from neural crest cells migrating along specific routes colonize the epidermis of the skin. More recently some data suggests that a second population of melanocytes are normal inhabitants of the dermis (Fernandes et al. 2004). These cells are derived from Schwann cell precursors migrating along peripheral nerves into the dermis and reside in the dermis in the adventitia of nerve fibers and other adnexa.

Factors distinguishing distinct subtypes of melanocytic neoplasms of the skin and ultimately affecting the clinical and histologic presentation include derivation from epithelial-associated melanocytes versus nonepithelial-associated melanocytes and specific genetic alterations. Most data correlating genetic features to morphology suggest that primary activating mutations are most strongly correlated to morphologic and clinical features. Some exceptions to this rule exist, primarily in various patterns of combined nevi where a second clonal population defined by a subsequent genetic alteration is seen. Initiating mutations in melanocytic neoplasms are typically activating point mutations in the mitogen-activated protein (MAP) kinase pathway or translocations in receptor tyrosine kinases (RTKs), which typically occur in a mutually exclusive pattern. This chapter will primarily discuss common acquired, congenital, and blue nevi and the

most common forms of melanoma, including melanocytic cell type of origin and initiating driver mutations and how these relates to morphologic and clinical features.

Common Acquired and Dysplastic Nevi

Definition

By definition, a nevus is a benign clonal proliferation of melanocytes arranged in nests. A nest is defined by three or more aggregated melanocytes. In contrast to melanoma, nevi are organized, limited proliferations of melanocytes typically arranged in a predominance of nests over single cells and have some level of symmetry and reasonably well-defined borders (Fig. 1).

Clinical and Histologic Features

The development of nevi is a function of environmental and genetic factors. The only known modifiable environmental variable is ultraviolet (UV) radiation exposure. Multiple genetic factors are involved: (1) skin type along with MCR1 receptor type and other pigmentary related genes, which all influence an individual's response to UV and ability to protect themselves from UV radiation through melanin production; (2) germline mutations in tumor suppressor genes, which may influence whether senescence of newly proliferating melanocytic neoplasms is immediate or delayed; and (3) immune-related genes, which can also influence immune-mediated clearance of newly initiated melanocytic neoplasia. Two different twin studies demonstrate

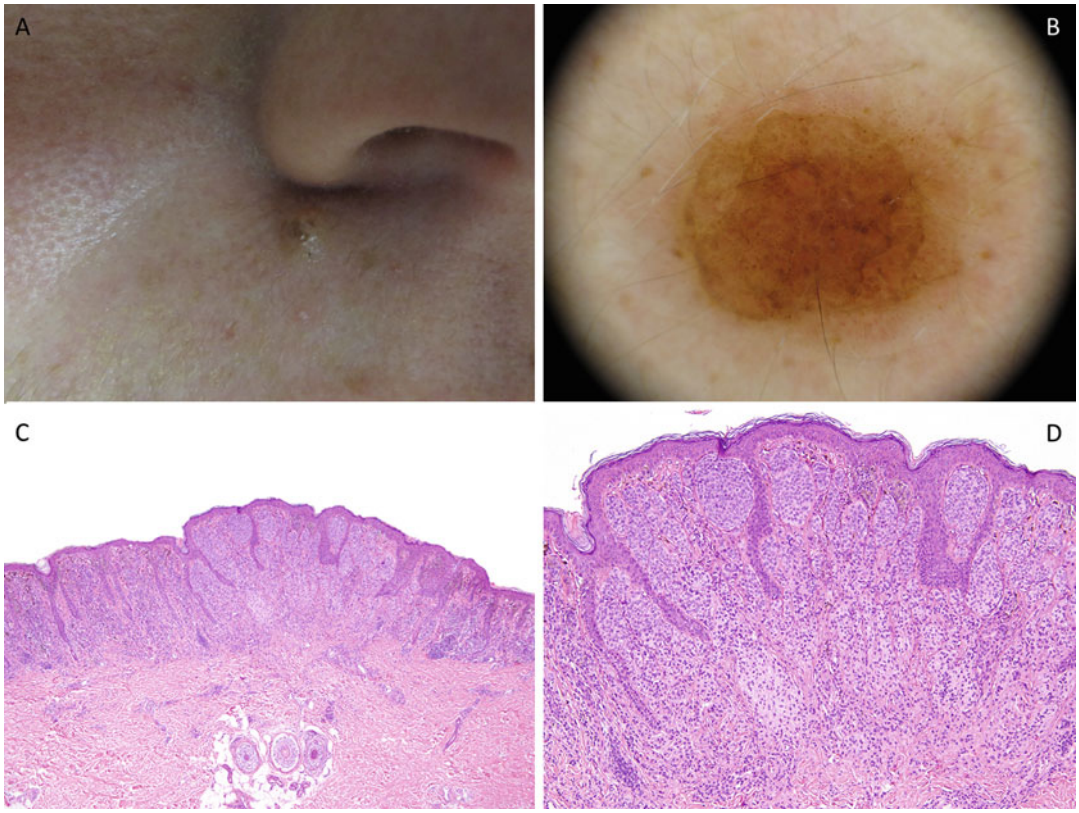

Fig. 1 This is an example of a common acquired nevus from the face of a 30-year-old woman. (**a**) Clinically, it is a 4 mm brown well-circumscribed exophytic papule. (**b**) Dermoscopically, one sees a rather uniform globular pattern. (**c** and **d**) Histologically, there is a predominantly nested pattern of melanocytes involving both the epidermis and dermis. The dermal component shows good maturation and the cells lack significant atypia.

a significant heritability component to total nevus counts. In both studies, monozygotic twins had significantly greater similarity in total nevus counts in comparison to dizygotic twins (Wachsmuth et al. 2001; Lee et al. 2016). Both studies also concluded that genetics play a greater role in nevus counts than sun exposure history. However, there is also convincing epidemiologic data documenting a relationship between history of ultraviolet radiation exposure and nevus counts (Dulon et al. 2002; Wiecker et al. 2003; Aalborg et al. 2009). These studies demonstrate a relationship between total nevus counts and sun exposure history and the distribution of nevi and sun exposure history. Hence, the development of acquired nevi is a combined function of multiple genetic factors and ultraviolet exposure (Table 1).

Acquired nevi may be completely flat (macular) or raised (papular). Most common acquired nevi are less than 6 mm in size and are relatively symmetric and uniform in color. By dermoscopic assessment, most have either a reticular pattern or globular pattern. The reticular pattern is the result of melanocytes and their melanin pigmentation aggregating along rete ridges, while a globular pattern is the result of distinct pigmented nests of melanocytes. Histologically, nevi can have nests strictly associated with the epidermis (junctional), in both the epidermis and dermis (compound), or strictly in the dermis (dermal). In children, most nevi are compound or dermal, while junctional nevi are uncommon. In adults, all three types of nevi can be seen. Most acquired nevi occur during the first two decades of life, but new nevi may occur at any age. In a study of 182 adult patients followed in an outpatient dermatology setting, 50 (27%) developed at least 1 new nevus (Oliveria et al. 2013). Most of the newly occurring nevi were reticular or reticular-globular, which are patterns suggestive of junctional or compound nevi, respectively.

Two typical histomorphologic patterns of common acquired nevi have been designated as Miescher's or Unna's nevus. Unna's nevi are compound or dermal exophytic nevi with a mammillated surface with nests of melanocytes in the papillary dermis. Miescher's nevi are smooth, dome-shaped papules, which are typically entirely dermal. Miescher's and Unna's nevi typically lack significant nuclear atypia or mitotic activity, although mitoses may rarely be seen, often in females of gestational age. Microscopically the lesions are predominantly organized in nests, and the dermal component is characterized by good maturation with decreasing nest and cell size with descent into the dermis. Cells in the deep dermis typically have inconspicuous nucleoli and pigmentation. If there is significant melanin pigmentation, it is typically superficial and lost with descent.

The term dysplastic nevus is controversial. It was originally utilized to clinically describe the large and irregular nevi seen in cohorts of patients with familial melanoma. The term has evolved considerably over time. While the subset of nevi the term originally referred to was probably a lot more limited the way it is currently used in practice most Caucasian individuals would have at least one dysplastic nevus. The WHO has created histomorphologic criteria for the designation. This requires both major criteria and two of four minor criteria to be met.

Major criteria

1. Basilar proliferation of atypical melanocytes that extends at least 3 rete ridges beyond the dermal component.
2. Organization of this proliferation in a lentiginous or epithelioid cell pattern.

Minor criteria

1. Lamellar fibrosis or concentric eosinophilic fibrosis.
2. Neovascularization.
3. Host response.
4. Fusion of rete ridges.

As indicated in the major criteria listed above, in contrast to Unna's or Miescher's nevi, these lesions have a broader intraepidermal component that extends at least 3 rete ridges beyond the dermal component (Fig. 2). The presence of this broad intraepidermal component is significant as multiple studies have shown that having larger acquired nevi is linked to an elevated risk for melanoma. Dysplastic nevi often also have greater nuclear atypia and architectural disorder than Unna's or Miescher's nevi. This may include areas with considerable single cell lentiginous

Table 1 Characteristics of distinct subsets of melanocytic neoplasms

Entity	Cell of origin	Clinical presentation	Histologic features	Common initiating genomic event
• **Common acquired nevi**	• Mostly epidermal-derived melanocyte Some acquired nevi with a congenital histologic pattern may be from dermal-derived melanocyte	• Variable presentation that may include both macular and papular lesions typically less than 6 mm in size that are relatively symmetric and uniform in color. • Miescher's nevi are commonly smooth dome-shaped lesions, Unna's nevi have a exophytic mammillated surface	• May be junctional, compound, or dermal • Miescher's and Unna nevi do not have shouldering, have nests with melanocytes without significant atypia and normal maturation seen extending from epidermis to deeper dermis. Acquired nevi in later life may take the form of lentiginous junctional or lentiginous compound nevus. These are typically small <6 mm with predominance of melanocytes typically aggregated around the rete ridges and small nests of melanocytes are seen in the dermis	• BRAF (85%) • NRAS (5%)
• **Dysplastic nevi**	• Likely epidermal-derived melanocyte	• Usually have a macular component. May be >6 mm in size and may also have some slight color variation and border irregularity	• Junctional component extends at least 3 rete ridges beyond dermal component (shouldering). Have a nested or lentiginous proliferation of melanocytes in epidermis. Bridging, periretal fibroblasia, host response, and perivascularization are all common	
• **Congenital nevi**	• Dermal-derived melanocyte	• Nevi present at birth of within the first few months of life with variable color ranging from tan to black and often with irregular borders • Small – <1.5 cm • Medium – 1.5–20 cm • Large – 20–40 cm • Giant – >40 cm	• In contrast to common acquired nevi, congenital nevi tend to extend deeper into the dermis and subcutaneous tissue. Melanocytes tend to track along the neurovascular or adnexal structures and dissect the collagen bundles	• NRAS (80% large) • BRAF (60% small-medium)
• **Blue nevus** • **Conventional** • **Cellular blue** • **Plaque-type** • **Nevus of ito** • **Nevus of ota**	• Dermal-derived melanocyte	• Conventional – Dorsal surfaces of the extremities • Cellular type – Along the cranio-sacral axis • Plaque type – Segmental distribution • Nevus of ota – Involves the 1st and 2nd division of trigeminal nerve and can involve the cheek,	• Blue nevus – Dendritic shaped melanocytes with melanophages often in a sclerotic stroma • Cellular blue – In addition to dendritic melanocytes have nests and fascicles of oval to spindle shaped melanocytes often with intervening dendritic melanocytes and melanophages as seen in conventional blue nevi. The cellular fascicles often form	• GNAQ (65%) • GNA11 (10%)

(*continued*)

Table 1 (continued)

Entity	Cell of origin	Clinical presentation	Histologic features	Common initiating genomic event
		temple, conjunctiva, and retina • Nevus of Ito – Find the nerve associated with this, occurs on the shoulder, supraclavicular, or scapular region along areas of supraclavicular and lateral brachial cutaneous nerves	a buttress against the subcutaneous tissue	
• **Malignant blue nevi**	• Dermal-derived melanocyte	• Typically occur in the same distribution as cellular blue nevi along the cranio-sacral axis	• Nests of oval shaped melanocytes that become highly expansile • Morphologic clues to malignancy include frank epithelioid transformation, zones of necrosis, high grade nuclear atypia, and elevated mitotic activity	
• **Melanoma of non-CSD skin/** • **Intermittently sun-damaged skin**	• SSM – Epidermal based melanocytes • Nodular – Either epidermal or dermal-derived melanocytes	• SSM most likely to follow the ABCD rule • Nodular may present as amelanotic or pigmented nodule	• Lack of aggregates of amorphous solar elastotic bundles; more likely to have a nevus component • SSM have radial growth phase that extends at least 3 rete beyond dermal component • Nodular component directly enter vertical phase with no preceding radial growth but often do not meet full criteria for MIS in epidermis but may have small intraepidermal aggregates of atypical cells with lentiginous or pagetoid growth pattern	• BRAF (50%) • NRAS (30%)
• **Melanoma of CSD skin**	• Lentigo maligna and SSM – epidermal-derived melanocyte • Nodular – either dermal or epidermal • Desmoplastic or dermal spindle cell – likely epidermal-derived dermal, although not known with certainty	• Predominantly occur in the head and neck region or other areas with excessive UV exposure; typically diagnosed between 60–80 years • Lentigo maligna type appears as a variably pigmented patch on a background of poikilodermatous skin	• LMM often grows as single atypical melanocytes in a broad lentiginous growth pattern along the DEJ with extensive adnexal involvement. Nesting is often a later stage phenomenon	• KIT (20%, LMM pattern) • NF1 (25% desmoplastic melanoma; CSD) • BRAF (15%)

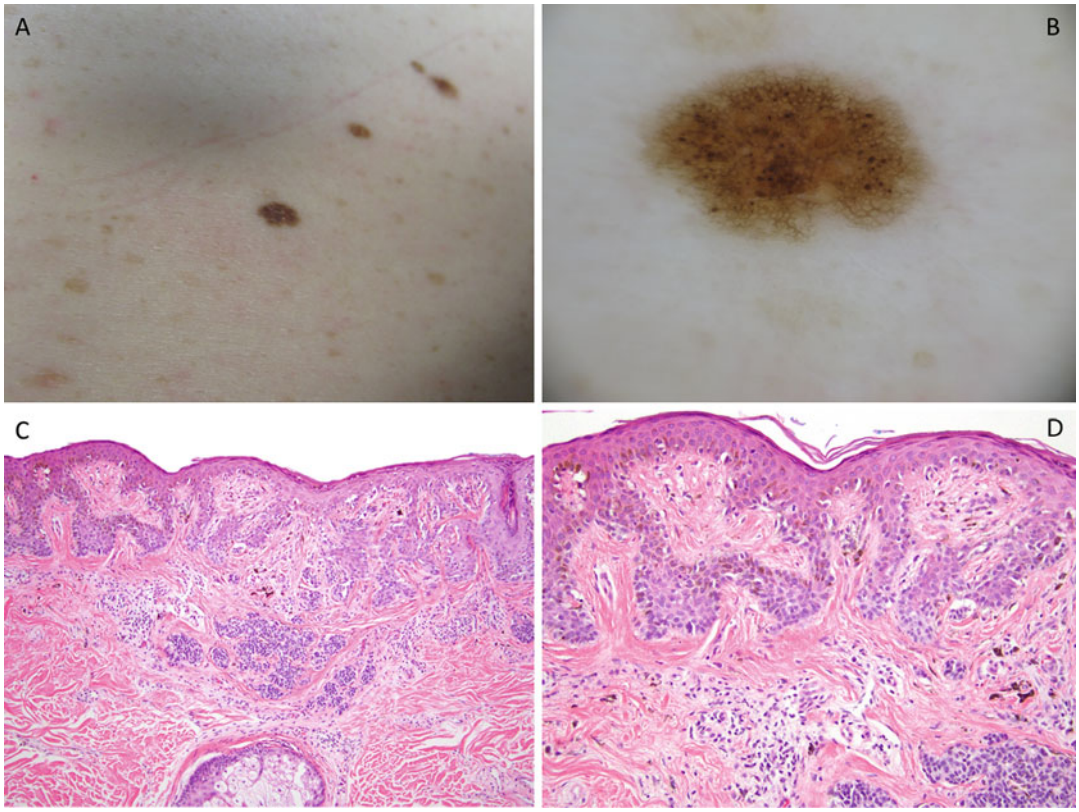

Fig. 2 (**a**) A clinically dysplastic nevus that measures to greater than 6 mm in size and has an irregular border grossly. (**b**) Dermoscopic assessment shows reticulated background with variably sized darker granules distributed throughout the whole lesion. There is slight loss of network towards the center of the lesion. (**c**) Low power magnification shows a dysplastic nevus with nests of melanocytes at the DEJ and within the dermis. There is shouldering, bridging, and periretal fibroplasia. (**d**) Higher power magnification showing typical architecture of a dysplastic nevus with bridging and periretal fibroplasia

growth along the dermal-epidermal junction, focal upward scatter or intraepidermal melanocytes (pagetoid spread), some asymmetry, less sharply defined lateral circumscription, and focal cell aggregates with considerable nuclear atypia. However, the association of other morphologic features of dysplastic nevi to an individual's risk for melanoma is more controversial (Elder 2016).

There can be morphologic overlap between the more atypical examples of dysplastic nevi and the radial growth phase of melanoma. The primary controversy over dysplastic nevi is in regards to whether they have a higher risk than other nevi to transform to melanoma or are intermediaries between common acquired nevi and melanoma. Strong evidence shows that individuals and families who possess clinically atypical nevi in higher numbers and of greater size are at significantly higher risk for melanoma overall (Goldgar et al. 1991; Tucker et al. 1997). Although in a study reviewing histologic dysplasia and diameter of melanocytic neoplasms, diameter of the lesion was the only variable which was statistically correlated with an individual's risk for melanoma (Shors et al. 2006). Genetic studies have found multiple pathogenic mutations in morphologically intermediate (i.e., dysplastic) nevi yet only a BRAF V600E mutation in unequivocally benign nevus cells (Shain et al. 2015). Hence, while theoretically one might expect dysplastic nevi to

have a higher risk for transformation, most epidemiologic data suggest they are for the most part relatively stable lesions with a very low risk for transformation to melanoma (Marks et al. 1990; Tucker et al. 2002; Tsao et al. 2003).

Initiating Oncogenic Events

The initiating driver mutation in common acquired nevi is approximately 85% BRAF and 5% NRAS (Pollock et al. 2003). While BRAF and NRAS mutant nevi may be seen in both sun exposed and sun protected areas, the epidemiologic correlation of nevus counts and distribution with sun exposure suggest there is a relationship between BRAF mutations and UV exposure (Thomas et al. 2007). However, the V600E mutation, which is by far the most common mutation seen in BRAF mutated common acquired nevi, is not a UV signature mutation (Landi et al. 2006; Nguyen et al. 2010).

Cell of Origin

Historically, it was proposed that all common acquired nevi have a life cycle beginning in the epidermis, melanocytes drop into the dermis to become compound, and later become dermal after fading of the junctional component (Unna 1893). This life cycle certainly occurs in some nevi and has been documented in studies and observed by most dermatologists following nevus patients with total body photography over time. However, the observation that dermal and compound nevi far outnumber junctional nevi in children while junctional nevi occur much more frequently in adults suggests that many nevi occurring in younger childhood may have a separate pattern of development. It may be that the melanocytic cell type of origin has an impact on this pattern of development. Specifically, if the cell of origin is an epithelial-derived melanocyte, this may result in a junctional or compound nevus, which may go through the above described life cycle. Common acquired nevi that have histomorphologic features of congenital nevi, such as tracing adnexa and deep extension between collagen, may originate from dermal-based melanocytes. This theory would be consistent with the finding that junctional nevi probably have the highest correlation to sun-exposed sites, whereas nevi that occur in sun-protected sites are often compound or dermal. However, this is not known with certainty.

Senescence and Other Factors Impacting Phenotype

Further mutagenic events may also impact the phenotypic features of common acquired nevi. In a study demonstrating the genetic evolution of benign nevi to melanoma, it was shown that morphologically intermediate lesions had more mutagenic events than obviously benign precursor lesions (Shain et al. 2015). This has also been demonstrated in the past with clonal nevi in which a secondary subclone with greater atypia emerges from an otherwise ordinary nevus (Ball and Golitz 1994). In contrast to initiating oncogenic events that are typically activating mutations, subsequent genomic events are frequently loss of function alterations in tumor suppressor genes.

Other factors that can impact the morphologic features of a nevus include many innate host factors such as the host's genetic, epigenetic (methylation changes), and immune control of senescence — basically how quickly the cell-intrinsic or extrinsic mechanisms can arrest the proliferation of the melanocytic cells. This may be particularly impactful on the size of the nevus. The mechanisms of senescence include: oncogene induced senescence, in which oncogenic activation of the MAP kinase pathway triggers growth arrest through the tumor suppressors p16 or p21; immunosurveillance-mediated senescence, in which the immune system removes neoplastic melanocytes; or replicative senescence, in which telomere shortening induces growth arrest. Telomerase lengthens the telomeres. It has recently become apparent that clonal TERT promoter mutations occur at quite an early stage in the genetic evolution of nevi to melanoma

(Shain et al. 2015). A selective growth advantage of nevus cells for TERT promoter mutations suggests that, even at the nevus level, the cells are turning over and replenishing themselves. Hence, arrest of nevus cells in a benign stage is continually dependent on the above-discussed mechanisms of growth arrest.

Risk for Melanoma

All melanocytic nevi can potentially be transformed to melanoma, and it has been demonstrated that the predominant manner in which this occurs is acquisition of additional genomic alterations as a result of UV mutagenesis. It is unclear if morphological clues can predict which lesions are at greatest risk for transformation. Many studies evaluating melanoma arising in nevi suggest that there is no greater risk of transformation of a dysplastic nevus than other common acquired nevi (Tsao et al. 2003). In fact, the most common subtype of nevus found in association with a melanoma is the conventional common acquired nevus (Marks et al. 1990). Although there may be some bias in that it may be more difficult to clearly delineate a dysplastic nevus from melanoma, compared to delineating other common acquired nevi from melanoma. In addition to primary activating oncogenic mutations at the molecular level, dysplastic nevi often also have loss of function mutations with loss of heterozygosity involving CDKN2A or TP53 (Hussein and Wood 2002). The ACS reports approximately 80,000 new melanomas per year and 30%, or 24,000, are estimated to arise from a precursor nevus (Siegel et al. 2016). When one considers the astronomical number of nevi that qualify for the current definition of "dysplastic," one can see that the per annum rate of transformation of any given dysplastic nevus to melanoma is quite small, which supports the idea that these lesions are stable neoplasms.

From an epidemiologic perspective, considering that among acquired benign melanocytic nevi 85% are BRAF mutated and 5% are NRAS mutated, while in malignant melanocytic neoplasms, 50% are BRAF mutated and nearly 30% are NRAS mutated, there is reason to suspect that NRAS mutated neoplasms have a higher risk of progression. From a molecular perspective, this would also be logical, since NRAS can simultaneously activate both the MAP kinase pathway and the PI3 kinase pathway, while BRAF is further downstream and only activates the MAP kinase pathway (Table 2).

Congenital Nevi

Definition

Congenital melanocytic nevi (CMN) are present at birth and occur in utero or within the first year of life and present in approximately 1% of infants. CMNs have been classified based on size as small (<1.5 cm in diameter in the adult), medium (1.5–20 cm), large (>20 cm), and sometimes giant (>40 cm) (Alikhan et al. 2012). In contrast to the common occurrence of small CMNs, larger CMNs have an estimated incidence of 1 in 20,000 individuals (Castilla et al. 1981). The term "tardive CMN" refers to nevi not present at birth but that become apparent within the first 2 years of life.

Clinical and Histologic Presentation

Most small and medium-sized congenital melanocytic nevi are fairly uniform in color with well circumscribed borders. They are often raised and sometimes can have a papillomatous appearance. Mature terminal hairs may be present. These lesions can occur on any area of the body. Special consideration needs to be given to giant congenital nevi particularly those in a craniosacral distribution. The melanocytic proliferation in these cases can extend into the meninges and may even involve the brain and spinal cord. This is referred to as leptomeningeal melanocytosis or neurocutaneous melanosis. Potential complications from this include hydrocephalus or primary leptomeningeal melanoma. The risk of neurocutaneous melanosis is particularly high for giant congenital nevi along the craniosacral axis with

Table 2 Distinct genomic pathways to melanoma

Gene	Type of alteration	Entities	Common secondary event involved in malignant transformation	Type of alteration
BRAF	Point mutation	Common acquired nevi Dysplastic nevi Congenital nevi Melanoma of non-CSD skin	CDKN2A	Deletion, mutation
			TERT	Mutation, amplification
			PTEN	Deletion, mutation
			ARID1A, 1B and 2	Deletion, mutation
			SMARCA4	Deletion, mutation
NRAS	Point mutation	Common acquired nevi Dysplastic nevi Congenital nevi Melanoma of non-CSD skin	CDKN2A	Deletion, mutation
			TERT	Mutation, amplification
			PTEN	Deletion, mutation
			ARID1A, 1B and 2	Deletion, mutation
			SMARCA4	Deletion, mutation
GNAQ/ GNA11	Point mutation	Blue nevi (cellular, plaque-type, nevus of ota, nevus of ito, Mongolian spots) Uveal melanoma	6p25	Gains
			SF3B1 (2q33)	Mutation
			BAP1	Deletion
			C-MYC	Amplification
KIT	Point mutation	Melanoma of CSD skin Acral melanoma[a] Vulvar melanoma[a] (Yelamos et al. 2016)	N/A	
NF1	Point mutation	Melanoma of CSD skin (desmoplastic and other CSD)	N/A	

[a]Not discussed in this chapter
N/A Not applicable

many satellite lesions (DeDavid et al. 1996; Marghoob et al. 2004; Kinsler et al. 2008).

By dermoscopy, small and medium-sized congenital nevi often show a regular reticular network, a globular pattern with a cobblestone appearance or just diffuse homogeneous pigmentation. Giant congenital nevi may have greater heterogeneity with distinct areas showing either a reticular, globular, or homogeneous pattern. Other common dermoscopic findings in congenital nevi include perifollicular hypo or hyperpigmentation and milia-like cysts. The characteristic histologic changes in congenital nevi include tracing of adnexal structures in the dermis with nests and aggregates of melanocytes in the hair follicle, eccrine glands, and neurovascular bundle. Single melanocytes can also be seen tracking deeply into the dermis and splaying between the collagen fibers (Fig. 3). Some nevi occurring later in life, including in adulthood, can have these histologic features but by history are not congenital in nature. These have been termed "nevi with a congenital pattern." It is likely that these are common acquired nevi that originate from a nonepithelial derived melanocyte, which results in the characteristic growth pattern of tracing adnexa.

Congenital nevi can develop benign nodular proliferations of melanocytes which can mimic melanoma known as proliferative nodules (Fig. 4). Proliferative nodules can develop in any

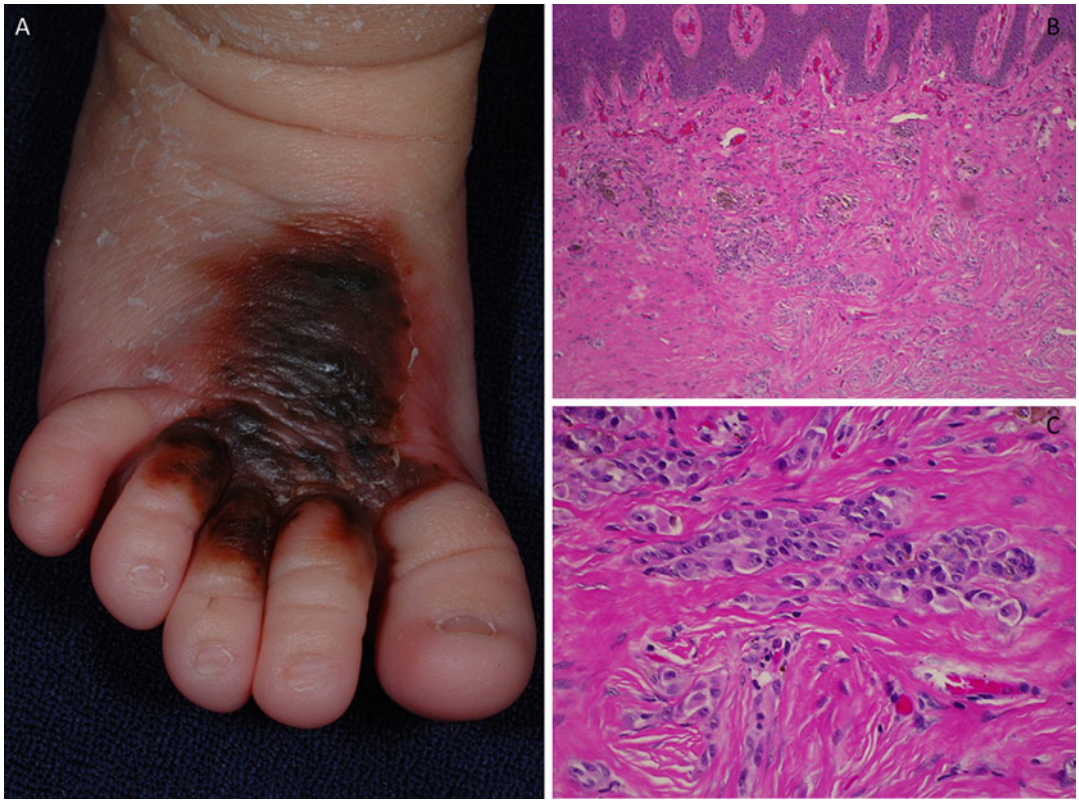

Fig. 3 (**a**) This is an example of a medium-sized (1.5–20 cm) congenital nevus on the dorsal foot of a newborn. (**b**) Low power histology shows small nests and single melanocytes along the DEJ with a predominance of nests in the dermis. (**c**) The dermal nests are seen dissecting through thick bundles of collagen extending through the reticular dermis.

sized congenital nevus and at any age, but are most characteristic of giant congenital nevi in early childhood or infancy (Phadke et al. 2011). Distinct morphologic patterns for proliferative nodules have been identified including nodular proliferations of epithelioid melanocytes, small round blue cell-like proliferations, neurocristic proliferations, spindle cell sarcomatous proliferations, the entire spectrum of blue nevus-like proliferations, and nevoid melanoma-like proliferations. Cytologic atypia and considerable mitotic activity greater than 15/mm^2 can be seen, particularly in small round blue cell or spindle cell sarcomatous patterns. Distinction from melanoma can be challenging. Features favoring melanoma include sharp demarcation from the congenital nevus component, high grade nuclear atypia throughout, high levels of mitotic activity greater than 5/mm^2 in a proliferation of epithelioid cells with high grade atypia, and zones of necrosis. Cytogenetically, proliferative nodules often have whole chromosomal copy number changes, while melanomas typically have clonal segmental chromosomal aberrations (Bastian et al. 2002; Yelamos et al. 2015a). The incidence of proliferative nodules in giant congenital nevi is estimated to be between 3 and 19%. In the author's experience, they are far more common than melanoma arising in a giant congenital nevus.

Initiating Oncogenic Event

Similar to common acquired nevi, the most common initiating oncogenic events in congenital nevi are BRAF or NRAS mutations. The frequency of these mutations varies depending on

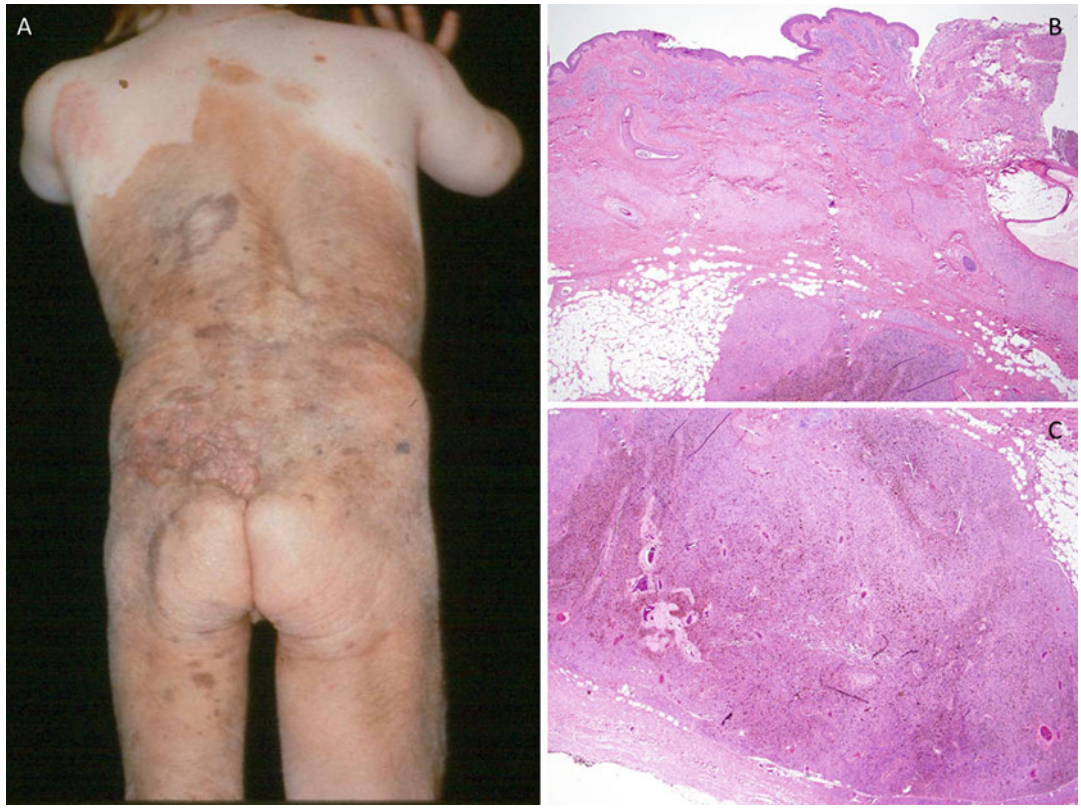

Fig. 4 (**a**) Giant congenital nevus in a truncal distribution with numerous papular and nodular proliferations of various sizes seen on the left lower back. This pattern of giant congenital nevus involving the cranio-sacral axis has a high risk for neural involvement. (**b**) The low power histology shows predominantly dermal involvement of the congenital nevus with melanocytes nesting around adnexal structures and dissecting collagen bundles. At the base, a proliferative nodule can be seen. (**c**) Higher power magnification reveals a benign proliferative nodule with a cellular blue nevus-like patter occurring in a giant congenital nevus

the size of the congenital nevus. In one study of 62 congenital nevi defined by presence at birth, the vast majority of large congenital nevi resulted from mutations in NRAS. In medium-sized congenital nevi, the ratio of NRAS:BRAF mutations was 5:3 and in small congenital nevi the ratio of NRAS:BRAF was 1:4 (Bauer et al. 2007; Ichii-Nakato et al. 2006). As seen by these ratios, the greater the size of the nevus, the higher the probability of an NRAS compared to BRAF mutation.

Cell of Origin

Morphologically, congenital nevi are typically compound or intradermal. In larger nevi, the cells can extend quite deeply into the soft tissues, including the meninges and CNS. This is referred to as neurocutaneous melanosis. The probability of neurocutaneous melanosis is greatest in giant congenital nevi and particularly those involving the trunk with many satellite lesions. Clearly, UV stimulation does not play a role in these lesions, which occur in utero. The melanocyte of origin is probably most often of dermal origin, as these lesions morphologically are characterized by deep dermal extension between collagen fibers and tracing of adnexal structures. However, these morphologic features are not specific to congenital nevi and can be seen in nevi, which demographically and historically are clearly common acquired nevi. Again, the authors theorize that it is the dermal origin of this subset of common acquired nevi that causes this morphologic pattern.

Senescence and Other Factors Impacting Morphology

Congenital nevi may develop benign secondary clonal proliferations, which, if limited, are often referred to as clonal nevi and, if extensive and highly proliferative, may be referred to as benign proliferative nodules. These clonal proliferations are the result of additional mutagenic events, such as loss of function mutations in critical tumor suppressor genes. Morphologically, proliferative nodules can raise significant concern for melanoma because of significant cytologic atypia, mitotic activity, and clonal copy number aberrations. However, these copy number aberrations are typically whole chromosomal aberrations rather than segmental gains or losses, as seen in melanoma. Morphologically these secondary proliferations can vary, with possibilities that include Spitzoid, a variety of blue nevus subtypes (epithelioid, cellular conventional blue, or DPN-like), to spindle cell, epithelioid, or small round blue cell like appearances. This is likely dependent on the subsequent mutagenic events taking place.

The large size of some congenital nevi is unique and, of course, not seen in acquired nevi. Additionally, as previously discussed, only NRAS mutant melanocytic proliferations tend to reach the size of giant congenital nevi (Bauer et al. 2007). Oncogene-induced senescence in NRAS may be a more delayed process in comparison to BRAF. Additionally, because of the young age of the patient there may be more replications allowed before replicative senescence takes effect and perhaps, the relatively immunosuppressed state of pregnancy and the in utero child allows for less immune surveillance-mediated senescence. While the precise reason leading to the ability of these nevi to reach such a large size is unknown, all of these factors could theoretically contribute to this process.

Melanoma Risk

The lifetime risk of melanoma in a congenital nevus is proportional to the size of the lesion, with giant congenital nevi having the greatest risk. In giant congenital nevi, the lifetime risk is approximately 5 to 10% with most cases occurring before age 18 (Ruiz-Maldonado et al. 1992; Bett 2005). Although controversial, there are several theoretical reasons to believe that congenital nevi have a higher risk to transform to melanoma than other nevi (Illig et al. 1985; Swerdlow et al. 1995; Rhodes et al. 1996). This includes more frequent NRAS mutations in comparison to BRAF mutations (Bauer et al. 2007; Kinsler et al. 2013). As discussed earlier, NRAS is upstream of BRAF, and activating mutations in NRAS can simultaneously activate both the MAP kinase and Phosphoinositol kinase pathways. Congenital nevi are usually present for longer periods of time than acquired nevi, and keeping in mind that even benign nevi are not static, in that melanocytic cells are undergoing ongoing death and replenishment over a lifetime, there is a higher probability for a secondary mutagenic event and more time to have potential UV exposure. Despite these theoretical reasons, the data suggest that if there is a greater risk for small congenital nevi, this is so small that it is difficult to quantify (Scalzo et al. 1997).

Blue Nevi

Definition

Blue nevi are one subtype of dermal melanocytosis. The term dermal melanocytosis refers to a proliferation of dermal melanocytes with predominantly dendritic cell morphology, often with many surrounding melanin-laden macrophages and devoid of a junctional component. The predominant presence of deeper melanin gives rise to the blue color. A discrete macule or papule with this morphologic pattern is referred to as a blue nevus. There are many subtypes of blue nevi, including epithelioid blue nevi, epithelioid blue nevus of chronically sun-damaged skin, cellular blue nevi, and plaque type blue nevus. Other patterns of dermal melanocytosis, which present more as a dermatomal patch or plaque of pigment, are referred to as "nevus of Ota" when involving the conjunctiva and periocular skin and "nevus of Ito" when involving the shoulder and upper back.

Newborns sometimes have faint blue patches of pigment on either the wrist, ankle, or buttocks region, colloquially referred to as "Mongolian spots."

Clinical and Histologic Presentation

Blue nevi may be congenital or acquired and occur most commonly on the dorsal wrist, dorsum of the feet, or in a head and neck distribution. They can occur in other organs other than the skin. In the CNS, they are referred to as melanocytomas. Most acquired blue nevi are clinically less than 1 cm in total diameter and have a uniform blue to blue-black color. Dermoscopic exam also reveals a uniform blue pigment. Histologic exam shows dendritic melanocytes and melanophages often in a somewhat sclerotic stroma (Fig. 5). Segmental distributions, if highly cellular, may be referred to as plaque type blue nevi, whereas less cellular segmental distributions around the orbit are referred to as "Nevus of Ota" and around the shoulder as "Nevus of Ito."

Epithelioid blue nevi have, in addition to the dendritic melanocytes, a majority of melanocytes which also maintain deep prominent melanin pigmentation but with an epithelioid morphology. These lesions may occur sporadically or with increased incidence in patients with Carney's syndrome, which consists of lentigines, myxomas, and epithelioid blue nevi. Epithelioid blue nevi do not have distinguishable clinical features. Cellular blue nevi often have the dendritic

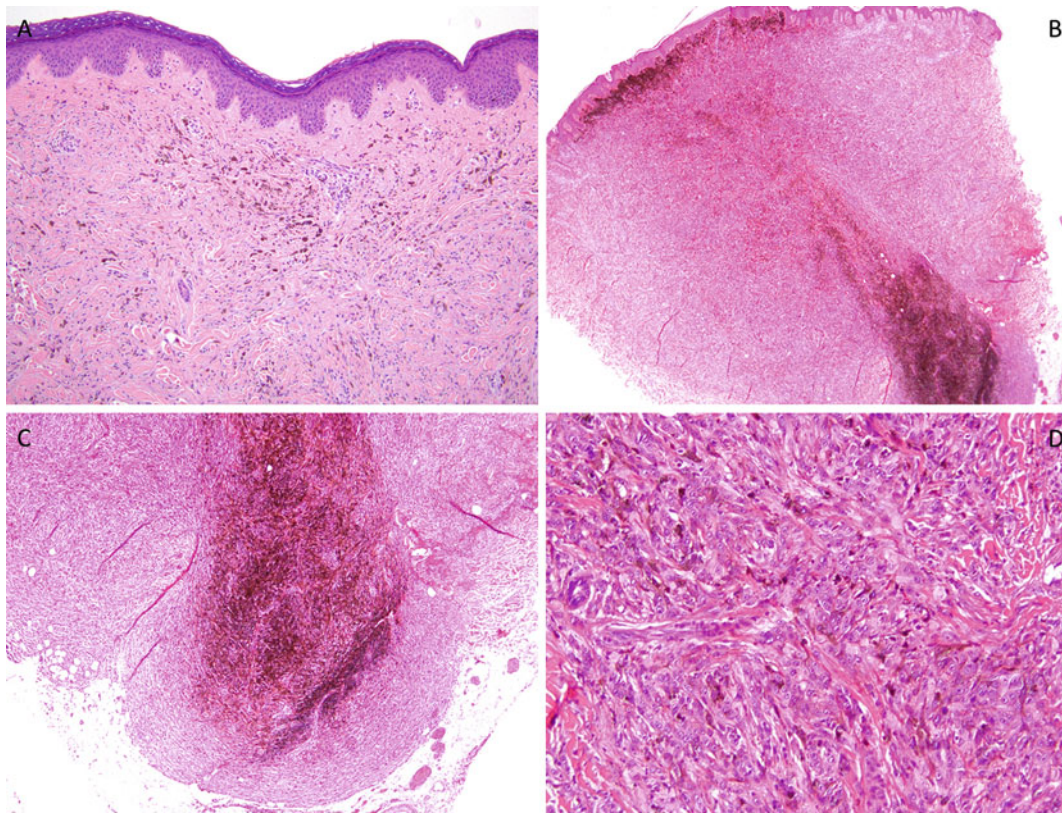

Fig. 5 (**a**) This image shows a dermal proliferation of dendritic and spindle shaped melanocytes in a fibrotic stroma with many intervening melanophages consistent with a conventional blue nevus. (**b**–**d**) The next images show a cellular blue nevus. The lower magnification shows the typical expansile cellular base which forms a buttress against the subcutaneous tissue. The highest power magnification shows fascicles of plump to oval spindle shaped melanocytes closely opposed to one another with only thin intervening strands of collagen.

melanocytic component but also have fascicles of oval to spindle-shaped cells, which often do not contain much melanin pigment, arranged in a plexiform pattern in the skin often forming a buttress against the subcutaneous tissue. Clinically, these lesions are typically raised, larger nodular lesions with a predilection for the cranio-sacral axis. Epithelioid and cellular blue nevi can often show benign involvement of lymph nodes.

Initiating Oncogenic Event

Activating mutations in the G-alpha Q family, GNAQ (65%) or GNA11 (9%), are seen in the majority of blue nevi (Van Raamsdonk et al. 2009). These genes encode for members of the q class of G protein alpha subunits and are involved in mediating signals between the G protein coupled receptors and downstream effectors which ultimately impact the MAP kinase pathway as in many of the other primary activating mutations in melanocytic neoplasms. The possible importance of the G-alpha Q family of proteins in blue nevi and other dermal melanocytosis was indicated by identifying hypomorphic germline mutations in these genes in a set of heavily pigmented mice (Van Raamsdonk et al. 2004). Histopathologic exam of these mice showed the same dendritic cell morphology of melanocytic proliferation as recognized in blue nevi and other dermal melanocytosis. It was not only shown that the majority of blue nevi have GNAQ or GNA11 mutations but that 46% of uveal melanomas do, as well (Van Raamsdonk et al. 2010). This is not surprising considering the overlapping morphology between the two entities. As is the case with most primary activating mutations, GNAQ and GNA11 mutations occur mutually exclusive of one another. Mutations in these genes either result in complete or partial loss of the protein's GTPase activity, leaving them constitutively activated. Epithelioid blue nevi result from a loss of heterozygosity in PRKAR1A in the context of an acquired nevus with a BRAF V600E mutation. This explains the increased incidence in Carney's complex, in which patients have a germline mutation in one copy of the PRKAR1A gene.

Cell of Origin

The distinctive common genetic alterations, histomorphology and lack of any epithelial involvement of blue nevi, dermal melanocytosis, and uveal melanoma have led to the proposal that they arise from a distinct type of melanocyte that does not reside within epithelia (Van Raamsdonk et al. 2010; Bastian 2014). These melanocytes are likely derived from the neural crest-derived bivalent precursor cell that can give rise to melanocytes and Schwann cells. These cells rely on endothelin signaling for differentiation and proliferation, which signals through the Gαq pathway, in which GNAQ and GNA11 operate. It is likely that these cells are the origin of blue nevi and their migration along the peripheral nerves explains their frequent presentation in sites such as the dorsal wrists and feet. Histologic exam often shows the dendritic melanocytes of blue nevus cells clustered around adventitia, such as hair follicles, which is also consistent with this theory of their origination. There are some tumors with composite features of blue nevi and various neural neoplasms, which have been designated as neurocristic hamartomas. The authors have personally noted some young children with composite tumors showing mixed differentiation, which include mixed morphologic and immunohistochemical staining patterns of blue nevi and neurofibromas. This further supports a similar derivation of blue nevus and Schwann cells.

Risk for Melanoma

Malignant transformation of dermal melanocytosis such as blue nevi, nevus of Ota, nevus of Ito, and Mongolian spots is uncommon, but may be no less frequent than in the much more common acquired nevi. It is difficult to estimate the incidence, as some of the largest case series of what has been referred to as malignant blue nevus or melanoma ex blue nevus typically include less than 20 cases (Connelly and Smith Jr. 1991; Costa et al. 2016) (Fig. 6). It is reasonable to conclude that malignant degeneration of these lesions undoubtedly happens but is a relatively rare

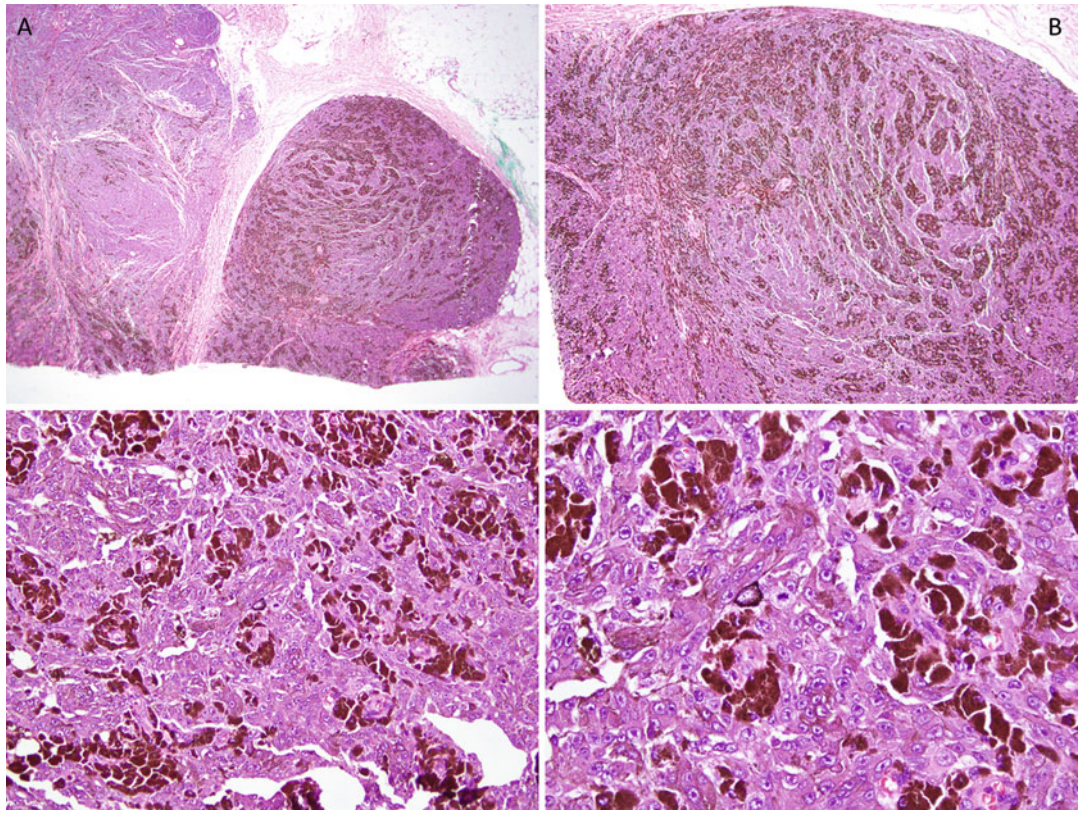

Fig. 6 (**a**) Low power view of a malignant blue nevus. Asymmetric expansile nodules of melanocytes with many melanophages abutting the subcutis. (**b**) While there are areas of residual conventional blue nevus in the background, this higher power magnification shows an area of epithelioid transformation. (**c** and **d**) At the highest magnification, one can see sheets of epithelioid melanocytes with notable nuclear atypia, prominent central nucleoli, and mitotic activity. Loss of BAP1 nuclear expression in these tumors has been shown to be an adverse prognostic parameter as in uveal melanoma.

event. Caucasian patients with nevus of Ota have an increased risk of uveal melanoma. When cutaneous melanoma occurs in these abovementioned lesions, they have similar genomic patterns and morphologic features to uveal melanoma, further underlining their close biologic relationship. It has long been recognized that the pattern of genomic alterations in uveal melanomas could be used to predict prognosis. Initially, this was done by cytogenetics. Cases with deletions involving 3p21 (BAP1) or gains/amplifications of 8q24 (c-Myc) had a significantly worse prognosis than cases without either of these alterations (Aalto et al. 2001; Harbour et al. 2010). More recently, it has been shown that while mutations in BAP1 are associated with aggressive disease, mutations in EIF1AX and SF3B1 may be good prognostic markers (Harbour and Chao 2014). Furthermore, not entirely surprisingly, in a recent study of cutaneous malignant blue nevi and cellular blue nevi, BAP1 mutations were similarly associated with aggressive disease, further establishing the similarities of melanomas arising from blue nevi and uveal melanoma (Costa et al. 2016).

Melanoma

Background Clinical and Histologic Features

The traditional classification of melanoma by Clark and colleagues distinguishes 4 major classes of cutaneous melanomas, which

includes superficial spreading type, nodular type, acral type, and lentigo maligna type. This classification system is primarily based on a combination of clinical and histomorphologic features.

Superficial Spreading Melanoma

Superficial spreading melanomas (SSM) are the most common subtype of melanoma accounting for approximately 60% of all melanomas (Singh et al. 2016). They may occur in all areas of the body but are most frequent on the trunk and extremities. This is the subtype of melanoma most likely to evolve from a precursor nevus and is the subtype of melanoma most linked to elevated nevus counts (Maldonado et al. 2003; Tsao et al. 2003). The ABCD rule is most useful and relevant to this subtype of melanoma. It suggests looking for asymmetric lesions, irregular borders, multiple colors, and size greater than 6 mm (Rigel et al. 2005). Common abnormal dermoscopic features include radial streaming, unilateral pseudopods, black blotches, and atypical network which are all features correlating to an atypical radial growth phase or in situ component. These lesions most often occur in areas of intermittently sun-damaged skin but can also occur in areas of chronic sun damage.

Histologically, these lesions are defined by having a radial growth phase with or without any vertical growth phase, but if there is a vertical growth phase, it is accompanied by an adjacent radial growth phase component that extends at least 3 rete ridges (by convention) beyond any vertical growth phase component. Prominent lentiginous growth of melanocytes along the dermal-epidermal junction with single melanocytes often predominating over nests is common. Lesions are often highly asymmetric and have poor lateral circumscription, often having irregular dispersion of single melanocytes at one lateral edge (Fig. 7). Pagetoid spread of melanocytes is common and may be widespread. Expansile junctional nesting with clustered mitoses and widespread nuclear atypia of melanocytes is commonly seen. A nevus remnant is present in up to one-third of cases.

Nodular Melanoma

Nodular melanomas may also occur both in areas of chronic and non-chronic/intermittently sun-damaged skin. A precursor nevus may be present but is less frequent than in the SSM subtype of melanoma (Pan et al. 2017; Yelamos et al. 2015b). Nodular melanomas are less likely to be identifiable by ABCD criteria. Lesions may be either symmetric, uniformly colored or asymmetric, multicolored nodular or papular lesions. Many cases are amelanotic and lack significant pigmentation and are often mistaken for basal or squamous cell carcinomas. There may be some dermoscopic clues, which include blue-white veil, multiple colors, the presence of shiny white streaks (Verzi et al. 2018), and a dot or polymorphous vascular pattern. While prognostically there is no difference between similarly staged SSM and NM, on average NM is diagnosed at a more advanced stage than SSM and is responsible for a disproportionate number of melanoma-related deaths (Mar et al. 2013). Studies suggest both the rapid growth of NM which goes directly into a vertical growth phase without a preceding radial growth phase and the difficulty in clinical recognition of these cases contributes to the advanced stage at diagnosis (Betti et al. 2008; Liu et al. 2008).

Histologically, nodular melanomas may have a junctional component, but they often do not have fully developed changes of melanoma in situ. By definition, if present, the junctional component does not extend more than 2 rete ridges beyond the dermal component. In NM the intraepidermal component may only have scattered signs of a more disconcerting process, such as focal areas of atypical junctional melanocytic hyperplasia, foci of pagetoid cells, or expansile nesting. In the dermis, there is often expansile nesting or sheet-like growth of melanocytes with nuclear atypia and mitotic activity. In histologic assessment of nodular melanomas arising from a nevus, melanocytes in the dermis often go through a transition, in which the cytology changes from that of a small, banal appearing cell with open chromatin and an unremarkable nucleolus to a large atypical cell with atypical nuclear features and a large prominent nucleolus with dusty,

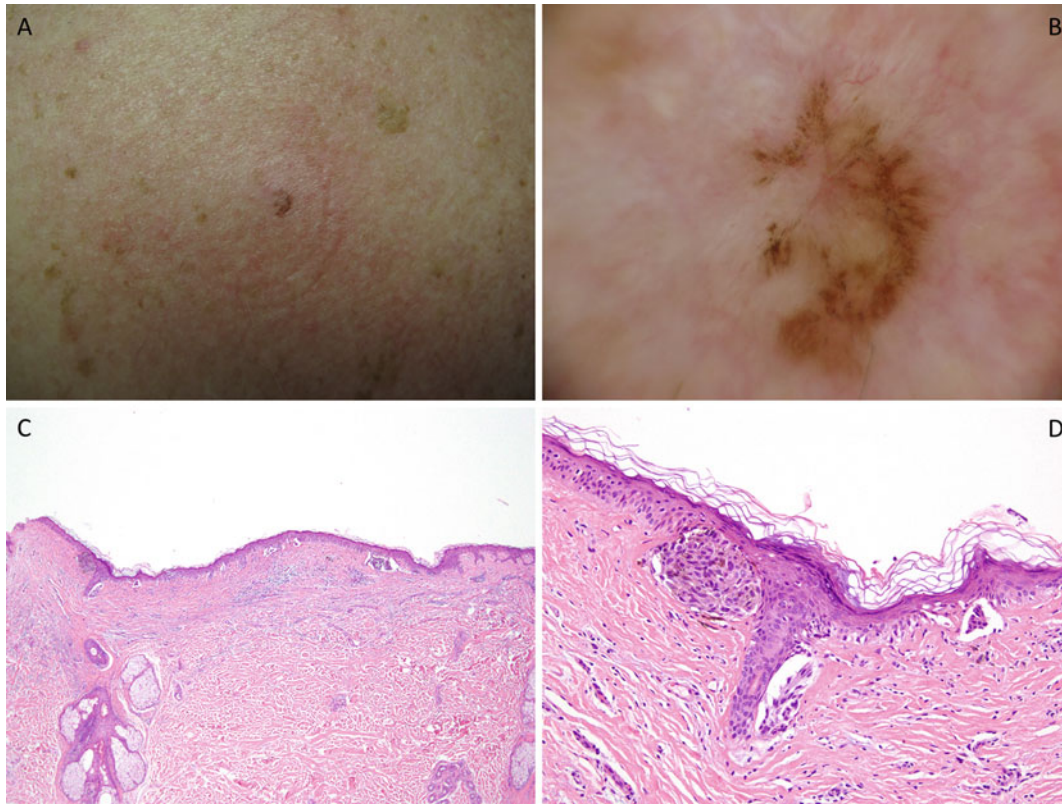

Fig. 7 (**a**) 65-year-old male with a new, irregular, multicolored macule measuring 5 mm on the back. (**b**) Dermoscopic assessment shows asymmetric streaks extending along the 1 o'clock to 5 o'clock edge. There are scar-like white areas consistent with regression, scattered asymmetric granules seen between 10 and 3 o'clock, and a focus of a residual distorted network around 6 o'clock. (**c**) Low power histology reveals an irregularly nested proliferation of highly atypical and pleomorphic melanocytes along the DEJ consistent with melanoma in situ and prominent underlying regression in the superficial dermis. (**d**) The higher magnification shows the irregularly nested atypical melanocytes with clefted spaces at the DEJ and broad underlying regression. This melanoma occurring on intermittently sun-damaged skin with a prominent nested pattern and highly pigmented melanocytes has a high likelihood of BRAF mutation.

pigmented cytoplasm. Even when an in situ melanoma component is present, it is unlikely that invasive melanoma always originates from the epidermis.

Lentigo Maligna Melanoma

Lentigo Maligna Melanoma (LMM) is a subtype of melanoma occurring exclusively in areas of chronically sun-damaged skin. Epidemiologically, melanomas of this type are most linked to lower intensity but prolonged and excessive UV exposure that results in the normal collagen bundles of the dermis developing into broad bands of grey solar elastotic material. They are predominantly seen in a head and neck distribution or on distal extremities and less commonly on the trunk. These melanomas often have a prolonged radial growth phase before entering a vertical growth phase. Lesions often appear as a variably pigmented patch on a background of poikilodermatous skin. Helpful dermoscopic features include asymmetric perifollicular pigmentation or perifollicular pigmented dots, rhomboidal structures or angulated lines, areas of homogeneous pigmentation, or essentially an asymmetric pigment blotch. In general, any

newly occurring melanocytic neoplasm occurring in a background of poikilodermatous/sun-damaged skin measuring 1 cm or greater in diameter should be considered highly suspicious for lentigo maligna.

The histologic growth pattern of LMM consists of single, variably atypical melanocytes, growing along the dermal-epidermal junction with less pagetoid spread than typically seen in superficial spreading melanomas. The epidermal rete ridges may or may not be effaced as a result of the extensive basal layer proliferation of atypical melanocytes. The lentiginous growth of single melanocytes may have florid extension into the adnexal epithelium (Fig. 8). A nevus remnant is not seen, but it is not uncommon to find small dermal nevic aggregates. These are not precursors but rather incidental benign nevi, as is commonly found in these anatomic sites. These melanomas are notorious for having a field effect as the direct result of mutagenic effects of ultraviolet exposure. This can result in melanocytic cells away from the primary focus carrying the same genetic alterations as those in the primary focus. Likewise, there can be skip lesions. Often, one may attempt a small incisional biopsy and find nondiagnostic changes. Clearance of these lesions can be difficult since areas of surrounding field effect can result in recurrences.

The dermal component may be a conventional epithelioid invasive melanoma or a desmoplastic spindle cell neurotropic melanoma. Desmoplastic spindle cell melanomas have hyperchromatic, atypical spindle shaped melanocytes often in a myxoid stroma with surrounding lymphoid aggregates, extending deep into the skin, often to or below the level of the subcutis. These hyperchromatic spindle cells may form fascicles deeply diving down into the dermis. There is often a sclerotic stroma, which pushes aside the solar elastosis, so that there is a rim of thick solar elastotic material around the periphery of the lesion. Neurotropism is common. Prognostic studies have shown that those cases with >90% desmoplastic pattern have low incidence of lymph node involvement, compared to cases that are more biphasic and have both a desmoplastic and solid epithelial component making up more than 10% of the lesion (Gyorki et al. 2003; Pawlik et al. 2006; George et al. 2009).

Acral Lentiginous Melanoma

Acral lentiginous melanoma (ALM) is a subtype of melanoma occurring on the volar surfaces of hands and feet and the nail apparatus. However, not all melanomas on acral surfaces are of the acral lentiginous subtype of melanoma. This subtype, like lentigo maligna, has a prominent radial growth phase component with single atypical cells along the dermal-epidermal junction. Importantly, because this subtype of melanoma has either limited or no UV signature mutations, UV exposure is not thought to be the predominant factor in most cases. More on acral melanomas is discussed in a separate chapter.

Initiating and Characteristic Oncogenic Events

Genomic changes resulting in malignant transformation of melanocytes is covered in detail in the ▶ Chap. 7, "Molecular Genetics of Melanocytic Neoplasia." In this section, we will focus on discussing the initiating genomic event most typical of each subclass of melanoma. There is not a perfect correlation of genomics with subtype or clinical and morphologic features, but there are some general trends. For example, factors such as younger age (<55 years of age) (Viros et al. 2008), involvement of skin without a high cumulative level of sun exposure (Broekaert et al. 2010), the presence of a precursor nevus, nest formation, notable melanin pigmentation, and a radial growth phase with notable pagetoid scatter have all been linked to higher likelihood of a BRAF mutation. These given clinical and morphologic features are most typical of the superficial spreading type of melanoma, and hence it follows that superficial spreading melanomas are the most likely type of melanoma among the 4 subtypes to have a BRAF mutation as the initiating genomic event. Approximately 52% of

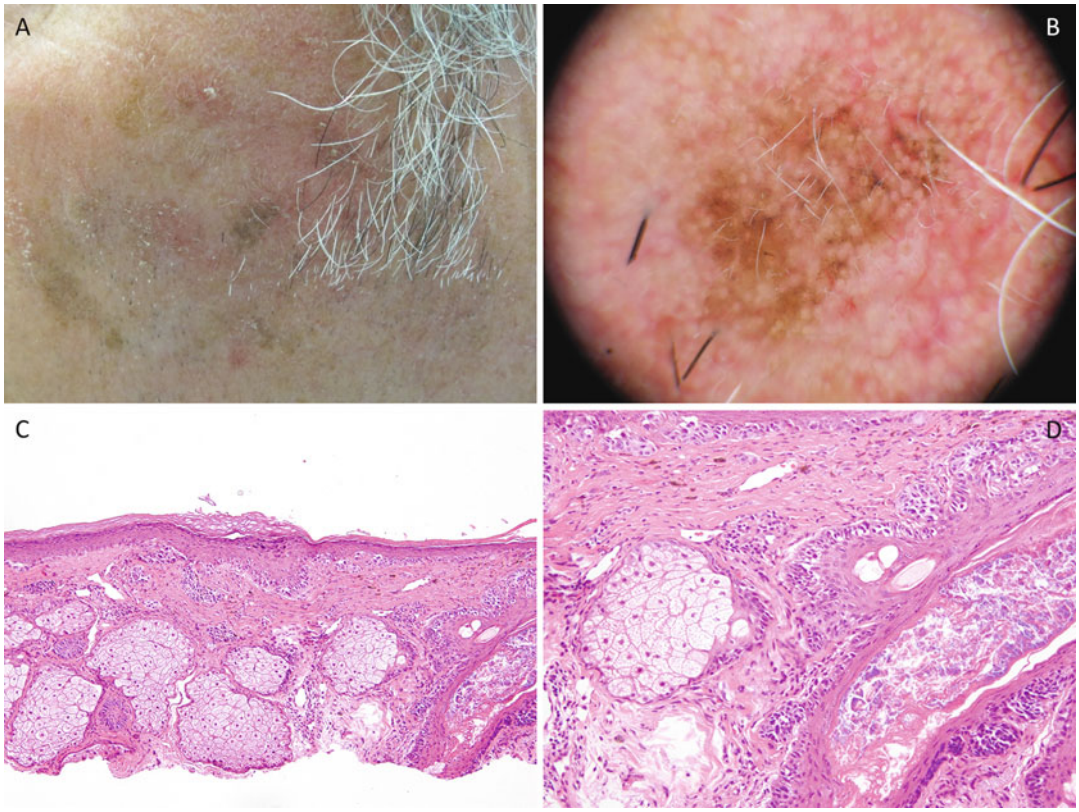

Fig. 8 (**a**) Poikilodermatous skin with a brown pigmented patch anterior to the sideburn in a 72-year-old male. (**b**) Dermoscopic assessment shows a pseudoreticulated pattern and the presence of asymmetric clusters of pigment granules in a perifollicular distribution, a feature often seen in lentigo maligna. (**c**) Low power histology of a lentigo maligna type of melanoma, which shows broad lentiginous growth of single melanocytes with small nests predominantly in the basal layer of the epidermis and within the follicular epithelium. (**d**) The higher magnification highlights the confluent single cell proliferation of atypical melanocytes in the follicular epithelium. This pattern of broad single cell growth of atypical melanocytes above chronically sun-damaged skin can be seen in KIT mutated melanomas. The melanocyte precursor in this case is an epithelial-derived melanocyte and likely has a high mutational burden.

SSM have BRAF and 20% have NRAS mutations (Viros et al. 2008).

The genomics of nodular melanomas may vary considerably, depending on whether they are nodular melanomas occurring in an area of the skin with high, low, or no cumulative sun exposure. Again younger age, location in areas of intermittently sun-damaged skin, and the presence of a precursor nevus increase the likelihood of a BRAF mutation. Conversely, the probability of an NRAS mutated NM increases with older age (Ellerhorst et al. 2011). The frequency of BRAF mutations in nodular melanomas in general is 41% and of NRAS is 27% (Lee et al. 2011). Another characteristic genomic alteration in amelanotic nodular melanomas is copy number gains in 8q24 at the C-Myc locus (Pouryazdanparast et al. 2012a; b). There is evidence that elevation in Myc can down regulate MITF, which is the master regulatory gene of pigmentary production. This results in decreased levels of tyrosinase, decreased melanin production, and an amelanotic appearance.

The initiating oncogenic event in LMM may involve NF1, c-Kit, NRAS, or BRAF. NF1 mutations are particularly common in those LMM

cases with a desmoplastic and spindle cell component (Gutzmer et al. 2000). Mutations in c-Kit can be seen in approximately 20% of melanomas of LMM and are most typical of those melanomas which begin with a broad lentiginous growth pattern along the dermal-epidermal junction (Curtin et al. 2006). Approximately 22% of LMM have BRAF mutations and 14% of LMM have NRAS mutations.

Approximately 20% of ALM have BRAF mutations, 30% have NRAS mutations (Haugh et al. 2018), 20% have c-KIT mutations (Curtin et al. 2006), and 17% have NF1 mutations (Moon et al. 2018). Structural aberrations with copy number gains in Cyclin D1 and CDK4 as well as deletions in CDKN2A are also particularly common in this subtype of melanoma (Bastian et al. 2000).

BRAF and NRAS mutations alone are insufficient for malignant transformation of melanocytes, and these mutations can be seen in nevi as well. It is the accumulation of additional genomic events over time, typically the result of UV exposure that results in the transformation of these lesions to melanoma (Shain et al. 2015). Some critical additional genomic events leading towards transformation with an initiating activating mutation in BRAF or NRAS include subsequent TERT promoter mutation or amplification, homozygous deletion or mutation in CDKN2A, or PTEN deletions or mutations (Tsao et al. 2004; Dankort et al. 2009; Huang et al. 2013). In ALM, which mostly occur independent of UV damage, structural aberrations in chromosomes leading to copy number gains in oncogenes or deletions of tumor suppressor genes have a greater role.

Cell of Origin

Superficial spreading, acral lentiginous, and lentigo maligna melanoma all likely originate from an epidermal-derived melanocyte as evidenced by the characteristic radial growth phase component seen in these tumors. The nodular types of melanoma may occur via an epidermal-derived melanocytic cell, which may explain the majority of nodular melanomas. However, some nodular melanomas have no junctional component and may evolve through a dermal derived melanocytic cell. In the author's experience, many nodular melanomas evolve from a conventional compound or dermal nevus. Hence, if the original nevus is compound and derived from an epidermal melanocyte, the subsequent melanoma would also be. Contrastingly, a melanoma arising from a dermal nevus with congenital features likely is originating from a melanocytic cell of dermal origin. Desmoplastic spindle cell melanomas which only have an overlying lentiginous melanoma in the epidermis in 50% of cases may originate from UV damage to a dermal melanocyte. Transformation of a dermal melanocyte having common origins with Schwann cells may explain the neural differentiation and neurotropism often seen in desmoplastic and spindle cell melanomas in chronically sun-damaged skin.

TCGA Classification

As described above, there is considerable genetic variability within the melanoma subtypes defined by Clark. With the emergence of genetic studies and recognition of important therapeutic implications of specific genetic aberrations in melanoma, other classification schemes have evolved. The TCGA of cutaneous melanomas excludes acral and mucosal melanoma and proposes a genetically based classification system which categorizes melanoma into four major groups: (1) BRAF mutated, (2) NRAS mutated, (3) NF1 mutated, and (4) triple wild type (i.e., wild type for BRAF, NRAS, and NF1). The last category is very heterogeneous and includes cases with c-KIT or GNAQ as well as other mutations and melanomas resulting from fusions or other structural aberrations. In the TCGA study, BRAF mutations were associated with younger age as in previous studies, as well as MITF amplifications. RAS mutations characteristically show elevated MAPK activation and AKT3 overexpression, while NF1 mutated melanomas were seen in older patients with higher mutational burden (Cancer Genome Atlas 2015).

An Integrated Taxonomy of Melanocytic Neoplasia

A taxonomy that integrates the clinical and histopathological features, genetic alterations, role of UV radiation, and epidemiological variation has been suggested by Bastian. This system classifies melanocytic neoplasms into two major categories: melanomas originating from melanocytes associated or not associated with epithelia such as epidermis or mucosa. Within each category, the classification distinguishes several classes of neoplasms that evolve from different types of precursor lesions to different melanoma subtypes through the progressive accumulation of genetic alterations (Bastian 2014).

The family originating from epithelia-associated melanocytes includes the following groups: (1) melanomas on sun-exposed skin without cumulative sun-induced damage (low-CSD melanomas) (Table 3). These melanomas lack marked solar elastosis in their surrounding skin, have frequent BRAF V600E mutations, and often arise from precursor nevi and affect the trunk and proximal extremities of patients under 55 years of age. (2) Melanomas on sun-exposed skin with high cumulative sun-induced damage (high-CSD melanomas). These melanomas show marked solar elastosis in their surrounding skin, have frequent NF1, NRAS, BRAF non-V600E, and KIT mutations, do not arise from precursor nevi, and affect the head and neck areas of patients over 55 years of age (3) acral melanoma, (4) mucosal melanoma, (5) desmoplastic melanoma, (6) Spitz melanoma, defined by specific genetic alterations such as HRAS mutation or kinase fusions. This

Table 3 Comparison of CSD and non-CSD melanoma

	Melanoma of non-CSD skin	Melanoma of CSD skin
Age at diagnosis (peak range)	40–50 years	60–80 years
Common sites of occurrence	Trunk and extremities or areas with intermittent bursts of UV exposure	Head and neck region or areas with prolonged and excessive UV exposure
Clinical presentation	ABCD rule – Most related to superficial spreading type of melanoma; lesions typically present with asymmetry, irregular borders, variegated color, and a diameter > 6 mm; often arise from a precursor nevus Nodular melanomas appear as either amelanotic or darkly pigmented, pedunculated, or polypoid nodules	Lentigo maligna melanoma often appears as a multicolored or darkly pigmented macular patch with variable pigmentation on a background of poikilodermatous skin Melanomas of SSM or nodular types appear similarly to those of non-CSD
Dermoscopic features	Typically include asymmetric blue-gray veil, unilateral pseudopods, radial streaming, irregular blue/black blotches and/or granules, shiny white streaks, or an atypical network; may also include dot or polymorphous vascular patterns	Typically include asymmetric perifollicular pigmentation, perifollicular pigmented dots, rhomboidal structures or angulated line, areas of homogeneous pigmentation
Histological characteristics	SSM – Broad radial growth phase extending at least three rete ridges beyond the dermal component; often single cells with lentiginous or pagetoid growth pattern predominating over nests Nodular – Lack a radial growth phase; have expansile nests or sheets of atypical mitotically active melanocytes in the dermis	Dermis has thick bundles of gray solar elastotic material LMM – Typically has a prominent basal layer proliferation of variably atypical melanocytes and an effacement of the rete ridge often with extensive involvement of the adnexa SSM and nodular possess similar characteristics to non-CSD melanoma
Common initiating genetic events	BRAF (50%) NRAS (30%)	NF1 (45% Desmoplastic melanoma) KIT (20%) NRAS (20%) BRAF (10–30%)

differs from Spitzoid melanoma, which is defined only by morphology, and has been shown to consist mostly of other melanomas (low-CSD) (cite PMID: 28186096).

The second category of melanocytic neoplasms arising from melanocytes not associated with epithelia consists of uveal melanoma, blue nevi and blue nevus-like melanomas, and melanocytomas of internal organs and related melanomas. These neoplasms are characterized by somatic mutations of the Gαq pathway, mostly at the level of GNAQ or GNA11. Also in this category fall bona fide congenital nevi and melanomas developing within leptomeninges. This classification system provides a more detailed subtyping of melanoma into groups that have greater homogeneity in underlying genetics and clinical behavior.

Staging of Melanoma

The American Joint Committee on Cancer has recently released the 8th edition of a Tumor, Nodes, Metastasis (TNM) staging system which includes specific changes to the T staging of melanoma. The primary factor in T staging is Breslow depth, which is a measurement from the granular layer of the epidermis to the deepest melanoma cell in the skin. Additional factors impacting the T stage include the presence of ulceration of the epidermis, which must be ulceration induced by excessive proliferation of melanoma cells near the surface of the skin and obviously does not include traumatically induced ulcers. Although in practice, it is not always simple to make this distinction. Mitotic count has been removed from the staging of T1 tumors. There is strong evidence in the literature linking mitotic activity to prognosis in general (Azzola et al. 2003; Francken et al. 2004; Thompson et al. 2011). However, the inclusion of mitoses as in the 7th edition as a discrete variable of absent or present was suboptimal. Numerous studies have also shown that the hot spot method of counting mitoses has considerable interobserver variability (Larsen et al. 1980; Heenan et al. 1984; Cook et al. 1996). Eventually, when more optimal cut off parameters for mitoses can be identified, it is likely that mitotic count will be re-introduced into the AJCC staging system.

In the 8th edition, T1a tumors are those that <0.8 mm in Breslow depth without ulceration. Tumors that are 0.8–1.00 mm with or without ulceration are T1b. Tumors ranging from >1.0 mm to 2.00 are T2, from >2.0 to 4.00 are T3, and those tumors greater than 4.00 mm are T4. The presence of ulceration moves the staging from T2a, T3a, or T4a to T2b, T3b, or T4b, respectively. According to the AJCC database of 23,001 patients stratified for T stage with no evidence of regional or distant metastasis at the time of diagnosis, the 10-year melanoma-specific survival was 98% for T1a, 96% for T1b, 92% for T2a, 88% for T2b, 88% for T3a, 81% for T3b, 83% for T4a, and 75% for T4b (Gershenwald et al. 2017.

The TNM classification of melanoma has contributed significantly to the ability to provide patients prognostic information about their disease, guide management, and standardize clinical trials. However, there remains a significant proportion of early stage patients who develop aggressive disease and patients with more advanced T stages including some with microscopic lymph node involvement who do well (Shaikh et al. 2016; Whiteman et al. 2015; Landow et al. 2017). Hence, there are limitations to traditional morphologic descriptors. Recently, a molecular-based staging system assessing mRNA expression of 31 distinct genes has emerged in clinical practice in the United States (Gerami et al. 2015a, b; Zager et al. 2018). This molecular test classifies melanoma into four categories: class 1a, 1b, 2a, and 2b and in retrospective studies has shown highly statistically significant correlation with outcome in multivariate analysis, independent of traditional prognostic markers.

Conclusion

In conclusion, this chapter describes the clinical and histologic features of melanoma and their relationship to the more recently described genomic alterations. Genetic changes are becoming increasingly important to assist in the diagnostic

classification of ambiguous melanocytic neoplasms. An example of this could be identifying a GNAQ mutation in a spindle and dendritic shaped melanocytic neoplasm with some atypia, which would favor blue nevus over the differential diagnosis of a desmoplastic melanoma. In malignant melanocytic neoplasms, the classification system is useful in predicting the pretest probability of finding genetic alterations predictive of response to targeted therapy. These are just two examples of how a classification system integrating genomics and melanocyte biology, as outlined in this chapter, could be utilized to better diagnose and predict the behavior of melanocytic neoplasms of the skin.

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Congenital Melanocytic Naevi

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Contents

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Abstract

The term congenital melanocytic naevi (CMN) covers a broad spectrum of clinical presentations, ranging from the common occurrence of small single CMN, to the rare occurrence of extensive and very numerous CMN accompanied by extra-cutaneous abnormalities. The study of these diseases is relevant to the wider understanding of naevogenesis and melanoma development and provides potentially powerful insights due to the lack of influence of ultraviolet radiation on the prenatal genetic events. Recent improved understanding of the pathogenesis of CMN, and of the relatively rare progression to melanoma, is contributing to the management of individuals affected by these conditions. In this chapter, current knowledge in this field and the authors' approach to management of this multifaceted disease will be reviewed.

Keywords

Congenital melanocytic naevus · CMN · *NRAS* · *BRAF* · Genetics · Pathogenesis · Management · Proliferative nodule · Melanoma · CNS

Introduction

Congenital melanocytic nevi (CMN) are benign melanocytic neoplasms whose origin is determined in utero. The traditional definition of a *congenital* naevus is that of a birthmark present at birth. However, based on their pathogenetic mechanisms (vide infra), as well as clinical observations, congenital naevi may be present at birth or appear in the course of the first year or so of life. Their development results from disorders in the proliferation and migration of melanocytic precursor cells. While most CMN behave in a banal fashion throughout the life of the individual, some are associated with symptoms such as pruritus and develop associated malignancies such as melanoma, and some are associated with cosmetic impairments. In addition, some individuals with CMN are at risk for harboring noncutaneous anomalies involving the central nervous system (CNS).

Classification

The classification of CMN has two main aims. The first is the standardization of phenotyping data collection, allowing clear communication

between clinicians and researchers, and comparison of research publication data. The second is classification for practical management in a clinical setting and the production of current clinical management guidelines.

For Publication, Research, and Sharing of Accurate Data

Cutaneous Classification

Classification of the cutaneous phenotype for these purposes has gone through many iterations over the last 30 years or so. The most recent version is detailed in Table 1, where each column is completed for each patient, resulting in a classification code. Full details of the classification are available in the original reference (Krengel et al. 2013). In addition, large CMN appear to follow specific patterns of anatomical distribution (Kinsler and Larue 2018; Martins da Silva et al. 2017). Evaluating only the phenotype of published cases of LCMN reveals that LCMN tend to be found on the upper back/neck (bolero distribution), mid back (back distribution), lower torso (bathing trunk distribution), breast/abdomen (breast/belly distribution), isolated to an extremity (body extremity distribution), or combination of bolero and bathing trunk distribution (body distribution) (Martins da Silva et al. 2017). Kinsler et al. subsequently provided a different set of patterns with a rational for the observed distribution of CMN and other congenital pigmentary disorders grounded in embryogenesis (Kinsler and Larue 2018). It is worth noting that the term "giant" can be perceived as derogatory by some patients/parents/physicians, and therefore the more general term of "large" or "extensive" may be more acceptable, in particular during patient interaction. For example of phenotypic variation of typical CMN see Fig. 1.

CMN Syndrome

Cutaneous classification however is only one part of a full phenotypic classification of patients with CMN. For anything other than single small CMN,

Table 1 Most recently proposed cutaneous classification of CMN. Each column to be scored separately, using letter/number codes where given

Projected adult size of largest CMN	Number of other CMN ("satellites")	Site of largest CMN	Color heterogeneity	Rugosity	Nodules	Hypertrichosis
Small (<1.5 cm)	0 (S0)	Head	None (C0)	None (R0)	None (N0)	None (H0)
Medium (M1) (1.5–10 cm)	1–20 (S1)	Trunk	Moderate (C1)	Moderate (R1)	Moderate (N1)	Moderate (H1)
Medium M2) (>10–20 cm)	>20–50 (S2)	Extremities	Marked (C2)	Marked (R2)	Marked (N2)	Marked (H2)
Large (L1) (>20–30 cm)	>50 (S3)					
Large (L2) (>30–40 cm)						
Giant (G1) (>40–60 cm)						
Giant (G2) (>60 cm)						
Multiple medium CMN (three or more medium CMN without an obvious predominant-sized CMN)						

Adapted from Krengel et al. (2013)

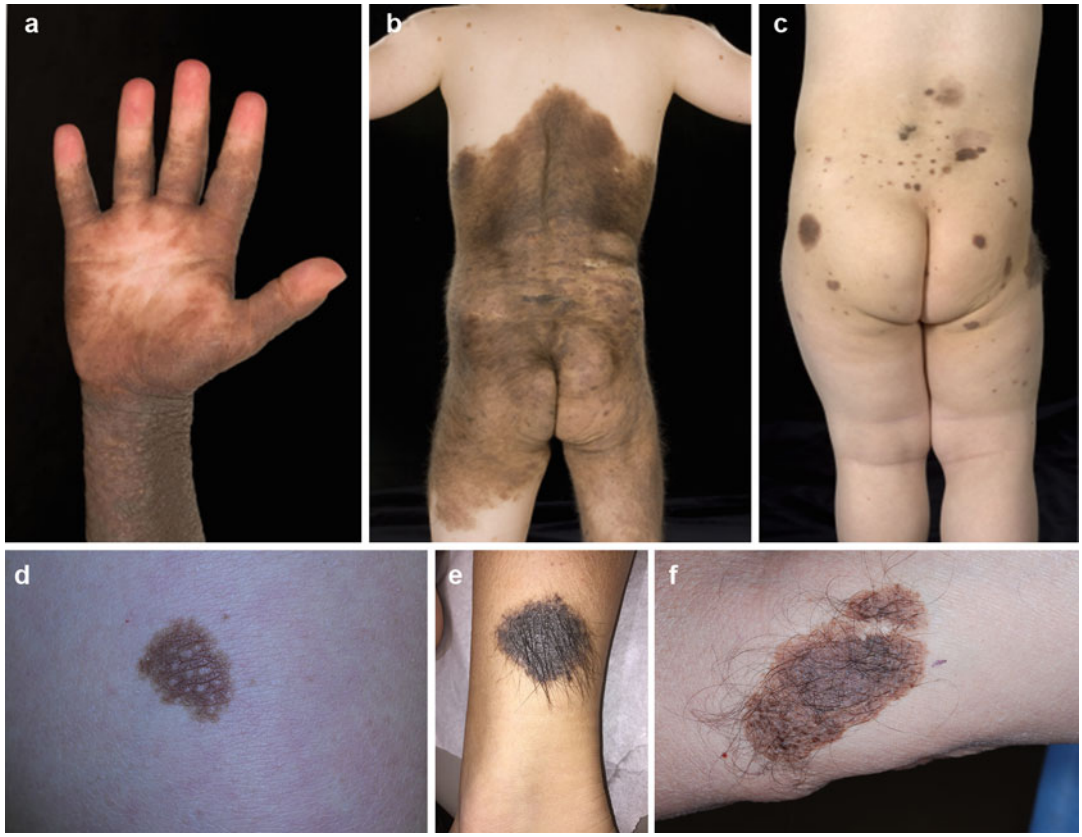

Fig. 1 Examples of cutaneous clinical phenotypic spectrum of CMN. (**a**) Large CMN arm and hand. (**b**) Multiple CMN with largest naevus >60 cm projected adult size. (**c**) Naevus spilus type CMN

it is important to include clinical neurological findings and radiological (MRI) neurological findings where appropriate, as well as the presence of characteristic facial features, clinical endocrinological or metabolic features, growth parameters in children, skeletal abnormalities, and the occurrence of malignancy. These individual features are described in more detail below. Where extra-cutaneous features are present the term CMN syndrome is used (Kinsler et al. 2012a), in line with the classification of other types of congenital naevi (Kinsler and Sebire 2016).

Genotypic Classification

In addition to this deep phenotyping approach, it is now possible to include the status of somatic *NRAS* codon 61 or *BRAF* codon 600 genotypes in a full classification of an individual with CMN. It still needs to be determined whether deep phenotyping and determining the mutational profile of CMN will improve upon risk stratification for developing complications including melanoma, CNS disease, and death.

For Practical Management in the Clinic

The classification required for clinical management of patients with CMN is based on evidence of which phenotypic variables are associated with adverse outcomes. The key medical adverse outcome metrics for individuals born with CMN are:

1. Neurologic symptoms or impaired neurodevelopment (e.g., delay in development, or seizures)

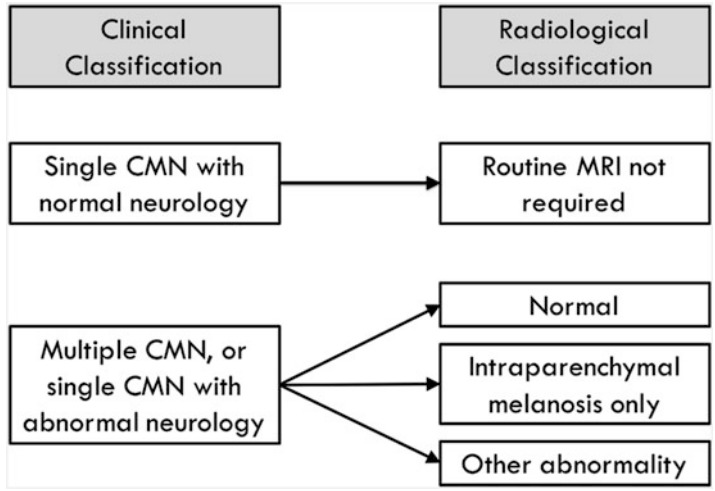

Fig. 2 Classification for practical management purposes in the clinic. Multiple CMN is defined as more than one at birth, of any size or site. Continued research is underway to help improve upon the risk satisfaction of patients with two or more CMN. While there may be a heightened risk for congenital neurological abnormalities in patients with 2 or more CMN followed in specialized clinics, it still needs to be determined what proportion of individuals with 2 or more CMN in the population at large that will never develop any complications. It is highly likely that the presence of only 2 small CMN (<1.5 cm in greatest diameter) carries with it a negligible risk of congenital neurological abnormalities as compared to those with larger CMN or those with more than 2 CMN, and improvements in classification and understanding will help substratify the current guidelines further

2. Development of melanoma within CMN, in uninvolved skin or in the CNS
3. Death

Early studies linking cutaneous phenotyping variables to these adverse outcome measures were retrospective, and not all phenotyping variables were included in a regression analysis of outcomes measures. As a result, an early suggestion was that the site of the largest CMN, in particular those overlying the brain or spine, was associated with a higher risk of neurological abnormalities. It has since become apparent that the site is in fact confounded by size, as CMN overlying the back are often larger and associated with multiple naevi. It is in fact the size (Kinsler et al. 2008) or number of naevi (Marghoob et al. 2004) which are linked to increased risk of neurological abnormalities. Site of the largest naevus is unconnected to any outcome measure at the current moment.

With further analysis of the size and number of CMN in prospective studies, it has been found that the strongest cutaneous phenotypic predictor of outcome measures is even more simple. Individuals with a single CMN are at extremely low risk for having radiological neurological abnormalities, or serious neurodevelopmental abnormalities. This means that for practical purposes in clinic there are two groups of patients – those with a single CMN (of any size or site) and those with multiple CMN (two or more CMN at birth, of any size or site). On the basis of currently available data, those with a single CMN do not require routine screening MRI examination. In contrast, individuals with multiple CMN are at risk of congenital neurological abnormalities and thus MRI scanning should be considered in these individuals (see "Management" section for further detail).

Further stratification of management based on clinical phenotype has then been shown to be best done with the results of the MRI of the CNS. In logistic regression analysis, MRI results were a better predictor of both neurological and malignant outcomes than size, number, and site of CMN. Other cutaneous phenotypic variables (e.g., color heterogeneity, rugosity) have not been shown thus far to be associated with any outcome measures. Hence, classification for the clinic management can be simplified as shown in Fig. 2.

Besides determining risk for developing CNS complications, it is also important to determine the risk for developing cutaneous melanoma in association with the CMN. Most studies assessing melanoma risk to date have classified nevi by size with small CMN being those <1.5 cm in diameter, medium being those with a diameter between 1.5 and 19.9 cm, and large being those greater than 20 cm in diameter. The overarching results disclose that melanoma can arise in association with any CMN but that the timing of melanoma development, location of melanoma within the CMN, and relative risk varies as a function of CMN size. It should be underscored that while the absolute risk for developing a cutaneous melanoma within any size of CMN at any age is likely less than 1–5%, the relative risk appears to be trivial for small CMN but higher for LCMN. While this is well established in childhood (Kinsler 2017), published prospective cohorts of adults are lacking. In addition, melanomas that develop in association with small CMN seem to do so after adolescence and often begin at the dermo-epidermal junction and towards the leading peripheral edge of the CMN. Thus, these melanomas can theoretically be discovered in their early stage via surveillance examinations and with the use of dermoscopy. In contrast, melanomas arising in large CMN usually do so before adolescence (Kinsler et al. 2017a) and often seem to develop deep to the dermo-epidermal junction, although prospective data on this are lacking. These melanomas are difficult to impossible to detect in their early stages via simple visual inspection and dermoscopy. Unfortunately, most of these melanomas are detected on palpation as a new or enlarging subcutaneous masses that carry with them a poor prognosis for the patient. It needs to be determined whether the narrower CMN size cut offs and other phenotypic features such as location and extent of rugosity, nodularity, color heterogeneity, and hypertrichosis (Table 1) can help improve the cutaneous melanoma risk stratification for individuals with CMN.

Clinical Subtypes

Naevus spilus, or speckled nevus, is a clinically distinctive variant presenting as a light brown macule with superimposed darker macular or papular areas. The café au lait macule-like background may be so light in color that it may be invisible at birth. The superimposed darker areas are often heterogeneous in color, appearance, size, and are composed of junctional or compound aggregates of nevus cells, similar as in common acquired or lentiginous melanocytic nevi. Even if the café -au-lait background is invisible at birth, the diagnosis is usually suggested by this clustering of macular or papular naevi. DNA sequence analysis of naevus spilus type CMN has identified a distinct genotype (see under "Pathogenesis" section below).

Tardive CMN are those which are not visible or only barely visible at birth and then appear within the first year or so of life, and progress over time to become classical CMN; although they usually do not attain an unduly large size. Tardive CMN are usually solitary lesions, and little information exists on their incidence. Aside from smaller so-called "satellite" nevi in children with large or multiple CMN, it is not uncommon for children develop a solitary melanocytic naevus in the first year of life, and these tend to be larger than the melanocytic naevi acquired later in life, and these nevi usually display congenital features such as nevoocytes splayed between collagen bundles of the dermis and clustered around dermal adnexal structures and neurovascular bundles. Certainly for anything other than small CMN the phenomenon of tardive nevi is relatively uncommon, being reported in just a few isolated cases. In all other respects, tardive CMN appear to be no different from CMN fully visible at birth, although genotyping has not yet been undertaken on these nevi. However, a recent study has shown that multiplicity of congenital or "prenatal" nevi, diagnosed using developmentally based criteria, increases with age, supporting the notion that these nevi start to develop early in life and take time to become clinically evident (Cramer et al. 2016).

Epidemiology

Incidence of CMN

Small single CMN are a common occurrence, with an incidence of 1–2% in neonates in all populations studied (Alper and Holmes 1983; Chaithirayanon and Chunharas 2013; Jacobs and Walton 1976). The incidence of multiple CMN as defined above is not known. The incidence of CMN greater than 20 cm in diameter has been estimated at 1 in 20,000 (Castilla et al. 1981). This may be an underestimate as severely affected fetuses could potentially be miscarried early in pregnancy, a fact suggested by the increased reports of threatened miscarriage in pregnancies carrying children with CMN(Kinsler et al. 2009).

Ethnicity, Gender, Environmental Factors

CMN are seen in all populations, however whether the incidence varies between ethnic groups has not been established. One large prospective study found a small increase in infants of African descent compared to those of South American descent(Castilla et al. 1981). This same study found an equal incidence between the sexes; however, most studies report a slightly higher incidence in females (Bittencourt et al. 2000; Kinsler et al. 2008; Ruiz-Maldonado et al. 1992).

There are no known links between CMN and environmental factors. Mothers pregnant with children with CMN were found however to be more likely to suffer a threatened miscarriage, severe nausea/vomiting, and high blood pressure than a group of controls, in a retrospective questionnaire study (Kinsler et al. 2009). How these factors are associated is not yet clear.

Melanoma in CMN

Melanoma in individuals with CMN can arise either within a CMN, or as a primary within the CNS, or very rarely in other organ systems. Accurate ascertainment of the incidence of melanoma has traditionally been hampered by a publishing bias towards cases of malignancy, with a clear inverse correlation between cohort size and incidence of melanoma (Krengel et al. 2006). In addition, it is difficult to decipher the risk of developing primary melanoma within the nevus versus within the CNS. From prospective studies and large literature reviews, it is now generally accepted that the risk of all types of melanoma in childhood is of the order of 0.1–2% (Bett 2005; Hale et al. 2005; Kinsler et al. 2009; Zaal et al. 2005) for CMN of any type. However, very large lesions or those with neurologic manifestations are likely at higher risk. The lifetime incidence of cutaneous melanoma in small single CMN is estimated at 0.1% (Krengel et al. 2006), and even in multiple and large CMN cutaneous melanoma is rare in childhood, perhaps of the order of 0–1% (Hale et al. 2005; Kinsler et al. 2009). However, in a prospective cohort study the incidence for melanoma was 10–15% for CMN of greater than 60 cm projected adult size (Kinsler et al. 2008) and this increased risk was primarily related to CNS melanoma. Early screening MRI of the CNS for complex congenital neurological disease in this study was a better predictor of melanoma than the cutaneous phenotype, presumably because it allows us to look at the full congenital phenotype, both skin and brain (Waelchli et al. 2015a). Radiological evidence of CNS involvement might also predict the risk for melanomas arising from involved skin, perhaps by acting as a surrogate measure of general disease severity, but the numbers for this remain small. Despite these published estimates, it remains difficult to obtain predictive models of melanoma risk in CMN due to the small numbers of cases. Further studies are required to determine whether other phenotypic characteristics of the CMN itself (Table 1) can help improve melanoma risk stratification.

From the most recent comprehensive literature review, the median age for melanoma development in CMN is 3 years (Neuhold et al. 2015); however, this is taken with the usual caveats regarding publishing bias, not only towards fatal cases but potentially also towards pediatric cases.

Pathogenesis

The pathogenesis of CMN was not until recently thought to be "genetic" in origin, as it is neither inherited nor passed down; a sporadic disorder. With advances in both the understanding and investigation of genetics however, it has become evident that many birthmarks, including CMN, are the visible result of mosaicism.

Mosaicism

The current consensus definition of a mosaic abnormality of the skin is the co-existence of at least 2 genotypes in an organism, at the time of birth, at least one of which is pathogenic, and which produces a disease phenotype (Kinsler et al. 2019). Practically speaking this means that the phenotype of CMN is caused by a mutation in the developing embryo, which affects the single cell hit by the mutation and any of its offspring, with the rest of the embryo being unaffected. The clinical phenotype of the disease will depend on a variety of factors surrounding the event, such as the timing of the mutation (earlier mutations are more likely to affect pluripotent cells), the cell lineage, the normal function of the gene during development, the expression of that gene in the tissues affected, and the exact mutation (Kinsler et al. 2019). Hence, a likely explanation for generating a single CMN as opposed to multiple CMN is that the former would occur much later in development when the melanocyte precursor cells were already committed to their fate in one particular area of skin. On the other hand, a mutation leading to multiple CMN (and its possible associations in the CNS) may be caused by an earlier mutation, when the affected cell could potentially differentiate into both neurological and cutaneous cell types.

Mutations in CMN

It is not possible to prove mosaicism or indeed causality on the basis of finding a mutation in a single CMN from one individual. Hence, for mutations found in single CMN, or in one CMN from an individual with multiple CMN, one can only draw certain tentative conclusions on the basis of data from different individuals with the same findings. These will be revisited later in the chapter.

When however exactly the same mutation is found in more than one cutaneous (or extracutaneous) lesion from a single individual, one can reasonably assume that this was originally a single post-zygotic mutation to one cell and that the appearance of multiple naevi was due to the dissemination of the progeny of that mutated cell. Thus, far mutations in two genes have been found to be the cause of multiple CMN in more than one patient, and of CMN syndrome. The most common gene is *NRAS*. Mutations in *NRAS* had earlier been described in individual samples of CMN (Bauer et al. 2007; Dessars et al. 2009; Papp et al. 1999; Papp et al. 2005; Phadke et al. 2011; Wu et al. 2011), along with mutations in *BRAF* (Dessars et al. 2007; Ichii-Nakato et al. 2006; Kumar et al. 2004; Papp et al. 1999; Papp et al. 2005; Pollock et al. 2003; Salgado et al. 2015a), *MC1R* (Kinsler et al. 2012b; Papp et al. 1999), *TP53* (Papp et al. 1999), and *GNAQ* (Phadke et al. 2011); however, there were no data as to causality at this stage. Thinking of this condition as post-zygotic mosaicism led to the finding of clonal oncogenic mutations in *NRAS* in more than one affected tissue from individuals with multiple CMN and CMN syndrome, and as these mutations were the same across different individuals with the same phenotype, this can be assumed to be a causal mutation (Kinsler et al. 2013). In this first description, two different missense mutations leading to amino acid changes at codon 61 were found (p.Q61K commonest, p.Q61R less so), but within any one individual the same mutation was detected in different tissues, whether cutaneous naevi or CNS abnormalities (Kinsler et al. 2013). Codon 61 *NRAS* mutations however were not found in all cases (Kinsler et al. 2013; Salgado et al. 2015a), suggesting there are other as yet undiscovered genes which can cause the same or a very similar phenotype; however, another study identified *NRAS* mutations in all samples examined (Charbel et al. 2014). This hotspot in codon

61 is also the mutation described in 15–20% of non-CMN-related melanoma (Forbes et al. 2015) and is known to cause complete inactivation of the *NRAS* GTPase, locking it in the active, GTP-bound conformation.

Naevus spilus type CMN interestingly have a distinct genotype, being caused by different *NRAS* missense mutations from the standard CMN phenotype. The commonest of these (Kinsler et al. 2014) leads to the p.Q61H amino acid change; however, p.G13R and p.Q61L (Krengel et al. 2016) have also been described in single patients.

BRAF p.(V600E) mutations have recently been described as causal in a single patient with multiple CMN, demonstrating that this is a rare cause of the congenital phenotype (Etchevers et al. 2018). This patient had a multinodular phenotype, as has previously been noted to be associated with BRAF mutations from single samples(Salgado et al. 2015a). In a recent large genotype-phenotype study, *BRAF* was found in 7% of cases, and a multinodular phenotype was again described in most of the patients (Polubothu et al. 2019). Importantly, this study did not find any differences in clinical outcomes with genotype *NRAS*, *BRAF*, or double wild-type, and routine genotyping is not therefore recommended for CMN at the moment. Genotyping however is useful in cases of suspected melanoma (*NRAS* and *BRAF* hotspots) to help direct targeted therapy (Kinsler et al. 2017a, b).

A single case each of a *RAF1* and *ALK* fusion have been described as present in more than one naevus from an individual with multiple CMN, suggesting causality. Further cases will be needed to determine the contribution of these genetic changes to the pathogenesis of CMN (Martins da Silva et al. 2019).

Potential Germline Predisposition

Notwithstanding that the ultimate causal mutation in CMN is somatic, a family history of CMN in first or second-degree relatives is found consistently in 25–30% of cases in a UK cohort. Although this could be due to recall bias, it is significantly more than one would expect statistically, given the incidence of small single CMN of 1%, even when accounting for an average number of first and second degree relatives. There may well be germline genetic factors that affect the penetrance of postzygotic *NRAS* mutations and the development or growth of *NRAS*-mutated cells. One such factor has been identified in UK CMN families compared to control groups, namely, compound heterozygosity and homozgyosity for germline variants in *MC1R* (Kinsler et al. 2012b). This again mimics the genetics of non-CMN-related melanoma and suggests that CMN may be a good genetic model for *NRAS*-mutated melanoma in general. There are likely to be other predisposing genetic influences, perhaps particularly between different ethnic groups.

Clinical Features

Cutaneous

Color, Texture, Hair

CMN are pigmented by production of melanin, and their color is usually therefore predominantly brown or black. At birth however they may appear red or purple, which can occasionally lead to diagnostic confusion with vascular birthmarks. CMN color often alters after birth and in particular can lighten quite substantially over a period of years. This is related to the natural genetic skin colour of the patient - lighter skin tone is associated with lighter final CMN colour (Polubothu and Kinsler 2019). Lightening is occasionally dramatic (Kinsler and Bulstrode 2009; Strauss and Newton Bishop 2008). While small CMN are usually uniform in color, larger CMN are very frequently heterogeneous in color, with many smaller patches of differently colored pigmented areas superimposed on the background pigmentation of the CMN. There have been no associations made thus far between the natural color of a CMN and its behavior, and depth or variability of color should not therefore be thought to be intrinsically concerning unless accompanied by other changes.

CMN are almost always palpable, and even when not palpable will have increased skin surface markings, distinguishing them from other macular birthmarks. CMN are more fragile than normal skin and can tear with minor trauma, particularly early in life. Dryness can be a feature in large CMN, particularly in those who are atopic, and eczema can develop within CMN. In rare cases individuals report hypersensitivity in an individual CMN. There are also anecdotal reports of decrease in sweating in large/giant CMN which could be attributed to the fact that the hamartomatous nature of the nevus cell proliferation disrupts the normal development and distribution of adnexal structures (e.g., sweat glands and sebaceous glands) in the involved skin, which frequently appear malformed or less in number in histologic analysis (see Histopathologic features below).

CMN are commonly hairy, although for non-scalp CMN this may not be apparent at birth. Scalp CMN often present with thick, sometimes coarse, and usually darkly pigmented hair at birth, and these often continue to grow hair at a faster rate than the surrounding scalp, necessitating increased cutting. However, sometimes CMN even on the scalp can be hairless or may grow hair that is lighter or similar in color than the surrounding scalp hair. Over time CMN may develop white hairs, and/or develop patchy hair loss, but total loss of hair over a CMN on the scalp is unusual. Of note, large/giant CMN with *BRAF* V600E mutation show a phenotype featuring dermal/subcutaneous nodules and less hair, with a statistically significant difference when compared with *NRAS* mutated naevi (Salgado et al. 2015a).

Proliferative Nodules and Other Benign Proliferations

The authors recognize many different types of proliferations clinically; however, there are at least three commonly seen types.

First, the classical focal proliferative nodules, present at birth, although they can develop at any age in childhood. They are typically superficial, well circumscribed, hairless, shiny, raised and domed, round to oval, uniformly colored (often but not always pink or a lighter color than the surrounding CMN), sometimes slightly lobulated, between 1 cm and 2 cm in most cases, and soft to firm (Fig. 3a). They often grow rapidly once they appear and then stabilize in size and appearance. They can bleed even if not clinically suspicious, so are often excised.

Second, the flat-topped proliferations, superficial, well circumscribed, flaccid, uniformly colored (often pinkish), round or oval, usually 0.5–5 cm in diameter, sometimes with a slightly raised edge/depressed center, and soft to touch. The larger ones are often present at birth and generally remain unchanged over time (Fig. 3b).

Third, the neuroid type proliferations, which are deeper, less well circumscribed, uniformly colored, often the same color as the surrounding CMN (or alternatively pinkish), round or spindle-shaped, and soft to firm. They are most requent in

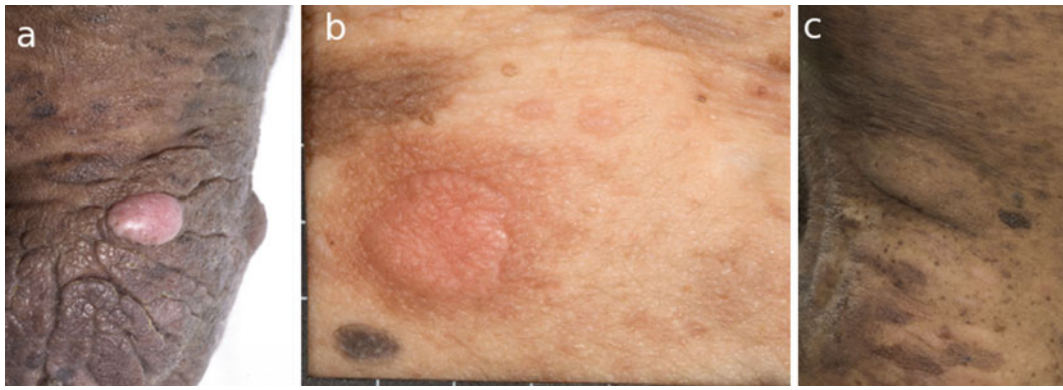

Fig. 3 Proliferative nodules of different types within CMN

the flanks in bathing trunk nevi (Fig. 3c), but can occur in other areas. They are not usually present at birth, and once they appear they can grow slowly, sometimes particularly around puberty. They range from 3 to 20 cm in diameter and can be pendulous when larger. Resection of these improves body contour, particularly when they are better circumscribed, although they often regrow within weeks or months. Histology reveals neurofibroma-like differentiation (so-called "neurotization") within the CMN.

It should be mentioned that even among experts there are discrepancies in definition of proliferative nodules due to an enormous variety. In general, benign proliferations of all types are unusual in small CMN, but a common occurrence in large CMN, particularly in certain individuals, with no more exact figures on incidence or epidemiology in current data.

Cutaneous Melanoma

Melanomas arising in association with small CMN tend to develop focally at or near the dermo-epidermal junction and towards the peripheral edge of the CMN. This makes it feasible to detect these melanomas at an early stage based on observed clinical changes or based on diagnostic features seen with dermoscopy. In addition, melanomas arising in smaller CMN tend to do so during adulthood and therefore if prophylactic surgery is being contemplated, it can wait until the patient is mature enough to be fully engaged in the decision-making process. In contrast, melanomas arising in large CMN often develop early in life and arise deeper in the skin or soft tissue, which makes it challenging to detect these malignancies at an early stage. In addition, there can be difficulty in differentiation from benign proliferations. While the normal rules of ABCDE do not apply to the morphology of CMN per se, these can be helpful in the context of change. Furthermore, the behavior of the new lump is a useful discriminator, as melanoma will not stabilize in growth, whereas a proliferative nodule will. Finally, it should be mentioned that the risk of melanoma in smaller "satellite" CMN is thought to be extremely low.

Neurological Manifestations

Terminology

The terminology for the association of neurological disease with CMN is changing, from "neurocutaneous melanosis" to CMN syndrome. The reason for this change is two-fold, firstly to encompass the nonmelanotic neurological abnormalities seen, and secondly to bring the classification of CMN in line with other types of congenital naevi (e.g., epidermal naevi associated with extracutaneous abnormalities are termed epidermal naevus syndrome [Kinsler and Sebire 2016]).

Clinical neurological history and examination is an important part of any assessment of a child with CMN, as neurological abnormalities are the commonest associated adverse outcome (Kinsler et al. 2009). Clinical neurological symptoms used to be considered to be a poor prognostic feature; however, this is likely to have been due to an amalgamation of data from benign congenital neurological abnormalities and malignant disease within the CNS.

Clinical Symptoms

The commonest clinical symptoms are of mild developmental delay, particularly speech delay; however, developmental delay can be moderate to severe in all modalities. Attention deficit hyperactivity disorder and autistic spectrum disorder have also been described. Seizures are rare and usually present as temporal lobe epilepsy, which can progress to generalized seizures. These symptoms usually present by school-age, and therefore neurological history and examination are particularly important in this early age group. Clearly some diagnoses can only be made at certain ages or developmental stages. Clinical neurological symptoms attributable to CMN can sometimes be seen with a normal MRI of the CNS, but in these instances the symptoms described are usually mild.

Congenital Neurological Disease

Congenital neurological disease in CMN is underpinned by the same somatic mutation that causes the cutaneous disease in an individual (Kinsler et al. 2013). Congenital neurological

disease visible on MRI scan has not so far been described in the literature in an individual with only a single CMN. All abnormalities listed below therefore are seen in the context of multiple CMN.

Intraparenchymal Melanosis/Melanocytosis

The commonest manifestation of congenital neurological disease is benign "intraparenchymal melanosis," which is an abnormal melanin-containing focus within the brain parenchyma, with a characteristic hyperintensity on T1 weighting on MRI (Barkovich et al. 1994). Rare histological specimens of these foci demonstrate melanin and melanosomes within mature neurons and glial cells and subtle cortical dysplasia (Kinsler et al. 2012c). These foci are most commonly seen in the mesial temporal lobes in the area of the amygdala (Barkovich et al. 1994; Frieden et al. 1994; Kinsler et al. 2008) and can be unilateral or bilateral. Foci can be present on histology but below the resolution of MRI (Kinsler et al. 2012c), which possibly explains the occasional occurrence of clinical neurological symptoms in the absence of MRI abnormalities. Intraparenchymal melanosis when not accompanied by any other abnormalities on MRI is associated with an increased incidence of developmental delay and of seizures, but is not associated with a poor prognosis in terms of melanoma or life expectancy, at least in childhood (Waelchli et al. 2015a).

Other Congenital Neurological Abnormalities on MRI

A wide variety of other congenital neurological abnormalities have been described on MRI, many of which are unique to the individual, and each case should be considered separately. These diagnoses include malformations such as Dandy-Walker malformation (hypoplastic of absent cerebellar vermis, dilatated IV ventricle and enlarged posterior fossa) and Arnold-Chiari malformation (Chiari malformation type II, showing downward displacement of the vermis, cerebellar tonsils, and spinal chord, usually associated with hydrocephalus) and very rarely benign tumors such as ependymoma and astrocytoma (Kadonaga et al. 1992; Kinsler et al. 2008). Given the rarity of these diagnoses assessment by experts in pediatric neurology and neurosurgery are required, repeat MR imaging is usually required to establish the behavior of the lesions, and biopsy may be needed. This group includes complex combined neurological abnormalities and frequently involves leptomeningeal disease. Leptomeningeal disease can be either focal or diffuse, stable, or progressive. Diffuse disease causes symptoms and signs of hydrocephalus, which may present in the absence of visible disease on imaging, or may appear as communicating hydrocephalus. With leptomeningeal disease, there is a spectrum ranging from stable benign congenital CNS disease (usually focal), through slowly progressive diffuse leptomeningeal disease, to rapidly progressive melanoma of the CNS (usually widespread diffuse). It is therefore unsurprising that patients in this group, as classified by screening MRI in the first 6 months of life, have an increased incidence of not only symptomatic neurological disease (developmental delay, seizures, etc.), but also of requirement for neurosurgery, and possibly an increased risk of death from melanoma in childhood (Waelchli et al. 2015a).

Malignant Disease of the CNS

Primary melanoma can occur within the brain parenchyma or within the leptomeninges in individuals with CMN. Exact analysis of data on melanoma is hampered by the rarity of the condition, as the overall incidence in the skin or CNS is around 1–2% over a lifetime (see above). CNS melanoma however is over-represented in children with extensive neurological disease on MRI, and extremely rare in those without(Waelchli et al. 2015a). Cutaneous melanoma in CNM is considered to be more common than primary CNS melanoma, but this is likely to be confounded by the old practice of publishing fatal neurological cases as "symptomatic neurocutaneous melanosis."

Any individual with CMN who presents with new neurological symptoms or signs at any age should have a full neurological history and examination, and there should be a very low threshold for performing an MRI of the whole CNS with gadolinium contrast. This is independent of whatever findings there were on baseline screening

MRI in the first 6 months of life, if this was performed. Symptoms of CNS melanoma at presentation are often of raised intracranial pressure, and/or seizures. Radiologists should be alerted to look for (1) communicating hydrocephalus, (2) leptomeningeal enhancement, (3) new space occupying lesion. Sometimes a parenchymal space occupying lesion can also be accompanied by leptomeningeal disease, and the two may either be in communication or entirely separate (Ramaswamy et al. 2012; Reyes-Mugica et al. 1993; Waelchli et al. 2015a). In cases of suspected melanoma, a biopsy should be performed for both histopathology and genetics. Genotyping of *NRAS* and *BRAF* hotspot mutations, and array CGH or FISH for copy number may be helpful (Kinsler, unpublished data). Genomic rearrangements that give rise to relevant gene fusions such as involving the BRAF kinase require special consideration.

Facial Involvement

As with many congenital syndromes, children with CMN can have characteristic facial features. These features have been defined using standardized published measures of facial morphology (Kinsler et al. 2012a), and facial features which are age-related were excluded. Facial features in CMN have only so far been studied in a White UK cohort, using a large Dutch cohort of normal children as the control population, and three or more characteristic facial features were found in approximately 70% of cases. There was no relationship found between the facial features and the cutaneous or neurological phenotype. The most common facial features are listed in Table 2.

The mechanism underlying this phenomenon could be the influence of the mutation in *NRAS* on the development of the bones and cartilage of the face, structures known to originate from the neural crest in humans, although no biopsies of bone or cartilage have been performed so it is not possible to be certain. Germline mutations in *NRAS* and in closely related pathway genes (the RASopathy genes) are involved in facial development. Furthermore, there are several other mosaic conditions which are described with characteristic facial features, such as Pallister Killian syndrome, Cornelia de Lange syndrome, and the *PIK3CA*-related overgrowth syndromes.

Table 2 Recurrent characteristic facial features seen in children with CMN. Three or more features are considered to constitute a relevant clustering (Kinsler et al. 2012a)

Facial features
Wide or prominent forehead
Hypertelorism
Eyebrow variants
Periorbital fullness
Small/short nose
Narrow nasal ridge
Broad nasal tip
Broad or round face
Full cheeks
Prominent premaxilla
Prominent/long philtrum
Everted lower lip

Endocrinological/Metabolic Manifestations

Disorders of these systems are relatively frequent within germline RASopathies, implying that the RAS signaling pathway is important in postnatal endocrinological and metabolic control. These are a newly described association of CMN in one large childhood cohort and therefore a new feature of CMN syndrome (Waelchli et al. 2015b). As expected in mosaic disorders the phenotype of endocrinological and metabolic abnormalities was found to vary from individual to individual. When the cohort was examined as a group, however, it was clear that although prenatal growth was normal, there was a clear tendency to gain weight in postnatal life, identified as attributable to adiposity rather than muscle or bone mass. This weight gain occurred at approximately twice the rate of the normal UK childhood population over the same period and was associated with a measurable tendency to insulin resistance (Waelchli et al. 2015b). Neither the underlying cause of weight gain nor the cause and effect relationship with insulin resistance have been established. It is

however clinically relevant to be vigilant of growth parameters in children with CMN and to institute appropriate dietary and exercise advice as for all overweight individuals.

Other clinical features which have been described are premature thelarche in girls, undescended testes in boys, and localized underdevelopment of both fat and muscle underlying some large CMN. Anterior pituitary hormonal measurements show variable subtle abnormalities of G-protein coupled receptor-binding hormones, most commonly suppression of LH (Waelchli et al. 2015b). These may be related to the clinical features seen, but do not seem to have a long-term effect on progression to pituitary, or to fertility.

Vitamin D resistant rickets is more commonly associated with nonmelanocytic congenital naevus syndromes; however, it has been described rarely in the literature. Of note the *NRAS* codon 61 mutation has not been looked for in the bone of the affected individuals, and in both cases the children also had evidence of epidermal naevi (Lim et al. 2014). Whether this is a true association with CMN syndrome per se is therefore not yet clear. Where there is clinical suspicion of calcium/phosphate metabolism, this should be investigated by serum and urinary measurements.

Other Clinical Associations

Other Tumors

Rhabdomyosarcoma (RMS) is the most recurrently described nonmelanocytic tumor to arise within a CMN (Cohen et al. 1996; Hendrickson and Ross 1981; Hoang et al. 2002; Ilyas et al. 2004). Genetic studies on these tumors are so far lacking in the literature. It is interesting to speculate whether *NRAS* hotspot mutations could be a driving mutation for RMS development in this context, as RAS mutations including those in *NRAS* have been found in RMS outside the context of CMN (Stratton et al. 1989).

There can rarely be primary tumors within the CNS of nonmelanocytic origin in patients with CMN, for example, astrocytoma, meningioma, and ependymoma (Kinsler et al. 2008; Waelchli et al. 2015a) and sarcoma Not Otherwise Specified (Reyes-Mugica, unpublished observations).

Dermoscopy Features

Dermoscopy allows clinicians to visualize structures within the epidermis and papillary dermis that are not discernable by the naked eye. These structures create patterns that together can aid in differentiating CMN from melanoma. Knowledge of the dermoscopic structures and patterns common to CMN can assist physicians in following these lesions and recognizing aberrancy that may be suggestive of melanoma. Thus, if a dermoscopic pattern does not conform to one of the patterns commonly encountered in CMN or if focal atypical dermoscopic structural changes develop, then a biopsy or an excision may be warranted. It is important to acknowledge that although most small and medium CMN are fairly homogeneous both clinically and dermoscopically, large CMN are often heterogeneous, displaying multiple islands of color and irregular topography. This together with the fact that most melanomas arising in large CMN are located below the papillary dermis results in barriers to their early detection via visual inspection and dermoscopy. However, since melanomas in small CMN tend to arise at the dermo-epidermal junction, dermoscopy is the ideal looking-glass to help in their early detection.

Dermoscopic evaluation of a CMN begins by analyzing the dermoscopic features present in the lesion (Table 3).

After identifying the local dermoscopic features commonly seen in CMN, it becomes apparent that these naevi often form specific dermoscopic patterns. These patterns are comprised of structures that are generally distributed in an organized and symmetrical manner. The five primary global patterns are (Fig. 4): reticular, globular, reticulo-globular (symmetrical), diffuse brown pigmentation, and multicomponent. A biopsy should be considered for any CMN that reveals structures and patterns other than the ones mentioned above.

It has been observed that anatomical location dictates, to a great extent, the

Table 3 Dermoscopic structures seen in CMN

Features	Characteristics	Histopathological correlation
Pigment network	Fine or thick lines that intersect creating a honeycomb-like network pattern. The distribution of the network can be present throughout the lesion in a homogeneous distribution or can be present focally or in a patchy distribution. In addition, in some small CMN the network can be present at the periphery with globules located in the center. At times one can only see linear network fragments or branched streaks that resemble hyphal elements (i.e., resemblance to the tubular branching of fungal hyphae)	Pigmented lines correspond to rete ridges, and the holes of the network corresponds to the suprapapillary plate overlying the dermal papillae
Globules	Small to large sharply circumscribed, round to oval brown aggregates. The distribution can be central or diffuse. While most globules are round they can also be somewhat polygonal in shape creating a cobblestone like arrangement. At times the globules appear to be surrounded by a halo producing a target-like appearance. The target-like appearance is created when the globule is centered in the "hole" of the network, corresponding to nests of melanocytes in the dermal papillae	Nests of melanin containing nevomelanocytes within the dermis
Diffuse brown pigmentation	Lesion has a homogeneous brown color. On close inspection, focal network fragments and sparse small globules may be seen	Diffuse distribution of melanin in the epidermis and dermis
Hypertrichosis	Increased number of terminal hairs, often with perifollicular hyper or hypo-pigmentation	
Blood vessels	The morphology of vessels seen in CMN includes comma, dotted, linear, serpentine, coiled, and hairpin vessels	
Milia like cyst	White to yellow, rounded, often hazy structures	Intraepidermal keratin cysts/pseudocysts

dermoscopic pattern observed in congenital naevi. CMN on the extremities usually have a reticular pattern and CMN on the torso, head, and neck usually have a globular pattern. The theory to account for the variation in dermoscopic pattern as relates to anatomical location hinges on presumed migratory pathways that melanoblasts may take during embryogenesis. Melanoblasts destined for the skin of the extremities are presumed to preferentially migrate along the dorsolateral route, which happens to be more "superficial," thus accounting for the predominant reticular pattern seen in CMN located on the extremity. In contrast, melanoblasts destined for the skin of the torso, head, and neck are presumed to preferentially migrate along the ventral route, which happens to correspond to the pathway of nerve trunks. The ventral pathway is "deeper" and this helps explain why globular CMN in these locations often have a globular component.

Histopathological Features

The histological appearance of CMN reveals their malformative, hamartomatous nature, and in parallel with their clinical appearance, it may vary with age. Typical appearances at birth are characterized by richly cellular lesions composed of melanocytic elements that may adopt a range of phenotypes. The bulk of naevus cells occupies deep dermal layers of the skin (reticular dermis) and frequently the subcutaneous tissue, with predominantly spindly elements that commonly

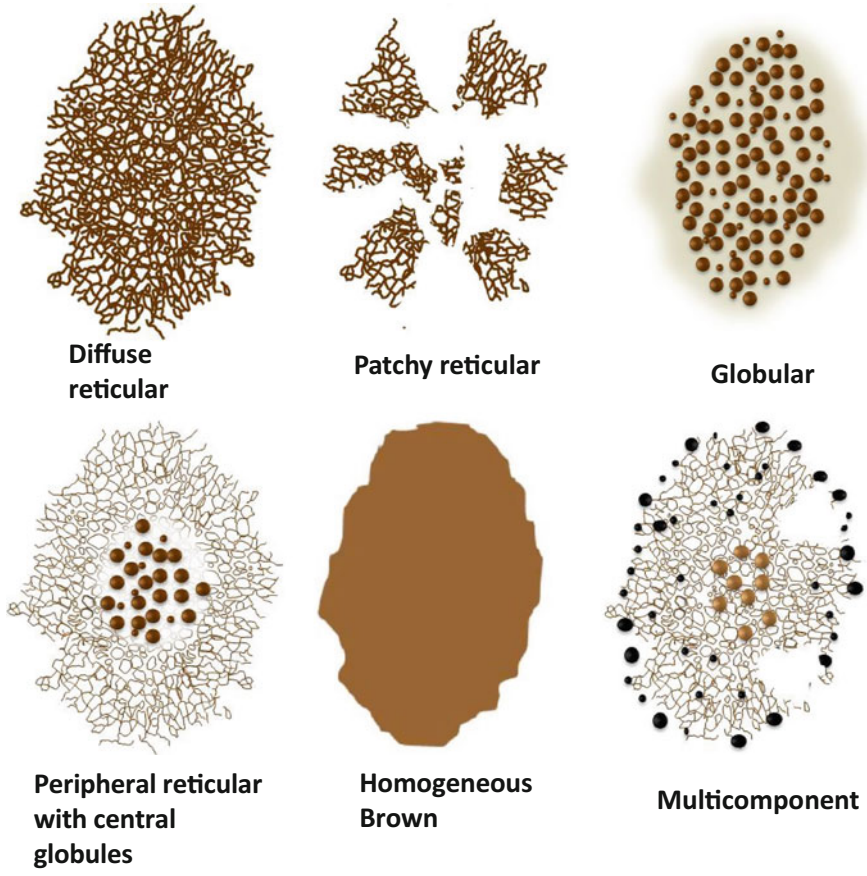

Fig. 4 The five primary dermoscopic patterns seen in CMN

adopt a "neuroid" look consistent with their presumed neural crest origin and/or peripheral nerve sheath common ancestry. These deeply situated cells are mid-size, with poorly defined cytoplasm, and uncommonly pigmented. Neuroid bodies, also known as Wegner-Meissner or Masson bodies (lames folicée), are characteristic of these lesions, explaining the designation of "neurotized naevi" (Figs. 5, 6, 7, and 8). The mid-portion of these typically thick lesions is occupied by compact sheets of rounder elements, alternating with occasional intervening lobules of fat. Entrapped and distorted adnexal structures, which are extensively infiltrated by the naevus cells, are very common. More superficial layers of the naevus show larger cells with a more epithelioid appearance, more abundant cytoplasm, and occasional intracytoplasmic melanin pigment. Not uncommonly, in these superficial layers, there are scattered clusters of heavily pigmented melanophages. Many congenital naevi show a compound histologic pattern, although a large proportion of them revel a Grenz zone within the papillary dermis. The overlying epidermis features variable degrees of atrophy of the rete ridges, although papillomatosis and verrucoid features are not uncommon (Fig. 9). In newborns, large congenital naevi may reveal striking pagetoid patterns, atypia, and even ulceration, features that should not prompt the diagnosis of melanoma in the absence of documented metastasis. However, most lesions show no significant atypia or pleomorphism. Naevus cells adopt a single file distribution within the dermis and are frequently observed splaying collagen fibers and penetrating underneath the endothelium of local vasculature.

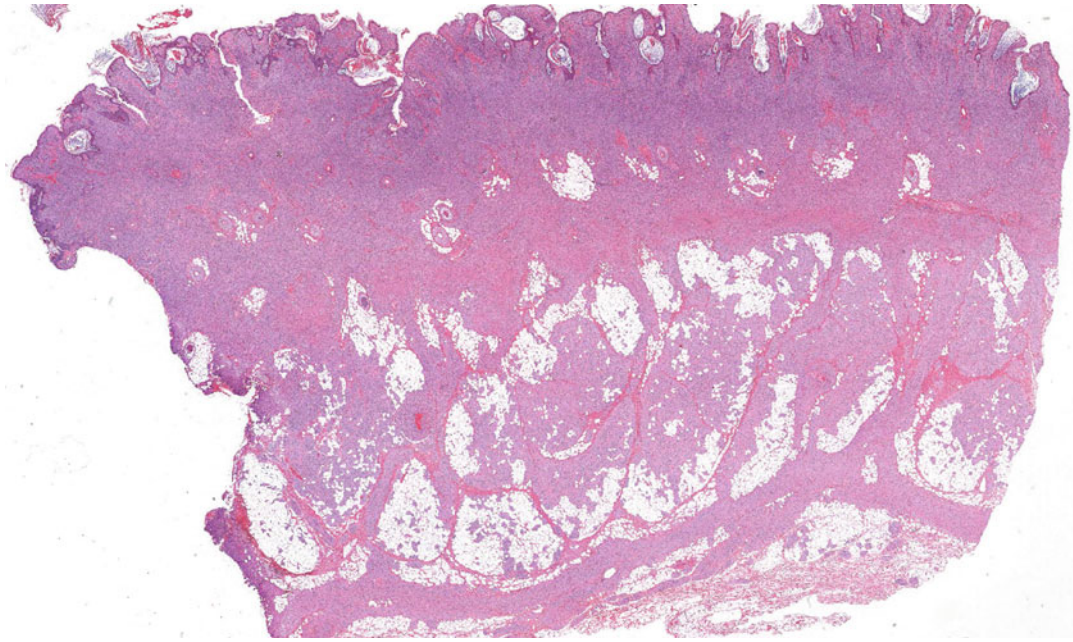

Fig. 5 Low power view (6×) of a giant congenital melanocytic nevus. The nevus cell proliferation is extremely thick involving from the base at the level of the fascia and subcutaneous tissue, replacing completely the reticular and papillary dermis. Skin adnexa are overwhelmed by the nevus cell proliferation, leaving only a few hair follicles. The epidermal surface is verrucous

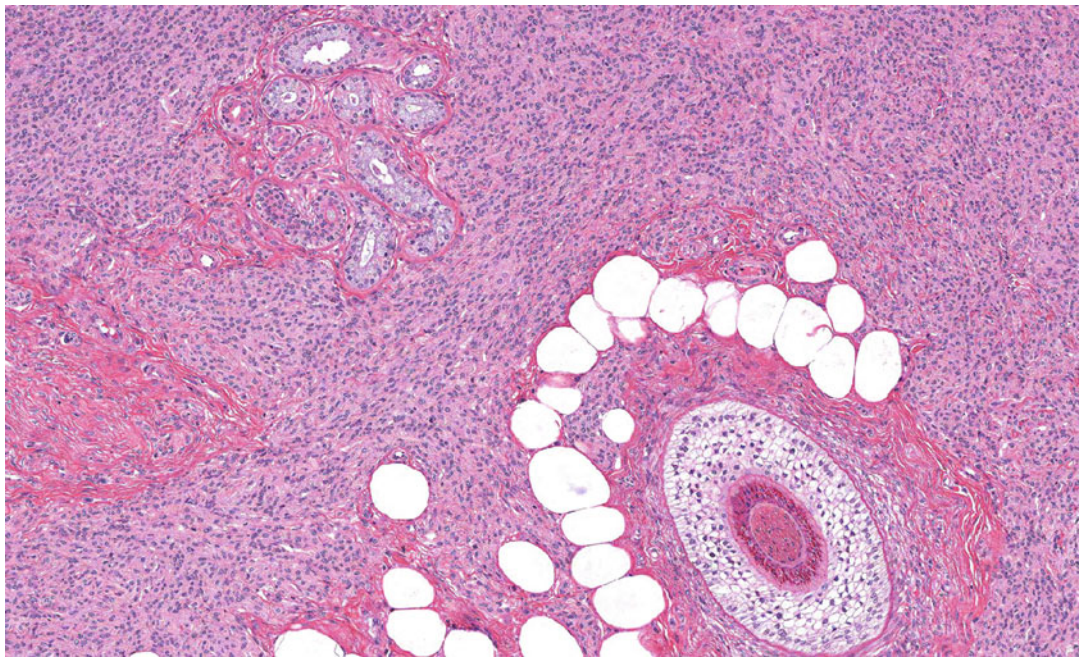

Fig. 6 Higher magnification (40×) from the nevus shown in MRM 1. Note the entrapped sweat glands surrounded by nevus cells. The hair follicle on the lower right shows infiltration by nevus cells, a classic feature of congenital nevi

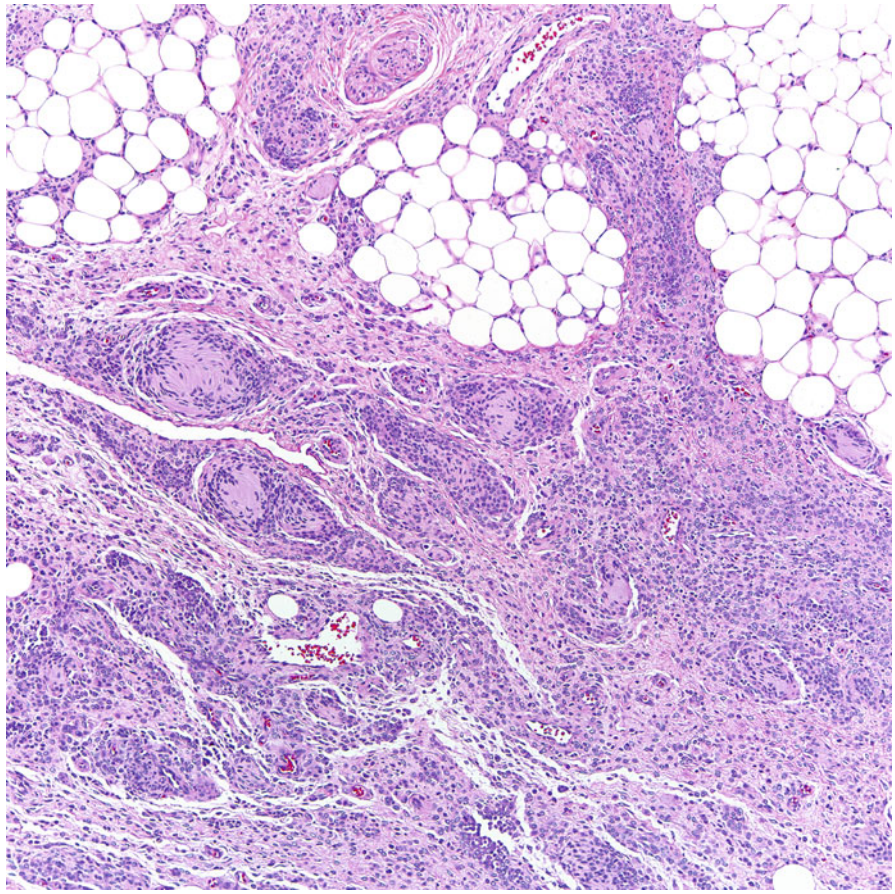

Fig. 7 Giant congenital melanocytic nevus with Wagner-Meissner or Masson bodies, evidence of so-called "neurotization," revealing the neural crest origins of the lesion (100×)

Additional Cell Populations and Tumors Arising in CMNs

The most important lesion due to its frequency arising in L/GCMNs is the so-called "proliferative nodule" (Fig. 10). Histopathologically these are well-delimited nodular growths surrounded by conventional naevus (Figs. 10 and 9, respectively). As mentioned above, histological features typically associated with malignant behavior in adult melanocytic lesions such as ulceration, pagetoid proliferation, and increased mitotic activity may occur in benign melanocytic lesions in infancy, in particular in proliferative nodules. When these features are present, the nodule should be classified as "atypical," and subjected to additional genetic studies, including comparative genomic hybridization and FISH.

Recapitulating their presumed neural crest origin, nevus cells reveal aberrant lines of differentiation (heterologous elements) and some lesions have excess adipose tissue and other mesenchymal elements, including cartilage, aberrant vasculature, etc. This may explain why, with some frequency, these lesions harbor malignant mesenchymal tumors such as rhabdomyosarcoma, liposarcoma, peripheral nerve sheath tumors, and others.

An inconspicuous, although seemingly important population is represented by statistically significant increase in the number of mast cells

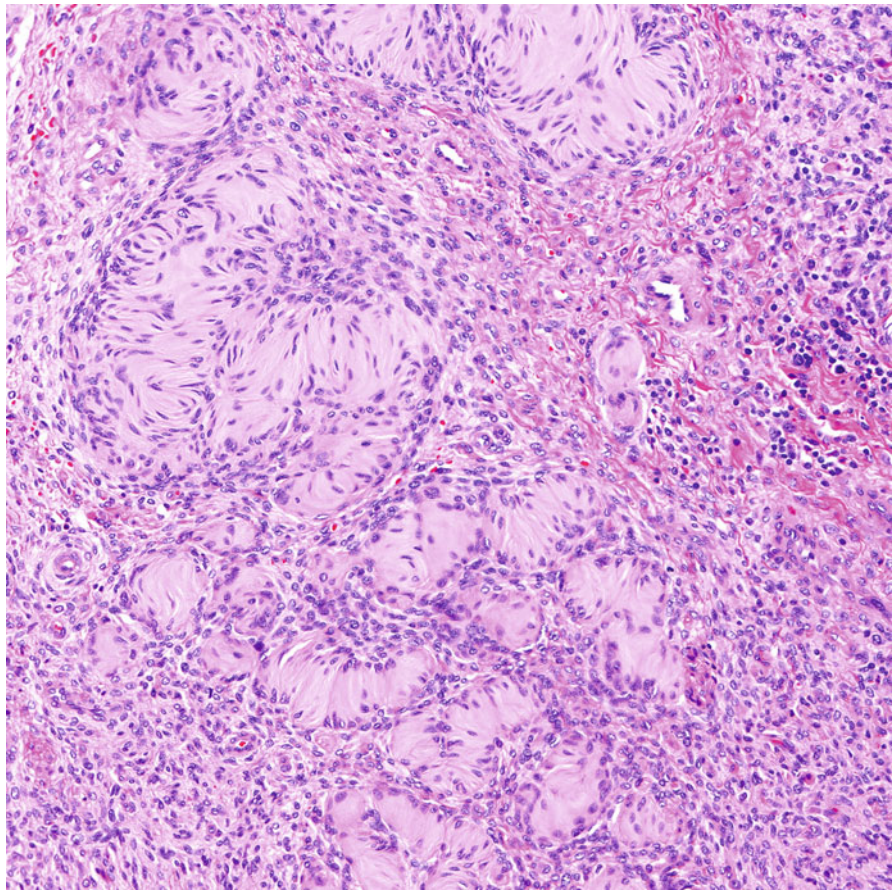

Fig. 8 Higher magnification (200×) from the nevus shown in MRM 3. The neurotization evidenced by the schwannian appearance of the *lame foliacée* is better appreciated

within the naevus skin and also in other skin areas in patients harboring L/GCMNs (Salgado et al. 2014). This mast cell hyperplasia may explain some of the symptoms occasionally associated with L/GCMNs such as incontrollable pruritus (Feng et al. 2005; Frigon and Desparmet 2006) and possibly excess fibrosis/scarring in naevus areas.

Small congenital nevi share some of the above-mentioned features but with the obvious difference in scale. These lesions are frequently wedged-shaped, with lichenoid pattern featuring nevus cells in a band-like distribution. The nevus cells surround and frequently penetrate skin adnexae and neurovascular bundles. Nevus cells adopt a single-cell or cord pattern, splaying collagen fibers (Figs. 11 and 12).

Genetic Testing

In the Absence of Malignancy

Genetic testing in the absence of malignancy does not currently alter management and does not need to be performed routinely. If genotyping is requested or desirable for extra information, this requires a punch biopsy from affected skin as the postzygotic mutation is not detectable from a blood sample. Alternatively, if the patient is having a naevus or part of a naevus removed for

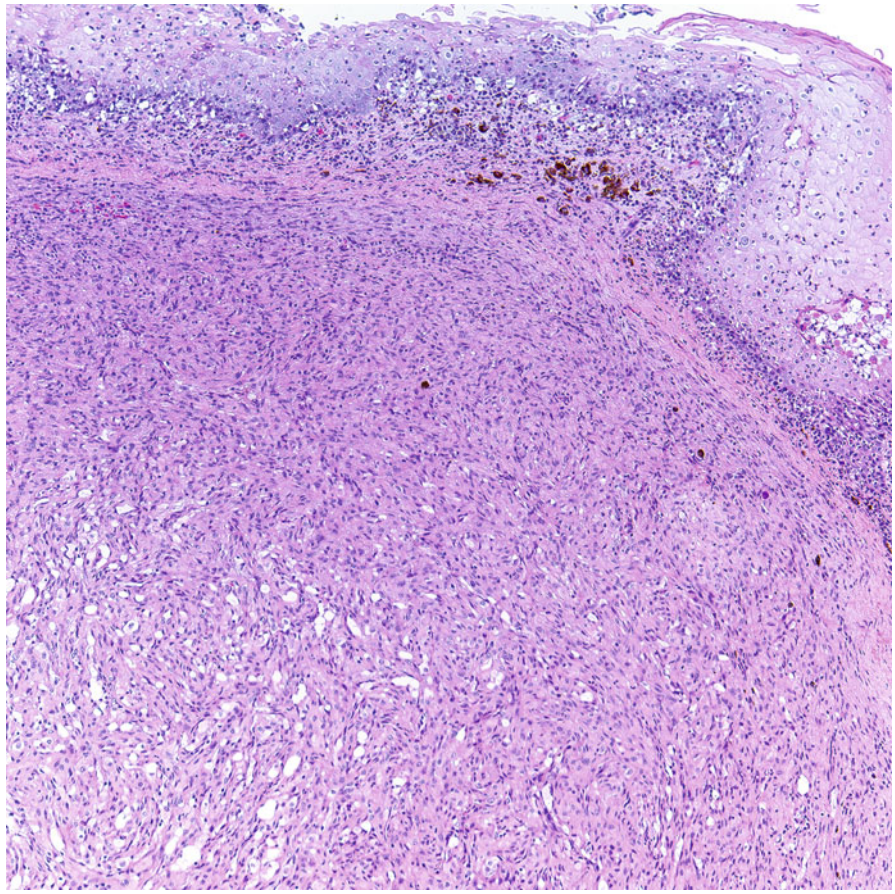

Fig. 9 Higher magnification of the lesion shown in MRM 5. Note the spindle appearance of the proliferating nevus cells, the clear demarcation, and the increased vascularity in the lower left area. Between the epidermis and the nodule, there is an area of heavily pigmented melanophages (100×)

cosmetic reasons, this sample could be used for genotyping if wished. Increasingly, well-informed patients are interested in knowing whether their CMN is *NRAS* mutation positive or negative. DNA should be extracted directly from the biopsy rather than from fibroblast culture, as the mutation is unlikely to be present in fibroblasts. *NRAS* codon 61 mutations should be looked for using sufficiently sensitive techniques to detect a mutant allele load of 10% in the first instance. If negative, further techniques such as next generation sequencing can be used to increase sensitivity. *BRAF* hotspot genotyping should also be undertaken if *NRAS* is wildtype, as this has recently been shown to be a rare cause of multiple CMN. *BRAF* fusions and other rearrangements have previously been described in single samples of congenital nevi, which open the possibility of targeted therapy if found to be causative in a particular patient (Botton et al. 2013; Dessars et al. 2007).

In Suspected Malignancy

Genetic testing in the clinical situation of suspected malignancy is not only helpful but mandatory if at all possible. If the malignancy has arisen within a naevus in the skin, then testing of the nevus in parallel with the malignancy can be helpful to look genetic alterations associated with progression. Clinically relevant testing can be divided into two areas, namely, driver mutation analysis and copy number analysis.

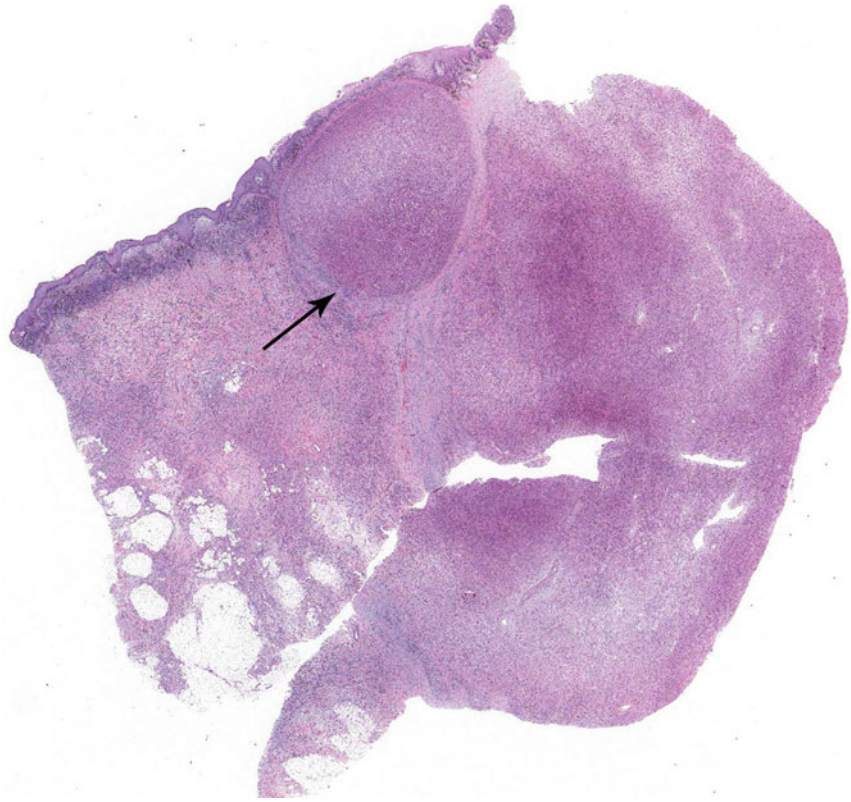

Fig. 10 Low-power view of a proliferative nodule arising in a giant congenital melanocytis nevus. The epidermis overlies a cellular nevus lesion in which one clearly demarcated nodule (arrow) is seen. The cellularity in the nodule is higher than in the conventional nevus that surrounds it. Deeper to the nodule there is another vaguely nodular area composed of proliferating spindle nevus elements (60×)

Driver Mutation Analysis

NRAS and *BRAF* hotspot mutation analysis is available in many diagnostic laboratories and can help guide clinical management in this difficult situation. Importantly however this should be performed on DNA directly extracted from a skin biopsy or excision, rather than from cultured fibroblasts, as these may not contain the mutation. Similarly, it is important to use adequately sensitive techniques for mutation detection, as standard Sanger sequencing is often too insensitive to be reliable.

If heterozygosity for *NRAS* codon 61 mutations are found in the nevus and in the suspected melanoma no conclusion can be drawn about whether the lesion is malignant, however this result will still guide management of malignant lesions. BRAF-inhibitors are contraindicated in *NRAS*-mutated melanoma; however, MEK inhibition may be a viable option. Homozygosity for *NRAS* codon 61 mutations has been described in cutaneous melanoma arising in a CMN which was heterozygous before malignant transformation (Kinsler et al. 2013) (Fig. 8). This finding may therefore be of assistance in assessing malignant status, but a larger series is needed before this can be confirmed. Another recently described mechanism underlying malignant transformation is *NRAS* mutation with amplification (Salgado et al. 2015b), which can be detected using quantitative real time PCR, or on high resolution copy number arrays (see below).

BRAF codon 600 mutations have not thus far been described in melanoma in individuals with CMN, but at this stage in our knowledge of mutations in CMN these are, in the authors' opinion,

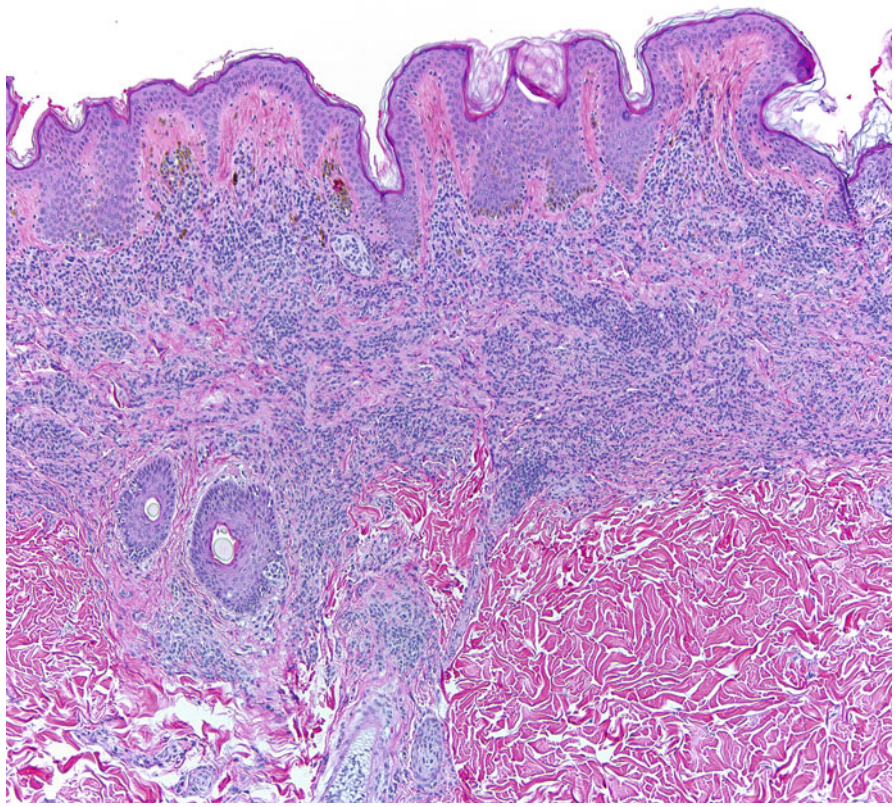

Fig. 11 Small congenital melanocytic nevus showing a lichenoid, band-like distribution of nevus cells in the papillary and reticular dermis, with a vaguely wedge shape and involvement of the skin adnexa (100×)

still worth testing for due to the success of BRAF inhibition.

Progression from a benign nodule with benign histology and a heterozygous *NRAS* c.181C>A, p.Q61K mutation (upper panel), which evolved to melanoma 5 years later, in which the NRAS mutation is now homozygous (lower panel). Reproduced with permission from *Journal of Investigative Dermatology* (Kinsler et al. 2013).

Whole Genome Copy Number Analysis

Whole genome copy number analysis was the first genetic test to differentiate between CMN and cutaneous melanoma (Bastian et al. 2002). This was initially performed using array comparative genomic hybridization (array CGH), but can also be done using SNP arrays, or fluorescent in situ hybridization (FISH). Benign CMN exhibit none or few copy number changes, as would a normal tissue. Proliferative nodules can demonstrate numeric copy number change of whole chromosomes. Melanoma on the other hand demonstrates multiple gains and/or losses of whole or parts of chromosomes. This has been confirmed recently not only in the skin but in the CNS melanomas of patients with CMN (Kinsler, unpublished data).

Genetic Counseling

Parents of children severely affected by CMN may request or require genetic counseling, despite the fact that the phenotype is ultimately caused by a postzygotic mutation. Recurrence rates for

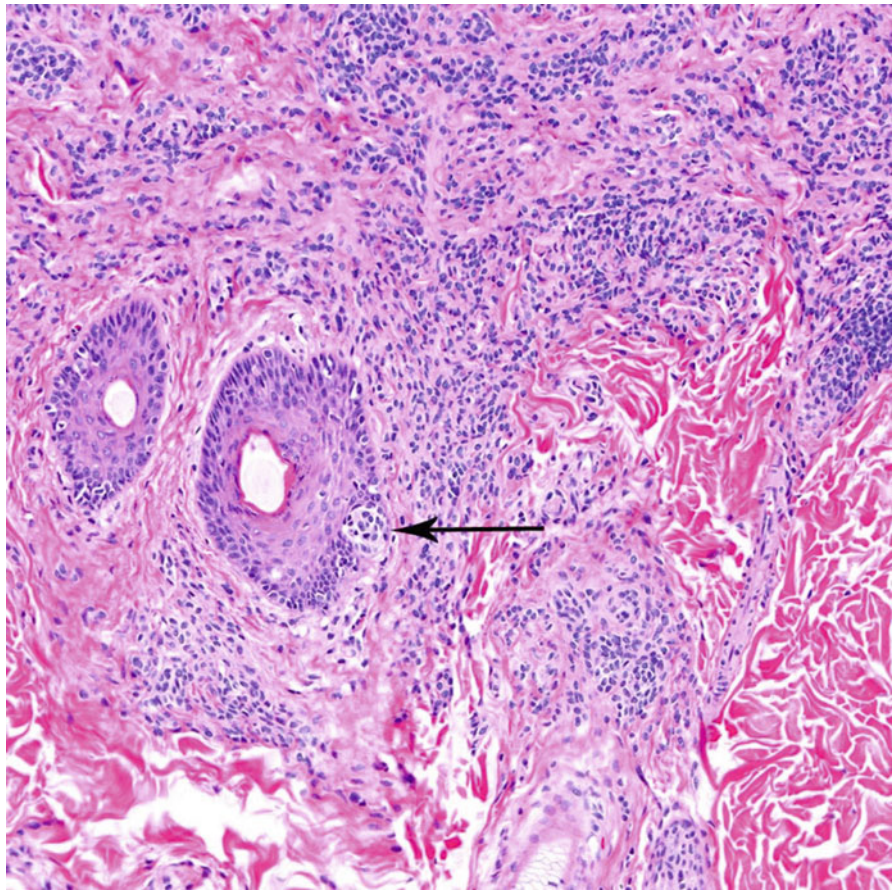

Fig. 12 Small congenial nevus shown involving adnexa in reticular dermis. A nest of nevus cells (arrow) is seen within the epithelial cells of the external follicular sheath, the equivalent of junctional activity in a hair follicle (200×)

future pregnancies should in theory be no different from the incidence in the normal population; however, as previously mentioned there is a loose family history in some families, and in very rare instances a clearer tendency to develop CMN in successive generations. These cases are presumably due to an as yet unidentified germline predisposition to somatic mutation, or possibly to a germline mutation which in itself produces a cutaneous phenotype indistinguishable from the usual. Such families would benefit from interaction with a clinical geneticist.

In addition, for individuals who are severely affected by CMN themselves the current advice would be that they are not at increased risk of having a child with CMN, as descriptions in the literature of instances of a parent and child being affected are extremely rare. Anecodatally the miscarriage rate in individuals with CMN does not seem to be raised. This is either due to the fact that gonadal mosaicism is not present in these individuals, or that there is a very low additional miscarriage rate due to passing on the *NRAS* or other causative mutation in the full heterozygous state.

Management

General Skin Care

CMN skin is relatively fragile, for reasons which are not clear. After birth there are not infrequently erosions present in large CMN, particularly on the back. These should be treated conservatively with

low-adherence dressings until healed. At older ages fragility can be a problem in areas prone to injury, such as the forehead or knees in young children. However, naevi do not usually bleed more profusely than normal skin, and with pressure applied in the usual way healing is usually good with minimal scarring.

CMN are prone to dryness, again for reasons which are not clear. However, some have speculated that this may be due to lack of or dysfunction of adnexal structures including sweat glands and sebaceous glands. General advice should be given about avoidance of soaps, and the use of moisturisers where required. Some individuals with CMN have chronic pruritus, which may or may not be associated with eczema in the CMN. In most cases pruritus can be managed as for dryness; however, in some cases topical steroids are required for symptom control, even in the absence of frank eczema. Pruritus generally improves with age. In rare instances severe and refractory pruritus is associated with induration and erythema within the CMN, and sometimes with a multinodular phenotype. In these cases, surgical excision can be the only option for symptom relief.

In rare instances CMN can be or can become over time very folded on the surface, in a cerebriform type of pattern. This is most often (but not exclusively) seen in scalp CMN. The "gyri" of the cerebriform morphology tend to lose hair growth and pigmentation, becoming pink in color, whereas the "sulci" harbor the remaining hairs. In some cases these cerebriform naevi also become multinodular. These naevi are extremely difficult to keep clean and can smell due to yeast and bacterial over growth. Where surgical excision is a viable option this should be considered.

Management of Patients with Solitary CMN

A thorough examination should be performed on every child born with a CMN, to establish how many naevi are present, taking into consideration that small naevi can be very pale at birth. If there is only a single CMN, irrespective of size or site, and if the neurological examination is normal, then no further investigation is required.

Whenever possible baseline photographs should be acquired since these can prove useful during subsequent surveillance examinations. If photographs are not obtained, then a thorough description of the nevus should be recorded including its texture, size, nodularity, rugosity, and color.

Prophylactic excision of solitary CMN with the aim of preventing the development of melanoma within the CMN remains an area of controversy. While a host of reasons, including anxiety of developing melanoma and cosmetic concerns, may prompt an individual to seek excision of their CMN, there is fairly consistent agreement among experts that prophylactic excision of small to medium size CMN during infancy is not absolutely necessary since the risk for developing cutaneous melanoma during preadolescence is extremely low. During later adulthood there is a small risk for developing melanoma in association with small to medium CMN. These melanomas tend to develop at the periphery of the CMN and arise from the dermoepidermal junction, thus making them amenable to early detection via periodic surveillance examinations. We recommend that adults with small to medium CMN examine their moles on a monthly basis and if they notice any focal changes to alert their physicians. Some patients may also find comfort in having a dermatologist periodically examine their skin. CMN developing concerning changes should be biopsied and if the presence of melanoma confirmed, the CMN together with the malignancy should be excised and the melanoma treated as would any other primary cutaneous melanoma of similar thickness.

While there is fairly good agreement among experts against routine prophylactic excision of small CMN, the topic of prophylactic excision of large CMN remains an area of ongoing debate, which at times can become quite contentious. From published data, it is irrefutable that melanoma can develop within large CMN and unlike small CMN these melanomas often present during

the preadolescent years of life. However, the benefits and harms from a watch and wait approach versus whole scale prophylactic excision of such nevi during infancy remain to be elucidated. Proponents against recommending prophylactic surgery for large CMN rely on the following rational:

1. The risk for developing a cutaneous melanoma in association with multiple CMN, no matter how large the principal CMN, if there is a normal MRI scan after birth, is approximately 2% in the largest prospective study of a severe phenotypic cohort, and is therefore likely to be less in the whole CMN population(Kinsler 2017). Compared to risks of melanoma in the normal population.
2. Lack of studies that definitively prove that prophylactic excision reduces the risk of developing melanoma.
3. The morbidity associated with surgery and scars can be significant and at times debilitating.
4. Possible adverse effects of general anesthesia on the developing brain (Rappaport et al. 2015) in individuals under the age of 3 years.
5. Complete excision of large or multiple CMN is often not feasible and melanoma can develop from residual nevus cells.
6. Surgical manipulation of the large CMN may heighten the risk for developing melanoma.

Proponents advocating prophylactic excision of large CMN rely on the following rational:

1. The heightened risk of developing both benign and malignant tumors within large CMN together with an effort to improve cosmesis warrants consideration for prophylactic excision at a young age.
2. Most melanomas arising within large CMN develop below the dermo-epidermal junction and thus are not amenable to early detection via visual surveillance examinations. Since these melanomas often present at an advanced and often incurable stage, prophylactic excision affords the only viable opportunity to prevent death related to these melanomas.
3. The absence of evidence is not evidence of absence of the potential therapeutic benefits of excision of large CMN. Since melanomas are arising from the malignant degeneration of melanocytes within the large CMN, it stands to reason that if these cells are removed prior to development of any transformative mutations that this will prevent or at least lower the risk for developing a fatal melanoma.

Unfortunately, there are no studies that will definitively help resolve the aforementioned debate within the foreseeable future. However, with the evolution of imaging technologies, molecular techniques, improved risk stratification based on nevus phenotype, and evaluation of outcome studies designed to investigate the potential benefits and harms associated with extensive surgical procedures, it is highly likely that the debate will become less contentious over time.

Management of Patients with Multiple CMN

Multiple CMN is defined as two or more at birth. A single screening MRI of the whole CNS with gadolinium contrast injection to look for the neurological anomalies is recommended from the largest prospective cohort study of children with multiple CMN. This can usually be done without general anesthesia, when done within the first year of life, and protocols for sedation have recently been published (Plumptre et al. 2019). Ideally screening MRI scans should be performed within the first 6 months of life to reduce the influence of myelinization on the appearance of melanosis. On the basis of the findings, individuals are divided into three management groups (Fig. 9). Those with a normal MRI do not need it repeated and can have a low-frequency follow up schedule (80% of those with multiple CMN). Those with the common finding of intraparenchymal melanosis only (approximately 10% of cases) do not need a repeat MRI but should have yearly neurodevelopmental assessment by a local pediatrician until school age, to pick up any issues that may

need intervention early on. The small minority (approximately 10% of cases) who have more complex radiological changes should be managed in a multidisciplinary team setting and have regular follow up for both skin and CNS examinations.

Suggested management of CMN based on classification known to differentiate clinical outcome. Reproduced with permission from the *British Journal of Dermatology* (Waelchli et al. 2015a).

Of a New Lump or Visible Change Within a CMN

As these are frequent, particularly in certain individuals, it is neither practical nor desirable to biopsy or surgically remove every change in a CMN. The authors would therefore recommend the following as a guideline, although individual cases should be managed at the physician's discretion. New lumps or changes should be photographed with color and size references, dermoscopy performed and recorded, and thorough examination should be performed for local lymphadenopathy. If there is a high index of suspicion for malignancy, it should be biopsied. If the index of suspicion is low, the patient should be seen again within 4 weeks, and appearances compared to the baseline photographs and dermoscopy. In addition, the lesion should be palpated to determine whether any palpable changes have developed including degree of firmness, nodularity, and size. If there has been no change at that stage, the patient should be seen again within a similar time frame. If however there has been clear change macroscopically or concerning changes dermoscopically, the lesion should be biopsied and sent for an expert histological opinion.

Of a New Neurological Presentation

The presentation of new neurological symptoms or signs in a child with CMN should prompt an MRI of the whole CNS with contrast enhancement, independent of what the initial screening MRI showed (if performed). This is to look for a primary CNS melanoma or other tumors or anomalies. If the MRI is normal, the patient can be referred to Neurology for further diagnosis.

Of Primary Cutaneous Melanoma

Very little hard data exist on management of melanoma in CMN. What follows is the distillation of the authors' collective experience of this disease, and a knowledge of existing literature.

Once a diagnosis of cutaneous melanoma is established on the basis of histology and/or genetic testing, management should ideally be discussed in a multidisciplinary team, especially if the melanoma is advanced or has developed within a large or multiple CMN. Early malignancies in adulthood in small CMN can often be resolved by complete excision; however, this has not been demonstrated for melanomas developing in large or multiple CMN in childhood in particular. In general, melanomas in large CMN are highly aggressive for reasons which are not yet understood. A very high index of suspicion should be maintained at all times in this situation. There is no evidence of clinical utility of sentinel node biopsy and this should therefore follow local protocols.

PET scanning should be considered for all cases of melanomas associated with large or multiple CMN at diagnosis, even with no clinical indication of spread. CMN do not appear on PET, and therefore, this investigation is thought to be clinically useful. In contrast, radiological scanning of patients with melanoma in association with smaller CMN in adulthood is usually not warranted.

Of Primary CNS Melanoma

Once a diagnosis of CNS melanoma is established, management should be within a multidisciplinary team, and biopsies should ideally have genetic analysis (see above). This

disease has so far been universally fatal, usually within 6 months from diagnosis. If leptomeningeal disease is prominent then a ventriculo-peritoneal shunt is in our experience required to manage symptoms of raised intracranial pressure, and despite the fact that very rarely melanoma has been shown to metastasize via a shunt. Oral corticosteroid therapy can be very helpful symptomatically. Radiotherapy to the leptomeninges can also be very helpful symptomatically, although this is a palliative measure.

A long-line can be indispensable in children to minimize trauma from repeated phlebotomy and intravenous drug administration when required. Ipilimumab has been tried with limited success in one case; however, MEK inhibition has been tried for *NRAS*-mutation positive melanoma in CMN, and symptomatic relief at least has been documented (unpublished data).

Of the Esthetic Aspects of CMN

Clearly, removing or de-bulking a large CMN will lower the melanocytic burden. While it may be intuitive that this in turn should lower the risk for developing cutaneous melanoma, there is no conclusive evidence that removal of CMN reduces melanoma risk. This may be because nevi which are small enough to be entirely resected have a very low malignancy risk. Similarly, those which are large and multiple with a more concerning malignancy risk are not only not amenable to be completely resected, but can develop CNS melanoma rather than cutaneous.

The aesthetic aspects of CMN however should not be underestimated, both for the patient and at least initially for their parents, and each patient will be different. This problem should be addressed early on and continue to be discussed at all appointments. Simple measures include shaving, for which a beard-trimmer is recommended to avoid pruritus on regrowth, and hair removal creams should be avoided due to fragility of the CMN.

CMN that are relatively easily resected with resulting improvements in cosmetic appearance can clearly be removed if desired, and the pros and cons of timing discussed on an individual basis. The problems tend to arise however with large or multiple CMN where complete excision is often not a viable option. In this situation, the pros and cons of any aesthetic improvement have to be balanced against the toll of the operations, and waiting until the child is old enough to participate in the decision should be considered. Superficial removal techniques such as curettage, dermabrasion, and laser all suffer from the same problem, which is that pigmentation recurs, and final colour is not altered (Polubothu and Kinsler 2019).

Conclusions

The understanding of CMN as a mosaic disorder has helped to clarify why there is such a variable spectrum of phenotypes and associations. Other than small single CMN this is a rare disease, and interaction between physicians in different institutions is often beneficial in managing difficult cases.

The key differentiators in routine clinical management of the patient are firstly whether there is a single CMN at birth or more than one (multiple) and secondly the results of a single screening MRI of the CNS under the age of 1 year (ideally in the first 6 months). Using these statistically supported classifiers of phenotype, most individuals can be reassured that they have a low long-term risk of adverse outcomes, and the minority of patients identified at higher risk can be followed up and investigated appropriately.

Outside of routine clinical management certain presentations should alert the physician to potentially dangerous issues. These are the development of new neurological symptoms or signs, and the appearance of new lumps in the CMN. Expert opinions in dermoscopy, histopathology, and radiology should be utilized to improve management of individual cases. The increasing availability of genetic testing and improved understanding of the differentiators between benign and malignant lesions will help with diagnosis and choice of treatments.

Online Resources

UK based patient support group www.caringmattersnow.co.uk

UK based research laboratory for CMN http://www.ucl.ac.uk/ich/research/genetics-genomic-medicine/genetics-epigenitics-health-disease/research-groups/veronica-kinsler

US based patient support group www.naevus.org

US based research laboratory for Large/Giant Congenital Melanocytic Naevi and Associated Disorders https://www.givetochildrens.org/GCMN-NCM

OMIM #249400, #137550, ∗164790

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Spitz Tumors

18

Iwei Yeh and Boris C. Bastian

Contents

Abstract

Spitz tumors constitute melanocytic tumors with distinctive epithelioid and spindled melanocytes that typically are benign, but can occasionally metastasize to regional lymph nodes, or in rare situations lead to widespread metastatic dissemination and death. They are characterized by specific genetic alterations such as activating mutations in HRAS or fusion genes that involve kinases such as ALK, ROS1, NTRK1, NTRK3, MET, RET, and BRAF, but

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lack BRAF V600E and NRAS mutations found in acquired nevi and GNAQ and GNA11 mutations found in blue nevi.

Keywords
Spitz nevus · Atypical Spitz tumor · Spitzoid melanoma · Fusion kinase · HRAS · BAP1

Introduction

The term Spitz nevus references Sophie Spitz's description of "juvenile melanoma" in 1948. She described neoplasms in children that were diagnosed as melanoma, but demonstrated distinctive histopathologic characteristics and mostly benign biologic behavior (Spitz 1948). In her original description, Spitz identified the characteristic features as melanocytes that are large and polygonal (epithelioid) or spindled with increased amounts of cytoplasm as compared to the smaller melanocytes in "common" or conventional nevi, thickening of the overlying epidermis, clefting around individual junctional melanocytes or nest of melanocytes, scatter of single melanocytes into the upper levels of the epidermis, and superficial dermal edema.

It later became clear that neoplasms with these morphologic characteristics fall onto a spectrum from benign to malignant, with an intermediate category that is difficult to classify in terms of prognosis (Barnhill et al. 1999; Cerroni et al. 2010a). Here the authors use the term Spitz tumor to denote the entire spectrum of melanocytic neoplasms with spitzoid morphology: Spitz nevi for lesions at the benign end of the spectrum, malignant Spitz tumor for those with unequivocal malignant features, and atypical Spitz tumor to designate lesions that show overlapping features of benign and malignant.

As outlined below, Spitz tumors represent a heterogeneous group of conditions with a range of different genetic alterations that initiate transformation, with subsequent alterations promoting transformation to a fully evolved malignancy with lethal potential. The authors anticipate that as the specific natures of the alterations unfold Spitz tumors will ultimately be subdivided into distinct classes, with most classes having a benign (nevus) and/or intermediate stage and a malignant (melanoma) stage.

The current methods in classifying and grading Spitz tumors, even with the use of ancillary genetic tests such as fluorescence in situ hybridization and comparative genomic hybridization, are suboptimal in their ability to accurately predict the biologic potential of individual lesions. As a consequence, in particular lesions designated as atypical Spitz tumor likely represent a mixture of biologically benign, intermediate, and malignant tumors.

Spitz Nevus

Clinical Features

Spitz nevi occur most frequently in the first decade of life, and their incidence decreases with age, though they are reported in patients of all ages (Weedon and Little 1977; Paniago-Pereira et al. 1978; Herreid and Shapiro 1996). They typically appear as pink to red-brown dome-shaped papules that are smooth with sharply delimited edges, with a predilection for the lower extremities (Fig. 1). Typically less than 1 cm in size, Spitz nevi commonly have a short period of rapid growth over months before becoming stable in size (Requena et al. 2009).

Some Spitz nevi have a vascular appearance or a verrucous surface. The clinical differential diagnosis often includes dermatofibroma, hemangioma, viral wart, pseudolymphoma, xanthogranuloma, and keloid (Requena et al. 2009; Tlougan et al. 2013).

Spitz nevi are typically solitary but rarely multiple Spitz nevi may occur. Multiple Spitz nevi may be disseminated or grouped. Grouped or agminated Spitz nevi may occur in a segmental or dermatomal distribution within a background of hyperpigmented or hypopigmented skin. About a third of cases occur within an area of hyperpigmentation, such as a café-au-lait macule or nevus spilus.

Involution of Spitz nevi over time has been reported based on a survey of pediatric

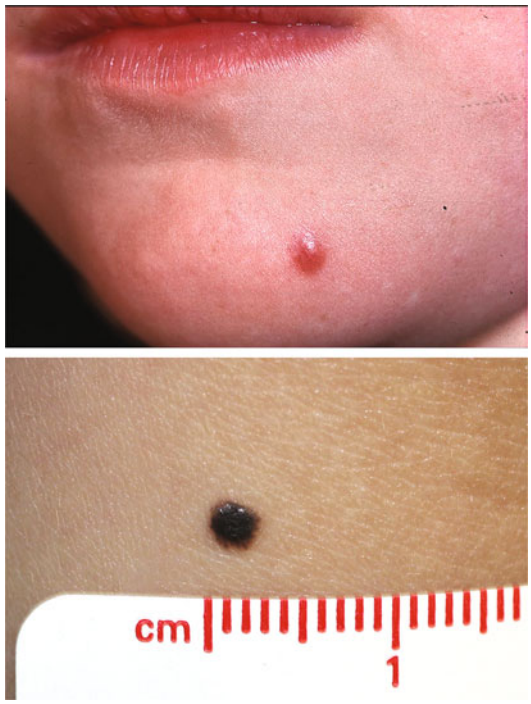

Fig. 1 Clinical appearance of Spitz nevi. Top: A pink brown papule on the chin (Courtesy of Dermatolgy Department University of Würzburg). Bottom: A darkly pigmented papule on the arm (Courtesy of Dr. Ilona Frieden)

dermatologists as well as a longitudinal clinical study following the dermoscopic features of suspected Spitz nevi in children (Argenziano et al. 2011; Tlougan et al. 2013).

Histopathologic Features and Subtypes

While the common defining feature of the Spitz nevus is its composition of large spindled and/or epithelioid melanocytes, there are additional histopathologic features that are characteristic. Thickening of the epidermis is typically seen in Spitz nevi if there are melanocytes at the dermo-epidermal junction, often with elongated rete ridges and expansion of the superficial layers of the epidermis (hypergranulosis and hyperkeratosis). Junctional melanocytes display clefts between melanocytes and between the melanocytes and surrounding keratinocytes due to a shrinkage artifact during tissue processing that

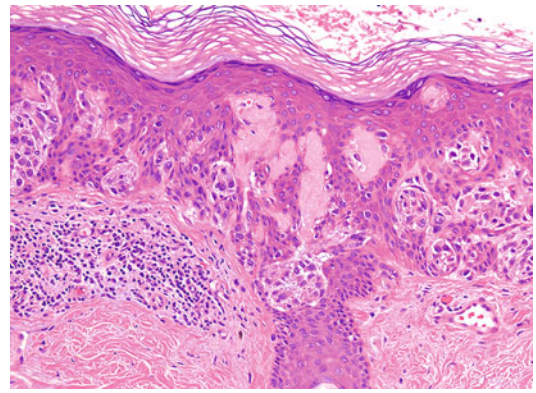

Fig. 2 Epidermal component of compound Spitz nevus illustrating epithelioid and spindled melanocytes, epidermal hyperplasia, clefting around melanocytes, and pale acellular eosinophilic material within the epidermis (Kamino bodies) (Courtesy of Dr. Philip LeBoit)

likely reflects decreased cell-cell adhesion. Kamino bodies are round to scalloped dull pink (by routine hematoxylin and eosin staining) homogenous aggregates that occur within the epidermis of Spitz tumors (Fig. 2). They are observed in over 60% of junctional and compound of Spitz nevi, but less frequently in intradermal nevi (Kamino et al. 1979). Kamino bodies are PAS-positive and diastase-resistant and ultrastructurally contain basement membrane material (Schmoeckel et al. 1990).

Within the dermis, the melanocytes of Spitz nevi demonstrate "maturation," typified by decreasing cell size, pigmentation, and size of dermal nests with increasing depth into the dermis. The architecture of the lesion is important, and a Spitz nevus should display sharp lateral demarcation and symmetry of the junctional component as well as even maturation across the lesion.

Spitz nevi often demonstrate mitotic activity within the dermis. Even deep mitoses near the bottom of the lesion may be present in Spitz nevi, in contrast to common acquired nevi in which dermal mitoses are rare and, when present, are typically confined to the superficial aspect of the tumor. The relatively frequent occurrence of mitoses in Spitz nevi suggests that many biopsies of Spitz nevi occur during the initial period of rapid growth.

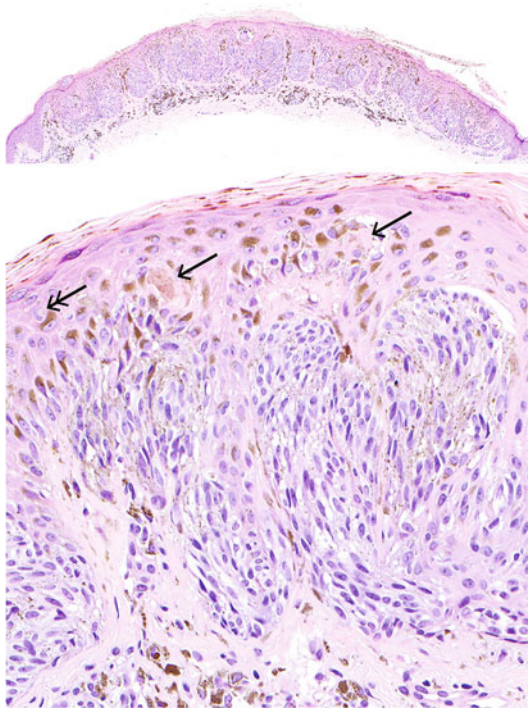

Fig. 3 Histopathology of pigmented spindle cell nevus. Top. Low power view demonstrates a symmetric, well-circumscribed junctional proliferation of melanocytes within epidermal hyperplasia. Bottom. High power view shows nests of pigmented and spindled melanocytes in the epidermis with Kamino bodies (single arrow) and scatter of single melanocytes into the upper epidermis (double arrow)

There are several histopathologic patterns of Spitz nevus, including junctional, compound, intradermal, and desmoplastic variants (Massi and LeBoit 2014). A distinctive variant is the pigmented spindle cell nevus (Reed nevus), which is typically junctional and contains heavily pigmented melanocytes that are predominantly spindled rather than epithelioid and smaller than those classically seen in Spitz nevus (Fig. 3).

Clinical Management

In children, the prevalence of Spitz nevus is much greater than the prevalence of melanoma with a dramatic inversion of this ratio in adults (Herreid and Shapiro 1996). As Spitz nevi commonly arise

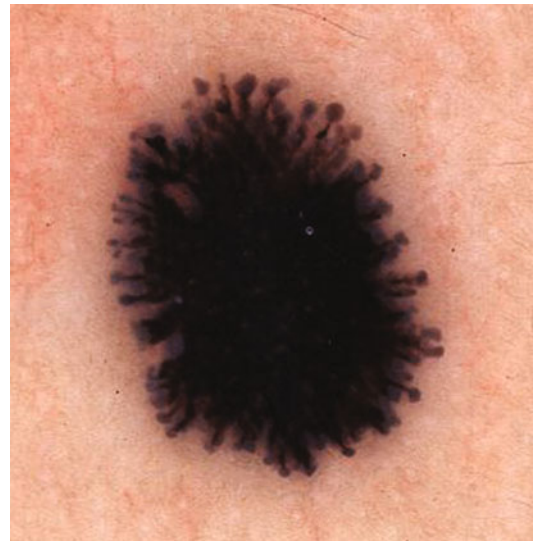

Fig. 4 Dermoscopy of a pigmented spindle cell nevus demonstrates a starburst pattern with radiating pseudopods at the periphery (Courtesy of Dermatolgy Department University of Würzburg)

on the face of children, conservative measures have been adopted, with some experts performing partial biopsies for diagnostic confirmation and other practitioners advocating clinical monitoring in diagnostically unequivocal cases (Tlougan et al. 2013).

Dermoscopy or epiluminescent microscopy allows for the assessment of colors and microstructures in the epidermis and superficial dermis and has been proposed as a method to aid in evaluation and monitoring of pigmented lesions. With dermoscopy, clinicians can identify specific structures and patterns of structures that are commonly observed in Spitz nevi and evaluate their distribution. The starburst pattern with radiating pseudopods at the periphery is characteristic of a variant of Spitz nevus also known as pigmented spindle cell nevus or Reed nevus (Steiner et al. 1992) (Fig. 4). Pigment globules and an inverse network is a common pattern in Spitz nevi, and nonpigmented Spitz nevi often have a symmetric pattern of dotted vessels (Pellacani et al. 2009). However, in some Spitz nevi, dermoscopic features specific to melanocytic tumors are not present (Fig. 5). Whereas dermoscopic monitoring of Spitz nevi

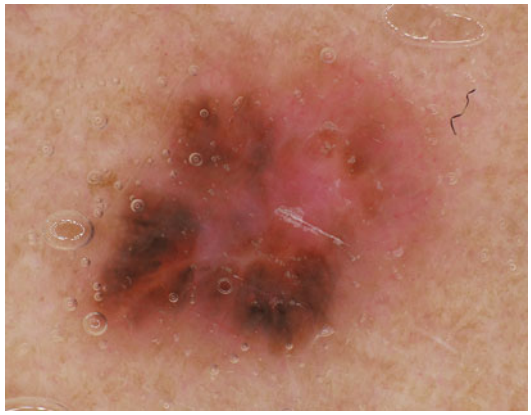

Fig. 5 Spitz nevus with features of dermatofibroma on dermoscopy (Courtesy of Dr. Giuseppe Argenziano)

is a generally accepted practice for children, a recent study demonstrated a greater than 10% risk of melanoma in symmetric lesions with dermoscopic features of Spitz nevus in patients over 12 years old (Lallas et al. 2015). In adults, excisional biopsy of suspected Spitz nevi is recommended, and many practitioners perform re-excision of incompletely excised Spitz nevi (Luo et al. 2011).

Atypical Spitz Tumors

Atypical Spitz tumor is a designation for tumors with Spitz nevus-like features that show changes that are incompatible with an unequivocally benign lesion such as increased proliferation rate, high cellularity, asymmetry and morphologically distinct tumor cell populations. Clinically and dermoscopically atypical Spitz tumors can resemble Spitz nevi (Fig. 6). Theoretically the category of atypical Spitz tumor would be for lesions that are biologically closer to melanoma than Spitz nevi, but have not yet reached the fully transformed state that would qualify them as melanomas. Practically, until better diagnostic methods have been developed, this intermediate category is a provisional "home" for unusual Spitz nevi, true biologically intermediate tumors, and unusual spitzoid melanomas (Barnhill et al. 1999; Cerroni et al. 2010b; Massi and LeBoit 2014).

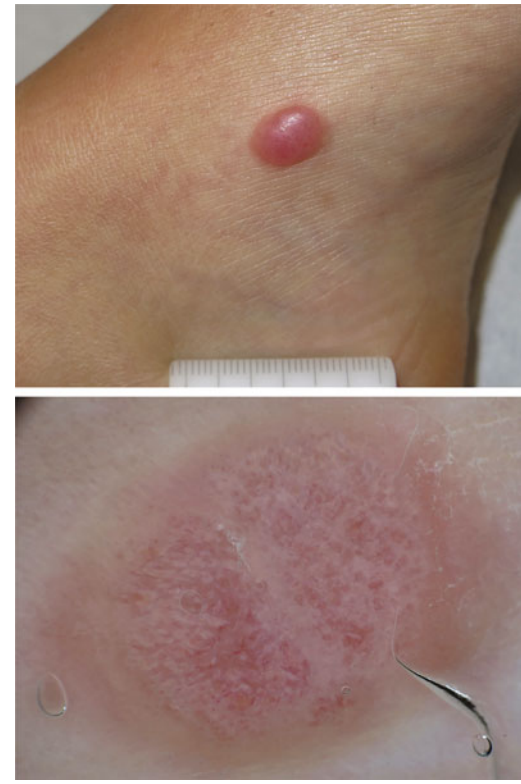

Fig. 6 Clinical and dermoscopic appearance of an atypical Spitz tumor. Top: A pink dome-shaped papule ~1 cm in greatest diameter. Bottom: By dermoscopy, there is an inverse pigment network as well as pigment globules (Courtesy of Dr. Giuseppe Argenziano)

Clinical Management

Reflecting the limitation of morphological classification of Spitz tumors, lesions classified as Spitz nevi and atypical Spitz tumors even by expert dermatopathologists have led to deaths in rare cases. A grading system based on histopathologic and clinical features was proposed for risk stratification of atypical Spitz tumors in children and adolescents (Spatz 1999) that considers age, clinical size, ulceration, depth of extension in the skin, and mitotic activity. However, this method also fell short of correctly identifying all lethal tumors in the initial cohort and awaits independent validation.

Some clinicians pursued sentinel lymph node biopsy as a diagnostic procedure for atypical Spitz tumors, under the assumption that any tumor deposit in the sentinel lymph node would provide

evidence that the primary tumor was biologically malignant (Su et al. 2003; Gamblin et al. 2006). A positive sentinel lymph node biopsy in this clinical setting was often followed by completion lymphadenectomy. Recent studies have demonstrated that lymph node metastases are found by sentinel lymph node biopsy in 39% of patients (Lohmann et al. 2002; Busam et al. 2009; Lallas et al. 2014). However, in contrast to cutaneous melanoma in which a positive sentinel lymph node purports a bad prognosis, the overwhelming majority of atypical Spitz tumors with positive sentinel lymph nodes do not progress to metastatic disease. Only one of 119 patients with atypical Spitz tumors who had a positive sentinel lymph node died of disease, after a mean follow-up of 59 months (Lallas et al. 2014). In contrast, less than 20% of patients with conventional melanoma have a positive sentinel lymph node, and those that do have a significantly increased risk for distant metastasis and death (Balch et al. 2009; Morton et al. 2014). Based on the indolent behavior of metastatic deposits in regional lymph nodes, completion lymphadenectomy for patients with atypical Spitz tumors and positive sentinel lymph nodes does not appear indicated.

The fact that regional metastases in atypical Spitz tumors are not a predictor of widespread metastasis and death appears counterintuitive. However, metastatic dissemination of benign melanocytic neoplasms is common. The presence of incidental benign melanocytic nevus cells (typically with the cellular morphology of common acquired, congenital, or blue nevi) in lymph nodes has been well-documented (Biddle et al. 2003). These aggregates typically reside within the lymph node capsule or trabeculae, suggesting they travel to the lymph node via lymphatic channels. This common finding implies that for melanocytic neoplasms, malignant transformation is not a requirement for migration of neoplastic cells to the lymph node. The frequency of sentinel lymph node positivity for Spitz nevus is not established, so that it is unclear whether their rate of sentinel lymph node involvement is lower or similar to that of atypical Spitz tumors.

Based on these considerations, sentinel lymph node biopsy is no longer recommended as a diagnostic or prognostic maneuver for patients with atypical Spitz tumors (Luo et al. 2011). The current management guidelines for atypical Spitz tumor call for complete excision. There is still uncertainty as to the diagnostic and prognostic significance of palpable regional lymphadenopathy and its clinical management.

Malignant Spitz Tumor

We use the term malignant Spitz tumor for melanomas with spitzoid morphology and the typical oncogenic alterations found in Spitz nevi and atypical Spitz tumor. Malignancy is defined either through the presence of frankly malignant histopathologic features or through their behavior with distant metastasis or death. This is a narrower definition than that of spitzoid melanoma, which is a less well-defined diagnostic entity based purely on cytomorphology. A recent study has shown that the majority of spitzoid melanomas defined solely on morphologic grounds have genetic alterations – BRAF V600E or NRAS mutations and a high mutation burden – similar to cutaneous melanomas arising on skin without chronic sun-induced damage (low-CSD melanomas) (Lazova et al. 2017) and thus are unrelated to Spitz tumors. The definition of malignant Spitz tumor is thus reserved for lesions that constitute the fully transformed stage of lesions that started as Spitz nevi or atypical Spitz tumor. Unfortunately, the term spitzoid melanoma has been used inconsistently and also inappropriately for atypical Spitz tumors that based on a positive sentinel lymph node biopsy have been upstaged as melanoma. These cases would no longer be considered as malignant Spitz tumors given the current understanding of the significance of positive sentinel lymph node status in atypical Spitz tumors. Due to the diagnostic difficulties in separating Spitz tumors into unequivocally benign or malignant forms, the diagnosis of malignancy is often made in retrospect, once distant metastases have been identified. Studies to date of spitzoid melanoma do not categorize tumors on the basis of their initiating oncogene, and the prevalence of BRAF and NRAS activating point mutations

varies widely, ranging from 3–64% to 3–19%, respectively (Palmedo et al. 2004; van Dijk et al. 2005; Fullen et al. 2006; Lee et al. 2006; Da Forno et al. 2009).

In the authors' clinical practice, the term "spitzoid melanoma, childhood type" is used for spitzoid tumors with striking nuclear pleomorphism and deep mitoses, but the term malignant Spitz tumor better captures their unique features and avoids confounding with conventional melanomas. In the authors' experience, these types of tumors have a better outcome than conventional melanomas of similar stage but this remains to be further studied.

Clinical Features

Malignant Spitz tumors typically lack the defining clinical features of cutaneous melanoma such as uneven pigmentation with asymmetry, irregular borders. They usually present as rapidly growing nodules and are not reliably distinguishable from Spitz nevi or atypical Spitz tumors on a clinical basis.

Histopathologic Features

Malignant Spitz tumors are comprised of large epithelioid and/or spindled melanocytes similar as Spitz nevi and atypical Spitz tumors, but often show additional features of malignancy such as cells arranged in large cohesive sheets, an increased proliferation rate, marked pleomorphism, and ulceration. Frequently malignant Spitz tumors do not display distinguishing characteristics of Spitz nevi such as Kamino bodies and epidermal hyperplasia.

Clinical Management

Spitzoid melanoma is currently not a subtype of melanoma listed in the WHO classification of Skin Tumors (2005) and is not included as a listed subtype in the AJCC staging protocol for primary cutaneous melanoma. The term malignant Spitz tumor will be included in the updated version of the WHO classification. Due to the limitations of histopathology to define malignant Spitz tumors morphologically in the absence of outcome, information distinguishing it from atypical Spitz tumor data about its clinical behavior and rationally based guidelines for clinical management is highly limited. Studies comparing conventional and spitzoid melanoma in children identified a trend toward better outcomes in spitzoid melanoma (Pol-Rodriquez et al. 2007; Paradela et al. 2013); however, this could be due to limited diagnostic ability to distinguish atypical Spitz tumors from spitzoid melanomas.

Genetics

Initiating Genetic Alterations in Spitz Nevi

Concordant with their distinctive histopathological features Spitz nevi have a nonoverlapping spectrum of oncogenic alterations with other nevi. They very rarely harbor BRAF or NRAS mutations (Yazdi et al. 2003; Palmedo et al. 2004; Gill et al. 2004a, b; van Dijk et al. 2005; Da Forno et al. 2009), which are the most common oncogenic alterations in cutaneous melanoma, in which they are considered initiating events that are sufficient for the formation of benign nevi but require additional subsequent mutations for transformation to melanoma. Instead, Spitz tumors harbor mutations in HRAS or rearrangements that activate receptor tyrosine (ALK, ROS1, MET, NTRK1, NTRK3, RET) or serine/threonine (BRAF) kinases through fusion of their respective kinase domain to the N-terminal portion of another gene product. In keeping with the findings of the genetic progression in other types of melanocytic neoplasms Spitz tumors that carry only a single alteration, typically one of the activated oncogenes listed above, would be considered benign. By contrast the presence of additional genetic alterations would indicate progression to either an intermediate state between benign and malignant or a fully transformed and therefore malignant tumor. Data to date indicates that the secondary and tertiary alterations in Spitz tumors are quite similar to those in other types of

melanocytic neoplasms and include loss of the tumor suppressor genes CDKN2A and mutation of the TERT promoter. In most instances, the spitzoid phenotype is a result of the initiating oncogenic alteration. However, there are exceptions in which spitzoid phenotype is a result of additional genetic alterations. Bi-allelic loss of BAP1 in a common acquired nevus initiated by BRAF V600E or sometimes NRAS mutation results in a change in cytomorphology that leads to enlargement of the small melanocytes of the nevus that is reminiscent of a Spitz tumor. As these lesions represent a special progression trajectory of common acquired nevi, the authors use the term BAP1-inactivated spitzoid nevi proposed by Vilian and colleagues and do not consider them as bona fide Spitz tumors (Vilain et al. 2015). The authors propose a similar distinction for malignant tumors and use the term malignant Spitz tumor for malignant tumors that originated from a bona fide Spitz nevus or atypical Spitz tumor, as determined by histology and characteristic genetic alterations and the term spitzoid melanoma for melanomas with some histopathological features of Spitz tumors but with different genetic alterations such as BRAF or NRAS point mutations (Lazova et al. 2017).

HRAS

Approximately 20% of Spitz nevi harbor oncogenic mutations of HRAS, often accompanied by copy-number increases of the entire short arm of the chromosome 11 harboring the mutant HRAS allele (Bastian et al. 2000). The mutations disrupt the intrinsic GTPase activity of HRAS, resulting in constitutively active HRAS signaling through the MAP kinase and PI3 (phosphoinositide3) kinase pathways. Exon 3 mutations at position Q61 of HRAS appear to be more common than mutations in exon 2 at positions G12 or G13, possibly indicating a different effect of these ras mutations as has been found for other ras family members in other cancers. NRAS mutations in melanoma and nevi also occur more frequently at position Q61 (Burd et al. 2014).

While not all HRAS mutated Spitz nevi demonstrate copy number gain of HRAS, a recent case report of agminated Spitz nevi arising within nevus spilus found an HRAS G13R mutation in the light brown macular portion of the nevus spilus and copy number increase of HRAS G13R in the multiple papular Spitz nevi within, suggesting that increased gene dosage of mutant HRAS may be required for Spitz nevus initiation (Sarin et al. 2013). HRAS mutant Spitz nevi are distinct from other Spitz nevi as they are predominantly intradermal lesions that are wider than they are deep in which the dermal component is comprised of nests and cords of melanocytes that are interspersed between thickened collagen bundles (Fig. 7) (Bastian et al. 2000; van Engen-van Grunsven et al. 2010).

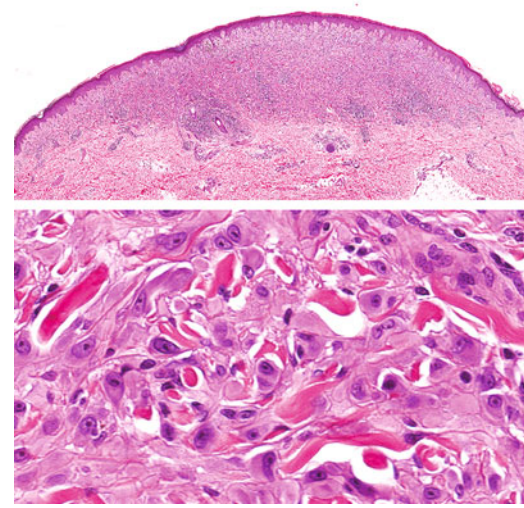

Fig. 7 Spitz nevus with HRAS mutation. Top: Low power view shows a predominantly intradermal melanocytic tumor that is more broad than deep, with a flat lower border. Bottom: High power view shows epithelioid and fusiform melanocytes intercalated between sclerotic collagen bundles

Kinase Fusions

Fusions of the serine/threonine kinase BRAF and the receptor tyrosine kinases (RTKs) ALK, MET, NTRK1, NTRK3, RET, and ROS1 are other initiating oncogenic alterations in Spitz nevi (Botton et al. 2013; Wiesner et al. 2014; Yeh et al. 2015a, 2016). Fusion kinases result from genomic

rearrangements that fuse the intact kinase domain in frame to a 5′ fusion partner. The resulting fusion kinase lacks the N-terminal autoinhibitory domain of the native kinase and is therefore constitutively active. The spectrum of 5′ fusion partners in Spitz tumors is quite broad and includes genes partaking in similar rearrangements in other cancers such as lung, colon, hematologic, and thyroid malignancies and several novel fusion partners. Most of the 5′ fusion partners contribute coiled-coil domains to the fusion kinase, likely promoting dimerization and kinase activity. While fusions involving the BRAF kinase domain are thought to only activate the MAP kinase pathway, fusions of RTKs activate pathways in addition to the MAP kinase pathway such as the PI3 kinase, STAT (signal transducer and activator of transcription), and phospholipase C pathways (Wiesner et al. 2014).

Some of the RTKs rearranged in Spitz nevi normally are expressed only during neural crest development, and are silenced in adult tissues. As the expression of the fusion genes encoding the fusion kinases are regulated by the promoter of the 5′ fusion partner, the rearrangements lead simultaneously to dysregulated expression and kinase activation, which provides a compelling explanation as to why rearrangements rather than point mutations are the predominant mode of activation of these genes in melanocytic neoplasms. Expression of the kinase domain of ALK, MET, NTRK1, and ROS1 above the usual expression level in melanocytes can be used as a marker for the presence of a kinase fusion in Spitz tumors. Kinase domain expression is a particularly useful indicator of aberrant RTK expression when the RTK is not expressed in mature melanocytes, as is the case for ALK and ROS1.

Neoplastic diseases that are initiated by structural rearrangements resulting in gene fusions are generally more common in children (Jones et al. 2008; Takeuchi et al. 2012; Del Castillo et al. 2015) and have been linked to the high proliferation rate of cells during organismal development. The increased incidence of Spitz nevi in childhood may thus also be related to an increased rate of structural rearrangement due to expansion of the melanocyte population during childhood.

Spitz nevi with ALK fusions typically are compound lesions with a wedge-shaped silhouette, in which large vertically oriented nests of fusiform melanocytes "rain down" from the superficial skin towards the apex of the wedge in the deep dermis. Single melanocytes or small clusters of them are often interspersed at a distance at the periphery of Spitz nevi with ALK fusions, suggesting that ALK signaling may promote melanocyte migration (Busam et al. 2014; Yeh et al. 2015b). This fascicular growth pattern was more frequently observed in Spitz nevi with ALK as compared to NTRK1 fusions (Kiuru et al. 2016), indicating that different fusion kinases may result in different histopathologic features.

ALK fusions appear to be sufficient to induce the morphologic features described above without the need for additional genetic alterations to occur. The high cellularity, rapid growth, and presence of mitoses would qualify many of them as atypical Spitz tumors on morphological grounds (Fig. 8), although their genetics may not constitute an intermediate state.

Secondary Genetic Alterations

CDKN2A and TERT

Thus far, oncogenic progression events identified in Spitz tumors include homozygous deletion of CDKN2A and TERT promoter hotspot mutations. p16, one of the two proteins encoded by CDKN2A, is a critical component of the G1/S cell cycle checkpoint and loss of p16 contributes to unchecked proliferation of melanocytes (Sviderskaya et al. 2003). Homozygous deletion of 9p21 which includes the CKDN2A locus is present in approximately 20% of atypical Spitz tumors and may be more common in tumors that develop distant metastases (Gerami et al. 2013; Lee et al. 2015). Hotspot mutations of the TERT promoter were identified in a small fraction of Spitz tumors and claimed to be predictive of lethal disease (Lee et al. 2015). However, only one of the four cases with TERT promoter mutations had genetic alterations typical of Spitz tumors (a BRAF fusion) so that it is not clear whether these cases were spitzoid melanomas, i.e.,

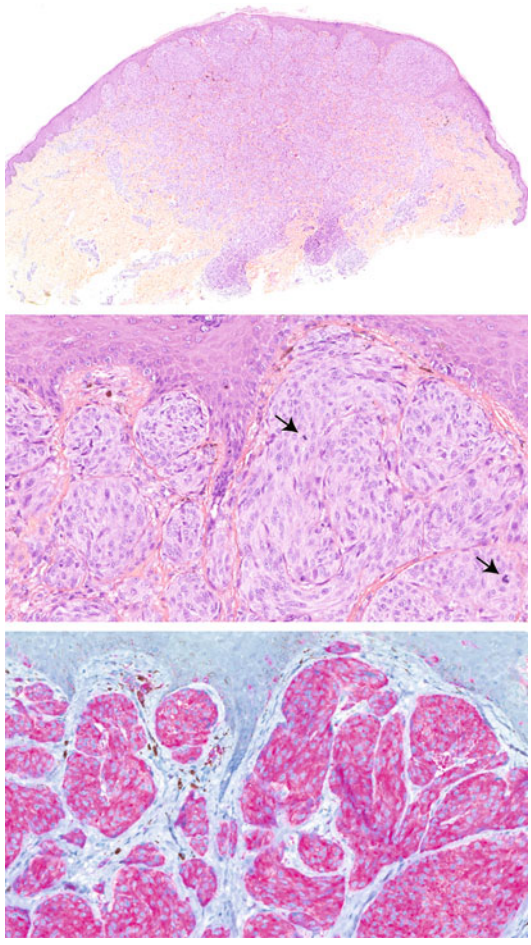

Fig. 8 Atypical Spitz tumor with ALK fusion. AST from the elbow of a 5-year-old girl. Top: Low power view shows a predominantly intradermal tumor extending into the subcutis with a wedge-shaped profile. Middle: Medium power view shows large elongated nests of melanocytes in radial orientation, pointing towards the apex of the wedge. Multiple mitotic figures are present (arrows). Bottom: The melanocytes express the kinase domain of ALK by immunohistochemistry (Courtesy of Dr. Arnaud de la Fouchardière)

melanomas with some morphological features of Spitz tumors, or bona fide malignant Spitz tumors. With these limitations, the findings indicate that disruption of the G1/S cell cycle checkpoint by loss of p16 and avoidance of critical telomere shortening though TERT promoter mutation and increased TERT expression are genetic progression events in Spitz tumors similar to most other forms of melanoma.

BAP1

A subset of tumors often categorized as atypical Spitz tumors acquire their spitzoid features not through the initiating oncogene itself but through a genetic alteration that occurs after the initiating oncogene led to the initial formation of a neoplasia. These tumors harbor BRAF or NRAS mutations as initiating events, and often are hybrid lesions in which a common acquired nevus can be recognized as a precursor lesion from which a component with spitzoid morphology arose. The spitzoid component of these lesions is characterized by biallelic loss of BAP1 (Fig. 9). While these neoplasms have epithelioid melanocytes similar to bona fide Spitz tumors, distinguishing features include epidermal thinning – as opposed to the epidermal hyperplasia present in Spitz nevi – often with an attenuated rete ridge pattern, and the frequent presence of a residual portion of common acquired nevus which represents a precursor lesion. The epithelioid melanocytes frequently display variable cell size, marked nuclear pleomorphism, amphophilic cytoplasm, well-defined cellular membranes, and eccentrically placed nuclei. Vilain and colleagues propose the term BAP1-inactivated spitzoid nevi for these tumors, a term which captures the notion that they share features with Spitz tumors, but represent a distinct clinical and diagnostic entity.

BAP1-inactivated spitzoid nevi are usually solitary lesions, but have important clinical implications when they occur in multiplicity. When present in the germline, BAP1 loss of function mutations are associated with an autosomal dominant syndrome characterized by multiple unpigmented papular melanocytic tumors that occasionally arise within a pigmented nevus. Individuals with germline mutations in BAP1 have a cancer predisposition syndrome, with increased incidence of uveal melanoma, mesothelioma, clear-cell renal cell carcinoma, and other cancers that is sometimes accompanied by multiple BAP1-inactivated spitzoid nevi (Testa et al. 2011; Abdel-Rahman et al. 2011; Farley et al. 2013).

BAP1-inactivated spitzoid nevi seem to develop more slowly and have fewer mitoses that are limited to the superficial aspect of the

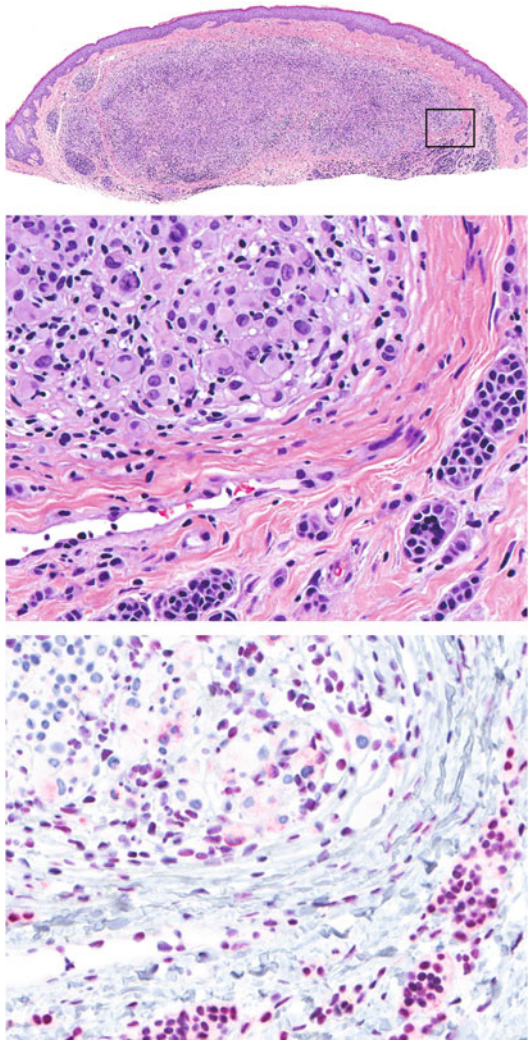

Fig. 9 BAP1-inactivated spitzoid nevus. Top panel: Low power demonstrates a predominantly intradermal proliferation of melanocytes with smaller melanocytes present at the periphery of the central dermal nodule. Middle panel: High power view of marked region from top panel shows that the central dermal nodule is composed of epithelioid melanocytes with enlarged nuclei and abundant cytoplasm. There are nests of small round melanocytes at the periphery. Bottom panel: BAP1 immunohistochemistry demonstrates nuclear staining in the small round melanocytes but loss in the epithelioid melanocytes. Admixed inflammatory cells retain nuclear BAP1

tumor as compared to Spitz nevi (Wiesner et al. 2012; Busam et al. 2013). Their biologic behavior is not well studied, with fewer than 10 sporadic cases in the literature and limited follow-up information. However, it is noteworthy that the one lethal atypical Spitz tumor classified as low-risk by the grading system of Spatz and colleagues had histopathologic characteristics of a BAP1-inactivated spitzoid tumor. The colloquial term BAPoma has been used for these lesions and they are considered as benign by most clinicians. However, they are genetically more advanced than conventional nevi as the sporadic versions have acquired at least the two additional genetic alterations necessary to disrupt both BAP1 alleles, and thus may have an increased risk for progression to melanoma. That risk may still be very small, but it seems appropriate to consider them as biologically intermediate neoplasms.

DNA Copy Number Changes

Cutaneous melanoma often demonstrates multiple copy number alterations and specific regions of the genome are recurrently gained or lost in melanoma indicating that these losses and gains are biologically significant (Curtin et al. 2005).

A limited number of studies have examined the copy number profile of Spitz tumors, in particular of "spitzoid melanoma." In contrast to common acquired nevi, which typically do not harbor copy number aberrations, Spitz nevi often harbor copy number alterations that increase the gene dosage of the initiating oncogene. Gains of chromosome 11p are frequent in Spitz nevi with HRAS mutation and affect the chromosomal arm that harbors the mutant HRAS allele (Bastian et al. 1999, 2000). Rearrangements such as deletions and tandem duplications that produce fusion genes often also result in copy number changes. Examples include deletion of a small 0.7 Mb region of chromosome 1q between LMNA and NTRK1 producing the LMNA-NTRK1 fusion; a deletion of 44 Mb fragment of chromosome 2p between DCTN1 and ALK producing the DCTN1-ALK fusion; and tandem duplication of the region on chromosome 15q between MYO5A and NTRK3 producing the MYO5A-NTRK3 fusion.

In other cases, the fusion-generating rearrangement appears to occur due to a complex genomic rearrangement suggestive of chromothripsis with multiple discrete regions of a single chromosome

affected by copy number changes of similar magnitude.

The authors have observed many cases of Spitz tumors in which copy number alterations increased the gene dosage of the fusion kinase gene. The patterns of copy number changes are quite diverse, as individual kinase genes can fuse with a range of 5′ partners. In one Spitz tumor an ALK fusion arose through the formation of a circular acentromeric DNA fragment (double minute), which was subsequently amplified to increase the gene dosage of the ALK fusion gene (Yeh et al. 2015b). The dosage of a given fusion kinase that is selected during tumor evolution is in part dependent on the expression level of the 5′ partner gene, and a more weakly expressed fusion kinase may require additional copy number changes to ramp up effective expression levels. It is currently unclear whether additional genetic alterations that target the initiating oncogene have any prognostic relevance. A Spitz tumor driven by a rearrangement of a kinase in which the fusion kinase gene was subsequently amplified may still have the same biological features as a Spitz tumor in which the fusion kinase gene was not amplified. However, it is also conceivable that such subsequent copy number changes increase the chance of events that would lead to additional genetic alterations that would advance the progression of the neoplasm. Further work remains to determine the diagnostic and prognostic significance of copy number alterations in Spitz tumors, and copy number alterations will most likely need to be evaluated in the context of structural variations and point mutations.

Targeted Therapy

Treatment of $BRAF^{V600E}$ mutant melanomas with BRAF inhibitors has clinical efficacy and there is promise of synergistic activity with immunotherapy regimens. As malignant Spitz tumors by definition do not harbor BRAF or NRAS mutations but instead a different subset of initiating oncogenes, the authors will briefly review potential targeted therapies of Spitz initiating oncogenes.

HRAS is the only ras isoform whose localization to the plasma membrane can be blocked by farnesyltransferase inhibitors (Chen et al. 2014). Currently farnesyltransferase inhibitors are in development for other indications, including AML and remain to be investigated for HRAS mutant melanoma.

Small molecule inhibition of BRAF fusions may be efficacious in melanoma. Notably, the BRAF fusions contain the wild-type kinase domain of BRAF and in vitro studies indicate that melanoma cell lines with BRAF fusions are relatively insensitive to vemurafenib, a class I inhibitor active against the activated form of BRAF, but sensitive to sorafenib, a class II inhibitor active against the resting conformation of BRAF (Botton et al. 2013; Botton T, unpublished 2017). There are a few case reports of patients with BRAF fusions responding to class II BRAF inhibitors or MEK inhibitors (Menzies et al. 2015).

Inhibitors of ALK and ROS1 are FDA approved for use in ALK and ROS1 rearranged nonsquamous cell lung cancer and in some cases patients with metastatic disease had complete responses to therapy with these drugs. Inhibitors of RET, NTRK1, and NTRK3 have also been reported to result in dramatic responses in patients with solid tumors harboring RET, NTRK1, or NTRK3 fusions. These agents have yet to be tested in melanomas with the respective fusions.

Conclusion

Recent studies have identified a diverse spectrum of initiating oncogenes in Spitz tumors. The majority of these initiating oncogenes are fusion kinases, which arise from structural rearrangements. Perhaps this mutational mechanism contributes in part to the propensity of Spitz tumors to occur in children. Early studies indicate that tumors driven by rearrangement of different kinases have different histopathologic appearances, likely accounting for the diagnostic difficulty of this class of tumors. The authors propose that the spectrum of Spitz tumors from benign to malignant be referred to as Spitz nevi, atypical Spitz tumors, and malignant Spitz tumor and

refer only to tumors initiated by Spitz oncogenes (HRAS, BRAF fusions, RTK fusions). Notably, some tumors previously classified as Spitz tumors do not fall in this category, namely BAP1-inactivated spitzoid nevi.

With a better understanding of the initiating genetic events in Spitz nevi, further refinement of diagnostic criteria for benign and malignant tumors is needed, based on both histopathologic and genetic features. Future studies with clinical follow-up information and genetic analysis are needed.

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Acral Melanoma

19

Melanoma Affecting Volar Skin and Nail Apparatus

Toshiaki Saida

Contents

Abstract

Acral melanoma is defined as melanoma affecting the palms, soles, and nail apparatus. This is not a synonym of acral lentiginous melanoma in Clark's classification, which is defined histopathologically. Acral melanoma is characterized by peculiar chromosomal and genetic alterations distinct from other subtypes of melanoma. These characteristics certainly reflect its unique pathogenesis, in which sun exposure is not a major causative factor. In this chapter, genetic characteristics of acral melanoma were first summarized and its molecular pathogenesis was discussed. Amplification of *CCND1* and *TERT* may be early events in the development of acral melanoma. Mutations of *BRAF/NRAS* may be also involved in the early developmental phase of acral melanoma along with *KIT* mutations/amplification, which can be utilized as targets by small molecular inhibitors for the treatment of advanced

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acral melanoma. Next, essential points in the clinical,dermoscopic, and histopathologic diagnoses of acral melanoma were described. Particularly, dermoscopy is very helpful in diagnosing primary lesions of acral melanoma by the parallel ridge pattern, which contrasts with the parallel furrow pattern found in the vast majority of acral nevi. These different pigmentation patterns suggest de novo genesis of acral melanoma, namely, melanoma and nevus arise independently in this anatomical site. Finally, management of acral melanoma is discussed, including some suggestions in the surgical treatment and recently introduced molecular targeting therapies.

Keywords

Acral melanoma · Acral lentiginous melanoma · Molecular pathogenesis · Clinical diagnosis · Dermoscopic diagnosis · Histopathologic diagnosis · Management and prognosis

Definition and Epidemiology of Acral Melanoma

Clark's histopathologic classification of melanoma was first described in 1969, in which the following three subtypes were proposed: the superficial spreading melanoma (SSM), lentigo maligna melanoma (LMM), and nodular melanoma (NM). Thereafter, Reed (1976) introduced the concept of acral lentiginous melanoma (ALM). Already in 1974, however, Seiji, a Japanese dermatologist renowned for his melanosome studies, reported that plantar and subungual melanomas often showed a distinctive adjacent intraepidermal proliferation of atypical melanocytes (Seiji and Takahashi 1974). ALM was soon later incorporated into Clark's classification.

ALM is defined by characteristic histopathologic features, namely, proliferation of atypical melanocytes in a lentiginous pattern along the basal layer of the peripheral epidermis, which usually shows moderate acanthosis and rete ridge elongation (Fig. 1). ALM is seen on acral volar skin (glabrous/non-hair-bearing skin) but can be seen on other anatomical sites such as on the dorsum of the foot. Furthermore, other subtypes, such as SSM and NM, can occur on the acral skin. In 2005, Bastian's group proposed a new classification system of melanomas based on their data of chromosomal and genetic investigation (Curtin et al. 2005). They identified the following four types: (1) melanoma on non-chronic sun-damaged skin (non-CSD melanoma), (2) melanoma on chronic sun-damaged skin (CSD melanoma), (3) acral melanoma (Fig. 1), and (4) mucosal melanoma. The non-CSD melanoma mostly corresponds to SSM, the CSD melanoma to LMM, and acral melanoma to ALM. But in a strict sense, acral melanoma is not identical to ALM. Acral melanoma was defined as melanoma affecting the palms, soles, or nail apparatus, irrespective of histopathologic features. Of note, according to the supplemental data in the paper by Bastian's group, 5 of 36 acral melanomas were SSM in Clark's subtypes. The study by Bastian's group has revealed that acral melanoma is genetically characterized by rare mutations of *BRAF* and *NRAS* and by chromosomal gains on 6p, 7, 8q, 17q, and 20q and loss on 6q, 9p, 10, and 21q. Acral melanoma is particularly unique in that gene amplifications are frequently detected throughout the genome, including *CCND1* (11q13), *TERT* (5p15), *RICTOR* (5p13), *KIT* (4q12), and *CDK4* (12q14) (Curtin et al. 2005). In this chapter, the author adopts the terms and concept defined by Bastian's group but uses the terms of Clark's classification when they were used in the past literatures. In the genetic classification put forth in The Cancer Genome Atlas Network, acral melanoma mainly falls into the category of "triple wild-type" melanomas (2015).

Incidence and subtypes of malignant melanoma are substantially different among races. While non-CSD melanoma is the most prevalent type in White populations, acral melanoma is a predominant subtype in other world populations. The proportion of acral melanoma/ALM in all cutaneous melanomas is highly variable among races, accounting for approximately 5% in White people, over 80% in Black people, and around

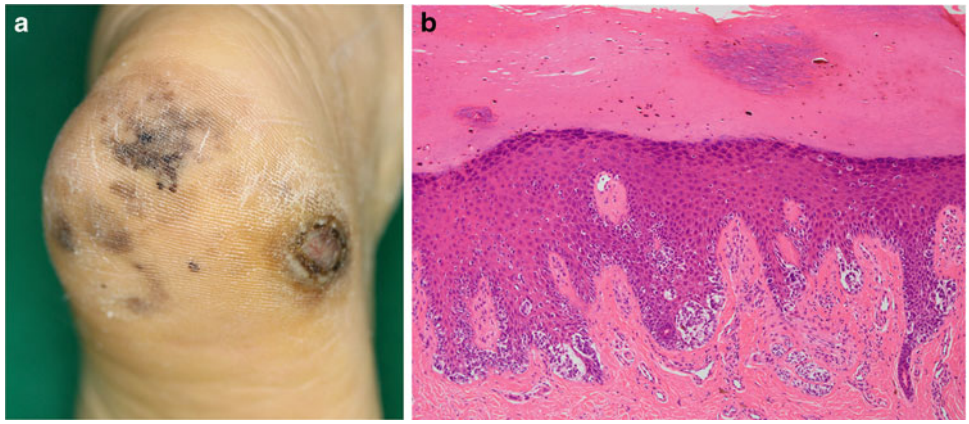

Fig. 1 Typical clinical and histopathologic features of acral melanoma. Clinically, this lesion on the heel exhibits an ulcerated nodule, accompanying ill-defined brown macules with variable shades, interposed by seemingly nonpigmented areas (**a**). Macular portions histopathologically show proliferation of atypical melanocytes as solitary units and in nests of variable size within the epidermis (**b**). Upward migration of atypical melanocytes is detected in the hyperplastic epidermis with some elongation of the rete ridges

50% in Asian people. Given that acral volar skin (palms and soles) occupies only about 5% of the total body surface area, the abovementioned site predilection of melanoma in dark-skinned peoples is surprising and indicates that the transformation risk of melanocytes in acral volar skin is elevated compared to melanocytes of non-glabrous skin. Moreover, the absolute incidence of acral melanoma was reported to be similar among all races (Stevens et al. 1990), and the very low relative proportion of acral melanoma in White populations is mainly due to increased incidence of the non-CSD melanoma in Whites. The major causative factor of the non-CSD melanoma is suspected to be sun exposure, particularly intermittent intense sun exposure. The fair skin of White people contains little melanin with a decreased eumelanin to pheomelanin ratio, resulting in reduced UV shielding. Thereby, melanocytes situated in the epidermal basal layer of fair skin are vulnerable to sun damage, and thus DNA of melanocytes is easily damaged by ultraviolet radiation. These may be the major reasons for the higher incidence of non-CSD melanomas in White people. In contrast, sun exposure is not a major causative factor of acral melanoma. This is reflected in a significantly lower somatic mutation burden in acral melanomas compared to non-CSD melanoma or CDS-melanoma (Turajlic et al. 2012; Hayward et al. 2017). Acral melanoma has distinctive chromosomal and genetic aberrations, the details of which are discussed in the following section.

Pathogenesis and Genetic Alterations of Acral Melanoma

Pathogenesis of Acral Melanoma

The age distribution of patients with acral melanoma peaks in the seventh decade, irrespective of races. The most prevalent site is the sole. In Japanese, plantar melanomas account for approximately 30% of all cutaneous melanomas. Plantar melanoma is about ten times more prevalent than palmar melanoma. In a study of a total of 177 acral melanomas in Koreans, the most prevalent subsites of acral melanoma were physically stressed sites, such as the center of the heels and inner forefoot (Jung et al. 2013). Among melanomas of the nail apparatus, the finger nails are more frequently affected than toe nails, with the thumbnail being the most frequently affected site. These data suggest that chronic mechanical pressure and/or repeated minor trauma could be a causative factor of acral melanoma (Saida 2007). In addition, the low melanin content in the epidermis of

these anatomical sites could contribute to transformation of melanocytes because of decreased antioxidant effects.

Genetic Alterations of Acral Melanoma

While initial studies have analyzed the genetic evolution of non-acral cutaneous melanomas from pre-neoplastic lesions (Shain et al. 2015), the genetic evolution of acral melanoma remains to be explored. Here, the author summarizes characteristic genetic and molecular alterations of acral melanoma described in the literatures to date.

Summing up the genetic findings described below, the author proposes a molecular/genetic model of development and progression of acral melanoma as illustrated in Fig. 2, though it is very preliminary.

CDKN2A

It is well known that *CDKN2A* located at 9p21 is an important gene responsible for melanomagenesis. Germline mutation of this gene was reported in families prone to develop non-CSD melanoma. *CDKN2A* deletion seems related to the invasive stage of melanoma. In the recent study by Bastian's group, *CDKN2A* aberrations were detected in invasive melanoma in most cases (Shain et al. 2015). In SMYM-PRGP, an acral melanoma cell line we established from the radial growth phase of plantar melanoma, neither mutation nor copy number loss of *CDKN2A* was detected (Murata et al. 2007), which suggests, also in acral melanoma, *CDKN2A* deletion may be a later occurring event.

CCND1

Acral melanoma is characterized by focal amplifications of various chromosomal loci. Particularly, amplification of 11q13, where cyclin D1 gene/*CCND1* is located, is frequently detected (Curtin et al. 2005). *CCND1* positively regulates the activity of *CDKs*, leading to phosphorylation of Rb and promoting entry into mitosis. Amplification of *CCND1* is detected in about 45% of acral melanoma (Sauter et al. 2002). More recent study showed that *CCND1* amplifications were found in 10 of 14 (71%) invasive and 4 out of 5 (80%) in situ lesions of acral melanoma (North et al. 2008). An immunohistochemical study revealed *CCND1* was expressed in 68% of acral melanoma.

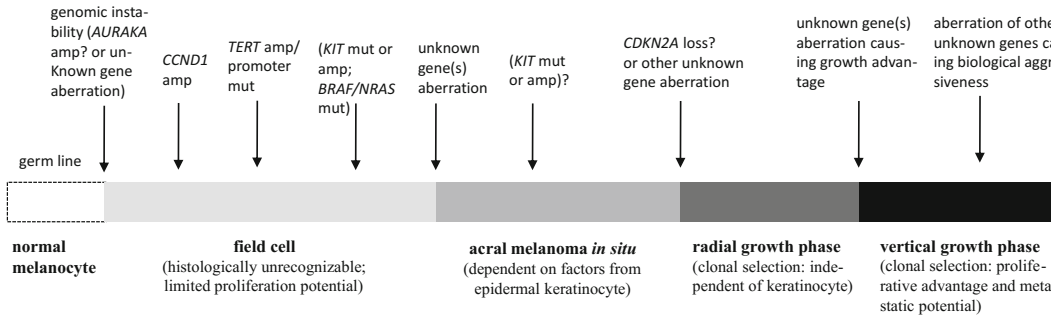

Fig. 2 Genetic developmental model of acral melanoma: preliminary hypothesis. Most acral melanomas arises de novo, not in association with a preceding melanocytic nevus. The first event may be transformation of epidermal melanocytes though the initial driver gene(s), probably related to genomic instability, is not yet identified. Thereafter, the melanocytes acquire amplification (amp) of *CCND1* and *TERT* and spread along the basal layer of the epidermis as the "field cells." *KIT* mutations (mut) or amplification and *BRAF*/*NRAS* mutations may be also involved in the early developmental phase. With some unknown gene(s) aberration, the field cells become morphologically atypical melanocytes, and a histopathologically diagnosable lesion of acral melanoma in situ appears. In this phase, the melanocytes are dependent on some factor(s) from keratinocytes, preventing them to invade the dermis. Loss of *CDKNA2* and/or aberration of other unknown gene(s) may contribute to transition from the in situ phase to the radial growth phase, making the cells independent of epidermal keratinocytes. In the following vertical growth phase, cell clones with a proliferative advantage and metastatic potential appear within the lesion. Other various genetic aberrations are responsible for the later progression phase

Although, in cancers of other organs, gene amplifications are generally found in later progression stages, *CCND1* amplifications in acral melanoma are detected in earlier developmental stages including acral melanoma in situ (Bastian et al. 2000). Furthermore, copy number increase of *CCND1* was detected in non-atypical melanocytes located in the peripheral epidermis beyond the histopathologically recognizable margin of acral melanoma (North et al. 2008). Bastian (2003) called these genetically aberrant cells with normal morphology "field cells" and proposed that they represent an early progression phase preceding the histopathologically apparent stages of melanoma in situ. Such field cells in acral melanoma occasionally extend far beyond the histopathological margin, and the extent does not correlate with tumor depth or diameter of the lesion (North et al. 2008). The concept of field cells is important in the diagnosis and treatment of acral melanoma. Amplification of *CCND1* was detected in an early acral melanoma cell line, SMYM-PRGP (Murata et al. 2007). These data strongly suggest the *CCND1* amplification is one of the earliest events in the development of acral melanoma and identify *CCND1* as a possibly driver gene relevant to the early evolving phase of acral melanoma.

TERT and AURKA

Activation of telomerase by the upregulation of human telomerase reverse transcriptase gene (*TERT*), located at chromosome 5p15.33, can immortalize somatic cells through extension of telomeres. *TERT* may play an important role in oncogenesis of acral melanoma. It was reported that *TERT* amplifications were detected in 7 of 14 (50%) invasive and 4 out of 5 (80%) in situ lesions of acral melanoma (North et al. 2008). Another study showed that gains of *TERT* were detected in 31.2% of 17 primary ALM lesions (Puig-Butillé et al. 2013). *TERT* promoter mutation was reported to be uncommon in ALM (6%, 2/32), while the mutation was detected in 33% (3/9) in non-acral melanomas (Liau et al. 2014). In a larger cohort of non-acral melanomas, *TERT* promoter mutations were detected in a total of 77% of areas of in situ or intermediate lesions (Shain et al. 2015). These data suggest that the *TERT* promoter mutations are caused mostly by UV exposure. An early acral melanoma cell line, SMYM-PRGP, shows distinctive amplification of *TERT* and *CCND1* (Murata et al. 2007), while it is wild in *BRAF* and *NRAS*. These data suggests importance of *TERT* amplification in the early developmental phase of acral melanoma.

TERT aberrations may have clinical significance. It was found that fluorescence in situ hybridization (FISH) analysis using *CCND1*, *TERT*, and *AURKA* (a gene encoding Aurora A kinase which is a member of a family of mitotic serine/threonine kinases) probes could improve sensitivity of histopathologic diagnosis for acral melanomas (sensitivity 97%; specificity 100%) (Diaz et al. 2014a). In this study, amplification of *AURKA* was detected in 2 of 34 (6%) primary lesions of ALM. Note that *AURKA* amplification was reported to contribute increased chromosomal instability in cancer cells. Another study revealed that amplification of *TERT* was associated with poor outcome of patients with ALM (Diaz et al. 2014b).

KIT

KIT encodes a receptor tyrosine kinase, whose ligand is stem cell factor (SCF). KIT–SCF signaling is essential for melanocyte to differentiate, proliferate, migrate, and survive in the fetal and postnatal tissue. In 2006, Curtin, Busam, Pinkel, and Bastian reported an important role of *KIT* in subsets of melanomas. They found mutations of *KIT* in 3 of 24 (12%) of acral melanomas, 8 of 38 (21%) of mucosal melanomas, and 4 of 18 (22%) of CSD melanomas. By contrast, none of the non-CSD melanomas had *KIT* aberrations. Later immunohistochemical studies detected expression of *KIT* in 40–80% of acral/mucosal melanomas. The rate of *KIT* mutations in acral melanoma seems to be less frequent in White persons: 15% of acral/mucosal melanomas in patients seen at MD Anderson Cancer Center (Torres-Cabala et al. 2009) and 6.8% of acral/mucosal melanomas (4.2% of acral melanoma) in a Canadian population (Abu-Abed et al. 2012). The mutation rates of *KIT* seem higher in Asians: 25% of nail apparatus melanomas and

15.6% of melanomas on palms and soles in Japanese (Sakaizawa et al. 2015), 33% of amelanotic acral melanomas in Koreans (Choi et al. 2013), and 23% of acral melanomas in Chinese (Dai et al. 2013). These data suggest that *KIT* is an important oncogene in the development of acral/mucosal melanoma and could be used as a therapeutic target of this type of melanoma.

In vitro studies have shown that growth of melanoma cells harboring *KIT* mutations is suppressed with small molecular inhibitors targeting *KIT*, such as imatinib and sunitinib (Ashida et al. 2009). More importantly, oral administration of imatinib exerted dramatic clinical effects on acral/mucosal melanomas with *KIT* mutations (to be discussed later in detail).

BRAF and NRAS

BRAF is the most commonly aberrated gene in non-CSD melanomas. Mutations of *BRAF* are detected in around 70% of this type of melanoma (Curtin et al. 2005), and 90% of the mutations are V600E. On the other hand, *NRAS* mutations have been found in about 15% of non-CSD melanoma. *BRAF* and *NRAS* mutations in melanomas are mutually exclusive in most cases. In contrast, in acral melanoma, mutation rates of *BRAF* and *NRAS* are reported to be much lower, around 10% and 5%, respectively. In a Spanish population, with the multiple-ligation-dependent probe amplification method on frozen samples, no *BRAF* mutations were detected in 17 primary lesions of ALM, but *NRAS* mutations were detected in 17% of them (Puig-Butillé et al. 2013). According to a study of 88 Swedish patients with ALM, *BRAF* mutations were detected in 17% and *NRAS* mutations were in 15% (Zebary et al. 2013). In a Japanese series, *BRAF* mutation was detected in 8.9% (4/45) of melanomas on palms and soles and in 12.5% (3/24) of nail apparatus melanomas (Sakaizawa et al. 2015). In the series, *NRAS* mutations were detected in 20% of melanomas on palms and soles. In another study in Japan, *BRAF* mutations were detected in 18.8% of ALM (9.5% in stage I/II, 36.4% in stage III/IV) (Yamazaki et al. 2015). In the latter Japanese series, *BRAF* mutations were detected in 64.7% of SSM, 50% in LMM, and 20% in NM. In a Korean subject, *BRAF* V600E mutation was detected in 19.4% (7/36) of patients with ALM.

Collectively, the mutation rate of *BRAF* in acral melanoma is lower than in non-CSD melanoma, but the rates are substantially variable among studies, the highest rate being 36.4% in Japanese patients with stage III/IV ALM. The mutation rate of *NRAS* in acral melanoma is around 15–20%. These data suggest MAPK inhibitors can be effective in selected patients with acral melanoma.

NUAK2

Chromosome 1q32 has been known to be frequently altered in melanoma cells. It was also reported that genomic gains of this locus were associated with tumor thickness of melanoma. *NUAK2*, located at this locus, was recently implicated as a relevant gene of melanoma (Namiki et al. 2011). *NUAC2* is a member of the AMP-activated protein kinase (AMPK) family of serine/threonine protein kinase. It was shown that knockdown of *NUAK2* induced senescence of melanoma cells and suppressed tumor growth in mice. Expression degrees of *NUAC2* were significantly related to survivals of patients with acral melanoma. Risk of relapse was greater in acral melanoma with high levels of *NUAK2* expression than in that with low expression level. These findings indicate *NUAC2* plays an important role in acral melanoma, particularly in later progressed stages.

The Mutational Landscape of Acral Melanoma

Using next-generation sequencing, whole genomic data were obtained in a case of acral melanoma (Turajlic et al. 2012). Compared to the high-frequency genomic changes in melanoma on sun-exposed sites, the rates of somatic mutation in the acral melanoma were lower, mostly comparable to the rates reported in cancer genomes not associated with mutagenic exposure. Another study was performed in six cell lines of acral melanoma using the techniques of whole-exome sequencing and array comparative genomic hybridization (Furney

et al. 2012). The cell lines display a mutation rate comparable to that revealed in the above case. Mutations were identified in oncogenes and tumor suppressors previously linked to melanoma including *BRAF*, *NRAS*, *KIT*, *PTEN*, and *TP53*. Mutations were detected in some cancer genes not previously linked to melanoma and in genes linked to DNA repair such as *BRCA1* and *BRCA2*.

According to the recent extensive study conducted by Australian research group, acral and mucosal melanomas showed a markedly different whole-genome landscape from melanomas on sun-exposed sites (Hayward et al. 2017). Acral melanomas were characterized by frequent structural variants (deletions, duplications, fold-back inversions) and complex rearrangements. Many acral melanomas had high-level amplifications on the long arm of chromosome 11, often targeting *CCND1*. Significantly mutated genes in acral melanoma were *BRAF*, *KIT*, *MAP2K2*, *NF1*, and *NRAS*, though mutations attributable to ultraviolet radiation were rare. The completely different whole-genome landscape of acral melanoma from melanomas on sun-exposed skin indicates that genomic aberrations in acral melanoma are not caused by UV radiation but by carcinogenic factors shared with cancers of internal organs without exposure to highly mutagenic agents.

Clinical, Dermoscopic, and Histopathologic Diagnoses of Acral Melanoma

Acral melanoma is typically detected at a later stage, explaining its overall poor prognosis. Early detection therefore is important to improve the prognosis. Almost all patients with acral melanoma in situ can be cured by excision, usually without functional or cosmetic impairment. Here, we describe clinical characteristics of melanomas on the palms and soles and those affecting nail apparatus separately because they are substantially different in anatomic and histologic structures.

Acral Melanoma on the Palms and Soles

Clinical Diagnosis of Melanoma on the Palms and Soles

Clinical diagnosis of advanced melanoma on the palms and soles is not difficult in most cases. As same to advanced melanoma on other anatomical sites, they are seen as a brownish black nodule or plaque, often partly eroded or ulcerated, and surrounded by a pigmented macule with variable shades of brown (Fig. 3). The lesions are typically large in size, often exceeding 2 cm in maximum diameter, and irregular and asymmetric in shape. Note that on the palms and soles, other common pigmented lesions such as seborrheic keratoses and basal cell carcinomas, which are important differential diagnoses of melanoma on other anatomical sites, are rare. However, melanocytic nevi are commonly seen on the palms and soles not only in dark-skinned people but also in Whites. About 10% of Japanese have melanocytic nevi on acral volar skin (Saida et al. 2011a). Acral nevi are typically small, mostly 7 mm or less in diameter, symmetric in color distribution and shape and thereby easily differentiated from invasive acral melanoma in most cases. However, clinical differentiation between early acral melanoma and acral nevus is sometimes very difficult because both can present as brownish macules. Acral melanoma in situ on the palms and soles exhibits the following characteristics: (a) a pigmented macule with asymmetric and irregular shape, often accompanied by notching at the periphery, (b) brown color with variable shades from tan to black, and (c) a diameter that exceeds 7 mm (Fig. 4). The last size criterion was proposed based on a study by the author's group, which had revealed that the vast majority of acquired acral nevi were 7 mm or less in diameter. Of course, these criteria are not absolute. Occasionally, melanocytic nevi are somewhat irregular in shape and color. Regarding the size, congenital acral nevus is often larger than 7 mm and even acquired acral nevus can become more than 7 mm.

Acral melanomas can occasionally be unpigmented (amelanotic/hypomelanotic) (Fig. 5); the rates of amelanotic/hypomelanotic

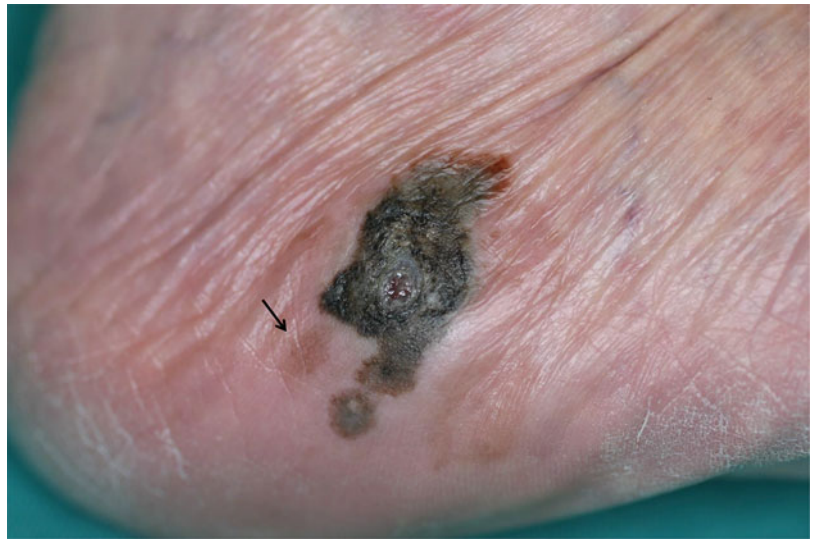

Fig. 3 Clinical features of advanced acral melanoma. This advanced acral melanoma on the sole is seen as a large, slightly elevated brownish black lesion with asymmetrical, irregular borders. There is an ulcerated nodule in the center. In addition, a light brown macule is detected in the left lower portion (arrow)

Fig. 4 Clinical features of early acral melanoma. This acral melanoma in situ on the sole, 9 mm in maximum diameter, presents as a brownish macule with variable shades from tan to black. The shape is asymmetric and irregular, and the border is ill-defined particularly in the right lower portion

acral melanomas seem different among races, varying from 30% in a French population and around 10% in Japanese. Differential diagnoses of amelanotic/hypomelanotic acral melanoma on the palms and soles include squamous cell carcinoma (SCC), eccrine poroma, pyogenic granuloma, and various kinds of ulcerated lesions. In rare cases, acral melanomas are hyperkeratotic or verrucous, but most of them are suspected to be melanoma because of brownish black color detected at least focally. However, clinical diagnosis of hyperkeratotic and completely amelanotic acral melanomas can be very difficult to distinguish from other hyperkeratotic lesions such as SCC, verruca vulgaris, and tylosis/clavus.

Dermoscopic Diagnosis of Melanoma on the Palms and Soles

Dermoscopic Features of Melanocytic Lesions on the Palms and Soles

Dermoscopic features of advanced primary acral melanomas on the palms and soles are same as those of melanomas affecting other anatomical sites. These non-site-specific melanoma criteria include irregular blotches with variegated shades of brown, abrupt edge, blue-white veil, and regression structures with whitish or grayish color (Fig. 6). Irregular streaks and irregular dots/globules are also important clues to the diagnosis of advanced acral melanoma. More importantly, the parallel ridge pattern (PRP), that is, striped pigmentation along the ridges of the skin markings, is detected in macular portions within the advanced melanomas (Fig. 7) (described in detail in the next paragraph). In contrast, an atypical pigment network is rare, except for lesions located on the transitional zone between the

glabrous and non-glabrous skin. In amelanotic or hypomelanotic acral melanomas, vascular patterns are helpful in determining diagnosis dermoscopically. They are polymorphous vessels, particularly combination of irregular linear or dotted vessels, and milky red areas that can be readily discerned due to the decreased or absent pigmentation.

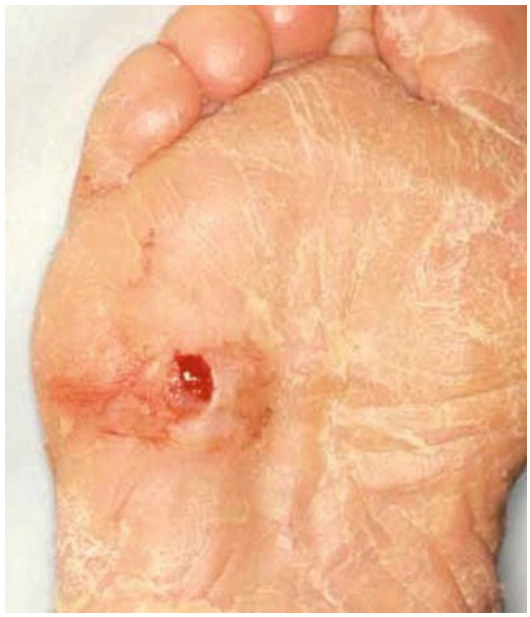

Fig. 5 Clinical features of amelanotic acral melanoma. The lesion on the sole is seen as a reddish plaque partly ulcerated with some bleeding. Note that, according to the patient's memory, the lesion was first recognized as a brownish macule and later lost the pigmentation

Dermoscopy is very helpful in the differentiation between early forms of acral melanoma and acral nevi, which can be difficult clinically. Surface skin markings or dermatoglyphs on the palms and soles run in a parallel linear or curvilinear fashion. Acral melanoma in situ in this area shows a unique dermoscopic pattern called the parallel ridge pattern (PRP) (Fig. 8) (Saida et al. 2004; Phan et al. 2010). In this pattern, stripes of pigmentation are detected along the dermatoglyphic ridges and are absent along the sulci. In our study of a total of 712 acral melanocytic lesions including 67 invasive and 36 in situ acral melanomas, diagnostic sensitivity and specificity of the PRP for acral melanoma were 86% and 99%, respectively. Diagnostic performance of the PRP is similarly very high for acral melanoma in situ. Irregular diffuse pigmentation is another important dermoscopic finding of acral melanoma on the palms and soles (Fig. 9); however, this feature is more frequently detected in advanced acral melanomas (Saida et al. 2011a).

By contrast, the major dermoscopic patterns seen in acral nevi are the parallel furrow, lattice-like, and fibrillar patterns (Fig. 10) (Saida et al. 2011a). The parallel furrow pattern (PFP) shows brownish linear pigmentation along the sulci of the surface skin markings (Fig. 10a). There are several variants in the parallel furrow pattern

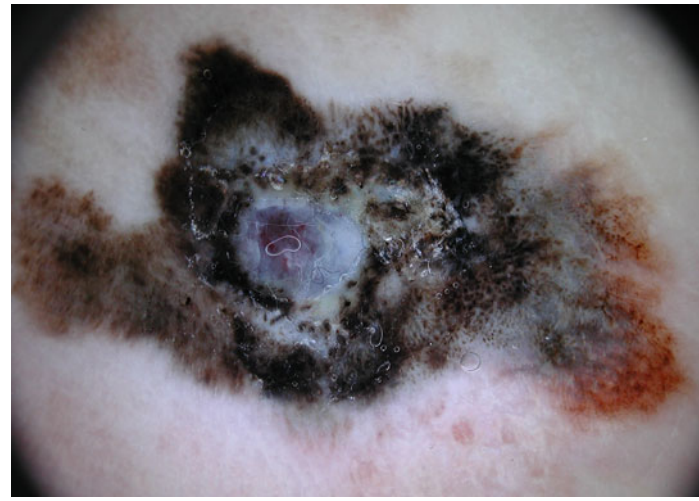

Fig. 6 Dermoscopic features of an advanced primary lesion of acral melanoma. Irregular blotches with variegated shades of brown, abrupt edge, blue-white veil, irregular streaks, and irregular dots/globules can be seen, similar to melanomas on other anatomic sites (this is a dermoscopic image of the lesion illustrated in Fig. 3)

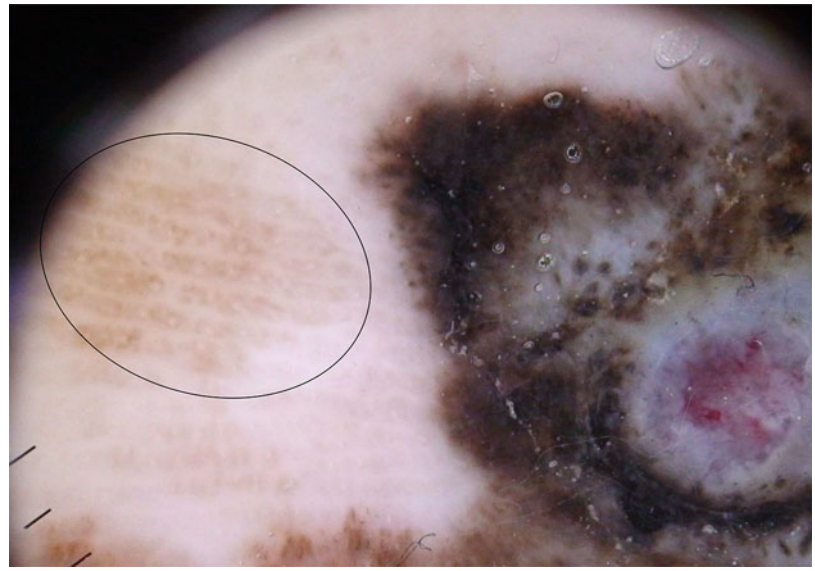

Fig. 7 Dermoscopy showing the parallel ridge pattern in the macular portions of an advanced acral melanoma. The parallel ridge pattern is detected in the left portion (circle), corresponding to the clinically light brown macular area of the lesion (indicated with the arrow in Fig. 3) (Reproduced with permission from Saida et al. 2011a)

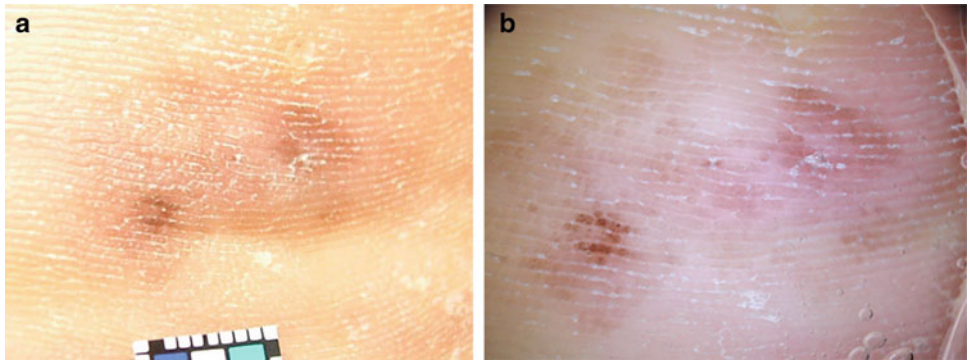

Fig. 8 Clinical and dermoscopic features of early acral melanoma. Clinically, this acral melanoma in situ on the sole presents as a light-brown macule, 21 × 16 mm in size (**a**). Dermoscopically, the parallel ridge pattern with variable shades of brown is detected throughout the lesion (**b**) (Reproduced with permission from Saida et al. 2007)

such as dotted line and double-line variants (Fig. 10a and b).The lattice-like and the fibrillar patterns are modifications of the PFP (Saida and Koga 2007). The lattice-like pattern is composed of parallel pigmented lines along the sulci as well as lines crossing the parallel lines (Fig. 10d). The fibrillar pattern shows densely packed, fine pigmented lines, usually arranged in the direction crossing the skin markings (Fig. 10e). Among these, the PFP is the major dermoscopic pattern most frequently seen in acral nevi, accounting for 40–60% of all acral nevi. The prevalence of the lattice-like pattern is 10–15% and that of the fibrillar pattern is 10–20%. The prevalence of these patterns in acral nevi are similar across races. Finally, there are several minor dermoscopic patterns in acral nevi, such as homogeneous/structureless (Fig. 10f), globular, and reticular patterns (Saida et al. 2011a).

Dermoscopic Guidelines for Detection of Melanoma on the Palms and Soles

Two kinds of dermoscopic guidelines have been proposed for effective detection of melanoma on the palms and soles: the three-step algorithm and the BRAAFF checklist. These may be helpful for clinicians in their daily practice.

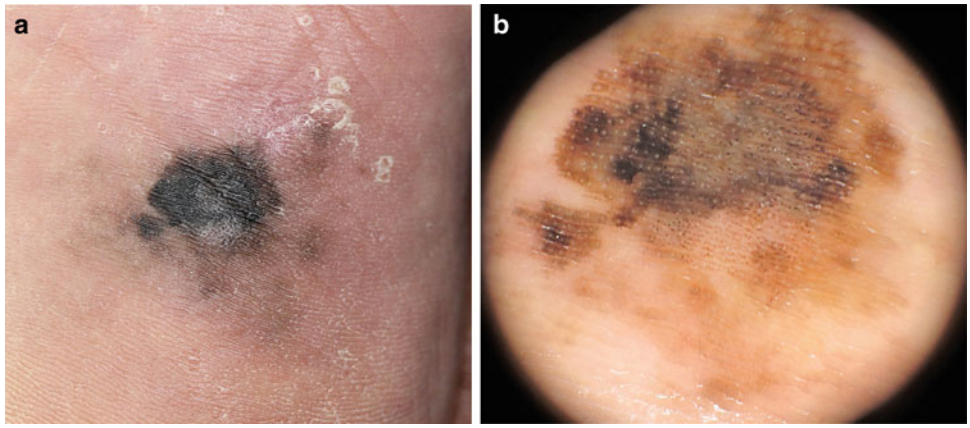

Fig. 9 Clinical and dermoscopic features of acral melanoma partly invading superficial dermis. Clinically, this ill-defined broad macular lesion on the sole is asymmetric in shape (**a**). The color is brownish black with variable shades. Dermoscopically, irregular diffuse pigmentation with variable shades of brown is recognized. In addition, a hint of the parallel ridge pattern is detected within the lesion

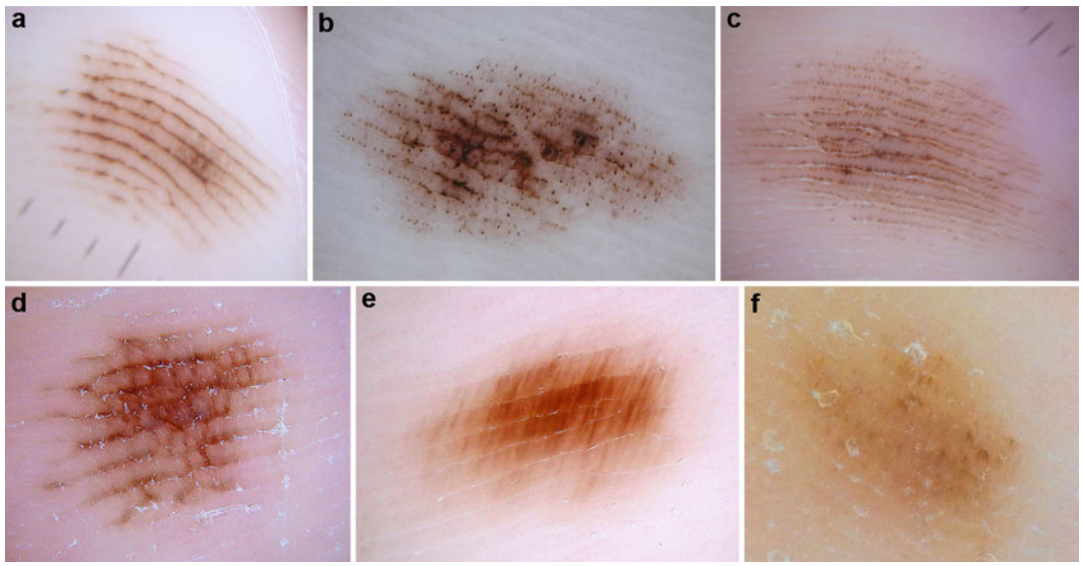

Fig. 10 Dermoscopic patterns of acral nevus on the palms and soles. Major dermoscopic patterns seen in acral nevus are the parallel furrow pattern (**a**–**c**) and its modifications, the lattice-like (**d**) and fibrillar patterns (**e**). There are additional variants in the parallel furrow pattern such as dotted-line variant (**b**) and double-line variant (**c**). In addition, several minor patterns such as homogeneous pattern (**f**) are detected in acral nevus (Fig. 10b and c was reproduced with permission from Saida and Koga 2013)

The Three-Step Algorithm

In 2007, our group proposed the original three-step algorithm for effective detection of melanomas on the palms and soles (Saida and Koga 2007) and revised it in 2011 (Koga and Saida 2011). The algorithm proceeds as follows (Fig. 11) (Saida et al. 2011a; Saida and Koga 2013):

Step 1: The lesion on the palms and soles is examined for the presence of the PRP. If the PRP is found in any part of the lesion, it should

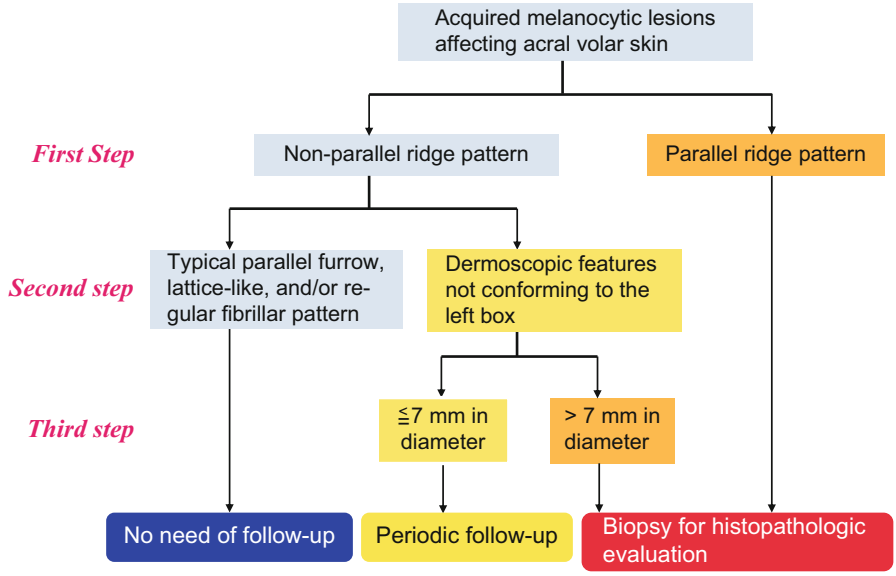

Fig. 11 **The three-step dermoscopic algorithm for the effective detection of acral melanoma on the palms and soles** (see the text in detail) (Reproduced with permission from Saida et al. 2011a)

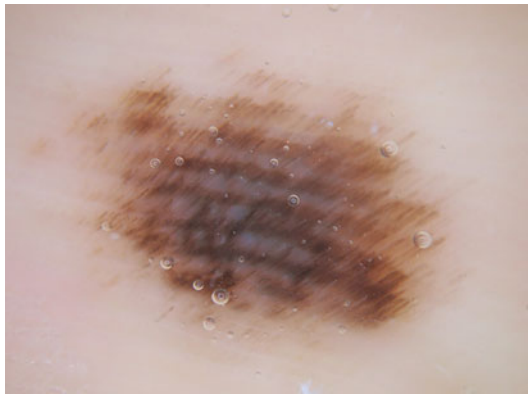

Fig. 12 **Combination of two dermoscopic patterns in acral nevus.** In this acral nevus on the heel, an orderly combination of the parallel furrow pattern and the fibrillar pattern is observed

be biopsied regardless of the size. If the lesion does not show the PRP, proceed to Step 2.

Step 2: The lesion is examined for the presence of the typical benign dermoscopic patterns (i.e., typical PFP, typical lattice-like pattern, regular fibrillar pattern). If the lesion shows one or orderly combination of two or three typical benign patterns (Fig. 12), further dermoscopic follow-up is not needed because they are certainly benign acral nevus without risk of developing to melanoma. If the lesion shows equivocal dermoscopic features, proceed to Step 3.

Step 3: The maximum diameter of lesions that do not show typical benign dermoscopic patterns is measured. Lesions >7 mm should be excised or biopsied for histopathologic evaluation. Lesions ≤7 mm in maximum diameter should be monitored clinically and dermoscopically at 3- to 6-month intervals.

There are several notes in the application of this algorithm (Saida and Koga 2013):

1. Congenital acral nevi have to be excluded. Congenital acral nevi are often but not always larger than 7 mm in diameter. Dermoscopic features of the congenital acral nevi are the typical PFP, the crista dotted pattern, and the peas-in-a-pod pattern (Fig. 13). The crista dotted pattern consists of brown dots/globules regularly distributed on the ridges of the skin markings. The peas-in-a-pod pattern is a combination of the parallel furrow and the crista dotted patterns. Nonetheless, it is not rare to see acral nevi whose type (acquired or congenital) cannot be determined; however the three-

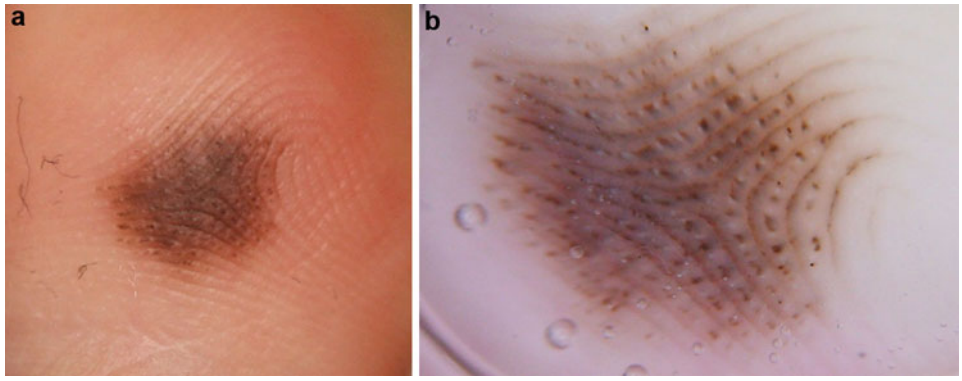

Fig. 13 Dermoscopic features of congenital acral nevus. In this congenital lesion on the volar aspect of the fourth toe (**a**: clinical features), in addition to the parallel furrow pattern, dots/globules are regularly distributed on the ridge of the skin markings, exhibiting the typical features of the peas-in-a pod pattern (**b**)

step algorithm can be used for such indeterminate lesions.

2. It is crucial to correctly identify the dermatoglyphic furrows and ridges, which can be facilitated by performing the furrow ink test (Uhara et al. 2009). The peripheral areas of the lesion are marked with a whiteboard marker pen, preferably blue or green in color, and then the skin surface is gently wiped with a dry paper towel in the direction crossing the skin markings. The furrows retain the blue or green ink and become clearly visible on dermoscopic examination as thin inked lines and distinguish PRP and PFP (Fig. 14). The ink can be easily removed with a wet paper towel.
3. Note that the PRP or its similar features could be detected in several benign conditions (Saida and Koga 2013). They include volar macules of Peutz–Jeghers or Laugier–Hunziker syndromes, acral pigmentation due to anticancer drugs, pigmented and ridged plantar warts, volar melanotic macules, subcorneal hemorrhage (e.g., so-called black heel due to friction with shoes and PlayStation purpura due to friction with the game controller), and pigmentation due to external pigment such as paraphenylenediamine (Fig. 15). However, most of these conditions can be easily differentiated from early acral melanoma by evaluating the clinical characteristics, number of lesions (single or multiple), personal and/or family history, and other associated clinical signs and symptoms (Saida et al. 2011a; Saida and Koga 2013).
4. In the second step, the clinician must assess whether the benign patterns are typical/regular. Typical parallel furrow or lattice-like patterns are symmetrically and evenly distributed across the lesion. The criteria for classifying a fibrillar pattern as regular are (a) symmetrical and regular overall arrangement of the fibrillar pigmentation, (b) even thickness and length of each fibril, and (c) alignment of the starting points of the fibrils on a surface furrow (Fig. 16a). In contrast, the irregular fibrillar pattern seen in acral melanoma exhibits asymmetrical arrangement of the fibrillar pigmentation and the fibrils vary in thickness and color (Fig. 16b).

BRAAFF Checklist

Several recent studies found that the sensitivity of the PRP for acral melanoma is only around 60%. This can be explained by the fact that the PRP is a characteristic feature of early acral melanoma and becomes obliterated as the lesions progress. The BRAAFF checklist was proposed for improved dermoscopic detection of acral melanoma (Table 1) (Lallas et al. 2015). This algorithm was based on the analysis of a total 603 acral melanocytic lesions including 131 acral melanomas (42 of them were in situ melanoma). In this study, the checklist shown in Table 1 diagnosed

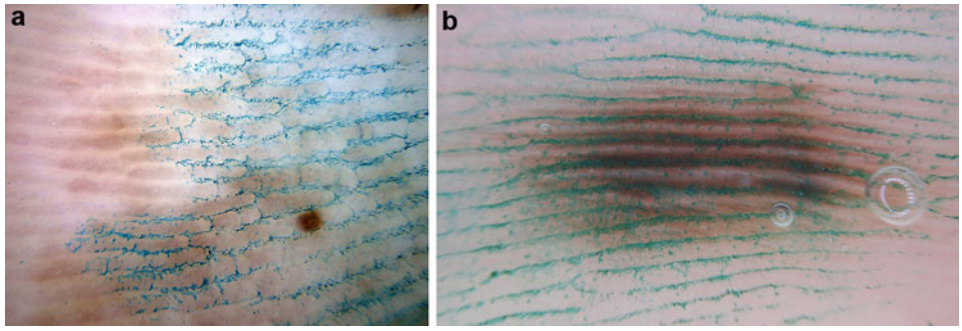

Fig. 14 The furrow ink test is useful in the determination of the dermoscopic patterns of acral lesions. The ink is seen as parallel green lines in the furrows. Thereby, the dermoscopic features illustrated in (**a**) are determined as the parallel ridge pattern of acral melanoma and those in (**b**) as the parallel furrow pattern of acral nevus (see the text in detail) (Fig. 14a was reproduced with permission from Saida and Koga 2013)

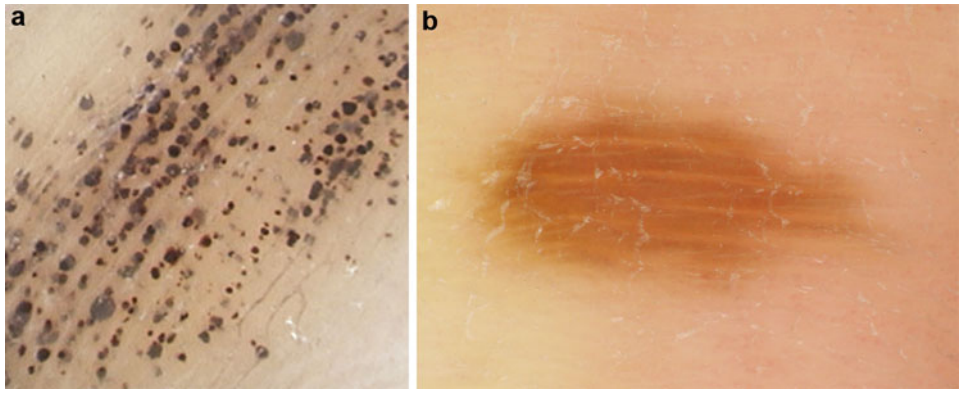

Fig. 15 Benign acral lesions which show dermoscopic features mimicking the parallel ridge pattern. There are several benign conditions which exhibit dermoscopic features mimicking the parallel ridge pattern, such as the so-called black heel (calcaneal petechia), known as the dermoscopic features of the "pebbles on the ridges" (**a**) and acral pigmented macules due to chemotherapeutic drugs such as 5-fluorouracil (**b**) (Fig. 15a was reproduced with permission from Saida and Koga 2013)

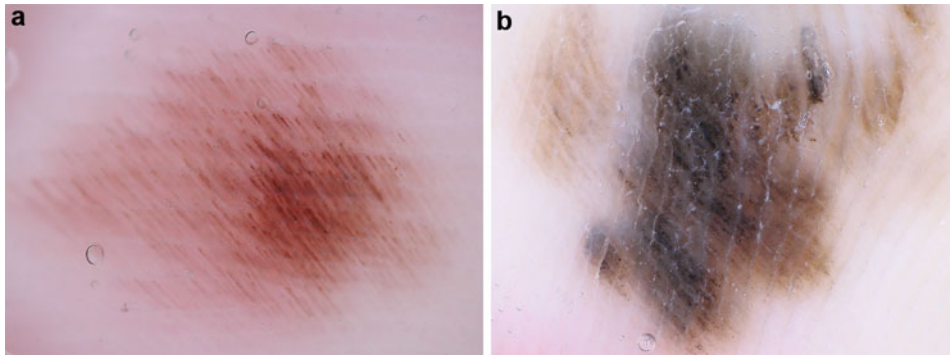

Fig. 16 Regular fibrillar pattern and irregular fibrillar pattern. Regular fibrillar pattern seen in benign acral nevus (**a**) and irregular fibrillar pattern in acral melanoma (**b**) (see the text in detail)

Table 1 The BRAAFF checklist for the dermoscopic diagnosis of acral melanoma. A total of ≧1 is needed for a diagnosis of melanoma

Acronym	Criterion	Points
B	Irregular blotch	+1
R	Parallel ridge pattern	+3
A	Asymmetry of structures	+1
A	Asymmetry of color	+1
F	Parallel furrow pattern	−1
F	Fibrillar pattern	−1

Reproduced from Lallas et al. 2015

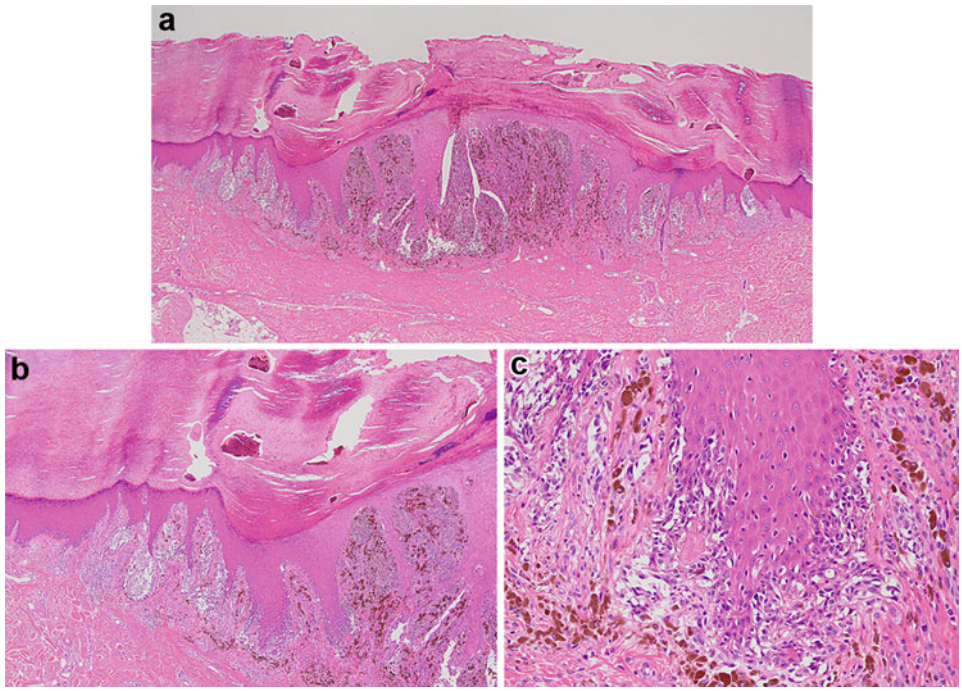

Fig. 17 Histopathologic features of advanced primary acral melanoma. Florid random proliferation of atypical melanocytes is detected not only in the epidermis but also in the dermis. Sheetlike dermal invasion is seen in the center of the lesion (**a**). The melanocytes proliferate as solitary units as well as in nests with variable shapes and sizes (**b**). Focally, solitary arranged melanocytes are prominent within the epidermis. The melanocytes are different from pagetoid cells commonly seen in SSM/low-CSD melanoma (**c**)

acral melanoma with 93.1% sensitivity and 86.7% specificity.

Clinical applicability and usefulness of the three-step algorithm and of the BRAAFF checklist in daily practice must be investigated and compared in further studies.

Histopathologic Diagnosis of Melanoma on the Palms and Soles

Clinically equivocal acral lesions are biopsied and evaluated histoapthologically. Histopathologic diagnosis of advanced melanoma on the palms and soles is not difficult. The criteria for the diagnosis are common to those for other subtypes of melanoma, but compared with SSM/non-CSD melanoma, intraepidermal upward migration of melanocytes is not prominent in most cases of acral melanoma. The neoplastic cells are typically small oval or dendritic rather than pagetoid, and their cytoplasmic melanin granules are not as fine and dusty as those seen in melanocytes of SSM/non-CSD melanoma (Fig. 17).

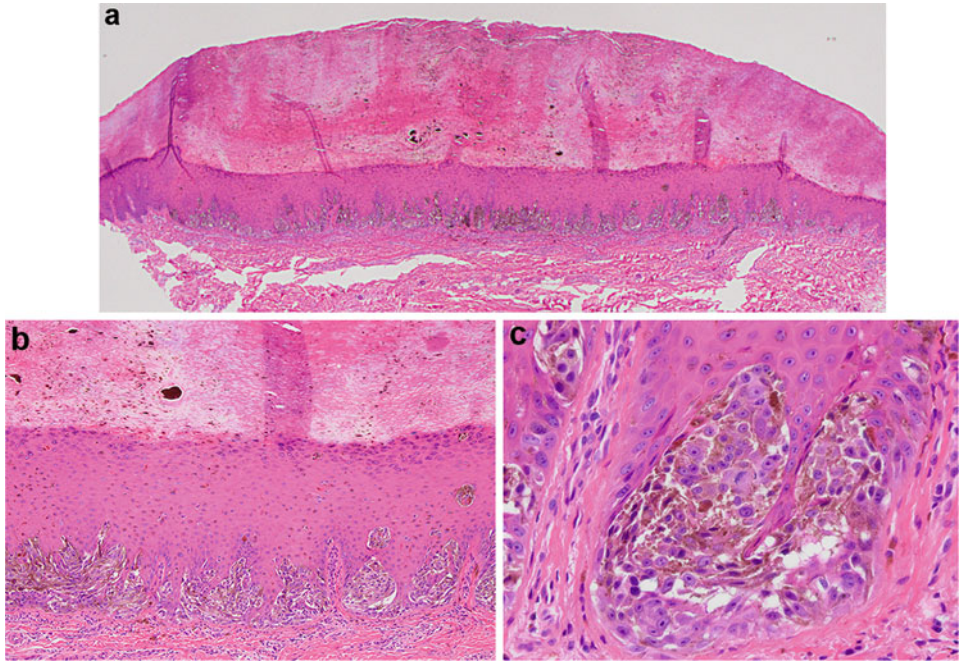

Fig. 18 Histopathologic features of Spitz nevus on acral volar skin. Symmetrical proliferation of melanocytes in the hyperplastic epidermis (**a**, **b**). Although the melanocytes are large and atypical (**c**), they are mostly arranged in nests located in the lower epidermis, and the nests are sharply demarcated (**b**)

One important histopathologic differential diagnosis of acral melanoma is Spitz nevus, which is not rare on the palms and soles. The histopathologic criteria for the differentiation are similar as for differentiation between both entities on non-glabrous skin. Spitz nevi are symmetric with an evenly hyperplastic epidermis, and melanocytes are arranged mainly in nests, which are situated mostly in the lower epidermis. The nests are sharply demarcated, with artifactual clefts separating them from the surrounding epidermis (Fig. 18). The intradermal component (when present) shows "maturation" with cells becoming smaller in size in the deeper portions, and at the bottom of the lesion, nevus cells tend to be arranged as solitary units among collagen bundles.

In contrast to the advanced lesions, histopathologic diagnosis of early acral melanoma on the palms and soles is sometimes very difficult (Saida 1989) because benign acral nevi not infrequently show prominent proliferation of melanocytes as solitary units within the epidermis and the proliferation occasionally reaches the upper epidermis, mimicking the features of melanoma in situ. Such confusing histopathologic features are particularly prominent in the tissue sections cut in the direction parallel to the skin markings (Fig. 19). Thus, when we histopathologically evaluate melanocytic lesions on the palms and soles, the tissue specimen should be cut perpendicularly to the skin markings. In such a section, we can recognize two kinds of epidermal rete ridges, one is under the surface furrows and the other under the surface ridges. In early acral melanoma in situ, solitary melanocytes are concentrated in the epidermal rete ridges underlying the surface ridges (Fig. 20a), corresponding to the dermoscopic PRP. In contrast, in most acral melanocytic nevi, nevus cells arranged in nests are mainly detected in the epidermal rete ridges underlying the surface furrow (Fig. 20b), corresponding to the dermoscopic PFP. However, in some cases, nevus cells are detected also in

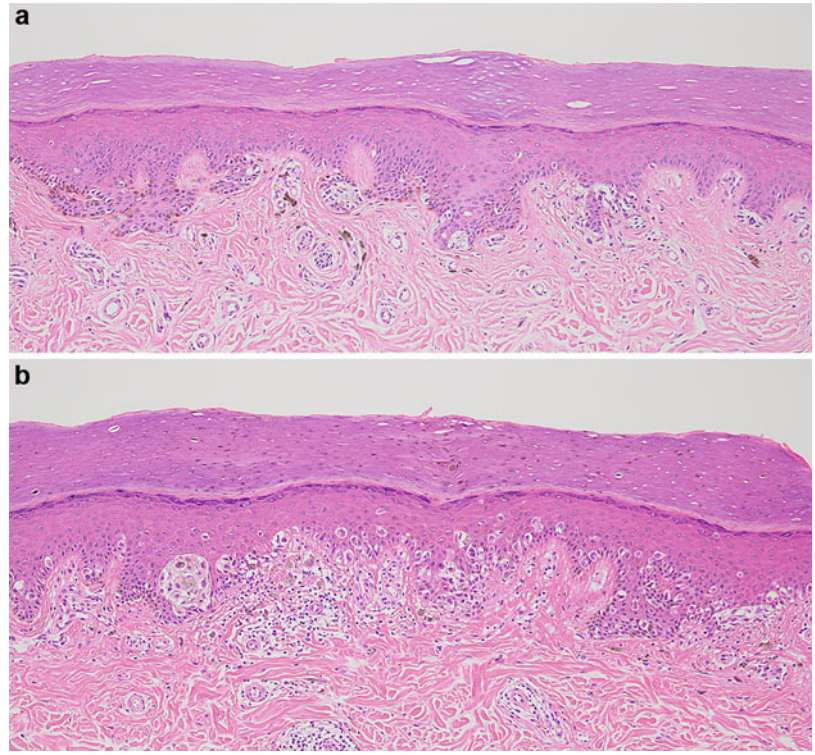

Fig. 19 Effect of sectioning orientation on the histopathologic features of acral nevus. These two figures show histopathologic features of the same acral nevus on the sole. (**a**) In the tissue section cut perpendicularly to the surface skin markings, most melanocytes are arranged in nests mainly located at the dermo-epidermal junction, indicating that this is a benign acral nevus. (**b**) In contrast, in the tissue section cut parallel to the skin markings, many melanocytes are seen as solitary units within the epidermis, simulating the histopathologic features of acral melanoma in situ

epidermal rete ridges underlying the surface ridges. Even in such cases, melanin granules in the cornified layer are arranged in a columnar fashion selectively under the surface furrow (Saida et al. 2011b). This finding aids in the histopathologic differentiation of acral nevus from melanoma in situ.

Melanocytic nevi located on the transitional zones between glabrous and non-glabrous skin (i.e., far peripheral areas of the palms and soles, lateral aspects of fingers and toes, and webs) not infrequently show prominent proliferation of melanocytes arranged as solitary units within the epidermis, histopathologically mimicking melanoma in situ. This is probably due to the complex structures of the epidermal rete ridges in these areas. Similar prominent solitary arrangement of melanocytes is occasionally found also in acral nevi located on the arch area. We consider these histopathologically confusing acral nevi to be a pseudomelanoma. The differentiating features of these nevi from melanoma are a symmetrical, orderly intraepidermal distribution of melanocytes without nuclear atypia and typical nevus cells in the dermis, when there is an intradermal component (Fig. 21).

Histopathologic changes of early acral melanoma in situ on the palms and soles are sometimes very subtle, showing only a slightly increased number of melanocytes in the epidermis. In 1994, several cases of problematic plantar pigmented lesions were described (Nogita et al. 1994). The brownish macular lesions were large in size and irregular in color and shape, fulfilling the clinical criteria for melanoma in situ. However, histopathologically, the lesions showed only a slightly increased number of melanocytes at the basal layer of the epidermis, which did not fulfill the histopathologic criteria for acral melanoma in situ. The authors considered these lesions could not be diagnosed as acral melanoma in situ and called these lesions atypical melanosis of the foot, implying their biologic nature was uncertain (Fig. 22). Later, however, it

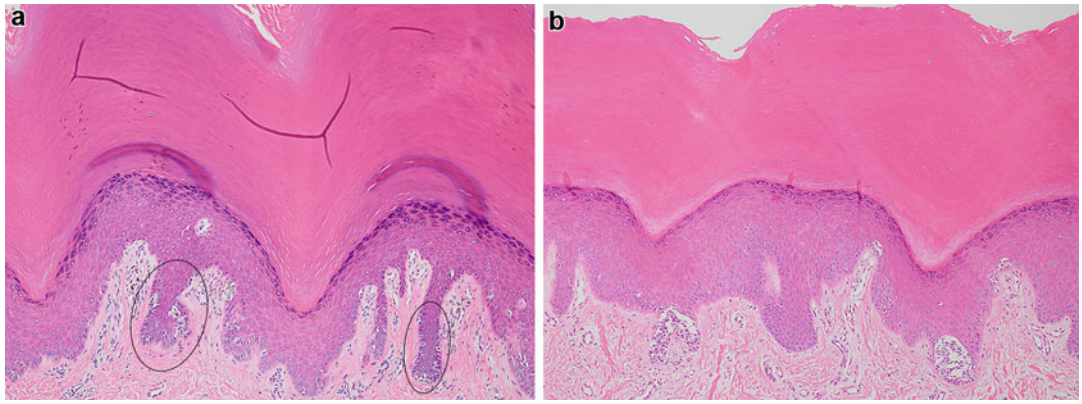

Fig. 20 Differential location of neoplastic melanocytes at the dermo-epidermal junction in early acral melanoma and acral nevus. In early acral melanoma on the palms and soles, increased numbers of solitary arranged melanocytes are mainly located in the epidermal rete ridges underlying the surface ridges (**a**: circles). In contrast, in acral nevus, melanocytes arranged in well-demarcated nests are situated in the epidermal rete ridges underlying the surface furrows (**b**)

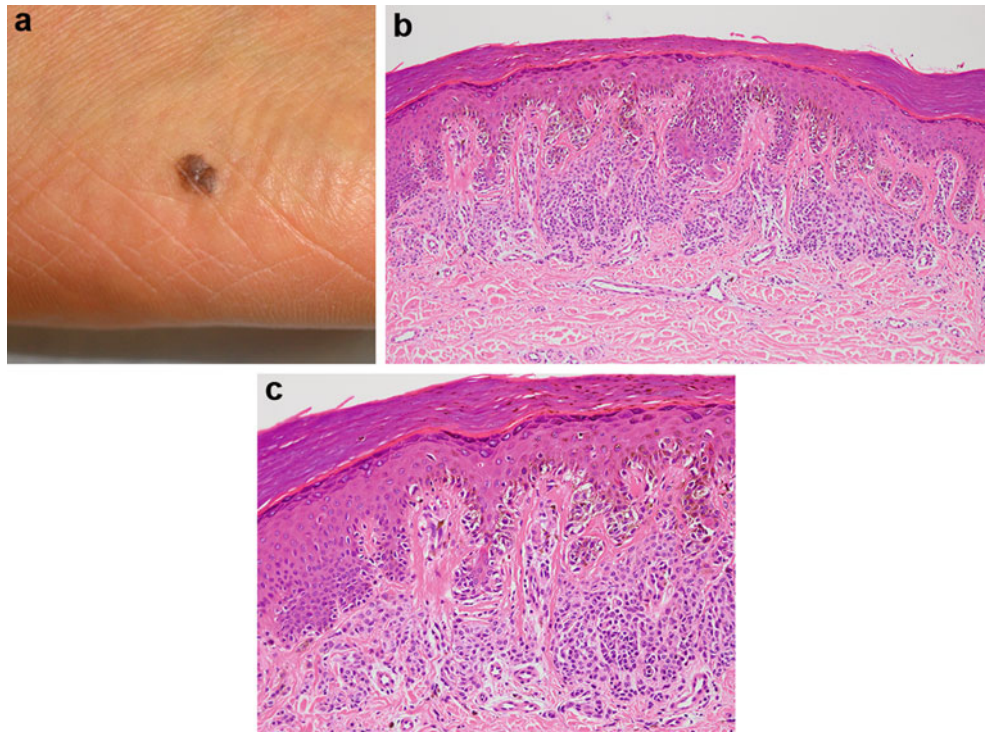

Fig. 21 Acral nevus located on the transitional zone between sole and dorsum of the foot. This nevus is located on the lateral aspect of the foot (**a**). Histopathologically, melanocytes are randomly arranged as solitary units within the epidermis, mimicking the histopathologic features of acral melanoma (**b**, **c**). However, the overall histopathologic structure is symmetrical and regular, and the proliferation of melanocytes is mostly limited to the lower portion of the epidermis (**b**). In addition, nevus cells in the dermis are small and without nuclear atypia (**c**)

was revealed that most of these lesions showed the PRP on dermoscopy, strongly suggesting these are acral melanoma in situ. Moreover, in recent years, it was shown that plantar lesions originally diagnosed as the atypical melanosis of the foot later developed into apparent acral

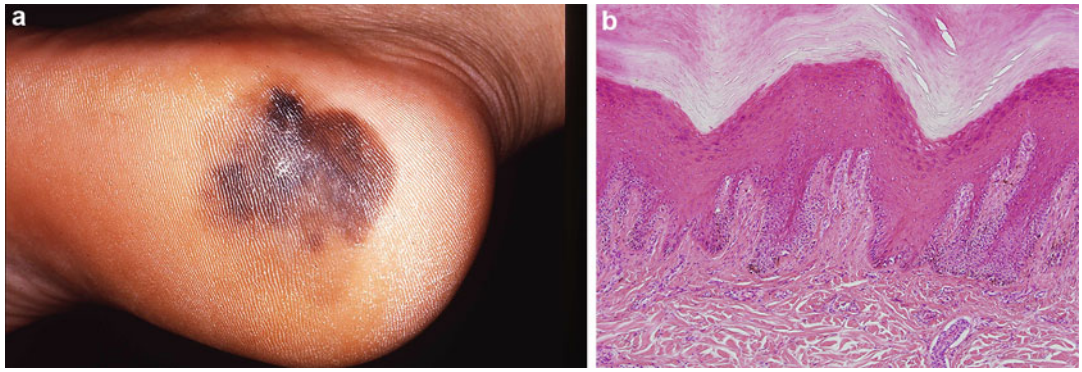

Fig. 22 Clinical and histopathologic features of so-called atypical melanosis of the foot. Clinically, this brownish macule on the heel is large and asymmetrical in shape and color distribution (**a**), suggesting the diagnosis of acral melanoma. However, histopathologically, only a slightly increased number of melanocytes are detected at the epidermal basal layer (**b**)

melanoma (Kilinc Karaarslan et al. 2007; Chiu et al. 2008). We now realize that most lesions of the atypical melanosis of the foot represent a special subtype of slowly evolving acral melanoma in situ.

Biological Meanings of the Dermoscopic Parallel Ridge Pattern

Histopathological findings indicate that the differential dermoscopic pattern in acral nevi and melanoma in situ are due to differential localization of neoplastic melanocytes in the epidermis. This difference strongly suggests that acral melanoma and acral nevus develop independently, supporting the concept of a de novo genesis of acral melanoma as opposed to an evolution from a preexisting nevus.

The preferential localization of melanocytes in the epidermal rete ridges under the surface ridges rather than the surface furrows in evolving acral melanoma could be explained by their emergence from melanocytes from a particular stem cell niche. As opposed to non-glabrous skin, where the stem cell niche is in the bulge of hair follicles, the stem cell niche for melanocytes is located in the secretory portion of eccrine sweat glands (Okamoto et al. 2014). The ducts of these glands ascend and pass through the epidermis and open at the center of the surface ridges. In acral melanoma in situ, neoplastic melanocytes with stem cell-like features can be detected in the secretary portions of eccrine glands as well as in intradermal eccrine ducts. These melanocytes are small and unpigmented but express MART1 and MCM2, markers for melanocytes and the non-G_0 of the cell cycle, respectively, and show amplification of *CCND1*, confirming their neoplastic nature. In contrast, the MART1 and MCM2 positive melanocytes with amplified *CCND1* were not detected in the sweat glands of normal acral skin tissues nor in those of benign acral nevus lesions (unpublished data).

What are the biological meanings of these findings? The concept of cancer stem cell could explain pathogenesis of the PRP. Although melanoma stem cells have been not yet biologically delineated definitely, altered melanocytes in early stages of melanoma in situ could possess some common biological and molecular properties to melanocyte stem cells. In a lesion of evolving acral melanoma in situ, the altered/transformed melanocytes can be maintained and proliferate preferentially in the stem cell niche, which is located in eccrine sweat glands connecting to the epidermal rete ridges underlying the surface ridges through intradermal eccrine ducts. This could be a reason why early acral melanoma in situ specifically exhibits the PRP on dermoscopy. It is still unclear whether acral melanoma cells originate from a melanocyte stem cell in the niche within the eccrine gland or they are transformed epidermal melanocytes which acquire genetically and/or biologically common natures to melanocyte stem cells.

Acral Melanoma of the Nail Apparatus

The nail apparatus is anatomically complex. Although melanomas affecting the nail apparatus have been called subungual melanoma, the term "nail apparatus melanoma" may be preferable (Saida 1992). They typically first manifest as longitudinal melanonychia, reflecting increased amount of melanin granules in the nail plate, which are produced by transformed melanocytes in the nail matrix.

Clinical Diagnosis of Nail Apparatus Melanoma

Clinical diagnosis of advanced lesions of nail apparatus melanoma is not difficult. It is recognized as a brownish black lesion broadly involving and/or destroying the nail plate (Fig. 23). Nodules may be detected within the lesion, often partly eroded or ulcerated. In addition, in most cases, irregular pigmented macules extend on to the nail fold, which is a diagnostically useful clue for melanoma (Hutchinson's sign). Nail apparatus melanoma is occasionally amelanotic (Fig. 24) and/or hyperkeratotic, which must be differentiated from SCC, viral wart, and dystrophic lesions of tinea unguium. Dermoscopy is useful in the differentiation of these nonpigmented lesions, just as described in amelanotic melanoma of the palms and soles.

Early nail apparatus melanomas are recognized as longitudinal melanonychia, that is, band-like pigmentation running from proximal areas to distal ends of the nail plate (Fig. 25). Various benign conditions such as melanocytic nevus, ethnic-type melanonychia, Addison's disease, and subungual hematoma also exhibit longitudinal pigmentation of the nail plate. Clinical differentiation of these conditions is very important in daily practice, because biopsy or excision of the nail apparatus often leads to nail deformities. In 1989, the author proposed clinical criteria for early detection of nail apparatus melanoma (Saida and Oshima 1989). Vast majority of early nail apparatus melanoma is seen as monodactylic longitudinal melanonychia that manifests during adulthood and shows one or more following criteria: (a) the width of the lesion is at least 6 mm, (b) the brownish color variegated from tan to black, and (c) occasionally accompanied by pigmentation on the nail fold (Hutchinson's sign). The main

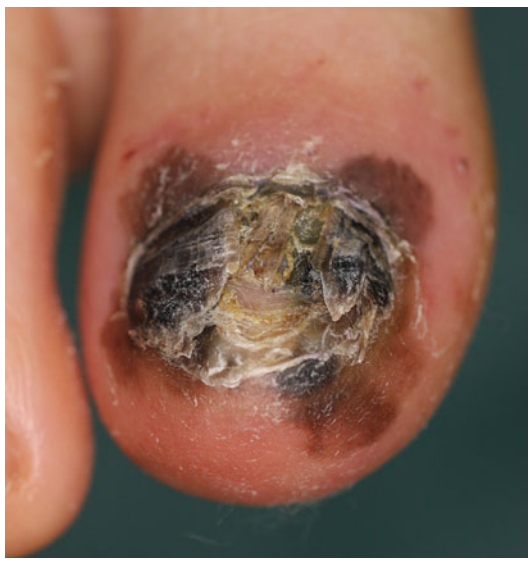

Fig. 23 Clinical features of advanced nail apparatus melanoma affecting the great toe. The nail plate is destroyed by black nodular lesion. Brownish macules are present on the periungual skin (Hutchinson's sign)

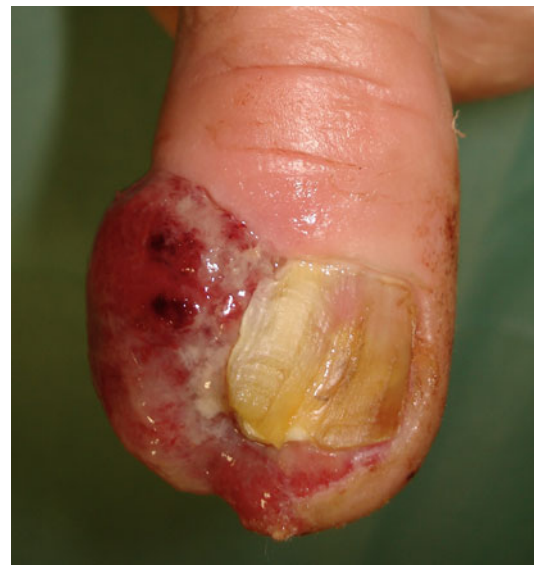

Fig. 24 Clinical features of advanced amelanotic nail apparatus melanoma affecting the left thumb. The nail plate is displaced and destroyed by the big ulcerated red nodule devoid of pigment

differential diagnosis to melanoma is melanocytic nevus of the nail apparatus, which typically presents as narrower longitudinal melanonychia less than 4 mm in width, and the color is uniform as opposed to variegated (Fig. 26a).

Note that broad variegated melanonychia is not infrequently observed in the digits of infants or young children, even with a Hutchinson's sign (Fig. 27a). Histopathologic examination of such nail lesions in infancy reveals increased number of solitary-arranged melanocytes in the epithelium of the nail matrix and nail bed, histopathologically also mimicking acral melanoma in situ. Some investigators considered such lesions to be authentic acral melanoma in situ. Importantly, however, the broad irregular nail pigmentation in children regresses spontaneously in most cases by the end of adolescence (Fig. 27b), confirming this is not a melanoma but a peculiar type of melanocytic nevus. Long-term dermoscopic follow-up of these ambiguous nail lesions in children is a reasonable choice of management (see below).

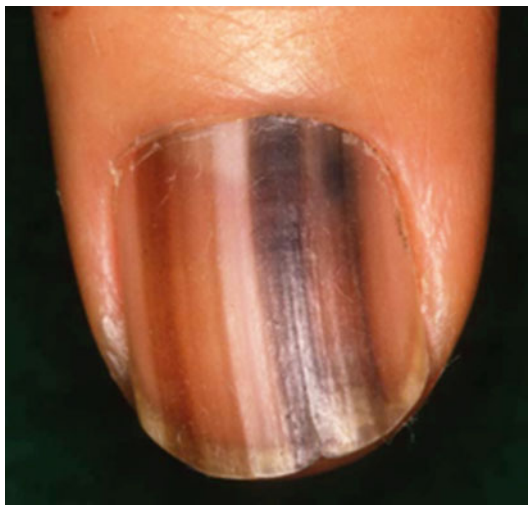

Fig. 25 Clinical features of early nail apparatus melanoma affecting the thumbnail. In this lesion, although no apparent destruction of the nail plate is observed, the longitudinal melanonychia is broad, covering almost the entire nail plate, and the pigmented lines constituting the melanonychia are variable in color and width and are arranged asymmetrically

Dermoscopic Diagnosis of Nail Apparatus Melanoma

Thomas' group proposed the following dermoscopic criteria for nail apparatus melanoma (Fig. 28): (a) light to dark brown coloration of the background; (b) presence of longitudinal brown to black lines that are irregular in color, spacing, orientation, and thickness (irregular lines), and (c) micro-Hutchinson's sign (subtle pigmentation of the cuticle that is only visible by dermoscopy) (Ronger et al. 2002). In contrast, melanocytic nevi of the nail apparatus dermoscopically show regular lines (Fig. 26b). In addition, when the pigmentation of the nail lesion spreads to the hyponychial skin, dermoscopy of any hyponychial involvement provides helpful clues such as the PRP indicative of melanoma and the PFP or its modified patterns indicative of acral nevus. Thomas' criteria are useful; however, the irregularity of longitudinal pigmented lines can be equivocal,

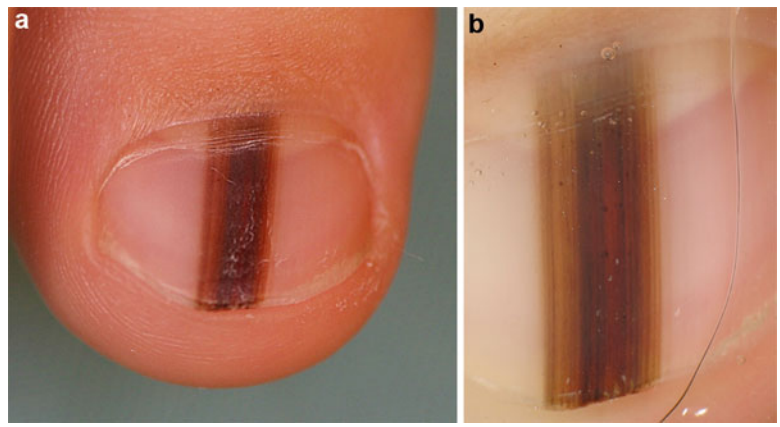

Fig. 26. Clinical and dermoscopic features of melanocytic nevus affecting the nail. Clinically, the longitudinal melanonychia is narrow in width, and the pigmentation is mostly symmetrical (**a**). Dermoscopically, the pigmented lines constituting the melanonychia are arranged regularly and symmetrically (**b**)

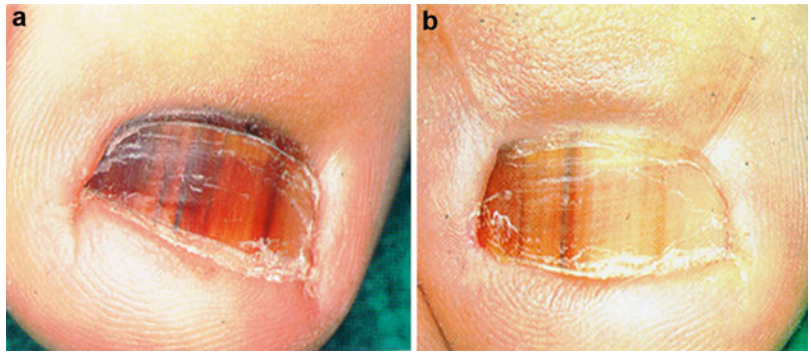

Fig. 27 Unique clinical presentation of melanocytic nevus affecting the nail of a child. This longitudinal melanonychia seen in a 10-year-old boy is broad and highly irregular, which was clinically suspected to be acral melanoma (**a**). However, 2 years later, the melanonychia mostly faded out (**b**), confirming that this was a benign nevus.

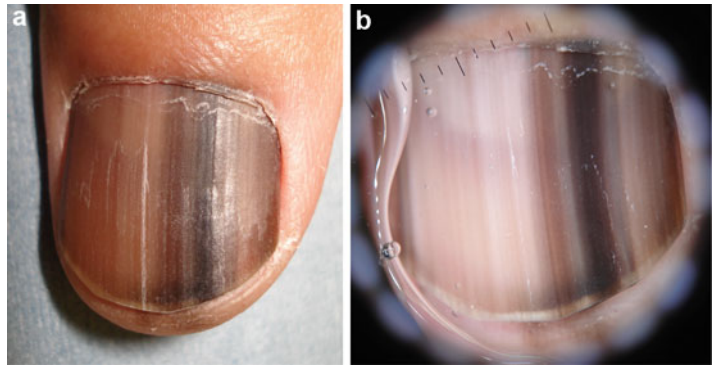

Fig. 28 Clinical and dermoscopic features of an early lesion of nail apparatus melanoma. Broad longitudinal melanonychia with some color variegation with a mostly intact nail plate (**a**). Dermoscopically, the pigmented lines constituting the melanonychia are irregularly arranged and highly variable in color and width (**b**)

not infrequently posing troubles in determining the diagnosis (Koga et al. 2011).

We have recently proposed a melanoma discrimination index for the diagnosis of early nail apparatus melanoma. The index represents randomness of colors in dermoscopic images of longitudinal melanonychia. The index is automatically calculated on a computer installed with an application we have developed. In our preliminary study, the index achieved a high level of diagnostic accuracy, seemingly superior to dermoscopic diagnosis by experts. In our study, diagnostic performance of this diagnostic method was 83% in specificity and 92% in sensitivity in the diagnosis of early nail apparatus melanoma (Koga et al. 2014). This objective evaluation system is also useful in monitoring ambiguous nail lesions seen in infants as well as in adults. While the index of benign nail lesions is static or decreases during the course, the index steadily increases in most cases of early nail apparatus melanoma during the follow-up periods.

Histopathologic Diagnosis of Nail Apparatus Melanoma

Histopathologic characteristics of nail apparatus melanoma are very similar to those of melanoma on the palms and soles. Proliferation of atypical melanocytes are detected in the epithelium of the nail matrix and nail bed as solitary units as well as in nests of variable shapes. Atypical melanocytes also extend into the dermis, arranged in nests or sheets. In advanced lesions, the nail apparatus may be focally or totally destroyed by the proliferation of atypical melanocytes, and in

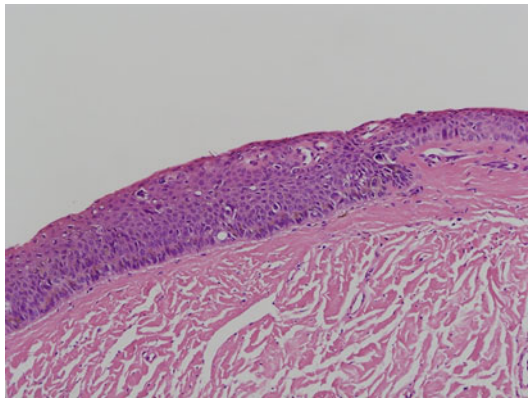

Fig. 29 Histopathologic features of early melanoma in situ affecting nail apparatus. Slightly increased numbers of melanocytes are arranged as solitary units within the epithelium of the nail bed. The melanocytes are present in the upper epithelium, and the nuclei of melanocytes are large and hyperchromatic

further advanced cases, the underlying bone tissue can be invaded and destroyed. Immunostaining using S-100 or MART-1/Melan-A is useful in diagnosing amelanotic cases.

Histopathologic diagnosis of nail apparatus melanoma in situ is sometimes very difficult. Occasionally only a slightly increased number of melanocytes are detected at the basal layer of the nail matrix and/or nail bed (Fig. 29) (Saida and Oshima 1989). Clues for diagnosis of early nail apparatus melanoma are focal upward migration of melanocytes in the epithelium, uneven distribution of melanocytes, and nuclear atypia of the melanocytes. In addition, melanocytes with long dendrites or dendrites of uneven thickness can be detected in the epithelium. Immunostaining using S-100, MART-1/Melan-A, or HMB-45 is useful to visualize the irregular distribution of melanocytes within the nail epithelium.

Management and Prognosis of Acral Melanoma

Diagnostic Workup and Staging of Acral Melanoma

The AJCC (American Joint Committee on Cancer) staging system is used also for acral melanoma. Thorough physical examination is essential at the beginning of diagnostic workup along with precise histopathologic evaluation of the primary lesion, including presence of ulceration, tumor thickness, and number of mitotic figures, which are necessary to determine the T category. Routine laboratory examinations, sentinel lymph node biopsy, MRI, and/or PET/CT scan is performed in selected cases to determine the stage of the disease.

Surgery of Acral Melanoma

Surgical treatment of acral melanoma is based on similar principles than that of melanoma in other anatomical sites, with some adaptation considering the anatomical differences of acral sites. Recommended surgical margins of a primary lesion are 3–5 mm free margin for melanoma in situ, about 1 cm margin for melanomas with 2 mm or less in thickness, and about 2 cm free margin for lesions more than 2 mm in thickness (Haigh et al. 2003). Due to functional or cosmetic considerations, the width can be reduced in certain situations.

When the primary lesions are located on the heel directly receiving body weight pressure, a medial plantar flap is used to reconstruct the tissue defect after excision of the primary lesion. If a digit has to be amputated to advanced nail apparatus melanoma, ray amputation should be considered if possible, which may contribute to better functions and cosmesis. Amputation can typically be avoided for melanoma in situ or early invasive lesions (Sureda et al. 2011). In such cases, the nail apparatus is excised including periosteum but preserving the underlying bone. Thereafter, artificial dermis is temporarily applied onto the defect until granulation tissue has formed and skin grafting can be performed. Functional and cosmetic outcomes are excellent with this approach (Fig. 30).

Conventional Chemotherapy and Radiation Therapy of Metastatic Acral Melanoma

Therapeutic guidelines for metastatic lesions of acral melanoma are basically same to those of

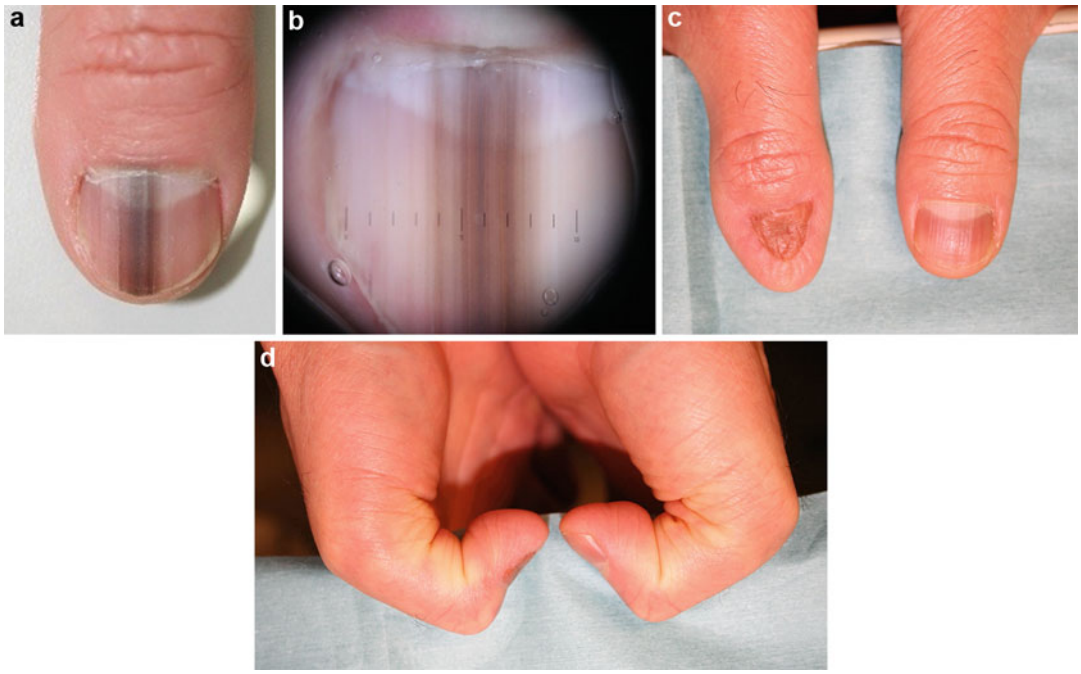

Fig. 30 Surgical treatment of melanoma in situ affecting the right thumbnail. Longitudinal melanonychia on the right thumbnail of a 42-year-old man that is narrow and without marked irregularity of pigmentation (**a**). Dermoscopy however revealed broad irregular lines (**b**), suggesting the diagnosis of melanoma in situ, which was confirmed histopathologically upon excision. Resection of the entire nail apparatus including the nail bed and nail matrix was performed along with the periosteum of the distal phalanx, but the digital bone was preserved. Artificial dermis was applied on the tissue defect after surgery. After formation of granulation tissue, full-thickness skin grafting was performed. One year after the surgery, the appearance (**c**) and function (**d**) of the thumb are excellent

other subtypes of melanoma. If metastasis is solitary or only a few in number, limited to one organ and static for a while, feasibility of surgical resection is evaluated. Surgical resection of such lesions could prolong survival time of the patients.

Clinical effect of conventional chemotherapy on the patients with advanced acral melanoma with multiple metastases is limited. For a long time, dacarbazine was a standard chemotherapeutic agent for patients with metastatic melanoma. However, the response rate with this drug is around 15–20% and long-term remissions are very rare. Various kinds of combination chemotherapy and biochemotherapy including interleukin-2 and interferon-a have been tried. Higher response rates were reported in some regimens; however, all of them failed to show significant improvement of survival. Note that these combination therapies increased incidence and severity of adverse effects.

Radiation therapy can be used as a palliative therapy. Stereotactic radiosurgery for cerebral metastatic melanoma is a choice of treatment for palliation. Pain from bone metastases can be transiently relieved with radiation therapy.

Immunotherapy and Molecular Targeting Therapy for Advanced Acral Melanoma

Among recently introduced new therapies for advanced melanoma, immune checkpoint inhibitors, such as ipilimumab and nivolumab, are effective not only for cutaneous melanomas

from sun-exposed skin but also for acral melanoma (Johnson et al. 2015). The opportunity for targeted therapy with BRAF and MEK inhibitors is limited by lower frequency of *BRAF* mutations in acral melanoma (10–20%).

KIT mutations are another therapeutic target. In 43 patients with metastatic melanoma harboring *KIT* mutation or amplification, imatinib therapy was effective, 23% overall response rate, with significantly longer survival times (Guo et al. 2011). A multicenter phase II trial of imatinib for *KIT*-mutated or *KIT*-amplified acral/mucosal melanomas revealed that overall response rates were 54% (7/13) in *KIT*-mutated melanomas, whereas no response were noted (0/11) in melanomas with KIT amplification only, indicating *KIT* gene mutations are a marker of good response with imatinib (Hodi et al. 2013). According to another preliminary trial, response rates with sunitinib were 75% (1 complete response and 2 partial responses in 4 patients) in acral/mucosal melanomas with *KIT* mutations but 17% (1 partial response in 6 patients) in those with *KIT* amplification only. A recent multicenter phase II uncontrolled trial of sunitinib showed improved survival of patients with acral/mucosal melanoma that was better than expected based on historic controls (overall disease control rate, 44%; 2-month progression-free survival, 52%). *KIT* mutation status did not influence on the effect (Buchbinder et al. 2015). Further clinical studies are necessary to confirm clinical significance and predictive biomarkers of different *KIT* inhibitors in the treatment of acral melanoma.

Prognostic Data of Acral Melanoma

Several papers have reported that acral melanoma/ALM is biologically aggressive and the prognosis of patients with acral melanoma is worse compared with other subtypes of melanoma. A recent study from a group of Memorial Sloan Kettering Cancer Center showed that prognosis of patients with acral melanoma was worse compared with that of patients with extremity non-acral melanoma (Bello et al. 2013). However, in our study of a total of 801 acral melanomas in Japan, 5-year survival rates according to the AJCC staging were as follows: stage IA, 98.1%; stage IB, 95.8%; stage IIA, 93.8%; stage IIB, 73.4%; stage IIC, 64.2%; stage IIIA, 48.0%; stage IIIB, 39.4%; stage IIIC, 44.1%; and stage IV, 16.0% (unpublished data). The survival rates in stages IIA, IIB, IIC, and IIIC appear to be slightly better than those reported in the USA, whose patients were mainly White persons suffering from SSM/non-CSD melanoma.

Conclusion

Acral melanoma is clinically and biologically distinct melanoma subtype that affects all world populations irrespective of skin complexion with similar incidence. Most acral melanomas arise de novo, rather than from melanocytic nevi. Its low mutation burden with a relative absence of UV signature mutations along with the presence of structural rearrangements and numerous copy number change including focal amplifications and deletions indicates that a distinct, yet to be discovered, mutational mechanism drives the molecular evolution of these neoplasms.

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Mucosal Melanoma

20

Tjoson Tjoa and Kevin Emerick

Contents

Abstract

Mucosal melanoma (MM) is a distinct entity with unique pathologic features and clinical behavior. It is a rare entity accounting for only 1–2% of all melanoma cases. Head and neck region mucosa comprises the majority of MMs. The nose and paranasal sinuses are by far the most common site representing 59–80% of all Head and Neck MM and oral cavity tumors representing the majority of the remaining cases. The pathogenesis of these tumors is poorly understood. Premalignant pigmented mucosal lesions have been identified and may evolve similar to cutaneous melanoma. Presentation of MM varies greatly depending on the location of the primary. Because a lesion may remain undetected for long periods of time in the nasal cavity, for example, MM commonly presents at an

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advanced stage. However, even patients who initially present with small localized disease have a high risk of distant relapse making survival rates for MM very low. The mainstay of treatment is surgical removal of the primary melanoma, which is not always possible in certain locations. While adjuvant radiation is often considered, definitive data showing clinical benefit is lacking. Recent progress in immunotherapy for cutaneous melanoma has yet to be fully assessed and understood for MM. Mucosal melanomas have distinct genetic alterations, including activating mutations of KIT, which offer an opportunity for targeted therapy in a subset of cases.

Keywords
Mucosal melanoma · Head and neck · Paranasal sinus · Pigmented mucosa · Vulvovaginal mucosa · c-KIT mutation

Introduction

Primary MMs were first described by Weber in 1859 (Weber 1859). MM account for approximately 0.8–1.8% of all melanomas in the US (Chang et al. 1998; Batsakis et al. 1982; Mendenhall 2005; McLaughlin et al. 2005), with a higher proportion in Asian and African populations. Any mucosal surface can be affected, with the majority arising from the head and neck, the anorectal, and the vulvovaginal mucosa. MM has been documented to arise in the mucosa of other tissues, including the urinary tract, the penis, gallbladder, esophagus, and intestines. However, these are exceedingly rare and it can be difficult to distinguish primary lesions of the gastrointestinal tract from metastatic lesions. MMs display distinctive behavior, epidemiology, and prognosis from cutaneous melanoma (CM), and despite a relatively stable incidence and increasing knowledge about these tumors, they continue to result in poor survival rates (Postow et al. 2012). This chapter examines the diagnostic, treatment, and outcome characteristics of MM, focusing particularly on sites within the head and neck, which comprise the majority of MMs.

Pigmented Lesions on the Mucosa

Melanin is the pigment derivative of tyrosine, synthesized by melanocytes, which typically reside in the basal cell layer of the epithelium (Westerhof 2006). Melanocytes, derived from neural crest tissue, are distributed throughout the upper respiratory tract and oral cavity. Although they are found within the mucosa of all races, they are found in much higher numbers in dark-skinned individuals (Thompson et al. 2003; Zak and Lawson 1974). While their action of cytoprotection against ultraviolet radiation is known in the skin, their role within mucosal epithelium is unclear. Though depositions of melanin within epithelium do not always have clinical consequence, both solitary and multifocal pigmented lesions should be investigated. Pathologic melanin production within the upper aerodigestive tract can be associated with a variety of etiologies, which can be reactive, neoplastic, and a result of systemic disease (Alawi 2013).

Melanotic Macule

The melanotic macule describes a single, well-circumscribed blue or brown-to-black lesion that is homogeneously colored and less than 1 cm in diameter (Meleti et al. 2008). They represent up to 86% of solitary pigmented lesions, representing the most common solitary pigmented mucosal lesion (Kaugars et al. 1993; Buchner et al. 2004). Some studies have suggested a female preponderance; however, this is not fully established. There is a predilection for the lower lip, gingiva, and palate (Alawi 2013). Functionally, they are caused by hyperactivity of the melanocytes, resulting in increased melanin production. Histologically, they are characterized by increased melanin in the basal cell layer, with incontinence of melanin into the submucosa and melanin within macrophages in the upper lamina propria (Buchner et al. 2004). There is no hyperplasia, or increase in the number of melanocytes. These lesions should be biopsied to confirm that there is in fact no hyperplasia. If melanocytic hyperplasia is found, incipient mucosal melanoma

in situ has to be considered and complete excision should be considered.

Oral Melanoacanthoma

Typically, a larger lesion than the melanotic macule, the oral melanoacanthoma is a rare, acquired lesion, often involving rapid, diffuse pigmentation of a large mucosal area (Buchner et al. 2004). It has a tendency to occur in younger black females on the buccal mucosa. It is thought to be a result of a reactive process, unrelated to the cutaneous melanoacanthoma, a form of seborrhoic keratosis, which is a benign neoplasm. These lesions are typically self-limiting and may spontaneously resolve without any intervention (Alawi 2013). However, they can grow very rapidly, so an incisional biopsy is typically required to rule out a neoplastic process. Histologically, they are characterized by spongiotic epithelium with dendritic pigmented melanocytes throughout the full epidermal thickness, with a mild inflammatory infiltrate (Alawi 2013). Once diagnosis is established, no further treatment is indicated.

Smoker's Melanosis

Cigarette smoking is known to induce oral mucosal pigmentation, though smoker's melanosis is not considered a preneoplastic condition. Rather, it is thought to represent a reactive mucosal response to the heat or irritants within a cigarette (Alawi 2013). It typically manifests as diffuse patchy irregular pigmentation along the maxillary and mandibular gingival. It occurs in approximately 21.5% of smokers, with intensity of pigmentation directly correlated to the amount and duration of cigarettes consumed (Taybos 2003). It occurs exclusively in smokers, and is more common in females. If it presents as a solitary lesion, a biopsy is indicated to rule out melanoma. Histologically, it appears very similar to a melanotic macule, with increased melanin within the basal layer of the epithelium without melanocytic hyperplasia. Cessation of smoking typically results in resolution of the pigmentation within 3–36 months (Taybos 2003).

Other Reactive Pigmentation

Hyperpigmentation can result from a variety of local reactive mechanisms. Inflammation-associated hyperpigmentation typically develops in an area of trauma or irritation. It may be focal or diffuse and patchy, and more commonly develops in dark-skinned individuals. It is characterized histopathologically by the presence of melanophages in the submucosa, and treatment is aimed at decreasing the inflammation or irritative stimulus.

Certain medications can also induce mucocutaneous pigmentation by induction of melanin production. Such medications include antimalarial drugs, phenothiazines, oral contraceptives, and various cytotoxic medications. The pigmentation may be either localized to one mucosal region or multifocal. The pigmentation is typically macular and irregular (Alawi 2013). If the melanosis can be temporally associated with the onset of a specific medication, then no further intervention or biopsy is indicated, and the pigmentation typically resolves within months after discontinuation of the drug (Dereure 2001). If a diagnosis cannot be achieved based on clinical history alone, a biopsy is warranted to rule out malignant melanoma.

Systemic Causes of Multifocal Pigmentation

Physiologic pigmentation of mucosal surfaces can commonly occur, and typically manifests as multifocal or diffuse mucosal pigmentation. However, several pathologic conditions can also result in mucosal pigmentation, including endocrinopathies and genetic disorders. Specifically, dysfunction along the pituitary-adrenal axis as a result of either adrenal insufficiency or Cushing disease can result in diffuse mucocutaneous pigmentation. This occurs because adrenocorticotropic hormone (ACTH), secreted by the anterior

pituitary gland, originates from the same gene as the alpha-melanocyte-stimulating hormone (alpha-MSH) through alternative splicing. As serum ACTH levels rise, there is a simultaneous increase in alpha-MSH secretion, which directly stimulates melanocytes.

Clinically, this manifests as diffuse mucocutaneous pigmentation, which is one of the earliest signs of hypoadrenocorticism. This is a potentially serious disease that can result in hypotension, hyponatremia, and hyperkalemia. Laboratory testing, including serum electrolyte and cortisol levels, is indicated if there is suspicion for adrenal insufficiency. Pigmentation and other signs and symptoms of this typically resolve with steroid replacement therapy. Cushing disease can result from continuous secretion of ACTH and alpha-MSH. Again, diffuse mucocutaneous pigmentation can be one of the first manifestations of this disease. Serum laboratory values will show elevated steroid levels and likely high levels of other hormones.

Peutz-Jeghers syndrome is a rare autosomal dominant genetic disorder that is characterized by intestinal polyposis and increased susceptibility for certain cancer types, including pancreatic, gastrointestinal tract, cervical, ovarian, and breast. One of the earliest clinical manifestations of the disorder in these patients is a highly distinctive labial and perioral pigmentation in a spotty pattern. Sometimes, these small, dark lentigines also involve the eye lids, nostrils, anus, and hands and feet. They appear in childhood and can persist throughout the patient's lifetime. A high index of suspicion for Peutz-Jeghers syndrome is necessary in a child with this pigmentation, and treatment is directed at symptomatic relief of gastrointestinal symptoms and cancer surveillance.

Neoplastic Pigmentation

Melanocytic nevi are a diverse group of benign tumors that arise from melanocytic proliferation induced by oncogenic mutations. They tend to occur more commonly on cutaneous surfaces, but can occasionally involve the mucosa. They are typically identified in patients over the age of 30 as a small, solitary brown or blue, well-circumscribed nodule or macule. The hard palate, buccal, and gingivolabial surfaces are most commonly affected, though any mucosal surface can be involved. Histologically, these nevi are proliferations of round, ovoid, or spindle-shaped melanocytes. Because the differential diagnosis of these focally pigmented lesions involves mucosal melanoma, biopsy is indicated for accurate diagnosis.

Mucosal Melanoma

MMs are comparatively rare, accounting for 0.8–1.8% of all melanomas in the US (Chang et al. 1998; Batsakis et al. 1982; Mendenhall 2005; McLaughlin et al. 2005), with an estimated absolute incidence of one in 2 million (Gal 2011). Interestingly, while the incidence of CM is increasing at a rate greater than any other cancer in the United States, the incidence of MM has remained stable over time (Carvajal et al. 2012). Due to its rarity, it is difficult to conduct large randomized controlled studies regarding this disease. Much of the knowledge regarding MM is based on data compiled from retrospective case series and analyses.

Epidemiology

The head and neck region comprises the majority of MM. Head and Neck MM (HNMM) can be further subtyped based on the anatomic location of the primary tumor. The nose and paranasal sinuses (SNMM) are by far the most common subsite, with large database studies and case series demonstrating SNMMs to represent 59–80% of all HNMMs (Postow et al. 2012; Patel et al. 2002; Bachar 2008; Jethanamest 2011). The oral cavity is the second most common subsite, representing 16–41% of all HNMMs. Other subtypes within the head and neck have been rarely reported, and include the nasopharynx, oropharynx, and larynx. These sites together account for less than 10% of all HNMMs (Carvajal et al. 2012). Vulvovaginal (VVMMs) and anorectal (ARMMs) melanomas

account for 18% and 24% of all MMs, respectively. Approximately 2–3% of MMs occur in the urinary tract (Chang et al. 1998; McLaughlin et al. 2005). Because of the relative infrequency of these subsites, the majority of this chapter will focus on HNMMs. Specific considerations regarding VVMMs and ARMMs will be discussed at the end of the chapter.

Unfortunately, robust epidemiological data is scarce due to the rarity of this tumor type. The current data is typically focused on specific subsites as described above. Therefore, even common epidemiological data such as age at presentation, gender, or ethnic predispositions are difficult to reliably define. The literature available shows MMs generally present within the fifth to eighth decades of life, with a median age of onset around 60–70 years old. There appears to be a female to male ratio of 1.85:1, largely due to the incidence of VVMM, which is the most common subtype in women (Postow et al. 2012). Head and Neck MM appears to occur in a similar distribution between genders, with perhaps a slight predominance in men (Chang et al. 1998; Moreno 2010; Patrick 2007; Bachar 2008). The absolute incidence of MM is higher in Whites than Blacks (2:1) (McLaughlin et al. 2005). A few publications have shown that within the oral cavity subsite, oral cavity malignancy melanoma (OCMM) is more common in Japanese. Among the Japanese population, 34% of MM occur in the oral cavity and it accounts for 7.5% of all melanomas in this population (Batsakis et al. 1982; Mendenhall 2005). By comparison, in Caucasians, OCMMs account for only around 1% of all melanomas. Similarly, in Ugandan Africans, sinonasal melanoma (SNMM) has been reported to represent 2.6% of all melanomas (Broomhall and Lewis 1967).

There is even less epidemiological data for nonHN sites. ARMMs may occur at a slightly higher rate in women than in men. However, this may be explained by earlier and more frequent detection due to routine gynecologic examinations. The median age at presentation is 70, similarly to HNMM. VVMM occurs at a rate of approximately 0.2 per 100,000 women each year. The majority occur in the vulvar mucosa, with only a 5% occurring within the vaginal mucosa. Vulvar MM tends to occur in older women, with an average age at diagnosis of 60–80 versus 50–70 in vaginal MM. The limited nature of the available epidemiological data makes it difficult to create a clear picture for HNMM, VVMM, and ARMM, respectively, but even more so for MM as a single entity.

Clinical Presentation

Many MM appear as flat, pigmented lesions on the mucosal surface. These often have characteristics similar to cutaneous melanoma such as areas of increased darkness, irregular borders, and areas with increased thickness. These are commonly isolated lesions surrounded by normal appearing pink mucosa. However, they may present similar to other cutaneous melanomas where there is a field of pigmentation and within this area a distinct increased pigmentation, nodularity, or thickness develops. However, even when the lesion appears to be isolated and surrounded by healthy pink mucosa pathologic changes are almost always seen in the surrounding tissue at the time of resection. Unlike cutaneous melanoma, MM can sometimes have an appearance more like a soft tissue mass. In the sinonasal cavity, MM may present as polyp, typically, but not always, with at least focal pigmenation. These masses can take various shapes and sizes based on their anatomic location. They are often soft and friable unlike other solid tumors. Beyond this initial description, the clinical presentation is dependent on the anatomic location.

SNMMs typically manifest as nonspecific nasal or ocular complaints. The most common presenting symptom among these SNMMs is epistaxis, with as many as 82% of patients seeking initial medical consult due to this (Batsakis et al. 1982: 82%). Other complaints at time of diagnosis are related to an enlarging mass and include nasal obstruction, facial pain and swelling, and vision changes. Proptosis, diplopia, and neurologic symptoms appear in more locally advanced tumor stages when tumors extend from the sinonasal cavity into the orbit (Lopez 2016).

Typically, nasal cavity MMs become symptomatic with epistaxis or nasal obstruction and are identified at an earlier stage than MMs of the paranasal sinuses. Paranasal sinus tumors can remain asymptomatic and difficult to visualize until tumors reach a very large size with invasion of nearby structures such as the orbit and skull base.

The most common sites of SNMMs include the lateral nasal wall and nasal septum (Thompson et al. 2003; Lopez 2016), with melanomas arising from the lateral nasal wall accounting for up to 50% of SNMM patients (Moreno 2012). Melanomas arising from the septum have a more favorable prognosis than those arising from other subsites (Dauer 2008; Moreno 2012). The middle and inferior turbinates, as well as the nasal vestibule, are other possible sites within the nasal cavity. Regarding the sinuses, the maxillary sinus is the most commonly affected cavity, followed by the ethmoid, frontal, and sphenoid, in order of decreasing incidence. SNMMs are frequently found to be a polypoid, fleshy mass within the nasal cavity (Batsakis et al. 1982). Therefore, any atypical polyp or polypoid mass in the sinonasal region warrants a biopsy.

OCMMs, which represent approximately 0.5% of all oral malignancies and <1% of total melanoma cases, are typically asymptomatic in their early stages. The hard palate and maxillary gingiva are the most frequently affected sites, but the buccal mucosa, lips, tongue, floor of mouth, and uvula can also be affected. Since these areas can be visualized by patients as well as during routine dental or medical exams, they are most commonly identified by observation rather than overt symptoms. However, some lesions due cause other symptoms that lead to the diagnosis including pain, bleeding, ulceration, and poorly-fitting dentures. In up to 10% of cases, OCMM is lacking pigment (amelanotic) (Alawi 2013; Lopez 2016). When it is pigmented, OCMM can present as a macule, plaque, or a mass. It can be either well-circumscribed or irregular. Because of its variety of appearances, any solitary pigmented lesion in the oral cavity should be biopsied. Biopsies stain positive for S-100, vimentin, and HMB-45 (Wagner 2008).

Once a suspicious lesion is confirmed upon biopsy, the diagnostic evaluation should also include flexible fiberoptic nasopharyngoscopy to optimally visualize the nasal and paranasal sinus mucosa. The primary tumor should then be further evaluated with computed tomography (CT). This can aid in assessing invasion of the surrounding bone including the skull base. Within the sinonasal cavity, magnetic resonance imaging (MRI) can be valuable in defining the locoregional extent of the tumor. Mucosal melanoma tends to have low signal on T2-weighted images and enhancement on T1 precontrast weighted images. MRI may be helpful when assessing orbit, perineural, and central nervous system invasion. These images can be critical in determining resectability (Lopez 2016). Systemic staging should also be performed at this early stage of the process before undertaking any type of surgical resection. PET CT, including the neck, chest, and abdomen, as well as a brain MRI, is the standard imaging systemic workup for these patients.

For ARMM, the most commonly affected areas include the anal canal and the anal verge/perianal area. The most common initial symptom at presentation was rectal bleeding, with other symptoms including a palpable mass, incontinence, pruritis, and a change in bowel habits. VVMM are most commonly recognized during routine exams; however, patients may note symptoms including a vulvar mass, pain, bleeding, or itching.

Tumor Behavior

It is generally reported that HNMMs exhibit highly aggressive behavior, with a median time between diagnosis and death due to disease of 19 months (Dauer 2008). This is partly due to the fact that some mucosal sites are obscured, resulting in more advanced stage at diagnosis, particularly in the paranasal sinuses (Chang et al. 1998, Papaspyrou 2011, Postow et al. 2012). Additionally, the paranasal sinus subsites are in close proximity to the skull base, orbit, and facial soft tissue. Similarly, the alveolar ridges and

palate subsites within the oral cavity typically present with early invasion of the underlying bone given the thin nature of the tissue. This may also help account for their poor prognosis (Lopez 2016)

Despite having a poor prognosis, the majority of patients with HNMM initially present with localized disease. This is especially the case in nasal cavity melanoma, where >75% are diagnosed with clinically localized disease (Lopez 2016). Overall, SNMMs present with lymphatic metastases in 6–20% of cases, and distant metastases in <10% of patients. OCMM patients are more likely to present with nodal involvement, with studies reporting up to 25% of patients having cervical lymph node metastasis at the time of presentation (refs). This likelihood increases when the tumor thickness is more than 5 mm (Patel et al. 2002).

Despite the predominance of localized disease, a metastatic disease workup should be performed at time of initial diagnosis. An additional 20% of SNMM patients can expect to develop nodal metastasis at some point during the course of the disease after initial presentation, and 40–50% will develop distant metastases. Predilection sites for distant metastasis include the lungs, brain, bone, and liver (Medhi 2012).

This high rate of distant metastasis leads to very poor survival in patients with MM. The largest database study found a 25% 5-year survival within all mucosal sites (Chang et al. 1998). Within the head and neck, 5-year overall survival (OS) ranges from 8% to 45% in the literature (Bachar 2008; Manolidis 1997; Owens 2003; Temam 2004; Gal 2011; Jethanamest, Gilligan 1991). In general, melanomas arising in the nasal cavity have a slightly better overall survival than oral cavity, likely owing to the earlier detection (Loree 1999; Wagner 2008; Jethanamest 2011). Sinonasal and nasopharyngeal MMs are associated with inferior survival rates. Various studies have implicated other factors such as age >70 years, tumor size, tumor thickness, presence of vascular invasion, nodal status, and distant metastasis status as independent factors for survival (Patel 2002; Jethanamest 2011). The median survival time after distant metastasis is detected as only 3 months (Dauer 2008).

Unlike HNMM which predominantly presents with local disease only, 61% of patients with ARMM present with regional nodal involvement, and 20% with distant disease at the time of diagnosis.. The overall prognosis is also poor in VVMM, but considerably worse in vaginal MM, with a 5-year OS of 19%, versus 50% in vulvar MM.

Staging

The rarity of HNMM has made it difficult to implement universal staging systems. In 1970, Ballentyne introduced a clinical staging system for CMs and HNMMs that comprises three stages: stage I for local disease only, stage II for regional disease, and stage III for distant disease (Ballentyne 1970). Clinical stage at presentation has been found in some studies to be a predictor of overall survival in HNMM (Patel 2002; Krengli 2006). Additionally, the advantage of this staging system is that it can be used in all MM subsites. However, due to the fact that 75% or greater of HNMM patients present with localized, stage I disease, it is difficult to establish a prognosis on this system alone.

Thompson et al. performed a clinicopathologic study and noted that the presence of metastatic disease was the most important factor in predicting patient outcome (Thompson et al. 2003). They proposed a TNM staging system for SNMM and nasopharyngeal MM. This system divided primary site into T1: single anatomic site and T2: two or more anatomic sites. Any lymph node metastasis was given N1 and distant metastasis was given M1. In this study, it was found that patient outcome was stratifiable by grouping these TNM characteristics into an overall stage (I, II, or III–IV). Around the same time, the AJCC published a staging system meant for all sinonasal malignancies, including SNMM (Table 1). This system was validated to provide an even distribution of stages with accurate, stage-specific prognostic information in a retrospective review by Moreno et al. at MD Anderson (Moreno 2012).

It was recommended that it could be used as the primary staging system for patients with SNMM. However, the obvious limitation of this system is that it is only applicable to one MM subsite.

Table 1

To remedy this, a histologic staging system has been proposed by Prasad et al. who suggested a system based on the depth of invasion into tissue compartments within the mucosa, analogous to Breslow or Clarke's levels for CM (Prasad et al. 2004). Level I was defined as in situ mucosal melanoma without invasion; Level II was invasion into the lamina propria; Level III was invasion into submucosa, bone, cartilage, or skeletal muscle. It was found that there were statistically significant differences in disease-specific survival based on these three levels. The difficulty with this system is that the level can only be determined after surgery, and, even then, histologic landmarks can be lacking and prevent accurate levels of invasion.

The most recent AJCC staging manual (7th edition, Edge 2011) includes a specific HNMM staging system (Table 2). The most striking feature in this staging system is that there is no stage I or II disease, reflecting its advanced stage at diagnosis. The staging system is applicable to all MM subsites, so it is not dependent on any specific anatomic boundaries or landmarks. All patients with regional nodal disease are automatically categorized as stage IV disease. Gal et al. performed a Surveillance, Epidemiology, and End Results tumor registry study to evaluate the AJCC staging 6th and 7th editions' impact on survival, focusing on SNMM, and advocated the use of the 7th edition. More recently, Michel assessed the prognostic value of the various staging systems for SNMM and found that the AJCC 6th edition was the only one that was significantly correlated with both overall survival and disease-free survival (Michel et al. 2014). Because of this, some recommend that the AJCC 6th edition staging system should be the main staging system for patients with SNMMs. However, this is a site-specific staging system that is not applicable to all other MM subsites, making its wide application across all MMs impossible. Clearly, a staging system that accurately reflects both the biology of MMs and the prognosis of its clinical behavior is still needed.

Table 2

Because the long-term survival of ARMM patients is very poor, the traditional Ballentyne staging of I: local, II: regional, and III: distant is commonly used, but not associated with overall prognosis. However, VVMM has been correlated with the 2002 AJCC staging system. This appears to be the best predictor of recurrence-free survival for vulvar MM. However, similar to ARMM, the Ballentyne staging system is used for vaginal MM.

Pathogenesis

MMs have no known association with sun exposure. Some MMs occur in locations with preexisting melanotic macules, but it is not firmly established where melanotic macules play any role as a preneoplastic lesions. Smoker's melanosis has been postulated as a risk factor, and it has been reported that up to 70% of HNMM patients have a history of smoking, but a direct link has yet to have been established (Reuter 1986; Postow 2012).

The pattern of somatic genetic alterations in mucosal melanoma differs from that of cutaneous melanoma (Curtin 2005). Mutations in BRAF are significantly less common (Cohen 2004, Curtin 2005; Maldonado 2003). Instead mutations in NRAS are found in 14–29% of MMs (Laurenco 2014). Activating mutations of KIT have been observed in 10–20% of cases (Curtin 2006; Satzger 2008; Carvajal et al. 2011). A small subset had SF3B1 mutations, similar as in uveal melanoma (Hintzsche et al. 2017; Hayward et al. 2017). Mucosal melanomas have a low mutation burden with UV signature mutations typically lacking. Instead, mucosal melanomas, like acral melanoma, have a high degree of structural rearrangements with a high frequency of amplifications and deletions (Curtin 2005, Hayward et al. 2017, PMID: 12508243). Frequently amplified loci include the sites of the CCDN1, CDK4, and MDM2 genes.

Treatment

Surgery

Complete surgical resection is the standard initial treatment for surgically resectable disease. However, the therapeutic strategy should be tailored individually according to tumor stage, location, and whether or not metastases are present. Establishing local control with curative intent is the goal of primary treatment without detectable metastases. However, because most patients advanced primary tumors ultimately develop metastatic disease, the patient preference and quality of life issues have to be carefully considered when considering aggressive treatment of the primary tumor.

For SNMM, wide surgical resection with postoperative radiotherapy is the common treatment. The resection has traditionally involved open approaches, including anterior craniofacial resection for tumors involving the cribriform plate, orbital exenteration for orbital involvement, and radical nasal exenteration for diffuse mucosal disease (Carvajal et al. 2012). Tumors involving the maxillary sinus require partial or total maxillectomy often extending into the adjacent structures of the orbit and nasal cavity. If the tumor does not involve the brain, masticator space, central skull base, dura, carotid artery, cranial nerves IX through XII, or the prevertebral space, the lesion is considered potentially resectable (Khalil 2014). These open surgical resections come with significant risk of complications, including wound complications, intracranial hemorrhage, cerebrospinal fluid leak, meningitis, and orbital injury (Ganly 2007). At a minimum, patients experience long-term dryness and crusting. More severe consequences are obvious from surgery such as orbital exenteration. Maxillectomy defects typically lead to a large opening from the oral cavity to the nasal cavity and maxillary sinus requiring free flap reconstruction or obturation. Cheek numbness, visual changes, enopthalmos are also possible long-term sequelae.

Over the last couple decades, there has been increasing adoption of endoscopic approaches in the management of sinonasal tumors, including SNMM. It has been shown that an endoscopic approach may reduce morbidity and improve functional outcomes, without affecting survival outcomes (Hanna 2009; Swegal and Burkey 2013; Lombardi 2016). One study with long-term follow-up after endoscopic resection reported a 5- and 10- year OS of 56% and 39%, which may represent a selection bias of smaller tumors than those that were treated by open approaches. Endoscopic approaches may not be applicable across tumors of all stages (Lund 2015; Rawal 2012). Thus, open surgery remains the gold standard for local control of SNMMs.

With regards to OCMM, open resection with gross negative margins is a common initial treatment for small and moderate size lesions. However, as previously mentioned complete systemic workup and multidisciplinary evaluation is critical prior to any surgical intervention. Fortunately, most OCMM are readily accessible via transoral approaches with limited morbidity. For larger and more extensive tumors more complex resections may be needed. This may involve a marginal or segmental mandibulectomy, partial maxillectomy, total maxillectomy with or without orbital exenteration. These types of defects also require more complex reconstruction including possible free tissue transfer. As the complexity and of resection and reconstruction increases, multidisciplinary teams must continue to weigh the morbidity of treatment versus the short and long-term control of the disease and impact on survival.

Management of regional nodal disease for HNMM is somewhat controversial. Most authors advocate surgical treatment of clinically or radiographically apparent disease. Since OCMM has a higher propensity than SNMM for nodal disease (25% vs. 6%), some advocate for upfront elective treatment of the neck in OCMM (Medina 2003; Mendenhall 2005; Krengli 2006; Wagner 2008). This involves a selective neck dissection of the submandibular and jugular chain levels II–IV lymph node regions. However, the data does not show a difference in 5-year OS in patients who do and do not recur in the lymph nodes. This has led most authors and management teams to not perform elective treatment of the clinically N0 neck (Manolidis 1997; Postow 2012). Sentinel lymph

node biopsies have been reported to assist in staging of SNMMs and may provide an alternative means of identifying those patients that would benefit from a therapeutic neck dissection (Starek 2006; Benlyazid et al. 2010). However, the radiotracer injections in the sinonasal cavity are logistically challenging and the overall utility of the findings from the procedure are not clear.

In patients with recurrent local or regional disease, in the absence of distant metastases, a second salvage surgical procedure is considered the best option if the tumor is resectable and an extensive restaging workup has been performed (Postow 2012; Lopez 2016). Surgery for recurrent disease is capable of salvaging up to 25% of patients, but failure to achieve adequate local control at the time of initial resection has consistently been shown to be a poor prognostic factor in terms of distant disease and overall survival (Lopez 2016).

Traditionally, ARMMs were managed with aggressive surgery, which involved abdominopelvic resections. However, local and distant recurrence rates remained high, and overall survival was not affected by the extent of surgical resection. Thus, the surgical treatment for these lesions has been scaled back to wide local excisions with 1 cm margins, with no significant effect on OS. It is accepted that a therapeutic lymph node dissection be performed for clinically apparent nodes, but there is no currently defined role for either an elective lymph node dissection or a sentinel node biopsy in patients with clinically negative regional disease.

As with other MM, VVMM is commonly treated with surgery as the initial treatment. Similarly, surgery was traditionally aggressive, but has transitioned to conservative approaches, as the extent of surgery does not change overall survival. Thus, a wide local excision is favored over radical vulvectomy. A regional lymph node dissection is indicated only if there are clinically apparent nodes, which occurs approximately 23% of the time at presentation. As with HNMM and ARMM, nodal status is not a prognosticator for disease-free or overall survival limiting the benefit any staging lymphadectomy. Additionally, the presence of distant metastases in the absence of nodal involvement is a well-documented phenomenon.

Radiation

Radiotherapy for MM has a very limited role. Historical response rates are very low. With the advent of new techniques enabling higher conformality, such as intensity-modulated radiation therapy (IMRT) and proton therapy, higher radiation volumes can be given to local targets while limiting toxicity and damage to surrounding structures (Lopez 2016). While this is appealing in the HN region, the lack of response by MM to radiation still makes its use in the primary treatment very limited. This has been further limited in recent years with the introduction of additional systemic treatment options. Radiation may have previously been considered in unresectable or marginally resectable tumors. However, given the available systemic treatments available this would often be a preferred treatment option.

Radiotherapy is much more commonly used as an adjuvant treatment in the postoperative setting for HNMM. However, clear indications for its usage have yet to be developed. Most agree that adjuvant radiation can improve local control when there is a positive or close margin on the initial resection (Patel 2002; Owens 2003; Temam 2005; Krengli 2006; Benlyazid et al. 2010). Others have advocated that radiation be used for perineural invasion, known regional lymphatic metastasis, and large tumors. Some have even recommended its consideration in all cases, given the aggressive nature of MM (Wagner 2008). Optimal dose and fractionation schemes have not yet been established, but evidence has shown that total dose greater than 54 Gy and hypofractionation may improve local control (Moreno 2010). If radiotherapy is to be used, the treatment volume for definitive radiotherapy should include the primary tumor with a wide margin and all of the at-risk cervical lymph node regions.

However, despite improvement in local and regional control, it has yet to be shown that postoperative radiation treatment improves either the rate of distant metastasis or OS (Manolidis 1997; Patel 2002; Meleti 2008; Wu 2010; Gal 2011).

While there may be selection bias towards more aggressive and advanced tumors, the lack of improvement in survival with adjuvant radiotherapy highlights the high risk of hematogenous spread and the poor prognosis associated with incomplete surgical resection of the primary MM. The role of adjuvant radiation may continue to decrease as better adjuvant systemic treatments become available.

Systemic Treatment

Given the high rate of recurrence and distant metastasis, even with aggressive bimodality treatment to the locoregional area, systemic therapy is always considered for these patients. Unfortunately, to date, there has been a paucity of clinical data to support chemotherapy, and no systemic therapy regimen has been proven to be effective for HNMMs (Lopez 2016). Because the rarity and poor prognosis of MM make accrual of patients for prospective clinical trials very difficult, much of the data on systemic therapies is extrapolated from work that has been done in CM. Chemotherapeutic and immunotherapeutic agents that are effective in CM have been utilized in a noncontrolled fashion as adjuvant or palliative treatment for MM without considerable success.

Until 2011, the only FDA-approved agents in the United States for the treatment of advanced melanoma of any subtype were limited to dacarbazine and high-dose interleukin (IL)-2, with limited benefit to OS associated with either drug (Carvajal 2012). These drugs were approved primarily on the basis of trials that included patients with CM, and their efficacy in MM. In a trial out of MD Anderson of 15 patients with advanced, either distantly metastatic or locoregionally recurrent HNMM, 18% of patients showed complete remission and 27% showed partial response after treatment with this combination, along with cisplatin, vinblastin, and interferon (Bartell 2008). However, due to the high toxicity and limited efficacy of this regimen, it is rarely used today. Other studies have shown that various combinations of these biochemotherapeutics are an independent predictor of overall survival, either in CM or MM (Bedikian 2008; Ahn 2010). Because of these results, systemic therapy using chemotherapeutics is currently considered in advanced, widely disseminated disease as an adjunct or for palliation (Lopez 2016).

As with all melanoma subtypes, MM has the ability to evade the natural protection of the immune system by inducing peripheral T-cell exhaustion. The development of immunotherapeutics to overcome this tumor-mediated exhaustion has been studied for decades and is beyond the scope of this chapter. In 2011, the FDA approved ipilimumab, an anticytotoxic T lymphocyte antigen 4 monoclonal antibody, based on phase 3 clinical trial data showing its benefits on overall survival in patients with CM (Hodi 2010). Unfortunately, ipilimumab was not shown to have the same benefit in a multicenter retrospective study involving metastatic or unresectable MM, with a median survival benefit of only 6.4 months (Postow 2013).

Molecular alterations that can serve as therapeutic target include mutations in BRAF and KIT. Only a small fraction of patients have BRAF V600E mutations, but those are expected to respond to a combination of BRAF and MEK inhibitors.

KIT mutations are found in approximately 15% of MMs. There are several kinase inhibitors that affect KIT, which have been tested in MM patients with KIT mutations, and inhibitors specifically targeting KIT are in development. Imatinib is an inhibitor of KIT and has demonstrated clinical activity in other cancers with KIT mutations such as gastrointestinal stromal tumors. It was first shown to have substantial benefit in patients with ARMM in isolated case reports (Lutzky, Hodi, Satzger), and has since been shown to have responses in both CM and MM patient populations with activating KIT mutations (Carvajal 2011; Guo 2011). A phase 2 study in 43 patients with unresectable MM showed that 23% of KIT-mutant MMs achieved partial response, and 30% of patients showed stable disease with a median duration of OS at 15 months in responders versus 9 months in nonresponders. 90% of the patients who demonstrated partial response had mutations in

exons 11 and 13, which appear to predict clinical response (Guo 2011).

Unfortunately, the durable response time to imatinib is relatively short, and it is unlikely as a monotherapy to offer significant curative benefit to patients with advanced MM. Several newer generation KIT inhibitors targeting specific alterations are currently in phase 2 trials, including dasatinib, sorafenib, nilotinib, sunitinib, and masitinib. Despite encouraging initial results, more understanding of the biological significance of each KIT mutation is needed in order to characterize the responses to each inhibitor. In addition, longitudinal studies and prospective trials are essential to determine mechanisms of resistance and the long-term prognostic implications of these treatments. Ultimately, a combination of targeted agents to a variety of signaling pathways along with immunotherapies will likely be necessary to gain adequate effectiveness in the systemic treatment of advanced metastatic MM.

Conclusion

Overall, MM is a rare form that is distinct from cutaneous melanoma, with its characteristic genetic alterations, clinical behavior, and epidemiology. It is highly aggressive, and despite multimodality approaches, has a very poor 5-year overall survival due to high rates of recurrence and hematogenous spread. No single staging system based on disease characteristics has yet succeeded in stratifying its overall prognosis. Current standard of care is surgical management of the primary lesion and clinically apparent regional disease, with or without postoperative radiotherapy. The role of immune checkpoint blockade effective in other melanoma subtypes has yet to be established and the characterization of molecular targets for therapy is ongoing.

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Ocular Melanoma

21

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Abstract

Approximately 5% of all melanomas arise in the eye. Of these ocular melanomas, about 97% are of uveal origin and 3% are conjunctival. Uveal and conjunctival melanomas are biologically and clinically distinct diseases. Uveal melanomas are mostly initiated by GNAQ and GNA11 mutations and progress via SF3B1, EIF1AX, and BAP1 mutations, the latter greatly enhancing metastatic potential. Conjunctival melanomas are biologically similar to cutaneous melanomas, with common mutations of BRAF or NRAS as initiating mutations. The first choice of treatment for primary uveal melanomas is radiotherapy in most centers, whereas conjunctival melanomas are treated by excision with adjunctive radiotherapy and/or topical chemotherapy. With uveal melanomas, the prognosis for survival is mostly based on genetic studies, whereas with conjunctival melanomas tumor size and location are more predictive. There currently is no effective treatment for metastatic uveal melanoma, whereas conjunctival melanomas, like cutaneous melanomas, can be treated with targeted therapy and/or immunotherapy.

Keywords

Ocular melanoma · Uveal melanoma · Conjunctival melanoma · Targeted therapy · Immunotherapy · Genetics · Histology · Diagnosis · Treatment

Introduction

Approximately 5% of all melanomas are ocular, with about 97% of these occurring in the uvea, within the eye, and the remainder arising in conjunctiva. The two kinds of tumor are genetically distinct and behave very differently from each other, with uveal melanomas showing similarities to blue nevus-like melanoma and primary leptomeningeal melanomas and conjunctival melanomas being similar to cutaneous melanoma (Shain and Bastian 2016).

The aims of this chapter are to describe the molecular and clinical pathology of ocular melanomas and to discuss the management of patients with these tumors.

Epidemiology

The incidence of uveal melanomas in the USA is approximately 6 per million per year and is stable, unlike conjunctival melanomas, whose incidence is increasing (Larsen 2016). Both kinds of melanoma are most common in individuals with a fair complexion and both affect men and women in equal numbers (Damato and Coupland 2012; Larsen 2016). Presentation peaks at around 60 years and is rare before adulthood. Risk factors for uveal melanoma also include congenital ocular

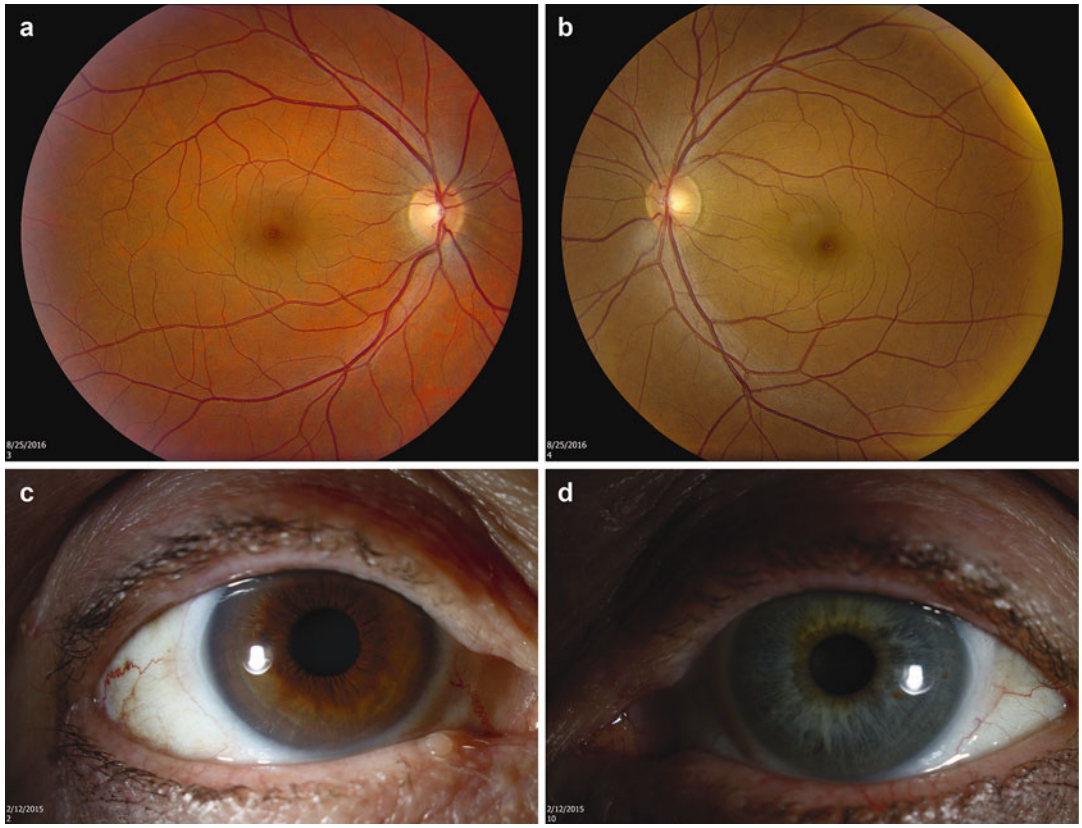

Fig. 1 (**a**) Right and (**b**) left fundus of a 36-year-old man with congenital ocular melanosis of the left eye. (**c**) Right and (**d**) left irides of a 69-year-old woman with congenital ocular melanocytosis of the right eye

melanocytosis (Fig. 1), uveal melanocytoma (Fig. 2a, b) and other nevi (Fig. 2c, d), and germline mutations in the BAP1 (BRCA1-associated protein 1) tumor suppressor gene (Wang et al. 2016). A meta-analysis did not find chronic ultraviolet exposure to be a significant risk factor for uveal melanoma (Shah et al. 2005). The growing incidence of conjunctival melanomas has been attributed to tumors in sun-exposed conjunctiva (i.e., bulbar conjunctiva, plica, and caruncle).

Molecular Pathology

Uveal Melanoma

GNAQ and GNA11 mutations are believed to be the first genetic alterations in the pathogenesis of uveal melanoma and are present in approximately 85% of tumors (Van Raamsdonk et al. 2009). These mutations activate the MAPK (mitogen-activated protein kinase) signaling pathway as well as the Hippo, PKC (protein kinase C), and AKT (v-akt murine thymoma viral oncogene) pathways. Less common mutations in this pathway include the G-protein coupled leukotriene receptor, CYSLTR2, immediately upstream of GNAQ/11 and in the downstream effector, PLCB4 (phospholipase C beta 4) (Moore et al. 2016). Mutations in these four genes occur in a mutually exclusive pattern. Without additional alterations in other genes they induce benign neoplastic melanocytic proliferations, which include uveal nevi. These nevi are biologically similar to cutaneous blue nevi of the skin and melanocytomas of the central nervous system (Shain and Bastian 2016).

BAP1 is associated associated with a high risk of metastasis from uveal melanoma (van Essen et al. 2014). BAP1 is a nuclear deubiquitinase (DUB), which acts on lysine 119 of histone 2A as part of a complex (PR-DUB) (Wang et al. 2016). Disruption of the PR-DUB complex by loss of BAP1 is thought to result in altered histone modifications and a deregulated gene expression pattern. BAP1 is located at 3p21.1, and biallelic BAP1 inactivation is the major selective force behind chromosome 3 loss in uveal melanoma. Some patients with uveal melanoma have a germline BAP1 mutation and are predisposed to a variety of tumors, such as mesothelioma, cutaneous melanoma, atypical Spitz tumors, thyroid cancer, and renal cell carcinoma (Wang et al. 2016).

SF3B1 (splicing factor 3B subunit 1) is associated with a relatively good prognosis in patients with uveal melanoma (Harbour et al. 2013). It influences the splicing of precursor mRNA and hence the production of mature RNA; SF3B1 mutations cause aberrant splicing to result in abnormal cell proliferation and differentiation. Recurrent SF3B1 mutations occur in approximately 20% of uveal melanomas and in myelodysplastic syndrome, chronic lymphocytic leukemia, breast cancer, and pancreatic cancer.

EIF1AX (eukaryotic translation initiation factor 1A, X-linked) is located on the X-chromosome. EIF1AX is of critical importance in the initiation of transcription, stimulating the binding of Met-tRNAi to the small (40S) ribosomal subunit and the formation of the preinitiation complex. The precise effects of EIF1AX mutations on cellular processes are currently not understood. In any case, these aberrations are associated with a good prognosis in patients with uveal melanoma (Decatur et al. 2016).

PRAME (preferentially expressed antigen in melanoma) enhances cancer cell growth by suppressing retinoic acid receptor (RAR) signaling (Epping et al. 2005). RAR normally induces proliferation arrest, differentiation, and apoptosis. PRAME was originally discovered as a melanoma antigen and was later found to be expressed in several other cancers but not nevi or normal tissues, except testis. PRAME expression in uveal melanomas is associated with poor survival probability (Field et al. 2016).

NRAS (neuroblastoma rat sarcoma viral oncogene homolog), CDKN2A (cyclin-dependent kinase inhibitor 2A), and BRAF (rapidly associated fibrosarcoma, homolog B) mutations are absent in ciliary body and choroidal melanomas. Genetic studies on iris melanomas are limited because of the rarity of these tumors but appear to show similar findings to posterior uveal melanomas (Krishna et al. 2016). Interestingly, one study has reported BRAF mutations in some iris melanomas (Henriquez et al. 2007).

Conjunctival Melanoma

BRAF mutations are present in approximately 30–50% of conjunctival melanomas and in 50% of conjunctival nevi. They activate the MAPK pathway. BRAF V600E and V600K have a ratio of about 4 to 1. These mutations are more common in sun-exposed conjunctiva (i.e., bulbar conjunctiva) and are in keeping with evidence of UV-induced DNA damage in these tumors (Larsen et al. 2016). They are also more common in conjunctival melanomas arising from nevi than from conjunctival melanocytic intra-epithelial neoplasia (CMIN) (Larsen et al. 2016). BRAF mutations in conjunctival melanomas are more prevalent in younger individuals, particularly males (Larsen et al. 2016). They have no prognostic value but indicate likely responsiveness to drugs such as vemurafenib and dabrafenib.

Activating KIT mutations, encoding receptor tyrosine kinase, are rare in conjunctival melanomas arising in Caucasians but more common in ethnic Chinese patients (Sheng et al. 2015). NRAS is reported in 20% of cases (Griewank et al. 2013).

Gene Expression Profile

Uveal melanomas have been categorized as class 1a, class1b, and class2 according to their gene expression profile; these classes respectively indicating minimal, intermediate, and high risk for

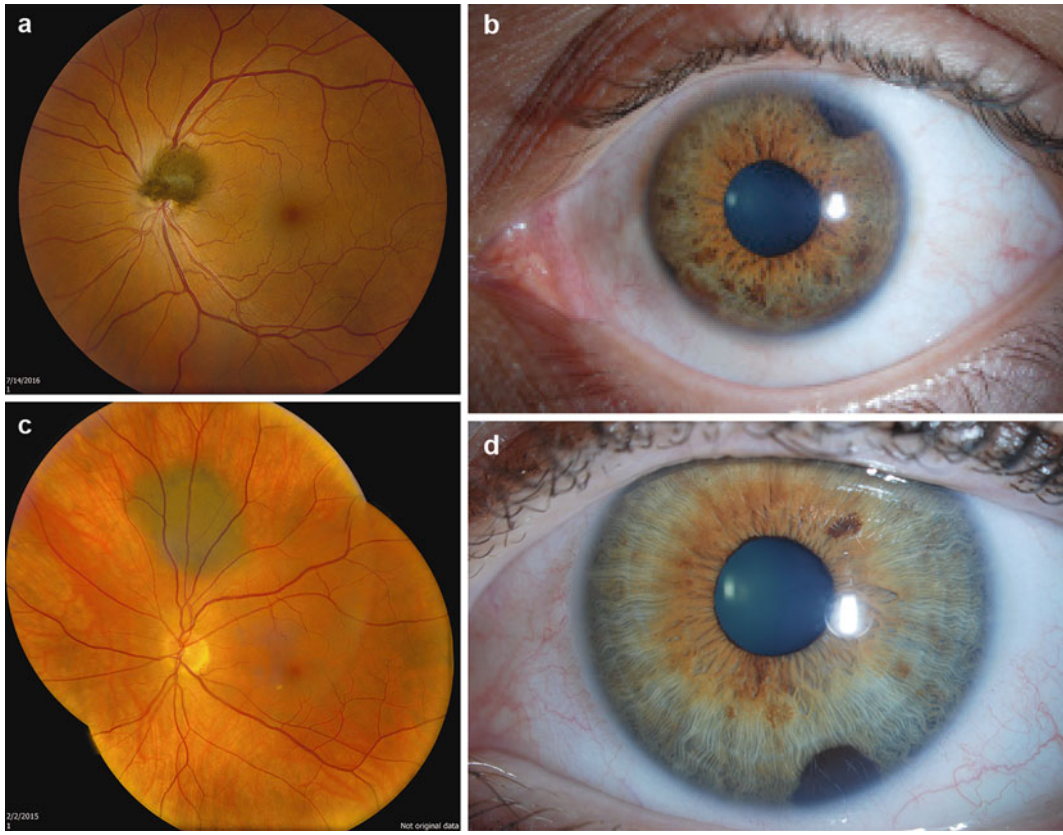

Fig. 2 (**a**) Melanocytoma of the left optic disk in a 48-year-old man. (**b**) Melanoctyoma of the right iris with histologically proven transformation to melanoma in a 30-year-old man. (**c**) Choroidal nevus, with a flat, featureless surface, in a 68-year-old woman. (**d**) Iris nevus in a 46-year-old woman

metastasis (Onken et al. 2004). It has recently been reported that metastatic disease in class1 tumors is associated with PRAME expression (Field et al. 2016).

To the authors' knowledge, no clinically relevant gene expression profiles have been established in conjunctival melanomas.

Cytogenetic Abnormalities

Uveal melanomas develop several recurring cytogenetic abnormalities, which include partial or total chromosome 3 loss ("monosomy 3"), isodisomy 3, isochromosome 8q, isochromosome 6p, chromosome 1p loss, and chromosome 9p loss (Coupland et al. 2013). Metastatic disease occurs almost exclusively in patients with chromosome 3 loss, which is associated with high mortality. The survival probability is worse when monosomy 3 and chromosome 8q gain coexist (Damato et al. 2010). Conversely, chromosome 6p gain is associated with a relatively good prognosis (Damato et al. 2010).

Conjunctival melanomas show the same cytogenetic abnormalities as cutaneous melanomas (Griewank et al. 2013).

Histopathology

Uveal Melanoma

Histologically, uveal melanomas are categorized as "spindle-cell," "epithelioid," or "mixed." Epithelioid cytomorphology is associated with more

aggressive disease and higher mortality; however, the distinction between spindle and epithelioid melanoma cells can be inconsistent and there is no consensus as to the number of epithelioid cells required for classification as "mixed" or "epithelioid." Mitotic counts correlate with mortality but there is inter-observer variability, particularly when special stains are not deployed. Uveal melanomas show a variety of "extravascular matrix patterns," with "closed loops" being associated with the highest mortality (Kivelä et al. 2004). Other factors known to be associated with higher mortality include IGF-1 (insulin-like growth factor 1) receptor upregulation, microvascular density, and increased accumulations of tumor-infiltrating lymphocytes (de la Cruz et al. 1990; Makitie et al. 1999, 2001; All-Ericsson et al. 2002; Al-Jamal and Kivelä 2011). Immunohistochemical staining for BAP1 protein is being performed more widely and may be superior to mutation analysis in identifying some lethal tumors (Kalirai et al. 2014).

Conjunctival Melanoma

Most conjunctival melanomas develop from CMIN, the remainder arising from nevi or *de novo*. CMIN can occur with or without atypia (Damato and Coupland 2008a, b). In the absence of atypia, the melanocytes have normal nuclei and are all located in the basal layer of the epithelium. Atypia is said to be present when the melanocytes develop cytomorphological features of malignancy (i.e., large nuclei, prominent nucleoli, abundant cytoplasm, and mitotic figures) and when they invade the more superficial epithelial layers, initially as isolated cells, then forming clumps, and eventually replacing the entire epithelium. To avoid ambiguous terms such as "mild," "moderate," or "severe," a scoring system has been developed, which grades the degree of malignancy according to the pattern of melanocytic proliferation, extent of vertical spread, and degree of cellular atypia (Damato and Coupland 2008a, b). A score of 0 indicates no cellular atypia, whereas a score of 5 or more suggests melanoma *in situ*, which corresponds to confluent proliferation of atypical melanocytes involving more than 50% of the thickness of the epithelium.

Invasive conjunctival melanomas are described histologically according to tumor thickness, surface ulceration, cytomorphology, mitotic count, microsatellites, as well as vascular and lymphatic invasion (Damato and Coupland 2008a, b; Larsen 2016). Recognition of melanoma cells is assisted by immunohistochemistry using melanocyte markers such as S100, human melanoma black 45 (HMB-45), SRY-box containing gene 10 (SOX-10), and Melan-A. BRAF V600E oncoprotein can be detected by immunohistochemistry in recent samples but molecular analysis is preferred because this also detects other BRAF mutations such as V600K (Larsen et al. 2016).

Clinical Features of the Primary Tumor

Uveal Melanoma

Approximately 90% of all uveal melanomas involve the choroid, the remainder being confined to ciliary body and/or iris (Damato and Coupland 2012). Choroidal melanomas are grey or brown, even if amelanotic, because of proliferation of the overlying retinal pigment epithelium (RPE). RPE disruption causes serous retinal detachment and accumulation of lipofuscin ("orange pigment") (Fig. 3a). These features help distinguish large, benign nevi from small, malignant melanomas. Choroidal melanomas tend to be dome-shaped, multinodular, or diffuse (Fig. 3b). If they rupture Bruch's membrane (i.e., the rigid basement membrane of the RPE), they grow into the retina, developing a pathognomonic mushroom shape. Ciliary body melanomas can press on the lens, to cause cataract, and they can invade the anterior chamber (Fig. 3c). Iris melanomas are mostly nodular, the few diffuse tumors showing higher mortality (Fig. 3d). At any stage, uveal melanomas can spread extraocularly into the orbit or subconjunctival tissues, through

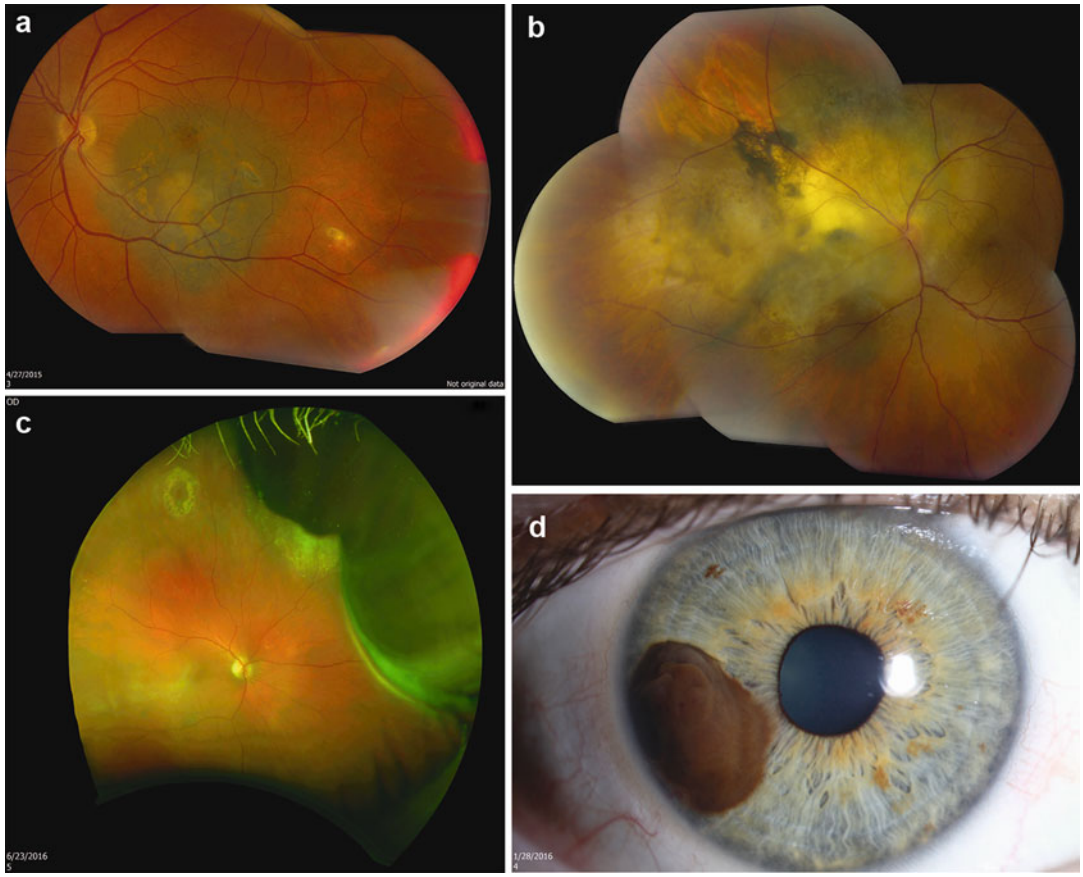

Fig. 3 (**a**) Dome-shaped choroidal melanoma, with clumps of orange (lipofuscin) pigment in a 60-year-old man. (**b**) Diffuse choroidal melanoma surrounding the left optic disc in a 52-year-old woman. (**c**) Large, superonasal ciliary body melanoma in the right eye of a 72-year-old woman. (**d**) Large iris melanoma in the right eye of a 30-year-old man

channels for arteries, veins, and nerves (Coupland et al. 2008). Although many choroidal melanomas encircle the optic disc, it is extremely rare for them to invade the optic nerve itself. If left untreated, uveal melanomas can result in a blind, inflamed, and painful eye, with proptosis if there is extensive extraocular spread.

Most patients present with symptoms such as blurred or distorted vision, visual field loss and photopsia, or the perception of light flashes (Damato 2001). About 40% are asymptomatic and detected on routine examination. Approximately 23% of all symptomatic patients report that their tumor was initially missed and these patients are more likely to lose useful vision and the eye. Further studies are needed to determine whether they are also more likely to die of metastatic disease.

Conjunctival Melanoma

Clinically, conjunctival melanosis appears as one or more brown areas of conjunctiva not present at birth and not secondary to systemic disease. Such "primary acquired melanosis (PAM)" has irregular margins and variable degrees of pigmentation (Fig. 4a). It is not possible to determine whether or not atypia is present unless biopsy is performed. CMIN must be distinguished from the scleral

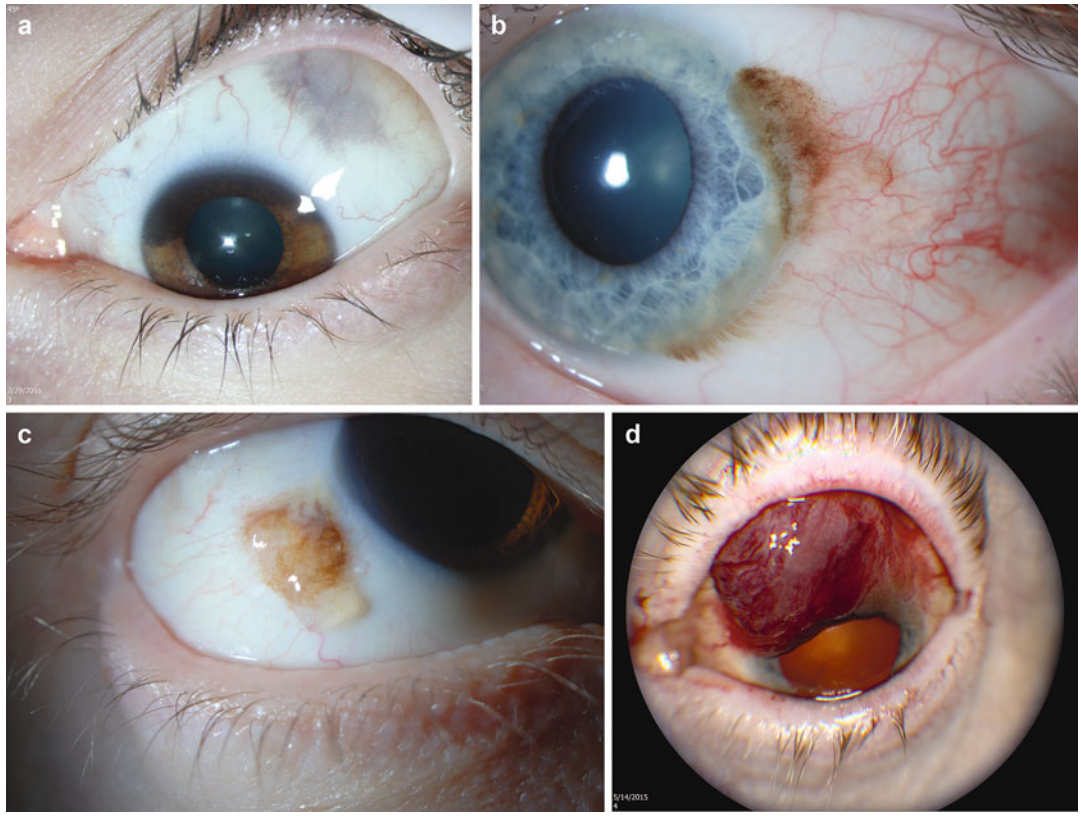

Fig. 4 (**a**) Congenital ocular melanocytosis of the left eye of a 39-year-old woman, with scleral pigmentation superotemporally and iris hyper-pigmentation superiorly. (**b**) Primary acquired melanosis of the left eye, which was histologically shown to consist of conjunctival melanocytic intraepithelial neoplasia with severe atypia in a 57-year-old man. (**c**) Conjunctival nevus of the right eye of a 49-year-old man, with intralesional cysts. (**d**) Large melanoma arising in the superior bulbar conjunctiva of the left eye of a 76-year-old woman. The patient was successfully treated by local excision and adjunctive radiotherapy

pigmentation of congenital ocular melanocytosis (Fig. 4b) and from nevi, which are usually nodular with visible intralesional cysts (Fig. 4c).

Invasive conjunctival melanomas can be: nodular or diffuse; deeply pigmented, lightly pigmented, or amelanotic; unifocal or multifocal; and with or without adjacent melanosis (Fig. 4d). Feeder vessels are usually present. Most tumors are located in the bulbar conjunctiva, usually involving the limbus (Damato and Coupland 2008a, b). Less commonly, tumors develop in the palpebral or forniceal conjunctiva, plica semilunaris, or caruncle. Regional lymph nodes may be affected at presentation. Advanced tumors can invade the eyelids, orbit, nasolacrimal duct, nasal sinuses, and even the intracranial cavity. Intraocular invasion is rare, unless the protective Bowman's layer in the cornea has been disrupted by previous surgery.

Ocular Investigations

Uveal Melanoma

Most uveal melanomas are diagnosed by slit-lamp examination or ophthalmoscopy. With choroidal melanomas, auto-fluorescence imaging reveals lipofuscin pigment and optical coherence tomography demonstrates subretinal fluid, both of which suggest malignancy. Ultrasonography is useful for measuring tumor dimensions and demonstrating

any extraocular extension, occasionally helping to distinguish melanomas from other kinds of tumor by virtue of their internal acoustic reflectivity. When these investigations are inconclusive, it is conventional to delay treatment for months or years until growth has been documented by sequential imaging; however, the risk of metastatic spread occurring during such procrastination is unknown. In some centers, therefore, transretinal biopsy is offered to the patient, relying on cytology and genetic findings to differentiate between melanomas and nevi (Sen et al. 2006).

Conjunctival Melanoma

CMIN and invasive melanoma are documented by drawings and color photography (Damato and Coupland 2008a, b).

Incisional biopsy of CMIN is required to determine the degree of malignancy; however, incisional biopsy of nodular melanomas has been associated with increased mortality and is therefore contraindicated (Larsen 2016). Unfortunately, many patients reach an ocular oncology center (often with local seeding) only after their local ophthalmologist has performed inexpert excision (i.e., without following a no-touch technique and without using fresh instruments for wound closure) or after incisional biopsy.

Some centers perform sentinel node biopsy to aid decisions regarding systemic surveillance, neck dissection, and participation in systemic adjuvant therapy trials (Aziz et al. 2015). The value of this procedure in patients with conjunctival melanoma has yet to be demonstrated.

Ocular Treatment

Uveal Melanoma

In most centers, the first choice of treatment is radiotherapy, consisting of plaque brachytherapy, proton beam radiotherapy, or some form of stereotactic radiotherapy (Stannard et al. 2013). If the tumor is not treatable with such methods, then enucleation is performed (Damato and Lecuona 2004). In some centers, there is expertise for transretinal endoresection or transscleral exoresection, which may be preferred for juxtapapillary and large tumors, respectively (Damato et al. 2013). Phototherapy, consisting of transpupillary thermotherapy or photodynamic therapy, is mostly administered as an adjunct to radiotherapy, to reduce exudation from the irradiated tumor. Other treatment for such morbidity includes intraocular injection of antiangiogenic agents and surgical resection of the irradiated "toxic tumor." Where choice exists, treatment selection is personalized according to the tumor features as well as the needs, wishes, and fears of the patient (Damato and Heimann 2013).

Ocular conservation is attempted in approximately 70% of patients and is successful in about 90% of cases. The chances of conserving useful vision depend on tumor size and location, with the best results occurring with small tumors not extending close to optic nerve and fovea.

The impact of ocular treatment on survival has been debated for centuries. In the 1970s, Zimmerman, an eminent US pathologist, hypothesized that enucleation accelerated metastatic death (Zimmerman et al. 1978). At the same time, Manschot, an equally respected Dutch pathologist, condemned radiotherapy of uveal melanomas as unsafe (Manschot et al. 1995). To resolve this dispute, the Collaborative Ocular Melanoma Study (COMS) conducted two randomized clinical trials to determine whether pre-enucleation radiotherapy prevented metastasis and whether plaque radiotherapy was as effective as enucleation in prolonging life (Hawkins and Collaborative Ocular Melanoma Study 2004; Collaborative Ocular Melanoma Study 2006). Lack of any significant statistical differences between the treatment arms profoundly influenced clinical care, promoting radiotherapy for medium-sized tumors and discrediting pre-enucleation radiotherapy. However, both studies were statistically inconclusive because so many patients should have been excluded, because they died soon after treatment, indicating that metastases must already have been present at the time of diagnosis (Damato 2007). When cytogenetic data became available, some authors

suggested that disomy-3 and monosomy-3 melanomas were distinct from their inception (Parrella et al. 1999; Tschentscher et al. 2003; Hoglund et al. 2004). This implied that ocular treatment was only palliative. Callejo et al. (2011) reported a patient whose tumor appeared to transform from disomy 3 to monosomy 3 while under observation (Callejo et al. 2011). Local tumor recurrence is associated with increased mortality (Ophthalmic Oncology Task 2016). Whether local treatment failure causes metastatic disease or whether it merely indicates higher grade of malignancy is debatable. Procedures such as endoresection without neoadjuvant radiotherapy are still controversial because of concerns that the piecemeal tumor removal might cause metastasis (Damato 2008). Many of these controversies persist because there is debate as to whether metastatic spread occurs early or late, that is, before or after a tumor grows large. Data from cytogenetic studies suggest that chromosomal abnormalities accumulate in random order, suggesting that monosomy 3 develops early in some patients, late in others, and not at all in a fortunate minority (Damato et al. 2010). According to this hypothesis, treatment prevents metastatic spread only if administered early in the few patients whose tumor would otherwise develop monosomy 3 and start metastasizing at a relatively late stage.

Conjunctival Melanoma

CMIN with atypia is treated by excision if resectable. Cryotherapy for extensive CMIN has been replaced by topical mitomycin C chemotherapy.

Invasive conjunctival melanomas are excised. Surgical resection alone or with cryotherapy is followed by high recurrence rates in the region of 30–60% (Shields et al. 2000). Such local treatment failure is associated with increased mortality (Larsen 2016). Adjunctive radiotherapy and topical mitomycin C therapy are highly effective for deep tumor remnants and pagetoid spread, respectively (Damato and Coupland 2009a). Good vision is usually retained, unless limbal stem cell deficiency results in corneal opacification. Exenteration for uncontrollable disease has become rare.

Prognostication

Uveal Melanoma

As with other cancers, prognostication identifies high-risk patients with special needs (e.g., counseling, more intensive surveillance, and enrolment in any clinical trials evaluating systemic adjuvant therapy), while reassuring patients with favorable survival prospects (Damato et al. 2011).

Conventionally, prognostication for patients with choroidal and/or ciliary body melanoma is based on the anatomic extent of the tumor, using the TNM (Tumor, Node, Metastasis) staging system of the AJCC (American Joint Committee on Cancer) (Kujala et al. 2013). This method lacks accuracy, however, because tumors within a particular TNM category vary greatly in their histologic degree of malignancy and their lethality as determined by genetic studies (Damato and Coupland 2009b)

Since 1999, one of the authors (BD) has offered patients genetic tumor typing to inform them whether their tumor has metastatic potential. Initially, Damato and associates in Liverpool, UK, used FISH (fluorescence in situ hybridization) but this was replaced by MLPA (multiplex ligation-dependent probe amplification), which provides more data using smaller samples, and by MSA (microsatellite analysis) when tumor samples were insufficient for MLPA (Damato et al. 2007). Elsewhere, genetic typing is performed with gene expression profiling, array comparative genomic hybridization (CGH), and other methods. Such genetic tumor typing enables surveillance for metastatic disease to be individualized according to risk, sparing patients from unnecessary investigations.

In patients undergoing radiotherapy, biopsy for prognostic tumor analysis is performed transretinally or transsclerally, depending on tumor location, either with a fine-needle aspiration or a vitreous cutter. The most common complications are insufficient sampling and vitreous hemorrhage, with rare problems including endophthalmitis, retinal detachment, and tumor seeding (Raja et al. 2011; Grixti et al. 2014; Sellam et al. 2016). One of the authors (BD) has performed genetic analysis of tumor samples obtained soon after proton beam radiotherapy, with apparent success (Hussain et al. 2016).

Further studies are needed to validate these results with other forms of radiotherapy and other genetic tests.

Damato and associates in Liverpool, UK, have developed an online tool that enhances prognostic accuracy by multivariate analysis that includes TNM stage, histologic grade of malignancy, and genetic tumor type, also taking account of normal life expectancy as estimated by the patient's age and sex (www.ocularmelanomaonline.org) (Eleuteri et al. 2012). This has recently been validated at UCSF (Sarah DeParis et al. 2016).

With iris melanomas, risk factors for metastasis include diffuse spread, irido-corneal angle involvement, and secondary glaucoma. Further studies are needed to determine the prognostic value of genetic analysis of iris melanomas (Krishna et al. 2016).

Conjunctival Melanoma

Clinical predictors of metastasis include large tumor size and nonbulbar conjunctival involvement, particularly caruncular tumor location, which form the basis of the TNM staging system (Tuomaala et al. 2002; Damato and Coupland 2008a, b). Mortality is also higher in patients who develop local tumor recurrence (Larsen 2016). Histologic predictors of metastasis include epithelioid cytomorphology, high mitotic count, and lymphatic invasion (Larsen 2016). As mentioned, the role of sentinel lymph-node biopsy has yet to be determined, since improved survival with this approach has not been conclusively demonstrated (Aziz et al. 2015). BRAF mutation has been associated with higher mortality, but only in univariate analysis (Larsen et al. 2016). Identification of BRAF status is useful in predicting response to treatment with BRAF inhibitors, such as vemurafenib.

Metastatic Disease

Uveal Melanoma

Nearly 50% of patients with uveal melanoma develop metastatic disease, which usually develops from the second postoperative year onwards. Less than 1% of all patients have detectable metastases when their ocular tumor is diagnosed and treated. In over 90% of patients, metastatic disease involves the liver, less common sites being the lung, skin, and bone (Collaborative Ocular Melanoma Study 2001). An algorithm has been developed to predict the length of survival in such patients (Kivelä et al. 2016).

Conjunctival Melanoma

Like their cutaneous counterparts, conjunctival melanomas frequently metastasize to the regional lymph nodes which is associated with a poor prognosis. Up to 30% of patients develop systemic metastases alone without any clinical evidence of regional metastases (Missotten et al. 2005).

Systemic Investigations

Uveal Melanoma

As metastatic disease almost always presents with liver involvement, most centers image only the liver without performing chest radiography or any other systemic imaging, if there are no suspicious symptoms. Liver function tests have low sensitivity, becoming abnormal only when hepatic metastases are advanced. Liver ultrasonography is operator dependent and may fail if the patient is obese. CT and PET/CT expose patients to ionizing radiation, which is cumulative. MRI performed every 6 months has been shown to detect metastatic disease in 92% of patients before the onset of symptoms, with almost half these patients having fewer than five lesions measuring less than 2 cm in diameter (Marshall et al. 2013).

It is conventional practice to screen for systemic metastases before starting ocular treatment; however, unless the presence of metastases is likely to alter patient management, one of the authors (BD) prefers to perform such investigation preoperatively only if a tumor diameter exceeding 17 mm indicates an increased risk of metastasis. If metastatic disease is found, the main

objective of ocular treatment is to remove any risk of the eye becoming acutely painful when the patient has developed terminal illness.

Evidence-based guidelines for systemic surveillance have been prepared in the UK (Nathan et al. 2015). Nevertheless, there is no consensus as to which patients should be investigated, how often, and for how long. Some oncologists recommend the same surveillance program to all patients and others restrict this to patients considered to be at high risk of metastasis because of large tumor size and/or lethal genetic aberrations in the tumor.

There is little if any evidence that systemic surveillance ever results in prolongation of life (Kim et al. 2010; Augsburger et al. 2011). However, early detection enhances prospects for participation in any ongoing clinical trials. A normal liver scan reassures patients that they are unlikely to develop symptomatic metastases within the next few months, even if genetic typing indicates that their prognosis is poor.

Conjunctival Melanoma

Surveillance after treatment of conjunctival melanoma follows the same principles as cutaneous melanoma.

Treatment of Metastatic Disease

Uveal Melanoma

A wide variety of treatments have been evaluated for metastatic uveal melanoma (Blum et al. 2016; Chattopadhyay et al. 2016; Goh and Layton 2016). Briefly, these include: (1) hepatic therapy (e.g., metastatectomy, radiofrequency ablation, intraarterial chemotherapy, isolated hepatic perfusion, yttrium-90 radioembolization); (2) systemic chemotherapy (e.g., fotemustine, temozolamide); (3) targeted therapies (e.g., tyrosine kinase inhibitors, sunitinib and sorafenib, as well as MEK (mitogen-activated protein kinase) inhibitors, selumetinib and trametinib); and (4) immunotherapy with CTLA-4 (cytotoxic T-lymphocyte associated protein-4) and PD-1 (programmed death ligand-1) inhibitors, such as ipilimumab, nivolumab, and pembrolizumab.

To the authors' knowledge, no studies have demonstrated prolonged survival except perhaps after resection of isolated hepatic metastases (Gomez et al. 2014). It is uncertain whether such apparently good outcomes after hepatic metastatectomy are the result of treatment, patient selection, or less aggressive malignancy.

Following impressive results with cutaneous melanoma, there were hopes that immune checkpoint blockade would be equally effective with uveal melanomas, but results have been disappointing (Blum et al. 2016). Such reduced efficacy may be caused by the relatively low neoantigen expression on uveal melanoma cells, possibly related to the small number of mutations in these tumors. Another possible explanation is ocular immune privilege, which influences immunoresponsiveness, not only intraocularly but also systemically (Oliva et al. 2016).

Animal studies suggesting that IGF-1R may be a useful therapeutic target have led to a clinical trial evaluating an anti-IGF-1R monoclonal antibody, cixutumumab, the results of which are awaited (Goh and Layton 2016). Various antiangiogenic agents, such as bevacizumab and axitinib, are also being investigated

To the authors' knowledge, no completed trials have shown any benefit from systemic adjuvant therapy, with agents such as fotemustine, checkpoint inhibitors, and autologous dendritic cells. Some studies have failed because they selected patients with large tumors, not all of which would have had monosomy 3 or a class 2 gene expression profile (Voelter et al. 2008). Several studies are ongoing, assessing agents such as ipilimumab, sunitinib, crizotinib, and HDAC (histone deacetylase) inhibitors, such as vorinostat (Blum et al. 2016; Chattopadhyay et al. 2016; Goh and Layton 2016).

Conjunctival Melanoma

Treatment for metastatic disease is similar to cutaneous melanoma and may include BRAF inhibitors if mutations of this gene are present. Considering the similarity to cutaneous

melanoma, immune checkpoint blockade therapy is also expected to be effective.

Conclusions

Uveal and conjunctival melanomas are two distinct forms of ocular melanoma. With uveal melanomas, genetics have transformed prognostication but not treatment; with conjunctival melanomas, genetics have enhanced treatment but not prognostication.

Genetic insights into uveal melanomas have not resolved the debate regarding the impact of ocular treatment on survival, so that controversy surrounds many aspects of patient care, such as delayed treatment of small melanomas, primary endoresection of choroidal melanomas without neoadjuvant radiotherapy, and surveillance for systemic metastases.

There is scope for large, multicenter, long-term studies that would continue for several decades, until the cause of death of all patients is known. These objectives would require the development of methods of data collection that are not only scientifically robust but also affordable, given the rarity of ocular melanomas.

Since efforts to conserve the eye and vision are aimed at enhancing patients' quality of life, there is also scope for psychological studies that would enhance the individualization of care according to each patient's needs, wishes, and fears.

A major cause of suboptimal care is that most patients with ocular melanoma are managed by ophthalmologists who have little or no training in oncology, working in ophthalmic departments that lack resources for psychological support and other aspects of holistic care that are normally provided in oncology units.

A survey conducted by the Ocular Melanoma Foundation reveals that many patients are dissatisfied because the consent they had provided for their care was not as informed as they had believed, particularly with respect to genetic tumor analysis and the therapeutic options available for their particular condition.

The management of patients with ocular melanoma would inevitably improve if providers were able to agree on minimum standards of care or if they were obligated to comply with a patient-defined "bill of rights."

Although this chapter has focused on research, ocular outcomes and probably survival would improve greatly through better education. For example, general ophthalmologists need to be aware of the dangers of incisional biopsy and inexpert excision of conjunctival melanomas. As with other cancers, the success of treatment is greatly enhanced by early diagnosis and treatment so that ophthalmologists and optometrists need to have the skills and equipment to differentiate small melanomas from large nevi and other conditions. Importantly, general oncologists need to understand the difference between uveal and cutaneous melanomas.

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Desmoplastic Melanoma

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Abstract

Desmoplastic melanoma is a rare subtype of melanoma accounting for 1–4% of all melanomas. There are a number of clinical and pathological features that distinguish desmoplastic melanoma from conventional melanoma. Desmoplastic melanoma usually occurs in the elderly, particularly males, and on chronically sun-damaged skin, especially on the head and neck region. Lesions often present in an innocuous fashion, and are frequently unrecognized or misdiagnosed clinically. The pathological diagnosis of desmoplastic melanoma can also be very challenging, because the tumor is often very subtle and its morphological features overlap with nonneoplastic conditions such as scars, as well as benign and malignant neoplasms. Histologically, desmoplastic melanoma is characterized by malignant spindle cells in which individual tumor cells are separated by collagen fibers. There are two subtypes of desmoplastic melanoma, namely pure and mixed. In the pure subtype, the overwhelming majority of invasive tumor is desmoplastic, while in the mixed subtype (which may consist of spindled or epithelioid cells, or both), the desmoplastic areas account for less than 90% of the invasive tumor. Desmoplastic melanoma has an affinity for nerves and a propensity for local recurrence. Compared with other melanoma subtypes, desmoplastic melanoma more frequently metastasizes to the lungs and less frequently to lymph nodes. The molecular landscape of desmoplastic melanoma is quite different from that of conventional melanoma; it has a higher mutation rate and almost always lacks BRAF, NRAS, or KIT mutations, commonly present in other subtypes. Recent genomic studies have highlighted the very high DNA mutation load of desmoplastic melanoma and clinical studies suggest patients with metastatic desmoplastic melanoma have higher response rates to immunotherapy. Primary desmoplastic melanoma should be treated by wide local excision of the primary tumor. The role of sentinel lymph node biopsy in patients with desmoplastic melanoma is controversial; a number of early studies showed the rate of sentinel lymph node metastasis in patients with pure subtype desmoplastic melanoma was very low (approximately 1%), while much higher rates have been reported in more recent studies.

Keywords

Desmoplastic · Spindle cell · Neurotropism · Sentinel lymph node

Introduction

Desmoplastic melanoma was first described by Conley and colleagues in 1971. They reported a small series of patients with particular clinical and pathological features who had poor clinical outcomes compared to other melanoma patients. These patients were older males with chronically sun-damaged skin who presented with amelanotic lesions, which were characterized microscopically by malignant spindle cells in a collagen-rich stroma. Clear surgical margins were difficult to achieve, they had more frequent nerve involvement, and local recurrence was more common (McCarthy et al. 2004).

The origin of the desmoplastic stroma in desmoplastic melanoma has been the subject of much controversy. While some authors have suggested that the tumor cells elicit neighboring fibroblasts to proliferate and lay down abundant collagen, others argue that the tumor cells themselves possess an intrinsic ability to produce collagen. While the latter hypothesis was previously supported by ultrastructural examination findings, more recent studies from melanoma cell lines reveal that fibroblasts are responsible for the

production of the collagen rather than melanoma cells, thus favoring the former hypothesis (McCarthy et al. 2004).

Clinical Features

Desmoplastic melanoma arises most commonly in elderly patients (mean age at diagnosis = 65 years). This is approximately 10 years older than for conventional melanoma. It has a predilection for Caucasian males (M: F = 2: 1). Chronic sun exposure is a strong predisposing risk factor and in order of decreasing frequency, the commonest anatomic sites affected are the head and neck region (~50%), extremities (~30%), and trunk (~20%). However, desmoplastic melanoma can involve any site (Chen et al. 2013).

Unlike conventional melanoma, desmoplastic melanoma often lacks the clinical ABCDE warning signs (asymmetry, border irregularity, color variation, diameter enlargement, and evolution/history of change) of melanoma (Oakley 2017). Patients usually present with a slow-growing painless indurated plaque or nodule, which is amelanotic in more than one half of cases. Some lesions may begin as a small papule. Pigmentation, when present, is usually due to an accompanying melanoma in situ component (often a lentigo maligna), which coexists in up to 50% of cases. Palpation of a lentigo maligna lesion may disclose an underlying thickening or nodularity, which may be a clue to an unsuspected desmoplastic melanoma (Chen et al. 2013).

Desmoplastic melanoma may be mistaken on pathological examination for other disease processes such as a scar, nodular fasciitis, dermatofibroma, or neurofibroma. Under-recognition and low clinical suspicion can result in delayed diagnosis and management. Tumors can be thick before they come to clinical attention; lesions have a median Breslow thickness of 2.5 mm at the time of diagnosis (McCarthy et al. 2004).

Dermoscopy reveals pigmented globules in about one half of cases. There may be features of regression including scar-like areas and gray dots and an atypical or polymorphous vascular pattern can be present (Oakley 2017).

The typical clinical course of desmoplastic melanoma differs from that of conventional melanoma in a number of ways. There is a greater propensity for nerve involvement, achieving clear surgical margins can be difficult, and the risk of local recurrence is higher. On the other hand, the incidence of regional lymph node metastasis is lower than in conventional melanoma. Distant metastases typically occur in the lung or bone as a result of hematogenous spread (Murali et al. 2011).

Histopathological Features

Desmoplastic melanoma is a variant of spindle cell melanoma initially centered predominantly in the dermis but often extending more deeply into the subcutis and occasionally involving underlying structures. Desmoplasia refers to the growth of fibrous or connective tissue. Desmoplastic melanoma is characterized by relatively paucicellular amelanotic malignant spindle cells associated with abundant collagen fibers that separate individual tumor cells. On hematoxylin- and eosin-stained tissue sections, the predominant stromal component often gives rise to a poorly defined area of pink change at scanning magnification, and is often accompanied by myxoid change. Desmoplastic melanoma tends to infiltrate the dermis and deeper structures in an irregular or tentacular pattern and the microscopic features can be very subtle (McCarthy et al. 2004) (See Figs. 1, 2, 3, and 4).

Criteria proposed by Busam et al. from Memorial Sloan Kettering Cancer Center defined two subtypes of desmoplastic melanoma: pure and mixed (or "combined"). In pure desmoplastic melanoma, the overwhelming majority of invasive tumor (i.e., more than 90%) is desmoplastic. If desmoplastic areas account for less than this, then it is considered a mixed desmoplastic melanoma. The latter subtype consists of both desmoplastic and nondesmoplastic components. The latter may be formed by spindled or

epithelioid cells, or both (Murali et al. 2011). Compared to the pure subtype, mixed desmoplastic melanoma has an increased risk of sentinel node positivity as well as of local and distant recurrence, but these rates are intermediate between those of pure desmoplastic melanoma and conventional melanoma (Scolyer and Thompson 2005).

With a strong association with chronic UV radiation, concomitant melanoma in situ (often a lentigo maligna) in the overlying epidermis is present in up to 50% of cases of desmoplastic melanoma (see Figs. 1 and 2). However, an epidermal component is not essential for the diagnosis. Regardless, the background skin typically shows features of severe sun damage, including epidermal atrophy and moderate to marked solar elastosis. Ulceration is not a common finding (McCarthy et al. 2004).

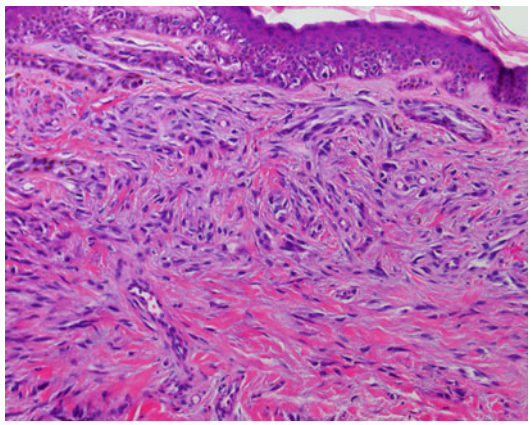

Fig. 1 Desmoplastic melanoma with overlying melanoma in situ, lentigo maligna type (H&E)

The tumor cells are fusiform, with epithelioid morphology seen only in mixed subtypes. While the degree of cytological atypia is variable, more often than not the tumor cells show only mild nuclear variability with enlargement and hyperchromasia and perhaps only focal pleomorphism (see Fig. 4c). The cells have elongated nuclei, irregular nuclear contours, and an open chromatin pattern with small but distinct nucleoli. The cytoplasm is poorly defined. Melanin pigment is inconspicuous or absent. Mitoses are usually infrequent but occasionally prominent. Given the mild cytological atypia, the tumor cells can be easily overlooked or misinterpreted as fibroblasts or spindle cells of another histogenesis (McCarthy et al. 2004). Immunohistochemical stains for S100 and Sox10 can be helpful in difficult cases but should be used judiciously (see further details below).

One of the most helpful clues to pathological diagnosis is the presence of lymphoid aggregates, with or without accompanying plasma cells, at the periphery of the tumor or within it. These lymphoid aggregates are often located in the deep

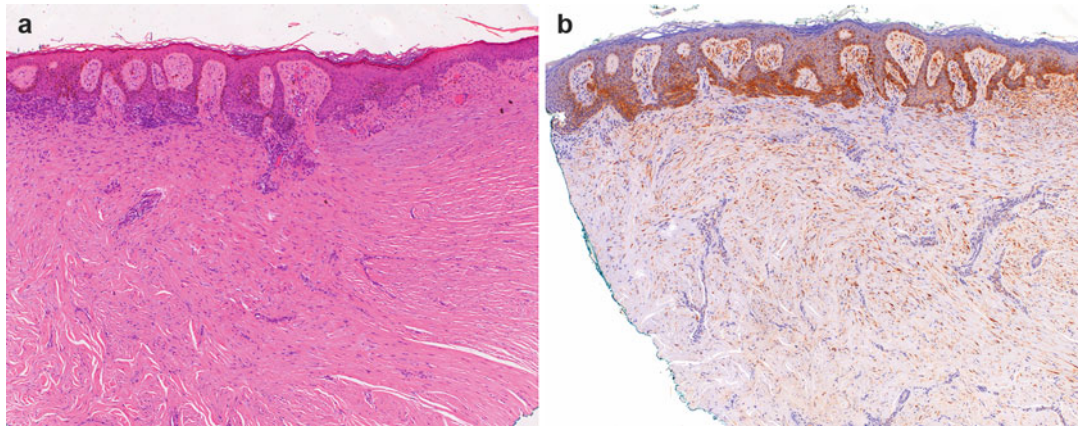

Fig. 2 (**a**) Desmoplastic melanoma, pure type (H&E). The predominant stromal component often gives rise to a poorly defined area of pink change at scanning magnification. (**b**) Both invasive and overlying in situ components are highlighted by S100 immunohistochemistry

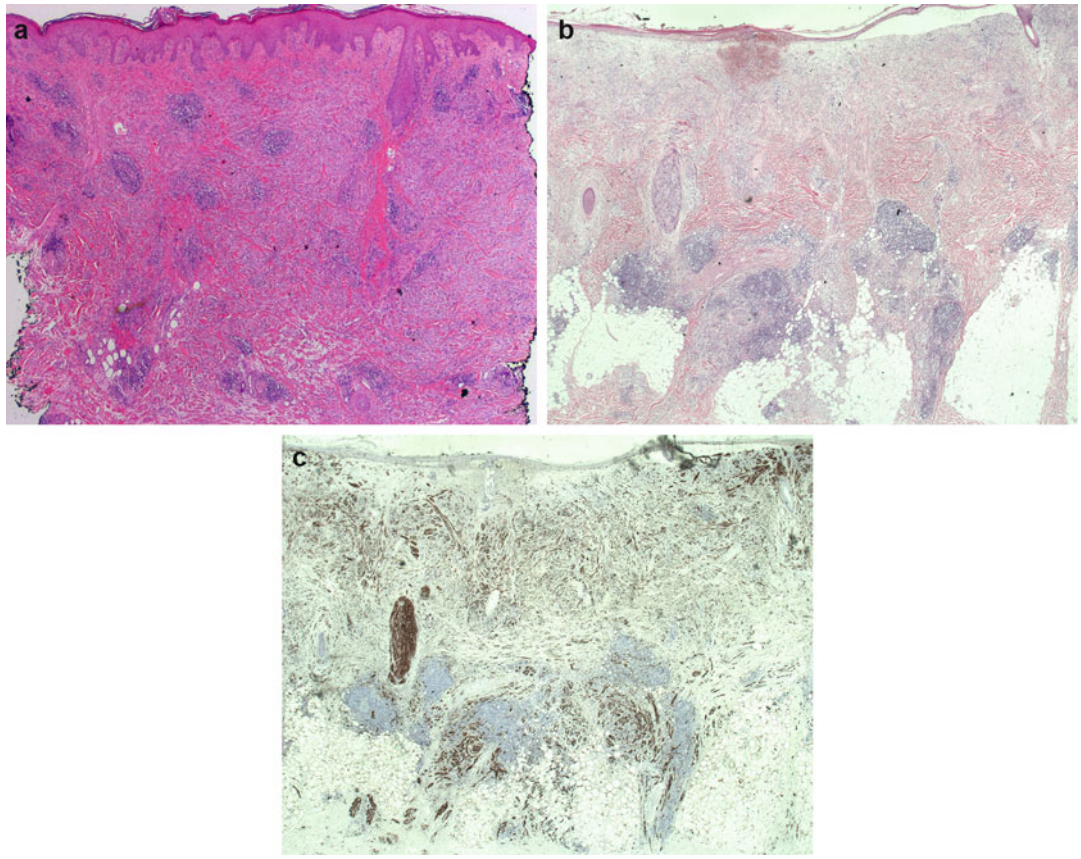

Fig. 3 (**a** and **b**) Desmoplastic melanoma involving the dermis and subcutaneous tissue. There are a number of associated lymphoid aggregates (H&E). (**c**) S100 immunostaining highlights the tentacular infiltration of desmoplastic melanoma

dermis or subcutis (Chen et al. 2013). However, lymphoid aggregates are not ubiquitous in desmoplastic melanoma and may not be captured in small biopsies. Furthermore, they may, on occasion, accompany other benign processes that may enter the differential diagnosis such as scars, sclerosing nevi, or neurofibromas.

Neurotropism, seen in about 30% of cases, is more common in desmoplastic melanoma compared with conventional melanoma (where it is present in up to 5%) (McCarthy et al. 2004). Three main patterns of nerve involvement have been described: perineural invasion, intraneural (or endoneural) invasion, and neural transformation, although it is somewhat controversial whether the latter should be regarded as a form of neurotropism (Varey et al. 2017). Perineural and intraneural invasion are terms referring to the extension of tumor cells around and within preexisting nerve structures, respectively (see Fig. 5a, b). In contrast, neural transformation occurs when tumor cells adopt the cytological characteristics of nerve cells, often in the form of nerve twigs. Although neurotropism does not influence survival directly, it is associated with a significantly increased risk of local recurrence because of the difficulty in achieving margin control (Murali et al. 2011). When neurotropism is present, postoperative radiotherapy may reduce the risk of local recurrence, particularly when there is difficulty achieving adequate surgical clearance margins (Chen et al. 2013).

Lymphovascular space invasion is rare. Occasionally, angioinvasion may be seen, as this is the presumed route for distant metastatic spread (Murali et al. 2010).

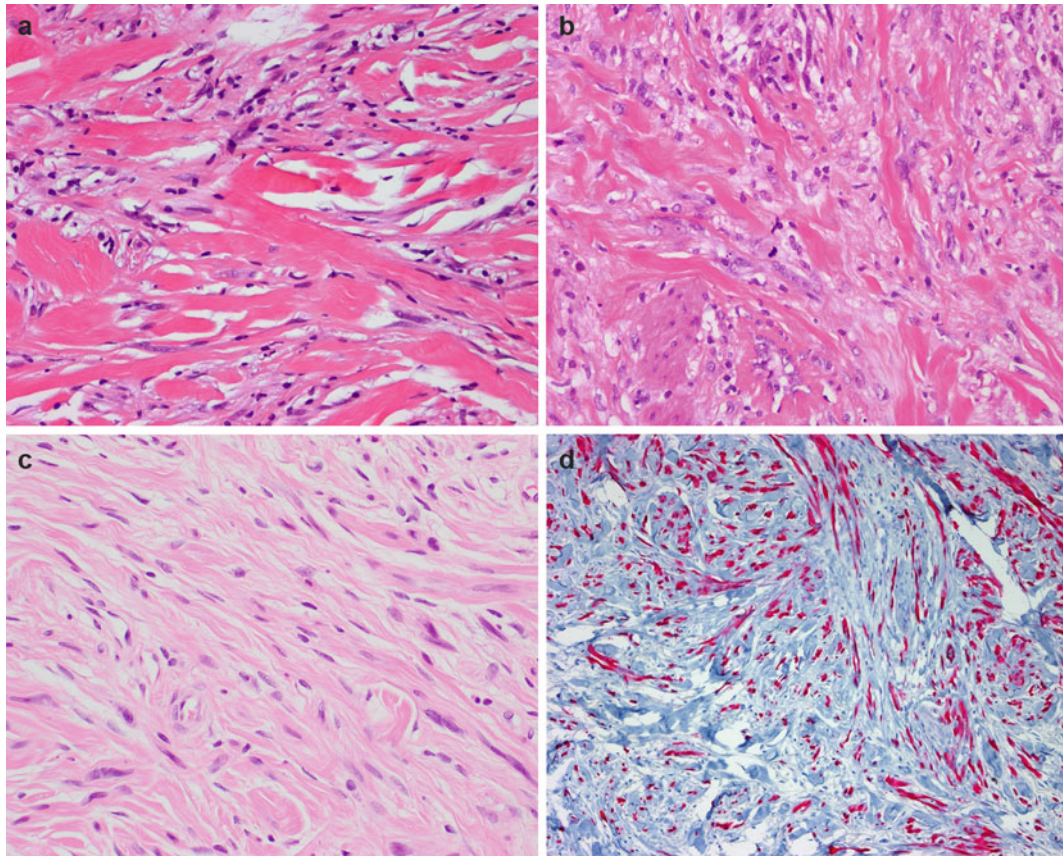

Fig. 4 (**a** and **b**) Desmoplastic melanoma, pure type (H&E). Abundant collagen fibers separate individual tumor cells. (**c**) Desmoplastic melanoma, pure type (H&E). The tumor cells are fusiform, showing mild nuclear variability with enlargement and hyperchromasia. The cytoplasm is poorly defined and melanin pigment is absent. (**d**) Immunohistochemistry can be helpful in subtle cases. Here, S100 is positive in the tumor nuclei and cytoplasm. Other markers of melanocytic differentiation including HMB45, MelanA/MART1, tyrosinase, and MITF are usually negative or only focally positive

Fine Needle Biopsy Features

Fine needle biopsy (FNB) is frequently used to diagnose metastatic melanoma, including desmoplastic melanoma, but it has only a very limited role in the assessment of primary tumors. FNB specimens from desmoplastic melanoma are often paucicellular due to the significant stromal content. Diagnosis can be even more challenging considering that the tumor cells tend to lack significant cytological atypia and mitotic activity. Compared to conventional melanoma, the tumor cells less frequently contain intranuclear cytoplasmic invaginations or intracytoplasmic melanin pigment (Murali et al. 2008). In the event of an insufficient or nondiagnostic cytology specimen and ongoing clinical suspicion, formal tissue biopsy for histopathological assessment is warranted.

Immunohistochemistry

Desmoplastic melanoma is typically diffusely positive for S100 protein and Sox10 (see Figs. 2, 3, and 4). S100 stains positively in both the cytoplasm and nuclei of desmoplastic melanoma cells and can be useful in highlighting their dendritic appearance, while Sox10 is restricted to staining nuclei. In contrast to conventional melanoma, other markers of melanocytic differentiation including HMB45, MelanA/MART1, tyrosinase, and MITF are usually negative or only focally

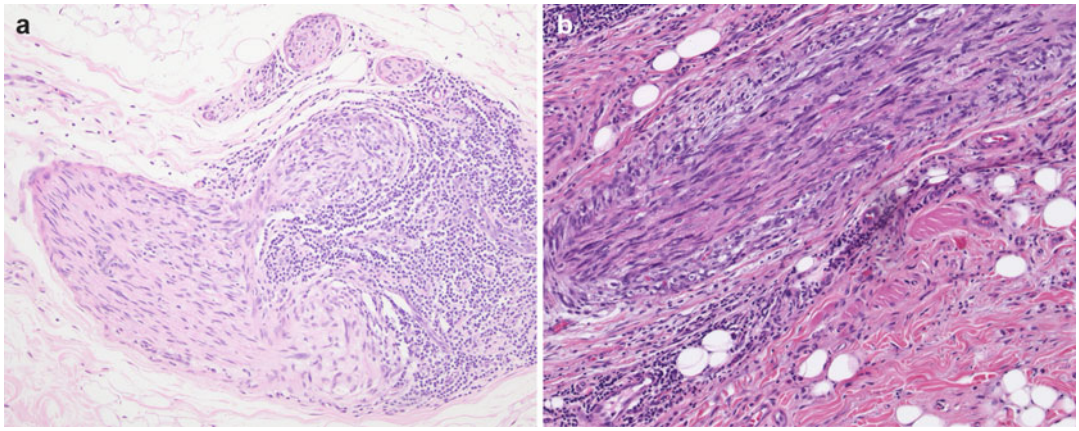

Fig. 5 (**a** and **b**) Neurotropism is present in about one third of cases of desmoplastic melanoma. Tumor cells may extend around and/or within preexisting nerve structures (H&E)

positive. These latter markers are more likely to be positive in epithelioid cells of the mixed subtype of desmoplastic melanoma (McCarthy et al. 2004; Murali et al. 2008).

A potential pitfall in the interpretation of immunohistochemical stains, especially in post-biopsy specimens, is that fibroblasts in scars are also immunoreactive with S100 and Sox10. Therefore, pathologists should not rely solely upon this ancillary method for diagnosis – careful attention to morphological detail is of paramount importance as the immunostains need to be interpreted in the appropriate context (Murali et al. 2011).

Desmoplastic melanoma may also exhibit a nonspecific staining pattern for SMA, CD10, and CD34. It has been suggested that CD34 can be useful in distinguishing desmoplastic melanoma from neurofibroma, with the latter showing a "fingerprint distribution" (Yeh and McCalmont 2011); however, it is our experience that this pattern is not uncommon in desmoplastic melanoma and has little practical value.

Genetic Features

Understanding of the genomic landscape of melanoma (including desmoplastic melanoma) has expanded rapidly in recent years with the discovery of significant genetic alterations that underlie the pathogenesis of the disease. Desmoplastic melanoma is among the most highly mutated of all human neoplasms. With more than 60 mutations per megabase of DNA, it has an exceptionally high mutation burden and DNA signatures have strongly implicated ultraviolet radiation as the dominant etiological cause. This lends support to the theory of a superficially located cell of origin and is consistent with the presence of associated melanoma in situ (lentigo maligna) in a significant proportion of cases (Hunter Shain et al. 2015).

Recently, recurrent NFKBIE promoter mutations and diverse activating mutations in the MAPK pathway have been identified using exome sequencing. The MAPK pathway mutations are mutually exclusive of those well recognized in conventional melanomas (namely BRAF and NRAS). Instead, other mutations involving activation of the MAPK and PI3K signaling cascades, including CDKN2A and NF1, are relatively common. Mutations of the tumor suppressor gene NF1 are usually nonsense mutations with premature stop codons, leading to lack of NF1 protein expression. TP53 mutations are also found in a large proportion of desmoplastic melanomas, in contrast to conventional melanomas (see Fig. 6) (Hunter Shain et al. 2015).

Differential Diagnosis

Given the wide spectrum of overlapping and often subtle clinicopathological features that may occur in desmoplastic melanoma, its diagnosis can be challenging. It can mimic a range of conditions

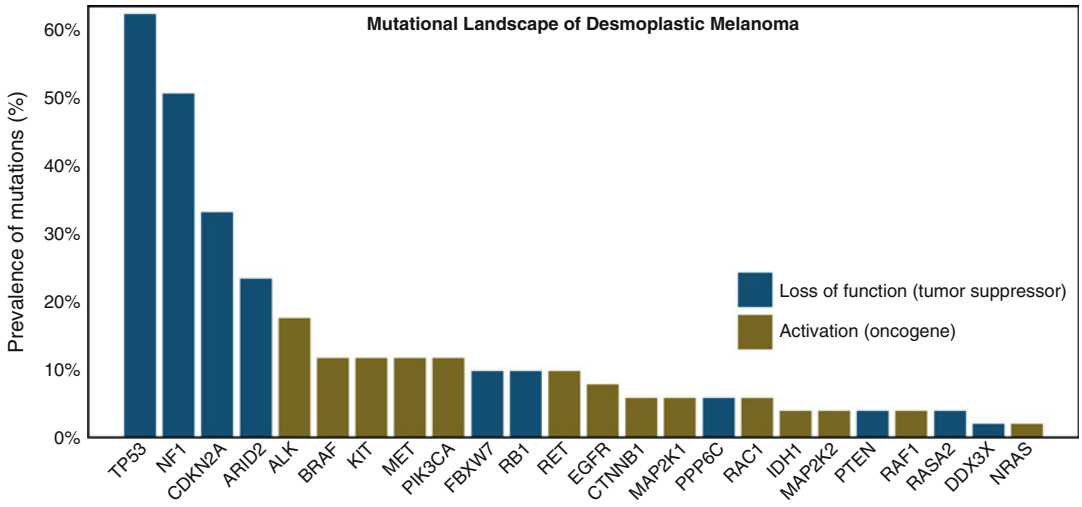

Fig. 6 Meta-analysis of the prevalence of melanoma driver mutations present in desmoplastic melanomas from two large melanoma genome sequencing studies (Hayward et al. 2017; Hunter Shain et al. 2015). (Image courtesy of Dr. James Wilmott)

including both completely benign entities and malignant processes. A previous history of melanoma should always prompt consideration of recurrence or a new primary melanoma. Diagnosis may be inadvertently delayed if clinical suspicion is lacking and a biopsy is not performed, or if the pathologist fails to recognize the subtle histopathological features of desmoplastic melanoma. As a consequence, desmoplastic melanoma is often not diagnosed until it is at an advanced stage. In the setting of chronically sun-damaged skin, pathologists should always consider the possibility of desmoplastic melanoma and search carefully for any of the aforementioned constellation of histopathological features. Clinical mimics include scar, desmoplastic nevus, dermatofibroma (fibrous histiocytoma), and neurofibroma (McCarthy et al. 2004).

Dermal scar is probably the most common diagnostic dilemma. While the dermal tumor cells in desmoplastic melanoma are distributed haphazardly among collagen, the spindle-shaped dermal fibroblasts in scars are typically aligned parallel to the overlying epidermis. A potential hazard in distinguishing scars from desmoplastic melanoma lies in the misinterpretation of immunohistochemical stains as S100 and Sox10 can be expressed in both, albeit usually only focally in the former. Careful evaluation of the architectural and cytological features is required to avoid this mistake (Chen et al. 2008).

Desmoplastic nevi typically arise at a younger age than desmoplastic melanoma. Microscopically these lesions usually display symmetry, are confined to the superficial dermis, and few, if any, associated lymphocytes are present. In addition to S100 and Sox10 immunoreactivity, they also express other melanocytic markers such as MelanA/MART1, in contrast to desmoplastic melanoma (McCarthy et al. 2004).

Dermatofibromas also occur at a younger age and commonly involve the legs of young female adults. The histopathological findings are those of a circumscribed dermal proliferation of spindle cells with a fibrohistiocytic appearance and dermal collagen entrapment at the periphery of the lesion. Associated diffuse epidermal hyperplasia with increased basal pigmentation is also usually present. While assessment can be challenging on a small biopsy where the entire lesion has not been sampled, positive immunostaining for Factor XIIIa and SMA and absent staining for S100 and Sox10 should clinch the diagnosis of dermatofibroma (McCarthy et al. 2004).

Neurofibroma and desmoplastic melanoma can share similar clinical and pathological features. Both usually present as amelanotic papules or nodules and both are characterized

microscopically by a spindle cell proliferation with associated collagenous stroma. Unfortunately, immunohistochemistry is not as helpful in distinguishing these two entities, as both are positive for S100 and Sox10 and negative for MelanA/MART1 and HMB45. Therefore, close attention to the morphological findings is required to establish a diagnosis. The growth pattern of neurofibroma is generally more circumscribed (except in the diffuse variant), the lesion is usually symmetrical and the lesional spindle cells tend to have more tapered nuclear contours (Murali et al. 2008).

Treatment

The mainstay of management of biopsy-proven desmoplastic melanoma is wide surgical excision with the same recommended clearance margins as for conventional melanoma, which vary according to the primary tumor thickness. The role of sentinel lymph node biopsy in desmoplastic melanoma has been questioned due to the lower incidence of positivity in both pure (1–5%) and mixed (5–20%) subtypes. Nevertheless, this procedure forms part of routine practice in many melanoma treatment centers. Local recurrence rates are higher in desmoplastic melanoma compared to conventional melanoma. Adjuvant radiotherapy may be offered in certain circumstances, usually when margin control is difficult to achieve or when there is nerve involvement. Systemic therapy options continue to evolve. The exceedingly high mutation burden found in desmoplastic melanoma makes it a promising candidate for immune checkpoint blockade therapy and preliminary data suggest that higher response rates are observed in desmoplastic melanoma compared with conventional melanoma.

Surgery

Like other types of melanoma, surgery is the mainstay of treatment of desmoplastic melanoma. This involves wide local excision aiming to achieve clear peripheral margins of 1 cm for tumor thickness less than or equal to 1 mm, 1–2 cm for tumors 1–2 mm thick, and at least 2 cm for tumors greater than 2 mm thick. It is also important to achieve adequate deep margins, as there is often extension into the subcutis or even deep fascia and underlying tissues. Achieving clear margins is not always possible on the initial excision due to the insidiously infiltrative nature of the tumor (Varey et al. 2017).

Sentinel Lymph Node Biopsy

The role of sentinel lymph node biopsy in patients with desmoplastic melanoma is somewhat controversial. The rate of sentinel lymph node positivity at the time of diagnosis when matched for tumor thickness is lower (1–5% for pure subtype desmoplastic melanoma) than conventional melanoma (about 16%) (Scolyer and Thompson 2005; Dunne et al. 2017). While some advocate the routine practice of sentinel lymph node biopsy, others propose that the frequency of sentinel lymph node metastasis is so low that the procedure can be safely avoided (Gyorki et al. 2003). At Melanoma Institute Australia, we continue to offer sentinel lymph node biopsy to all patients with melanoma tumor thickness > 1 mm. With recent clinical trials reporting reduced rates of recurrence in stage III melanoma patients who received adjuvant targeted and immune therapies, it would appear that the role of sentinel lymph node biopsy in desmoplastic melanoma might expand in centers where this staging procedure is not currently part of the routine management of patients with desmoplastic melanoma.

Radiotherapy

Radiotherapy may be beneficial in some cases of desmoplastic melanoma, particularly when it is difficult to achieve clear surgical margins or when there is nerve involvement. In the event of positive or narrow margins or extensive neurotropism and where functional and/or cosmetic reasons preclude wider re-excision, radiation in a dose of 20–40 gray over six fractions can be applied to reduce the risk of local recurrence.

This is particularly relevant for lesions arising in the head and neck region (Chen et al. 2008).

Systemic Therapy

The exceedingly high mutation burden found in desmoplastic melanoma makes it a promising candidate for immune checkpoint blockade therapy. Emerging data have shown that patients with desmoplastic melanoma respond well to anti-PD1 agents. Response rates are in the order of about 70%, significantly higher than those for conventional melanoma (40%). The presumed reason for this higher response rate is that desmoplastic melanoma expresses high levels of PD-L1. In their study, Eroglu et al. (2018) found that single agent anti-PD1 therapy, rather than the more toxic combination immunotherapy involving both anti-PD1 and anti-CTLA-4 inhibition, might be sufficient for patients with desmoplastic melanoma.

Prognosis

In 1988, Egbert et al. asserted that the clinical outcome for desmoplastic melanoma was far worse than for other types of melanoma, but failed to consider important pathologic prognostic features in their analyses (Scolyer and Thompson 2005). In contrast, recent studies have found that when matched for tumor thickness, survival times were actually longer. Some studies have shown a poorer prognosis for patients with desmoplastic melanoma in cases of male gender, older age, and head and neck location (McCarthy et al. 2004).

By definition, recurrence is local when at or near the surgical excision site (<5 cm), in transit when >5 cm from the surgical excision site but not beyond the draining lymph node basin, and regional when it has reached the draining lymph node basin. Local recurrences are common in desmoplastic melanoma compared to conventional melanoma, especially following incomplete or narrow (<10 mm) surgical excision or when there is neurotropism. Most recurrences (78.2%) occur within the first 2 years. Distant metastases occur in 11–40% of patients with desmoplastic melanoma (Chen et al. 2008).

Conclusion

Desmoplastic melanoma is a rare subtype of spindle cell melanoma with a strong association with high cumulative UV exposure and a predilection to involve the head and neck region. It affects males almost twice as often as females and occurs with a median age of diagnosis approximately 10 years later than conventional melanoma. Desmoplastic melanoma is a great mimicker both clinically and pathologically and as such, a high index of suspicion, especially in patients with a previous melanoma history, is essential to avoid misdiagnosis. Immunohistochemistry for S100 and Sox10 can be employed as an ancillary test but must be interpreted with caution, as the staining pattern in desmoplastic melanoma is not specific.

Desmoplastic melanoma is among the most highly mutated of all human neoplasms. Recurrent mutations in NF1, TP53, and NFKBIE have been documented in desmoplastic melanoma. This contrasts with the prevalent BRAF and NRAS mutations identified in conventional melanoma.

The mainstay of treatment is surgical excision with a surrounding wide margin of normal tissue. Desmoplastic melanoma infiltrates deeply, sometimes making adequate excision difficult, and it has a propensity for nerve involvement. There is a high risk of local recurrence if peripheral and deep excision margins are narrow or incomplete. Compared to conventional melanoma, there is a reduced likelihood of sentinel node positivity, especially in the pure subtype. When matched for stage, however, prognosis is comparable between conventional and desmoplastic melanomas.

Desmoplastic melanoma	
Clinical features	**Differential diagnosis**
Elderly (median age 65 years) Chronically sun-exposed skin (especially head and neck) Indurated plaque or amelanotic papule/nodule	Scar Sclerosing nevus Neurofibroma Spindle cell carcinoma Dermatofibrosarcoma protuberans Malignant peripheral nerve sheath tumor
Histopathological features	**Genetic features**
Dermal-based spindle cell lesion with abundant pink collagen and infiltrative borders into subcutis Associated melanoma in situ (lentigo maligna) present in up to half of cases Pure (spindle cells) or mixed (spindle and epithelioid cells) Pigment absent or sparse Mild to moderate cytological atypia Mitoses rare Lymphoid aggregates at periphery or within tumor Neurotropism (30%)	Exceptionally high mutation burden (>60 mutations per Mb DNA) Recurrent mutations in NF1, TP53, NFKBIE
Immunohistochemistry	**Treatment**
S100 and Sox10 positive HMB45, MelanA/MART1, tyrosinase, and MITF negative or only focally positive	Surgery: Wide local excision mainstay of treatment Sentinel lymph node biopsy: Role controversial; lower rates of positivity, but routinely offered in most melanoma referral centers Radiotherapy: Adjunct for margin control or when there is neurotropism Systemic therapy: Emerging data suggest high response rates to PD1 immunotherapy
	Prognosis
	Higher risk of local recurrence Lower risk of regional and distant metastasis Overall survival comparable to conventional melanoma when matched for tumor thickness

	Desmoplastic melanoma	Nondesmoplastic/conventional melanoma
Age (median)	65 years	55 years
Site	Head and neck region	Any cutaneous or acral site, mucous membranes
Clinical features	Typically nonpigmented (unless overlying melanoma in situ)	Typically pigmented
Pathologic features	Dermal cellular proliferation associated with collagen which separates individual tumor cells Pure (spindle) or mixed (spindle and epithelioid) Mild to moderate cytological atypia Mitoses rare Lymphoid aggregates at periphery or within tumor Neurotropism (30%)	Dermally invasive component with or without a junctional component Epithelioid, spindled, nevoid, or a combination of these cell types Usually significant cytological atypia Mitoses common Neurotropism (up to 5%)
Immunohistochemistry	S100 and Sox10	S100, Sox10, MelanA/MART1, HMB45, tyrosinase, MITF
Mutations	NF1, TP53, NFKBIE	BRAF, NRAS, CDKN2A (p16), others, e.g., KIT (mucosal), CCND1 (acral), GNAQ (uveal), ALK (Spitz)
Sentinel lymph node positivity rate	5.4% (pure subtype) 13.8% (mixed subtype)	15–20%
Common sites of metastases	Lung, bone	Skin, regional lymph nodes, liver, lungs, bone, brain
Prognosis	Overall survival comparable to conventional melanoma when matched for tumor thickness	
Treatment	Wide local excision ± Sentinel lymph node biopsy ± Immunotherapy, radiotherapy	Wide local excision ± Sentinel lymph node biopsy ± Immunotherapy, targeted molecular therapy, radiotherapy, chemotherapy

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Part III

Clinical Management

Melanoma Clinical Staging (Historical and Current)

23

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Contents

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Abstract

Melanoma staging has evolved as our understanding of clinical and pathological risk factors have improved and surgical staging strategies have matured. The current American Joint Committee on Cancer (AJCC) melanoma staging system is based on the tumor (T), node (N), metastasis (M) system, similar to most other solid tumors; criteria that define TNM

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have changed over time. The T category is determined by primary tumor thickness and presence or absence of ulceration; the N category takes into account both the number of clinically occult and clinically detected lymph node metastases, as well as the presence or absence of non-nodal regional metastases. The M category is defined by anatomic site of disease and lactate dehydrogenase levels. Sentinel lymph node biopsy has become a standard assessment technique by which T2-T4 melanomas, and some T1 melanomas, are staged. Taken together, the melanoma staging system allows for accurate risk stratification of large subsets of melanoma patients that can help guide clinicians and patients regarding prognosis. In the future, melanoma staging may be complemented by validated clinical tools based on multiple clinical, pathological, and molecular risk factors, and may provide a more precise individualized risk assessment for melanoma patients.

Keywords
Melanoma · Staging · Sentinel lymph node biopsy · Prognosis · Metastasis · Lymphadenectomy · Lymph node dissection · Risk assessment

Introduction

The melanoma staging system is based on pathological characteristics of the primary tumor; extent of regional disease, if any; and the absence or presence of distant metastasis. Since the late 1970s, melanoma has been staged according to the American Joint Committee on Cancer (AJCC) melanoma staging system, a TNM-based system that designates tumor (T), regional nodal (N), and distant metastasis (M) classifications based on pathological tumor characteristics of the primary melanoma (T), the number of lymph nodes involved and/or other evidence of regional disease (N), and the presence of distant metastatic disease (M). Stages I and II, Stage III, and Stage IV comprise patients with localized disease, regional disease, and distant metastases, respectively (Table 1). Criteria used to define the AJCC staging system have evolved over time, utilizing an improved understanding of the biology of melanoma, more accurate and less-invasive staging procedures, and identification of factors that better stratify patients according to risk. In this chapter, we review historical aspects of melanoma staging, new changes to the 8th Edition AJCC melanoma staging system and their rationale, future directions in staging classification and risk stratification, and the development of clinical tools that may enhance clinical decision-making.

Primary Tumor Assessment

Primary Tumor Thickness

Solid tumors are most commonly characterized by primary tumor size to determine T category. Melanoma size is assessed by the extent of tumor penetrance from the skin surface, rather than the surface diameter of the lesion. In 1969, Clark et al. first proposed classification of level of invasion based on the relationship of the primary melanoma to the papillary and reticular dermis and which was defined by five levels (I–V) (Clark et al. 1969). Shortly thereafter, Breslow proposed measuring tumor thickness by depth of invasion from the skin surface using an ocular micrometer (Breslow 1970). This measurement, referred to as the Breslow thickness (or, commonly, tumor thickness), is taken from the top of the granular layer of the epidermis to the deepest invasive cell across the broad base of the tumor. When a primary tumor is ulcerated, the tumor thickness measurement is made from the base of the ulcer. Initially, cutpoints of 0.75, 1.50, 2.25, and 3.0 mm were used to stratify patients (Breslow 1970). Breslow thickness provided a more objective, reproducible measure of tumor thickness and could more accurately risk stratify patients with Clark level III and IV primary melanomas, who were observed to have a wide range of prognoses (Breslow 1975).

Initially, Clark level and Breslow thickness complemented each other and were used together to stage patients with primary cutaneous

Table 1 TNM staging for cutaneous melanoma, 8th Edition AJCC

T classification	Thickness (mm)	Ulceration status
T0: no evidence of primary tumor	NA	NA
Tis (melanoma in situ)	NA	NA
T1	≤1.0	a: <0.8 mm without ulceration
		b: 0.8–1.0 mm with or without ulceration
T2	>1.0–2.0	a: Without ulceration
		b: With ulceration
T3	>2.0–4.0	a: Without ulceration
		b: With ulceration
T4	>4.0	a: Without ulceration
		b: With ulceration
N classification	**Number of metastatic nodes**	**Nodal metastatic burden**
N0	No regional metastases detected	NA
N1	1 tumor-involved node or in-transit, satellite, and/or microsatellite metastases with no tumor-involved nodes	a: Clinically occult (i.e., detected by SLN biopsy)
		b: Clinically detected
		c: No regional nodal disease
N2	2–3 tumor-involved nodes or in-transit, satellite, and/or microsatellite metastases with 1 tumor-involved node	a: Clinically occult (i.e., detected by SLN biopsy)
		b: At least one of the two to three nodes clinically detected
		c: 1 clinically occult or clinically detected node with in-transit, satellite, and/or microsatellite metastases
N3	4+ metastatic nodes; in-transit, satellite, and/or microsatellite metastases with 2 or more tumor-involved nodes; or any number of matted nodes without or with in-transit, satellite, and/or microsatellite metastases	a: 4+ clinically occult nodes (i.e., detected by SLN biopsy)
		b: 4+ nodes, at least one of which was clinically detected, or presence of any number of matted nodes
		c: 2+ clinically occult or clinically detected and/or presence of any number of matted nodes in the presence of in-transit, satellite, and/or microsatellite metastases
M classification	**Site**	**Serum LDH**
M0	No distant metastases	NA
M1a	Distant metastasis to the skin and soft tissue including the muscle and/or nonregional nodal metastases	0: not elevated
		1: elevated
M1b	Lung metastases with or without M1a sites of disease	0: not elevated
		1: elevated
M1c	Non-CNS visceral metastases with or without M1a or M1b sites of disease	0: not elevated
		1: elevated
M1d	CNS metastases with or without M1a, M1b, or M1c sites of disease	0: not elevated
		1: elevated

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Clinically occult are diagnosed after sentinel lymph node biopsy

Clinically detected are defined as clinically detectable nodal metastases confirmed pathologically

NA not applicable, *CNS* central nervous system, *LDH* lactate dehydrogenase

Suffixes for M category: (0) LDH not elevated, (1) LDH elevated. No suffix is used if LDH is not recorded or is unspecified

melanoma (Beahrs et al. 1992; Fleming et al. 1997). Over time, however, Breslow thickness became the more widely used method and is evidenced by a gradual evolution in the importance placed on these two measurements in the AJCC melanoma staging system. In earlier editions, Clark level and Breslow thickness were both used to determine T category (Wanebo et al. 1975; Balch et al. 1978, 1979). In the 6th Edition (2002) AJCC melanoma staging system, Breslow thickness became the primary T category criterion, using tumor thickness cutpoints of 1.0, 2.0, and 4.0 mm. Clark level was used only to subcategorize T1 lesions (Balch et al. 2001a). In the 7th Edition, mitotic rate (discussed below) replaced Clark level of invasion as a criterion to help define a T1b melanoma (Balch et al. 2009; Edge 2010).

In the 8th Edition (2017) AJCC melanoma staging system, tumor thickness cutpoints of 1.0, 2.0, and 4.0 mm continue to define T1, T2, T3, and T4 primary melanoma (Gershenwald et al. 2017a). As in the 7th Edition, T1 tumors are subcategorized according to the presence or absence of primary tumor ulceration, and new to the 8th Edition, a T1b tumor is also defined by any primary melanoma that is 0.8–1.0 mm in tumor thickness regardless of ulceration status (Gershenwald et al. 2017a). Also new to the 8th Edition AJCC melanoma staging system, Breslow thickness measurements are to be recorded to the nearest 0.1 mm (rather than to the nearest 0.01 mm) (Gershenwald et al. 2017a). This change was made in an effort to avoid clustering of reported measurements around critical cutpoints for staging classification, which has been demonstrated to have bias with implications for staging (Ge et al. 2016).

Primary Tumor Ulceration

Primary tumor ulceration is a well-established pathological risk factor associated with adverse survival in patients with cutaneous melanoma. Ulceration is defined microscopically as a full-thickness epidermal defect with evidence of reactive changes and thinning, effacement, or reactive hyperplasia of the surrounding epidermis (Smoller et al. 2016; Edge 2010). The incidence of ulceration increases with increasing Breslow thickness (Balch et al. 2009, 1980; White et al. 2011). The 6th Edition (2002) AJCC melanoma staging system was the first to designate the T category as "a" or "b" based on the absence or presence of ulceration, respectively (Balch et al. 2001a). Multiple studies have found that primary tumor ulceration is associated with worse survival across all tumor thickness groups – essentially "upstaging" a patient to the next highest T category with a tumor that is not ulcerated (Balch et al. 2001a, 2009). For example, a clinically node-negative patient with a T2 primary melanoma that is ulcerated (T2b) has approximately the same survival as a patient with a T3 tumor that is not ulcerated (T3a); stage groupings are discussed below (Balch et al. 2001b, 2009). In patients with tumor-negative sentinel lymph nodes (SLN), ulceration has been shown to be an independent predictor of increased risk of locoregional and distant recurrence and worse melanoma-specific survival, with a relative increase in risk of recurrence or death two to three times that of patients whose primary tumors are non-ulcerated (Balch et al. 2009; Yee et al. 2005; Egger et al. 2016). Primary tumor ulceration is also an important adverse prognostic factor even among patients with Stage III (regional) disease. Primary tumor ulceration has also been shown to impact survival among patients with regional metastasis. Accordingly, in both the 7th and 8th Editions of the AJCC melanoma staging system, the presence or absence of ulceration contributes to the subgrouping of node-positive patients (Gershenwald et al. 2017a; Edge 2010). In summary, primary tumor ulceration is an important staging element for patients with cutaneous melanoma and offers insights into the patient's risk of recurrence and death.

Mitotic Rate

Mitotic rate is a pathological feature of the primary tumor that has also been used to stage patients with primary cutaneous melanoma. Mitotic rate is defined as the number of mitoses

per mm^2 using the dermal "hot spot" method (Edge 2010). Clark et al. identified mitotic rate as an important risk factor in localized cutaneous melanoma in the 1980s (Clark et al. 1989). Mitotic rate was introduced into the 7th Edition AJCC melanoma T category assessment of primary melanoma for patients with "thin" T1 melanoma based on a series of tumor thickness-stratified multivariable models (Balch et al. 2009; Edge 2010). Higher mitotic rate has been shown to be an independent risk factor for death from melanoma and was more important than ulceration in some studies (Barnhill et al. 2005; Azzola et al. 2003). Using both Surveillance, Epidemiology, and End Results (SEER) data and a single-institution database, Gimotty et al. demonstrated that a classification system using mitotic rate greater than zero (i.e., as a dichotomous putative prognostic factor), among other factors, was able to stratify patients with thin, non-ulcerated melanomas into groups with significantly different survival rates (Gimotty et al. 2007). Kesmodel et al. reported that a mitotic rate greater than zero was an independent predictor of a tumor-positive SLN in patients with thin (Breslow thickness $\leq$1.0 mm) melanoma (Kesmodel et al. 2005). Using the 7th Edition AJCC melanoma staging database, Thompson et al. showed that mitotic rate was an independent adverse predictor of survival in localized (Stages I and II) cutaneous melanoma; it was the strongest predictor of survival outcome after Breslow thickness (Thompson et al. 2011). Among patients with Stage III cutaneous melanoma in the same database, Balch et al. observed that mitotic rate was an independent adverse predictor of survival in patients with nodal micrometastases (i.e., from a positive SLN or historically from tumor-involved nodes identified at elective lymph node dissection), but not among patients with nodal macrometastases (i.e., clinically evident) (Balch et al. 2010).

The 7th Edition AJCC melanoma staging committee evaluated mitotic rate as a dichotomous variable (i.e., $<$1 mitosis/mm^2 versus $\geq$1 mitosis/mm^2) within each AJCC tumor thickness group and determined that it was an independent adverse predictor of survival among patients with T1 melanomas. As a result, mitotic rate was introduced into the 7th Edition AJCC staging system as a T1 ($\leq$1.0 mm) primary melanoma criterion; the presence of ulceration and/or a mitotic rate of $\geq$1/mm^2 defined T1b (Balch et al. 2009). However, in the 8th Edition AJCC staging system, mitotic rate is no longer used to subcategorize T1 (Gershenwald et al. 2017a, b). While ulceration continues to be used to subcategorize melanoma, a new approach based on tumor thickness among patients with a thin melanoma is used to define T1 subcategories in the 8th Edition. In particular, mitotic rate was removed as a T1 criterion because analysis of patients with melanomas whose primary tumor thickness was $\leq$1 mm in the international database demonstrated that tumor thickness itself (stratified as $<$0.8 mm vs. 0.8–1.0 mm) was more prognostically important with respect to melanoma-specific survival than was mitotic rate (as a dichotomous variable as employed in the 7th Edition) (Gershenwald et al. 2017a, b).

Importantly, the 8th Edition AJCC melanoma expert panel strongly recommends that mitotic rate continue to be recorded for all patients with a primary cutaneous melanoma and notes that when explored using the mitotic rate continuum, it has been associated with survival across the tumor thickness continuum (Thompson et al. 2011; Gershenwald et al. 2017a, b). Although not a formal component of 8th Edition AJCC melanoma staging system, mitotic rate remains an important component of overall risk assessment and will likely be incorporated into the future development of clinical tools to aid in clinical decision-making through improved risk stratification and prognostic assessment (Gershenwald et al. 2017a).

Regional Lymph Node Assessment

The N category is the next component of melanoma staging and documents the absence or presence of regional lymph node and/or nonnodal locoregional (i.e., microsatellites, satellites, and/or in-transit) melanoma metastasis. The surgical approaches and pathological assessment of the regional lymph nodes have been refined over

the past three decades, with important implications for staging, risk stratification, and assessment for surgical, adjuvant, and other treatment decisions.

Historical: Approach to the Regional Nodal Basin

The role of lymph node dissection for staging purposes, particularly among clinically node-negative patients, has evolved over the years as new surgical techniques evolved, including the technique of lymphatic mapping and sentinel lymph node (SLN) biopsy (Gershenwald et al. 1999). Regional lymph node basins are initially routinely assessed by clinical exam. Clinically suspicious lymph nodes can be biopsied by fine needle aspiration, often using ultrasound guidance, to confirm metastatic disease. In the presence of pathologically confirmed, clinically evident lymph node metastasis, a therapeutic lymph node dissection is generally performed. From a staging perspective, such an approach affords an assessment of the regional nodal basin and an accurate count of the number of nodal metastases to determine N category criteria. Prior to the development of the SLN biopsy technique, an elective lymph node dissection was sometimes performed in patients with intermediate (1–4 mm) tumor thickness primary melanoma and clinically negative nodes to identify microscopic regional nodal metastasis and accurately determine the N category.

Current Approach to the Patient with Clinically Negative Regional Lymph Nodes: Rationale for Lymphatic Mapping and Sentinel Lymph Node (SLN) Biopsy

The technique of lymphatic mapping and SLN biopsy was introduced by Morton and colleagues in 1992, and its prognostic significance was validated by Gershenwald and colleagues in a 1999 multi-institutional study (Morton et al. 1992; Gershenwald et al. 1999). The rationale for this approach is based on the concept that for a given area of the skin, there is at least one regional lymph node that receives direct afferent lymphatic drainage from the primary tumor site – the "sentinel node" – prior to the rest of the regional nodal basin. Morton and colleagues initially demonstrated that the SLN is the most likely first site of metastasis to the regional nodal basin if any are involved, and if the SLN is negative, the remaining regional basin nodes are unlikely to harbor microscopic melanoma metastasis (Ross et al. 1993; Reintgen et al. 1994; Thompson et al. 1995). First incorporated into the 6th Edition (2002) AJCC melanoma staging system, the technique's accuracy has been validated in multiple multi-institutional studies (Balch et al. 2001a; Gershenwald et al. 1999; Morton et al. 1999). Over the past two decades, SLN biopsy has become an important cornerstone for the accurate assessment of many patients with at-risk melanoma who have clinically negative regional lymph nodes.

The principal purpose of the technique of lymphatic mapping and SLN biopsy for staging purposes is to identify microscopic regional lymph node metastases in clinically node-negative patients. The decision to perform lymphatic mapping and SLN biopsy for staging is based on the predicted risk of clinically occult regional node disease. Primary tumor factors, such as Breslow thickness, ulceration, and mitotic rate (discussed above), can be used to inform this decision-making (Kesmodel et al. 2005; Rousseau et al. 2003; McMasters et al. 2001; Sondak et al. 2004). Based on the associations of these primary tumor factors with microscopic regional lymph node metastasis, SLN biopsy is required for staging patients with clinically negative lymph node basins with T2, T3, and T4 melanomas to be included in the 8th Edition AJCC staging system; selective consideration of SLN biopsy for patients with T1b melanoma is permitted (Gershenwald et al. 2017a, b). Metastases identified by SLN biopsy are defined as "clinically occult" and designated with an "a" suffix in the AJCC N category. Metastases that are clinically evident and confirmed pathologically are considered "clinically detected" and designated with a "b" suffix in the

AJCC N category. The 8th Edition AJCC melanoma staging system defines N category as N1 (one positive lymph node), N2 (two to three positive lymph nodes), or N3 (four or more positive lymph nodes); in-transit, satellite, and/or microsatellite metastases can be categorized as N1c, N2c, or N3c, depending on the number of regional lymph nodes involved (see discussion below) (Gershenwald et al. 2017a). As in the 7th Edition, N category suffixes "a" or "b" continue to denote clinically occult or clinically evident, respectively (see also section below on "Non-nodal Locoregional Disease").

Non-nodal Locoregional Disease

Non-nodal regional disease – including microsatellites, satellite lesions, or in-transit metastases – represents an additional component of the AJCC N category staging criteria. In-transit metastases have been classically defined as cutaneous or subcutaneous metastases located greater than 2 cm from the primary tumor site, between the primary tumor and a draining regional nodal basin. Satellite lesions have a similar clinical definition except they are located within 2 cm of the primary tumor. In contemporary practice, however, the distinction between in-transit and satellite metastases is not clinically relevant, as they are equivalent from a staging perspective (i.e., both examples of non-nodal regional disease) and are generally considered in the same context for clinical decision-making. As for patients with regional node metastasis, prognosis in patients with satellite or in-transit metastasis is also informed by primary tumor characteristics and the presence of regional lymph node metastases (Shaikh et al. 2005; Bartlett et al. 2014; Read et al. 2015).

Microsatellite disease, another type of non-nodal regional metastasis, is a microscopic cutaneous and/or subcutaneous metastasis adjacent or deep to, and discontinuous from, a primary melanoma on pathological examination of the primary tumor site (Gershenwald et al. 2017a, b). The presence of microsatellites is also a risk factor for regional node metastasis (Kimsey et al. 2009).

From a staging perspective, patients with satellite, microsatellite, or in-transit metastasis without regional lymph node metastasis are categorized as N1c, where the "c" designation denotes satellite, microsatellite, or in-transit metastases. In the 7th Edition AJCC melanoma staging system, these patients were all designated as N2c. Patients with regional nodal metastasis who also have satellite, microsatellite, or in-transit metastasis are categorized as N2c or N3c, depending on the number of regional nodal metastases: N2c if there is one regional metastatic node and N3c if there are two or more tumor-involved regional nodes.

Assessment of Distant Metastasis

In the AJCC melanoma staging system, the M category denotes distant metastatic disease: M1 if present and M0 if absent. Overall, M category criteria are based on anatomic site(s) of distant metastasis as well as serum lactate dehydrogenase (LDH) levels.

Site of Distant Metastasis

In the 8th Edition AJCC melanoma staging system, M1a denotes metastatic disease confined to distant skin or subcutaneous tissues (including the muscle) or distant nodal metastasis (Gershenwald et al. 2017a). In general, a nodal metastasis is characterized as M1a disease when located beyond the regional nodal basin(s) of the primary tumor. For example, in the setting of a lower extremity primary, metastasis to the ipsilateral inguinal nodal basin is considered Stage III disease, but metastasis to the axilla is considered M1a. Metastasis confined to distant skin, subcutaneous tissues, or distant lymph nodes is generally associated with a more favorable survival compared to other sites of distant metastasis (Balch et al. 1983, 2009; Bowen et al. 2000; Barth et al. 1995).

M1b is defined as metastasis to the lung, with or without the presence of distant skin or subcutaneous metastasis or distant nodal disease

(Gershenwald et al. 2017a). Overall, these patients have been shown to have a somewhat worse prognosis compared to patients with M1a disease but more favorable survival compared to patients with nonpulmonary visceral metastases (Balch et al. 1983, 2009; Barth et al. 1995).

In the 8th Edition AJCC melanoma staging system, M1c is defined as noncentral nervous system (CNS) visceral metastases (Gershenwald et al. 2017a). Previously, in the 7th Edition, M1c was defined as any nonpulmonary visceral metastasis, including CNS metastasis. The 7th Edition M1c definition was refined as noted above, and a new M subcategory, M1d, has been introduced in the 8th Edition to denote metastasis to the brain, including CNS metastasis. As such, patients with CNS disease, regardless of whether other sites of metastasis are involved, will be categorized as M1d. Overall, patients with CNS metastasis have been noted to have a prognosis worse than patients without CNS metastasis, with median survival historically reported to be less than 1 year and 5-year survival rates $<10\%$ (Barth et al. 1995; Balch et al. 1983). CNS involvement is also frequently used as an inclusion or exclusion criterion for clinical trial eligibility, as well as a component of clinical trial stratification and analysis. In patients with multiple sites of distant metastases, the highest M subcategory corresponding to the anatomic site(s) of distant metastasis is used for staging purposes.

Laboratory Markers

It is generally uncommon for a cancer staging system to use serum markers for staging; however, for patients with melanoma, an elevated serum lactate dehydrogenase (LDH) level at the time of diagnosis of distant metastasis has been shown to be a strong adverse predictor of survival, regardless of anatomic site (Sirott et al. 1993; Eton et al. 1998; Deichmann et al. 1999). In the 8th Edition AJCC melanoma staging system, a suffix of "(0)" or "(1)" further characterizes M1a, M1b, M1c, and M1d disease with non-elevated (0) or elevated (1) LDH levels, respectively. The underlying mechanism for the association of elevated LDH levels with prognosis in metastatic melanoma is incompletely understood, but it remains an important tool to assess prognosis. LDH levels have also been shown to be associated with response to some of the targeted therapies for patients with metastatic or unresectable melanoma; normal LDH levels have been associated with a long-term response without progression to combination BRAF/MEK inhibition (hazard ratio for normal LDH for overall survival in the trial was 0.21) (Long et al. 2016).

Melanoma Stage Groupings

Once the T, N, and M categories are known, a patient's stage grouping (I–IV) can be determined. The 8th Edition AJCC staging system employs both a clinical and pathological classification system (Table 2). Clinical classification is performed after the biopsy of the primary tumor has been performed with clinical or biopsy assessment of the regional lymph nodes. The only assessment of the lymph nodes required for clinical staging is physical examination. The primary tumor pathological features Breslow thickness and ulceration define clinical Stages IA, IB, IIA, IIB, and IIC. Clinically evident regional lymph node and/or non-nodal regional disease identified in the clinical staging of a patient designates a patient as clinical Stage III, without consideration of the number of positive nodes. Clinical Stage IV includes patients who have distant metastasis at the time of diagnosis.

Pathological stage groups are determined after the status of the regional lymph nodes is determined after either SLN biopsy or completion lymph node dissection. Pathological classification uses information from additional microstaging of the primary tumor after biopsy and wide excision and from assessment of the regional nodal basin by either SLN or complete lymph node dissection; although SLN biopsy may be performed for some patients with T1 melanoma and clinically negative lymph nodes, SLN biopsy is not required for AJCC staging for patients with a T1 melanoma. Primary tumor thickness and ulceration define pathological Stages IA, IB, IIA, IIB, and IIC.

Table 2 Stage groupings for cutaneous melanoma, 8th Edition AJCC

Clinical stage				Pathological staging			
	T	N	M		T	N	M
0	Tis	N0	M0	0	Tis	N0	M0
IA	T1a	N0	M0	IA	T1a	N0	M0
					T1b	N0	M0
IB	T1b	N0	M0	IB	T2a	N0	M0
	T2a	N0	M0				
IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
IIC	T4b	N0	M0	IIC	T4b	N0	M0
III	Any T	N ≥1	M0	IIIA	T1a/b-T2a	N1a or N2a	M0
				IIIB	T0	N1b or N1c	M0
					T1a/b-T2a	N1b/c or N2b	M0
					T2b/T3a	N1a-N2b	M0
				IIIC	T0	N2b, N2c, N3b, or N3c	M0
					T1a-T3a	N2c or N3a/b/c	M0
					T3b/T4a	Any N ≥ N1	M0
					T4b	N1a-N2c	M0
				IIID	T4b	N3a/b/c	M0
IV	Any T	Any N	M1	IV	Any T	Any N	M1

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Pathological Stage III is reserved for patients with nodal or non-nodal regional disease. Stages IIIA, IIIB, IIIC, and IIID are determined by primary tumor thickness, ulceration, and the N categorization for nodal or non-nodal regional disease. Pathological Stage IV is for any M1 disease; there are no Stage IV substages.

Future Directions

Current staging criteria and classification continue to evolve. Reflective of the desire to develop and mature a framework by which additional known or putative prognostic elements can be collected for analysis and the development of improved clinical tools, the AJCC charges each of its expert panels to recommend which additional primary tumor, nodal, and/or distant disease factors be collected. Although these factors are not used to assign stage grouping, they may be of relevance to individual risk assessment; several such elements are discussed below. Prognostic models that take into account a multitude of patient and tumor characteristics can be used to personalize risk assessment and for the development of validated clinical tools.

Primary Tumor Assessment

Pathological assessment of the primary tumor includes more features than are included to define the AJCC T category. Some such factors include mitotic rate as a continuous variable and across all tumor thickness categories, level of invasion, regression, lymphovascular invasion, tumor-infiltrating lymphocytes, and neurotropism. While these factors may contribute information to risk assessment for an individual patient,

their influence on survival, independent of the more established pathological prognostic factors – Breslow thickness, mitotic rate, and ulceration – has not been unequivocally established. As such, these factors are not included in AJCC staging, but should continue to be collected for ongoing and future research into individual risk assessment models.

Efforts have also been made to subclassify cutaneous melanoma based on histologic subtypes and molecular profiles. Cutaneous melanoma has been classically divided into five histologic subtypes: superficial spreading, nodular, lentigo maligna, acral lentiginous, and desmoplastic. The most common subtype is superficial spreading. While AJCC staging does not currently incorporate these histologic subtypes, variations in biological behavior of the different subtypes can potentially be used to inform future staging and development of clinical tools. In exploratory studies, molecular classification of primary melanomas (e.g., by differential gene expressions) has identified possible strategies to inform clinical outcome (Bittner et al. 2000; Jaeger et al. 2007; Gerami et al. 2015; Koh et al. 2012; Rajkumar and Watson 2016). These approaches have not been sufficiently validated for clinical use nor have they been implemented into AJCC staging criteria, but taken together represent an area of opportunity to develop clinical tools that may improve risk stratification and enhance clinical decision-making.

N Category

The 8th Edition AJCC staging system incorporates the pathological status of SLNs without consideration for the extent of microscopic tumor burden in positive SLNs. Several studies support that both volume and distribution of microscopic disease have prognostic significance and that all positive SLNs should not therefore be considered at equal risk for non-SLN metastases and death from melanoma. Various measures of metastatic SLN tumor burden have been proposed, including measurement of the diameter of the SLN metastasis, depth of SLN tumor invasion, and anatomic distribution of the metastasis within the SLN (Ranieri et al. 2002; Carlson et al. 2003; Debarbieux et al. 2007; van Akkooi et al. 2008; Dewar et al. 2004; Starz et al. 2004). In general, several assessments of microscopic tumor burden have been shown to be associated with non-SLN metastases among patients who have a completion lymph node dissection, as well as survival. Maximum diameter of the largest metastatic focus has become the most common measurement used in clinical practice, given its reported prognostic significance, ease of measurement, and reproducibility. Although such measurements are currently not yet incorporated into AJCC melanoma staging, the 8th Edition AJCC melanoma staging system recommends that the SLN tumor burden be recorded. In the future, these measures may be incorporated into prognostic models and clinical tools (Gershenwald et al. 2017a, b).

Molecular and immunological analyses have been explored to further refine the assessment of SLNs in an attempt to identify patients at high and low clinical risk. Reverse transcriptase polymerase chain reaction (RT-PCR)-based and other techniques have been employed over the past two decades as a way to detect submicroscopic and otherwise undetectable metastatic melanoma using putative surrogate markers of melanoma (Wang et al. 1994; Van der Velde-Zimmermann et al. 1996; Goydos et al. 1998). Early observational studies evaluating the use of RT-PCR-based techniques suggested that this type of so-called molecular staging was prognostically significant. Confounding such early reports, however, other studies reported contrary findings, suggesting that RT-PCR-based analysis does not refine prognostic abilities beyond standard pathological analysis (Shivers et al. 1998; Bostick et al. 1999; Blaheta et al. 2000; Hochberg et al. 2002; Kuo et al. 2003; Ribuffo et al. 2003; Ulrich et al. 2004; Romanini et al. 2005; Kammula et al. 2004; Mangas et al. 2006; Hilari et al. 2009). A multi-institutional randomized clinical trial reported no difference in overall survival between patients with pathologically negative SLNs whose SLNs were "RT-PCR positive" only and whose regional nodal basins were observed and similar patients who

underwent complete lymph node dissection of the mapped nodal basin, with or without adjuvant interferon therapy (Scoggins et al. 2006; McMasters et al. 2016). Currently, this molecular approach is not employed for risk stratification for routine clinical care in cutaneous melanoma and is not a component of AJCC staging guidelines. The immunological milieu of the SLN has also been explored. Tumor-mediated immune modulation may, for example, render a lymph node more or less susceptible to the establishment of metastases (Cochran et al. 2006). Moreover, markers of immune response in the SLN may identify patients at increased risk of recurrence (Ma et al. 2012; Vallacchi et al. 2014). Currently, neither RT-PCR-based nor immunologic assessment of the SLN is included in AJCC melanoma staging; studies are ongoing using contemporary approaches such as next-generation sequencing, etc., to further assess possible roles for molecular profiling in the risk assessment of patients with cutaneous melanoma.

M Category

The melanoma M staging category currently includes site of disease and serum LDH levels. Novel ways to assess the risk of progression and potential response to therapy have been proposed for patients with metastatic disease. In the rapidly evolving era of mutation-targeted therapy and immunotherapy, genomic profiling of metastatic melanoma plays an important role in the assessment of patients with Stage IV melanoma to determine suitability for enrollment in clinical trials or other treatment options. These measures may someday play a role in staging.

Estimates of metastatic tumor burden (e.g., number of metastases, size of metastases, change in tumor burden over time) have been shown to correlate with prognosis in patients with Stage IV melanoma (Gaudy-Marqueste et al. 2014; Panasiti et al. 2013). The AJCC melanoma expert panel recognizes that the number of distant metastases has prognostic value; however, such measures have not been incorporated into the staging system because of the variability in the use of imaging to identify metastatic disease and inconsistent and nonuniform inclusion in many institutional melanoma databases that have been used to inform AJCC staging. Fold elevation of serum LDH and exploratory studies of alternative tumor markers such as S100B and YKL-40 have also been associated with prognosis and treatment response (Egberts et al. 2012; Dick et al. 2016; Simeone et al. 2014). Changes in the serum levels of these tumor markers have been shown to be associated with responses to targeted BRAF agents and immunotherapy (Abusaif et al. 2013; Diem et al. 2016). Elevated LDH has been shown to correlate with poor survival in patients treated with the anti-CTLA-4 immunotherapy drug ipilimumab (Kelderman et al. 2014).

Contemporary molecular techniques may also risk stratify patients with metastatic melanoma. Investigators have attempted to correlate circulating markers of immune response, such as neutrophil to lymphocyte ratio, or receptor expression on $CD4^+$ and $CD8^+$ T lymphocytes, with survival (Jacquelot et al. 2016; Gandini et al. 2016). Sera samples can be tested using RT-PCR or melanoma-specific antigen-detecting platforms to quantify minute expression levels of melanoma-associated cells. This so-called "liquid biopsy" approach can identify circulating tumor cells, cell-free circulating DNA, or cell-free circulating microRNA that has been shown to correlate with survival in Stage III and Stage IV patients (Huang and Hoon 2016).

The current (8th) edition AJCC melanoma staging system does not provide specific recommendations regarding the use of mutational testing for staging; nonetheless, it is clear that there is utility in assessing for somatic mutations among patients with unresectable disease or distant metastasis to help inform therapeutic options. High-throughput gene sequencing, termed next-generation sequencing, can identify genetic mutations that can be used to select targeted therapy, potentially classify patients into prognostic groups, and predict response to immunotherapy (Castiglione et al. 2016). These approaches, while not part of the current (8th) AJCC staging system, represent areas of ongoing investigation that may improve individualized

risk assessment for patients to help guide decision-making in the future.

Personalized Risk Assessment Versus Staging

The complex interplay between multiple risk factors, the wide range of prognoses within stage groups (e.g., heterogeneity of Stage III melanoma), and the power of computer-based analysis provide opportunities to further refine individualized risk assessment beyond TNM. One must understand that cancer staging and personalized risk assessment serve different roles. Staging classifies patients into large groups of generally similar risk. Staging is useful to inform clinical decision-making, to compare patients across clinical trials, and for other research and reporting efforts. The current staging system is necessarily constrained under a TNM-based system and therefore does not allow the inclusion of other risk factors that can potentially provide a more personalized individual risk assessment. This precise (or imprecise) estimation is more suitably determined by clinically validated prognostic tools (Collins et al. 2015; Kattan et al. 2016). These tools use multiple clinical and pathological features to estimate a single individual's risk of melanoma recurrence and death.

Several risk calculators are available online that use a composite of clinical and pathological factors to provide patients and clinicians with personalized risk assessment (Soong et al. 2010; Callender et al. 2012). In principle, such models can be useful clinical tools to improve clinical decision-making and risk assessment. However, one must be mindful of the shortcomings of currently available clinical prognostic tools and discuss such limitations with patients (Mahar et al. 2016). Issues include both the internal and external validity of the studies used to build the predictive models, either of which may limit the applicability of the tools to certain patient populations. Moreover, the data used to build the models may be somewhat dated, and the clinical risk assessments do not take into account newer therapies and improved diagnostic techniques. The 8th Edition AJCC Precision Medicine Core has developed criteria by which clinical prognostic tools can be critically assessed in an effort to inform both the professional and lay users of these tools (Kattan et al. 2016). Moving forward, AJCC staging guidelines and clinical prognostic tools will likely both play important roles in the study and management of patients with cutaneous melanoma.

The AJCC melanoma staging system is based on estimates of survival at the time of diagnosis based on clinicopathological data available at that time. A complementary approach to survival analysis is the concept of conditional survival. This type of survival estimation is based on a premise that a patient has already survived for a specific period of time following initial diagnosis. Given that they are alive for a certain period of time after diagnosis, their likelihood of survival has improved. Conditional survival has been explored for melanoma across all AJCC stages. These studies demonstrate improved conditional survival over time for AJCC Stage II, III, and IV patients, but not for Stage I patients (Xing et al. 2010; van der Leest et al. 2014). The implication is that the prognosis for localized Stage I disease is overall quite favorable and generally constant over time, while for patients with more advanced locoregional or distant metastatic disease, prognosis improves over time as a patient survives longer following the initial diagnosis. Conditional survival models can be used for all stages in melanoma to improve risk assessment. This approach takes advantage of information gained over time and offers a dynamic complement to the AJCC staging system and associated prognostic models based on the time of diagnosis. It is likely that conditional survival analyses will be explored using contemporary analytic approaches going forward.

Conclusion

Contemporary AJCC staging for cutaneous melanoma incorporates a TNM-based assessment of the primary tumor (T), nodal and non-nodal regional metastasis (N), and distant metastases (M). Primary tumor thickness and ulceration are

important prognostic factors that are important to prognosis in both localized and regionally advanced disease. Regionally metastatic disease exists across a spectrum of microscopic tumor deposits in single lymph nodes to non-nodal regional metastases and bulky, clinically apparent nodal metastases. N stage groups stratify these differences in an effort to risk cohort patients with Stage III disease. Patients with distant metastatic melanoma can be risk stratified according to their anatomic site of disease and serum LDH levels. Taken together, these factors can be used to predict the risk of melanoma-related death. As our understanding of melanoma evolves, so too will the potential factors – clinical, pathological, molecular, immunologic, etc. – and tools that can be utilized to assess risk. Clinicians must continue to collect data on clinical and pathological risk factors so that predictive models and staging system can be critically appraised and updated.

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Clinical Management of Primary Cutaneous Melanoma

24

Sonia Cohen and Kenneth Tanabe

Contents

Abstract

Surgical management continues to provide the mainstay of treatment for patients with early melanoma. In this chapter the authors describe the surgical approach to primary cutaneous melanoma lesions, including sentinel lymph node biopsy. These techniques are not only potentially curative but also provide the prognostic information necessary for subsequent treatment decisions. The current recommendations for the surgical management of early melanoma based on randomized prospective clinical trials, as well as future directions, are reviewed.

Keywords

Surgery · Melanoma · Excision margins · Wide local excision · Sentinel lymph node biopsy

Introduction

The incidence of cutaneous melanoma has steadily increased over recent decades to 21.6 per 100,000 individuals per year and now represents the sixth most common cancer in the United States (http://seer.cancer.gov). It is estimated that there were over 76,000 cases of melanoma diagnosed in 2016, corresponding to 4.5% of new

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cancers among males and females of all races, and that 2.1% of the population will be diagnosed with melanoma at some point during their lifetime. At the time of diagnosis, 84% of melanomas show no evidence of regional or distant metastases. The American Joint Commission on Cancer (AJCC) staging system for melanoma identifies several prognostic factors for these early-stage melanomas which aid in predicting survival, all of which are based on biopsy results. These include primary tumor thickness, the presence of ulceration in the primary lesion, the mitotic rate of tumor cells, and the presence of micrometastases identified by analysis of regional lymph nodes. Initial clinical management of most cases of invasive melanoma is guided by biopsy of suspicious lesions for thorough histopathologic assessment. Once the diagnosis of melanoma is confirmed, the patient will undergo surgical resection of the primary lesion, as well as biopsy of regional lymph nodes to detect metastatic disease when indicated. For localized tumors, resection of the primary tumor is potentially curative and provides an excellent prognosis, with 5-year survival rates as high as 98%. For tumors with evidence of regional metastatic disease, resection of the primary lesion and lymphadenectomy for nodal metastases provide further diagnostic and prognostic information, reduce tumor burden, and may extend overall survival. Understanding the data that guide this surgical management of melanoma is essential to providing optimal care for melanoma patients, as well as for designing new strategies to improve future outcomes.

Historical Overview

Melanoma was first described as a disease entity in the English literature in 1820 by W. Norris, who described tumors arising from pigmented lesions in two separate families (Hecht 1989). Over the subsequent century, the management of melanoma evolved as this disease was further characterized. The observation that there was a high local recurrence rate even after excision prompted the recommendation for aggressive, wide excision of the skin and subcutaneous tissues surrounding the primary lesion. The propensity of melanoma to metastasize led to the recommendation for early surgical intervention accompanied by regional lymphadenectomy at the time of initial resection. By the early 1900s, invasive melanoma was treated with surgical resection of the primary lesion with at least 5 cm margins in all directions, based on the observation of atypical melanocytes up to 5 cm from the edge of a primary lesion (Wong 1970). Primary excision was accompanied by complete regional lymph node dissection for all patients. Due to the frequent need for skin grafts for wound closure as well as wound complications and lymphedema arising from complete lymphadenectomy, this aggressive approach resulted in significant morbidity. Over the past several decades, the surgical management of malignant melanoma was refined using outcomes from clinical trials. While today surgery remains the mainstay of treatment of melanoma, current recommendations tailor treatment based on studies that balance efficacy with morbidity.

Excision of Primary Lesion

The goal of surgical excision of a primary melanoma lesion is durable disease control at the tumor site. This is of particular importance in the large majority of melanoma patients who are free from micrometastases at regional or distant sites. Potential mechanisms of local recurrence include incomplete excision of the primary tumor, incomplete excision of separate nests of melanoma cells (e.g., satellites or in-transit metastases), second primary melanomas, and hematogenous dissemination of cells back to the original excision site. Excision with wider margins may be an effective strategy to combat the first two mechanisms of local recurrence by facilitating complete excision of a primary tumor and any nearby micrometastases. Wider excision could also have a modest impact on the development of second primary melanomas by removal of additional skin affected by an oncogenic field defect. However, large excisions may be prone to poor healing or surgical site infections, require skin grafting for closure, and lead to impaired function and

Table 1 Current recommendations for margins of excision of primary cutaneous melanoma

	Tumor thickness (mm)	Circumferential excision margin (cm)
Thin melanoma	<1	1
Intermediate melanoma	1–2	2[a]
	2–4	2
Thick melanoma	>4	2

[a]1 cm margin may be acceptable if significantly less morbid than 2 cm margin

mobility. Thus, clinical trials have been used to determine the minimum safe excision margins that are sufficient for cancer treatment while minimizing functional and cosmetic impairments. The current recommendations for surgical margins (Table 1) are based on the results of a series of prospective randomized trials.

Margins of Surgical Resection Are Tailored to Melanoma Thickness

Tumor thickness is a prognostic factor for local recurrence. The overall recurrence rate for melanomas <1 mm thick after excision is less than 6% across a number of studies, suggesting that extensive resection might be unnecessary for these thin tumors. As surgical practice shifted toward narrower margins in this context, initial retrospective reviews of patient outcomes in the treatment of thin melanomas found that the rate of local recurrence was not affected. For example, in one series of 936 patients with thin tumors in which 62% underwent excision with margins of 2 cm or less, not a single case of local recurrence was documented over 5 years of observation (Urist et al. 1985). These data suggested that reducing excision margins for low-risk melanomas could be safe. However, the retrospective nature of these studies and the resultant variability in treatment combined with the overall low rate of local recurrence may have obscured any effect of narrow excision margins on oncologic outcomes.

To better address the safety of narrow excision margins for thin melanomas, the World Health Organization (WHO) Melanoma Program conducted a randomized prospective trial comparing 1 cm versus 3 cm clinical margins for primary melanomas less than 2 mm thick (Veronesi et al. 1988). Six hundred twelve patients with localized, biopsy-confirmed thin melanoma were randomly assigned to either wide or narrow excision and then followed for evidence of relapse or death. After a median follow-up of 55 months, there was no difference in overall, disease-free survival, or local recurrence among the two groups. There were three cases of isolated local recurrence, and all occurred in the narrow excision margin group. However, the overall rate of local recurrence remained too low (2.7%) for this difference between groups to be of statistical significance. Interestingly, all three local recurrences occurred in patients with melanomas with greater than 1 mm thickness, suggesting that excision margins of 1 cm are safe for thin melanomas, but should perhaps be limited to tumors less than 1 mm thick. No randomized prospective trial since has readdressed the excision margin for melanomas <1 mm thick. Thus, current guidelines continue to recommend a 1 cm margin of surgical resection for melanomas less than 1 mm thick and are supported by case-control series (MacKenzie Ross et al. 2016). For melanomas between 1 and 2 mm in thickness, some surgeons are reluctant to use a margin of only 1 cm because of the trend toward increased local recurrence observed in the 1 cm margin group in the WHO trial. However, in cases in which a 1 cm margin could be achieved with substantially less morbidity than with a wider margin, the WHO clinical trial data suggest that the use of a 1 cm margin leads to the same overall survival and perhaps only a slight increased risk for local recurrence. Therefore, current guidelines accept a 1 cm margin of excision if this will result in significantly less morbidity than a wider margin, requiring intraoperative judgment to balance the risk and benefit on a case-by-case basis.

The Intergroup Melanoma Surgical Trial was the first randomized prospective trial to address the safety of narrow excision margins for intermediate-thickness melanomas (1–4 mm

thickness). Four hundred eighty-six patients with intermediate-thickness lesions were randomized to undergo excision with either 2 cm or 4 cm margins. After a median follow-up of 72 months, no significant difference in recurrence rate or survival was observed between the two arms (Balch et al. 1993). Increasing tumor thickness, the presence of ulceration, and truncal location of the tumor did correlate with decreased survival, but the margin of excision did not, even after adjusting for these other prognostic factors. Importantly, there was a statistically significant decrease in the rate of skin grafting required to close the excision site in patients who underwent surgical resection with narrow margins (11% vs. 46% in patients with 4 cm excision margins). Lower rates of skin grafting led to significantly lower rates of wound infection and shorter hospital stays. Even after 10 years of follow-up, there remained no statistically significant difference in local recurrence, 10-year disease-specific survival, and overall survival (Balch et al. 2000), supporting the long-term safety of narrow excision margins. Moreover, the short-term decrease in morbidity associated with wider excision suggested an overall advantage to treatment of intermediate melanomas with narrow margins.

Subsequent large, randomized, prospective studies specifically addressed the safety of narrow margins in subsets of these intermediate-thickness melanomas. The Swedish Melanoma Study Group trial examined cutaneous melanoma between 0.8 and 2 mm in thickness (Ringborg et al. 1996). One subgroup of patients with clinically suspected melanoma underwent initial excision with a 2 cm margin – following this initial excision and analysis of tumor depth, patients with tumors between 0.8 and 2 mm thick were then randomized to either undergo subsequent wide excision of the scar with 3 cm margins (for a total of 5 cm) or no further intervention. These data were combined with those from patients where the initial diagnosis of melanoma was made via excisional biopsy, and patients with tumors of the appropriate depth were then randomized to surgical resection of the scar with either 2 or 5 cm margins. All surgical interventions were completed within 6 weeks of the initial diagnostic procedure. A total of 989 patients ultimately participated with a median follow-up of 11 years (Cohn-Cedermark et al. 2000). The observed rates of local and distant melanoma recurrence, as well as disease-free and overall survival, were not significantly different between those randomized to 2 cm rather than 5 cm margins. A similar prospective study by the French Melanoma Group which randomized 337 patients with melanomas less than 2.1 mm thick to excision with 2 cm or 5 cm margins confirmed no differences in rates of recurrence or disease-free or overall survival after a median follow-up of 16 years (Khayat et al. 2003). These studies are consistent with the Intergroup Trial results demonstrating that a 2 cm margin is adequate for all intermediate-thickness melanomas.

Two trials have focused on the safety of narrow margin excisions in cutaneous melanoma 2 mm or greater in thickness. As reviewed above, the Intergroup Melanoma Surgical Trial concluded that 2 cm margins of excision should be safe for all tumors less than 4 mm thick. However, to specifically address recommendations for tumors thicker than 2 mm, Gillgren et al. analyzed 2 cm versus 4 cm excision margins in this patient group (Gillgren et al. 2011). Nine hundred thirty-six patients with tumors of the trunk or extremity were included. There was no difference observed in the overall or disease-free survival between the 2 cm and 4 cm excision groups. The authors did find a trend toward an increase in local recurrence in the 2 cm margin group, although this did not reach statistical significance ($p = 0.06$). In a second study, 900 patients were randomized to excision of melanomas greater than 2 mm thick with 1 cm or 3 cm margins (Thomas et al. 2004). In this study, locoregional relapses were redefined at interim analyses to be inclusive of local recurrence, satellite, in-transit, and regional lymph node metastases. With this new definition including lymph node metastases, the observed increase in the rate of locoregional recurrence identified in the population treated with 1 cm margins of excision (37% vs. 32% in those treated with a 3 cm excision margin) reached statistical significance at a median follow-up of 60 months. By a median follow-up of 106 months, this had translated into a

significantly higher risk of death from melanoma in the 1 cm margin group as compared to the 3 cm group (HR 1.24, 95% CI 1.01–1.53, $p = 0.041$) (Hayes et al. 2016). Notably individuals with tumors greater than 2 mm thick would typically undergo sentinel node biopsy (see below), but patients in this study were treated without sentinel node biopsy. Thus, many of the locoregional recurrences potentially could have been prevented with sentinel node biopsy. This is supported by the finding that the statistical significance of the observed difference in locoregional recurrences between the 1 cm and 3 cm groups is lost when nodal events are taken out of the analysis. Overall these trial results are consistent with the WHO Melanoma Program trial results, summarized above, which suggested that excision with only 1 cm margins is insufficient for tumors greater than 1 mm thick due to a trend toward an increase in the rate of local recurrence (Veronesi et al. 1988). It is therefore not surprising that a 1 cm margin of excision would also be insufficient for tumors greater than 2 mm thick. And for melanomas >2 mm in thickness, the Intergroup Trial results demonstrate that a 4 cm margin is no better than a 2 cm margin, resulting in the current recommendation of 2 cm excision margins for melanoma between 2 and 4 mm thick (Table 1).

Inclusion of all tumors greater than 2 mm in a clinical trial may be too broad a cohort to detect significant differences between excision margin groups. It is possible that melanomas greater than 4 mm in thickness could require more aggressive excision margins than those closer to 2 mm in thickness. Several studies have found that the thickness of tumor (along with the presence of ulceration) correlates with the risk of locoregional recurrence of primary cutaneous melanoma (Urist et al. 1984; Balch et al. 1993; Karakousis et al. 1996), so inclusion of all tumors greater than 2 mm in a single cohort may prevent investigators from identifying significant differences within treatment arms. It seems reasonable to entertain the idea that the thickest tumors may require wider margins of excision. Most melanomas are less than 2 mm thick at the time of diagnosis; thus, the number of very thick primary cutaneous melanomas without clinical evidence of metastatic disease at the time of diagnosis is relatively small. No randomized prospective trial has examined resection margins in only thick melanomas. One retrospective study examining resection of tumors greater than 4 mm thick with margins of excision either less than or greater than 2 cm found no significant difference in locoregional recurrence or survival (Heaton et al. 1998). However, in another retrospective analysis, Pasquali et al. found that patients with melanomas greater than 4 mm thick with a pathologically determined margin of less than 1.6 cm (corresponding to a fresh tissue margin of about 2 cm) had a significantly increased risk of local recurrence compared to patients whose pathologically determined margin was greater than 1.6 cm ($p = 0.01$, with a hazard ration of 2.41 and confidence interval of 1.23–4.73) (Pasquali et al. 2013). Thus, 2 cm margins of surgical resection may be safe for any cutaneous melanoma with a Breslow thickness greater than 2 mm, but further investigation using specific thickness subgroups in a prospective randomized trial is needed to definitively tailor recommendations.

Given the potential difficulty in detecting differences in outcomes between narrow and wide excision margins due to low rates of local recurrence in thin melanomas and relatively few cases of thick melanomas, a number of meta-analyses have been undertaken of the studies reviewed above (Haigh et al. 2003; Sladden et al. 2009; Mocellin et al. 2011; Wheatley et al. 2016). These analyses have the advantage of increased statistical power based on larger combined sample sizes, but the disadvantage of combining heterogenous datasets. The most recent meta-analysis which had access to all the trials reviewed found no significantly increased risk of locoregional recurrence or overall survival between narrow margin and wider margin groups. Importantly, however, this conclusion was based on the grouping of both 1 cm and 2 cm margins as "narrow" excisions. When trials with identical arms were combined for analysis, only overall survival was reported, despite the suggestion that locoregional recurrence may be the most affected outcome. Moreover, there was no attempt to analyze the data by specific subgroup of tumor thickness. As

discussed above, this heterogeneity in the comparison groups makes it difficult to interpret the conclusions reached by this and previous meta-analyses, supporting a need for further investigation. Additionally, thicker tumors have higher rates of regional metastases at the time of diagnosis (Morton et al. 2014), suggesting that locoregional recurrence is dependent on control of these metastases in addition to excision of the primary lesion (reviewed below). Analysis of locoregional recurrence in patients with thicker melanomas without accounting for this difference in tumor stage likely confounds the results.

To summarize, current recommendations based on the data reviewed above are the use of a 1 cm margin for melanomas <1 mm in thickness and 2 cm margin for melanomas >2 mm in thickness. For melanomas with thickness between 1 and 2 mm, ideally a 2 cm margin would be used. However, in instances in which this margin is associated with significantly greater morbidity compared to the use of 1 cm margin, then the use of a 1 cm margin is appropriate (Table 1).

Excision Technique

The importance of obtaining an adequate biopsy in the diagnosis of melanoma cannot be overemphasized. Tissue samples are examined by a pathologist for the presence of malignant-appearing cells, which can be confirmed using immunohistochemistry to detect the presence of cellular markers of melanoma. As discussed previously, the thickness of the melanoma itself, in addition to the mitotic rate of the melanoma cells, and the presence of ulceration within the biopsied lesion are all characteristics of the tumor which provide important prognostic information that drive subsequent treatment decisions. Mutational analysis can also be performed from the tissue obtained to help determine the need and utility of systemic therapies in cases of high-risk or late-stage melanoma. Suspicious lesions are most often identified and biopsied in an office setting by a dermatologist or general practitioner, and it is critical that the appropriate technique is used for the initial biopsy to ensure that the tissue sample can be thoroughly characterized. Shave biopsies which take a tangential biopsy of the lesion are often insufficient as they may not sample the complete thickness of the lesion. Shave biopsies should therefore be performed only if the suspicion for and risk of melanoma are very low or the shave is very deep. In contrast, punch or excisional biopsies remove a full-thickness sample of the skin and are the preferred method for sampling any suspected melanoma as they can provide more accurate assessment of tumor thickness.

Once a melanoma has been identified by biopsy, the patient will undergo wide local excision to ensure that the lesion has been completely removed with adequate margins (Fig. 1). Wide local excision is often performed under local anesthesia or regional anesthesia in cases where sentinel node biopsy or lymphadenectomy is not planned; otherwise, general anesthesia is used. Recommended excision margins (Table 1) are clinically determined margins measured from the edge of the lesion or prior biopsy scar and do not refer to the width of the margin assessed by the pathologist. By convention the muscle fascia serves as the deep margin, though there are not data to provide guidance on this matter. Excision of the muscular fascia itself is recommended only in cases of fascial involvement by tumor. Specimens are then submitted for permanent pathology as frozen analysis has not proven reliable for melanoma.

A number of techniques are used to close the wound primarily after excision, including the use of an elliptical incision to prevent “dog ears” and raising skin flaps if needed to reduce tension during closure. The excision site (or “wound edge”) is then closed in layers to reduce the potential space and prevent seroma formation. In the case of excision of a lesion with significant tension or in a difficult anatomical area (e.g., the head or neck), the use of skin grafts or local flaps may

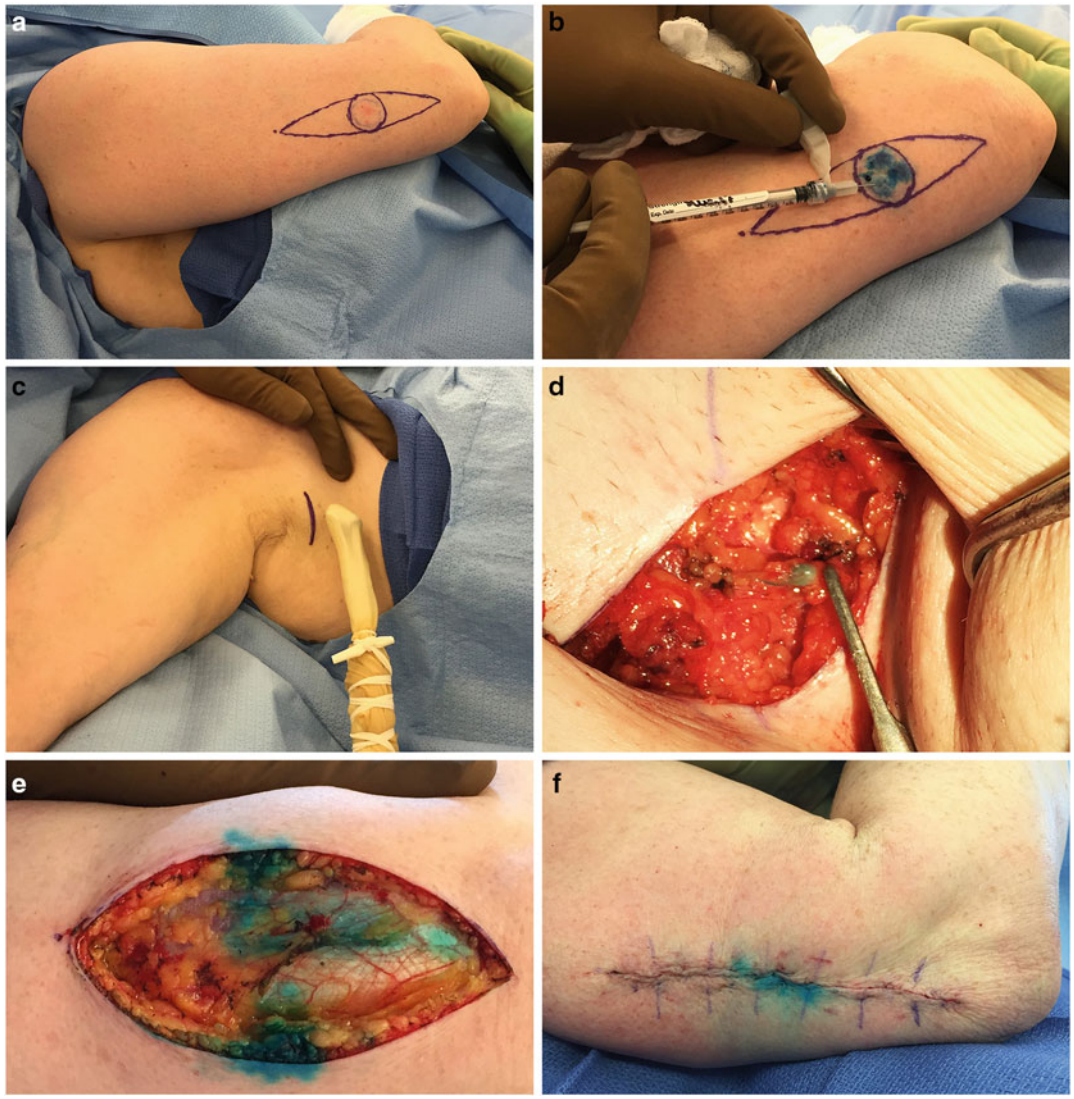

Fig. 1 Wide local excision of right arm melanoma with right axillary sentinel node biopsy. The site of the previously biopsied primary cutaneous melanoma on the right upper extremity has been sterilized and draped. The right axilla has been included in the operative field for planned sentinel node biopsy. The site of the previous biopsy has been marked with a circumferential 1 cm margin (blue circle surrounding scar in **a**) to delineate the planned margin of wide local excision. The incision will be extended into a longitudinal ellipse to reduce the size of "dog ears" on the ends (blue ellipse in **a**). Orienting the excision longitudinally will also help minimize future tissue loss if re-excision is necessary. For sentinel node biopsy, the dermis surrounding the lesion is injected preoperatively with a radioactive tracer, technetium-99 sulfur colloid. This dye is taken up by the dermal lymphatics which label the drainage basin (in this case the right axilla). The nodes are also labeled with intradermal injection of isosulfan blue (**b**) prior to the start of the procedure. The sentinel node biopsy precedes excision of the primary lesion so as not to disrupt the lymphatic drainage from the lesion. A handheld gamma probe is used to guide the initial incision (**c**). Identification of the sentinel node(s) is made by the presence of radioactivity and the blue coloration of the node (**d**). After dissection of the sentinel node is complete, the primary lesion is excised including all subcutaneous tissues down to the muscle fascia (**e**). The elliptical excision site is then closed primarily (**f**)

prove helpful. In some cases where the surgeon is not confident that the excision margin is free of cancer, or where narrow margins are necessitated by anatomy, the wound may be left open. Alternatively, a temporary wound closure device (e.g., a wound vacuum) can be placed until pathology results are available. If the pathological margins prove to be negative, then a skin graft or local flap can be used to close the excision.

A final important consideration for both the initial biopsy (if incisional) and wide local excision is the orientation of the scar that is formed. Because the margins of any necessary re-excision will extend circumferentially along the entire length of the scar, the initial scar should be oriented accordingly. For example, the initial incisional biopsy or wide local excision of a lesion on an extremity should be oriented longitudinally along the long axis of the extremity. If re-excision is required (such as in a case where what was thought to be a thin melanoma on initial biopsy is found to be of intermediate thickness after complete excision), then a longitudinal orientation along the extremity will maximize the chances that the scar can be removed with adequate margins and still allow for primary closure of the wound. The possible need for re-excision should be considered when determining the best approach to excision of every lesion based on both size and location.

Role of Lymphadenectomy in Primary Cutaneous Melanoma

Early observations suggested that metastatic cutaneous melanoma initially spreads through intradermal lymphatics to regional nodal basins and then to more distant sites. As early as the 1890s, it was recognized that individuals with clinically evident nodal disease were more likely to have distant metastases. As a result routine, early elective complete lymphadenectomy evolved as part of standard surgical management of intermediate-thickness primary cutaneous melanomas to try to prevent distant spread of metastatic disease. Unfortunately analyses of the nodes excised revealed that only 20% of patients undergoing elective lymphadenectomy had nodal metastases at the time of resection (Beitsch and Balch 1992), exposing 80% of patients undergoing this procedure to the associated risks without an obvious benefit. Moreover, there was no survival benefit when elective early lymphadenectomy was compared to performing complete lymphadenectomy only once a patient had developed clinically palpable nodal disease (Balch 1999; Balch et al. 1996). However, the alternative of nodal observation with lymphadenectomy only once a patient developed clinically evident nodal disease was thought to potentially compromise long-term control of metastatic disease (Balch et al. 2010; Cascinelli 1998; Morton et al. 2006). Lymphatic mapping and sentinel lymph node biopsy was developed by Morton and colleagues as a method to try to identify in a less-invasive manner which patients had nodal metastases (Wong et al. 1991). In this setting, completion lymphadenectomy could be limited to individuals with clinically occult metastatic disease where the goal would be to prevent the progression to clinically evident nodal disease. Multiple studies have since demonstrated the prognostic value of sentinel node biopsy. However, completion lymphadenectomy based on the presence of sentinel lymph node metastases without clinically evident nodal disease has not been definitively shown to improve melanoma-specific survival.

Technique of Sentinel Lymph Node Biopsy

Sentinel lymph node biopsy is based on the premise that lymphatic channels draining from specific cutaneous sites drain to specific first, or sentinel, lymph nodes that can be identified and resected. The presence or absence of melanoma metastases in these sentinel nodes accurately correlates with the presence or absence of metastatic melanoma in the entire nodal basin.

Sentinel node biopsy is performed using preoperative injection of a radioactive tracer, technetium-99 sulfur colloid, into the dermis surrounding a lesion or biopsy scar on the day of wide local excision and sentinel lymph node

biopsy. This dye is taken up by the dermal lymphatics which label the drainage basin. Deep injection below the dermis may map the wrong lymphatic channels and lead to the harvesting of the incorrect lymph nodes or prevent migration of the isotope to a regional lymphatic basin. Subcutaneous injection should be suspected if subsequent imaging does not reveal a draining nodal basin. Following injection, a scintillation camera may be used to identify patterns of lymphatic drainage and sentinel nodal basin (s) (Fig. 2). Labeled lymph nodes are apparent within 30 min of injection, and the radioactive signal persists for several hours. This technique

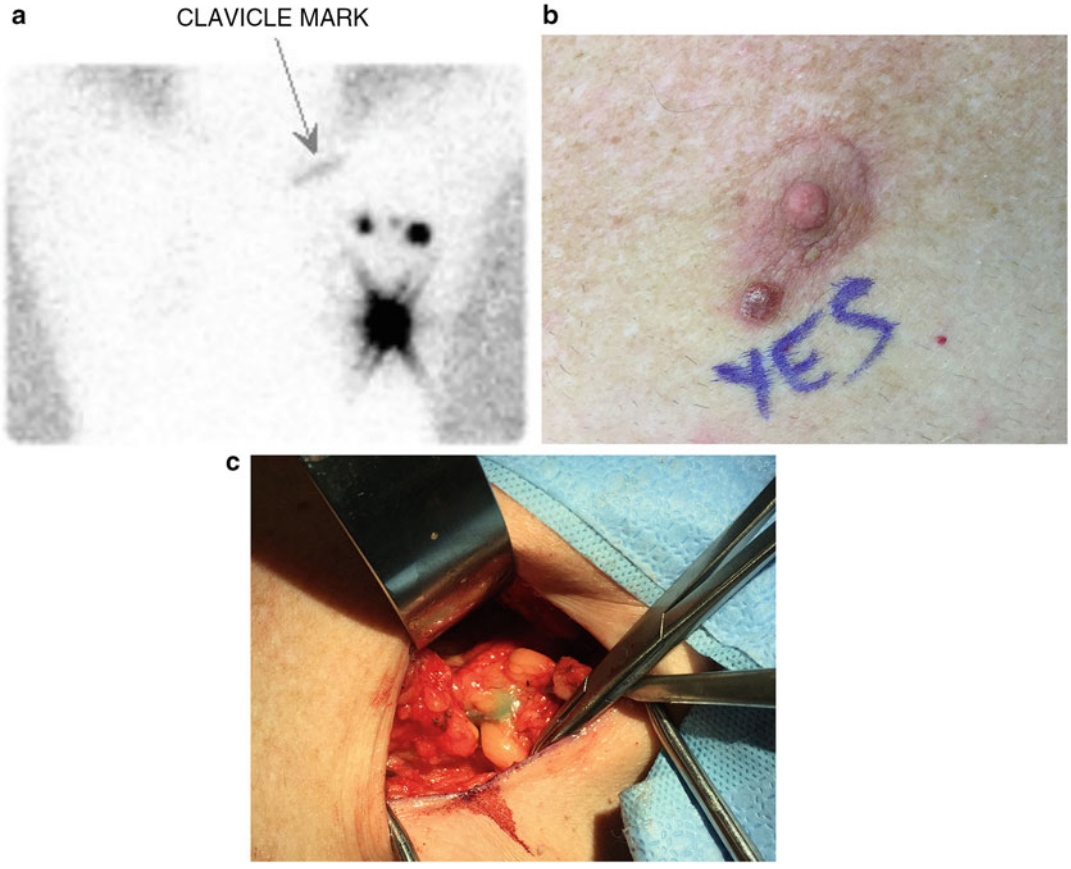

Fig. 2 Lymphoscintigram localizes regional drainage basin containing sentinel nodes. Sentinel node biopsy is performed using preoperative injection of a radioactive tracer, technetium-99 sulfur colloid, into the dermis surrounding a lesion. This dye is taken up by the dermal lymphatics which label the drainage basin. Following injection, a scintillation camera is used to identify patterns of lymphatic drainage and sentinel nodal basin(s) by imaging the radioactive signal, as shown here. (**a**) **Lymphoscintigram of chest and bilateral axillae**. Lymphoscintigram taken 5 min after injection of technetium-99 adjacent to the melanoma excision scar located on the left anterior chest (marked with white * on image). The additional foci of radioactive uptake represent three sentinel nodes within the left axilla. Patterns of lymphatic drainage are not predictable for non-extremity lesions and may even involve contralateral nodes, making preoperative lymphoscintigraphy helpful in focusing intraoperative dissection efforts. In this case, the left anterior chest lesion drained to the left axillary nodal basin. (**b**) **Left chest melanoma prior to resection**. Intraoperative photo of left chest melanoma which has been labeled with technetium-99 sulfur colloid (see lymphoscintigram in **a**). Sentinel nodes were double labeled with radioisotope and isosulfan blue prior to resection to aid in their identification (**c**). Tumor is marked "YES" as part of the preoperative universal protocol prior to induction of anesthesia to ensure resection of the correct lesion. (**c**) **Left axillary sentinel node**. Intraoperative photo of left axillary sentinel node draining left chest melanoma (**b**) identified both by the presence of radioactivity as demonstrated in lymphoscintigram (**a**) and by the presence of isosulfan blue

may also identify interval or in-transit nodes. Patterns of lymphatic drainage are not reliably predictable for non-extremity lesions and may even involve contralateral nodes, making preoperative lymphoscintigraphy helpful in focusing intraoperative dissection efforts. Intradermal injection of isosulfan blue at the lesion or biopsy scar further helps guide dissection (Fig. 1). The injected site is typically resected as part of the wide local excision; however, if this is not planned (e.g., when sentinel node biopsy is performed after wide local excision), it should be kept in mind that the isosulfan blue injection may leave behind a small but permanent tattoo. A handheld gamma probe and results of lymphoscintigraphy guide the initial target area for incision, while blue lymphatic channels help lead the dissection to the sentinel node(s). Using this double labeling technique, the sentinel node is defined by its blue color as well as by its radioactivity (Figs. 1 and 2). All nodes with radioactivity count at least 10% of the most radioactive node are defined as sentinel nodes and harvested, a technique which minimizes the rate of false-negative sentinel lymph node biopsy results (Luo et al. 2015). The sentinel lymph node can be successfully identified and removed in more than 99% of patients (Gershenwald et al. 1998). Usually between one and three sentinel nodes are identified per basin and sent for permanent pathology to evaluate for the presence of micrometastases using H&E staining and immunohistochemistry of multiple sections. When occurring as part of the same procedure, sentinel lymph node dissection is often performed prior to wide local excision of the primary lesion to prevent disruption of the labeled lymphatics that help to identify the sentinel node(s). However, in some cases, it is beneficial to reverse this sequence to prevent radiation from injection of the primary tumor site from interfering with localization of the sentinel node.

Sentinel Lymph Node Biopsy Provides Prognostic and Staging Information

Currently the results of sentinel lymph node biopsy are used for accurate staging and prognosis and to help determine whether completion lymphadenectomy or adjuvant therapy would be of benefit. Sentinel lymph node biopsy at the time of wide local excision is recommended for any patients with melanomas greater than 1 mm thick, as well as for melanomas equal to or less than 1 mm thick which have other high-risk features such as ulceration, a high rate of mitoses, or lymphovascular invasion. In terms of prognosis, it has been estimated that individuals with negative sentinel lymph node biopsies have a 90% 3-year disease-free survival, which decreases to 60% if they are found to have positive sentinel lymph nodes (Gershenwald et al. 1999). Moreover, a number of studies have shown that the histological status of the sentinel lymph node is the best predictor of survival in clinically node negative melanoma patients (Table 2).

The Multicenter Selective Lymphadenectomy Trial (MSLT-I) was a phase 3 trial designed to determine whether identifying patients with clinically occult nodal melanoma metastases via sentinel node biopsy and then performing an immediate completion lymphadenectomy in those patients improved outcomes (Morton et al. 2014). A total of 2001 patients were enrolled, and ultimately 1270 patients with intermediate-thickness tumors between 1.2 and 3.5 mm thick completed the trial. Another 314 had thicker

Table 2 Multiple multivariate analyses suggest that the presence of regional node metastases are the most important prognostic factors in early-stage melanoma and most reliably predict survival across studies

Prognostic factor
Node status
Number of involved nodes[a, b, c]
Tumor burden within nodes[b]
Primary tumor thickness[a, b, c]
Ulceration[b]
Site of primary lesion[a, b]
Patient age[b]

An individual patient's risk of sentinel lymph node metastases can be calculated using a number of available tools (Mahar et al. 2016), including the Memorial Sloan Kettering Cancer Center Melanoma Nomogram which is available at https://www.mskcc.org/nomograms/melanoma (Wong et al. 2005)

Based on [a]Morton et al. (1991), [b]Balch et al. (2001), and [c]Gershenwald et al. (1999)

primary melanomas. Of the individuals enrolled in the trial, 60% were randomized to wide local excision with 2–3 cm excision margins as well as sentinel lymph node biopsy, while the remaining 40% of patients enrolled underwent wide excision with nodal observation. A positive sentinel node biopsy triggered immediate completion lymphadenectomy. Otherwise, patients were observed and underwent lymphadenectomy only in the case of clinically evident nodal recurrence.

As predicted by previous studies, MSLT-I demonstrated that in the biopsy group, patients with sentinel node metastases had worse outcomes as compared to those without evidence of metastatic disease. In those with intermediate-thickness tumors, the 10-year melanoma-specific survival rate was 62.1% in node-positive patients, compared to 85.1% in patients without a positive sentinel lymph node biopsy ($p < 0.001$). For patients with thick tumors, the respective rates were 48% and 64.6% ($p = 0.03$). While there seemed to be little debate regarding the prognostic value of the sentinel lymph node biopsy, there remained significant controversy regarding whether SLNB itself actually reduces rates of recurrence and improves disease-free survival.

Much of the controversy surrounding MSLT-I stemmed from the fact that the trial was ultimately insufficiently powered to address the primary endpoint of melanoma-specific survival in all randomized subjects. This was due to the fact that the majority of patients with intermediate-thickness melanomas, 80%, demonstrated no nodal metastases – the survival of this group therefore could not be expected to be improved by early nodal excision, making it difficult to detect a significant benefit of sentinel node biopsy across the entire population. However, when subgroups were analyzed to examine the 20% of patients who ultimately developed nodal metastases (either demonstrated by initial sentinel node biopsy or during the observation period), immediate lymphadenectomy was suggested to improve outcomes. Individuals with intermediate-thickness melanomas and node-positive disease demonstrated a 10-year melanoma-specific survival benefit with early removal of nodal metastases (62.1% in biopsy group vs. 41.5% in observation group, hazard ratio 0.56, $p = 0.006$). Disease-free survival was also significantly improved (hazard ratio 0.62, $p = 0.02$). There was no treatment-related difference demonstrated among those individuals without nodal metastases at sentinel node biopsy or during the observation period. These results suggested that sentinel node biopsy and early completion lymphadenectomy might provide survival benefit to patients with intermediate-thickness melanoma.

A positive result on pathological examination of the sentinel node(s) indicates that the patient has had clinically occult spread of their melanoma into the lymphatic drainage basin examined. Given the aggressive nature of metastatic melanoma until very recently, the standard of care for a patient with a positive sentinel node biopsy was to offer completion lymphadenectomy, which involves dissection of the remainder of the regional lymphatic tissue to remove any other occult disease that may be present. Complete regional lymphadenectomy can be complicated by wound infection and seroma in the short term, as well as chronic lymphedema and neuronal dysfunction, prompting the need to ensure that this relatively morbid procedure results in improved outcomes.

The DeCOG-SLT study randomized patients with sentinel node-positive melanoma to close clinical observation of the nodal basin or completion lymphadenectomy (Leiter et al. 2016). Four hundred eighty-three patients were randomized, and as a whole, they had low risk of harboring disease in non-sentinel lymph nodes, as nearly 70% of the patients had less than 1 mm of sentinel lymph node tumor burden. The study was underpowered, and insufficient events were recorded to reach statistical significance. No melanoma-specific survival difference was observed after a median follow-up of 3 years, despite a significant increase in the nodal basin recurrence rate in the patients randomized to nodal basin observation. Patients randomized to completion lymphadenectomy had more frequent adverse events – primarily wound complications and lymphedema – compared to those in the observation arm.

MSLT-II was a randomized, prospective trial designed to specifically address whether patients with intermediate-thickness melanomas and sentinel node metastases would incur a survival benefit from immediate completion lymphadenectomy (Faries et al. 2017). One thousand nine hundred thirty-four individuals with a positive sentinel node biopsy were assigned to undergo either dissection of the affected lymph node basin or close observation with clinical examination and nodal ultrasonography. Completion lymphadenectomy did provide additional prognostic information in terms of the pathologic status of the non-sentinel nodes and led to a reduction in locoregional recurrence by about 70%. Despite these findings, with relatively short median follow-up of 43 months, there was no significant survival benefit with completion lymphadenectomy as compared to the observation group.

Together these data suggest that patients with melanoma metastatic to a sentinel lymph node are just as likely to have systemic metastases as they are to have metastases to the remainder of the lymph node basin. Completion lymphadenectomy therefore provides no therapeutic advantage over sentinel lymph node biopsy itself. While there are some complications associated with the sentinel lymph node biopsy, including wound infection and seroma formation, multiple studies comparing the rates of postoperative complication demonstrate that the risk is significantly lower for sentinel node biopsy alone as compared to completion lymphadenectomy (10% vs. 37% in MSLT-I, 24% vs. 6% MSLT-II, and 4.6% vs. 23.2% in the Sunbelt Melanoma Trial (Wrightson et al. 2003)). Therefore, as sentinel node biopsy provides equivalent benefit in terms of survival, completion lymph node dissection should not be recommended in patients who can undergo close clinical and ultrasonographic observation. In this new era of effective systemic treatments for melanoma, the true utility of sentinel lymph node biopsy will likely derive not from selecting patients for early completion lymphadenectomy but from identifying patients who will benefit from aggressive systemic therapies.

Conclusions and Future Directions

Despite the increase in the incidence of malignant melanoma, there have been dramatic improvements in the diagnosis and treatment of patients with melanoma in recent years. Surgical resection of early disease remains the mainstay of curative treatment. Current margin guidelines are derived from randomized, controlled studies and are based on tumor thickness. Reduction in surgical margins over the past several decades has limited the need for skin grafting, resulted in fewer wound complications, and led to faster recovery times without compromising disease-free or overall survival. The development of sentinel node biopsy has provided essential prognostic information and may prove to provide sufficient debulking of regional metastatic disease to make completion lymphadenectomy unnecessary. Moving forward, as our understanding of the molecular basis of malignant melanoma evolves, we will be even better able to predict disease behavior based on a tumor's molecular profile. Understanding which markers confer increased risk for metastatic disease will provide the information needed to further tailor surgical management, reserving aggressive surgical resection for those individuals at highest risk. Even with the development of targeted therapies that are transforming the landscape of care for advanced melanoma, surgical management will continue to provide the mainstay of curative treatment for patients with early disease.

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Management of Locally and Regionally Recurrent Melanoma

25

Cristina O'Donoghue, Jonathan S. Zager, and Vernon K. Sondak

Contents

Abstract

Locally and regionally recurrent melanoma is a recurrence at the site of primary disease, the regional draining lymph node basin, or in between, described as satellite or in-transit metastases. Close follow-up of melanoma patients is merited as most recurrence is diagnosed from a physical exam. Adjunct imaging is important to determine the extent of disease and to rule out distant metastases. The treatment options for locoregional recurrent melanoma include: surgery, intra-arterial regional therapy, intralesional and topical therapies, radiation and systemic therapies. Factors such as time to recurrence, previous treatments, patient age, recurrence size, location and number should be considered to determine the best treatment option or sequence of options. Patients with melanoma recurrence should be discussed in a multidisciplinary tumor board especially as treatment options continue to develop and evolve.

Keywords

Melanoma · In-transit · Satellitosis · Intra-arterial therapy · Isolated limb infusion · Intralesional therapy

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Introduction

Locoregional recurrence of melanoma is defined as recurrence locally at the site of the primary excision scar, regionally within the draining lymph node basin, or in between, described as satellite or in-transit metastases. Satellite metastases by convention are those occurring within 2 cm of the primary tumor, whereas in-transit metastases are any dermal or subcutaneous metastases 2 cm or more from the primary tumor but not beyond the draining regional node basin (see Fig. 1) (Amin et al. 2017). Local recurrence can represent persistent disease following a margin-positive initial excision or recurrence in or adjacent to the primary excision scar after a margin-negative prior excision (Karakousis et al. 1996). Clarification of the nature of the local recurrence may be important for prognosis and may have implications for nodal staging as well. Satellite metastases are considered intralymphatic spread of the primary tumor and in-transit metastases are tumor deposits growing along routes of lymphatic drainage (Speicher et al. 2015). Locoregional disease is staged by the American Joint Committee on Cancer (AJCC) as stage III disease, with satellite and/or in-transit metastases defined as a component of nodal (N) staging and subclassified depending on the absence or presence of lymph node involvement (AJCC 2017).

Up to 25% of melanoma patients develop locoregional recurrent disease. The likelihood of recurrence is impacted by initial stage at presentation, occurring in approximately 14% of stage I and II patients (Meier et al. 2002; Statius Muller et al. 2002) but in up to 47% of stage III melanoma patients (Dalal et al. 2007). As practice patterns change with recent randomized controlled trial results, such as the Multicenter Selective Lymphadenectomy Trial II (MSLT-II) (Faries et al. 2017) and DECOG trial (Leiter et al. 2016), which demonstrated immediate completion lymph node dissection for sentinel lymph node positive disease does not improve survival but increases regional control, there may be an increase in regional nodal recurrence as more patients forgo complete lymph node dissections. Elderly patients (>70 years) may have higher rates of local and in-transit recurrence despite a lower incidence of sentinel lymph node metastases, and poorer disease-specific mortality (Balch et al. 2013; Macdonald et al. 2011). Primary sites with higher risk for recurrence include melanomas on the head and neck and lower extremity, especially acral lentiginous primaries. Histopathologic features contributing to the risk for local, in-transit, and nodal basin recurrence include ulceration, thickness of primary (especially >4 mm), positive margin, microsatellitosis and nodal involvement, especially with multiple nodes, extracapsular extension or large nodal metastases (Karakousis et al. 1996; Speicher et al. 2015; Pidhorecky et al. 2001; Leon et al. 1991). Microsatellitosis as a recurrence risk factor was demonstrated in the Intergroup Melanoma Trial, which showed local recurrence risk to be 32% in patients with microsatellitosis versus 2% in those without (Karakousis et al. 1996). After recurrence, patients have a reported 44–74% 5-year survival depending on type of locoregional recurrence (Francken et al. 2008), (Bartlett et al.

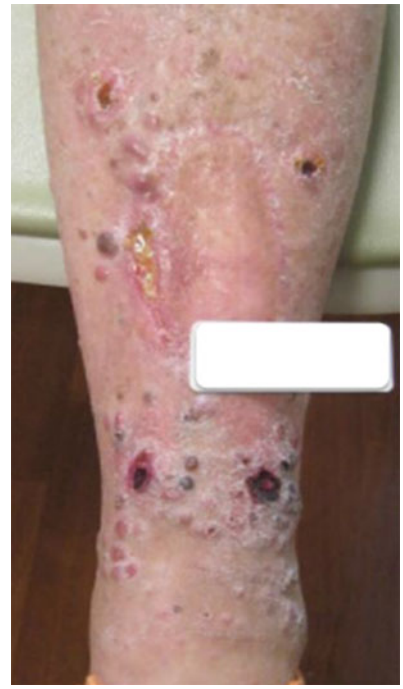

Fig. 1 In-transit metastases from a melanoma of the lower extremity

2014). Patients with locoregional disease should be discussed in a multidisciplinary tumor board to review evaluation, imaging and treatment options taking into account the patient's age and comorbidities, pattern of recurrence, previous treatments and the overall burden of disease.

Initial Evaluation of Locoregional Recurrent Melanoma

When locoregional metastasis or recurrence is suspected, the first step is typically to biopsy the lesion by fine needle aspiration, core, incisional or excisional biopsy as clinically indicated to confirm melanoma recurrence (Coit et al. 2018). Pathologic analysis may clarify recurrence versus the development of a new primary melanoma, but there can be cases where the distinction between cutaneous metastases and a new primary melanoma can be very difficult to make. A review of the initial primary excision pathology may identify risk factors present for recurrence or recognize margin-positive excisions. Biopsies can be facilitated with the use of ultrasound if the suspected recurrence is difficult to palpate in the subcutaneous tissue or in regional lymph nodes.

Ultrasound imaging is a fast, inexpensive diagnostic modality to evaluate local recurrence or regionally metastatic disease. For local and in-transit disease, ultrasound is more sensitive and specific for detecting satellite and in-transit lesions than a physical examination (Solivetti et al. 2006; Blum et al. 2006). On ultrasonography, dermal metastases appear as subcutaneous hypoechoic nodules with irregular or lobulated margins. For regional disease, ultrasound is also more sensitive than palpation (89% vs. 71%) for detecting tumor-involved lymph nodes (Blum et al. 2000). Additionally, it can be valuable for the evaluation of minor nodal basins, including epitrochlear and popliteal nodes, and other nodes located outside the conventional cervical, axillary and inguinal node basins (McMasters et al. 2002). For non-extremity melanomas, ultrasound evaluation of bilateral nodal basins should be considered when the possibility exists they may metastasize to contralateral locations. For example, for suspected recurrence of near-midline melanomas in the midportion of the trunk (back or abdomen), ultrasound of bilateral axillary and/or inguinal basins should be considered; and for suspected recurrence of near-midline head/neck melanomas, ultrasound of bilateral parotid, neck and supraclavicular nodal basins should be considered on a case-by-case basis.

Lymphoscintigraphy is another diagnostic tool that can be used to evaluate recurrent melanoma in several ways. As stated above, melanomas at highest risk for contralateral metastases include those located near the midline on the trunk and head/neck. Reviewing the original lymphoscintigraphy may help ascertain if all visualized sentinel lymph nodes were biopsied. Additionally, if a primary melanoma with potentially ambiguous drainage is shown to have metastasized to one nodal basin that will be treated with a lymph node dissection, preoperative lymphoscintigraphy may be useful to evaluate if there is drainage to the contralateral side, which could harbor micrometastatic disease (Norman et al. 1991). Repeating lymphoscintigraphy for recurrent melanoma is controversial, but there is evidence that it is technically feasible for patients with local recurrence or even in-transit disease. Beasley et al. studied 33 patients with in-transit or local recurrence who underwent lymphoscintigraphy, of whom 79% had undergone a sentinel lymph node biopsy previously (Beasley et al. 2014). At least 1 lymph node was present in 30 of 33 cases, with one third of those being positive. Yao et al. reviewed 30 patients who had recurrent melanoma and found 47% of them had a positive sentinel lymph node (Yao et al. 2003). Repeat lymphoscintigraphy and sentinel lymph node biopsy has been shown to be feasible, but the clinical utility of repeat sentinel lymph node biopsy remains uncertain (discussed subsequently).

Because local, regional and distant sites of disease often are present simultaneously, identification of melanoma recurrence in one location should prompt a careful evaluation for other areas of disease. Local and in-transit recurrences,

Table 1 Method of detection of the initial recurrence in 198 patients with clinical stage I or II cutaneous melanoma that recurred after wide excision and sentinel lymph node biopsy

		Method of detection			
		Self		Physician	
Type of recurrence	No. of patients	Symptom	Physical finding	Physical finding	Tests
Local	11	0	4	7	0
In-transit	44	1	26	17	0
Nodal	42	0	25	13	4
Systemic	101	38	15	9	39
Total	198	39	70	46	43

Tests defined as CT, chest x-ray or PET. Reprinted from Moore Dalal et al. 2008, with permission of Springer

however, are rarely initially detected by diagnostic imaging, but rather by patient or physician examination (Table 1). Whole-body staging for metastatic disease is indicated for stage III melanoma with satellite, in-transit or nodal disease. National Comprehensive Cancer Network (NCCN) recommended modalities include computerized tomography of the chest, abdomen and pelvis with intravenous (IV) contrast or whole-body PET/CT (Coit et al. 2018). If CT is performed, a CT of the neck may be useful if the primary was located on the head and neck or upper torso. PET/CT has been found to have a higher sensitivity in detecting distant metastases, especially bone and subcutaneous sites, as well as a higher specificity and diagnostic odds ratio (Aukema et al. 2010; Reinhardt et al. 2006; Bastiaannet et al. 2009; Xing et al. 2011). Studies have shown PET/CT changing treatment decisions in 19–37% of cases by finding distant metastatic disease (Aukema et al. 2010; Bastiaannet et al. 2009). The cost-effectiveness of PET/CT has been studied in Canada (Hong et al. 2015). This study showed that a PET/CT scan cost $22,570 Canadian dollars more for each accurate diagnosis achieved compared to CT alone, but resulted in fewer lymphadenectomies and more accurate diagnoses. PET/CT is not as useful for brain metastasis due to the brain's high physiologic uptake of ^{18}F-fluorodeoxyglucose, which limits its sensitivity. Magnetic resonance imaging (MRI) scan of the brain with IV contrast is generally recommended in addition to cross-sectional imaging of the rest of the body for patients with recurrent melanoma (Schellinger et al. 1999).

Evaluation of an Unknown Primary

A special case of locoregional disease is that of lymph nodes found to have melanoma without a known primary site. Plausible explanations for this presentation include: removal of the original primary on the skin without recognizing it as melanoma (e.g., cryoablation of a presumed non-melanoma skin cancer, unrecognized accidental or traumatic amputation of the primary, misdiagnosis of a prior biopsy as benign), immune-mediated regression of the original primary with persistence of metastatic cells in the regional node and primary malignant degeneration of intranodal melanocytes (nodal nevus cells). Patients without a known primary now constitute approximately 13–17% of melanoma patients presenting with clinically positive lymph nodes (Cormier et al. 2006; Lee et al. 2008). A thorough history including questions about prior biopsies, skin treatments and lesions that have regressed is an important first step in evaluation. For unknown primary melanoma presenting in inguinal nodes, questions should include any history of hemorrhoids or genital lesions, and the physical examination should include rectal and (for women) pelvic examinations. For cervical nodes, endoscopy should be considered for the possibility of a nasal or oropharyngeal mucosal primary. Evaluation of an unknown primary melanoma patient routinely includes imaging for occult metastatic disease and determining resectability. Often, we add ultrasound evaluation of other nodal basins, particularly if the original primary lesion may have arisen in an area with ambiguous lymphatic drainage. The outcome for these unknown primary patients

has been shown to be slightly better than that for all patients with macroscopic stage III melanoma from a known primary site (Cormier et al. 2006; Lee et al. 2008).

Treatment Options

Treatment options for locoregionally recurrent melanoma include excision, isolated limb infusion (ILI), hyperthermic isolated limb perfusion (HILP), intralesional injections, topical therapies, radiation and systemic therapies (Table 2). The last decade has seen a surge in the adoption of new therapies for metastatic melanoma, which has resulted in patients living longer with stage IV melanoma (Ugurel et al. 2017). As survival for melanoma increases, so does the importance of locoregional control. Factors that contribute to the decision-making regarding treatment include consideration of therapies the patient has already received, the patient's age and comorbidities, and the size, location and number of identified tumor deposits.

In evaluating a recurrence, it is important to know the therapies that have already been received. Understanding what therapies the patient has already had determines what therapies will potentially be useful for new disease. Understanding the patient's initial surgery to excise the primary, if they had a sentinel lymph node biopsy or a complete lymph node dissection and the number of nodes that were excised all inform decision-making in redo surgeries. Knowing the BRAF and C-KIT mutation status of a recurrence and whether the patient already received a BRAF inhibitor with or without a MEK inhibitor will inform additional treatment options with targeted therapies as well as eligibility for participation in specific clinical trials (Coit et al. 2018; Long et al. 2011). Some patients may have received radiation, which will limit the amount of radiation, if any, they can receive to treat a recurrence. These are a few examples of prior treatments affecting treatment planning and options for further therapy with recurrence.

Surgery

Complete surgical excision with negative margins is the preferred approach if the entire recurrence can be excised and the patient rendered disease free (Coit et al. 2018; Squires III and Delman 2013). Surgical excision to clear margins is generally thought to offer the best chance for long-

Table 2 Selected treatment options for locoregional recurrent melanoma

Treatment options for locoregional recurrent melanoma	
Surgery	Radical wide excision
Intra-arterial regional therapy	Hyperthermic isolated limb perfusion Isolated limb infusion
Intralesional and topical therapies	BCG IL-2 GM-CSF TVEC PV-10 Topical imiquimod Topical diphenylcyclopropenone
Radiation therapy	Adjuvant postoperative radiation Palliative radiation
Systemic therapy	Molecularly targeted agents Anti-CTLA4 antibodies (ipilimumab) Anti-PD1 antibodies (pembrolizumab, nivolumab) Chemotherapy Dacarbazine (DTIC) Temozolomide (TMZ) Carboplatin/paclitaxel

term disease-free survival (Squires III and Delman 2013). Dong et al. studied a series of 648 patients with primary melanomas and subsequent local recurrence initially treated with surgical excision and found 124 patients (19%) had no further recurrences (Dong et al. 2000). One hundred and ninety-six (30%) developed another local recurrence, 178 (27%) developed in-transit disease, and 150 (23%) eventually developed systemic disease. This demonstrates that close to 20% of patients with a local recurrence are likely to derive long-term benefit from resection. Many of the patients who progressed went on to receive additional treatments including additional surgery or intra-arterial perfusion, and over 50% of the patients in the series were alive at 5 years, with an estimated 10-year survival of 34.9% (Dong et al. 2000).

Surgical considerations include the location of the recurrence with relation to important structures, and the morbidity that would be involved in excising to clear margins. The aim of surgical excision for recurrence is of course to have a clear margin, but the exact specified margin of excision or even the consequences of resection with a microscopically positive margin have never been adequately defined. In our center, we aim for 1 cm grossly negative radial margins, but do not insist on a defined margin for recurrent or metastatic disease as long the excision ultimately has histologically negative margins. For large defects or areas difficult to close that may need a skin graft or flap coverage, deferred closure pending final pathologic assessment of the margins may be important. Homograft skin or acellular dermis (AlloDerm) reconstruction provides temporary coverage alternatives. Additionally, AlloDerm can be used with adjuvant radiation and may not require any further reconstruction (Deneve et al. 2013).

Patients with overwhelming locoregional recurrence are occasionally referred to surgeons for amputation as a last resort. Amputation generally should not be advocated as other forms of aggressive treatment for local control of disease are preferred, such as regional chemotherapy and intralesional therapy that will be discussed below. However, amputations may be considered when limb-preserving strategies have been exhausted or to palliate patients in the case of a dysfunctional limb or uncontrollable pain. Read et al. described the Melanoma Institute Australia experience with 55 cases (17 upper limb, 38 lower limb) (Read et al. 2015a). The most common indications for amputation were progressive in-transit metastases (67%), problematic limb metastases from distant sites (14%), pain or ulceration after regional chemotherapy (14%) and otherwise inoperable regional recurrence (6%). Most patients in their series (58%) had failed prior limb perfusion or infusion. The overall 5-year survival rate from time of amputation was 22.8%. Stage III patients who had all known disease resected at the time of amputation had a 5-year survival of 38.4% (Read et al. 2015a).

Repeat Sentinel Lymph Node Biopsy

In-transit disease has a high risk for regional lymph node involvement and at this time the NCCN recommends considering sentinel lymph node biopsy for resectable in-transit disease, as a "category 2B" recommendation (Coit et al. 2018). Ultrasonography is a good first step in evaluating lymph node involvement of a recurrence and should be considered prior to a repeat sentinel lymph node biopsy. In one institutional series of 31 patients undergoing elective lymph node dissection after the development of clinically node-negative in-transit metastasis, the authors found 19.4% of patients had tumor-involved lymph nodes in the dissection, but (like in primary melanomas) an elective lymph node dissection did not have a significant impact on overall 5-year survival (Read et al. 2015b). As mentioned previously, lymphatic mapping and repeat sentinel lymph node biopsy are feasible in patients with local and in-transit recurrent melanoma (Table 3) (Beasley et al. 2014; Yao et al. 2003; Beasley and Tyler 2015). Evidence from these studies shows that patients with clinically occult lymph node involvement along with in-transit metastases have a shorter time to develop metastatic disease; however, it has never been shown that repeating a sentinel lymph node biopsy at the time of recurrence improves disease free or overall survival. Repeat sentinel lymph node biopsy, if considered, should only be performed for resectable disease or

Table 3 Series reporting sentinel lymph node (SLN) biopsy for melanoma locoregional recurrence, either as an initial nodal staging procedure or as a repeat procedure after prior negative SLN biopsy

	% Successful SLN biopsies	Patients with prior SLN biopsy	Patients with any positive SLN
Yao et al. (2003)	100% (30/30)	33% (10/30)	47% (14/30)
Coventry et al. (2004)	92% (11/12)	0% (0/12)	33% (4/12)
Beasley et al. (2014)	91% (30/33)	73% (24/32)	33% (10/30)

for disease confined to an extremity that can be definitively treated with regional therapy (Beasley et al. 2014). An alternative approach to repeat sentinel lymph node biopsy is to follow clinically negative but at-risk regional nodal basins with ultrasound, and this is our institutional preference. We will, however, perform a sentinel lymph node biopsy for locally recurrent melanoma in patients who did not initially have a sentinel lymph node biopsy performed at the time of their primary excision.

Intra-Arterial Regional Therapies

Hyperthermic Isolated Limb Perfusion

Hyperthermic isolated limb perfusion (HILP) and isolated limb infusion (ILI) are two techniques used for advanced locoregional disease limited to an extremity, by isolating the extremity from systemic circulation and giving high-dose chemotherapy intra-arterially. Such regional therapies are considered when the extent of local recurrence is beyond what can reasonably be managed surgically, or if resection fails and the patient has persistent disease or subsequent local recurrence.

HILP involves surgically dissecting and isolating the femoral or external iliac vessels for lower extremity disease or the subclavian or axillary vessels for upper extremity disease (Creech et al. 1958). Regional lymphadenectomy can be done at the time of vascular dissection if indicated. Vessels are directly cannulated and the limb is isolated via ligation of collateral vessels and a tourniquet. Chemotherapy is then infused and circulated throughout the limb for 60–90 min by a cardiopulmonary bypass machine, which heats and oxygenates the perfusate with goal flow rates of 400–600 mL/min. Limb temperature is set to reach 39–41 °C, which is thought to amplify the effects of the melphalan-based chemotherapy (Fraker et al. 1996; Fraker 1999, 2004; Noorda et al. 2004). At the end of the circulation time, the extremity is then washed out with 2 L of electrolyte solution (Fraker 2004). With HILP, drug concentrations are 15–25 times higher in the target tissue but the patient is largely spared from toxicities associated with systemic chemotherapy.

Results for HILP have shown objective response rates (ORR) of 60–90% with complete response (CR) rates as high as 70% (Fraker et al. 1996; Fraker 1999, 2004; Noorda et al. 2004, b; Eggermont et al. 1996; Kroon et al. 2002; Grünhagen et al. 2005; Cornett et al. 2006; Aloia et al. 2005; Raymond et al. 2011; Lienard et al. 1994, 1999). The chemotherapeutic agents most widely used for HILP are melphalan in the United States and melphalan combined with tumor necrosis factor-alpha (TNF-α) in Europe. Melphalan for HILP is dosed at 13 mg/L of limb volume for the lower extremity and 10 mg/L for the upper extremity (Raymond et al. 2011).

The addition of TNF-α to melphalan in European studies has shown CR rates of 60–80%, but the data are not consistently reproducible (Fraker et al. 1996; Noorda et al. 2004; Grünhagen et al. 2005; Lienard et al. 1994, 1999). Moreover, randomized clinical trials in the United States and Europe failed to show significant differences in CR rates between HILP using melphalan versus melphalan plus TNF-α (Eggermont et al. 1996; Cornett et al. 2006). The American College of Surgeons Oncology Group (ACOSOG) Z0020 trial showed no significant differences between the melphalan and melphalan plus TNF-α arms in terms of either ORR (64% vs. 69%, respectively) or CR rates (25% vs. 26%) at 3 months after the procedure. However, the

TNF-α arm experienced significantly higher complication rates (16% vs. 4% grade IV adverse events, $p = 0.04$) (Cornett et al. 2006). In the European study, melphalan plus TNF-α was associated with more CRs but the difference was not significant, with CR rates of 45% for melphalan alone versus 59% for melphalan plus TNF-α ($p = 0.14$) (Noorda et al. 2004). Other drugs have been studied in HILP, including the addition of interferon-γ, but without demonstrated benefit (Lienard et al. 1999).

Isolated Limb Infusion

ILI, developed at the Melanoma Institute Australia by Thompson and colleagues, is often used as the first regional therapy because it is less invasive than HILP. ILI uses percutaneous catheters (5 or 6 French) fluoroscopically placed into the involved limb and a tourniquet to isolate the limb (Fig. 2) (Thompson et al. 1998). ILI uses a low flow rate (typically 80–120 mL/min) in an acidotic, hypoxic environment, as opposed to HILP. A combination of melphalan and actinomycin D chemotherapy is used and dosed by extremity volume. The chemotherapy is infused over 2–5 min to avoid toxicity from peak perfusate concentrations. In ILI, less melphalan is used (10 mg/L and 7.5 mg/L for lower and upper extremity, respectively) than in HILP, with many centers adjusting the dose for ideal body weight (Rashid et al. 2014). Limb temperatures are measured by subcutaneous and intramuscular temperature probes, with a goal temperature of 37 °C. A tourniquet is placed around the proximal aspect of the limb after systemic heparin is given, and isolation of the limb from the systemic circulation is tested using a Doppler. Once the limb temperature is 37 °C, the chemotherapy is circulated through an extracorporeal circuit with a heating coil, then into the arterial catheter and then removed through the venous catheter in a closed circuit for 30 min (Thompson et al. 1998; Thompson and Kam 2004; Gimbel et al. 2008; Beasley et al. 2008). If a concomitant lymph node dissection is indicated, at our institution we perform the dissection after the ILI procedure to monitor bleeding and so that a fresh surgical wound is not exposed to systemic heparin during the time of ILI.

ILI studies have reported CR rates of 23–44% and partial response (PR) rates from 27% to 56%, with a median duration of responses between 12 and 18 months (Beasley et al. 2008; Kroon et al. 2008; Santillan et al. 2009; Beasley et al. 2009; O'Donoghue et al. 2017a). In a retrospective review, Dossett et al. compared the response rates of HILP and ILI and found that although the ORR was higher for HILP (80%) compared with ILI (53%), the median overall survival (OS) was not significantly different (40 months for HILP vs. 46 months with ILI, $p = 0.31$) despite a higher burden of disease in the ILI patients (Dossett et al. 2016). The largest published study of ILI is from an Australian multicenter experience that reported the results of 316 first ILI procedures performed between 1992 to 2008 and showed an ORR of 75% (CR rate 33%, PR rate 42%) (Kroon et al. 2016). Median survival was 80 months for those with a CR. Another large, single institution study reporting experience with 107 patients in 2013 (Wong et al. 2013) recently updated their results to include 163 patients undergoing 205 procedures of initial and repeat treatment for melanoma, sarcoma, squamous cell carcinoma, and Merkel cell carcinoma (O'Donoghue et al. 2017b). For melanoma, they reported an ORR of 59.0%, with a CR rate of 25.7%. Responders had significantly longer in-field progression free-survival (14.1 vs. 3.2 months, $p < 0.001$), distant metastatic disease free-survival (not reached vs. 25.8 months, $p = 0.006$) and OS (56.0 vs. 26.7 months, $p = 0.0004$) compared to nonresponders. The initial burden of locoregional disease predicted response, as patients with lower burden of disease had an increased CR rate (50%), ORR (73%) and improved PFS (Muilenburg et al. 2015). Another multi-institutional study demonstrated that resection of all residual disease after an ILI results in progression-free and overall survival similar to that for patients achieving a CR after ILI (Wong et al. 2014).

A benefit of ILI compared to HILP is the ability to do repeat procedures on the same limb with ILI. Treatment algorithms for regional perfusions for extremity in-transit melanoma have been proposed by Chai et al. and Beasley et al., noting the role of repeat regional therapy

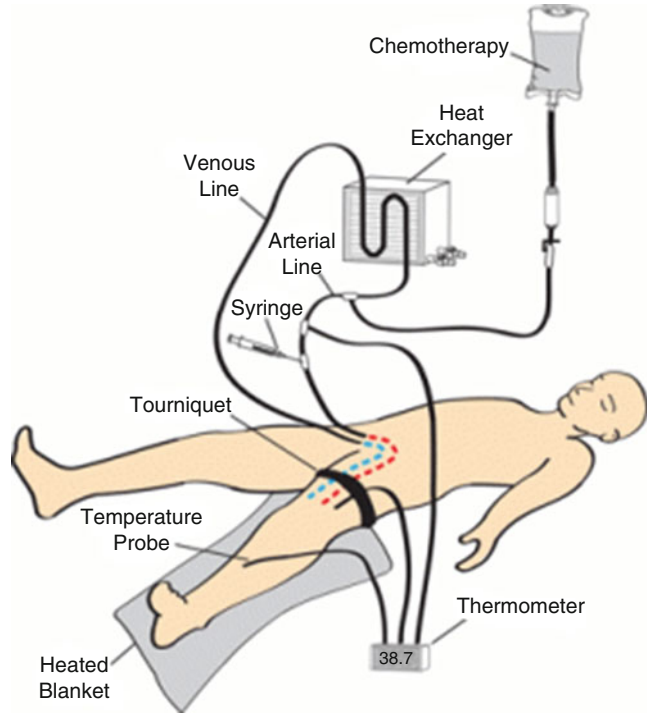

Fig. 2 Isolated limb infusion (ILI) circuit. Catheters are placed into the contralateral femoral artery and vein and directed into the involved limb. A tourniquet isolates the limb, and a circuit is established to deliver melphalan and actinomycin D chemotherapy, which is hand-circulated at low flow rates using a syringe. (Image courtesy Jeffrey M. Farma, MD, Fox Chase Cancer Center.)

(Beasley et al. 2009; Chai et al. 2012). Chai et al. recommends ILI be used in most cases for initial regional perfusions and HILP be used initially for high volume in-transit disease and as a salvage regional perfusion procedure for patients who progressed rapidly after ILI. However, if the patient had a good response with the first ILI and then relapsed, repeat ILI is technically much easier to perform and better tolerated than salvage HILP (Chai et al. 2012). Response rates from repeat regional chemotherapy can be as high as 60–83% for overall response and 20–40% for CR (O'Donoghue et al. 2017a; Chai et al. 2012; Kroon et al. 2009).

Morbidity from HILP and ILI

Complications from HILP and ILI are multifactorial and can be related to either the surgery or the chemotherapy secondary to local or systemic leakage (Vrouenraets et al. 1999; Thompson et al. 1996; Möller et al. 2008). Either procedure in combination with a lymph node basin dissection will have associated surgical morbidities including infection, lymphedema and paresthesias. Vascular complications including venous thromboembolism occur in 1–10% of HILP patients (Möller et al. 2008). Given that ILI requires percutaneous access as opposed to surgical dissection, patients with medical comorbidities that preclude HILP can still undergo ILI. Both procedures incur a risk of local effects of the chemotherapy, including skin and soft-tissue damage. Erythema and edema of the extremity are the most common morbidities and occur in 12–36% of patients (Thompson et al. 1998; Möller et al. 2008). Toxicity can range from mild erythema and epidermolysis to extensive tissue damage requiring fasciotomy or even limb amputation (Möller et al. 2008; Noorda et al. 2002). Risk factors for developing local tissue damage include temperatures higher than 40 °C and a higher concentration of chemotherapy administered (Thompson et al. 1998). A scale designed to measure the limb toxicity of limb perfusions, the Wieberdink grading system (Wieberdink et al. 1982) has been found applicable to patients undergoing ILI as well (Dossett et al. 2016). A multi-institutional study of 171 patients undergoing ILI found that 32% of patients had mild (grades I-II) and 32% had severe

(grades >III) toxicity using the Wieberdink grading system, with one patient requiring an amputation. A multi-institutional study in the USA with 128 patients found that papaverine used for vasodilation significantly improved response rate at the expense of increased toxicity (Beasley et al. 2009). This study also found that limb toxicity was reduced with correction of melphalan dose for ideal body weight without altering the CR rate. Studies have shown that high creatinine phosphokinase levels are associated with higher local toxicity, but not perfusate gas analysis at 30 min (pH, PaO_2 and base excess), limb temperature or ischemia time (Santillan et al. 2009; Beasley et al. 2009). Systemic toxicity occurs when chemotherapy leaks from the isolated limb or because of inadequate washout, and is a greater risk for HILP (Sonneveld et al. 1996). Melphalan chemotoxicity can cause gastrointestinal disturbance, myelosuppression and hypotension (Möller et al. 2008). Systemic leakage can be continuously monitored during HILP using radiolabeled red blood cells that can be detected by a precordial probe (Sonneveld et al. 1996).

Intralesional and Topical Therapies

Intralesional therapy has been used since the 1960s for the treatment of intradermal and subcutaneous melanoma metastases (Morton et al. 1974). Tumor lysis resulting from the injected agent and/or the resultant immune infiltrate is thought to promote tumor antigen presentation to the immune system and potentially induce a bystander response in which uninjected distant lesions regress from the resultant systemic immunity (Thompson et al. 2008, 2015; Andtbacka et al. 2016). The bystander effect is most commonly seen in close-by uninjected skin and subcutaneous lesions, but uninjected visceral metastases have been documented to regress after intralesional injection of remote lesions. Talimogene laherparepvec (TVEC) is the only intralesional agent approved by the US Food and Drug Administration (FDA), with other therapies listed in NCCN guidelines as supported by category 2B evidence (Coit et al. 2018). Intralesional therapies are used for patients with unresectable melanoma in stages IIIB/C and IV (M1a), recognizing that at times the only distinction between stage III and IV injectable skin or subcutaneous metastases is the location (extremity or non-extremity) and/or the relationship of the disease to the regional nodal basins.

Bacille Calmette-Guérin

Bacille Calmette-Guérin (BCG) was the first commonly used intralesional therapy in the setting of in-transit metastases, with Morton et al. in 1974 reporting regression in 90% of BCG-injected cutaneous lesions and 17% of uninjected nodules. Complete regression of all injected disease was noted in 31% of patients with a duration of response of 6–74 months (Morton et al. 1974). Its side effect profile includes severe injection site reactions, fevers, chills, seroconversion, rare systemic infections, pneumonitis, BCG granulomas, hypotension and disseminated intravascular coagulation (Cohen et al. 1991; Robinson 1977; Agarwala et al. 2004). With the development of newer agents, most clinicians feel there is no longer a role for BCG, and it is not used in our institution.

Intralesional Cytokines

Interleukin-2 (IL-2) as systemic therapy is FDA-approved for the treatment of metastatic melanoma, but this cytokine can also be used intralesionally for patients with locally recurrent and in-transit metastases. In 2003, Radny et al. conducted a phase II trial involving 24 patients treated with intralesional IL-2, given 2–3 times weekly over 1–57 weeks, as salvage therapy. Participants had previously failed surgery, regional perfusion, radiation therapy or systemic chemotherapy. CR was achieved in 15 patients (62.5%) and PR in 5 (21%) additional patients (Radny et al. 2003). Toxicity is mainly flu-like symptoms or grades 1 and 2 local toxicities. IL-2 has not been definitely reported to induce an immune bystander response like other intralesional agents, but formal prospective trials have not been conducted to ascertain whether this is a fundamental property of intralesional IL-2. The utilization of IL-2 is limited both by its high cost and the

need for frequent injections, and its use has largely been superseded by newer agents.

Intralesional granulocyte-macrophage colony-stimulating factor (GM-CSF) is thought to induce an immunotherapeutic response to treat melanoma by improving antigen-presenting cells activity and stimulating dendritic cells that are deficient in peritumoral tissue (Ridolfi et al. 2001). A study of intralesional GM-CSF in subcutaneous melanoma metastases showed a PR in 3 of 13 patients (23.0%) with 15–50 mg doses of GM-CSF (Si et al. 1996). Responders had increased T cell and Langerhans' cell infiltrates into the tumor. One study looked at GM-CSF followed by subcutaneous IL-2 and found 2 patients to have a partial response out of 14 patients (14%) (Ridolfi et al. 2001). They found that some response was seen in non-injected lesions and that the treatment was well tolerated, with most patients having mild fever and one patient experiencing muscle pain and arthralgia.

Talimogene Laherparepvec

Talimogene laherparepvec (TVEC) is a newer intralesional agent, a genetically modified oncolytic herpes simplex type 1 virus (Gangi and Zager 2017). TVEC is designed to replicate within and lyse tumor cells and has been genetically engineered to contain the DNA sequence coding for the GM-CSF protein, which is intended to promote a systemic antitumor immune response. A multi-center phase III trial, the OncoVex Pivotal Trial in Melanoma (OPTiM), randomized 435 patients with unresectable stage IIIB to IV melanoma with no or only limited visceral disease in a 2:1 ratio into two arms: TVEC (295 patients) and systemically (not intralesionally) administered GM-CSF (141 patients) (Andtbacka et al. 2015). The primary endpoint of the study was the durable response rate (DRR), which was defined as an objective response (CR or PR) lasting $\geq$6 months. Secondary endpoints were ORR, OS and safety. A significantly higher DRR was seen with TVEC compared to GM-CSF, 16.3% versus 2.1% ($p < 0.001$). The ORR was also significantly higher in the TVEC arm at 26.4% compared to 5.7% in the GM-CSF arm ($p < 0.001$). Bystander effects were observed, with regression of both uninjected nodules and visceral sites of disease in some patients. A CR was seen in 32 TVEC patients (10.8%) but in only 1 GM-CSF patient (<1%). Median follow-up was 44.4 months, with patients in the TVEC arm having a longer median survival of 23.3 months versus 18.9 months in the GM-CSF arm, with the difference reaching borderline statistical significance ($p = 0.051$). Differences in DRR between the study arms were more pronounced in stage IIIB or IIIC (33% vs. 0%) and IV (M1a) patients (16% vs. 2%) in contrast to stage IV (M1b) (3% vs. 4%) and IV (M1c) disease (8% vs. 3%). Treatment naïve patients receiving TVEC as a first-line therapy were more likely to respond than those receiving treatment as second-line or subsequent therapy (24% vs. 10%). Median survival for treatment-naïve patients receiving TVEC was 33.1 months versus 17.0 months with GM-CSF, compared to second-line therapy where median survival was 17.1 months with TVEC versus 23.2 months with GM-CSF. Median survival for stage IIIB, IIIC or IV (M1a) disease was 41.1 months with TVEC versus 21.5 months with GM-CSF, compared to stage IV (M1b) and IV (M1c) disease where there was a median survival of 13.4 months with TVEC versus 15.9 months with GM-CSF (Andtbacka et al. 2015). The most common side effects included pyrexia, chills, fatigue, nausea and injection site pain (Andtbacka et al. 2015; Hu et al. 2006). The results of the OPTiM trial led the FDA to approve TVEC as Imlygic in 2015.

Following FDA approval, interest in TVEC intralesional therapy has increased and the NCCN melanoma clinical practice guidelines list TVEC as an option supported by category 1 evidence (Coit et al. 2018). Treatment involves intratumoral injection of up to 4 mL of 10^6 plaque-forming units (PFU)/mL for the initial injection (plaque-forming units are a measure of the total number of viral particles in a specific volume). The amount that is injected into each lesion is based on the lesion diameter. The largest lesion is injected first and then smaller lesions are injected until the total injectate volume has been used or there are no more lesions to inject. Three

weeks later, the dose is increased based on the expectation that the patient will have seroconverted to react to the virus: up to 4 mL of 10^8 PFU/mL is injected intralesionally every 2 weeks for a suggested minimum of 6 months or until there is no residual tumor to inject. New lesions are injected first, followed by the largest lesions until all lesions are injected or the total injectate volume has been used. Future directions for TVEC include combination with other immunotherapies such as anti-CTLA4 (Puzanov et al. 2016) and anti-PD1 antibodies (discussed further below).

Allovectin-7

Allovectin-7 (Vical Inc) has been studied as an intralesional treatment. It consists of an injectable formulation of plasmid DNA encoding the human leukocyte antigen (HLA)-B7 and β2 microglobulin complex, with the hope of augmenting immune recognition of the injected tumor and potentially triggering a powerful immune-mediated "rejection" reaction (Bedikian and Del Vecchio 2008). A phase II study was conducted of Allovectin-7 among 133 patients with stage IIIB/C or IV (M1a/b) injectable cutaneous, subcutaneous, or nodal melanoma that was recurrent or unresponsive to prior therapy (Bedikian et al. 2010). Patients received 6 weekly intralesional injections followed by 3 weeks of observation and evaluation. Patients with stable or responding disease were eligible to receive additional cycles of Allovectin-7. An objective response was seen in 15 patients (11.8%) with a median duration of response of 13.8 months and a median time to progression of 1.6 months. Regression was observed in uninjected lesions in 9 of 42 patients (21%) with stage IV disease (Bedikian et al. 2010). A phase III study, Allovectin Immunotherapy for *M*etastatic *M*elanoma (AIMM), randomized 390 patients 2:1 to intralesional injection of 2 mg Allovectin-7 versus systemically administered dacarbazine or temozolomide (DTIC-TMZ) chemotherapy. The trial failed to show that Allovectin-7 was associated with a significant improvement in ORR or OS compared to the chemotherapy arm. The primary outcome of durable and sustained regional response at 24 weeks in fact showed that DTIC-TMZ was more effective than Allovectin-7, 12.3% versus 4.6%, respectively ($p = 0.01$), though responses were more durable for Allovectin-7 (Agarwala 2015). The results of AIMM trial also highlight that chemotherapy with either DTIC or TMZ, although better than Allovectin-7, is not very effective for potentially injectable recurrent or metastatic melanoma.

PV-10 (Rose Bengal)

PV-10 (Provectus, Inc.) is a 10% solution of rose bengal, a water-soluble xanthene dye that has been used historically for various medical applications but has now been repurposed for intralesional injection into tumors. Intralesional PV-10 differs from the other intralesional agents described in that it induces tumor lysis via non-immune mechanisms, but the resultant local inflammation facilitates exposure of tumor antigens to antigen-presenting cells. A phase II trial of 80 patients with measurable stage III–IV melanoma with a median of 6 prior interventions receiving PV-10 into up to 20 cutaneous and subcutaneous lesions up to four times over a 16-week period showed an ORR of 51% with a 33% CR rate in patients. Of 21 patients with evaluable bystander lesions, 33% achieved a CR in those lesions (Thompson et al. 2015; Agarwala et al. 2010). No grade 4 or 5 adverse events were reported, and side effects included transient pain at injection sites, local edema and vesicles. In a subgroup of 28 patients who received PV-10 into all existing melanoma lesions, the ORR was 71% with CR in 50% (Agarwala et al. 2014). PV-10 tends to work quickly, with rapid shrinkage of tumor and formation of an eschar at the injection site, but with a pink discoloration to the skin that can last for several weeks or longer (Fig. 3). Sarnaik et al. demonstrated in a small study that intralesional PV-10 was associated with a concomitant immune response, with an increase in circulating cytotoxic $CD3^+/CD8^+$ T cells and tumor-specific interferon-gamma release (Sarnaik et al. 2014). The study showed a 50% CR rate (4/8) in injected and uninjected lesions, with all eight patients showing at least partial regression of injected lesions. Six of eight patients had metastatic disease refractory to immunotherapy. Four

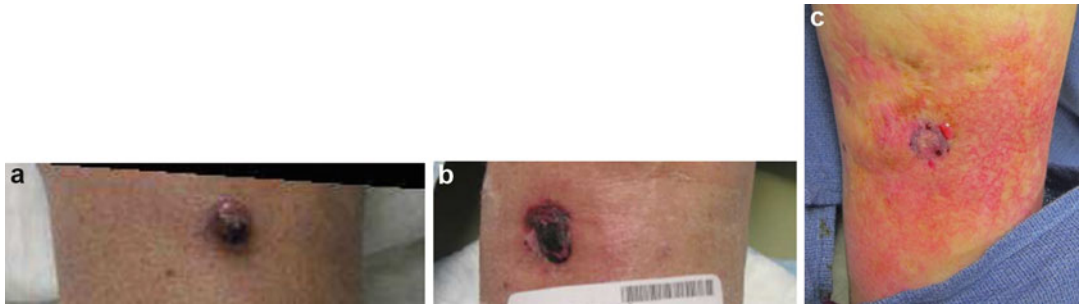

Fig. 3 (**a**) A 1 cm dermal nodule of metastatic melanoma on the lower extremity, prior to intralesional therapy with PV-10 (10% rose bengal). (**b**) The same site 3 weeks later, showing complete regression of the tumor with formation of an overlying eschar. Note the residual pink discoloration of the skin that gradually faded away. (**c**) A different lesion from another patient, photgraphed 4 h after PV-10 injection, demonstrating the bright pink temporary discoloration associated with PV-10 injection. (Photographs courtesy of Amod Sarnaik, MD, Department of Cutaneous Oncology, Moffitt Cancer Center)

of the patients who were refractory to prior ipilimumab, anti-PD1 and/or vemurafenib therapy had pathologic CRs in both the injected and uninjected lesions. In 2015, a phase III trial began comparing intralesional PV-10 to systemic DTIC or TMZ for locally advanced cutaneous melanoma in patients who are BRAF V600 wild-type and who failed or are not candidates for checkpoint inhibitor immunotherapy.

Topical Agents

Imiquimod and diphenylcyclopropenone (DPCP, also referred to as diphencyprone) are topical immunomodulatory creams that have been used in the treatment of cutaneous metastatic melanoma lesions. Imiquimod is a toll-like receptor agonist thought to elicit cell-mediated antitumor immune responses through toll-like receptor 7 (Tomai et al. 1995). Additional mechanisms of imiquimod's effects include activating dendritic cells (Tomai et al. 1995) and proapoptotic activity toward epithelial cancer cells (Schön et al. 2004). One clinical report using imiquimod 5% cream (Aldara) twice daily under occlusive conditions for 21–28 weeks in three patients with cutaneous in-transit melanoma metastases (>15 lesions each) had two patients with >90% regression of treated lesions (Bong et al. 2002). Another case series studied the topical administration of imiquimod and 5-fluorouracil in 5 patients with a combined total of 45 cutaneous metastases (Florin et al. 2012). A clinical response was seen in 44 of the 45 lesions treated, with complete regression of 19 lesions. No patients developed new lesions during treatment.

DPCP is hypothesized to work via activation of TH17 lymphocytes (Damian and Thompson 2007). The largest series of patients treated with DPCP was reported by Damian et al., who studied 50 patients with locally recurrent, in-transit or dermal metastatic melanoma (Damian et al. 2014). A CR was seen in 46% (23/50) of patients and 38% (19/50) had a PR. Patients with superficial disease had a better response, with a CR rate of 61%, compared to those with deeper or more bulky disease, of whom only 21% had a CR. Regression of bystander lesions or distant metastases has not to our knowledge been reported with the use of topical agents.

Radiation Therapy

Radiation is sometimes considered as adjuvant treatment for locoregional control of high-risk primary melanomas and nodal basins following resection of lymph nodes at high-risk of relapse. Adjuvant radiation has a demonstrated role in the primary treatment of desmoplastic melanoma, particularly if neurotropism is present or the primary was resected with narrow margins (Coit et al. 2018; Strom et al. 2014). Radiation therapy can also be used as a palliative option for unresectable locally recurrent or in-transit

metastatic melanoma of any histologic type, particularly if other modalities have failed.

High-risk factors for regional relapse following radical lymphadenectomy include the finding of extranodal extension, increasing number of involved nodes and larger size of tumor-containing nodes, and adjuvant radiation to the nodal basin has been shown to reduce the risk of recurrence within the radiated field (Agrawal et al. 2009; Burmeister et al. 2012; Strom et al. 2017). A randomized controlled trial of 123 patients randomized 1:1 to receive adjuvant radiotherapy of 48 Gray in 20 fractions or observation, after a median follow-up of 40 months, found that the risk of lymph node field relapse was significantly reduced in the adjuvant radiotherapy group compared with the observation group (Burmeister et al. 2012). However, there was no significant difference in relapse free or overall survival.

Systemic Therapy

Newer systemic therapies for melanoma have greatly changed the overall prognosis for patients with unresectable metastatic disease. Such systemic treatments include BRAF inhibitors (vemurafenib and dabrafenib) and MEK inhibitors (trametinib and cobimetinib) and immunotherapeutic agents including ipilimumab and anti-PD1 antibodies (pembrolizumab, nivolumab) (Hodi et al. 2010; Flaherty et al. 2012a; Falchook et al. 2012; Flaherty et al. 2012b; Larkin et al. 2015; Long et al. 2014).

Approximately 45–50% of cutaneous melanomas contain BRAF mutations – although locoregional recurrences are somewhat less likely to be BRAF mutant, potentially due to the fact that melanomas in populations at highest risk to recur in this fashion (such as those in the elderly, and acral lentiginous and head/neck primaries) have a lower incidence of BRAF mutations. In 2011, the FDA approved vemurafenib for metastatic melanoma carrying BRAF V600 mutations (Ascierto et al. 2012). Combined BRAF and MEK inhibition has been shown to improve ORR and OS compared to BRAF inhibition alone (Robert et al. 2015; Johnson et al. 2014; Long et al. 2014). Long et al. published a phase III study that demonstrated a response rate of 67% in patients treated with dabrafenib-trametinib versus 51% in patients treated with dabrafenib only ($p = 0.002$). With such results, there is the potential to use targeted therapies as neoadjuvant treatment, particularly for borderline or unresectable stage III patients. Moffitt Cancer Center reported its experience with 15 patients with locoregional melanoma treated with BRAF inhibition alone (vemurafenib, 11 patients) or combination therapy (dabrafenib plus trametinib, four patients) (Sloot et al. 2016). Using RECIST criteria, 11 patients (73%) had an objective response. Six patients underwent resection of any remaining disease, with two complete and two partial pathologic responses. In a randomized phase 2 trial involving 21 patients with high-risk resectable stage III-IV melanoma, researchers at MD Anderson Cancer Center showed that neoadjuvant targeted therapy with BRAF and MEK inhibitors significantly improved event-free survival versus standard of care (19.7 months vs 2.9 months) (Amaria et al. 2018). Neoadjuvant protocols vary in the length of treatment before surgery, but we generally use 6 months of preoperative therapy and may not continue treatment postoperatively in case of a pathologic CR or near CR.

The role of adjuvant use of dabrafenib plus trametinib in resected stage III melanoma with BRAF V600E or V600 K mutations has also been studied. A recent phase III randomized, placebo-controlled trial showed that patients who received oral dabrafenib (150 mg twice daily) plus trametinib (2 mg daily) had a significantly lower risk of recurrence compared to those treated with a placebo control (Long et al. 2017). The estimated 3-year rate of relapse-free survival was 58% in the combination therapy group and 39% in the placebo group (hazard ratio for relapse or death 0.47, $p < 0.001$). OS was also improved in the combination arm, with estimated survival rates of 97% at 1 year, 91% at 2 years and 86% at 3 years compared to the placebo arm estimated rates of 94%, 83% and 77%, respectively (hazard ratio for death 0.57, $p = 0.0006$), but the survival difference did not cross the prespecified interim analysis boundary of $p = 0.000019$. More mature survival data from this trial are eagerly awaited, and FDA approval of the combination for

adjuvant therapy of resected stage III BRAF-mutant melanoma was granted in the spring of 2018.

Options for adjuvant immunotherapy treatment after resection of stage III and IV disease are also evolving, as several phase III trials have shown benefit (Luu et al. 2016). In 2015, high dose ipilimumab was approved by the FDA for adjuvant treatment of resected stage III melanoma, specifically stage IIIA with sentinel node metastases >1 mm, stage IIIB-C or resected nodal recurrence. Approval was based on a phase III trial evaluating adjuvant ipilimumab versus placebo after resection of high-risk stage III melanoma (EORTC 18071), which enrolled 951 patients and found improved recurrence-free and overall survival in the ipilimumab group after a median follow-up of 5.3 years (Eggermont et al. 2016). Patients in the ipilimumab arm received intravenous infusions of 10 mg/kg ipilimumab every 3 weeks (which is higher than the 3 mg/kg dose used for systemic disease) for four doses, then every 3 months for up to 3 years. Grade 3–4 toxicities occurred in 54% of the ipilimumab group, with five drug-related deaths (1.1%). Adverse events led to discontinuation of treatment in 245 (52%) of patients in the ipilimumab group (Eggermont et al. 2015).

In 2017, the CheckMate 238 trial showed that adjuvant therapy with the anti-PD1 antibody nivolumab significantly increased recurrence-free survival among patients after resection of stage IIIB, IIIC or IV melanoma, with a lower rate of grade 3 or 4 adverse events, compared with adjuvant therapy with ipilimumab (Weber et al. 2017). The randomized, double-blind phase III trial assigned patients to nivolumab at a standard dose of 3 mg/kg every 2 weeks (453 patients) or ipilimumab at the higher 10 mg/kg dose every 3 weeks for four doses and then every 12 weeks (453 patients). The rate of recurrence-free survival at 1 year was 70.5% (95% CI, 66.1–74.5) in the nivolumab group and 60.8% (95% CI, 56.0–65.2) in the ipilimumab group. Grade 3 or 4 treatment-related adverse events were reported in 14.4% of the patients in the nivolumab group and 45.9% in the ipilimumab group. Although survival data is not yet available from this trial, the FDA has now approved nivolumab as an adjuvant treatment option for patients with resected high-risk stage III-IV melanoma.

Combination Treatments

Combining intralesional therapies such as TVEC with systemic immunotherapies is increasingly being studied. A phase Ib multicenter trial was designed to evaluate the safety and efficacy of TVEC plus ipilimumab (Puzanov et al. 2016). Ipilimumab (3 mg/kg IV) was given every 3 weeks for four doses starting week 6, after the first two TVEC injection sessions. Nineteen patients were assessed and the ORR was 40%, with 44% having a durable response lasting ≥6 months. With a median follow-up of 20 months, the 18-month progression free survival was 50% and 18-month survival was 67%.

Another combination trial investigated pembrolizumab with TVEC for stage IIIB/IIIC and IV melanoma (NCT02263508) (Ribas et al. 2015). Patients received TVEC injections then started pembrolizumab 200 mg IV on day 36 for up to 2 years. Twenty-one patients were enrolled. Six patients had grade 3 adverse events (generalized rash, anemia, hyperglycemia, macular rash, and headache); no treatment-related grade 4 adverse events were reported (Long et al. 2015). Efficacy endpoints have not been reported at the time of this writing.

PV-10 is also under investigation as part of combination therapy (Agarwala et al. 2017). A phase Ib/II study of PV-10 in combination with the anti-PD1 antibody pembrolizumab, PV-10-MM-1201 (NCT02557321), evaluated stage IV melanoma patients with at least one injectable lesion and a visceral lesion who could receive pembrolizumab. Initial data from 12 patients shows acceptable tolerability and a 50% response rate, with one CR (Agarwala et al. 2017).

Conclusion

Locoregional recurrent melanoma continues to be a difficult disease to treat, but the armamentarium of options has expanded in recent years. The challenge physicians face is to determine what treatments to offer and the timing or sequence of treatments. Patients' individual clinical factors

(e.g., time to recurrence, previous treatment, patient age, recurrence size, location, and number) should be discussed in a multidisciplinary setting to weigh the benefits and timing of treatment options. Clinical trials are integral to the advancement the treatment of locoregional melanoma. As new therapies emerge and evidence improves on the efficacy of current treatments, recommendations will continue to evolve for locoregional recurrent melanoma.

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Melanoma Brain Metastasis: Insights, Progress, Challenges, and Opportunities

26

Grant M. Fischer and Michael A. Davies

Contents

Abstract

Brain metastasis is a frequent and often devastating complication of metastatic melanoma. Melanoma has one of the highest rates of brain metastasis among all solid tumors. Historically the survival for melanoma patients with brain metastases has been less than 6 months. However, the development of improved CNS-directed and systemic therapies appears to be resulting in rapidly improving outcomes in these patients. In parallel, there is growing understanding of the unique features of brain metastases that contribute to their aggressive behavior, providing new opportunities to develop additional strategies

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to further improve patient outcomes. This chapter will review the current understanding of the pathogenesis of melanoma brain metastases and the treatments used for this disease to provide a context for future investigations in this area.

Keywords
Brain metastasis · Melanoma · Whole brain radiation therapy · Stereotactic radiosurgery · Targeted therapy · Immunotherapy

Introduction

The development of more effective strategies to prevent and treat brain metastases is an increasingly important challenge in oncology. It is estimated that approximately 10% of all cancer patients will be diagnosed with brain metastases and up to 40% of cancer patients with metastatic disease (Bollig-Fischer et al. 2013; Maher et al. 2009). Brain metastases are associated with significant morbidity and mortality, and it is estimated that ~100,000 cancer patients die from brain metastases each year.

The most common sources of brain metastases are lung cancer, breast cancer, and melanoma. Melanoma is much less common than lung and breast cancer. Thus, melanoma has the highest risk of brain metastasis among all common solid tumors. Brain metastases are detected in 20–40% of melanoma patients at the diagnosis of stage IV disease, and autopsy studies detected CNS metastases in ~70% of patients who died from this disease (Cohen et al. 2016). Historically the median survival of metastatic melanoma patients with brain metastases was ~4 months (Glitza et al. 2016). However, new insights into the molecular biology and immunology of this disease have led to the development of new targeted therapies and immune therapies that have achieved very promising results in recent clinical trials. Despite this progress, many of these therapies have demonstrated less efficacy in patients with brain metastases than in patients with extracranial tumors only, and the brain is a frequent initial site of treatment failure for contemporary treatments.

The development of more effective prevention and treatment for melanoma brain metastases (MBMs) will be facilitated by an improved understanding of the molecular features and drivers of these tumors. Thus, in this chapter the current understanding of the pathogenesis of melanoma brain metastases will be reviewed. Outcomes with historical and current therapies will also be summarized, to provide an integrated view of the key challenges that exist in this field.

The Pathogenesis of Melanoma Brain Metastases

Metastasis of cancer cells to the brain is a complex, multi-step process that involves invasion of tissues surrounding the primary tumor; access to and survival in the lymphatic system and bloodstream; arrest in brain capillaries and extravasation into the brain parenchyma; and growth and survival in the brain (Table 1). Understanding the features that are critical to each of these steps, and the drivers that support the maintenance and aggressive behavior of MBMs, will facilitate the development of rational, more effective treatments for patients. Notably, many of these studies suggest that MBMs have distinct characteristics that may contribute to the distinctly poor outcomes associated with these tumors.

Comprehensive Molecular Analyses of Melanoma Brain Metastases

While limited, currently available global molecular characterization efforts suggest that MBMs possess unique molecular features compared to metastases that form at other anatomical sites. Gene expression microarray studies have identified numerous differentially expressed genes between MBMs and extracranial metastases (ECMs) (Chen et al. 2014; Hamilton et al. 2013). However, the microarray studies featured a relatively small number of samples, including very few patient-matched metastases, and overall failed to identify significantly enriched

Table 1 Key steps in brain metastasis formation and maintenance

Steps	Description
Growth of primary tumor	Genetic aberrations drive unregulated growth of transformed melanocytes; accumulation of mutations results in creation of subclones with increased metastatic propensity
Migration/invasion of surrounding tissues	Loss of adherence to cells in primary tumors; increased communication with extracellular matrix and surrounding stroma; digestion of extracellular matrix; increased cellular motility
Intravasation/spread to regional lymph nodes	Acquisition of access to lymphatic vessels and growth in regional lymph nodes, the site to which most melanomas spread to initially
Survival in bloodstream	Survival in harsh environment and shear stress inherent to circulatory system; ability to form emboli increases survival
Arrest in brain capillary beds	Mechanical arrest in the capillary beds of brain; mediated by interactions between adhesion molecules expressed on blood vessels of brain capillaries and tumor cells
Extravasation	Exit from the circulatory system across the BBB; mediated by factors able to digest tight junctions of the BBB
Angiogenesis	Acquisition of vital nutrients via formation of new blood vessels or co-option of existing blood vessels in the brain
Evasion of immune system	Evasion of immune attack facilitates tumor growth

pathways. Further analysis demonstrated significant heterogeneity of immune-related gene sets between MBMs from different patients and identified a significant correlation between immune-related BioCarta gene sets and prolonged overall survival (OS) (Hamilton et al. 2013). Whole exome sequencing (WES) analysis of multiple tumor types has identified significant differences between BMs and primary tumors, including three tumors from three melanoma patients (Brastianos et al. 2015). The analyses demonstrated significantly divergent evolution of BMs following metastasis from the primary tumors yet minimal divergent evolution between multiple BMs from the same patient.

Gene expression microarray analyses of breast, lung, colorectal, and melanoma cell lines suggest that the brain tumor microenvironment (TME) can trigger a fundamental reprogramming of cell lines grown in mouse brains compared to those grown in subcutaneous tissue or orthotopic sites (Park et al. 2011). Cells grown in the brain overexpressed thousands of genes and acquired neuronal cell characteristics following epigenetic changes induced by interactions with surrounding astrocytes. Interestingly, co-culturing tumor cells with astrocytes replicated this reprogramming. These studies highlighted the importance of the TME in regulating tumor cell biology.

Signaling Pathways Implicated in Melanoma Brain Metastases

Despite limited global profiling efforts, focused studies have identified numerous signaling pathways that likely contribute to MBM pathogenesis, including the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase-protein kinase B (PI3K-AKT), Janus kinase-signal transducer and activator of transcription (JAK-STAT), vascular endothelial growth factor (VEGF), and endothelin pathways. Additional molecules and pathways have been implicated in many of the steps that are critical to the establishment and maintenance of MBMs.

MAPK Pathway

MAPK pathway hyperactivation occurs in over 90% of cutaneous melanomas (Cancer Genome Atlas Network 2015). Somatic mutations in v-Raf murine sarcoma viral oncogene homolog (*BRAF*) (35–50%) and neuroblastoma RAS viral oncogene homolog (*NRAS*) (10–25%), and loss of function mutations affecting Neurofibromin 1 (*NF1*) (~15%), primarily drive MAPK pathway activation. Point mutation analysis of patient-matched MBMs and ECMs demonstrated no significant differences in the prevalence of *BRAF* mutations between anatomical sites (Chen et al.

2014). Data regarding *NRAS* mutation concordance between anatomical sites differs depending on if the comparison occurs between MBMs and ECMs or between MBMs and primary tumors. Analysis of patient-matched MBMs and ECMs identified 100% concordance in *NRAS* mutations, while significantly lower concordance (80%) was observed between patient-matched MBMs and primary tumors (Chen et al. 2014; Colombino et al. 2012). While no concordance data exist for *NF1* mutations, it is clear that MAPK pathway hyperactivation is highly prevalent in MBMs, which is consistent with the fact that activating *BRAF* and *NRAS* mutations occur early in melanoma progression and are likely not selected against during the brain metastasis cascade (Pollock et al. 2003; Shain et al. 2015).

PI3K-AKT Pathway

PI3K-AKT pathway activation occurs frequently during tumorigenesis in multiple cancer types and promotes the malignant phenotype through multiple effectors (Davies 2011; Kwong and Davies 2014). The pathway can be activated via receptor tyrosine kinases (RTKs) following stimulation by extracellular ligands [e.g., hepatocyte growth factor (HGF) activating tyrosine-protein kinase Met (c-MET)], point mutations in RTKs [e.g., epidermal growth factor receptor (*EGFR*) in non-small-cell lung cancers], and/or gene amplifications in RTKs [e.g., receptor tyrosine-protein kinase erbB-2 (*HER2/neu*) in breast cancer]. The pathway can also be activated by mutations in key effectors, including point mutations in phosphatidylinositol 3-kinase, catalytic subunit alpha (*PIK3CA*) (encodes the catalytic subunit of PI3K), *AKT1*, and *AKT3*. Constitutive activation of the pathway also results from loss of function of the tumor suppressor phosphatase and tensin homolog (PTEN), which dephosphorylates lipids to counteract the activity of PI3K.

In melanoma, point mutations rarely activate the pathway, affecting *AKT1* and *AKT3* in 1–2% of cases and *PIK3CA* in 3% (Omholt et al. 2006; Davies et al. 2008; Cancer Genome Atlas Network 2015). Mutations more commonly affect the *NRAS* gene, primarily at codons Q60/61 and G12/13, which results in constitutive activation of both the MAPK and PI3K-AKT pathways. Additionally, complete loss of PTEN has been detected in 10–30% of melanomas, generally in tumors with concurrent *BRAF* mutations, and thus mutually exclusive with *NRAS* mutations (Bucheit et al. 2014; Davies et al. 2009). Missense mutations that inhibit the tumor suppressor function of PTEN have been recorded and typically occur in exon 5 of the *PTEN* gene, which encodes the phosphatase domain of the protein. Nonsense mutations are more common genetic causes of loss of PTEN function in melanomas (Aguissa-Toure and Li 2012). Interestingly, genetic aberrations might not explain all instances of loss of PTEN expression in melanomas. Epigenetic events including miRNA signaling and promoter methylation can silence PTEN expression and may facilitate PI3K-AKT pathway hyperactivation in melanomas (Mirmohammadsadegh et al. 2006; Zhang et al. 2015). However, more recent genomic studies of the melanoma TCGA database that employed optimized criteria for identifying copy number alterations demonstrated that only point mutations or high-medium-amplitude focal deletions corresponded with complete loss of PTEN protein expression and significant increases in P-AKT levels (Roszik et al. 2016).

Unlike the MAPK pathway, a significant difference in PI3K-AKT pathway activation has been observed in MBMs compared to metastases at other sites. Reverse phase protein arrays (RPPA) analysis demonstrated significantly higher expression of numerous markers of PI3K-AKT activation and lower expression of PTEN in MBM specimens compared to lung and liver metastases (Davies et al. 2009). Subsequent RPPA analysis of patient-matched MBMs and ECMs confirmed PI3K-AKT pathway hyperactivation in MBMs yet failed to demonstrate significant differences in PTEN protein levels (Chen et al. 2014). Immunohistochemistry (IHC) of an independent cohort of patient-matched samples also demonstrated PI3K-AKT pathway hyperactivation in MBMs, with some (but not all) of the brain lesions demonstrating decreased PTEN expression compared to the patient-matched ECMs (Niessner et al. 2013). Together,

these findings suggest that PI3K-AKT pathway hyperactivation plays an important role in MBM pathogenesis and that pathway activation can occur independently of loss of PTEN expression.

PI3K-AKT hyperactivation plays multiple roles in MBM pathogenesis by facilitating cell adhesion, extravasation, and angiogenesis. One study of patients with stage III melanoma determined that loss of PTEN expression correlated with significantly increased risk of MBM formation (Bucheit et al. 2014). Similarly, experiments by Cho et al. using a genetically engineered mouse model showed that PI3K-AKT pathway activation promoted MBM formation. Loss of Pten in melanocytes with concurrent $Braf^{V600E}$ mutations and cyclin-dependent kinase inhibitor 2A (*Cdkn2a*) deletions formed primary melanomas in 100% of mice but infrequently caused distant metastases. However, introduction of a constitutively active Akt1 protein promoted MBM formation in 80% of mice (Cho et al. 2015). Together, these findings suggest that PI3K-AKT pathway hyperactivation promotes MBM formation, and primary tumors and ECMs enriched in this pathway may metastasize to the brain more frequently.

The TME also appears to play a critical role in mediating PI3K-AKT pathway hyperactivation. Zhang et al. demonstrated that loss of PTEN expression occurred following intracarotid injection of PTEN-intact cell lines from multiple tumor types, including B16F10 melanoma cells (Zhang et al. 2015). Astrocytes in contact with the tumor cells transferred miRNAs that suppressed expression of PTEN via exosomes. Interestingly, loss of PTEN expression promoted secretion of the cytokine C–C motif chemokine ligand 2 (CCL2), which recruited Iba1+ myeloid cells that promoted growth of the tumor cells. In contrast, the authors failed to observe differences in metastatic incidence between B16F10 cells expressing PTEN and those in which PTEN was silenced. Further, Seifert et al. provided additional evidence that cerebrospinal fluid (CSF), which bathes the brain, can promote PI3K-AKT pathway activation (Seifert et al. 2016). Culturing melanoma cells in the presence of CSF caused significant activation of the PI3K-AKT pathway and mediated resistance to targeted therapies without causing a loss of PTEN expression.

JAK-STAT Pathway

The JAK-STAT pathway transduces signals from a variety of ligands – often cytokines such as interleukins and interferons – from the cell surface to the nucleus of tumor cells. Unlike RTKs, cytokine receptors typically lack intrinsic kinase activity. Instead, ligand binding triggers dimerization of receptors, which recruits and activates JAKs in the cytoplasm. In turn, JAKs phosphorylate the receptor, which facilitates binding of STAT proteins to the receptor. JAKs then phosphorylate and activate the STATs, which dimerize and move into the nucleus to drive transcription of genes (Jatiani et al. 2010).

STAT3 activity facilitates transcription of tumor cell survival, growth, angiogenesis, and immunosuppressive genes such as B-cell lymphoma 2 (*BCL2*), *c-MYC*, *Cyclin D1 (CCND1)*, *VEGF-A*, and interleukin-10 (*IL-10*) (Yu and Jove 2004). STAT3 has also been implicated in MBM pathogenesis. Xie et al.'s analysis of MBMs demonstrated significantly stronger staining for phosphorylated STAT3 (P-STAT3) in MBMs compared to primary tumors. In addition, the introduction of a constitutively active form of P-STAT3 transformed nonmetastatic A375P cells into highly brain-metastatic cells but did not alter the growth rates of cells implanted subcutaneously. Likewise, brain-metastatic TXM-18 melanoma cells lost their brain-metastatic capacity following suppression of P-STAT3 (Xie et al. 2006). Huang et al. demonstrated that overexpression of suppressor of cytokine signaling 1 (*SOCS1*), a negative regulator of JAK-STAT signaling, prevented brain-metastatic melanoma cell lines from forming MBMs following intracarotid injections (Huang et al. 2008). Mechanistic studies determined that JAK-STAT signaling promoted beneficial angiogenesis and invasion signaling cascades by increasing transcription of *VEGF-A* and matrix metalloprotease-2 (*MMP-2*) (Xie et al. 2006; Huang et al. 2008). While these findings implicate JAK-STAT signaling in MBM pathogenesis, enforced expression of P-STAT3 also promoted

melanoma cell metastasis to the lungs of mice, while suppression of P-STAT3 prevented lung metastasis formation (Xie et al. 2006). Together, these findings suggest that JAK-STAT signaling may promote the general process of melanoma metastasis instead of metastasis specifically to the brain.

VEGF

The VEGF signaling cascade is mostly mediated through the binding of the VEGF-A (VEGF) ligand to the VEGFR2 receptor on endothelial cells (Ferrara et al. 2003). Binding of the ligand to the receptor triggers activation of the PI3K-AKT, phospholipase Cγ, and Src family kinase signaling pathway to promote angiogenesis and vascular permeability (Guo et al. 1995; Eliceiri et al. 1999). Yano et al. used a panel of cell lines from multiple tumor types – including melanoma – to demonstrate that VEGF expression correlates with brain metastatic capacity *in vivo*. Cells expressing high levels of VEGF formed large, rapidly growing metastases, while cells lacking VEGF expression formed small, poorly growing metastases (Yano et al. 2000). Additionally, forced overexpression of VEGF facilitated growth of MBMs formed by MEL57 cells following intracarotid injection. Interestingly, VEGF expression did not induce the formation of new vessels but rather caused beneficial co-option of existing blood vessels (Kusters et al. 2002). This propensity for vascular co-option agrees with pathological assessments of human brain metastases that determined vascular co-option occurs more often in MBMs relative to metastases of other tumor types (Berghoff et al. 2013).

Endothelins

Endothelin signaling appears to facilitate spread of melanomas to the brain. Cruz-Munoz et al. established a mouse model of spontaneous melanoma metastasis and determined via microarray analysis that brain-metastatic WM239 variants significantly overexpressed endothelin receptor B (EDNRB) compared to visceral metastatic variants. However, the visceral variants did not overexpress EDNRB compared to nonmetastatic variants. Forced overexpression of EDNRB in the visceral metastatic variants increased overall metastatic incidence and promoted metastasis to the brain. Pharmacological inhibition of EDNRB inhibited lung metastasis formation and brain metastasis growth. Together, these findings suggest that EDNRB promotes a general metastatic phenotype but plays a critical role in the formation of MBMs (Cruz-Munoz et al. 2012).

Factors That Promote Penetration of the Blood-Brain Barrier (BBB)

The BBB is a network designed to protect the brain by restricting free access of blood products to the brain parenchyma. Brain capillary walls are composed of endothelial cells connected via tight junctions, end-foot process of astrocytes surrounding the capillaries, and pericytes embedded in the basement membrane of the capillaries (Daneman and Prat 2015). Together, this BBB limits free passage into the brain to small (<400 Da) lipophilic molecules (Pardridge 2003). Circulating melanoma cells must cross this BBB to access the brain parenchyma during a process known as extravasation. As described below, a variety of factors appear capable of contributing to this process (Kircher et al. 2016; Chen and Davies 2012). Very late antigen 4 (VLA-4) mediates adhesion of melanoma cells to vascular cell adhesion molecule 1 (VCAM-1) on the surface of endothelial cells, which facilitates arrest in capillaries and successful extravasation. VLA-4 expression promoted melanoma cell migration across several immortalized endothelial cell lines, and VLA-4 inhibition prevented the formation of melanoma lung metastases *in vivo* (Garcia-Martin et al. 2018; Schlesinger et al. 2014). To assess the role of VLA-4 in MBM pathogenesis, Garcia-Martin et al. utilized a tissue microarray and determined that over 90% of MBMs express VLA-4. They subsequently constructed a mouse *in vitro* BBB model and observed that inhibition of VLA-4 prevented melanoma cells from adhering to the endothelial cells and migrating across the BBB (Garcia-Martin et al. 2018). The exact route by which melanoma cells transverse the BBB remains unclear. Melanoma cells might

utilize the paracellular route by degrading tight junction proteins via serine proteases such as seprase. Analyses of A2058 and B16-F10 melanoma cells demonstrated that secretion of seprase allowed these cells to migrate across an *in vitro* model of the BBB. Pharmacological and siRNA-mediated inhibition of seprase effectively prevented their transmigration across the BBB (Fazakas et al. 2011). However, the role of seprase in mediating extravasation has not been confirmed *in vivo*. Heparanase (HPSE) is an enzyme that degrades heparan sulfate chains in endothelial cell layers and appears to mediate extravasation. Murine (B16B15b) and human (70W) cells selected for their brain-metastatic capacity displayed significantly higher HPSE production and activity compared to their parental cell lines (B16F1 and MEWO) (Marchetti 1997). Additionally, *in vitro* treatment of B16B15b cells with HPSE increased their ability to invade a brain slide model. Pharmacological inhibition of HPSE mitigated this effect (Murry et al. 2006). Interestingly, co-culturing brain-metastatic melanoma cells with syngeneic astrocytes or nerve growth factor (NGF) – a growth factor secreted in the brain – significantly enhanced their secretion of HPSE and invasiveness, indicating that normal brain tissue plays a role in facilitating the entrance of metastatic melanoma cells into the parenchyma (Marchetti et al. 1993, 2000). To date, *in vivo* studies assessing HPSE's role in BM pathogenesis have been limited to breast cancer. Zhang et al. determined that breast cancer brain metastases lose expression of miRNA-1258, which is a key suppressor of HPSE production. Further, microRNA-1258 expression levels negatively correlated with the brain-metastatic capacity of breast cancer cells. Transfection of miRNA-1258 in breast cancer cells significantly inhibited brain metastasis formation in an experimental model of metastasis (Zhang et al. 2011). Interest in the role of pleckstrin homology domain containing A5 (PLEKHA5) in MBMs arose following Jilaveanu et al.'s integrative comparative analysis of a brain-metastatic A375P subclone (A375Br) and the parental line and between ECMs from patients that did and did not develop MBMs (Jilaveanu et al. 2015). Gene expression profiling determined that PLEKHA5 expression was significantly higher in A375Br cells and in ECMs from patients that developed MBMs. Interestingly, PLEKHA5 expression correlated only with formation of metastases in the brain. PLEKHA5 suppression inhibited survival of A375Br cells and their ability to invade an *in vitro* BBB model. However, these findings have not been confirmed *in vivo*.

Studies of the A375 cell line's interactions with an *in vitro* BBB demonstrated that secretion of S100A4 calcium-binding protein A4 (S100A4) mediated BBB invasion by facilitating a loss of tight junction integrity (Herwig et al. 2016). Binding of S100A4 to its receptor [receptor for advanced glycation end products (RAGE)] suppressed expression of the tight junction proteins occludin and VE-cadherin. These findings were confirmed *in vivo* where forced overexpression of S100A4 increased brain metastasis formation of A375 cells following intracardiac injection. However, S100A4 overexpression also stimulated metastasis to other anatomical sites, suggesting that S100A4 promotes a generic pro-metastatic phenotype as opposed to a brain-metastatic phenotype.

Studies across multiple tumor types have also implicated MMPs in tumor cell invasion and metastasis through their ability to proteolytically degrade components of the extracellular matrix (Gialeli et al. 2011). MMP-2 appears to mediate extravasation across the BBB. MMP-2 catalyzes the breakdown of type IV collagen, which forms a critical component of the basement membrane of endothelial cells in the BBB (Gialeli et al. 2011). Correlation studies determined that tumor MMP-2 expression levels predict worse survival in patients (Rotte et al. 2012). Gene expression studies established that MMP-2 is expressed significantly higher in primary melanoma cultures able to invade an *in vitro* model of the BBB compared to poorly invasive primary melanoma cultures (Rizzo et al. 2015). Interestingly, astrocytes facilitate expression of MMP-2 in melanoma cells by secreting interleukin-23 (IL-23) (Klein et al. 2015). Tang et al. determined that IL-23 stimulates secretion of IL-17 by γδ T cells. The IL-17 drives increased levels of P-STAT3 through

interleukin-6 (IL-6) and is likely the means by which this signaling axis promotes MMP-2 transcription (Tang et al. 2013).

Homing to the Brain

Circulating melanoma cells arrest in brain capillaries once they exceed the capillary in size (Kienast et al. 2010). Additionally, they express numerous adhesion factors including integrins (including VLA-4, as previously described) and selectins that promote the arrest of the cells within the capillaries. Thus, mechanical factors promote the spread of melanoma cells to the brain. Consistent with Stephen Paget's "seed and soil" hypothesis, however, numerous additional factors (including the PI3K-AKT pathway, as previously described) appear to promote metastasis specifically to the brain.

Using miRNA expression profiling and three cohorts of clinical primary melanomas, Hanniford et al. derived a miRNA signature (*miR-150–5p, miR-15b-5p, miR-16–5p*, and *miR-374b-3p*) that predicted the likelihood of brain metastasis formation (Hanniford et al. 2015). Further, their signature distinguished primary melanomas that initially metastasized to the brain from those that metastasized to the brain along with other extracranial sites, suggesting that their signature might predict brain tropism. Zhang et al. demonstrated that TGF-β signaling is necessary for metastasis to the brain parenchyma (Zhang et al. 2009). K1735 cells exclusively formed metastases in the brains of mice following intracarotid injections while B16F10 cells formed metastases in the ventricles. K1735 cells expressed high levels of TGF-β2 while B16F10 cells lacked expression of TGF-β2. Forced overexpression of TGF-β2 in B16F10 cells promoted metastasis to the parenchyma while suppression of TGF-β2 in K1735 cells inhibited metastasis to the brain parenchyma. Gene expression studies identified significantly higher levels of C–C chemokine receptor 4 (CCR4) in cell lines from MBMs compared to cell lines from cutaneous tumors (Izraely et al. 2010). Klein et al. confirmed these findings by comparing flow cytometry results between YDFR.CB4, M12.CB3, and M16.CB2 brain-metastatic variants and their parental cell lines (YDFR.C, M12.C, and M16.C). They also determined that astrocytes and microglia secrete known CCR4 ligands and that incubation of these glial cells in brain metastasizing melanoma cell-conditioned media upregulates their secretion of CCR4 ligands. Further, incubation of YDFR.C cells overexpressing CCR4 with CCR4 ligands facilitated transmigration across an *in vitro* BBB model, and pharmacological inhibition of CCR4 prevented metastasis of these cells to the brain metastases *in vivo*. Together, these studies strongly implicate CCR4 in MBM pathogenesis (Izraely et al. 2010). However, analysis of clinical specimens determined that both MBMs and lymph node metastases expressed CCR4 at significantly higher levels than primary tumors, but CCR4 expression did not differ between MBMs and lymph node metastases, suggesting that CCR4 might not mediate expression exclusively to the brain (Klein et al. 2017). In contrast, brain-metastatic YDFR cells express significantly less claudin-1 (CLDN1) than their parental cells (Izraely et al. 2012). Forced overexpression of CLDN1 in brain-metastatic YDFR cells significantly inhibited the formation of MBMs *in vivo* but had no effect on the formation of lung metastases, indicating that CLDN1 promotes melanoma metastasis exclusively to the brain (Izraely et al. 2015).

Immunology of Melanoma Brain Metastases

There is a growing need to improve our understanding of the immunological features of MBMs because of the rapidly expanding role of immunotherapy in the treatment of this disease. To date, Kluger et al. have performed the most comprehensive analysis of the immunological features of MBMs (Kluger et al. 2015). Utilizing a tumor microarray (TMA) of 95 metastatic melanomas, they identified significantly fewer T cells (total and $CD8^+$) in MBMs compared to metastases at other anatomical sites. Furthermore, they observed significant heterogeneity in the extent of MBM T cell infiltrate and determined that the amount of T cell infiltrate correlated significantly

with improved survival. While MBM T cell infiltrate correlated significantly with PD-L1 expression, they observed no significant differences in PD-L1 staining between MBMs and metastases from other sites. Finally, T cell infiltrate at extracranial metastases correlated significantly with the amount of time to brain metastasis diagnosis. The findings of Kluger et al. agree with Hamilton et al.'s gene expression microarray of MBMs, which identified a significant correlation between immune-related expression signatures and patient survival (Hamilton et al. 2013). Further, *miR-150–5p* (one component of Hanniford et al.'s miRNA signature that predicts metastasis to the brain) was significantly downregulated in brain-metastatic primary melanomas and correlated with $CD45^+$ lymphocyte infiltrate, further implicating immunosuppression in MBM pathogenesis (Hanniford et al. 2015).

The cause for MBM immunosuppression remains unclear. While it might be tempting to blame the BBB for physically impeding immune cells from accessing MBMs, several lines of evidence challenge this conclusion. BBB disruption occurs in numerous neurological diseases such as multiple sclerosis, stroke, and epilepsy. In each case, the BBB is unable to restrict the entry of immune cells into the brain parenchyma and prevent an inflammatory state as it typically does in a normal, healthy brain (Obermeier et al. 2013). MBMs induce significant leakage in the BBB during the processes of extravasation and angiogenesis. The previously described factors HPSE, MMP-2, and S100A4 damage the integrity of the BBB to promote extravasation into the parenchyma, and VEGF causes vessels to leak as it stimulates angiogenesis. Clinical imaging findings confirm MBM-induced BBB leakage. For example, MBMs take up gadolinium contrast agent during MRI imaging while normal brain parenchyma fails to do so. Additionally, as described later in this chapter, immunotherapy with anti-CTLA4 and anti-PD1 antibodies achieves clinical responses in patients with MBMs (Goldberg et al. 2016; Margolin et al. 2012). Both of these treatments depend on T cells crossing the BBB and accessing the tumor. Clinical responses to these agents indicate that the BBB is not able to prevent the entry of T cells into the brain parenchyma. Together, these findings indicate that physical barriers cannot explain immunosuppression in MBMs.

Several key signaling pathways mediate immunosuppression in melanoma ECMs and play critical roles in MBM pathogenesis. For example, Peng et al. observed that loss of PTEN stimulates PI3K-AKT pathway activation and secretion of VEGF in melanomas (Peng et al. 2016). Expression levels of VEGF inversely correlated with intratumoral T cell infiltrate and response to anti-PD1 immunotherapy. Further, treatment of PTEN-null melanomas with the selective PI3KB inhibitor GSK2636771 overcame the immunosuppression and promoted response to immunotherapy. As multiple investigators have observed increased levels of P-AKT in MBMs compared to metastases from other sites, further studies are needed to directly evaluate the relationship between PI3K-AKT pathway activation and suppression of the immune response in MBMs. The immunosuppressive effects of TGF-β signaling have been extensively characterized (Yang et al. 2010; Gigante et al. 2012). Additionally, Walentynowicz et al. demonstrated that TGF-β2 polarizes glioma-associated macrophages into tumor-supporting cells (Walentynowicz et al. 2018). It is possible that the TGF-β2 signaling axis which is necessary for formation of parenchymal metastases also mediates MBM immunosuppression. Further studies would be necessary to confirm this possibility and to elucidate whether or not the mechanisms of TGF-β2-mediated immunosuppression are the same in MBMs as in ECMs. It is also possible that stromal cells may cause immunosuppression in MBMs via STAT3 signaling. Priego et al. performed IHC on 91 BMs, including 2 MBMs, and identified positive P-STAT3 staining in the peritumoral reactive astrocytes of 89% of the BMs. Further, strong P-STAT3 staining in reactive astrocytes significantly correlated with patient survival. Mechanistic studies determined that the BMs induced P-STAT3 signaling in the astrocytes, which in turn inhibited the activation of $CD8^+$ T cells and promoted the activity of pro-tumor macrophages/microglia

expressing cluster of differentiation 74 (CD74). Importantly, inhibition of P-STAT3 signaling in reactive astrocytes inhibited the intracranial growth of the B16/F10-BrM melanoma cell line *in vivo*, suggesting that targeting this signaling axis in reactive astrocytes could be beneficial for patients with MBMs (Priego et al. 2018).

In summary, many new insights have been made into the pathogenesis of brain metastases from melanoma. As described below, this has occurred in parallel to the development of new therapeutic approaches with improved efficacy in these patients. A key challenge remains to translate these new insights into rational, more effective treatment approaches.

Treatment of Melanoma Brain Metastases

There are multiple therapeutic approaches available for the treatment of MBMs (Table 2). In the past era of ineffective systemic therapies, most treatments for patients with MBMs were locally directed therapies, such as surgery and radiation, which initially consisted of whole brain radiation therapy (WBXRT). Over time, technological advances led to the development of stereotactic radiosurgery (SRS) techniques, which are increasingly used instead of WBXRT. Similarly, systemic treatments for MBMs initially consisted of chemotherapies, particularly ones that were able to penetrate the BBB. However, such treatments have now been largely replaced by molecularly targeted therapies and antibody-based immune therapies, which can have activity simultaneously on brain and extracranial disease. With these many options, the management of patients with MBMs utilizes multidisciplinary evaluation to select among these varied, and very different, treatment options. Further, available data strongly supports the rationale to evaluate the safety and efficacy of combining different modalities together.

Local Therapies: Surgery and Radiation

The role of locally directed therapies for MBMs is evolving. As noted above, surgery previously was a mainstay in the treatment of these

Table 2 Current treatments for melanoma brain metastasis

Treatment modality	Strengths	Limitations
Surgery	Often necessary to control large or hemorrhagic brain metastases, which may enable the use of other treatment modalities	Morbidity of surgery; risk of recurrence both locally and elsewhere in the brain; no impact on extracranial tumors
Whole brain radiation therapy (WBXRT)	Can provide rapid relief in patients with widespread, symptomatic brain metastases; reduce the risk of progression in the brain when used in the adjuvant setting	Generally short duration of disease control when used alone to treat MBMs; significant risk of cognitive decline; no clear impact on extracranial tumors
Stereotactic radiosurgery (SRS)	High rates of disease control of radiated brain metastases; rapid onset of effects; decreased surgical morbidity for non-superficial lesions compared to surgery	Efficacy decreased for tumors >2–3 cm with current technologies; lack of impact on lesions (i.e., micrometastases) that are not irradiated; no clear impact on extracranial tumors
Targeted therapy – BRAF + MEK inhibitors	High rates of initial intracranial tumor response and disease control; generally rapid onset of effects; can be used in patients with symptomatic lesions that require steroids; concurrent treatment of intracranial and extracranial disease	Can only be used in patients with targetable mutations (i.e., $BRAF^{V600}$ for BRAF + MEK inhibitors); durability of responses short compared to extracranial tumors and compared to immunotherapy
Immunotherapy – anti-PD-1 + anti-CTLA-4 antibodies	High rates of intracranial responses in asymptomatic patients, the majority of which appear to be durable (>6 months or more); use not restricted to specific molecularly defined subpopulations; concurrent treatment of intracranial and extracranial disease	Limited data about efficacy in patients with symptomatic MBMs (i.e., requiring steroids); lower disease control rates than SRS or targeted therapy

patients. Survival in most patients treated with neurosurgical approaches was poor in the overwhelming majority of patients, as most patients with MBMs present with concurrent disease elsewhere. Thus, even if surgery could address the brain metastases, the lack of effective therapies to control other tumors resulted in limited survival. However, the subset of patients that presented with brain metastases only could achieve long-term survival (Sampson et al. 1998). Thus, when treating for curative intent, surgical resection can be considered in patients with oligometastatic (<4) brain metastases if extracranial disease is absent or controlled. Surgery can also have a palliative role in the management of symptomatic brain metastases, particularly those with significant hemorrhage or that have been refractory to other treatments (i.e., radiation). Surgical resection is also indicated in patients who present with brain metastases without a known cancer diagnosis, or in patients with more than one cancer type that can metastasize to the brain, to help clarify the tumor of origin and guide further treatment strategies.

The initial form of radiation used in the management of melanoma patients with brain metastases was WBXRT. In historical series, WBXRT resulted in some prolongation of survival, but it very rarely achieved durable control of MBMs (Davies et al. 2011). As such, it was primarily considered for patients with too many brain metastases or tumor locations that precluded surgical resection. WBXRT was shown in studies to improve overall survival when used as an adjuvant treatment after the surgical resection of brain metastases from multiple tumor types (Gaspar et al. 2010). Unfortunately, WBXRT frequently causes significant cognitive decline due to damage to normal brain tissue from this radiation technique (Laack and Brown 2004; Brown et al. 2016). In the era in which treatments for patients with MBMs were largely considered palliative due to the lack of effective systemic therapies, such declines could be considered acceptable due to the short survival of many of these patients, particularly as the onset of cognitive effects may be delayed. However, in an era in which new therapies are providing markedly increased chances of durable survival, the clinical significance of the cognitive decline caused by WBXRT has increased. Thus, the use of WBXRT in the current era has become more limited, generally reserved for patients who have brain metastases that by size and/or number are not amenable to newer, focused radiation approaches, particularly in the setting of tumor-related neurological symptoms. New WBXRT techniques are also being evaluated to see if the neurotoxicity of this treatment modality can be reduced.

Stereotactic radiosurgery (SRS) is a technique in which many low-intensity beams of radiation are delivered so that they intersect at the site of a tumor. This technique delivers a high dose of radiation to that specific area that is targeted while delivering much lower doses of radiation of other areas, including normal brain tissue. This technique is highly effective at achieving durable control in tumors that are less that 2–3 cm in maximum diameter, with local disease control rates of >80% at 1 year (Ajithkumar et al. 2015). Importantly, SRS also causes less cognitive effects than WBXRT and thus has significant advantages. While technical limitations initially made it possible to only treat a limited (i.e., <4) brain metastases in a single day, new techniques, such as gamma knife radiosurgery, can allow for many more tumors to be treated expediently, markedly expanding the clinical utilization of SRS. Thus, SRS has largely replaced WBXRT in the management of patients with MBMs despite the lack of head-to-head prospective trials specifically in this disease. Notably, the sparing of normal brain tissue from toxicity has also made SRS a standard option even in patients that have tumors that could be addressed with surgical resection, which thus is often reserved for MBMs that progress despite SRS treatment.

While SRS is highly effective against small MBMs, its ultimate utility in larger tumors is less established. Further, while SRS causes less cognitive decline than WBXRT, it is not as effective at preventing the development of other tumors in the brain (Chang et al. 2009). However, it is possible that this deficit may now be addressable with systemic therapies.

Chemotherapy

Traditionally the use of chemotherapy for brain metastases has prioritized agents that can cross the BBB. However, the need for this requirement is unclear, as there is significant evidence that brain metastases cause significant disruption of the BBB (Gerstner and Fine 2007).

The chemotherapy agent tested most extensively in MBM patients is temozolomide (TMZ). TMZ is metabolized to the same active metabolite as dacarbazine (DTIC), a DNA alkylator which was approved for the treatment of stage IV metastatic melanoma patients in 1975. In contrast to DTIC, TMZ has very good penetration of the BBB, and it is FDA-approved for the treatment of primary brain tumors. However, TMZ achieved intracranial responses in 7% of patients with previously untreated MBM, and in only 3% of patients with previously treated MBMs, in a phase II trial that included 151 patients (Agarwala et al. 2004). TMZ has also been evaluated in multiple small phase II studies in combination with a variety of other agents, without convincing evidence of significant benefit (Glitza et al. 2016).

Targeted Therapy

As noted earlier in this chapter, approximately 50% of cutaneous melanomas harbor a mutation in the *BRAF* proto-oncogene that result in substitutions of the V600 residue of the protein and constitutive activation of its serine-threonine kinase activity. In 2011, vemurafenib, a selective small molecule inhibitor of the activated mutant form of BRAF, became the first targeted therapy to be approved for patients with metastatic melanoma. The approval was limited to patients with a $BRAF^{V600}$ mutation, as both preclinical studies and early-phase clinical trials showed no significant activity in melanomas without this mutation and even the potential to accelerate the growth of such tumors. Dabrafenib was the second such inhibitor to be approved for patients with a $BRAF^{V600}$ mutation, in 2014. Despite being the second agent approved, dabrafenib was the first BRAF inhibitor to be evaluated in patients with MBMs.

Dabrafenib was selected in part for clinical development based on the fact that it did not cross the BBB in preclinical models, which was thought to be advantageous by minimizing the risk of neurological side effects caused by the drug. Despite this property, intracranial tumor shrinkage was observed in nine out of ten patients, with seven patients achieving confirmed responses, with MBMs in the phase I clinical trial of dabrafenib (Falchook et al. 2012). Based on this promising results, the BREAK-MB phase II trial evaluated the safety and efficacy of dabrafenib in 89 patients with previously untreated MBMs and 83 patients with new or progressing MBMs after previous CNS local therapies (Long et al. 2012). All patients had either mutation resulting either a $BRAF^{V600E}$ or a $BRAF^{V600K}$ substitution, which are the two most common forms seen (~70% and ~20% of *Braf* mutations, respectively). In addition, all patients were required to have no active neurological symptoms from their MBMs; patients who required corticosteroids to control such symptoms were eligible as long as the steroid dosing regimen was stable or decreasing. Dabrafenib achieved confirmed intracranial responses in 30–40%, intracranial disease control in 80–90%, and a median OS of ~8 months in MBM patients with a $BRAF^{V600E}$ mutation. Slightly lower rates were seen in patients with a $BRAF^{V600K}$ substitution. While no randomized trial was performed to directly compare dabrafenib versus TMZ, the results with targeted therapy were clearly superior, establishing dabrafenib as a treatment options for MBM patients with a $BRAF^{V600}$ mutation. Notably, a phase II study of vemurafenib in 146 MBM patients reported several years later demonstrated similar intracranial activity and overall survival as was observed with dabrafenib (McArthur et al. 2017).

Several clinical trials in metastatic melanoma patients with a $BRAF^{V600}$ mutation but without MBMs showed that combined treatment with a BRAF and a MEK inhibitor was superior to single-agent BRAF inhibitor therapy (Luke et al. 2017). These trials led to the approval of three

different targeted therapy combinations for metastatic melanoma patients with a $BRAF^{V600}$ mutation: dabrafenib and trametinib (2014), vemurafenib and cobimetinib (2015), and encorafenib and binimetinib (2018). In 2017, the results of the first clinical trial with such combination in MBM patients were reported. COMBI-MB was a phase II trial to evaluate the safety and efficacy of the FDA-approved dosing of dabrafenib and trametinib in patients with new or progressing MBMs (Davies et al. 2017). The primary goal of the study was to evaluate dabrafenib and trametinib in patients with a $BRAF^{V600E}$ mutation, asymptomatic MBMs (steroids allowed), and no prior CNS-directed therapies, and the trial included 76 such patients (Cohort A). The trial also included small, exploratory cohorts (16 patients each) of patients with previous CNS-directed therapies and with a $BRAF^{V600K}$ mutation, and one cohort of patients with active neurological symptoms not controlled by steroids ($n = 17$). The treatment was well-tolerated in all cohorts, with no new or increased toxicities compared to what had been seen with this regimen in previous trials. For patients in Cohort A, the intracranial response rate was 58% and the intracranial disease control rate was 78%; similar rates were observed in the other smaller cohorts in the study. These initial response rates were promising and were only slightly lower than the rates previously reported in patients without MBMs. However, the median duration of responses for the MBMs in the patients in cohort A was only 6.5 months. This was approximately half as long as had been reported in the previous phase III trials of dabrafenib and trametinib in extracranial metastases (Long et al. 2016). Further supporting less durable responses in the MBMs, overall approximately 50% of all of the patients in the COMBI-MB study developed progressive disease in the brain before showing evidence of disease progression in their extracranial tumors (Davies et al. 2017). Finally, the median OS for patients in Cohort A was 10.8 months, which was again markedly less than previously reported in clinical trials with dabrafenib and trametinib in patients without MBMs (median 25.6 months) (Long et al. 2016).

At this time the cause of the shorter durability of responses of MBMs with dabrafenib and trametinib is unknown. Previous studies with both single-agent BRAF inhibitors and with BRAF and MEK inhibitor combinations have demonstrated the importance of the degree of MAPK pathway inhibition achieved in tumor cells to the clinical benefits from these regimens (Bollag et al. 2010; Flaherty et al. 2012). While it is clear that BRAF and MEK inhibitors are penetrating the BBB due to the high rates of clinical response and disease control, it is currently unknown whether similar drug levels and MAPK pathway inhibition are being achieved in MBMs compared to ECMs. If inferior pathway inhibition is being achieved in MBMs, then perhaps outcomes could be improved with alternative dosing regimens, or agents with improved BBB penetration, that achieve higher drug levels in the brain. However, as discussed previously, there is also evidence that MBMs can have significant molecular and immune differences compared to ECMs, which could also explain or contribute to the differential activity observed. For example, multiple studies have shown that activation of the PI3K-AKT pathway, either by loss of PTEN or by activation of receptor tyrosine kinases (RTKs), can cause resistance to BRAF and MEK inhibitors in melanomas with a $BRAF^{V600}$ mutation (McQuade and Davies 2015). In addition to identifying hyperactivation of the PI3K-AKT pathway in MBM clinical samples, independent studies also demonstrated clinical activity of PI3K inhibitors in preclinical models of MBMs, including in combination with BRAF inhibitors (Chen et al. 2014; Niessner et al. 2016). While the PI3K-AKT pathway is thus an attractive combinatorial target for MBMs, successful inhibition of the pathway in patients has been challenging due to toxicities. Approaches being explored to overcome this limitation include isoform-selective kinase inhibitors and intermittent dosing strategies.

In addition to molecular differences, immunological differences could also play a role in the shorter durability of intracranial responses with targeted therapy. Experiments in preclinical models strongly support that the immune system

plays an active role in the clinical benefit achieved by BRAF inhibitors (Knight et al. 2013). Further, the degree of immune cell infiltration in tumors at baseline has been shown to correlate with the duration of responses achieved in metastatic melanoma patients treated with BRAF inhibitors (Kwong et al. 2015). Together the findings support the rationale to evaluate combinations of targeted and immune therapies, which are primarily being explored at this time in patients without MBMs.

Immunotherapy

The brain is a site that is relatively protected from the immune system, as inflammation of the brain can cause significant morbidity. The use of interleukin-2, the first immunotherapy approved for patients with metastatic melanoma, in patients with brain metastases was further complicated by the need for aggressive fluid hydration to treat the vasodilatory effects of this cytokine, which can increase the risk of cerebral edema. Initial clinical trials also showed that the presence of brain metastases predicted very poor outcomes with interleukin-2 treatment in melanoma patients (Phan et al. 2001). However, much more promising safety and efficacy have been demonstrated in clinical trials with checkpoint inhibitor immunotherapies.

Ipilimumab, which is a monoclonal antibody against CTLA-4 on the surface of immune cells, was the first immune checkpoint inhibitor to be approved for the treatment of metastatic melanoma patients. It was also the first checkpoint inhibitor to be evaluated in melanoma patients with brain metastases. A phase II study evaluated the safety and efficacy of ipilimumab, given at a dose of 10 mg/kg, to 72 MBM patients, who were divided into two cohorts (Margolin et al. 2012). The larger cohort, Cohort A, included patients with small, asymptomatic brain metastases that did not require steroids ($n = 51$). The objective response rate in the brain for these patients was 18%, and 24% achieved disease control. Although long-term follow-up has yet to be reported, the responses appeared to be durable. Cohort B ($n = 21$) included patients with symptomatic brain metastases that required steroids to control symptoms from cerebral edema. Only 5% of patients in Cohort B achieved intracranial responses, and the intracranial disease control rate was 10%. Together, the study provided an important proof-of-principle result that immune checkpoint inhibitors could safely achieve durable clinical responses in MBM patients. The results also suggested that efficacy may be much lower in patients requiring steroids to control cerebral edema from MBMs. Thus, most subsequent studies of immunotherapies in patients with brain metastases have excluded patients that require steroids. However, it is unknown whether the poor outcomes in Cohort B observed in this study were due to steroids antagonizing the effects of the ipilimumab or whether those patients did poorly because they had more aggressive tumors.

Multiple clinical trials in metastatic melanoma patients without CNS involvement demonstrated that anti-PD-1 checkpoint inhibitor antibodies were both more effective and safer than ipilimumab (Luke et al. 2017). The first clinical trial to evaluate anti-PD-1 immunotherapy in patients with brain metastases was a phase II study of pembrolizumab that included both lung cancer ($n = 18$) and melanoma ($n = 18$) patients (Goldberg et al. 2016). None of the patients were on steroids at the start of treatment, but many patients had received prior radiation and/or ipilimumab. The intracranial response rate among the melanoma patients was 22%, and recent reports have demonstrated that virtually all of these responses have been durable (Kluger et al. 2019). The Anti-PD1 Brain Collaboration (ABC) study reported a very similar intracranial response rate with the anti-PD-1 antibody nivolumab of 20% in 25 patients with previously untreated MBMs, with again most responses appearing to be durable (Long et al. 2018). While the results again suggested very promising results in a subset of patients, the response rates did appear to be lower than those observed in patients in previous studies who did not have CNS metastases (i.e., response rate of 45% for nivolumab) (Larkin et al. 2015).

Combined treatment with ipilimumab and nivolumab is a highly active therapy that has reported clinical response rates of close to 60% in metastatic melanoma patients without CNS metastases, with most responses occurring very quickly (Larkin et al. 2015; Luke et al. 2017). However, it is also associated with significantly more toxicity than single-agent checkpoint inhibitor therapy. The CheckMate 204 study evaluated this combination in 94 metastatic melanoma patients with asymptomatic brain metastases (Tawbi et al. 2018). The initial report of this study demonstrated an impressive intracranial response rate of 55%, with highly concordant results in the extracranial metastases of these patients, and 90% of the responses were ongoing at the time the results were presented. Impressively, the 12-month overall survival was 81.5%. The ABC study reported a slightly lower intracranial response rate of 46% among 35 asymptomatic patients without prior CNS-directed therapy treated with the same regimen of ipilimumab with nivolumab, with a 6-month overall survival rate of 78% (Long et al. 2018). Both studies reported significant toxicities in ~50% of patients, which is very similar to results observed in patients without CNS disease, and there was no apparent increase in neurotoxicity observed.

The results with ipilimumab and nivolumab quickly established this regimen as an important option to consider in melanoma patients with brain metastases. However, it must also be acknowledged that the results may not apply to all patients. In particular, many patients with brain metastases are diagnosed due to the presence of neurological symptoms and often require steroids to control them. As noted above, the pembrolizumab and CheckMate 204 studies excluded such patients (Tawbi et al. 2018; Goldberg et al. 2016). The ABC trial did report an exploratory cohort of 12 patients who had previous CNS-directed treatment or required steroids for neurological symptoms who were treated with single-agent nivolumab, which achieved an intracranial response in only 1 patient (6%) (Long et al. 2018). The CheckMate 204 will report in the future the outcomes of a cohort of patients treated with ipilimumab and nivolumab who required concurrent steroids (up to 4 mg per day of dexamethasone) for symptom control. Thus, the optimal management for patients with symptomatic brain metastases remains unclear, and the outcomes of those patients remains poor. Possible strategies for such patients include upfront radiation, particularly stereotactic radiosurgery. Alternatively, for patients with a $BRAF^{V600}$ mutation, initial treatment with targeted therapies could be used based on the high initial response and disease control rates they achieve.

Combinations

The durability of responses achieved recently with immunotherapy both intracranially and extracranially in metastatic melanoma patients with brain metastases supports a prominent role in the management of these patients moving forward. A key question is how to build upon the initial results that have been observed, particularly with ipilimumab and nivolumab. Notably, while the overwhelming majority of patients who have responded to this treatment appear to achieve durable disease control and survival, 40–50% of patients failed to respond to this treatment, including a significant subset who were removed from the study before they could even be formally assessed for treatment response. In addition, while toxicities are more likely to be tolerated by practitioners in patients who have poor prognosis, such as those with CNS involvement, ultimately it would be beneficial to achieve similar results with less toxic regimens. Many new immunotherapy combinations are now being evaluated to see if similar efficacy can be achieved as that observed with ipilimumab and nivolumab but with less toxicity. However, almost none of these trials include patients with active brain metastases.

One potential strategy to build upon recent advances is to evaluate regimens that combine different treatment modalities. As described earlier, SRS achieves very high rates of disease control in treated lesions. Combining this approach with immunotherapy could potentially result in nearly uniform initial disease control

intracranially, particularly in patients with a relatively small number of brain metastases. As there is also evidence that radiation can boost the antitumor immune response, there is also reason to hope that combinations of radiation and immunotherapy may result in therapeutic synergy (Postow et al. 2012; Vanpouille-Box et al. 2017). Supporting this possibility, several retrospective series have reported impressive outcomes in MBM patients that received both checkpoint inhibitors and SRS, and prospective clinical trials are underway or in planning stages (Cohen et al. 2016). While this approach is quite promising, retrospective series also indicate that this approach may increase the risk of radiation necrosis, a known complication of radiation therapy that can cause significant morbidity.

As noted above, there is also clinical rationale to combine targeted therapy with immunotherapy. The rapid onset and high rate of initial disease control with dabrafenib and trametinib in MBM patients with a $BRAF^{V600}$ mutation could potentially provide an opportunity to further improve response rates with immunotherapy. Further, as there is significant preclinical data supporting the importance of the immune system in clinical responses to BRAF and MEK inhibitor targeted therapy, it is possible that checkpoint inhibitors may extend the durability of responses achieved with those targeted therapy agents. Similar to radiation, clinical trials combining anti-PD-1 agents with BRAF and MEK inhibitors have been initiated.

There are additional potential combinatorial approaches based on molecular features and drivers of brain metastases that have been identified. For example, multiple studies have indicated that MBMs frequently demonstrate increased activation of the PI3K-AKT pathway. Activation of the PI3K pathway, and particularly loss of PTEN, has also been shown in this disease to contribute to resistance to BRAF inhibitors, MEK inhibitors, and checkpoint inhibitor immunotherapy, which can be overcome with PI3K pathway inhibitors (Chen et al. 2014; Niessner et al. 2016; Peng et al. 2016). As the PI3K-AKT pathway appears to be activated in MBMs even when it does not appear to be activated in ECMs in individual patients, agents targeting this pathway may have the greatest clinical impact in patients with CNS involvement. Interestingly, preclinical models suggest that loss of PTEN promotes resistance to immunotherapy in part by inducing the expression of VEGF, which impedes the trafficking of immune cells into regions in which it is expressed (Peng et al. 2016). Increased VEGF has been implicated in MBMs independently, and both antibodies and small molecules are available to inhibit VEGF signaling (Yano et al. 2000). Notably, VEGF inhibition is also utilized clinically to control cerebral edema, particularly in patients in whom steroids fail to be effective. Thus, VEGF inhibition may have steroid-sparing effects that may again help to augment the efficacy of immunotherapy in patients with aggressive brain metastases.

Conclusions

There has been tremendous progress in the management of melanoma. While brain metastasis remains an all too common problem in this disease, a new era has dawned in which the understanding and treatments of these tumors has improved dramatically. However, significant work remains to be done.

Research has identified multiple molecules and pathways that contribute to the pathogenesis of MBMs. Additional investigation is needed to improve our understanding of the global status of these tumors, to parallel work conducted over the last several years in extracranial tumors and particularly to compare brain metastases to primary tumors and extracranial metastases from the same patients. Notably, there is also an important need to understand how the molecular and immune features of MBMs relate to prognosis and therapeutic resistance in the current era. While multiple studies have shed insight into the selective pressures current therapies exert on extracranial tumors, currently very little is known about the features and/or changes that occur in brain tumors that cause resistance to immune and targeted therapies. Importantly, as oncology increasingly evolves toward defining cancer patients and

their tumors based on molecular and/or immune features, a key challenge in MBM patients will be to define key features of these tumors non-invasively, as obtaining biopsies of intracranial lesions is difficult and frequently risky.

Similarly, the new therapeutic landscape of melanoma is providing new hope to patients, with marked improvements in outcomes being achieved with SRS, targeted therapy, and immunotherapy (Table 2). While each modality has demonstrated impressive results, a key question is whether combining these different therapeutic modalities together will improve outcomes further, or whether they should be used in specific sequences. As research has also identified multiple pathways that are activated in MBMs that are potential therapeutic targets, there are now also opportunities to utilize new agents as additional approaches to further improve outcomes. Another key challenge is to make new therapies more accessible to MBM patients. Historically, patients with brain metastases were often excluded from clinical trials, including all of the registration trials for currently approved immune and targeted therapies for this disease. However, the results of clinical trials conducted in the last several years have demonstrated the feasibility of clinical investigations in this population. Clinical investigators need to continue to support the development of clinical trials for this population with new and emerging agents and particularly investigations into their combinatorial effects with other relevant treatment modalities.

Finally, as noted at the beginning of this chapter, brain metastasis is an important and common problem in multiple other cancers, particularly lung and breast cancer. As progress is made in the understanding and treatment of brain metastases from melanoma, an open question is whether the same pathways and approaches are relevant to brain metastases from other tumor types. Similarly, it will be important to determine if features and strategies identified in other cancers can accelerate progress for MBM patients. Ultimately, a broad and comprehensive approach that integrates basic science, multiple treatment modalities, and investigations across cancer types may be the most effective way to overcome this daunting challenge and further improve outcomes.

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Imaging Melanoma

27

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Abstract

The role of noninvasive imaging in melanoma differs depending on the clinical circumstance. At diagnosis, no or very limited systemic imaging for tumor staging is performed if patients have no clinical evidence of metastatic disease to lymph nodes or systemically. However, imaging is increasingly used to guide radionuclide sentinel lymph node biopsy procedures, especially if complex drainage routes are expected such as in the head and neck where SPECT/CT can be valuable.

Ultrasound can be used to assess and follow regional lymph nodes in patients in whom sentinel node biopsy is not successful or not performed. In higher-risk patients if there is tumor involvement in lymph nodes at sentinel node imaging, or clinically, more extensive whole-body imaging including CT, FDG PET/CT, and MRI of the brain are commonly performed. Surveillance with these methods at regular intervals is recommended by several groups for several years post diagnosis, though guidance varies and is based on the risk of recurrence.

Imaging, especially with PET/CT, is often used to assess suitability for surgical resection. Treatment response assessments by imaging are performed at more frequent intervals related to the timing of the specific therapy. Special attention in imaging patients treated with immune checkpoint inhibitors is necessary. CT, MRI, and PET/CT can have pseudo-progression, where responding tumors can transiently grow in size and metabolic activity, as well as apparent number, before response occurs. A delay of 1–2 months before repeat imaging to confirm progression or response is often needed. Use of imaging in melanoma still requires careful assessment for appropriateness to assure avoidance of overuse and attendant radiation and economic costs. Noninvasive imaging is a crucial part of the management of melanoma at multiple time points across the history of the illness. As new therapies evolve, our understanding of the best imaging methods and timing of imaging will evolve as well.

Keywords

Imaging · PET/CT · Sentinel-node · FDG surveillance · MRI

Overall Rationale for Imaging

Melanoma is increasing in frequency across the world. While totally curable if diagnosed early, melanoma is highly lethal if systemically disseminated. Melanoma commonly spreads first to regional lymph nodes, then systemically. In general, if there is a very low likelihood of spread to regional lymph nodes (primary tumor <0.75 mm) and negative clinical nodes, no sentinel node assessment is performed and no systemic imaging studies are undertaken. However, in intermediate and thicker melanomas, assessment of the regional draining lymph nodes for tumor involvement for prognostic purposes is important. Even in these patients, only about 20% have nodal metastases. Patients with tumor involvement in the lymph nodes are much more likely to have systemic metastases and to die from melanoma than patients who do not have lymph node involvement with tumor.

Diagnostic lymph node dissections have been replaced with sentinel lymph node diagnostic procedures in most clinical settings. The sentinel node procedures provide important prognostic information and appear to have modest therapeutic benefit in selected patient groups when coupled

with lymph node dissections in patients with positive SLNs (Morton et al. 2014).

Sentinel node sampling can be informed by imaging. If there is evidence of disease in lymph nodes, especially of any substantial tumor burden, whole-body imaging is more commonly performed to identify metastatic disease. If there is measurable disease and the patient is undergoing surgical or medical treatment, imaging is used to assess the completeness of resection as well as response to therapy. Imaging to assess treatment response provides evidence of efficacy/inefficacy of therapy before it is continued or changed.

In patients at high risk of tumor recurrence, whole-body (or nodal) imaging is performed periodically as surveillance, as there is emerging evidence that resection of low tumor volume disease is more effective than if high tumor volume is resected. In addition, low tumor volumes appear to be more responsive to therapies such as immunotherapies than higher tumor volumes. The case for aggressive tumor surveillance is made more robust the higher the risk of tumor recurrence, and the more effective therapies become. With the rapid change in melanoma therapies, more imaging is necessary, but therapies have evolved so rapidly that precise guidance on when imaging is most necessary continues to evolve, especially with checkpoint inhibitor therapy.

Imaging Methods

There is a wide range of noninvasive imaging tests available for patients with the diagnosis of melanoma. Their use is detailed in the individual sections of this chapter. Some of the methods involve ionizing radiation (CT, PET/CT), while others do not (e.g., ultrasound, MRI).

Chest X-Ray

The PA and lateral chest radiograph is a mainstay of imaging in humans. Chest radiographs are typically done in the PA and lateral views and are acquired in most instances using digital techniques. While the chest X-ray is relatively inexpensive and of rather low radiation dose, it is a relatively insensitive tool for finding small pulmonary lesions and can fail to detect small malignant pulmonary nodules.

CT Scan

The CT scan is an anatomic imaging tool which detects masses and lesions that differ in X-ray absorption from surrounding normal tissues. CT scans typically have higher radiation doses than X-rays and cost more, but with modern digital detectors, iterative reconstruction algorithms, and low energy CT beams, the radiation dose from CT scans has been decreased versus that used previously. CT scan detection of melanoma can be enhanced in some instances with the use of IV contrast. For pulmonary nodule diagnosis, non-contrast, thin-section, breath-hold CT is a superior diagnostic technique to a chest radiograph and is also superior to PET imaging alone for detecting small pulmonary nodules. It is the preferred method for evaluating the presence/absence of lung lesions.

MRI Scanning

MRI takes advantage of differences in magnetic susceptibility among tissues to identify tumors. MRI can be improved in sensitivity, especially in the brain, by the use of IV contrast media which alter MRI contrast and lesion detectability that is dependent on the permeability of the blood-brain barrier. MRI scans are less sensitive than CT for identifying lung lesions but may be somewhat superior to CT for identifying liver lesions. MRI imaging takes a fairly long period of time, often an hour, and is relatively expensive. It is not optimally suited to evaluations of the entire body. MRI is the preferred technique for identifying brain metastases. A variety of pulse sequences can be used, but typically T1-weighted, T2-weighed, and diffusion-weighted images are

obtained, including some sequences before and after IV contrast.

Ultrasound

Ultrasound depends on the reflected characteristics of emitted sound waves which are detected by a transducer array system. These systems can have high frequency and very high resolution. While ultrasound can assess many areas of the body, it is degraded by bone and in very large patients. It is well suited to evaluate the size of small lymph nodes and to determine if they have changed in size. Ultrasound can be done in real time and can direct biopsies. This method is quite user dependent, but in expert hands it can identify suspicious nodal lesions 5 mm in size and sometimes smaller, for biopsy. While ultrasound contrast agents have recently been approved, they have not yet been systematically evaluated in the diagnosis of melanoma. Ultrasound tends to be used more in countries where there is less access to CT, MRI, or PET imaging, but tends to have its major niche in evaluating nodal groups, especially those patients who cannot undergo, or did not undergo, sentinel lymph node sampling.

A specialized research use of ultrasound, which is in early clinical evaluations, is photoacoustic imaging, in which a bright light source is applied to a structure with either intrinsic or dye contrast being excited and emits sound waves. These sound waves can be detected as so-called photoacoustic imaging. Recently, elastography has been explored as an adjunct to purely anatomic imaging, as size alone does not determine the presence/absence of cancer in nodes (Uematsu et al. 2013).

PET/CT

Positron emission tomography (PET) has substantially been replaced by PET/CT and, to a lesser extent, PET/MRI imaging in melanoma. The PET method in melanoma typically involves taking advantage of the typically very high glucose metabolism in melanoma. Early studies by Gritters et al. showed that FDG PET can detect a substantial number of tumor lesions not detected by anatomic imaging methods alone (Gritters et al. 1993).

A cyclotron-produced positron emitting radioactive glucose analog, ^{18}F-Fluoro-2-deoxy-D-glucose, is given intravenously. About 1 h later, melanomas have selectively accumulated the FDG versus most normal tissues. This increased FDG uptake can be detected by a combined PET/CT scanner which can give high-resolution CT and PET images and display hybrid or individual images. PET/CT is the single most powerful tool for detecting metastatic melanoma, but is limited in that the PET scan often fails to detect tumors under 5–6 mm in diameter. Small pulmonary metastases are better detected on CT (especially if there is a breath hold). If background tracer activity is high, such as in the brain, metastases can fail to be detected by PET, hence the need for MRI to detect brain metastases.

While other PET tracers than FDG have been investigated, FDG is the preferred radiotracer for melanoma detection in most instances. In general, a PET/CT scan requires close to 2 h of patient time, 1 h for tracer uptake, and 20–40 min for imaging. These scans must be done in fasted patients as high serum glucose levels can interfere with scan quality. A variety of other radiotracers have been explored in PET imaging of melanoma, but so far are not compellingly superior to FDG (Ren et al. 2010).

Single-Photon Imaging and Single-Photon Emission Computed Tomography/CT (SPECT/CT)

Similar to PET imaging, when radionuclides are injected, images can be obtained by a gamma camera. This gamma camera can be stationary or can move around the patient and provide multiple image views that can be reconstructed to 3D images (SPECT). Recently, like PET/CT, SPECT has been combined with CT to produce SPECT/CT images, which allow the display of the radionuclide images on the anatomic background of a CT scan.

For many years, the radionuclide bone scan was the main method for detecting metastatic disease to bone. While still available, it has been substantially replaced by the PET/CT scan with FDG, which appears to adequately assess bone metastases.

A more commonly deployed single-photon imaging method is in the form of lymphoscintigraphy, typically performed in the context of sentinel lymph node imaging with a radioactive tracer that is often a radiocolloid (Scarsbrook et al. 2007). The lymphoscintigraphic images can follow the lymphatic channels and identify lymph nodes at risk of having metastases. Since the lymph node metastases are often very small, e.g., 1 or 2 mm in size, the imaging tool only identifies the flow and uptake to the nodes and not the presence/absence of nodal metastases. This approach with imaging is variably practiced.

In situations in which nodal drainage may be expected to go to many sites, such as a vertex lesion of the scalp or a mid-back lesion, the imaging study can be very informative in identifying the at-risk nodal basins (e.g., one or both axilla, left/right neck nodes, axilla or groin, etc.). In some institutions, marking the nodes with an external marker is used to help direct the surgeon to the highest yield location for SLN identification/resection.

In the past several years, there has been more and more interest in 3D identification of the location of sentinel nodes in the neck to minimize surgical morbidity and shorten procedures. The use of SPECT/CT has helped in this domain (Fig. 1).

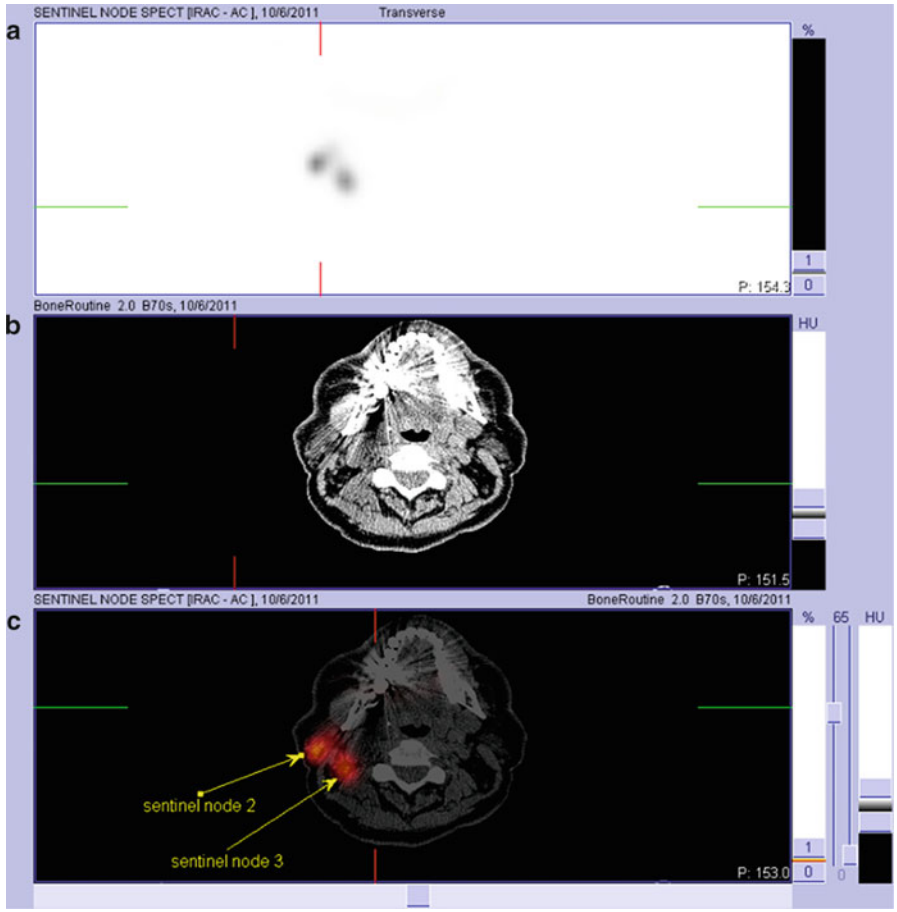

Fig. 1 Transverse SPECT/CT imaging of lymph nodes identified as sentinel nodes on ^{99m}Tc-sulfur colloid SPECT/CT imaging. Two nodes are identified

The radiotracer imaging agent used for lymphoscintigraphy differs by location in the world and regional approvals by regulatory agencies. ^{99m}Tc-tilmanocept, which binds to CD206-positive cells in lymph nodes, has been approved in the USA. Filtered and unfiltered Tc-sulfur colloid is often used in the USA as well (Sondak et al. 2013). In the rest of the world, agents such as albumin microcolloids and antimony ^{99m}Tc colloids have been used, as well as ^{99m}Tc-human serum albumin. In general, the larger the particle size, the less migration there is from the injection site and the greater the retention is in the proximal nodal group (the true SLN).

Since most studies have shown that blue dye alone shows fewer SLN than radionuclide studies, it is common in many locales to use a dye, such as blue dye, radioactive imaging (lymphoscintigraphy), and a radioactive probe detector (or intraoperative gamma camera) to identify nodal groups for resection as sentinel nodes (Niebling et al. 2016). There has also been recent growth in using combined approaches such as an indocyanine green dye mixed with ^{99m}Tc-albumin microcolloid, to allow photoacoustic, fluorescent, and radionuclide imaging of nodes (Fig. 2). Virtually all of the light-based methods deteriorate in the presence of obesity, so radionuclide imaging remains relevant for SLN procedures. It is notable, however, than in some situations, that imaging adds little beyond what is possible simply using a radionuclide probe system.

Radiation Dose

It would be ideal if all imaging of melanoma could be done with non-ionizing radiation. This is not the case, at present, but careful attention to radiation dose is essential. PET/CT and CT are the two highest systemic radiation dose methods. Sentinel node imaging delivers a high radiation dose to the injected skin and to the nodes, but the skin and nodes are most commonly removed surgically, limiting the relevance of this local dose. There have been major efforts to drive down radiation dose through the use of more sensitive PET scanners, through PET/MRI, and by more dose-efficient CT scanners. Despite this, caution is in order if many repeated scans are to be performed, especially in the setting of surveillance and in younger patients. It is, for example, perhaps reasonable to limit evaluation of the pelvis in a patient who has had a head and neck melanoma, as the risk of metastases to the pelvis is low.

Emerging Methods

Optical and photoacoustic methods as well as contrast-enhanced ultrasound are of potential interest at least in the setting of defining the extent of regional disease. It is too early to recommend these techniques for routine approaches, but as our database grows, they may be more appropriate for broader dissemination (Stoffels et al. 2015). The nonradioactive indocyaninine (ICG) dye method is showing promise versus a blue dye optical technique (Korn et al. 2014). There are

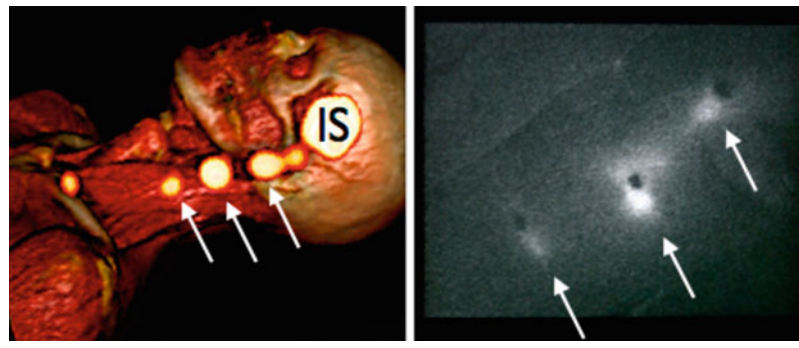

Fig. 2 Lateral view of neck showing both ^{99m}Tc-albumin colloid and ICG photoacoustic imaging of the neck showing sentinel nodes (van den Berg et al. 2015 reproduced from Radiology)

imaging methods under investigation, specifically for pigmented suspicious skin lesions, which are in evolution and include confocal microscopy, spectroscopic imaging, and combined methodology approaches (Smith and Macneil 2011; Wang and Hashemi 2010).

Imaging for Regional Metastases

The sentinel node procedure is considered the reference standard for N staging for melanoma and can be applied when there is no clinical evidence of regional nodal metastasis. This procedure is commonly performed for primary melanomas of >0.75 mm in thickness, but its use and precise method of deployment is variable. There are some controversies, addressed elsewhere, over precisely which patients need a SLN procedure, on the need for complete node dissection post positive SLN, and whether the procedure is truly beneficial to survival (Cordeiro et al. 2016; Madu et al. 2017). The procedure clearly provides prognostic information. Sentinel node procedures are performed in various ways, some of which include no imaging beforehand, but which follow an intraoperative injection of a dye or which follow the injection of a radiocolloid before surgery. In the latter instance, imaging is commonly performed with a gamma camera before the surgery. In many centers, the location of the sentinel node(s) is marked to assist in expediting the surgical procedure to remove the sentinel node.

In some instances, if there is clinical suspicion of metastatic adenopathy, ultrasound of the nodal basin with fine-needle aspiration (FNA) or biopsy of suspiciously enlarged nodes can be performed. There is then the possibility of proceeding directly to formal regional lymphadenectomy. In the case of palpable nodes, FDG PET imaging has been shown to be reasonably reliable in determining if the nodal basin is involved with tumor, but tissue is essential to confirm the presence of metastatic disease (Macfarlane et al. 1998). In the event of a negative sentinel lymph node biopsy, the patient is considered to have clinical stage I or II disease. No systemic imaging is indicated for these patients.

Increasing evidence suggests early diagnosis and resection of tumor-involved sentinel nodes is of value both in reducing local recurrence rates and, seemingly, in increasing survival of patients with intermediate-thickness melanomas versus a delayed observation strategy. While the differences in survival are modest, they might be expected to increase as newer therapies of melanoma are being deployed such as checkpoint inhibitors, including those for lower-volume stage III disease (Balch and Gershenwald 2014; Morton et al. 2014).

All imaging methods, including US, CT, and PET-CT, have limited utility for the early stages of melanoma, with very low diagnostic yield and a burden of false-positive results leading to unnecessary workup. Positive sentinel lymph node biopsy indicates clinical stage III disease. The next step in management usually includes complete formal regional lymphadenectomy.

Newly diagnosed low metastatic burden, i.e., stage IIIA, disease may or may not require systemic imaging. While yields from systemic imaging are low, failing to detect systemic metastatic disease may be problematic as it relates to delayed care and increased morbidity. Conversely, clinically-evident stage III disease requires baseline imaging to detect the possibility of clinically occult stage IV disease. This is of particular importance if the disease is considered to be resectable or amenable to other locoregional treatment, such as isolated limb infusion with chemotherapy or more radical surgical excision (Patnana et al. 2011).

Some groups have evaluated less invasive methods of lymph node evaluation, such as ultrasound. This tool can perform well in experienced hands. However, SLN biopsy is typically well tolerated, especially in comparison to lymph node excision; it contributes little additional morbidity to wide local excision, though it is resource consumptive. At present, an experienced sonographer is able to identify only those SLN metastases that have reached a minimum diameter of approximately 4–5 mm, which represents a significant burden of disease that itself is associated

with a relatively high rate of future distant disease. False-positive enlarged nodes are common as well. It is important to contrast this minimum threshold for detection to the experience of (Gershenwald and Ross) who indicated the median largest SLN metastatic focus was only 1 mm for patients who had a positive SLN biopsy and fewer than one in five patients had a largest SLN metastatic focus >4 mm.

Such limited sensitivity in detecting SLN disease argues against the routine use of sonography as the sole surveillance tool for early detection of clinically occult regional nodal disease – assuming detecting early metastases to lymph nodes is important prognostically (Ross and Gershenwald 2013). Several groups have used SPECT/CT imaging to detect and locate sentinel lymph nodes in patients with melanoma.

In 35 patients with a primary melanoma who were scheduled for wide local excision and sentinel node biopsy, conventional lymphoscintigraphy and subsequently SPECT/CT were performed. SPECT/CT depicted the same 69 sentinel nodes detected through conventional lymphoscintigraphy in the 35 patients, and identified 8 additional sentinel nodes in 7 patients (20%). In two of these patients (5.7%), an additional nodal basin had to be explored to find the extra sentinel nodes. SPECT/CT provided additional anatomic information that was helpful to the surgeon in 11 patients (31%), which led to an adjustment of the surgical approach in 10 patients (29%). Overall, SPECT/CT provided relevant additional information in 16 (46%) of the 35 patients (Veenstra et al. 2012).

In head and neck nodal staging in 20 patients, SPECT/CT was compared to planar imaging. SPECT/CT depicted an additional sentinel node in 16% of the patients and clearly showed the anatomic location of the hot nodes in all patients. The surgical approach was adjusted on the basis of SPECT/CT images in 11 patients (55%) (Vermeeren et al. 2011). In a small series, SPECT/CT lymphoscintigraphy of the head and neck resulted in a change in surgical approach in over 60% of cases (Lopez-Rodriguez et al. 2016).

Zender et al. studied 14 patients with melanoma who had sentinel nodes located near the parotid glands, and lymphatic drainage to the parotid region was suspected. They had received lymphoscintigraphy followed by SPECT/CT prior to surgical excision and SLN biopsy. Retrospective analyses showed SPECT/CT provided data which changed management in 57% of patients. The authors concluded the distinction between level II and parotid sentinel lymph nodes which was clearly identified through SPECT/CT images (Zender et al. 2014).

Stoffel et al. evaluated metastatic node detection and disease-free survival using single-photon emission computed tomography/computed tomography (SPECT/CT)-aided sentinel lymph node excision (SLNE) versus standard SLNE in patients with melanoma. A total of 403 patients with clinically-negative lymph nodes, who underwent SLNE with or without preoperative SPECT/CT, qualified for subsequent analysis. Two hundred fifty-four patients underwent the standard SLNE technique. Subsequently 149 patients underwent the SPECT/CT technique.

Using SPECT/CT allowed SLNE in the head and neck area more frequently (2.0% for standard versus 23.5% for SPECT/CT; difference, 21.1%; 95% CI, 14.1%–28.2%; $P < 0.001$). In the SPECT/CT cohort, more sentinel lymph nodes per patient were detected than in the standard cohort (2.40 vs. 1.87; 95% CI, 1.93–2.18; $P < 0.001$). The number of positive sentinel lymph nodes per patient was significantly higher in the SPECT/CT cohort than in the standard cohort (0.34 vs. 0.21; 95% CI, 0.21–0.31; $P = 0.04$). The local relapse rate in the SPECT/CT cohort was lower than in the standard cohort (6.8% vs. 23.8%, $P = 0.03$), with prolonged 4-year disease-free survival (93.9% vs. 79.2%; $P = 0.02$).

The authors concluded that in patients with clinically lymph node-negative melanoma, the use of SPECT/CT-aided SLNE compared with SLNE alone was associated with a higher frequency of metastatic involvement and a higher rate of disease-free survival (Stoffels JAMA (Stoffels et al. 2014)).

These same patients were studied for cost-effectiveness of the SLN procedure augmented or not augmented by SPECT/CT imaging. There

was a mean cost saving of <euro> 710.50 when SPECT/CT was added to preoperative imaging. This was achieved by a reduction in operative time (median 40 min vs. 45 min; $p = 0.002$), hospital stay duration (5 days vs. 8 days; $p < 0.001$), and more frequent use of local anesthesia (90.6% vs. 70.5%; $p < 0.001$). The median cost of SLNE using SPECT/CT was <euro> 1,619.7 and of SLNE without SPECT/CT was <euro> 2,330.2), a cost saving of 30.5%.

These authors concluded that in patients with cutaneous melanoma, the use of preoperative SPECT/CT-aided SLNE compared with standard SLNE was associated not only with higher detection of metastatic involvement but also with a significant cost reduction. Whether these results will extend to the USA and other systems is uncertain, as the duration of the inpatient stay is substantially longer than the stays typical in the USA, especially with some of the procedures being done as fully outpatient procedures in the USA (Stoffels et al. 2012, 2014).

From these limited new data, if the technology is available, routine use of SPECT/CT in addition to conventional lymphoscintigraphy is recommended in melanoma patients undergoing lymphatic mapping. This is especially true in the head and neck or other areas where the anatomy may be complex and surgical risks more substantial. Some authors have suggested that the algorithm for imaging for lymphoscintigraphy, even for relatively straightforward indications for sentinel node location (like extremities) aberrant basins, including epitrochlear, popliteal, intermuscular, retroperitoneal and in transit nodes are seen best with imaging, especially SPECT/CT as a complement to the sentinel node detection.

This recent improvement in imaging is leading to a new paradigm of "see and open" in contraposition to the former "open and see" in the SN procedure. The new mixed reality protocols which are able to transfer SPECT/CT to the operating room for surgical navigation will reinforce this "see and open" concept (Valdes Olmos et al. 2014).

It should be noted that there are continuing advancements in surgical navigation in the operating room. While "hot nodes" have been typically detected with a radiation-sensitive probe system, 3D navigation systems have been developed. For example, a "free hand SPECT" system has been developed for the operating room. This system has been piloted in a small number of centers, but is not yet the widespread standard of care. The approach appears at least as good as planar imaging and may be superior to an intraoperative probe system. One disadvantage of these systems is that the time for acquisition of images may be longer than those to obtain radioactive probe counts (Mihaljevic et al. 2014). This area of technology is advancing rapidly.

FDG PET for Evaluating Sentinel Nodes

There was initially great enthusiasm for FDG PET imaging of regional nodal metastases (Gritters et al. 1993; Macfarlane et al. 1998). The early studies were performed in patients with palpable lymph nodes, and in such settings lesion detection was quite accurate. However, larger studies have shown FDG PET can miss many small nodal lesions in patients with melanoma; hence the use of FDG PET cannot be recommended as a tool for nodal staging in the clinically N0 nodal basins, in contrast to the SLN procedures discussed in this section (Friedman and Wahl 2004; Perng et al. 2015).

Wagner et al. and Crippa reported that the sensitivity of FDG-PET for detection of metastatic melanoma in lymph nodes depends on sufficient tumor volume. FDG-PET begins to reliably detect metastatic tumor in lymph nodes at volumes greater than approximately 80 mm^3, but sensitivity falls rapidly below this.

The experience of Singh et al. is representative (Singh et al. 2008). They evaluated the role of preoperative ^{18}F-fluorodeoxyglucose-positron emission tomography/computed tomography (FDG/PET-CT) scanning, preoperative lymphoscintigraphy (LS), and sentinel lymph node biopsy in 52 patients with malignant melanoma. None of the study patients had clinical or radiological evidence of regional lymph node metastatic disease. At least one sentinel node was

identified in all patients. Preoperative LS detected a total of 111 sentinel lymph nodes (average 2.13 sentinel lymph node per patient) and demonstrated a single nodal draining basin in 38 patients (73%) and multiple (two to three draining basins) in the remaining 14 patients (27%). Fourteen out of the 52 patients (27%) had at least one involved sentinel node. Positron emission tomography showed true positive in two patients with a sentinel node greater than 1 cm and false positive in two other patients. In this study, the detection of sentinel lymph node by LS and gamma probe had a sensitivity of 100%. In contrast, ^{18}F-FDG-PET imaging demonstrated very low sensitivity (14.3%; 95% CI, 2.5–44%) and positive predictive value (50%; 95% CI, 9–90%) for localizing the subclinical nodal metastases.

They concluded that in patients with non-palpable nodes, FDG PET/CT is not able to replace LS/sentinel lymph node biopsy in patients at stage I or II (Singh et al. 2008). While the Singh study is small and the positive predictive value of PET is lower than in some other series, this study clearly indicates the challenge of using PET to noninvasively assess palpably normal lymph node basins.

Ultrasound for Imaging Sentinel Nodes

Ultrasound can be used to assess regional lymph nodes and guide nodal biopsies. It is not a substitute for sentinel node imaging or sampling, but can be a useful adjunct. It has a particular role in patients in whom sentinel node imaging is not successful or if patients refuse to have a sentinel node sampling procedure performed. In such settings, sequential ultrasound imaging may be very helpful in identifying interval nodal growth which may indicate early growth of tumors in lymph nodes and suggest the need for biopsy.

Xing conducted a meta-analysis of imaging methods for locoregional nodal disease and systemic disease imaging methods including ultrasonography, computed tomography (CT), positron emission tomography (PET), and a combination of both PET and CT for the staging and surveillance of melanoma patients (Xing et al. 2011).

Patient-level data from 74 studies containing 10,528 patients (between January 1, 1990, and June, 30, 2009) were used to derive characteristics of the diagnostic tests used. Among the four imaging methods examined for the staging of regional lymph nodes, ultrasonography had the highest sensitivity (60%, 95% CrI = 33–83%), specificity (97%, 95% CrI = 88–99%), and diagnostic odds ratio (42, 95% CrI = 8.08–249.8). For staging of distant metastases, PET-CT had the highest sensitivity (80%, 95% CrI = 53–93%), specificity (87%, 95% CrI = 54–97%), and diagnostic odds ratio (25, 95% CrI = 3.58–198.7). Similar trends were observed for melanoma surveillance of lymph node involvement, with ultrasonography having the highest sensitivity (96%, 95% CrI = 85–99%), specificity (99%, 95% CrI = 95–100%), and diagnostic odds ratio (1675, 95% CrI = 226.6–15,920).

Positive predictive values were likewise highest for ultrasonography in lymph node staging and for PET-CT in detecting distant metastases. Among the compared modalities, ultrasonography was superior for detecting lymph node metastases, and PET-CT was superior for the detection of distant metastases in both the staging and surveillance of melanoma patients.

Because US is less sensitive than SLN imaging, SLN procedures will provide greater sensitivity and specificity, though at the price of an invasive procedure and considerable expense. In some settings US is used instead of SLN, with SLN reserved for cases where there is nodal growth on US surveillance (Xing et al. 2011; Rueth et al. 2014; Trotter et al. 2013).

Emerging Imaging Approaches to Identify and Characterize the Sentinel Node

A combined imaging agent with -^{99m}Tc and indocyanine as well as a separate blue dye injection were prospectively compared in 104 patients with newly diagnosed melanoma to determine efficacy in detecting sentinel lymph nodes. Following intradermal hybrid tracer administration, lymphoscintigraphy and single-photon emission

computed tomography/computed tomography were performed. Blue dye was intradermally injected prior to the start of the surgical operation (excluding patients with a facial melanoma). Intraoperatively, SNs were initially pursued by using gamma tracing followed by fluorescence imaging (FI) and, when applicable, blue dye detection. A portable gamma camera was used to confirm SN removal. Collected data included number and location of the preoperatively and intraoperatively identified SNs and the intraoperative number of SNs that were radioactive, fluorescent, and blue.

Preoperative imaging revealed 2.4 SNs (range, 1–6) per patient. Intraoperatively, 93.8% (286 of 305) of the SNs were radioactive, 96.7% (295 of 305) of the SNs were fluorescent, while only 61.7% (116 of 188) of the SNs stained blue ($P < 0.0001$). Fluorescent imaging (FI) was of value for identification of near-injection-site SNs (two patients), SNs located in complex anatomic areas (head and neck [28 patients]), and SNs that failed to accumulate blue dye (19 patients). This study is intriguing, and while the imaging agent is not FDA approved in the USA, it illustrates that radioactive and optical dyes may be complementary to one another, with the combination clearly superior to using only the blue dye optical approaches for SLN visualization (van den Berg et al. 2015).

In a combined in vitro and in vivo study, Stoffels et al. were able, using multispectral optoacoustic imaging (MSOT), to detect melanin content in excised sentinel nodes from melanoma patients. They relied on different spectral characteristics for melanin versus other nodal tissues. MSOT significantly improved the tumor metastasis detection rate in excised SLN (506 SLNs from 214 melanoma patients) compared with the conventional EORTC (European Organisation for Research and Treatment of Cancer) Melanoma Group in vitro protocol (22.9% vs. 14.2%). MSOT identified cancer-free SLNs in vivo and ex vivo without a single false negative (189 total lymph nodes), with 100% sensitivity and 48–62% specificity, suggesting this approach may have a role in vitro and possibly in vivo in assessing SLNs noninvasively. MSOT combined with the near-infrared fluorophore indocyanine green reliably visualized SLNs in vivo in 20 patients, up to 5 cm depth of visualization penetration and with 100% concordance with ^{99m}Tc-marked SLN lymphoscintigraphy (Stoffels et al. 2015).

Photoacoustic imaging and optoacoustic imaging are promising tools for imaging small nodal metastases, at least in animal models. Preliminary data suggest this tool may be superior to FDG PET/CT (Neuschmelting et al. 2016).

Imaging for Initial Staging and Follow-Up

While 80% of melanoma patients have no nodal metastases at presentation, 10–15% do have nodal metastases, and up to 5% of patients have systemic metastases. In general, stage I and II patients do not need systemic staging. But, many stage III and suspected stage IV patients will typically have systemic imaging. If there are palpable lymph nodes, biopsy-proven sentinel lymph nodes involved with cancer, an elevated LDH, an elevated serum S100 protein, a high mitotic rate, tumor ulceration, and/or a deep primary tumor, the probability of systemic metastases at staging increases.

The NCCN guidelines offer flexibility in terms of which patients should have systemic staging procedures. Staging guidelines differ substantially by country (Trotter et al. 2013). At some point, the yield of whole-body staging becomes sufficiently low that it is not cost-effective to undertake the requisite imaging.

Since FDG PET was introduced for imaging areas of the whole body in 1993 and showed superior performance to CT imaging, many studies have shown the superiority of FDG PET/CT to CT and other methods in detecting most visceral metastases of melanoma (Gritters et al. 1993).

Niebling et al. examined the prognostic value of staging in FDG-PET and CT-negative or FDG-PET and CT-positive melanoma patients to assess which factors have an independent prognostic impact on survival of these patients. Patients with palpable and histologically or cytologically proven LNM of melanoma, referred to

participating hospitals for examination with FDG-PET and CT, were selected from a previous study. For all 252 patients selected, 5-year melanoma-specific survival was 38.2%. For FDG-PET and CT-negative and FDG-PET and CT-positive patients, 5-year MSS was 47.6 and 16.9%, respectively.

The disease-free period for FDG-PET and CT-negative patients was 46.0% after 5 years. Gender, a positive FDG-PET and CT, LNM in the axilla compared to the head or neck, and the presence of extranodal growth were independent factors for worse MSS in all patients. Positive FDG-PET and CT was the most important prognostic factor for MSS with a hazard ratio of 2.54 (95% CI, 1.55–4.17, $P < 0.001$).

These authors concluded that staging melanoma patients with palpable LNM was more accurate when whole-body FDG-PET and CT is added to the diagnostic workup and recommended the tests in the staging of clinical stage III melanoma patients (Niebling et al. 2013).

In a retrospective and blinded study of 250 consecutive patients who underwent FDG-PET/CT for staging of cutaneous melanoma at different time points in the course of disease. Diagnostic accuracy for N- and M-staging was determined for CT alone, PET alone, and PET/CT. PET/CT detected significantly more visceral and non-visceral metastases than PET alone and CT alone (98.7%, 88.8%, and 69.7%, respectively). PET/CT imaging provided significantly more accurate interpretations regarding overall N- and M-staging than PET alone and CT alone. Overall N- and M-stage was correctly determined by PET/CT in 243 of 250 patients (97.2%; 95% CI, 95.2–99.4%) compared with 232 patients (92.8%; 95% CI, 89.6–96.0%) by PET, and 197 patients (78.8%; 95% CI, 73.7–83.9%) by CT. All differences were significant. The accuracy of PET/CT was significantly higher than that of PET and CT for M-staging (0.98 vs. 0.93 and 0.84) and significantly higher than that of CT for N-staging (0.98 vs. 0.86). Change of treatment according to PET/CT findings occurred in 121 patients (48.4%). This high sensitivity is probably biased by including patients with more advanced disease (Reinhardt et al. 2006).

Fuster et al. determined the accuracy of ^{18}F-FDG PET in detecting recurrent melanoma. In 156 patients with confirmed melanoma and recurrence suspected by clinical examination, 184 PET scans were retrospectively reviewed. Histology or clinical follow-up was used for the final diagnosis. They found the sensitivity and specificity of PET for detecting lesions on an individual patient basis were 74% and 86%, respectively, compared with values of 58% and 45% for conventional imaging alone. The overall accuracy for PET was 81%, compared with 52% for other methods. PET was more accurate (91% vs. 67%) than conventional imaging in detecting locoregional disease and distant metastases (85% vs. 55%), and PET results led to a change in the planned clinical management of 36% of patients included in this study.

PET was more accurate than CT in detecting skin lesions, malignant lymph nodes, and metastases to the abdomen, liver, and bone. In the assessment of pulmonary disease, PET showed higher specificity (92% vs. 70%) than CT for the detection of lung parenchyma lesions; however, the sensitivity was better for CT (93%) than for PET (57%). They concluded PET was better than conventional imaging in detecting locoregional disease and distant metastases in all sites except the lung, where it appears to be a useful adjunct to CT (Fuster et al. 2004).

In a meta-analysis of over 10,000 patients, to determine if distant metastases were present, PET-CT had the highest sensitivity (86%, 95% CrI = 76–93%), specificity (91%, 95% CrI = 79–97%), and diagnostic odds ratio (67, 95% CrI = 20.42–229.7) in comparison to CT or ultrasound (Xing et al. 2011).

In a systematic review, Schröer-Günther and colleagues reported finding no randomized clinical trials investigating the patient-relevant benefit of PET(/CT) and no prognostic accuracy studies. Seventeen diagnostic accuracy studies of varying quality were identified. For patients with American Joint Committee on Cancer (AJCC) stages I and II, sensitivity mostly ranged from 0% to 67%, again reflecting the lower sensitivity of PET for nodal metastases, which are often small. Specificity ranged from 77% to 100%. For AJCC stages

III and IV, sensitivity ranged from 68% to 87% and specificity from 92% to 98%.

They concluded that there was currently no evidence of a patient-relevant benefit of PET (/CT) in the primary staging of malignant melanoma and suggested the opportunity for RCTs investigating patient-relevant outcomes. Of course, the lack of RCT evidence does not mean that PET is not valuable, and for nearly 15 years FDG PET/CT has been reimbursed by CMS in the USA (Schroer-Gunther et al. 2012). Rohren has also reviewed the role of FDG PET/CT in tumor staging and outcomes, as the disease evolves (Rohren 2015). Examples of positive FDG PET/CT scans are shown in Figs. 3a–c.

There is clear evidence that FDG PET/CT can meaningfully change management of patients. Gulec et al. reported on 49 patients with known or suspected metastatic melanoma who underwent diagnostic evaluations using computerized tomography (CT) of the chest, abdomen, and pelvis and magnetic resonance imaging (MRI) of the brain (Gulec et al. 2003). After formulation of an initial treatment plan, the patients underwent FDG-PET imaging. Treatment plans pre- and post-PET were compared. The PET scan identified more metastatic sites in 27 of 49 patients (55%). In 6 of those 27 patients, PET-detected disease outside the fields of CT and MRI. Fifty-one lesions were resected surgically. Of these, 44 were pathologically confirmed to be melanoma. The results of PET led to treatment changes in 24 patients (49%). Eighteen of these changes (75%) were surgical. In 12 cases (67%), the planned operative procedure was cancelled, and in 6 cases (33%), an additional operation was performed. In 6 of 24 patients (25%), systemic therapy was prompted by identification of new focus of disease. Significant surgical and medical treatment alterations were made based on the PET results.

It is important to note that FDG PET/CT can fail to detect small lung metastases (on the PET portion of the exam) and brain metastases. Close examination of the CT scans of the thorax (breath hold) and of brain MRI are essential for optimal evaluation.

The NCCN guidelines version 1-2017 offer flexibility on which studies to include in imaging for staging and when to image, but, they say, to include the chest, abdomen, and pelvis with CT and IV contrast and/or PET/CT of the whole body. MRI is required to exclude brain metastases, if suspected.

Friedman et al. stated cumulative data suggests that FDG-PET is the modality of choice for evaluating patients who fit into one of four categories: (1) individuals with a high risk for distant metastases based on the extent of locoregional disease, (2) patients with findings that are suspicious for distant metastases, (3) individuals with known distant tumor deposits who still stand to benefit from customized therapies if new lesions are discovered or treated lesions regress, and (4) patients at high risk for systemic relapse who are considering aggressive medical therapy (Friedman and Wahl 2004).

Despite the overall superiority of FDG-PET in the detection of melanoma metastases, limitations exist with respect to detection of small (Uematsu et al. 2013) lung nodules and brain metastases, which are better evaluated by computed tomography and magnetic resonance imaging, respectively (Friedman and Wahl 2004). In some instances, CT or MRI may be more sensitive for liver metastases than FDG PET/CT (Friedman and Wahl 2004). In some settings with different availability of resources, ultrasound may be a useful tool for evaluating the liver for metastases (Ulrich et al. 2011).

Imaging in Assessment of Treatment Response

Until a few years ago, imaging melanomas for treatment response was not a major part of the use of imaging, as many melanomas did not respond to standard treatments. In the past few years, there has been great progress in melanoma therapy, and imaging has had a growing role to help monitor and guide therapies. In general, PET/CT or CT (or MRI if a brain lesion) is used to follow treatment response. Response criteria such as RECIST 1.1 and PERCIST 1.0 are now

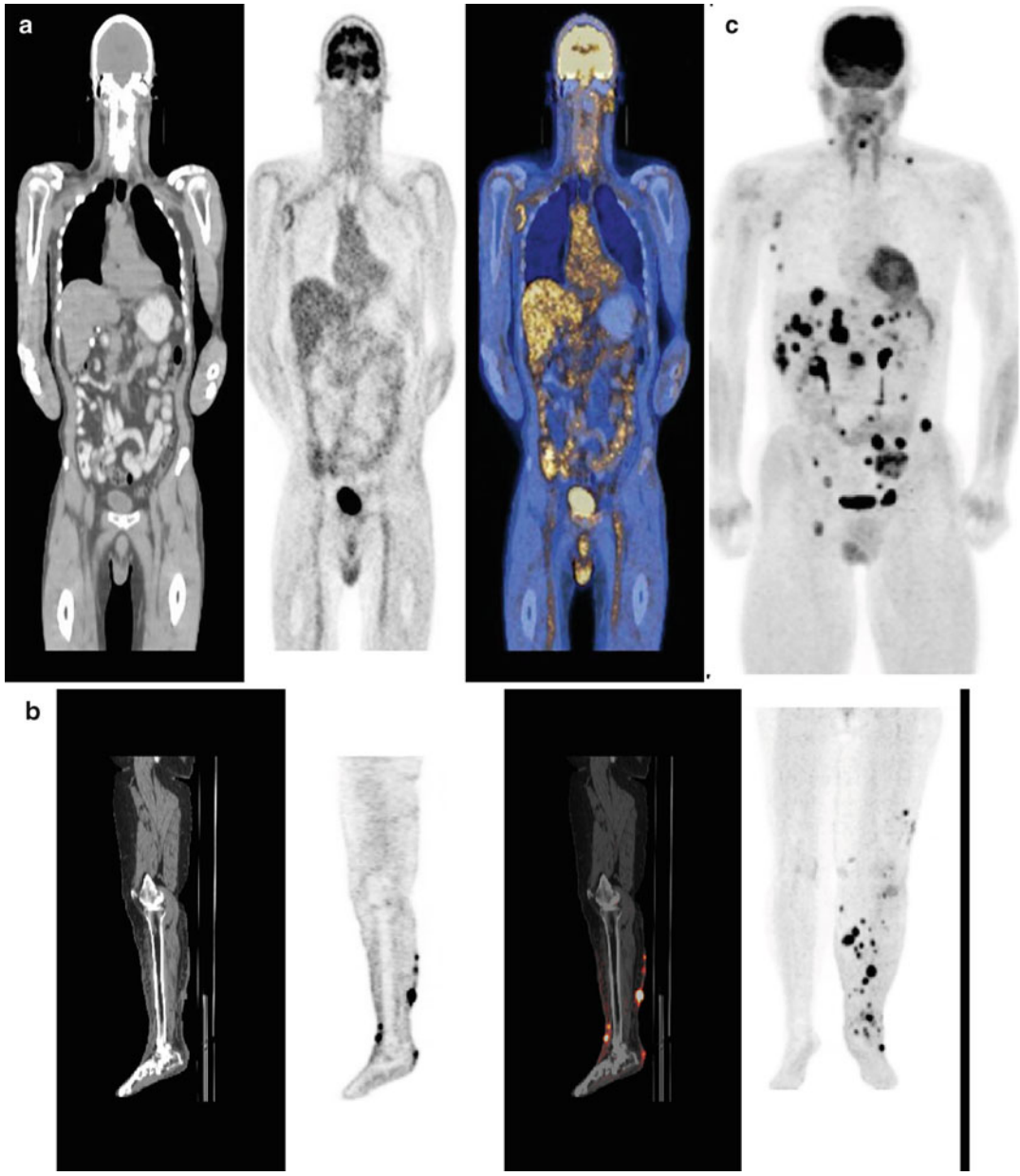

Fig. 3 (**a**) FDG PET whole-body imaging mainly showing intense uptake in cutaneous tumor foci in the left leg. (**b**) FDG PET whole-body imaging showing a suspicious lesion in right axilla, proven to be metastatic melanoma to the axilla. (**c**) FDG PET whole-body imaging showing extensive systemic metastases, including in the liver, in a patient with stage IV melanoma

increasingly applied, at least in clinical trials of therapy (Eisenhauer et al. 2009; Wahl et al. 2009). However, immune responses differ and additional modifications of response criteria have become necessary.

Chemotherapy

For chemotherapy, the reality is most treatments do not work well in melanoma. However, PET has been used extensively in assessment of treatment

response in a wide range of cancer types, and those results directly relate to PET in treatment response. Strobel and Kalff compared ^{18}F-FDG-PET/CT, CT, brain MRI, and the tumor marker S-100B in assessment of the response to chemotherapy for 25 stage IV melanoma patients (Strobel et al. 2008). These tests were obtained at baseline and after 2–3 months (three cycles) of chemotherapy. They then linked these early findings to longer-term outcomes. In patients with a clinical suspicion for brain metastases, MRI or CCT was performed. They found general agreement between FDG-PET/CT and CT regarding response to chemotherapy in all patients. There was a clear trend to a longer overall survival of PET/CT responders ($n = 10$) compared with PET/CT non-responders ($n = 15$; $p = 0.072$) with better 1-year OS of 80% compared to 40% ($p = 0.048$). There was a significant longer PFS of PET/CT responders compared with PET/CT non-responders ($p = 0.002$). S-100B was normal at baseline in 8 of 22 patients where it was available.

Chemotherapy response assessment with S-100B failed to show correlation with OS or PFS. Eleven patients developed brain metastases during treatment, first detected by PET/CT in two and by MRI or CCT in 9 of 11 patients. Appearance of brain metastases was associated with a poor survival. These authors concluded ^{18}F-FDG-PET/CT and CT alone are equally suitable for assessment of response to chemotherapy in melanoma patients and clearly superior to S-100B. PET/CT responders have better early survival, but this is short-lived due to late therapy failure – often with brain recurrence. Additional brain MRI for assessment of response to therapy in such high-risk patients is mandatory to detect brain metastases missed by PET/CT.

In contrast to systemic chemotherapy, regional delivery of chemotherapy with isolated limb perfusion, radiation therapy, and hyperthermia can be of value for regional disease. In a two-center trial, Beasley et al. evaluated 97 patients with stage IIIB/IIIC extremity melanoma undergoing isolated limb infusion (ILI). They had whole-body FDG-PET/CT scans before and every 3 months after treatment. Clinical response was determined at 3 months by Response Evaluation Criteria in Solid Tumors (Beasley et al. 2012; McArthur et al. 2012).

Consistent with the greater efficacy of regional delivery, complete response (CR) after ILI occurred in 33% (32/97) of patients. FDG-PET/CT accurately identified 59% of patients who were CRs (19/32), whereas 41% (13/32) had residual metabolic activity in the extremity that was histologically negative for melanoma. The 3-year disease-free rate was 62.2% for those patients who were CRs by both clinical/pathologic examination and FDG-PET/CT ($n = 19$) compared to only 29.4% of those CRs who still had residual FDG-PET/CT activity ($n = 13$). FDG-PET/CT was utilized for surveillance of disease recurrence outside the regional field of treatment. Fifty-two percent of patients developed disease outside the extremity at a median time of 212 days from pre-ILI FDG-PET/CT. In 47% of these cases, the recurrence was resected.

Although FDG-PET/CT did not appear to accurately identify patients who appear to be CRs to ILI, it appeared to identify a subgroup of patients whose regional progression-free survival was markedly worse. In these patients, FDG-PET/CT was an excellent method for surveillance in stage IIIB/IIIC patients after regional chemotherapy infusion with an ability to identify surgically resectable recurrent disease in these high-risk patients. A challenge in studies using qualitative assessments is that there is relatively little background radiotracer uptake. This means that achieving a "negative" scan versus background can be difficult and qualitative readings of a dichotomous yes/no to increased tracer uptake versus in an extremity are inherently subjective.

BRAF Inhibitors

Inhibitors of driver mutations of cancer are promising therapeutics. These agents have a role in the treatment of melanomas having the relevant mutations. Consistent with results in other tumor types, rapid declines in FDG uptake are seen when these agents are used in patients with sensitive tumors.

McArthur et al. (2012) and Beasley et al. reported on 31 melanoma patients with BRAF mutations. Baseline and day 15 FDG-PET was evaluated mainly at doses expected to be therapeutic with vemurafenib (PLX06-02). In the 27 patients treated at potentially therapeutic levels, at least a partial metabolic response was found in 24, and 3 patients achieved a complete metabolic response. In the 27 patients, there was an 80 ± 3% reduction in the maximum standardized uptake value (SUVmax) of target lesions. There was a positive correlation between %ID in all identified disease and target-lesion SUVmax ($r(2) = 0.66$; $P < 0.001$). This indicated a significant homogeneity of the response between lesions in individual patients.

Although no relationship was found between the reduction in target lesion SUVmax and the best response according to RECIST (Response Evaluation Criteria in Solid Tumors), there was a trend for patients with greater reductions in uptake of FDG to have longer progression-free survival. These data suggest rapid changes in glycolysis, and presumably to some extent tumor viability, are seen with this treatment. These results seem similar to those that have been reported in GIST with FDG and imatinib therapy. It is quite possible, however, that drops in tumor metabolism do not mean the tumors have died.

Immunotherapy

There has been a revolution in the past decade in the use of immune checkpoint inhibitors to treat melanoma such as anti-CTLA-4 and anti-PD-1 agents. These treatments are discussed elsewhere in the text, but it is important to note that some of the responses to these treatments may be much different in their kinetics than responses to chemotherapy. The RECIST criteria were developed mainly based on chemotherapeutic approaches. Given that the biological mechanism of response in immunotherapy differs greatly from that of traditional cytotoxic chemotherapy, these RECIST criteria may not be appropriate for immunotherapy response (Wolchok et al. 2009).

Wolchok et al. described the following four types of responses associated with favorable survival in melanoma patients undergoing therapy with ipilimumab: (1) shrinking the baseline target lesion with no new lesions, (2) durable stability with subsequent a slow gradual decline in tumor burden, (3) an initial increase in tumor burden with subsequent shrinkage, and (4) shrinkage of baseline target lesions with new interval lesions.

They proposed that the four types of responses can be unified into one general category of controlled disease. It is notable that only the first of the proposed criteria would be a response by RECIST and the last two would be progressive disease by RECIST. It is presumed that tumor enlargement seen in patients who do well may be related to infiltration of tumors by reactive activated lymphocytes that increases lesion size.

Nishino et al. have studied immune-related responses by CT quite extensively. In addition to the conventional response pattern of a decrease in tumor burden, irRC describes two additional patterns of immune-related response specific to immunotherapy, including (1) responses after an initial increase in total tumor burden and (2) reduction in total tumor burden during or after the appearance of new lesions at time points later than 12 weeks since the initiation of therapy. These additional patterns of response are likely due to the activation of T cell immunity caused by ipilimumab. In a case study of an ipilimumab-treated patient with apparent increase of tumor burden at 12 weeks of therapy, histologic analyses showed that the increase in lesion size was due to T-cell infiltration rather than tumor cell proliferation [17].To capture these immune-related response patterns, irRC requires confirmation for progressive disease by a repeat consecutive assessment no less than 4 weeks from the first documentation (Nishino et al. 2015).

Brain Tumor Assessments

Pseudoprogression can also occur anatomically in brain tumors. It has been suggested that the anatomic RANO criteria be modified to the I-RANO

criteria (van den Bent et al. 2011; Okada et al. 2015). In patients who have imaging findings that meet RANO criteria for progressive disease within 6 months of starting immunotherapy, including the development of new lesions or confirmation of radiographic progression on follow-up imaging, before defining the patient as non-responsive to treatment, provided that the patient does not have new or substantially worse neurological deficits, longer observation may be required. For example, such patients might be allowed a window of 3 months before confirming disease progression with the scan that first showed initial progressive changes as the new reference scan for comparison with subsequent imaging studies. If RANO criteria for progressive disease are met on the follow-up scan 3 months later, non-responsiveness to treatment should be assumed, and the date of progressive disease should be back-dated to the initial date when it was first identified. This area is under further study and evolving, but indicates caution needs to be in order when it comes to assessing brain tumor response to immunotherapy (Okada et al. 2015).

FDG PET in Immunotherapy Response

Sachpekidis and Haberkorn evaluated FDG PET/CT performed at baseline and after two and four cycles of ipilimumab to predict the final response to ipilimumab (anti-CTLA-4) therapy in 22 patients with unresectable metastatic melanoma (Sachpekidis et al. 2015). Evaluation of the patient response to treatment assessed with PET was based on the 1999 criteria of the European Organization for Research and Treatment of Cancer. After completing treatment, 15 patients were characterized as having progressive metabolic disease (PMD) and five as having stable metabolic disease (SMD), and two patients showed a partial metabolic response (PMR). Early PET/CT performed after two ipilimumab cycles predicted treatment response in 13 of the 15 PMD patients, in five of the five SMD patients and in neither of the two PMR patients. Both patients with PMR showed pseudoprogression after the second cycle and were therefore wrongly classified.

According to the patients' clinical outcome, patients with early PMD had a median PFS of 2.7 months (mean 5.5 months) and patients with early SMD had a median PFS of 6.3 months (mean 7.5 months). The difference in PFS between the two groups was statistically significant (log-rank $p < 0.001$). The median OS among patients with early PMD was 8.8 months (mean 12.0 months) and among those with early SMD 9.8 months (mean 10.0 months). The difference in OS between the two groups was statistically significant (log-rank $p < 0.001$). While the authors concluded (^{18})F-FDG PET/CT after two cycles of ipilimumab was highly predictive of the final treatment outcome in patients with PMD and SMD, it should be noted that the only two PMR patients were misclassified as progression due to pseudoprogression. Thus, FDG has some challenges in this population, though it appears that progression after two cycles on PET is often, though not always, associated with a less favorable outcome.

From these limited data, and preliminary data from Cho (2017) et al., a repeat PET/CT scan after 40.8 weeks more of therapy is suggested to more confidently assess treatment response by PET and to avoid erroneously confusing the favorable response of pseudoprogression with true progression that requires additional treatment.

The challenge of pseudoprogression has been reviewed by Chiou et al. (Chiou and Burotto 2015). They pointed out that 9.7% of patients with melanoma had clinical responses (PR and SD) that would have been misclassified as progression by anatomic criteria with ipilimumab. Similar figures of 4–12% of atypical responses potentially classified as progression (pseudoprogression) were seen in preliminary reports on melanomas treated with anti-PD-1 antibodies (Chiou et al.). There is much more to be learned in this area of study, but it is clear that a small, but not insubstantial, fraction of patients with an ultimately good response to checkpoint inhibitor therapy will have transient tumor "progression" before their good response is apparent.

Imaging the Toxicity of Immunotherapy

In addition to the changes in tumors treated with immunotherapy, there can be immunological side effects from these treatments imaged on anatomic or functional imaging (Bronstein et al. 2011). Bronstein et al. retrospectively reviewed the images and medical records of 119 patients with metastatic melanoma treated with anti-CTLA-4 for the presence of radiologic manifestations of immune-related adverse events and the clinical responses to therapy.

The responses were categorized as progressive or controlled disease. Radiologic manifestations of immune-related adverse events were found in 20 patients (16.8%). Clinically evident manifestations included colitis, hypophysitis, thyroiditis, and arthritis. Clinically silent manifestations were benign lymphadenopathy and inflammatory changes in the soft tissues, such as myositis, fasciitis, and retroperitoneal fat haziness. There was a significant association between the incidence of radiologic manifestations of immune-related adverse events and clinical responses to anti-CTLA-4 therapy.

The disease control rates were 18% for the entire group, 55% for the group with radiologic manifestations, and 10% for the group without radiologic manifestations of immune-related adverse events. In three patients (2.5%), lymphadenopathy related to radiologic manifestations of immune-related adverse events was interpreted as suspected metastasis but was proved benign at biopsy. Radiologic manifestations of immune-related adverse events are associated with significant clinical benefit of anti-CTLA-4 therapy. In the era of developing immune checkpoint-targeted therapy for metastatic melanoma, radiologists should be alert to the possibility of these manifestations, which can mimic radiologic disease progression (Bronstein).

Much less is reported related to FDG PET and toxicity. Wachsman et al. have reported that four patients with immune-mediated side effects were identified among the patients being treated with ipilimumab who underwent ^{18}F-FDG PET-CT for monitoring therapeutic effects (Wachsmann et al. 2017). These immune-mediated side effects include new findings of abnormal increased FDG uptake associated with immune-mediated pancreatitis and hypophysitis, as well as immune-mediated thyroiditis and colitis (Fig. 4). Attention must be paid to both CT and PET/CT for findings that suggest an immunological side effect.

If and When to Image a Patient with Melanoma

Early detection of a melanoma recurrence is a major concern for the clinician. However, the follow-up care of melanoma patients lacks a uniform approach. Different dermatological and oncological organizations have developed their own strategies of follow-up management that vary by specialty and methods of screening for recurrence (Trotter et al). It is important to develop an effective, evidence-based approach to melanoma clinical follow-up care. However, such evidence is impacted by the therapeutic options available, which have been in evolution. Guidelines based on nihilistic approaches to metastatic melanoma are no longer relevant given the multiple new effective treatments now available and emerging.

It is largely agreed that most patients with stage I and II melanomas need no systemic imaging. Gold et al. reviewed an 8-year experience of 181 patients who had a positive SLNB (Gold et al. 2007). At least one study (computed tomography or magnetic resonance imaging of the brain; chest X-ray; computed tomography of the thorax, abdomen, or pelvis; positron emission tomography scan; or bone scan) was obtained around the time of SLNB in 178 patients (98%).

Studies were obtained after SLNB in 107 patients (59%). Studies ordered after SLNB resulted in indeterminate findings in 51 patients (48% of those studied). Among patients tested after SLNB, four were found to have metastatic disease (positive rate 3.7%). All of these patients had both a thick melanoma and macrometastasis within the SLN. The number of patients with indeterminate findings

would be decreased, and the yield of the workup increased fourfold by restricting the workup to those with thick melanoma and macrometastasis.

These authors propose restricting workup to patients with thick melanoma and macrometastasis on SLNB to spare patients from indeterminate findings and increase the yield of the whole-body imaging evaluation (Gold et al. 2007).

Danielsen et al. reviewed the records of 167 patients with newly diagnosed "high-risk" primary cutaneous melanoma who underwent a PET/CT scan performed as part of their initial staging (Danielsen et al. 2016). Clinical and histologic factors were evaluated as possible predictors of melanoma metastasis identified on PET/CT scanning. Thirty-two patients (19.2%) had a positive PET/CT finding of metastatic melanoma. In more than half of these patients (56.3%), PET/CT scanning identified disease that was not detectable on clinical examination. Mitotic rate, tumor thickness, lymphadenopathy, and bleeding were significantly predictive of PET/CT positivity. A combinatorial index constructed from these factors revealed a significant association between the number of high-risk factors observed and the prevalence of PET/CT positivity, which increased from 5.8% (with the presence of 0–2 factors) to 100.0%, when all four factors were present.

These results indicate that combining clinical and histologic prognostic factors enables the identification of patients with a higher likelihood of a positive PET/CT scan. It appears that "high-risk" melanoma patients can be identified who are more appropriate candidates for PET/CT imaging, though how reliable such stratification algorithms are in practice remains to be determined (Danielsen et al. 2016).

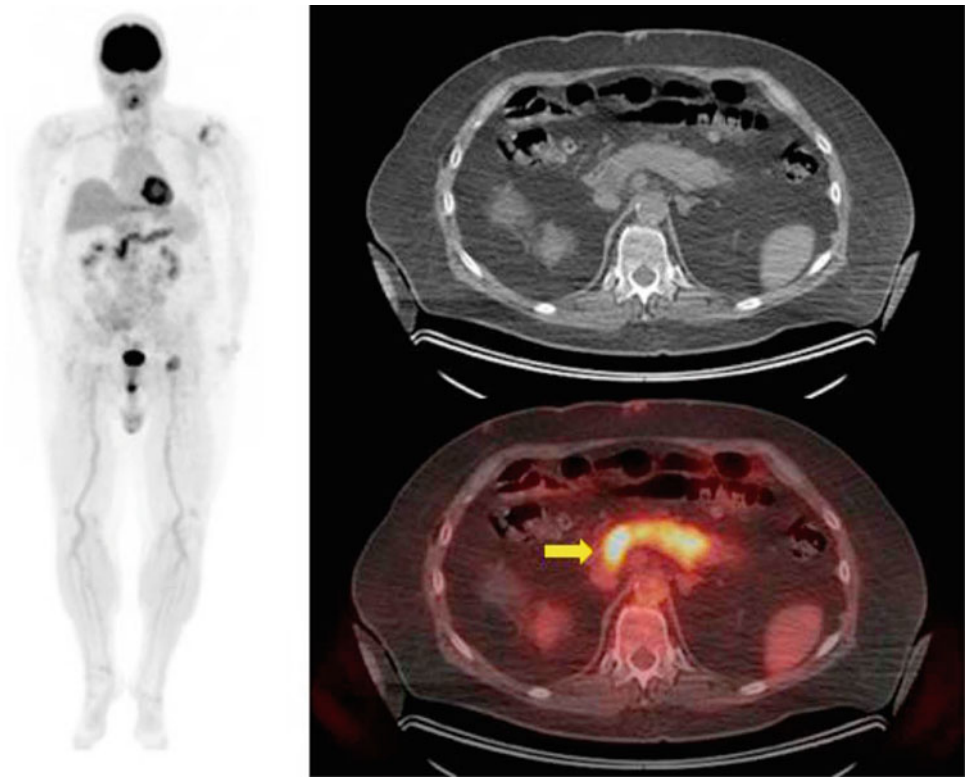

Fig. 4 FDG PET imaging showing diffuse pancreatic FDG uptake indicative of active pancreatitis associated with immune check-point therapy (Reproduced from Wachsmann et al. 2017)

In stage III and IV melanoma, the role of PET/CT and imaging at presentation seems well established. Krug et al. estimated the diagnostic performance of ^{18}F- fluorodeoxyglucose (FDG) positron emission tomographic (PET) imaging in the initial staging of cutaneous malignant melanoma (CMM). They identified 28 studies involving 2,905 patients who met the inclusion criteria. The pooled estimates of FDG PET performance for the detection of metastasis in the initial staging of CMM were sensitivity, 83%, and specificity, 85%; positive likelihood ratio (LR), 4.56; negative LR, 0; and diagnostic odds ratio, 19.8. Results from eight studies suggested that FDG PET was associated with 33% disease management changes (range, 15–64%). These authors pointed out the need for prospective studies in this space (Krug et al. 2008).

Mena et al. reviewed clinical records of PET/CT scans of 232 biopsy-proven melanoma patients who underwent FDG-PET/CT scans. Of these, 71 patients had 4 or more follow-up FDG-PET/CT scans after completion of primary treatment, with a total of 246 fourth or subsequent follow-up PET/CT scans.

The added value of each follow-up PET/CT scan to the patient's clinical assessment and treatment management was established. Of the 246 fourth and subsequent follow-up PET/CT scans, 61% (150/246) were negative for malignancy, and 39.0% (96/246) were positive for recurrence/metastases. The PET/CT scan resulted in change of the patient's management in approximately 16.7% (41/246) of the scans. Change in management was significantly greater in patients whose scans were done with prior clinical signs suggestive of malignancy or for therapy assessment than without prior clinical suspicion (29.3% vs. 4.1%; $P < 0.0001$).

The authors concluded the fourth and subsequent FDG-PET/CT scans obtained after completion of primary treatment added value to clinical assessment in patients with melanoma, especially in patients with clinical signs suggestive of recurrence or metastases. Those being monitored for treatment response are more likely to benefit from the fourth or subsequent FDG PET/CT than those without prior clinical suspicion (Mena et al. 2016).

Extent of Examination

Although pelvic computed tomography (CT) scans are frequently performed as a part of routine surveillance, the evidence for or against the routine use of these scans in patients with primary melanoma in the head and neck is weak. Alvrado et al. conducted a retrospective study to evaluate the value of pelvic CT scans as routine surveillance in patients with primary melanoma in the head and neck in 146 patients with either primary or mucosal primary melanoma who had adequate follow-up evaluation for at least 5 years in institution. A total of 82 patients (56%) had eventually developed distant metastases, but only 10 (7%) had developed metastases in the pelvis, and none had developed pelvic metastases as the first and the only site of recurrence. This study suggests that the routine use of a pelvic CT scan as a surveillance method does not have any impact on the management in patients with primary melanoma in the head and neck (Alvarado et al. 2011).

Imaging to Determine Suitability for Removal of Lung Metastases

Complete surgical resection of melanomas remains a proven therapy. It appears that lung metastases with low Glut 1 expression and slow proliferation may be the most appropriate for resection (of course these parameters can be best known after the tissue is removed). However, performing a pulmonary metastectomy is likely not sensible if there is extrapulmonary non-resected metastatic disease.

Krug et al. developed a cost-effectiveness analysis, using a Markov model over a 10-year period, which was performed to compare two different surveillance programs, either PET-CT or whole-body CT, in patients with suspected pulmonary metastasized melanoma. The PET-CT strategy provided 86.29 life-months gained (LMG) at a

discounted cost of euro 3,974, while the conventional strategy provided 86.08 LMG at a discounted cost of euro 5,022. This PET-CT strategy resulted in a net saving of euro 1,048 with a gain of 0.2 LMG. Based on PET-CT findings, 20% of futile surgeries could be avoided.

The authors concluded that integrating PET-CT in the management of patients with high-risk MM appears to be less costly and more accurate by avoiding futile thoracotomies in one of five patients as well as by providing a small survival benefit at 10 years (Krug et al. 2010).

Controversies in Surveillance

There is no international consensus on optimal follow-up schedules, and supplementary tests should be used after resection of a primary melanoma. Recommendations vary drastically by region and by stage of melanoma. Low-risk, SLN negative patients require no imaging and only regular skin exams for new lesions and exams for lymph node growth. Abbott et al. found that for microscopic node + SLN, the role of surveillance FDG PET had a lower yield than performing annual surveillance PET in patients with substantial lymph node burden, but in both groups, recurrences were identified in 10% or more of patients. By contrast, patients with more advanced melanoma may benefit from intensive surveillance using advanced imaging tools. Podlipnik et al. prospectively evaluated 290 consecutive patients with a diagnosis of stage IIB, IIC, and III melanoma. Patients were followed up with an intensive protocol based on imaging studies (computed tomography of the chest, abdomen, and pelvis, and brain magnetic resonance imaging), periodic laboratory tests, regular physical examinations, and patient self-examinations. A total of 2,382 clinical examinations and 3,069 imaging tests were performed. The patients completed 899.8 person-years of follow-up, with a median of 2.5 years. In all, 115 recurrences in 290 patients were recorded, of which computed tomography detected 48.3%; brain magnetic resonance imaging, 7.6%; laboratory test, 2.5%; physician, 23.7%; and patient, 17.8%. The authors concluded that intensive monitoring is appropriate for early detection of recurrence in stage IIB, IIC, and III melanoma, though they did not use PET/CT extensively (Podlipnik et al. 2016). Baker et al., in a small retrospective study, found a low utility of follow-up PET/CT in a small group of SLN+ patients any occurred recurrences were missed by PET and challenges with false-positive studies, and the a-H were not convinced of the utility of FDG/PET for finding early recurrence (Baker et al. 2014).

Peric Zagar et al. examined the role of serum marker addition in melanoma surveillance and concluded that adding an S-100B serum level enhanced the value of PET/CT imaging in surveillance of patients for recurrence (115 patients), again indicating that imaging is a very important part of assessing patients for recurrence, but not the only tool available to clinicians (Peric et al. 2011).

Danielsen et al. reviewed the performance of FDG PET in follow-up programs for asymptomatic patients at high risk of relapse to detect systemic recurrences. Their systematic literature search in PUBMED, EMBASE, and the Cochrane Controlled Trials Register identified seven original studies on the diagnostic value of FDG-PET in the follow-up of CMM. Sensitivity, specificity, and positive and negative predictive values were calculated to examine PET's diagnostic value in detecting relapse. The mean sensitivity of PET was 96% and the specificity was 92%. The positive and negative predictive values were, respectively, 92% and 95%. Overall, PET has a high diagnostic value and the many advantages of PET indicate utility in the routine follow-up program of CMM. However, the number of prospective studies of high quality is scarce, and as the use of PET and PET/CT is becoming more widespread and the technology is expensive, these authors suggested there is an urgent need for systematic assessment of the diagnostic value (Danielsen et al. 2013, 2016).

Leiter has presented intriguing data suggesting patient survival is increased by aggressive surveillance. This assertion is based on a long-term survival analysis of 1,969 patients with stage I-III CM documented during 1996–1998 in the frame

of a prospective surveillance study. Development of metastatic spread was detected in 112 patients during this period and classified as early phase or advanced phase based on tumor load and operability. Of 59 patients with metastases detected in an early phase of development, 64.4% died of CM, of 43 patients with advanced phase metastases 86% died ($P = 0.013$). The 10-year overall survival probability was 42.6% for early and 25.6% for advanced phase metastases ($P = 0.012$). This comparison remained significant after adjustment for sojourn time. Multivariate analysis identified detection of early phase metastases ($P = 0.022$) and stage at primary diagnosis ($P < 0.0001$) as independent prognostic factors. This study did not use PET to a significant extent, but it is intriguing nonetheless (Leiter et al. 2010).

Summary

The role of noninvasive imaging in melanoma differs depending on the clinical circumstance. At diagnosis, no or very limited systemic imaging for tumor staging is performed if patients have no clinical evidence of metastatic disease to lymph nodes. However, imaging increasingly is used to guide radionuclide sentinel lymph node biopsy procedures, especially if complex drainage routes are expected such as in the head and neck where SPECT/CT can be valuable.

Ultrasound can be used to assess and follow regional lymph nodes in patients in whom sentinel node biopsy is not successful or not performed. If there is tumor involvement in lymph nodes at sentinel node imaging, or clinically, at least in higher-risk patients, more extensive whole-body imaging including CT of the thorax, FDG PET/CT, and MRI of the brain is commonly performed in the USA. Surveillance with these methods at regular intervals is recommended by several groups for several years post diagnosis, though guidance varies and is informed by risks of recurrence and must be balanced against radiation dose and costs.

Imaging, especially with PET/CT, is often used to assess treatment response and is performed at more frequent intervals related to the timing of the therapy. Special cautions in imaging patients treated with immune checkpoint inhibitors is necessary, as CT, US, MRI, and PET/CT can have pseudo-progression, where lymphocytic infiltration in tumors can cause growth in size and metabolic activity transiently, as well as apparent number, before response occurs. One to 2 month delayed repeat imaging to confirm progression/response is often needed. Use of imaging in melanoma still requires careful assessment for appropriateness to assure avoidance of overuse and attendant radiation and economic costs.

Noninvasive imaging is a crucial part of the management of melanoma at multiple time points across the history of the illness. Since therapies have been changing so rapidly, the use of imaging is currently informed by expert opinion and small trials, as opposed to prospective randomized trials, which should eventually be performed to better refine the best utilization of imaging techniques in this disease.

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Genetic Counseling

28

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Abstract

Since the first international consensus paper in 2009, genetic counseling and testing options for melanoma have progressed rapidly. The number of known high-penetrance genes has expanded from *P16/CDKN2A*, *P14^{arf}/CDKN2A*, and *CDK4*, to include *BAP1*, and several telomere-related genes, including *TERT*, *POT1*, *ACD*, and *TERF21P*. In addition, moderate- and low-penetrance genes have been added to *MC1R* as contributors to overall risk for melanoma, including: *MITF*, *HERC2/OCA2*, *TYR*, *TYRP1*, *SLC45A2*, and *ASIP*. Besides these genes that increase the inherited risk for melanoma, there are other genes that have well-established roles as high-penetrance genes for other cancer syndromes that appear to also serve as less-penetrant melanoma predisposition genes as well. These other cancer predisposition genes include *BRCA1*, *BRCA2*, *PTEN*, and *CHEK2*. (The xeroderma pigmentosum spectrum genes are not included in this chapter because genetic counseling for this subset of disorders requires additional considerations specific to the syndrome.) This chapter reviews the data supporting high-, moderate- and low-penetrance genes as relevant melanoma susceptibility genes, suggests a standardized protocol for risk assessment, and presents referral criteria for genetic assessment. The chapter also reviews rapidly progressing genetic testing technologies with an emphasis on selecting the most appropriate test and tailoring the testing process to each individual. Finally, a discussion of nuances related to genetic test interpretation, insurance discrimination, and the unique impact that genetic testing can have on individuals and society is explored.

Keywords

Melanoma · Genetics · Counseling · Testing · FAMMM · Inherited · Familial · Predisposition · Susceptibility

Introduction

Melanoma risk is conferred through a complex and overlapping set of genetic features and modified by the inherent genetic background and environmental exposures sustained by an individual over time (Berwick et al. 2016; Goldstein et al. 2007; Lo 2014). For example, inherited features that create a melanoma-vulnerable phenotype such as red hair, lightly colored skin, and freckles not only increase sensitivity to damage from ultraviolet radiation (UVR) but will also be modified by inherited factors such as DNA damage and repair gene polymorphisms (Berwick et al. 2016). Rarely, families are at substantially increased risk for melanoma due to inherited mutations in cancer predisposition genes (Ransohoff et al. 2016). These genes have higher penetrance for melanoma than the more common skin or hair color genes but are also likely modified by other inherited and environmental factors. In 1994, *p16/CDKN2A* was identified as the first melanoma predisposition gene (Kamb et al. 1994). Since that time, multiple genetic factors contributing to melanoma risk have been identified, and genetic testing has evolved to allow for a more thorough analysis of high-risk families. This review addresses new developments in the genetics of melanoma, incorporating risk assessment into practice, and current and evolving practice for genetic counseling and testing.

Melanoma Predisposition Genes

P16/CDKN2A

CDKN2A, which encodes for the p16 protein, is the most significant melanoma predisposition identified to date, with mutations accounting for 20–40% of high-risk families (Goldstein et al. 2007, 2006). Estimates of risk associated with *CDKN2A* mutations have been variable due to

differences in the populations tested and inclusion criteria. Initial studies of *CDKN2A* mutation carriers enrolled families with multiple cases and found a high lifetime melanoma risk. Lifetime risks for *CDKN2A* mutation carriers in Europe, the USA, and Australia were 58%, 76%, and 91% respectively (Bishop et al. 2002). However, population-based ascertainment resulted in much lower, but still significant, risk estimate of 28% (Begg et al. 2005). Ascertainment strategies likely account for this wide range of penetrance estimates. Families selected on the basis of multiple cases of melanoma may be more likely to have co-inheritance of additional risk factors. For example, carrying a melanocortin 1 receptor (*MC1R*) variant, along with a *CDKN2A* mutation, significantly increases melanoma risk (Box et al. 2001; Demenais et al. 2010). Members of multiple-case, *CDKN2A* families, who test negative for the familial mutation, have been found to have residual elevated risk, providing further evidence of that high-penetrance families, and may have multiple risk factors interacting with *CDKN2A* (Florell et al. 2008; Hansen et al. 2004). *CDKN2A* mutation status alone may not be sufficient for determining specific risk estimates, and clinical counseling of patients should incorporate the family history and phenotype.

The interaction between *CDKN2A* and ultraviolet (UV) radiation exposure has also been investigated. Bishop et al. found differences in penetrance that corresponded with geographical differences in UV intensity, with the United Kingdom (UK) having the lowest UV intensity and the lowest penetrance, Australia having both the highest UV intensity and penetrance estimates, and the USA being intermediate in both factors. More recent comparisons between countries, using population-based recruitment, did not detect significant difference in risk between carriers in the United Kingdom compared to those in Australia (or between high and low ambient areas of Australia), with lifetime penetrance estimates of 45% and 52%, respectively (Cust et al. 2011), thereby calling into question whether UV exposure impacts melanoma risk in *CDKN2A* mutation carriers. However, while the interaction between UV and *CDKN2A* is uncertain, UV is a well-established contributor to melanoma risk in the general population (Gandini et al. 2005a; Green et al. 2011; Hodis et al. 2012). In vitro assays have also demonstrated that p16, in complex with CDK4 and Sp1, is involved in regulation of cell response to UV-induced DNA damage (Al-Khalaf et al. 2013). Further study is needed to clarify the extent of interaction between germ line *CKND2A* mutations and UV exposure, but mutation carriers should be counseled to minimize UV exposure in avoiding further enhancement of melanoma risk.

Familial atypical multiple mole melanoma (FAMMM) syndrome was the diagnosis given to families presenting with numerous, large, atypical nevi and a high melanoma risk. Families with this phenotype were selected for inclusion in the original studies to identify the genetic contributions to melanoma risk. Some families with the FAMMM phenotype were found to have *CDKN2A* mutations, while others were not. Of note, even within a single family, some mutation carriers will have a FAMMM phenotype, while others will not (Florell et al. 2008). Other studies have found that the presence of multiple atypical nevi alone is not a strong predictor of carrying a *CDKN2A* mutation (Celebi et al. 2005; de Snoo et al. 2007). It is likely that additional factors, possibly interacting with or independent from *CDKN2A*, are required for the FAMMM phenotype. Therefore, genetic testing for *CDKN2A* is appropriate for families with multiple cases of melanoma (Table 1), regardless of whether they express the FAMMM phenotype. Conversely, relatives who test negative for familial mutations, but have a high-risk features, should still be considered at increased risk and screened appropriately. In addition to melanoma, *CDKN2A* predisposes to pancreatic cancer (up to 25% lifetime risk) (Parker et al. 2003; de Snoo et al. 2008). Vasen et al. followed 178 *CDKN2A* mutation carriers with annual endoscopic ultrasound and/or magnetic resonance cholangiopancreatography (MRCP). During this study, 13 (7.3%) developed pancreatic cancer, 75% were diagnosed at a resectable stage, and the 5-year survival rate was 24% (Vasen et al. 2016). This and other studies suggest that screening can make modest improvements in the outcome of pancreatic cancer in these high-risk individuals, and the International Pancreas

Table 1 Genetic testing criteria for hereditary melanoma (Leachman et al. 2009)

Criteria for high-incidence areas[a]	Criteria for low-incidence areas[b]
≥3[c] case of melanoma and/or[c] pancreatic cancer among first- and second-degree relatives on the same side of the family	≥2[3] cases of melanoma and/or[c] pancreatic cancer among first- and second-degree relatives on the same side of the family
≥3 synchronous or metachronous melanomas in an individual	≥2 synchronous or metachronous melanomas in an individual

[a]High incidence ≥10 cases/100,000
[b]Low incidence <10 cases/100,000
[c]At least one case must be melanoma

Screening consortium recommends that *CDKN2A* mutation carriers, with a first-degree relative with pancreatic cancer, have screening with annual endoscopic ultrasound or magnetic resonance cholangiopancreatography beginning at age 50 (or earlier if there has been an early diagnosis in the family) (Canto et al. 2013).

P14^{arf}/CDKN2A

CDKN2A encodes two proteins, p16 and p14^{arf}. Mutations in the gene can affect either one or both of these proteins. No significant differences in risk or cancer spectrum have been identified between mutations disrupting just p16 or p16 and p14^{arf}. Mutations that disrupt only p14^{arf} are rare. Families segregating both melanoma and a neural tumor, particularly astrocytoma, have been reported and linked to the chromosomal location of *CDKN2A* (19p23.1). Analysis of the few such reported cases has suggested that this combination of tumor risks may result from mutations affecting p14^{arf}, though undetected alterations in other gene products offer an alternative explanation. A 2001 case report described a family with melanoma and neural tumors that segregated with a deletion that affected only p14^{arf} (Randerson-Moor et al. 2001). Overall, mutations in p14^{arf} are so rare; it has been difficult to confirm this genotype/phenotype association (Goldstein et al. 2006).

CDK4

CDK4 has also been identified as a very rare cause of hereditary melanoma. Unlike the majority of cancer predisposition genes, *CDK4* is a proto-oncogene which becomes activated by a mutation, mostly commonly Arg24His or Arg24Cys (Soura et al. 2016). Specific lifetime risk estimates have not been calculated, but a 2013 review of 89 mutation carriers and 79 mutation-negative family members found that of those who developed melanoma, 41% developed a second melanoma. They also found a higher prevalence of atypical nevi (approximately 70%) compared to the mutation-negative family members (27%) (Puntervoll et al. 2013). Because CDK4 functions in a complex with p16, it would be anticipated that mutations in CDK4 would result in a similar phenotype as *CDKN2A* mutations (Al-Khalaf et al. 2013). However, an association with pancreatic or other cancers has not yet been confirmed.

BAP1

BAP1 is a tumor suppressor gene that has been shown to have several roles in the cell, which, if disrupted, could contribute to carcinogenesis. *BAP1* has several functions, that if impaired could impact cancer risk, including chromatin remodeling, regulation of cell cycle progression, differentiation, and DNA damage response (Soura et al. 2016). *BAP1* was initially identified through the study of families presenting with a hereditary pattern of uveal melanoma and mesothelioma. Other risks related to *BAP1* mutations include cutaneous melanoma and renal cancer. Review of *BAP1* mutation carriers indicates that 13% (23/174) have been diagnosed with cutaneous melanoma, and of those with melanoma, 22% (5/23) had multiple primary melanoma (Rai et al.

2015). *BAP1* is commonly somatically mutated in uveal melanoma (Nasu et al. 2015). However, germ line mutations in *BAP1* are rare, accounting for only 3–4% of uveal melanomas and slightly larger portion of metastatic tumors. A study looking at germ line incidence found *BAP1* mutations in 4/50 metastatic cases versus 0/50 non-metastatic cases (Njauw et al. 2012).

BAP1 mutations may also cause an increased risk for non-melanoma skin cancer. Basal cell carcinoma (BCC) has been reported in 11/174 mutation carriers. Analysis of BCC in mutation carriers has demonstrated loss of heterozygosity of *BAP1*, suggesting that these lesions are related to the underlying predisposition rather than reflective of the relatively high general population risk for BCC (Rai et al. 2015).

In addition to malignancies, *BAP1* mutations are also associated with atypical melanocytic lesions. These lesions have been described as ranging from pink to tan in color and size from 0.2 to 1 cm (Rai et al. 2015; Soura et al. 2016). They can begin to develop during the first decade of life and become numerous (range 5–50). Clinically, the appearance is similar to a dermal nevus, but they have unique histological and molecular features which have led to the suggestions that these lesions be designated as melanocytic BAP1-mutated, atypical intradermal tumors (MBAITs) or atypical Spitz tumors (ASTs). Histologically, they are characterized by large epithelioid and spindled melanocytes, cytologic atypia, and pleomorphic hyperchromatic nuclei. These lesions may occur sporadically as well as due to an underlying *BAP1* mutation. Immunohistochemical staining of the BAP1 protein can be performed on biopsied tissue. Loss of *BAP1* expression detected on immunohistochemistry would be an indication to pursue germ line genetic testing. Characterization of the metastatic potential of these lesions is underway, and the prognosis is unclear. Until these lesions are better characterized, management with conservative re-excision and consideration of sentinel lymph node biopsy for lesions that fulfill criteria for melanoma are appropriate.

Consensus guidelines for *BAP1* mutation carriers have not been established, but management strategies have been derived based on the literature. Rai and Pilarski propose that *BAP1* mutation carriers have annual dilated eye exams beginning at age 11, screening for cutaneous malignancies with annual total body skin exam and monthly self-skin exams beginning by age 20, avoid UV exposure, and have annual abdominal imaging for renal cancer screening (Rai et al. 2015).

Telomere-Related Genes

Several genes related to telomere maintenance, *TERT*, *POT1*, *ACD*, and *TERF21P*, have been implicated in conferring melanoma risk. Mutations in these genes are very rare causes of hereditary melanoma, with each only accounting for a few families (Soura et al. 2016). The families found to carry mutations in these genes have generally been high-penetrance families with multiple cases of melanoma. However, numbers are insufficient at this time to derive specific penetrance estimates or establish definitive associations with non-melanoma cancers.

TERT encodes the enzyme, telomerase reverse transcriptase, which is a component of the telomerase complex that is responsible for adding a TTAGGG repeat to keep the ends from prematurely shortening. Overexpression of telomerase is key to the uncontrolled growth of cancer cells (Harland et al. 2016). One family reported to have a mutation in the promoter of *TERT* had 14 cases of melanoma. Two affected individuals in this family also developed ovarian cancer, and a third member of the family had five primary cancers including ovarian, renal, bladder, breast, and lung. Another family with a *TERT* mutation was identified after screening 273 families with three or more cases of melanoma. This family had seven members affected with melanoma. One member also had bladder and basal cell carcinoma.

POT1, *ACD*, and *TERF21P* form the shelterin complex which also contributes to telomere maintenance. Mutations in these genes are collectively thought to account for about 1% of hereditary melanoma (Potrony et al. 2015). Families with mutations in these genes have been reported to have had onset of melanoma as early as

adolescence (Soura et al. 2016). Other cancers such as breast, prostate, and lung have been described, but it has not been confirmed that the other cancers in these families are related to the underlying mutations.

At this time clinical genetic testing for telomere maintenance genes related to melanoma risk is predominately restricted to research. These genes are not generally included in clinical test offerings at this time.

Low- and Moderate-Penetrance Genes

A significant portion of familial melanoma cannot be accounted for by the high-risk genes identified to date. Low- and moderate-penetrance genes are likely factors in these families. While these genetic variants individually have small effects on risk, some combinations of genetic factors may be additive and, when inherited together, could confer greatly increased risk.

Melanocortin 1 Receptor (MC1R)

MC1R, previously noted to enhance the risks associated with *CDKN2A* mutations, has been a prominent target for evaluation. Outstanding reviews have been published summarizing the structure and function of *MC1R*, which highlight the mechanism of action of the receptor on a variety of nonpigmentary antitumor effects including nucleotide excision repair, apoptosis, and oxidative stress pathway control (Swope 2016; Wolf Horrell and D'Orazio 2016). Some variants in *MC1R* affect the ratio between eumelanin and pheomelanin, leading to the red hair and pinkish skin tone in carriers of these variants. Additional overlapping *MC1R* variants have been associated with melanoma, and it is not completely clear if certain functional *MC1R* mutations are capable of conferring a predisposition to melanoma without red hair. Heterozygote carriers of these variants are predisposed to melanoma but frequently do not have red hair or a photo-vulnerable phenotype. These individuals tan normally and appear to have a higher risk for melanoma than heterozygotes with red hair and photo-vulnerability (Tagliabue et al. 2016). It has been hypothesized that heterozygotes without red hair or photo-vulnerability may be at higher risk because of behavioral issues associated with tolerance to greater sun exposure (Tagliabue et al. 2016).

Microphthalmia-Associated Transcription Factor (MITF)

MITF is a helix-loop-helix transcription factor and transcriptional repressor that plays a central regulatory role in melanocyte development and pigmentation. Inheritance of a germ line mutation at a sumoylation site of the protein, E318K, is associated with an increased risk of melanoma in a relatively small number of families worldwide (Ghiorzo et al. 2013). As with the high-penetrance genes described above, definitive penetrance estimates are difficult to establish but have been reported to range from about three- to fivefold increased risk of melanoma. Of interest, E318K *MITF* mutations also lead to an increased risk of renal cell carcinoma at a rate similar to that of melanoma, and pancreatic cancer, breast cancer, and lymphoma have also been reported in association with this mutation. It has been reported that the phenotype associated with the *MITF* E318K mutation includes increased number and size of nevi; increased numbers of large, atypical nevi; young age of onset of melanoma (<40); non-blue eye color, amelanotic melanoma formation; and no apparent association with skin or hair color or freckling. When observed under dermoscopy, a reticular pattern has been noted in the nevi.

Other Less Penetrant Melanoma Predisposition Genes

Additional, more common, but less penetrant genes have been identified through case-control and genome-wide association studies (e.g., *HERC2/OCA2*, *TYR*, *TYRP1*, *SLC45A2*, and *ASIP*). However, it is premature to include these as part of routine clinical genetic testing because

the impact they have on melanoma penetrance and risk for other cancers is yet to be determined.

Cancer Predisposition Genes with Effects on Melanoma Risk

Hereditary Breast/Ovarian Cancer

Women with a pathogenic variant in *BRCA1* or *BRCA2* have an average lifetime risk (to age 70) of 60% to develop breast cancer and 59% to develop ovarian cancer (Mavaddat et al. 2013). Men with a pathogenic variant in *BRCA2* have a 6–9% lifetime risk (to age 80) to develop male breast cancer (Evans et al. 2010) and an increased risk to develop prostate cancer (Narod et al. 2008). The Breast Cancer Linkage Consortium documented a 2.58-fold increased risk for melanoma in individuals with a pathogenic variant in *BRCA2* (CI = 1.28–5.17). More recently, a study from the University of Texas MD Anderson Cancer Center revealed a trend toward increased melanoma with *BRCA1* pathogenic variants (Mersch et al. 2015). The National Comprehensive Cancer Network (NCCN) publishes management recommendations for individuals with a *BRCA1* or *BRCA2* pathogenic variant (Genetic/Familial High-Risk Assessment: Breast and Ovarian Cancer Version 2.2016 2016). At this time, although noting a full-body skin and eye exam could be considered, the NCCN has no specific screening guideline for melanoma in individuals with a *BRCA* mutation (Genetic/Familial High-Risk Assessment: Breast and Ovarian Cancer Version 2.2016, 2016).

PTEN Hamartoma Syndrome

Another established inherited cancer syndrome that has been associated with an increased risk for melanoma is *PTEN* hamartoma tumor syndrome, also called Cowden syndrome, caused by pathogenic variants in the *PTEN* gene. Individuals with *PTEN* have an average lifetime risk (to age 70) of >60% to develop female breast cancer, and increased risks to develop thyroid, endometrial, kidney, and colorectal cancers, and an increased risk of up to 6% to develop melanoma (Pilarski et al. 2013). As for individuals with a *BRCA* pathogenic variant, the NCCN publishes management recommendations for individuals with a *PTEN* pathogenic variant and does not detail specific screening guideline for melanoma but includes "dermatologic management may be indicated for some patients" (Genetic/Familial High-Risk Assessment: Breast and Ovarian Cancer Version 2.2016, 2016).

CHEK2

Pathogenic variants in the *CHEK2* gene have been associated with an increased risk of breast, prostate, kidney, thyroid, and colon cancers (Cybulski et al. 2004). A study investigating one of the most common pathogenic variants in *CHEK2*, 1100delC, and the risk of melanoma revealed a twofold increased risk for melanoma (Weischer et al. 2012). The authors have observed melanocytic neoplasms and melanoma in patients with a *CHEK2* pathogenic variant. At the time of this publication, melanoma risk and management are not addressed in the NCCN guidelines (Daly et al. 2016).

Risk Assessment

Risk assessment for hereditary melanoma begins with taking a personal and family history. In a busy clinical practice, taking time to review family history can be challenging. The use of questionnaires that patients can fill out prior to appointments can help to ensure that information is available to quickly review. Also, allowing patients to have some time to think about the history and confer with relatives will generate more complete, accurate information. Individuals who have features suggestive of an inherited cancer predisposition will benefit from a referral for genetic counseling. Genetic counselors will expand and verify the family history information in order to determine if genetic testing is appropriate.

When evaluating family history in clinic, key elements to assess and document include (Lu et al. 2014):

- The patient's personal history of cancer
- Their first- and second-degree relatives
  - First-degree relatives – parents, siblings, and children
  - Second-degree relatives – grandparents, aunts, uncles, grandchildren, and nieces
- Ethnicity
- For each cancer reported in the family, document the type of cancer, age at diagnosis, and linage

Criteria for referral for genetics evaluation for hereditary melanoma have focused on identifying families with a significant probability of harboring a *CDKN2A* mutation. The "Rule of 3s" criteria can be met by identification of three or more cases of invasive melanoma or pancreatic adenocarcinoma in an individual or a family (Table 1). These guidelines were suggested for areas of high melanoma incidence. When evaluating individuals from populations with a low incidence of melanoma, only two cases of melanoma or pancreatic cancer are needed to warrant referral ("Rule of 2s") (Leachman et al. 2009).

However, as previously described, melanoma is a component of multiple genetic syndromes that have tumor spectrums that go beyond that of *CDKN2A*. The Rule of 3s will not identify these families. To date, the diagnosis of melanoma has not been incorporated into the testing criteria for other hereditary cancer syndromes. The NCCN guidelines for genetics evaluation include melanoma, along with breast, ovarian, brain, pancreatic, prostate, endometrial, gastric, thyroid, and kidney cancers, leukemia, and sarcoma, in the constellation of cancers that may signify a hereditary cancer predisposition (Daly et al. 2016). However, NCCN's guidelines for genetic testing for hereditary breast/ovarian cancer syndrome, of which many of the causative genes also confer melanoma risk, do not include melanoma in the testing criteria. This may change in the future as research continues to expand the phenotypes associated with genetic mutations.

Therefore, clinicians should take a comprehensive family history that not only includes family history of melanoma but other cancers as well. Patients with family histories exhibiting features of hereditary cancer (Table 2) should be referred for genetic counseling because determining whether the pattern of cancer in the family is related to an identifiable genetic cause will likely lead to more accurate risk assessment and more tailored screening. For example, consider a patient presenting for routine dermatology care who reports that her mother and sister had breast cancer in their 40s and that her brother had melanoma. Her risk for melanoma may be increased twofold because she has a first-degree relative with melanoma, and her risk may be higher than that if the cancer in the family is related to a BRCA2 mutation which she has also inherited or close to average risk if the family history is due to a genetic mutation she did not inherit.

Multiple, specific guidelines have been developed for the multitude of hereditary cancer syndromes that have been identified. Determining whether patients meet detailed guidelines, such as the NCCN's guidelines for hereditary breast/ovarian cancer, PTEN hamartoma syndrome, Li-Fraumeni syndrome, etc., may not be feasible in general clinical practice. The American Society of Clinical Oncology has suggested some general red flags that are easily evaluated in clinical practice (Lu et al. 2014) (Table 2). Earlier than average age of onset is a red flag for most common adult forms of cancer. It is important to remember that melanoma can occur in young adults and even in children. Therefore melanoma occurring at a young age is not on its own a strong predictor of genetic risk. However, for cancers in which the median age is later in life, such as breast or colon cancer, a diagnosis under the age of 50 is highly suggestive of a genetic cause. Families with multiple individuals diagnosed with the same type of cancer or types of cancer that are known to share a genetic link are also candidates for genetics evaluation. Three or more cases of similar or related cancers on the same side of the family would warrant an evaluation, but fewer cases would be needed if one or more of the diagnoses occurred at young ages. Finally, there are certain types of

Table 2 General red flags for identifying common patients appropriate for a genetics referral for common hereditary cancer syndromes (Lu et al. 2014)

Criteria	Examples
Multiple relatives on the same side of the family with similar or related types of cancer	Three relatives with breast cancer at any age
	Three relatives on the same side of the family with prostate cancer at any age
Development of cancer at a younger than average age	Breast or colon cancer <50 years of age
Individuals with multiple primary cancers	Bilateral breast cancer
	Breast and ovarian cancer
	Colon and endometrial
	Melanoma and pancreatic cancer
	Melanoma and mesothelioma
Certain cancers warrant a genetics evaluation regardless of additional history	Epithelial ovarian cancer
	Triple-negative[a] breast cancer <60
	Paraganglioma/pheochromocytoma
	Retinoblastoma
	Colorectal and endometrial cancers exhibiting microsatellite instability
	Medullary thyroid cancer
	Acoustic or vestibular schwannoma
	Atypical teratoid/rhabdoid tumor
	Optic pathway tumor
	Malignant peripheral nerve sheath tumor
	Juvenile myelomonocytic leukemia

[a]Triple-negative breast cancers lack expression of estrogen and progesterone receptor and overexpression of Her2/neu

cancer that are so closely associated with a genetic syndrome that a genetics referral is warranted regardless of age or whether there is additional genetic testing (Table 2).

Genetic Testing

Genetic testing technology is evolving rapidly. This provides greater opportunities for determining the cause of melanoma risk in families, but challenges come along with these benefits including selection of the appropriate test, interpreting the results and appropriately consenting patients.

Picking a Test

Genetic counseling and risk assessment for inherited cancer syndromes have historically been offered to individuals at an increased risk for an inherited cancer syndrome based on their personal and/or family history and established testing criteria. The testing most often included one or a few genes associated with a well-established inherited cancer syndrome. For example, women with a personal history of premenopausal breast cancer and a first-degree relative diagnosed with ovarian cancer were offered genetic testing for *BRCA1* and *BRCA2*, the two genes known to account for most inherited breast and ovarian cancers. If that testing did not reveal a mutation, the family would be counseled that the cancers were most likely not due to a known hereditary cancer syndrome, and risk for additional cancers was based on empiric estimates from family history data.

The advent of next-generation sequencing techniques now allows for multiple genes or even entire exomes or genomes, to be sequenced for a fraction of the cost of traditional sequencing. Elimination of genetic sequence patents has also contributed to increased competition, lower costs, and more options for testing. However, not all genetic testing offerings are equal. Several areas of service may vary between laboratories including level of

Table 3 Resources for finding genetic services and genetic discrimination protections

Resource	Services	Website
National Society of Genetic Counselors	Search for genetic counselors by specialty throughout the USA and internationally	www.nsgc.org
	Patient-friendly resources for explaining genetic testing	
National Cancer Institute Genetic Services Providers Directory	Search for cancer genetics providers nationally and internationally	https://www.cancer.gov/about-cancer/causes-prevention/genetics/directory
GenoMEL	An international hereditary melanoma research consortium	http://www.genomel.org/
	Genetic counseling guides and management recommendations for patients with hereditary melanoma	
Health Insurance Portability and Accountability Act	Information on legal protections for patients	http://www.hhs.gov/ocr/privacy/hipaa/understanding.index.html
Genetic Information Nondiscrimination Act	Information on legal protections for patients	http://www.genome.gov/PolicyEthics/LegDatabase/pubserach.cfm
Affordable Care Act	Information about mandated coverage of preventive services	https://www.healthcare.gov/get-answers/
	Resources for uninsured patients	

sequencing coverage (this determines the likelihood that a mutation will be detected if present), whether large deletions or genomic rearrangements can be detected, the genes offered, variant classification approaches, policies for notification about reclassifications, and insurance authorization support. Providers should be aware of a laboratory's methods and policies before ordering a test or refer patients to genetic counselors who are knowledgeable about laboratory options (Table 3).

To date, whole exome sequencing (evaluation of the areas of genetic material that code for proteins) and whole genome sequencing (evaluation of both coding and noncoding areas of genome) are being applied to tumors for treatment planning but are rarely used in cancer predisposition testing. A more common approach for predisposition testing of panels of genes is associated with cancer predisposition. Tests may be targeted toward a few genes related to a specific cancer type, such as melanoma or breast cancer, or the tests may be broad and include a range of cancer predisposition genes. Not all genes available through panels have been well studied. Some may only have very preliminary data regarding penetrance and tumor spectrum, and many do not have established management guidelines. The potential for increased mutation detection needs to be balanced with the increased chance of receiving results of uncertain clinical significance, which can be challenging to explain to patients.

Pretest counseling is an important step in the selection of a test. During this process, the provider and patient should discuss the purpose of the testing (what is the question trying to be answered?) and the patient's interest in evaluating additional risks.

Interpretation of Test Result

There are three possible findings from genetic testing. First, one or more pathogenic mutations may be detected. The identification of a pathogenic mutation in an individual often determines the ongoing clinical management and enables familial mutation testing in family members. Historically, family members that test negative for the familial mutation are given the recommendations for screening based on their own age and personal medical history, no longer considering the family history of cancer. However, when a mutation is

identified in a moderate-risk or limited evidence gene, result interpretation for the proband and their family members may not be clear. Available data on the particular gene and variant identified should be reviewed. As has been described with *CDKN2A*, penetrance for other genes has been reported to vary based on family history.

The second possible outcome is that no mutation is identified. This result cannot rule out an increased familial risk. Even though testing may include multiple genes, the genetic factors known to be associated with cancer risk still only account for a portion of familial risk. A negative test result in a proband does not eliminate risk for them or relatives. Individuals with a prior melanoma have a ninefold risk of developing a second melanoma, and unaffected individuals with a first-degree relative (parent, siblings, child) with melanoma have approximately a twofold increased risk for melanoma (Gandini et al. 2005b).

Finally, testing may result in a variant of uncertain significance (VUS). These are genetic variants for which there is insufficient data to determine if they are simply part of normal human variation or if they are related to disease risk. Variants of uncertain significance are identified in approximately 30% of multigene panel test (Tung et al. 2016). Findings classified as VUS are not clinically actionable, meaning that management recommendations do not change based on the test result; management recommendations remain based on personal and family history (Lindor et al. 2013). Further, family members are not offered testing for a VUS as the result should not be used to alter their management either. Over time, variants may be reclassified as pathogenic or benign. As discussed previously, laboratory efforts regarding variant classification differ. Tracking and review of new data on VUS are difficult and time-consuming. Working with laboratories that have an active process for reclassifying variants over time and that have policies in place for notifying clinicians of new information is often the best way to ensure that management is being determined based on the most accurate information.

Insurance Discrimination

There are both national and state laws that protect most individuals from health insurance discrimination based on genetic test results. The national laws include the Health Insurance Portability and Accountability Act (HIPAA) and the Genetic Information Nondiscrimination Act (GINA), and state laws vary by state. HIPAA ensures that individuals can keep continuity of health insurance and helps to ensure health information is kept private and secure. It was amended in 2013 to prohibit the use of genetic information in underwriting. Information regarding the protections provided by HIPAA can be found at http://www.hhs.gov/ocr/privacy/hipaa/understanding.index.html. Signed into law in 2008, GINA prohibits the use of genetic information by health insurers or employers. Genetic information is defined broadly and includes information about risk based on family history as well as test results. When there are discrepancies between federal and state laws, whichever law offers the greatest protection takes precedence. Information about state laws can be found at http://www.genome.gov/PolicyEthics/LegDatabase/pubserach.cfm. Federal government employees and military personnel are not covered by GINA. The protections provided by GINA predominately benefit unaffected individuals who may have a genetic predisposition but do not yet have any features of the disease. The Affordable Care Act, which limits exclusions based on preexisting conditions, now offers protection for those who are already symptomatic. Information about the Affordable Care Act can be found at https://www.healthcare.gov/get-answers/. However, to date laws do not address the use of genetic information by life or disability insurers.

The Unique Impact of Genetic Testing

Because risk for melanoma can be conferred by phenotype and family history, the argument has been raised that genetic testing does not add significantly to assessment of risk (Kefford et al.

2002). However, research has shown that the process of receiving genetic test results may have a unique impact on risk perception and screening behaviors beyond family history-based risk assessment alone. A study of patients from high-risk families compared screening and preventive behaviors before and after receiving results from testing for familial *CDKN2A* mutations. Two years following genetic testing, unaffected, mutation carriers' adherence to recommendations for total body skin exams (TBSE) increased from 40% to 70% (Aspinwall et al. 2013a). Those who tested negative for the familial mutation demonstrated significant reduction in the frequency of skin exams. However, this was appropriate given their reduced level of risk. Unaffected, mutation carriers also reported increased thoroughness of self-skin examinations. In this same cohort, unaffected, mutation carriers also demonstrated increase in sun protection, with greater than 96% utilizing at least one sun protection strategy per day, up from 76% at baseline (Aspinwall et al. 2014).

Despite being aware of the family history of melanoma, baseline compliance in these high-risk families was low, and receiving genetic counseling and testing prompted greater adherence. Evaluation of the psychological impact of receiving genetic test results found that identifying a mutation did not result in fatalistic attitudes; rather 93% reported that they could take actions to help prevent or decrease the likelihood of developing melanoma (Aspinwall et al. 2015). Receiving genetic test results has also not been associated with significant increases in anxiety or depression in the short or long term (Aspinwall et al. 2013b).

However, a limitation of this research has been that genetic test results were provided in the context of thorough education and counseling. Therefore, the effects of the education and genetic testing on attitudes and behavior cannot be evaluated independently. To address this, a recent study compared two cohorts of patients, one group were unaffected members of families with known *CDKN2A* mutation and the other group were from families with multiple cases of melanoma, but no identifiable genetic etiology. Both groups received similar, thorough education about risk, screening, and prevention measure, but only those from *CDKN2A* families received personal test results. Comparison of these two groups at 1 month found that those who received genetic testing reported greater understanding of risk as well as greater applicability of the information compared to those receiving family history-based counseling alone. Those in the testing group were less likely to discount or downplay risk information. These findings suggest that inclusion of personalized genetic information may provide benefits beyond just intensive education based on less specific risk factors (Taber et al. 2015).

The highly personal and technical nature of genetic testing may lead to this information being uniquely valued by patients. Not all studies have consistently found that receiving genetic testing results will lead to behavioral changes such as smoking cessation or diet changes (Hollands et al. 2016). However, further study regarding the optimal ways to communicate genetic information in order to maximize the benefit on behavior is warranted.

Conclusion

We are currently in an era of rapid evolution with respect to genetic testing. The technology applied to genetic testing is transitioning from single-gene based tests into whole exome and whole genome sequencing. Molecular tumor boards are becoming more prevalent within genetic testing centers and will be invaluable as we move into personalized medicine at an individual level. Although it is likely that these sequencing technologies and molecular evaluations of genetic alterations will be the gold-standard of the future, we are currently in a transitional era of panel testing. In this era, it remains critical to identify which genes have data to support the value of testing, which patients fulfill the criteria for testing, and which test/s should be performed. It is paramount to balance the application of powerful technological tools for the benefit of clinical care with the need to assure that valid, useful information is provided to the patients. Nowhere more than the field of genetic

counseling/testing are technological "growing pains" being felt; yet, it is an essential evolution of the field that holds exceptional promise for the benefit of mankind.

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Artificial Intelligence Approach in Melanoma

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Abstract

Since its inception in the mid-twentieth century, the field of artificial intelligence (AI) has undergone numerous transformations and retreats. Using large datasets, powerful computers, and modern computational methods, the subset of AI known as machine learning can identify complex patterns in real-world data, yielding observations, associations, and predictions that can match or exceed human capabilities. After decades of promise, the field stands poised to influence a broad range of human endeavors, from the most complex strategic games to autonomous vehicle navigation, financial engineering, and health care. Therefore, the purpose of this chapter is to provide an introduction to AI approaches and medical applications while elaborating on the role of AI in malignant melanoma detection and diagnosis from a healthcare provider and consumer perspective. It is critical that we continue to balance the opportunity and threat of AI in malignant melanoma, as this technology becomes more robust to maximize an effective implementation.

Keywords

Artificial intelligence · Machine learning · Dermatology · Dermoscopy · Medical imaging · Imaging databases · Melanoma · Skin cancer

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Introduction

Since its inception in the mid-twentieth century, the field of artificial intelligence (AI) has undergone numerous transformations and retreats prior to reaching its current state (LeCun et al. 2015). Using large datasets, powerful computers, and modern computational methods, the subset of AI known as machine learning can identify complex patterns in real-world data, yielding observations, associations, and predictions that can match or

exceed human capabilities. After decades of promise, the field stands poised to influence a broad range of human endeavors, from the most complex strategic games (Silver et al. 2017) to autonomous vehicle navigation (Fagnant and Kockelman 2015), financial engineering (Heaton et al. 2016), and health care (Jha and Topol 2016).

As images on computer screens represent a discrete set of data points, a subset of AI known as computer vision has been applied to image analysis, with remarkable results. The analysis of dermatology images in general and melanoma images in particular represent a natural application of this field. Machine learning can be used to generate diagnoses (i.e., melanocytic nevus vs. melanoma) (Esteva et al. 2017). However, it can also be used to improve temporal tracking of lesions and provide results that are more robust to changes in lighting and angle (Li et al. 2016). Furthermore, the detection of subtle patterns in visual data may yield new insights that have yet to be discovered. For example, AI-led analysis of retinal images can predict cardiovascular risk status and even the gender of patients, the latter a heretofore-unknown association (Poplin et al. 2018). Similar insights into melanoma biology and outcomes may yet be garnered from AI-led analysis of the skin. Finally, AI can demonstrate enormous scalability, as these algorithms can be downloaded onto smartphones and used all over the world. With increasing data collection, the performance of the algorithms is expected to continuously improve, driving greater adoption and increased data collection.

In the sections below, we provide a general overview of the field of artificial intelligence. We will introduce relevant concepts, such as supervised learning, training versus validation datasets, neural networks, backpropagation, and deep learning. Next, we will discuss the use of AI in various medical applications, ranging from electronic medical records to the prediction of hospital mortality. As large datasets lie at the core of many recent advances, we will discuss the creation and curation of effective shared datasets with melanoma cases, along with the current state of the art in image classification as applied to dermatology, with an emphasis in melanoma. As with all scientific innovations, however, AI has significant shortcomings, ranging from interpretability to bias. We will review these challenges before discussing the technology's relevance to patients as end-users and the potential limitations of such an approach.

What is Artificial Intelligence?

Artificial intelligence (AI), also known as machine intelligence, has many definitions. This is not surprising as the concept of intelligence itself has many definitions (Legg and Hutter 2007). In fact, according to the leading psychologist Robert Sternberg, "viewed narrowly, there seem to be almost as many definitions of intelligence as there were experts asked to define it." An intuitive definition of AI, a term coined in 1956, is "the study of how to make computers do things at which, at the moment, people do better" (Rich 1987). AI is a vast field and composed of six main areas according to Russell and Norvig (2016): natural language processing, knowledge representation, automated reasoning, machine learning, computer vision, and robotics. In the following, we will briefly discuss two of these fields, namely, machine learning (ML) and computer vision (CV), as they are the most pertinent to the application of AI to melanoma diagnosis. Russell and Norvig also enumerate the fields that contributed ideas, viewpoints, and techniques to AI as follows: philosophy, mathematics, economics, neuroscience, psychology, computer engineering, control theory and cybernetics, and linguistics (2016). Many experts, however, consider AI to be mainly a subfield of Computer Science (CS).

As mentioned earlier in this chapter, we will focus on ML and CV primarily as they relate to melanoma. Mitchell defines *learning* in the context of machines as "a computer program is said to learn from experience E with respect to some class of tasks T and performance measure P if its performance at tasks in T, as measured by P, improves with experience E" (Mitchell 1997). Two fields that heavily overlap with ML, and thus with AI, are pattern recognition and data mining. Jain et al. define pattern recognition (PR), also known as

pattern classification (Duda et al. 2007) or pattern analysis, as "the study of how machines can observe the environment, learn to distinguish patterns of interest from their background, and make sound and reasonable decisions about the categories of the patterns" (Jain et al. 2000). Data mining (DM), on the other hand, can be defined as "automated or convenient extraction of patterns representing knowledge implicitly stored or captured in large databases, data warehouses, the web, other massive information repositories, or data streams" (Han et al. 2012). As mentioned before, ML, PR, and DM are substantially overlapping fields. Generally speaking, the common theme in these three fields is learning, where statistical approaches play a dominant role (Hand 1998).

There are two major paradigms in ML: supervised learning (SL) and unsupervised learning (UL). Here, "supervision" refers to the availability of class labels, or established, "ground truth" for the examples of interest. In dermatology, the quality of this label may vary from a dermatologist's clinical impression to the lesions' histopathological diagnosis to molecular data and clinical history.

It is often convenient to assume that the available samples are represented as D-dimensional numerical feature vectors, where D is referred to as the "dimensionality" of the data. In many applications, D is a large number because practitioners do not have a good understanding of which features may be relevant to the learning task; some features may also be redundant or noisy. As a concrete example, consider a biopsy algorithm for dermoscopic images of pigmented lesions, with four dichotomous features: the presence or absence of a blue-white veil, peripheral streaking, size greater than 6 mm, and the clinical information of age greater than 65 years. Each lesion is positioned in a four-dimensional space according to these various features, and if we have the histopathological diagnosis associated with each photographed lesion, we can then perform supervised learning on our dataset. This type of SL is referred to as "classification."

So, as one can see, features may represent visual information, such as pixels in an image, or metadata, defined as "data about data." Metadata is usually text-based information that describes the dermatologic imaging study (Caffery et al. 2018) and includes both clinical data (i.e., age, skin type, clinical history) as well as contextual data (i.e., temporal evolution of a lesion or a rash) (Caffery et al. 2018). In earlier forms of machine learning, experts might pick the various relevant features that would be analyzed by the algorithm.

Classification has two main phases: training and testing. Training is the process of building a classification model based on training data (in our example, the biopsy-proven skin lesions). Training implicitly or explicitly entails the partitioning of the feature space (see Fig. 1 for a two-dimensional illustration). Testing, on the other hand, is the process of evaluating the model on as-yet-unseen (test) data. The fundamental goal of training is to generalize beyond the samples in the training data. In other words, we would like to obtain a classification model that characterizes the training data well, but, more importantly, produces accurate predictions on the test data. Algorithms for classification are called classifiers, of which there are many techniques in machine learning, like nearest neighbors, decision trees, artificial neural networks, support vector machines, and ensemble classifiers (Fernández-Delgado et al. 2014).

Modern versions of ML-algorithms can be applied to complex information with high dimensionality, incorporating thousands of features. While earlier expert systems relied on extensive domain expertise to determine which features were relevant, this process is laborious and limited to one specific problem. Furthermore, in the example above, the algorithm relies on an expert to not only identify the important features but also then to subclassify patterns of pixels in each individual picture into the various categories (i.e., presence or absence of a blue-white veil). In the context of supervised learning, convolutional neural networks (CNNs) have recently become immensely popular, especially in computer vision applications (Guo et al. 2016; Litjens et al. 2017). CNNs alleviate the ML expert's burden of "feature engineering" by automatically discovering high-level abstractions from "low-level" data, such as image pixels (Goodfellow et al. 2016). Figure 2 schematically

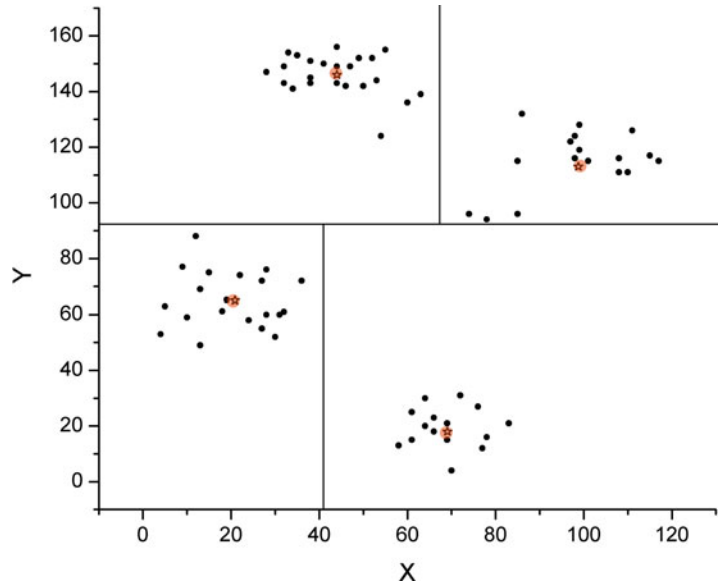

Fig. 1 Illustration of a simple four-class classification model for a two-dimensional data set (filled circles denote the samples, whereas stars denote the centroid of each class). Note that, in general, the decision boundaries are not necessarily parallel to the coordinate axes; they can be oblique or even curvilinear

illustrates how a CNN processes single pixels layer by layer to abstract shapes to reach a final class decision. Modeled on the relationship of neurons in the visual cortex, a CNN is a series of mathematical functions that can accept individual pixels as inputs. These are multiplied by various weights and summed, "firing" if they exceed the function's threshold. These neurons are then arrayed in multiple layers, allowing the algorithm to find complex patterns in pixels and how they relate to each other, proceeding from detecting edges of objects to curves, shapes, objects themselves, and complex categories, such as "basal cell carcinoma" and "dermatofibroma." The algorithm yields an output which is then compared to the image's "ground truth" label. During "training," the algorithm automatically adjusts the weights of its neurons, based on the difference between the "output" and "ground-truth." This iterative, computationally intensive process requires powerful modern processors and enormous datasets, and is an example of an approach referred to as "deep learning" (LeCun et al. 2015), which is capable of extraordinarily complex classification tasks (Krizhevsky et al. 2017).

UL (Celebi and Aydin 2016), that is, learning without class labels, is the second major paradigm in ML. The prototypical example of UL is clustering (Jain and Dubes 1988), also known as cluster analysis (Anderberg 1973), which can be defined as "grouping a set of samples in such a way that those in the same group (cluster) are similar, whereas those in different groups are dissimilar." Primary goals of clustering include gaining insight into data, classifying data, and compressing data. Clustering algorithms can be broadly classified into two groups: hierarchical and partitional. Hierarchical algorithms (Murtagh 1983) find nested clusters either in a top-down or bottom-up fashion. In contrast, partitional algorithms (Celebi 2014) find all the clusters simultaneously as a partition of the data and do not impose a hierarchical structure. Hierarchical algorithms are often unsuitable for large data sets. Partitional algorithms, on the other hand, typically require careful initialization (Celebi et al. 2013). Regardless of the nature of learning, supervised or unsupervised, it is crucial to have relevant, independent, and noise-free features; otherwise, we are at risk of "garbage in, garbage out." Two common problems that plague classification applications are overfitting (capturing random quirks, rather than general trends, in the training data), and the curse of dimensionality (difficulty of generalization in complex high dimensional spaces, where the fixed-size training data covers only a negligible fraction of the feature space) (Domingos 2012). For example, if a study

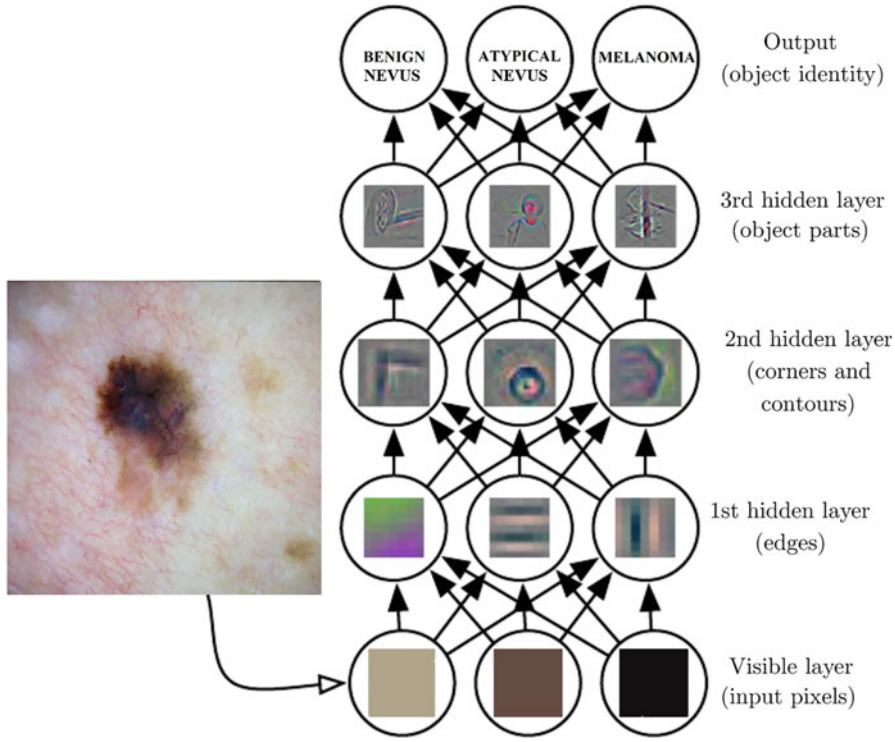

Fig. 2 Illustration of a deep learning classification model. As we move from the bottom (visible layer) towards the top (output layer), the classifier extracts increasingly abstract features from the input image. (Reproduced with permission from Goodfellow et al. 2016)

tracking the natural history of benign nevi has a unique ruler or size marker in those images, and if the benign images with this ruler comprise a significant number of benign lesions within a dataset, an algorithm may "learn" that lesions with that ruler or size marker are more likely to be benign. For this reason and many others, it is ideal to obtain transparent, understandable, and explainable models (Goebel et al. 2018), especially in medical applications. A less frequently problem encountered in ML and melanoma is underfitting, by failing to include relevant data with features indicative of MM diagnosis.

Artificial Intelligence Approaches and Medical Applications

There is a long history of using machine learning for artificial intelligence in biomedical applications. The spectrum of machine learning applications (Beam and Kohane 2018) ranges from clinical decision rules that utilize patient demographics and clinical measurements (e.g., the CHA_2DS_2-VASc clinical risk score (Lip et al. 2010) for stroke prediction in patients with atrial fibrillation), to statistical classifiers that rely on hand-crafted features derived from images or texts, to more recent deep learning-based computer-aided systems for diagnosis, prognosis, or treatment planning. A few examples of major machine learning applications, primarily focused on deep learning, that have started to demonstrate or could have clinical impact are discussed below (Fenton 2015).

Electronic Health Records

Electronic health records (EHRs) include both structured data (e.g., diagnoses, medications, laboratory measurements) and unstructured data (e.g., free-text clinical notes for admission, discharge, or transfer). In particular, better machine

understanding of these unstructured "free-text" can help improve the search of patient records for specific medical concepts (i.e., entities of interest such as medical problems, disorders, and treatment) and analysis of such concepts for epidemiological studies. Prior works have attempted to address this by extracting medical concepts with ML approaches, assigning notions of time to each extraction (Bethard et al. 2016), identifying structured relationships between different medical concepts (Uzuner et al. 2011), or disambiguating medical abbreviations (Pakhomov et al. 2005). Another primary goal of applying ML to EHR data is for clinical decision support, such as for predicting suicide risk (Tran et al. 2015) and other future adverse outcomes (Miotto et al. 2016; Nguyen et al. 2017; Rajkomar et al. 2018). One recent example demonstrated a novel method to standardize representation of raw EHR data and used this approach to develop deep learning models to make effective predictions for length of stay, future illness, readmission, and mortality across multiple institutions (Rajkomar et al. 2018). In addition, the large volume of EHR data has made it possible to learn richer, data-driven descriptions of illnesses (Che et al. 2015), representing a step towards the eventual goal of personalized precision healthcare (Fig. 3).

An emerging research area for ML in EHR applications is the generation of clinical notes. In the current clinical workflow, clinicians spend significant time documenting patient encounters. Although the use of voice recognition tools can help improve report turnaround time, they can result in higher error rates than medical transcription services (Hodgson and Coiera 2015). Deep learning has been shown to significantly reduce the error rates of automated speech recognition systems (Abdel-Hamid et al. 2014) and dramatically improve the usability of these systems in practice. These are encouraging results for future research development.

Medical Imaging

Medical imaging is one of the primary beneficiaries of recent advances in ML, in particular, the CNN-based methods, to address a variety of image analysis tasks. Typical tasks include: (1) image-level diagnoses, i.e., prediction of disease state from one or multiple images, (2) organ segmentation, i.e., outlining the precise boundaries of the organ of interest, and (3) image registration, i.e., spatial

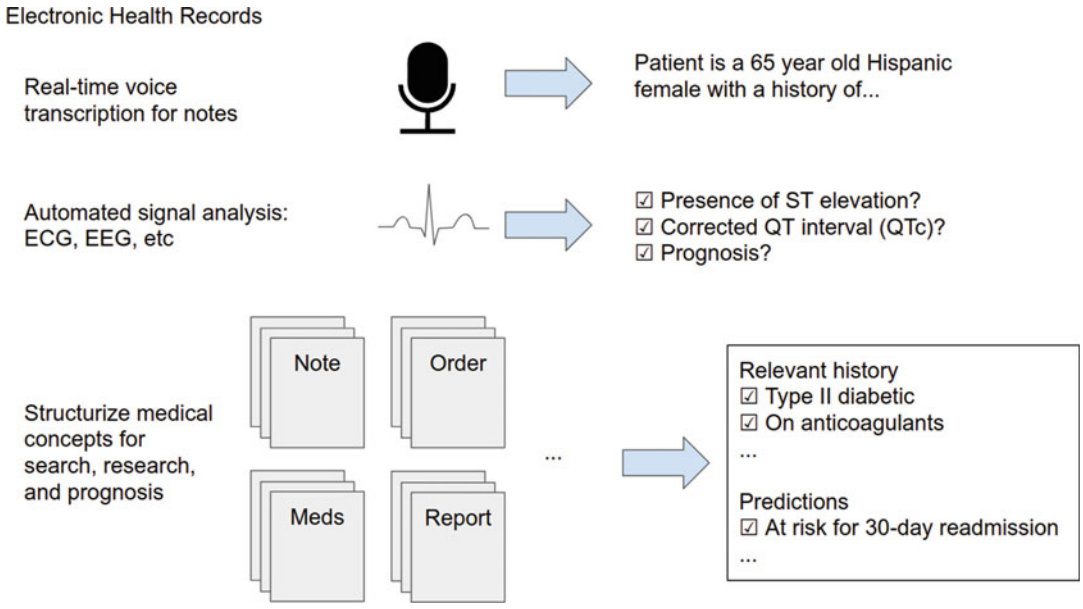

Fig. 3 Examples of applications of machine learning for electronic health records

alignment of two or more images into the same coordinate system. Many studies have demonstrated promising results in different domains of medical imaging, spanning from radiology, ophthalmology, pathology, to dermatology, as will be illustrated below in each subsection.

Radiology

Radiology is one of the earliest application domains for ML methods in medical imaging, particularly for the classification of disorders or the prediction of disease progression (Fig. 4). For example, researchers have been applying

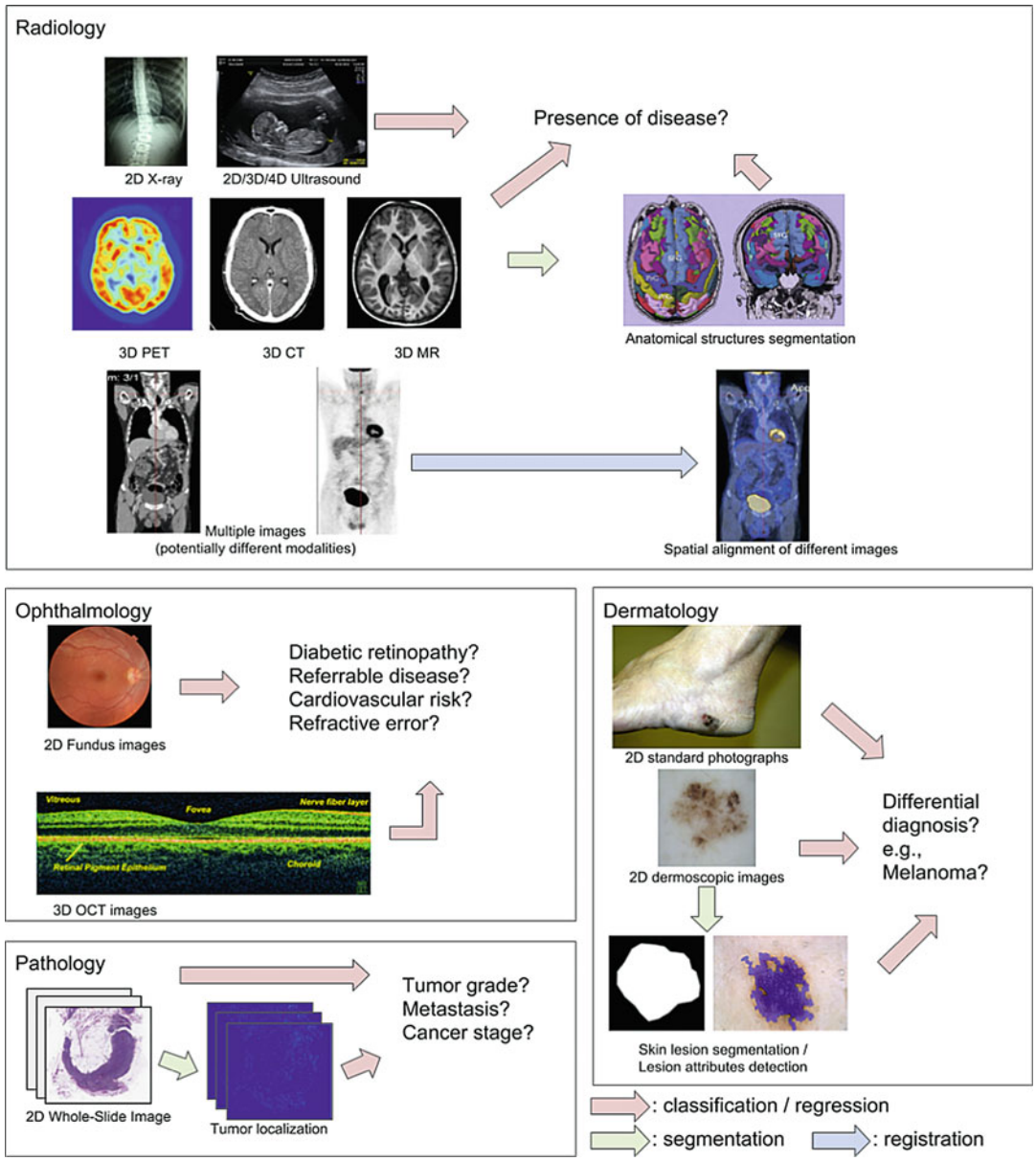

Fig. 4 Sample applications of machine learning for medical imaging. (Radiology images reproduced from Wikimedia; ophthalmology images reproduced from Wikimedia; pathology images derived from CAMELYON16: https://camelyon16.grand-challenge.org/; dermatology images reproduced from Wikimedia and ISIC Skin Image Analysis Workshop: https://workshop2018.isic-archive.com/)

such approaches to chest X-rays to detect common thoracic diseases like pneumonia (Wang et al. 2017b), as well as to assess the age of skeletal bones (Spampinato et al. 2017). Another active field is in neuroimaging for the early diagnosis of Alzheimer's disease in subjects with mild cognitive impairment based on structural magnetic resonance imaging (MRI), thanks to the large public dataset provided by the Alzheimer's Disease Neuroimaging Initiative (ADNI) (Alzheimer's Disease Neuroimaging Initiative 2018). Recently, ML was employed to identify abnormalities such as the subtypes of intracranial hemorrhage and fractures, using more than 300,000 cranial computed tomography (cCT) images (Chilamkurthy et al. 2018). Another large body of work focuses on segmenting organs or lesions using scans from one or more modalities: MRI, CT, and positron emission tomography (PET). Such segmentation allows quantitative analysis of clinical parameters and empowers the development of computer-aided systems for applications such as surgical planning or radiation treatment planning. The use of deep learning for segmentation has achieved state-of-the-art results in many public challenges. For example, the top three entries among 50+ participating teams in the 2017 brain tumor segmentation challenge ("BRATS") leveraged deep learning (Multimodal Brain Tumor Segmentation Challenge 2017 2017).

Ophthalmology

In diabetic eye disease screening, early detection can prevent or delay the onset of blindness. However, one of the biggest challenges in the development of screening programs is to train and retain skilled personnel to review the fundus images for signs of diabetic retinopathy or macular edema and refer eligible patients for further follow-up if indicated (Freudenstein and Verne 2001). Recently, deep learning algorithms have been introduced to assist graders in this repetitive task and have been shown to perform comparably with retinal specialists (Gulshan et al. 2016; Ting et al. 2017; Krause et al. 2018). More promisingly, these results have borne out prospectively (Abràmoff et al. 2018), and one approach has gained U.S. Food and Drug Administration (FDA) approval (Office of the Commissioner 2018). Although hurdles such as integration into the clinical workflow remain, the use of these technologies at scale to reduce preventable blindness may soon be within reach. Tantalizingly, similar algorithms have also been developed to quantify previously unknown associations such as cardiovascular risk (Poplin et al. 2018) and refractive error (Varadarajan et al. 2018). These findings suggest that retinal fundus photographs, which provide cheap and noninvasive imaging of vasculature, could be used to extract biomarkers for multiple conditions simultaneously.

Pathology

Pathology has had a slower start to medical image analysis, in part because of the lack of digitized images and the technical difficulty of working with the resultant gigapixel-sized images (each digitized slide is equivalent to 1,000 smartphone photographs). However, with the approval and gradual implementation of digital pathology scanners (Mukhopadhyay et al. 2018), adoption of digital pathology is growing, and with it the efforts in image analysis. One particular use case involves detection of lymph node metastases. Part of the challenge lies in the relative length scales; tissue samples (e.g., 1–2 cm across) appear as large expanses when viewed under the microscope, but tumors can be as small as one-ten-thousandth of the tissue area (e.g., 0.0004 cm^2). Thus nodal metastasis detection is tedious and potentially error prone for small tumor foci. Driven by the "CAMELYON16" challenge that provided hundreds of gigapixel images, deep learning algorithms have been derived and validated for detecting metastatic breast cancer (Bejnordi et al. 2017; Liu et al. 2018). Encouragingly, using one such algorithm to help review slides with small tumor foci halved the review time and false negative rate (Steiner et al. 2018). Importantly, the study also showed that the algorithm-assisted pathologists were more accurate than either unassisted pathologists or the algorithm alone, suggesting that humans and algorithms can work together effectively. Though promising, more work remains to study the

impact of using such algorithms in real clinical workflows.

Dermatology

Application of computational intelligence methods in dermatology traces back to 1987 (Cascinelli et al. 1987) and has gained increasing attention over the years. Most works so far focus on recognizing skin cancers in dermoscopic images (Celebi et al. 2007b) and even standard photographic images (Esteva et al. 2017). Work related to melanoma will be covered in more detail in subsequent sections. For other skin disorders, Han et al. (2018) trained a region-based classifier using clinical photographs to identify onychomycosis. Yang et al. (2018) presented a new visual representation to diagnose up to 198 skin lesions using a dataset of 6,584 clinical images (Sun et al. 2016) uploaded by dermatologists or patients. Despite the equivalent or even superior classification accuracy against experts reported by many papers, a direct measure of the impact of their work on clinical workflow is still lacking at this time.

Summary

Propelled by the latest development of deep learning technology and increasingly vast amounts of patient data, there has been a drastic rise in the number of publications in AI for healthcare, spanning a variety of medical applications from EHR to imaging, bioinformatics, genomics, and more. AI has been considered a potential game changer in healthcare in the near future, for example, by improving patient outcomes or reducing costs. However, most of the examples described above are still early-phase, ranging from "basic science" (i.e., model development or targeting a similar nonmedical application) to "preclinical" (i.e., model validation or studies in idealized settings that show efficacy). Although many studies have reported "clinician-level" performance, this is far from sufficient. Many nuanced issues remain, such as intended use and interpretability. Subsequent sections will discuss in detail challenges and barriers ahead in adopting AI for dermatology, and melanoma in particular.

Role of Artificial Intelligence in Malignant Melanoma

Due to a large prevalence, and a continuously growing incidence of skin cancer, its automated detection is currently the most prominent application in the field of automated diagnostics in dermatology. Especially early detection of melanoma has raised interest because of a drastic reduction of mortality through early diagnosis and broad knowledge of morphologic distinct patterns seen by dermoscopy. After a first wave of research on artificial intelligence (and neural network) – based classifiers in the late 1990s including a controlled prospective clinical trial (Dreiseitl et al. 2009), current improvements of image classification with convolutional neural networks have restarted scientific interest in the field. Since these models need robust and accurate collections of images to be trained effectively, creation and collection of appropriate image-databases was – and is –a critical element for their development and described below.

Imaging Databases: Relevance and Current State

In the current era of sophisticated AI technologies, the need for large data pipelines is one of the primary barriers to developing clinical level AI, as the development and training of such algorithms requires large datasets of images tagged with a "ground truth" diagnosis. The "ground truth" tagging can include a histological or clinical diagnosis of the lesion in the image for training of UL algorithms, or more detailed annotations on the image itself (e.g., annotations of specific dermoscopic structures such as pigmented network) to be used for SL algorithms.

Challenges and Risk of Bias

There are several challenges in developing large image databases. First is the lack of imaging and

metadata standards in dermatology. While there have been some efforts to create guidelines and standards for skin image acquisition and storage (Finnane et al. 2017), they are not easy to implement and require extensive time and resources. In addition, current dermatology databases (see below) typically do not include image metadata, while EHR systems are not data driven and fail to integrate this data in an easily extracted format. Image metadata is important for interpreting dermatologic images and its absence may decrease diagnostic accuracy, as two seemingly identical skin lesions can have different meanings in different clinical contexts (e.g., a new spitzoid lesion that appears on the skin of a young child as opposed to an elderly individual).

Second, there are unique privacy issues associated with skin/naked images. Many concerns have been raised about obtaining and storing such images in clinical settings and in secured closed systems. Some have even suggested that written consent be obtained from each patient before acquiring skin images (Kunde et al. 2013). These concerns are even greater when the images are uploaded to a public domain.

A third challenge is posed by image copyright issues. Whether the image owner is the patient, the person who took the image or the medical center delivering the care, is a matter of continued debate. In addition, while some databases are free and open to the public, many others are private and require permission and/or payment to access. Even in public databases, images can have different licensures, e.g., commercial versus noncommercial Creative Commons licenses, which may prohibit commercial companies from using these images to develop AI algorithms.

A fourth major challenge is the diagnostic "ground truth." The "ground truth" diagnosis of skin lesions is determined by histopathological characteristics and in an ideal world by long-term clinical behavior. Dermatologists have a high clinical sensitivity for the majority of benign skin lesions, and so these lesions are not routinely biopsied (Corbo et al. 2012). If databases only include images that have a histopathological diagnosis, they will contain a severe underrepresentation of the most common skin lesions seen in clinical practice. This can lead to a much lower diagnostic accuracy of AI systems in clinical settings despite their good performance in experimental settings. On the other hand, the inclusion of metadata that is not based on "ground truth" histopathology can lead to misclassified images and reduced accuracy of training AI algorithms.

Databases are susceptible to bias (lack of generalizability) if they are not representative of all skin types, cultures, geography, and disease distributions. Most of the current existing databases include a high representation of Fitzpatrick skin types I–III. Since AI algorithms are only as good as the data from which they learn, their accuracy on darker skin may be hampered (Adamson and Smith 2018). This was recently demonstrated when an algorithm that was trained on databases with a high proportion of Asian patients did not perform as well on images from Caucasian patients (Navarrete-Dechent et al. 2018; Han et al. 2018).

Another potential bias is complete absence of many diagnoses from image databases. While dermatology has over 3,000 entities identified in the literature (Lim et al. 2017), a large portion of the existing image databases includes only 2–14 entities. The discrepancy between the limited number of entities included in the databases that are used to train the AI algorithms and the variety of entities in real life again may hamper the performance of such algorithms in clinical settings as compared to experimental conditions (Tschandl et al. 2018c).

Current State of Image Databases

The numerous dermatological image databases that currently exist can be divided into three categories:

1. Dermatology atlases – These websites comprise the majority of image databases available online. These sites contain primarily clinical (as opposed to dermoscopic) images of a large number of entities along with an

explanation about each entity. Examples include: www.dermIS.net, www.dermnetnz.org, www.derm101.com, and www.dermatlas.net. Some atlases, such as www.Dermnet.com, specifically state that the use of their images for algorithm analysis or image processing without written consent is prohibited.

2. Private image databases from universities, medical centers, and private individuals. These databases contain clinical and dermoscopic images obtained from patients during clinical work. The number of images is variable, ranging from a few dozen to hundreds of thousands. The databases are not available online and can be accessed by obtaining permission from their owners. Examples include the Asan medical center database and the Hallym University database (Han et al. 2018).
3. Free online dermatologic image databases that include clinical and dermoscopic images. These databases are available for the public to download and use. The most notable example is the International Skin Imaging Collaboration Archive (see below), which glues together a collection of Creative Commons licensed datasets from the MSKCC, the SONIC study and the HAM10000-dataset. Additional examples include The University of Edinburgh database (https://licensing.eri.ed.ac.uk/i/software/dermofit-image-library.html) and MEDNODE database (http://www.cs.rug.nl/~imaging/databases/melanoma_naevi).

The International Skin Imaging Collaboration (ISIC) is a combined academia and industry effort aimed at improving melanoma diagnoses and reducing melanoma mortality by facilitating the application of digital skin imaging technologies. ISIC has developed and is expanding the largest public archive (www.isic-archive.com) of dermoscopic and clinical images of skin lesions, with over 40,000 images from leading clinical centers across the globe. The images are acquired from a variety of devices within each center and are screened for both privacy and quality assurance. Most images have associated clinical metadata, which has been vetted by recognized melanoma experts. The images on the ISIC archive are free and available for everyone to use and download. The archive's software infrastructure is open source and is freely available to the public as well.

All of the above-mentioned databases include only clinical and dermoscopic images. In addition, there are several databases that include histologic images of dermatologic diagnoses. They are generally educational databases and fall into the first (atlases) and second (private databases) categories. Some examples include https://digitalpathologyassociation.org, https://atlases.muni.cz, and the University of Michigan Virtual Slide Box (https://www.pathology.med.umich.edu/slides/search.php?collection=DermPath&dxview=show).

Medical image databases are not limited to the field of dermatology, and similar initiatives have been undertaken in other medical specialties. One of the best examples is the "Alzheimer's Disease Neuroimaging Initiative" in the field of neuroradiology (Alzheimer's Disease Neuroimaging Initiative 2018). It is an initiative that aims to promote the early detection of Alzheimer's disease, and it includes a large database of both MRI and PET images that are free and publically available.

Artificial Intelligence Applications in Malignant Melanoma Based on Clinical Photography and Dermoscopy

The current field of research in AI applied to melanoma detection is broad: methods span across multiple imaging modalities, including dermoscopy, clinical photographs, confocal microscopy, and pathology. Most recent work has been primarily centered on dermoscopy and clinical photographs, for which data is simplest, least costly to acquire, and in many instances, available for public use as mentioned in section "Current State of Imaging Databases." Therefore, these modalities comprise the focus of the rest of this section. For historical surveys, the reader is referred to (Korotkov and Garcia 2012; Scharcanski and

Celebi 2013; Celebi et al. 2015b, 2019; Mishra and Celebi 2016).

A dermatoscope captures digital photographs of suspicious skin lesions at a magnification of usually 10X with, or without, a polarized lighting system to assist in lesion diagnosis and tracking (Errichetti and Stinco 2016; Bakos et al. 2018). Clinical interpretation is simplified, as images are acquired with consistent lighting, magnification, and reduced skin reflectance. Dermoscopy has been repeatedly shown to increase sensitivity, specificity, and accuracy of melanoma detection with training (Vestergaard et al. 2008; Kittler et al. 2002; Binder 1995).

Within dermoscopy, automated recognition of melanoma has involved several image analysis tasks, including lesion segmentation, feature detection, and disease diagnosis. In segmentation, the area of skin within the dermoscopic field-of-view constituting the lesion is separated from background (Celebi et al. 2009, 2015a). In feature detection, the goal is to identify and localize dermoscopic feature patterns inside the lesion area, such as dots, globules, streaks, pigment network, etc. (Argenziano et al. 2003). For disease diagnosis, classification approaches may be directly applied to image pixel data, or additionally make use of segmentation data, dermoscopic feature data, or other available metadata. As the number of independent works in each of these tasks is incredibly large, segmentation and feature extraction will only be covered in this section as they relate to the subsequent classification of melanoma.

Within clinical photography, particularly in reference to imaging of individual lesions, automated recognition of melanoma may involve the image analysis tasks of skin area segmentation, lesion detection, and disease classification. Skin area segmentation is the only task which is not also a component of automated analysis of dermoscopic images. While the dermatoscope is usually in contact with skin, clinical photography may include background, garment and other artifacts, depending on the distance of the camera to the patient. However, until now, only one work is known to address this problem in a patient cohort with varying demographics and disease states (Codella et al. 2018a), and no known works yet study the use of skin segmentation for subsequent melanoma classification in clinical photography.

Historically, solutions to image analysis tasks have involved various classical computer vision and machine learning techniques (see section "What is Artificial Intelligence?"). These studies typically involved segmentation (particularly, clustering and thresholding techniques (Celebi et al. 2009, 2015a)), low-level shape/texture/color features (Barata et al. 2018) extracted from the lesion of interest (e.g., area/diameter of the lesion, statistical texture features extracted from the gray-level co-occurrence matrix, and mean/variance of select color channels), and traditional classifiers (e.g., decision trees, artificial neural networks, and support vector machines), with the resulting systems often evaluated on proprietary datasets (Celebi et al. 2007a, b; 2008; Celebi and Zornberg 2014; Barata et al. 2016, 2017). Later attempts were made at establishing a standard dataset for evaluation and comparison between methods (Mendonca et al. 2013; Barata et al. 2013, 2014), but the dataset was limited in size and scope (200 images) (see section "Challenges and Risk of Bias").

In recent years, dermoscopy and clinical photography image analysis tasks have been revolutionized by the advances in deep learning. The most common group of topologies used for study involve the CNN, of which several varieties have been developed over the years, including AlexNet (Krizhevsky et al. 2017), Inception (Szegedy et al. 2015), ResNet (He et al. 2016), and DenseNet (Huang et al. 2017) for classification, and U-Net (Ronneberger et al. 2015) for segmentation.

In the first work to apply current deep learning techniques to dermoscopic image analysis for the detection of melanoma (Codella et al. 2015) used a dataset of 2,624 dermoscopic images from the ISIC Archive consisting of 334 melanomas, 144 atypical nevi, and 2,146 benign nevi. The method used an ensemble of multiple machine learning classifiers in order to make predictions. Measured accuracy reached 93.1%, with sensitivity and specificity reported at 94.9% and 92.8%,

respectively. Deep features alone were found to achieve the best performance among all the individual features, at 91.9% accuracy, 90.3% sensitivity, and 92.1% specificity. Shortly after this work, an additional study on 1,760 dermoscopic images demonstrated a 5.8% improvement in melanoma recognition AUC by using classical image analysis techniques to align images according to the major axis of skin lesions prior to classification via a CNN (Yoshida et al. 2016).

Since that time, significant efforts have been undertaken by the International Skin Imaging Collaboration (ISIC) to host large-scale public challenges for melanoma detection in dermoscopic images. The first such challenge was hosted in 2016 at the IEEE International Symposium on Biomedical Imaging (ISBI), comprising 900 images for training and 250 for testing (Codella et al. 2018b). The challenge received 324 registrations and 65 final submissions, across three tasks of lesion segmentation, dermoscopic feature detection, and disease classification. For the disease classification task, participants were asked to build algorithms to discriminate between two disease categories: malignant and benign. The winning team for the disease classification task (Yu et al. 2017), as determined by average precision (AP), implemented a two-stage framework consisting of a network for segmentation, followed by a network for disease classification. The segmentation network localizes the lesion, which is then rescaled to fixed image dimensions before being input into the classification framework. Classification output was computed by a fusion of softmax scores from the disease classification layer as well as a support vector machine trained on top of network outputs. The resulting performance was 0.637 AP, 85.5% accuracy, and 0.804 area-under-curve (AUC). Performance degraded when removing the segmentation network or by using softmax alone.

Later in the same year, the first work to demonstrate an automated melanoma classification accuracy higher than the average expert dermatologist in dermoscopic images was released in preprint (Codella et al. 2017). The dataset used for experimentation came from the 2016 ISIC Challenge (Codella et al. 2018b). Similar to Codella et al. (2015), an ensemble of multiple machine learning approaches was employed; however, a U-Net variant was used to first segment the lesion, and features were extracted at two scales: from the whole image level and from the cropped lesion area after segmentation. Features extracted included hand-designed features, sparse codes, and deep learning features from AlexNet, ResNet, as well as the fully connected bottleneck layer from the U-Net used for lesion segmentation. Compared to the average of eight expert dermatologists on a subset of 100 images from the ISIC 2016 test dataset, the ensemble system achieved 76% accuracy, versus 70.5%. On the entire test dataset, a 0.649 AP was achieved, with a 0.843 AUC.

Notable advancements for analysis of clinical photographs began to be developed around the same time frame. For example, a standardized dataset for clinical photography, called SD-198, containing 6,584 images across 198 disease classes, was released, along with baseline experiments incorporating a variety of classical computer vision approaches and early convolutional neural networks (Sun et al. 2016). Highest performances were reported at approximately 50% multiclass accuracy.

In 2017, the largest scale joint clinical photography and dermoscopy dataset, containing 129,450 clinical images and 3,374 dermoscopy images, with 2,032 different disease diagnoses arranged in a hierarchical taxonomy, was conducted (Fig. 5). This work studied the performance of the GoogLeNet Inception v3 architecture in this setting and compared against the performance of expert clinicians (Esteva et al. 2017). Data was acquired from a variety of sources, including, Stanford hospital, the ISIC Archive, and the Edinburgh Dermofit Library. Around 127,463 images were split for training and the remaining 1,942 images used for test. In ninefold training validation, the algorithm was tasked to classify the first two hierarchy levels, including three disease states of benign lesions, malignant lesions, and non-neoplastic lesions, followed by nine disease states. For the first validation task, the algorithm achieved 72.1% accuracy, and on the second task, the algorithm

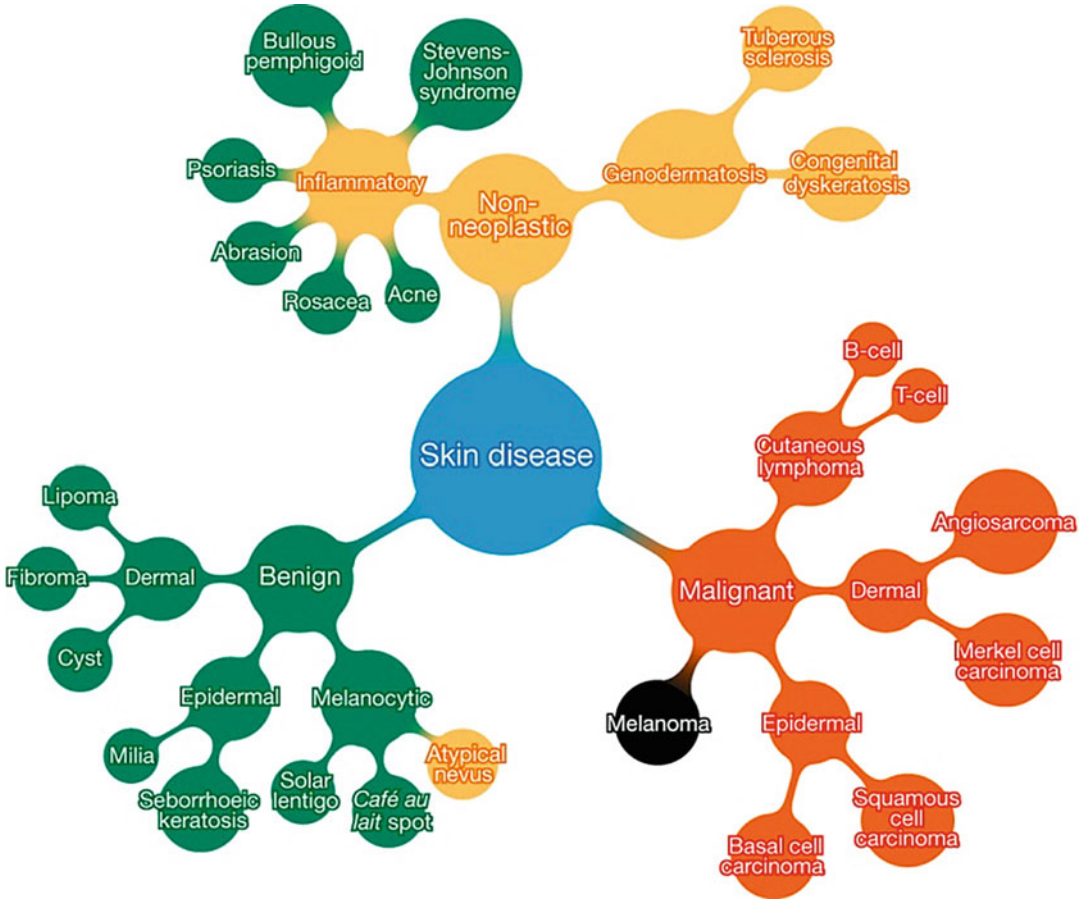

Fig. 5 A partial example of the hierarchical taxonomy constructed from 2,032 disease diagnoses and labels. (Reproduced from Esteva et al. 2017)

achieved 55.4%. Two dermatologists tested on a subset of the training data were measured at 65.56% and 66.0%, and 53.3% and 55.0%, respectively. Subsequently, a direct head-to-head comparison between algorithms and 21 dermatologists was conducted on a subset of the test dataset. The tasks included detection of carcinoma from clinical images (135 total), melanoma from clinical images (130 total), and melanoma from dermoscopy (111 total), with algorithm ROC curves falling consistently above average dermatologist operating points (Fig. 6).

Between 2017 and 2018, the number of published works on skin image analysis in dermoscopy and clinical photographs grew remarkably, in part due to the organization of the following three events: (1) The 2017 ISIC Challenge of Skin Image Analysis for Melanoma Detection at the International Symposium on Biomedical Imaging 2017, (2) The Special Issue on Skin Lesion Image Analysis for Melanoma Detection in the IEEE Journal of Biomedical and Health Informatics (JBHI), and (3) the ISIC Skin Image Analysis Workshop and Challenge at the Conference for Medical Image Computing and Computer Aided Intervention (MICCAI) 2018, hosted in Granada, Spain.

At the 2017 ISIC Challenge, 593 registrations, 81 presubmissions, and 46 finalized submissions were received across three tasks of lesion segmentation, dermoscopic feature detection, and disease classification. All finalized submissions

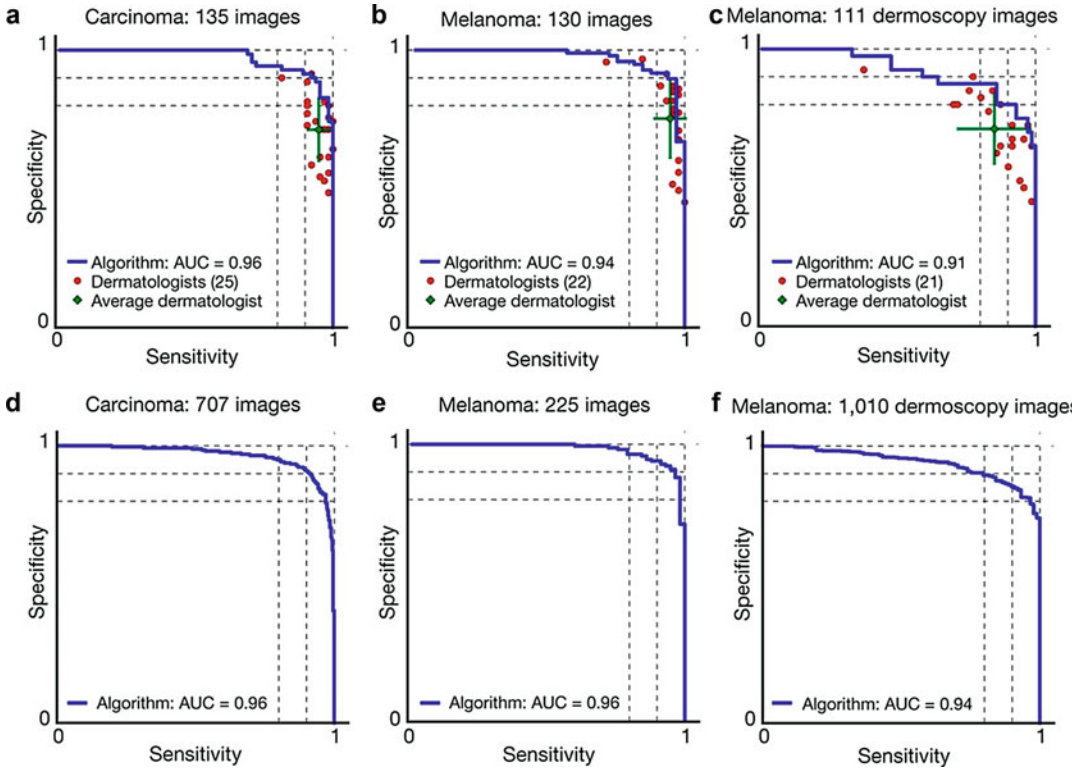

Fig. 6 Performance of convolutional neural network versus 21 dermatologists on binary classification of lesions: "biopsy/treat versus reassure." (**a**) It represents the performance of the dermatologist cohort and the algorithm on clinical images of keratinocyte carcinomas versus benign lesions, while **b** and **c** represent algorithm and cohort performance on 130 clinical and 111 dermoscopic images of pigmented lesions, respectively. The *bold line* represents the algorithm's performance at varying diagnostic thresholds. In all three tests, the AUC is greater than 0.9, with performance comparable to the average dermatologist (green point). (**d**–**f**) It represent the algorithm's performance on an expanded test set, with comparable performance. (Reproduced from Esteva et al. 2017)

required the inclusion of a four-page manuscript describing the methodology behind the submission. For the disease classification task, participants were asked to build algorithms to discriminate between three disease categories: melanoma, seborrheic keratosis, and nevus. The winning submission for the disease classification task (Matsunaga et al. 2017) used an approach that first normalized inputs, then input augmented samples into parallel ensemble 50-layer ResNets. Two classifiers were created, one for each disease state of the challenge (Seborrheic Keratosis, Melanoma). The outputs were fed into a simple logic system using thresholds that take into account age and gender information when available. Codella, Gutman, et al. performed a meta-study that demonstrated fusions of all participant submissions outperformed any single system alone (2018b).

In the Special Issue on Skin Lesion Image Analysis for Melanoma Detection in JBHI, 12 works were published, including 2 on melanoma classification from dermoscopic images (Gonzalez Diaz 2018; Kawahara et al. 2018) that will be covered here. The remainder of the works covered segmentation (6) (Yuan and Lo 2017; Riaz et al. 2018; Guarracino and Maddalena 2018; Navarro et al. 2018; Jahanifar et al. 2018; Li et al. 2018a), feature extraction (3) (Saez et al. 2018; Sabbaghi et al. 2018; Kawahara and Hamarneh 2018), image registration for lesion tracking using total body photography (1) (Korotkov et al. 2018), and diagnosis in microscopy (Argenziano et al. 1998).

In DermaKNet (Gonzalez Diaz 2018), dermoscopic classification of melanoma is carried out in conjunction with both segmentation and dermoscopic feature classification. Images are first segmented using a fully convolutional network (FCN). The segmented images then go through a cropping and data augmentation step, which is then fed to a dermoscopic structure segmentation network (DSSN), which is trained with weak image-level annotations rather than image masks. Finally, the diagnosis network takes the previous steps as input and computes a diagnosis, using a variation of a ResNet architecture. The technique was evaluated on the 2017 ISIC Challenge and achieved state-of-art results for seborrheic keratosis and average AUC among all disease classes.

In the work by Kawahara et al. (2018), classification is carried out in conjunction with automatic extraction of 7-point checklist criteria (Argenziano et al. 1998), using a multimodal dataset consisting of 1,011 cases containing both dermoscopic and clinical close-up images, as well as metadata (gender, lesion location, and lesion elevation). A multimodal multitask objective loss function is employed to train on various combinations of modalities, making the approach robust to potentially missing data. In addition, the method can produce a melanoma classification score either directly from classification network outputs or as a result of inference from the 7-point checklist. While direct classification achieves the best performance (AUC 86.3), inference surprisingly performs competitively (AUC 81.6), making this perhaps the first interpretable disease diagnosis approach in this field.

At the 2018 ISIC Challenge at MICCAI 2018, approximately 900 users registered for data download, 115 submitted to the lesion segmentation task, 25 submitted to the lesion attribute detection task, and 159 submitted to the disease classification task, all with supplied manuscripts describing each the approaches (https://challenge2018.isic-archive.com/). For the disease classification task, participants were asked to build algorithms to discriminate between seven disease categories, including intraepithelial carcinoma, basal cell carcinoma, benign keratotic lesions, dermatofibroma, melanoma, melanocytic nevus, and vascular lesions. Over 10,000 images were made available for training, and 1,511 images were held-out for testing (Tschandl et al. 2018c). The winning top performing submission (Nozdryn-Plotnicki et al. 2018) achieved a normalized multiclass accuracy (equivalent to the mean sensitivities for every class) of 88.5%. This approach utilized the given training dataset (10,015 images), data from the ISIC Archive (4,163 images), as well as a proprietary dataset (33,644 images). In addition, 19 different models were trained on this data, including variants of ResNet, DenseNet, and others. The outputs of each of the models were fused together in a meta-learning approach. Classification scores were reweighted to enforce a balanced prior distribution among classes.

At the 2018 ISIC Workshop at MICCAI 2018, 10 papers unrelated to the challenge were accepted and published in proceedings. Three papers covered the topic of lesion segmentation (Li et al. 2018b; Venkatesh et al. 2018; Vesal et al. 2018), two papers explored the generation of synthetic images using a recent technology referred to as Generative Adversarial Networks (Bissoto et al. 2018; Baur et al. 2018), two papers covered nonmelanoma disease classification (Burlina et al. 2018; Pal et al. 2018), and three papers covered melanoma classification (Gu et al. 2018; Perez et al. 2018; Thandiackal and Goksel 2018).

Additional works related to melanoma detection have continued to be published across other major medical imaging conferences and workshops. One common thread being explored more recently is the development of classification systems that are interpretable or provide some evidence or justification for decisions that can be independently verified by the user. Aside from Gonzalez Diaz (2018) and Kawahara et al. (2018), which have been previously discussed, (Yang et al.) presents a system for classification developed on features meant to mimic manual measurements taken by clinicians, such a asymmetry, color, texture, border irregularity. Experiments were performed on the SD-198 dataset (Sun et al. 2016). New state-of-the-art performance was achieved at 57.62% accuracy. In Ge et al. (2017), a multimodal neural network architecture was

proposed that incorporated class activation maps (CAM) to localize image regions most critical for resultant classification decisions. In Sadeghi et al. (2018), a content-based image retrieval (CBIR) was studied for its efficacy of improving the diagnostic capability of novice users, and Tschandl et al. (2018a) showed equivalent diagnostic accuracy of CBIR to softmax-based classification. In Noel, Codella, Lin et al., a CBIR system is built that can jointly learn from both disease labels of images as well as additional annotations of similarity between images as defined by untrained human observation (2018c). For a given query, the CBIR system both returns similar images as well as activation maps highlighting the regions of images used to measure similarity. Results demonstrated that using both sets of annotation, melanoma classification performance improves according to both criteria of classification AUC, as well as human-measured similarity of results.

The Role of Artificial Intelligence in Melanoma Diagnosis: The Consumer Perspective

With the refinement of AI and the frenetic pace of mobile application (app) development, early detection of melanoma has fallen directly into the hands of consumers. According to a Pew Research Center survey in January 2018, 77% of adults in the United States own smart phones. Ownership rates exceeded 90% in three groups: persons aged 18–29 years, those with annual household incomes >$75,000, and college graduates (Mobile Fact Sheet). As of August 2017, 526 dermatology apps were available for Apple, Android, and Windows users. This total represented growth of more than 80% since the last comprehensive review in 2014. Ninety (17%) of those apps pertained to self-surveillance and diagnosis (Flaten et al. 2018). A 2014 survey estimated that 1 in 5 dermatology patients under the age of 50 years had used a smart phone to diagnose a skin problem. Of those who tried to self-diagnose, they most commonly sought information about skin cancer, moles, or concerning spots. Interestingly, these attempts at self-diagnosis in young patients did not correlate with distance traveled to the clinic or insurance status (Wolf et al. 2015).

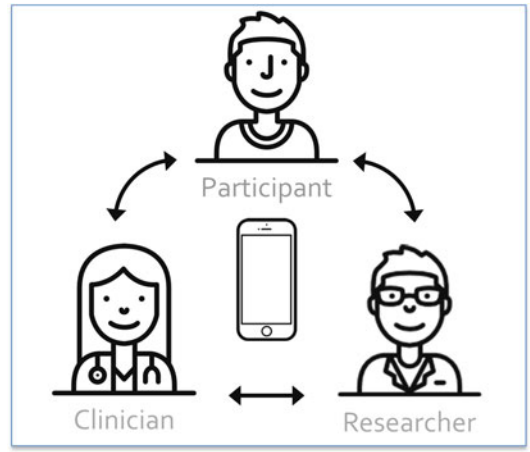

Fig. 7 Mobile apps designed to diagnose melanoma early have the potential to benefit patients, clinicians, and researchers

Mobile technology designed to provide lesion assessments has the potential to improve the dynamic among consumers, clinicians, and researchers (Fig. 7). Apps equipped with AI risk stratification algorithms may flag concerning lesions and alert the consumer to seek care. Indeed, apps with validated algorithms could serve as inexpensive triage tools to ensure all potentially malignant lesions are seen in person (Chao et al. 2017). Additionally, these apps could streamline clinic visits by giving consumers and providers a quantitative, impartial measure on which to make informed decisions. With the rich dataset of images gathered from this experience, these apps could foster further research to allow the process to continue to improve. Successful future apps will need to optimize consumer safety without impeding the opportunity and duty to expand care to those with limited access to dermatologists.

Consumer Opinion

To date, no published studies have directly examined the consumer's opinion on the role of

AI in diagnosing their skin lesions. However, the abundant research surrounding the consumer perspective on teledermatology and teledermoscopy may provide insight. Teledermatology and teledermoscopy remove the barriers of time and space by sending images to experts for evaluation. Many studies have demonstrated that mobile teledermatology and teledermoscopy are feasible and well-accepted (Horsham et al. 2016; Spinks et al. 2016; Mounessa et al. 2018; Warshaw et al. 2011). In fact, some consumers have reported that they are as satisfied with telemedicine care as they are with face-to-face dermatology examination (Coates et al. 2015). Rapid diagnosis, ease of use, and increased awareness of the importance of skin self-monitoring have traditionally been drivers of consumer satisfaction (Horsham et al. 2016). As AI algorithms in apps become more mainstream, further research will be essential to address the knowledge gap that exists surrounding consumer perception of AI for melanoma diagnosis.

Barriers to Implementation and Limitations

Despite rapid growth of mobile technology, several important barriers have prevented routine implementation of AI algorithms for lesion risk stratification, including technology literacy, access, trust, and confidentiality (including compliance with US government regulations as discussed below). Although technology literacy is improving, smartphone ownership remains inversely associated with age. Only 73% of those 50–64 years of age and 46% of those over 65 years of age owned smartphones in early 2018 (Mobile Fact Sheet). A number of studies have identified difficulty downloading and using apps as a major obstacle for those in the older population (Dehzad et al. 2014; Hall and Murchie 2014; Horsham et al. 2016). Additionally, if smartphone ownership can serve as a surrogate marker for access to this revolutionary technology, some traditionally disadvantaged populations are still not being reached. Only 65% adults in rural areas own smartphones. Decreased rates of ownership also track with lower education levels and socioeconomic status. Of note, smart phone ownership does not appear to significantly vary by race (Mobile Fact Sheet)

Trust is another important limitation to adoption. Nearly half of participants in a survey by Horsham‘s group reported that they did not completely trust telediagnosis when compared to in-person dermatology examination (Horsham et al. 2016). Although Anyanwu et al. found 27% of dermatologists reported patient concerns about the storage and sharing of their photographs, the study comprised a small cross-sectional survey in Philadelphia (Anyanwu and Lipoff 2015). Several other studies have raised concerns about the security and confidentiality of submitted photos (Chao et al. 2017; Hamilton and Brady 2012). Creators of the Molemapper™ app discovered that 26% of images submitted for research had identifiable characteristics of the app users that required removal from the public data set (Webster et al. 2017). The US government is now working through two of its regulatory agencies to protect consumers as discussed below.

Lesions selected by consumers and the quality of the images they submit for evaluation can impair the ability of these apps to accurately risk stratify moles and melanoma. Janda et al. report that the lay members of their teledermoscopy study primarily selected benign lesions to photograph rather than suspicious lesions (2014). Furthermore, dermatologists conducting in-person follow-up examinations detected additional high-risk lesions that had not been identified by the patients themselves (Janda et al. 2014). Even medical personnel outside of dermatology have difficulty selecting appropriate lesions for telediagnosis (Gendreau et al. 2017). Melanoma can arise in any location, but certain body sites are extremely difficult to visualize and photograph, including the ears, nail bed, mucosa, genitals, and hair-bearing areas (Horsham et al. 2016; March et al. 2015; Janda et al. 2014). Lesions that are ulcerated, on tanned skin, or in the midst of several background lesions can also prove troublesome for AI algorithms (Rat et al. 2018). Ten percent of images submitted to the Molemapper™ study were of insufficient quality for research and

many images were too blurry or did not contain skin (Webster et al. 2017). Until consumer app-based examinations improve in both lesion selection and photo quality, machine learning and AI will fall short of histopathology as the current gold-standard diagnostic tool.

Potential Harms

Initial worry about a skin lesion may lead a person to seek assistance from an app. However, perpetuating and increasing consumer anxiety when an app incorrectly categorizes a benign lesion as high-risk remains a major concern. In this situation, the psychological and emotional burden may be excessive while waiting for formal evaluation (Wang et al. 2017a; Chao et al. 2017). Wolf et al. surveyed patients who had recently used mobile technology to evaluate their skin lesions. The team asked participants to rate their anticipated degree of worry on a five-point scale if the Internet or smartphone app flagged a mole as high risk. Overall, the median worry rating increased from three to five, indicating the profound impact these apps have on consumer anxiety. Importantly, this study did not quantify relief from worry in the case of individuals who received reassurance that the lesion submitted was low-risk (Wolf et al. 2015).

Even more concerning is the situation in which an AI algorithm incorrectly labels a high-risk lesion as benign, which could potentially lead to life-threatening delays in melanoma diagnosis. The historically poor sensitivity and specificity of these apps can lead to false reassurance of consumers and have sparked an outcry from the medical community (Hamilton and Brady 2012; Wolf et al. 2013, 2015; Wang et al. 2017a; Chao et al. 2017; Rat et al. 2018; Robson et al. 2012; Dorairaj et al. 2017; Ngoo et al. 2018; Ferrero et al. 2013; Maier et al. 2015; Zouridakis et al. 2015). Experts worry that AI algorithms have been too rapidly deployed to the consumer without involvement of dermatologists and appropriate validation. In a critical evaluation of dermatology app advertising material, one group found that 36% of apps failed to mention involvement of any medical team in the development (Hamilton and Brady 2012). Five published studies that analyzed apps exclusively advertising the capability to diagnose melanoma or risk stratify moles were unreliable, with wide-ranging sensitivities (6.8–80%) and specificities (9–100%) (Wolf et al. 2013; Robson et al. 2012; Dorairaj et al. 2017; Ngoo et al. 2018; Maier et al. 2015; Rat et al. 2018). Some apps incorrectly reported 30% or more of melanomas as low-risk lesions (Wolf et al. 2013). One app only classified 10.8% of melanomas as high-risk lesions (Ferrero et al. 2013). As Wang and colleagues wrote in their 2016 editorial, the most important question surrounding future development and distribution of this technology is "Are we doing 'good'?"(Wang et al. 2017a).

Regulation

In February of 2015, the Food and Drug Administration (FDA) labeled mobile apps "performing patient-specific analysis and providing patient-specific diagnosis, or treatment recommendations" as regulated medical devices (U.S. Food and Drug 2015). Concurrently, the Federal Trade Commission (FTC) cracked down on app producers that made unfounded claims to diagnose melanoma. MelApp and Mole Detective settled with the FTC for $17,963 and $3,930, respectively (Federal Trade Commission 2015). The commissioner of the FDA, Scott Gottlieb, has underscored the importance of fostering "greater innovation in digital health, including the use of Artificial Intelligence" (Gottlieb 2018). In the spring of 2018, the FDA launched the Digital Health Action plan that outlined a pilot for a "reimagined" expedited review process for mobile health technology (U.S. Food and Drug 2018). Despite these initial steps, no formal regulatory structure exits and there are no quality standards governing development of these apps (Chao et al. 2017). As the AI algorithms improve and this technology becomes more widely accepted, government officials, healthcare providers, and researchers must work together to ensure the proper regulatory framework is in place to prioritize consumer safety and outcomes above all else.

Additional Considerations and Challenges Ahead

Within this chapter, we provided an overview on current automated diagnostics through artificial intelligence, its potential roles, and its current state of application. To conclude the topic, we will provide a brief overview of challenges and additional considerations (see Marcus (2018) for an in-depth review).

Comprehensive Data

Current artificial intelligence systems, among these CNNs are limited to prediction of classes they have seen during "training." Since all current systems require training data, many of the previous advances in the field were built upon large, well annotated image collections. One of the best known collections in the field of modern ML, ImageNet, contains over 14 million hand-labeled images, currently comprising more than 20,000 categories (Russakovsky et al. 2015). Obtaining and labelling hundreds or thousands of pictures as dog, cat, or fish lends itself to crowd sourcing and can be easily validated. As the task becomes harder, such as labeling individual breeds of dogs or cats, the complexity of the task and availability of domain experts can become limiting.

Thus, until recently, public datasets were limited in size and mostly confined to nevi and melanomas (e.g., PH2 [Mendonca et al. 2013]), and as a consequence, automated skin lesion analysis has historically been performed on pigmented skin lesions only (Dreiseitl et al. 2009). Newer dataset initiatives incentives, such as the ISIC-archive (see chapter "Current State of Imaging Databases") or the HAM10000-dataset (Tschandl et al. 2018c) try to close this gap for pigmented skin lesions, but probably still have too few – or too biased – examples of common benign diseases such as dermatofibromas and angiomas.

A bigger challenge remains a good collection of nonpigmented skin lesions. The ML system of a recent study (Tschandl et al. 2018b) performed well only on common malignant diseases such as basal cell carcinomas prevalent in their dataset. Other common diagnoses such as nonpigmented dermatofibroma or clear-cell-acanthoma were not predicted at all because they were simply missing in the training data.

As recently discussed, algorithms will likely produce biased or erroneous results when applied generally (Adamson and Smith 2018; Takeshita 2018), which may also be the case if a system has not been trained on images obtained from patients of different skin types/colors. Therefore, in early adoption, and until the applied clinical performance is robust enough, the proposed algorithms will likely require inclusion criteria for lesion selection.

Several major trends may lead to more robust image databases in the future. An increasing number of dermatologists and institutions are using photography for the diagnosis and follow-up of skin lesions (Milam and Leger 2018), creating a much larger pool of clinical and dermoscopic images. These images are expected to have more accurate metadata tagging due to the implementation of standard formats such as the Digital Imaging Communication in Medicine (DICOM) format. This format was specified by the DICOM standard, an international, interoperability standard for the storage and transmission of digital medical images. It includes two components: the first is text-based metadata describing patient, study, acquisition, and image attributes, and the second is the pixel data of the image (Finnane et al. 2017). In addition, there ongoing international effort to standardized image acquisition at the community and institutional level (Finnane et al. 2017; Katragadda et al. 2016). Finally, resolving the regulatory and legal issues will remove the obstacle of adding more images to the databases from variable sources. Together, these trends will make it possible to include many more accurately tagged images of variable skin lesions from different skin types, cultures, and geographies and will help create larger and more comprehensive image databases, ultimately leading to enhanced AI diagnostic systems for melanoma diagnosis.

Resilience and Generalizability

Current deep learning constructs can be fooled by small, and sometimes perceptibly insignificant, perturbations to images, mistakenly producing high probability results for incorrect classifications (Szegedy et al. 2013). Known as "adversarial examples," Nguyen et al. provides many examples of these inaccurate, and high confidence classifications for images, and illustrates the insidious nature of relying on hidden features in cases where reliability matters (2015). One such example of misclassification leading to potentially disastrous results was illustrated by Eykholt et al. where images of stop signs were altered in such a way where most humans would still recognize the sign, yet it was identified as a speed limit sign by a deep learning system (see Fig. 8) (2017). If we intend to use such algorithms in cases where clinical decisions are being made, human review is still essential.

Generalizability of systems is another significant problem. As an example, deep learning systems were successfully built to win in multiple video games (Mnih et al. 2015), yet similar algorithms failed miserably when challenged with minor changes to game structure, thus illustrating the inability to extrapolate success in response to minor game perturbations (Kansky et al. 2017). This has clear implications for the analysis of dermatology images in a clinical setting. In most cases, numerous transformations occur between the photons hitting the imaging sensor and the final saved image. Since it is common to use "off the shelf" cameras to obtain clinical images, compression settings and built-in preprocessing steps can potentially induce imperceptible changes to the human eye that may produce unpredictable changes to machine-generated predictions.

Clinical Integration

It is improbable that an automated classification system on digital images will meaningfully outperform experienced physicians in a routine skin examination, especially in specialized centers that have demonstrated excellent diagnostic performance when implementing dermoscopy assessment (Salerni et al. 2012). Therefore, in this setting, the benefit of an automated system will be less in increasing accuracy and more in enhancing efficacy and throughput of patients by prescreening straightforward cases, or improving the use of total-body photography. On the other hand, for general providers or physician extenders, like a general practitioner confronted with an occasional patient with a skin lesion in question, or assessment of multiple lesions in a given individual, accuracy is more important than high-volume screening. Safety and interpretability of confidence become a bigger issue, as the user is less experienced in the topic, making it less probable that an error of an automated system will be uncovered through follow-up examinations. These dangers can probably be overcome if a CNN-based classifier is not used for a final diagnoses but rather for referral stratification, similar to teledermatology as used by Börve et al. (2015).

Given previously mentioned shortcomings, as well as the inability of CNNs to dynamically integrate clinical findings, situations or patient

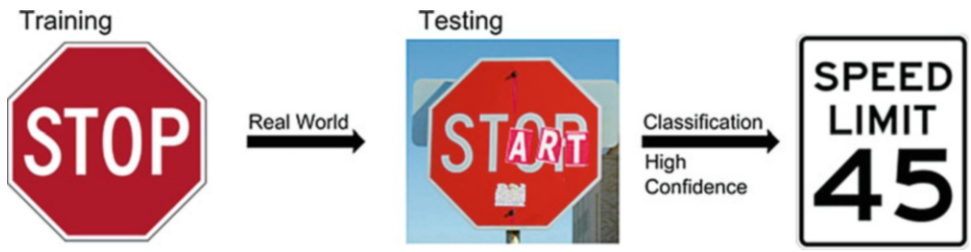

Fig. 8 Application of image classification systems into the real world may be hindered by unexpected variations not represented within the training set. High confidence misclassification may lead to serious consequences such as traffic accidents or medical malpractice. (Figure adapted from Eykholt et al. 2017)

preferences, the primary objective of AI in skin cancer detection today is to "augment" our current practice and not necessarily to replace the role of the medical provider. Therefore, it is of critical importance for ongoing and future AI efforts to specifically assess its influence on medical decision-making, practice flow, and patient outcome and satisfaction. A critical element in this development process relates to the need to enhance medical provider performance through a feedback mechanism based on accurate AI results. Only then will AI be effectively implemented in our field.

This refers to one last, pressing, problem which is the lack of clinical testing of CNN architectures in the field of dermatology. It remains unclear whether reported claims of "expert-level" or "superhuman" performance in experimental studies actually transform to clinical practice. One previous prospective clinical trial using a neural network to analyze a digital dermoscopic image (Dreiseitl et al. 2009) found a marked decrease of diagnostic values for the binary distinction of nevus versus melanoma. An FDA-approved device with promising experimental data eventually failed in clinical practice (see Excursus: MelaFind). Until further controlled and prospective studies are commenced with current state-of-the-art CNNs, as has occurred in the field of ophthalmology (Abràmoff et al. 2018), we will not know if promises from experimental studies hold and prove of benefit in practice.

Conclusion

Moving forward, the challenges in the clinical application of AI to melanoma detection are as follows: (1) the continued creation of larger, more comprehensive, and less biased datasets, both for training and evaluation, that capture a sufficient representation of the standardized full spectrum of patients for which the system will be used (age, gender, ethnicity, genetics, actinic damage, etc.); (2) the development of AI systems that provide interpretable explanation or justification that can be independently verified by a nonexpert user; (3) the development of AI systems that are robust and self-aware in the sense of being able to recognize images or disease states for which it has not been trained sufficiently; and (4) evidence-based, impactful, and safe implementations in specified clinical scenarios.

Excursus: MelaFind

MelaFind was an FDA-approved device developed to aid dermatologists in their decision to biopsy suspicious lesions which received FDA approval (Fink et al. 2017). MelaFind generates a score based on a proprietary algorithm with scores ≥ 2 representing a probability of melanoma greater than 6% (Winkelmann et al. 2014). Its high sensitivity and low specificity for malignant melanoma detection in the prospective study, 98.4% and 10.5%, respectively, showed promising clinical implications (Monheit et al. 2011). A study by Wells et al. replicated high sensitivity rates but showed lackluster specificity measurements when compared to dermatologists (estimated biopsy specificity of 0.08 vs. 0.43, respectively) (2012). MelaFind recommended biopsy on 44 out of 47 lesions, with a sensitivity of 96% (Cukras 2013). A later study by Fink et al. demonstrated a melanoma detection specificity in biopsied lesions of 5.5%, and diagnostic accuracy of 2.3% using a cutoff score of 2 (2017). Given its high sensitivity and low specificity, it was suggested that MelaFind may be a good candidate as a clinical pretest to rule out melanoma. This was consistent with results from the pilot study suggesting that MelaFind was more sensitive than dermatologists, 98.4% versus 78% (Monheit et al. 2011). On March 2017, notice was sent to all 90 MelaFind owners that support would no longer be offered for the device after September 2017 (STRATA Skin Sciences 2017). While MelaFind likely did not incorporate modern deep learning algorithms, it highlights potential issues related to determining the appropriate balance of sensitivity and specificity for a clinically useful device, as well as how to incorporate machine-based assessments into the clinical decision process.

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Molecular Diagnostics in Melanocytic Neoplasia

30

Jeffrey P. North

Contents

Abstract

Ancillary molecular tests have been developed to assist in the diagnosis of histopathologically ambiguous tumors and as prognostic tools in melanoma. These include DNA-based assays such as comparative genomic hybridization (CGH), fluorescent in situ hybridization (FISH), and next generation sequencing, as well as RNA-based tests including gene expression profiling and microRNA analysis. Protein-based techniques such as immunohistochemistry and mass spectrometry are also available, with immunohistochemistry representing the mostly widely available and highly utilized modality in melanoma diagnostic testing. Each type of test has strengths and limitations. Many of them are expensive (>$1000) and require proper resources and expertise to perform. Familiarity with the available testing options combined with knowledge of genetic and histopathologic features of the various types of melanocytic tumors allows for judicious use of molecular testing to increase diagnostic accuracy and provide valuable prognostic information. Molecular tests can also be used to guide treatment decisions in the expanding era of precision medicine where

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treatment is based on individual tumor characteristics rather than summary clinical trial data.

Keywords
Comparative genomic hybridization · CGH · Fluorescent in situ hybridization · FISH · DNA sequencing · Gene expression analysis · Immunohistochemistry · MicroRNA · Mass spectrometry

Introduction

Accurate diagnosis in melanocytic neoplasia requires incorporation of clinical features (patient age, lesion size, and clinical evolution), histopathologic characteristics, and genomic abnormalities. Despite continued research developments that provide insight into various distinguishing features between melanocytic nevi and melanomas, there is considerable variability among pathologists in the diagnosis of melanocytic tumors, with discordance rates ranging from 15% in routine referral cases, to as high as 38% when focusing on histopathologically challenging biopsies (Shoo et al. 2010; Farmer et al. 1996). This diagnostic imprecision continues to hinder optimal treatment of patients. Ancillary molecular tests have been developed to assist with histopathologically ambiguous tumors in hopes of attaining more accurate diagnoses. While some experts have advocated the idea of a dichomatous diagnostic world in which melanocytic neoplasms are either benign melanocytic nevi or malignant melanoma, increasing evidence supports the paradigm that melanocytic neoplasia progresses through a spectrum that begins with unequivocally benign nevi initiated by a single activating mutation or translocation in a proliferative oncogene (Shain et al. 2015). Additional mutations in oncogenes and tumor suppressor genes (e.g., TERT promoter, CDKN2A) produce intermediate neoplasms with varying degrees of histopathologic atypia that generate high degrees of diagnostic discordance. Unequivocal melanomas then develop with accumulation of additional mutations and chromosomal aberrations.

Multiple molecular tests are now available to assist in the diagnosis of ambiguous melanocytic neoplasms that cannot be reliably classified based on clinical and histopathologic features alone. These include DNA, RNA, and protein based platforms. While there is overlap in the genetic changes that generate most types of melanocytic tumors (e.g., MAP kinase pathway activation), continuing research of the genetic landscape of melanocytic neoplasia indicates there is significant heterogeneity in the evolution of melanocytic neoplasms. Given this heterogeneity, caution is advised for any test claiming the ability to distinguish between all types of melanocytic tumors. Each molecular test available has strengths and limitations. Knowledge of the genetic changes that correlate with histopathologic phenotypes can help inform decisions on which molecular test to obtain for various tumor types.

DNA-Based Testing

Comparative Genomic Hybridization (CGH)

The vast majority of melanomas show genetic instability in the form of multiple chromosomal gains and losses indicative of failure of the innate cellular checkpoints that maintain a normal diploid state (Bastian et al. 1998). These chromosomal gains and losses are not randomly distributed in the genome, but are selected for when growth advantage is conferred by gain of an oncogene or loss of a tumor suppressor gene. Gains at chromosomes 1q, 5p, 6p, 7, 8q, 11q, 17q, and 20 are most common among melanoma, while losses commonly occur at chromosomes 6q, 8p, 9p, 9q, 10q, and 11q (Fig. 1) (Bastian et al. 1998). Solitary chromosomal abnormalities can be seen in subsets of nevi (e.g., 11p gain in *HRAS* mutant Spitz nevi (Fig. 2), and loss of the BAP1 locus on chromosome 3p in BAP1-inactivated melanocytomas (BAP1-inactivated Spitzoid tumors (Fig. 3)), but multiple gains and losses are typical of melanoma and are generally not found in melanocytic nevi.

Comparative genomic hybridization (CGH) is one method for copy number alteration (CNA)

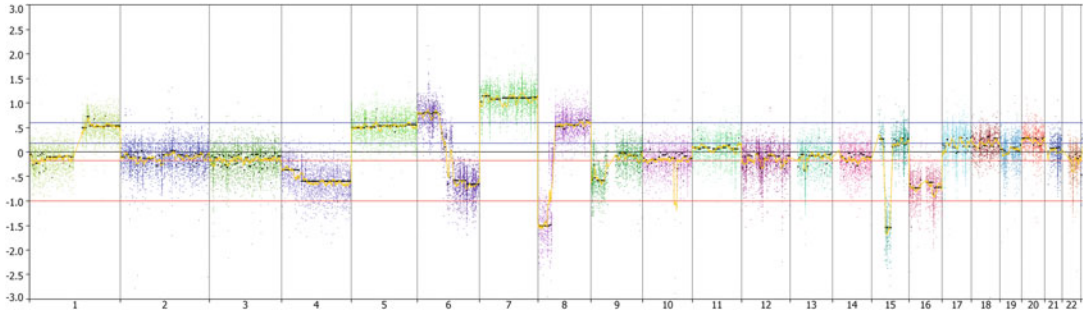

Fig. 1 CGH analysis of chromosomal copy number alterations (CNAs) in melanoma. Chromosome number/location is listed along the X axis. Deviations below the 0 value on the Y axis indicate copy number loss, while those above 0 indicate copy number gain. This tracing illustrates many of the common melanoma CNAs with chromosome 1q, 6p, 7, 8q, and 20 gain, and chromosome 6q, 8p, 9p loss. Additional losses of chromosome 4, 15, and 16 are also present

assessment. CGH involves fluorescent labeling of normal control diploid DNA in one color and tumor DNA in a different color. The differentially labeled DNA samples are then hybridized to microarrays containing DNA probes covering the genome at different densities. Tumor and control DNA compete for binding sites on the array, and neoplasms with copy number gains show brighter tumor signals at array probes corresponding to regions of copy number gain compared to the control DNA. Brighter control DNA signal intensity is seen at array probes corresponding to regions of copy number loss in the neoplasm. DNA contamination from non-neoplastic cells in the tumor sample can mask CNAs. Microdissection of the tumor cells from the surrounding tissue is recommended before DNA extraction to minimize the amount of non-neoplastic DNA and provide optimal results. Biopsies with low tumor volume or heavily inflamed neoplasms are best analyzed with other molecular tests such as fluorescent in situ hybridization or next generation sequencing. CGH microarrays incorporating probes for single nucleotide polymorphisms (SNP) can also be used in melanoma diagnostics (Carter et al. 2018). Such SNP arrays also provide allelic ratios to help identify copy number-neutral loss of heterozygosity, which cannot be detected with traditional CGH microarrays.

Both the number of CNAs and their chromosomal location can assist in diagnosing melanocytic tumors. As noted above, gains of chromosome 11p occur in *HRAS* mutant Spitz nevi where increased copy number of mutated *HRAS* confers growth advantage. Losses of the *BAP1* locus on chromosome 3 are seen in BAP1 inactivated melanocytomas, uveal melanoma, and blue nevus-like melanomas. The losses tend to be focused in BAP1 inactivated melanocytomas, and mainly present as loss of the entire chromosome (monosomy 3) in the latter entities. Chromosomal rearrangements involving multiple tyrosine kinases as well as *BRAF* have been identified as a common initiating event in spitzoid neoplasms and occasionally other types of melanomas (Table 1) (Wiesner et al. 2014; Yeh et al. 2019; Ablain et al. 2018). The resulting fusion genes are often subject to subsequent copy number increases. The location of CNAs at specific chromosomal loci can be a clue to the presence of such a rearrangement. For example, CNAs on chromosome 7q34 in Spitz tumors can indicate an underlying *BRAF* fusion event (Fig. 4).

Distribution and quantity of CNAs has been shown to vary based on the degree of UV exposure. One of the earliest molecular melanoma classification schemes is based on differential CNA patterns which distinguish melanomas occurring on chronically sun exposed skin, intermittently sun exposed skin, acral skin, and mucosal sites (Curtin et al. 2005). Melanomas on sun protected sites (i.e., acral and mucosal melanomas) have a particularly high number of CNAs, including high numbers of chromosomal amplifications, while their genomic mutation burden is much lower than other types of melanomas (Curtin et al. 2005; Genomic

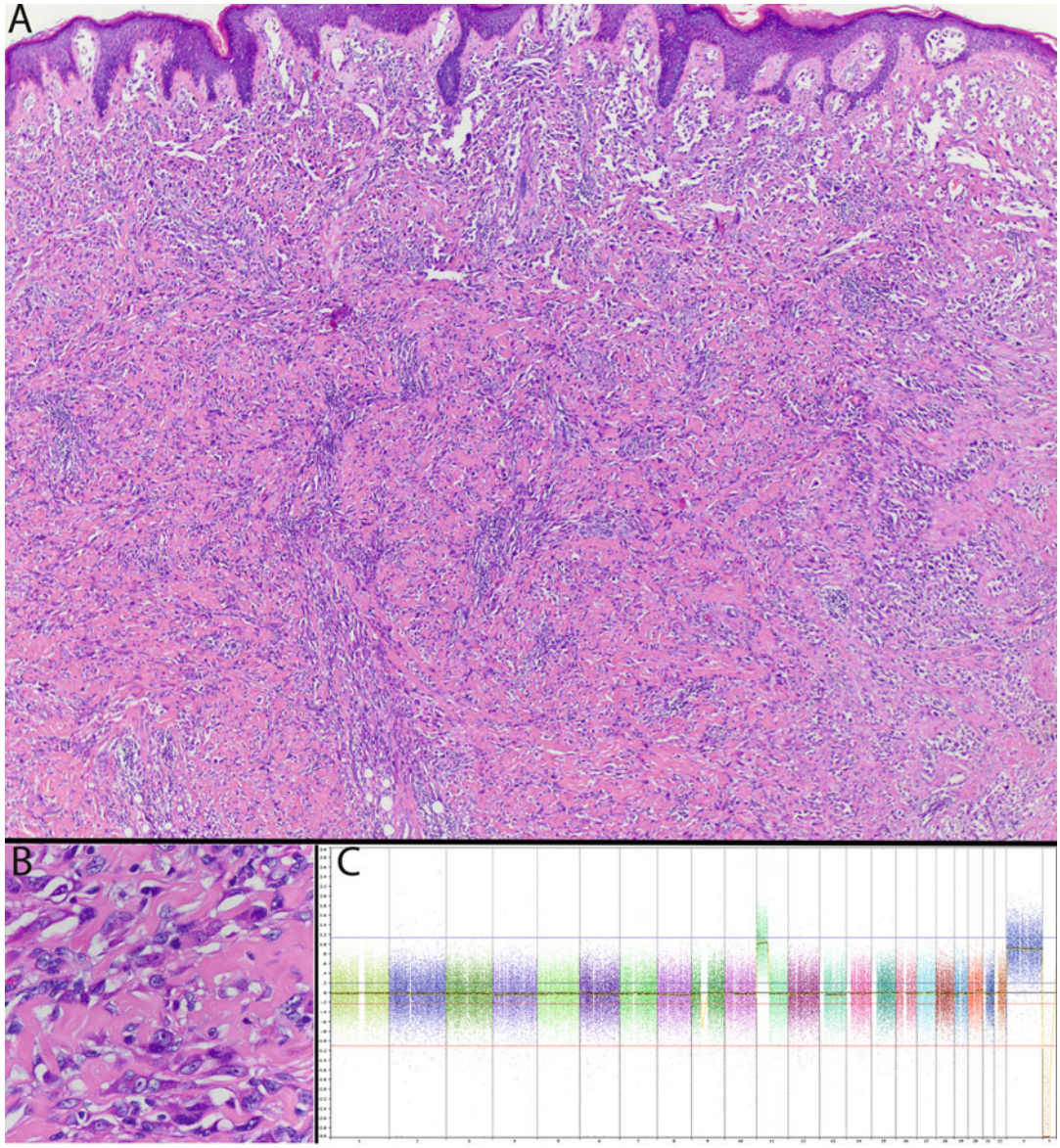

Fig. 2 *HRAS* mutant Spitz nevus in 2-month-old baby. (**a**) The neoplasm has a symmetrical appearance with prominent desmoplastic stroma (H&E 20x). (**b**) *HRAS*-mutant Spitz nevi have characteristic features including epithelioid melanocytes with sclerotic stroma and infiltrative appearance in the dermis (H&E 200x). (**c**) CGH analysis shows a single aberration with gain of chromosome 11p where *HRAS* is located

Classification of Cutaneous Melanoma 2015; Hayward et al. 2017). Ninety percent of acral melanomas have focal chromosomal amplifications involving oncogenes such as the genes encoding cyclin D1 (*CCND1* on 11q13), CDK4 (12q14), and telomerase (*TERT* on 5p15). Such amplifications are infrequent in melanomas on sun exposed skin. As these amplifications in acral melanoma can be detected early in tumor progression, including in melanoma in situ and precursor field cells (North et al. 2008), they represent a distinguishing feature of acral melanoma that can be assessed for in DNA-based assays such as CGH and fluorescent in situ hybridization (FISH).

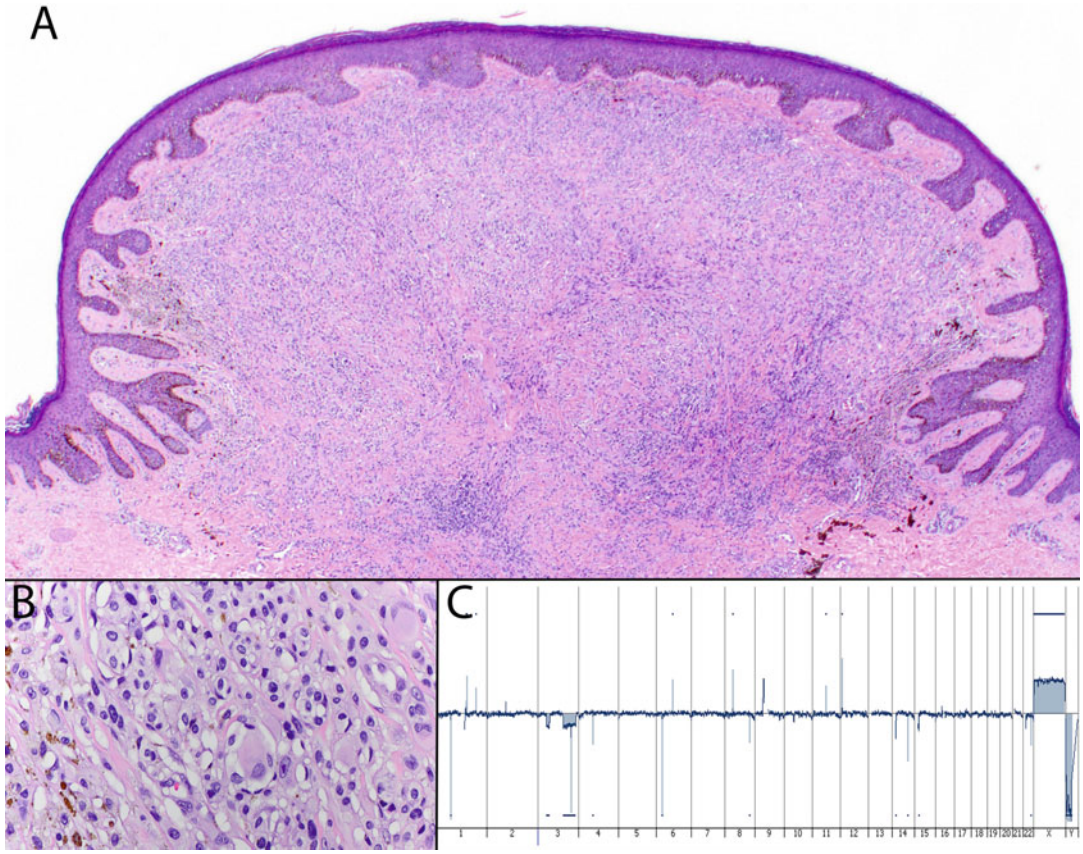

Fig. 3 Combined melanocytic nevus with BAP1 loss (BAP1-inactivated melanocytoma). (**a**) A biphasic proliferation of melanocytes is present with a large central population of amelanotic cells and small peripheral collections of pigmented melanocytes (H&E 20x). (**b**) The amelanotic melanocytes have larger nuclei, abundant eosinophilic cytoplasm, and numerous multinucleated melanocytes are present (right side). Smaller melanocytes with more pigmented cytoplasm are present on the left (H&E 200x). (**c**) CGH analysis shows solitary loss of chromosome 3 where *BAP1* resides

Table 1 Common sites of translocation in Spitz nevi and other spitzoid melanocytic neoplasms

Gene	Chromosome
ALK	2p23
BRAF	7q34
NTRK1	1q23
NTRK3	15q
MET	7q31
RET	10q11
ROS1	1q21

As molecular profiling of melanocytic tumors has progressed, increasing evidence has been found that malignant transformation of melanocytic neoplasms progresses in stepwise fashion through the accumulations of mutations, structural rearrangements, and chromosomal gains or losses that overcome cellular checkpoints that normally prevent oncogenesis. These phases of progression from unequivocally benign to partially transformed to overtly malignant can be molecularly traced in biopsies that contain melanomas with adjacent precursor nevi (Shain et al. 2015). In general, increasing numbers of CNAs parallels the degree of histopathologic atypia. This pattern of increasing CNAs has been demonstrated across various types of melanocytic tumors, including tumors of the blue nevus family. Common or cellular blue nevi show no detectable CNAs, whereas blue nevus-like neoplasms with ambiguous/atypical features have 0–3 CNAs, consistent with partial transformation. Unequivocal blue nevus-like melanomas show a

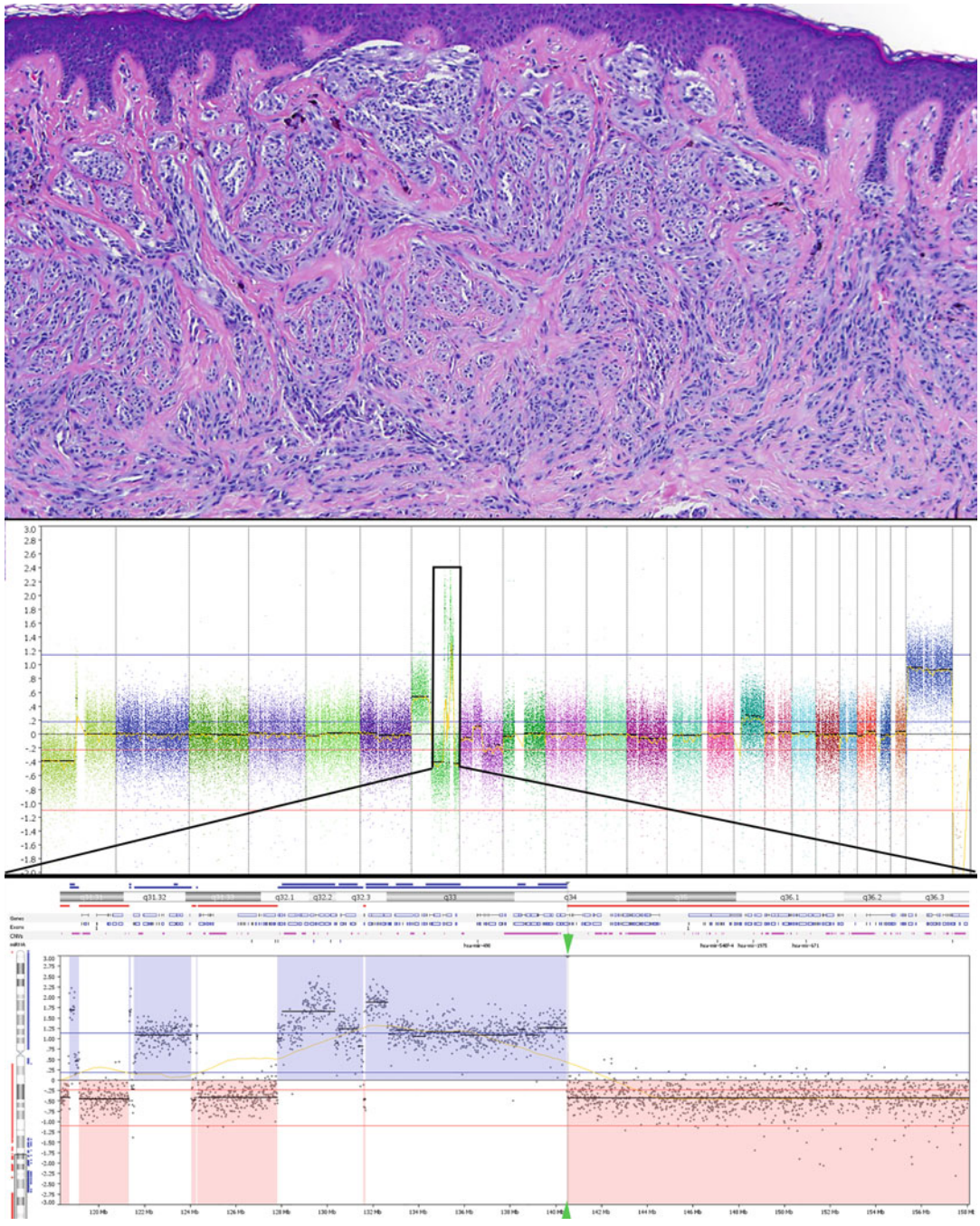

Fig. 4 Spitz tumor with *BRAF* fusion. (**Top**) Epithelioid melanocytes form nests and fascicles with prominent clefts and associated epidermal hyperplasia (H&E 40x). (**Middle**) CGH analysis shows a complex pattern of gains and losses on chromosome 7q with additional losses on chromosome 1 and 8 and gains on 7p and 15. Multiple CNAs are indicative of genomic instability and indicate a differential diagnosis of atypical Spitz tumor and spitzoid melanoma. (**Bottom**) Close up view of chromosome 7q where multiple short chromosomal gains and losses are clustered. Green arrowheads highlight locus 7q34 where *BRAF* is located. The sharp transition from chromosomal gain to loss within the *BRAF* gene is a clue to the presence of a *BRAF* gene fusion event

greater degree of genomic instability with >3 CNAs (Costa et al. 2016; Maize et al. 2005). Similar to uveal melanomas which are genetically related to blue nevi in that they share mutations in the Gαq signaling pathway, loss of *BAP1* on chromosome 3 in tumors with a blue nevus phenotype is associated with aggressive disease and poor prognosis (Costa et al. 2016).

A similar pattern of increasing CNAs has been documented in Spitz tumors, which frequently cause diagnostic uncertainty due to ambiguous histopathologic features. One study documented 0–1 CNAs in unambiguous Spitz nevi and 1–8 CNAs in atypical Spitz tumors and spitzoid melanomas (Raskin et al. 2011). Ambiguous spitzoid neoplasms are the most common tumors for which ancillary molecular testing is requested (North et al. 2014). Unfortunately, outside of a few distinct scenarios such as isolated chromosome 11p gain in *HRAS* mutant Spitz nevi (Fig. 2) and isolated losses on chromosome 3p in BAP1-inactivated melanocytomas (Fig. 3), there is a dearth of evidence regarding the reliability of ancillary molecular tests in this setting. Practically speaking, ambiguous spitzoid tumors with CGH testing showing solitary chromosomal abnormalities at loci which are not typically associated with melanoma such as chromosome gain at 11p or 7q can be regarded as benign, while spitzoid tumors with multiple melanoma-associated CNAs should be regarded as melanoma. An intermediate category of spitzoid tumors may exist in children which have small numbers of CNAs that are not commonly found in melanoma. These tumors frequently metastasize to the regional lymph nodes, but risk of metastasis beyond regional lymph nodes is low. Such atypical Spitz tumors could represent partially transformed neoplasms, but current understanding of such cases is limited.

Another setting which causes diagnostic uncertainty is the development of hypercellular, mitotically active nodules within a preexisting congenital melanocytic nevus. CGH testing of such proliferative nodules frequently shows gains or losses of entire chromosomes, particularly loss of chromosomes 7, 9, or 10, rather than the segmental chromosomal gains and losses seen in melanomas arising in congenital nevi (Bastian et al. 2002). However, a case of melanoma arising in a giant congenital nevus with only whole chromosome gains has been reported (Machan et al. 2015), and proliferative nodules with partial chromosomal losses involving chromosomes 7, 10, and 11 have also been reported (Yélamos et al. 2015a). Hypercellular nodules can also arise within congenital plaque type blue nevi (plaque type blue nevus with subcutaneous cellular nodules). In contrast to the benign proliferative nodules seen in conventional congenital nevi, CGH analysis of these nodules suggests that they often represent bona fide melanomas arising within blue nevi with classical melanoma associated CNAs such as chromosome 6p gain and 6q loss (North et al. 2012).

Prognosis

In addition to functioning as a diagnostic aid in ambiguous melanocytic neoplasms, CGH may also provide prognostic information. A study comparing 10 lethal melanomas to 10 melanomas with favorable outcomes found a relationship between number of CNAs and prognosis. Lethal melanomas had a mean CNA count of 14 compared to a mean of 2 CNAs in the nonlethal melanomas (Hirsch et al. 2012). Specific CNAs can also have prognostic implications, such as chromosome 3 loss as a poor prognostic indicator in blue nevus-like melanoma and uveal melanoma (Costa et al. 2016; Sisley et al. 1997). Chromosome 8q gain is also associated with aggressive disease in uveal melanoma (Sisley et al. 1997).

Limitations of CGH

- High cost and limited availability of CGH testing
- Limitations in assessing clonal heterogeneity within a tumor
- Relatively large amount of tumor DNA required
- Possible false negative results through normal cell contamination
- No mutation information
- Relatively long term around time (≥2 weeks)

Fluorescence In Situ Hybridization (FISH)

The discovery of recurrent CNAs in melanoma by CGH led to the development of FISH probe sets targeting those melanoma associated CNAs. In FISH, fluorescently labeled nucleic acid probes target chromosomal loci of interest and can be used to assess for CNAs and/or chromosomal translocations. These probes are hybridized to tissue sections on glass slides where fluorescent signals can be counted in individual tumor cells through a fluorescent microscope. Signals are enumerated in randomly selected nuclei in the most suspicious area of the lesion. When the percentage of nuclei with deviating signal counts exceeds the preset threshold (Table 2), the results support a diagnosis of melanoma (Figs. 5 and 6). Familiarity with the FISH technique is critical to ensure that only tumor cells are counted, and that counts from overly truncated nuclei are excluded.

The first study assessing multiple FISH probes for distinguishing nevi from melanomas evaluated FISH probes targeted to chromosomal regions that prior CGH studies had identified as most frequently altered in melanoma. In this study, a probe set targeting 6p25, 6q23, and 11q13, with a reference centromere 6 probe to assess for relative 6p gain and 6q loss, yielded the best results (Gerami et al. 2009a). This original probe set discriminated definitive melanomas from nevi with 87% sensitivity and 95% specificity, and correctly identified all 6 of 27 ambiguous primary tumors with long-term clinical follow-up that later metastasized. Subsequent studies validated the high sensitivity and specificity of this probe set in distinguishing blue nevi from blue nevus-like melanoma (Gammon et al. 2011), lentiginous melanocytic neoplasms (Newman et al. 2009), nodal nevi from metastatic melanoma (Dalton et al. 2010), nevoid melanoma from mitotically active nevi (Gerami et al. 2009b), conjunctival nevi from conjunctival melanoma (Busam et al. 2010), and atypical intraepidermal melanocytic neoplasms (Gerami et al. 2010). While maintaining high specificity in desmoplastic nevi, the sensitivity for detecting desmoplastic melanomas in this probe set was only 47% in one study (Gerami et al. 2011a).

While FISH performs with high sensitivity and specificity in studies of unequivocal nevi and melanomas, the limited number of studies with ambiguous neoplasms with known follow-up indicates caution is warranted when interpreting FISH results. A sensitivity of 43% was reported for the detection of lymph node or distant metastatic spread in a cohort of 90 ambiguous melanocytic tumors which contained a large percentage of spitzoid tumors (Vergier et al. 2011). In an effort to address this, addition of a probe for detection of chromosome 9p21 (*CDKN2A*) loss was shown to increase sensitivity from 70% to 85% in a study of 43 spitzoid melanomas (Gammon et al. 2012). Homozygous 9p21 loss was also found to be particularly significant in a cohort of 75 atypical Spitz tumors in which 6/8 patients who developed stage 4 disease and 3/3 patients who died of metastatic melanoma had homozygous 9p21 loss (Gerami et al. 2013). These results justify addition of a 9p probe to assess for *CDKN2A* loss, particularly for spitzoid tumors.

A second FISH probe set incorporating homozygous 9p21 loss with 6p25, 11q13, and 8q24 gain outperformed the original probe set in distinguishing melanoma from nevi in one study,

Table 2 Criteria for positive FISH testing in melanocytic tumors

	6p gain	Rel. 6p gain[b]	6q loss[c]	11q gain	8q gain	Homozygous 9p loss
Gerami et al. 2009a	>29%	>55%	>40%	>38%	N/A	N/A
Gerami et al. 2012	>29%	N/A	N/A	>29%	>29%	>29%
Neogenomics NeoSITE	>29% 17–29% b-line[a]	N/A	N/A	>29% 20–29% b-line[a]	>29% 11–29% b-line[a]	>29% 11–29% b-line[a]

[a]Borderline positive
[b]Relative 6p gain determined by the number of nuclei with 6p signal count greater than reference centromere 6 count
[c]6q loss determined by the number of nuclei with 6q count less than centromere 6 count

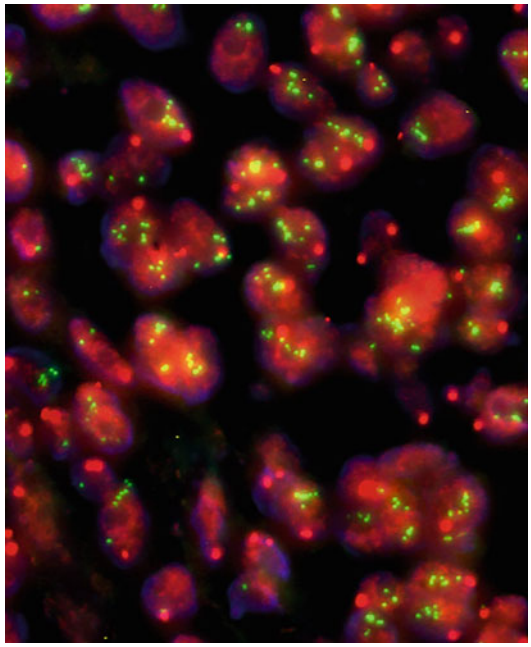

Fig. 5 FISH showing 11q13 gain in a melanoma. Neoplastic cells show ≥3 green signals per nucleus indicative of chromosome 11q13 gain. FISH 400x: Green probe- 11q13, red probe- 6p25

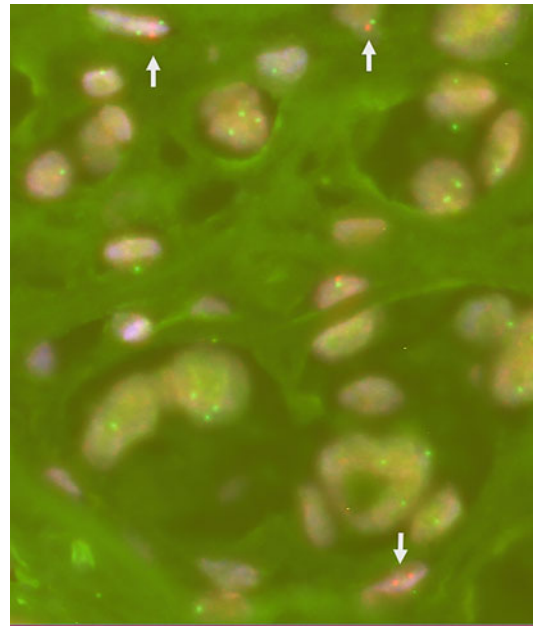

Fig. 6 FISH showing homozygous 9p21 loss indicative of *CDKN2A* loss. Neoplastic cells have no detectable 9p21 signal (red), but show 1–2 signal counts for the control green probe (centromere 9). Stromal cells show 1–2 red signals (white arrows). FISH 400x: Green probe – centromere 9, red probe 9p21

showing 94% sensitivity and 98% specificity (Gerami et al. 2012). As this second probe set targets four different chromosomes compared to two in the original set, it permits better detection of polyploidy. Polyploidy can be found in both melanomas and nevi, particularly Spitz nevi, and can generate false positive FISH results (Fig. 7) (Zembowicz et al. 2012). The addition of an 8q24 probe appears to be particularly useful in acral and nevoid melanomas, both of which harbor frequent 8q24 gains (Su et al. 2017; Yélamos et al. 2015b). Similar >90% sensitivity and specificity for the second probe set was also found in 39 unequivocal melanomas and nevi in one study (Minca et al. 2016). However, sensitivity and specificity dropped dramatically (56% and 83%, respectively) when testing ambiguous cases, and one additional study of the second probe set involving 37 challenging melanocytic tumors showed an even lower sensitivity (39%) (Al-Rohil et al. 2016). While the lack of correlation with long -term follow-up and metastatic spread was a limitation in both these studies, their results combined with the paucity of other studies comparing the original and second probe sets makes it difficult to determine which is superior. While a single probe set suitable for all types of melanocytic tumors would be ideal, it is more likely that a tailored approach with different FISH probes targeting the most common CNAs in the type of neoplasm being tested would bring the highest sensitivity and specificity.

Prognosis

FISH has also demonstrated prognostic value in the assessment of melanocytic tumors. In a cohort of 144 primary melanomas of at least 2 mm thickness, positive FISH testing with the original chromosome 6 and 11 probe set was associated with increased risk of metastasis (hazard ratio 5.9) even after controlling for other known prognostic factors such sentinel lymph node status, ulceration, Breslow depth, and patient age (North et al. 2011). Gain of 11q13 (CCND1) and 8q24 (MYC) have been associated with metastatic potential (Gerami et al. 2011b). As previously mentioned,

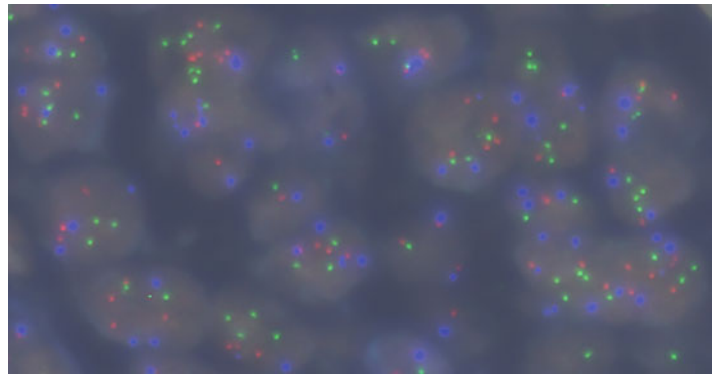

Fig. 7 Tetraploidy in FISH. Tetraploidy should be suspected in FISH when 3–4 probe signals are seen for all probes in the cells of interest. As only partial sections of nuclei are present in FISH sections, not all cells will show four signals in tetraploid states. In this case, many cells have 3–4 signals of green (11q13), red (6p25), and blue (centromere 6). While the signal count reaches the threshold for a positive FISH test, tetraploidy can be seen in both nevi and melanomas and should not be reported as positive (FISH 400x)

homozygous loss of 9p21 was associated with metastatic and lethal spitzoid melanomas, while spitzoid neoplasms with solitary 6q23 loss appear to have a good prognosis with low rates of spread beyond regional lymph nodes (Shen et al. 2013, 23).

Limitations of FISH

- Copy number assessment limited to only a small number of chromosomal loci
- Requires expertise and special equipment
- Different laboratories use differing thresholds and include borderline positive categories which impairs comparison of test results between laboratories (Table 2)
- False positive tests due to polyploidy

Next Generation (Massive Parallel) Sequencing

The development of a melanocytic neoplasm, whether it is a nevus or melanoma, requires an initial mutation that stimulates cell proliferation (driver mutation). Driver mutations in melanocytic tumors most frequently involve the MAP-kinase pathway, with *BRAF*, *NRAS,* and *KIT* mutations being among the most common. Such mutations are easily detectable through DNA sequencing and can provide treatment guidance in directing targeted therapy of advanced stage melanoma (e.g., BRAF inhibitors). However, detection of these driver mutations holds minimal benefit for the diagnosis of ambiguous melanocytic tumors given the shared presence of these mutations in both nevi and melanomas. The development of massive parallel/next generation (next gen) sequencing has revolutionized molecular testing and our understanding of melanocytic neoplasia as it allows for a more comprehensive assessment, namely the identification of secondary and tertiary mutations that mark the transition to melanoma. This type of sequencing can be tailored for analysis of the whole genome, the exome (i.e., all protein-encoding sequences of genes), or any desired panel of cancer-associated genes. Data produced from next generation sequencing not only provides mutation analysis of cancer associated genes; it can identify chromosomal rearrangements, and simultaneously provide chromosomal copy number information to indicate the presence of CNAs (Shain et al. 2015). Next gen sequencing can be used for in depth analysis of clonal evolution within a heterogeneous tumor through microdissection of different cell populations and sequencing these distinct areas (Fig. 8).

While the detection of a MAP-kinase driver mutation cannot distinguish a nevus from melanoma, detection of such a mutation in combination with additional mutations in tumor suppressor and other genes can be informative in distinguishing

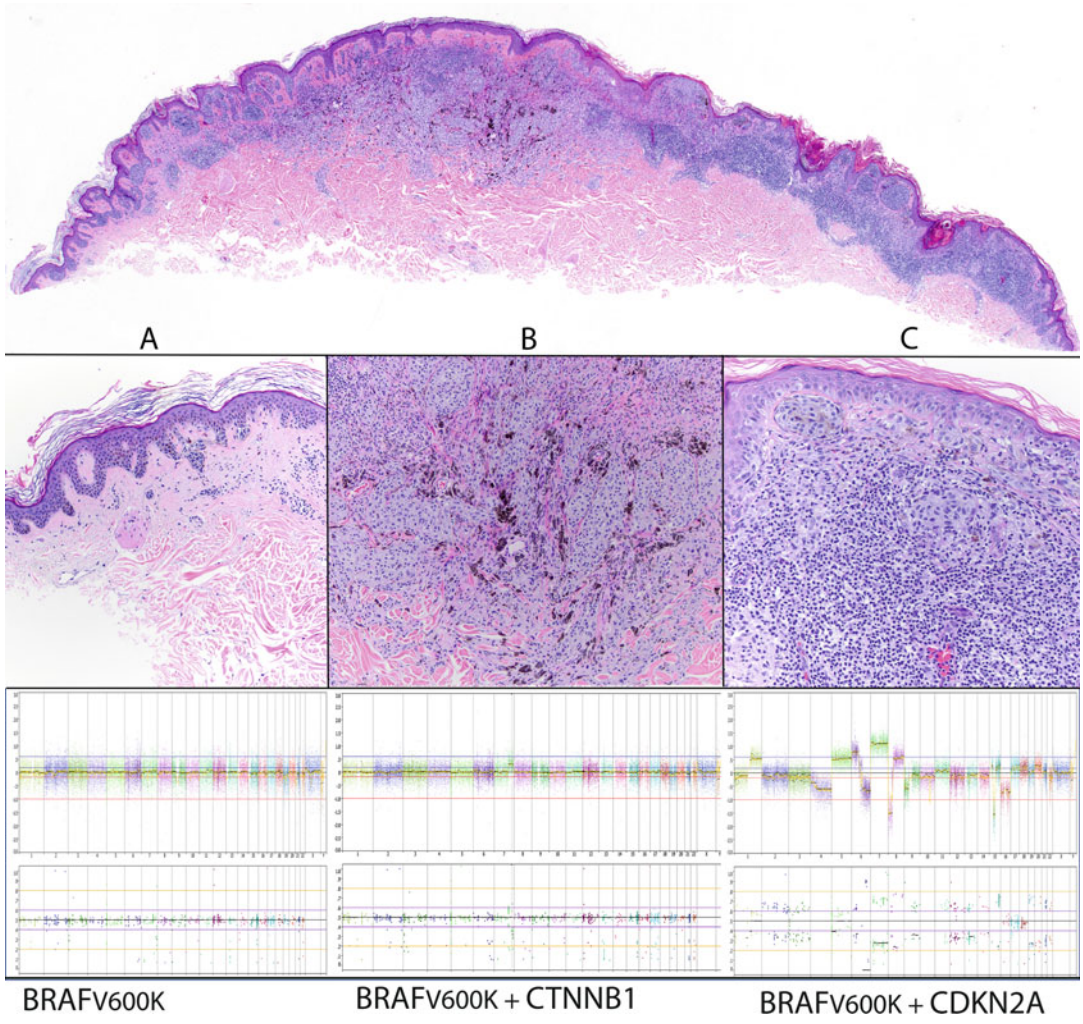

Fig. 8 Next gen sequencing in a complicated melanocytic neoplasm. (**Top**) A large, heterogeneous melanocytic neoplasm shows three distinct populations of melanocytes labeled (**a**), (**b**), and (**c**). Each area was microdissected and analyzed with next gen sequencing. Copy number analysis and salient mutations for each area are located in the lower panels. (**a**) An area of unequivocal melanocytic nevus with nests of small melanocytes is present in the epidermis. Next gen sequencing shows no CNAs with a solitary *BRAF*^{V600K} mutation. (**b**) Centrally, larger melanocytes form fascicles in the dermis with numerous melanophages (depth 2.5 mm). Next gen sequencing shows *BRAF*^{V600K} mutation with additional chromosome 7q gain where *BRAF* is located, and mutation of the *CTNNB1* gene encoding beta-catenin. (**c**) On the right, large melanocytes are irregularly distributed in the epidermis with pagetoid scatter. Nests of similar cells are present in the dermis (depth 0.7 mm) with a florid lymphocytic inflammatory reaction. Next gen sequencing shows *BRAF*^{V600K} mutation with an additional *CDKN2A* mutation, but no *CTNNB1* mutation. Numerous CNAs are present including gains of chromosome 1q, 5, 6p, 7, 8q, 15, 18, and 20 and losses of 4, 6q, 9p, and 16. Incorporating both the molecular and histopathologic features leads to the correct diagnosis: Melanoma, 0.7 mm thickness, arising in a *BRAF*^{V600K} nevus with a separate deep penetrating nevus that arose independently from the same precursor *BRAF* mutant nevus. Top – H&E 20x, Middle – H&E 100x

benign and malignant melanocytic neoplasms, particularly when accompanied by CNA assessment. A study of melanomas arising within precursor nevi indicates unequivocal melanocytic nevi frequently possess only a single driver mutation (most often *BRAF* ^{V600E}) with no additional mutations or CNAs (Shain et al. 2015). Meanwhile nevi with some degree of histopathologic atypia that

generate diagnostic uncertainty often harbor *NRAS* or *BRAF*$^{\text{non-V600E}}$ mutations with some additional mutations such as heterozygous *CDKN2A* or *TERT* promoter mutations. Further mutations in tumor suppressors such as *PTEN, TP53, ARID1/2,* and homozygous mutation/loss of *CDKN2A* arise during progression to invasive melanoma. Next generation sequencing provides a complete picture of both initiating and subsequent mutations as well as CNAs, and thus represents an improved test for assessing ambiguous melanocytic neoplasms. Due to the high cost and labor intensive nature of next gen sequencing, there is a lack of studies assessing the value of this molecular test in this capacity. However, as illustrated by the tumor in Fig. 8, next gen sequencing can be an extremely valuable adjunct to assist in accurate classification of difficult melanocytic neoplasms. Next gen sequencing also provides valuable information to guide treatment. In a study of targeted next gen sequencing for 274 consecutive melanomas, actionable mutations were detected in 72% of tumors, highlighting the utility of such analysis for guiding therapy (Leichsenring et al. 2018). Next gen sequencing also provides information on mutational burden allowing an estimate of the neoantigen load to help assess the likelihood of response to immune checkpoint blockade therapy. The greater the mutation burden and neoantigen load in a melanoma, the more likely the tumor is to respond to such immunotherapies (Van Allen et al. 2015).

Prognosis

Numerous studies have assessed mutation status with prognosis. While *BRAF* mutation has no implications in the distinction of melanocytic nevi from melanomas, the presence of *BRAF* mutations in melanomas has been associated with more aggressive disease when compared to *BRAF* wild type melanomas in some studies (Nagore et al. 2014; Long et al. 2011). *TERT* promoter mutations have also been associated with a poor prognosis in non-acral cutaneous melanomas (Griewank et al. 2014). Regarding spitzoid neoplasms, *TERT* promoter mutations were found in the only four lethal melanomas in a study of 56 atypical Spitz tumors and spitzoid melanomas, while all nonlethal tumors lacked the mutation (Lee et al. 2015a). Bi-allelic inactivation of *BAP1*, often through a combination of mutation and chromosome 3 loss, is a poor prognostic indicator in uveal and blue nevus-like melanoma (Harbour et al. 2010), while mutations in *SF3B1* or *EIF1AX* are associated with less aggressive uveal melanomas (Harbour et al. 2013; Martin et al. 2013).

Limitations

- Requires special equipment and bioinformatic infrastructure
- Expertise required to determine which DNA alterations represent true pathogenic mutations

RNA-Based Testing

Gene Expression Analysis

Commercially available gene expression tests are available for both diagnostic and prognostic assistance in melanocytic tumors. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) is used to assess mRNA levels in select genes from formalin fixed, paraffin embedded tissue. A 23 gene panel with a reported sensitivity and specificity of approximately 90% in distinguishing unequivocal nevi from melanomas is commercially available from Myriad (myPath® Melanoma) to assist in the diagnosis of ambiguous melanocytic neoplasms (Clarke et al. 2015). mRNA levels of 23 genes including one melanocytic differentiation gene (*PRAME*), 8 immune-related genes, 5 cell signaling genes, and 9 housekeeping genes are assessed in a proprietary algorithm, and a score from −16.7 to 11.1 is generated (Table 3). Scores from –16.7 to −2.1 are considered likely benign, scores from −2.0 to −0.1 are indeterminate, and scores from 0.0 to +11.1 are considered likely malignant. Large validation cohorts have been studied with this test with a range of different types of nevi, but the vast majority of melanomas assessed include only superficial spreading, lentigo maligna, and nodular melanomas (Clarke et al. 2015, 2017a). Lower sensitivity of 75% was reported in a later study on desmoplastic melanoma and desmoplastic nevi, while specificity remained

Table 3 Myriad myPath gene expression panel

PRAME	*CLTC*[a]
S100A7	*MRFAPI*[a]
S100A8	*PPP2CA*[a]
S100A9	*PSMA1*[a]
S100A12	*RPL13A*[a]
PI3	*RPL8*[a]
CCL5	*RPS29*[a]
CD38	*SLC25A3*[a]
CXCL9	*TXNLI*[a]
CXCL10	
IRF1	
LCP2	
PTPRC	
SELL	

[a]Control genes

high in this setting (100%) (Clarke et al. 2017b). Limited numbers of acral, spitzoid, nevoid, and blue nevus-like melanomas have been assessed. Case reports of false negative myPath® results in blue nevus-like melanomas indicate caution is warranted when testing less common variants of melanoma with this assay (Castillo et al. 2018). A significant limitation for incorporating this gene expression test into clinical practice is the lack of data on performance in ambiguous melanocytic tumors with known clinical outcomes. As the test is intended for use in such ambiguous tumors and not unequivocal nevi and melanomas, further studies are needed to determine the sensitivity and specificity in ambiguous neoplasms.

myPath® Gene Expression Versus FISH

In a head-to-head study of FISH and the myPath® gene expression test, FISH outperformed myPath® with 93% sensitivity, 100% specificity in unequivocal nevi and melanomas compared to 62% sensitivity, 97% specificity for myPath® (Minca et al. 2016). Performance of both tests decreased for histopathologically ambiguous cases with sensitivity and specificity of 52% and 80% for myPath® compared to 56% and 83% for FISH, with 15% of cases yielding indeterminate readings for myPath®. Sensitivity was particularly poor in spitzoid tumors (30% myPath, 50% FISH). A similar study comparing myPath® with FISH showed 72% sensitivity and 94% specificity for myPath in an initial cohort of unambiguous nevi and melanomas, which decreased to 50% sensitivity and 96% specificity in tumors with ambiguous histopathologic features (Reimann et al. 2018). FISH was not tested in the initial unambiguous cohort, but showed 61% sensitivity, 100% specificity in the ambiguous tumors. A limited number of the ambiguous cases were also tested with SNP array CGH. Overall, SNP array CGH had the best correlation with consensus diagnoses, followed by FISH and then myPath®. A major limitation of these studies is the lack of clinical outcome data for the ambiguous tumors.

Noninvasive Gene Expression Testing

In addition to extracting RNA from a skin biopsy, RNA can also be isolated from the stratum corneum of the epidermis through a tape stripping technique. A customized adhesive is applied to the skin and when removed, pulls a portion of the outer cornified layer off that can be assessed with RT-PCR for gene expression. Early testing of this technique generated a 17 gene expression panel that distinguished between melanoma and nevi in a validation cohort with a sensitivity and specificity of 100% and 88% respectively (Wachsman et al. 2011). Follow-up studies found a simplified two gene assay targeting expression levels of *LINC00518* and *PRAME* had sensitivity and specificity of 91% and 69%, respectively (Gerami et al. 2017). Specificity appears to be highest when expression levels for both genes test positive (Ferris et al. 2018). This testing is commercially available as the pigmented lesion assay (PLA) from DermTech. The number of studies assessing the clinical utility of this technique is limited.

Limitations

- Diagnostic gene expression tests are only validated for primary biopsies of primary tumors and are not recommended for re-excision specimens
- Additionally, tape stripping analysis is not validated for use on mucosal surfaces, acral sites, areas where non-vellus hair cannot be trimmed (e.g., scalp), bleeding or ulcerated lesions, pediatric patients, patients with a Fitzpatrick

skin type IV or higher, and nonpigmented lesions
- Reports indicate poor sensitivity for spitzoid and blue nevus-type tumors
- A significant percentage of cases return an indeterminate result for myPath®

Prognosis

Commercially available prognostic gene expression tests are available for uveal melanoma (Castle Biosciences, DecisionDx-UM) and cutaneous melanoma (Castle Biosciences, DecisionDx-Melanoma). Both tests divide melanomas into prognostic classes based on differential gene expression patterns. The uveal DecisionDx-UM test utilizes a 15-gene panel to identify tumors as class 1A with a 2% chance of metastasis within 5 years, class 1B with a 21% chance, and class 2 with a 72% chance (Gill and Char 2012). The DecisionDx-Melanoma test uses a 31-gene panel to determine prognosis for cutaneous melanoma (Table 4) (Gerami et al. 2015). Cutaneous class 1 melanomas have an 8% risk of metastasis within 5 years, while class 2 tumors have a 38% risk (Zager et al. 2018). The prognostic value of these gene expression tests appears to be independent of other known prognostic factors including tumor thickness and sentinel lymph node status. In an effort to further subclassify prognostic groups, class 1 and 2 were split into classes 1A, 1B, 2A, and 2B. This results in greater separation between prognostic groups 1A and 2B, but creates a more confusing classification for 1B and 2A, where class 1B tumors can have a worse prognosis than 2A tumors (Zager et al. 2018). Concern has also been raised regarding the use of the test in early stage melanoma, as there could be potential harm from overtreatment and emotional distress for patients with class 2b results that still have >85% five year survival rates (Marchetti et al. 2018). The cost of the test (~$8000) is also a potential concern. The 2018 recommendations from the American Joint Committee on Cancer (AJCC) did not find sufficient evidence to recommend gene expression profiling for staging of cutaneous melanoma. Prospective clinical trials are needed to demonstrate test benefits outweigh potential harms and high costs.

MicroRNA

MicroRNAs (miRNA) are small, noncoding RNA molecules that regulate gene expression by binding messenger RNA (mRNA) and preventing protein translation. Hundreds of miRNA genes have been discovered, indicating broad involvement of this type of RNA in cellular function. Additionally, dysregulation of miRNA has been demonstrated in various cancers, including melanoma (Lu et al. 2005). A small number of studies have looked at the differential expression of miRNAs in different types of melanocytic tumors (Table 5). Significant increase in miR-21-5p and miR-424-5p has been found in invasive melanoma compared with in situ melanoma, while let-7b levels were decreased in invasive and in situ melanomas compared with melanocytic nevi (Babapoor et al. 2017). miR-21 and miR-155 have been reported as two of the most highly upregulated miRNAs in melanoma and borderline melanocytic neoplasms compared to nevi (Grignol et al. 2011). Spitzoid melanomas have increased miR-21, miR-150, miR-155, and miR-200c levels, while Spitz nevi

Table 4 DecisionDx-Melanoma prognostic gene expression panel

BAP1	*SAP130*	*CRABP2*	*TRIM29*
MGP	*ARG1*	*KRT14*	*AQP3*
SPP1	*KRT6B*	*ROBO1*	*TYRP1*
CXCL14	*GJA1*	*RBM23*	*PPL*
CLCA2	*ID2*	*TACSTD2*	*LTA4H*
S100A8	*EIF1B*	*DSC1*	*CST6*
S100A9	*BTG1*	*SPRR1B*	
HNRPNL[a]	*YKT6*[a]	*FXR1*[a]	

[a]Control genes

Table 5 MicroRNA levels in melanocytic neoplasms

Upregulated in melanoma	Up in nevi
miR-17-5p	Let-7b
miR-21	miR-22
miR-107	miR-211
miR-130	
miR-150	
miR-155	
miR-181-b	
miR-200c	
miR-221	
miR-424-5p	

have upregulation of miR-22 (Latchana et al. 2017). miRNA levels can be quantified with RT-PCR, or in situ hybridization can also be used to assess miRNA expression levels in routine biopsies. Due to the limited amount of study data, miRNA analysis is not currently routinely used in clinical practice for evaluating melanocytic tumors.

Protein-Based Testing

Immunohistochemistry

Immunohistochemistry involves the use of monoclonal or polyclonal antibodies to assess for the presence of a target protein. A direct method can be employed in which the primary antibodies are conjugated to a signaling molecule such as a fluorescent tag which can be visualized with microscopy. An indirect method utilizes an unlabeled primary antibody followed by a secondary antibody that binds the Fc portion of the primary antibody. The secondary antibody is linked to a marker molecule or enzyme that catalyzes a detection signal (e.g., peroxidase). The indirect method has the advantage of signal amplification, where multiple secondary antibodies will bind the primary antibody and amplify the signal.

Immunohistochemical staining (immunostaining) is routinely used by pathologists to aid in the diagnosis of melanocytic neoplasms. Immunostaining with antibodies to proteins such as SOX10, S100, Melan-A, tyrosinase, HMB45, and MITF can be used to assess for melanocytic differentiation in a tumor. SOX10 and S100 stains offer high sensitivity for the detection of melanocytic tumors, but lack specificity. Immunostains targeting melanosome-associated proteins such as Melan-A and HMB45 are more specific, but are frequently negative in poorly differentiated melanocytic tumors (e.g., desmoplastic melanoma). Dermal maturation gradients that are typically found in melanocytic nevi and not in melanoma can also be assessed for with HMB45 staining.

In addition to identifying melanocytic lineage, immunostains are also used as diagnostic adjuncts in the assessment of ambiguous melanocytic neoplasms. Markers of cellular proliferation and mitosis such as Ki-67 and phosphohistone H3 show increased labeling in melanomas compared to nevi. Assessing their expression in the context of other clinical and histopathologic features can add value in distinguishing melanocytic nevi and melanomas and can also add prognostic value (Ladstein et al. 2010; Nielsen et al. 2013). Loss of tumor suppressor proteins such as p15, p16, and p21, which play critical roles in the prevention of melanoma development, can also be assessed with immunohistochemistry. Semiquantitative or quantitative analysis of p16 staining can provide insight into hetero- or homozygous loss of the *CDKN2A* gene (Shain et al. 2015). Loss of p16 expression has been found in multiple studies as a distinguishing feature between melanoma (absent p16 expression) and Spitz nevi (Harms et al. 2016; Wiedemeyer et al. 2018)

Recently, an antibody for PRAME was developed to assist in distinguishing nevi from melanomas. PRAME was first discovered as a protein in metastatic melanoma, but it has subsequently been identified as a tumor antigen in cancers of numerous organ systems. *PRAME* expression levels are a component of multiple RNA expression assays used as diagnostic and prognostic tests in the assessment of melanocytic tumors (see GENE EXPRESSION ANALYSIS section). Positive immunostaining for PRAME has recently been reported as ~85% sensitive for the detection of melanoma in a cohort of 255 primary and metastatic melanomas (Lezcano et al. 2018).

Sensitivity was high for acral, superficial spreading, nodular, and lentigo maligna melanoma subtypes, while only 35% of desmoplastic melanomas were positive. PRAME expression may be useful to assess surgical margins for subtle melanoma in situ as well. Approximately 15% of melanocytic nevi are PRAME positive, typically showing only focal staining for PRAME.

Immunohistochemistry and Epigenetics

As understanding of the epigenetics of melanocytic neoplasia has increased, immunostains have been developed to target epigenetic differences between melanocytic nevi and melanomas. Loss of the epigenetic marker 5-hydroxymethylcytosine (5-hmC) has been reported as a distinguishing feature of melanoma that can be assessed by immunostaining (Lian et al. 2012). Numerous studies have shown sensitivity and specificity >90% for 5-hmC staining in the diagnosis of various types of nevi including conventional nevi, Spitz nevi, dysplastic nevi, blue nevi, deep penetrating nevi, and intranodal nevi and various types of melanoma including melanomas on acral skin, skin from both low and high cumulative sun exposure, mucosal melanomas, and metastatic melanomas (Lee et al. 2015b, 2017; Uchiyama et al. 2014). 5-hmC staining in histopathologically ambiguous neoplasms has been reported in one study and appears less definitive showing intermediate levels of 5-hmC expression.

An additional epigenetic alteration reported to assist in the diagnosis of melanomas is loss of trimethylation at lysine 27 of histone H3 (H3K27me3). Distinguishing spindle cell and desmoplastic melanomas from malignant peripheral nerve sheath tumors (MPNSTs) can be exceedingly difficult. Loss of H3K27me3 was initially reported as a highly specific feature of higher grade MPNSTs that was not seen in spindle cell melanomas (Schaefer et al. 2016). However, a larger follow-up study of 122 MPNSTs and 265 melanomas did not support this high specificity, with 72% of MPNSTs showing complete loss of expression and 37% of melanomas also showing complete loss of expression (Le Guellec et al. 2017). The lack of specificity of H3K27me3 loss in this differential diagnosis limits the clinical utility of this stain.

Immunohistochemistry for the Detection of Genetic Alterations

Immunohistochemistry can also serve as a screening tool for genetic alterations in melanocytic tumors. As previously mentioned, loss of p16 expression can indicate inactivation/loss of *CDKN2A*. Loss of nuclear BAP1 expression is indicative of bi-allelic inactivation. When the nuclear localization sequence of BAP1 is disrupted, BAP1 protein can be seen accumulating in the cytoplasm outside the nuclear membrane by BAP1 immunostaining (Fig. 9). However, some inactivating mutations of BAP1 still show preserved immunoreactivity, limiting the sensitivity of the stain. Immunostains have also been developed to detect common conserved mutations in melanoma such as $BRAF^{V600E}$ and $NRAS^{Q61R}$ with very high sensitivity and specificity (Anwar et al. 2016; Massi et al. 2015). Similarly, some kinase fusions can be assessed for using immunostains. ALK immunostaining is highly effective for the detection of *ALK* fusions, as ALK protein is not normally expressed in melanocytes (Busam et al. 2014). Of note, a small percentage of melanocytic neoplasms without *ALK* gene fusion can activate ALK through alternative transcript

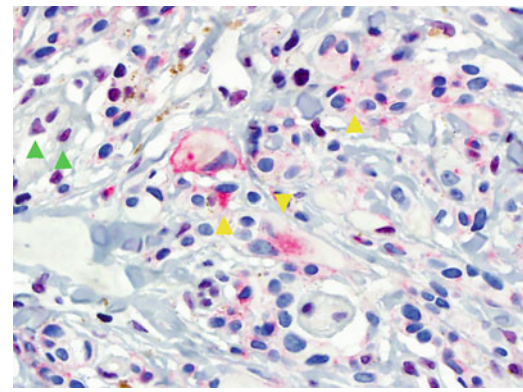

Fig. 9 Immunohistochemistry of BAP1 inactivation. The large neoplastic melanocytes show negative nuclear staining, while positive staining is present in the cytoplasm adjacent to the nucleus (yellow arrows). Nonneoplastic stromal cells have normal nuclear expression of BAP1 (green arrows). BAP1 stain 600x, red chromagen

activation of ALK, which can also produce positive ALK immunostaining (Wiesner et al. 2015). NTRK1 staining can be helpful in detecting *NTRK* fusions, but is more difficult to interpret due to basal expression of NTRK proteins in normal melanocytes. ROS1 and MET stains are also available, and high expression levels can indicate the presence of the respective fusion kinase.

Immunohistochemistry for Immune Checkpoint Blockade

Recent advances in immunotherapy for late-stage melanoma have revolutionized treatment and increased survival for patients. Given the high cost and potential serious side effects of these new immunologic therapies such as the immune checkpoint blockade agents targeting PD1 and PDL1, there is great interest in biomarkers that can indicate likelihood of tumor response to therapy. Dense infiltrates of CD8+ cytotoxic T lymphocytes have been observed in biopsies of patients experiencing tumor regression after anti-PD1 therapy (Hamid et al. 2013). When assessing pretreatment biopsies, the presence higher numbers of CD8+, PD-1+, and PD-L1+ cells at the invasive tumor margin has been associated with favorable treatment responses, with CD8 expression at the invasive margin being the most significant predictor in one study (Tumeh et al. 2014). Expression of cell surface PDL1 by at least 5% of tumor cells has been shown to indicate higher likelihood of treatment response to anti-PD1 therapy (Topalian et al. 2012). However, variable tumor PDL1 expression can be seen in multiple biopsies from the same patient, indicating this method is problematic to predict treatment response based on a single biopsy. Additionally, while greater expression of PDL1 appears to consistently indicate a higher likelihood of treatment response (~45%) for both anti-PD1 and anti-PDL1 therapy across multiple studies, approximately 15% of tumors that lack expression of PDL1 also respond to treatment (Sunshine and Taube 2015). Standardization of scoring and staining for PDL1 expression also presents challenges. While there does appear to be some utility in immunostaining to predict treatment response, it remains an imperfect modality and cannot be relied on as a sole predictor to guide therapeutic decisions.

Mass Spectrometry

Mass spectrometry involves separation of a target sample into its constituent parts based on mass and charge. In a biopsy, this technique can be used to identify different proteins in a given piece of tissue and create multi-protein spectral plots for comparison between nevi and melanomas. A pilot study using matrix-assisted laser desorption ionization (MALDI) mass spectrometry to assess spitzoid melanocytic neoplasms was reported in 2012 (Lazova et al. 2012). By analyzing both the tumor and the adjacent tumor microenvironment, this technique was able to differentiate between Spitz nevi and spitzoid melanoma with 97% sensitivity and 90% specificity. Two of the protein peaks in the spectra used for distinguishing the neoplasms were identified as actin and vimentin. However, follow-up studies using immunohistochemistry did not show any significant difference in the expression of these proteins between the nevi and melanomas (Alomari et al. 2015). Additional reports indicate promise for this test in atypical spitzoid tumors with long-term follow-up and anecdotally in a congenital nevus and proliferative nodule (Lazova et al. 2016, 2017). The test is now commercially available. However, given the limited amount of published data, which comes exclusively from the group that developed and commercialized the test, clinical utility of this test remains uncertain.

Conclusion

Multiple molecular tests are available to assist in the diagnosis of melanocytic tumors. Immunohistochemistry has become fundamental in all pathology labs and is used daily to assist pathologists in the diagnosis of melanocytic tumors. In cases where a definitive diagnosis is not achieved from histopathologic and immunohistochemical assessment, the DNA-based modalities of CGH and FISH have the most study data to support their

utility. Both can provide valuable information regarding DNA copy number changes in a melanocytic neoplasm, with CGH providing a comprehensive view of the genome and FISH assessing for targeted CNAs associated with melanoma. Next gen sequencing provides the most comprehensive genomic information, but presents the most challenges with data interpretation. Gene expression panels and mass spectrometry are commercially available for both diagnostic and prognostic use, but their niche in ancillary testing of melanocytic tumors is less well defined. Further studies are necessary to identify scenarios in which these tests would be preferred.

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Melanoma Immunology and Immunotherapy

31

Ryan J. Sullivan and F. Stephen Hodi

Contents

Abstract

Immunotherapy has been a mainstay for decades, however in recent years a number of new approaches to harness the immune system have been developed and revolutionized the treatment of this disease. This chapter serves as an introduction to immunotherapy efforts in melanoma that includes a description of the immune system elements and tumor immune microenvironment and the justification for their targeting, presentation of proof of concept examples of effective immunotherapy, and a discussion of some of the present dilemmas in the field that need to be sorted out over the coming decade.

Keywords

PD-1 inhibitor · Interleukin 2 · CTLA4 · Immune related adverse events · Biomarkers

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Introduction

In the modern history of cancer therapy, distinct eras can be defined going back to the nineteenth century. With the rise of antiseptic technique, "heroic" and radical surgeries were made possible that focused on removing solid tumors and often the draining basin lymph nodes. In this era of surgery, the morbidity of surgery was significant, and it was not until nearly a full century had passed before modified surgeries, often followed by adjuvant radiation therapy to optimize local control, became the new standard of care (Fisher et al. 1989). While surgery and radiation therapy were well-established methods of controlling locoregional disease and, if performed "early," or perhaps more accurately in patients with favorable biology, could be associated with improved survival, systemic therapy was always designed to treat disseminated disease. The early days of cytotoxic therapy led to the development of regimens that were designed to target different points in the cell division machinery and combined agents with non-overlapping, dose-limiting toxicities (Frei et al. 1958). During the era of chemotherapy, the care of hematologic malignancies and many solid tumors (e.g., testicular cancer, ovarian cancer, breast cancer) was revolutionized; however these efforts only minimally impacted the care of patients with certain tumor types, such as melanoma, and further discoveries were required to definitively impact the survival of these patients (Korn et al. 2008).

Over the past 20 years, so-called targeted therapies have been developed to treat a number of diseases, including melanoma. These include monoclonal antibodies that inhibit cell surface markers (CD-20; rituximab), cell surface receptors (erb-B2 receptor kinase (ERBB2/HER2); trastuzumab), and growth factors associated with angiogenesis (vascular endothelial growth factor (VEGF); bevacizumab), as well as small molecule inhibitors of a number of cellular targets (Imai and Takaoka 2006). This latter category includes inhibitors of driving oncoproteins including BRAF in melanoma and lung cancer, epidermal growth factor receptor (EGFR) in lung cancer, KIT in gastrointestinal stromal tumor (GIST), and many others. However, and with few exceptions, developing therapeutic regimens that include multiple targeted agents, whether monoclonal antibodies or small molecules, has been challenging due to overlapping toxicity, and these approaches tend to be associated with transient control of disease but rarely, if ever, cure.

The search for truly curative therapy has been the goal of cancer treatment efforts since the days of the morbid, radical surgeries defined above. Curiously, the origin of revolutionary therapies aimed at inspiring the patient's immune system to kill the cancer rather than deliver agents designed to kill cancer cells directly has their roots in the era of surgery. Indeed, a surgeon named Thomas Coley observed near the turn of the twentieth century a curious, albeit rare, phenomenon of complete and durable tumor response during the recovery of a patient with a head and neck tumor and a life-threatening bacterial infection (Coley 1910). Subsequently, Coley attempted to harness the mediators of this type of response by designing and delivering a "toxin" derived from *Streptococcus*. While this ultimately was not proven effective for the treatment of cancer generally, it offered a proof of concept that the immune system could indeed be weaponized against an active and life-threatening malignancy. In the subsequent century, a fuller understanding of the immune system has been gained and more recently translated into the treatment of therapies that have efficacy in a number of indications.

A disease at the center of nearly all immunotherapy development is melanoma, a malignancy notoriously resistant to chemotherapy. In fact, nearly every type of immune therapy developed to date – cytokines, adoptive cell therapy (ACT), vaccines, checkpoint inhibitors, bi-specific antibodies – has been tested with some effectiveness in patients with melanoma. The initial efforts with the cytokines, interferon alpha 2b, and interleukin 2 (IL-2) were effective in a small percentage of patients and led to FDA approval of each agent in the adjuvant and metastatic setting, respectively (Kirkwood et al. 1996, 2004; Atkins et al. 1999). More importantly, the results with these agents set the stage for melanoma to be the training ground for new immunotherapy strategies. Building on

the results of IL-2, ACT was developed by investigators at the National Cancer Institute and, initially, involved isolating tumor-infiltrating T cells (TIL), growing and activating these ex vivo, and then giving these back to the patient in the context of IL-2 following lymphodepleting chemotherapy (Rosenberg et al. 2011). Building upon this approach, cellular engineering to both TIL and peripherally isolated lymphocytes has led to new products called chimeric antigen receptor (CAR)-modified T cells and T-cell receptor (TCR)-engineered cells that provide more specific T-cell activation and perhaps more robust responses than generic TIL (Maus and June 2016; Robbins et al. 2011). And yet, perhaps the most important translatable discovery was the identification of the regulatory elements around T-cell activation and effector function that, when inhibited, led to dramatic responses in a significant percentage of patients (Hodi et al. 2010; Topalian et al. 2012). These so-called immune checkpoint inhibitors do not require any ex vivo work or cell engineering but rather are given by infusion every 2–3 weeks. More importantly, these agents have activity in a broad range of malignancies and have changed the field of oncology more than any class of agents to date.

What follows is an introduction to immunotherapy efforts in melanoma that includes a description of the immune system elements and tumor immune microenvironment and the justification for their targeting, presentation of proof-of-concept examples of effective immunotherapy, and discussion of some of the present dilemmas in the field that need to be sorted out over the coming decade.

Requirements for Anti-melanoma Immunity

Cancer in its development in a host has developed the ability to progress despite doing so within the context of an active immune system. Schreiber and colleagues described the three "Es" of the interaction between malignant cells and immune cells that either lead to the **e**limination of the tumor, a state of **e**quilibrium between tumor and host immune system, or **e**scape from immune regulation leading to the propagation of the malignant phenotype (Dunn et al. 2002). This process is, of course, hopelessly more complex that this simple model suggests, and an iterative process of malignant alterations, immune system adaptation to these alterations, leading to more malignant cell alterations and on and on. In melanoma, there are two unique aspects to the natural history of the disease that highlight this concept. First, it is a well-known phenomenon that long latencies may occur from the diagnosis of primary disease to widespread metastases. While the majority of patients with melanoma who relapse do so in the first 3 years from diagnosis, a small minority recur more than a decade afterward (Ossowski and Aguirre-Ghiso 2010). While this may be related to a number of factors, tumor-immune equilibrium remains a likely contributor to these types of cases. A second important aspect to melanoma natural history is that in a small percentage of cases, spontaneous tumor regression has been noted (summarized nicely by William Coley himself) and more commonly histologic regression in the primary tumor site (Coley and Hoguet 1916). This latter finding is also the theoretical reason behind the thinking that melanoma of unknown primary is typically considered to be from a cutaneous primary that has undergone complete regression. Further support for this theory is that genetic analyses of these unknown primary melanomas more closely match the pattern of driving mutations seen in cutaneous melanoma than in acral, mucosal, or uveal melanoma (Siroy et al. 2015). Interestingly, in the cases of melanoma of unknown primary, by definition, the elimination of the primary tumor is not associated with the prevention of metastatic disease, as some tumor cells were able to escape immune destruction in order to develop into a clinically identifiable tumor.

In essence, the mechanism of action of immunotherapy is to tip the balance of this interaction and lead to the elimination of escaped tumors or at least trigger a period of sustained equilibrium. However, the bulk of immunotherapy development has aimed to improve the activity of effector T lymphocytes that leads to more

effective cell-mediated immunity. Chen and Mellman famously have described this process in a figure that has been utilized in seemingly every immunotherapy lecture since its publication in 2013 (Chen and Mellman 2013). The concept is that tumor immunity, and in particular T-cell immunity generated against tumor cells, involves a cyclical process that involves a number of critical steps that are all potentially druggable. The first steps involve the release of cancer antigens and their processing and presentation by antigen-presenting cells (APCs) in the context of major histocompatibility (MHC) molecules. T cells, via interaction of their T-cell receptor (TCR) with antigen, then undergo priming and activation. This process is highly regulated by a number of molecules, known as immune checkpoints, that either positively or negatively affect the activation status of the T cell. Once activated, T cells migrate to and infiltrate into tumors where they recognize tumor cells in an antigen-dependent manner, again through TCR interaction with antigen in the context of MGH, and then kill tumor cells.

At every step, this process can be altered or evaded (Chen and Mellman 2013, 2017). Negative regulators of T-cell priming and activation, such as the cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), can reduce populations of antigen-specific T cells that are capable of leading to tumor elimination. Certain tumors, including some with genetic aberrations in beta-catenin signaling, exclude either T-cell trafficking or infiltration, leading to a tumor microenvironment state known as an immune desert devoid of immune elements (Spranger et al. 2015). Once present, activated T cells may be thwarted by tumors that have either downregulated antigen expression or have been enriched by regulatory elements such as T-regulatory cells (Tregs) and/or myeloid-derived suppressor cells (MDSCs) that impair effector T-cell function. However, it seems that the lowest common denominator required to protect the tumor from immune-mediated destruction is the expression (either on the tumor cells or in the immune microenvironment) of proteins that are capable of suppressing the activity of activated effector T cells. The most common of these are the programmed death 1 receptor ligands 1 and 2 (PD-L1, PD-L2) which interact with the programmed death 1 receptor (PD-1) that is expressed on activated T cells. When this interaction occurs, effector T cells are rendered ineffective. However, when this interaction is disrupted pharmacologically, for example, with monoclonal antibodies that inhibit PD-1 or PD-L1, tumors utilizing this mechanism to prevent immunologic destruction become vulnerable to antigen-specific T-cell-mediated immunity. It is this last concept that has led to revolutionary advances in immunotherapy, as anti-PD-1/PD-L1 agents have become the most effective immune oncology agents developed to date (Chen and Mellman 2017).

Immunotherapy Proof-of-Concept Examples in Melanoma

High-Dose Interleukin 2

The first "modern" immunotherapy developed and subsequently approved to treat metastatic melanoma was high-dose interleukin 2 (HD IL-2). Originally discovered as (and named) T-cell growth factor, IL-2 quickly was moved into the clinic (Mier and Gallo 1980). In patients with melanoma and renal cell carcinoma, complete and durable remissions were seen in a small minority of patients (Atkins et al. 1999; Fyfe et al. 1995). In a collection of 270 patients with metastatic melanoma treated at a number of specialized centers, the response rate was 16% with durable and complete responses seen in approximately 6% of patients. Additionally, the median overall survival was 11.4 months, longer than other contemporary studies, although patients enrolled tended to have better prognosis features due to the potential for life-threatening toxicity (Atkins et al. 1999). In fact, due to its limited efficacy and high toxicity, namely, a capillary leak syndrome that is associated with hypotension, renal insufficiency, edema, and neurologic toxicity, HD IL-2 was not widely adopted. Still, specialized centers continued to treat patients, typically younger and with lower tumor volume, excellent performance status, and

excellent cardiac and pulmonary function. More contemporary datasets have corroborated response rates in the 16–20% range with durable benefit (progression-free at 18 months or greater) seen in 5–10% of patients, although in this era of more effective alternative systemic therapies, as expected, the overall survival of patients is improved to the historical dataset (Alva et al. 2016; Curti et al. 2017; Joseph et al. 2012). While this data is not nearly as strong as that seen with anti-PD-1 therapy in patients with metastatic melanoma, HD IL-2 is the original therapy that led to a discussion about considering where the "tail" on the survival curve is when determining the value of immunotherapy.

Interestingly, the major limitation to efficacy appears to be the fact that IL-2 receptors are found on both effector and regulatory T cells (Fontenot et al. 2005). This fact may be exploitable with certain modifications to IL-2, such as pegylation (PEG), and a number of modified IL-2s are in clinical trials as single agents and in combination with immune checkpoint inhibitors (NCT02350673, NCT02869295, NCT02983045, NCT03138889, NCT02799095) (Charych et al. 2017). Still, HD IL-2 is the original therapy that led to a discussion about considering where the "tail" on the survival curve is when determining the value of immunotherapy. The earliest data to emerge from these new takes on an old agent has been with NKTR-214, a 6-PEG IL-2 that preferentially binds to the beta-gamma subunits of the IL-2 receptor and not the alpha, which results in biased signaling of effector and natural killer T cells as opposed to regulatory T cells (Alva et al. 2016). In the Phase I single-agent study, NKTR-214 was considered to be safe and without the dose-limiting toxicities of high-dose IL-2 which allows for outpatient dosing (Bernatchez et al. 2017). Unfortunately, in this population of typically heavily pretreated patients, a clinical efficacy signal of single-agent therapy did not emerge. However, the initial data with NKTR-214 in combination with nivolumab was more encouraging. Specifically, in the first 41 patients treated at the recommended Phase II dose, responses were seen in 20 of the 38 evaluable patients, with 9 (24%) experiencing a complete response (Diab et al. 2018a). In the larger group of treated patients with a spectrum of solid tumor malignancies, it is important to note that the grade 3 or higher rate of immune-related adverse events is at least no different and numerically lower than predicted from single-agent nivolumab (Diab et al. 2018a, b). Based on the early results of this study, a randomized Phase III trial was launched (NCT03635983).

Anti-CTLA4 Inhibition

The first immune checkpoint molecule identified was CTLA4, and its inhibition was associated with significant preclinical activity in immunocompetent mouse models (Leach et al. 1996). Specifically, the activity of CTLA4 blockade became evident when combined with a GM-CSF secreting B16 murine tumor vaccine (Soiffer et al. 2003). The potential for inflammatory events was noted as approximately half the mice lost pigment in their fur from this combination treatment. As a single agent in early clinical trials, ipilimumab demonstrated a single-digit response rate in previously treated patients with melanoma (Weber et al. 2008). As CTLA4 is a major regulator of T-cell activation, it was felt that the use of anti-CTLA therapy might be most useful in conjunction with a vaccination strategy (Quezada et al. 2006). Treatment with ipilimumab importantly demonstrated an influx of a variety of immune effector cells into the tumor microenvironment as a function of treatment (Hodi et al. 2003). Many of the first clinical efforts with ipilimumab included combination with or subsequent to a vaccination strategy (Hodi et al. 2008, 2010). Interestingly, it became clear that targeting CTLA4 might be sufficient, and a three-armed randomized trial of ipilimumab, ipilimumab plus a gp100 peptide vaccine, and gp100 peptide vaccine alone demonstrated clear superiority of the ipilimumab-containing regimens (hazard ratio (HR) for death 0.68, p-value <0.001), but no advantage of gp100 vaccination in combination with ipilimumab (HR 1.04, p-value $= 0.76$) (Hodi et al. 2010). This was the first study to show an improvement in overall survival in the setting of

metastatic melanoma and led to the regulatory approval of ipilimumab including US FDA approval in 2011. In subsequent studies, it appears that treatment with ipilimumab is associated with long-term survival in approximately 22% of patients, marking a new benchmark for the "tail on the curve" for patients with metastatic melanoma (Schadendorf et al. 2015). Another important aspect to anti-CTLA4 inhibitors is the constellation of autoimmune toxicities that never before has been seen with conventional antineoplastic therapy. These so-called immune-related adverse events (irAEs) can be severe and even fatal, often require treatment with immunosuppression, and represent the major limitation of immune checkpoint inhibition (Weber et al. 2013).

Anti-PD-1 Inhibition

With the development of ipilimumab, the field of tumor immunology had a clear success, but it was of limited clinical value to the majority of patients. However, the initial data from the Phase I trial of nivolumab suggested that targeting anti-PD-1 might be a more effective strategy with less toxicity (Topalian et al. 2012). Specifically, responses in over 30% of patients with melanoma, all of whom had been treated with prior therapy, established a new benchmark for immunotherapy response rates. Indeed, both PD-1 inhibitors nivolumab and pembrolizumab were associated with response rates in the 30% range following ipilimumab, leading to regulatory approval of both agents in that patient population based on this data from open-label dosing with pembro (Keynote, KN, 001) and randomized trials of single-agent anti-PD versus chemotherapy (KN002, Checkmate, CM, 037) (Hamid et al. 2013; Weber et al. 2015; Ribas et al. 2015). However, in the frontline setting, as demonstrated in KN006 and CM067, these agents are clearly superior to ipilimumab with response rates in the 40–45% range, 1-year progression-free rates of approximately 40%, 2-year overall survival of 56–58%, and irAE rates of less than 20% (Larkin et al. 2015; Robert et al. 2015a). As with ipilimumab before, the data with these two anti-PD-1 inhibitors reset the bar for efficacy in this disease, and single-agent therapy with either of these agents became the indisputable frontline immunotherapy for patients with metastatic melanoma.

Combination Immune Checkpoint Inhibition

A consistent practice in the era of chemotherapy was to build combinatorial regimens of therapies with slightly different mechanisms of action and single-agent efficacy. With the development of ipilimumab and anti-PD-1 antibodies, it was a logical next step to evaluate the combination of the two in melanoma. In this case, there was strong preclinical data supporting the combination of ipilimumab and nivolumab (ipi/nivo) (Curran et al. 2010). The phase I trial of this regimen showed that ipilimumab 3 mg/kg in combination with nivolumab 1 mg/kg given every three weeks times four doses followed by full dose nivolumab every two weeks, was associated with unprecedented efficacy, response rates in excess of 50%, and toxicity and treatment-related irAE rates greater than 55%, as a clear majority of patients had an objective response to therapy and a majority of patients had severe or life-threatening irAEs (Wolchok et al. 2013). Based on this data, both randomized Phase II trial, CM069, comparing ipi/nivo with single-agent ipilimumab, and three-armed randomized Phase III trial, CM067, comparing the combination versus both single-agent ipilimumab and single-agent nivolumab, were conducted (Larkin et al. 2015; Postow et al. 2015). In both Phase II and III trials, the combination was shown to be superior to single-agent ipilimumab. However, it is less clear whether ipi/nivo is substantially better than single-agent nivolumab to justify the substantial increase in toxicity. For example, with at least 3 years of follow-up, the progression-free and overall survival rates, respectively, of the three arms were 39% and 58% for combination ipi/nivo, 32% and 52% for single-agent nivolumab, and 10% and 34% for ipilimumab. As this trial was not powered

to compare the two nivolumab-containing regimens, these differences in those two arms are not statistically significant. However, the development of this combination marks an important milestone in the treatment of melanoma, and the lessons learned with this regimen in melanoma have been applied to a number of other malignancies where this combination (at various doses) is being evaluated.

In melanoma, the use of low-dose ipilimumab (typically 1 mg/kg) given in combination with standard-dose anti-PD-1 therapy has been explored in a number of studies. In the most recent update of KN029, a Phase I/II trial of standard-dose pembrolizumab with low-dose ipilimumab, the rate of grade 3/4 toxicity was 47%, with 26% rate of irAEs, as well as a 62% response rate and a 27% complete response rate (Long et al. 2018a). In CM511, alternative dosing regimens of ipi/nivo were compared. Specifically, the standard induction dosing (IPI 3/NIVO 1 every 3 weeks) was compared to NIVO 3/IPI1 followed by standard nivolumab maintenance (Lebbé et al. 2018). Not surprisingly, the rate of irAEs is lower in patients who received low-dose ipilimumab, but the preliminary progression-free and overall survival of the two cohorts were no different.

Oncolytic Viruses

The first routinely used immune therapy in melanoma was intralesional bacillus Calmette-Guerin (BCG), a tuberculosis vaccine that causes a robust T-cell-mediated response at the site of injection (Morton et al. 1974). While this often was associated with regression of tumors, in the setting of suboptimal systemic therapies, the true value of this approach was very limited. In recent years, renewed interest in intralesional therapies facilitated the development of talimogene laherparepvec (TVEC), an oncolytic herpesvirus engineered to secrete GM-CSF (Hu et al. 2006). In a Phase III trial (OPTIM), 436 patients with advanced Stage III or IV melanoma with palpable, injectable lesions were randomized, in a 2:1 fashion, to receive intralesional TVEC versus subcutaneous GM-CSF (Andtbacka et al. 2015). With respect to the primary end-point, durable response rate, TVEC was superior to GM-CSF in patients with local-regional or limited metastatic disease, leading to its approval (Andtbacka et al. 2015). There was also a strong trend to improved overall survival (HR 0.79, 95% confidence interval 0.62–1.00, p-value = 0.051), a key secondary end-point, in patients randomized to TVEC. Importantly, the early data with combination with ipilimumab or pembrolizumab looks promising; suggesting that the ideal use of this agent may be in combination with immune checkpoint inhibition (Chesney et al. 2017; Ribas et al. 2017). For example, in the Phase I trial of ipilimumab plus TVEC, the response rate was 50%, a finding that led to a trial randomizing 175 patients to receive ipilimumab with or without TVEC (Chesney et al. 2017; Puzanov et al. 2016). While this study demonstrated a doubling of the response rate with combination therapy (39% vs. 18%), the progression-free survival of both cohorts was similar. More recently, the combination of pembrolizumab plus TVEC has been reported in a Phase I/II study (Ribas et al. 2017). The response rate was confirmed to be over 60%, with response seen in patients with and without pretreatment tumor characteristics associated, in other studies, with response (CD8 density, tumoral PD-L1 expression, interferon gamma gene expression profile scoring). Not surprisingly, there is a lot of interest in this approach generally, and it appears that TVEC is just the beginning, with a number of intralesional therapies, most commonly oncolytic viruses entering the clinic for the treatment of melanoma and other solid tumor malignancies.

Current Dilemmas and Future Directions of Immunotherapy for Patients with Melanoma

Predictive Biomarkers/Patient Selection Strategies

The most pressing clinical dilemma is determining selecting the correct treatment for the right patient. In oncology, this invariably includes histopathology and immunohistochemistry

(IHC) to identify the specific type of malignancy (e.g., carcinoma of the lung) or, better yet, pathologic subset that may behave differently (e.g., small cell vs. non-small cell lung carcinoma). More recently, large-scale genomic testing has been performed in most cancers and helped to identify molecularly defined subsets that may be clinically relevant. In cutaneous melanoma, for example, four molecularly defined subsets have been identified by the type of driving genetic alteration (either oncogene or tumor suppressor gene) that is or is not present (Cancer Genome Atlas Network 2015). These include (Fisher et al. 1989) BRAF mutant (~50%), (Frei et al. 1958) NRAS mutant (~25%), (Korn et al. 2008) NF1 mutant (10–15%), and (Imai and Takaoka 2006) triple wild-type (~10–15%). In only the BRAF mutant population, and more specifically patients with a $BRAF^{V600}$ mutation, are there agents that are proven to benefit this and only this population. These are the BRAF and MEK inhibitors, typically given in combination such as dabrafenib plus trametinib, vemurafenib plus cobimetinib, and encorafenib plus binimetinib (Dummer et al. 2018; Larkin et al. 2014; Long et al. 2015; Robert et al. 2015b). With respect to immunotherapy, there does not appear to be a major difference in outcomes with immunotherapy in patients with BRAF mutant or non-BRAF mutant melanoma (IL-2, ipi, nivo/pembro, ipi/nivo); however there are some data that patients with NRAS mutant melanoma may be more likely to benefit from IL-2 and immune checkpoint inhibitors, although this was not corroborated in a small study looking at using a next-generation sequencing (NGS) platform in patients treated with anti-PD-1/PD-L1 inhibitors (Joseph et al. 2012; Shahabi et al. 2012; Johnson et al. 2015, 2016). In that same NGS analysis, patients with NF1 mutations had a higher response rate but also had a significantly higher tumor mutational burden (TMB), which had been shown in this and other reports to be a strong predictor of response to immune checkpoint inhibitors (Johnson et al. 2016; Van Allen et al. 2015). Interestingly, in NSCLC, molecular defined populations where targeted therapy is available, namely, patients with either EGFR, ROS, or ALK aberrancy, anti-PD-1/PD-L1 therapy is ineffective (Gainor et al. 2016).

Beyond evaluating for specific genetic aberrations, such as activating (oncogenes) or inactivating (tumor suppressor gene) mutations, there have been a number of efforts to evaluate tumors and blood to help predict response to immune checkpoint inhibitor therapy. As described above, TMB has been associated with improved efficacy in patients with metastatic melanoma treated with immune checkpoint inhibitors (Johnson et al. 2016; Van Allen et al. 2015). This is predictable since TMB is a reflection of how altered from self is the tumor. The model follows that the more altered a tumor is, the more likely that the tumor would have immunogenic neoantigens produced, expressed on the cell surface in the context of major histocompatibility complex (MHC), and attract tumor-specific effector T cells. The subsequent response to immune cell infiltration may be expression of PD-L1, either in the tumor cells or induced in the tumor microenvironment, that will engage with PD-1 on the surface of activated effector T cells.

Given that PD-L1 expression seemingly is the final step to blunting a tumor-antigen specific response in the above model and that PD-L1 is involved in the mechanism of action of anti-PD-1 and PD-L1 inhibitors, it logically was the first tissue-based biomarker evaluated for responsiveness in melanoma and other diseases. The clear conclusion from the preponderance of evidence is that the presence of PD-L1 staining above a 1% cut-off is associated with improved outcomes, both in terms of response and survival endpoints. Interestingly, this is true for the anti-PD-1 antibodies pembrolizumab (Daud, JCO, 2016) and nivolumab (CM067, NEJM 2017) and the combination of ipilimumab plus nivolumab (CM067), but not with ipilimumab single-agent therapy (Daud et al. 2016; Wolchok et al. 2017). Specifically in 451 patients with melanoma enrolled onto the Phase I trial of pembrolizumab (KN001), which included first-line and previously treated patients, response rates in patients with below 1% were 10% and above 39% (Daud et al. 2016). In a frontline

study comparing ipilimumab, nivolumab, and the combination of the two (Checkmate 067), the response rates in patients with <1% staining were 18%, 35%, and 54%, respectively, and in patients with ≥1%, they were 19%, 54%, and 65% (Wolchok et al. 2017). Still, given that a quarter to a third of patients with PD-L1 <1% respond to single-agent pembrolizumab or nivolumab, the value of PD-L1 staining is not strong enough to be used to exclude the use of these treatments. One possible use of PD-L1 staining in melanoma could be to discriminate those who might be offered single-agent versus combination ipilimumab and nivolumab, although in the Checkmate 067 study, generation of a receiver operating curve indicated that the use PD-L1 status to predict overall survival of either treatment was poor (Wolchok et al. 2017).

A number of markers independent to tissue PD-L1 status have been explored as a potential biomarker of efficacy in patients with melanoma. The data looks promising with a number of tissue markers including presence of CD8+ T cells interacting with PD-L1 at the leading edge of the tumor, interferon gamma signature, MHC I and II expression, and TMB (Johnson et al. 2016; Van Allen et al. 2015; Tumeh et al. 2014; Taube et al. 2012). More recently the presence of specific CD8 + T-cell subsets, particularly those expressing the transcription factor TCF7, has been identified from signal cell RNA sequencing (scRNA seq) data, demonstrated to be present with a validated immunofluorescence assay, and shown to predict responsiveness to PD-1 inhibition in patients with melanoma (Sade-Feldman et al. 2018). Additionally, certain markers have been associated with poor outcomes including absence of CD-8 positive cells, PTEN loss, wnt/beta-catenin pathway genetic aberrations, and a resistance gene expression program identified from scRNA seq and validated in bulk RNA sequencing data (Spranger et al. 2015; Peng et al. 2016; Jerby-Arnon et al. 2018). In the blood, a number of markers seem to be prognostic, including serum LDH, total lymphocyte count, and a serum proteomic signature (Martens et al. 2016; Weber et al. 2018). To date, however, none of these tests have been validated in large trials as a biomarker that can truly predict efficacy or non-efficacy.

Prediction/Mitigation of Toxicity

Perhaps the best sign to herald the coming wave of data demonstrating the efficacy immune checkpoint inhibitors in patients was the reports of true autoimmune toxicity in patients receiving ipilimumab (Beck et al. 2006; Maker et al. 2005; Yang et al. 2007). Since there have been volumes of literature written on the topic, and with the widespread approval of anti-PD-1/PD-L1 monoclonal antibodies for a variety of indications, the use of immune checkpoint inhibition is no longer limited to a smaller number of centers with experience using these agents. Thus, understanding the nature of common and rare toxicities is critical, as is the development of strategies to diagnose and treat or better yet predict and prevent these toxicities.

Immune checkpoint inhibitor toxicity may be mild or fatal and can involve every organ system (Postow et al. 2018). In general, tissue infiltration and/or expansion of inflammatory cells, typically T cells, can cause tissue damage that can manifest clinically. Early diagnosis and treatment is critical, since more severe toxicity will not resolve with discontinuation of the immune checkpoint inhibitor and rather can only be reversed with immune suppression (Puzanov et al. 2017). The best analogy to this is in oncology graft-versus-host disease (GVHD) seen with hematopoietic stem cell transplantation, although many of the autoimmune toxicities seen with checkpoint inhibitors mimic well-described autoimmune disease such as inflammatory bowel disease and inflammatory arthritis. As such, the treatment of these conditions has been crafted with the corresponding autoimmune condition. For example, the treatment of checkpoint inhibitor colitis involves corticosteroids followed by monoclonal antibodies against TNF, agents that are approved for the use of IBD (Puzanov et al. 2017). The concern with immunosuppressive therapy is that the introduction of these agents early into therapy may mitigate efficacy. While this is a theoretical

concern, a fair amount of data supports the conclusion that immune suppression in the setting of checkpoint inhibitor toxicity is not associated with poorer outcomes.

One of the key issues in the field today is to better educate clinicians as to the potentially serious risks of these agents and to develop algorithms to work up and treat these toxicities. Over the past few years, professional societies such as the Society for Immunotherapy of Cancer (SITC) have been instrumental in providing educational material and published guidelines (Puzanov et al. 2017). More recently, the American Society of Clinical Oncology (ASCO) has also drafted and published guidelines for the diagnosis and management of these toxicities (Brahmer et al. 2018). These efforts are critical, but there is an opportunity, in the coming years, to leverage emerging technologies, such as single-cell RNA sequencing and wide-scale proteomic arrays, in order to have a deeper understanding of the driving forces in specific types of toxicity (e.g., colitis vs. pneumonitis vs. inflammatory arthritis). Once this has occurred, strategies may then be implemented to predict, monitor for the development of, better treat, and even prevent checkpoint inhibitor toxicity.

New Combinations

As opposed to blocking the brakes to the immune system, new immune targets are being explored including agonists such as OX40, GITR, CD137, and CD40. These agents have the potential to improve antigen presentation and immune activation, as well as deplete cells in the tumor microenvironment with immune regulatory function. With the efficacy of immune checkpoint blockade, there remains the constant effort to improve upon results through combination therapies. These include immuno-immuno combinations such as checkpoint blockade with agonist targets, priming with a variety of vaccination strategies including neoantigen vaccines, improving immune priming with TLR agonists, and combinations with targeted therapies (BRAF and MEK inhibition). Additional methods in attempt to improve current immune therapy strategies include targeting immunosuppressive metabolic pathways such as indoleamine 2,3-dioxygenase 1 (IDO1), transforming growth factor beta (TGF-beta), and nitric oxide, recognizing the importance of innate immunity by attempting to shift the myeloid cell populations in the tumor microenvironment to more tumoricidal that is immune suppressive as well as investigating means to improve NK cell function. These approaches likely represent the next wave of immunologic attempts to improve patient outcomes.

While there is excitement about a number of approaches to target alternative immune checkpoints (e.g., TIM3, LAG3, GITR, TIGIT, CD137, OX40/OX40L, etc.), microenvironment factors (IDO, TGF-beta, etc.), or other immune cells (Tregs, myeloid cells, B cells, etc.), it is important to remember the basics of drug development when moving potentially promising combination therapies forward. This is of particular importance in the setting of the data from the ECHO-301/KN-252 study that randomized 706 patients with previously untreated advanced melanoma to either pembrolizumab or pembrolizumab in combination with the IDO1 inhibitor epacadostat. The basis of this trial was promising data from the Phase I/II trial of this combination (ECHO-202/KN-037) demonstrating a response rate of 55% in the cohort of patients with treatment-naïve melanoma without substantial additional toxicity (Hamid et al. 2017). However, in the randomized trial, the addition of epacadostat to pembrolizumab was not associated with improvement in the primary endpoints, progression-free (HR 1.00) and overall survival (HR 1.13), or an improved response rate (Long et al. 2018b). In trying to figure out why a combination therapy with promising preliminary evidence could fail so spectacularly, it is clear that several key tenets of drug development were not followed. In fact, the supporting data failed to meet any of the following criteria that need to be met (at least one of the criteria) prior to advancing with a registration trial of a combination therapy. They are (Fisher et al. 1989) single-agent efficacy of both agents in the population being studied, (Frei et al. 1958) identification of a well-defined biomarker population that gleans the most benefit

from the combination, (Korn et al. 2008) data showing improved efficacy of surrogate markers in a small randomized study, and (Imai and Takaoka 2006) efficacy with the combination in a population of patients resistant/refractory to one of the agents in the combination. Hopefully the field will learn from this study and vet promising combinations more thoroughly in the future.

Role of Cellular Therapy

The actual "drug" for high-dose IL-2, ipilimumab, and anti-PD-1/PD-L1 inhibitors is the effector T cell, meaning that each of these agents works by better enabling T cells to directly kill cancer cells. This concept has always been known, and furthermore the identification of tumor infiltrating lymphocytes (TIL) had been described many decades before the development of immune checkpoint inhibitors (Rosenberg et al. 1982). With the development of high-dose IL-2, it became clear that immunotherapy was feasible, but not effective for the majority of patients (Atkins et al. 1999). In an attempt to enhance the effects of IL-2, the NCI, under the leadership of Dr. Steven Rosenberg, launched a new type of immune therapy that involved the harvesting of tumors to isolate TIL, grow and expand these ex vivo, and then infuse back into the patient and treatment with IL-2 (Rosenberg et al. 2011; Rosenberg 2011). It became clear early on that lymphodepleting chemotherapy was required to enhance efficacy. With this inclusion, the basic framework of adoptive cell therapy (ACT) was in place. Since the early trials of basic TIL therapy, a number of newer approaches have been pursued. These include better selection of higher-affinity or effective TIL clones, as well as cellular engineering of TIL and, more recently, peripheral blood lymphocytes to insert receptors that better recognize tumor-expressed antigens. These constructs include engineering T cells to express tumor-specific T-cell receptors (TCR) against a shared (across patients) tumor antigen (e.g., MAGE A10) or to insert chimeric antigen receptors (CAR) that can recognize surface-expressed proteins that are not processed and expressed in the context of MHC (Maus and June 2016). The challenge with any engineered T cell, be it a TCR-T or CAR-T cell, is the specificity. Mainly, the target antigen must only be expressed tumor cells or only on tumor cells and host cells that are not critical for survival. For example, CAR-T cells against CD-19 lead to dramatic results in patients with B-cell malignancies; however a CAR-T against ERBB2 leads to rapid, lethal toxicity (Maude et al. 2018; Neelapu et al. 2017; Morgan et al. 2010). In melanoma, a number of ACT studies are ongoing, and those that have concluded show responses in the 40–50% range (Rosenberg et al. 2011). These include TIL therapy and TCR-T cell therapies against shared antigens such as MAGE, MART1, and gp100. However, to date, these approaches appear to be more complicated and, at best, no more effective than immune checkpoint inhibition. Thus, ACT only is being tested in the checkpoint inhibitor refractory population, although combination therapy with immune checkpoint inhibitors is a logical strategy in the future.

Conclusion

From Coley to combined immune checkpoint inhibition, the field of cancer immunotherapy has captured the minds of patients and doctors. With recent advances, the concept has become the most exciting type of anticancer therapy. However, with this success have arisen new challenges, namely, innate and acquired resistance to therapy as well as diagnosis and management of toxicity, that will surely be the focus of future work.

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Targeted Therapy in Advanced Melanoma

32

Jeffrey A. Sosman and Douglas B. Johnson

Contents

Abstract

Molecularly targeted therapy, specifically small molecule therapeutics against particular oncogenes, has transformed the treatment landscape in melanoma and other cancers. Activating mutations in *BRAF*^{V600} produce constitutive activation of the mitogen-activated protein kinase (MAPK) pathway, causing unrestrained growth in nearly half of all

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melanomas. In turn, therapeutic blockade of this pathway through BRAF inhibitors produces dramatic clinical responses and improved survival compared to traditional cytotoxic chemotherapy. The addition of downstream MAPK blockade via MEK inhibition has further improved clinical outcomes. Although initial responses are impressive in most patients, and durable responses occasionally occur, acquired resistance remains a major barrier to long-term efficacy with these agents. A number of other potential therapeutic targets have been identified among other subsets of melanoma, including those with *NRAS* mutations, *KIT* mutations, and atypical *BRAF* alterations. Further, combinatorial regimens targeted MAPK and other pathways (including CDK4/6 and PI3K/AKT pathways) have shown early promise. This chapter reviews the development, current clinical activity, and future development directions for targeted therapy in melanoma.

Keywords
BRAF · Targeted therapy · NRAS · KIT · Vemurafenib · Dabrafenib · Trametinib

Introduction

Historically, advanced melanoma has been associated with a poor prognosis and a median survival of 6–9 months (Balch et al. 2009). This was, in part, explained by a notorious lack of efficacy of traditional cytotoxic chemotherapy in patients with this disease. As such, a powerful drive to identify novel therapeutic strategies for advanced melanoma has persisted among the melanoma community.

Targeting specific genetic alterations that fuel cancer cell growth and division has led to major advances across several cancer types, and is now a cornerstone of melanoma therapeutics. A prerequisite for effective targeted therapy, however, is the identification of an appropriate "target." Several characteristics make particular molecular changes attractive for targeting by anticancer agents. First, it should be cancer specific, with limited or no expression on healthy tissues. This issue surfaces more frequently when targeting cell surface molecules, and less often presents a problem when targeting cancer-specific mutations. Second, it needs to contribute to cancer growth and progression. Genomic alterations that are fundamental to tumor growth are preferable than those that arise following malignant transformation or metastatic spread. Third, it should occur in a "targetable" protein. Transcription factors and GTPase proteins have proven difficult to target, whereas kinases are more amenable to therapeutic modulation. Fourth, the cancer cell would be highly dependent on the target, with a dearth of co-occurring, functionally redundant mutations. Finally, it would occur at a high frequency in a particular cancer type or across cancers. While frequency is not necessary for successful targeting, it provides enormous advantages in terms of detection, characterization, and drug development. A recurrent mutation in the 600th codon of BRAF was identified in 2002 in approximately half of melanoma tumors, fulfilling all of the above criteria except the fourth (as melanoma is among the most highly mutated of human cancers) (Alexandrov et al. 2013).

While early targeted therapy efforts predated 2002, the discovery of these recurrent $BRAF^{V600}$ mutations sparked the first sustained and successful rational targeted therapy approaches in this disease (Davies et al. 2002). A number of comprehensive sequencing efforts have subsequently identified numerous potential genetic and non-genetic candidates for targeted therapies beyond *BRAF* mutations (discussed in "Melanoma Genetics" below). With the description of these recurrent genetic alterations, a novel classification schema was born, derived not from histologic or anatomic features, but from the genetic makeup of the tumor. The development of active targeted therapies has highlighted the clear clinical applications of this novel classification scheme.

Melanoma Genetics

A review of the genetics of melanoma as described by The Cancer Genome Atlas (TCGA) provides an ideal basis for a discussion of targeted

therapy of melanomas (Cancer Genome Atlas Network 2015). The TCGA is based on melanoma tissue obtained from regional lymph nodes and primary melanomas that were all cutaneous in origin and included no melanomas originating from mucosal surfaces, acral surfaces, or uveal origin. The TCGA provides an overall framework for melanoma genomic classification based on presumed driver mutations. This approach has classified melanoma by the predominant driver, *BRAF*, *NRAS*, *NF1* or "Triple Wild Type" (TWT). The major driver oncogenes and several of those present within the TWT population strongly support mitogen-activated protein kinase (MAPK) pathway inhibition as an important component of any targeted therapy, since over 90% of melanomas in the TCGA have driver genes activating this pathway (Cancer Genome Atlas Network 2015).

As mentioned, recurrent mutations in the serine threonine kinase BRAF at the 600th codon offered a previously unrecognized approach in treating this disease. Most commonly, this mutation involved a valine to glutamine substitution (V600E), but also included other changes (most often valine to arginine – V600 K) (Davies et al. 2002). Other, non-V600 mutations were also identified in a smaller proportion of melanomas (roughly 5%), primarily in exons 11 and 15, and most commonly codons 466, 469, 597, and 601 (Wan et al. 2004). These mutations lock BRAF into its active conformation, inducing constitutive downstream MAPK signaling through MEK1/2 and ERK independent of upstream RAS signaling. Other alterations in *BRAF* have also been identified at lower frequencies, including fusions, gene amplifications, and kinase domain duplications. The discovery of recurrent *NRAS* mutations at codons 12, 13, and 61 actually predated the identification of recurrent *BRAF* mutations. These changes are present in 15–20% of melanomas, with codon 61 mutations predominating, and less frequent codon 12 and 13 mutations. Less than 5% of the RAS-mutated melanomas involve *KRAS* or *HRAS*. This contrasts with mutation patterns in *KRAS* in other cancers (colon, lung adenocarcinomas), where codon 12 and 13 mutations predominate. Loss of function and truncating mutations in the tumor suppressor gene *NF1* were also identified in approximately 15% of melanomas (Hodis et al. 2012; Krauthammer et al. 2012). *NF1* mutations often co-occur with other MAPK activating mutations, suggesting that although they promote MAPK signaling, additional alterations are required for oncogenic pathway activation. Other low frequency mutations that promote MAPK signaling were also identified, including those in *KRAS*, *HRAS*, *MAP2K1*, *CRAF*, and various receptor tyrosine kinase encoding genes. Notably, these "driver" type mutations are largely mutually exclusive (with the exception of *NF1*). Thus, MAPK signaling is dysregulated and promoted in nearly all melanomas, suggesting that targeting this pathway could be effective in multiple genetically defined subtypes. See Fig. 1 for potential therapeutic targets and agents.

The other cohort of cutaneous melanomas has been identified as triple wild type (TWT) melanomas. Oncogenic drivers in this group are diverse, and include *KIT*, *GNAQ,* and *GNA11* (drivers of uveal melanoma); *PDGFRα* amplifications; *CTNNB1*; and *EZH2* mutations (Cancer Genome Atlas Network 2015). The overall frequency of a UV signature (C to T inversion or CC to TT inversion) is seen in less than 30% of TWT melanomas while >90% of those from the other genetic classifications demonstrate a UV signature. Finally, copy number alterations (CNA) and other structural events such as TERT, CCND1, CDK4, MDM2, MITF, and PDL1 amplifications may also occur. Despite the somewhat distinct genetic makeup of this group, evidence of MAPK activation is still present in most TWT melanomas.

The stereotypic oncogenic "driver" mutations such as *BRAF* and *NRAS* were often found to coexist with cooperating genetic alterations that promote an invasive phenotype. These include *CDKN2A* loss or mutations, *TP53* mutations, *TERT* promoter mutations, *PTEN* loss, or other alterations in the PI3K-AKT pathway (Cancer Genome Atlas Network 2015; Hodis et al. 2012). A large, elegant study performed sequencing of numerous pre-malignant and primary melanoma tumors, and showed that these changes

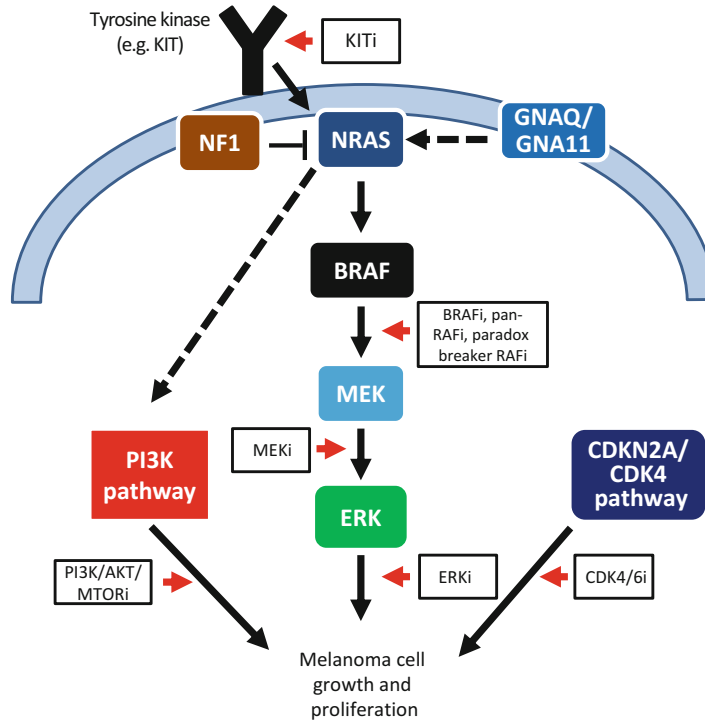

Fig. 1 Simplified schematic of relevant cell signaling pathways in melanoma and approved or experimental therapeutic approaches

accumulate in a predictable, stepwise fashion as melanomas evolve from pre-neoplastic lesions (Shain et al. 2015). *BRAF*^{V600E} mutations occurred in benign nevi, whereas *NRAS* mutations and several other drivers occurred in intermediate lesions. These intermediate lesions and melanoma in situ also frequently harbored *TERT* promoter mutations. *CDKN2A* deletions, *PTEN* loss, and *TP53* mutations occurred only in invasive melanomas. Furthermore, total mutational burden, the total number of somatic nucleotide variants identified in the tumor, accumulated with malignant progression. These co-occurring mutations may provide additional therapeutic targets.

Oncogenic driver mutations are also associated with unique clinical patterns. *BRAF* mutations occur more commonly in melanomas from skin with intermittent sun exposure, and are infrequent in melanomas arising from skin with chronic sun damage (CSD) (Curtin et al. 2005). If *BRAF* mutations are identified in skin with CSD, they frequently are V600 K rather than V600E. *NRAS* mutations occur in a relatively predictable 15–20%, regardless of anatomic location (with the exception of uveal melanoma). These alterations are also correlated with an inferior overall prognosis and thicker primary tumors. *KIT* mutations are present in 15–20% of acral and mucosal melanomas and 2% of CSD melanomas, but rarely in other cutaneous melanomas. *GNAQ* and *GNA11* mutations occur in nearly 90% of uveal melanomas, but rarely in other subtypes (Van Raamsdonk et al. 2009, 2010). *NF1* mutations also commonly occur in skin with CSD and are associated with a high overall burden of somatic mutations (Krauthammer et al. 2012).

BRAF Inhibitors

BRAF is a canonical member of the mitogen activated protein kinase (MAPK) signaling pathway. Mutations in codon V600 produce constitutive activation and MAPK signaling independent of upstream RAS stimulation. Since this mutation is present in nearly half of all melanomas, this represented a potential high frequency target with plausible biologic rationale. Initial trials of the putative BRAF inhibitor sorafenib showed disappointing clinical activity despite

promising preclinical results in *BRAF* mutant cell lines. The infrequent responses observed were thought to arise from the anti-angiogenic and multikinase inhibitory effects of sorafenib, rather than to direct inhibition of mutant *BRAF*.

A novel set of small molecules that directly target BRAF were subsequently developed. The first of these with preclinical validation and to enter clinical trials was vemurafenib (also known as PLX4032 and RG7204). The first few patients were treated with a crystalline formulation that inhibited ERK in on-treatment tumor biopsies, but failed to cause tumor regression and had unfavorable pharmacokinetics. The agent was reformulated as an amorphous formulation that enabled higher drug exposures and clinical responses (Bollag et al. 2010). Initially, several patients without *BRAF* mutations were included in the phase I portion, none of whom responded to treatment. Thereafter, only patients with $BRAF^{V600}$ mutations received vemurafenib.

The phase I study of vemurafenib produced remarkable clinical activity comparable to the most effective targeted therapies of the time (Flaherty et al. 2010). Many patients experienced dramatic clinical improvement even within hours to days of commencing treatment. In a small expansion cohort, the objective (unconfirmed) response rate was 81%, with a median progression-free survival (PFS) of approximately 7 months. Furthermore, nearly all patients experienced some degree of tumor regression, including those that did not achieve a true partial response.

Follow-up studies were similarly encouraging. A large phase III study was conducted, comparing vemurafenib with the alkylating agent dacarbazine in 675 patients with $BRAF^{V600E}$ mutant melanoma. This study demonstrated a dramatic improvement in clinical outcomes with vemurafenib, including confirmed response rates (48% vs. 5%), PFS (hazard ratio [HR] for progression = 0.26; median 5.3 months vs. 1.6 months), and OS (HR for death = 0.37; 6 month OS 84% vs. 64%, all p values <0.001) (Chapman et al. 2011). Benefits were observed in essentially all subgroups, including in both patients with poor traditional prognostic features (elevated lactate dehydrogenase [LDH], visceral disease involvement, advanced age) and those with favorable clinical prognosis (normal LDH, nonvisceral metastases, younger age). The phase II study of vemurafenib was subsequently reported with a longer duration of follow-up. This study demonstrated a 53% ORR and a median overall survival of 15.9 months among previously treated patients (Sosman et al. 2012). The median duration of response in this study was 6.7 months, although some patients remained on study for a longer duration (>12 months). In context, the historical median survival in advanced melanoma ranged from 6 to 9 months in prior series. See Table 1 for clinical activity of approved and experimental targeted therapies.

Subsequently, dabrafenib, another BRAF inhibitor, entered clinical development. A phase III clinical trial was the first large published experience with this agent, and confirmed clinical efficacy that was comparable with vemurafenib (Hauschild et al. 2012). This study randomized 250 patients at a 2:1 ratio to receive either dabrafenib or dacarbazine, and noted that dabrafenib produced superior ORR (50% vs. 7%) and PFS (median 5.1 months vs. 2.7 months, $p < 0.001$). Crossover to dabrafenib was permitted after progression on chemotherapy, decreasing the likelihood of observing an overall survival difference (which was not observed on this trial).

The original vemurafenib studies limited treatment to patients specifically with the *BRAF* V600E mutation. While V600E is the most common BRAF mutation, approximately 20% of mutations at the same codon involve alternative substitutions, most often valine to lysine (V600 K). Most subsequent studies included both mutations, however, found similar benefit for V600E and V600 K mutations. Several case series later showed that less common *BRAF* V600 mutations could also respond to treatment, including V600R and V600 M (Klein et al. 2013). Thus, many clinical trials (and clinical guidelines) now consider alternate V600 mutations as likely to benefit from BRAF (+/− MEK) inhibition. By contrast, non-V600 mutations appear to be insensitive to mutant-specific BRAF inhibitors (see "MEK Inhibitors" section below).

Table 1 Available targeted therapy options for melanoma

Agent	Indication	Response rate	PFS (median)	OS (median)	Reference
Vemurafenib	*BRAF*-mutant	48%	5.3 months	13.6 months	
Vemurafenib + cobimetinib	*BRAF*-mutant	68%	9.9 months	81% at 9 months	
Dabrafenib	*BRAF*-mutant	50%	5.1 months	Not reported	
Trametinib	*BRAF*-mutant	22%	4.8 months	81% at 6 months	
Dabrafenib + trametinib	*BRAF*-mutant	64%	11.4 months	72% at 12 months	
Binimetinib[a,b]	*NRAS*-mutant	20%	3.7 months	Not reported	
Imatinib[a]	*KIT*-mutant	16–29%[c]	2.9–3.5 months	10.7 months	
Nilotinib[a]	*KIT*-mutant, imatinib refractory	11% (2/19 patients)	3.3 months	9.1 months	
Trametinib[a]	Atypical *BRAF*-mutant	Case reports, but numerous responses reported for $BRAF^{L597}$ and $BRAF^{K601}$ mutations			

[a]Not FDA approved for this indication
[b]Not clinically available at the time of publication
[c]Responses appear be in the 30–50% range for exon 11 and 15 mutations, <10% for amplifications

Most initial studies also excluded patients with active brain metastases. Separate phase II studies were performed specifically for patients with brain involvement. In the BREAK-MB study, 172 patients with at least one asymptomatic brain metastasis between 0.5 and 4 cm were treated with dabrafenib (Long et al. 2012). Patients were divided into those naïve to local treatment (e.g., radiation; cohort A) or patients with prior treatment (cohort B). Among those with $BRAF^{V600E}$ mutations, 39% and 31% of patients in cohorts A and B experienced intracranial disease responses. Concordance between intracranial and extracranial responses was generally high, although some patients did experience intracranial disease progression despite systemic control. Retrospective data also suggest that vemurafenib is also active in the setting of brain metastases (Harding et al. 2015). Thus, BRAF inhibitors can be an important therapeutic tool when patients develop melanoma brain metastases.

BRAF inhibitors were generally well tolerated but were associated with a characteristic toxicity profile. Chronic effects included arthralgias, fevers, gastrointestinal symptoms, and numerous cutaneous toxicities (Chapman et al. 2011; Hauschild et al. 2012; Sosman et al. 2012). The skin effects included various hyperproliferative skin lesions, including papular rashes, papillomas, and most notably, cutaneous squamous cell carcinomas (cuSCCs). In most cases, patients were able to continue therapy following surgical resection. When sequenced, these cuSCCs were found to harbor RAS mutations (Su et al. 2012a). Interestingly, BRAF inhibitors appeared to neither initiate nor promote carcinogenesis in these tumors. Instead, they paradoxically promoted MAPK signaling, thus hastening tumor growth in these RAS-mutated cells. Several other rare cases of other RAS mutant cancers arising on BRAF inhibitors have also been described.

Although vemurafenib and dabrafenib had relatively similar toxicity profiles, several key differences were observed. Vemurafenib was generally associated with higher rates of phototoxicity, whereas dabrafenib caused more pyrexia. Overall, these agents are generally thought to have equivalent efficacy, and can thus be chosen based on tolerability and physician or patient preference. At this time however, BRAF inhibitors are rarely used as single agents, and are generally combined with MEK inhibitors.

Other BRAF Inhibitors

Encorafenib (LGX818) is a highly potent BRAF inhibitor currently under clinical development.

While early trials demonstrated clinical activity in small numbers of patients, further studies are primarily focusing on combining this agent with the MEK inhibitor binimetinib (see BRAF + MEK Inhibitors). PLX8394 is a structurally distinct agent termed a "paradox-breaker" BRAF inhibitor. This agent, in preclinical models, inhibits BRAF without inducing paradoxical MAPK signaling in RAS-mutant cells (Zhang et al. 2015). PLX8394 is currently in phase I/II clinical trials. Other multikinase inhibitors with some degree of specificity for BRAF, including RAF265, are also undergoing clinical development (Su et al. 2012b).

MEK Inhibitors

MEK is immediately downstream of RAF in the MAPK pathway. As such, it was hoped that inhibition of this signaling node would have activity in both *RAF* and *RAS* mutant cancers. A phase I/II study was conducted that included patients with a variety of malignancies. A large subset of melanoma was enrolled, consisting of 36 patients with *BRAF* mutations, 10 with *NRAS* mutations, and 29 with *BRAF*/*NRAS* wild-type disease (Falchook et al. 2012). Among the *BRAF* mutant cohort, the response rate was 33% and the median PFS was 5.7 months. Notably, no responses were observed among the six patients who previously received a BRAF inhibitor. No patients with *NRAS* mutations responded to therapy, and 10% of the *BRAF*/*NRAS* WT group responded. Two of these patients were later found to have atypical non-V600 *BRAF* mutations, suggesting that only a small proportion of truly *BRAF* wild-type melanomas would benefit from trametinib.

To follow up this study, a phase III study comparing trametinib to investigator's choice chemotherapy was performed in 322 patients (Flaherty et al. 2012b). The median PFS was substantially higher in patients treated with trametinib (4.8 vs. 1.5 months, $p < 0.001$). In addition, OS was superior, despite allowing for crossover to trametinib for patients who progressed on chemotherapy (6 month OS rate 81% vs. 67%, $p = 0.01$). Based on these data, trametinib received FDA approval for advanced, *BRAF*-mutated melanoma. Given the marginally inferior outcomes compared to BRAF inhibition (albeit comparing across trials), trametinib has not been extensively used as a single agent in this setting. Several other MEK inhibitors have undergone clinical development (see "*NRAS* Mutant Melanoma" and "Uveal Melanoma" sections).

The toxicities observed with trametinib were quite distinct from those of BRAF inhibitors. Cutaneous events were also present, but stemmed from hypoproliferative effects on keratinocytes. Clinically, this manifests as an acneiform rash similar to that observed with EGFR inhibitors (e.g., erlotinib, gefitinib). Ocular toxicities, including central serous retinopathy and retinal vein occlusion, were observed occasionally, but at much lower rates than earlier generation MEK inhibitors. Cardiomyopathy, which usually reversed following treatment, was also observed infrequently. Gastrointestinal symptoms, edema, and joint pain also occurred and were generally of low-grade and clinically manageable.

BRAF + MEK Inhibition

A number of sequencing efforts demonstrated that most tumors resistant to BRAF inhibitors had incomplete MAPK blockade (see BRAF resistance, below) (Shi et al. 2014). Further, both BRAF and MEK inhibitors demonstrated substantial clinical activity as single agents at distinct nodes in the MAPK pathway. Thus, combining these agents was thought to be a rational therapeutic strategy for the *BRAF* mutant cohort. A phase I/II trial was conducted with dabrafenib and trametinib. In a randomized portion of this study, dabrafenib and trametinib were compared with dabrafenib monotherapy (Flaherty et al. 2012a). Improvements were noted in ORR (76% vs. 54%, $p = 0.03$) and median PFS (9.4 vs. 5.8 months, $p < 0.001$) with the highest dose of the combination (dabrafenib 150 mg twice daily and trametinib 2 mg daily). Based on these data, the combination of dabrafenib and trametinib received FDA approval in 2014.

Several follow-up phase III studies have verified and extended these findings in larger

populations. Dabrafenib and trametinib were compared with dabrafenib (COMBI-D study) in 423 patients with advanced, *BRAF*-mutant melanoma (Long et al. 2015). The combination resulted in superior PFS (median 11 vs. 8.8 months, $p < 0.001$) and OS (median 25.1 vs. 18. months, $p = 0.01$). Survival at 1 year (74% vs. 68%) and 2 years (51% vs. 42%) were also improved in the combination arm. In parallel, the COMBI-V study compared the same combination with vemurafenib in 704 patients (Robert et al. 2014). Similar findings were reported in this study, with improved 12 month OS (72% vs. 65%, $p = 0.005$), median PFS (11.4 vs. 7.3 months, $p < 0.001$), and response rates (64% vs. 51%).

Vemurafenib has also been evaluated in combination with another MEK inhibitor, cobimetinib. After promising response rates and PFS were observed in early phase trials, a phase III study comparing this combination with vemurafenib monotherapy was conducted (Larkin et al. 2014). Similar to the other BRAF + MEK inhibitor regimen, the combination produced superior PFS (median 9.9 vs. 6.2 months, $p < 0.001$) and response rate (68% vs. 45%, $p < 0.001$). Vemurafenib and cobimetinib received regulatory approval in November 2015 for treatment of *BRAF* V600 mutant melanoma. Based on these data, combined BRAF and MEK inhibition with either dabrafenib + trametinib or vemurafenib + cobimetinib is preferred over single-agent therapy and is now widely used.

Combined BRAF/MEK inhibition produced a unique toxicity profile. Stereotypic MEK inhibitor effects were observed at expected rates (cardiac, ocular) (Larkin et al. 2014; Long et al. 2015; Robert et al. 2014). Fevers also emerged as the most problematic, stereotypic toxicity with dabrafenib + trametinib. In most cases, these were manageable with nonsteroidal anti-inflammatory drugs (NSAIDs) and/or corticosteroids. Intriguingly, the cutaneous effects of either single agent were attenuated by combining agents. This apparent "cancelling out" of toxicities is attributed to blockade of paradoxical MAPK activation by the concurrent MEK inhibition in BRAF WT skin cells. The combination of vemurafenib and cobimetinib was not associated with pyrexia, but photosensitivity, diarrhea, and elevated creatinine phosphokinase levels were observed. Overall, the toxicity profiles of either combination are generally viewed as equivalent or potentially even superior compared to BRAF or MEK inhibitor monotherapy.

Another BRAF and MEK inhibitor combination is also undergoing clinical development: encorafenib and binimetinib. Early results suggest that response rates, progression-free survival, and incidence of toxicities are relatively comparable to the approved combinations. A randomized phase III study is currently comparing encorafenib and binimetinib with vemurafenib.

Therapy Selection

The data presented above clearly demonstrates superiority for combined BRAF + MEK inhibitors compared to single agent BRAF inhibitors. Therapy selection between BRAF + MEK inhibitors and immune therapy, however, is not so clear. In fact, this decision presents a common conundrum for patients with $BRAF^{V600}$ mutations. The clinical efficacy of immune checkpoint inhibitors extends to patients with and without *BRAF* mutations, providing a compelling alternative clinical option for these patients. A cooperative group trial sponsored by the US National Cancer Institute is currently evaluating dabrafenib + trametinib compared to ipilimumab + nivolumab with crossover to the alternative group at the time of progression (https://clinicaltrials.gov/ct2/show/NCT02224781). This study will provide more definitive guidance for clinicians about the most appropriate first-line selection. In the interim, there are several principals to guide this decision.

In general, BRAF-directed therapies are associated with high response rates and clinical benefit in nearly all patients. Therefore, for patients who are highly symptomatic and "need a response," many clinicians will choose BRAF + MEK inhibition as the initial therapy over immune checkpoint inhibition. Unfortunately, this group of patients generally has poor outcomes overall,

with a limited duration of benefit from BRAF + MEK inhibitors. By contrast, immune therapies may provide durable benefit for a sizable minority of patients (or perhaps even a majority for ipilimumab + nivolumab). Thus, many clinicians prefer an immune approach for most patients initially and reserve BRAF-directed therapies for the salvage setting.

Recent long-term data from early dabrafenib + trametinib studies add complexity to this decision (Long et al. 2016). Patients with low LDH or <3 sites of disease had outstanding outcomes to targeted therapy, with 3-year overall survival rates of 62% and 58%, respectively. This is a population that typically performs well with immune therapy as well. By contrast, patients with high LDH and those with ≥3 sites of disease involvement had dismal long-term outcomes with dabrafenib + trametinib, with 3-year OS of 5% and 19%, respectively. Thus, the traditional melanoma risk factors (metastatic stage, LDH) appear to correlate with outcomes for both immune and targeted therapies. Better therapies and improved selection markers are needed, particularly for the poor-prognostic subgroups.

Long-Term Outcomes

The traditional dogma has been that BRAF-directed therapy produces responses of limited duration with inevitable onset of acquired resistance. The long-term outcome data has challenged these assumptions, with extended follow-up from BRAF inhibitor monotherapy and BRAF+MEK combination studies. Particularly with the combination, a 3–4 year survival of approximately 20% appears to be emerging, with a "tail of the curve" appearance to the PFS figures (i.e., very few patients have disease progression between 2.5 and 4 years) (Long et al. 2016). While it is unclear whether these patients will have truly long-lasting responses, the lack of delayed progression argues for durability. Our own clinical experience mirrors these studies, with a small but definite patient population still receiving treatment many years after beginning therapy.

BRAF Inhibitor Resistance

Unfortunately, long-term PFS with BRAF-directed therapy is the exception rather than the rule. While this topic is comprehensively covered in another chapter, the core signaling nodes and concepts that underpin resistance are briefly reviewed here. Although nearly all patients receive clinical benefit and tumor regression from BRAF inhibitors, most ultimately develop acquired resistance to therapy causing disease progression. Posttreatment biopsies, obtained at the time of disease progression, revealed that MAPK signaling was reactivated in most progressing tumors despite ongoing BRAF inhibition. Intensive sequencing efforts by a number of groups revealed numerous and recurrent genetic and transcriptomic changes that reinvigorate MAPK signaling (Rizos et al. 2014; Shi et al. 2014; Van Allen et al. 2014). These included *NRAS* mutations, *BRAF* amplification, alternate splicing of *BRAF* (causing dimerization and efficient signaling), *MEK1/2* mutations, and *COT* overexpression. A smaller set of resistant samples displays MAPK-independent resistance mechanisms, including PI3K-AKT pathway changes or receptor tyrosine kinase upregulation. Overall, though, the majority of these changes leading to BRAF inhibitor resistance involved MAPK pathway reactivation. Hence, combined BRAF and MEK inhibition was considered to more completely extinguish MAPK signaling. Several studies have also shown, however, that genetic changes promoting MAPK signaling drive combination therapy resistance as well. More recently, a number of nongenetic and immune correlates of resistance have been identified which may further complicate attempts to target resistant pathways and impact immune therapy approaches (Hugo et al. 2015). Further, substantial heterogeneity within and across progressing tumors has been described. This diverse and complex landscape of resistance has decreased our optimism that targeting canonical signaling pathways can reverse or overcome resistance. A number of different approaches, however, have been attempted or are ongoing.

Targeting BRAF Inhibitor Resistance

One initial trial assessed MEK inhibition with trametinib following resistance to dabrafenib or vemurafenib. In a phase II study, 40 patients who failed BRAF inhibition received trametinib; none responded to therapy, and the median PFS was a dismal 1.8 months (Kim et al. 2013). Combined BRAF and MEK inhibition, which clearly delayed the onset of acquired resistance and improved clinical outcomes compared to monotherapy, was assessed in the BRAF inhibitor resistant setting. As part of the initial phase II study of dabrafenib + trametinib, a cohort of patients who failed vemurafenib or dabrafenib was included. These patients had markedly inferior outcomes to patients treated in the naïve setting, with median PFS of 3.6 months and a response rate of 12% (Johnson et al. 2014). Similar results were observed with vemurafenib and cobimetinib (Ribas et al. 2014). Thus, this approach is not commonly used.

Other single agents or combinations of targeted therapies have produced promising preclinical results, but this has yet to translate into clear clinical efficacy. ERK inhibitors may have a role in overcoming BRAF inhibitor resistance. The final canonical member of the MAPK pathway, however, has been challenging to target. Several inhibitors are now in clinical trials, with some early and modest signs of efficacy. Various combinations with preclinical support are also being tested to prevent or overcome resistance, including combinations of BRAF +/− MEK inhibitors with inhibitors of the PI3K/AKT pathway, heat shock proteins, MDM2 (a protein that interacts with p53), and autophagy (hydroxychloroquine).

Another intriguing strategy to delay resistance is intermittent dosing of BRAF inhibitors. While this approach has not been confirmed clinically, preclinical data suggests that drug dependence develops with continuous dosing of vemurafenib (Das Thakur et al. 2013). Discontinuous dosing, by contrast, exploits this dependency and forestalls the onset of resistance. A US National Cancer Institute-sponsored cooperative group study is currently testing this strategy with dabrafenib and trametinib. (https://clinicaltrials.gov/ct2/show/NCT02196181). In this study, patients receive continuous dosing for 8 weeks. Nonprogressing patients are then randomized to a 5 weeks on and 3 weeks off treatment schedule or continuous dosing. While the intermittent dosing schedule has not been validated for routine use, many experts prefer this strategy to repeated dose decreases in the presence of toxicities.

Finally, combining targeted and immune therapies, the two active therapeutic classes in melanoma, remains of great interest. Several elegant preclinical studies have suggested that BRAF and/or MEK inhibitors have various effects on the tumor microenvironment (preclinical and clinical data reviewed in Robert et al. 2016). Specifically, BRAF inhibitors have been associated with upregulation of melanoma differentiation antigens, major histocompatibility complexes, and immunogenic cytokines. MEK inhibitors have been shown to dampen T cell proliferation, which could either compromise immune therapy activity, or suppress toxicities. The combination has also demonstrated increased tumor infiltrating lymphocytes with increased clonality (suggesting a more specific antitumor response), and increase in PD-1/PD-L1 expression.

Clinically, combined BRAF-directed and immune therapies have had mixed results. The combination of vemurafenib and ipilimumab produced unacceptable hepatotoxicity, and the initial experience with the triple combination of dabrafenib, trametinib, and ipilimumab was complicated by frequent colitis and bowel perforations. While these initial studies have demonstrated the unpredictable nature of these toxicities, several subsequent studies have been more promising. Dabrafenib and ipilimumab appear to have a tolerable side effect profile in early results. Several other studies combining anti-PD-1/PD-L1 directed agents (atezolizumab, pembrolizumab) with BRAF+MEK inhibitors have shown signs of efficacy without substantial additive toxicity. Interestingly, a run-in period of vemurafenib and cobimetinib (for 28 or 56 days) appeared to produce superior activity (responses in 14 of 16 patients vs. 1 of 3 patients) and toxicity (grade 3/4 toxicities in 5 of 14 vs. 2 of 3) compared to concurrent administration with

atezolizumab. The median PFS in these few patients was 10.9 months, however, which is similar to vemurafenib + cobimetinib alone. Further study is needed to assess these novel approaches.

Therapy of Melanomas Without *BRAF*^{V600} Mutations

Genetics of *BRAF* V600 Wild-Type Melanomas

Following *BRAF*, *RAS* is the next most frequent driver mutation making up approximately 20% of the cases of cutaneous melanoma in numerous studies (Cancer Genome Atlas Network 2015; Hodis et al. 2012; Krauthammer et al. 2012). *NF1* loss of function (LOF) mutations make up 14% of melanomas analyzed with some overlap with BRAF-non-V600 mutations. The tumors which have *NF1* mutations also have the highest overall somatic mutation burden, likely reflecting their primaries on chronically sun-exposed sites from older patients (Krauthammer et al. 2012). Other recurrent alterations in *RAC1*, *PPP6C*, *ARID2*, *IDH1, RB1*, and *DDX3X* have been identified. Finally, there are a number of candidate fusion drivers in cases involving *BRAF* and other genes (Hutchinson et al. 2013). Many of these fusions have intact kinase domains without the regulatory sequences, leading to constitutive kinase activation.

Another way of looking at the TCGA would be the class of the genetic alteration in terms of the strength of evidence for them to be actionable targets. **Class 1** would be those which are known to be **clinically targetable** including those responsive to BRAF inhibition or MEK inhibition, CDK inhibition, MDM2/p53 inhibition, PI3K/Akt, mTOR inhibitors; or **class 2 translational actionable**, ERK inhibitors, MEK inhibitors, IDH1 inhibitors, EZH2 inhibitors and even Aurora kinase inhibitors (PPP6C) and **class 3 preclinical**, chromatin remodelers inhibition, BH3 mimetics, and others. The TCGA report also included an extensive gene expression and protein expression analysis (Cancer Genome Atlas Network 2015). This was most revealing for the presence of a strong immune RNA expression signature with overexpression of genes associated with T cells, B cells, NK cells, chemokines, cytokines, and immune signaling molecules and inhibitory checkpoint proteins in melanomas from all of the genetically classified TCGA melanoma cohorts. The presence of an immune signature was associated with an improved prognosis independent of any treatment. This likely is of greatest relevance to the responsiveness to immunotherapy, although it may also have implications in targeted therapies. The remainder of this chapter will focus on defined subsets of *BRAF* V600 WT melanomas including those with mutations in *NRAS*, *NF1*, TWT and followed by uveal, mucosal, and acral, subtypes not represented in the TCGA.

NRAS-Mutated Melanomas

NRAS mutant melanomas present with several specific clinical features (Thomas et al. 2015). Primary tumors have been associated with regions of chronic sun damage, increase in number of mitoses, decrease in tumor infiltrating lymphocytes, and frequently upstaged primaries. At diagnosis of stage IV (M1) disease *NRAS* melanomas have a worse prognosis to TWT melanomas and more likely involvement of the central nervous system.

Directly targeting *NRAS* has been a challenge, since it is not a kinase and because of the very tight RAS-GTP binding (Stephen et al. 2014). NRAS-mediated activation involves the reversible exchange of GDP for GTP whereas turning off RAS involves hydrolysis of GTP to GDP. Guanine nucleotide exchange factors (GEFs) accelerate the activation of NRAS, while GTPase activating proteins (GAP) accelerate the off switch. The downstream effectors include ERK through the MAP kinase pathway, PI3K and PDK1, RALGDS, RAL guanine nucleotide dissociation stimulator, PLD phospholipase D (PLD), Phospholipase C (PLC), protein kinase C (PKC), and T-cell lymphoma invasion and metastasis 1 (TIAM1) (Johnson and Puzanov 2015). In preclinical studies completely knocking down

(extinguishing) PIK3CA, p110α can reduce both NRAS- and KRAS-driven tumors formation. These results highlight the need to simultaneously inhibit other pathways beyond MAPK signaling for *NRAS*-mutated melanomas. Most of the downstream pathways, however, are not tumor-specific targets which may lead to significant side effects. As always, well-designed clinical studies with both pharmacokinetics and pharmacodynamic endpoints are needed for combination therapy.

Numerous attempts have been made including preventing the binding of NRAS from the cell membrane with initially farnesyltransferase inhibitors, and following their failure, the targeting of the geranyl-geranyl-transferase, inhibition of RAS-SOS protein interaction with small molecules, and inhibiting the binding of RAS to the RAF molecule (s) (Gajewski et al. 2012). Since *NRAS* mutant melanomas predominantly have a Q61 mutation, the cysteine reactive molecules selective for *KRAS* G12C binds to another pocket and would not be effective (Burd et al. 2014). This has led to a focus on MEK inhibition downstream from RAS-RAF, which is already known to be problematic due to the greater ERK reactivation feedback that it induces. Nevertheless an oral MEK inhibitor (MEK162, Binimetinib), a non-competitive ATP inhibitor of MEK1 and MEK2, has been tested clinically in patients with metastatic melanoma harboring BRAF or NRAS mutations. Early encouraging results in NRAS-mutated patients have been noted, with an objective response rate of 20%, of which only 10% were confirmed with imaging (Ascierto et al. 2013). The median PFS was 3.7 months with a median duration of response of 7.6 weeks. This is consistent with rapid development of acquired resistance, even for responding patients. This phase I/II study led to a phase III trial comparing binimetinib versus chemotherapy enrolling 402 NRAS-mutant melanoma patients with a 2:1 randomization favoring binimetinib. The study was recently reported at the 2016 ASCO meeting. Patients were required to have a Q61 NRAS mutation and towards the completion of accrual, prior immunotherapy was allowed. Only 20% had prior checkpoint inhibitors, including 13% with ipilimumab and only a few percent (5–6%) receiving prior anti-PD1. The study met its primary endpoint with an improvement of PFS (HR = 0.62, $p = 0.001$). However, the increase in median PFS was not especially impressive, increasing from 1.5 to 2.8 months, and the ORR favored binimetinib 15% versus 7%. The PFS benefit appeared to be more pronounced in those patients with poor prognostic factors including those with stage IV M1c, more numbers of organ involved, presence of visceral disease, and elevated serum LDH. Interestingly, even though those with prior immunotherapy represented only 20% of the patients, they had the most obvious benefit in terms of median PFS, increasing from 1.6 months to 5.5 months. The median overall survival for all patients enrolled was no different (11 vs. 10.1 months, HR = 1.0). Of note 45% of both groups received immunotherapy following protocol treatment with those who received binimetinib being slightly more likely to have PD as their best response to immunotherapy. Results of this trial may lead to approval of the first targeted agent for *NRAS* mutant melanoma, but the results are below the hoped-for improvement. It may provide a component of therapy in the future. The results with those patients who have had prior immunotherapy is the most promising aspect of the trial, since nearly all patients in the future would be treated in this order, even with the availability of a MEK inhibitor.

Combination therapy with a MEK inhibitor backbone has also been a treatment strategy of interest. Generally, combined PI3K and MEK inhibitor therapy has seemed to be feasible with manageable safety and toxicity profile. The most common adverse events (AEs) of therapy include diarrhea, rash, fatigue, vomiting, and hyperglycemia. The clinical activity of GDC-0973 (MEK1/2 inhibitor) and GDC-0941 (class I PI3K inhibitor) was studied in 78 patients with advanced solid tumors (Asati et al. 2016). Daily dosing of BKM120 (pan-PI3K inhibitor) and trametinib (MEK inhibitor) was evaluated with 49 patients with advanced RAS- or B-RAF-mutant cancers. In another combination study, 49 patients were treated with the pan-PI3K inhibitor copanlisib and the MEK inhibitor refametinib (Asati et al. 2016). The combination of BYL719 (PI3Kα

inhibitor) and binimetinib (MEK inhibitor) was studied in 58 patients with advanced solid tumors with RAS or B-RAF mutations (Asati et al. 2016). None of these trials with MEK inhibitors and PI-3 kinase/mTOR inhibitors generated promising enough results to pursue phase II trials in melanoma, including in *NRAS* mutant melanoma. Toxicity of the regimen, while tolerable, never allowed dose escalation required to see the promising clinical effects in patients.

Interestingly, hyperactivation of AKT and loss of PTEN expression dominates the picture in brain metastasis versus other sites of disease (Davies et al. 2009). Brain-derived factors appear to induce hyperactivation of the AKT survival pathway and to promote the survival and drug resistance of melanoma cells in the brain. Thus, inhibition of PI3K-AKT signaling shows potential for enhancing and/or prolonging the antitumor effect of MEK inhibitors in melanoma brain metastases.

In all of non-*BRAF* ^{V600} mutant melanoma (NRAS, NF1, TWT), interest in combining MEK inhibitors with CDK4/6 inhibitors has the frequent dysregulation of the CDK4/6-RB1 pathway. This occurs through several mechanisms, including overexpression and/or amplification of D-type cyclins, mutation or amplification of *CDK4/6*, or loss of cyclin D-CDK4/6 negative regulators such as p16INK4A (Hodis et al. 2012). In mouse models of *NRAS* mutant melanoma, including xenograft and syngeneic models, combined MAPK and CDK4/6 inhibition has been promising (Kwong et al. 2012). Inhibition of MEK activates apoptosis, but not cell-cycle arrest. Therefore, cell death is balanced by continued proliferation, leading to tumor stasis in vivo. In contrast, the knock out (extinguishing) of *NRAS* induces apoptosis and cell cycle arrest. CDK4 was identified as the critical driver of this differential phenotype. The predominant cytostatic effects of CDK4/6 inhibition, when combined with MEK inhibition, led to apoptosis with blockade of continued proliferation, resulting in net tumor regression and substantial synergy in therapeutic efficacy. Consistent with these results, combined CDK4 and MEK inhibition led to increased apoptosis and/or reduced viability in colony formation assays in human melanoma and pancreatic cancer cell lines.

Combination of CDK4/6 inhibitors with RAS/RAF/MEK/ERK pathway inhibitors is a promising therapeutic approach, particularly in patients with melanoma. Identifying the optimal dose and schedule to maximally inhibit both pathways while minimizing toxicity remains an elusive goal. Treatment-related toxicities were common and included creatinine phosphokinase elevation, rash, edema, anemia, nausea, diarrhea, and fatigue. Clinical studies have included binimetinib + ribociclib, trametinib + palbociclib, and PD901+ palbociclib (Sosman et al. 2014). There is the most experience with the combination of binimetinib + ribociclib, first with a 28-day cycle of ribociclib (3 weeks on and 1 week off) and continuous binimetinib. Twenty-two patients were enrolled with some significant toxicities including renal, creatinine phosphokinase elevations, anemia, atrial fibrillation with five DLTs including an intracranial bleed. Only well below single-agent MTD could be assessed for ribociclib and binimetinib, but still clinical activity was extremely encouraging with five confirmed PR, four unconfirmed PR, and eight SD – 9/22 (41%) ORR. The duration of response was 56–351 days and overall median PFS was 6.7 months. Due to toxicity, a 21-day cycle of ribociclib (14 days on, 7 days off) was assessed enrolling 22 patients with 4 objective responses and a median PFS of 4 months. An additional 7 patients were enrolled to receive 200 mg ribociclib daily for 14 of 28 days/45 mg BID binimetinib. Ultimately, this was the recommended phase 2 dose. Cumulatively, 6 responses were seen out of 16 treated patients at this selected dose and level with an overall RR of 35% (10/29), median DOR of 5.0 months, and median PFS of 6.4 months. All of these metrics appeared superior to binimetinib alone. While trametinib plus palbociclib has completed a phase I trial, the heterogenous composition of patients did not allow any assessment of clinical activity in NRAS mutant melanoma.

There have been numerous approaches that are currently being tested in clinical trials, but too early to provide a clear idea of the clinical activity. One approach of great interest is ERK inhibition

which would conceptually prevent the ERK-induced feedback seen with MEK inhibitors. Three ERK inhibitors have entered clinical trial development. SCH772984 and RG7842 (GDC0994; Genentech/Roche) are currently being tested in Phase I clinical trials (Morris et al. 2013). BVD-523 (ulixertinib; Biomed Valley Discoveries), a novel ERK1/2 kinase inhibitor, has recently entered phase I clinical trials with responses noted in three patients with BRAF mutant melanoma, including one refractory to prior BRAF/MEK inhibition. Expansion cohorts have included over 20 patients with *NRAS* mutant melanoma, but no results have been reported at this time.

For RAS-mutant tumors, effective inhibition of MAPK signaling remains a major challenge. MEK inhibitors have shown definite modest clinical activity in this setting and they may be more effective if combined with either CDK4/6 inhibitors, PI3K/mTOR inhibitors, or other approaches. ERK inhibitors or RAF inhibitors that can overcome resistance due to RAF dimerization or even potential RAS inhibitors may ultimately become a reality.

Other potential approaches include c-Met inhibition, based on ex vivo work demonstrating increased C-Met phosphorylation in *NRAS* mutant melanoma. Inhibition of NRAS decreases C-MET responsiveness to HGF, and *NRAS* mutant tumors were more sensitive to c-Met blockade (Chattopadhyay et al. 2012). RAF paradox breakers (PLX7904 and PLX8394) are RAF inhibitors that evade paradoxical MAPK pathway activation with no pERK induction in RAS mutant tumors, and pan-RAF inhibitors are in early clinical trials (Zhang et al. 2015). Finally, inhibitors of polo-like kinase (PLK1), a molecule which is overexpressed in *NRAS* ^{Q61} mutant melanoma, when combined with MEK inhibitors, lead to major reduction in cell cycle-related genes including CCND1, CDC25A, CHEK2, E2F1, causing dual cell cycle arrest (Posch et al. 2015). Results suggest that cells escaping G1 arrest induced by MEK inhibition, or cells escaping G2/M arrest from PLK1 inhibition, maintain their susceptibility to the other drugs in combination. This will have to be tested in the clinic but remains of interest. Additional targets include MDM2, through its effect on p53, BET inhibitors, where members of family of BET proteins (especially BRD4 and BRD2) are overexpressed in many melanomas, and MITF, due to the finding that MEK inhibition increased MITF expression, which in turn elevated levels of PGC1a (Fedorenko et al. 2012). A HIV1 protease nelfinavir suppresses both MITF and PAX3 and inhibits growth. Finally, the combination of metformin and trametinib has a synergistic effect in *NRAS* mutant tumors and reduces tumor size in a xenograft model (Smith et al. 2016). This is likely a result of suppressing the phosphorylation of NRAS effector proteins ERK and S6 decreasing cell viability.

Targeting of the NF1 Loss of Function (LOF) Melanomas

In the TCGA and the Yale cohort (213 melanomas), three genes are mutated with an incidence greater than 10%: *BRAF* and *NRAS*, with known recurrent activating mutations, and *NF1*. *NF1* had a high number of inactivating or damaging mutations; 90% are nonsense, splice-site variant or insertion-deletion (indel) and LOH (loss of the other allele in most of these cases) (Hodis et al. 2012; Krauthammer et al. 2012). These are cases where *NF1* was presumably the driver of the melanoma without activations through *BRAF* or *NRAS* mutations. Inactivating *NF1* mutations were present in 46% of melanomas expressing wild-type *BRAF* and wild-type *RAS*, occurred in older patients, harbored many more somatic mutations throughout the entire exome, and had an OS similar to BRAF, NRAS, and TWT melanomas. NF1 is a GTPase-activating protein, a GAP protein that suppresses RAS function. NF1 suppression leads to increased RAS activation in a large percentage of melanomas (Krauthammer et al. 2012). Loss of *NF1*, however, does not predict sensitivity to MEK or ERK inhibitors. These tumors showed a distinct pattern of co-mutation with other genes related to RAS pathways such as RASopathy gene mutations (Krauthammer et al. 2012). These are included in (15/26) 60% with RASopathy gene mutations in *RASA2* (9 cases),

PTPN11 (4), *SOS1* (2), *RAF1* (2), and *SPRED1* (2) that may enhance its role in melanoma development.

In terms of therapy specific for *NF1* mutant melanomas, there is very little information. One preclinical study suggested that MEK inhibition could be effective, although we do not yet have clinical experience demonstrating this finding (Nissan et al. 2014). However, in neurofibromatosis type 1, where the *NF1* gene is damaged in the germline, patients are predisposed to cutaneous neurofibromas, plexiform neurofibromas (PNFs), and malignant peripheral nerve sheath tumors (MPNSTs) among other neoplasias and manifestations. In these cases, MEK inhibition has shown substantial benefit (Dombi et al. 2016).

BRAF/NRAS wild-type melanomas are highly sensitive to the MEK inhibitor, trametinib, ex vivo, but the loss of *NF1* protein expression alone does not select for sensitive cell lines. In a review of "exceptional responses" (objective response or SD > 6 months) to everolimus, one patient with head and neck cancer with a LOF *NF1* mutation had PFS of nearly 10 months (Lim et al. 2016). Ultimately, the most effective targeted therapy approaches for melanomas with *NF1* mutations has yet to be defined.

BRAF-resistant cell lines are sensitive to AZ628, an inhibitor of $BRAF^{V600E}$, WT *BRAF*, and *CRAF* (a so-called pan-RAF inhibitor) (Peng et al. 2015). This inhibitor was combined with the MEK inhibitor selumetinib with near complete pERK decrease and improved responses in resistant cell lines through the loss-of-function mutations in NF1 (LOXIMVI, HCT116). It should be noted that AZ628, RAF265, and MLN2480 are all type II RAF inhibitors (pan-RAF), whereas vemurafenib and dabrafenib are type I inhibitors. These agents could potentially have activity in NRAS, NF1, and TWT melanomas.

Targeting Atypical BRAF Mutant Melanoma (Non-V600)

Approximately 5% of all melanomas harbor mutations in *BRAF* at loci other than *V600 (BRAF non-V600 mutations*) (Cancer Genome Atlas Network 2015). These mutations are often not detected by testing platforms commonly used in clinical practice that assess only codon 600 for mutations. However, they are detected by sequencing all the *BRAF* exons. A number of these *BRAF* non-V600 mutations result in increased kinase activity of the BRAF protein in vitro (i.e., L597 V, K601E, G469A). In addition, other mutations that do not increase the catalytic activity of BRAF (including G466E, D594V, G596R) appear to increase MAPK pathway activity through protein-protein interactions with CRAF or wild-type BRAF (Wan et al. 2004). This occurs in the setting of upstream activation (NRAS mutation or receptor tyrosine kinase (RTK) activation). Furthermore, BRAF fusions which activate MAPK signaling have been identified in a small percentage of melanomas (Hutchinson et al. 2013). MEK inhibitors have demonstrated activity in vitro in melanomas with high activity non-V600 BRAF mutations, and individual patients with these mutations have demonstrated significant clinical responses to treatment with MEK inhibitors. A phase II trial is currently ongoing to assess the activity of trametinib across the spectrum of atypical BRAF mutations and fusions.

Targeting KIT

KIT mutations and/or amplifications are rare in melanoma, although 5–20% of melanomas originating on mucosal, acral, and chronic sun damage (CSD) surfaces demonstrate KIT genetic abnormalities (Curtin et al. 2006). By contrast, these alterations are almost never seen originating from other cutaneous areas without CSD. The mutant allele is sometimes amplified and in some tumors the wild-type KIT locus is amplified. Given the experience with gastrointestinal stromal tumors, inhibition of KIT was thought to be an attractive therapeutic strategy. Exon 11 and 13 are the most sensitive and the most abundant mutations are at L576P and K642E. The first large cohort from the USA enrolled patients with mutations and/or amplifications in KIT (Carvajal et al. 2011). Two hundred and ninety-seven patients were screened

with melanomas originating in one of these three sites and only 51 were found to have mutations and/or amplifications in KIT, with only 28 enrolled onto imatinib treatment over 3 years. Of 25 evaluable patients treated at 400 mg daily imatinib, there were four objective responses, (2CR and 2PR; ORR 16%) and all were durable >12 months. All responders had mutations (rather than amplifications) and those were at codons L576 and K642 in two of four patients. Of the 209 actually screened, 18/84 acral melanoma, 17/93 mucosal melanoma, and 5/32 CSD had *KIT* mutations. In another trial undertaken in China, 43 patients were enrolled with either *KIT* amplification or mutation or both and received imatinib at 400 mg/day which could be increased to 800 mg q day at progression (Guo et al. 2011). In this study, there were 10 PR and no CR; 9/10 responses had mutations at exon 11 or 13 (9/26), while 1 of 3 patients with amplified *KIT* responded. More recently, Hodi and colleagues published on 25 treated patients out of 213 screened over 5 years (Hodi et al. 2013). Of 24 patients with *KIT* alterations, 8 had KIT mutations alone, 5 had mutations+ amplifications of *KIT*, and 11 had only KIT amplification. Seven patients experienced PRs, but these only occurred in *KIT*-mutated melanomas. The ORR was 29%, but in 13 patients with mutations, the ORR was over 50% (7/13), and 6 responses had exon 11 or 13 mutations (of which 4 had L576P and K642E mutations). However, only 1/7 patients had response duration >12 months with one ongoing response at 27+ months. Two other studies of nilotinib or sunitinib were performed. In patients who were refractory or with intolerable side effect on imatinib, only 2/11 had a response in second line to nilotinib (Carvajal et al. 2015). Finally, 52 patients with acral or mucosal melanoma were treated with sunitinib and only 13 of the 44 patients whose tumors were tested for *KIT* mutations. 1/13 with mutations responded while 3/31 WT responded to sunitinib (Buchbinder et al. 2015). In this study, the presence of mutations did not correlate with response rate and all responses were 5–10 months in duration. In summary, the primary drug studied in *KIT* mutant/amplified melanoma has been imatinib, and overall responses were almost always observed in those with mutations in exon 11 and exon 13 (particularly L576P and K642E). Although some responses were very durable, most lasted less than 12 months. The ORR has been in the range of 15–25%, leaving plenty of room for further improvement.

Uveal melanomas have a distinct biology from cutaneous melanoma with a low mutation burden without a UV signature and absence of mutations of *BRAF*, *NRAS*, *KIT*, or *NF1*. In over 80% of tumors, either *GNAQ* or *GNA11* is mutated in a mutually exclusive fashion. These genes appear to activate the MAP kinase pathway through PKC and the RAS-GEF RasGRP3 (Chen et al. 2017; Van Raamsdonk et al. 2009, 2010). More recently the YAP- hippo pathway has also been implicated in uveal melanomas (Feng et al. 2014). About 40–50% of uveal melanoma have LOF mutations or deletions in the *BAP1* gene, which is associated with inferior prognosis and high likelihood of metastases (Harbour et al. 2010). *SF3B1*, a splicing factor, is another recurrently mutated gene which occurs in 15% of uveal melanomas and is associated with a good prognosis (Harbour et al. 2013). Therapy with interferon in the adjuvant setting and checkpoint inhibitors in the metastatic setting have been very disappointing (Luke et al. 2013). One promising lead had been seen with a MEK inhibitor, selumetinib. A randomized phase II trial showed a RR of 14% versus 0% for dacarbazine chemotherapy and a doubling of PFS, increasing from a median of 7 weeks to 15.9 weeks (Carvajal et al. 2014). However in a follow-up study with selumetinib + dacarbazine versus dacarbazine alone, the PFS endpoint was not met (Komatsubara et al. 2016). Finally, more disappointment occurred with a recent study presentation where a MEK inhibitor, trametinib alone or with an AKTi, demonstrated only one objective response in 20 patients, and the study was closed early due to lack of efficacy. Other approaches are ongoing with a MEK inhibitor and a PKC inhibitor, and targeting BAP1 with EZH2 inhibitor, targeting of YAP, or inhibition of the hippo pathway. Certainly therapy of uveal melanoma has been stagnant without any real evidence of efficacy with either targeted or immunotherapy. New

approaches targeting the biology of uveal melanoma are desperately needed.

Conclusions

Ultimately, targeted therapy in melanoma has been a qualified success story thus far. The dramatic clinical efficacy of BRAF and MEK inhibitors in *BRAF* mutant melanoma greatly benefits patients harboring these mutations, although acquired resistance limits the duration of benefit. Novel strategies to overcome resistance in the *BRAF* cohort, to identify tractable therapeutic targets in the *BRAF* WT population, and to design effective combinatorial strategies remain urgent needs.

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Combinatorial Approach to Treatment of Melanoma

33

Kamran Abri and Adil I. Daud

Contents

Abstract

There are now multiple effective, well-tolerated, FDA-approved systemic therapy options for the treatment of advanced melanoma, as well as exciting new immunotherapy and targeted therapy agents currently in clinical trials. Both traditional cytotoxic chemotherapy and targeted BRAF inhibitors can increase antigen presentation and can rebalance the intratumoral immune milieu. The combination of pulsed cytotoxic therapy and immunotherapy is a logical next step forward in designing treatment regimens. Combination radiotherapy and immunotherapy also has experimental and clinical support. Not all combinations are likely to be additive or synergistic; indeed in

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some murine models, BRAF inhibitors may be more cytostatic than cytotoxic in tumors with PTEN loss, and may actually deter immune response. Additionally, combinations of drugs with apparently nonoverlapping toxicities can lead to unexpected adverse events, as has been shown in ipilimumab (Yervoy) + vemurafenib (Zelboraf). The standard of care for patients with advanced melanoma remains participation in clinical trials in order to enhance our understanding of the effectiveness and toxicities of combination regimens, and there remains a need for correlative studies (e.g., serial biopsies) to further elucidate the alterations in the tumor microenvironment engendered by combination therapy and allow for customization of regimens to tumor genotype.

Keywords
Melanoma · BRAF · MEK · Immunotherapy · Combination therapy · Targeted therapy

Key Points

- Given the multiple FDA-approved therapies for patients with advanced melanoma, the next logical step is exploration of combination therapy.
- Cytotoxic therapies, including targeted therapies, increase antigen presentation and in many cases create a favorable environment for the action of immune therapy.
- Response to the combination of targeted therapy and immunotherapy may vary by tumor genotype (e.g., concurrent PTEN loss).
- Exploration of combination therapy should be explored in a clinical trial setting due to possible unanticipated adverse effects, to maximize scientific understanding through correlative studies.
- Clinical trials have been moving away from exploration of chemotherapy (alone and in combination) in melanoma in favor of immunotherapy and targeted therapy.

Introduction

The past several years have seen a rapid increase in effective and tolerable treatment options for patients with advanced melanoma across the major modalities in systemic therapy: we have progressed from cytotoxic chemotherapy to targeted therapy to dramatic improvements in immunotherapy. Cytotoxic chemotherapy, despite multiple clinical trials of various combination regimens, has not proven effective in increasing overall survival but can lead to an objective response in a fraction of patients. Historically, trials of biochemotherapy (cytokine therapy + chemotherapy) regimens in advanced melanoma have not demonstrated improved overall survival as compared to chemotherapy (Sasse et al. 2007; Ives et al. 2007). Targeted therapy has an excellent response rate in BRAF $V600^{E/K}$-mutant advanced melanoma, up to 76% when given as combination BRAF + MEK inhibition, but the duration of response is limited, with the longest median time to progression reported so far at 9.4 months (Flaherty et al. 2012). Immunotherapy with new anti-PD1 antibodies has shown a $\geq$30% response rate, displaying even higher response rates when given in combination (Wolchok et al. 2013) with anti-CTLA4 antibody ipilimumab (Yervoy), with durable responses in most patients. Research into combinations of chemotherapy, targeted therapy, and immunotherapy must be expanded so that we might achieve both a higher overall response rate and a prolonged duration of response for our patients with advanced melanoma; however, the past several years have seen a drastic decrease in scientific attention to chemotherapy as a treatment class for melanoma.

Combination Immunotherapy + Chemotherapy

Rationale

Cytotoxic chemotherapy can act in two complementary ways: direct damage and death of cancer cells and the attraction and activation of cytotoxic

immune cells. Cell death triggered by treatment with chemotherapy has been shown to be immunogenic and lead to dendritic cell activation and subsequent activation of tumor antigen-specific T cells (Hannani et al. 2011; Green et al. 2009; Kepp et al. 2011). After administration of dacarbazine (DTIC), activation of genes involved in cytokine production, leukocyte activation, immune response, and cell motility has been observed, changes thought to create a favorable environment for tumor antigen-specific $CD8^+$ T-cell responses (Nisticò et al. 2009). In another study, a low dose of melphalan (Alkeran) was shown to induce tumor expression of chemokines that lead to enhanced recruitment of tumor-reactive T cells (Hong et al. 2015) and improved response to anti-CTLA4 therapy (Mokyr et al. 1998). Additionally, in examination of pre-treatment biopsy specimens, a tumor microenvironment with infiltrate featuring $CD8^+$ T cells has been associated with an improved response rate to chemotherapy (DeNardo et al. 2011).

Clinical Trials

Phase II–III trials have shown early evidence of tolerability and efficacy with the combination of ipilimumab with cytotoxic drugs such as alkylating agents dacarbazine and temozolomide, which is also active in the central nervous system, or fotemustine, a drug available in Europe (Table 1).

Combination Immunotherapy + Targeted Therapy

Rationale

The combination of targeted therapy and immunotherapy appears to have a pleasing symmetry: high response rate and rapid onset of action in targeted therapy, with hope of long-term response to slower-acting immunotherapy. There is little evidence that patients with $BRAF^{V600E}$ mutations are less responsive to immunotherapy (Shahabi et al. 2012). Vemurafenib (Zelboraf) and the biochemically similar compound, PLX4720, have been shown to have a cytotoxic effect in melanoma (Lee et al. 2010), and as previously discussed with chemotherapy, drugs with cytotoxic effect can enhance immune activity. BRAF inhibition has been shown to enhance T-cell recognition of melanoma cells and not interfere with lymphocyte functioning (Boni et al. 2010). Review of tumor biopsies from patients treated with BRAF inhibitors vemurafenib or dabrafenib (Tafinlar) showed enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma (Wilmott et al. 2012; Frederick et al. 2013). Response to ipilimumab is linked to an immune-active tumor microenvironment (Ji et al. 2012).

Preclinical testing of combination immunotherapy + targeted therapy has been done in the context of an anti-CTLA4 monoclonal antibody + PLX4720 (vemurafenib precursor) in an immunocompetent mouse model of $BRAF^{V600E}/PTEN^{-/-}$ mutant melanoma. In contrast to the observed cytotoxicity of BRAF inhibitors reported in human patients with melanoma (Lee et al. 2010), treatment in this mouse model does not cause cell death but leads to decreased tumor proliferation (Hooijkaas et al. 2012). Decreased tumor-resident lymphocyte frequencies were observed after treatment with PLX4720 (decreased $CD45^+$ leukocytes, $CD8^+$ T cells, $CD4^+$ T cells, Tregs; no change in B lymphocytes; slightly increased NK cells, myeloid-derived suppressor cells, macrophages), along with decrease in visible inflammation at tumor sites. These changes in T-lymphocyte frequencies were not found elsewhere in the mouse tissues (lymph nodes or spleen) or in similarly treated mice with BRAF-WT tumors, arguing against a direct effect of PLX4720 on the T cells. No synergy was observed in the combination of PLX4720 and anti-CTLA4 mAb in this study. Concurrent treatment with PLX4720 and the anti-CTLA4 mAb did not restore the intratumoral immune milieu. Of note, in a group of human patients with $BRAF^{V600E}$ mutations treated with dabrafenib, those with concurrent PTEN loss had decreased median

Table 1 Ipilimumab + cytotoxic chemotherapy

Regimen	Phase	N	ORR	PFS	OS
Ipilimumab + dacarbazine versus ipilimumab (Hersh et al. 2011)	II	35 versus 37	14.3 versus 5.4%	No difference	14.3 months versus 11.4 months; 1-year OS 62 versus 45%, 2-year OS 24 versus 21%, 3-year OS 20 versus 9%
Ipilimumab + dacarbazine versus dacarbazine (Robert et al. 2011)	III	196 versus 218	38 versus 26%; 4% CR, 34% PR, and 45% SD versus 2% CR, 24% PR, 50% SD	No difference	1-year OS 47.3 versus 36.3%, 2-year OS 28.5 versus 17.9%, 3-year OS 20.8 versus 12.2%
Ipilimumab + temozolomide (Patel and Hwu 2012; Wang et al. 2012)	II	64	28.1%: 10 CR, 8 PR	5.1 months	NR
Ipilimumab + fotemustine (Di Giacomo et al. 2012a, b)	II	86	irORR 29.1%; 5 CR, 20 PR	irPFS 5.3 months	1-year OS 51.8%

progression-free survival (Flaherty et al. 2010; Nathanson et al. 2013). One interpretation from this may be that in patients with PTEN loss, a tendency for BRAF inhibitors to have a more cytostatic than cytotoxic effect may lead to decreased efficacy, and there may also be a deleterious effect on the immune tumor microenvironment that actually decreases likelihood of response to anti-CTLA therapy. This will need further examination on clinical trials of the combination of BRAF inhibitors and the anti-PD1 antibodies, with studies of the correlation between observed clinical responses, tumor genetics, and lymphocyte profiling. It should be noted that PLX4720 has a less dramatic effect in mouse models than vemurafenib does in human melanoma.

Ipilimumab + Vemurafenib

A phase I study of concurrent vemurafenib + ipilimumab showed dose-limiting toxicity of grades III–IV hepatotoxicity in 50% of patients, asymptomatic LFT elevations that were reversible with dose interruption or administration of corticosteroids (Ribas et al. 2013), and the study was closed to further accrual. Efficacy outcomes were not reported. The authors recommend well-designed clinical trials to examine future combinations of even approved therapies with nonoverlapping toxicities and separate mechanisms of action.

Interestingly, a more recent phase I study of vemurafenib followed by ipilimumab has shown an absence of the hepatotoxicity seen in the combination (Amin and Lawson 2015).

Sequential Therapy

Rationale

In clinical oncology, combinations regimens are often developed to maximize response, but these can be limited by overlapping toxicities, and patients often proceed from one regimen sequentially to another at the time of progression of disease or end of tolerability. In the past, we have only seen the efficacy of sequential therapies through retrospective analysis of patients moving naturally from one trial to another. Recent studies, however, have directly examined the role of immunotherapy versus chemotherapy in pretreated patients, clarifying our knowledge of secondary and tertiary lines of treatment in melanoma.

As monotherapy (e.g., choosing between available open clinical trials) has often been the only option at a given point in a patient's care, targeted

therapy may be the first option chosen for its high response rate and rapid onset of action, for example, in a patient presenting with widely metastatic disease or bulky symptomatic disease causing pain or threatening organ function, while immune therapy may be chosen up front for patients with a low burden of disease who are able to tolerate a delayed onset of action and trade a relatively lower response rate for the opportunity at a long-term response.

Experience and Next Directions

An interesting dichotomy of response has been revealed in patients at the time of discontinuation of BRAF inhibitor therapy. A retrospective review of 28 patients first treated with a BRAF inhibitor (vemurafenib or dabrafenib) followed by ipilimumab reported that 43% of the patients experienced rapid progression of disease when the BRAF inhibitor was discontinued, and this prevented successful completion of planned treatment with ipilimumab (Ascierto et al. 2012, 2014). In another series of patients, however, a proportion was observed to have tumor shrinkage at the time of discontinuation of BRAF inhibitor (Stuart 2013). The response to BRAF inhibitors can be rescued in some patients with reinitiation of treatment after an interruption (Romano et al. 2013). This has led to exploration of intermittent dosing of BRAF inhibitors in an effort to delay or overcome resistance (Das Thakur et al. 2013). A strategy of continuous targeted therapy followed by abrupt discontinuation of treatment at time of progression and then initiation of immunotherapy is unlikely to be successful in many patients, and combination strategies (+/− intermittent dosing of BRAF inhibitor) are more likely to yield long-lasting benefits.

We have also begun to see increasing clarity in sequential therapies post immunotherapy failure. One phase II trial of 540 patients examined response rates of patients treated with pembrolizumab versus chemotherapy in an ipilimumab refractory population. The results were decisive: 6-month progression-free survival for those treated with 2 mg/kg and 10 mg/kg of the PD-1 inhibitor were 34% and 38%, respectively, compared to 16% in the chemotherapy group (Ribas et al. 2015). Several other trials have confirmed the efficacy of following immunotherapy with immunotherapy (Robert et al. 2014; Weber et al. 2015), signaling the increasing relevance of checkpoint inhibitors in melanoma and driving chemotherapy further into the realm of situational, palliative, or salvage therapy.

Another recently published paper indicated impressive efficacy of nab-paclitaxel in melanoma, possibly pointing to this as a therapy to be used prior to the oft used dacarbazine (Leon-Ferre and Markovic 2015); it is reasonable to expect that nab-paclitaxel may be increasingly tested in new combinations in melanoma as well. However, these authors also concede to the increasing use of immunotherapy in the first and second lines and advocate for nab-paclitaxel to be used as a salvage therapy in heavily pretreated populations.

Recent Trials of Other Combinations across Modalities or Pathways

Rationale

Given the complexity of cell signaling and the multitude of genetic errors characteristic of advanced melanoma tumors, trials are ongoing with various combinations of agents designed to hit multiple targets of growth signaling, combine cytotoxicity with immunotherapy, or otherwise take advantage of synergy between agents with different mechanisms.

Clinical Trials

See Table 2

Conclusion

This is an exciting time in the field of treatment of advanced melanoma, and the aim of therapy has shifted from temporary response in a small

Table 2 Other combination trials (2012–present)

Regimen	Phase	N	ORR	PFS	OS
Preclinical studies revealed that the proteasome inhibitor bortezomib + cytokine interferon-α (IFN-α) synergistically induce apoptosis in human melanoma cells, and combined treatment in a murine model led to improved survival. A phase I study showed (Markowitz et al. 2014):					
IFN α-2B + bortezomib	I	16	1 PR, 7 SD, 8 PD	2.5 months	10.3 months
Preclinical studies showed that bortezomib and sorafenib, a multikinase inhibitor that blocks tumor growth and angiogenesis, modulate expression of BCL-family members and augment cytotoxicity in cell lines. A phase I study showed (Sullivan et al. 2013):					
Bortezomib + sorafenib	I	11	2 SD, 9 PD	NR	NR
The MET receptor tyrosine kinase is activated in NRAS-mutant melanoma. Oral MET inhibitor tivantinib was studied in combination with sorafenib. Preclinical data indicated synergy between these two agents. A phase I study (Means-Powell et al. 2012) performed in patients with NRAS-mutant or NRAS-WT melanoma showed:					
Sorafenib + tivantinib	I	11	1 CR, 3 PR, 3 SD	5.3 months	NR
NRAS-mutant patients only		8	1 CR, 1 PR, 2 SD	9.2 months	NR
A double-blind, randomized, placebo-controlled phase III study showed no improvement in OS with the addition of sorafenib to cytotoxic chemotherapy with carboplatin + paclitaxel (Flaherty et al. 2013)					
Sorafenib + carboplatin + paclitaxel versus carboplatin + paclitaxel	III	823	20 versus 18%, $p = 0.427$	4.9 versus 4.2 months	11.3 versus 11.1 months
A phase II trial in patients with metastatic uveal melanoma was closed early for lack of response (Bhatia et al. 2012)					
Sorafenib + carboplatin + paclitaxel	II	24	0	4 months; 6 months PFS 29%	11 months
Multikinase inhibitor sorafenib was the foundation of two compared regimens: sorafenib + mTOR inhibitor temsirolimus and sorafenib + tipifarnib, an oral farnesyl transferase inhibitor in a randomized phase II trial (Margolin et al. 2012). Neither combination showed sufficient activity to merit further use					
Sorafenib + temsirolimus versus sorafenib + tipifarnib	II	63 versus 39	3 PR versus 1 PR	2.1 versus 1.8 months	7 versus 7 months
In preclinical models, bevacizumab, a mAb to VEGF inhibiting angiogenesis and tissue growth, suppressed growth and hepatic establishment of micrometastases, and a potential clinical benefit of the combination of bevacizumab + alkylating agent dacarbazine. A phase II trial (BEVATEM) showed (Piperno-Neumann et al. 2012, 2013):					
Bevacizumab + temozolomide	II	35	9/35 SD	3 months; 6-month PFS 26%	12 months
Temsirolimus is a targeted inhibitor of mTOR kinase activity, blocking progression of the cell cycle past G1 phase. A phase II trial of the combination of temsirolimus and bevacizumab showed (Slingluff et al. 2013):					
Bevacizumab + temsirolimus	II	16	3 PR in BRAF-WT pts, 9 SD; one response duration >3 years	NR	NR
A phase II trial evaluated bevacizumab in combination with oral alkylating agent temozolomide (von Moos et al. 2012)					
Bevacizumab + temozolomide	II	62	1 CR, 9 PR (16.1%)	4.2 months	9.6 months; BRAF WT 12 months, $BRAF^{V600E}$ 9.2 months, $p = 0.014$
A phase II trial compared bevacizumab as the foundation of two regimens: bevacizumab + temozolomide (BT) and nab-paclitaxel + bevacizumab + carboplatin (ABC) (Kottschade et al. 2013)					
BT versus ABC	II	42 versus 51	1 CR, 9 PR (23.8%) versus 0 CR, 17 PR (33.3%)	3.8 versus 6.7 months; 6-month PFS 32.8 versus 56.1%	12.3 months versus 13.9 months
Another randomized phase II study evaluated carboplatin + bevacizumab + paclitaxel (CBP) versus carboplatin + paclitaxel (CP) alone (Kim et al. 2012)					

(*continued*)

Table 2 (continued)

Regimen	Phase	N	ORR	PFS	OS
CBP versus CP	II	143 versus 71	25.5% versus 16.4%, $p = 0.1577$	5.6 versus 4.2 months, $p = 0.1414$	12.3 versus 8.6 months, $p = 0.0366$
Hypomethylating agent decitabine was evaluated in combination with temozolomide, an oral alkylating agent, in a phase I/II trial (Tawbi et al. 2013)					
Decitabine + temozolomide	I/II	35	2 CR, 4 PR, 14 SD	3.4 months; 6-month PFS 32%	12.4 months; 1-year OS 56%
ALT-801 is a recombinant human IL-2 fused to a single-chain T-cell receptor specific to human p53 peptide antigen presented in the setting of HLA-A2 positivity. This fusion protein showed activity as monotherapy and synergy with cisplatin in melanoma xenograft mouse models. This phase Ib study showed (Milhem et al. 2012):					
Cisplatin + ALT-801	Ib	22	NR	NR	6-month PFS 87%; 12-month PFS 58%
Angiogenesis inhibitor rh-endostatin in combination with dacarbazine was compared to dacarbazine monotherapy in this randomized, placebo-controlled phase II Chinese trial (Guo et al. 2012) that showed:					
Dacarbazine + rh-endostatin versus dacarbazine alone	II	110	NR	5 versus 1.5 months	16 versus 7 months; 1-year OS 51 versus 22%
Lenvatinib is an oral, receptor TKI targeting VEGFR1-3, FGFR1-4, RET, KIT, and PDGFRβ. Dacarbazine (DTIC) upregulates VEGF and has been shown to confer resistance in cell lines. A phase II study was performed to investigate if combination of anti-angiogenic drug could potentiate DTIC (Maio et al. 2013)					
Lenvatinib + DTIC versus DTIC	II	78	NR	19.1 weeks versus 7 weeks	NR
Lenvatinib was given at 24 mg PO daily + temozolomide 150 mg/m^2 PO d1-5/28 in this phase Ib trial (Hong et al. 2015)					
Lenvatinib + temozolomide	Ib	32	6 PR	5.4 months; 6-month PFS 37%	NR
Plitidepsin is a synthetic form of a peptide isolated from *Aplidium albicans* which triggers apoptosis and block VEGF secretion in tumor models. A phase I/II trial of dacarbazine + plitidepsin versus plitidepsin alone showed (Plummer et al. 2013a):					
Dacarbazine + plitidepsin versus plitidepsin	I/II	28 versus 16	1 CR, 5 PR, 9 SD versus 0 CR, 0 PR, 2 SD	3.3 versus 1.5 months	NR
Histone deacetylase inhibitor (HDAC inhibitor) panobinostat and demethylating agent decitabine were given in combination with temozolomide in an effort to overcome development of epigenetically mediated temozolomide resistance in this phase I/II trial (Xia et al. 2012). The MTD of this combination has not yet been reached					
Decitabine + panobinostat + temozolomide	I/II	17	NR	NR	NR
ERK1/2 is constitutively active in melanoma cells regardless of BRAF mutation status; selumetinib is a highly selective allosteric inhibitor of MEK1/2, suppressing pERK levels in melanoma independent of BRAF and NRAS mutation status. Selumetinib and docetaxel have demonstrated synergy in xenograft models of melanoma. A randomized phase II trial (DOC-MEK) showed (Gupta et al. 2014):					
Selumetinib + docetaxel versus docetaxel	II	83	32 versus 14%	6-month PFS 40% versus 26%	NR
Selumetinib was also tested in combination with cytotoxic alkylating agent dacarbazine versus dacarbazine alone in a phase II double-blind randomized study (Robert et al. 2013)					
Selumetinib + dacarbazine versus dacarbazine	II	45 versus 46	1 CR, 17 PR, 13 SD versus 1 CR, 11 PR, 10 SD	5.6 versus 3 months, $p = 0.021$	13.9 months versus 10.5 months, $p = 0.39$
YM155 is an inhibitor of survivin, a microtubule-associated protein overexpressed in melanoma and associated with cell viability and regulation of mitosis. An open-label phase II study (Steinberg et al. 2012) of YM155 in combination with microtubule-stabilizing chemotherapy agent docetaxel showed:					
Docetaxel + YM155	II	64	8 PR (12.5%), 33 SD (51.6%)	6 month PFS 34.8%	1-year OS 50.5%

(*continued*)

Table 2 (continued)

Regimen	Phase	N	ORR	PFS	OS
Everolimus is an oral inhibitor of mTOR, a component of the PI3k/AKT pathway, and has single-agent activity in advanced melanoma. A phase II trial tested everolimus in combination with chemotherapy with DNA cross-linking agent carboplatin and microtubule-stabilizing agent paclitaxel (Hauke et al. 2013). As this was not a marked improvement over previously published data with carboplatin + paclitaxel alone, further development was not recommended					
Everolimus + carboplatin + paclitaxel	II	70	12 PR, 42 SD	4 months	10 months
Pazopanib is an antiangiogenic inhibitor of VEGFR-1,2,3, PDGFR-B, and c-KIT with activity in melanoma tumor xenografts. A phase II study of pazopanib given in combination with paclitaxel showed (Ein-Gal et al. 2013):					
Pazopanib + paclitaxel	II	31	32%: 1 CR, 9 PR, 13 SD, 8 PD	NR	NR
PARP inhibitor rucaparib was examined in combination with oral alkylating agent temozolomide in a phase II trial that showed (Plummer et al. 2013b):					
Rucaparib + temozolomide	II	46	8 PR 17.4%, 8 SD	3.5 months; 6-month PFS 36%	9.9 months
Preclinical data indicated that Src inhibitors sensitize cells to the effects of cytotoxic chemotherapy. Src and c-Kit inhibitor dasatinib was combined with dacarbazine in a phase I trial (Algazi et al. 2012)					
Dasatinib (70 mg PO BID cohort) + dacarbazine	I	29	4 PR, 17 SD	6-month PFS 20.7%	12-month OS 34.5%
Ramucirumab is a fully humanized monoclonal antibody targeting VEGFR-2. It was tested as monotherapy and compared to a combination with dacarbazine in a phase II trial (Carvajal et al. 2014)					
Ramucirumab + dacarbazine versus ramucirumab	II	52 versus 50	9 PR, 19 SD versus 2 PR, 21 SD	2.6 months versus 1.7 months	8.7 months versus 11.1 months
Based on data suggesting that VEGF expression may be prediction of clinical outcomes to CTLA-4 blockade, ipilimumab was combined with bevacizumab in this phase I trial (Hodi et al. 2014)					
Ipilimumab + bevacizumab	I	46	8 PR, 22 SD	9.0 months	25.1 months
Autophagy inhibition has been proposed as a potential mechanism to augment the cytotoxicity of targeted therapies. In this phase I trial, the autophagy inhibitor hydroxychloroquinone is used in combination with temsirolimus, an mTOR inhibitor, in a variety of cancers (melanoma results below) (Rangwala et al. 2014a)					
Hydroxychloroquinone + temsirolimus	I	19	14 SD	3.5 months	NR
In similar concept to the above trial, this phase I trial combined hydroxychloroquinone with temozolomide in advanced solid tumors (melanoma results below) (Rangwala et al. 2014b):					
Hydroxychloroquinone + temozolomide	I	22	3 PR, 6 SD	NR	NR

N number of treated patients, *ORR* overall response rate, CR + PR, *CR* complete response, *PR* partial response, *PFS* median progression-free survival, *OS* median overall survival, *NR* not reported, *irORR* immune-related overall response rate, *irPFS* immune-related progression-free survival

minority of patients to meaningful durable complete or partial responses. While the excitement over targeted kinase inhibitors continues, it is clear that these drugs do not lead to durable remissions except in a small number of patients. Immunotherapy has also experienced rapid advances recently but still leaves many tumor anergic patients without response. Ultimately, combination therapy may deliver this goal better than either cytotoxic or targeted or immunotherapy is able to.

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Viral-Based Therapies in Melanoma

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Abstract

Viruses are microscopic organisms that can mediate antitumor activity by commandeering their natural ability to induce innate and adaptive immune responses and through genetic engineering, therapeutic transgene expression by the viral genome. Melanoma is especially well suited for viral-based therapeutics due to the underlying immunogenicity of melanoma cells and the relatively easy ability to inject established tumors in patients. Pharmacologic development of viral therapy in melanoma has focused on viral-based vaccines and oncolytic

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immunotherapy. In fact, the first approved oncologic application of viral-based agents has been the oncolytic virus, talimogene laherparepvec, for the treatment of advanced melanoma. This chapter will provide the biologic rationale and preclinical proof of concept for viral therapy, describe recent clinical trial results, and discuss some of the unique logistical and biosafety issues associated with the clinical application of viral-based therapeutics. The versatility of viruses as therapeutic agents coupled with a highly tolerable safety profile suggests that viral-based therapies may be important agents for further drug development alone and as part of multicomponent treatment regimens for patients with melanoma.

Keywords
Immunotherapy · Intratumoral treatment · Melanoma · Oncolytic virus · Vaccine

Introduction

Viral-based therapy represents a new class of antineoplastic treatment and is based on adapting biologic features of viruses to kill tumor cells and enhance host antitumor immunity. Viruses possess several unique features that makes them well suited for melanoma therapy, including an ability to selectively replicate in and kill tumor cells, activate host immunity and express antineoplastic and/or immune modulating transgenes (Bommareddy et al. 2018). The first viral-based therapy to achieve approval is talimogene laherparepvec (T-VEC), an oncolytic herpes simplex virus, type 1 (HSV-1) encoding granulocyte-macrophage colony stimulating factor (GM-CSF) for the treatment of advanced cutaneous, subcutaneous, and nodal melanoma that recurs after initial excision. The approval of T-VEC was based on improved durable and objective response rates, improved progression-free survival and enhanced overall survival, especially in patients with unresectable stage III or IVM1a disease, based on a prospective, randomized clinical trial (Andtbacka et al. 2015). These data provided clinical validation that viral-based therapies are clinically meaningful in melanoma and has generate considerable interest in expanding the role of viral therapy for the treatment of melanoma.

Viruses are microscopic organisms that are composed of nucleic acid core and typically surrounded by a protein or glycoprotein coat. There are approximately 320,000 known mammalian viruses and these can be broadly classified as DNA or RNA viruses based on their core nucleic acid composition (Anthony et al. 2013). All viruses replicate in host cells and each virus follows a specific life cycle that utilizes a combination of enzymatic proteins carried or encoded by the virus and from infected host cells to replicate progeny viral particles, and typically induces cell lysis with release of new viruses to infect other host cells. In this way, viruses propagate and expand within a single host and can be transmitted to other hosts based on the anatomic location of the viral infection and biologic features of each virus. Some viruses have been associated with zoonotic or human diseases that range from mild infections, such as the common cold, to uniformly fatal syndromes, such as Ebola. The host immune system plays an important role in preventing ongoing viral infection and includes innate viral sensing within eukaryotic cells through a set of intracellular proteins collectively known as the antiviral machinery. Once engaged, the machinery will alert the host immune system and a more orchestrated induction of innate and adaptive immune responses occurs to eradicate the virus and focusing the immune response on identifying virus-infected cells since viruses exist largely as intracellular microbes.

The dependency on host cells for replication and their usual detection by the intracellular antiviral mechanisms explains, in part, the early observations dating back to the early part of the twentieth century that some tumor cells can be killed by viruses (Pelner et al. 1958). Indeed, contemporary studies have shown that viruses can replicate more efficiently in host cells undergoing rapid cell division due to the presence of excess nucleic acid production that can be commandeered for viral replication (Kaufman et al. 2015). Further, many tumor cells have defects in antiviral machinery elements that

permit more selective replication, especially for attenuated viruses. Some viruses exhibit innate tropism for neoplastic cells and exhibit preferential replication in such cells, whereas other viruses can be genetically engineered to effectively replicate in tumor cells and not in normal host cells. While many viruses can replicate efficiently in tumor cells, even under the best conditions, ongoing viral infection and replication is ultimately halted by the immune system. This occurs because viruses are highly immunogenic and are potent activators of immune responses, and this characteristic can be exploited for melanoma therapy as well.

While most viruses induce humoral immune responses as evidenced by antibody production against virion particles, the obligate intracellular life cycle of viruses also results in strong cellular immune responses. Indeed, viruses are among the most potent inducers of host cellular immunity, a feature that can be utilized for melanoma immunotherapy. From an evolutionary perspective, viruses have had a long relationship with host immune systems and many have evolved elaborate molecular mechanisms for escaping immune detection. For example, the vaccinia virus B18R gene encodes an interferon receptor decoy protein that can bind and neutralize type 1 interferons, which allows further viral propagation even after antiviral immunity has been initiated (Symons et al. 1995). Herpes simplex viruses encodes an infected cell protein 47 (ICP47) that blocks the entry of viral peptides into the antigen-processing machinery and prevents binding of viral peptides to host cell major histocompatibility complex (MHC) class I molecules allowing the virus to replicate undetected by circulating T cells (Galocha et al. 1997). Since melanoma immunotherapy is dependent on the presence of CD8+ T cells in the tumor microenvironment (Riaz et al. 2017), the use of viruses to induce cellular immunity can potentially augment T cell responses and can theoretically enhance antitumor immunity. Oncolytic viruses have, in fact, demonstrated increased recruitment of T cells to the tumor microenvironment in melanoma patients, and this was associated with improved therapeutic responses to systemic immunotherapy (Ribas et al. 2017a). Achieving a balance between the antiviral and antitumor immune response is critical when considering viral-based therapy since immune activation against viral and tumor-associated antigens may be linked, and premature viral clearance may circumvent effective antitumor immunity. Further, weakened antiviral immunity could potentially cause toxicity due to uncontrolled viral infection. The ability to select native viruses or genetically engineer viruses has allowed the development of viral agents that exhibit appropriate balance between antiviral and antitumor immunity.

An additional feature of viruses is the ability of some viruses to encode eukaryotic transgenes and express them upon infection. Among the first such constructs was a vaccinia virus engineered to express the human carcinoembryonic antigen (CEA) gene (Kaufman et al. 1991). The virus was used to immunize patients with CEA-expressing adenocarcinomas and generate CEA-specific T cell responses but therapeutic activity was not observed (McAneny et al. 1996). In addition to single transgenes, it is now well established that certain large viruses can accommodate multiple eukaryotic transgenes (Kaufman et al. 2002; Duggan et al. 2016). Thus, genes encoding cytotoxic agents could be delivered directly into tumor cells by viruses, and additional immune modulating genes can be expressed within the local tumor microenvironment or utilized for systemic vaccination purposes.

Viral-based therapy for melanoma has emerged as a new class of therapeutic agents receiving increasing attention. There are two major approaches, one uses viruses as oncolytic agents to directly infect and induce immunogenic tumor cell death and the other using viruses as expression vehicles for systemic immunization or delivery of antineoplastic genes to tumor cells. In this chapter, we will discuss the biologic rationale for using viruses as therapeutic agents in melanoma, describe some of the important preclinical and clinical studies supporting viral-based therapeutics, discuss some of the unique logistical and biosafety issues associated with administering viruses in the clinic setting, and provide insights into high-priority areas for future investigation in this rapidly emerging field.

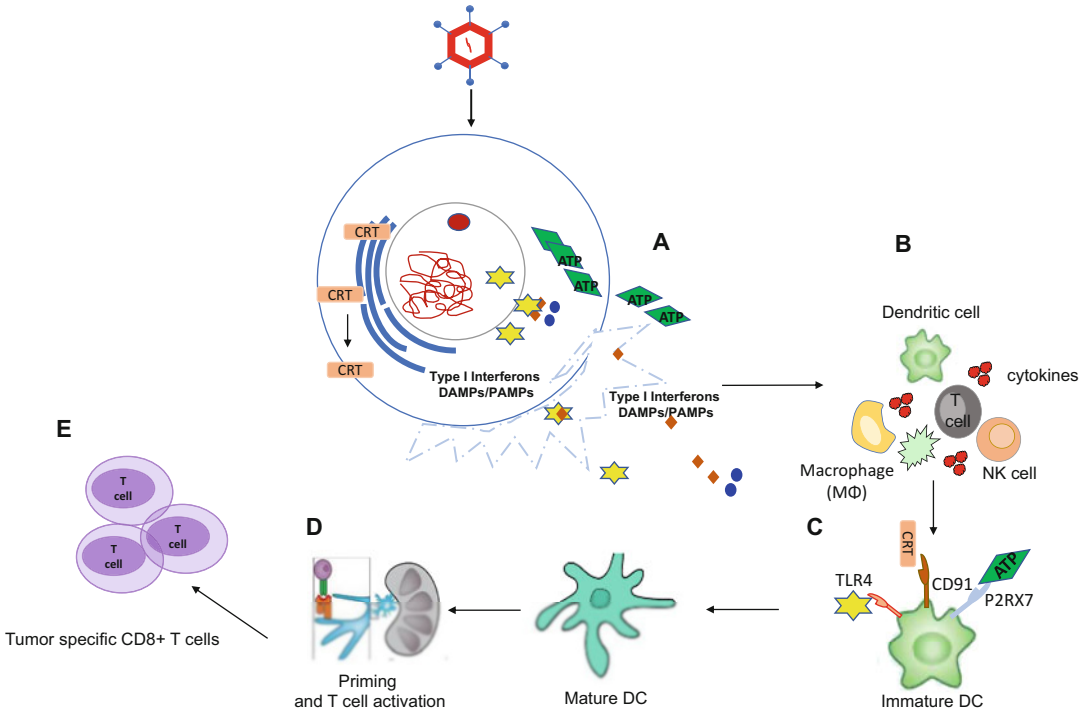

Fig. 1 Schematic showing the basic mechanisms of action for oncolytic viruses. Oncolytic viruses mediate antitumor activity through preferential infection and replication in tumor cells (**a**), induction of immunogenic cell death and release of soluble tumor-associated antigens and danger associated molecular pattern (DAMP) factors (**b**), recruitment of dendritic cells and innate immune cells (**c**), cross presentation of tumor antigens, and (**d**) generation of antigen specific T cells

Preclinical Studies of Oncolytic Immunotherapy

A number of preclinical studies in animal models and tumor cell lines have demonstrated the potential therapeutic benefit of oncolytic immunotherapy and improved our understanding of the mechanisms of action while guiding clinical development. In this section, we will review some of the more pertinent preclinical data. Small molecules and monoclonal antibodies can be used to induce oncolytic effects, but the oncolytic platform in melanoma has largely focused on viruses due to their preferential replication in melanoma cells, ability to trigger multiple immune modulatory pathways, and tolerable safety profile (Fig. 1). Although many oncolytic viruses can infect normal cells, most oncolytic viruses are unable to replicate efficiently in normal cells. This may be due to altered viral receptor expression between tumor and normal cells, defective intracellular antiviral machinery elements in cancer cells or due to genetic manipulation of the viral genome through deletions of nonessential viral genes or inclusion of transgenes that can limit replication in normal cells.

Tumor Tropism

Some viruses exhibit innate tropism for tumor cells, while other viruses can be selected or genetically modified to promote and/or enhance tumor tropism. For example, selection of group I adenoviruses that contain fiber knobs with highly specific CD46-binding properties has been shown to enter and lyse CD46-expressing tumor cells with higher efficiency than other adenovirus strains (Tuve et al. 2006). Deletion of the HSV-1 infected cell protein 34.5 (ICP34.5) decreases

pathogenicity since the ICP34.5 gene product mediates neurotoxicity, but deletion also confers a selective replication advantage to HSV-1 in tumor cells (Bommareddy et al. 2018). Furthermore, preclinical studies have shown that downregulation of protein kinase R (PKR), part of the antiviral machinery, can help some strains of HSV-1 replicate better in tumors with RAS mutations (Bommareddy et al. 2017). In contrast to tumor cells, normal cells while susceptible to HSV-1 infection, will have normal PKR levels that undergoes autophosphorylation upon viral detection, and then further phosphorylation of eukaryotic translation initiation factor (EIF2a) occurs and inhibits protein synthesis with selective restriction of viral infection in normal cells (Cassady and Gross 2002).

Adenoviruses generally require that quiescent cells enter the cell cycle in order to propagate. Thus, oncolytic adenoviruses can take advantage of tumor cells harboring mutations in the Rb tumor suppressor gene. In normal cells, E2F drives cell cycle entry and the process is regulated by Rb binding and inactivation of E2F. Preclinical studies have confirmed that oncolytic adenovirus infection of Rb-mutated tumor cells resulted in rapid viral replication and highly efficient cell killing, which was not observed in cells containing intact Rb function (Pelka et al. 2011; Savontaus et al. 2002). Another approach that enhances tumor selective replication has been by regulating viral replication component genes by tumor tissue-specific promoters. For example, an oncolytic adenovirus encoding CD40L used the human reverse transcriptase (hTERT) promoter to restrict replication to tumor cells (Zafar et al. 2017). Other tumor- and tissue-specific promoters such as c-myc and prostate-specific antigen (PSA) have also been used to limit oncolytic virus replication to specific tumor cells and/or prostate tissue respectively.

Immunogenic Cell Death and Activation of Innate of Adaptive Immune Responses

The role of oncolytic viruses in inducing immunogenic cell death has been well documented but the mechanism(s) through which this occurs are not as well defined (see Fig. 1). In one study, an oncolytic reovirus was shown to enhance killing of BRAF-mutant melanoma cells treated with BRAF and MEK inhibitors through reovirus-induced endoplasmic reticulum (ER) stress-mediated apoptosis (Roulstone et al. 2015). In another study, an oncolytic vaccinia virus encoding a CXCL4 antagonist was able to mediate antitumor activity in a chemoresistant ovarian melanoma cell model by inducing tumor immunity via the oncolytic effect of the vaccinia virus and reprogramming the suppressive immune network in the tumor microenvironment through the antagonistic activity of the anti-CXCL4 (Komorowski et al. 2016). Immunogenic cell death induced by oncolytic viruses is often accompanied by the release of DAMPs from tumor cells – such as adenosine triphosphate (ATP), uric acid, HMGB1, and the translocation of calreticulin (CALR), an ER-associated chaperone – to the cell surface (Nishio et al. 2014). Extracellular ATP can act as a chemoattractant for several immune cells and also plays a major role in dendritic cell activation (Ottolino-Perry et al. 2010). HMGB1 and CALR can act as ligands for TLR4 receptors on dendritic cells resulting in conditioning of dendritic cells, which help prime T cell activation (Galluzzi et al. 2017). CALR neutralize CD47 receptors present on tumors cells, which serve as an eat-me-not signals, thus promoting tumor cell engulfment by macrophages and dendritic cells (Sick et al. 2012). The release of DAMPs helps promote activation of tumor-specific dendritic cells and orchestrate priming and recruitment of tumor-specific T cells into the tumor microenvironment.

Toll-like receptors (TLRs) are sensing devices of the innate immune system which helps the host recognize and fight microbial pathogens and infections. The TLR family was initially discovered as part of the essential gene components involved in Drosophila development (Anderson et al. 1985). Immune activation occurs when TLRs detect defined structures of pathogens called pathogen-associated molecular patterns (PAMPs). In humans, ten different kinds of TLRs have been identified, each of which

have unique functions designed to identify and eradicate specific pathogens. Some TLRs exist as cell surface receptors and others are located within the cytoplasm of host cells, including tumor cells (Salaun et al. 2007). Intracellular pathogens and RNA viruses are frequently "sensed" via intracellular TLRs, which is among the earliest pathways to initiate antimicrobial immunity. Therapeutically, TLR agonists are being actively investigated as agents alone or in combination to promote melanoma immunotherapy. TLR agonists often aid the production of pro-inflammatory cytokines and chemokines (e.g., IL-1, TNF, IL-6), which can help mature dendritic cells, enhance antigen presentation and activate adaptive T cell antitumor immunity. Several TLR agonists such as TLR3, TLR4, and TLR7/8 agonists are currently being investigated as agents that can activate innate and adaptive immune responses based on promising preclinical studies. A detailed review of each of the TLR agonists and their mechanism of action can be found elsewhere (Gnjatic et al. 2010).

In contrast to TLRs which recognize RNA viral genome segments, DNA viruses are detected by specialized sensing complex collectively known as the cyclic GMP-AMP synthase and stimulatory of interferon gene expression (cGAS-STING) complex. cGAS binding of DNA elements produces cyclic dinucleotides 2′3′-cGAMP, which can bind to STING directly, and upon activation, STING translocates from the ER to the Golgi apparatus where it binds to tank-binding kinase 1 (TBK1), which leads to STING phosphorylation. Phosphorylation of STING results in the recruitment of interferon regulatory factor 3 (IRF3) to the complex, which phosphorylates IRF3. Following this, the complex enters the cell nucleus and induces transcription of interferon-related genes, such as IFNB1, tumor necrosis factor (TNF), and interleukin 6 (IL-6) (Corrales et al. 2016). STING appears to also play an important role in mediating antitumor immunity as shown by studies in which STING expression is deficient and this results in a lymphoid deficient tumor microenvironment, which could be restored by local expression of STING within tumor cells (Corrales et al. 2017). Preclinical studies using STING agonists as intratumoral therapies have shown promising results and seem to be dependent on CD8+ T cells (Corrales et al. 2015). In these studies, the effects of STING agonists were partially abrogated in RAG2$^{-/-}$ mice, which lack functional T cells. While the mechanism of action of STING agonists are complex and generally thought to be mediated through recruitment and activation of tumor-specific T cells, other studies have suggested a role for TNF-mediated vascular destruction in clearing established tumors in vivo (Corrales et al. 2015).

Viral Vaccines

In addition to oncolytic viruses, nononcolytic viruses – often termed as viral vaccines – may also be considered for the treatment of melanoma (Fig. 2). These nononcolytic viruses can be utilized as vaccination strategies to activate antitumor responses through expression of defined tumor-associated antigens by encoding the antigen genes under a viral promoter. Other strategies have used viruses to deliver cytotoxic agents and/or immune stimulatory cytokines directly into the tumor microenvironment, where native antigens including neoantigens can be presented in a more immunogenic manner. Viral vaccines carrying defined melanoma or neoantigens can serve to activate host antitumor immunity. While many nonreplicating or heat-inactivated viruses have been evaluated in the past for their potential to serve as a therapeutic melanoma vaccine, most studies conducted, to date, have not been clearly associated with clinical benefit. Today, armed with a better understanding of tumor immunology and the opportunity to combine viral-based vaccines with immune checkpoint blockade, there has been renewed interested in the role of viral vaccines for melanoma treatment. Thus, we will limit our discussion to the more recent approaches under clinical development.

MG1-MAGEA3 is an oncolytic Maraba virus encoding melanoma antigen family A3 (MAGEA3), a cancer-testis antigen that is frequently expressed in melanoma (Pol et al. 2018). The goal of this vaccine strategy is to deliver the

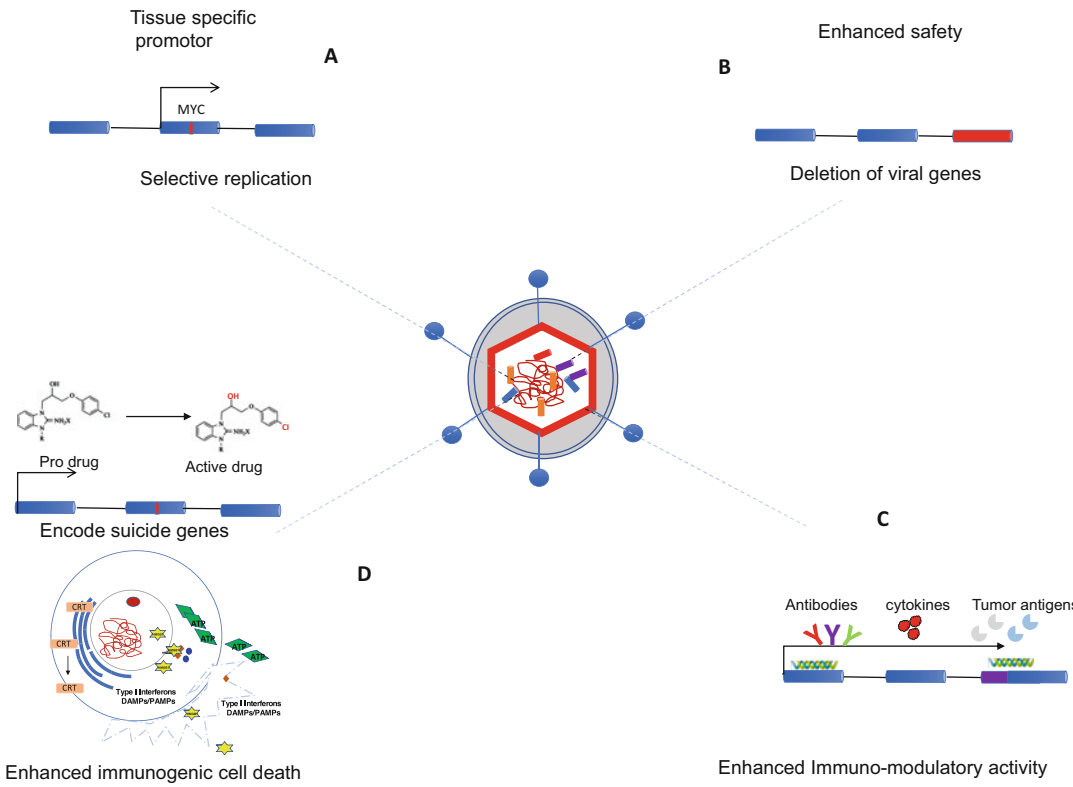

Fig. 2 Schematic showing how viruses can be modified to enhance safety and can be used to encode tumor-associated antigens to induce systemic immunization. (**a**) Viral replication genes can be placed under tumor specific promotors for selective replication in tumor cells; (**b**) Deletion of nonessential viral genes to enhance to safety profile; (**c**) Encoding blocking antibodies, immune stimulatory ligands, cytokines, and/or tumor associated antigens to enhance immune-modulatory functions; and (**d**) Encoding tumor cytotoxic agents that can induce immunogenic cell death and soluble antigen release

virus to the tumor where MAGEA3 expression should generate MAGEA3-specific CD8+ T cell, which can initiate tumor eradiation and presumably induce antigen spreading to other tumor-related neoantigen responses. This approach has been especially interesting in preclinical tumor models when used in a heterologous prime-boost strategy (Pol et al. 2014; Aitken et al. 2018). In studies using a murine melanoma model, a combination of adenovirus and Maraba virus MG1 demonstrated significant therapeutic responses, which related to the tumor accumulation and oncolytic activity of Maraba virus coupled with the ability of adenovirus to boost immune responses (Pol et al. 2014). This concept has also been extended to MG1 and *Listeria monocytogenes* (Aitken et al. 2018). MG1 can also be engineered to deliver other desired tumor antigens. The strategy has demonstrated activation of dendritic cells and natural killer (NK) cells in preclinical models and human clinical trials are anticipated in the near future (Zhang et al. 2014; Alkayyal et al. 2017).

Toca 511 is a nonlytic murine leukemia virus (MLV) engineered to encode yeast cytosine deaminase, which when expressed results in conversion of the precursor drug 5-flurocytosine (5-FC) to the antineoplastic agent 5-fluorouracil (5-FU) (Perez et al. 2012). This virus preferentially replicates in actively dividing cells making it particularly attractive in targeting melanoma cells. In a study of immunocompetent mice implanted with human brain tumors, infection with Toca 511 following pretreatment with 5-FC led to reduction in tumor

burden and a survival advantage (Hiraoka et al. 2017). A unique advantage of this system is the ability of locally produced 5-FU to cross the cell membrane and diffuse to nearby tumor cells that remain uninfected, leading to a pronounced bystander effect. Furthermore, the nonlytic nature of the virus confers a continued reservoir of virus available for ongoing infection of new tumor cells which can be reignited by further rounds of treatment with 5-FC prodrug (Twitty et al. 2016). Further improvements in the therapeutic effectiveness of the Toca 511 virus were observed in a human glioblastoma cell line when viral therapy was combined with radiation treatment (Takahashi et al. 2014).

Other strategies for enhancing the therapeutic potential of oncolytic immunotherapy have relied on mathematical models (Mahasa et al. 2017). These models have considered various factors associated with oncolytic immunotherapy and have suggested new approaches, including lower viral doses with oncolytic viruses may be associated with better production of new virions and higher overall infection rates within a tumor mass. Computational models have also shed light on the possibility of amplifying the antitumor immune response by dual infection with two different oncolytic viruses (Eftimie et al. 2011).

Despite these encouraging preclinical results, there are many challenges inherent to current preclinical models. For some viruses, murine models have been complicated by a lack of viral tropism, which may be high in humans but not in rodents. For example, HSV-1 is fairly resistant to infection of murine tumor cells whereas human cells are typically highly sensitive. Likewise, subtle differences in viral entry receptors among tumors and preclinical models may render studies of therapeutic activity and toxicity unreliable. The impact of prior exposure to specific viral agents may also be an important determinant of clinical response, and it is unclear how well animal models can replicate prior viral exposure in humans in order to test therapeutic strategies of oncolytic immunotherapy in the face of preexisting antiviral immunity. Nonetheless, progress in preclinical studies of oncolytic immunotherapy have supported early phase clinical development, which is allowing new insights into how oncolytic immunotherapy can be used in treating melanoma.

Clinical Studies of Oncolytic Viruses for Melanoma

Melanoma, with its tendency to form satellite, in transit and subcutaneous metastases in approximately one third of patients, is suitable for study of intratumoral virus delivery. The first oncolytic virus to achieve regulatory approval in the United States was talimogene laherperepvec (T-VEC) for advanced melanoma treatment in 2015. T-VEC was subsequently approved in Europe and Australia as well. T-VEC is administered by injection directly into the tumor. Clinical studies of various oncolytic viruses have used intravenous, intramuscular, intratumoral, and intravesicular approaches. In this section, selected viruses for melanoma and methods for drug delivery will be discussed.

Viral-based vaccines have been administered through a variety of delivery sites, including intradermal, subcutaneous, intramuscular, mucosal, intranodal, and intravenous routes. While there is limited consensus on the optimal delivery approach, considerations include the type of virus, anatomic location of tumor, potential targeting of individual immune elements (i.e., lymph node or tissue resident dendritic cells, tumor cells, mucosal epithelium, endothelium) depending on the presumed mechanism(s) of immune modulation, ease of clinical administration, anticipated safety and shedding potential, and cost. Although viral vaccines used for systemic immunization can be administered through multiple administrative routes, most oncolytic viruses have been restricted to two major delivery pathways, intravenous and intratumoral.

Intravenous (IV) delivery has obvious advantages and disadvantages. First, IV delivery can be used for any patient and is not limited to those with anatomically accessible tumors. Secondly, IV dosing is straightforward – either flat-dose or weight-based dosing can be used.

With intratumoral administration, dosing is more complicated because the administered volume is usually adjusted for the size of the tumor, with a larger volume for larger tumors. IV delivery has potential disadvantages, however. The virus may not reach the tumor because of rapid clearance from the circulation through sequestration in the liver and spleen or binding to circulating antibodies or serum proteins. In addition, effective viral delivery to tumor sites may be further decreased by dilution in the peripheral blood compartment. In general, IV-delivered viruses may be associated with a higher incidence and severity of adverse events (AEs). Although there are limited comparative studies of IV vs. IT (intratumoral) administration, the incidence of fevers and other flu-like symptoms were greater following IV administration (Pandha et al. 2017a) appears to be greater compared to intratumoral administration of coxsackievirus CVA21 (Silk et al. 2017).

Intratumoral delivery, including intravesicular administration for bladder cancer, has distinct advantages. First, as mentioned above, there may be less systemic toxicity. There may also be immune advantages. Administering the virus directly into the tumor conveys specificity by promoting a more prominent local inflammatory response, which may increase the presentation of tumor-associated antigens by antigen presenting cells. In this way, intratumoral administration is serving as a personalized in situ vaccination allowing tumor-associated defined antigens, and possibly neoantigens, to achieve optimal processing and presentation (Toda et al. 1999). Some preclinical models demonstrate that intratumoral administration of immune stimulants may be more effective than systemic administration (Sagiv-Barfi et al. 2018).

Talimogene Laherparepvec

Talimogene laherparepvec (T-VEC) is an attenuated herpes simplex virus type 1 (HSV-1) encoding human granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF recruits and activates antigen presenting cells which can process and present tumor-derived antigens to promote effector T cell responses. Normal cells are able to protect against T-VEC infection as they have intact antiviral defense mechanisms, and T-VEC will undergo an aborted infection in normal cells. In contrast, cancer cells are susceptible to lysis following infection. T-VEC has been further modified by functional deletion of two non-essential viral genes – ICP34.5 that encodes the neurovirulence factor and ICP47 that encodes a viral protein that blocks peptide loading onto MHC class I complexes. The deletions of ICP34.5 and ICP47 allow selective replication in tumor tissue and prevent inhibition of antigen processing and presentation, respectively.

In a phase II study of 50 patients with advanced melanoma, an initial objective response rate of 26% was reported with adverse events largely consisting of low-grade constitutional symptoms and local injection site reactions (Senzer et al. 2009). Notably, responses were durable, some lasting at least 2 years. Activity was evident in injected lesions as well as those not directly injected, suggestive of an anenestic effect (Marabelle et al. 2018). This was followed by a prospectively randomized phase III study known as OPTiM which became the first study of an oncolytic immunotherapy to demonstrate a statistically significant benefit for the treatment of melanoma (Andtbacka et al. 2015). In this open-label study, T-VEC was compared with recombinant GM-CSF in 436 patients with unresectable stage IIIB, IIIC, and IV melanoma. Patients were assigned in a 2:1 randomization to receive intratumoral T-VEC or subcutaneous recombinant GM-CSF. The primary endpoint was durable response rate, defined as a response initiating within the first 12 months of therapy and lasting at least 6 months. Analysis of efficacy demonstrated that durable response rate was significantly higher in the T-VEC arm compared with the GM-CSF arm (16.3% vs. 2.1%, $p < 0.001$). In addition, an improvement in objective response rate (26.4% vs. 5.7) and the median overall survival was 23.3 months in the T-VEC arm compared with 18.9 months in the control arm, which approached but did not cross the significance threshold (hazard ratio 0.79, $p = 0.051$) on initial analysis. The greatest benefits were seen in

patients with less advanced disease. In a subset analysis of patients with IIIB, IIIC, or IVM1a disease versus M1b/c, overall survival was significantly improved (hazard ratio 0.57, $p < 0.001$). With further follow-up at a median of 49 months, the median OS for T-VEC was 24.5 months compared to 18.9 months for patients in the GM-CSF arm (HR, 0.78; 95% CI, 0.61–0.99; $P = 0.0439$) (PMID:pending). Thus, OPTiM serves as a proof of concept for oncolytic viral immunotherapy in melanoma and led to the FDA and EMA approval of T-VEC.

Overall, most adverse events (AEs) reported in subjects administered with T-VEC were mild. In the phase III study, the three most frequent AEs observed in the T-VEC group were fatigue (36.2% GM-CSF, 50.3% T-VEC), chills (8.7%, 48.6%), and pyrexia (8.7%, 42.8%). The only grade 3 or 4 adverse event that occurred in more than 2% of patients was cellulitis, which occurred in 2.1% of T-VEC treated patients. There were no treatment-related deaths in the phase III trial (Andtbacka et al. 2015).

T-VEC works better locally then it works systemically. In a lesion-by-lesion analysis of the phase III patient data, injected tumors were more likely to respond and responded more quickly compared with uninjected visceral metastases (Kaufman et al. 2016). Among lesions directly injected with T-VEC, 86/128 (67.2%) responded, including 59/128 (46.1%) completely resolved. Among uninjected nonvisceral lesions, 60/146 (41.1%) responded, including 44/146 (30.1%) which completely resolved. While the response rate in injected and uninjected nonvisceral lesions was impressive, the response rate in visceral lesions was poor. Four out of 32 (12.5%) responded, with only 3/32 (9.4%) complete responses. The median time to response in injected tumors versus visceral uninjected tumors was 18.4 versus 51.3 weeks, respectively.

To optimize the systemic activity of T-VEC, combination approaches with immune checkpoint inhibitors have been under clinical development. In a study of advanced melanoma patients treated with T-VEC and ipilimumab, the initial response rate was 39% compared to 18% with ipilimumab alone (Chesney et al. 2018). In a phase I study of T-VEC and systemic pembrolizumab in 21 patients, responses were seen in 62% of the patients with a 33% complete response rate. Although the number of patients was small, responses were observed even in tumors with low PD-L1 expression and low interferon-gamma gene expression signatures, suggesting that T-VEC can introduce inflammation into a "cold" tumor, priming it for response to immune checkpoint blockade (Ribas et al. 2017b). This approach is currently being tested in a large randomized phase III study that has fully accrued over 700 melanoma patients, but results are not yet available.

Coxsackievirus

Coxsackievirus A21 (CVA21) is a naturally occurring strain of coxsackievirus, a single stranded RNA virus. The CVA21 strain was bio selected for its oncolytic properties and has been studied for the past 7 years using both intravenous and intratumoral approaches in multiple tumor types, especially melanoma. In a phase I study, advanced melanoma patients were treated with IV coxsackievirus A21 (Pandha et al. 2017b). Post-treatment tumor samples demonstrated an increase in CD8+ T cell infiltration and an increase in PD-L1 expression. Follow-up studies in melanomas are pairing intratumoral CVA21 with systemic ipilimumab (Curti et al. 2017) and with systemic pembrolizumab (Silk et al. 2017). Common adverse events (>10%) were injection site pain, pyrexia, fatigue, chills, myalgia, and headache. There have been no reports of myocarditis or pericarditis.

Rigvir

Rigvir, a wild type ECHO-7 virus, was first approved in Latvia in 2004 and was tested in melanoma and other solid tumors (Alberts et al. 2018). Rigvir was administered after surgical resection of early stage melanoma with multiple intramuscular doses over three years (Donina et al. 2015). The study was not well done and

lacked a control group, so the treatment group was compared to a retrospective cohort of melanoma patients. The Rigvir virus did not garner much support outside of Latvia until it was approved by the country of Georgia in 2015 and later in several other Eastern European countries.

Adenoviruses

Multiple types of recombinant adenoviruses have been tested against melanomas including a serotype 5/3 chimeric adenovirus encoding GM-CSF, which was tested in nine melanoma patients with activity in at least one patient (Bramante et al. 2015). In another phase I study, an adenovirus engineered to express IL-12 under the control of an oral activator ligand, Ad-RTS-hIL-12 showed clinical activity in five of seven patients treated by intratumoral injection (Linette et al. 2013). Serum levels of IL-12 and IFN-γ increased with treatment and absolute numbers of CD3+ and CD8+ T cells increased sevenfold.

Clinical Studies of Other Viral Therapies for Melanoma

Viral Delivery of Defined Melanoma Antigens

In contrast to oncolytic viruses which generate tumor-specific immune response by exposing tumor-derived soluble antigens after lysis and promoting a proinflammatory tumor microenvironment, viruses can also be used to generate systemic immunogenicity through expressing defined antigens or delivery of immunomodulatory genes into the tumor microenvironment. Large DNA viruses, such as vaccinia and HSV viruses, can be engineered with multiple payload transgenes for cancer immunization (Kaufman et al. 2005). An example of this is a recombinant UV-inactivated vaccinia virus expressing five engineered genes, including three HLA-A0201-restricted tumor-associated epitopes (Melan-A/MART-$1_{27–35}$, $gp100_{280–288}$, and $tyrosinase_{1–9}$), as well as two costimulatory proteins, CD80 and CD86 (Zajac et al. 2003). This virus was tested intradermally in a phase I/II trial in melanoma patients without clinically significant toxicity. The authors observed regression of individual metastases in 3 of 18 patients. In the peripheral blood, 43% of subjects with stage III melanoma demonstrated induction of cytotoxic T lymphocytes specific for all three epitopes; however, none of the three subjects with stage IV disease demonstrated a response to the desired epitopes. The same viral construct was tested intranodally in a follow-up study, again in melanoma patients (Adamina et al. 2010). The vaccine was immunogenic, but the clinical activity was still very modest, so there does not appear to be an advantage to intranodal injection over intradermal. Although there have not been any large phase III studies to date, the recombinant DNA viral constructs are versatile and promising, and studies are ongoing in melanoma and other solid tumors.

A vaccinia virus engineered to express the B7.1 (CD80) costimulatory molecule was tested in a small phase 1 trial in patients with melanoma (Kaufman et al. 2005). The virus was injected intratumorally to induce tumor lysis and release of antigen in the context of local B7.1 cell surface expression to provide additional costimulation for responding T cells. Treatment was associated with low grade fever, fatigue, and myalgias. Although the sample size was small, of 12 patients treated, two had disease stabilization and one had a complete response that was ongoing at 59 months following injection. Treatment effects correlated with the appearance of memory gp100- and MART-1-specific CD8+ T cells and local accumulation of CD8+ T cells and interferon-γ. To improve upon these outcomes, a vaccinia virus encoding three costimulatory molecules, including B7.1, ICAM-1, and LFA-3, was generated and tested in a similar phase I study (Kaufman et al. 2006). A similar adverse event profile was seen with mild fatigue and myalgias being the most common effects observed. In addition, one patient developed post-treatment autoimmune vitiligo. There was a 30.7% objective response rate with one patient achieving a complete response that was ongoing at 22 months.

Virus-Engineered Dendritic Cells

Viral-based vaccines have also been introduced into cell products as another strategy to avoid neutralization in the systemic circulation and enhance the induction of antitumor immunity. Retroviruses have the ability to transfer engineered genes into critical antigen presenting cells, such as dendritic cells. Successful transduction of dendritic cells with a lentivirus that expresses MAGE-A3 has been accomplished in the laboratory (Lin et al. 2014). Clinical studies using dendritic cells with engineered viruses and with personalized neoantigens are underway. In addition, various nanodelivery techniques are also in development for optimizing tumor delivery of oncolytic and nononcolytic virus particles (Badrinath et al. 2018).

Safety, Biosafety, and Logistical Issues Related to Viral Therapies

As a new class of anticancer agents, viruses pose several unique challenges with respect to safety and side effect management as well as biosafety and logistical issues when integrating viral treatment into busy ambulatory clinics. Table 1 lists the adverse events associated with viral-based therapeutics and provides guidelines for initial management. To date, most viral-based therapeutics have exhibited tolerable safety profiles with adverse events largely consisting of low-grade generalized constitutional symptoms, such as fatigue, fever, chills, anorexia, and myalgias. These are often preventable with early use of analgesics and antipyretics, such as acetaminophen. Oncolytic viruses have also been associated with low-grade injection site reactions, which may include localized pain, tenderness, warmth, and erythema at sites of injection that begin around 6–12 h after injection and may last 24–72 h. Such reactions may respond to local cold compresses and analgesics. In cases where erythema and tenderness do not recede, work-up for secondary bacterial cellulitis may be indicated. In general, clinically symptomatic viremia has not been observed even in patients with advanced malignancies. Many viruses retain sensitivity to antiviral agents, such as acyclovir, but this depends on the individual virus and physicians using viral agents should be aware of the antiviral sensitivity profile of individual viruses. Although contact transmission to close household contacts is possible, no such cases have been reported for viral-based therapeutics. In the event of such transmission, infected individuals should be closely monitored, and if symptoms develop, antiviral medications can be used. In some cases where viruses are resistant to antiviral medications or systemic viremia is present, treatment with immune globulin may be useful (Wittek 2006).

While patient safety has not been a significant issue with viral-based therapeutics, additional attention to biosafety is necessary since most of the viruses retain replicative ability and could theoretically be transmitted from patients or materials contaminated with drug to other people, such as healthcare providers or household contacts, or into the environment. To date, there have been several cases of viral transmission to healthcare workers, but no evidence has emerged for transmission to close household contacts or into the environment (Lewis et al. 2006). In the case of accidental exposure, antiviral medications, such as acyclovir, may be appropriate if the virus retains sensitivity to treatment, as is the case for most herpes viruses. Alternatively, immune globulin may be indicated for treatment of viremia with some viruses, such as vaccinia virus. Because of the small but real risk to healthcare providers preparing viral products, injecting virus, or working with viruses in the laboratory, strict adherence to standard universal precautions and practices designed to avoid needlestick injuries should be utilized. A formal educational training program may also be helpful. Healthcare providers with immunosuppressive conditions, including pregnancy, should probably not work directly with viruses. A plan for rapid assessment and initiation of prophylactic treatment following exposure should also be implemented at sites using viral-based therapeutics.

In addition to following standard operating procedures for use of oncolytic viruses, attention

Table 1 Adverse events associated with viral-based therapies and management guidelines

Adverse event	Incidence	Management
Constitutional symptoms Fatigue Fever Chills Myalgias	Common	Mild analgesics, such as acetaminophen; usually self-limited to 24–48 h
Gastrointestinal complaints Nausea Vomiting	Common	Usually mild, consider antiemetics if persistent
Local reactions Injection site pain Tenderness Erythema	Common	Cold compresses, analgesics Consider bacterial cellulitis if not resolved within 48–72 h
Injection site cellulitis	Rare	Cultures, IV antibiotics
Viremia	Very rare	Antiviral medications, if sensitive Immune globulin
Contact transmission	Not reported	Antiviral prophylaxis, if sensitive

to proper drug administration, injection site management, and waste disposal is important. Most oncolytic viruses are neutralized by 10% bleach solution and this can be used to clean spills and disinfect patient areas following exposure to viral products, although confirmation of effective disinfectant practices should be sought for individual viral strains. Proper management of injection sites includes washing the area prior to injection with an aseptic solution, such as alcohol wipes or betadine solution. Following injection and withdrawal of the needle, the site injected should be covered with a small dry gauze and an occlusive bandage, such as Tegaderm™. Prior studies have shown Tegaderm™ is an effective barrier to viral penetration. In addition, patient education to wash hands before and after dressing changes is helpful. Patients can be given instructions for placing soiled dressings in a small biohazard plastic bag for return to the institution or appropriate disposal and provided with fresh dressing materials should a change be necessary. Depending on the expected persistence of the virus, sites should be dressed up until the risk of continued virus is minimal, usually around 5–7 days. Most viral waste can be disposed of in biohazard waste receptacles or biohazard sharp containers for needles or other sharp objects.

In addition to establishing standard procedures for dealing with biosafety issues, the implementation of an oncolytic virus or viral-based therapeutic program requires logistical considerations for most ambulatory clinics. There are numerous guidelines that have been published providing recommendations for establishing such clinics (Harrington et al. 2017; Collichio et al. 2018; Larson et al. 2018). Since patients may require tumor measurements prior to defining the injection volume on any given treatment day, establishing clinic flow that allows for rapid patient assessment prior to drug preparation and delivery can allow an efficient clinic experience for patients and staff. A single room on specific days can be dedicated to viral injections and terminal cleaning with 10% bleach solution or other appropriate disinfectant can be completed at the end of the treatment visit. We have found that a cart dedicated to oncolytic virus administration, which contains gowns, gloves, masks, eye protection, gauze, bandages, and solid and sharp biohazard waste receptacles, is helpful and can be moved from room to room if a dedicated area is not available. Additional education on infection control procedures and injection technique for healthcare providers can also minimize anxiety and confusion related to caring for patients undergoing viral-based therapy. Finally, as some studies

are now exploring image-guided delivery of viral products, similar logistical implementation can be utilized for interventional radiology suites.

Conclusions and Future Directions in Viral Therapies for Melanoma

Viruses are versatile genetic agents that can be harnessed for therapeutic purposes in the treatment of cancer. The use of the native ability of viruses to induce host innate and adaptive immunity can be utilized to target cancer through expression of tumor-associated antigens and through induction of immunogenic cell death by oncolytic virus infection of tumor cells. The approach has now been clinically validated in melanoma with the approval of intratumoral talimogene laherparepvec, an oncolytic HSV-1 virus encoding GM-CSF. While many viruses are under clinical development for melanoma and other cancers, progress in expanding the immunogenic potential of oncolytic viruses is being supported by combination studies with immune checkpoint inhibitors with early data showing additive, if not even synergistic, activity. Given these preliminary data and the tolerable safety profile demonstrated with most viral therapeutics, additional combination studies of oncolytic viruses and other immunotherapy agents, cytotoxic chemotherapy, targeted therapy, and radiation therapy are high priorities for clinical translation.

Although the first oncolytic virus has been approved for the treatment of melanoma, there is little information on the optimal dosing, schedule, and sequencing of oncolytic viruses or viral vaccines in the management of patients with cancer. Viruses are well suited for neoadjuvant treatment and this may be another important setting to develop virus-based therapeutics. Similar to other immuno-oncology agents, the identification of predictive biomarkers of oncolytic virus therapy is a critical unmet need, which could help identify which patients will benefit from such treatment and could help guide personalized combination approaches. The importance of tumor neoantigens has been recently stressed in immunotherapy treatment (Schumacher and Schreiber 2015), and viruses are well positioned as a platform to encode individual patient tumor-derived neoantigens, or alternatively using oncolytic viruses to provide an in situ source of neoantigen release. Although viruses are best known as human pathogens, an improved understanding of the molecular virology and tumor immunology has resulted in the ability to utilize viruses as therapeutic agents for the treatment of cancer, and this appears to be especially potent for patients with melanoma.

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Adjuvant Therapy

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Diwakar Davar and John M. Kirkwood

Contents

Abstract

Of the estimated 76,380 patients who will be diagnosed with melanoma in the USA in 2016, only 4% will have metastatic disease, while the proportion of patients with localized and regional disease is substantially larger at 84% and 9%, respectively. It is in this group of patients who are candidates for adjuvant therapy given that they are at elevated risk of relapse and subsequent mortality. Given the substantially lower burden of disease in the adjuvant setting, treatments that have a limited role in the advanced setting may be significantly more effective in the former setting. This principle of adjuvant therapy underlies why effective immune inhibitor and targeted therapies have been studied in trials designed to evaluate their potential adjuvant benefit. Although only interferons (IFN-α and pegylated IFN-α) and anti-CTLA-4 blocking antibody ipilimumab have reached regulatory approval for adjuvant therapy, trials evaluating anti-PD-1 blocking antibodies and BRAF as well as BRAF+MEK inhibitors have been conducted, with early results suggesting significantly increased activity for which definitive results are anticipated shortly.

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Introduction

Data from the surveillance, epidemiology, and end results (SEER) program of the National Cancer Institute suggest that in 2016 an estimated 76,380 patients will be diagnosed with melanoma of whom 10,130 will die in the USA (Melanoma of the Skin – SEER Stat Fact Sheets [Internet]). The incidence of melanoma is rapidly increasing in both sexes: among men, melanoma is rising more than any other malignancy, and among women it is rising more than all except lung cancer (Melanoma of the Skin – SEER Stat Fact Sheets [Internet]). The current median age at diagnosis of melanoma is 59 years, and the lifetime risk is 1:34 (women) and 1:53 (men), so that melanoma results in a greater loss of productive years of potential life (20.4 years) than many other more common malignancies that occur later in life (16.6 years) (Melanoma of the Skin – SEER Stat Fact Sheets [Internet]).

In the USA, the majority of patients (84%) are diagnosed with localized disease, while 9% and 4% of patients present with regional and distant metastatic disease, respectively, with associated 5-year disease-specific survivals of 39–97% (stages IIC–IA), 39–70% (stages IIIC–IIIA), and 7–20% (stage IV, substages A–C) (Balch et al. 2009). Although regional and metastatic disease account for the minority of incident cases, they account for the vast majority of treatment-related expense. The goal of adjuvant therapy has been to identify patients who are at the highest risk of recurrence and to intervene with treatment that may reduce or eliminate the likelihood of recurrence. In this chapter, we focus on the rationale and indications for adjuvant therapy while summarizing the recent studies in the areas of immunotherapy, radiation therapy and molecularly targeted therapy. The results of the pivotal studies and pending studies that have been conducted for adjuvant therapy are reviewed.

Prognostication and Indications for Adjuvant Therapy

The consideration of therapy in the adjuvant setting is predicated on the assessment of the risk of disease recurrence and mortality. Consensus recommendations regarding risk stratification and adjuvant therapy for surgically resected high-risk melanoma have been published by the Society for Immunotherapy of Cancer (SITC) and National Comprehensive Cancer Network (NCCN) (Coit et al. 2016; Kaufman et al. 2013). In patients with operable locoregional disease, primary tumor characteristics (depth, ulceration status, and mitotic rate) and the extent of regional lymph node involvement determine stage – as delineated by the tumor, node, and metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) (Balch et al. 2009, 2011).

Primary Breslow tumor depth in millimeters partially predicts the risk of nodal involvement, 1% (Breslow depth <0.75 mm), 8% (Breslow depth 0.75–1.5 mm), 23% (Breslow depth 1.5–4.0 mm), and 36% (Breslow depth >4.0 mm), and is reflected in the "T" substaging categories of both the 6th and 7th editions of the AJCC staging system (Balch et al. 2009, 2001). The risk of sentinel lymph node (SLN) involvement in thin (<1.0 mm) melanomas has historically been considered to be low, and these patients are generally therefore not offered SLN evaluation. However, the incidence of SLN involvement in thin (<1.00 mm) melanomas varies significantly between 1% and 5% in reported series, risk factors for which include Breslow tumor depth (particularly >0.75 mm), Clark level IV/V, mitoses, and microsatellites (Balch et al. 2011; Wong et al. 2012; Cordeiro et al. 2016). Lack of consensus regarding prognostication of the T1A substage led to changes in T1A/T1B designations between the 6th (ulceration and Clark level) and 7th (ulceration and mitoses) editions of AJCC staging system. These disparate factors are reconciled in the 8th edition which gives primacy to Breslow tumor depth in defining T1A (<0.80 mm) and T1B (0.81–1.00 mm) melanoma (see Table 1). Consensus guidelines recommend

Table 1 "T" stage migration in AJCC cutaneous melanoma staging systems

	6th edition AJCC (Balch et al. 2001)	7th edition AJCC (Balch et al. 2009)	8th edition AJCC
T stage			
T1	<1.00 mm	<1.00 mm	<1.00 mm
A	Non-ulcerated and level II/III	Non-ulcerated and mitoses $<1/mm^2$	<0.8 mm
B	Ulcerated and/or level IV/V	Ulcerated and/or mitoses $\geq 1/mm^2$	0.81–1.00 mm
T2	1.01–2.00 mm	1.01–2.00 mm	1.01–2.00 mm
A	Non-ulcerated	Non-ulcerated	Non-ulcerated
B	Ulcerated	Ulcerated	Ulcerated
T3	2.01–4.00 mm	2.01–4.00 mm	2.01–4.00 mm
A	Non-ulcerated	Non-ulcerated	Non-ulcerated
B	Ulcerated	Ulcerated	Ulcerated
T4	>4.00 mm	>4.00 mm	>4.00 mm
A	Non-ulcerated	Non-ulcerated	Non-ulcerated
B	Ulcerated	Ulcerated	Ulcerated

SLN evaluation in ≥T2 melanomas. Following SLN evaluation, patients can be more comprehensively stratified for risk as follows:

- Low-risk node-negative (stages IA–IIA)
- Elevated-risk node-negative (stages IIB–IIC)
- Microscopic node-positive (IIIA)
- Macroscopic node-positive or multiple node involvement (IIIB–IIIC)

Up till relatively recently, patients with positive SLN were usually advised to undergo completion lymph node dissection (CLND) although no prospective data existed to support the efficacy of this approach. The Multicenter Selective Lymphadenectomy Trial II (MSLT-II) was a large randomized phase III study that evaluated melanoma-specific survival (MSS) in 1934 melanoma patients randomized to either CLND or nodal surveillance following SLN (Faries et al. 2017). In the subgroup of 608 SLN-positive patients, 3-year MSS was similar in both CLND (86 ± 1.3%) and nodal surveillance groups (86 ± 1.2%). The risk of regional recurrence was unsurprisingly higher in the surveillance group at 23% (compared to 8% with CLND); the similar MSS across groups indicated that the majority of regional recurrences could be salvaged (Faries et al. 2017). Although MSLT-II patients were highly selected with a relatively low SLN disease burden, these practice-changing results suggest that CLND should be optional in this patient population.

In summary, current consensus recommendations argue against considering adjuvant systemic therapy in patients with low-risk node-negative (stages IA–IIA) disease. There is strong consensus to support consideration of adjuvant systemic therapy in patients with high-risk node-negative (stages IIB–IIC) as well as microscopic node-positive (IIIA) and macroscopic node-positive disease (IIIB–IIIC). Adjuvant therapy trials aiming to address the highest-risk populations have also more recently included resectable stage IV (M1A/B).

Predictive and Prognostic Biomarkers

Patients with node-negative (stages IA–IIC) disease have a generally lower risk of disease progression but comprise a heterogeneous group among which a 5-/10-year survival ranges from 97%/93% (IA) to 53%/39% (IIC) (Balch et al. 2011). Efforts aimed at developing predictive and prognostic models to guide clinical decision-making for patients and providers have generally taken two approaches. Predictive algorithms largely based on logistic regression analysis of factors identified by the AJCC staging system have been developed to estimate the risk of SLN involvement (Memorial Sloan Kettering (MSK) sentinel node metastasis prediction tool) and

5-/10-year survival (AJCC Individualized Melanoma Patient Outcome Prediction Tool) with a high degree of accuracy (Wong et al. 2005; AJCC – American Joint Committee on Cancer [Internet]). The MSK SLN nomogram has been validated in 979 patients who underwent SLN biopsy at a single Irish institution and accurately predicted SLN involvement with a slightly higher concordance index compared to the AJCC staging system (Woods et al. 2015). Separately, other investigators have evaluated microarray expression data to generate prognostic gene signatures. Utilizing data from published genomic analyses of cutaneous melanomas, Gerami and colleagues identified 28 prognostic genes and 3 control genes and created a 31-gene genetic signature using radial basis machine (RBM) modeling (DecisionDx-Melanoma, Castle Biosciences Inc.) (Gerami et al. 2015a). In a test cohort comprising 107 stage I/II cutaneous melanoma patients, this signature dichotomized low-risk (class I) and high-risk (class II) patients (Gerami et al. 2015a). A second cohort comprising SLN-positive and SLN-negative patients demonstrated that although the 31-gene signature had similar positive predictive value (for recurrence and distant metastases) as a positive SLN, the signature had better negative predictive value than negative SLN (Gerami et al. 2015b). Genetic signatures such as these would be most clinically useful to *either* predict SLN status in patients pre-SLN evaluation *or* predict recurrence in patients with negative SLN, thereby guiding decision-making regarding adjuvant therapy decision – the lack of prospective validation of this signature in either setting argues against its use at this time.

Although prognostic biomarkers such as DecisionDx-Melanoma (Castle Biosciences Inc.) exist, these have not been prospectively validated in appropriate risk cohorts, and hence their use cannot be recommended outside of a clinical trial.

Adjuvant Therapy: Radiotherapy

Melanoma has historically been considered refractory to radiotherapy (RT), but RT has been considered in the adjuvant management of melanoma patients in particular settings. Desmoplastic melanoma (DM) is a rare variant of melanoma commonly found in chronic sun-damaged (CSD) skin, particularly in the head and neck region. Histopathologically distinguished by the presence of abundant stromal collagen, one subtype of neurotropic DM (NDM) is associated with extension along cutaneous nerves and tends to recur locally (Quinn et al. 1998). Retrospective data suggests that postoperative RT can improve local control rates in DM particularly for lesions with certain characteristics: location in the head/neck region, primary Breslow thickness $\geq$4 mm, and/or exhibiting neutropism (Vongtama et al. 2003; Arora et al. 2005). RT has also been considered for locally recurrent DM and/or DM that has not been resected with adequate margins (Coit et al. 2016).

Retrospective data suggests that adjuvant RT following definitive lymph node dissection reduces regional recurrence after controlling for other factors on multivariate analysis, albeit at the cost of increased treatment-related morbidity especially lymphedema (Agrawal et al. 2009). The only prospective data comes from a prospective multicenter study conducted by the Australia and New Zealand Melanoma Trials Group (ANZMTG) in coordination with Trans-Tasman Radiation Oncology Group (TROG). ANZMTG 01.02/TROG 02.01 was a prospective multicenter phase III study that randomized 250 patients with high risk including clinically node-positive melanoma (N1b, N2b or N3) to receive either observation or regional nodal basin RT (48 Gy in 20 fractions) within 12 weeks of definitive lymph node dissection (Henderson et al. 2015). At a median follow-up time of 73 months, RT significantly reduced local relapse (HR 0.52, 95% CI 0.31–0.88, $p = 0.023$) although overall survival (OS) and relapse-free survival (RFS) did not differ significantly between the treated and observed groups. A follow-up TROG (RTN2, NCT00975520) study selectively evaluating adjuvant RT following completion lymph node dissection in head/neck primary melanomas with neurotropic features is currently in accrual.

Although early studies suggested that melanoma was a tumor refractory to RT, recent work

has established that RT is well tolerated and demonstrates moderate efficacy in the disease. Various fractionation schedules have been studied with no apparent difference in efficacy between standard and hypofractionated schemes, although the latter has a slightly lower incidence of toxicity (Hallemeier et al. 2013). The vast majority of patients with primary melanoma will undergo definitive surgical resection; and RT can be considered an adjunct to surgery in patients with DM (particularly NDM) and/or in primaries located in the head/neck region, especially when surgical margins have not been possible to obtain. Adjuvant RT reduces incidence of regional lymph node relapse in patients with high-risk clinically palpable LN involvement based on ANZMTG 01.02/TROG 02.01. Unfortunately, the toxicity with radiation fibrosis and local symptoms, with the lack of overall RFS/OS benefit, has qualified the adoption of RT in the adjuvant setting.

Adjuvant Therapy: IFN-α and Biochemotherapy

Traditional adjuvant chemotherapeutic approaches have been ineffective to improve either RFS or OS in melanoma. Interferons (IFNs) comprise a large group of structurally related molecules with diverse properties and actions. Type 1 IFNs (IFN-α, IFN-β, IFN-ω, IFN-ε, and IFN-κ) are primarily produced by dendritic cells responding to infectious agents, where this family links the adaptive and innate arms of the immune response. Canonical type I IFN-α signaling involves the cell surface IFN-α receptor (IFN-αR) and signaling via the JAK/STAT pathway (JAK1/TYK2) and IFN-α regulatory factor (IRF)-9 which binds the cytosolic STAT1-STAT2 complex that subsequently migrates to the nucleus and induces expression of genes with either interferon-stimulated response elements (ISREs) or gamma interferon activation site (GAS) elements (Ivashkiv and Donlin 2014). Of the various type 1 IFNs studied as anticancer agents, IFN-α-2b is the most extensively described with potent activity against multiple malignancies (Ivashkiv and Donlin 2014).

The mechanism of action of IFN-α is thought to be immunomodulatory rather than directly cytotoxic or anti-angiogenic. IFN-α has disparate effects on antitumor immunity, **augmenting** T-cell-mediated antitumor responses through MHC class I/II upregulation, constitutive activation of STAT3, and improved T-cell recruitment and dendritic cell-mediated T-cell priming while **concurrently increasing** expression of inhibitory T-cell markers such as PD-L1 (Ivashkiv and Donlin 2014; Bellucci et al. 2015). Collectively, IFN-α administration appears to promote antitumor immunity. Following initial reports of activity in advanced disease, IFN-α were studied as an adjuvant therapy in high-risk resected melanoma.

Although many trials have tested the broad swath of IFN-α dosages, schedules, routes, as well as durations of therapy, high-dose IFN-α (HDI) has been the most widely used regimen in the adjuvant arena in the USA. HDI has been approved by regulatory agencies worldwide for adjuvant therapy. HDI comprises an initial 1-month-long intravenous IFN-α-2b 20 MU/m^2/daily 5 days per week for 4 weeks (**induction phase**), followed by 11-month-long subcutaneous maintenance IFN-α-2b 10 MU/m^2/daily 3 days per week (**maintenance phase**). HDI has been studied in six large prospective randomized clinical trials (see Table 2) that enrolled stage IIB–IIIC patients, although ECOG E2696 allowed patients with very-high-risk disease (in-transit N3 and resectable distant metastatic M1A) and Sunbelt Melanoma Trial accrued only patients with at least one positive sentinel lymph node (N1A).

North Central Cancer Treatment Group (NCCTG) 83-0752 and Eastern Cooperative Oncology Group (ECOG) E1684 were the first prospective randomized phase III studies to test high-dose IFN-α-2b for the adjuvant therapy of high-risk resected melanoma (Kirkwood et al. 1996; Creagan et al. 1995). NCCTG 83-0752 tested a truncated high-dose IFN-α-2a regimen (20 MU/m^2 thrice weekly IM for 12 weeks) against observation in 262 stage II/III patients (61% lymph node positive) and reported nonsignificantly improved RFS/OS. Rather than IFN-α-2a for 12 weeks, ECOG E1684 tested a

Table 2 Phase II/III studies of adjuvant IFN-α in high-risk resected melanoma

Study reference	Number of patients eligible for analysis	Stage	% node positive	Therapy and IFN subspecies	Dose and schedule – treatment arm	Median follow-up at time of reporting (years)	DFS/RFS	OS
High dose								
NCCTG 83-7052 (Kirkwood et al. 1996)	262	IIA–IIIC	61%	IFN-α-2a vs. observation	IM 20 MU/m^2 thrice weekly for 4 months	6.1	HR: 1.20 (HDI vs. observation) (NS)	HR: 1.11 (HDI vs. observation) (NS)
							Median RFS: not reported	Median OS: not reported
ECOG E1684 (Creagan et al. 1995)	287	IIB–IIIC	89%	IFN-α-2b vs. observation	High-dose IFN (HDI)	12.6	HR	HR
					Induction – IV 20 MU/m^2 5 days a week for 4 weeks		1.38 (HDI vs. obs) (S)	1.22 (HDI vs. obs) (S at 6.9 years but NS at 12.6 years)
					Maintenance – SC 10 MU/m^2 3 days a week for 48 weeks		Median RFS: not reported	Median OS: not reported
ECOG E1690 (Kirkwood et al. 2000)	642	IIB–IIIC	74%	IFN-α-2b high dose vs. low dose vs. observation	HDI as above	4.3	HR	HR
					Low-dose IFN: C. 3 MU/m^2 2 days a week for 2 years		1.28 (HDI vs. obs) (S)	1.0 (HDI vs. obs) (NS)
							1.19 (LDI vs. obs) (NS)	1.04 (LDI vs. obs) (NS)
							Median RFS	Median OS
							44% (HDI) vs. 40% (LDI) vs. 35% (obs)	52% (HDI) vs. 53% (LDI) vs. 55% (obs)
ECOG E1694 (Kirkwood et al. 2001a)	774	IIB–IIIC	77%	IFN-α-2b vs. GMK vaccine	HDI as above	2.1	HR	HR
							1.49 (HDI vs. GMK) (S)	1.38 (HDI vs. GMK) (S)
							Median RFS	Median OS
							25% (HDI) vs. 39% (GMK)	78% (HDI) vs. 73% (GMK)

ECOG E2696 (Kirkwood et al. 2001b)	107	IIB–resected stage IV	Not available	GMK vaccination with concurrent HDI (arm A)	HDI as above	2.4	HR	HR
				vs. GMK vaccination with HDI beginning D28 (arm B)	GMK vaccination:		1.75 (C vs. A) (S)	Not reported
				vs. GMK vaccination alone (arm C)	GM2-KLH/QS-21 on D1, 8, 15, and 22 and then weeks 12, 24, and 36		1.96 (C vs. B) (S)	Median OS
							Median RFS	Not reached (A, B, or C)
							Not reached (A)	
							vs. 30.72 months (B)	
							vs. 14.85 months (C)	
Sunbelt Melanoma Trial (McMasters et al. 2016)	218	IIIA–IIIC	100%	Protocol A: histologically positive sentinel lymph node biopsy, followed by completion lymph node dissection and then randomized to HDI vs. observation	HDI as above	5.9	HR: 0.82 (HDI vs. obs) (NS)	HR: 1.10 (HDI vs. obs) (NS)
							Median RFS: not reported	Median OS: not reported
Oxford phase II (Payne et al. 2014)	194	IIB–IIIC	77%	IFN-α-2b high dose without maintenance vs. with maintenance	HDI as above without maintenance (arm A) vs. with maintenance (arm B)	3.3	HR: 0.8 (arm B vs. arm A) (NS)	HR: 0.59 (arm B vs. arm A) (S)
							Median RFS: 22.7 months (arm A) vs. 33.3 (arm B) (NS)	Median OS: 41.5 months (arm A) vs. not reported (arm B) (S)
Hellenic He13A/98 (Pectasides et al. 2009)	364	IIB–IIIC	58%	Modified IFN-α-2b induction-only (arm A) vs. modified IFN-α-2b induction and maintenance (arm B)	Non-inferiority design	5.3	Median RFS: 24.1 months (arm A) vs. 27.9 (arm B) (NS, primary non-inferiority endpoint met)	Median OS: 64.4 months (arm A) vs. 65.3 (arm B) (NS)
					Modified induction			
					IV 15 MU/m^2 5 days a week for 4 weeks			
					Modified maintenance			
					SC 10 MU 3 days a week for 48 weeks			
E1697 (Agarwala et al. 2017)	1150	IIA–IIIA	18%	IFN-α-2b high dose without maintenance vs. observation	HDI as above without maintenance (IFN) vs. observation (obs)	7.0	HR: 0.98 (IFN vs. obs) (NS)	HR: 1.08 (IFN vs. obs) (NS)
							5-year RFS: 0.70 (IFN) vs. 0.70 (obs) (NS)	Median RFS: 0.83 (IFN) vs. 0.83 (obs) (NS)

(*continued*)

Table 2 (continued)

Study reference	Number of patients eligible for analysis	Stage	% node positive	Therapy and IFN subspecies	Dose and schedule – treatment arm	Median follow-up at time of reporting (years)	DFS/RFS	OS
S0008 (Flaherty et al. 2014)	402	IIIA–IIIC	24%	HDI vs. biochemotherapy (IL-2, IFN, cisplatin, vinblastine, and dacarbazine)	HDI as above	7.2	Median RFS: 4.0 years (biochemotherapy) vs. 1.9 years (HDI)	Median OS: 9.9 years (biochemotherapy) vs. 6.7 years (HDI)
					Biochemotherapy		(S)	(S)
					Dacarbazine 800 mg/m^2 (day 1), cisplatin 20 mg/m^2, vinblastine 1.2 mg/m^2, IL-2 at 9 MU/m^2 continuous IV infusion (days 1–4), IFN 5 MU/m^2 (day 1–5, 8, 10, 12). Repeated every 21 days for 3 cycles		5-year RFS: 39% (biochemotherapy) vs. 48% (HDI)	5-year OS: 56% (biochemotherapy) vs. 56% (HDI)
							(S)	(NS)
Italian Melanoma Intergroup (Chiarion-Sileni et al. 2011)	330	IIIA–IIIC	100%	Intensified IFN-α-2b (IHDI) every other month vs. IFN-α-2b for 1 year	IHDI	5.0	Median RFS: 47.9 months (IHDI)	Median OS: 88.7 months (IHDI)
					IV 20 MU/m^2 5 days a week for 4 weeks every other month for 4 cycles		vs. 35.6 months (HDI) (NS)	vs. 82.6 months (HDI) (NS)
					HDI as above		5-year RFS	5-year OS
							45.8% (IHDI) vs.	60.1% (IHDI) vs.
							44.3% (HDI) (NS)	52.7% (HDI) (NS)

Intermediate dose								
EORTC 18952 (Eggermont et al. 2012, 2005)	1388	IIIA–IIIC	74%	Modified IFN-α-2b for 1 year vs. 2 years vs. observation	Modified HDI induction	11.0	RFS	OS
					IV 10 MU 5 days a week for 4 weeks		IFN (13 months) vs. obs: 0.94 (NS)	IFN (13 months) vs. obs: 0.95 (NS)
					Modified maintenance		IFN (25 months) vs. obs: 0.84 (NS)	IFN (25 months) vs. obs: 0.84 (NS)
					SC 10 MU 3 days a week for 48 weeks or SC 5 MU 3 days a week for 96 weeks		DMFS	
							IFN (13 months) vs. obs: 0.95 (NS)	
							IFN (25 months) vs. obs: 0.84 (NS)	
							Ulcerated primaries	Ulcerated primaries
							RFS	OS
							IFN (13 months) vs. obs: 0.82 (NS)	IFN (13 months) vs. obs: 0.80 (NS)
							IFN (25 months) vs. obs: 0.61 (S)	IFN (25 months) vs. obs: 0.59 (S)
							DMFS	
							IFN (13 months) vs. obs: 0.76 (NS)	
							IFN (25 months) vs. obs: 0.57 (S)	
EORTC 18991 (Eggermont et al. 2008, 2016a)	1256	IIIA–IIIC	100%	Pegylated IFN-α-2b for 5 years vs. observation	PEG-IFN induction – SC 6 μg/kg/week for 8 weeks	7.6	34.8 months (IFN)	47.8% (IFN)
					PEG-IFN maintenance – SC 3 μg/kg/week for 5 years		vs. 25.6 months (obs); S	vs. 46.4% (obs); NS

(*continued*)

Table 2 (continued)

Study reference	Number of patients eligible for analysis	Stage	% node positive	Therapy and IFN subspecies	Dose and schedule – treatment arm	Median follow-up at time of reporting (years)	DFS/RFS	OS
Nordic IFN (Hansson et al. 2011)	855	IIB–IIIB	81%	Modified IFN-α-2b for 1 year vs. 2 years vs. observation	Modified HDI induction	6.0	23.2 months (A)	56.1 months (A)
					IV 10 MU 5 days a week for 4 weeks		vs. 37.8 months (B) vs. 28.6 months (C)	vs. 72.1 months (B) vs. 64.3 months (C) (NS)
					Modified maintenance		IFN vs. obs and IFN 1 year vs. obs (S)	
					SC 10 MU 3 days a week for 48 or 96 weeks		IFN 2 years vs. obs (NS)	
					Arms: A (observation), B (4 weeks induction, 48 weeks maintenance) and C (4 weeks induction, 96 weeks maintenance)			
Low dose								
Austrian Melanoma Cooperative Group (AMCG) (Pehamberger et al. 1998)	311	IB–IIC	0%	IFN-α-2a vs. observation	SC 3 MU 7 days a week for 3 weeks → then →	3.4	RFS/DMFS not reported	Not available
					SC 3 MU 3 days a week for 1 year		Rate of relapse: (24.0% LDI vs. 36.3% obs)	
French Melanoma Cooperative Group (FCGM) (Grob et al. 1998)	499	IB–IIC	0%	IFN-α-2a vs. observation	SC 3 MU 3 days a week for 18 months	>3	HR	HR
							0.74 (LDI vs. obs) (S)	0.70 (LDI vs. obs) (S)

WHO Melanoma Program Trial 16 (Cascinelli et al. 2001)	444	IIIA–IIIC	100%	IFN-α-2a vs. observation	SC 3 MU 3 days a week for 36 months	7.3	NS	NS
Scottish Melanoma Cooperative Group (Cameron et al. 2001)	96	IIA–IIIC	Not available	IFN-α-2a vs. observation	SC 3 MU 3 days a week for 6 months	>6	NS	NS
UKCCCR-AIM HIGH (Hancock et al. 2004)	674	IIA–IIIC	Not available	IFN-α-2a vs. observation	SC 3 MU 3 days a week for 24 months	3.1	NS	NS
DeCOG (Garbe et al. 2008)	840	IIIA–IIIC	Not available	IFN-α-2a	SC 3 MU 3 days a week for 18 months (A) vs 5 years (B)	4.3	5-year DMFS 81.9% (A)	5-year OS 85.9% (A)
							vs. 79.7% (B) (NS)	vs. 84.9% (B) (NS)
DeCOG (Hauschild et al. 2010)	444	IIIA–IIIC	100%	IFN-α-2a	SC 3 MU 3 days a week for 24 months (A)	3.9	HR	HR
					vs. SC 3 MU 3 days a week for 24 months + DTIC 850 mg/m^2 every 4–8 weeks for 24 months (B)		0.69 (A)	0.62 (A)
					vs. observation (C)		vs. 1.01 (B) vs. 1.0 (C)	vs. 0.96 (B)
								vs. 1.0 (C)
Very low dose								
EORTC 18871/ DKG 80-1 (Kleeberg et al. 2004)	728	IIA–IIIC	58%	IFN-α-2b vs. IFN-γ vs. ISCADOR M® vs. observation	IFN-α-2b	8.2	NS	NS
					SC 1 MU every other day for 12 months			
					IFN-γ			
					SC 0.2 mg every other day for 12 months			

DFS disease-free survival, *DMFS* distant metastases-free survival, *HDI* high-dose IFN, *LDI* low-dose IFN, *NS* not significant, *OS* overall survival, *obs* observation, *RFS* relapse-free survival, *S* significant

longer IFN-α-2b regimen comprising *induction* (IV 20 MU/m^2 daily for 5 days for 4 weeks) and *maintenance* (SC 10 MU/m^2 thrice weekly for 48 weeks) phases in 287 stage IIB–IIIC patients (89% lymph node positive). When initially reported at 6.9 years median follow-up, HDI significantly improved both RFS (5-year RFS rate 37% vs. 26%) and OS (5-year OS rate 46% vs. 37%). Toxicity was considerable – with most patients experiencing constitutional adverse events (AEs) that were rapidly reversible with treatment delays and/or dose reductions. These results prompted the Food and Drug Administration (FDA) in the USA and the European Medicines Agency (EMA) in Europe to approve HDI for the adjuvant treatment of high-risk resected melanoma in 1995.

Five other studies have studied the same regimen against various comparators including lower IFN-α doses (E1690), adjuvant ganglioside vaccine GMK (E1694 and E2696), and observation (E1690 and Sunbelt Melanoma Trial – Protocol A) (Kirkwood et al. 1996, 2000, 2001a, b; Creagan et al. 1995; McMasters et al. 2016). Results from these studies vary slightly but overall suggest that HDI improves RFS consistently and significantly, with less consistent benefit in terms of OS. When RFS/OS data from E1684, E1690, E1694, and E2696 were reanalyzed at 12.6 years median follow-up, RFS improvement was shown to be durably sustained although OS benefit was not significant on this reanalysis – possibly due to competing causes of death. The variability in OS benefit across these studies suggests that the benefit of HDI is likely limited to a subset of patients – although it remains unclear how to identify this subset of patients.

Efforts to improve the toxicity/benefit profile of HDI, while maintaining RFS/OS benefits, have prompted multiple investigators to design alternate regimens of lower intensity, utilizing different schedules and/or shorter durations of therapy. Lower-intensity regimens include very-low-dose regimens (1 MU SC every other day) in the European Organisation for Research and Treatment of Cancer (EORTC) 18871; low-dose regimen (3 MU SC thrice weekly) tested in WHO Melanoma Program Trial 16, ECOG E1690, UKCCCR AIM-High trial, Scottish trial, and German DeCOG 2008 and DeCOG 2010 studies (Pehamberger et al. 1998; Grob et al. 1998; Cascinelli et al. 2001; Cameron et al. 2001; Hancock et al. 2004; Garbe et al. 2008; Hauschild et al. 2010; Kleeberg et al. 2004); and intermediate-dose regimens tested in EORTC 18952, EORTC 18991, and Nordic IFN-α trials (Eggermont et al. 2005, 2008, 2012, 2016a; Hansson et al. 2011). Significant inter-study heterogeneity precludes cross-trial comparisons, and it remains unclear which of these regimens, if any, provide clinical benefit that equals or exceeds that seen with standard HDI. At the present time none of these are recommended outside of a clinical trial.

Analyses of the RFS benefit in E1684 suggested that the improvement in RFS occurred early with treatment, perhaps even in the first month of therapy – suggesting that the overall RFS benefit of this regimen might be attributable to the intravenous "induction" phase. Three prospective randomized studies have now evaluated the value of a truncated HDI regimen, compared either to observation (E1697) or standard/modified 1-year-long regimen (Hellenic He13A/98 and Oxford phase II study) (Payne et al. 2014; Pectasides et al. 2009; Agarwala et al. 2017). E1697 compared the standard HDI induction regimen to observation alone in resected stage IIA–IIIB patients. Interim analyses of 1150 patients of a planned enrollment of 1420 (19% lymph node positive) were presented at ASCO 2011 – where investigators reported no improvement in RFS or 5-year OS for the truncated schedule following which the study was terminated for futility. The Oxford phase II study randomized 194 patients (77% lymph node positive) to either HDI induction alone or standard HDI and concluded that HDI induction alone lacked any benefit. Hellenic He13A/98 utilized a non-inferiority design that tested a modified induction/maintenance dosage in stages IIB–IIIC (58% lymph node positive) and concluded that the modified induction-only regimen was not inferior – although the nonstandard doses and small stage III enrollment without a control arm qualify these results. A meta-analysis that included several of

these studies concluded that the truncated regimens did not meet the prespecified non-inferiority bar for either RFS or OS (Malczewski et al. 2016).

Other investigators have attempted to answer the antipodal question of whether prolonging duration of therapy confers greater treatment benefit. To improve the tolerability of longer regimens, studies have used either lower doses of IFN alpha-2b or pegylated interferon alpha-2b (PEG-IFN-α) – the latter being a mainstay of therapy for chronic viral hepatitis prior to the advent of more highly effective nucleotide analogs. These studies included ECOG E1690, WHO 16, EORTC 18952/Nordic IFN-α trial (IFN-α-2b for 1 year vs. 2 years vs. observation), and EORTC 18991 (PEG-IFN-α for 5 years vs. observation) (Eggermont et al. 2005, 2008, 2012, 2016a; Hansson et al. 2011). Neither WHO trial 16 nor ECOG trial E1690 demonstrated RFS/OS benefit with 2–3 years of lower-dosage IFN alpha-2a or 2b (3 MU two to three times weekly). Although EORTC 18952 and Nordic IFN-α trial were similar in many respects, the maintenance IFN-α doses on the 2-year arms of these trials were different: 5 MU 3/week in EORTC 18952 but 10 MU 3/week in Nordic IFN-α study. At initial reporting after ~5 years of median follow-up, both the Nordic IFN-α trial and EORTC 18952 concluded that adjuvant IFN-α for 1 year improved RFS but not OS without incremental benefit derived from additional treatment. Interestingly, following extended follow-up (median 11 years), EORTC 18952 showed that RFS, distant metastases-free survival (DMFS), and/or OS *were not significantly different* in either treatment arm compared to observation in the primary analysis but that patients with ulcerated primaries benefited from longer therapy in unplanned subgroup analyses. EORTC 18991 reported that PEG-IFN-α given for 5 years improved RFS (HR 0.87; 7-year RFS 39.1% vs. 34.6%) but has shown no impact on OS in stage III melanoma. Toxicity of PEG-IFN-α resulted in treatment discontinuation among 37% of patients. Similar to the observations in EORTC 18952, PEG-IFN-α therapy resulted in greater RFS, DMFS, and OS benefits in patients with ulcerated primaries and N1a disease. Selective benefits in patients with ulcerated primaries at initial reporting (median follow-up 3.8 years) were still present at subsequent reporting (median follow-up 7.6 years). These observations have spurred EORTC to evaluate PEG-IFN-α for 2 years against observation in patients with ulcerated primaries and/or microscopic nodal metastases in EORTC 18081.

Efforts to augment the benefits of HDI either by dose intensification or with the addition of chemotherapy have generally demonstrated disappointing results, with high toxicity rates and no greater impact upon overall survival. Southwest Oncology Group's (SWOG) S0008 evaluated an intensive biochemotherapy regimen comprising IL-2, IFN-α, cisplatin, vinblastine, and dacarbazine given for 3 months, compared to standard HDI for 1 year among 402 patients with stage III (24% IIIC) melanoma (Flaherty et al. 2014). Notably, biochemotherapy improved RFS at a median follow-up of 7.2 years without any improvement in OS. Toxicity was considerable in both arms although grade 4 toxicity rates were greater with biochemotherapy than for HDI (40% vs. 7%). Treatment discontinuation rates were similar in both cohorts, and further evaluation of this regimen is not planned. The Italian Melanoma Intergroup (IMI) evaluated a dose-intense HDI schedule (comprising standard HDI induction given every other month for four courses – intermittent HDI, IHDI) in 336 stage IIIA–IIIC patients (Chiarion-Sileni et al. 2011). The incidence of grade 3/4 toxicity and treatment-related discontinuation was balanced in both arms, but more IHDI patients completed therapy compared to HDI patients (66% vs. 49%). RFS and 5-year RFS statistics were not significantly different for the two arms, suggesting that intensive HDI is feasible and increases overall drug exposure.

Multiple systemic reviews, pooled individual patient data analysis, and pooled meta-analyses have examined the results of IFN-α treatment upon disease-free survival (DFS) and OS in patients with resected high-risk cutaneous melanoma (Lens and Dawes 2002; Garbe et al. 2011; Wheatley et al. 2003, 2007; Mocellin et al. 2010, 2013). Collectively these studies have concluded that IFN-α-based adjuvant therapy regardless of

the dosage tested consistently improves RFS by 17% (HR 0.83, 95% CI 0.78–0.87, p-value significant), with 9% OS improvement (HR 0.91, 95% CI 0.85–0.97, p-value significant) in the most recent Cochrane database review by Mocellin and colleagues (2013). HDI and PEG-IFN-α are approved by American, European (HDI only, not PEG-IFN-α), and Australian regulatory authorities for the adjuvant treatment of high-risk resected melanoma defined as node-positive disease or node-negative disease with a primary Breslow thickness of T3b or greater. Both yearlong HDI and 5-year PEG-IFN-α regimens improve RFS (30% HDI; 13% PEG-IFN-α) with lesser effects upon OS, or no detectable effect upon OS (PEG-IFN-α). Therapy-related toxicity was considerable with both regimens and required delays or discontinuation in ∼50% of treated patients?

Adjuvant Therapy: Vaccines

Anecdotal reports of spontaneous antitumor immunity with subsequent tumor eradication have spawned multiple attempts at cancer vaccination. Early vaccines tested non-mutated immunogenic tumor-associated antigens and demonstrated rare clinical responses. Cancer vaccines can be categorized based on the antigen and/or cell incorporated – whole cell/cell lysate (autologous, allogeneic), dendritic cell (DC), peptide, ganglioside, DNA, and viral vectors. Early vaccine studies tested non-specific targets in highly heterogeneous patient populations and occasionally yielded early responses that could not be reproduced in large studies, and this data is summarized elsewhere (Ozao-Choy et al. 2014; Weiss et al. 2014).

Two vaccines with promising positive results in early-phase studies have recently reported negative results in definitive phase III studies: Vical's Allovectin-7® and GlaxoSmithKline's MAGE-A3 antigen-specific cancer immunotherapeutic (ASCI). Allovectin-7® is a DNA-liposome complex containing a plasmid that encoded major histocompatibility complex (MHC) class I (HLA-B7/B-2 microglobulin) encased in a cationic lipid vector. Early-phase studies of intralesional Allovectin-7® injections were promising with several partial responses (Stopeck et al. 1997, 2001). In the pivotal phase III study, Allovectin-7® was compared to first-line chemotherapy (Dacarbazine or Temolozomide) in 390 patients with previously untreated unresectable stage III/IV melanoma where Allovectin-7® failed to demonstrate significant improvements for either the primary response endpoint or secondary OS endpoint. No further studies are planned. MAGE-A3 ASCI consists of recombinant MAGE-A3 protein and a novel immunostimulant AS15 (QS-21 Stimulon® adjuvant, monophosphoryl lipid A, and TLR-9 agonist CpG7909 in a liposomal formulation). MAGE-A3 ASCI was studied in 1351 patients with MAGE-A3+ high-risk stage III melanoma with macroscopic lymph node involvement. Compared to placebo, MAGE-A3 ASCI failed to improve DFS at first interim analysis which led to early study termination. Follow-up is ongoing to evaluate the co-primary endpoint of DFS in patients positive for a gene signature, expression of which was associated with clinical benefit in patients treated with MAGE-A3 ASCI in early-phase studies (Ulloa-Montoya et al. 2013).

Amgen's talimogene laherparepvec (T-VEC, Imlygic®) is a first-in-class oncolytic virus based on a modified herpes simplex virus (HSV) type 1 designed to selectively replicate in and lyse tumor cells while encouraging regional and systemic antitumor immunity following intralesional injection. T-VEC lacks herpes virus neurovirulence factor ICP34.5 and ICP47 genes. ICP34.5 deletion attenuates virulence while enhancing tumor-selective replication, while ICP47 deletion increases antigen presentation and increases expression of the HSV US11 gene which in turn increases replication and oncolysis. T-VEC also contains a gene encoding human granulocyte-macrophage colony-stimulating factor (GM-CSF) which increases local GM-CSF production with resultant recruitment and activation of antigen-presenting cells. Earlier proof-of-principle studies demonstrated immunogenicity and clinical responses in virus-injected and non-injected lesions (Kaufman et al. 2016). The registration phase III OPTiM study randomized 436 unresected

stage IIIB–IV (M1A–C) melanoma patients 2:1 to receive either T-VEC or subcutaneous GM-CSF powered for a primary endpoint of independently assessed durable (≥6 months) response rate (DRR) (Andtbacka et al. 2015). Although this study permitted stage IIIB–IV (M1A-C) patients to enroll, M1B/C patients were limited to 40% of enrollment with response stratified by presence/absence of liver metastases. T-VEC significantly improved DRR over GM-CSF (16.3% vs. 2.1%) with nonsignificantly improved OS (HR 0.79, 95% CI 0.62–1.00, $p = 0.051$). T-VEC was most effective in treatment-naive patients and those with stage IIIB/C or M1A disease. Currently approved for local treatment of unresectable and nodal disease in patients with recurrent melanoma, T-VEC is being evaluated as a neoadjuvant treatment prior to surgery for completely resectable stage IIIB/IIIC/M1A melanoma (NCT02211131).

Adjuvant Therapy: Checkpoint Inhibitors

CTLA-4 and PD-1 are negative regulatory checkpoints that restrict T-cell activation. Blocking antibodies to CTLA-4 and PD-1 demonstrated potent antitumor activity in murine models and led to evaluation in patients with advanced melanoma. Two phase III trials evaluated the CTLA-4 blocking antibody ipilimumab with improved responses and overall survival compared to GP-100 (MDX010-020) in previously treated patients and dacarbazine (CA184-024) in previously untreated patients (Hodi et al. 2010; Robert et al. 2011). Notably, these studies tested different doses and schedules (020 trial, 3 mg/kg for four doses; 024 trial, 10 mg/kg for four doses and then 10 mg/kg maintenance till progression) and reported relatively similar rates of response but significantly greater rates of toxicity with higher doses of ipilimumab. Response rates with ipilimumab are 10–15%, and at 3 years, 21% of patients remain alive with a plateau in survival curves, suggesting that responders at this time point have durable benefit (Maio et al. 2015). PD-1 inhibitors nivolumab and pembrolizumab are associated with response rates of 35–50%, while the ipilimumab/nivolumab combination produces even greater response rates (58%) compared to single-agent therapy with either nivolumab (44%) or ipilimumab (19%) albeit with significantly increased toxicity (55% grade 3/4 AEs) (Hodi et al. 2010; Larkin et al. 2015a; Robert et al. 2015a, b). Similar to ipilimumab, responses seen with nivolumab and pembrolizumab and the combination ipilimumab/nivolumab are exceedingly durable (Larkin et al. 2015b; Ribas et al. 2016; Wolchok et al. 2017). The efficacy of these agents has prompted a spate of adjuvant studies (see Table 3).

EORTC 18071 tested adjuvant ipilimumab against placebo in a randomized blinded phase III study that enrolled 951 patients with stage III melanoma, although patients with <1.0 mm lymph node involvement or in-transit disease were excluded. The dose of ipilimumab used in this adjuvant trial was higher (10 mg/kg every 3 weeks) than that approved for use in advanced disease in the USA, and additional maintenance treatment was specified (10 mg/kg every 3 months for 3 years) (Eggermont et al. 2015). At a median follow-up of 2.7 years, adjuvant ipilimumab 10 mg/kg significantly improved RFS (26.1 vs. 17.1 months) and 3-year RFS (46.5% vs. 34.8%) albeit with significant attendant toxicity: 54% of patients had grade 3/4 toxicity, and 52% discontinued treatment due to toxicity, with five deaths (1%). More recently, a report performed at a median of 5.3 years follow-up suggested that this RFS benefit was sustained with additional benefit in OS (hazard ratio for death 0.72) (Eggermont et al. 2016b). On the basis of these results, the FDA and EMA approved adjuvant ipilimumab at 10 mg/kg dose for the adjuvant treatment of stage III melanoma following surgery. Unfortunately, EORTC 18071 did not clarify the role of adjuvant therapy in patients with resected distant metastases (M1A/M1B) nor whether the less intense but more tolerable regimen tested in MDX010-020 was similarly efficacious.

ECOG's intergroup trial E1609 was a phase III study that tested adjuvant ipilimumab at 10 mg/kg and 3 mg/kg against to HDI in both high-risk (stage IIIC/IIIC) and very high-risk (stage IV

Table 3 Phase III studies of adjuvant checkpoint inhibition in high-risk resected melanoma

Study reference	Number of patients eligible for analysis	Stage	Agents	Dose and schedule – treatment arm	Median follow-up at time of reporting (years)	DFS/RFS	OS
EORTC 18071/CA 184-029 (Eggermont et al. 2015, 2016b)	951	IIIA (T1–4AN1AM0) to IIIC (T1–4BN1B–3M0) excluding in-transit disease	Ipilimumab vs. placebo	Ipilimumab	5.3	Median RFS (ipi vs. placebo): 27.6 vs. 17.1 months	Median OS (ipi vs. placebo): not reported
				Induction: 10 mg/kg q3 weeks ×4		5-year RFS rate: 40.5% (ipi) vs. 30.3% (placebo)	5-year OS rate: 65.4% (ipi) vs. 54.4% (placebo)
				Maintenance: 10 mg/kg q3 months for 3 years		HR: 0.76 (S)	HR: 0.72 (S)
ECOG E1609 (Tarhini et al. 2017)	1670	IIIB (T1–4BN1A–2AM0 or T1–4AN1B–N2CM0), IIIC (T1–4BN1B–N3M0) and IVA/B (TxNxM1A–B)	Ipilimumab 10 mg/kg or 3 mg/kg vs. HDI	Ipilimumab	3.1	Median RFS: not reported	Not reported
				Induction: 3 or 10 mg/kg q3 weeks or 4 doses		3-year RFS rate: 54% (ipilimumab-10) vs. 56% (ipilimumab-3); not reported for HDI	
				Maintenance: 3 or 10 mg/kg q3 months for 4 doses			
				HDI as above			

CheckMate 238 (Weber et al. 2017)		IIIB–IIIC; IV–A; IV–B; IV–C	Nivolumab vs. ipilimumab 10 mg/kg	Ipilimumab	1.6	Median RFS: not reached in either group	Not reported
				Induction: 3 or 10 mg/kg q3 weeks or 4 doses		1-year RFS rate: 71% (nivolumab) vs. 61% (ipilimumab-10)	
				Maintenance: 3 or 10 mg/kg q3 months for 4 doses		18-month RFS rate: 66% (nivolumab) vs. 53% (ipilimumab-10)	
				Nivolumab: 2 mg/kg every 2 weeks for 1 year		HR: 0.65 (S)	
CheckMate 915 (NCT03068455)	900 (planned)	IIIB–IIIC; IV–A; IV–B; IV–C	Nivolumab vs. ipilimumab 10 mg/kg vs. ipilimumab	Ipilimumab: as in CheckMate 238	Pending	Pending	Pending
				Nivolumab: as in CheckMate 238			
				Ipilimumab/ Nivolumab: ipilimumab induction with nivolumab as in CheckMate 238			
SWOG S1404 (NCT02506153)	1378 (accrued)	IIIB (T1–4BN2AM0 or T1–4AN1B–N2CM0), IIIC (T1–4BN1B–N3M0), and IVA/B (TxNxM1A–B)	Pembrolizumab vs. investigator's choice HDI or ipilimumab	Pembrolizumab: 200 mg q3 weeks for 1 year	Pending	Pending	Pending
				HDI as above			
				Ipilimumab as in ECOG E1609			

DFS disease-free survival, *DMFS* distant metastases-free survival, *HDI* high-dose IFN, *NS* not significant, *OS* overall survival, *obs* observation, *RFS* relapse-free survival, *S* significant

M1A/B) resected melanoma. Ipilimumab was dosed at 3 mg/kg or 10 mg/kg every 3 weeks for four doses (induction) followed by a shorter maintenance phase (3 mg/kg or 10 mg/kg every 3 months for four cycles) than that which was utilized in EORTC 18071. The study was originally powered to evaluate the co-primary endpoints of RFS and OS in the three arms by allocating type I error in a hierarchical fashion initially between IFN-α and ipilimumab arms, permitting a comparison between ipilimumab-3 and ipilimumab-10 *only if* this former analysis was statistically significant. However, with the interim approval of adjuvant ipilimumab-10 based on EORTC 18071 and its' attendant toxicity, there was a pressing need to evaluate the relative efficacy of ipilimumab-3 schedule as tested in ECOG E1609. At ASCO 2017, authors presented an unplanned interim analysis of RFS in ipilimumab-3 and ipilimumab-10 (Tarhini et al. 2017). At a median of 3.1 years follow-up, 3-year RFS rates were similar in both ipilimumab-3 and ipilimumab-10 (54% vs. 56%, respectively) patients although patients treated with ipilimumab-10 had a much higher rate of treatment discontinuation compared to ipilimumab-3 (54% vs. 35%, respectively). Given the unplanned and interim nature of the analyses, neither OS data nor RFS/OS data comparing either ipilimumab dose to the HDI comparator were provided.

Given the favorable safety profile and better efficacy reported with PD-1 inhibitors compared to ipilimumab in unresectable stage IV melanoma, multiple studies were launched to evaluate these agents as adjuvant therapy in patients with resected stage IIIC and IV melanoma. CheckMate 238 was a randomized, double-blind, phase III trial that compared nivolumab (3 mg/kg every 2 weeks for 1 year) to ipilimumab (10 mg/kg every 3 weeks for four doses and then every 12 weeks for up to 1 year) in patients with resected stage IIIB, IIIC, or IV (M1A/B/C) melanoma (Weber et al. 2017). Randomization was stratified by stage (IIIB/C vs. M1A/B vs. M1C) and PD-L1 staining using a 5% cutoff on tumor cells (negative vs. intermediate vs. positive). Patients with stage IV disease accounted for ~20% of enrollment in both arms and mostly comprised M1A patients (~60%). Prespecified interim analyses at 19.5 months of follow-up showed that 12-month RFS rate was significantly greater with nivolumab compared to ipilimumab (71% vs. 61%) with significantly less treatment-related toxicity (14% vs. 46% grade 3/4 AEs).

A similar study was conducted by the Southwest Oncology Group (SWOG) in cooperation with ECOG to test the efficacy of pembrolizumab against investigator's choice of either ipilimumab (EORTC 18071 schedule) or HDI in resected stage IIIA–IV melanoma – SWOG S1404. Patients with stage IIIA disease are required to have at least N2 disease for eligibility, and only stage IV A/B patients are permitted to enroll. Accrual in this study is complete, and results will shed light on the comparative efficacy of PD-1 inhibition against CTLA-4 inhibition and IFN-α in the adjuvant arena.

To summarize, adjuvant ipilimumab as tested in EORTC 18071 significantly improves RFS and OS with significant attendant toxicity. Ipilimumab given at a lower dose for a shorter duration may be similarly efficacious (ECOG E1609), but reported analyses are very preliminary. CheckMate 238 demonstrated that adjuvant nivolumab was clearly superior to ipilimumab in stage III/IV melanoma in both RFS and OS terms. Based on these results, the FDA has granted a priority review to a supplemental biologics license application (sBLA) for nivolumab in this indication. The results of SWOG S1404 and CheckMate 915 (nivolumab vs. ipilimumab vs. ipilimumab/nivolumab) are eagerly awaited.

Adjuvant Therapy: Signaling Inhibitors

Deep sequencing data has identified several driver mutations in distinct subsets of melanoma including *BRAF/NRAS/NF1* (cutaneous), *GNA11/GNAQ* (uveal), and *KIT* (mucosal/acral). Activating *BRAF* mutations are present in 40–60% of cutaneous melanomas – mostly comprising a glutamic acid for valine substitution at amino acid 600 (V600E) (Curtin et al. 2005, 2006). *BRAF*-mutated melanoma is associated with

certain features (truncal primary, lack of chronic sun damage (CSD), and earlier age of onset) and an aggressive clinical course. BRAF inhibitors result in dramatic responses and prolonged survival in a large fraction of patients. Combining BRAF inhibitors with inhibitors of downstream MEK yields higher response rates and longer RFS/OS compared with BRAF inhibition alone (Chapman et al. 2011; Flaherty et al. 2012; Larkin et al. 2014; Long et al. 2014, 2015). BRAF and MEK inhibitors are being evaluated as adjuvant options in *BRAF V600E/K* mutant high-risk resected melanoma in several trials (see Table 4).

The role of single-agent BRAF inhibition in the adjuvant setting was tested in BRIM8: a phase III, randomized, double-blind, placebo-controlled study of 1 year of vemurafenib adjuvant therapy in patients with surgically resected stage II and III melanoma (Lewis et al. 2017). BRIM8 included patients with deep/ulcerated primaries that were lymph node-negative (stage IIC) in addition to lymph node-positive (stage III A/B/C) patients but analyzed patients in two cohorts: lower-risk stage IIC–IIIB (cohort 1) and higher-risk stage IIIC (cohort 2). BRIM8 was powered on a primary endpoint of RFS that was evaluated in a hierarchical fashion in which cohort 2 was required to meet the primary endpoint before cohort 1 could be analyzed. Although vemurafenib monotherapy improved RFS in cohort 2 (hazard ratio for relapse 0.80), this was nonsignificant and precluded formally analyzing RFS data in cohort 1 (hazard ratio for relapse 0.54).

COMBI-AD was a randomized, placebo-controlled, double-blinded, phase III trial that studied the combination of oral dabrafenib/trametinib compared to placebo for 1 year in BRAF V600E/K-mutated patients with stage III (A/B/C) melanoma following definitive surgery (Long et al. 2017). Enrollment was stratified by BRAF mutation status (V600E or V600K) and stage (IIIA, IIIB, or IIIC). In an interim analysis at a median follow-up of 2.8 years, adjuvant dabrafenib/trametinib significantly improved RFS (58% vs. 39%) and OS. Of note, 3-year OS rate was greater (86% vs. 77%) with dabrafenib/trametinib although this difference did not meet the prespecified threshold to claim statistical significance.

Unlike *BRAF/NRAS/NF1* mutations, *KIT* mutations and amplification of wild-type KIT are typically observed in mucosal, acral, or CSD melanomas. *KIT* mutations in melanoma are typically located in the juxtamembrane domain with constitutive kinase activity – functionally similar to the activating mutations associated with gastrointestinal stromal tumors (GIST). Although ineffective in unselected patients, dramatic responses have been reported with the use of small molecular KIT inhibitors imatinib and dasatinib in patients with activating *KIT* mutations (Wyman et al. 2006; Lutzky et al. 2008; Woodman et al. 2009). A phase II study comparing adjuvant imatinib 400 mg daily for 1 year to modified HDI in a cohort of *KIT*-mutated stage IIB–IIIC Chinese patients was launched in 2012. As this study only enrolled seven patients at 2013 when it was initially reported, the limited sample size and follow-up preclude definitive conclusions being drawn regarding the use of adjuvant KIT inhibition in *KIT*-mutated melanoma (A phase II randomized study of adjuvant imatinib versus high-dose interferon alpha-2b for resected high-risk c-kit mutated melanoma 2013).

In summary, adjuvant BRAF and MEK inhibition appears to meaningfully improve RFS in stage III melanoma. However, the role of BRAF monotherapy and/or KIT inhibition in *BRAF*-mutated or *KIT*-mutated melanoma at high risk of recurrence following resection remains investigational at this time.

Conclusion

Data from multiple phase III studies definitively demonstrate the impact of adjuvant IFN-α on RFS, while the impact on OS is less significant and diminishes after 10 years. PEG-IFN-α exclusively improves RFS with no OS benefit. Some features including systemic autoimmunity and certain features of the primary tumor (ulcerated primaries and/or microscopic node-positive disease) appear to be predictive biomarkers of IFN-α benefit; and the latter will be prospectively validated in an EORTC study (18081) (Gogas et al. 2006).

Table 4 Phase III studies of adjuvant targeted therapies in high-risk resected melanoma

Study reference	Number of patients to be enrolled	Stage	Agents	Dose and schedule – treatment arm	Median follow-up at time of reporting (years)	RFS	OS
BRIM8 (Lewis et al. 2017)	475	IIC or IIIA–C BRAF V600E/K mutated melanoma in two cohorts: lower-risk stages IIC–IIIB (cohort 1) and higher-risk stage IIIC (cohort 2)	Vemurafenib vs. placebo	Vemurafenib 960 mg BID	2.7	Median RFS (cohort 1): not reported (V) vs. 36.9 months (placebo)	Not reached
		Hierarchical analyses of primary RFS endpoint required cohort 2 to meet prior to analyses of cohort 1				HR (cohort 1): 0.54 (S but not interpretable)	
						Median RFS (cohort 2): not reported	
						HR (cohort 2): 0.80 (NS)	
COMBI-AD (Long et al. 2017)	870	IIIA–IIIC BRAF V600E/K-mutated melanoma	Dabrafenib/ trametinib vs. placebo	Dabrafenib 150 mg BID and trametinib 2 mg daily	2.8	Median RFS: not reached (D/T) vs. 16.6 months (placebo)	Median OS: not reached
						1-year: 88% (D/T) vs. 56% (placebo)	1-year: 97% (D/T) vs. 94% (placebo)
						2-year: 67% (D/T) vs. 44% (placebo)	2-year: 91% (D/T) vs. 83% (placebo)
						3-year: 58% (D/T) vs. 39% (placebo)	3-year: 86% (D/T) vs. 77% (placebo)
						HR: 0.47 (S)	HR: 0.57 (NS)

D/T Dabrafenib/trametinib, *NS* not significant, *OS* overall survival, *obs* observation, *RFS* relapse-free survival, *S* significant, *V* vemurafenib

Adjuvant ipilimumab at 10 mg/kg as tested in EORTC 18071 significantly improves RFS and OS, albeit with significant attendant toxicity. Although approved for this indication, how ipilimumab performs against HDI and whether lower doses are just as effective are unknown. ECOG E1609 suggests that ipilimumab 3 mg/kg is as efficacious as 10 mg/kg although the unplanned and interim nature of the reported analyses suggests data must be interpreted cautiously. In comparison, CheckMate 238 demonstrated that adjuvant nivolumab for 1 year significantly and meaningfully improved RFS and OS over ipilimumab in stage III and IV melanoma. Whether the ipilimumab/nivolumab combination further raises the bar over nivolumab monotherapy remains to be seen (CheckMate 915).

Molecularly targeted therapy with BRAF/MEK inhibitors and to a lesser extent KIT inhibitors has demonstrated high response rates and survival benefits in metastatic melanoma, prompting evaluation in the adjuvant setting. While adjuvant BRAF/MEK inhibition is superior to placebo in BRAF-mutated melanoma, the impact of BRAF monotherapy is less clear.

To accelerate progress in this arena, trials of candidate adjuvant interventions in the neoadjuvant setting are vital. The advantages of this approach include the ability to evaluate tissue samples prior to and following 1–2 months of therapy, the mechanism of action of therapies, the clinical antitumor effects of the neoadjuvant intervention, and the evaluation of alternative endpoints (pathological complete response and others) that may be surrogates for RFS and OS endpoints. This approach offers a solution to the long window of time that adjuvant trials typically require for maturity. Separately, combinations of PD-1 inhibitors with BRAF/MEK inhibitors, small molecular inhibitors of immunomodulatory enzymes (IDO), and other immune checkpoint inhibitors (TIM3, LAG-3, TIGIT) are being considered and may raise the bar further. Biomarkers of prognostic utility, as well as biomarkers that may predict the therapeutic benefit or toxic events that are likely from adjuvant therapy, are increasingly important for adjuvant therapy discussions with patients.

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Cutaneous Adverse Events of Systemic Melanoma Treatments

36

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Abstract

Cutaneous adverse events (AEs) are frequent with systemic melanoma treatments. As a result of a paradigmatic shift in melanoma management from traditional cytotoxic chemotherapy to immunotherapies and targeted therapies as first-line treatment, the spectrum of skin AEs to these treatments has significantly broadened. Cutaneous toxicities from anticancer therapy manifest as doubly burdensome as visible stigmatization often carries profound psychosocial implications. Early detection and treatment help to minimize a reduction in patients' quality of life and maximize anticancer treatment adherence and outcome. The knowledge of typical presentations associated with the specific drug regimen administered to the patient is essential for timely management of these conditions. A dermatological evaluation of the skin condition appears to be essential for an interdisciplinary approach as very often even dramatic skin presentations do not necessitate a cessation of the potentially lifesaving antineoplastic drug. Since the onset of AEs of some therapies can take up to several months or years and may also occur in cancer survivors long after completion of their therapy, thorough dermatological follow-up may be advised even after successful completion of antineoplastic treatments.

Keywords

Cutaneous toxicities · Immune checkpoint inhibitor (ICI) · BRAF inhibitor (BRAFi) · MEK inhibitor (MEKi) · Chemotherapy · Adverse events (AEs)

Abbreviations

AD	Atopic dermatitis
ADLs	Activities of daily living
AE	Adverse event
AH	Antihistamines
AK	Actinic keratosis
BP	Bullous pemphigoid
BPAG	Bullous pemphigoid antigen
BRAF wt	BRAF wild-type mutation
BRAFi	BRAF inhibitor
BSA	Body surface area
CIA	Chemotherapy-induced alopecia
CsA	Cyclosporin A
CTCAE	Common Terminology Criteria for Adverse Events
CTLA-4	Cytotoxic T-lymphocyte antigen-4
cuSCC/SCC	(Cutaneous) Squamous cell carcinoma
DEJ	Dermo-epidermal junction
DRESS	Drug reaction with eosinophilia and systemic symptoms
DTIC	5-(3,3-Dimethyl-1-triazeno) imidazole-4-carboxamide
EGFRi	Epidermal growth factor receptor inhibitor
FDA	Food and Drug Administration
H&E	Hematoxylin and Eosin stain
HDAC	Histone deacetylase
HFS	Hand-foot syndrome
ICI	Immune checkpoint inhibitor
IDO	Indoleamine-pyrrole 2,3-dioxygenase
ircAE	Immune-related cutaneous adverse event
irAE	Immune-related adverse event
KA	Keratoacanthoma
LP	Lichen planus

MAPK pathway	Mitogen-activated protein kinase pathway
MEKi	MEK inhibitor
MMF	Mycophenolate mofetil
MTIC	5-3-Methyltriazen-1-yl-imidazo-4-carboxamide
MTX	Methotrexate
NSCLC	Non-small cell lung cancer
OS	Overall survival
OTC	Over-the-counter
PD-1	Programmed cell death protein-1
PD-L1	Programmed cell death ligand-1
PFS	Progression-free survival
QoL	Quality of life
RCC	Renal cell carcinoma
RR	Response rate
SCAR	Severe cutaneous adverse reaction
SJS	Stevens-Johnson syndrome
TCR	T-cell receptor
TEN	Toxic epidermolytic necrolysis

Introduction

The therapy profile of advanced malignant melanoma has rapidly evolved from chemotherapy regimens with low response rates and no survival benefit toward an immunotherapy-based model beginning in 2011 after the introduction of the cytotoxic T-lymphocyte antigen-4 (CTLA-4) inhibitor ipilimumab and the development of targeted therapies such as BRAF and MEK inhibitors. These new therapies have shown a dramatic increase in response rates (RR), progression-free survival (PFS), and overall survival (OS). Hence, formerly utilized chemotherapies such as dacarbazine have acquired a second-line role in the therapeutic landscape of metastatic melanoma. Thus, the spectrum of therapy-associated adverse events (AEs) encountered in daily practice has shifted to phenomena associated with immune response augmentation or mitogen-activated protein kinase (MAPK) pathway inhibition exploited by modern melanoma therapy agents. The introduction of novel mechanisms of modern tumor therapies has also necessitated a rethinking of the management of their AEs.

Grading of Dermatologic Toxicities

Accurate grading is critical to assess response to antitoxicity interventions and impact on patients (Lynch et al. 2007). The most widely used system to grade toxicities in clinical trials is the **Common Terminology Criteria for Adverse Events (CTCAE)** in its current version 5.0, published by the US Department of Health and Human Services on November 27, 2017. Adverse events are graded from 1 to 5, taking into consideration the severity of symptoms and the impact an AE has on patient functional status, capsulized as activities of daily living (ADLs) (Table 1).

Immunotherapy-Related Cutaneous Adverse Events (ircAEs)

In 2011, ipilimumab, an anti-CTLA-4 monoclonal antibody, was the first checkpoint inhibitor to be approved by the US Food and Drug Administration (FDA) for the treatment of metastatic melanoma. The concept of immunotherapy as a means of harnessing the patient's own immune system in the fight against cancer represents a paradigm shift from traditional chemotherapy regimens, which had always demonstrated low response rates below 20% and no proven overall survival benefit. Given the remarkable effect that ipilimumab had on melanoma patient outcomes, immunotherapy quickly became one of the main pillars in first-line melanoma therapy and has been adapted for a variety of solid tumors as well. Soon after ipilimumab, new immune checkpoint inhibitors (ICIs) were developed, including those that block programmed cell death protein-1 (PD-1; nivolumab and pembrolizumab), and those that block programmed cell death ligand-1 (PD-L1; avelumab, atezolizumab, and durvalumab). Now, combination therapy with the use of multiple ICIs is common in the treatment of advanced malignancies with the benefit of increased survival. However, with immunotherapy, the upregulation of the immune system also meant a new host of adverse reactions secondary to the disinhibition of tightly regulated immune processes. Given the differences

Table 1 Common Terminology Criteria for Adverse Events (CTCAE) grading system for adverse events, version 5.0

Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated	Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental ADL	Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care ADL	Life-threatening consequences; urgent intervention indicated	Death related to AE

Table 2 Skin toxicity data of FDA-approved immunotherapies for advanced melanoma. (Adapted from Hassel et al. 2017)

Drug	Ipilimumab[a] (*N*=357)	Nivolumab[b] (*N*=787)	Pembrolizumab[c] (*N*=786)	Ipilimumab/nivolumab[a] (*N*=407)
Target molecule	CTLA-4	PD-1	PD-1	CTLA-4 and PD-1
Skin toxicity (any grade)	61.9%	38.4%	28.3%	64%
Skin toxicity (grade ≥3)	6.4%	1.1%	0.02%	7%
Rash (any grade)	31%	16.9%	13.4–16.1%	30%
Rash (grade ≥3)	3.2%	0.4%	0–0.2%	3%
Pruritus (any grade)	33.4%	18.4%	14.1–17.7%	35%
Pruritus (grade ≥3)	1.7%	0.1%	0%	2%
Vitiligo (any grade)	7.6%	8.8%	4.7–11.2%	9%

[a]Data was obtained from CheckMate-067 and CheckMate-069 (ipilimumab 3 mg/kg plus nivolumab 1 mg/kg q3w in stage IV melanoma)
[b]Data from CheckMate-037, CheckMate-066, and CheckMate-067 (nivolumab 3 mg/kg q2w in stage IV melanoma)
[c]Data obtained from KEYNOTE-006 and KEYNOTE-054 (pembrolizumab 2 mg/kg q3w in stage IV melanoma and resected stage III melanoma) (Eggermont et al. 2018; Robert et al. 2015a)

in underlying mechanisms of these drugs, as well as the variability in which they are used in combination, we are still learning about the different immune-related cutaneous adverse events (ircAEs) that are possible. Each drug has its own profile and likelihood of producing a certain set of ircAEs, and when used in combination, the expected frequencies can be quite different (Khoja et al. 2017).

Overarching Principles

ircAEs are the most common and earliest AEs to manifest from immunotherapy (Habre et al. 2016; Weber et al. 2012). All-grade rash and pruritus have been reported to occur in up to 62% of subjects (Table 2) (Larkin et al. 2015). However, incidences vary depending on the drug used and the type of tumor treated. In an article by Khoja et al, ircAEs were found to occur more frequently with ipilimumab than newer ICIs. Additionally, ircAEs were also more commonly seen when ICIs were used to treat melanoma versus non-small cell lung cancer (NSCLC) or renal cell carcinoma (RCC) (Khoja et al. 2017).

Given that systemic immunomodulators – in particular oral corticosteroids – have been positioned as a mainstay in the management of moderate to severe ircAEs, clinicians need to remain cognizant of the theoretical concern that systemic immunosuppression could cause unwanted blunting of the original ICI's antitumor activity. While early reports reassured oncologists that the use of systemic steroids did not affect

overall prognosis for this population (Horvat et al. 2015), recent reports have evidenced a deleterious effect of systemic corticosteroids in the efficacy of ICIs when used in high doses or during ICI initiation (Arbour et al. 2018). Specifically, systemic steroids used in high doses to treat ipilimumab-related hypophysitis in melanoma patients has been associated with worse prognosis (Faje et al. 2018), opening the possibility that a similar detrimental effect on the antitumor response may be seen with systemic steroids used in the course of ircAE management.

Ultimately, as ICI use continues to expand into new settings, the changing landscape of the safety profile begets a dynamic interpretation of AE diagnosis and management. Herein we present the established and common ircAEs seen with immune checkpoint blockade in the treatment of melanoma, with the caveat that this field is constantly changing (Table 3).

Rash

The frequently cited irAE "rash" in fact encompasses a variety of possible cutaneous presentations of reactions of immunotherapy. The most common morphologies are eczematous eruptions (Hwang et al. 2016; Min Lee et al. 2018), morbilliform eruptions (Mochel et al. 2016), and erythroderma (Coleman et al. 2018). Notably, eczematous eruptions were one of the top three ircAEs seen with blockade of PD-1 (Hwang et al. 2016). Eczematous eruptions, along with lichenoid eruptions, were associated with improved overall prognosis (Min Lee et al. 2018). Lichenoid eruptions will be discussed below.

Depending on the type of eruption, the clinical presentation can differ and may or may not be associated with pruritus (Lacouture et al. 2014). Morbilliform eruptions are 3–4 mm papules that coalesce into plaques, commonly considered the classic "exanthematous drug eruption" (Chen et al. 2017). Eczematous eruptions are described as erythematous plaques with collarettes of scale, occasionally preceded by small papules in the same distribution. Eczematous plaques can be moist and weepy if acute but can also become lichenified with accentuated skin markings if in the chronic phase. Erythroderma, by definition, involves diffuse erythema in the skin covering over 80% of the body surface area (BSA) (Chen et al. 2017). A pityriasis rubra pilaris (PRP)-like pattern has also been described (Coleman et al. 2018).

Clinicians should be cognizant of systemic symptoms or laboratory abnormalities that may accompany immunotherapy-related rash in order to early recognize severe cutaneous adverse reactions (SCARs) (see section "Pruritus").

The rash observed with ICIs resembles the maculopapular drug rash seen with antibiotics, NSAIDs, or atopic dermatitis (AD) where increased T-cell activation and cytokine secretion with subsequent inflammatory infiltrate lead to a type IV-like hypersensitivity reaction (Lacouture et al. 2014).

Typical histologic findings include a superficial and deep perivascular lymphocytic infiltrate occasionally accompanied by a prominent eosinophilic component. Eczematous eruptions would usually show spongiosis (edema) in the epidermis with overlying acanthosis and hyperkeratosis. Erythroderma can have many of the aforementioned findings but sometimes are present to a milder extent.

As with most dermatologic drug eruptions, first-line therapy includes topical corticosteroids, especially in grade 1 or 2 cutaneous eruptions (Lacouture et al. 2014; Chen et al. 2017). If necessary, Class I superpotent steroids (such as clobetasol propionate 0.05% ointment) can be trialed up to twice daily for 2 weeks. Milder eruptions can be managed with milder steroids (such as triamcinolone acetonide 0.1% ointment) twice daily monitoring for improvement after 2 weeks. Care should be taken not to overuse topical steroids given the risk of skin atrophy and striae.

Pruritus

Patients receiving immunotherapy can develop pruritus without rash (Collins et al. 2017). Despite the lack of skin findings, the pruritus can be

Table 3 CTCAE v5.0 grades of most common adverse events under immunotherapy with suggested management. Grades range from 1 to 5 with grade 1 only causing mild symptoms and grade 5 describing fatality due to adverse events. (Adapted from (Dermatologic principles and practice in oncology: conditions of the skin, hair and nails in cancer patients 2014; Lacouture et al. 2019))

CTCAE term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Rash maculopapular	Macules/papules covering <10% BSA with or without symptoms (e.g., pruritus, burning, tightness)	Macules/papules covering 10–30% BSA with or without symptoms (e.g., pruritus, burning, tightness); limiting instrumental ADL; rash covering > 30% BSA with or without mild symptoms	Macules/papules covering >30% BSA with moderate or severe symptoms; limiting self-care ADL		
	Continue ICIs at current dose; topical mild corticosteroids (hydrocortisone 2.5% cream) to face AND mid-strength (triamcinolone 0.1% cream) to body BID	Continue ICIs at current dose; topical mild corticosteroids to face AND mid-strength to body BID	Interrupt ICI treatment until severity decreases to grade 1–2; topical mild corticosteroids (hydrocortisone 2.5% cream) to face AND potent corticosteroids (fluocinonide 0.1% cream) to body BID AND prednisone 0.5 mg/kg for 10 days; if no improvement or deterioration consider dose interruption or discontinuation		
Eczema	Asymptomatic or mild symptoms; additional medical intervention over baseline not indicated	Moderate; topical or oral intervention indicated; additional medical intervention over baseline indicated	Severe or medically significant but not immediately life-threatening; IV intervention indicated		
	Continue ICIs at current dose; avoid skin irritants, OTC topical emollients	Continue ICI at current dose; same as grade 1 AND topical mild corticosteroids (hydrocortisone 2.5% cream) to face AND mid-strength corticosteroids (triamcinolone 0.1% cream) to body BID; AHs for itch	Interrupt ICI treatment until severity decreases to grade 1–2; same as grade 2 AND 0.5–1 mg/kg prednisone (or equivalent dose of methylprednisolone) for 3 days and then wean over 1–2 weeks		

Erythroderma		Erythema covering >90% BSA without associated symptoms; limiting instrumental ADL	Erythema covering >90% BSA with associated symptoms (e.g., pruritus or tenderness); limiting self-care ADL	Erythema covering >90% BSA with associated fluid or electrolyte abnormalities; ICU care or burn unit indicated	Death
		Continue ICI at current dose; topical mild corticosteroids (hydrocortisone 2.5% cream) to face AND mid-strength corticosteroids (triamcinolone 0.1% cream) to body BID; additional topical emollients 1–2×/days	Interrupt ICI treatment until severity decreases to grade 1–2; same as grade 2 AND AHs for itch AND 0.5–1 mg/kg prednisone (or equivalent dose of methylprednisolone) for 3 days and then wean over 1–2 weeks	Discontinue ICI treatment; topical therapy as grade 2–3; AHs for itch AND 1–2 mg/kg prednisone (or equivalent dose of methylprednisolone) for 3 days and then wean over 1–2 weeks; IV fluids/ICU care	
Dry skin	Covering <10% BSA and no associated erythema or pruritus	Covering 10–30% BSA and associated with erythema or pruritus; limiting instrumental ADL	Covering >30% BSA and associated with pruritus; limiting self-care ADL		
	Prophylactic/supportive treatment: use of bath oils and mild soaps; daily moisturizing creams, avoidance of extreme temperatures and direct sunlight				
	Continue ICIs at current dose; OTC emollient or ointment to face BID AND ammonium lactate 12% cream or urea 10% ointment to body BID	Continue ICIs at current dose; OTC emollient or ointment to face BID AND ammonium lactate 12% OR urea 10% ointment OR salicylic acid 6% cream to body BID	Interrupt ICI treatment until severity decreases to grade 1–2; same topicals as grade 2 AND topical mild corticosteroids (hydrocortisone 2.5% cream) to face AND mid-strength corticosteroids (triamcinolone 0.1% cream) to body BID		

(*continued*)

Table 3 (continued)

CTCAE term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Pruritus	Mild or localized; topical intervention indicated	Widespread and intermittent; skin changes from scratching (e.g., edema, papulation, excoriations, lichenification, oozing/crusts); oral intervention indicated; limiting instrumental ADL	Widespread and constant; limiting self-care ADL or sleep; systemic corticosteroid or immunosuppressive therapy indicated		
	Continue ICIs at current dose; topical doxepin AND/OR medium to high-potency corticosteroids (triamcinolone acetonide 0.025%, desonide 0.05%, fluticasone propionate 0.05%, alclometasone 0.05%)	Continue ICIs at current dose; topical treatments as grade 1, oral AHs	Consider interruption of ICI treatment/dose modification until severity decreases to grade 1–2; topical treatments as grade 1–2, oral AHs, gabapentin/pregabalin, doxepin, prednisone		
Skin hypopigmentation (e.g., vitiligo)	Hypopigmentation or depigmentation covering <10% BSA; no psychosocial impact	Hypopigmentation or depigmentation covering >10% BSA; associated psychosocial impact			
	Continue ICIs at current dose; no treatment necessary	Continue ICIs at current dose; consider psychological guidance and patient education about association with better response rate; mid- to high-potency corticosteroids can be tried BID on hypopigmented lesions			

Bullous dermatitis	Asymptomatic; blisters covering <10% BSA	Blisters covering 10–30% BSA; painful blisters; limiting instrumental ADL	Blisters covering >30% BSA; limiting self-care ADL	Blisters covering >30% BSA; associated with fluid or electrolyte abnormalities; ICU care or burn unit indicated	Death
	Continue ICIs at current dose; topical potent steroids (e.g., clobetasol propionate ointment BID); non-adherent wound covering for erosions	Continue ICIs at current dose; topical potent corticosteroids (e.g., clobetasol propionate ointment BID); non-adherent wound covering for erosions	Interrupt ICI treatment until severity decreases to grade 1–2; 1–2 mg/kg prednisone (or equivalent dose of methylprednisolone), wean over 4–8 weeks, local wound care, pain medication as needed	Discontinue ICI; treat as grade 3 AND ICU care/burn unit	
Stevens-Johnson syndrome (SJS)/ toxic epidermal necrolysis (TEN)			Skin sloughing covering <10% BSA with associated signs (e.g., erythema, purpura, epidermal detachment, and mucous membrane detachment)	SJS-TEN overlap, skin sloughing covering 10–30% BSA with associated signs (e.g., erythema, purpura, epidermal detachment, and mucous membrane detachment); TEN, skin sloughing covering ≥30% BSA with associated symptoms (e.g., erythema, purpura, or epidermal detachment)	Death
			Discontinue ICI treatment; ICU care/burn unit, IV fluids, IVIGs >2 g/kg total dose over 3–4 days, additional systemic immunosuppressants	Same as grade 3	

extremely disconcerting and bothersome, impact sleep quality, functioning, and have a great negative impact on the quality of life (QoL) (Lacouture et al. 2014). With ICIs, pruritus appears to be a direct result of enhanced unmyelinated C fiber stimulation by disinhibited T cells (Ensslin et al. 2013).

Patients can present with pruritus in association with skin findings; however, for the purposes of this section, we will focus on the pruritus without rash. It was reported that the immune-related pruritus QoL burden was greater than the one found in hemodialysis patients with chronic itch and that the QoL burden was the same (no statistically significant difference) between patients with pruritus and rash and patients with just pruritus (Phillips et al. 2018). As with most patients with chronic itch, patients often report worsening of pruritus in the evening. Patients may present with secondary changes from pruritus, including excoriated prurigo-like papules, hemorrhagic crusting at sites of self-induced trauma, and lichenified skin from constant rubbing.

Of note, the process of immunosenescence can also lead to pruritus without rash and should be considered in the geriatric population as a potential cause outside of direct effects of immunotherapy (Berger and Steinhoff 2011). As is seen in immunosenescence, it is important to consider certain organic dermatologic processes, such as a pre-bullous bullous pemphigoid, which is more likely in immunotherapy (see below).

In the case of pruritus without rash, the biopsy may show normal skin histology or may show a superficial and deep perivascular infiltrate with possible eosinophils if a subclinical allergic response is occurring. In the case of a pre-bullous bullous pemphigoid, typical findings of this autoimmune condition (subepidermal blister with positive direct immunofluorescence at the dermal-epidermal junction positive for IgG and C3) are possible.

In a first attempt to treat patients with pruritus, emollients, antihistamines (AH), and menthol containing products can be tried (Lacouture et al. 2014). Further treatment of pruritus in immunotherapy recipients may include neuromodulatory medications that may help blunt the transmission of itch stimulus to the central nervous system. Medications that have been trialed include GABA analogues (gabapentin, pregabalin) and mirtazapine (Lacouture et al. 2014). There have also been reports of the neurokinin-1 inhibitor aprepitant being useful in pruritus triggered by ICI (Ito et al. 2017).

Vitiligo

Vitiligo, the depigmentation of the skin from the autoimmune destruction of melanocytes, has been reported with use of immunotherapy in particular in patients treated for melanoma (Choi 2014; Teulings et al. 2015; Hua et al. 2016). Vitiligo has been reported in 5–25% of patients among all ICI classes, and the presence of vitiligo has been associated with an improved PFS and OS for the patient (Choi 2014; Teulings et al. 2015; Hua et al. 2016).

Although immunotherapy-related vitiligo has been rarely reported in patients with other tumor entities, the incidence is much higher in melanoma patients. It has been hypothesized that shared antigens (e.g., tyrosinase-related protein-2 [TRP-2]) on melanoma oncocytes and melanocytes may cause cross reactivity among CD8+ T cells in the context of checkpoint blockade. This proxy of immune activation might underscore the strong correlation between vitiligo and tumor response to immunotherapy (Sanlorenzo et al. 2015; Freeman-Keller et al. 2016). Histopathological examination of immunotherapy-related vitiligo shows skin lesions infiltrated with CD8+ T cells expressing C-X-C motif receptor 3, with elevated levels of INF-g and TNFa (Uenami et al. 2017; Yin et al. 2017).

Vitiligo is not associated with other symptoms, but patients may suffer from its appearance in terms of cosmesis. As a treatment, topical steroids can be trialed; however, response is highly variable. If further treatment is desired, referral to a dermatologist would be prudent (Fig. 1).

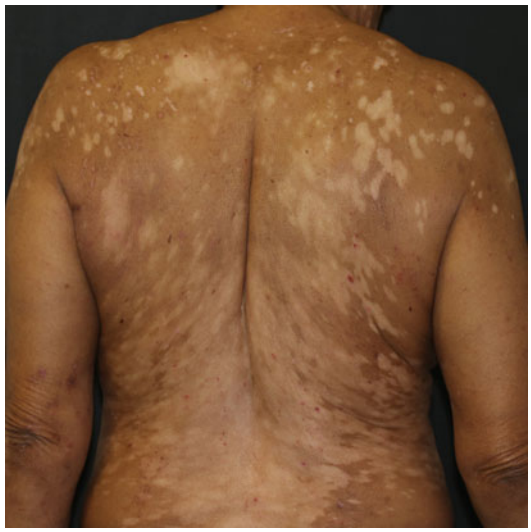

Fig. 1 Vitiligo due to immunotherapy with ipilimumab and later pembrolizumab for malignant melanoma

Lichen Planus (LP)/Lichenoid Eruption

Lichenoid, or lichen planus (LP)-like, eruptions are among the most common skin manifestations in the setting of ICI use (Min Lee et al. 2018; Sibaud 2018). Usually, these eruptions are mild and can be managed with topical steroids. There are severe cases that require systemic immunosuppression or ICI discontinuation, as is further detailed below.

The clinical appearance of LP or lichenoid eruptions is that of the purple to pink, planar (flat topped), polygonal, pruritic papules which may be associated with intense itch. Lesions are typically found on the torso and the extremities but may present in any location. In one case series, lichenoid eruption occurred on average 42 days after ICI initiation (Tetzlaff et al. 2017); however, a recent article demonstrated delayed cutaneous eruptions, sometimes even after ICIs had already been stopped (Wang et al. 2018). Some cases co-occur with a lichenoid mucositis, which has similar histologic findings when biopsied (Enomoto et al. 2018).

In severe cases, skin lesions can be hypertrophic and can mimic squamous cell carcinomas (SCCs) (Fontecilla et al. 2018), especially if biopsy is too superficial. Cases of lichenoid eruptions with suprabasilar clefting mimicking a clinical appearance of paraneoplastic pemphigus have also been reported (Chou et al. 2017).

Mucocutaneous lesions characteristically have a lichenoid interface dermatitis: a dense band-like infiltrate of lymphocytes in the superficial dermis. Hyperkeratosis, hypergranulosis, dyskeratosis, and saw-tooth rete ridge pattern are frequently seen, occasionally accompanied by eosinophils (Tetzlaff et al. 2017) (Enomoto et al. 2018). Lichenoid rash in patients treated with ICIs is very similar to idiopathic LP, except for a slightly increased abundance of CD163-positive cells indicating a macrophage-monocyte lineage (Lacouture and Sibaud 2018).

Grade 1 and 2 lichenoid eruptions are usually treated with topical corticosteroids. However, systemic corticosteroids may be required for grade 3 and 4 eruptions, especially those that show hypertrophic or vesiculobullous variants or present with mucositis. In severe cases, ICI discontinuation may be required after discussion between the oncologist, the dermatologist, and the patient.

Bullous Pemphigoid (BP)-Like Reaction

Bullous pemphigoid (BP) is an autoimmune blistering disorder reported with all ICI classes but most commonly with PD-1 and PD-L1 inhibitors (Naidoo et al. 2016; Jour et al. 2016; Hanley et al. 2018). One case series from Yale estimated BP to occur in approximately one percent of patients treated with PD-1 and PD-L1 inhibitors (Siegel et al. 2018). Since vast bullous disease with multiple erosions compromises the QoL of patients and results in life-threatening conditions such as infections and sepsis, ICI discontinuation is often advised.

BP may present with intense pruritus that evolves into eczematous plaques or urticaria that eventually form tense, fluid-filled vesicles and bullae, with negative Nikolsky sign. With time, vesicles and bullae may become purulent and flaccid with rupture (Chen et al. 2017). Mucosal lesions can occur in a minority of cases. Laboratory studies may reveal an eosinophilia and positive serum BP 120/180/BPAGs.

Although ircAE mechanisms are primarily thought to involve T-cell hyperactivation, primary autoimmune bullous pemphigoid is considered a largely B-cell-mediated process, whereby autoantibodies cause disruption of hemidesmosomes in the basal layer of the epidermis; hence, it has been hypothesized that B cells may be implicated in ircAEs such as immunotherapy-related BP (Chen et al. 2017).

On hematoxylin and eosin stain (H&E), a split is seen in the subepidermal space with a spongiotic eosinophilic infiltrate. Direct immunofluorescence shows linear deposition of IgG and C3 at the dermo-epidermal junction (DEJ).

Mild cases of BP can be managed with potent topical steroids, which has demonstrated comparable efficacy to systemic steroids with a milder side effect profile (Joly et al. 2002). However, in our experience, many of our patients who develop BP from ICI have a more severe course which requires systemic agents. In the Yale series, all seven BP patients required systemic corticosteroids and interruption in ICI use (Siegel et al. 2018). Steroid-sparing immunomodulatory agents used in the course of BP include intravenous immunoglobulin (IVIG), dapsone, doxycycline/niacinamide, mycophenolate mofetil (MMF), cyclosporin A, and methotrexate (MTX); however, these agents have not been assessed prospectively for efficacy and safety in immunotherapy-related BP. Given its progressive course and impact on QoL and ICI administration, consultation with dermatology for bullous reactions to immunotherapy is advised (Fig. 2).

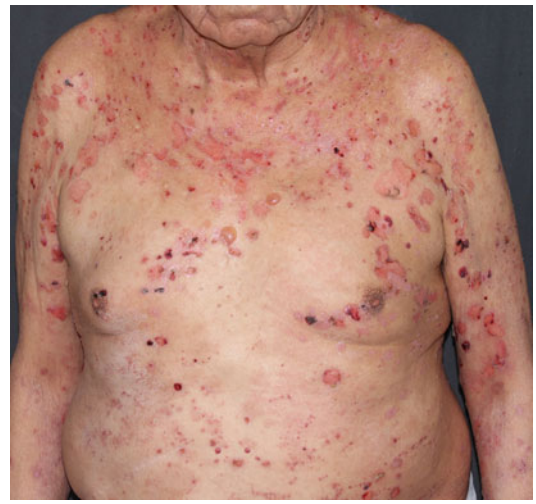

Fig. 2 Bullous pemphigoid in patient on nivolumab therapy showing intact, tense bullae and ruptured bullae with multiple erosions

Severe Cutaneous Adverse Reactions (SCARs)

Although rare (<1%), immunotherapy can induce severe cutaneous adverse reactions (SCARs) which are potentially life-threatening. SCARs comprise drug rash with eosinophilia with systemic symptoms (DRESS), Stevens-Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN) and may initially present as a less concerning morphology like a maculopapular rash (Chen et al. 2017). SJS/TEN and DRESS have been reported in association with all classes of ICIs but are still rare in frequency (Chirasuthat and Chayavichitsilp 2018; Hwang et al. 2018; Nayar et al. 2016; Dika et al. 2017). SCARs may have a latency of several weeks between drug exposure and clinical presentation, taking up to several weeks.

SJS and TEN typically show multiple targetoid eruptions. The lesions usually involve acral surfaces including palms and soles and classically present with a severe mucositis. Nikolsky sign may be positive (rubbing the skin next to a blister or erosion causes an additional blister to form). Patients often complain of an ocular foreign body sensation, dysuria, dysphagia, and odynophagia given the mucosal involvement commonly seen. Body surface area (BSA) involvement dictates diagnosis: SJS is diagnosed when there is less than 10% BSA skin detachment, TEN requires over 30% BSA detachment, and 10–30% BSA involvement is termed SJS/TEN overlap syndrome. In SJS/DRESS clinical and histopathological findings are essential to make the diagnosis. Yet, elevation of serum urea, creatinine, and glucose levels, as well as neutropenia, lymphopenia, and thrombocytopenia have been linked to a poor outcome. In DRESS, laboratory testing may reveal eosinophilia, transaminitis, atypical lymphocytes, and cytopenias. Vital

signs could show hypotension, fever, or other aberrations and patients are usually quite ill (Chen et al. 2017).

The pathophysiology of SJS and TEN remains under debate. Hypothesized mechanisms include fatty acid synthetase (Fas) and Fas Ligand interactions leading to caspase activation and keratinocyte apoptosis (Chen et al. 2017). A systematic histologic analysis of a small series of patients showed signs of cytotoxic lesions characterized by an accumulation of CD8+ T cells at the DEJ and CD8+ T-cell exocytosis into the epidermis with apoptotic keratinocytes. Similar features can also be observed in severe immune-mediated skin diseases, such as acute GVHD. Gene expression analysis of lesional skin from anti-PD-1-treated patients revealed a gene expression profile resembling SJS/TEN with an upregulation of major inflammatory chemokines and cytotoxic mediators as well as the pro-apoptotic molecule FASLG. These data suggest that, at least in some patients, anti-PD-1 antibody can induce SJS/TEN-like adverse cutaneous reactions (Goldinger et al. 2016). In the case of DRESS, there is some evidence implicating HHV-6 in the aberrant systemic inflammatory response (Chen et al. 2017).

Histopathological findings in SJS and TEN show full-thickness epidermal necrosis with prominent interface dermatitis and necrotic/apoptotic keratinocytes. SJS/TEN is thought to represent a pauci-inflammatory process; however, there are many cases of SJS and TEN that show an inflammatory infiltrate of lymphocytes. The histopathological findings in DRESS are similar to eczematous eruptions, and as such, clinical findings are critical to diagnosis.

Universally, SCARs require management that involves systemic immunosuppression and ICI discontinuation, given the high risk of morbidity and mortality associated with these reactions (Chen et al. 2017). In classic cases of SJS and TEN, systemic steroids were sometimes avoided given the increased risk of infection with systemic immunosuppression. Instead, the use of steroid sparing agents has been attempted, including the use of cyclosporine, etanercept, thalidomide, IVIG, and only supportive care (Chen et al. 2017). There have been no convincing studies to support one treatment over another. However, with ICI as the inciting agent, there may be more of a pathophysiologic basis for warranting use of systemic corticosteroids. While there are no prospective, randomized studies to suggest treatment superiority, prove efficacy, or determine optimal dosing, typically 0.5–1 mg/kg/day of prednisone is a standard starting dose. Dermatologic and ophthalmologic consultations are highly advised, with consideration of gynecologic consultation (for female patients) or urologic consultation (for male patients). ICI should be permanently discontinued without possibility of ICI retrial after SCAR resolution.

Targeted Therapy-induced Cutaneous Adverse Events

The mitogen-activated protein kinase (MAPK) pathway is one of the most frequently deregulated pathways in melanoma explaining the profound efficacy of BRAF and MEK inhibition in the treatment of advanced disease with overall response rates of BRAFi/MEKi combinations of around 70% and improved PFS and OS (Long et al. 2015; Ascierto et al. 2016; Dummer et al. 2018a). BRAF mutations are found in about 50% of all melanomas (Curtin et al. 2005). Combined inhibition of BRAF and MEK in the RAS-BRAF-MEK-ERK pathway with multi-agent therapy has a reduced side effect profile and less potential for development of tumor resistance to treatment as compared to BRAF inhibition alone (Long et al. 2017a). Yet, mutations in one of the mediators along the pathway often lead to resistance against the antiproliferative medication or result in aberrant keratinocytic proliferation as one of the major side effects of targeted therapies. Thus, PFS tends to drop to about 50% after 12 months and 20–30% after 3 years of BRAFi/MEKi therapy (Ascierto et al. 2016; Dummer et al. 2018a; Long et al. 2017a).

The skin is the organ most frequently affected by AEs during targeted therapy for advanced melanoma. Cutaneous AEs were registered in over 95% of patients treated with BRAFi and

over 90% of those treated with MEKi monotherapy. Whereas some of the AEs such as keratinocytic proliferations can be seen with all BRAFi agents, others are specific to one drug class such as photosensitivity for vemurafenib or pyrexia for dabrafenib (Belum et al. 2013). Since the introduction of combination therapies of BRAFi/MEKi, a significant reduction of AEs was achieved (Robert et al. 2015b; Keating 2016). Most reported AEs with BRAFi/MEKi occur within the first 3 months after therapy initiation and tend to be grade 1 or 2 without the need for dose interruption or discontinuation.

Medication combinations approved by the FDA for the treatment of advanced melanoma comprise vemurafenib plus cobimetinib, dabrafenib plus trametinib, and most recently, encorafenib plus binimetinib (Dummer et al. 2018b).

Additional targeted therapies such as indoleamine-pyrrole 2,3-dioxygenase (IDO) and histone deacetylase (HDAC) inhibitors in combination with checkpoint inhibitors are still under investigation and are not yet established in routine melanoma regimens. In patients with tumors harboring a c-Kit mutation more individualized therapeutic approaches such as the multi-kinase inhibitor imatinib may be considered (Guo et al. 2011). While these agents have their own distinct sets of AEs, the following paragraph focuses on the cutaneous AEs of targeted therapies approved for stage IV melanoma by the FDA, e.g., BRAFi/MEKi (Table 4).

Rash

The major phase III studies of BRAFi/MEKi therapy for treatment of metastatic melanoma did not distinguish between maculopapular, eczematous, erythematous, and other exanthematous skin manifestations, instead referring to them as "rash" (Ascierto et al. 2016; Long et al. 2017a). Rashes were reported in up to 70% of both, patients receiving BRAFi and the BRAFi/MEKi combination, throughout the first few weeks of therapy (Ascierto et al. 2016; Flaherty et al. 2012b). Thus, rashes are one of the most frequent and earliest cutaneous AEs from targeted therapies. Skin eruptions may start in the face or trunk and disseminate to the rest of the body. Erythematous, maculopapular, and papulopustular presentations have been described with acneiform eruptions being more frequent under MEKi therapy (see below) (Long et al. 2015; Flaherty et al. 2012b). Maculopapular rash was specifically reported in 30% of patients receiving dabrafenib, 4–21% of patients receiving vemurafenib, and in 10% of patients receiving encorafenib (Lacouture and Sibaud 2018; Chapman et al. 2011). The severity of the exanthem appears to be dose-dependent (Amitay-Laish et al. 2011). In rare cases the occurrence of very severe skin reactions such as a Stevens-Johnson syndrome (SJS), drug reaction with eosinophilia and systemic symptoms (DRESS) or toxic epidermal necrolysis (TEN) necessitates systemic corticosteroids and the permanent discontinuation of the responsible agent (Yorio et al. 2014; Minor et al. 2012; Wenk et al. 2013).

Usually within the first weeks to months after the start of therapy, eruptive folliculocentric papules on the torso and extremities may emerge and coalesce into broad morbilliform erythematous plaques.

Exanthematous drug eruptions have been described for numerous drugs as cell-mediated hypersensitivity reactions. The sensitization to and presentation of the drug antigen to T lymphocytes lead to a cytotoxic reaction with inflammation and destruction of cutaneous keratinocytes.

Histopathology shows features of exanthematous drug eruption with perivascular and interstitial lymphocytic infiltrate, interface changes, and possibly some eosinophils.

Emollients and observation may be sufficient for grade 1 eruptions; grade 2 and 3 eruptions may require antihistamines and mild to medium strength topical corticosteroids. In severe cases, a short course of systemic corticosteroid and interruption of treatment may be necessary. Rare, grade 4 eruptions demand additional hospital admission, administration of i.v. fluids, and drug discontinuation.

Table 4 Skin toxicity data of FDA-approved targeted therapies (BRAF/MEKi monotherapies and combination) for advanced melanoma. Adapted from (Long et al. 2015; Ascierto et al. 2016; Dummer et al. 2018a; Long et al. 2017b; Flaherty et al. 2012a)

	Vemurafenib (Columbus)		Dabrafenib (Combi-d)		Encorafenib (Columbus)		Trametinib (METRIC)		Dabrafenib/ trametinib (COMBI-d/ COMBI-AD*)		Vemurafenib/ cobimetinib (CoBRIM)		Encorafenib/ binimetinib (Columbus)	
	960 mg BID		150 mg BID		300 mg 1×/day		2 mg 1×/day		150 mg BID plus 2 mg 1×/day		960 mg BID plus 60 mg 1×/day over 21 days		450 mg 1×/day plus 45 mg BID	
	(*N*=186)		(*N*=211)		(*N*=192)		(*N=221*)		(*N*=209/*644)		(*N*=247)		(*N*=192)	
Severity grade	All grades	Grade ≥3	All grades	Grade ≥3	All grades	Grade ≥3	All grades	Grade ≥3	All grades	Grade ≥3	All grades	Grade ≥3	All grades	Grade ≥3
Dry skin	23%	0%	9%	0%	30%	0%	N/A	N/A	13%	0%	N/A	N/A	14%	0
Hyperkeratosis	29%	0%	6%	0%	38%	4%	0%	0%	33%	<1%	10%	<1%	14%	1%
Rash	29%	3%	24%	0%	21%	2%	55%	7%	23%	<1%	72%	17%	14%	1%
Alopecia	37%	0%	5%	0%	56%	0%	16%	<1%	26%	0%	17%	<1%	14%	0
Pruritus	11%	0%	7%	0%	22%	1%	N/A	N/A	11%	0%	N/A	N/A	11%	1%
Hand-foot syndr.	14%	1%	6%	<1%	51%	14%	N/A	N/A	27%	<1%	N/A	N/A	7%	0
Skin papilloma	17%	0%	1%	0%	9%	0%	0%	0%	19%	0%	N/A	N/A	6%	0
Photosensitivity	24%	1%	N/A	N/A	4%	0%	N/A	N/A	N/A	N/A	N/A	N/A	4%	1%
Acneiform rash	N/A	N/A	8%	0%	N/A	N/A	18%	<1%	9%	<1%	N/A	N/A	N/A	N/A
CuSCC or KA	28%	20%	3%	3%	14%	8%	0%	0%	4%	4%	6%	5%	5%	3%

CuSCC cutaneous squamous cell carcinomas, *KA* keratoacanthoma, * adjuvant data

Folliculitis and Acneiform Rash

Acneiform rash is the most frequent AE seen with MEKi monotherapy (77%) but also occurs in about 10% of patients treated with BRAFi monotherapy (Balagula et al. 2011; Kim et al. 2013). Its appearance resembles the acneiform eruptions seen with epidermal growth factor inhibitors (EGFRi), most often seen 1–4 weeks after the start of therapy.

Papules, pustules, and inflammatory erythema with possibly cyst formation are observed predominantly on the seborrheic areas, e.g., face, chest, upper back, and shoulders (Balagula et al. 2011). The eruptions may be accompanied by extreme pruritus and even skin pain. Notably, there are no comedones. About a third of patients are reported to develop secondary infections at the sites of acneiform eruptions.

While the exact pathomechanism remains poorly understood, it is not surprising that MEKis show similar cutaneous side effects as EGFRi as in both substance classes the MAP kinase pathway is inhibited. It has been shown that EGFRi-induced chemokine production in epidermal keratinocytes affects the inhibition of *Staphylococcus aureus* colony formation. Furthermore, cutaneous inflammation, immunomodulatory effects, neutrophil accumulation, epidermal keratinocyte proliferation, and erosion of the stratum corneum appear to contribute to the acneiform morphology (Lacouture and Sibaud 2018). More precisely, the release of pro-inflammatory cytokines may lead to recruitment of leukocytes with subsequent tissue damage and apoptosis, hence forming the inflammatory papules and pustules seen in papulopustular eruptions. Immunohistochemical analyses of the acute phase of acneiform eruptions show apoptotic keratinocytes with increased p53 expression and focal perifollicular inflammatory infiltrates of mostly neutrophils and sparse lymphocytes (Schad et al. 2010).

In a Phase II study of EGFRi-treated patients, the prophylactic use of systemic doxycycline, topical corticosteroids, sunscreen, and moisturizer led to a fivefold reduction of the incidence of dermatological toxicities (Lacouture et al. 2010). Reactive therapy approaches utilized for EGFRi-induced rashes are also useful in the management of MEKi-induced cutaneous AEs including mild syndets, topical steroids, topical antibiotics, emollients, and systemic tetracyclines such as doxycycline. Retinoids are preserved for treatment-resistant cases in this setting. Interruption of culprit medications often leads to rapid improvement in cutaneous AEs (Fig. 3).

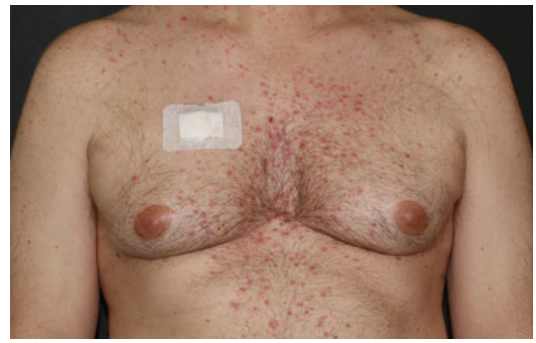

Fig. 3 Acneiform rash induced by the MEKi trametinib

Paronychia

Paronychia, an acute or chronic inflammation of the nail fold characterized by painful edema and erythema, is a nail toxicity mostly seen with MEKis but may also appear with BRAFis in rare cases. The incidence is estimated at a slightly lower rate as in EGFRi (17.8%) but was never reconfirmed in a prospective trial (Robert et al. 2015c).

In the chronic form, a periungual pyogenic granuloma in the form of a hyperemic friable papule may evolve from unremitting nail fold inflammation. Impaired function and restrictions in ADLs are not uncommon especially with hand nail involvement.

The exact pathomechanism underlying the development of paronychia in targeted therapeutics is unknown. MAP kinase pathway inhibition may lead to changes in differentiation and migration of epidermal cells, compromising the skin barrier, which may subsequently lead to piercing of the paronychium by the lateral edges of the nail plate resulting in a foreign body reaction (Robert et al. 2015c). The pyogenic granuloma form evolves from a pre-existing injury or

irritation, limited capacity for growth, and reactive neovascularization.

Histologically, paronychia findings are nonspecific and concordant with reactive inflammation. Pyogenic granulomas are characterized by a proliferation of small capillaries, often arranged in a lobular pattern, with thick, intervening bands of fibrous tissue.

Patients should be educated about appropriate nail care and apply emollients to protect nail folds. In mild cases of paronychia, the topical application of 2% povidone-iodine has shown efficacy in 76% of patients on MEKi over the course of a 6-week treatment (Capriotti et al. 2017). In addition, topical formulations combining glucocorticoids and fusidinic acid have proven more efficacious than topical antibiotics alone (Wollina 2001). If pyogenic granuloma appears, ablative procedures or surgery can increase the rate of healing. Superinfection may warrant the use of systemic antibiotics (Fig. 4).

Hair Changes

Alopecia has been reported in up to a quarter of patients receiving BRAFi monotherapy for advanced melanoma with a lower incidence of 6–13% with the use of the combination of BRAFi and MEKi (Lacouture and Sibaud 2018). Usually, the hair changes represent a non-scarring diffuse alopecia with possible hair shaft changes including increased curling or altered hair color (Anforth et al. 2013). The extent of hair loss is mild and fully reversible after the medication is discontinued.

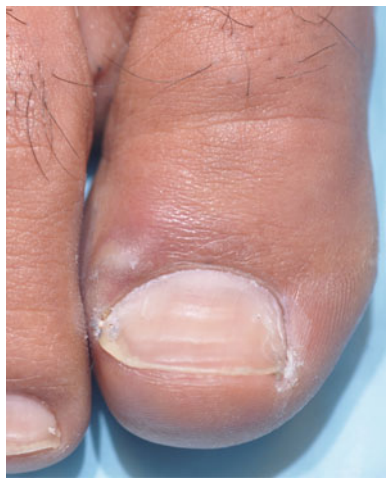

Fig. 4 Paronychia induced by the MEKi trametinib

The mechanism leading to follicular changes is unknown. Similar to nail changes, the perturbation of the MAP kinase pathway and subsequent disturbances in proliferation and differentiation of keratinic cells in the epithelium may explain the follicular changes seen in BRAFi and MEKi.

Application of topical minoxidil may improve impaired hair growth if alopecia is bothersome to the patient.

Epidermal Tumors

BRAFi therapy targets the mutations V600E and V600K in melanoma cells and leads to cell cycle arrest by interrupting the mitogen-activated protein kinase (MAPK) pathway. Paradoxically, BRAFi can lead to a mild activation of the MAP kinase signal pathway in normal body cells, supporting the growth of mucocutaneous tumors. Keratinocytic lesions appear in over 50% of patients under BRAFi monotherapy and range from benign verrucal keratoses to neoplasms of malignant potential such as actinic keratoses, keratoacanthomas, and squamous cell carcinomas (SCCs) (Lacouture et al. 2013). Keratinocytic verrucal hyperproliferations are the most frequent BRAFi-induced cutaneous AEs with 29–50% of patients affected under BRAFi monotherapy (Anforth et al. 2015). SCC are reported to develop in up to 12% under BRAFi monotherapy. Of concern, new melanomas lacking a BRAF mutation may emerge from preexistent melanocytic nevi (see below) (Su et al. 2012; Zimmer et al. 2012a).

New lesions usually develop within the first 2–3 months of therapy and can be appreciated anywhere on the body including the oral mucosa. The clinical picture resembles that of other epitheliomas in non-BRAFi-exposed patients (Lacouture and Sibaud 2018). Histopathologically, 80% of verrucous lesions were found to

have slight to moderate atypia and KRAS and HRAS mutations, which have also been found in association with BRAFi-induced SCCs (Anforth et al. 2015). Transformation from verrucous lesions into malignant neoplasms has not been reported. SCCs occurring under BRAFi tend to be clinically well-defined and histologically well-differentiated (Anforth et al. 2013).

MEKi is efficacious in downregulating the MAPK pathway not only in BRAF-mutated cells but also wt melanoma cells. Therefore, the rate of skin tumors has drastically decreased since the regular use of BRAFi plus MEKi combination therapy (Ascierto et al. 2016; Long et al. 2017a). Monotherapy with dabrafenib leads to SCC in 19%, hyperkeratoses in 30%, and papillomas in 15% of patients. Meanwhile the combination with trametinib leads to relatively lower rates of 7%, 9%, and 4%, respectively. Whereas the BRAFi molecule causes hetero- and homodimerization of RAF isotypes promoting the paradoxical activation of the MAPK pathway in RAS-mutated BRAF wt cells, the MEKi is able to interrupt this cascade (Robert et al. 2015b).

The most frequent manifestations are benign verrucous keratoses, and also SCCs are usually well-differentiated (Chu et al. 2012). Excision is the therapy of choice for newly discovered tumors if suspicious for malignancy. Regular whole-body skin exams every 4–6 weeks should be conducted as part of the follow-ups (Fig. 5).

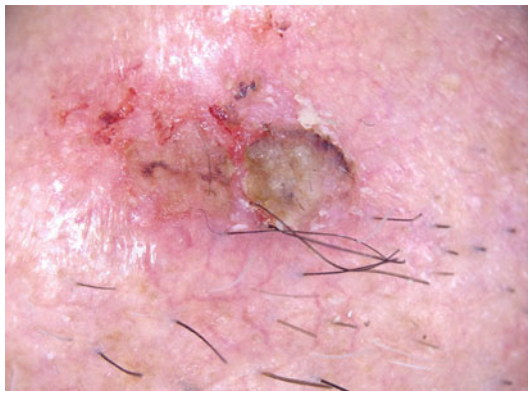

Fig. 5 Squamous cell carcinoma in patient receiving the BRAFi vemurafenib

Melanocytic Proliferations and New Primary Melanomas

Similar to keratinocytic proliferation, albeit more seldomly, paradoxical MAPK pathway activation from BRAFi in BRAF wild-type cells can lead to increased melanocytic proliferation. The development of new melanocytic nevi as well as changes in shape and color of pre-existing lesions have been described. About 50% of pre-existing pigmented lesions were found to demonstrate changes after initiation of treatment with vemurafenib (Lacouture and Sibaud 2018). However, only 1.2% of these altered melanocytic lesions transform into actual melanomas (Lacouture and Sibaud 2018). The incidence of new primary melanomas in BRAFi-treated patients (approximately 2.5%) is equal to the incidence of secondary melanomas in non-BRAFi-treated melanoma patients. New primary melanomas are mostly seen within the first few months of therapy with greater frequency in sun-exposed regions (Zimmer et al. 2012a). The treatment of therapy-induced melanomas does not differ from sporadic cases.

Hand-Foot Reaction (Hyperkeratosis)

Hand-foot reaction, affecting up to 30% of patients under BRAFi monotherapy, appears exclusively in mechanically used areas of the skin exposed to friction or pressure, such as the hands and feet (Lacouture and Sibaud 2018). With the addition of MEKi, the incidence has decreased to 6–10% (Long et al. 2015; Ascierto et al. 2016).

Typically, several weeks after the initiation of therapy, hyperkeratotic areas can form in areas prone to friction such as the palms and soles. There may be mild inflammation with some erythema, which can lead to pain and discomfort in the affected areas. Dysesthesia has also been reported (Anforth et al. 2013).

Similar to epidermal tumor formation, BRAF/MEK inhibition can result in a paradoxical activation of the MAPK pathway by mutated

RAS activation. Augmented proliferation of keratinocytes ensues, resulting in palmoplantar hyperkeratosis, follicular hyperkeratosis, milia, and follicular keratosis, some of which may progress to keratinocytic tumors.

Histopathology shows necrosis of keratinocytes with perivascular inflammatory infiltrates.

Prophylactic treatment with emollients as well as avoidance of pressure and mechanical stress by means such as spacious footwear or cushioned gloves is recommended. Ointments containing salicylic acid or urea can help reduce existing hyperkeratosis. If inflammation and pain are prominent, medium strength topical steroids and keratolytic topicals such as urea 10–40% ointment may be considered. Fissures can be addressed by local therapy, e.g., tissue glue (dermabond) or topical silver nitrate. Otherwise, dose reduction or interruption of the responsible therapy may be necessary for severe or unremitting cases.

Photosensitivity

Photosensitivity almost exclusively occurs with the use of vemurafenib (all grades, 41%; grade 3–4, 4%) (Dummer et al. 2012) as photosensitivity reactions are seldomly observed in patients under dabrafenib (1–3%) and encorafenib (4%) therapies (Hauschild et al. 2012; Dummer et al. 2018c). Even minor doses of UVA transmitted through window glass can initiate an inflammatory reaction of the skin (Chu et al. 2012). In fact, a lower threshold for UVA tolerance and normal UVB tolerance have been demonstrated in patients with vemurafenib through minimal erythema dose testing (Dummer et al. 2012). It is one of the earliest AEs – usually noticed within the first two weeks after initiation of therapy – and it is fully reversible after therapy cessation.

Patients report a burning sensation and redness without recalling direct sun exposure. Grade 3 photosensitive dermatitis can present with blistering and painful erythema. The onset of the inflammatory reaction may only take minutes after UVA exposure, which differs from classic drug-induced phototoxicity where the skin reaction takes several hours to develop.

It is unclear how the chemical structure of vemurafenib leads to the difference in photosensitivity as compared to the other BRAFis. A protoporphyrin and niacin-dependent mechanism has been proposed where delta-aminolevulinate synthase 2 (ALAS2) activation by vemurafenib catalyzes heme biosynthesis, leading to an increase in erythrocyte porphyrin. At the same time, vemurafenib has been observed to decrease vitamin B3 (niacin) which interferes with the tryptophan-kynurenine-niacin pathway (Gelot et al. 2013).

Histologically, necrotic keratinocytes, edema, a sparse dermal lymphocytic infiltrate, and vasodilation can be appreciated.

Strict sun protection is mandatory in patients receiving vemurafenib. Patients should avoid the sun, wear photoprotective clothing, and use broad-spectrum sunscreen with potent UVA-blocking features on a daily basis even under cloudy weather conditions. UVA-blocking agents include titanium dioxide, zinc oxide, ecamsule, and avobenzone (Macdonald et al. 2015). It is important to educate patients about the penetration of UVA radiation through window glass. UV-blocking window films can be applied to car, home, and office windows for further protection. Serum vitamin D levels should be monitored on a regular basis in these patients.

Radiation Sensitivity and Radiation Recall Dermatitis

BRAFi can lead to more pronounced inflammatory reactions in concurrently irradiated areas as well as induce a dermatitis in areas previously exposed to radiation therapy (Boussemart et al. 2013).

Radiation sensitivity or radiation recall is characterized by a well-demarcated erythematous area with edema, dry or moist desquamation, and bullae in severe cases, restricted to an area of (prior) irradiation. Skin symptoms can include burning or itching.

The mechanism underlying the increased sensitivity to irradiation is unknown. In chemotherapy-related radio-sensitization, theories implicate vascular damage during antineoplastic therapy with proliferative changes, depletion of epithelial stem cells, sensitization of the epithelial stem cells, or a drug hypersensitivity reaction (Camidge and Price 2001).

Radiation dermatitis shows ballooning degeneration of epidermal keratinocytes, arterioles obstructed by fibrin thrombi, mixed inflammatory infiltrate, and prominent edema.

In order to prevent radiation sensitivity and radiation recall dermatitis, the BRAFi may be paused 5–7 days prior to radiotherapy. If this is not feasible, regular skin checks should be performed every 1–2 days throughout the course of radiotherapy. In the event of radiodermatitis, the interruption of BRAFi therapy is recommended. Topical corticosteroids can be used to hasten reconstitution of the skin. If persistent radiation recall dermatitis poses a problem, a dose reduction of the BRAFi may be necessary.

Panniculitis

Although a very rare AE, neutrophilic panniculitis has been described in both BRAFi- and MEKi-treated patients, especially on the extensor areas of the extremities in female patients receiving targeted therapy.

Subcutaneously palpable, erythematous to purplish nodules appear in the extensor areas of arms and legs that may be tender to palpation. Sometimes arthralgia and concomitant edema can affect the extremity's range of motion. The nodules may be intermittent and improve without any adjustments to the underlying agent.

Although the pathomechanism causing this condition remains unknown, the symptoms could be linked to a systemic inflammatory reaction to the drug, to the melanoma (i.e., a paraneoplastic panniculitis), or to a dysregulation of neutrophil migration (Vazquez-Osorio et al. 2016).

Histologically, a lobular panniculitis with neutrophilic infiltrate and non-caseating granulomas within fat lobules can be appreciated with possible small vessel perivascular inflammation. Similarities to erythema nodosum, sweet syndrome, or granulomatous inflammatory dermatoses have been described (Zimmer et al. 2012b; Park et al. 2014; Pattanaprichakul et al. 2014).

The therapy of targeted therapy-induced panniculitis comprises a similar approach as in the case of autoimmune panniculitis. Anti-inflammatory treatment with systemic COX-2 inhibitors or nonsteroidal anti-inflammatory drugs (NSAIDs) is recommended. For persisting cases, systemic corticosteroids and cessation of the causative agent may be considered (Fig. 6 and Table 5).

Chemotherapy-Induced Cutaneous Adverse Events

Traditional cytotoxic chemotherapy plays a relatively minor role reserved for unique cases in the first-line setting of advanced melanoma therapy since the emergence of targeted and immunotherapy. RR for all chemotherapies regardless of the agent used have shown rates between 10% and 20%; furthermore, no OS benefit has ever been demonstrated with any chemotherapy regimen throughout their vast historical use for metastatic melanoma (Pasquali et al. 2018; Crosby et al. 2000). However, chemotherapy, especially the alkylating agent dacarbazine, clearly holds an important role in the palliative setting (Luke and Schwartz 2013). Other chemotherapeutic agents that have been used in advanced melanoma and

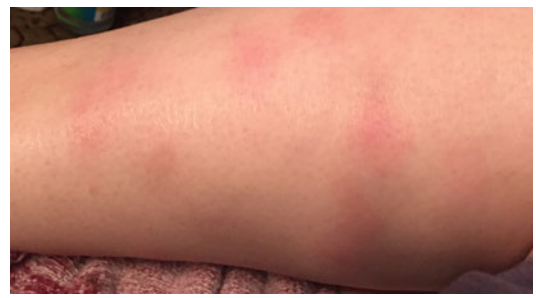

Fig. 6 Vemurafenib-/trametinib-induced drug-induced panniculitis on the lower leg

Table 5 CTCAE v5.0 grading of the most common AEs under targeted therapy for advanced melanoma with corresponding recommended management. (Adapted from (Dermatologic principles and practice in oncology: conditions of the skin, hair and nails in cancer patients 2014; Lacouture et al. 2019)

CTCAE term	Grade 1	Grade 2	Grade 3	Grade 4	Grade5
Rash	Macules/papules covering <10% BSA with or without symptoms (e.g., pruritus, burning, tightness)	Macules/papules covering 10–30% BSA with or without symptoms (e.g., pruritus, burning, tightness); limiting instrumental ADL; rash covering >30% BSA with or without mild symptoms	Macules/papules covering >30% BSA with moderate or severe symptoms; limiting self-care ADL		
	Prophylaxis with moisturizing creams; gentle skin care instructions given				
	Continue BRAFi/MEKi at current dose; topical mild corticosteroids (hydrocortisone 2.5% cream) to face AND mid-strength (triamcinolone 0.1% cream) to body BID	Continue BRAFi/MEKi at current dose; topical mild corticosteroids to face AND mid-strength to body BID	Interrupt BRAFi/MEKi treatment until severity decreases to grade 1–2; topical mild corticosteroids (hydrocortisone 2.5% cream) to face AND potent corticosteroids (fluocinonide 0.1% cream) to body BID AND prednisone 0.5 mg/kg for 10 days; if no improvement or deterioration consider dose reduction or discontinuation		
Hand-foot reaction (hyperkeratosis)	Minimal skin changes or dermatitis (e.g., erythema, edema, or hyperkeratosis) without pain	Skin changes (e.g., peeling, blisters, bleeding, edema, or hyperkeratosis) with pain; limiting instrumental ADL	Severe skin changes (e.g., peeling, blisters, bleeding, edema, or hyperkeratosis) with pain; limiting self-care ADL		
	Prophylaxis with ammonium lactate 12% cream BID OR heavy moisturizer (e.g., Vaseline) BID; orthopedic shoe inserts				
	Continue BRAFi/MEKi at current dose; keratolytic topicals (e.g., salicylic acid 6% or urea 10–40% or ammonium lactate 12%), potent topical corticosteroid (clobetasol propionate 0.05%)	Continue BRAFi/MEKi at current dose; keratolytic topicals (e.g., salicylic acid 6% or urea 10–40% or ammonium lactate 12%), potent topical corticosteroid (clobetasol propionate 0.05%), topical analgesics (e.g., lidocaine 3%, EMLA, diclofenac gel)	Interrupt BRAFi/MEKi treatment until severity decreases to grade 1–2; potent topical corticosteroid (clobetasol propionate 0.05%), topical analgesics (e.g., lidocaine 3%, EMLA, diclofenac gel); systemic analgesics (NSAIDs, GABA agonists, opioids as needed); if no improvement or deterioration, consider dose reduction or discontinuation		

(*continued*)

Table 5 (continued)

CTCAE term	Grade 1	Grade 2	Grade 3	Grade 4	Grade5
Paronychia	Nail fold edema or erythema; disruption of the cuticle	Local intervention indicated; oral intervention indicated (e.g., antibiotic, antifungal, antiviral); nail fold edema or erythema with pain; associated with discharge or nail plate separation; limiting instrumental ADL	Operative intervention indicated; IV antibiotics indicated; limiting self-care ADL		
	Prophylaxis with moisturizing creams; manicure/pedicure; gentle skin care; avoid traumatization of nail structure				
	Continue BRAFi/MEKi at current dose; topical 2% povidone-iodine or hydrocortisone plus topical fusidinic acid	Continue BRAFi/MEKi at current dose; topical 2% povidone-iodine or hydrocortisone plus topical fusidinic acid cream, silver nitrate application on pyogenic granuloma weekly	Interrupt BRAFi/MEKi treatment until severity decreases to grade 1–2; surgery or laser surgery; consider dose reduction or discontinuation if no improvement or deterioration		
Rash acneiform/ folliculitis	Papules and/or pustules covering <10% BSA, which may or may not be associated with symptoms of pruritus or tenderness	Papules and/or pustules covering 10–30% BSA, which may or may not be associated with symptoms of pruritus or tenderness; associated with psychosocial impact; limiting instrumental ADL; papules and/or pustules covering > 30% BSA with or without mild symptoms	Papules and/or pustules covering >30% BSA with moderate or severe symptoms; limiting self-care ADL; associated with local superinfection with oral antibiotics indicated	Life-threatening consequences; papules and/or pustules covering any % BSA, which may or may not be associated with symptoms of pruritus or tenderness and are associated with extensive superinfection with IV antibiotics indicated	Death
	Prophylactic therapy: sunscreen (SPF ≥30), hydrocortisone 2.5% cream AND clindamycin 1% gel or dapsone 5% gel AND doxycycline 100 mg BID or minocycline 100 mg QD for the first 6–8 weeks				
	Continue BRAFi/MEKi at current dose; hydrocortisone 2.5% cream AND clindamycin 1% gel or dapsone 5% gel	Continue BRAFi/MEKi at current dose, hydrocortisone 2.5% cream or alclometasone 0.05% cream or fluocinonide 0.05% cream BID AND doxycycline 100 mg BID or minocycline 100 mg QD	Interrupt BRAFi/MEKi treatment until severity decreases to grade 1–2; hydrocortisone 2.5% cream or alclometasone 0.05% cream or fluocinonide 0.05% cream BID AND doxycycline 100 mg or minocycline 100 mg BID AND oral prednisone 0.5 mg/kg for 5 days; if no improvement or	Discontinue BRAFi/MEKi, prednisone 1–2 mg/kg (or methylprednisolone equivalent), broad-spectrum antibiotics, hospitalization	

			deterioration, consider dose reduction or discontinuation		
Photosensitivity	Painless erythema and erythema covering <10% BSA	Tender erythema covering 10–30% BSA	Erythema covering >30% BSA and erythema with blistering; photosensitivity; oral corticosteroid therapy indicated; pain control indicated (e.g., narcotics or NSAIDs)	Life-threatening consequences; urgent intervention indicated	Death
	Prophylactic use of sun protection; patients should be educated about the use of broad-spectrum sunscreen, sun-protective clothing, broad-brimmed hats, and avoidance of sun exposure during peak UV hours but also through glass of windows (UVA penetration)				
	Continue BRAFi/MEKi at current dose; topical mild corticosteroids (hydrocortisone 2.5% cream) to face AND mid-strength (triamcinolone 0.1% cream) to body BID	Continue BRAFi/MEKi at current dose; topical mild corticosteroids (hydrocortisone 2.5% cream) to face AND potent corticosteroids (fluocinonide 0.1% cream) to body BID	Interrupt BRAFi/MEKi treatment until severity decreases to grade 1–2; topical mild corticosteroids (hydrocortisone 2.5% cream) to face AND potent corticosteroids (fluocinonide 0.1% cream) to body BID AND prednisone 0.5 mg/kg × 10 days (or equivalent of methylprednisolone); if no improvement or deterioration, consider dose reduction or discontinuation	Transfer to burn unit/ICU	
Radiation dermatitis/ radiation recall	Faint erythema or dry desquamation	Moderate to brisk erythema; patchy moist desquamation, mostly defined to skin folds and creases; moderate edema	Moist desquamation other than skin folds and creases; bleeding induced by minor trauma or abrasion	Skin necrosis or ulceration of full-thickness dermis; spontaneous bleeding from involved site	
	Prophylaxis with mometasone 0.1% cream BID throughout therapy, BRAFi may be paused 5–7 days prior to radiotherapy				
	Continue BRAFi/MEKi at current dose; topical mid-strength corticosteroid (mometasone furoate 0.1% cream) BID	Continue BRAFi/MEKi at current dose; topical mid-strength corticosteroid (mometasone furoate 0.1% cream) BID AND silver sulfadiazine 1% cream BID to open areas	Interrupt BRAFi/MEKi treatment until severity decreases to grade 1–2; topical mid-strength corticosteroid (mometasone furoate 0.1% cream) BID AND silver sulfadiazine 1% cream BID to open AND pain control with NSAIDs/GABA agonists/ narcotics; consider dose reduction or discontinuation if no improvement or deterioration		

have shown comparable activity to dacarbazine are temozolomide (MTIC), carmustine (BCNU), lomustine (CCNU), carboplatin, paclitaxel, and docetaxel. A difference in toxicity-related adverse events has led to the preference of monotherapy with dacarbazine over polychemotherapy regimens and other cytotoxic drugs (Pasquali et al. 2018; Luke and Schwartz 2013).

Dacarbazine or 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide (DTIC) is also the only chemotherapeutic drug which has ever acquired approval by the FDA for use in advanced malignant melanoma since 1975. While each chemotherapy has its own distinct set of AEs, the following paragraph focuses on the cutaneous AEs encountered with dacarbazine, which is the most frequently used chemotherapy as second- or third-line treatment in stage IV melanoma. Compared to gastrointestinal and hematopoietic AEs which are seen in about 90% of patients, cutaneous AEs are rarely seen and mainly comprise hypersensitivity and phototoxic reactions (Palathinkal et al. 2014).

Phototoxicity

Several case reports have shown rash-like eruptions in the sun-exposed areas shortly after receiving chemotherapy with intravenous dacarbazine (DTIC) (Kunze et al. 1980; Yung et al. 1981).

The range of onset was reported at various time points, ranging from very early after the first or second session of therapy to the 16th session with a median of about 6 sessions (Treudler et al. 2004).

UV exposure caused pruritic erythematous urticarial macules or papules confined to those areas that had been in contact with UV radiation, which occurred approximately 12 h after drug application and lasted about 72 h with full recovery after this time (Yung et al. 1981). It is known that DTIC is unstable in solutions, showing rapid photodegradation after exposure to sunlight into 2-azahypoxanthine, the apparent culprit photosensitizer (Treudler et al. 2004). As in phototoxic reactions seen with other agents such as the BRAFi vemurafenib, investigations have revealed increased sensitivity to UVA after dacarbazine (Treudler et al. 2004). Since patch testing in these investigations was negative, a phototoxic rather than a photoallergic mechanism is suspected. Additionally, the accumulation of phototoxic products in the skin may be linked to a decrease in enzymes which metabolize DTIC to MTIC (5-3-methyltriazen-1-yl-imidazo-4-carboxamide).

Sun exposure should be avoided during the first 3 days after dacarbazine infusion. Photoprotective clothing and use of broad-spectrum sunscreen with potent UVA-blocking features even during cloudy weather are recommended. The penetration of UVA radiation through window glass should be kept in mind and protective measures should be planned accordingly.

Hypersensitivity

Hypersensitivity reactions during or shortly after infusions are most frequent with platinum-based antineoplastic drugs (after several cycles), taxanes (at the first or second cycle), and liposomal doxorubicin (Sibaud et al. 2016) but have also been reported with dacarbazine (Levy et al. 2006). Hypersensitivity reactions can present in the form of flush, urticaria, morbilliform exanthem, pruritus, and angioedema accompanied by systemic symptoms such as dyspnea, pain, coughing, tachycardia, abdominal pain, and hypertension, potentially deteriorating to severe anaphylaxis necessitating intensive care. Although the reactions resemble type I hypersensitivity reactions, no prior sensitization is required as opposed to a classic allergic reaction (Sibaud et al. 2016). The infusion should be immediately stopped, and symptoms should be addressed accordingly. Premedication, reduction of infusion speed, and desensitization may be required to prevent recurrence.

Conclusion

Cutaneous toxicities profoundly diminish the quality of life (QoL) of affected patients and impairment seems to be more severe in patients receiving modern melanoma therapies such as targeted therapy as compared to conventional chemotherapy (Rosen et al. 2013). Aside from

significant symptoms and functional burden, cutaneous AEs make the anticancer therapy visible, leaving patients with a profound psychosocial burden that may impact therapy administration. Prompt diagnosis, severity assessment, and management are therefore crucial for the well-being and conservation of QoL in oncological patients. At times the severity and the impact of cutaneous adverse events are overestimated by oncological specialists not trained in dermatology (Barrios et al. 2017). Since this may lead to the interruption or even discontinuation of potentially lifesaving therapies, an interdisciplinary approach including a dermatologist is recommended in the case of cutaneous adverse events to ensure the best possible outcome for the oncological patient.

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Side Effects of Systemic Therapy and Their Clinical Management

37

Suthee Rapisuwon and Geoffrey T. Gibney

Contents

Abstract

Understanding when to interrupt or permanently discontinue systemic therapy in patients with advanced melanoma is dependent on the class of therapy, type of adverse event, and severity. Criteria for resuming therapy following adverse events differs among drug classes. While patients can be re-challenged with reduced doses of BRAF and MEK inhibitors after improvement or resolution of toxicities, grade 2 or higher immune related adverse events (irAEs) associated with immune checkpoint inhibitor therapy usually requires drug interruption or discontinuation and upfront

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corticosteroids. This chapter will discuss toxicities of BRAF-targeted therapy, current immune checkpoint inhibitors, and injectable oncolytic viral therapy as well as toxicity management guidelines. The toxicities associated with targeted and immune checkpoint inhibitor therapies in patients with advanced melanoma present unique challenges to providers and patients.

Keywords
Cutaneous toxicities · Immune checkpoint inhibitor (ICI) · BRAF inhibitor (BRAFi) · MEK inhibitor (MEKi) · Chemotherapy · Adverse events (AEs) · Immune related adverse events (irAEs)

Introduction

Prior to 2011, relatively, few systemic therapies were approved for use in patients with high-risk resected and advanced melanoma. These included dacarbazine, interferon (IFN)-alpha2b, and interleukin-2 (IL-2) (Gibney and Atkins 2015). While most oncologists have been well versed in the management of side effects with dacarbazine and IFN, high-dose IL-2 presented unique challenges due to the need for repeated intravenous infusion every 8 h for up to 15 doses and the severity of adverse events due in a large part to a capillary leak syndrome. Significant toxicities with IL-2 such as hypotension, arrhythmias, respiratory failure, and acute kidney injury have required aggressive supportive care (Atkins et al. 1999). As a result, the use of IL-2 has been limited to the inpatient setting administered by experienced providers.

In recent years, more effective therapeutics have been developed and approved by the FDA for use in patients with melanoma. These include selective inhibitors of the mitogen-activated protein kinase (MAPK) pathway in BRAF-mutant melanoma (dabrafenib, trametinib, vemurafenib, and cobimetinib), immune checkpoint inhibitors (ipilimumab, pembrolizumab, and nivolumab), and a genetically engineered oncolytic virus (talimogene laherparepvec). Consequently, the spectrum of treatment-related side effects in melanoma has also expanded. Recognition and management of these toxicities require a new set of skills by treating providers, especially with the emergence of immune-related adverse events with checkpoint immunotherapy.

In this chapter, we focus on the management of side effects associated with newer targeted therapies and immunotherapies in patients with advanced melanoma. Toxicity management is largely based on grading the severity of adverse events using the Common Terminology Criteria for Adverse Events (CTCAE) that was developed by the US Department of Health and Human Services (Common Terminology of Clinical Adverse Events (CTCAE)). Providers should familiarize themselves with the CTCAE grading schemas. The following sections discuss adverse events seen with these approved therapies and their general management. For more information and specifics on dosing, it is recommended to refer to the updated package inserts available for each therapy.

Targeted Therapy

Currently, FDA-approved targeted therapies in patients with BRAF V600-mutant melanoma include the selective BRAF V600-mutant inhibitors dabrafenib and vemurafenib and the MEK inhibitors trametinib and cobimetinib. Both BRAF and MEK inhibitors have been studied as single agents and show significant clinical activity and favorable toxicity profiles in this patient population (Chapman et al. 2011; Flaherty et al. 2012; Hauschild et al. 2012). Of note, less data on cobimetinib as monotherapy is available, and it is approved for use only in combination with vemurafenib (Rosen et al. 2016). The combinations of dabrafenib plus trametinib and vemurafenib plus cobimetinib have become the standard of care for BRAF-targeted therapy based on superior response rates and survival seen in three randomized phase III trials (COMBI-d, COMBI-v, and coBRIM) (Larkin et al. 2014; Long et al. 2015; Robert et al. 2015a).

In comparison to BRAF inhibitor monotherapy, the rates of severe toxicities (grade 3–4

events) are not substantially different with combination BRAF plus MEK inhibition. A grade 3 adverse event rate of 32% was seen in patients receiving dabrafenib plus trametinib, whereas the rate was 33% in patients receiving dabrafenib monotherapy on the COMBI-d study (of note, updated data reported treatment-related rates of 23% and 24%, respectively) (Long et al. 2015; Flaherty et al. 2016). Similarly, equivalent rates of grade 3 adverse events and fewer grade 4 adverse events were seen with vemurafenib plus cobimetinib compared to vemurafenib monotherapy in the coBRIM study (Larkin et al. 2014). Although BRAF plus MEK inhibitor therapy is generally well tolerated, dose interruptions and reductions are relatively common. For example, up to 55% of patients receiving dabrafenib plus trametinib required a dose interruption, and 33% of patients required a dose reduction as reported on the COMBI-v study (Robert et al. 2015a). However, only 13% of patients required permanent discontinuation, which was similar to the COMBI-d study (Long et al. 2015). While there are slight differences in the toxicity profiles for dabrafenib plus trametinib and vemurafenib plus cobimetinib, the two regimens have not been directly compared head to head making it difficult to determine if one regimen is better tolerated than the other (Table 1).

Table 1 Select reported adverse events (treatment-emergent) in patients treated with BRAF plus MEK inhibitor combinations on phase III studies

Adverse event	Dabrafenib plus trametinib (COMBI-d) Long et al. (2015)		Dabrafenib plus trametinib (COMBI-v) Robert et al. (2015a)		Vemurafenib plus cobimetinib (coBRIM) Larkin et al. (2014)	
	All grades	Grade 3	All grades	Grade 3	All grades	Grade 3
Any	87%	32%	98%	48%	96%	49%
Pyrexia	52%	7%	53%	4%	26%	2%
Chills	28%	0%	31%	1%	NR	NR
Fatigue	27%	2%	NR	NR	32%	4%
Rash	24%	0%	22%	1%	38%	5%
Nausea	20%	0%	35%	<1%	39%	1%
Headache	19%	0%	NR	NR	NR	NR
Diarrhea	18%	<1%	32%	1%	57%	6%
Vomiting	14%	<1%	29%	1%	21%	1%
Increased AST	11%	3%	NR	NR	22%	8%
Peripheral edema	11%	1%	NR	NR	NR	NR
Increased ALT	10%	2%	NR	NR	23%	11%
Pruritus	7%	0%	NR	NR	NR	NR
Hyperkeratosis	6%	0%	4%	0%	10%	0%
HFS	6%	<1%	4%	0%	NR	NR
Alopecia	5%	0%	6%	0%	14%	<1%
Arthralgias	NR	NR	24%	1%	33%	2%
Photosensitivity reaction	NR	NR	NR	NR	28%	2%
Increased CK	NR	NR	NR	NR	26%	7%
cuSCC	3%[a]		1%[a]		3%	
Keratoacanthoma	NR		NR		1%	
Chorioretinopathy	<1%	0%	1%	0%	12%	<1%
Retinal detachment	NR	NR	NR	NR	8%	2%
Decreased ejection fraction	4%	3%	8%	4%	7%	1%
QT interval prolongation	NR	NR	NR	NR	4%	<1%

AST Aspartate aminotransferase, *ALT* alanine aminotransferase, *HFS* hand-foot syndrome or palmar-plantar erythrodysesthesia, *CK* creatine kinase, *cuSCC* cutaneous squamous cell carcinoma, *NR* not reported

[a]Includes both cuSCC and keratoacanthomas

One of the unusual findings seen with selective BRAF inhibition using vemurafenib or dabrafenib monotherapy is a paradoxical activation of the MAPK pathway in BRAF wild-type cells (Gibney et al. 2013). This is thought to occur through BRAF inhibitor-mediated homodimerization or heterodimerization of the nonmutant RAF isoforms, which activates MEK and ERK signaling. This process may be further accelerated in cells harboring RAS mutations. Proliferative skin events ranging from benign such as hyperkeratosis and verrucal keratoses to malignant such as keratoacanthomas/squamous cell carcinomas and new primary melanomas have been reported (Anforth et al. 2013). In addition, gastric and colonic polyps and non-skin secondary malignancies have been reported, which have also been attributed to BRAF inhibitor-mediated paradoxical activation of the MAPK pathway. The latter include reports on the emergence of leukemia, colorectal cancer, pancreatic cancer, breast cancer, and other secondary malignancies in patients with advanced melanoma on BRAF inhibitor therapy (Long et al. 2015; Gibney et al. 2013; Grey et al. 2014). The addition of the MEK inhibitor appears to significantly reduce the paradoxical activation of the MAPK pathway and the resultant proliferative skin events. For example, markedly fewer events of hyperkeratosis, hand-foot syndrome, keratoacanthomas, and cutaneous SCCs were reported in patients treated with BRAF plus MEK inhibitor combination therapy compared to BRAF inhibitor monotherapy in all phase III trials (Larkin et al. 2014; Long et al. 2015; Robert et al. 2015a).

In addition to the proliferative events seen with BRAF inhibitor therapy, there are other toxicities that are unique to either BRAF or MEK inhibitor classes, which has implications for dose interruptions and modifications. In particular, BRAF inhibitors have been associated more with pyrexia, photosensitivity reactions, arthralgias, and QT interval prolongation, whereas MEK inhibitors have been more associated with peripheral edema, serous retinopathy, retinal vein occlusion, and decreased ejection fraction (Flaherty et al. 2012; Larkin et al. 2014; Long et al. 2015). Other toxicities may have significant overlap between the two drug classes, making it difficult to know if one or both agents are causing the adverse event(s). In general, if a patient experiences a grade 3–4 event, then the BRAF-targeted therapy would need to be held until improvement to grade 1 or resolution. Typically, one or both drugs would be dose reduced upon restarting. In some cases, re-treatment is not advised. Exceptions do exist, and drug interruptions may be necessary for lower-grade events (such as ocular and cardiac toxicities). The following subsections address management of important adverse events seen with combination of BRAF plus MEK inhibitor therapy in patients with advanced melanoma.

Pyrexia

Pyrexia, defined as a temperature over 38 °C/100.4 °F, was seen in 52–53% of patients treated with dabrafenib plus trametinib in the COMBI-d and COMBI-v studies (Long et al. 2015; Robert et al. 2015a). In contrast, vemurafenib plus cobimetinib appeared to have a lower rate of pyrexia (26% in the coBRIM study) (Larkin et al. 2014). Grade 3 pyrexia (temperature >40 °C/102.3 °F for ≤24 h) was seen in 4–7% of patients treated with dabrafenib plus trametinib and 2% of patients treated with vemurafenib plus cobimetinib. Of note, rates of pyrexia for dabrafenib and vemurafenib monotherapies in these studies were 25% and 21–22%, respectively. The underlying mechanism(s) for the pyrexia remains unknown. In the phase I/II study of dabrafenib plus trametinib, there was a trend for pyrexia in patients achieving higher blood levels of dabrafenib, whereas baseline characteristics were not associated with pyrexia (Menzies et al. 2015). The median timing to first pyrexia event was 19 days, with subsequent events frequently observed.

Guidelines on management of pyrexia have evolved with more experience on using dabrafenib plus trametinib (Menzies et al. 2015). For cases of pyrexia, it is recommended to temporarily hold dabrafenib. For grade 3 pyrexia (or pyrexia associated with other signs/symptoms such as rigors or hypotension), both dabrafenib and trametinib should be held. Symptom management with acetaminophen or NSAIDs can be

administered. An infectious workup may also be appropriate, especially in more severe cases. Drug therapy can be reinstituted once pyrexia has resolved for at least 24 h. Dose reductions are appropriate in patients with recurrent or more severe pyrexia episodes. Low-dose corticosteroids can be considered in patients who do not respond to acetaminophen or NSAIDs. Premedication with antipyretic agents may also be useful in patients with recurrent pyrexia. A similar approach can be utilized for managing pyrexia in patients receiving vemurafenib plus cobimetinib.

Skin Toxicity

A range of cutaneous adverse events have been reported in patients treated with BRAF-targeted therapies. These primarily include proliferative skin events, photosensitivity, and rash. The former is largely related to the paradoxical MAPK activation as described earlier. In patients who do develop uncomplicated secondary cutaneous neoplastic disease on combination BRAF/MEK inhibitor therapy, such as the 1–3% of patients with keratoacanthomas or squamous cell carcinomas (Larkin et al. 2014; Long et al. 2015; Robert et al. 2015a), local management with cryotherapy or excision is usually feasible, and patients can continue on treatment (Anforth et al. 2013). With regard to photosensitivity, this is more common in patients treated with vemurafenib. In the coBRIM study, 28% of patients treated with vemurafenib plus cobimetinib reported photosensitivity, with a grade 3 rate of 2% (Larkin et al. 2014). Patients can experience a painful burning sensation that evolves into an erythematous skin reaction similar to serious sunburns when exposed to the sun or other ultraviolet light sources. This appears to be more due to UVA rather than UVB exposure, and the burning sensation can last up to 10 min after exposure even in less severe cases (Anforth et al. 2013). Patient education and protective measures (sun avoidance along with topical sunblock and sun-protective clothing) are important. In severe cases, temporary drug hold is warranted until resolution and dose reductions when restarting therapy may be considered (Vemurafenib package insert).

Both BRAF and MEK inhibitor monotherapies have also been associated with rash. An erythematous maculopapular rash that is either confluent or patchy in distribution is often described with dabrafenib and vemurafenib. Other cases that are potentially severe, such as erythema nodosum, DRESS syndrome, Sweet's syndrome, and toxic epidermal necrolysis (TEN)/Stevens-Johnson syndrome, have been reported (Jeudy et al. 2015; Mossner et al. 2015; Sinha et al. 2015; Wenk et al. 2013; Yorio et al. 2014). With regard to MEK inhibitor monotherapy, an acneiform-type rash has also been described (Flaherty et al. 2012; Rosen et al. 2016). In the phase III studies of dabrafenib plus trametinib and vemurafenib plus cobimetinib, rash has been observed in 22–24% and 38% of melanoma patients, respectively (Larkin et al. 2014; Long et al. 2015; Robert et al. 2015a). Fortunately, only 5% or less of treated patients experienced a grade 3 rash covering more than 30% of the body surface area (BSA). Drug interruption or discontinuation is usually necessary for grade 3 or higher rash. As it is difficult to determine which drug is primarily responsible, typically both the BRAF and MEK inhibitors are held. Supportive therapy may be helpful (such as topical/oral corticosteroids or antibiotics depending on the situation). In less severe cases, patients who have recovered can be restarted on BRAF-targeted therapy at reduced doses. However, permanent discontinuation may be necessary in patients who experience severe life-threatening rashes. Interestingly, in patients with BRAF-induced TEN/Stevens-Johnson syndrome, there are reports of successful desensitization and switching from one BRAF agent to another (Jeudy et al. 2015; Minor et al. 2012).

Ocular Toxicities

In early- and late-phase development of BRAF and MEK inhibitor-targeted therapies, infrequent but significant ocular toxicities have been observed. In a retrospective review of 568 patients treated with vemurafenib, ocular adverse events were noted in 22% of patients (Choe et al. 2014). The most common were uveitis (4%),

conjunctivitis (3%), and dry eyes (2%). Similar events have been reported for dabrafenib. With MEK inhibitor therapy, blurred vision and dry eyes have been reported, as well as rare cases of central serous retinopathy and retinal vein occlusion (Rosen et al. 2016; Infante et al. 2012). Serous retinopathy appears more common with BRAF plus MEK inhibitor therapy, which was reported in 26% of patients treated with vemurafenib plus cobimetinib from the coBRIM study where serial ophthalmic exams were required (De la Cruz-Merino et al. 2015). Most were grade 1 events and did not require dose interruption; dose modification or interruption of cobimetinib was sufficient in most grade 2 and 3 events. Chorioretinopathy, which represents a subset of serous retinopathy, has been reported in 13% of patients receiving vemurafenib plus cobimetinib and less than 1% of patients treated with dabrafenib plus trametinib – although the divergence in rates may be due to differences in monitoring/reporting (Long et al. 2015; Robert et al. 2015a). Patients on BRAF-targeted therapy should be monitored for ocular events. For most clinically significant toxicities, treatment should be interrupted, and patients should be managed by an ophthalmologist. Permanent discontinuation of BRAF-targeted therapy may be necessary.

Cardiac Toxicities

Prolongation of the QT interval has been reported in 4–5% of patients receiving vemurafenib alone or in combination with cobimetinib on the coBRIM study and 7% of patients receiving vemurafenib in an expanded access program where cardiac events were closely monitored (Larkin et al. 2014; Flaherty et al. 2014). Less than 1–3% of patients on treatment were identified to have a QT interval of $\geq$500 ms (grade 3), and no related significant arrhythmias were reported. Similar rates of grade 3 prolongation of the QT interval have been reported in the package insert for dabrafenib alone or in combination with trametinib (Dabrafenib package insert). The addition of MEK inhibitor therapy does not appear to increase this risk of significant QT interval prolongation, which is supported by a phase I study of trametinib where even supratherapeutic dosing did not affect the QT interval (Patnaik et al. 2016). Monitoring by electrocardiogram while on therapy is recommended, and treatment should be held in patients with QT intervals $\geq$500 ms or uncontrolled arrhythmias.

Cardiomyopathy, mainly documented as left ventricular dysfunction or decreased ejection fraction (EF) by echocardiogram, has been a concern with MEK inhibitor therapy. In the phase I trial of trametinib, this was reported in 8% of patients (Infante et al. 2012). Less data is available on cardiomyopathy with cobimetinib monotherapy. Decreased ejection fraction has also been reported in patients receiving vemurafenib and dabrafenib monotherapies (0–3% and 3%, respectively) (Larkin et al. 2014; Long et al. 2015; Robert et al. 2015a). In patients receiving combination of BRAF plus MEK inhibitor therapy, the rates were 7% with vemurafenib plus cobimetinib and 4–8% with dabrafenib plus trametinib, which may not be substantially increased over MEK inhibitor monotherapy. Grade 3 events (EF of 20–39% or a $>$20% drop from baseline) have been reported in 1–4% of patients. Monitoring by echocardiogram is recommended. Interruption of MEK inhibitor therapy should be considered in patients with a decrease of 10% or greater in EF or a symptomatic decrease in EF (i.e., presence of signs/symptoms of heart failure). Permanent discontinuation of BRAF-targeted therapy should also be considered in severe cases. Re-initiation of BRAF-targeted therapy (dose reduced) is dependent on the severity of the event and recovery of cardiac function.

Immune Checkpoint Inhibitors

Focus on immunotherapies in melanoma has largely shifted toward immune checkpoint inhibition. The first approved agent in this class is ipilimumab, which is a fully humanized monoclonal IgG1 antibody against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). CTLA-4 functions as a potent immune inhibitory receptor on activated T lymphocytes where it has a higher affinity for B7-1 or B7-2 proteins on

antigen-presenting cells than the co-stimulatory molecule CD28 (Baksh and Weber 2015; Yao et al. 2013). It plays diverse roles in regulating immune responses in normal immune homeostasis as well as antitumor responses. The second approved agents, nivolumab and pembrolizumab, are monoclonal antibodies targeting the programmed death-1 (PD-1) receptor, which is also a cell surface inhibitory molecule in the B7-CD28 family. PD-1 receptor has two known ligands: PD-L1 (B7-H1) and PD-L2 (B7-DC) – both of which have distinct expression patterns on tumor cells, antigen-presenting cells, and other cell populations (Dong et al. 1999; Latchman et al. 2001). PD-1 plays a role in the induction and maintenance of normal T-cell tolerance, which can also lead to T-cell exhaustion and tumor immune evasion (primarily via PD-1/PD-L1 interaction) (Baksh and Weber 2015; Yao et al. 2013). When either CTLA-4 or PD-1 is genetically knocked out in mouse models, autoimmune disorders develop, such as rapid lymphocytic proliferation and myocarditis with CTLA-4 loss or glomerulonephritis, arthritis, and cardiomyopathy with PD-1 loss (Nishimura et al. 1999, 2001; Waterhouse et al. 1995). Not surprisingly, immune-related adverse events (irAEs) are observed in patients treated with immune checkpoint inhibitor therapy, in addition to traditional constitutional symptoms.

Almost every patient treated with ipilimumab reported at least one adverse event (97%) in the phase III trial that led to its FDA approval (Hodi et al. 2010). In this study and others, treatment-related grade 3 and 4 adverse events have been reported in 23–27% of patients (Table 2; Larkin et al. 2015; Postow et al. 2015). The most common reported events were rash, pruritus, fatigue, nausea, diarrhea, and decreased appetite. One of the more worrisome toxicities has been immune-mediated colitis, with grade 3–4 cases seen in up to 9% of patients. Similar to ipilimumab,

Table 2 Select reported adverse events (treatment-related) in patients treated with immune checkpoint inhibitor therapy

	Ipilimumab Hodi et al. (2010), Larkin et al. (2015), Postow et al. (2015), and Robert et al. (2015b)		Pembrolizumab or nivolumab Larkin et al. (2015), Robert et al. (2014, 2015b)		Nivolumab plus ipilimumab Larkin et al. (2015) and Postow et al. (2015)	
Adverse event	All grades	Grades 3–4	All grades	Grades 3–4	All grades	Grades 3–4
Any	73–93%	20–27%	73–82%	10–16%	91–96%	54–55%
Fatigue	15–43%	1–7%	19–34%	0–1%	35–39%	4–5%
Decreased appetite	8–27%	0–2%	5–11%	0%	15–18%	0–1%
Headache	4–15%	0–2%	2–7%	0%	10–14%	0.3–2%
Pyrexia	2–15%	0–0.3%	1–7%	0%	19–20%	0–1%
Pruritus	24–35%	0–0.4%	14–19%	0–1%	33–35%	1–2%
Rash	15–33%	0–2%	13–26%	0–1%	40–41%	5%
Vitiligo	2–9%	0%	7–11%	0–0.3%	7–11%	0%
Nausea	9–35%	0.4–2%	10–17%	0%	22–26%	1–2%
Vomiting	6–24%	0–2%	6%	0.3–1%	14–15%	1–3%
Diarrhea	33–37%	3–11%	14–19%	1–3%	44–45%	9–11%
Colitis	8–13%	5–9%	1–3%	1–2%	12–23%	8–17%
Increased ALT	2–4%	0–2%	1–4%	0–1%	18–22%	8–11%
Increased AST	2–4%	0–2%	1–5%	0–1%	15–21%	6–7%
Arthralgia	6–9%	0–1%	8–12%	0–0.4%	11%	0–0.3%
Dyspnea	1–15%	0–4%	1–5%	0–0.4%	10%	1–3%
Pneumonitis	0.4–4%	0.3–2%	0.4–2%	0–0.4%	6–11%	1–2%
Hypothyroidism	2–15%	0%	4–9%	0–0.4%	15–16%	0–0.3%
Hyperthyroidism	1–2%	0–0.4%	3–6%	0%	4–10%	0–1%
Hypophysitis	2–4%	0–2%	0.4–1%	0–1%	8–12%	2%

AST Aspartate aminotransferase, *ALT* alanine aminotransferase

most patients treated with either nivolumab or pembrolizumab have reported adverse events during therapy (Larkin et al. 2015; Robert et al. 2014, 2015b). The most common reported events were fatigue/asthenia, rash, pruritus, vitiligo, arthralgias, diarrhea, constipation, and nausea. However, treatment-related grade 3 and 4 adverse events have been seen in 13–16% of patients. The most common grade 3–4 adverse events reported have been fatigue (up to 2%) and diarrhea (up to 2%).

Combination immunotherapy with nivolumab plus ipilimumab has demonstrated higher response rates (up to 60%) and longer survival over ipilimumab and nivolumab monotherapy strategies in patients with advanced melanoma based on results from the Checkmate-069 and Checkmate-067 studies (Larkin et al. 2015; Postow et al. 2015). In addition to the increased antitumor activity, higher rates of grade 3–4 adverse events have been reported as well (54–55%). The most common treatment-related adverse events included diarrhea, rash, fatigue, pruritus, colitis, nausea, elevated transaminase levels, pyrexia, and hypothyroidism. Other important irAEs that were captured in over 10% of patients include thyroiditis and hypophysitis (resulting in hypothyroidism and adrenal insufficiency), pneumonitis, and arthralgias (related to arthritis). In addition, elevated serum lipase, including 9% of patients with grade 3–4 levels, was seen in these studies, but most have not been associated with clinically relevant pancreatitis. The greater incidence of elevated transaminase level (or hepatitis) and diarrhea/colitis grade 3–4 events accounted for a major proportion of the increased severe adverse event rate. Rates of treatment discontinuation due to adverse events with combination nivolumab plus ipilimumab therapy were 36–47% compared to 8% with nivolumab monotherapy and 15–17% with ipilimumab monotherapy in the two studies.

The recognition and optimal management of irAEs require vigilance on behalf of patients and providers, along with familiarity of recommended toxicity management algorithms. In contrast to most chemotherapy and targeted therapy adverse events, immune checkpoint inhibitor interruption alone usually does not result in improvement or resolution of significant irAEs. Typically, toxicities reaching a grade 3–4 level require treatment with high-dose corticosteroids (e.g., prednisone or equivalent at 1–2 mg/kg body weight administered daily and tapered over 4–5 weeks). In certain cases, less severe toxicities, such as grade 2 pneumonitis or other persistent grade 2 irAEs, will also require this approach. Situations that are refractory to corticosteroids may require additional immunomodulatory agents, such as infliximab or mycophenolate mofetil. After recovery, a decision needs to be made with the patient whether or not to restart the immune checkpoint inhibitor therapy based on the type and degree of toxicity treated. Dose reductions are not recommended, and recurrence of the toxicity can occur if the same regimen is restarted. Fortunately, antitumor activity achieved in patients who discontinue immune checkpoint inhibitor therapy and receive immunomodulatory agents appears equivalent to that achieved by patients who remain on active treatment (Postow et al. 2015; Horvat et al. 2015; Weber et al. 2015a). Also, even though irAEs overlap between anti-CTLA-4 and anti-PD-1 therapies and are managed similarly, patients can move from one immune checkpoint inhibitor class to another if needed with a low likelihood of experiencing a recurrence of the prior irAE. Below is further information on specific irAEs and their management.

Skin Toxicity

Dermatologic side effects (primarily rash, pruritus, and vitiligo) are one of the most common categories of adverse reactions from anti-CTLA-4 and anti-PD-1 antibody therapies. With ipilimumab monotherapy, rates of rash have been reported in 15–33% of melanoma patients, pruritus in 24–35%, and vitiligo in up to 9% (Hodi et al. 2010; Larkin et al. 2015; Postow et al. 2015; Robert et al. 2015b). Similarly, melanoma patients who received anti-PD-1 therapy with either pembrolizumab or nivolumab reported rash in 13–26% of patients, pruritus in 17–27%, and vitiligo in up to 11% (Larkin et al. 2015; Robert et al. 2014, 2015b). With combination nivolumab plus ipilimumab, rates of rash and pruritus are even

higher (rash 40–41%, pruritus 33–35%). While rates of severe rash or pruritus are relatively low overall, up to 5% of patients treated with nivolumab plus ipilimumab have reported grade 3–4 rash. The symptoms usually present within the first month.

Exacerbation of autoimmune skin conditions, e.g., psoriasis, has been reported with both nivolumab and pembrolizumab (Hofmann et al. 2016; Kato et al. 2015; Sahuquillo-Torralba et al. 2016). Other uncommon dermatologic side effects reported with anti-PD-1 antibody have ranged from eczema, lichenoid skin reaction (lichen sclerosus et atrophicus, lichen planus), and bullous skin eruption (bullous pemphigoid and bullous erythema multiforme) (Hofmann et al. 2016; Carlos et al. 2015; Hwang et al. 2016; Jour et al. 2016; Naidoo et al. 2016). Similarly, cases of TEN/Stevens-Johnson syndrome, DRESS syndrome, and Sweet's syndrome have been reported with ipilimumab (Gormley et al. 2014; Ipilimumab package insert; Kyllo et al. 2014; Voskens et al. 2013).

Referral to dermatology and biopsy of skin reactions may be warranted to clarify the diagnosis. Microscopically, the maculopapular or lichenoid rashes have been described as showing perivascular lymphocytic infiltration extending from the dermis into the epidermal layer with primarily CD4+ and CD8+ T cells, as well as the presence of occasional eosinophils and apoptotic keratinocytes (Goldinger et al. 2016; Joseph et al. 2015; Weber et al. 2012). PD-L1 positivity has been reported in 10–20% of the T-cell infiltrate. Furthermore, melan-A or gp100-specific CD8+ T cells may cross-react against both melanocytes and melanoma cells, which could explain the frequent loss of pigmentation or vitiligo (Weber et al. 2012; Downey et al. 2007).

While the majority of cutaneous toxicities require no treatment (such as grade 1 rash or grade 1–2 vitiligo), oral antihistaminergic agents (e.g., diphenhydramine) and/or topical corticosteroid or emollient creams may be helpful in treating patients with more diffuse or symptomatic rashes. Low-potency topical steroids such as 1% hydrocortisone can be used initially, but higher-potency topical steroids such as 0.1% triamcinolone or 0.05% clobetasol may be necessary in refractory cases. However, once an immune-related rash has become grade 3 or the emergence of more serious cases as described above, the immune checkpoint inhibitor (s) should be held and systemic high-dose corticosteroids administered. After resolution or improvement to grade 1 (<10% BSA), restarting the immunotherapy can be considered in select cases after the corticosteroid taper.

Gastrointestinal Toxicity

Gastrointestinal adverse effects from immune checkpoint inhibitor therapy are relatively common. Treatment-related diarrhea has been reported in 33–37% among subjects who received ipilimumab, 14–19% for anti-PD-1 antibody, and 44–45% for nivolumab plus ipilimumab (Hodi et al. 2010; Larkin et al. 2015; Postow et al. 2015; Robert et al. 2014, 2015b). Severe cases of diarrhea (grade 3–4; ≥7 stools per day over baseline, incontinence, hospitalization indicated, or life-threatening consequences) are most often seen with ipilimumab regimens – up to 11% with both ipilimumab monotherapy and combined with nivolumab. Of note, there may be some overlap with the reporting between colitis and diarrhea in the referenced studies. Diarrhea associated with signs/symptoms such as abdominal pain, blood in the stool, and/or confirmation by endoscopy and biopsy is usually referred to as colitis. Some patients may have predominantly enteritis with inflammation affecting only the small bowel. Pathology shows either a neutrophilic, lymphocytic, or mixed neutrophilic-lymphocytic infiltrate (Weber et al. 2012). Grade 3–4 colitis (severe abdominal pain, peritoneal signs, medical intervention indicated, or life-threatening consequences) has been observed in up to 9% of patients treated with ipilimumab, 2% with anti-PD-1 monotherapy, and 17% with nivolumab plus ipilimumab. Because of the potential for intestinal perforation, obstruction, and death with severe cases of diarrhea/colitis, careful attention and management are necessary. Other gastrointestinal symptoms, such as nausea and vomiting, can be seen in up to a third of patients treated with immune checkpoint therapy and usually can be managed with antiemetic therapy.

Patients who experience mild diarrhea or colitis (grade 1; increase of <4 stools per day over baseline or mild increase in ostomy output over baseline or diagnostic evidence of inflammation in an asymptomatic patient) can be treated with the oral anti-motility agent loperamide, dosed to achieve a normal stool output. Oral hydration is important, and alternative anti-motility agents, such as diphenoxylate-atropine, can be used. Stool studies to rule out infection (including routine bacterial and *C. difficile* (+/− parasite) assays) should be performed in patients with prolonged mild diarrhea and in those with more significant cases. Immune checkpoint inhibitor therapy can usually be continued with grade 1 diarrhea/colitis, but patients need to be monitored closely. In patients with grade 2 diarrhea or colitis (four to six stools per day over baseline or moderate increase in ostomy output compared to baseline or diarrhea accompanied by abdominal pain/blood in stool), the immune checkpoint inhibitor therapy should be held and patients managed as above.

In patients with grade 2 diarrhea/colitis lasting more than several days or in patients presenting with grade 3–4 diarrhea/colitis, systemic corticosteroids also should be initiated. Hospitalization for supportive care (intravenous hydration, temporary bowel rest, and antidiarrheal/emetic agents) and intravenous methylprednisolone may be necessary in more severe cases. Urgent sigmoidoscopy or colonoscopy is recommended in most cases to confirm the diagnosis but is not always feasible. CT scan of the abdomen and pelvis may also be helpful in looking for bowel inflammation.

In patients without clinical improvement in 48 h after starting corticosteroids, the diagnosis should be confirmed by endoscopy (if not already done), and ensure infectious and other causes are ruled out. For this situation, escalation of immunomodulatory agents should be pursued, such as the addition of the anti-TNFα inhibitor infliximab (Weber et al. 2012). Repeated dosing of infliximab can be considered every 2 weeks. Prior to infliximab, many centers rule out latent tuberculosis due to the potential for reactivation. Optimized management of diarrhea/colitis is currently being studied with the use of upfront infliximab plus a lower dose of corticosteroids versus high-dose corticosteroids alone (NCT02763761). More aggressive approaches, including resection of inflamed bowel segments and diverting colostomies, have been needed in rare cases. Tapering of the corticosteroids usually starts after there is substantial clinical improvement in the patient and needs to be extended to 4 weeks or longer to prevent recurrence of symptoms.

Ipilimumab should not be restarted in patients after recovery from grade 3 to 4 diarrhea/colitis and steroid taper. In patients treated for grade 2 toxicity, restarting ipilimumab can be considered after improvement to <grade 1 symptoms and corticosteroid taper (per package insert) (Ipilimumab package insert). Restarting treatment with anti-PD-1 monotherapy can be considered after recovery from either grade 2 or 3 diarrhea/colitis (per package inserts) (Nivolumab package insert; Pembrolizumab package insert). However, both scenarios pose a risk to the patient for recurrence of the same toxicity and must be approached with caution. In patients who experience grade 2–3 diarrhea/colitis during the induction phase of combined nivolumab and ipilimumab, some experts feel comfortable with restarting treatment in the nivolumab maintenance phase after recovery due to the likelihood that the diarrhea/colitis was largely driven by the ipilimumab.

Prior efforts to prevent immune-related diarrhea and colitis were unsuccessful in a randomized phase II ipilimumab study using oral budesonide (Weber et al. 2009). This trial of patients with advanced melanoma, both previously treated and treatment-naïve and randomized to ipilimumab 10 mg/kg plus either daily oral budesonide or placebo, resulted in no substantial difference in the incidence of diarrhea or colitis. Use of prophylactic anti-motility agents or systemic corticosteroids in asymptomatic patients is not recommended.

Hepatic Toxicity

Hepatic toxicity attributed to immune-mediated injury within the hepatobiliary system is usually

captured by either elevation in blood levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), or bilirubin. Additionally, some studies have also included hepatitis in adverse event reporting. Typically, patients with hepatic toxicity are found to have elevated ALT and/or AST on blood work and are asymptomatic (lack of related nausea, vomiting, decreased appetite, or jaundice). Bilirubin elevation is not common but may be a sign of severe liver injury or a nonimmune-mediated cause such as biliary obstruction. Treatment-related increased ALT or AST has been reported in 1–5% of patients treated with ipilimumab or anti-PD-1 monotherapy (Hodi et al. 2010; Larkin et al. 2015; Postow et al. 2015; Robert et al. 2014, 2015b). Grade 3–4 events are rare. However, in patients treated with the combination nivolumab plus ipilimumab, the risk is much greater. As many as 22% of patients have been reported to have elevated ALT or AST, which includes up to 11% at the grade 3–4 level. Histologically, the hepatocytes are surrounded by diffuse T-cell infiltration, which can be difficult to distinguish from other drug-induced hepatitis, viral hepatitis, and other etiologies (Weber et al. 2012).

Patients with presumed treatment-related grade 1 elevation of ALT and/or AST (up to 3 × the upper limit of normal) or bilirubin (up to 1.5 × the upper limit of normal) can generally continue immune checkpoint inhibitor therapy but require close monitoring for worsening of the blood levels. Grade 2 elevation in levels (ALT/AST = 3–5 × the upper limit of normal; bilirubin = 1.5–3 × the upper limit of normal) or greater requires holding immune checkpoint inhibitor therapy and warrants further investigation with imaging studies, such as a right upper quadrant ultrasound, to evaluate for nontreatment-related causes. Concurrent medications should be reviewed for possible contribution to liver toxicity and viral hepatitis screen considered. Liver biopsy may be helpful to clarify the diagnosis, especially in patients with pre-existing liver metastases. In patients with grade 2 or greater immune-related liver toxicity, initiation of corticosteroids is warranted. Frequent monitoring of the liver function tests should be performed during the initial management until significant improvement is seen. Patients who recover from grade 2 toxicity and are tapered off corticosteroids may be considered for retreatment. However, retreatment after recovery from grade 3 to 4 toxicity is generally not advised.

Not all cases are straightforward. Some patients have elevated baseline liver function tests due to underlying disease. In these patients, careful monitoring during immune checkpoint inhibitor therapy is important. While standard ranges utilized by the CTCAE for grading may not apply, substantial elevations in ALT, AST, and/or bilirubin should be treated as grade 3–4 hepatic toxicity unless due to disease progression or another nonimmune-related cause. In addition, some patients with immune-related hepatic toxicity may not respond appropriately to high-dose corticosteroids. If there is no improvement in 48–72 h,, mycophenolate mofetil is usually added to the treatment regimen. Treatment with mycophenolate mofetil should be continued until toxicity decreases to grade 1 level and then tapered after the corticosteroid has been discontinued. Use of three drug immunomodulatory regimens, such as corticosteroid, mycophenolate mofetil, and antithymocyte globulin, has been reported in recalcitrant cases (Ahmed et al. 2015; Chmiel et al. 2011).

Pulmonary Toxicity

While dyspnea has been reported as a treatment-related adverse event in 1–15% of patients treated with ipilimumab, 1–5% of patients treated with anti-PD-1 monotherapy, and 10% of patients treated with nivolumab plus ipilimumab (Hodi et al. 2010; Larkin et al. 2015; Postow et al. 2015; Robert et al. 2014, 2015b), the major concern for patients is the development of treatment-related pneumonitis. Treatment-related pneumonitis has been reported in up to 4% of melanoma patients treated with ipilimumab or anti-PD-1 monotherapy, although severe cases are rare. With nivolumab plus ipilimumab, the risk appears greater with pneumonitis reported in 6–11% of patients (1–2% severe cases). Patients can present

with dyspnea and a dry cough and are usually afebrile. On imaging studies, the inflammatory response may be characterized as patchy diffuse infiltrative process, ground-glass appearance, or focal infiltrative patterns that can mirror edema, pneumonia, or even fungal infection. It may be difficult to discern pneumonitis from other diagnoses by radiographic appearance alone.

While asymptomatic cases of pneumonitis can be detected during restaging scans (grade 1), the emergence of symptoms possibly related to pneumonitis warrants further investigation. This would include clinical exam, pulse oximetry, and imaging studies (either chest x-ray or CT scan). Bronchoscopy with washings or lung biopsy has been recommended by some to look for signs of inflammation and to rule out infection. In patients with respiratory symptoms and signs of pneumonitis on imaging studies, systemic corticosteroids should be administered. In patients with hypoxia and/or severe symptoms (grade 3–4), hospitalization should be considered for supplemental oxygen, supportive care, and intravenous methylprednisolone. Additional immune modulatory agents, such as infliximab or mycophenolate mofetil, may be required if there is no clinical improvement in the first 48 h. Retreatment with the immune checkpoint inhibitor therapy may be considered in patients with grade 2 pneumonitis (mild to moderate symptoms, no oxygen requirement) after recovery and corticosteroid taper.

Endocrine Toxicity

Endocrine side effects can range from asymptomatic hypothyroidism or hyperthyroidism to more serious cases of adrenal crisis. These usually result from inflammation in the thyroid, pituitary, or adrenal glands and can present with an acute excess or deficiency in hormone levels. The average time to onset of endocrine toxicity is approximately 6 weeks with ipilimumab (Weber et al. 2012) and approximately 10 weeks in PD-1-treated patients (Weber et al. 2015b). Co-management with an endocrinologist is recommended.

Hypothyroidism has been reported in 2–15% of patients treated with ipilimumab and 4–9% of patients treated with either pembrolizumab or nivolumab (Hodi et al. 2010; Larkin et al. 2015; Postow et al. 2015; Robert et al. 2014, 2015b). The rate is up to 16% in patients treated with nivolumab plus ipilimumab. Most patients with immune-related hypothyroidism have grade 1–2 toxicity levels where only levothyroxine replacement is necessary. However, more severe cases of hypothyroidism have been reported. The starting dose of levothyroxine is dependent on clinical presentation and hormone levels. Complete weight-based replacement of thyroid hormone and interruption/discontinuation of immune checkpoint inhibitor therapy are usually not necessary. It is important to determine if the patient is experiencing primary versus central hypothyroidism. The latter would be due to inflammation at the pituitary level and will likely require hormone replacement beyond the thyroid axis as well.

Hyperthyroidism tends to be less common with an overall incidence of up to 10% with nivolumab plus ipilimumab (Larkin et al. 2015; Postow et al. 2015). Most patients develop subclinical hyperthyroidism with mild elevation of serum-free T4, although cases of overt hyperthyroidism have been documented. The patients with symptomatic immune-related hyperthyroidism can be treated with beta-blockers, such as propranolol. Occasionally, more severe situations require additional inhibition of thyroid peroxidase (e.g., methimazole or propylthiouracil). The use of high-dose corticosteroids may be indicated in grade 3–4 toxicity situations. Patients with symptomatic hyperthyroidism should withhold their treatment with immune checkpoint blockade and resume the treatment when the side effects reduced to grade 1 or less. It is important to keep in mind that most patients with hyperthyroidism will eventually develop hypothyroidism. Because thyroid dysfunction is commonly seen in both anti-CTLA-4 and anti-PD-1therapy, routine monitoring is indicated during treatment.

Inflammation of the pituitary gland, termed hypophysitis in most studies, has been captured at low rates with immune checkpoint inhibitor

monotherapy and up to 12% of patients treated with nivolumab plus ipilimumab (Larkin et al. 2015; Postow et al. 2015). Presenting symptoms of hypophysitis can sometimes be nonspecific such as fatigue, decreased appetite, or mild nausea. Headache and visual disturbance can occur in more serious cases due to swelling of pituitary gland, or others can have significant hemodynamic/electrolyte changes with adrenal crisis. When hypophysitis is suspected, laboratory workup should include morning ACTH and cortisol levels, as well as TSH and T3/T4 levels. Other hormone levels can be assessed, including prolactin, LH/FSH, and testosterone (in males). MRI with cuts of the sella turcica and para-sella areas may show edema of the pituitary gland and be helpful in confirming the diagnosis. However, a normal-appearing pituitary does not rule out hypophysitis. High-dose corticosteroid and interruption of immune checkpoint inhibitor therapy are usually indicated in grade 3–4 hypophysitis. Otherwise, only hormone replacement of the deficient axis is necessary. Recovery of endocrine gland function after an immune-related event is not common, and long-term hormone replacement is usually required.

Less Common Toxicities

In preclinical mouse models with CTLA-4 and PD-1 gene knockout experiments, inflammatory responses were seen in both renal and cardiac organ systems (Nishimura et al. 1999, 2001; Waterhouse et al. 1995). Fortunately, severe renal or cardiac toxicities in patients treated with immune checkpoint inhibitor therapy are rare. With regard to renal events, proteinuria and interstitial nephritis have been reported, along with a case report of lupus nephritis in association with ipilimumab (Hofmann et al. 2016; Fadel et al. 2009). Elevation of serum creatinine is usually the first sign of immune-related renal toxicity in the absence of other obvious causes (such as volume depletion or obstruction). Further workup includes urine assessment and imaging studies of the renal system. Referral to nephrology is recommended in severe cases. The presence of urine eosinophils helps support the diagnosis but does not rule it out or exclude other causes. Renal biopsy may be indicated to clarify the diagnosis. Management for immune-related renal toxicities grade 2 or greater includes interruption/discontinuation of immune checkpoint inhibitor therapy and administration of high-dose systemic corticosteroids followed by a taper.

Cases of severe cardiac toxicities have been reported in patients receiving anti-CTLA-4 and anti-PD-1 therapies. In a case series of pooled data from six cancer centers, eight cases of immune-related cardiotoxicity were identified (Heinzerling et al. 2016). These included myocarditis, myocardial fibrosis, cardiomyopathy, heart failure, and cardiac arrest. Two cases were fatal. Other cardiovascular toxicities that have been reported in patients receiving anti-PD-1 therapy include angina, sinus tachycardia, hypertension, atrial flutter, ventricular arrhythmia, and asystole (Zimmer et al. 2016). Some may be indirectly related, such as hyperthyroidism, from thyroiditis that leads to a hyperdynamic state. In the asystole case, workup revealed moderately reduced left ventricular function resembling takotsubo cardiomyopathy. Cardiac biopsy in patients suspected of myocarditis can show interstitial inflammation with lymphocytes (predominantly CD8+ T cells) and fibrosis (Heinzerling et al. 2016; Laubli et al. 2015). Workup of suspected cardiac toxicity typically includes cardiac serologic markers, electrocardiogram, and echocardiogram. Further investigation may be necessary such as cardiac catheterization and biopsy to clarify the diagnosis. Prompt treatment with high-dose corticosteroids and discontinuation of immune checkpoint inhibitor therapy is indicated. Early escalation of care with further immune modulatory agents and transfer of care to a cardiac transplant center with experience in managing graft rejection may be necessary.

Another potentially serious but rare complication of immune checkpoint inhibitor therapy is the development of grade 3–4 neurotoxicities. Cases of meningoencephalitis and aseptic meningitis, necrotic myelopathy, Guillain-Barre syndrome (GBS), transverse myelitis, and chronic inflammatory demyelinating polyneuropathy have been

reported in association with ipilimumab treatment (Abdallah et al. 2016; Bot et al. 2013; Gaudy-Marqueste et al. 2013; Liao et al. 2014; Stein et al. 2015; Wilgenhof and Neyns 2011). Similarly, cases of peripheral neuropathy, seizure, myasthenia gravis, GBS, and multifocal central nervous system demyelination have been attributed to anti-PD-1 therapy (Zimmer et al. 2016; Mandel et al. 2014). Prompt recognition and management are critical in grade 3–4 situations. Corticosteroids should be administered and immune checkpoint inhibitor therapy discontinued. Plasmapheresis or intravenous immunoglobulin administration for myasthenia gravis, GBS, and chronic intermittent demyelinating polyneuropathy resistant to high-dose corticosteroid has been recommended.

Oncolytic Viral Therapy

Genetically modified oncolytic viral therapies for advanced melanoma have been under investigation for almost 20 years. Talimogene laherparepvec (T-VEC), a second-generation oncolytic virus (herpes simplex virus JS1 strain with compound deletion of its neurovirulent factor, ICP34.5, and a protein essential for immune evasion, ICP-47, with the gene producing human GM-CSF), was approved by the FDA in 2015 as the first oncolytic viral therapy for patients with melanoma based on a randomized phase III trial (OPTiM) (Andtbacka et al. 2015). Adverse events occurring more frequently in the T-VEC arm compared to subcutaneous GM-CSF were chills (49%), pyrexia (43%), injection-site pain (28%), nausea (36%), influenza-like illness (30%), and fatigue (50%). The incidence of grade 3 or higher treatment-related adverse events was 11% in the T-VEC arm compared to 5% in the GM-CSF arm. Cellulitis was the only grade 3–4 adverse event reported in more than 2% in T-VEC arm (2.1%). There were no patient deaths attributed to T-VEC in this study. Hematologic dissemination and urine shedding of the virus appear to be transient with no clear clinical impact based on earlier studies (Hu et al. 2006; Senzer et al. 2009). Serious immune-related adverse events similar to immune checkpoint inhibitor therapy have not been reported, although immune-mediated vitiligo has been observed with T-VEC. Management of severe adverse events is usually limited to drug hold and supportive care.

Conclusions

The toxicities associated with targeted and immune checkpoint inhibitor therapies in patients with advanced melanoma present unique challenges to providers and patients. Understanding when to interrupt or permanently discontinue therapy is dependent on the class of therapy, type of adverse event, and severity. With BRAF-targeted therapies, drug interruption and supportive care are indicated for some recurrent grade 2 and most grade 3–4 adverse events. Additional management is usually limited to supportive care. Patients can be rechallenged with reduced doses of BRAF and MEK inhibitors after improvement or resolution of the toxicities. On the contrary, grade 2 or higher irAEs associated with immune checkpoint inhibitor therapy require drug interruption or discontinuation and upfront corticosteroids in most cases. Without prompt initiation of corticosteroids, irAEs can escalate leading to fatal situations. In patients that do not respond to high-dose corticosteroids, additional immunomodulating agents are necessary. Upon improvement or resolution of irAEs, providers can consider rechallenging with the immune checkpoint inhibitor therapy in select situations. Dose reductions are not recommended. While T-VEC is generally well tolerated, patients may be treated in close temporal proximity to targeted and immune checkpoint inhibitor therapies where overlapping toxicities might exist.

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Evolving Role of the Oncology Nurse in the Care of Patients with Melanoma

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Contents

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Abstract

Over the past several years, treatment for melanoma has undergone a revolutionary change. A virtual explosion of scientific knowledge has given rise to a plethora of novel therapies as evidenced by the FDA approval of 14 new drugs and/or combinations since 2011. Patients have derived tremendous benefit from this, and the survival statistics have beneficially changed transforming a once uniformly fatal disease to one with the hope of sustained control, possibly even cure. However, the complex nature of melanoma makes it one of the most challenging malignancies to treat, and it is recognized that patients with melanoma have considerable unmet needs. This chapter will describe the evolving role of oncology nursing in the patient's journey through the melanoma disease spectrum. The various components of the nursing role will be illustrated in the context of the disease trajectory including that of communicator, patient advocate, educator, coordinator, and often as a coach for the patient and caregiver/family. Furthermore, the value of nursing presence on the interdisciplinary care team will be highlighted, as well as the ever-important role of toxicity management.

Keywords

Metastatic melanoma · Disease spectrum · Evolving role · Nursing care · Nursing role · Oncology nurse · Immune checkpoints · Immune toxicity · Multidisciplinary

Introduction

Melanoma is a notoriously aggressive cancer that can metastasize even at the earliest stages. It is a malignancy resistant to traditional anticancer therapies such as chemotherapy and radiotherapy. Fortunately, about 90% of melanomas are diagnosed as primary tumors without any evidence of metastasis (Garbe et al. 2016) and are likely cured with surgical intervention only. Decades of research have led to breakthroughs in treatment as the result of increased understanding of the underlying biology of melanoma and role of the immune system and cancer. This has resulted in the development of novel therapies dramatically improving the treatment landscape including the introduction of immune checkpoint inhibitors (ICIs) and molecularly targeted agents. As the field advances, there is excitement among the melanoma community. These novel therapies offer the promise of hope. Improved disease and survival outcomes are a reality, and the momentum of successes is driving the field forward. However, toxicities associated with these agents not only differ among class but differ from traditional chemotherapy, and management is often complex and time-consuming, with some patients requiring significant support (Lomax et al. 2017).

Despite the historic paradigm change, melanoma remains a challenging malignancy. Heterogenous in nature and complex in behavior, the clinical management of an individual with advanced melanoma often presents a clinical conundrum for treating physicians. Only a subset of patients will derive long-term benefit from ICIs, and for patients eligible for targeted therapy, the benefit does not continue beyond a year or two (Ugurel et al. 2017). Unanswered questions remain about ideal patient selection, sequencing, and best treatment approaches, notably whether all stage III or M1a patients should receive adjuvant therapy (Dimitriou et al. 2018). Predictive biomarkers have yet to be established. As such, a multidisciplinary approach to patient care is recommended, wherein individual patients are discussed among diverse specialists and a consensus decision regarding management is reached (Dummer et al. 2017).

Nurses are an essential member of the multidisciplinary team. They are often the most accessible, play a variety of roles, and provide support beyond care directly related to the illness (Cooper and de Lord 2018). Nurses play a unique role in the care and management of patients with cancer, one of the most important is providing knowledge and assistance, since patients can become overwhelmed by the care and information they receive. Thus, patients and families must be supported in times of their vulnerabilities (Cooper and de Lord 2018: Tariman et al. 2016).

Furthermore, with the emergence of new and novel treatments including ICIs and targeted therapies, it is essential that oncology nurses are knowledgeable and skilled in assessing toxicities and following agreed pathways in order to manage the complexity of various toxicities, leading to improved outcomes

Diagnosis

A diagnosis of melanoma is devastating. For most individuals, such a diagnosis unleashes a vast array of emotions. It is important to recognize approximately 30% of all patients diagnosed with melanoma, including long-term survivors, report levels of psychological distress, notably anxiety and depression (Vogel et al. 2017; Kasparian et al. 2009; Beesley et al. 2015; Tan et al. 2014). A sense of uncertainty frequently accompanies a diagnosis, not only for the patient but also the family and/or caregiver(s) as they face unfamiliar, complex, and potentially life-threatening experiences (Kasparian et al. 2016; Stamataki et al. 2015). It is not uncommon for multiple providers of various specialties to be involved in the care of patients with melanoma (e.g., dermatology, surgical oncology, medical oncology, radiation oncology, and occasionally plastic surgery). Each provider has a distinct role in the care; nonetheless, individual care is often uncoordinated, and it is easy for patients to become confused and overwhelmed if left to navigate a highly specialized but fragmented care system.

A component of the oncology nurse role is coordination among various providers. In addition, nurses support and assist patients as they learn to navigate the healthcare system, offer reassurance, answer questions, address concerns, and ensure patients' needs are met (Tariman et al. 2016). Every interaction provides an exchange of information which enables the nurse to get to know a patient and their caregiver(s) via assessment of their physical and emotional state, past health history, health practices, and beliefs. This exchange begins the formation of the ever-important therapeutic relationship establishing the foundation from which patient-centered care evolves.

Treatment of Melanoma

Surgery

Surgery is the most common treatment for melanoma and spans the spectrum from wide excision of a primary lesion with curative intent to palliative resection. It is the preferred treatment for local and regional melanoma. Fortunately for most patients presenting with a primary tumor, surgical resection will represent definitive treatment without the need for any further treatment. Despite a favorable prognosis, it is imperative providers be cognizant of the emotional, social, and psychological consequences that may result from cancer surgery. Findings from multiple qualitative studies reveal dissatisfaction with scar appearance of (Vogel et al. 2017; Kasparian et al. 2009; Tan et al. 2014), especially among patients who underwent resections on the head or neck as the scars prove more difficult to disguise with clothing (Vogel et al. 2017; Kasparian et al. 2009). The visible nature of cancer, associated scarring and/or disfigurement, and altered body image that may occur are identified as significant concerns expressed by melanoma patients (Tan et al. 2014; Oliveria et al. 2011). Stamataki et al. (2015) describe how, for some patients, altered body image was linked to a disparity in the pre- and postsurgery expectations of scar appearance. Respondents felt they had not been fully prepared for the reality of the scar appearance despite speaking to health professionals beforehand. Furthermore, the authors highlight disparity between doctors' perceptions of a healing scar and the language used to describe it, compared to patients' perceptions of their healing scar and language they (the patient) would use to describe it (Stamataki et al. 2015).

Nurses with awareness of these findings can minimize distress in the postsurgical setting by anticipating such concerns and importantly setting expectations about the scar and other physical sequelae. Including family/caregivers in these

discussions improves communication and patient/caregiver satisfaction. Interventions such as showing photos or thoroughly describing the process of wound healing can decrease distress about scar appearance (Cassileth et al. 1983). Anticipatory guidance about postoperative expectations for pain; need for medications, drain, and/or wound care; and recognizing complications are important information to provide for both patients and caregivers (Tan et al. 2014). Tailored information should be provided about what patients can expect in terms of functional capacity, limitations, or restrictions on activity (Tan et al. 2014) such as driving; lifting; ability to weight-bear; ability to work, drive, and care for children; etc. as these may negatively affect recovery if patients are not adequately prepared.

For patients whom sentinel lymph node biopsy (SLNB) is advised, reiterate that the procedure is a staging procedure and will involve a team of providers from both nuclear medicine and surgery. Some patients may benefit from a detailed description of how the procedure is performed, while others may want only minimal information; discussions should be tailored accordingly. It is essential, however, to ensure all patients understand the procedure objectives and rationale, and what to expect postoperatively, including when pathology results may be available and what they mean. Providing as much information as possible and setting expectations will minimize anxiety.

Historically, patients with a positive SLNB were advised to proceed with immediate completion lymph node dissection (CLND). Despite significant associated morbidity, the procedure was recommended because it provided increased regional disease control and additional prognostic information (Faries et al. 2017) such as number and extent of nodal involvement. However, recent evidence demonstrates a lack of a survival advantage with immediate CLND (Faries et al. 2017; Leiter et al. 2016). Findings from the Multicenter Selective Lymphadenectomy Trial (MSLT-II) (Faries et al. 2017) and from German Dermatologic Cooperative Oncology Group (DeCOG) (Leiter et al. 2016) demonstrate similar survival outcomes whether nodal dissection is immediate or when/if a nodal relapse is detected. These findings provide unequivocal evidence that not all patients with a positive sentinel node biopsy require CLND. These findings support a change in practice, not proceeding immediately to CLND but, instead, following closely with the aid of frequent nodal ultrasound, reserving nodal dissection for patients in whom clinically detected nodal recurrence had developed (Faries et al. 2017). Such practice spares those patients who *do not* relapse from a morbid (and unnecessary) surgery, and for those that *do* relapse, performing LND at that time of relapse does not negatively impact survival. Moreover, in the current era of effective adjuvant therapies (discussed below), foregoing immediate CLND facilitates earlier commencement of adjuvant systemic, augmenting the potential for long-term benefit.

For some patients, the idea of not pursuing CLND despite a positive sentinel node biopsy may be a difficult concept to grasp. Nurses must be prepared to provide support to patients and families through education and counseling, despite the prognostic significance of having an involved sentinel node(s). Preparing patients for potential outcomes through anticipatory guidance provided *prior to* the sentinel node biopsy procedure sets expectations. Early education prepares patients for what may be advised postoperatively; thus patients are better able to understand the recommendation for a "watch-and-wait" approach despite involved sentinel node(s). Reiterating the rationale for avoiding CLND to spare a potentially morbid surgery should be emphasized. Nurses should also stress the importance and rationale for ongoing patient self-examination as well as follow-up visits including the need for regular imaging (ultrasound to evaluate the nodal basin as well as systemic imaging to evaluate for distant relapse). For patients who do need lymph node dissection (LND), it is important they are prepared for the potential outcomes given the procedure can be associated with significant morbidities including development of seroma, infection, wound dehiscence, bleeding, deep vein thrombosis, and, notably, lymphedema (Ahmed et al. 2013).

Lymphedema is a significant health issue for cancer survivors with a considerable impact on

patients' health-related quality of life (Tan et al. 2014). The amount of edema can range from mild to severe and when left untreated can lead to skin changes, impaired function, loss of normal sensation, discomfort, pain, and chronic infections that affect the quality of life (Chang and Cormier 2013). Complete decongestive therapy (CDT) is the mainstay treatment for lymphedema and should only be performed by certified lymphedema therapist (Chang and Cormier 2013). Oncology nurses play a vital role in the prevention and management of this disorder by assessing for early signs and symptoms of lymphedema and prospectively identifying high-risk individuals. Early diagnosis is important because lymphedema is most successfully treated and complications minimized when therapies are introduced early (Chang and Cormier 2013). For example, patients who undergo inguinal LND are at greater risk of developing lymphedema compared with those who undergo axillary LND (Ahmed et al. 2013). Other factors contribute to increased risk including prior radiation to a lymphatic basin, development of a postoperative infection or seroma, and obesity (Ridner 2013). Obtaining preoperative limb measurements for high-risk individuals in comparing to postoperative measurements promotes early identification of lymphedema. Patients should be instructed to self-monitor for physical signs of lymphedema including noting the presence of edema during exercise, changes in skin texture, change in range of motion or skin tone, as well as sensation changes such as limb heaviness or numbness. During follow-up visits, query patients about "heaviness" or swelling area near resection site or if clothing or jewelry has become tighter (Ridner 2013). A positive response to the above inquiries should trigger a prompt referral to specialty care with a dedicated lymphedema specialist.

High-Risk Melanoma

The concept of "recurrence risk" is difficult for many patients to comprehend because they are disease-free but are being treated for a future statistical possibility of recurrence and death. Patients considered high risk include those with stage II and III disease. For patients with stage IIB/C disease [American Joint Committee on Cancer (AJCC) 8th edition] (between 2.0 and 4.0 mm with ulceration or >4.0 mm regardless of ulceration status), discussions regarding recurrence risk may be particularly difficult as they are often focused on the notion that "the cancer was removed, and there was no spread to lymph nodes" but are then surprised to hear about the underlying risk of relapse and death despite uninvolved nodes. Stage III disease represents a vastly heterogenous population encompassing resected nodal disease, as well as non-nodal locoregional sites (e.g., microsatellites, satellites, and in-transit metastases). Five-year survival rates vary greatly, from 93% for stage IIIA to 32% for IIID (Gershenwald et al. 2017). Treatment recommendations are based on stage and associated prognosis; thus patients may be faced with the decision to pursue adjuvant therapy, a difficult decision for many patients as they are unintentionally confronted with facing their own mortality.

Increasingly there has been a swing toward more active patient involvement in their own treatment making decisions. Stacey et al. (2010) found improved outcomes when patients were actively engaged in the decision-making process about cancer screening and treatment. This collaborative approach is known as "shared decision-making" (SDM). It is described by Tariman et al. (2016) as "the process of choosing between treatment alternatives or multiple treatment options. It is a complex process in which data are gathered and evaluated, information is exchanged between patients and clinicians, and a decision is mutually agreed upon." This approach implies that not only should patients be provided with the necessary information to make informed decisions about their healthcare but incorporates understanding about those factors that influence an individual's preferences and view on treatment based on their personal situation (Jansen et al. 2004). In other words, understanding the values of each individual patient enables providers to assess risk for that individual. To best support patients through this process, Jansen et al. (2004) emphasize the

importance of getting to know patients by taking the time to explore their willingness to accept side effects for a given therapy or to forego benefits of an alternative treatment. This decides the relative strength of their treatment preference. Each patient has a sense of what risk-benefit ratio is acceptable to them, and the threshold at which risk outweighs benefit will vary among individual patients as well as providers. Patients need information about what they can realistically expect in terms of toxicity, how it is managed, and how the treatment will impact his or her everyday life, as well as caregivers/family (Tariman and Szubski 2015). Case in point is the decision to pursue postoperative radiation in the setting of bulky, matted nodes noted upon LND. Weighing the risk of lymphedema against the benefit of increased local control may be a straightforward choice, but it needs to be clear to patients that the risk of systemic relapse is not reduced. Concern for disfigurement if a local relapse were to occur may influence one patient's decision to proceed with the radiation, while another patient may choose to forgo radiation due to the risk of lymphedema. Knowing a patient, encouraging that individual to take an active role in their own care, and advocating for their treatment preference will improve the quality of decision-making (Tariman and Szubski 2015). Shared decision-making contributes to shaping the patient experience. Cooper and de Lord (2018) call attention to the cancer patient experience being reported as on a par with clinical effectiveness [of treatment] in terms of importance.

Adjuvant Treatment

The landscape of adjuvant therapy has recently undergone a revolutionary transformation with results from two pivotal phase III clinical trials revealing practice-changing results (Eggermont and Dummer 2017). There has been a shift to PD-1 inhibitor therapy as the mainstay treatment for all patients, and for those with an identified v-Raf murine sarcoma viral oncogene homolog B (BRAF) mutation, treatment options may also include BRAF inhibitor-based therapy (with or without mitogen-activated protein kinase (MEK) inhibition (Eggermont and Dummer 2017)). The role of local therapy such as radiation or isolated limb perfusion/infusion remains unclear but may be appropriate in select instances. Adjuvant ipilimumab, on the other hand, has most certainly been replaced by nivolumab based on superior efficacy and an improved toxicity profile (Eggermont and Dummer 2017). Interferon too has also fallen out of form and will have little to no role in the adjuvant arena. Its utility should be limited to patients with ulcerated primary melanomas only, and this may remain the mainstay treatment in countries without access to the novel therapies (Eggermont and Dummer 2017). As the landscape continues to evolve, it is important that nurses remain knowledgeable about these therapies to appropriately counsel and support patients during their decision-making process. A brief review of currently available therapies will be discussed along with nursing considerations.

Locally Directed Therapy

Radiation

Adjuvant radiation may be recommended in select situations, for instance, in the treatment of patients with recurrent, in-transit, or nodal metastatic melanoma needing local control (Dimitriou et al. 2018; Garbe et al. 2016). Nurses provide support by ensuring patients and their family understand the goal of treatment and risks involved such as lymphedema and perform ongoing assessments of patient and caregiver coping. If applicable, nurses may facilitate referrals to the appropriate provider (s) such as to physical therapy for lymphedema management or to social work or other resources if a need has been identified by the patient or nurse. Nurses also aid in coordinating care among various providers, counsel patients, and family about proper skin care during radiotherapy and provide anticipatory guidance about treatment.

Limb Perfusion and Infusion

Isolated limb perfusion and isolated limb infusion (a simpler and less invasive procedure) are

therapies available for treatment of new or recurrent in-transit disease of an extremity. Both are highly specialized surgical procedures providing intravascular delivery of chemotherapy, most commonly melphalan, occasionally combined with tumor necrosis factor. These procedures are available only at select facilities with trained staff. For the most part, the direct care of patients receiving limb perfusion is delivered by perianesthesia and surgical nurses; however, oncology nurses should possess general knowledge and understanding of the procedures to provide support and anticipatory guidance before and after the procedure (Ashton 2012).

Systemic Therapy

Interferon

At present, there remains little to no role for use of adjuvant interferon. Instead, it has been replaced by more effective therapies such as anti-PD-1 agents and molecularly targeted therapies. In countries with only access to IFN, its use can be restricted to patients with ulcerated melanoma where the benefit is felt to be greatest (Eggermont and Dummer 2017).

Ipilimumab

Ipilimumab is a human IgG1 monoclonal antibody against cytotoxic T-lymphocyte antigen-4 (CTLA-4) and was approved by the FDA in late 2015 for use in the adjuvant setting. Known as an immune checkpoint inhibitor (ICI), ipilimumab is a novel therapy with a novel toxicity profile completely unlike traditional anticancer therapies. The unique mechanism of action directly leads to a characteristic and vast toxicity spectrum almost exclusively autoimmune-based referred to as immune-mediated adverse events (imAEs) or immune-related adverse events (irAEs). The dose of ipilimumab in the adjuvant setting (10 mg/kg) is higher than that approved in the metastatic setting (3 mg/kg). In the phase III trial, nearly half of the participants experienced severe (grade 3–4) toxicity and five deaths occurred from drug-related causes (Eggermont 2016) [Detailed discussion of specific ICI-related toxicity is discussed below in the section on checkpoints in metastatic disease.].

Nivolumab and Pembrolizumab

Nivolumab and pembrolizumab are human IgG4 monoclonal antibodies against programmed death 1 (PD-1). Like ipilimumab, nivolumab and pembrolizumab are known as immune checkpoint inhibitors (ICIs). Nivolumab was approved by the FDA in late 2017 for use in the adjuvant setting. The approval of nivolumab was based on data showing improved RFS and, notably, a lower rate of grade 3 or 4 adverse events when compared to adjuvant ipilimumab (Dimitriou et al. 2018; Eggermont and Dummer 2017). Checkmate 054, is a phase III trial enrolling patients with resected stage IIIA (limited to those with sentinel node tumor volume >1 mm) IIIB, IIIC, or IV and compared pembrolizumab to placebo. Findings demonstrated improved RFS, with a similar toxicity profile to that of nivolumab and no new toxicities (Eggermont et al. 2018). Based on these results reported in May 2018, pembrolizumab is expected to gain FDA approval sometime in the next year.

While generally well tolerated, the risk of severe and/or permanent toxicity makes the decision to recommend adjuvant immunotherapy a challenging one, a decision that should be reached together with the patient and family. It remains to be seen whether it is best to treat patients in the adjuvant setting versus waiting to see if relapse occurs. Data suggests that due to the primed immune system, toxicities may be greater in the adjuvant setting than in advanced disease setting (Napolitano et al. 2018). Conversely, a major advantage of adjuvant immunotherapy is the possibility to discontinue treatment and maintain antitumor responses. The immunological "memory" induced by the immunotherapy agent offers the potential for long-lasting, lifelong, therapeutic responses (Napolitano et al. 2018).

As with any adjuvant discussion, it must be made clear to patients that surgical resection alone may be curative. Adjuvant therapy recommendations are based *on a risk*: risk of relapse, notably risk of systemic (visceral) relapse. Local

relapse can be managed, for the most part, with additional surgical resection, but when the relapse is a systemic, management becomes complex. Each risk/benefit discussion must be based on the estimated *individualized risk* for a specific patient and should consider comorbidities or other related factors that may influence treatment outcomes (age, prior treatment, psychosocial issues, etc.) carefully weighed against the risk of both short- and long-term adverse events associated with PD-1 blockade (discussed in detail in the metastatic treatment section).

Given the lack of predictive and prognostic biomarkers or other means of ideal patient selection, questions will remain regarding best treatment approach. Until that time is reached, patients must be encouraged to be active participants in their care and treatment decisions and to partner with their oncology team to make decisions that are best for them. The process of shared decision-making is discussed in more detail below.

Dabrafenib and Trametinib

In April 2018, the FDA approved the combination of dabrafenib and trametinib (D/T) for the adjuvant treatment of patients with *BRAF* V600E- or V600 K-positive stage III melanoma following complete resection. This approval is the first oral molecularly targeted agents available as an adjuvant treatment option. The approval was based on results from the COMBI-AD trial, a phase III trial comparing D/T to placebo. Results demonstrate improved RFS without concern for additional toxicity than is seen in patients with unresectable stage III or stage IV (Long et al. 2017). Additional details and the nursing role in the care of patients receiving these agents will be discussed in the metastatic section below.

Future Considerations

Checkmate 915 is a phase III clinical trial, currently underway, comparing the combination of nivolumab plus ipilimumab versus nivolumab monotherapy in the adjuvant setting. The study enrolls patients after complete resection of stage IIIB/IIIC/IIID or stage IV melanoma, based on the AJCC 8th edition staging criteria. The rationale for this trial stems from the observation reported by Wolchok et al. (2017) that combination of CTLA-4 with PD-1 blockade augments the immune response when compared with each agent alone in metastatic melanoma (Dimitriou et al. 2018).

Follow-Up/Surveillance

Upon completion of definitive treatment (surgical or medical), recommendations for follow-up should be outlined and discussed with patients and their families. Nurses must be able to effectively convey the main objectives of follow-up care: early detection of relapse and identification of additional primary melanomas (Kurtz et al. 2017; Mrazek and Chao 2014; Garbe et al. 2016). An estimated 50% of recurrences in stages II and III melanoma are identified by clinical examination alone including physician detection, patient detection, or symptoms that prompt further testing (Kurtz et al. 2017). Of the relapses, approximately 50% recur in regional lymph nodes, 20% recur locally, and 30% recur at distant site(s) (Rueth et al. 2015). Ninety percent of relapses occur within 5 years (Garbe et al. 2016), with most relapses occurring within 2–3 years from diagnosis (Rueth et al. 2015; Kurtz et al. 2017). Early detection of recurrence is important as surgical resection of metastatic disease can provide a survival benefit in addition to the survival benefit of effective modern systemic therapy (Kurtz et al. 2017).

The survival advantage from early detection of relapses underscores the importance of patient education and establishing a strong patient-nurse relationship. Anticipatory guidance about what to expect at follow-up visits should be provided. Patients should know that a detailed history and review of symptoms is performed at every visit to identify early symptoms that may be suggestive of relapse. For the same reason, patients should be instructed to report any new symptoms that may develop in between visits. Instruction on skin and lymph node self-examination should be provided as well as counseling about proper UV/sun

protection (Garbe et al. 2016; Mrazek and Chao 2014). Nurses are the ideal member of the oncology team to provide this type of education. Nursing assessment of patient learning will help define best counseling methods and may include demonstration, video or web-based educational tools, or other strategies that best fit the patients' learning need. In addition, barriers to learning should be identified and addressed. Subsequent visits allow the nurse to reassess self-exam technique, and the visit also provides an opportunity for assessment of patient coping and their willingness to engage in self-care and evaluate adherence to follow-up recommendations.

In addition to history and physical exam, the use of surveillance imaging has an evolving role in management. Approximately 50% of asymptomatic recurrences are detected via imaging (Kurtz et al. 2017); however, there is controversy in defining optimal follow-up strategies (Kurtz et al. 2017; Garbe et al. 2016). Hence the frequency and intensity of follow-up should be determined based on estimated individual risk along with other factors, if present including history of multiple primary melanomas, the presence of clinically atypical nevi, family history of melanoma, patient anxiety, and the patient's awareness and ability to detect early signs and symptoms of disease (Garbe et al. 2016).

The period of follow-up, referred to by some as a time of "watch and wait," is often enormously challenging for patients (Boyle 2003) and family members. The so-called "active therapy" such as surgical resection or adjuvant therapy has been completed, and many patients report significant anxiety as they feel they are "waiting for the other shoe to drop." Numerous qualitative studies examining the supportive care needs of patients with melanoma demonstrate a consistent need for disease-specific information for both the patient and their caregivers, provided in a timely and easy to understand language (Beesley et al. 2015; Kasparian et al. 2016; Stamataki et al. 2015). Findings also reveal concerns including anxiety, fear of relapse, and what to expect if recurrence occurs (Stamataki et al. 2015; Beesley et al. 2015). Emotional support for physical symptoms (e.g., lymphedema, pain, fatigue) was also identified as important to respondents. Setting expectations, providing anticipatory guidance, and simply "being present" improve the understanding of disease management. It is also a time to clarify patient or family misunderstandings or misconceptions regarding diagnosis, prognosis, or treatment plans. Through active listening and encouraging patients to express underlying fears, concerns, and worries, nurses can assess patient coping level of distress and, when necessary, refer for emotional or psychological supports. As reported by Stamataki et al. (2015), patients describe improved outcomes when provided with an opportunity to meet individually for 1 h with a melanoma nurse specialist. Patients valued the time spent, reported feeling better informed, and experienced decreased stress and anxiety.

Recurrent Melanoma

Recurrent melanoma is devastating and may be accompanied by anxiety, fear, and, once again, uncertainty for the future. When a recurrence is suspected or confirmed, additional diagnostic and/or genomic testing may be required to ascertain the extent of the relapse and determine treatment options. The primary role of the oncology nurse is supportive: ensuring patients understand why certain testing is being performed, what results mean, and assessing the psychosocial impact. Tariman et al. (2016) emphasize that many patients are facing life-changing illness; are often overwhelmed and overburdened with the diagnosis, treatment decisions, and overall healthcare system; and thus should be supported in times of their vulnerabilities. Nurses ensure patients' needs are met, their concerns addressed, and their questions answered (Tariman et al. 2016).

Treatment of Metastatic Melanoma

Chemotherapy

Up until recently, a diagnosis of metastatic melanoma equated a dismal survival of less than 1 year and was considered an incurable malignancy

without effective treatments. Various chemotherapy regimens have been tried including dacarbazine, temozolomide, and carboplatin/paclitaxel among others. Responses are marginal at best, typically short-lived, and without demonstrated survival advantage. Combination regimens have slightly better responses but with increased toxicity. In the current arena, chemotherapy may be considered in second and third line in patients with resistance to immunotherapy and targeted therapy (Garbe et al. 2016). In the rare instance chemotherapy is used, the primary nursing role is providing support and education regarding expectations of treatment and toxicity management.

Interleukin-2

Interleukin-2 (IL-2), a cytokine, received FDA approval in 1998 for the treatment of unresectable or metastatic melanoma. It is historically one of the few treatments that could produce complete responses (CRs) that were often durable for decades without further therapy (Atkins et al. 1999). However, only highly selected patients with excellent organ function and performance status are candidates for treatment (Atkins et al. 1999) which entails 2 near-week-long inpatient admissions. Treatment is associated with significant toxicity often requiring intensive care level of support, and for that reason, availability is limited to specialized centers with highly trained staff expert in administration and management (Atkins et al. 1999). Given the historical lack of effective treatments for advanced melanoma, oncology nurses may have little experience in caring for this patient population. Instead, the experience may be limited to nurses in academic medical centers or institutions heavily involved in clinical research. In the community setting, care may be limited to patients receiving salvage chemotherapy and/or palliative/supportive care.

Immune Checkpoint Inhibitors

The integration of ICIs into clinical practice has led to a dramatic change in practice with the contemporary melanoma clinic strikingly different than a few years ago. The anti-CTLA-4 antibody ipilimumab and the PD-1 antibodies pembrolizumab and nivolumab are now what should be considered standard of care treatments. Their use either as monotherapy or in combination has redefined the meaning of long-term survival for a population of patients with little to no effective therapies. As the field continues to advance with newer therapies and combination approaches evolving, the ability to tailor treatment and improve quality of life continues to expand. As a group, oncologists tend to direct efforts to risk-benefit assessment and the ultimate treatment goal of improved overall and disease-free survival. Yet they caution that less attention directed toward safety assessments and delayed recognition of symptoms can lead to increased morbidity, prolonged hospitalizations, nonadherence, premature termination of treatment, and potentially lethal outcomes (Gordon et al. 2017; Kirkwood and Ribas 2017).

In what has now been deemed an era of immunotherapy, oncology nurses in a variety of settings are more likely to be involved in the care of patients with metastatic melanoma receiving ICIs. Early identification and management of toxicity are essential to achieving all treatment outcomes (Kirkwood and Ribas 2017). Dummer et al. (2017) foresee oncology nurses increasingly being integrated into the treatment processes and be given major responsibilities, especially in keeping regular contact with patients regarding irAEs. The authors also highlight that many patients feel more comfortable and are more likely to call a nurse than the physician; hence, nurses could provide open and easy contact with the treatment team. As such, nurses must possess a thorough understanding of how ICIs work and truly comprehend how distinctly different these agents are from traditional chemotherapy. Insight into when certain toxicities are more likely to appear, and which patients are at greater risk, will maximize prompt diagnosis. Published algorithms and consensus on guidelines for management of irAEs are available from organizations including European Society of Medical Oncology, Society for Immunotherapy of Cancer, and American Society of Clinical Oncology-National

Comprehensive Cancer Network. Nursing-specific information is available through the Oncology Nursing Society and the Society for Immunotherapy of Cancer, and an entire nurse-centric educational initiative was developed by the Melanoma Nursing Initiative. Nurses should be familiar with these valuable multimodality resources which offer tools, strategies, and interventions to maximize patient care, including best practices for telephone triage, a critical skill for nurses caring for these patients.

Nurses cognizant of the wide spectrum of toxicities associated with ICIs will anticipate and recognize subtle signs and facilitate appropriate intervention. Due diligence warrants thoughtful assessment of symptoms. Once an irAE has been identified, a suitable intervention will depend on the severity, or grade, as defined by the most current version of Common Toxicity Criteria for Adverse Events (CTCAE). This process allows for interpretation of subjective symptoms in an objective manner (Rubin 2017a) by employing a grading system from 1 (mild) to 5 (death) to represent symptoms defined by specific parameters based on the organ system involved (National Cancer Institute (NCI) 2017). Interventions are based on the assigned grade; therefore, nurses must be able to identify and grade symptoms. A key strategy is establishing and documenting patients' "baseline" health. It must be made clear to patients how important it is to report any change in baseline health, no matter how subtle or seemingly insignificant (McGettigan and Rubin 2017). It is imperative patients understand toxicity from ICIs can be atypical in presentation and easily overlooked. While most toxicities occur within the first 4 months of therapy, they can occur at any time, including during and after completion of therapy (Puzanov et al. 2017; Weber et al. 2015).

The most common irAEs affect the skin (rash, pruritus), gastrointestinal organs (diarrhea, colitis), endocrine, and hepatic systems; these are discussed below. Neurologic events occur with less frequency (Friedman et al. 2016) but may have dire consequences if not identified. Toxicity from ipilimumab appears to be dose related (Michot et al. 2016; Weber et al. 2015). Notably, the approved dose of ipilimumab in the metastatic setting is 3 mg/kg, while the approved dose in the adjuvant setting is 10 mg/kg. Nurses must be mindful of the differences in doses when educating patients and families regarding treatment and when assessing for toxicity. It is not unusual to see an earlier onset of toxicity with higher [10 mg/kg] doses of ipilimumab (Madden and Hoffner 2017). Importantly, nurses must keep in mind the incidence of irAEs is higher with combination of ipilimumab plus nivolumab with either agent alone (Wolchok et al. 2017).

Unlike traditional cancer therapies, dose reductions are *not* strategies employed as management of ICIs. Generally, mild irAEs are managed conservatively without the need for treatment interruption; however, close monitoring is necessary to assess for increasing severity. Persistent mild or moderate toxicity requires initiation of oral corticosteroids and a hold of the immunotherapy and, for the most part, should be manageable on an outpatient basis (Friedman et al. 2016; Weber et al. 2015). Severe or life-threatening irAEs require immediate hospitalization, in some cases intensive care, and prompt initiation of high-dose intravenous (IV) corticosteroids and permanent discontinuation of the immunotherapy. In rare instances, toxicities such hepatitis or colitis not responding to IV steroids after 3–5 days require additional immunosuppression such as mycophenolate mofetil or infliximab (Weber et al. 2015; Friedman et al. 2016). For many of the common toxicities, algorithms have been developed with detailed management guidelines to help clinicians manage and treat the most common irAEs. The key management strategies employed by oncology nurses include ongoing assessment and vigilant toxicity screening. Bottom-line, prompt intervention is crucial as outcomes depend on how quickly irAEs are recognized, reported, and treated.

The potential for toxicity underscores the importance of open communication among the patient, family, and treating oncology team. Patient education is paramount. Ideally, every patient has a pretreatment comprehensive education session with the nurse or nurse practitioner.

This session would be dedicated to the provision of ICI-specific education tailored to individual patients including a discussion of safe sexual practices, pregnancy avoidance, and fertility preservation for appropriate patients with referral to a fertility specialist as necessary *prior to* starting treatment. Toxicity-specific counseling includes direction about when patients should call, how to call (e.g., how to contact the provider outside of office hours), and specifically what to say when they call. This is of particular importance if on-call providers are not familiar with ICI toxicity. Further complicating such a scenario is if patients are not taught to correctly refer to treatment by the drug name or as "immunotherapy" and instead refer to it as "chemotherapy." Interventions for "chemotherapy-related diarrhea" vastly differ from interventions for "ipilimumab-related diarrhea." Such detail is not trivial.

The pretreatment visit also offers opportunity for nurses to assess patients' understanding of their disease, recommended treatment, and goals of care and whether they demonstrate a clear understanding of risks and benefits of treatment, including the risk of serious toxicity. It is also a chance for nurses to identify potential barriers to treatment or adherence (e.g., lack of physical resources such as transportation or telephone) (Madden and Hoffner 2017) and to identify comorbid conditions that may negatively affect treatment outcomes (e.g., current or prior history of serious mental illness, cognitive deficit, substance abuse, underlying autoimmune disease).

Specific irAEs

Skin-related adverse events are the most common and typically the earliest to develop (Puzanov et al. 2017; Weber et al. 2015; Friedman et al. 2016). Nurses must educate patients about cutaneous irAEs, implement self-care strategies for at-risk individuals, and minimize the severity of irAEs that do develop (Madden and Hoffner 2017). Rash and/or pruritus are seen in approximately half the patients (Weber et al. 2015) and are typically mild to moderate in severity. Symptoms are managed conservatively with over-the-counter moisturizers, antihistamines, and topical corticosteroids. Pruritus is managed conservatively with moisturizers and antipruritus medications as the mainstay. However, pruritus can be very distressing and therefore requires prompt intervention and aggressive management even in the absence of rash. Grade 3 dermatitis requires oral or IV systemic corticosteroids, and grade 4 symptoms require hospitalization with the initiation of intravenous (IV) corticosteroids.

Nurses should encourage patients to employ proactive prevention strategies including skin hygiene with regular use of a gentle skin cleanser (non-soap), moisturizing once or twice daily with nonsteroidal emollients or creams, vigilant sun protection, and treating existing xerosis (McGettigan and Rubin 2017). In some cases, providing a prescription for a topical corticosteroid can expedite treatment but will require specific instructions for use. Ongoing assessment of cutaneous toxicity is necessary as serious events have been reported including Stevens-Johnson syndrome and toxic epidermal necrolysis (Puzanov et al. 2017; Friedman et al. 2016). Nurses play a crucial role in counseling patients, providing individualized interventions, assessing patient and caregiver understanding, assessing treatment adherence and barriers to treatment, and assessing skin integrity. Dermatologic toxicities can have a detrimental impact on quality of life. Pruritus can be incredibly distressing and be distracting to patients (McGettigan and Rubin 2017); therefore proactive and aggressive management of cutaneous toxicity including early referral to dermatology will improve outcomes.

Gastrointestinal (GI) effects are associated with ICIs. Diarrhea is one of the most common GI presentations. Abdominal pain, when accompanied by the presence of mucus and/or blood in the stool, is suggestive of colitis (Madden and Hoffner 2017). Recognizing symptoms early is critical to minimize the risk of bowel obstruction and/or perforation or other grave complications. Awareness that colitis is commonly seen 4–6 weeks from treatment start and is more likely in patients receiving ipilimumab (Weber et al. 2015) enables nurses to individualize assessments and education. Establishing patients' baseline

bowel pattern is crucial as a change from baseline may be indicative of evolving toxicity. Therefore, patients should be queried about change in bowel pattern, consistency or frequency, and/or other symptoms that may be indicative of evolving GI toxicity such as reflux, chance in appetite, abdominal pain, or cramping. A focused and detailed review of systems should include assessing for a recent change in diet, recent travel, and taking the time to fully review and reconcile each patient's medication list. Patients should be queried about all medications including prescription, over the counter, vitamins, minerals, herbals, and any type of supplement both current and recent. Due diligence warrants thoughtful assessment of symptoms and can sometimes reveal simple etiology of seemingly big problems. Case in point: a patient receiving anti-PD-1 monotherapy calls shortly after the second infusion to report diarrhea for 2 days. Via directed questioning and ROS, it was apparent the symptoms were a result of overuse of laxative stool softeners resulting in symptoms mimicking colitis. The patient had not mentioned he was taking the bowel agents as he did not consider them "medications."

Autoimmune hepatitis is a less common but notable toxicity because the majority of clinical presentations are characterized by asymptomatic elevations in liver function tests (LFTs) including aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) and may or may not include elevations in bilirubin (Puzanov et al. 2017; Friedman et al. 2016; Weber et al. 2012). Rarely, patients may report nonspecific symptoms such as fever, fatigue, nausea, and abdominal pain making a prompt diagnosis more challenging. However, the knowledge that median onset of hepatitis is approximately 6–14 weeks after starting therapy and is most commonly seen in patients receiving combined anti-CTLA-4 and anti-PD-1 provides insight (Puzanov et al. 2017; Friedman et al. 2016) and enables nurses to facilitate expedited work-up by narrowing down vague symptoms. Nurses should ensure baseline LFTs are obtained before starting ICI therapy and reassessed prior to each cycle. Recognition of even mild increases from baseline, when low grade, should prompt intervention such as querying patients more in-depth about alcohol intake and use of hepatoxic medications (Friedman et al. 2016; Madden and Hoffner, 2017). As applicable, patients should be instructed to abstain from alcohol, avoid or minimize acetaminophen, and consider withholding statin. Repeating LFTs a few days later to determine trajectory is recommended. Higher-grade elevations require withholding the ICI and corticosteroids started. Abdominal imaging and/or liver biopsy may be warranted in certain instances (Puzanov et al. 2017; Weber et al. 2012), and referral to hepatologist or GI specialist may be beneficial (Madden and Hoffner 2017; McGettigan and Rubin 2017).

Pneumonitis is a rare but potentially fatal irAE seen with ICIs that can be fatal if not recognized early (Friedman et al. 2016; Puzanov et al. 2017). It is more prevalent with PD-1 inhibitors than with ipilimumab, but it is most likely to occur with combination of CTLA-4 and PD-1 inhibition (Friedman et al. 2016). Onset tends to be later than other irAEs, occurring several months after the start of treatment, though it may occur at any time (Friedman et al. 2016; Madden and Hoffner 2017). Presentations vary; symptoms include dyspnea, dry cough, tachypnea, tachycardia, fatigue, or less commonly fevers and chills (Puzanov et al. 2017; Friedman et al. 2016; Weber et al. 2015). When pneumonitis is suspected, a chest CT scan is indicated (Friedman et al. 2016; Michot et al. 2016). Importantly, clinical and radiographic findings may closely mimic pneumonia or disease progression (Puzanov et al. 2017) which may lead to improper treatment. Findings may be described as "ground-glass opacities" or "multifocal consolidation." Any new findings suggestive of an infiltrative process predominately in the lower lobes (Michot et al. 2016; Friedman et al. 2016) should be presumed to be pneumonitis in patients receiving (or having received) ICIs.

Treatment for pneumonitis is based on severity and includes oral or IV corticosteroids. ICI will be held. In some cases, bronchoscopy with or without lung biopsy may be performed to exclude infectious etiologies before starting immunosuppression. In severe cases, patients should be hospitalized, and treatment with high-dose

corticosteroids (e.g., methylprednisolone 2–4 mg/kg/d) should be initiated. For refractory cases, additional immunosuppression, including mycophenolate mofetil, cyclophosphamide, and infliximab, can be administered. Severe cases require permanent discontinuation of ICIs (Friedman et al. 2016; Michot et al. 2016; Weber et al. 2015).

Patient outcomes are influenced by vigilant nursing assessment as early management is associated with improved prognosis and reduced morbidity and mortality (Gordon et al. 2017). Baseline oxygen saturation should be documented and reassessed at every visit. Hypoxia (pulse oximetry <90%) is reported as a presenting symptom of pneumonitis (Weber et al. 2015; Friedman et al. 2016); however, nurses with knowledge of the spectrum of irAEs with ICIs would heed subtleties such as a slight decrease in oxygen saturation (rather than a decrease to <90%) resulting in diagnosis when low grade. Knowing patients allows nurses to identify a patient report of only being able to play a 9-hole round of golf rather than his usual 18 holes as a red flag, once again, possibly identifying pneumonitis when low grade. The ongoing, directed, and focused questioning as part of a nursing assessment allows for identification of seemingly innocuous signs and symptoms suggestive of evolving toxicity that otherwise would be easily overlooked. The key is maintaining a high index of clinical suspicion in any patient treated with ICIs (Gordon et al. 2017; McGettigan and Rubin, 2017) and the realization that attention to detail cannot be underestimated.

Endocrine-related toxicities, or endocrinopathies, tend to appear after the sixth or seventh week of treatment, with a median time to onset of 7–20 weeks (González-Rodríguez and Rodríguez-Eberu 2016). They occur in up to 1/3 of patients treated with ICIs (Alessandrino et al. 2018) and include thyroid dysfunction, hypophysitis, primary adrenal insufficiency (AI), and autoimmune diabetes mellitus (DM). Awareness is key. Endocrinopathy is outside of the typical side-effect profile of chemotherapies. If not identified early and promptly treated, endocrinopathies can progress and pose serious, possibly life-threatening consequences as in the case of adrenal insufficiency or adrenal crisis. However, nurses with awareness and understanding of these distinct toxicities can mitigate risk through vigilant screening, triage, and ready strategies to facilitate expedited work-up ensuring correct diagnosis and management. The result is decreased morbidity and increased likelihood of patients staying on treatment (Madden and Hoffner 2017; González-Rodríguez and Rodríguez-Eberu 2016).

Hypophysitis and thyroid dysfunction are the most common of the endocrinopathies (Alessandro et al. 2018). Nurses are frequently the primary and constant contact for patients (Sznol et al. 2017); therefore nurses must be mindful as hypophysitis can be challenging to recognize as signs and symptoms are often subtle, nonspecific in presentation (Alessandro et al. 2018), and can mimic symptoms common to patients with advanced cancer such as headaches, fatigue, nausea, and/or vomiting. It is most common in older males and in patients treated with combination of anti-CTLA-4 plus anti-PD-1. Incidence with anti-CTLA-4 is dose dependent; a higher incidence is seen in doses >3 mg/kg (Alessandro et al., 2018; González-Rodríguez and Rodríguez-Abreu 2016). Hypophysitis is rare with PD-1 monotherapy. If suspected, nurses should ensure treatment is held and facilitate obtaining proper labs and magnetic resonance imaging (MRI) with pituitary cuts. Results of hormone studies and MRI will confirm the diagnosis. Adrenal insufficiency or crisis is a potentially life-threatening condition. Patients require immediate intervention with stress-dose corticosteroids (e.g., hydrocortisone 100 mg IV immediately, followed by 50–100 mg every 8 h), hydration, and supportive care (Gordon et al. 2017; Sznol et al. 2017).

Management of hypophysitis includes decreasing the pituitary inflammation with steroids, which are then slowly tapered; and at the same time, hormone replacement of affected hypothalamic-pituitary axes should be started when a deficiency is present (cortisol, thyroxine, and testosterone/estradiol) (Iglesias 2018). For the most part, once side effects are controlled, and steroids are tapered to <10 mg prednisone or

equivalent per day, ICI therapy may be restarted (Iglesias, 2018; González-Rodríguez and Rodríguez-Abreu, 2016). These patients are usually followed by endocrinology with oncology nurses serving as a liaison between the patients, oncologists, and various specialty providers (Sznol et al. 2017). Patients and their families may require a great deal of support during this time given the often complex nature of endocrine toxicity and need for specialty involvement. Nurses provide emotional support, assess patient understanding and coping, and provide anticipatory guidance regarding disease and toxicity management.

Thyroid dysfunction or thyroiditis is seen more frequently with PD-1 antibodies than ipilimumab and more commonly in women (González-Rodríguez and Rodríguez-Abreu 2016; Alessandro et al. 2018). However, the incidence is highest in patients receiving combination of anti-CTLA-4 plus anti-PD-1. Thyroiditis manifests most commonly as hypothyroidism and less commonly hyperthyroidism. Median onset of hypothyroidism ranges from 1 to 5 months sometimes following a brief period of hyperthyroidism. Treatment involves replacing thyroid hormone (e.g., levothyroxine), while hyperthyroidism is treated with β-blockers in symptomatic cases, followed by levothyroxine for hypothyroidism that develops later (Sznol et al. 2017).

Type 1 diabetes results from complete insulin deficiency caused by autoimmune destruction of pancreatic beta cells (González-Rodríguez and Rodríguez-Abreu 2016). It has been reported in patients receiving PD-1 inhibitors as well as in patients receiving combination of anti-CTLA-4 plus anti-PD-1 (Iglesias 2018). Management requires insulin therapy. Nurses should ensure baseline glucose level is obtained and repeated regularly. Typically, these patients are referred to endocrinology or to a diabetes specialty provider (Madden and Hoffner 2017).

Unique from other irAEs, endocrinopathies typically do not resolve because the function of the gland rarely recovers. Lifelong hormone replacement is therefore required (Iglesias 2018; Sznol et al. 2017). Patient counseling must be provided regarding "sick-day rules" of steroid dosing for medical procedures or acute illness (fever or cases of nausea, vomiting, and diarrhea), and patients should be encouraged to obtain a medical alert necklace or bracelet (Sznol et al. 2017). In some cases, providing patients with a prescription and instructions for use of hydrocortisone emergency injections may be beneficial (González-Rodríguez and Rodríguez-Abreu 2016). It is also prudent to assess for barriers to medication adherence (e.g., inability to take oral medication, cognitive dysfunction, lack of caregiver resource, financial problems that may impact inability to afford medication). Due to the risk of adrenal crisis, medication adherence is critical (González-Rodríguez and Rodríguez-Abreu 2016).

Arthralgias and inflammatory arthritis are reported by approximately 10% of patients receiving ICIs, particularly PD-1 inhibitors, and those receiving combination of PD-1 and anti-CTLA-4 inhibition (Madden and Hoffner 2017). A thorough medical history and review of symptoms will highlight patients at greater risk including those with underlying joint inflammation from prior injury or overuse (tendonitis, bursitis) or those with underlying rheumatologic disorders such as polymyalgia rheumatica or rheumatoid arthritis. For example, a patient with a history of a rotator cuff injury, even decades prior, may develop recrudescence of tenderness, pain, or discomfort during treatment with ICIs. Since delayed diagnosis and treatment can lead to long-term disability, and disorders may become chronic and require ongoing immunosuppressive/immunomodulatory therapy, it is important to understand typical symptom presentation and recommended management (Puzanov et al. 2017).

Treatment is directed at managing the inflammation with resultant pain, maintaining or improving physical functioning and ability to perform activities of daily living (ADLs), and decreasing the impact on quality of life (Madden and Hoffner 2017; McGettigan and Rubin 2017; Puzanov et al. 2017). Most patients will find conservative management strategies effective; nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, or topical diclofenac gel applied to localized or limited sore joints, or for those who cannot tolerate

NSAIDs (Madden and Hoffner 2017; McGettigan and Rubin 2017). In some cases, low doses of prednisone may be necessary (Michot et al. 2016).

Rheumatologic consultation should be initiated for patients in whom symptoms do not improve with conservative management or for those where mobility or quality of life is impaired. Nurses should assess patients at risk of fall and implement safety strategies. Low-intensity physical activity should be encouraged to improve physical conditioning and sleep and decrease pain perception (Madden and Hoffner 2017; McGettigan and Rubin 2017).

Xerostomia and mucositis are less common rheumatologic irAEs seen with ICIs. Sicca syndrome with severe eye and mouth dryness and parotitis (Puzanov et al. 2017) and Sjögren's syndrome have been reported (Michot et al. 2016). Importantly, even mild symptoms may have a negative impact on quality of life and affect day-to-day functioning such as eating. Even mild symptoms can lead to more serious sequelae such as decreased oral intake, weight loss, or aspiration. Withholding the ICI may be indicated and will be determined on an individual basis. Prior to starting treatment, nurses should screen patients for preexisting symptoms, notably xerostomia, from prior surgery such as parotid dissection or parotid radiation. Review of medication list may reveal patients at risk including those with concomitant medications known to cause or contribute to xerostomia such as antihistamines. Because of the impact on quality of life, proactive and aggressive symptom management should be instituted by nurses. For dry eyes, lubricating eye drop (Michot et al. 2016) should be encouraged and consideration should be given to ophthalmological consultation if symptoms are impacting vision or for patients who wear contact lenses. Xerostomia and mucositis treatments may overlap and include oral moisturizing agents such as saliva substitutes or synthetic saliva and/or secretagogues both non-pharmacologic (sugarless gum, sour candies). If ineffective, nurses may advocate for pharmacologic agents (pilocarpine, cevimeline hydrochloride) depending on the severity of symptoms (Michot et al. 2016; Madden and Hoffner 2017; McGettigan and Rubin 2017). Patient education includes counseling regarding vigilant oral hygiene: increasing frequency of brushing to every 4 hours and at bedtime using a soft toothbrush, daily flossing, avoidance of alcohol-based mouthwashes, proper fit of dentures (if applicable), and regular dental exams. For patients unable to tolerate brushing, chlorhexidine gluconate 0.12% or sodium bicarbonate rinses (1 teaspoon baking soda in 8 ounces of water or 1/2 tsp. salt and 2 tablespoons sodium bicarbonate dissolved in 4 cups of water) can be effective. Encourage patients to sip cool water or crushed ice. Soft, bland, nonacidic foods are better tolerated. Advocate for use of pharmacologic agents for appropriate patients (Gelclair® and Zilactin®, 2% viscous lidocaine applied to lesions 15 minutes prior to meals, 2% morphine mouthwash, and 0.5% doxepin mouthwash or "miracle mouthwash" of diphenhydramine, lidocaine, and simethicone may be effective). Corticosteroid rinse (dexamethasone oral solution) can also be effective but will require prophylactic treatment for candidiasis such as weekly fluconazole (Madden and Hoffner 2017; McGettigan and Rubin 2017).

Neurologic toxicity is rare but can be seen with both anti-CTLA-4 and anti-PD-1. A range of neurologic conditions has been reported including Guillain-Barré syndrome, myasthenia gravis, encephalitis, motor dysfunction, meningitis, demyelination, neuropathy, and nerve paresis (Michot et al. 2016; Friedman et al. 2016; Weber et al. 2015; Puzanov et al. 2017). Because symptoms can be subtle, overlap, or be misinterpreted by patients, nurses should perform focused assessments and direct questioning at every visit to evaluate for changes from baseline neurologic functioning. Prompt referral to a neuromuscular specialist is indicated as accurate diagnosis can be delayed if not identified early leading to poorer outcomes (Madden and Hoffner 2017). Depending on presentation, patients may require neuroimaging, nerve conduction studies, and, potentially, nerve or muscle biopsy to arrive at a diagnosis (Puzanov et al. 2017). Once again, the key to prompt recognition of possibly early toxicity is maintaining a high index of clinical suspicion in any patient treated with ICIs (Gordon et al. 2017; McGettigan and Rubin 2017).

Nephritis, another rare toxicity, should be considered with an increase in creatinine. Nurses should ensure baseline renal function is documented and take note of change from baseline and notify the treating provider. Vigilant nursing assessment can reveal subtle changes in renal function enabling early intervention to minimize the risk of high-grade toxicity. Focused and detailed nursing assessment may reveal modifiable risks such as the use of NSAIDs or inadequate oral hydration around the time of routine scans. Appropriate counseling and intervention may mitigate such risks, thus optimizing treatment. However, a 1.5-fold increase from baseline creatinine or development of proteinuria requires further evaluation, referral to nephrology, and consideration of renal biopsy (Puzanov et al. 2017). Nurses should anticipate the use of high-dose corticosteroids to prevent late damage to the kidneys, need for IV hydration, and avoidance or modification(s) to nephrotoxic medications and contrast media (Madden and Hoffner 2017; McGettigan and Rubin 2017).

Ocular and orbital toxicity includes uveitis most commonly, and others include conjunctivitis, blepharitis, iritis, keratitis, scleritis, episcleritis, vitritis, choroiditis, and serous retinal detachment (Michot et al. 2016; Puzanov et al. 2017; Madden and Hoffner 2017). Symptoms include eye pain, redness, photophobia, excessive tearing, floaters, and decreased visual acuity (Gordon et al. 2017). Awareness that ocular toxicities are frequently accompanied by irAEs in other systems, especially colitis (Puzanov et al., 2017), improves vigilance. Prompt ophthalmologic evaluation is required.

Cardiotoxicity includes myocardial fibrosis, pericarditis, cardiomyopathy, heart block, and myocarditis. Though exceedingly rare, several cases of fatal myocarditis have been reported in patients treated with ICIs. Data reveals incidence and severity is greatest in patients who received combination ipilimumab and nivolumab (Mahood et al. 2018; Varricchi et al. 2017; Johnson et al. 2016) compared with monotherapy regimens. Overall incidence is reported from <1% (Johnson et al. 2016; Puzanov et al. 2017) to 2.4% with anti-PD-1/anti-CTLA-4 combined therapy (Mahood et al. 2018). However, Johnson et al. (2016) assert the true incidence of early and late cardiotoxicity is unknown and likely underestimated because routine cardiac monitoring with EKG and/or assessing troponin levels was not routinely performed in most of the immunotherapy trials (Varricchi et al. 2017).

Findings reported by Mahmood and colleagues (2018) provide important clinical feature clinicians to be aware with regard to myocarditis. These include time to onset of myocarditis from first ICI was 34 days, 81% of patients presented with 3 months of starting therapy, nearly all cases of myocarditis (94%) had an elevated troponin, and 89% had an abnormal ECG, both of which were normal prior to starting therapy. Furthermore, they also emphasize of the 51% of patients with myocarditis, LVEF was normal. Based on these findings, the authors do not recommend pretreatment screening EKG or ECHO; however, they do advise obtaining baseline troponin. Nurses, therefore, anticipate and ensure baseline troponin levels are obtained as part of the pretreatment laboratory assessment on all patients commencing ICI-based therapy.

Clinical suspicion must be maintained by nurses when patients report vague symptoms such as fatigue, weakness, muscle pain, or syncope, with higher suspicion with reports of more typical cardiac symptoms such as chest pain, shortness of breath, lower extremity edema, or palpitations. Patients with underlying cardiac dysfunction require careful monitoring as do patients with evidence of myocarditis, vasculitis, or myositis. Weights should be monitored. For patients presenting with symptoms or concern for evolving cardiotoxicity, nurses should anticipate obtaining N-terminal pro-B-type natriuretic peptide (NT pro-BNP), troponin, and EKG and assisting in the prompt referral to specialty care (Johnson et al. 2016; Madden and Hoffner 2017). Cardiac MRI and/or cardiac biopsy may be requested. The ICI will likely be withheld and, more likely, will be permanently discontinued depending on the severity.

Molecularly Targeted Therapies

The integration of molecularly targeted agents into the therapeutic landscape for melanoma has contributed to the successes seen not only in metastatic disease but also now in the adjuvant setting. Combination BRAF/MEK is now considered a standard treatment option for patients with unresectable metastatic melanoma with an identified BRAF V600E or V600 K mutation (Daud and Tsai 2017). At this time there are two combination regimens approved by the FDA: dabrafenib/trametinib and vemurafenib/cobimetinib. A third combination, encorafenib/binimetinib, has completed phase III trials with results demonstrating improved ORR and PFS as well as what appears to be a more favorable toxicity profile (Dummer et al. 2017; Daud and Tsai 2017; Flaherty 2017). Data is currently being evaluated by the FDA and is expected to be approved in summer 2018.

As with every cancer therapy, patient education is a fundamental component of the oncology nurse role. With targeted therapy, however, the need for counseling starts way before a treatment plan has been formulated. Anticipatory guidance about targeted therapies includes conveying the guiding principle of this treatment: the presence of a BRAF mutation. Identification of a BRAF mutation within the tumor by an FDA-approved test is critical to ensuring a patient is receiving appropriate therapy and education begins with this concept. It should also be made clear that a BRAF mutation is an acquired mutation, not somatic, as many patients fear a positive BRAF result implies increased risk to offspring.

There are currently two approved mutation tests: cobas® 4800 BRAF V600 which identifies V600E mutations (for vemurafenib plus cobimetinib) and THxID™ which identifies both V600E and V600K (for dabrafenib plus trametinib). Some institutions may use other assays based on sequencing methods due to improved sensitivity and ability to analyze multiple genes; however, insurance coverage may vary. Because BRAFi are contraindicated in patients with wild-type tumors as they may promote tumor growth by activating MAPK pathway signaling (Czupryn and Cisneros 2017; Rubin 2017b), nurses must be prepared to field questions regarding testing. Nurses are in key positions to anticipate and expedite specific BRAF testing (or retesting), necessary for intended treatment (Czupryn and Cisneros 2017). This is especially important for patients with aggressive disease or those with a heavy symptom burden who need to begin therapy as soon as possible. Minimizing delays may be a matter of life or death and is therefore of the utmost importance.

Targeted agents are administered until disease progression or unacceptable toxicity. The goal of treatment is to prolong survival with minimal impairment of quality of life; therefore, drug-related AEs require prompt management to ensure patients derive optimal benefit from therapy (Daud and Tsai 2017). BRAF and MEK inhibitors are generally very well tolerated. Several AEs associated with BRAFi or MEKi (e.g., fatigue, nausea, diarrhea) are common with other cancer treatments and have well-reported management strategies; however, there are distinct, class-specific AEs of BRAF inhibitors, MEK inhibitors, as well as characteristic AEs seen with combination BRAFi/MEKi (Czupryn and Cisneros 2017; Daud and Tsai 2017; Rubin 2017b). Anticipating, continuously assessing, promptly recognizing, and managing AEs are a key role of oncology nurses and require a comprehensive understanding of the MOA and etiology of AEs. Nurses with in-depth knowledge of the AE profiles and a thorough understanding of which AEs are most likely attributed to the BRAFi or MEKi possess skills necessary for early identification of AE prompt intervention, thus resultant improved outcomes as well as patient satisfaction.

Targeted therapies are oral agents, thus offering more convenient administration compared with injectable therapies. Furthermore, oral delivery allows alterations in treatment – interruptions and dose reductions – to be made readily (Flaherty 2017). Nonetheless oral delivery has its own challenges that may hamper appropriate use (Czupryn and Cisneros 2017), most notably patient adherence. Nurses improve outcomes by simply anticipating, assessing, and addressing both real and perceived barriers that may influence proper

administration and dosing. Barriers include delays in accessing drugs from specialty pharmacies, high copays, difficulties following complex dosing regimens, lack of social supports, reluctance to report AEs, and challenges associated with managing AEs (Kottschade and Reed 2017). When educating patients, nurses must include counseling on proper dosing and administration and drug and food interactions and provide anticipatory guidance about AEs. Counseling about safe sexual practices including pregnancy avoidance for appropriate patients should be a component of the education. Importantly, dabrafenib can interact with hormonal contraceptives (oral pills, injections, or patches); thus when necessary, patients should be referred to their primary care physician or to an obstetrician/gynecologist when necessary. Furthermore, nurses should be prepared to discuss fertility preservation options and/or provide referred to a fertility specialist as necessary *prior to* initiating therapy (Kottschade and Reed 2017).

Of the novel AEs associated with targeted therapies, pyrexia, by far, is identified as a characteristic and challenging AE associated with BRAFi-based therapy, primarily seen with combination dabrafenib/trametinib. Long et al. (2017) reported 5-year safety and efficacy outcomes for patients enrolled on the landmark BRF113220 phase II study. Pyrexia was the most commonly reported AE with the incidence of 69%. It was also the most frequent cause of treatment discontinuation. These findings were similar to those reported by Robert and colleagues (2015) in the COMBI-v trial, comparing combination dabrafenib plus trametinib with vemurafenib. In this study, pyrexia was the most commonly reported AE with combination D/T, and pyrexia was the most common reason for dose interruption or reduction, as well as most common reason for treatment discontinuation. Conversely, results from the COLUMBUS trial demonstrate a low frequency of pyrexia seen with encorafenib monotherapy, as well as when encorafenib was combined with binimetinib (Dummer et al. 2018; Flaherty 2017). The low rate of pyrexia seen with encorafenib and binimetinib (enco/bini) distinguishes this combination from the other combination targeted therapies and will likely have significant clinical implications.

Defined by oral temperature >38.5 °C (101.3 °F) in the absence of clinical or microbiologic evidence of infection (Rubin 2017b), the etiology of pyrexia is not well understood and does not appear to correlate with any predictive baseline characteristics or be predictive of clinical outcome or response to treatment (Daud and Tsai 2017). Some patients will often identify a prodrome prior to the development of fever or significant symptoms of pyrexia, and if this occurs, management should be the same as for established pyrexia syndrome (Atkinson et al. 2016). Management guidelines vary in the literature, but most agree on withholding both dabrafenib and trametinib (Daud and Tsai 2017) and restarting at the same dose once afebrile for 24 hours. Additional recommendations include the use of antipyretics (such as acetaminophen and/or nonsteroidal anti-inflammatories), supportive care strategies such as maintaining hydration, and ensuring patients are adhering to treatment recommendations, particularly those concerning treatment holds or dose adjustments (Czupryn and Cisneros 2017; Rubin 2017b). For recurrent episodes, scheduled administration of antipyretics continued upon reinitiating the targeted therapy can be effective. For patients with persistent pyrexia, a short course of corticosteroids (e.g., prednisone 10 mg daily for 5 days) may be considered. Alternatively, an intermittent dosing schedule can be effective employing full doses of medications. Expert opinion suggests that intermittent dosing is an effective management strategy that is unlikely to impact efficacy and is preferable to dose reduction although randomized clinical trial evidence is lacking (Atkinson et al. 2016; Daud and Tsai 2017). The authors provide an example: if a patient experiences pyrexia syndrome every 2–3 weeks, consider treating for 12 days followed by a 2-day break. Atkinson and colleagues also suggest considering prophylactic corticosteroids (e.g., prednisone 10–25 mg/day), with plans to taper if/when the patient has remained pyrexia free for at least 1 month. Dose reductions should be considered only if intermittent dosing and corticosteroid prophylaxis have failed, with an

attempt to escalate doses as tolerated (Daud and Tsai 2017).

Experience may influence how patients are managed. Nonetheless, effective management requires significant clinical support, commonly provided by nurses. This often involves daily phone calls for symptom assessment and management. In addition, frequent clinic visits may be necessary for assessment and/or supportive care (such as IV hydration), not to mention the psychosocial support patients and families should be provided through education and anticipatory guidance. Throughout the process, nurses should be managing expectations and assess coping to minimize anxiety. This level and degree of care is resource and time intensive and require nurses with knowledge, skill, and experience to manage this unique toxicity. The arduous clinical management makes the safety profile of encorafenib/binimetinib a particularly attractive treatment options given the low incidence of associated pyrexia.

Arthralgias are a common AE more common with BRAFi monotherapy and less so with combination BRAF/ MEKi (Rubin 2017b). Management depends on severity; therefore patient education requires reporting of symptoms including severity and how impactful symptoms are on quality of life. NSAIDs and/or acetaminophen is advised for mild to moderate symptoms, while more severe symptoms may require a dose interruption until symptoms improve and dose reduction when restarting. For recurrent pain, low-dose prednisone can be very effective (Rubin 2017b).

Cutaneous AEs are associated with the available BRAF/MEKi and include rash, pruritus, and photosensitivity (Daud and Tsai 2017). The photosensitivity associated with vemurafenib is of particular clinical significance as it can be seen with even short sun exposures and therefore requires dedicated significant patient education and intervention to minimize UV. Notably results from the COLUMBUS trial (Dummer et al. 2018) demonstrated *low incidence* of photosensitivity with enco/bini. This finding represents yet another distinctive clinical advantage of enco/bini compared to other available combinations in the same class; specifically, it provides a safer option for patients at greater risk of photosensitivity or unwilling to adhere to UV protection.

Nurses should be familiar with rash and pruritus management including generalized skin care strategies: avoidance of harsh soaps, use of tepid (not hot) water for bathing, and daily use of moisturizers to minimize irritation. Anti-itch interventions include adding menthol to moisturizers, cool cloths to focal areas of pruritus, antihistamines, and steroids (topical or oral). Patients should be counseled to avoid direct sun or ultraviolet (UV) exposure. Use of protective clothing and/or sunscreen should be employed to minimize UV exposure. Use of physical sunscreens is preferred over chemical sunscreens as they are less irritating and work as soon as they are applied avoiding the need to apply in advance of UV exposure (Rubin 2017b). Hyperproliferative skin disorders such as keratoacanthoma (KA) and KA-like squamous cell carcinomas (SCCs) are a class effect of BRAFi resulting from paradoxical activation of the MAPK pathway by BRAFi in BRAF wild-type cells. For this reason, it is important to counsel patients to report any new or changing skin lesions, and routine full-body skin examinations (including oral and genitalia) by a dermatology provider are advised. Some skin lesions may resolve without intervention. Other management modalities include excision, cryotherapy, and curettage (Czupryn and Cisneros 2017; Rubin 2017b).

Other AEs are associated with BRAF/MEKi requiring awareness by nurses. Ocular and cardiac effects are rare but warrant awareness to ensure signs and symptoms are recognized and managed appropriately. Guidelines for management are available and should be familiar to nurses. Uveitis and retinal disorders are associated with BRAFi and MEKi, respectively. Ophthalmologic screening is recommended at regular intervals for patients treated with MEKi with or without BRAFi, and patients should be counseled to report any visual symptoms including blurred or double vision, redness of the eyes, or any type of eye pain (Czupryn and Cisneros 2017; Rubin 2017b). Cardiac effects associated with BRAFi include QT prolongation, and with MEKi ventricular dysfunction can be seen. Nurses should ensure

baseline EKG and cardiac echocardiogram are obtained pretreatment and repeated at various intervals. Patients with an asymptomatic decrease in LVEF of 10% or greater from baseline require withholding the MEKi. If improvement is seen, agents can be restarted with appropriate dose reduction. If no improvement is seen, MEKi should be permanently discontinued (Daud and Tsai 2017). Anticipatory guidance regarding management of toxicity is essential to minimize patient dissatisfaction. Patients must understand the rationale for management and how it will impact overall treatment goals.

Oncolytic Viruses

Talimogene laherparepvec (TVEC) is first-in-class oncolytic virus derived from a herpes simplex virus-1 (HSV-1). It received FDA approval in October 2015 for local treatment of unresectable cutaneous, subcutaneous, and nodal melanoma metastases (Seery 2017; Rehman et al. 2016). TVEC is a live, attenuated virus, which has been modified to replicate specifically within tumor cells resulting in cell destruction (Seery 2017). The approval of TVEC offers a unique treatment option for a select subset of patients.

The logistics of TVEC administration requires a coordinated effort among multiple departments and personnel. While technically feasible for nurses to administer, at the centers currently offering this therapy, oncologists (medical or surgical) or advanced practice providers (APPs) tend to be the ones administering TVEC (personal communication with V. Seery; personal communication with H. Kaufman). Select nursing staff at various centers have been involved in coordinating and implementing TVEC into their institution and subsequently providing patient (and staff) education.

Providers who are pregnant or immunocompromised should not prepare or administer TVEC, touch a patient injection site(s) or dressing, or encounter any bodily fluids (Seery 2017). Treatment is contraindicated in pregnant or immunocompromised patients, in patients with clinical or laboratory evidence of an active herpetic infection, or in patients who require daily antiviral therapy such as acyclovir (Rehman et al. 2016). Patient education should include a description of treatment logistics, side effects, and postinjection care. The first injection consists of a lower concentration and is given initially to allow all patients to seroconvert if not previously exposed to HSV-1. The second injection is administered 3 weeks later at a higher concentration as are all subsequent injections. Treatment continues every 2 weeks, depending on treatment response and patient tolerability (Seery 2017; Rehman et al. 2016). An occlusive dressing is applied to the injection site(s), and patients should be instructed to keep site(s) covered for at least the first week after each treatment visit or longer if the injection site is weeping or oozing and to replace the dressing if it falls off. Patients should be instructed to avoid touching or scratching the injection sites, even if covered, to prevent the transfer of TVEC, and should be counseled to avoid kissing close contacts if either has an open mouth sore and to use condoms when engaging in sexual activity (Amgen 2017).

Adverse reactions are generally mild and resolve within 2–3 days. Common side effects include fatigue, chills, fever, nausea/vomiting, arthralgias and/or myalgias, and injection site pain (Seery 2017; Amgen 2017). Premedication with acetaminophen and/or nonsteroidal anti-inflammatories can prevent or minimize symptoms (Seery 2017); local anesthetics are not required but may be used if a patient has previously experienced significant pain during the injections (Rehman et al. 2016). In rare instances, cellulitis may develop at an injection site; therefore any reports of persistent or worsening erythema or edema or for a fever that persists beyond 48 h warrant evaluation (Seery 2017).

Central Nervous System Metastases

Approximately 50% of patients with stage IV melanoma will develop brain metastases (Olivia et al. 2017; Venur et al. 2017b; Flanigan et al. 2013) and up to 75% of patients in autopsy series

(Olivia et al. 2017). The presence of BRAF or NRAS mutations increases the risk of CNS metastases in patients with advanced melanoma by 24% and 23%, respectively, compared with a 12% rate in wild-type tumors (Venur et al. 2017b). Common clinical manifestations include headache, neurologic deficits, cognitive impairment, and seizure, yet many patients will be asymptomatic with metastases identified on routine imaging. Though an individual clinical course is difficult to predict, certain prognostic indicators determine survival and thus guide treatment/management decisions. These include age, performance status, comorbidities, extent of systemic (extracranial) disease, number and location of CNS metastases, time from primary tumor diagnosis, the presence of neurologic symptoms, and elevated lactate dehydrogenase (Venur et al. 2017a; Flanigan et al. 2013).

Management strategies are broadly divided into supportive and therapeutic (Venur et al. 2017a). Supportive treatments include corticosteroids to reduce peritumoral edema, antiepileptics for seizure control, and medications to preserve cognitive function. Traditionally, therapeutic strategies focused on local treatment, including surgery, whole-brain radiation therapy (WBRT), and stereotactic radiosurgery (SRS) and often a combination. Novel treatment approaches are being investigated, and early clinical trials with immunotherapy have shown encouraging intracranial activity in patients with asymptomatic brain metastases as have trials with BRAF-directed targeted therapies (Venur et al. 2017b). Combination of radiation and systemic therapy may be used to improve local control.

Caring for patients with CNS involvement is challenging and requires insight and experience by the nursing team. A clinical course for patients with CNS involvement varies considerably, and patients and families require much support. Nursing care must be comprehensive: meeting both the direct physical needs of patients and families (e.g., managing symptoms) while at the same time addressing the psychosocial and emotional responses to and spiritual aspects of cancer.

Palliative Care/End of Life

Immunotherapy brings with it the promise of meaningful benefit. However, despite advances that have dramatically improved outcomes for so many, there remain a significant number of patients who will not benefit from contemporary therapies and will die of melanoma. When treatment(s) fails, or if risks outweigh perceived benefits of treatment, patients must be informed. Nevidjon and Mayer (2012) advise frank, open, and ongoing discussions to ensure patients, their family/caregivers, and providers are all on the same page and goals of care are clearly described. They go on to explain if a patient does not have an honest picture of the prognosis, a realistic conversation can't even begin about end-of-life care. When it does, concerns about the financial impact of end-of-life care for the individual and his or her family, differences between patient and family wishes, and fears of abandonment from the healthcare providers can overshadow the quality-of-life desires of the individual. These conversations are difficult to conduct in a brief office or inpatient visit, especially if the health of the patient is deteriorating (Nevidjon and Mayer 2012). Ideally, these types of discussions are explored with patients prior to when answers are needed. Within the confines of a therapeutic nurse-patient relationship, nurses should be prepared to bring up difficult topics (Nevidjon and Mayer 2012) such as code status; preference for dying at home, hospital, or hospice house; and exploring the emotional and psychosocial implications of transitioning care from a disease focus to symptom focus.

Nurses provide support though simply listening and encouraging patients to identify what is important to them, correcting any misconceptions they may have about the process of dying, and inquiring about any religious, spiritual, and cultural aspects of care that are important to the patients and should advocate for inclusion into the plan of care. In knowing a patient, the nurse can provide or facilitate care tailored to the needs of the patient. This may include interfacing with family or other caregivers when needed and acting as an advocate for other healthcare providers.

Fundamental to the nursing role is symptom management. Unlike most other malignancies, melanoma commonly metastasizes to the skin, subcutaneous tissue, and lymph nodes. Malignant wounds often develop which can be intensely painful disfiguring tumors that are both physically and psychologically devastating to not only the patient but to caregivers/family (Young 2017). These lesions may weep and bleed and, due to necrosis, often have a terrible odor that causes intense distress and embarrassment. Often the care of the malignant wound takes over the lives of the individuals and their caregivers (Young 2017). Healing is often not a therapeutic reality, instead, the goal wound care with symptom control what Young (2017) calls "palliative wound care." This requires palliative care to be combined with effective wound management with priority given to symptom management and the relief/prevention of pain along with psychological, spiritual, and emotional support. Oncology nurses are ideally positioned to spearhead this type of effort through facilitating interdisciplinary collaboration among palliative care provider(s) and individuals providing wound care, whether that be as part of a home care team such as hospice or visiting nurse service. Goals of wound care must be defined with various providers on the same page regarding management strategies for both physical care and psychological support. When appropriate, oncology nurses provide guidance during the transition to the end of life care. Young (2017) emphasizes how even at the end of life, combining a palliative model of care with effective wound management can significantly enhance the patient's quality of life.

Survivorship

Cancer survivorship is a term that has gained popularity over the past few decades. It began with the establishment of the National Coalition of Cancer Survivors (NCCS) in 1986 and then later evolved to the Office of Cancer Survivorship at the National Cancer Institute (NCI). The primary intent was recognition of cancer survivors as a unique population with unique needs. Survivorship is defined by the NCI as "the health and life of a person with cancer post treatment until the end of life. It covers the physical, psychosocial, and economic issues of cancer, beyond the diagnosis and treatment phases." To formalize care, the Institute of Medicine defined four major components of survivorship care models: prevention, surveillance, interventions, and coordination.

There is a paucity of data in the literature about melanoma-specific survivorship. The available evidence is analogous to the components identified by IOM and, for the most part, is already included in standard melanoma follow-up care. It is worth mentioning that the term "survivor" has a different meaning to different people (Smith et al. 2015). Oliveria et al. (2011) conducted a series of focus groups with stages I–III melanoma patients diagnosed 1–10 years prior; none were receiving treatment. The respondents varied in how they perceived their diagnosis. Those who did not consider themselves to be a survivor provided reasons such as not a "real" cancer, nor was it as serious as other cancers. One respondent remarked she did not consider herself a survivor as it [the melanoma] was on "the outside of the body, never inside"; therefore it was not interpreted as serious. Another respondent provided a similar reason, stating because it was not metastatic, it was not "real." Some felt the label "survivor" should be reserved for individuals who received chemotherapy, radiation, or other extensive treatment. Another believed that one was not considered a survivor until reaching the 5-year mark from diagnosis (Oliveria et al. 2011; Vogel et al. 2017). Knowing how individuals see themselves influences follow-up care.

Prevention is a critical component of melanoma survivorship due to the higher risk of secondary melanoma and other skin cancers as discussed previously. Through assessment of existing knowledge, nurses can provide tailored education on proper use and application of sunscreens, use of UV protective clothing and hats, and eye protection. Education also includes strategies to reduce ambient and recreational sun exposure: seeking shade when possible, avoiding sunburns and tanning bed usage, and other safe sun practices (Mrazek and Chao 2014). Interestingly, Oliveria et al. (2011) found many survivors become less

stringent with UV protection with pasting time, the reason being the desire to "live life" normally by going outdoors, and felt barriers to sun protection presented by lack of convenience. Regular and ongoing nursing assessment identifies those at risk and allows for tailored strategies aimed at increasing independence adherence.

Hereditary forms of melanoma (those with germline mutations in CDKN2A or CDK4) are rare. However, these patients have a high risk of developing multiple primary melanomas as well as increased risk of additional malignancies including pancreatic, breast, and lung (Soura et al. 2016). These individuals are best managed by a multidisciplinary team to address all required subspecialty care and screenings. Nurses should facilitate any appropriate referrals and ensure patients understand the rationale for ongoing follow-up.

Surveillance is an important aspect of follow-up and stems from higher risk of additional skin cancer development (Kasparian et al. 2016; Mrazek and Chao 2014). If not already, patients should be referred to a pigmented lesion clinic or to a dermatology provider skilled in high-risk skin cancer assessment for ongoing surveillance. Patient education should include instructions on skin and lymph node self-examination. Ongoing assessment and reinforcement of proper technique will identify any barriers to adherence. It is also helpful to clarify when and why patients should contact their primary care provider versus oncologist provider (Grant et al. 2010). Interventions focus on symptom management or education for long-term effects of cancer and cancer treatments. Grant et al. (2010) illustrate how interventions can be organized in relation to four domains of quality of life: physical, psychological, social, and spiritual.

Physical interventions are directed at management of symptoms such as lymphedema, pain, or decreased range of motion or addressing physical consequences from disease or treatment such as scar or other body image issues identified (Tan et al. 2014; Oliveria et al. 2011).

Psychological support has been identified as a priority need among individuals with melanoma (Tan et al. 2014; Mrazek and Chao 2014). In a systematic review of published studies, Kasparian et al. (2009) found approximately 30% of patients diagnosed with melanoma, including long-term survivors, and 20–30% of cancer caregivers reported levels of psychological distress report indicative of the need for clinical intervention. Anxiety and depression are the most commonly reported emotions experienced by melanoma patients (Mrazek and Chao 2014). Despite limited data, results of multiple studies highlight the psychological challenges faced by individuals with or a survivor of melanoma.

The primary goal of melanoma follow-up care is early detection of recurrent disease or new primary melanoma (Mrazek and Chao 2014); however, best practice would suggest that psychological care and support be incorporated into standard follow-up. Interventions include ongoing screening for psychological distress at various time points throughout care, facilitating access to specialty care when necessary (e.g., social work, psycho-oncology, local mental health provider), and promoting effective communication between patients, family members, and healthcare professionals (Wouters et al. 2018).

A melanoma diagnosis and subsequent treatment can affect social supports and family dynamics, often adversely affecting patients and their caregivers. Caregivers may benefit from receiving information/support interventions to address caregiver burden resulting from new roles and responsibilities. Such interventions help caregivers maintain their own psychosocial well-being (Tan et al. 2014). Support groups are another means of effective social support. Group programs are cost-effective and provide a safe environment for patients and caregivers to meet to support each other, feel a sense of belonging, develop friendships, and share ideas and thoughts (Tan et al. 2014).

Religion and/or spiritual beliefs are a tremendous source of support for patients and families and can help them cope with cancer (Vogel et al. 2017; Tan et al. 2014). Religious and spiritual preferences should be incorporated into comprehensive care. Churches, synagogues, and other religious institutions are often sources of community support, and efforts should be made to facilitate inclusion into plans of care as appropriate.

Coordination

Oncology care does not end when treatment ends. Nurses have a significant role in the dissemination and coordination of information between the patient and other healthcare providers (Grant et al. 2010). Simple interventions such as ensuring visit notes are being sent to relevant providers will enhance effective communication. Helping patients to prioritize their health needs and navigate the healthcare system will reduce stress and assisting patients to coordinate appointments among the multiple providers.

Conclusion

The arsenal of effective treatments for melanoma is expected to expand as result of ongoing research into various combinations and as new agents become available. As treatment options for melanoma expand, so does the role of oncology nurses. Nurses are on the front lines of patient care, play an important role in the multidisciplinary team, and influence treatment adherence and completion of therapy yielding more successful outcomes across the entire disease spectrum. Quintessential to providing effective patient-centered care is taking the time to build a therapeutic nurse-patient relationship. This relationship affords the framework for care and is necessary to provide individualized care that meets the needs of patients and their families.

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