Molecular Pathology and Genomics of Melanoma

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C. M. Balch et al. (eds.), *Cutaneous Melanoma*,
https://doi.org/10.1007/978-3-030-05070-2_41

Abstract

Genetic aberrations, in consort with epigenetic factors and the tumor microenvironment, drive tumor progression that ultimately results in unlimited cell proliferation, avoidance of immune destruction, and metastatic potential. The characterization of the genomic landscape of melanocytic tumors has significantly improved our understanding of their pathogenesis. Many clinical and histologic subtypes of melanocytic tumors have been found to harbor characteristic genetic aberrations, which often correlate well with their clinical and pathologic phenotypes. In other cases, the detection of genetic aberrations has improved the genotype-based prognostic stratification of melanocytic tumors that are clinically or histologically similar but exhibit differing biological behavior. With the introduction of mechanism-based treatment strategies, genetic markers have also become important predictors of treatment responses, e.g., *BRAF* mutations determine the therapeutic response to BRAF inhibitors; or high mutational burden in melanoma is associated with favorable response to immunotherapy. However, despite considerable enthusiasm about our improved understanding of genetic aberrations in melanocytic tumors, there have also been sobering results. For example, many genetic tests aiming to better distinguish benign and malignant neoplasms have not fulfilled their expectations, in part, because numerous non-genetic factors, including epigenetic dynamics, cell identity, tumor microenvironment, and the immune system, also influence clinical behavior. Additionally, tumor progression, resulting from a stepwise acquisition of genetic aberrations, implies a continuous spectrum of biologic behavior in melanocytic tumors rather than a clear-cut binary division between "benign" and "malignant." Furthermore, although numerous recurrent gene alterations in melanocytic tumors have been identified, the functional, diagnostic, and prognostic implications of many of these alterations are yet to be elucidated.

Introduction

Melanocytic neoplasms range from benign lesions, termed melanocytic nevi, to malignant tumors, termed melanomas. The process of neoplastic transformation from normal benign to neoplastic, malignant cells results primarily from the sequential accumulation of genetic aberrations. Genetic aberrations alter genes involved in sustaining cell proliferation, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, activating cell invasion and metastasis, inducing angiogenesis, facilitating genome instability, deregulating cellular metabolism, and resisting cell death. These capabilities or traits of cancer cell are often encpacsulated by the term "hallmarks of cancer" (Hanahan and Weinberg 2011).

Despite recent advances in our understanding of the genetic aberrations in melanocytic tumors and the pathogenetic processes involved in tumor progression, the diagnosis of melanocytic tumors still relies primarily on pathologists' interpretation of histopathologic features in hematoxylin-and-eosin-stained slides. However, diagnosis based purely on morphology has limitations, and the ancillary use of molecular genetic techniques can aid in diagnosis, particularly of histopathologically challenging or ambiguous lesions. Ancillary diagnostic techniques are already utilized for making treatment decisions in patients with

advanced disease when considering mechanism-based systemic therapies, such as targeted agents and immunotherapies. A key example is the molecular detection of $BRAF^{V600E}$ mutations in metastasized melanoma as a prerequisite for treatment with MAPK inhibitors (MAPKi), such as dabrafenib and trametinib, or vemurafenib and cobimetinib (Haas et al. 2018).

Accurate detection and interpretation of molecular aberrations is critical to making correct diagnoses and devising optimal treatment plans. In order to maintain a high diagnostic and therapeutic standard, clinicians and pathologists have to understand the basic principles of genetics, the functional consequences of genetic aberrations, and the requirements and limitations of molecular genetic assays. Clinicians and pathologists should also correlate molecular findings with clinical and histopathologic data. This chapter presents an overview of cancer progression in melanocytic tumors, genetic aberrations present in benign and malignant melanocytic tumors, and genetic assays that are common ancillary tools for the diagnosis of melanocytic tumors.

The Beginnings of Cancer Genetics

Over a century ago, Peyton Rous and Thomas Boveri laid the groundwork for subsequent discoveries defining genetic aberrations, which initiate and sustain cancer. In 1911, Peyton Rous described a cell-free filterable substance with which he could transmit sarcoma from one fowl to the other (Rous 1911). Subsequently other viruses leading to tumor formation were identified (Weiss and Vogt 2011). However, despite intensive efforts, no viruses could be found in most neoplasms, suggesting other pathogenetic mechanisms. An important clue to this puzzle was the demonstration that related genes transmitted by these viruses are already present in the genomes of normal cells (Stehelin et al. 1976), such as the *RAS* family of genes. Later it was shown that distinct genetic aberrations in these genes can constitutively activate them and transform cells in the absence of a virus (Cancer Genome Atlas 2015; Parada et al. 1982).

In 1914, Thomas Boveri was the first to hypothesize that chromosomal aberrations play a role in cancer (Boveri 1914). At the time, his hypothesis was not easy to prove and was not commonly accepted in the scientific community. A century later, the role of chromosomal aberrations in pathogenesis of tumors is clearly established (Beroukhim et al. 2010). In melanocytic tumors, Boveri's observation prefigured the introduction of genetic assays to distinguish benign from malignant melanocytic proliferations based on the presence or absence of chromosomal aberrations (Bastian et al. 1998; Gerami et al. 2009).

Basic Principles and Terms in Cancer Genetics

The human genome is distributed among 46 chromosomes, 22 pairs of autosomal chromosomes, and 2 sex chromosomes. Biochemically, the genetic information is stored in the deoxyribonucleic acid (DNA), which is a sequence of 3 billion nucleotides. **Nucleotides** consist of one of four bases (A, adenine; T, thymine; C, cytosine; G, guanine) and a sugar-phosphate backbone. Two complementary, antiparallel DNA strands are bound together by hydrogen bonds to form a double helix. The hydrogen bonding is responsible for the pairing of complementary bases in the DNA: adenine (A) binds thymine (T), and cytosine (C) binds guanine (G). As a result of this complementarity, all the information in the DNA helix is duplicated on each strand, which is vital for DNA replication, DNA repair, and transcription. This complementary nature of base pairing is also the underlying principle for the majority of molecular methods such as the binding of primers in polymerase chain reaction (PCR) assays, binding of fluorescence-labeled probes in fluorescence in situ hybridization (FISH) assays, or binding of fluorescence-labeled nucleotides in sequencing assays (Fig. 1).

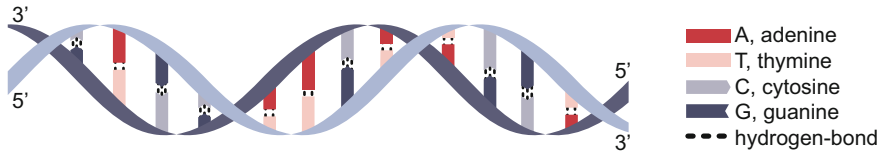


Fig. 1 Structure of deoxyribonucleic acid (DNA). DNA is composed of two polynucleotide chains that coil around each other to form a double helix. The genetic information is stored in the sequence of the nucleobases: adenine (A), thymine (T), cytosine (C), and guanine (G).

DNA is organized into linear domains of genes, regulatory elements, and repetitive sequences. Genes are the molecular units of heredity that encode proteins, but consist of many components of which the protein coding sequence is only a small part. **Transcription** of genes is performed by **RNA polymerase**, which binds to the promoter region and transcribes the DNA to the complementary pre-messenger RNA (pre-mRNA), except that the base uracil (U) in the RNA replaces thymine (T) in the DNA. The introns are then removed from the pre-mRNA, and the exons are spliced together to form mature **messenger RNA** (mRNA). To control the amount of mRNA, the transcription is tightly regulated by **promoter, enhancer, and repressor elements** that bind numerous regulatory proteins such as transcription factors (Fig. 2).

Translation into a protein begins at the mRNA's start codon and ends at the stop codon. Translation is facilitated by specialized protein complexes termed ribosomes, which add new amino acids to the growing polypeptide chain. The genetic code is read in three nucleotides (codons) at a time. Each codon interacts specifically with three complementary bases (anti-codon) on specialized RNA molecules, termed **transfer RNA** (tRNA), which are covalently attached to an amino acid specified by the codon. When the tRNA's anti-codon binds to its complementary codon on the mRNA, the ribosome attaches its amino acid cargo to the new polypeptide chain. The translation, stability of the mRNA, and consequently the protein amount are regulated by the **5′ and 3′ untranslated regions** (UTRs). **Proteins** are involved in the vast majority of functions within cells and tissues.

Types of Genetic Aberrations

Genetic aberrations (**mutations**) are permanent changes in the DNA's nucleotide sequence and usually result from DNA damage that is not repaired. Genetic aberrations can have either no effect or can change the function of a protein (**loss-of-function mutation**, **gain-of-function mutation**). When mutations affect one or a few nucleotides but code for the same amino acids and, thus, do not change the protein function, they are classified as **synonymous mutations**. **Non-synonymous mutations**, in contrast, change the amino acid sequence and may alter protein function. They are further classified into:

1. **Missense mutations** that result in the incorporation of a different amino acid.
2. **Nonsense mutations** (also termed truncating mutations) that result in a premature stop codon, leading to a truncated protein.
3. **Insertions** and **deletions** that add or remove one or more nucleotides, respectively. If insertions and deletions preserve the reading frame, they are termed in-frame mutations; if they shift the reading frame, they are termed frameshift mutations.

Large genetic aberrations affect chromosomal components and may alter the expression level of the involved genes. They are often classified as:

1. **Copy number gains** (**amplifications**) that increase the dosage of genes.
2. **Copy number losses** (**deletions**) that lead to a loss of genes; the deletion may involve only one copy of the gene (**heterozygous deletion**) or both copies (**homozygous deletion**, **deep deletion**).

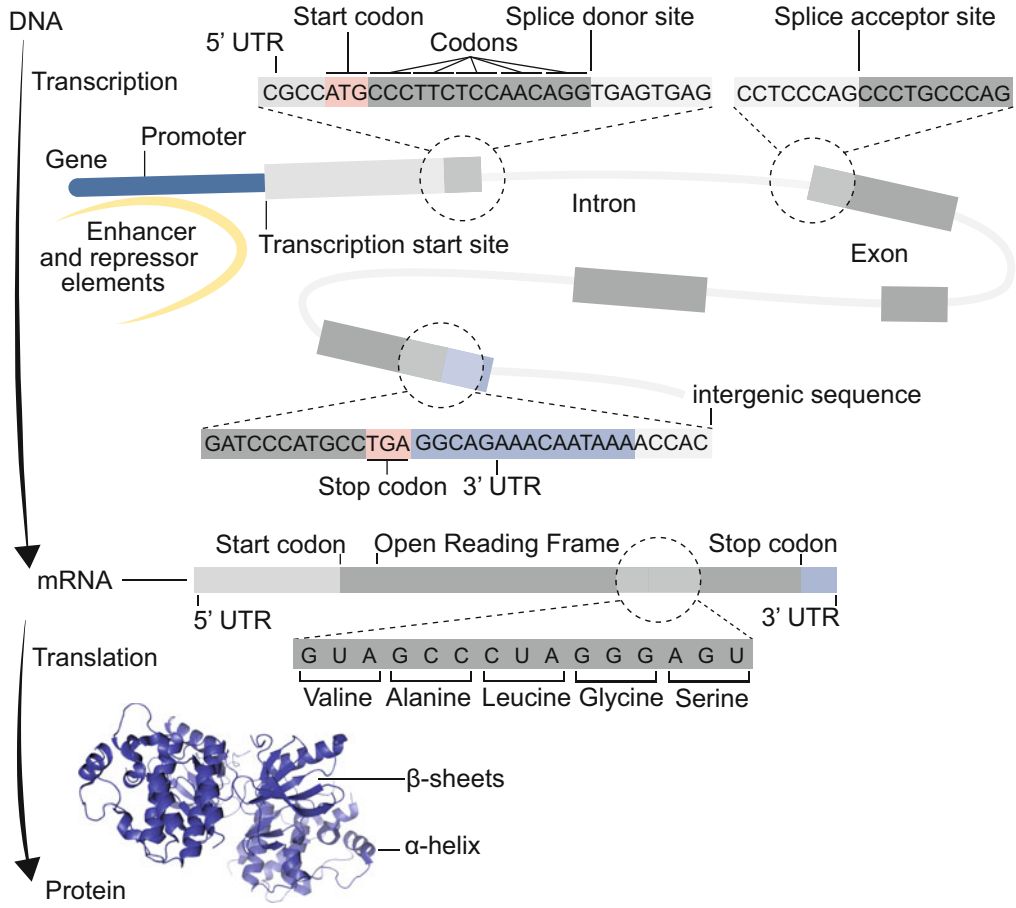


Fig. 2 Gene structure, regulatory elements, transcription, and translation. Genetic information is stored in genes. Genes contain regulatory sequences such as enhancers, repressive elements, and promoters, all of which control the transcription into mRNA. Only about 1.5% of the human genome consists of protein-coding exons. However, many DNA sequences that do not encode proteins regulate gene expression and may encode noncoding RNA molecules. The open reading frame (ORF) is the part of the mRNA that is translated into a protein, begins at the start codon (usually ATG), and ends with a stop codon (usually TGA). The genetic code is read in three nucleotides (codons) at a time; each codon corresponds to a specific amino acid (or a start/stop codon during translations). DNA and RNA molecules are written in a language of four nucleotides, while proteins are encoded by varying combinations of 20 amino acids. A protein is a sequence of amino acids that forms specific three-dimensional structures, such as α-helices or β-sheets. Mutations may change the amino acid sequence, which can result in altered protein structure, and may influence the protein function.

3. **Translocations** (**gene fusions**) that juxtapose previously separated DNA regions next to each other.

Recent Advances in Cancer Genetics

The development of increasingly sophisticated molecular techniques over the last few decades has led to an improved understanding of the pathogenesis of human cancers. Arguably, the most important technical advance has been **next-generation sequencing** (**NGS**; **massively parallel sequencing**). NGS approaches have led to the discovery of novel genetic aberrations and have helped clarify the pathogenesis of many cancer types. Technologic refinements and decreasing sequencing costs have resulted in

NGS becoming a common component of clinical management protocols in many cancer centers worldwide (Zehir et al. 2017). The flexibility of NGS techniques allows detection of mutations, insertions/deletions, translocations, and copy number aberrations of select genes or the entire genome. NGS techniques already play important roles in patient care and will likely gain more relevance in the future. While most centers currently use **targeted sequencing** panels to analyze a selection of potentially relevant genetic aberrations, it is likely that in the near future, more comprehensive NGS approaches, such as **whole-genome sequencing**, will be used routinely in clinical settings as diagnostic and prognostic aids, and to help with treatment decisions.

Combination targeted therapy with agents inhibiting single or multiple oncogenic signaling pathways is already in clinical use. For example, the combination of BRAF and MEK inhibitors has proven to be more effective than BRAF inhibition alone (Haas et al. 2018). As single-agent targeted therapies are frequently associated with relatively rapid development of resistance, it is likely the number of combination treatments will continue to increase in the future. As the number of targetable genetic aberrations increases, more comprehensive genetic analyses screening for these alterations will be required to plan optimal therapy.

Immunotherapy has also gained considerable momentum in the treatment of advanced melanoma, and efforts are under way to identify biomarkers predicting therapeutic responses (Haas et al. 2018). Approaches showing promise include assessment of a tumor's overall mutational burden, mutations altering protein-coding regions, or mutations resulting in aberration of presented antigens (Hellmann et al. 2018; Rizvi et al. 2018). Should such genetic approaches prove to be reliable predictive biomarkers for responses to immunotherapies with checkpoint inhibitors, such as anti-PD1, anti-PD-L1, or anti-CTLA4, NGS techniques will also become important for decision-making with regard to the use of immunotherapy.

Pathogenesis: Acquisition of Mutations

Ultraviolet radiation (UV) Pathogenesis

Epidemiologic data have long supported a key role of ultraviolet radiation (UV) exposure as a risk factor for cutaneous melanoma. Increased risk of developing melanoma is particularly associated with multiple intermittent UV exposures in childhood (Elwood and Jopson 1997). This is in contrast to non-melanoma skin cancer, in which cumulative UV exposure appears to be more important. Other factors such as a family history of melanoma, large numbers of nevi, light eye color (green or blue), light hair color (red > blond > brown), freckles, and minimal tanning response have been linked to melanoma risk (Tschandl and Wiesner 2018). Some of these traits are associated with an increased sensitivity to UV exposure and with the presence *of MC1R* variants, which appear to predispose to melanoma via UV-dependent and UV-independent mechanisms (Mitra et al. 2012).

Recent NGS studies have confirmed the epidemiologic data and further underlined the role of UV exposure in melanoma pathogenesis. Cutaneous neoplasms have been found to mostly demonstrate mutations with a **UV signature**, in particular C>T and CC>TT aberrations, the latter being considered pathognomonic for UV-induced aberrations (Brash et al. 1991; Pleasance et al. 2010). Recent studies have demonstrated that T>A transversions can also frequently occur in sun-exposed tumors (Hayward et al. 2017) suggesting that UV induction can cause the frequent *BRAF* V600E T>A transversion found in around 50% of melanomas.

Melanomas on average show a **mutation rate** of around 30 mutations per megabase of DNA (equivalent to 100,000 somatic mutations per genome), which represents a higher mutational burden than that seen in many other human malignancies (Fig. 3) (Lawrence et al. 2013). The number of mutations varies depending on UV

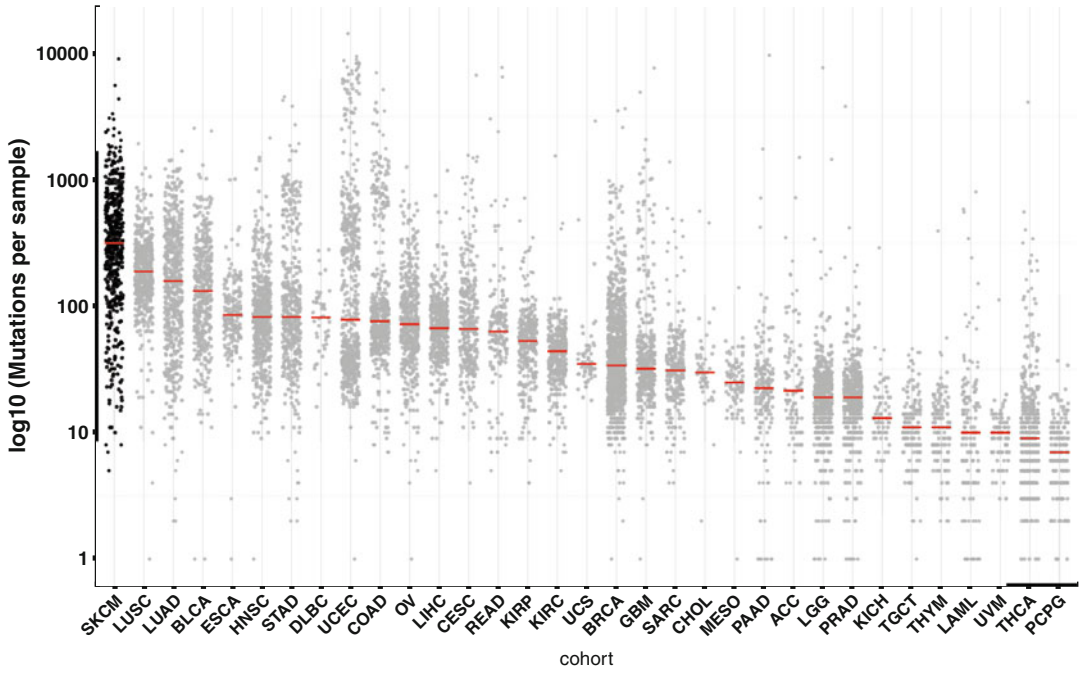


Fig. 3 Somatic mutation frequencies in various cancer types. Number of somatic mutations observed in exomes from 10,394 tumor-normal pairs. Each dot corresponds to a tumor-normal pair; the vertical position indicates the total frequency of somatic mutations in the exome. Tumor types are ordered by their median somatic mutation frequency with the highest frequencies on the left side with tumors induced by carcinogens such as tobacco smoke and ultraviolet light and the lowest frequency in hematologic and pediatric tumors on the right side. Mutation frequencies vary more than 1000-fold between different cancers and also within tumor types (note the logarithmic scale). ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; LGG, brain lower-grade glioma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THYM, thymoma; THCA, thyroid carcinoma; UCS, uterine carcinosarcoma; UCEC, uterine corpus endometrial carcinoma. The figure was created using a modified version of the tcgaCompare() function of the maftools Bioconductor package (http://bioconductor.org/packages/release/bioc/html/maftools.html) (Courtesy of Lukas Leiendecker, Research Institute of Molecular Pathology, Vienna, Austria).

exposure and melanoma subtype. Melanomas arising in heavily sun-damaged skin on the head, such as desmoplastic melanomas or lentigo maligna melanomas, have been reported to harbor up to 60 mutations per megabase (Eroglu et al. 2018). Melanomas on the trunk and the extremities harbor around 15 mutations per megabase, likely because these skin areas are not chronically exposed to the sun. Mucosal, acral, and uveal melanomas harbor considerably fewer mutations and lack a UV-signature mutation profile, in keeping with the low or absent levels of UV exposure associated with their pathogenesis. These tumors do however demonstrate higher numbers of structural aberrations and copy number alterations (Hayward et al. 2017).

Stepwise Progression of Melanocytic Tumors

Melanocytic tumors comprise several biologically distinct entities that differ in their clinical presentation, histologic appearance, and genetic aberrations (Table 1). Benign melanocytic tumors are caused by gain-of-function mutations in oncogenes that predominantly activate the mitogen-activated protein kinase (MAPK) pathway (Fig. 4). Depending on the underlying genetic aberrations and the cell of origin, melanocytic nevi present with characteristic clinical and histologic features (Table 2).

Various tumor-suppression mechanisms, including cell autonomous (e.g., cell cycle deregulation, telomere dysregulation, DNA damage response, epigenetic dysregulation) and non-cell autonomous (e.g., immune evasion mechanisms) mechanisms, inhibit the progression from nevi to melanoma (Fig. 5). The sequential acquisition of genetic aberrations can abrogate these tumor-suppression mechanisms, activate additional oncogenic pathways, or interfere with differentiation programs responsible for cell identity (Table 3).

In addition to genetic aberrations present in the neoplastic cells, tumors exhibit another dimension of complexity: they are intimately associated with a repertoire of recruited, ostensibly normal cells that establish a **tumor microenvironment**, which contribute to the acquisition of the hallmarks of cancer (Hanahan and Weinberg 2011). Together with the tumor microenvironment and interactions with the immune system, the acquisition of various combinations of genetic aberrations by melanocytes may ultimately lead to their malignant transformation.

Nevi

Nevi are benign neoplastic proliferations of melanocytes that usually appear in the first decades of life, but may also be present at birth (congenital nevi) or develop later in life. The most frequent nevi arising on the skin are designated **common acquired nevi**. Histologically, they are composed of small, oval, uniform melanocytes and are categorized into three subtypes, depending on whether they are confined to the epidermis (junctional nevi) and the dermis (dermal nevi) or show both epidermal and dermal components (compound nevi). Common acquired nevi harbor *BRAF* V600E mutations in more than 80% of cases (Pollock et al. 2003). The uniform morphology of the neoplastic cells, their symmetric

Table 1 Recurrent genetic aberrations in benign melanocytic neoplasms

Gene	Genetic aberration	Oncogenic signaling pathway	Tumor types
BRAF	Mutation V600, translocation	MAPK	Common acquired nevi (mutation) (Pollock et al. 2003) Spitz nevi (translocation) (Wiesner et al. 2014)
NRAS	Mutation Q61	MAPK and PI3K	Congenital nevi (Bauer et al. 2007)
HRAS	Mutation G12, G13	MAPK and PI3K	Spitz nevi (desmoplastic) (Bastian et al. 2000)
KRAS	Mutation G12, G13	MAPK and PI3K	Cutaneous melanoma (Cancer Genome Atlas 2015)
ALK	Translocation	MAPK and PI3K	Spitz nevi (plexiform) (Busam et al. 2014)
ROS1	Translocation	MAPK and PI3K	Spitz nevi (Wiesner et al. 2014)
RET	Translocation	MAPK and PI3K	Spitz nevi (Wiesner et al. 2014)
MET	Translocation	MAPK and PI3K	Spitz nevi (Yeh et al. 2015)
NTRK1	Translocation	MAPK and PI3K	Spitz nevi (Wiesner et al. 2014)
NTKR3	Translocation	MAPK and PI3K	Pigmented spindle cell nevus of Reed (VandenBoom et al. 2018)
GNAQ	Mutation Q209	MAP and Hippo-YAP	Blue nevi (Moller et al. 2016)
GNA11	Mutation Q209	MAP and Hippo-YAP	Blue nevi (Moller et al. 2016)
MAP2K1	Mutation P124, E203	MAPK	Deep penetrating nevi (some cases) (Yeh et al. 2017)

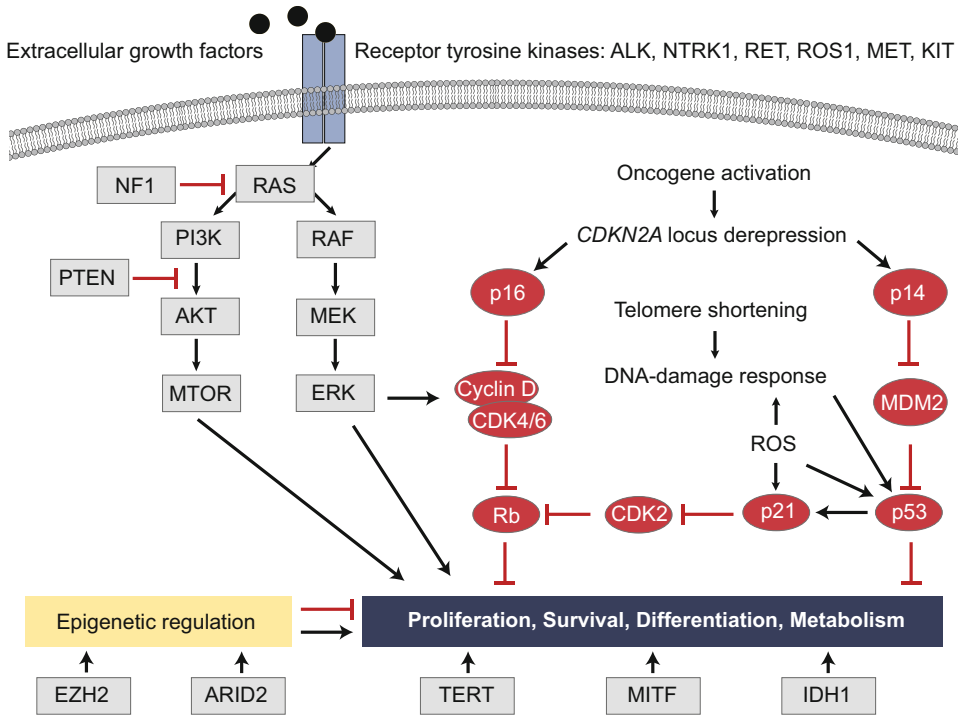


Fig. 4 Important signaling pathways in melanocytic tumors. Extracellular growth factors trigger the dimerization of receptor tyrosine kinases, leading to autophosphorylation and activation of intracellular signaling cascades such as the MAPK/ERK or the PI3K/AKT/mTOR pathway. Among a variety of other functions, these signaling pathways increase cell proliferation and survival. Many of the components, especially of the MAPK/ERK pathway, show genetic aberrations in melanocytic tumors, which lead to constitutive pathway activation. However, the activation of oncogenic pathways epigenetically de-represses the *CDKN2A* locus, encoding the proteins p16 and p14. Oncogene activation and cell proliferation may also lead to telomere shortening and increased concentrations of reactive oxygen species (ROS), which causes a DNA damage response and activates p53 and p21. The signaling cascades of the p16 and p53 pathway converge on the tumor suppressor retinoblastoma (Rb) and on cell cycle inhibitors. After initial cell proliferation, these signaling pathways cause a durable proliferative arrest, termed senescence.

distribution, and the presence of the same oncogenic driver *BRAF* V600E mutation in the neoplastic cells suggest that nevi result from the clonal expansion of a single cell (Bastian 2014).

Congenital nevi arise in utero or shortly after birth. They are significantly larger than common acquired nevi, can cover large portions of the body, and may also involve extracutaneous sites, including the central nervous system (neurocutaneous melanosis). In adults, congenital melanocytic nevi are categorized as small (<1.5 cm), intermediate (1.5–20 cm), or giant (>20 cm). Histologically, the small, oval, uniform melanocytes are often in close proximity to appendages and neurovascular bundles, splay between collagen bundles, and extend to the reticular dermis and sometimes also into the subcutis. In contrast to acquired nevi, in which *BRAF V600E* mutations predominate, congenital nevi harbor *NRAS* mutations in 80% of cases (Bauer et al. 2007). In patients with satellite lesions of congenital nevi, the same *NRAS* mutation is shared among all lesions. This observation indicates clonal expansion of a single melanocyte that acquired the *NRAS* mutation during its migration from the neural crest to the epidermis (Bastian 2014).

Blue nevi arise in the dermis and have a blue hue clinically, a result of the combination of the presence of deep-lying melanin pigment and the Tyndall effect. Blue nevi can be acquired or

Table 2 Subtypes of melanocytic neoplasms and their most common genetic aberrations

Melanocytic neoplasm	Frequent genetic aberrations	References
Common acquired melanocytic nevi	*BRAF* (>80%)	(Pollock et al. 2003)
Congenital nevi	*NRAS* (>80%)	(Bauer et al. 2007)
Spitz nevus	*HRAS* (20%), translocations of *ALK*, *ROS1*, *RET*, *NTRK1*, *NTRK3*, *BRAF* (50%)	(Bastian et al. 2000; Wiesner et al. 2014)
BAP1-inactivated melanocytic nevus	*BRAF* (>90%), *BAP1* (100%)	(Wiesner et al. 2012b)
Pigmented spindle cell nevus	*NTRK3* (50%)	(VandenBoom et al. 2018)
Deep penetrating nevus	*BRAF* (>90%), *MAP2K1* (30%), *CTNNB1* (80%)	(Yeh et al. 2017)
Pigmented epithelioid melanocytoma	*BRAF*, *PRKAR1A*	(Cohen et al. 2017)
Blue nevi, nevus of Ota, nevus of Ito	*GNAQ* (53%), *GNA11* (15%), *CYSLTR2* (1%), *PLCB4* (1%)	(Griewank et al. 2017)
Cutaneous melanoma	*BRAF* (50%), *NRAS* (25%), *NF1* (15%), *TERT* (70%), *CDKN2A* (20%), *PTEN* (15%), *TP53* (15%) High number of mutations, low number of copy number aberrations and structural aberrations	(Curtin et al. 2005; Hayward et al. 2017)
Mucosal/acral melanoma	*BRAF* (15%), *NRAS* (15%), *KIT* (15%), *TERT* (70%) Low number of mutations, high number of copy number aberrations and structural aberrations	(Curtin et al. 2005; Hayward et al. 2017)
Desmoplastic melanoma	*NF1* (60%), *TERT* (70%), high number mutations	(Krauthammer et al. 2015; Wiesner et al. 2015)
Lentigo maligna melanoma	*NF1* (60%), *TERT* (70%), high number mutations	(Krauthammer et al. 2015)
Uveal melanoma	*GNAQ* (33%), *GNA11* (40%), BAP1 (80%)	(Griewank et al. 2017; Harbour et al. 2010)

congenital. Congenital blue nevi include nevus of Ito, nevus of Ota, and Mongolian spots. Histologically, blue nevi are composed of dendritic, spindle, oval, or epithelioid melanocytes without significant epithelial involvement. Blue nevi harbor frequently *GNAQ* and *GNA11* mutations, and rarely *CYSLTR2* or *PLCB4* mutations, similar to uveal melanoma (Griewank et al. 2017; Moller et al. 2016). Blue nevi can also arise as part of Carney's complex, which is caused by germline mutations of the *PRKAR1A* gene (Bastian 2014).

Spitz nevi are a heterogeneous group of melanocytic tumors that occur mostly in young individuals and show an indolent clinical behavior. Spitz nevi are characterized histologically by a predominance of large spindle-shaped or epithelioid melanocytes with enlarged, vesicular chromatin and prominent nucleoli with minor histologic atypia. Spitzoid melanocytic lesions often present diagnostic problems, because they can be difficult to distinguish from melanoma. Spitz nevi harbor different genetic aberrations than common acquired, congenital, and blue nevi (Wiesner and Kutzner 2015). Many Spitz nevi are found to harbor translocations of receptor tyrosine kinases, including *ALK*, *ROS1*, *RET*, *NTRK1*, *NTRK3*, and *MET*, and the serine-threonine kinase *BRAF* (Busam et al. 2014; Kiuru et al. 2016; Wiesner et al. 2014).

Some of these translocations are associated with specific histologic features. **Plexiform Spitz nevi** exhibit a growth pattern of intersecting fascicles of predominantly fusiform melanocytes in the dermis. The melanocytes in plexiform Spitz nevi usually show smooth nuclear contours and a slightly vesicular chromatin pattern. Plexiform Spitz nevi often harbor *ALK* translocations. **Pigmented spindle cell nevi**, also referred to as

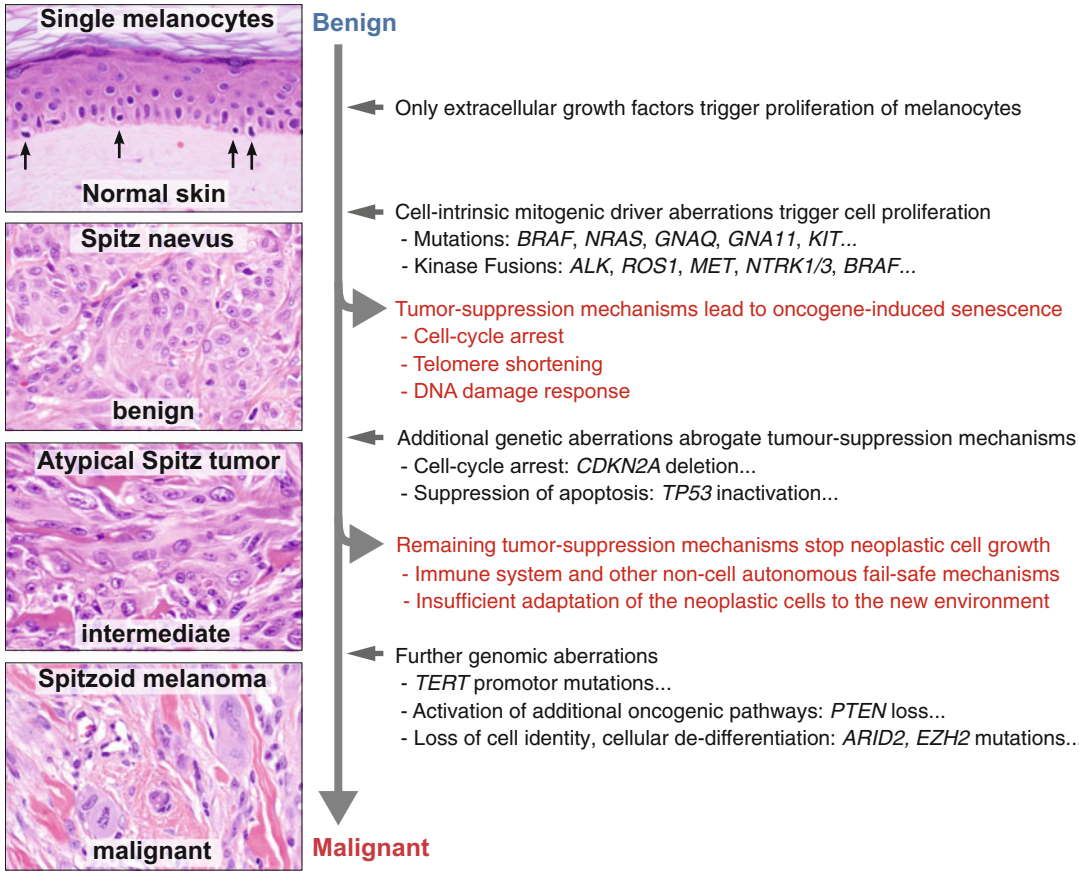


Fig. 5 Traditional tumor progression model. Many melanocytic tumors likely develop through sequential acquisition of genetic aberrations, suggesting a continuous biologic spectrum rather than a dichotomy between benign and malignant melanocytic tumors. The acquisition of genetic aberrations usually correlates with increased histologic atypia. The order of genetic aberrations displayed here denotes that melanomas have additional mutations, but not that all melanomas arise from nevi. Melanoma arising without an obvious antecedent nevus suggests that aberrations in the tumor-suppression mechanisms develop before the mitogenic driver mutation. Nevi usually only harbor a single mutation activating the MAPK pathway. These mitogenic genetic aberrations initiate tumor formation, but after initial cell proliferation, tumor-suppression mechanisms stably block further growth, and the cells become senescent (oncogene-induced senescence). Intermediate lesions, such as atypical Spitz tumors, abrogate some of these tumor-suppression mechanisms by gaining additional genetic aberrations so that the cells may continue to grow or to survive in distant organs. For example, aberrations of *CDKN2A*, *CDK4*, or *CCND1* undermine cell cycle arrest, and *TERT* promoter mutations may prevent telomere shortening and senescence. Melanomas acquire even more genetic and epigenetic aberrations, which may activate additional oncogenic pathways, affect the chromatin landscape, or reduce cell differentiation, so that the neoplastic cells may colonize and infiltrate other organs (metastasis).

Reed nevi, are heavily pigmented and spindle cell variants of Spitz nevi. Pigmented spindle cell Spitz nevi often show confluent and expansile junctional nesting, pagetoid dispersion, and frequently harbor *NTRK3* translocations (VandenBoom et al. 2018). **Desmoplastic Spitz** nevi usually present as intradermal proliferations of large epithelioid melanocytes that are dispersed between thickened collagen bundles and often harbor *HRAS* mutations and copy number gains of 11p (the locus of *HRAS*) (Bastian et al. 2000).

BAP1-inactivated melanocytic nevi were initially categorized as variants of Spitz nevi, but are grouped in the new WHO classification with

Table 3 Recurrent genetic aberrations abrogating tumor-suppression mechanisms or driving cancer progression in the four molecular subtypes of melanoma

Genetic subtype	BRAF 50%	RAS 25%	NF1 15%	Triple-WT 10%
Average mutations per megabase	12	17	39	9
UV signature	90%	95%	95%	30%
MAPK pathway	*BRAF* V600 mut	*NRAS* Q61 mut *HRAS* G12, G13 mut *KRAS* G12, G13 mut	*NF1* mut/loss	Kinase fusions, *KIT* mut, *GNAQ* mut, *GNA11* mut, *PDGFRa* amp
PI3K pathway	*PTEN* mut/loss (17%) *AKT3* overexpr. (<10%)	*PTEN* mut/loss (8%) *AKT3* overexpr. (40%)	*PTEN* mut/loss (7%) *AKT3* overexpr. (30%)	*PTEN* mut/loss (7%) *AKT3* overexpr. (20%)
Cell cycle dysregulation	*CDKN2A* mut/del (60%) *CCND1* amp (~3%) *CDK4* amp (3%) *RB1* mut (2%)	*CDKN2A* mut/del (70%) *CCND1* amp (8%) *CDK4* amp (7%) *RB1* mut (8%)	*CDKN2A* mut/del (70%) *CCDK4* amp (4%) *RB1* mut (11%)	*CDKN2A* mut/del (40%) *CCND1* amp (11%) *CDK4* amp (15%)
Telomerase dysregulation	*TERT* pro. mut (75%)	*TERT* pro. mut (~70%)	*TERT* pro. mut (~85%)	*TERT* pro. mut (10%) *TERT* amp (15%)
DNA damage response	*TP53* mut (10%) *MDM2* amp (1%)	*TP53* mut (20%) *MDM2* amp (3%)	*TP53* mut (30%) *MDM2* amp (4%)	*TP53* mut (7%) *MDM2* amp (15%)
Epigenetic dysregulation	*ARID2* (15%)	*ARID2* (15%)	*ARID2* (30%)	*IDH1* (rare)

Modified from (Rajkumar and Watson 2016)
Mut mutated, *del* deletion, *overexpr* overexpression, *amp* amplification, *pro*. promoter

combined nevi. BAP1-inactivated melanocytic nevi are histologically composed of epithelioid cells with well-defined cytoplasmic borders, amphophilic cytoplasm, vesicular nuclei, and prominent nucleoli. Other histologic features of Spitz nevi, such as epidermal hyperplasia, hypergranulosis, clefting around junctional nests, and Kamino bodies, are usually absent. All melanocytes in BAP1-inactivated nevi usually harbor *BRAF* V600E mutations, but only the epithelioid melanocytes show a loss of BAP1 expression, indicating that the epithelioid component represents a progression from the common acquired nevus component.

In conclusion, the distinct clinical and histologic appearance of several subtypes of melanocytic nevi often correlates well with the specific underlying genetic alterations (Tables 1 and 2). The number of melanocytic nevi in which no activating mutation is detectable is decreasing, and sensitivity limits of assays and the presence of very rare mutations/aberrations not tested for in routine panels presumably explain most cases in which no mutation is identified (Wiesner 2016).

Intermediate Melanocytic Tumors

As discussed above, benign melanocytic proliferations (nevi) usually exhibit only a single mutation (*NRAS*, *BRAF*, *GNAQ*, etc.) or translocation (*BRAF*, *ALK*, *ROS1*, etc.) in oncogenes that drive cell proliferation via activation of the MAPK signaling pathway (Fig. 4). By acquiring additional genetic aberrations, a nevus may progress to an intermediate melanocytic tumor (Fig. 5). **Intermediate melanocytic tumors** have morphologic features that overlap with melanoma, but lack sufficient histopathologic evidence for a definitive diagnosis of melanoma. These neoplasms are also termed low-grade malignancies, melanocytomas, melanocytic tumors of uncertain malignant potential, or, in the case of intermediate spitzoid tumors, "atypical Spitz tumors." Intermediate lesions may proliferate independently of cell-intrinsic and cell-extrinsic mechanisms that usually inhibit

uncontrolled cell growth. However, intermediate lesions usually do not show all hallmarks of cancer (Hanahan and Weinberg 2011) and usually lack overtly malignant features such as the ability to metastasize.

Array comparative genomic hybridization (CGH) shows that intermediate tumors show more DNA copy number aberrations than nevi, but fewer than melanoma (Fig. 6). Common genetic alterations present in intermediate lesions are *TERT* promoter mutations and loss of *CDKN2A*. Whereas loss of one copy of the *CDKN2A* gene seems to be associated with lower oncogenic potential, homozygous loss of *CDKN2A* indicates a more severe deregulation of cell cycle control (Gerami et al. 2012). These additional genetic aberrations may increase the malignant potential and the probability of metastasis. Nevertheless, no single genetic aberration is diagnostic of a malignant melanocytic proliferation. The gold standard for the diagnosis and classification of melanocytic tumors remains careful histopathologic examination in conjunction with consideration of the clinical characteristics and the judicious use of ancillary genetic assays. If a melanocytic neoplasm has two or fewer genetic aberrations, but is morphologically benign and clinically indolent, it should be classified as a nevus. Examples of nevi with two genetic aberrations, benign histology and indolent clinical behavior, include BAP1-inactivated melanocytic nevi and deep penetrating nevi (discussed below) (Wiesner et al. 2012b; Yeh et al. 2017).

BAP1-inactivated melanocytic tumors usually evolve by the sequential acquisition of *BRAF* V600E mutations (common acquired nevus component) and inactivation of the *BAP1* gene (in the epithelioid melanocytic component). Most of these lesions are indisputably indolent on histologic and clinical grounds and should be termed

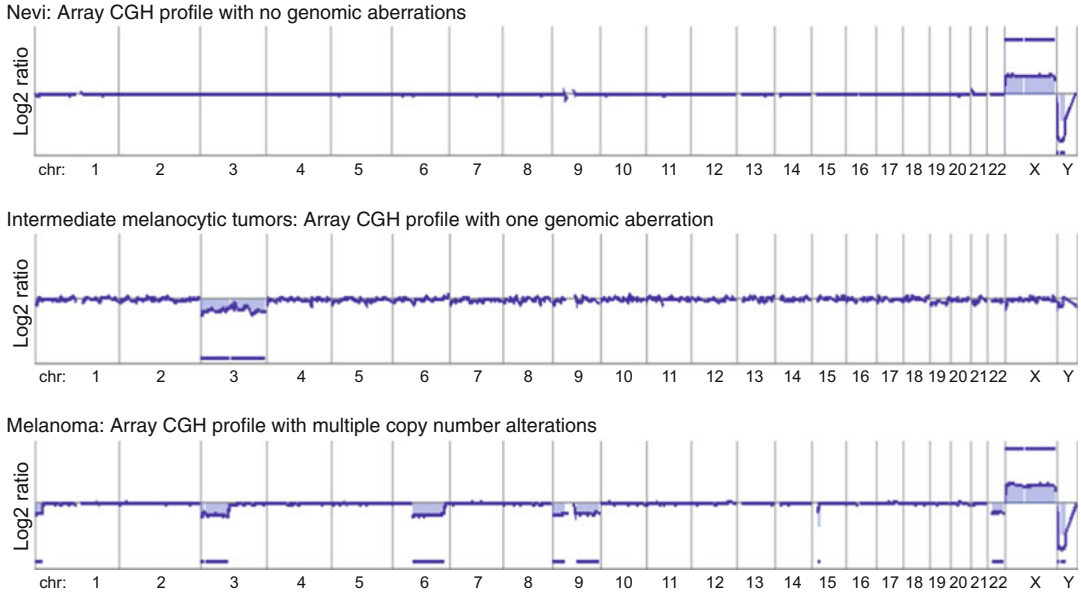


Fig. 6 Tumor progression model illustrated by means of array comparative genomic hybridization (CGH). The array CGH profile of benign melanocytic tumors (nevi) usually shows a flat line indicating no chromosomal gains or losses (top; the profile of the X and Y chromosome indicates that the tumor is from a female patient: two X chromosomes, no Y chromosome). Intermediate melanocytic tumors with histologic atypia have some histopathologic overlap with melanoma but lack sufficient evidence for a pathologic diagnosis of melanoma. These intermediate lesions usually show gains or losses of one or two chromosomes or chromosome arms, which are more than in nevi, but fewer than in melanoma (center; loss of the entire chromosome 3). Melanoma shows marked histologic atypia, pleomorphism, and numerous, atypical mitoses. Their array CGH profiles usually exhibit numerous chromosomal aberrations (bottom; loss of parts of chromosome 1, 3, 6, and 15 and loss of the entire chromosomes 9 and 22).

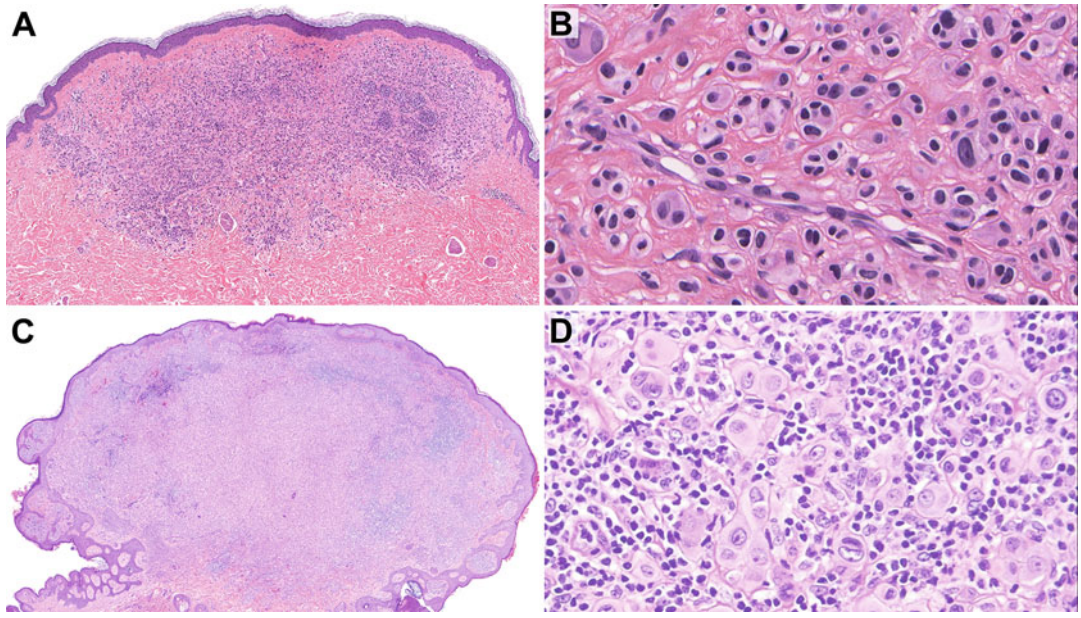


Fig. 7 Histologic spectrum of BAP1-inactivated nevi and tumor/melanocytoma. (**a**) Symmetrical compound nevus with (**b**) large epithelioid melanocytes and only mild histologic atypia. These lesions should be termed "BAP1-inactivated nevus" and usually harbor *BRAF* and *BAP1* mutations. (**c**) Large melanocytic, compound proliferation composed of (**d**) atypical melanocytes with abundant cytoplasm, pleomorphic nuclei with vesicular chromatin, and prominent nucleoli. Numerous tumor-infiltrating lymphocytes. These lesions have likely acquired genetic aberrations in addition to *BRAF* and *BAP1* mutations and should be termed "BAP1-inactivated tumor/melanocytoma."

BAP1-inactivated melanocytic nevi. However, some lesions may acquire additional genetic aberrations and exhibit greater histopathologic atypia and are classified as ***BAP1*-inactivated melanocytic tumor/melanocytoma** (Fig. 7) (Wiesner et al. 2011, 2016; Yeh et al. 2014).

Another nevus type with two genetic aberrations is the **deep penetrating nevus** (Seab et al. 1989). The genetic aberrations in deep penetrating nevi were recently identified and include an activating mutation in a component of the MAPK pathway, most frequently a *BRAF, MAP 2K1, or NRAS* mutation, and genetic alteration of the *CTNNB1* or *APC* gene (Yeh et al. 2017). Similar to *BAP1*-inactivated melanocytic nevi, the genetic alteration in the *CTNNB1* or *APC* gene explain the morphology of deep penetrating nevi. The vast majority of deep penetrating nevi have an indolent clinical course, but if they acquire additional genetic events, they may undergo transformation to melanoma (Yeh et al. 2017).

Pigmented epithelioid melanocytoma is also frequently grouped in the spectrum of tumors consisting of blue nevi, deep penetrating nevi, and spitzoid neoplasms. A recent study demonstrated that while many of these tumors harbor activating aberrations in the MAPK pathway, they also frequently show concomitant genomic aberrations in *PRKAR1A* or *PRKCA* (Cohen et al. 2017).

Melanoma

Up to 30% of primary melanomas are seen in association with melanocytic precursor lesions, including nevi or intermediate melanocytic tumors (Pampena et al. 2017; Shain and Bastian 2016). Similar to the stepwise progression observed in other cancer types, melanoma is also thought to arise through the sequential acquisition of genetic aberrations. It is thought that early mutations affect the MAPK pathway, leading to the formation of nevi (Fig. 5). When no other genomic aberration is present, melanocytes with

a MAPK pathway-activating mutation enter **oncogene-induced senescence**. If benign or intermediate melanocytic tumors acquire additional genetic aberrations in tumor suppressor genes, chromatin-modifying genes, or other cancer-related genes, they may progress further and eventually acquire metastatic potential (Shain et al. 2018).

However, only a minority of melanomas show melanocytic precursor lesions in their vicinity. One possible explanation for this observation could be that in these cases melanocytes first acquire genomic aberrations that lead to abrogation of the tumor-suppression mechanisms, such as dysregulation of their cell cycle, telomerase activity, epigenetic homeostasis, or DNA damage response. These genomic aberrations are usually not found in nevi and are therefore considered to be secondary events in the development of melanoma. Cells with these genetic aberrations may survive as single melanocytes in the epidermis, and when the MAPK pathway is activated in a later, additional genetic event, they immediately start to proliferate unrestrained as a melanoma (Fig. 5). Alternatively, the sequential acquisition of mutations may occur so rapidly that any precursor lesion that arises may exist too transiently to allow detection (Baca et al. 2013; Stephens et al. 2011).

Recently, it has been suggested that for conventional epidermal-derived melanocytic tumors, a minimum of three key mutations is necessary for progression to melanoma. In this study, exonic regions of 293 cancer genes were investigated, but the vast majority of the genome was not examined (Shain et al. 2015b). Most melanomas harbor high numbers of mutations and genetic aberrations (Cancer Genome Atlas 2015; Hayward et al. 2017), and while three genetic aberrations may indeed be sufficient for malignant progression in some cases, it seems likely that the number of genetic aberrations required for a melanocytic neoplasm to become fully malignant varies considerably; in many cases, malignant melanocytic tumors harbor many more than three genetic alterations. Whether a tumor becomes malignant is also highly dependent on the mutation profile and the cellular context. In dermal melanocytic proliferations such as blue nevi, activating mutations in either *GNAQ*, *GNA11*, *CYSLTR2*, or *PLCB4* as well as a co-occurring *SF3B1* or *BAP1* mutation may result in a malignant tumor (Griewank et al. 2017). However, the same *BAP1* mutation co-occurring with a *BRAF V600E* mutation is typical for BAP1-inactivated melanocytic nevi, which have a negligible risk of metastasis (Wiesner et al. 2011, 2012b).

In summary, the progression from benign to malignant melanocytic proliferations is associated with an increasing number of genetic aberrations. Although the characterization of the genetic events present in a melanocytic tumor may help classify the biologic behavior of melanocytic tumors in the future, it remains critical to correlate the histopathologic features with the genetic findings. Tumors found to harbor only one genetic event should not be considered benign if they show worrisome histologic traits such as high mitotic index and elevated proliferation index (e.g., Ki-67/MIB-1 expression) or areas of necrosis. Although histopathology remains the primary approach for classifying melanocytic tumors, morphologically innocuous tumors should be reviewed with care if they are found to have a genetic profile suggestive of a malignant melanocytic tumor (e.g., *GNAQ* and *BAP1* mutations in blue nevus-like melanocytic neoplasms).

Genetic Aberrations in Melanocytic Tumors

As in other cancer types, neoplastic transformation of melanocytes results in part from genetic mutations that lead to abnormal cell proliferation, survival, motility, differentiation, and identity. The genetic aberrations accumulate during this multistep development that ultimately culminates in the invasion of cancer cells into surrounding tissues and metastasis to distant organs. Whereas nevi usually contain only genetic aberrations affecting cell proliferation via activation of the MAPK pathway (Table 1), intermediate melanocytic tumors and melanoma show genetic aberrations that affect additional oncogenic

pathways, dysregulation of the cell cycle, telomerase, DNA damage response, or cell identity (Tables 2 and 3).

MAPK-Activating Aberrations

Various genetic mechanisms can lead to an activation of oncogenic signaling pathways. Gain-of-function mutations, usually missense mutations, lead to constitutively active proteins. Gene amplification often results in overexpressed proteins that can cause an activation of the corresponding downstream signaling pathway. Translocations can either lead to constitutively active, chimeric proteins (fusion genes) or to increased protein expression by combining coding genes with non-physiologic regulatory domains such as promoters or enhancers. Different genetic aberrations affecting the same gene can also be present simultaneously in a cancer cell (e.g., concurrent *KIT* mutations and amplifications) (Curtin et al. 2006).

The most prevalent activating events in cutaneous melanoma are aberrations increasing the signal output of the MAPK pathway (Fig. 4). Four genetically distinct groups of tumors have been proposed (Cancer Genome Atlas 2015; Hayward et al. 2017) (Table 3, Fig. 8):

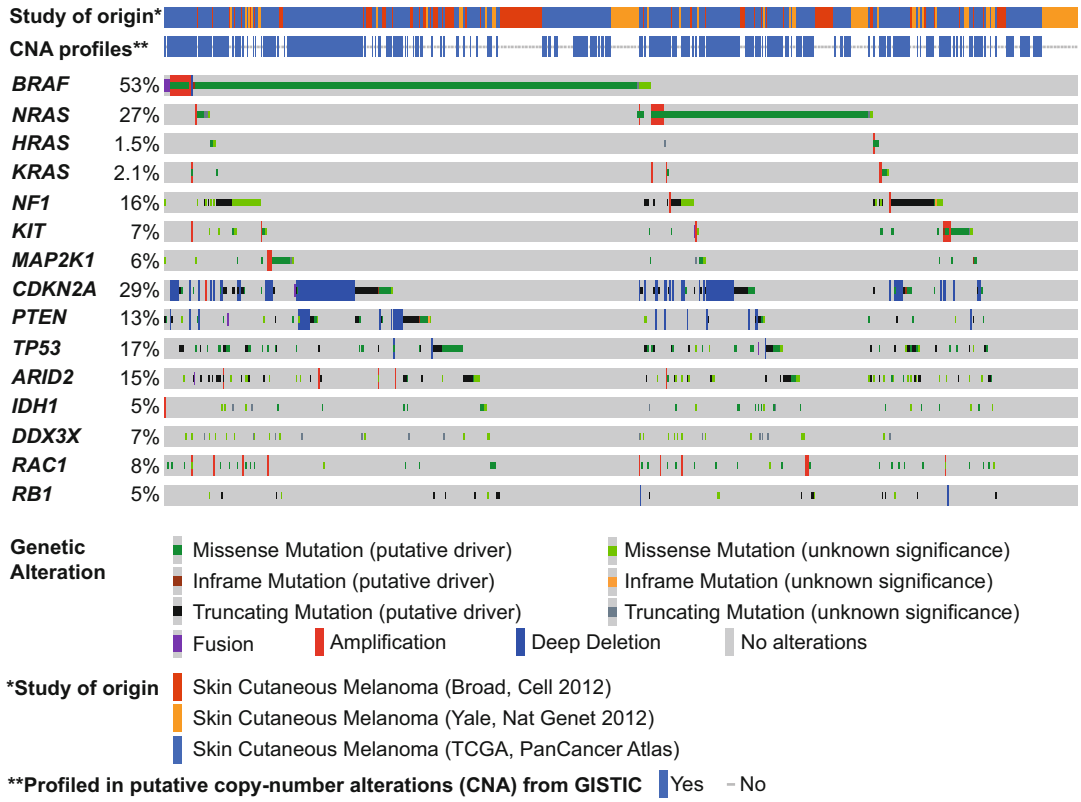


Fig. 8 Frequent genetic aberrations in cutaneous melanoma. Color-coded matrix of frequent genetic aberrations in cutaneous melanoma. The figure was created using the OncoPrint function in the cBioPortal (http://www.cbioportal.org/) (each row represents a gene, and each column represents a melanoma sample, which is not clearly visible due to the large number of samples). In total, the genetic aberrations of 660 melanoma samples were queried, which were previously reported in The Cancer Genome Atlas (TCGA), PanCancer Atlas (provisional), and 2 additional large studies characterizing the genetic landscape of melanoma (Hodis et al. 2012; Krauthammer et al. 2012).

1. ***BRAF*-mutated melanoma** accounts for about 50% of all melanoma.
2. ***RAS*-mutated melanoma** represents around 25% of melanoma, including most frequently *NRAS* and less frequently *KRAS* and *HRAS* mutations.
3. ***NF1*-mutated melanoma** accounts for 10–15% of melanoma. The high frequency of these mutations as well as their frequent occurrence in melanoma lacking *BRAF* and *NRAS* mutations has been appreciated in recent next-generation sequencing studies.
4. **Triple-wild-type melanoma** represents around 10% of melanoma and is defined as melanoma not harboring *BRAF*, *NRAS*, or *NF1* mutations. This heterogeneous group includes tumors harboring rarer activating genetic events such as mutations in *KIT*, *GNAQ,* and *GNA11* or translocations of kinases.

BRAF

BRAF belongs to the family of *RAF* genes. *RAF* was first detected as the cellular homologue of v-raf, a gene present in a transforming murine retrovirus (Rapp et al. 1983). The initial *RAF* isoform detected was *CRAF* (Jansen et al. 1983; Rapp et al. 1983). In vertebrates two other *RAF* isoforms exist, *ARAF* and *BRAF* (Wellbrock et al. 2004). In the signal transduction pathway, RAF proteins are downstream of the small G protein RAS and lead to activation and phosphorylation of ERK1/ERK2 (extracellular signal-regulated kinase) through activation of MEK1/2 (Fig. 4).

In 2002, *BRAF* mutations were identified in a genetic screen as a frequent event in human cancers, particularly in melanoma (Davies et al. 2002). Although functionally relevant mutations can occur in various regions of the gene, the overwhelming majority of mutations result in substitutions of the valine residue at the amino acid position 600 of the protein. The most frequent (~75%) of these mutations results in a V600E substitution (valine V is substituted by glutamic acid E). Mutations resulting in non-V600E protein aberrations at amino acid position 600 account for around 20% of mutations; most frequently (~17%) in V600 K substitutions (valine V to lysine K) (Menzies et al. 2012), less commonly in V600D (valine V to aspartic acid D), V600G (valine V to glycine G), and other V600 alterations also occur (Heinzerling et al. 2013). Mutations in other regions of the *BRAF* gene may also be pathogenic but are less well studied.

BRAF mutations are thought to be early events in the tumorigenesis of melanocytic tumors and are present in the majority of common acquired nevi (Pollock et al. 2003). Whereas individual reports have suggested that *BRAF* mutations are associated with an unfavorable prognosis in patients with melanoma (Long et al. 2011; Mann et al. 2012), this has not been definitively shown in other studies (Bhatia et al. 2015). With the advent of BRAF inhibitor therapies, it has become difficult to compare survival, as patients with and without *BRAF*-mutated melanoma no longer receive identical treatment. Genomic amplification of the *BRAF* locus and expression of *BRAF* splice variants confer resistance to MAPK inhibitors, demonstrating the importance of this node in the MAPK pathway. It is likely that these mechanisms of *BRAF* activation play an underappreciated role in melanoma pathogenesis.

NRAS

NRAS is a member of the *RAS* family of oncogenes and was initially detected in oncogenic viruses (Hall et al. 1983). Soon thereafter, *NRAS* was found to be mutated in melanoma cell lines (Albino et al. 1984). *NRAS* mutations occur in around 20% of melanomas and are evenly distributed among cutaneous, acral, and mucosal melanomas (Cancer Genome Atlas 2015; Hayward et al. 2017). Mutations in the other two RAS family members, *HRAS* and *KRAS*, are less common (each in 1–2% of melanoma) but are recurrent and generally occur in a mutually exclusive fashion with *NRAS* mutations.

Mutations in *RAS* genes typically affect codons 12, 13, and 61. Whereas the majority of mutations in *NRAS* affect the glutamine on position 61 (Q61), mutations in *KRAS* and *HRAS* are more often found at the glycine 12 and 13 (G12, G13). All three hotspot mutations (Q61, G12 and G13) lead to

GTPase inactivation, resulting in a constitutively active GTP-bound protein. Activation of the RAS protein signals through multiple oncogenic downstream pathways, including activation of the MAPK signaling pathway by triggering the RAS/RAF/MEK/ERK signaling cascade, and activation of the PI3K/AKT pathway via PI3K (phosphatidylinositol 3OH-kinase) phosphorylation (Fig. 4).

NF1

NF1 is a tumor suppressor gene that is inactivated by germline mutations in patients with the hereditary disease, type 1 neurofibromatosis (Wallace et al. 1990). Its recognition as an oncogene in various malignancies came later, when it was found to be inactivated in a number of human malignancies, including lung adenocarcinomas, neuroblastomas, and glioblastomas (Ding et al. 2008; Parsons et al. 2008). *NF1* encodes the protein neurofibromin which negatively regulates RAS by inducing hydrolysis of RAS-bound GTP to GDP (Martin et al. 1990). Functional inactivation of *NF1* leads to activation of RAS and its downstream signaling pathways including the MAPK, PI3K/AKT, and mTOR pathways (Fig. 4) (Dasgupta et al. 2005).

The role of *NF1* in melanoma was recognized when next-generation sequencing studies found inactivating *NF1* mutations in melanomas without *BRAF* and *NRAS* mutations. *NF1*-mutant melanoma represents the third class of melanoma harboring activating mutations in the MAPK pathway, accounting for around 10–15% of melanomas. *NF1* mutations are generally inactivating, frequently truncating mutations or losses, and occur throughout the gene, rather than in specific hotspots. Certain tumor types, particularly melanomas arising in heavily sun-damaged skin (lentigo maligna melanomas) or desmoplastic melanomas, show a particularly high rate of *NF1* mutations (Krauthammer et al. 2015; Shain et al. 2015a; Wiesner et al. 2015). Acral and mucosal melanomas, which have lower rates of *BRAF* and *NRAS* mutations also harbor *NF1* mutations more frequently (Cosgarea et al. 2017; Moon et al. 2018).

Studies have suggested that *NF1* may be associated with resistance to BRAF and MEK inhibitors (Maertens et al. 2013; Whittaker et al. 2013). However, *NF1*-mutated melanomas have also been associated with tumors with high tumor mutation burden that respond well to immunotherapy (Cirenajwis et al. 2017; Eroglu et al. 2018). These findings indicate that the *NF1* mutational status may be important for therapeutic decision-making.

MAP2K1/MAP2K2(MEK1/MEK2)

MAP2K1 and *MAP2K2* are also termed *MEK1 and MEK2*, respectively. They encode for protein kinases that are downstream of RAF proteins in the MAPK pathway, integrate multiple biochemical signals, and are considered as gatekeepers of ERK1/ERK2 activity. MEK2 forms a heterodimer with MEK1 that promotes extracellular signal-regulated kinase (ERK) phosphorylation (Caunt et al. 2015). *MAP2K1/MAP2K2* mutations have been shown to confer cross-resistance to RAF and MEK inhibitors in melanoma (Fig. 4) (Nikolaev et al. 2011; Van Allen et al. 2014).

KIT

The *KIT* gene encodes a receptor tyrosine kinase protein. *KIT* aberrations include mutations and copy number alterations and have been reported to occur in up to 30% of acral and mucosal melanomas, as well as in melanomas on chronically sun-damaged skin (Curtin et al. 2006). Aberrations of *KIT* are usually mutually exclusive of *BRAF*, *NRAS*, and *NF1* mutations. *KIT* mutations in melanoma are widely distributed across the gene, but frequently occur in exons 11 and 13. Tumors with L576P and K642E mutations respond more often to KIT inhibitors such as imatinib (Beadling et al. 2008; Hodi et al. 2008). However, these responses are largely not durable (Fig. 4) (Carvajal et al. 2011; Guo et al. 2011).

GNAQ/GNA11/CYSLTR2/PLCB4

GNAQ and *GNA11* mutations were first identified in blue nevi and uveal melanomas (Van Raamsdonk et al. 2009; Van Raamsdonk et al. 2010). The mutations were later also identified in central nervous system melanomas (Murali et al. 2012; van de Nes et al. 2016) and in blue nevus-like melanomas (Griewank et al. 2017).

Additionally, *CYSLTR2* and *PLCB4* mutations were also identified in these tumors. All these mutations are mutually exclusive of each other and *BRAF*, *NRAS*, and other activating mutations. These activating hotspot mutations all appear to activate the PLCβ, MAPK, and Hippo pathways (Feng et al. 2014; Van Raamsdonk et al. 2010). *GNAQ* and *GNA11* mutations frequently co-occur with *EIF1AX*, *SF3B1*, or *BAP1* mutations in malignant melanocytic tumors (Robertson et al. 2017).

Translocations

Translocations are an alternative mechanism to activate the MAPK and other oncogenic pathways. Long recognized in hematologic malignancies and various sarcomas, the presence of translocations in melanoma was not appreciated until relatively recently. In particular, melanocytic tumors with spitzoid morphology (Spitz nevi, atypical Spitz tumors, and spitzoid melanomas) were found to frequently harbor translocations, resulting in fusion genes of *ALK*, *ROS1*, *RET*, *BRAF*, *NTRK1*, *NTRK3*, and *MET* (Wiesner et al. 2014; Yeh et al. 2015, 2016). These translocations were found to be mutually exclusive of each other and of other MAPK-activating mutations, supporting the identified translocations as being important driver events.

The translocations fuse the kinase domains of various oncogenic proteins to a wide range of 5′ fusion partners, including novel fusion partners and genes involved in similar rearrangements in hematologic, lung, colon, and thyroid cancers. Most of the 5′ fusion partners have coiled-coil domains, suggesting that they allow the kinase domains to dimerize and autophosphorylate, resulting in ligand-independent constitutive activation of multiple oncogenic signaling pathways, including the MAPK and PI3K pathways. Many of the kinases involved in the translocations, such as *ALK*, *ROS1*, *RET*, and *NTRK1/NTRK3*, are expressed only during neural crest development and are silenced in adult tissues. The kinase fusions thus lead simultaneously to expression and kinase activation, which explains why rearrangements (rather than point mutations) are the predominant means of oncogenic activation of genes that are usually not expressed in adult tissues.

Whereas most translocations appear almost exclusively in spitzoid neoplasms, *BRAF* translocations also occur in conventional melanomas (Botton et al. 2013). The functional consequences vary depending on the translocation type and on the activated signaling pathway. However, in most cases, the translocations activate multiple oncogenic pathways, including the MAPK and PI3K signaling pathways (Wiesner et al. 2014). As inhibitors are available for some of these translocations, such as crizotinib for *ALK* and *ROS1* translocations, identifying the pathogenic translocation in melanocytic tumors can be of potential therapeutic significance for affected patients (Fig. 4).

Loss-of-Function Mutations

CDKN2A

CDKN2A was first identified as a gene locus on chromosome 9p21.3 linked to familial melanoma predisposition (Cannon-Albright et al. 1992). Later studies identified *CDKN2A* as an important gene in this locus, responsible for regulating the cell cycle (Kamb et al. 1994). *CDKN2A* is the most frequently affected tumor suppressor gene in sporadic melanoma (Curtin et al. 2005). *CDKN2A* losses, often biallelic, are found in 50–80% of sporadic melanoma (Cancer Genome Atlas 2015; Gast et al. 2010), and inactivating mutations or promoter methylation silencing of the gene have been described in 9–28% of tumors (Bennett 2008).

The *CDKN2A* gene has two alternate reading frames coding for p16 and $p14^{ARF}$ (Quelle et al. 1995). p16 maintains cell cycle control by downregulating cyclin-dependent kinases (CDK) 4 and 6. This blocks phosphorylation of the retinoblastoma protein (Rb) (Koh et al. 1995; Lukas et al. 1995). Rb phosphorylation releases the E2F transcription factor with cell cycle progression from G1 to S phase (Weinberg 1995). $p14^{ARF}$ inhibits the ubiquitin ligase MDM2 which targets proteasomal degradation of TP53. TP53 can control the cell cycle by inducing p21, inhibiting CDK2

and cyclin E-induced phosphorylation of Rb (Weinberg 1995) (Fig. 4).

PTEN

The PTEN protein functions as a tumor suppressor by inhibiting PI3K signaling. The lipid phosphatase domain of PTEN dephosphorylates PI3K 3-phosphoinositide products, resulting in inhibition of various oncogenic signaling pathways, most prominently the AKT pathway (Fig. 4).

PTEN has been reported to be lost or mutated in 43% (15/35) of melanoma cell lines and 38% of primary melanomas (Guldberg et al. 1997). Other studies have suggested higher frequencies of loss, in up to 70% of melanomas (Bastian et al. 1998; Cancer Genome Atlas 2015; Gast et al. 2010). Epigenetic silencing of *PTEN* may also play a role in melanoma (Lahtz et al. 2010; Mirmohammadsadegh et al. 2006). *PTEN* loss is more frequently identified in *BRAF*-mutant than in *NRAS*-mutant melanoma (Curtin et al. 2005; Gast et al. 2010), a finding consistent with PI3K/AKT pathway activation by *NRAS* but not *BRAF* mutations which require an additional hit. *PTEN* inactivation has been associated with resistance to BRAF inhibitors (Zuo et al. 2018) and immunotherapy (Peng et al. 2016) in melanoma patients.

Noncoding Genetic Aberrations

TERT Promoter Mutations

Activating mutations in the telomerase reverse transcriptase (*TERT*) promoter were identified in up to 71% of cutaneous melanomas (Horn et al. 2013; Huang et al. 2013) and a wide range of human tumors (Killela et al. 2013; Vinagre et al. 2013) representing the most frequent point mutation in human cancers. Mutations were shown to increase gene expression, with increased telomerase expression allowing neoplastic cells to continuously proliferate without entering senescence or apoptosis by maintaining telomere length and avoiding chromosomal instability. Functional studies demonstrated that the mutations led to increased binding of the GABP (GA-binding protein) transcription factor resulting in aberrant gene expression (Bell et al. 2015). Recently *TERT* promoter mutations were shown to promote tumorigenesis in two phases, primarily not by impeding telomere shortening but by repairing the shortest telomeres. Genetic instability then occurs, but later mutation-induced increased telomerase expression enables continued cell proliferation (Chiba et al. 2017).

Mutations in the *TERT* promoter occur primarily in a few mutational hotspots. The most frequent mutations occur at chr5:1295250C>T (−124C>T) or chr5:1295228C>T (−146C>T). In most cases, the identified mutations are mutually exclusive. Multiple mutations can occur, but this is rare. Somewhat less frequent, but also recurrent, are chr5:1295242_243CC>TT (−138_139CC>TT) and chr5:1295228_229CC>TT (−124_125CC>TT) aberrations. Reports have suggested that *TERT* promoter mutations are associated with more aggressive behavior and poorer prognosis (Griewank et al. 2014; Lu et al. 2015).

Mutations in Other Noncoding Regions of the DNA

With the recognition of *TERT* promoter mutations as common events in melanoma, the search for other pathogenetically relevant mutations in noncoding regions in melanoma intensified. A general challenge in melanoma is that they harbor numerous mutations, many of which are background/passenger mutations, and it is difficult to identify pathogenically relevant driver mutations. Proposed promoter mutations include *NFKBIE* mutations in desmoplastic melanomas (Shain et al. 2015a) as well as in other cutaneous melanomas (Hayward et al. 2017). Recurrent *SDHD* promoter mutations have also been reported (Weinhold et al. 2014), but have not been confirmed in other studies (Hayward et al. 2017; Scholz et al. 2015). The largest published whole-genome sequencing study of melanoma to date has reported a considerable number of additional mutations affecting transcription factor binding sites (Hayward et al. 2017). However, the pathogenic relevance of these mutations is still not clear and will need to be investigated in future studies.

Noncoding RNAs

Approximately 98% of genetic DNA does not code for proteins, and 90% of expressed RNAs are not transcribed into proteins (Carninci et al. 2005). Noncoding RNAs, such as micro RNA (miRNA) or long noncoding RNA (lncRNA), play incompletely understood roles in the pathogenesis of different cancer types by fine-tuning the expression of proteins and regulating biologic processes (Bartonicek et al. 2016). In recent years, the number of newly recognized noncoding RNAs has increased significantly, and the field is rapidly expanding. However, their role in melanoma pathogenesis still needs to be defined before their potential clinical value can be assessed.

Micro RNAs (miRNAs) are 21–23-nucleotide-long RNA sequences that primarily bind and influence the expression of coding mRNAs. miRNAs are frequently grouped together in loci and expressed together. To become active, miRNAs must first be processed with a number of different processing mechanisms including the Drosha and Dicer complex. They have been reported to be differentially expressed in benign and malignant lesions and associated with poor prognosis, and therapy resistance. Examples of miRNAs reported to be important in melanoma are the miRNAs 17–92 complex associated with melanoma progression (Greenberg et al. 2014); miRNA-125b reported to influence cell proliferation, migration, and senescence (Kappelmann et al. 2013); and miRNA-143 which regulates proliferation and apoptosis, is often expressed at lower levels in melanoma than in normal tissue, and is associated with improved survival when highly expressed (Li et al. 2014b; Segura et al. 2010). Signatures of miRNA expression have been applied to predict recurrence (Segura et al. 2010), as markers of metastasis (Hanniford et al. 2015a, b), or to distinguish melanoma subtypes (Chan et al. 2011).

Long noncoding RNA (lncRNA) is also differentially regulated in multiple cancer types (Yan et al. 2015). Mutations or SNPs can alter lncRNA processing, expression, secondary structure formation, and transcription factor regulation (Wan et al. 2014). Various lncRNAs which are relevant in melanoma pathogenesis have been described. One example is BANCR (*BRAF*-activated noncoding RNA), which is upregulated in *BRAF*-mutant melanoma, was found to be important for proliferation of cells in culture and associated with poorer patient survival when highly expressed (Li et al. 2014a). MALAT1 and HOTAIR were both found to be expressed at higher levels in metastatic melanoma than in primary melanoma and were reported to influence tumor migration (Tang et al. 2013; Tian et al. 2014). SAMMSON, another recently described lncRNA, was reported to be expressed in 90% of melanoma, but not in benign melanocytes and nevi. In future, lncRNAs may also be of diagnostic use for the prediction of clinical behavior of melanocytic tumors (Liu et al. 2016).

Genes Associated with Increased Melanoma Susceptibility

Familial melanoma is defined as melanoma occurring in two or more first-degree relatives (see also ▶ "Molecular Epidemiology of Melanoma"). The term familial melanoma excludes disorders such as xeroderma pigmentosum or albinism which are characterized by extreme sensitivity to UV radiation. The proportion of melanomas occurring in melanoma-prone families is ~7%. Generally, countries with higher levels of UV radiation exposure have a greater proportion of melanoma cases associated with a family history (e.g., Australia, ~10% vs. Denmark, ~3%) (Ransohoff et al. 2016; Read et al. 2016).

Melanoma susceptibility genes can be classified based on the extent to which they pose a risk of developing melanoma (see also ▶ "Clinical Genetics and Risk Assessment of Melanoma"). High-penetrance genes, which often cause melanoma when altered in the germline, are *CDKN2A*, *CDK4, BAP1, POT1, ACD, TERF2IP*, and the *TERT* promoter. These genes are associated with the development of melanoma in 60–90% of affected individuals (Ransohoff et al. 2016). Within this group, the most common susceptibility gene is *CDKN2A* (Cannon-Albright et al. 1992; Goldstein et al. 2007). Low-penetrance genes such as *MITF* and *MC1R* variants are

associated with a lower risk of developing melanoma. Other gene aberrations associated with an increased risk of developing various tumors, including melanoma, are *BRCA1*, *BRCA2*, *RB*, *TP53*, and various UV repair gene defects causing xeroderma pigmentosum.

Cell Cycle Genes: CDKN2A and CDK4

Mutations in the *CDKN2A* gene are the most frequent germline mutations predisposing to melanoma and are seen in 8–57% of familial melanomas (Eliason et al. 2006; Goldstein et al. 2007). Germline mutations in *CDKN2A* more frequently affect p16; however, cases harboring selective aberration of p14 have also been described (Garcia-Casado et al. 2009; Harland et al. 2005). Patients with *CDKN2A* mutations in p16 also have an increased risk of developing pancreatic cancer, and *CDKN2A* mutations affecting p14 also confer an increased risk of developing neural system tumors (Goldstein et al. 2006).

Another melanoma susceptibility gene affecting cell cycle control is cyclin-dependent kinase 4 (*CDK4*) (Tsao et al. 1998). CDK4 binds p16 and both regulate the downstream protein retinoblastoma. In the reported mutations to date, *CDK4* mutations alter the R24 residue, disrupting p16 protein binding and subsequent cell cycle control. *RB1* gene mutation carriers are also at a somewhat increased risk of developing melanoma (Fig. 4) (Chin et al. 2006).

Telomere-Associated Mutations: TERT, POT1, ACH, and TERF2IP

TERT promoter mutations were described in one family with very high penetrance for cutaneous melanoma at an early age (Horn et al. 2013). These mutations create new binding sites for *TERT*, increasing transcription, allowing unlimited cell division, and subsequently promoting cancer progression. Other families with *TERT* promoter mutations have been described; however, mutations in the *TERT* promoter appear to be rare events in families with melanoma predisposition (Harland et al. 2016). Interestingly, the mutations identified in familial melanoma (Chr.5:1295161C>T or −57C>T) are only rarely identified in sporadic melanoma.

Mutations in telomere-related genes, such as *POT1*, *ACD* (adrenocortical dysplasia homologue), and *TERF2IP* (telomeric repeat-binding factor 2, interacting protein), have also been identified in hereditary melanoma families. *POT1* founder mutations were detected in multiple cutaneous melanoma families in Italy (Robles-Espinoza et al. 2014; Shi et al. 2014). *POT1* encodes a protein, involved in a telomere-protecting complex (shelterin), which protects chromosomal ends from being damaged, degraded, or inappropriately processed by DNA repair mechanisms (Doksani and de Lange 2014). Mutations in other shelterin complex components were identified shortly afterward in families with predisposition to cutaneous melanoma (Aoude et al. 2015). Germline mutations in shelterin complex genes (i.e., *ACD*, *TERF2IP*, and *POT1*) and the *TERT* promoter are rare but are reported to account for ~10% of melanoma predisposition in families not demonstrating *CDKN2A* or *CDK4* mutations (Aoude et al. 2015).

BAP1

Inactivating *BAP1* mutations cause the *BAP1* tumor predisposition syndrome (BAP1-TPDS), which is inherited in an autosomal dominant pattern. Individuals carrying heterozygous *BAP1* mutations have an increased risk of developing various tumor types, most commonly BAP1-inactivated nevi/melanocytomas of the skin, uveal and cutaneous melanoma, peritoneal and pleural mesotheliomas, clear cell renal cell carcinoma and basal cell carcinoma, and possibly other cancer types (Wiesner et al. 2011, 2012a).

The most common manifestations of BAP1-TPDS are multiple BAP1-inactivated nevi/melanocytomas of the skin. Beginning in the second decade of life, affected patients usually develop multiple inconspicuous, skin-colored to reddish-brown, dome-shaped to pedunculated, well-circumscribed papules, predominantly on sun-exposed skin. Some of these BAP1-inactivated melanocytic tumors display small areas of brown color at their periphery, likely because they evolve from common acquired nevi. The number of lesions varies, typically ranging from a few to more than 50.

Histologically, BAP1-inactivated nevi/melanocytomas of the skin are predominantly composed of intradermal melanocytes with varying degrees of atypia, ranging from clearly benign lesions with nevoid cells and minimal atypia (BAP1-inactivated nevi) to atypical tumors with large epithelioid cells with well-defined cytoplasmic borders, abundant amphophilic cytoplasm, pleomorphic vesicular nuclei, and prominent nucleoli (BAP1-inactivated melanocytomas). Many of these skin lesions appear as combined melanocytic nevi with areas of small, oval melanocytes (a common nevus component) adjacent to the larger, epithelioid melanocytes. In most lesions, the wild-type *BAP1* allele is inactivated (by various somatic aberrations), resulting in loss of nuclear BAP1 expression by immunohistochemistry. Most lesions also harbor *BRAF* V600E mutations (Wiesner et al. 2011, 2012b).

MITF

Recurrent *MITF* mutations which impair the protein's physiologic sumoylation appear to be an intermediate risk factor for developing melanoma (Bertolotto et al. 2011; Yokoyama et al. 2011). The recurrent *MITF* E318K aberration has been shown to result in gene expression profile changes. The altered regulation of MITF targets leads to a biologic gain-of-function effect, which results in cells showing increased clonogenicity, migration, and invasion (Bertolotto et al. 2011; Yokoyama et al. 2011).

MC1R

The most prevalent low-risk genetic factors for melanoma are variants in melanocortin 1 receptor (*MC1R*) genes (Raimondi et al. 2008). The G protein-coupled receptor signals through cAMP and protein kinase A (PKA). There are numerous *MC1R* polymorphisms which are associated with skin pigmentation, hair color, and ability to tan (Scherer et al. 2008). The risk of developing melanoma differs considerably depending on the variant present and increases when multiple inactivating variants co-occur.

Although the risk of developing melanoma with *MC1R* variants was originally linked primarily to sun exposure, experimental evidence has demonstrated that *MC1R* variants increase melanoma risk independently of UV exposure (Jarrett et al. 2015; Mitra et al. 2012). Due to its low-risk association, *MC1R* is not generally considered a factor associated with hereditary melanoma – it is in the same category as low skin pigmentation with respect to its level of risk for melanoma.

Cancer Syndromes Associated with Increased Risk of Melanoma

There are multiple genes which are known to cause familial cancer syndromes and are associated with various malignancies, including melanoma. Patients affected by these predisposing conditions should receive regular skin checks to enable early identification and treatment of melanoma and potentially other cutaneous malignancies (see also chapter ▶ "Clinical Genetics and Risk Assessment of Melanoma").

Xeroderma pigmentosum is a genetic disease associated with increased occurrence of cutaneous neoplasms due to defects in DNA damage repair mechanisms. Various genes included in mismatch repair can be affected, including *XPA-XPG* (Lambert et al. 1995). While the risk per gene may vary, a general increase in melanoma risk is accepted (Paszkowska-Szczur et al. 2013).

Li-Fraumeni syndrome is caused by mutations in the tumor suppressor gene *TP53*. Affected patients have an increased risk of developing various types of sarcomas, leukemia, breast cancer, and others, frequently beginning at an early age (<30 years) (Li et al. 1988). These affected families also have an increased incidence of melanoma (Curiel-Lewandrowski et al. 2011; Hartley et al. 1987).

Germline mutations in *PTEN*, associated with Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome, are also associated with various cancers, including an increased risk for melanoma (Tan et al. 2012). The retinoblastoma protein is regulated in part by CDK4 and p16. *RB1* mutation carriers, as described in the section referring to gene aberrations affecting the cell cycle, have an increased risk of developing retinoblastoma, but also have an increased risk of developing epithelial tumors (e.g., lung, bladder, and breast cancer) and melanoma (Dommering et al. 2012; Marees et al. 2008).

BRCA1 and *BRCA2* mutation carriers have an increased risk of breast, ovarian, prostate, and pancreatic cancer. The precise level of risk for melanoma is not clear but appears to be modest (Mersch et al. 2015; van Asperen et al. 2005).

Genetic Tests in the Diagnosis of Melanoma

In the past 10 years, the analysis of genetic aberrations in melanocytic tumors has become more common, with the goals of improving diagnosis and prognostic prediction of melanocytic tumors and of aiding and optimizing treatment. While it is likely that NGS-based assays will be dominant in the near future, currently several techniques are still in use including reverse transcriptase polymerase chain reaction (RT-PCR), gene expression profiling (GEP), Sanger sequencing, fluorescence in situ hybridization (FISH), and comparative genomic hybridization (CGH), each with its own set of advantages and disadvantages (Table 4).

Polymerase Chain Reaction-Based Methods

PCR

The polymerase chain reaction (PCR) is a technique to amplify a few copies of a specific DNA region to several millions of copies. PCR-based methods revolutionized the field of molecular biology by allowing fast, cheap, and sensitive amplification of specific DNA fragments from a pool of DNA. PCR techniques are an indispensable component of a plethora of molecular biologic techniques and are widely used to detect pathogens, to sequence genes, and to quantify gene expression (Fig. 9).

PCR-based assays require four basic components: (1) template DNA; (2) primers, which are

Table 4 Ancillary genetic techniques in the diagnosis of cutaneous melanoma

Method	Advantages/benefits	Disadvantages/limitations
Next-generation sequencing	Detects all types of genetic aberrations Scalable to the entire genome (whole-genome sequencing) or to specific parts human genome (targeted sequencing) Can also determine the gene expression profile (RNAseq) or the chromatin landscape (ChIPseq)	Data analysis requires expertise in bioinformatics
Sanger sequencing	Easy and fast method for sequencing less than 1000 bp	No detection of copy number aberrations Expensive, time-consuming, and labor intensive for large genes Mutations with a low frequency (<25%) are difficult to detect
Array comparative genomic hybridization	Genome-wide screening without prior knowledge of genetic aberrations	Low tumor cell percentage may lead to false-negative results No detection of mutations (single nucleotide changes), small deletions or amplifications, translocations, or polyploidy
Fluorescence in situ hybridization	Each cell nucleus is evaluated independently Only small amounts of tissue required	Analyzes only a limited number (usually 4–6) of chromosomal loci No detection of mutations (single nucleotide changes) or small copy number aberrations
Gene expression-based assays	Only small amounts of tissue required	Evaluates gene expression, but not the underlying genetic aberrations
cfDNA (peripheral blood)	Monitors disease recurrence and therapy response	Still experimental; clinical trials are required before routine clinical use

short DNA fragments with a defined nucleotide sequence complementary to the target DNA to be amplified; (3) the four nucleotide components of DNA (A, T, C, G); and (4) DNA polymerase, an enzyme that connects the nucleotides to the PCR product (amplicon). A mix of these components is placed in a thermal cycler, which is a machine that raises and lowers the temperature in three predefined steps (Fig. 10):

1. **Denaturation**: The temperature is raised to ~95 °C, causing denaturation of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.
2. **Annealing**: The temperature is lowered to ~60 °C, allowing annealing of the primers to each of the single-stranded DNA fragments. Usually, one forward and one reverse primer are used – one for each of the two complementary DNA fragments containing the target region.
3. **Elongation**: The temperature is raised to ~72 °C, which is the optimal temperature for the DNA polymerase. In this step, the DNA polymerase binds to the primers and synthesizes the new DNA strand by adding nucleotides that are complementary to the DNA template. For quantitative PCR (qPCR), a dye, termed SYBR Green, is added to a sample, which binds to all double-stranded DNA. As PCR progresses and the number of PCR products increases, the fluorescence intensity increases proportionally allowing the initial DNA concentration to be calculated.

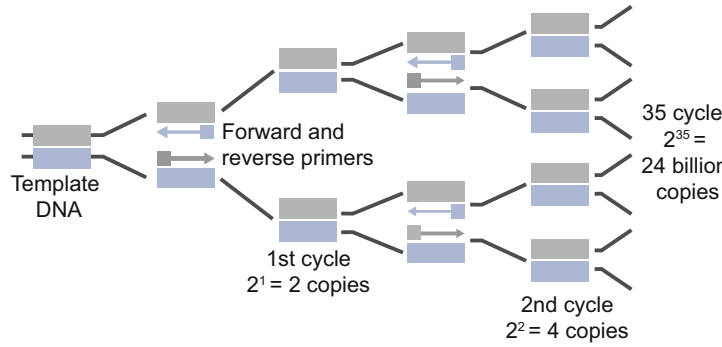


Fig. 9 Principle of polymerase chain reaction (PCR). PCR is an indispensable technique in molecular biology to amplify specific DNA fragments to several millions of copies, termed amplicons.

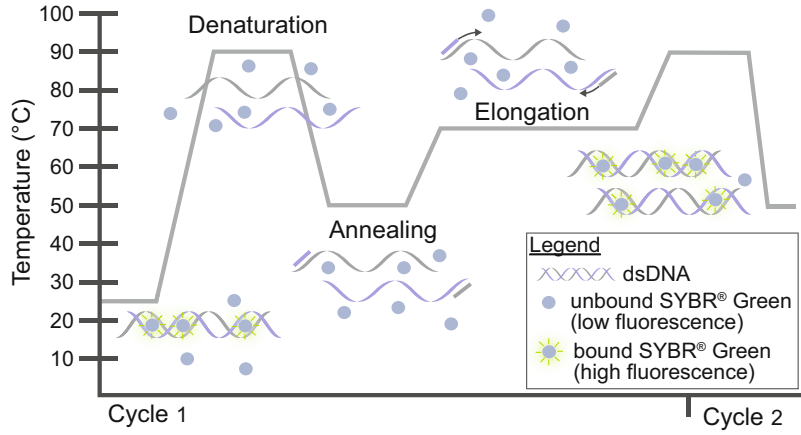


Fig. 10 PCR procedure and principle of quantitative PCR. Typically, PCR consists of a repeated set of temperature changes, called cycles. Each cycle consists of three discrete temperature steps: (1) denaturation: the DNA double helix separates to two single DNA strands; (2) annealing: the primers specifically bind to the single-stranded DNA fragments that are complementary to their own nucleotide sequence; (3) elongation: the DNA

nucleotides in the reaction mixture are added to the new, elongating DNA strand, which is complementary to the DNA template strand.

With each cycle of these three steps, the number of DNA molecules doubles, and billions of amplicons are generated after 20–40 PCR cycles. The amplicons are then visualized with agarose gel electrophoresis, which separates DNA products on the basis of size, or are further analyzed in various downstream applications.

PCR is a very sensitive method and is therefore prone to contamination. Quality controls, including negative and positive controls, are therefore essential at each step. The most common and serious problem is carryover contamination of amplicons from previous PCR reactions. Other difficulties in performing PCR result from low-quality DNA (often caused by inappropriate fixation of tissue specimens, e.g., in unbuffered formalin), non-specific PCR products due to non-specific binding of the primers, or incorporation of incorrect nucleotides by the DNA polymerases.

In addition to the traditional PCR technique described above, various PCR approaches are based on the same principle with slight modifications. In nested PCR assays, the sensitivity and specificity is further increased by amplifying the initial amplicon with a new set of primers intrinsic to the first set of primers. Multiplex PCR assays use two or more pairs of primers targeting different DNA regions in the same reaction mix, resulting in multiple amplicons.

Quantitative PCR and Gene Expression-Based Assays

Quantitative PCR (qPCR) allows for both detection and quantification of specific DNA sequences in real time. Two methods are commonly used for detection and quantification: the first method is based on a non-specific dye, usually SYBR Green, which binds to the double-stranded DNA amplicons in the qPCR reaction and emits fluorescent light. The number of amplicons is proportional to the increase of the fluorescence intensity, which is measured at each PCR cycle (Fig. 10). The second method uses probes that are labeled with fluorescent dyes to detect only amplicons containing the sequence complementary to the probe. The use of such reporter probes increases the specificity, and by using different-colored probes, several amplicons can be monitored simultaneously in the same tube (multiplex qPCR).

Benign and malignant melanocytic neoplasms often show different gene expression patterns. This finding led to the development of assays with the aim of better predicting the biologic behavior of melanocytic lesions or to improve their classification into benign and malignant categories. These gene expression profile (GEP) assays, which are commercialized under names such as melaGenix (Brunner et al. 2013), myPath® Melanoma (Clarke et al. 2015), or DecisionDx™ (Zager et al. 2018), evaluate the expression of 9, 23, and 31 genes, respectively. The genes assessed are specific to the assays offered by the companies and show little or only minor overlap. For these gene expression-based assays, mRNA is isolated and converted into cDNA by reverse transcription. The cDNA is then semiquantitatively analyzed in qPCR reactions to assess the expression levels of the mRNA and the corresponding genes. In unequivocally benign and malignant lesions, the sensitivity and specificity of these assays each seems to be ~80% (which is less than those of histopathologic classification), and their diagnostic value for histopathologically ambiguous melanocytic lesions remains to be determined.

DNA Sequencing Methods

Sequencing is a method to determine the order of nucleotides within a DNA strand (Fig. 11). The knowledge of nucleotide sequences is essential to detect mutations and to assess the status and function of oncogenes and tumor suppressor genes in tumors.

Sanger Sequencing

For more than 25 years, Sanger sequencing was the most widely used sequencing method, but since 2010, it has been rapidly replaced by NGS techniques. However, Sanger sequencing is still in

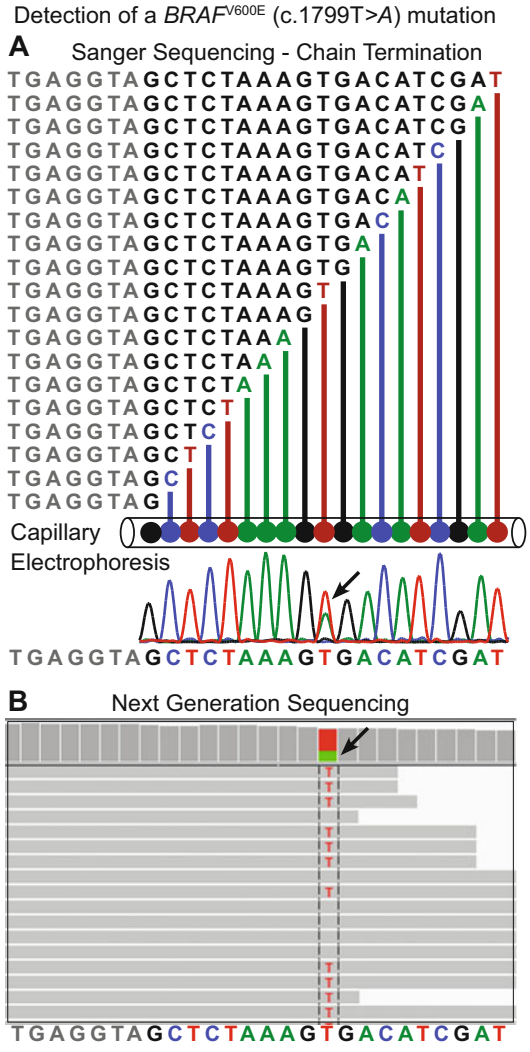


Fig. 11 DNA sequencing. (**a**) Principle of Sanger sequencing using chain termination and capillary electrophoresis resulting in a sequencing electropherogram (analog data). (**b**) Illustration of next-generation sequencing data. Each gray bar represents a sequencing read corresponding to a distinct DNA sequencing cluster on the flow cell (digital data).

use for projects in which only a few exons are sequenced, to validate NGS results and to obtain long contiguous sequence reads with a length of up to 1000 bp.

Sanger sequencing is based on the selective incorporation of chain-terminating nucleotides and requires five components: (1) template DNA, (2) primer, (3) polymerase, (4) a mix of the four normal nucleotides (A, T, G, C), and (5) four labeled nucleotides, which are each labeled with a different fluorescent dye and terminate the DNA strand elongation. During the sequencing reaction, the primer hybridizes to the single-stranded DNA template, and the polymerase synthesizes the complementary strand but is randomly interrupted by the fluorescent-labeled nucleotides leading to chain termination. The resulting DNA fragments, which are randomly terminated by the incorporated fluorescent-labeled nucleotides, have different lengths and are size-separated by capillary electrophoresis. During capillary electrophoresis, the negatively charged, fluorescently labeled DNA fragments are injected into capillaries and move through the capillaries toward the positive electrode, resulting in size separation. Then, the DNA fragments pass through a laser beam, generating fluorescence signals according to the labels on the nucleotides. The nucleotide sequence is computationally inferred from the optically detected fluorescence signal patterns (Fig. 11).

Next-Generation Sequencing

NGS (also termed massively parallel sequencing) has transformed the molecular analysis of cancers and has evolved to become the dominant technique in molecular diagnostics. NGS is more comprehensive than any other genetic assay, such as Sanger sequencing, array CGH, or FISH, each of which only assesses specific genetic regions or specific types of genetic aberrations. Using NGS assays, the entire genome can be evaluated for all types of genetic aberrations in a single experiment. NGS assays can be scaled to the required tasks, ranging from sequencing the entire genome (whole-genome sequencing, WGS) to sequencing only specific areas of interest (targeted sequencing). Targeted sequencing assays may include all protein-coding regions (whole-exome sequencing, WES; ~2% of the genome), only a few selected genes (e.g., cancer-related gene panels), or other genomic areas of interest. Targeted sequencing approaches are currently more cost-effective than WGS and allow to focus on specific regions of interest.

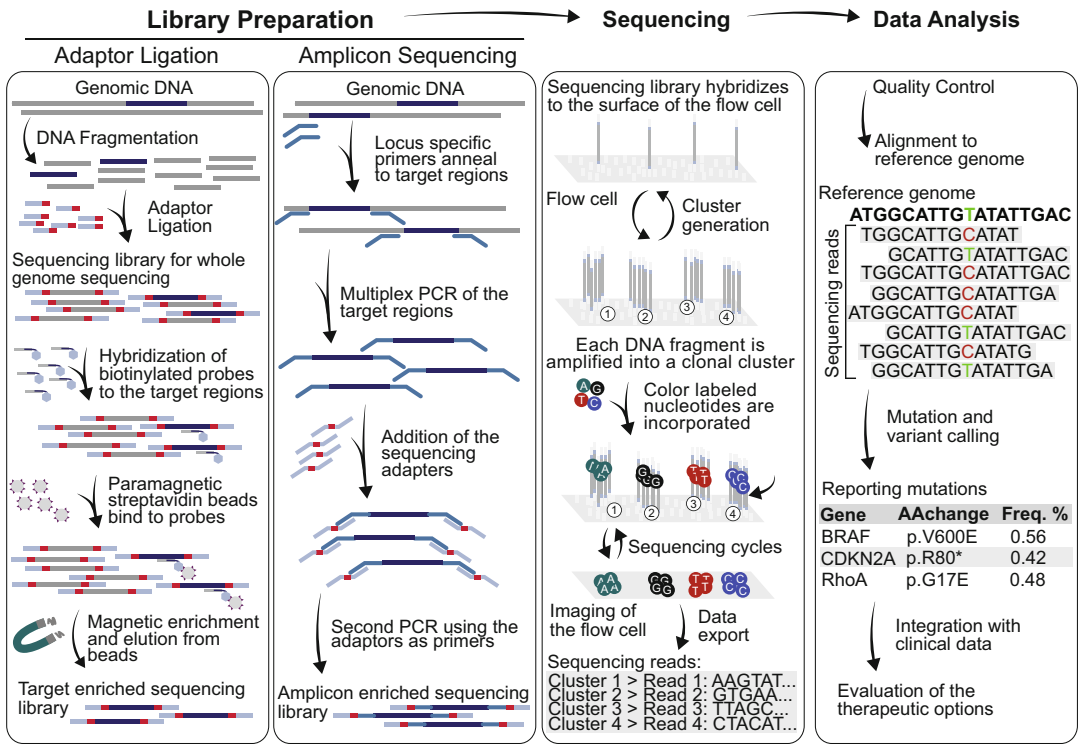


Fig. 12 Principles and workflow of next-generation sequencing (NGS). The NGS workflow on Illumina platforms can be divided into three steps: (1) library preparation – the test DNA is flanked with sequencing adaptors either by a ligation step (adaptor ligation) or a PCR step (amplicon sequencing). The DNA regions of interest can be enriched before (in case of amplicon sequencing) or after (in case of adaptor ligation) the addition of the sequencing adaptors. (2) Sequencing: the adaptor-flanked test DNA binds to oligonucleotides bound on the surface of the flow cell. The DNA polymerases catalyze the incorporation of fluorescently labeled nucleotides, which are identified by fluorophore excitation. After enzymatic removal of the fluorophores, a new fluorescently labeled nucleotide is incorporated and the nucleotide of the test DNA is read out. (3) Data analysis – after quality controls and alignment of the sequencing reads to reference genomes, the genetic aberrations are called and annotated using various computational algorithms.

The technology used for NGS varies among different platforms. Here, we discuss the dominant technology commercialized by Illumina Inc., which currently holds a global market share of >90% in this area. The concept is similar to Sanger sequencing, but instead of sequencing a single DNA fragment, NGS evaluates millions of DNA fragments at the same time (hence the term "massively parallel sequencing"): after ligating the test DNA to sequencing adaptors that bind to surface-bound oligonucleotides, the DNA polymerases catalyze the incorporation of fluorescently labeled nucleotides into DNA templates. The incorporated nucleotides are subjected to fluorophore excitation, and the resulting signals are detected with a high-resolution camera, after which the fluorophores are enzymatically removed, and the next cycle starts. The process can be divided into three steps (Fig. 12):

1. **Library preparation**: Two approaches are currently used to prepare the sequencing libraries. The first method (**adapter ligation**) randomly fragments the DNA, followed by ligating sequencing adapters to both ends of the DNA fragments. The adapter-ligated DNA fragments are then used directly for whole-genome sequencing or are further enriched for specific regions of interest (**target enrichment**). For target enrichment, the DNA

fragments are captured by hybridizing the sequencing library to biotinylated probes that specifically bind to the complementary DNA fragments containing the regions of interest. The biotinylated DNA probes are then isolated by magnetic pulldown, resulting in an enrichment of the DNA fragments with the regions of interest. Depending on the library preparation kit, target enrichment captures regions ranging between 1 and 70 megabases (Mbs). The second approach (**amplicon sequencing**) uses multiplex PCR to amplify DNA segments containing the regions of interest with multiple PCR primers and sequencing adapters.

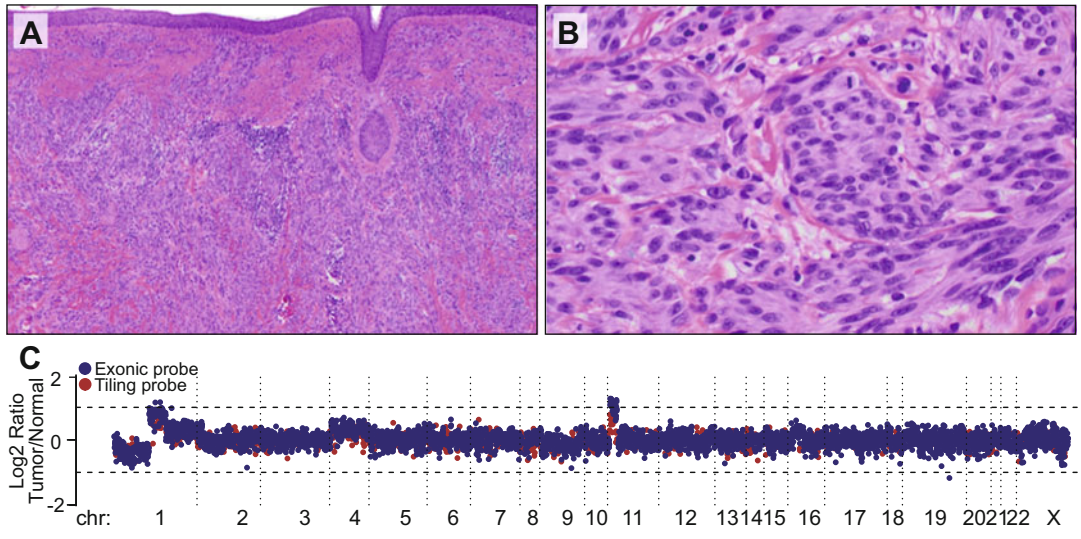
2. **Sequencing**: The prepared libraries are loaded on a glass slides, termed flow cells. Oligonucleotides, which are complementary to the sequencing adaptors, are bound on the surface of the flow cell and specifically bind the sequencing library. Each DNA fragment of the sequencing library is then amplified into a clonal cluster of DNA fragments on the surface of the flow cell (cluster generation) using a special PCR-based method, termed bridge amplification. After cluster generation, the four nucleotide types, labeled with four different fluorescent dyes and corresponding to A, T, G, and C, are added to the flow cell. The DNA strands are extended by only one nucleotide, as all nucleotides contain a terminal elongation blocker. After the nucleotides are incorporated into the DNA strands and the non-incorporated nucleotides are washed away, a camera captures images of the fluorescently labeled nucleotides to determine the base of the incorporated nucleotide in the clusters. Then the dye and the terminal elongation blocker are chemically removed from the DNA, and the next cycle starts.
3. **Data analysis**: The sequencing reads are computationally aligned to a reference genome, and various types of computational analyses are then performed to detect single nucleotide variants, large insertions and deletions, translocations, and DNA copy number variations.

NGS platforms enable a wide variety of analyses of the genome, transcriptome, or epigenome. The sequencing methods differ primarily in the preparation of the DNA or RNA samples and in the methods of data analysis. After the sequencing libraries are prepared, the actual sequencing stage remains essentially the same. Current challenges of NGS include the need for the sequencing infrastructure, considerable computing power, capacity for large-volume data storage, and expertise in bioinformatics and data interpretation. As NGS costs continue to decrease, its use as an ancillary method to help with diagnosis, prognostic assessment, and evaluation of therapeutic targets can be expected to increase in the future (Fig. 13).

Methods to Detect DNA Copy Number Aberrations

Array Comparative Genomic Hybridization

Array comparative genomic hybridization (CGH) is a technique that analyzes the entire genome for copy number alterations (CNAs) by comparing tumor DNA to a reference (non-neoplastic) DNA. To perform array CGH, DNA is isolated from a tumor, labeled with a fluorescent dye (usually green), and then mixed in a 1:1 ratio with reference DNA labeled in a different color (usually red). The differentially fluorescent-labeled DNA samples are then co-hybridized to a glass slide, on which several thousand DNA probes are spotted. Each spot corresponds to a specific locus in the human genome. During hybridization, the green-labeled tumor DNA competes with the red-labeled reference DNA for the binding site on each spot of the DNA microarray. After incubation and washing off the unbound DNA, the intensities of the red and green fluorescent dyes at each spot are measured, quantified, and normalized. The fluorescence intensities are proportional to the relative DNA copy number. If the green and red fluorescence intensities are equal, the genome region is interpreted as having equal quantity of DNA in the tumor and reference samples, i.e., the tumor has no CNA at this region. A shift toward green indicates a copy number gain, and a shift toward red indicates a copy number loss in the tumor. CNAs can be measured in a semiquantitative way, allowing detection of homozygous



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Chr	Start	Ref	Alt	Variant-Class	Gene	Exon	Transcript_ID	cDNAchange	AAchange	Freq (%)
5	176638939	A	G	NS_SNV	NSD1	exon5	NM_022455	c.3539A>G	p.K1180R	0.46
11	533875	G	T	NS_SNV	HRAS	exon3	NM_001130442	c.181C>A	p.Q61K	0.59
13	29001913	G	T	NS_SNV	FLT1	exon9	NM_002019	c.1252C>A	p.L418I	0.25
22	29121242	G	A	NS_SNV	CHEK2	exon3	NM_007194	c.433C>T	p.R145W	0.45
6	157099426	A	ACAGCAG	NF_insertion	ARID1B	exon1	NM_020732	c.363_364 insCAGCAG	p.Q121_Q122 insQQ	0.14

Fig. 13 Example of targeted next-generation sequencing for melanocytic tumors. Integrated molecular diagnostics with targeted next-generation sequencing for ambiguous melanocytic tumors. The tumor metastasized to regional lymph nodes. (**a**) Predominantly intradermal melanocytic tumor with large nests and desmoplasia. (**b**) Large, epithelioid melanocytes with vesicular chromatin, prominent nucleoli and mitotic figures. (**c**) A targeted next-generation sequencing assay, termed MSK-IMPACT, reveals loss of chromosome 1p and partial gains on chromosomes 1q and 11p, which is characteristic for desmoplastic Spitz tumors. The log2 ratio was calculated across all targeted regions by comparing the coverage in tumor versus matched normal DNA. (**d**) Mutational profiling of 341 genes revealed 5 somatically acquired mutations, including an activating *HRAS* Q61K mutation, which is in line with the 11p gain, and a non-frameshift insertion of 6 nucleotides in exon 1 of *ARID1B*. The three missense mutations exchange a single amino acid in the genes *NSD1*, *FLT1*, and *CHEK2*.

deletions, heterozygous losses, low-copy gains, and high-copy amplifications (Fig. 14).

Array CGH allows genome-wide testing for CNAs, which is in contrast to targeted approaches such as FISH. Array CGH works reasonably well on formalin-fixed, paraffin-embedded tissue, but it can be difficult to obtain reliable results in cases with suboptimal DNA quality. In contrast to FISH, which assesses the genetic aberrations at a single-cell level, array CGH provides information about pools of cells, which may cause false-negative results when the percentage of tumor cells in a specimen is below 50% (e.g., due to tumor-infiltrating lymphocytes or admixed stromal cells). Most importantly, array CGH only detects CNAs, but no other genetic aberrations such as small insertions/deletions, point mutations, or balanced translocations.

Most melanocytic nevi lack CNAs, but an isolated gain of 11p can be found in a subset of Spitz nevi (Bastian et al. 2000), and an isolated loss of chromosome 3 may be seen in epithelioid melanocytic nevi with *BAP1* loss (Wiesner et al. 2011). Most melanomas, in contrast, exhibit multiple CNAs of whole chromosomes and chromosomal subregions. Melanomas frequently have homozygous deletions of regions containing tumor suppressor genes (*CDKN2A*,

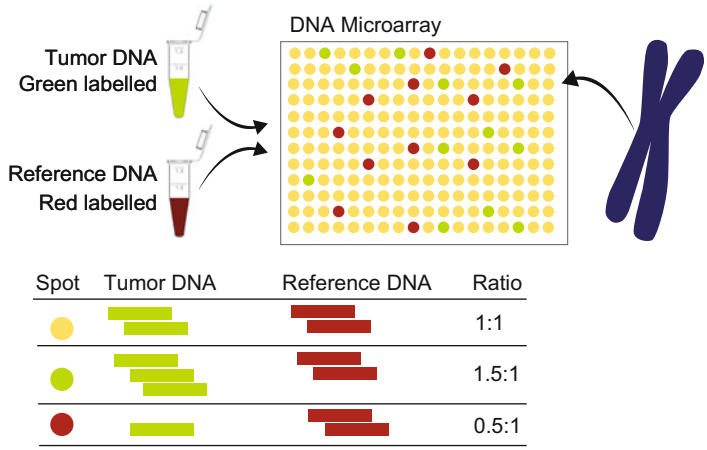

Fig. 14 Array comparative genomic hybridization (CGH). Array CGH analyses the entire genome for copy number aberrations by comparing the amount of tumor DNA, labeled with a green dye, to a reference DNA, labeled with a red dye. Tumor and reference DNA are mixed at equal molarity and are co-hybridized to DNA microarray, which is a glass slide, on which many thousand DNA probes are spotted. Each spot corresponds to a specific locus in the human genome, and the tumor and reference DNAs compete for the binding site on each spot on the DNA microarray. After washing off the unbound DNA, the intensities of the red and green fluorescent dye at each spot are proportional to the relative DNA copy number. Yellow spot: equal quantity of DNA. Green spot: copy number gain in tumor. Red spot: copy number loss in the tumor.

9p21; *PTEN*, 10q23) and hemizygous losses affecting entire chromosome parts, including 6q, 8p, 9p, and 10. Regions containing oncogenes (*BRAF*, 7q34; *MITF*, 3p13) are frequently amplified, and low-copy number gains of chromosome parts 6p, 7, and 8q are often observed. In addition, specific subtypes of melanoma are associated with distinct patterns of CNAs (Wiesner et al. 2016).

Since the majority of melanocytic tumors are histologically distinguishable as clearly benign or malignant, there is little need for performing array CGH on these tumors for diagnostic purposes. For difficult melanocytic lesions, few anecdotal published data on the use of array CGH are available. In our experience, melanocytic tumors with ambiguous microscopic features usually show more genetic aberrations than nevi, but fewer than melanomas (Fig. 6). Furthermore, the affected chromosomal regions may be different from those seen in melanomas. In conclusion, array CGH might be a useful ancillary diagnostic tool for melanocytic tumors in specialized centers, and in a subset of challenging cases, array CGH may help to delineate benign and malignant melanocytic tumors with greater accuracy.

SNP Arrays and Molecular Inversion Probes

Single-nucleotide polymorphism (SNP) arrays present another method to screen the genome for CNAs. SNPs are variations of single nucleotides that occur at specific positions in the genome, often affect nonprotein-coding regions, and are present in a certain proportion of the healthy population. Similar to array CGH, SNP arrays contain thousands of spots with oligonucleotide probes, which represent the varying alleles (allele A and B). In contrast to array CGH, only the tumor DNA is fluorescently labeled and hybridized (no reference DNA is required). The fluorescently labeled tumor DNA then binds to the oligonucleotide probes: if the tumor DNA is homozygous, it will bind only A or B probes, resulting in an AA or BB fluorescent signal; if it is heterozygous, it will bind both probes (AB fluorescent signal). CNAs are then calculated from the relative fluorescent

intensities at each of the oligonucleotide probe locations.

SNP arrays require excellent DNA quality and, therefore, do not work well with DNA isolated from formalin-fixed, paraffin-embedded tissue. To overcome these limitations, SNP arrays are often combined with molecular inversion probe technology, in which oligonucleotide probes bind upstream and downstream of the SNP regions on the tumor DNA. The gap between the bound probes is then filled with a PCR reaction so that its nucleotide sequence is complementary to the tumor DNA. Finally, the generated probes are fluorescently labeled, hybridized to a SNP array, and the allele status is calculated from the relative fluorescence intensities at each genetic location. Such assays are commercialized under the name OncoScan® and can detect DNA copy number aberrations, loss of heterozygosity, and also certain mutations.

Fluorescence In Situ Hybridization

The identification of chromosomal gains and losses in melanocytic tumors and their use as ancillary diagnostic tools led to the development of interphase fluorescence in situ hybridization (FISH) assays. To perform FISH, fluorescently labeled DNA probes are hybridized to formalin-fixed, paraffin-embedded histologic sections on glass slides. Denaturation of the specimen's DNA allows the FISH probes to bind to their complementary DNA sequences at the specific chromosomal loci. After washing away the unbound FISH probes and counterstaining the cell nuclei with a blue fluorescence dye, termed DAPI, the number and pattern of the fluorescent signals in each cell nucleus is evaluated under a fluorescence microscope. The number of fluorescent signals is equal to the copy number of the chromosomal regions targeted by the FISH probes (Fig. 15).

The first commercially available melanoma FISH assay used four probes to evaluate the *CCND1* locus (11q13, green) on chromosome 11 and the *RREB1* (6p25, red), centromere 6 (blue), and *MYB* (6q23, orange) loci on chromosome 6. This assay was reported to distinguish histologically unambiguous melanomas from nevi with a sensitivity of 87% and a specificity of 95% (Gerami et al. 2009). In an attempt to increase accuracy, FISH probes targeting 9p21 and centromere 9 were added to the original FISH panel, resulting in a 6-probe assay. Other labs omitted probes on chromosome 6 and instead added probes for *MYC* at 8q24, *CDKN2A* at 9p21, and centromere 9 (CEP9), resulting in a 5-probe assay. However, the sensitivity and specificity of all FISH assays in discriminating between melanoma and benign nevi seems to be quite similar and tends to be lower in spitzoid lesions and superficial spreading melanoma (Gerami et al. 2012).

Although a number of commercial labs and academic centers are promoting FISH as an ancillary diagnostic test for difficult melanocytic tumors, its value for diagnostic use is probably lower than advertised. FISH only evaluates four to six genetic loci for DNA copy number aberrations, which explains its limited sensitivity and specificity (Gaiser et al. 2010). Copy number aberrations in other chromosomal regions, and all other types of genetic aberrations, including mutations, small insertions/deletions, and genetic rearrangements, cannot be detected with FISH. Many FISH-negative tumors meet criteria for melanoma when analyzed with array CGH. Consequently, a negative FISH does not exclude melanoma if the histopathologic features are concerning for melanoma. Conversely, a positive FISH result does not necessarily imply malignancy, especially if only a single chromosomal region is affected, or if the enumerated gains or losses are near empirical cutoff values. Specific examples are melanocytic tumors with loss of *MYB* or *BAP1*, which usually show indolent clinical behavior. Lastly, the accuracy of FISH is highly operator dependent (e.g., due to factors such as biased selection of nuclei for evaluation and the presence of polyploidy), which is also exemplified by the fact that several different analytic algorithms are published. Based on the available evidence, FISH has limited value in the diagnostic evaluation of ambiguous melanocytic tumors.

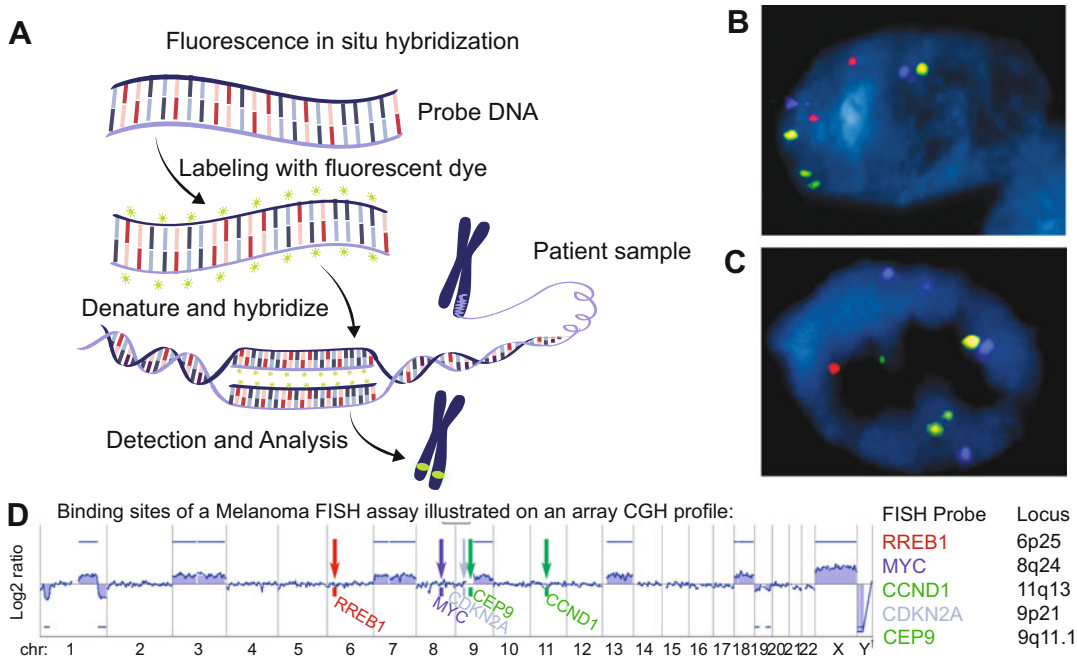

Fig. 15 Principle of fluorescence in situ hybridization (FISH). (**a**) Summary of steps in FISH. (**b**) In normal cells and cells without chromosomal gains and losses (melanocytic nevi), two signals of each probe are detected. (**c**) In melanoma cells, which usually harbor multiple chromosomal aberrations affecting the FISH probe-binding regions, losses or gains of the FISH signals can be observed (here, four aqua, three green, and one red and orange signal). (**d**) The array CGH profile of a melanoma exhibits several chromosomal aberrations involving chromosome 1, 3, 7, 9, 13, 18, and 19. The binding sites of a commercially used assay with FISH probes for *RREB1* (6p25), *MYC* (8q24), *CDKN2A* (9p21), centromere 9 (CEP 9), and *CCND1* (11q13) illustrate that the chromosomal aberrations detected by aCGH would be missed by FISH. This example demonstrates that FISH has major deficiencies in the diagnosis of melanoma.

Genetic Testing for Therapeutic Decision-Making

The introduction of the first effective mechanism-based therapy for advanced melanoma in 2010 has revolutionized the management of patients with metastasized melanoma (Haas et al. 2018). Cell signaling inhibitors downregulating aberrant BRAF signaling, as well as immunotherapies, have since been further refined (Larkin et al. 2015; Long et al. 2014). Despite great advances, which have increased the overall survival of patients with metastatic melanoma, not all patients benefit from these therapies. Biomarkers predicting responses to these therapies are the focus of ongoing research. Some of the findings to date are presented here.

BRAF and MAPK Inhibitors

BRAF inhibitors have now become a standard therapeutic option for advanced melanoma (see also ►"Targeted Therapies for BRAF-Mutant Metastatic Melanoma"). A prerequisite for therapy is the presence of a *BRAF* V600 mutation. Sequencing for *BRAF* mutation status has thus become a routine screening approach performed in centers treating patients with advanced melanoma. The response of patients to *BRAF* V600-mutant melanoma varies considerably. The number of patients benefiting from therapy, as well as the duration of therapy response, has increased with the addition of MEK inhibitors to BRAF inhibitor therapy (Long et al. 2014; Robert et al. 2015a). However, despite this improvement, the

duration of most responses is still limited, and some patients fail to respond at all.

Several genetic mechanisms associated with resistance have been identified. Additional aberrations in the MAPK pathway, including activating mutations in *NRAS* (Nazarian et al. 2010) and *MEK* (Wagle et al. 2011), inactivation of *NF1* (Maertens et al. 2013; Whittaker et al. 2013), and splice variations or amplifications of *BRAF* (Poulikakos et al. 2011; Shi et al. 2012; Whittaker et al. 2010) or *CRAF* (Montagut et al. 2008), form the genetic basis for resistance in some cases. Increased expression of COT (Johannessen et al. 2010), upregulation of receptor tyrosine kinases such as PDGFRβ (Nazarian et al. 2010) and IGF-1R (Villanueva et al. 2010), and activation of the PI3K/AKT pathway by losses of *PTEN* (Paraiso et al. 2011) have also been described as resistance mechanisms. In addition, changes in cell cycle control, in particular overexpression of *CCND1* (Smalley et al. 2008) and losses of *CDKN2A* (Yang et al. 2017), have been associated with resistance to BRAF inhibitors. Hepatocyte growth factor (HGF) signaling through its receptor c-MET was also shown to mediate BRAF inhibitor resistance (Straussman et al. 2012; Wilson et al. 2012). Other analyses have demonstrated that gene expression and methylation patterns can be associated with resistance to MAPK inhibitor therapies (Hugo et al. 2015). Drivers of resistance mechanisms include c-MET up-expression, infra-physiologic LEF1 down-expression and YAP1 signature enrichment (Hugo et al. 2015). These and other data argue that about half of the resistance mechanisms discovered to date are due at least in part to genetic events. Non-genetic factors probably play additional roles in resistance in tumors lacking genetic resistance mechanisms (Hugo et al. 2015). Although various resistance mechanisms are now known, therapeutic decision-making is currently still primarily based on *BRAF* mutation status alone.

Immunotherapy

Immunotherapies have arguably become the most popular systemic therapies for treatment of advanced melanoma (see also chapter ▶ "Checkpoint Inhibitors in the Treatment of Metastatic Melanoma"). With the shift from anti-CTLA-4 to anti-PD-1 therapies, the response rates have increased considerably, and the frequency and severity of side effects has decreased (Larkin et al. 2015; Robert et al. 2015b). Combining checkpoint inhibitors has further increased response rates, but also results in greater side effects (Larkin et al. 2015). Considerable effort has been invested in identifying biomarkers that predict response to immunotherapies. One of the most common approaches currently used is PD-L1 immunohistochemistry on tumor tissue, but this approach requires refinement, as there are many different assays and cutoffs, and reported results vary considerably (Sunshine et al. 2017; Zhao et al. 2017).

One reason cutaneous melanomas respond well to immunotherapies is because these tumors have a very high mutational burden, higher than most other major tumor types (Lawrence et al. 2013). For anti-CTLA-4 and anti-PD-1 therapies, therapy response has been associated with high mutational loads, both in melanoma and other major cancer types (McGranahan et al. 2016; Rizvi et al. 2015; Van Allen et al. 2015). This association has been reported with overall mutational burden, as well as high neo-antigen load. A direct link between mutational load and response to therapy was observed in DNA mismatch repair-deficient colorectal tumors, which acquire large numbers of DNA aberrations. DNA mismatch repair-deficient tumors responded better to PD-1 therapy than tumors with intact mismatch repair (Le et al. 2015). Clonal mutations are also associated with stronger responses to immune checkpoint blockade and prolonged patient survival (McGranahan et al. 2016). In summary, the existing data suggests that the presence of higher numbers of mutations, including immune-relevant mutations, affecting the majority of tumor cells, is predictive for a better response to immune checkpoint blockade.

Gene mutations can alter the amino acid sequence of proteins, resulting in peptides which elicit an immune response when presented to T cells (Schumacher and Schreiber 2015). These

altered peptides are largely presented on MHC class I molecules to CD8 T cells, although presentation on MHC class II molecules and recognition by CD4 T cells has also been described (Kreiter et al. 2015; Linnemann et al. 2015; Tran et al. 2014). CD4 T-cell responses may augment CD8 T-cell recognition of MHC class I neoantigens (Bobisse et al. 2016; Kreiter et al. 2015; Platten and Offringa 2015).

Genetic mechanisms of resistance to immunotherapy have been identified. For exsample, mutations affecting the JAK/STAT pathway and MHC class I presentation (β*2M*), may result in resistance to anti-PD-1 and other immunotherapies (Horn et al. 2018; Sucker et al. 2014). Furthermore, the interferon gamma signaling pathway has been reported to play a role in immunotherapy resistance (Sucker et al. 2017; Zaretsky et al. 2016). Multiple gene aberrations affecting this pathway were reported in experimental models (Patel et al. 2017) and in tumors not responding to anti-CTLA-4 therapy (Gao et al. 2016). Moreover, resistance to immunotherapies is associated with *PTEN* loss (Peng et al. 2016).

It is likely that a greater number of genetic aberrations associated with resistance to immunotherapies will be identified over the next few years. Understanding these mechanisms will be critical to further improve immunotherapy for patients with advanced melanoma. Currently, comprehensive sequencing with analysis of immune-relevant mutations is still not part of routine management protocols. It is however conceivable that, with further refinement of the assays and algorithms available, such approaches could enter routine clinical use within the foreseeable future.

Outlook

Molecular methods are already an integral part in the diagnostic work-up of melanocytic tumors and are essential to determine specific mutations so that patients can be treated with mechanism-based therapies such as MAPK inhibitors. However, the results of prognostic stratification of diagnostically challenging or histologically ambiguous melanocytic tumors using molecular methods have been mixed. Array CGH and FISH are quite proficient at separating tumors that are readily classifiable as benign or malignant by pathologists after microscopic evaluation, but they are of limited value for determining the dignity of histologically ambiguous melanocytic tumors, because they often yield ambiguous, false-negative, or false-positive results. The failure of these tests to permit a clear delineation between benign and malignant neoplasms is not unexpected, as our concept of tumor progression implies a stepwise acquisition of genetic aberrations and the existence of a continuous biologic spectrum rather than a clear binary separation between benign and malignant melanocytic tumors.

Furthermore, current genetic techniques are not able to capture the complexity of mechanisms involved in tumor progression. The morphologic phenotype, however, reflects the cumulative result of an enormous number of pathobiologic processes, including the concerted expression of genes, the sum of epigenetic effects, the tumor microenvironment, and the immune response with specific cellular infiltration patterns. This explains why the diagnosis of melanocytic tumors should still be based primarily on histopathologic features, in conjunction with the judicious use of additional immunophenotypic and genetic tests as ancillary aids in selected cases.

Nevertheless, with additional technological advances in the future, NGS technologies will likely help to accurately determine the genetic landscape of specific histologic subtypes of melanocytic tumors. We believe this will pave the way for a refined classification system that will improve diagnostic and prognostic accuracy and optimally select patients for mechanism-based therapies and immunotherapies.

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