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Ryan J. Sullivan *Editor*

BRAF Targets in Melanoma

Biological Mechanisms, Resistance, and Drug Discovery

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Chapter 1
Melanoma: Historical Context

Suraj Venna, Sekwon Jang and Michael Atkins

Abstract We are in the midst of a therapeutic revolution for patients with melanoma. This chapter reviews several topics on melanoma from epidemiologic trends, to the evolution of the surgical approach, to adjuvant treatment of melanoma, and also reviews various systemic therapies for metastatic melanoma. Each component of this chapter describes advances from a historical perspective, beginning with the first descriptions of melanoma in the literature, to the discovery of activating B-raf mutations in melanoma, and concluding with the current immune and targeted based therapies for advanced melanoma. It serves as a segue to the more detailed therapies and advances in the ensuing chapters.

Keywords B-raf · Checkpoint inhibition · Adjuvant therapy · Chemotherapy · Biochemotherapy · Immunotherapy · MC1R · Risk factors · Sentinel lymph node biopsy · Vaccines

1.1 Introduction

John Hunter in 1787 excised a tumor from the jaw of a young man and aptly described it as a "*cancerous fungous excrescence.*" Hunter detailed that the tumor recurred on the patients chin several years later, thought to perhaps have been incited by trauma as this young gentleman had partaken in a bar room brawl at that time. This specimen was preserved for nearly 200 years in the Hunterian Museum of the Royal College of Surgeons in London and is now specimen number 219 [1]. In 1968 the specimen was examined and verified to be melanoma. Rene Laennec in 1806 is credited as the first physician in modern times to describe melanoma as a disease and published this while still a medical student [2]. William Norris in 1820

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published his post-mortem description of a patient with atypical nevi who developed and died of metastatic melanoma: "*On making an incision through the original tumour, I found the texture to be heterogeneous; it was of a reddish and whitish brown tint throughout, not very unlike the internal structure of a nutmeg. The newly formed tumour, and the tubera around, though during life they wore a very different aspect, after death both exhibited the same dark-coloured appearance. On puncturing a considerable number of the different tubercles, a thick dark fluid was discharged from them* [3]." The first formal acknowledgement that melanoma, in advanced stages, is untreatable and a death sentence, was documented in 1844 by Samuel Cooper in his textbook *First lines of theory and practice of surgery* [4]. He published that the only chance for survival was early removal of the disease, stating that "*No remedy is known of, for melanosis….the only chance of benefit depends upon the early removal of the disease by operation*…." However, the earliest example of melanoma has been suggested to come from Mummified skeletal remains of Peruvian Incas dating to 2400 BC [5]. Over time, we have moved from simple descriptive terms such as *cancerous fungous excrescence*, to defining the molecular pathways responsible for the development of melanoma. This has elegantly been now translated into targeted therapies for melanoma that provide the expectation of controlling this often devastating disease in significant subsets of patients for increasingly extended periods of time.

After a nearly 15 year stand-still for the treatment of metastatic melanoma, we are in the midst of a virtual revolution in systemic treatment of patients with melanoma. The targeted therapy era for melanoma was initiated by the finding that a significant proportion of melanomas carry activating mutations in a component of the mitogen activated protein kinase (MAPK) signaling pathway. Activating mutations in B-raf were identified in 2002 and also found to occur in the vast majority of benign nevi (80 %) [6, 7]. These findings led to a resurgence of interest in melanoma but also led to continued interrogation of the MAPK pathway and other pathways. The discovery of B-raf mutations in benign nevi made it clear that this may be a necessary but early step in melanoma progression and other critical targets must also be involved.

1.2 Epidemiologic Trends

Melanoma accounts for 5 % of all skin cancers but is the major cause of death from skin cancer. In the year 2013, there were an estimated 76,690 new cases of invasive melanoma in the United States and over 9480 deaths attributable to melanoma [8]. This equates to one melanoma-specific death every hour. The number of annual new cases is likely underestimated given that in-situ lesions and thin invasive melanomas (Stage 1a) are not consistently reported to tumor registries, being excised in the outpatient and private practice settings [9]. The lifetime incidence of developing melanoma in the United States was 1/1500 for individuals born in the early 1900's [10]. Between 1950 and 2000, there was an explosive increase in melanoma

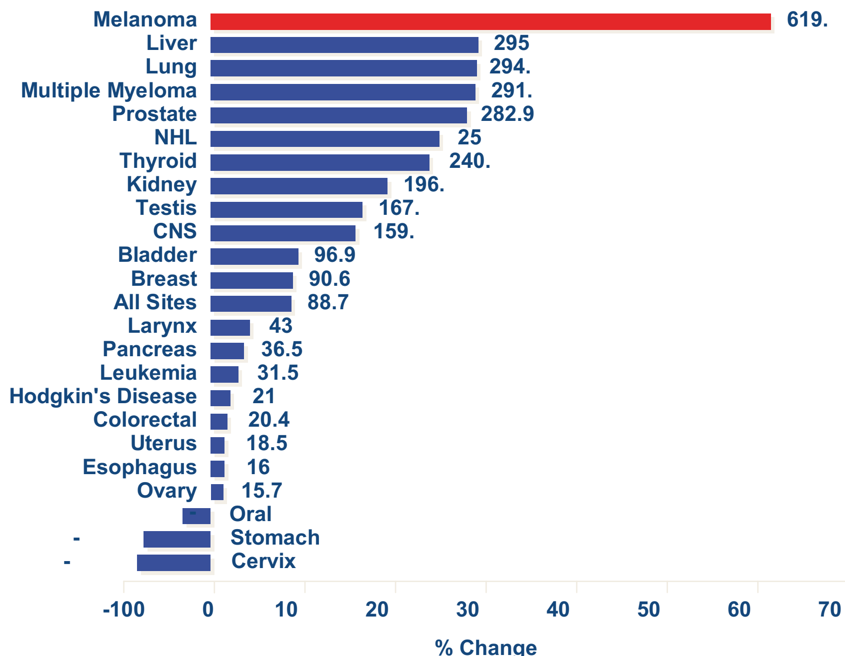

Fig. 1.1 Trends in cancer incidence SEER 1950–2000. (From http://seer.cancer.gov/archive/csr/1975_2000/results_merged/topic_inc_mor_trends.pdf)

incidence rate, outpacing all other tumors with a 619% increase during this 50-year interval (see Fig. 1.1). Today it is predicted that the lifetime incidence of developing invasive melanoma for a white man or woman is 1/50 or 2% of the population. All thicknesses of melanomas have contributed to this increased incidence [11]. During a 14 year period from 1992–2006, the annual rise in melanoma incidence was over 3% in non-hispanic whites [12]. Over the last 10 years the annual rise in cases has been about 2.6% [13]. Interestingly, death rates over the same period have remained stable (Fig. 1.2; [13]). It has been suggested that this rise in incidence may be due to diagnostic drift with a lower threshold for diagnosing melanoma histologically [14, 15]. However, incidence trends have found increases not just in thin melanomas, but also in thicker melanomas, for which diagnostic drift would be less likely [16].

Melanoma ranks 2nd only to leukemia in terms of years of productive life years lost (YPLL) [17]. A recent SEER analysis studied incidence trends of melanoma in young adults for the period of 1973 thru 2004. Age-adjusted annual incidence of melanoma among young men increased from 4.7 cases per 100,000 persons in 1973 to 7.7 per 100,000 in 2004. Among women, age-adjusted annual incidence per 100,000 increased from 5.5 in 1973 to 13.9 in 2004 [18]. Given that melanoma preferentially affects those during the most productive years of life there is a societal burden associated with this disease that exceeds its incidence. On average, an individual in the United States loses 20.4 years of potential life during

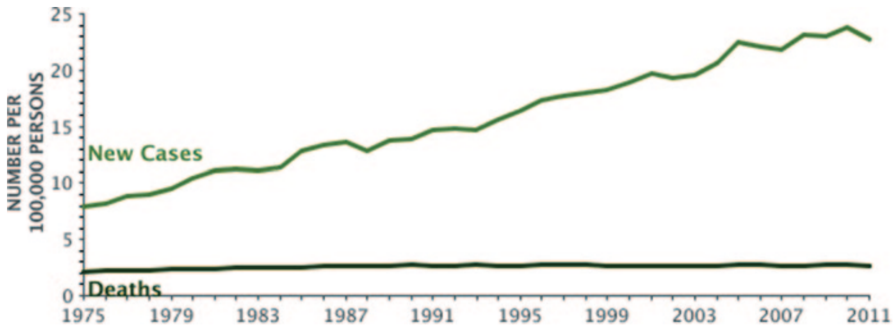

Fig. 1.2 During this period, new cases of melanoma increased significantly while death from melanoma remained relatively constant (From SEER Website: http://seer.cancer.gov/statfacts/html/melan.html)

their lifetime as a result of melanoma mortality compared with 16.6 years for all malignant cancers [19]. Among studies examining all stages of melanoma, annual treatment costs ranged from $ 44.9 million among Medicare patients with existing cases to $ 932.5 million among newly diagnosed cases across all age groups [20]. Melanoma mortality significantly impacts the US economy with a loss of $ 3.5 billion annually [19]. Given the substantial costs of treating melanoma, public health strategies should include efforts to enhance both primary prevention (reduction of ultraviolet light exposure for example) and secondary prevention (earlier detection) of melanoma.

Risk factors There are many risk factors for melanoma including phenotype, genotype, family history, and exposure to ultraviolet light (UVL) with varying effects on the relative risk of developing melanoma (Table 1.1). The focus on risk factors stems from awareness of increased risk, based on the host phenotype, such as those with fair skin, atypical moles and family history, which cannot be altered, to those risks which can be modified such as exposure to UVL, whether from natural or artificial sources. Up until recently, we relied heavily on indirect evidence of UVL being an important factor in the genesis of melanoma. In 2009, the first comprehensive analysis of the melanoma genome was undertaken based on the assessment of an immortalized melanoma cell line, COLO-829, derived from a 43 year old man who died of metastatic melanoma, from an unknown primary [21]. Over 33,000 somatic mutations were identified, including mutational signatures of UVL. Of the 510 dinucleotide substitutions, 360 were CC>TT/GG>AA, changes associated with UVL exposure. The risk factor of UVL exposure also relates to intrinsic factors that increase risk, such as variants in the melanocortin-1 receptor (MC1R), responsible for determining skin pigmentation and processing of UVL-induced skin damage [22]. Certain MC1R polymorphisms are associated with an increased risk of melanoma, and are considered low-penetrance melanoma susceptibility alleles [23, 24]. In one study, MC1R variants were associated with melanoma progression and thicker melanomas in both cases of sporadic and familial melanoma [25]. It has been shown that the more variants of MC1R a patient has, the greater the likelihood

Table 1.1 Relative risk of melanoma

Fair skin	2–18
Freckles	3–20
Blonde hair	2–10
Red hair	2–6
Inability to tan	2–5
Blue eyes	2–5
Constant sun	2–5
Intermittent sun	2–3
Immunosuppression	2–8
Tanning bed use	2–4
NMSC	3–17
Personal history of melanoma	9–10
Family history of melanoma	8
50–100 common nevi	2–64
1 or 2 atypical moles	2–11
Atypical mole syndrome patients	
No personal or family history of melanoma	2–92
Personal history, but no family history	8–127
1 family member with melanoma	33–444
2 family members with melanoma	85–1269

of their melanoma harboring a mutant B-Raf [26]. The MC1R-Braf association is now well established, with MC1R variants demonstrating an increased risk of B-Raf mutant melanomas based on having one or two variants [26, 27]. Multiple MC1R variants had up to a 15-fold increased risk of developing B-Raf mutant melanoma. There was no association between MC1R status with melanomas without B-Raf mutations. The mechanism behind this association remains to be elucidated.

Screening The goal of primary prevention is to prevent the development of a disease and in the case of melanoma, this may be accomplished by limiting UVL exposure. However, secondary prevention may be a more realistic approach to detect melanomas at early stages and cure the patient with a simple excision. The United States Preventative Services Task Force (USPSTF) last reviewed evidence for skin screenings in 2009 and they concluded that the evidence for or against total skin exams for the early detection of melanoma and non-melanoma skin cancer was insufficient, due mostly to limited high quality studies and lack of randomized controlled trials (RCT's) [28]. However two recent studies from Germany have reignited the debate and make the case for population based skin cancer screenings. One was an observational study involving over 360,000 screened participants from 1 region of Germany. The mortality rates were compared with 3 adjacent townships and the country of Denmark. The screened population demonstrated a 47 % decrease in melanoma mortality after this population-wide skin cancer screening

program when compared to the 4 other regions devoid of such a screening intervention [29]. In another study, the SCREEN (Skin Cancer Research to provide Evidence for Effectiveness of Screening in Northern Germany) project, melanoma incidence was determined before, during, and after skin cancer screening in the German state of Schleswig-Holstein [30]. The incidence of melanoma increased in both men and women during this screening effort: invasive melanoma in men: +4.0 per 100,000 (95 % CI: 1.6; 6.4); women: +8.9 per 100,000 (95 % confidence intervals (CI): 6.1; 11.7); and decreased afterwards (women: −10.6 per 100,000 (95 % CI: −13.3; −7.9); men: −4.1 per 100,000 (95 % CI: −6.5; −1.7). During that same period of time, these trends did not occur in another German state where the screenings were not being performed. On a practical level, RCT's for melanoma screening would be a large and at this time unrealistic undertaking. In order to prove that population based skin screenings can affect mortality from melanoma, it has been estimated that such a RCT would necessitate 800,000 screenings to generate adequate power to arrive at such conclusions [31]. Until such RCTs are completed, it should be duly noted that in the case of melanoma, screening strategies as a form of secondary prevention has value in that melanomas can be readily identifiable by educated patients and primary care providers. Unfortunately, it is estimated that only 30 % of dermatologists perform full skin screenings [32]. Finally, although dermatologists are the group best trained at identifying skin cancers, there is a shortage of these physicians in the workforce relative to the population [33–35]. Therefore it is important to identify those individuals at highest risk of developing melanoma and focus screening efforts among these groups. These would be people with a personal and/or family history (PH/FH) of melanoma as well as those with a phenotypic risks such as high nevus count, atypical nevi, fair skin; behavioral risks such as tanning bed use and indiscriminate UVL exposure; and those who have had non-melanoma skin cancers (NMSC), such as basal cell carcinoma and squamous cell carcinoma. In one study, those with a history of NMSC had a 17-fold increased risk of developing melanoma, with an average follow-up of over 9 years [36]. The vast majority of the melanomas in this study were detected on sun-protected sites with an average Breslow's depth of 0.70 mm. It is important to remember that about 50 % of newly diagnosed melanomas are detected by the patient upon the Self Skin Examination (SSE) [37, 38]. Therefore, physicians should continue to alert those at high risk and educate patients on what to look for. Although a significant portion of melanomas are detected by the patient, when melanomas are detected by dermatologists, they are significantly thinner than those detected by patients performing the SSE [39, 40].

Examination by a physician allows for anatomic sites to be evaluated that would be difficult for the patient, such as the back, scalp, and calves. Total body photography (TBP) is an important adjunct in the surveillance of patients to document stability of nevi and also to identify new lesions [41]. TBP has allowed for the detection of early stage melanomas that may otherwise have been missed and also allow the clinician to avoid unnecessary biopsies of benign lesions that are documented to have remained stable during follow up exams [42, 43]. Unfortunately, due to the related infrastructure for storage of digital images and retrieval, TBP is infrequently

used in private offices. In a recent survey of US dermatology training programs, the most common reasons for not using TBP were logistical and financial [44]. The most popular non-invasive bedside tool to evaluate nevi is surface microscopy or dermatoscopy. This is a hand-held device comprised of a series of non-polarized lights with magnification of 10x. The clinical diagnosis of melanoma with the unaided eye has a sensitivity of about 60 %. This can be significantly enhanced with dermatoscopy.

Dermatoscopy improves the ability to diagnose melanoma, and correctly identify benign skin lesions and forego unnecessary skin biopsy [45–47]. However, specialized training is needed in the use of this bedside aide [48]. In comparison to the unaided eye, dermatoscopy improves sensitivity by 20 % and specificity by 10 % [49]. Other techniques to improve early detection of melanoma are also being investigated. Computer assisted systems based on multi-spectral image analysis are available. The machine will image a mole or suspicious lesion and calculate a number of parameters such as color gradient, width, borders, and other morphologic features and then compare it to a database to determine if the combined parameters reach a threshold for melanoma, and therefore to biopsy. These types of devices based on pattern recognition have pitfalls such as in the atypical nevus patient. In a patient with atypical nevi, the computer-generated pattern can yield a worrisome score requesting biopsy, when in reality the patient has 50 other nevi with a similar pattern. This underscores a very important clinical principle when evaluating patients with many nevi, especially atypical: proceed with caution when considering skin biopsy and avoid making that decision on whether or not to biopsy a suspicious mole in isolation. In other words, that decision should be done in the context of multiple factors including the patient's phenotype and other risk factors. Confocal laser scanning microscopy (CLSM) is another method to evaluate nevi in a non-invasive manner where horizontal sections are visualized to a depth of the papillary dermis. Due to the fact that melanin and melanocytes offer strong contrast with this near-infrared device, it would theoretically be an effective technique for cutaneous melanoma diagnosis [50]. In one study CLSM had a higher sensitivity than dermatoscopy, but a lower specificity for diagnosing melanoma [51]. However the cost, time to image lesions, and most importantly the extensive training needed to capture and interpret images are major barriers to its widespread use. At the current time, CLSM is relegated to a few academic centers with very limited clinical utility. On a practical level, the clinical examination complemented with dermatoscopy should be considered the standard method to evaluate patients at risk for melanoma.

1.3 Evolution in Surgical Management

The nature of the extent of surgery has evolved slowly over the centuries as the understanding of the biology of melanoma has progressed. William Handley is credited with setting the course for the surgical treatment of melanoma for a 50-year period. In 1907 he advised that melanomas should be excised with 5 cm margins

down to the level of the fascia and that the regional nodes should be removed, sometimes termed in those days as *lymph node evacuations* [52]. In 1967, a Finnish surgeon, Grete Olsen, published data that she gathered from the Finsen Institute and Radium Center Copenhagen, Denmark, on 500 melanoma patients [52]. Metastases to regional nodes developed more frequently in those patients in whom the underlying fascia had been removed versus those in whom the fascia was intact, 45 % versus 8–14 %. She stated in this paper that "*when the fascia is excised ….there is now nothing to hinder the spreading of melanoma cells from the subcutis region to the deep subfascial lymphatic vessels*…." The theory was that the removal of the muscular fascia propagated metastatic melanoma, in that the fascia had a physical barrier role that would otherwise obstruct melanoma from metastasizing. And since Olsen's publication, the depth of melanoma excision has been through the subcutis with preservation of the muscular fascia. There have never been studies examining the optimal depth of excision for melanoma. The radial margins of excising melanoma have been reduced considerably since the initial guidelines set out by Handley in 1907. The margins of excision now recommended are designed to limit the risk of local recurrence with its potential effect on survival by capturing, in-theory stray melanoma cells with the radial margin. These margins are modified according to particular anatomic site. However, the guidelines for margins of excision for melanoma are based primarily on 5 multi-institutional trials, which compared excision of margins of 1 vs. 3 cm (2 studies), 2 vs. 4 cm (2 studies), and 2 vs. 5 cm (1 study) [53–57]. The wider margins did not improve overall survival; however, the current practice is to do no less than 1 cm resection margin for melanomas less than 1 mm in depth; 1–2 cm for melanomas measured between 1–2 mm; and at least 2 cm for melanomas measuring 2.01 mm or greater in thickness. The final exact margin is always decided in the context of the individual patient and anatomic location of the melanoma. The current evidence is insufficient to address the optimal excision margins for melanoma and less is known regarding the optimal depth of excision. However, it is clear at least, that very wide margins of 5 cm do not offer patients a survival advantage.

For over 100 years, radical en-bloc or 'gland excision' was carried out, advocated and published first by Dr. Herbert Snow in his lecture titled "Melanotic cancerous disease" in the Lancet, presented at the Cancer Hospital in London, February 5, 1892 [58]. In this published lecture, he aptly noted that the melanomas could arise from pre-existing nevi stating, "non-prominent moles or cutaneous stains may be antecedent to melanotic developments." And also assumed that the progression of melanoma was from skin to lymph nodes to systemic, and therefore advocated for complete node dissection along with wide local excision: "it is essential to remove, whenever possible, those lymph glands which first receive the infective protoplasm."

Once it became clear that the level and absolute depth of invasion of melanoma are key determinants of prognosis and likelihood of occult nodal disease, the role of these radical 'gland excisions' was called into question and ultimately evolved into a dramatic change in the approach to the assessment of nodal disease. In a paper published in 1979 titled "Melanoma Thickness and Surgical Treatment" the authors

set out to examine their experience with Stage 1 patients to determine the role of elective node dissection [59]. They found that melanoma thickness correlated with risk of nodal disease, with 62 % occurrence in melanomas greater than 4 mm, 57 % in lesions between 1.50–3.99 mm, 25 % in lesions between 0.77–1.49 mm, and 0 % in melanomas less than 0.77 mm. At that time, they concluded that at least in the thin melanomas (< 0.77 mm) elective lymph node dissection (ELND) was not justified. However, they recommended continued ELND for intermediate thickness melanomas 1.50–3.99 mm based on the significant difference in 5-year survival (83 % WLE + ELND vs. 37 % WLE alone). Interestingly, they also commented that for melanomas > 4 mm, "the potential benefits of immediate lymphadenectomy are much less because the incidence of simultaneous metastases at distant sites appear to diminish the beneficial effects of removing any regional metastases."

Dr. Donald Morton is credited with developing the technique of sentinel lymph node biopsy (SLNB) for melanoma, a minimally-invasive way to stage the regional nodes, and better stratifying patients into those who may benefit from a subsequent complete node dissection, thereafter. Since thick melanomas have a propensity for hematogenous spread, the largest prospective trial assessing the value of sentinel lymph node biopsy (SLNB) for melanoma focused primarily on intermediate thickness melanomas. A "final" analysis of the largest trial assessing the role of SLNB for intermediate thickness melanomas showed that there was no significant difference in the 10-year melanoma-specific survival when comparing those patients with or without sentinel lymph node procedures [60]. However, there was a significant improvement in the 10-year disease free interval in the SLNB group versus the observation group among patients with intermediate-thickness melanomas, defined as 1.20–3.50 mm (71.3 ± 1.8 % vs. 64.7 ± 2.3 %; hazard ratio for recurrence or metastasis, 0.76; *P* = 0.01), and those with thick melanomas, defined as > 3.50 mm (50.7 ± 4.0 % vs. 40.5 ± 4.7 %; hazard ratio, 0.70; *P* = 0.03). In the node positive patients, those who were diagnosed via SLNB as compared to macroscopic presentation (the observation group), the 10-year melanoma-specific survival rate was 62.1 ± 4.8 % versus 41.5 ± 5.6 % in the observation group (hazard ratio for death from melanoma, 0.56; 95 % CI, 0.37 to 0.84; *P* = 0.006). The final analysis established unequivocally, that (1) SLNB is accurate and provides prognostic information; (2) early intervention decreases the risk of nodal recurrence, distant metastases, and death from melanoma; (3) SLNB can identify patients with nodal disease who may benefit from immediate completion lymphadenectomy.

The role of surgery in patients with late stage melanoma continues to evolve especially in the current era, given the number of systemic treatment options that have recently become available. Recent data has also supported a limited role of metastasectomy. A SEER analysis of Stage IV patients undergoing metastasectomy found that patients who underwent metastasectomy (33.6 %) had an improved median (12 months versus 5 months) and 5-year overall survival (16 % versus 7 % (*P* < 0.001) as compared to patients who did not [61]. In patients with M1a disease (*n* = 1994), this improvement of survival following metastasectomy was enhanced; median survival of 14 months versus 6 months and 5-year overall survival of 20 % versus 9 % (*P* < 0.001).

The surgical management of melanoma has been steadily refined over the past century, with research efforts until recently having the luxury of being largely unencumbered by the confounding effects of effective systemic treatment approaches. With the advent of systemic therapies that unequivocally prolong survival in patients with stage IV melanoma, the integration of surgery with other effective treatment will likely need to be more actively considered.

1.4 Adjuvant Therapy

For the majority of patients presenting with melanoma, complete surgical excision will be possible and potentially curative. However, the risk of systemic recurrence is high among patients with thick primary lesions or positive lymph nodes. There has been considerable effort to assess adjuvant interventions including adjuvant chemotherapy, nonspecific immunostimulants or vaccines. However, none of these approaches, used either alone or in various combinations, proved beneficial when compared to either observation or placebo in randomized clinical trials. Adjuvant immunotherapy with high dose interferon alpha (IFNa) prolongs disease-free survival, and in some studies prevents relapse and death in as many as 25–33 % of patients at risk. High dose IFNa, and more recently pegylated IFNa received US Food and Drug Agency (FDA) approval as adjuvant treatments for patients stage IIB, IIC and III melanoma and are presently considered the standard of care. Nonetheless, a recent National Cancer Data Base analysis of over 34,000 patients with Stage III melanoma, suggest that less than one-third of patients eligible for such adjuvant treatment actually receive it [62]. Thus, there remains a need to develop adjuvant treatments with improved efficacy and/or reduced toxicity that can achieve general acceptability.

Cytotoxic Chemotherapy and Combination Chemotherapies Single-agent chemotherapy or combination chemotherapy regimens have been evaluated for the adjuvant treatment of patients with melanoma. In a randomized controlled trial, the administration of dacarbazine (DTIC) either alone or in combination with BCG after wide local excision and regional lymphadenectomy failed to show improvement in disease-free survival (DFS) or overall survival (OS) [63]. The combination chemotherapy regimen of carmustine, actinomycin-D, and vincristine administered for 6 months was compared to observation among 173 patients with resected stage III or stage IV melanoma [64]. This trial demonstrated a significant improvement in relapse-free survival (5-year Kaplan-Meier estimates of relapse-free survival of 29 % vs. 9 %; $p = 0.03$), however, there was no difference in overall survival. Given the small size of this trial and the lack of confirmatory results in larger trials, adjuvant chemotherapy is not currently advocated for treatment of patients with high-risk melanoma.

Nonspecific Immunostimulants and Vaccines Multiple different immunostimulant and vaccine strategies have been pursued as adjuvant therapy for patients with high

risk melanoma over the past 40 years with none showing convincing or reproducible benefits. Some of the most promising of these approaches are described below.

Observation of regression in intradermal metastases of melanoma after intralesional injection of Bacillus Calmette-Guerin (BCG) led to adjuvant trial with BCG in high-risk patients [65]. In the EORTC 18781 trial, 353 patients were randomized to two different BCG preparations or to follow-up only [66]. Although the treatment was generally well tolerated, there was no benefit in patient survival and time to relapse. *Corynebacterium parvum* is another micro-organism which stimulates the immune system. In a randomized clinical trial of *C. parvum* compared to observation in 260 patients with clinically localized melanoma, there was no significant difference in survival between the two treatment arms [67]. Levamisole, an antihelminthic agent with immunomodulatory effects, was tested in a few randomized controlled trials. It failed to show any benefit in all except one study. This study demonstrated statistically insignificant reduction in the death rate and the recurrence rate in levamisole group compared with observation [68]. Levamisole has never been adopted widespread as a therapeutic agent.

In the wake of negative studies with nonspecific immunostimulants, investigators switched course and attempted to develop vaccines capable of eliciting a specific host immune response against melanoma. A variety of vaccination strategies using autologous or allogeneic melanoma cells have been tested over the last few decades. Technical complexities inherent in harvesting tumor and preparing a vaccine made it difficult to test autologous cellular vaccine in large multi-institutional trials. Allogeneic tumor cell vaccines, conversely, are generally prepared from cultured cell lines or lysates allowing the conduct of large-scale, multi-institutional clinical trials. The Southwest Oncology Group (SWOG) conducted one such, large randomized trial of an allogenic melanoma vaccine (melacine) compared to observation in patients with intermediate-thickness, node-negative melanoma [69]. There was no evidence of improved disease-free survival among patients randomized to receive vaccine. Canvaxin, a polyvalent cell vaccine composed of a combination of allogeneic cell lines, showed great promise in a variety of phase II trials [70]. However, it also failed to show improvement in progression-free or overall survival in randomized phase 3 trials comparing canvaxin plus BCG to placebo plus BCG in patients with resected melanoma stage III and stage IV disease [71].

Melanoma vaccines based on peptides or gangliosides also have been developed and examined in clinical trials in the adjuvant setting. The GM2 ganglioside is expressed in the majority of melanomas and could induce an antibody response. A GM2 vaccine was shown to be associated with freedom from disease recurrence in patients who developed an antibody response to the vaccine. Combining the vaccine with GM2-KLH/QS-21 adjuvant led to enhanced immunogenicity suggesting it might be an even more potent adjuvant therapy. However a randomized phase III trial comparing standard HD IFN to the GM2/KLH/QS-21 vaccine in patients with Stage IIB and III melanoma (E1694) conducted in the US Intergroup, had to be closed early because there were 50 % more relapses and deaths on the vaccine arm relative to the IFNa arm [72]. In a second randomized phase II study, E2696, patients with stage III melanoma were randomized to receive two different

schedules of IFNa, IFNa + the GM2/KLH/QS-21 vaccine or the vaccine alone [73]. In this small study the two IFNa containing arms showed a significant improvement in relapse free survival (RFS) over the vaccine only arm. This same vaccine was also compared to placebo by the EORTC in a randomized Phase III trial involving 1314 patients with stage II melanoma [74]. A trend toward adverse overall survival outcome for the vaccine arm led to trial termination at the 2nd interim analysis; however, more mature data has suggested no significant difference in any outcome.

The majority of patients with melanoma have the MAGE-A3 antigen expression on the tumors and MAGE-3 vaccination is an attractive strategy. A phase I/II study demonstrated MAGE-3-specific antibody and T-cell responses following vaccination in patients with MAGE-3-positive tumors [75]. This led to a randomized phase III clinical trial (DERMA) in patients with stage III nodal metastases and detectable MAGE-3 expression in resected lymph nodes. A recent sponsor-led press release from September 2013 based on an independent analysis failed to show significant extension of DFS in Stage III patients with MAGE-A3 tumors who were on the vaccine versus placebo. However, the trial will continue to assess its second co-primary endpoint of DFS in the gene signature positive patients. Results from this analysis are expected in 2015. The National Cancer Institute surgery branch reported vaccination efforts in 95 HLA-A*0201 patients at high risk for recurrence of melanoma who received prolonged immunization with a peptide vaccine, gp100209-217 [76]. Vaccination was highly effective at inducing large numbers of self/tumor-Antigen reactive T cells, however, there was no difference in the levels of antitumor Antigen-specific T cells in patients who recurred compared with those who remained disease-free. Based on the results of this extensive research effort, one must conclude that adjuvant vaccine strategies in patients with resected high and intermediate risk melanoma have yet to show efficacy and newer approaches and a better understanding of tumor immunology are necessary to advance this field.

Interferon Type I IFNs, including IFNa, are natural proteins produced by immune cells in response to infectious agents. Durable responses seen in patients treated with IFNa for metastatic melanoma, particularly in those patients with small volume and soft tissue only disease, led to investigations in the adjuvant setting for patients with high-risk resected melanoma [77]. The majority of studies with high-dose IFNa have been conducted by the Eastern Cooperative Oncology Group (ECOG). The first trial (E1684) randomized 287 patients with resected Stage IIB or III melanoma to either observation or high-dose IFNa with an induction phase of daily intravenous IFN-a at 20 million international units (MU)/m^2 for 4 weeks followed by 48 weeks of maintenance therapy at 10 MU/m^2 subcutaneously 3 days a week [78].This study demonstrated statistically significant improvement in both relapsed free and overall survival (one-sided $p = 0.0237$) for the IFNa treated patients relative to those on observation at a median follow-up time of 6.9 years. On the basis of these results, the US FDA approved this high-dose IFNa regimen as the first postsurgical adjuvant therapy for stage IIB (T4) and III melanoma in 1996. However, the benefits of IFNa therapy on overall survival decreased, and eventually disappeared, in patients who were followed for a median of 12.6 years based on a pooled analysis [79]. This called into question the impact of high-dose IFNa

on overall survival. The controversy regarding the survival benefits of adjuvant IFNa was further heightened by subsequent ECOG led studies showing conflicting results. For example, E1690 randomized patients with Stage II and III melanoma to high-dose IFNa, lower dose IFNa or observation and showed an improvement in relapse free survival for the high-dose IFNa arm, but no difference in overall survival [80], while E1694 (as noted above) showed significant improvement in both relapse free and overall survival for high-dose IFNa compared to a ganglioside vaccine [72]. Large meta-analyses have tried to address this controversy. Mocellin et al confirmed that IFNa has a substantial, if limited, benefit [81]. This analysis, which included trials with high, intermediate, and low-dose interferon, showed an overall hazard ratio of 0.82 for relapse-free survival ($P<0.001$), with a smaller, but still significant risk reduction of 0.89 for overall survival ($P=0.002$). In the review, no optimal dose, treatment duration, or subset of patients was identified as being more responsive to adjuvant interferon therapy. More recently, the Melanoma Disease Site Group in Canada published an analysis of high-dose IFNa regimens and found a mean relapse free survival hazard ratio of 0.76 (95 % confidence interval 0.67, 0.87) and mean overall survival hazard ratio of 0.87 (95 % confidence interval 0.75, 1.01) which just failed to reach statistical significance [82]. Taken together these data suggest a risk reduction for relapse of around 25 % and for death of about 10 % associated with high-dose IFNa. However, the usefulness of this data is further compromised by the fact that these studies took place in the era before routine sentinel lymph node staging and therefore do not provide any information on patients with currently defined N1 (Stage IIIA) melanoma, the most commonly identified high risk population in the current era.

Efforts to improve upon the therapeutic index for high-dose IFNa have focused on the use of longer acting IFN compounds, such as Pegylated IFNa, and shorter duration treatment regimens. Pegylated IFNa has been used to treat hepatitis B or C, and EORTC 18991 investigated its use in patients with resected stage III melanoma in a randomized phase III trial compared to observation [83]. Pegylated IFNa was administered subcutaneously at a dose 6 µg/kg once a week for 8 weeks followed by 3 µg/kg for 5 years. Although there was no difference in overall survival or distant metastases-free survival (DMFS), pegylated IFNa improved recurrence free survival, which led to the approval of this agent for adjuvant treatment of stage III melanoma in the US in 2011. This benefit was particularly apparent in the subset of patients with microscopic involvement of 1 lymph node and ulcerated primaries. These retrospective subset analyses, however, have yet to have independent or prospective validation.

Two studies have looked at shorter duration regimens. A study conducted in Greece examined the use of a regimen in which patients with resected high-risk melanoma were randomized to receive either a year of high-dose IFNa or a truncated regimen in which IFNa was given for only the 4-week induction period [84]. At a median follow-up of 63 months (95 % CI 58.1—67.7), the median relapse free and overall survival were essentially equivalent between the two arms while patients in the 12-month treatment arm had more grade 1 to 2 hepatotoxicity, nausea/vomiting, alopecia, and neurologic toxicity. This study, while provocative, was felt to be

too small to confirm equivalence. To further investigate the utility of this shortened regimen, E1697 compared 4-week high-dose IFNa induction only with observation in 1150 patients with resected intermediate- and high-risk melanoma [85]. The median relapse-free survival was 7.3 years (95 % CI 5.3, 9.8) in the observation arm and 6.8 years (95 % CI 5.1, 9.0) for IFNa, while the 5-year overall survival rate was 85 % (95 % CI 81, 89) for observation and 82 % (95 % CI 78, 86) for IFNa. Because of the lack of any apparent treatment benefit, this trial was terminated early. These data call into question the value of abbreviated and modified IFN regimens and leave the original HD IFNa regimen as the, albeit controversial, standard of care for adjuvant treatment of patients with intermediate or high risk melanoma.

Biochemotherapy As another attempt to improve adjuvant treatment for high risk melanoma, patients with stage IIIB and IIIC melanoma were randomized to receive either a combination of biologics (IFNa, interleukin-2) and chemotherapy (cisplatin, vinblastine, DTIC), so called biochemotherapy, over a 9 week period or standard high-dose IFNa in an intergroup phase III study organized by the SWOG [86]. This study showed a significant improvement in relapse-free survival for the biochemotherapy arm but no improvement in overall survival. Considering the added toxicity and expense associated with the intensive inpatient biochemotherapy regimen and the lack of impact on overall survival, it is unlikely that this regimen will see much clinical application.

Other Regimens Ipilimumab is a CTLA-4 blocking monoclonal antibody which demonstrated improvement in overall survival compared to vaccine as well as chemotherapy for patients with unresectable or metastatic melanoma [87, 88]. Two large-scale Phase III trials are underway examining the value of adjuvant ipilimumab therapy, EORTC 18071 trial comparing adjuvant ipilimumab to placebo and E1609 is comparing two different doses of ipilimumab to high-dose IFNa. Accrual to these trials is now complete and results are eagerly anticipated.

1.5 Evolution of Systemic Treatment Approaches

The prognosis for patients with Stage IV melanoma has historically been poor with median survival less than a year and a 5-year overall survival rate of less than 10 %. Two US Food and Drug Administration (FDA) approved drugs had been used for the treatment of patients with Stage IV melanoma in the US prior to 2011, namely, DTIC and recombinant human interleukin-2 (IL-2). Recent advances in melanoma therapy have been dramatic with the approval of ipilimumab and vemurafenib in the US in 2011 followed by approval of dabrafenib and trametinib in 2013. Greater understanding of melanoma biology coupled with the successful development of novel treatments such as anti-PD-1 antibody and new combination regimens will further improve patient outcomes in the future.

Cytotoxic chemotherapy The objective response rate of DTIC is approximately 10–20 % with most responses ranging from 3 to 6 months, although long-term remis-

sions can occur in a small number of patients who achieve a complete response. Despite its FDA approval DTIC has never been shown to improve median progression free survival or overall survival compared to a control arm in any prospective randomized study. Although combinations of cytotoxic agents, including those containing DTIC or regimens adding either IFN or tamoxifen to DTIC have often produced higher response rates than DTIC alone, they also increased the toxicity without a significant improvement in survival compared to DTIC alone [89].

1.6 Immunotherapy

Interleukin-2 based therapy High-dose bolus interleukin 2 (HD IL-2) received FDA approval in 1998 for the treatment of patients with metastatic melanoma largely based on its ability to produce durable complete responses in 5–10% of patients. In a retrospective review of 270 patients treated on multiple Phase II studies, the objective response rate was 16%, with a median duration of 9 months (range 4 to 106+ months). Despite the low objective response rate, 59% of complete responders remained progression-free at 7 years and no patient responding for longer than 30 months had progressed, suggesting that some patients are "cured" [90]. Treatment, however, was associated with significant toxicity limiting its application to a select group of patients treated in specialized centers.

Efforts to improve upon the activity of IL-2 in patients with melanoma have included combinations with chemotherapy (biochemotherapy), vaccines and adoptive T cell therapy. Although several phase II trials, a small phase III trial and two meta-analyses suggested that combinations of IL-2 and cisplatin-based biochemotherapy offered benefit relative to either chemotherapy or IL-2 alone, several multi-institutional phase III trials have failed to confirm this benefit [91, 92].

Another approach to improving the activity of HD IL-2 involved the addition of a gp100 peptide vaccine. A phase III trial randomly assigned 185 patients with metastatic melanoma to HD IL-2 given alone every 3 weeks or in combination with a gp100 peptide vaccine [93]. Because of the restriction properties of the vaccine, enrollment was limited to patients who were shown to be HLA type A201. The study reported an objective response rate of 16% for the combination compared with 6% for HD IL-2 alone. There were eight complete responses (9%) in the combination arm, but only one (1%) among those treated with IL-2 alone. There was a trend toward increased overall survival (median 17.8 versus 11.1 months, $p=0.06$), although the trial was not adequately powered to assess this endpoint. The clinical significance of this finding is uncertain considering the relatively poor response rate in patients treated with HD IL-2 alone, the current lack of availability of the specific formulation of vaccine adjuvant used in this trial and the observations that this same vaccine did not improve the efficacy of ipilimumab in a phase III trial [94] (see below).

Others have explored the efficacy of HD IL-2 in combination with adoptive transfer of tumor derived tumor reactive T cells. These approaches have included preparative regimens involving myeloablative chemotherapy with or without total body irradiation (TBI) in order to delete host immune cells and promote engraftment of adoptively transferred tumor-reactive T cells [95]. Autologous hematopoietic progenitor cell support was used in patients who received TBI. The NCI Surgery Branch recently reported the combined results from 3 separate trials. There were 52 objective responses in 93 patients (56 % response rate), including 20 (22 %) complete responses. Complete responses were ongoing at 37–82 months in 19 of the 20 responders, and the three- and 5-year actuarial survival rates for patients achieving a complete response were 100 and 93 %, respectively. Efforts to confirm these results at other centers as well as to develop a more practical treatment regimen are currently underway.

Ipilimumab The CTLA-4 receptor on T lymphocytes is a negative regulator of T cell activation that blocks positive stimulatory effects to these cells mediated through their co-stimulatory and antigen specific T cell receptors. The monoclonal antibodies ipilimumab and tremelimumab bind to CTLA-4 and thus prevent this feedback inhibition. Both have been studied in patients with melanoma, with the most extensive data and promising results being observed with ipilimumab.

Ipilimumab was studied in a placebo-controlled phase III trial in which 676 patients with previously treated advanced melanoma were randomly assigned in a 3:1:1 ratio to ipilimumab plus gp100 peptide vaccine, ipilimumab alone or gp100 vaccine alone [94]. Ipilimumab (3 mg/kg) and/or vaccine were given every 3 weeks for four doses. Patients with confirmed partial or complete response or stable disease for 3 months or more after completion of the 12 week induction period were allowed to receive re-induction with their original treatment if they subsequently had disease progression.

In this study, overall survival was significantly increased in the two groups that received ipilimumab (median 10.0 and 10.1 versus 6.4 months, in the ipilimumab plus gp100, ipilimumab alone, and gp100 groups, hazard ratios for death 0.68 and 0.66 versus gp100 alone, respectively). Treatment benefits appeared to be independent of gender, age (≤ 65 or >65 years), stage at presentation (M0, M1a, and M1b versus M1c), baseline LDH or prior use of IL-2. Tumor response rate was also significantly improved in both groups of patients treated with ipilimumab compared to gp100 alone (5.7 and 10.9 versus 1.5 %, respectively). Further objective partial or complete responses were maintained for at least 2 years in 4 of 23 (17 %) patients treated with ipilimumab plus gp100 and 9 of 15 (60 %) with ipilimumab alone. Among 31 patients who initially received ipilimumab either alone or with gp100 and then underwent reinduction therapy with ipilimumab, six (21 %) had an objective response to retreatment, and 15 (48 %) had stable disease. Although this phase III trial limited enrollment to patients who were HLA-A*0201 positive, a retrospective analysis of four phase II trials involving ipilimumab alone showed similar activity regardless of HLA type [96]. Although patients on this trial did not have tumor profiling for BRAF mutations, recent data suggest that the activity of ipilimumab is independent of BRAF mutational status [97]. As a consequence of

this study, ipilimumab was approved for the treatment of all patients with advanced melanoma.

Ipilimumab's presumed mechanism of action is to break down tolerance to tumor-associated antigens in the melanoma. At the same time, this break down of tolerance may result in autoimmune reactions against self antigens. A wide range of immune-mediated adverse events have been observed. The most common serious manifestations include enterocolitis, hepatitis, dermatitis, and endocrinopathies. In this trial using a 3 mg/kg dose of ipilimumab immune-related adverse events occurred in approximately 60 % of patients treated with ipilimumab. Grade 3 or 4 toxicity was seen in 10–15 % of ipilimumab-treated patients, compared to 3 % of those receiving only gp100. These side effects were typically not seen until 6 or more weeks into therapy. A somewhat higher incidence of side effects was observed with a dose of 10 mg/kg every 3 weeks in the randomized phase II trial that assessed the effects of dose on activity and toxicity [98]. Several investigators have suggested that the development of immune related toxicities correlated with benefit from therapy; however, other studies have not confirmed this correlation.

Although patients with untreated brain metastases were excluded from the phase III trial, other studies have observed antitumor activity with ipilimumab treatment in patients with brain metastases [99]. Finally, data from phase II trials suggested that a number of patients (up to 10 % of those treated) exhibited apparent disease progression after 12 weeks of ipilimumab (with either larger lesions or new lesions), followed by subsequent disease regression. The overall survival outcome of these patients was similar to those exhibiting a tumor response. This led to the establishment of Immune-related Response Criteria that endeavored to capture these patients in the subset of patients achieving treatment benefit [100].

A second phase III trial involved previously untreated patients who were randomly assigned to dacarbazine plus either ipilimumab or placebo [101]. In this study, overall survival was significantly increased in patients assigned to the dacarbazine plus ipilimumab arm (median 11.2 versus 9.1 months). The overall incidence of grade 3 or 4 toxicity was significantly higher with dacarbazine plus ipilimumab (56 versus 28 %). In particular, hepatic toxicity was significantly more common with the combination than with dacarbazine alone or than that previously or subsequently observed with ipilimumab alone. The increase in hepatic toxicity relative to single agent ipilimumab may be due to the fact that dacarbazine is also known to be hepatotoxic. On other hand, the incidence of other immune related toxicities (colitis, rash, hypophysitis) was less than that seen in prior studies with ipilimumab alone, perhaps suggesting that dacarbazine may have blunted and/or the higher incidence of hepatotoxicity may have pre-empted the immune toxicity profile of ipilimumab. Whether this blunting of immune toxicity by dacarbazine might have also blunted the antitumor effect of ipilimumab is a matter of speculation. However, the overall pattern of toxicity and efficacy on this trial do not support the addition of dacarbazine to ipilimumab. The relative value of the use of ipilimumab at the 10 mg/kg dose used this study and in multiple phase II studies vs. the already approved 3 mg/kg dose awaits the completion of an ongoing Phase III trial directly comparing the two doses.

A recent report of long-term survival of patients receiving ipilimumab suggests that death rate for patients followed for more than 3 years declines dramatically and that 20–25 % of patients will achieve long term survival [102].

Anti-PD1 based therapy Another immune checkpoint, programmed death 1 (PD-1), acts as an inhibitory receptor of T cells similar to CTLA-4. However, in contrast to CTLA4, the ligand for PD-1 (PDL1) appears to be expressed almost exclusively at sites of inflammation, such as in the tumor microenvironment. This observation has raised the hope that blockade of PD1 binding with PDL1 might lead to more selective restoration of immunity within the tumor microenvironment and, therefore, less associated toxicity than seen with CTLA4 blockade. Early clinical trials investigating antibodies to PD-1 and PDL1 in patients with melanoma have shown response rates ranging from 25–50 % [103, 104]. In addition a study evaluating the concurrent administration of the combination of ipilimumab and the PD1 antibody nivolumab produced rapid and deep tumor responses in patients with metastatic melanoma and an overall response rate of 53 % in a small number of patients (103). The promising results seen with various anti-PD1 and PDL1 antibodies either alone or in combination with ipilimumab have led to multiple randomized clinical trials of comparing anti-PD-1 antibodies alone or in combination with ipilimumab to standard of care in patients metastatic melanoma. In addition, efforts are underway to study the optimal coordination of immunotherapy with molecularly targeted therapies in patients with BRAF mutant melanomas.

Treatment Selection options Considerable effort has focused on identifying patients who respond to immunotherapy in the hope or restricting such treatment to those most likely to benefit. IL-2 response has been shown to be more likely in patients with normal serum LDH, or low plasma VEGF and fibronectin levels [105]. In addition, response appears to be more frequent in patients whose tumors contain mutations in BRAF or NRAS, or possess an inflammatory gene expression signature [106]. More recent studies have suggested that response to IL-2 is associated with enhancement of a pre-existing gene expression pattern within the tumor associated with immune-mediated tissue-specific destruction under the control of IFNgamma [107]. Benefit from vaccination has also been linked to tumors expressing an IFN driven chemokine signature (107). Preliminary results suggest that both PD1 antibody responsiveness and IL-2 responsive in patients with RCC may be correlated with tumor cell surface expression of PDL1 (102, 108). Furthermore, research suggests that tumor PDL1 expression is not constitutive, but is related to the secretion of IFNgamma by of tumor reactive CD8 T cells in the microenvironment. Thus, effective immunotherapy may require pre-existence of tumor specific immunity within the microenvironment and the use of agents that can either drive T cell function (HD IL-2 or vaccines) or block inherent immunoregulatory signals (ipilimumab, or anti-PD1). Several current studies are underway to validate these predictive biomarkers for specific immunotherapies as well as to determine if combinations of immunotherapy with either other immunotherapies or molecularly targeted agents could convert non-immune responsive tumors into those capable of responding.

1.7 Conclusion

The diagnosis and treatment of patients with all stages of melanoma has continued to evolve over the course of the past century. Although until recently the most effective treatment approaches have been surgical, the greater understanding of the tumor microenvironment have led to advances in immune based systemic treatment options for patients with metastatic melanoma. The challenge now is to determine how best to use these agents alone, in sequence and in combination, how to predict patients destined to respond to therapies and determine timing and mechanisms of resistance and how to move these approaches into the adjuvant settings. In addition, considerable investigation is needed to determine how best to integrate these novel immune based therapies with the rapidly expanding knowledge of molecular changes within the tumor cells themselves and the treatment approaches being developed to target these oncogenic drivers that are described in this book.

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Chapter 2
Melanoma Pathogenesis

Jennifer A. Lo and David E. Fisher

Abstract Melanoma is an aggressive and heterogeneous disease with respect to clinical behavior and underlying genomic lesions. Melanoma development is multifactorial, and increased susceptibility is associated with sun exposure, fair pigmentation, family history, and melanocytic nevi. Major advances in our understanding of its molecular pathogenesis include the identification of recurrent mutations and aberrations in key signaling and developmental pathways. BRAF is the most commonly affected gene, with BRAF(V600E) mutations found in half of all melanomas. The discovery and characterization of oncogenic mutations in the MAPK, RB, p53, and MITF pathways have set the stage for clinically meaningful progress in the melanoma field.

Keywords Melanoma · Melanocyte · Pigmentation · Nevus · BRAF · MAPK · MITF · NRAS

2.1 Melanocyte Biology

Melanomas arise from the malignant transformation of melanocytes. Melanocytes are the pigment producing cells of the skin and are derived from neural crest stem cells. Their development is modulated by the receptor tyrosine kinase c-KIT and microphthalmia-associated transcription factor (MITF), two genes that are mutated or amplified in many melanomas [1].

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Melanocytes can produce multiple types of pigment, most obviously dark brown eumelanin and reddish pheomelanin. Pro-pigmentation signaling is initiated by binding of α-melanocyte stimulating hormone (α-MSH) to the melanocortin 1 receptor (MC1R) on the melanocyte cell surface. MC1R is a seven-transmembrane G-protein-coupled receptor that activates adenylate cyclase, leading to increased intracellular cAMP levels and expression of MITF. MITF in turn induces transcription of pigment synthesis genes and production of melanin [2]. Although many loci are involved in human pigmentation, MC1R is a major determinant of pigmentation phenotype. MC1R polymorphisms involving single amino-acid substitutions can reduce MC1R signaling, resulting in impaired eumelanin production and a red hair/fair skin phenotype [3].

In addition to basal pigmentation, acquired pigmentation can occur in response to stimuli such as ultraviolet radiation (UVR). Eumelanin is the pigment that provides UVR attenuation in darkly pigmented skin. The tanning response to UVR has been shown to involve p53 activation in keratinocytes following UV-induced DNA damage, leading to p53-mediated POMC/MSH expression. Secreted MSH stimulates MC1R in neighboring melanocytes and produces cutaneous pigmentation [4].

2.2 Melanoma Risk Factors

Melanoma pathogenesis is driven by both environmental and genetic factors. Epidemiologic studies have linked melanoma to geographic location and sun (UV) exposure, which is believed to be the most important environmental risk factor. In particular, severe sunburns early in life are associated with the highest risk for melanoma [5, 6]. Melanomas occur more frequently on sun-exposed regions of the body. However, unlike most keratinocyte skin cancers which are known to be products of UVR, melanoma primary tumors are not restricted to sun-exposed skin. Although individuals with fair skin are more susceptible, melanomas also arise in darkly pigmented individuals, most often at acral or mucosal sites. These observations suggest that sun exposure does not account for all melanoma risk.

Increased melanoma susceptibility is associated with family history, fair pigmentation phenotypes, and higher numbers of melanocytic nevi. A family history of melanoma confers an estimated twofold increase in melanoma risk, and approximately 10 % of melanoma patients have a family history of the disease [7]. Melanoma is considered familial if two first-degree relatives or three individuals in a family, irrespective of relationship, are diagnosed with melanoma. Familial melanoma is most often associated with dysregulation of cell cycle checkpoints due to mutations in cell cycle regulatory genes such as cyclin-dependent kinase 4 (CDK4) [8] and cyclin-dependent kinase inhibitor 2A (CDKN2A), which accounts for 40 % of cases and is the most common high-penetrance melanoma susceptibility locus [9]. Germline CDKN2A mutations are responsible for familial atypical multiple mole melanoma (FAMM) syndrome, an autosomal dominant genodermatosis characterized by increased incidence of melanocytic nevi and melanoma, and elevated

risk of other malignancies such as pancreatic cancer in some FAMM kindreds [10, 11]. A less common cause of familial melanoma is the recently reported E318K variant of MITF [12, 13]. This variant exhibited gain-of-function activity for MITF, which is a previously described amplified melanoma oncogene. Individuals carrying the allele exhibited elevated nevus counts and non-blue eye colors, together with increased melanoma risk. The risk was intermediate in nature in the general population (sporadic) and also segregated among many studied melanoma families in multiple continents. The E318K coding variant disrupts a sumoylation site on MITF [14], thereby inhibiting a functionally suppressive post-translational modification on MITF.

Heritable physical characteristics such as fair skin complexion, inability to tan, and blue eyes are associated with elevated melanoma susceptibility. Germline variants of pigmentation genes such as MC1R, agouti signaling protein (ASIP), and tyrosinase (TYR) confer low- or moderate-penetrance melanoma risk [15, 16]. Individuals with non-signaling variants of MC1R have the red hair/fair skin phenotype, characterized by fair pigmentation, freckling, and sun sensitivity, that is associated with the highest risk of melanoma of all pigmentation phenotypes [17]. MC1R coding variants are found in 80 % of individuals with red hair, less than 20 % of individuals with brown or black hair, and less than 4 % of individuals with a robust tanning response [3]. Comparison of melanomas in murine models of different pigmentation phenotypes has demonstrated that the synthesis pathway of the red pigment pheomelanin contributes to melanomagenesis via a UV-independent mechanism [18].

2.3 MAPK and PI3K Pathways

Oncogenic driver mutations have been identified in key signaling and developmental pathways that are involved in survival and proliferation of melanocytes. The most frequently observed recurrent mutations in melanoma occur within the mitogen-activated protein kinase (MAPK) signaling pathway, which promotes cell survival, cell cycle progression, and transformation (Fig. 2.1). In nonmalignant cells, the MAPK pathway is only activated in response to ligand binding to receptor tyrosine kinases or cytokine receptors. Stimulation of receptors leads to activation of RAS family members, monomeric G proteins that act as GTPase switch proteins. RAS-GTP promotes formation of signal-transduction complexes and activates a cascade of serine/threonine kinases culminating in activation of ERK, also known as MAPK. ERK is a serine/threonine kinase that can phosphorylate many targets such as transcription factors.

MAPK signaling is constitutively activated in almost all melanomas. The v-raf murine sarcoma viral oncogene homolog B1 (BRAF) is the dominant genetic target in this pathway, with 40–50 % of melanomas carrying a somatic mutation [19–22]. To a lesser extent, BRAF mutations are also observed in other cancers [23, 24]. BRAF is a serine/threonine kinase directly activated by RAS and is highly expressed in melanocytes, neuronal tissues, testis, and haematopoietic cells. Unlike

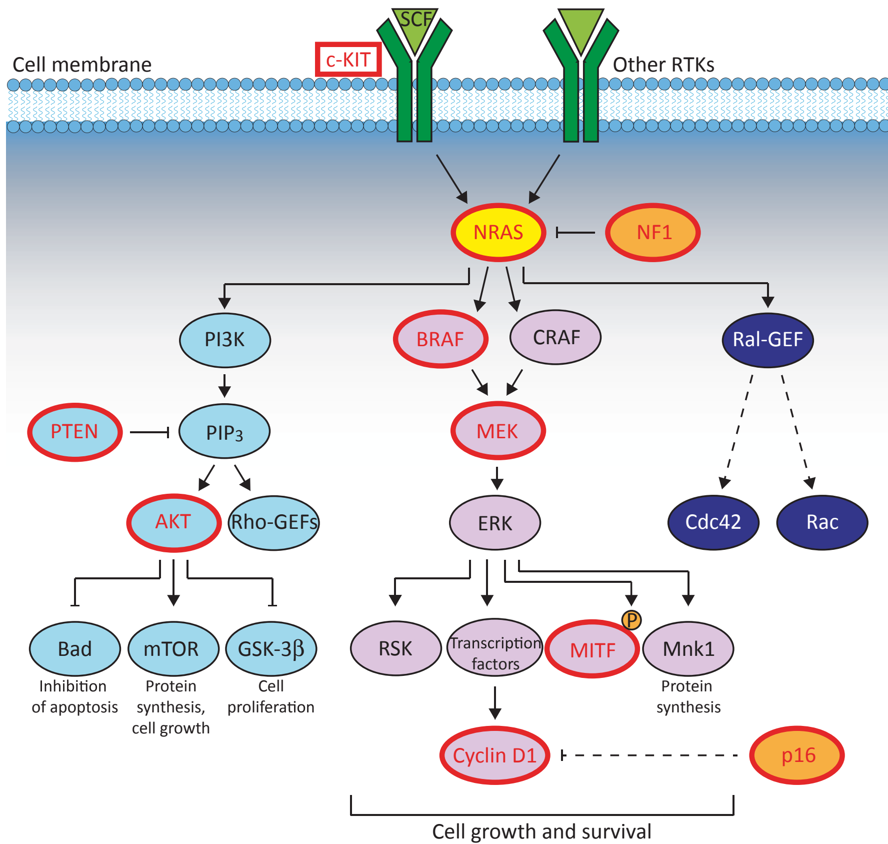

Fig. 2.1 RAS signaling. RAS family members are monomeric G proteins that are activated by receptor tyrosine kinases and signal through direct interaction with effector enzymes including phosphoinositide (PI) 3-kinases, RAF kinases, and Ral-guanine nucleotide exchange factors (*Ral-GEFs*). Although RAS mutations are less common in melanoma than other solid tumors, *NRAS* activating mutations are found in 10–20 % of melanomas. Mitogen-activated protein (MAP) kinase signaling in response to RAF kinase activity promotes cell growth and survival, and the MAPK pathway is constitutively activated in almost all melanomas. *BRAF* is the most frequently mutated gene in melanoma, with activating lesions found in 40–50 % of tumors. Melanoma oncogenes and tumor suppressors are labeled in red. Dotted lines represent omitted pathway components. *NF1* neurofibromatosis 1, *PTEN* phosphatase and tensin homolog, PIP_3 phosphatidylinositol-3,4,5-triphosphate, *mTOR* mammalian target of rapamycin, *GSK-3β* glycogen synthase kinase-3β, *RSK* ribosomal S6 kinase, *Mnk1* MAP kinase-interacting kinase 1, *Cdc42* cell division control protein 42 homolog

CRAF, which can participate in signaling events outside the MAPK pathway, BRAF's only known substrate is MEK/MAP2K. Phosphorylated MEK activates ERK by phosphorylation, leading to pro-growth and transforming effects that are critical in melanoma pathogenesis.

The most common BRAF mutation in melanoma, accounting for 90 % of variants, is a valine to glutamic acid substitution at codon 600 (V600E) in exon 15 [25].

This mutation constitutively activates the kinase domain. Other oncogenic BRAF mutations are found elsewhere in exon 15 or in exon 11, and most of the over 100 rare non-V600E mutations described occur in the glycine-rich loop and activation segment of the kinase domain. Mutations in these regions indirectly activate BRAF by disrupting the normal intramolecular interactions which hold BRAF in an inactive conformation [26].

An alternate oncogenic mechanism in melanoma involves rare BRAF mutants with low kinase activity. Although no CRAF activating mutations have been reported in melanoma [25, 27, 28], mutations such as G469E and D594G produce a BRAF that directly activates CRAF but minimally phosphorylates MEK. Melanoma lines with these low-activity BRAF mutations are dependent on CRAF for survival [29].

BRAF(V600E) mutations are observed much more frequently in melanomas arising in intermittently sun-exposed skin regions than acral or mucosal melanomas, suggesting that BRAF mutations may be linked to sun exposure. However, the thymidine to adenine (T>A) transversion at position 1799 that is responsible for the V600E substitution is not a typical UV-signature DNA mutation. It is possible that the transversion could result from a "non-classic" UV-induced DNA lesion or from secondary effects of UVR exposure such as generation of reactive oxygen species [30].

Mutations that increase RAS activity also promote cell proliferation. In comparison to other solid tumors, RAS mutations occur with relatively low frequency in melanomas. Only 10–20% of melanomas, most often amelanotic nodular subtypes, carry an activating RAS mutation. NRAS is the most commonly affected RAS family member in melanoma [31, 32], and NRAS activating mutations [33, 34] primarily involve glycine 12, glycine 13, and glutamine 61 and trap NRAS in its active, GTP-bound conformation. While BRAF mutations activate only MAPK signaling, NRAS activating mutations simultaneously activate the MAPK and phosphatidylinositide 3-kinase (PI3K) pathways.

Although oncogenic mutations are usually not stand-alone events in melanoma, some are thought to be mutually exclusive. For example, NRAS and BRAF mutations almost never occur concomitantly [35, 36], suggesting that NRAS and BRAF have overlapping oncogenic activities and either is sufficient for constitutive activation of the MAPK pathway. Both BRAF and NRAS mutations are associated with poorer clinical prognosis. In the rare cases when both BRAF and NRAS mutations are present in melanoma, the BRAF mutation is not the classic V600E substitution [37]. "Acquired" (or selected) NRAS mutations have also been observed simultaneously with BRAF(V600E) in the context of melanomas which initially responded to BRAF-targeted therapy but subsequently became resistant [38].

PI3K signaling results in increased activation of the serine/threonine kinase AKT (also known as protein kinase B), which is a major mediator of cell survival through activation of targets such as mammalian target of rapamycin (mTOR) and inhibition of pro-apoptotic signals. While PI3K itself is rarely mutated in melanoma [39], constitutive activation of NRAS, amplification of AKT3, or loss of the phosphatase and tensin homolog (PTEN) tumor suppressor can lead to dysregulation of the PI3K

pathway. PTEN encodes a lipid and protein phosphatase that negatively regulates signaling pathways which use the cytosolic second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3), such as the PI3K pathway. Lower levels of intracellular PIP_3 result in less downstream activating phosphorylation of AKT. Thus, loss of PTEN protein or function eliminates a mechanism of negative regulation of AKT and cell survival. Increased phospho-AKT levels are associated with poor melanoma prognosis [40].

PTEN can be lost upon chromosome 10q deletion. 50–60 % of melanomas contain hemizygous deletions or point mutations in 10q, while 10 % contain homozygous deletion [41]. Epigenetic silencing of PTEN has also been described [36, 42, 43]. Hemizygous PTEN deletions tend to occur with BRAF mutation [42, 44], suggesting that BRAF and PTEN can cooperate in melanomagenesis. This idea is supported by studies of a murine model of melanoma in the setting of BRAF(V600E) and PTEN inactivation [45].

Neoplastic transformation of melanocytes can give rise to benign nevi as well as malignant melanoma, and activating mutations in BRAF and NRAS are implicated in both. Activating BRAF mutations are found in 70–80 % of dysplastic nevi [22, 46–48], while NRAS mutations are rare in dysplastic nevi [49, 50] but present in most congenital nevi [50]. Mutation of HRAS is associated with Spitz nevi [51]. The BRAF(V600E) mutation induces nevus formation, involving initial cell proliferation followed by oncogene-induced senescence likely due in part to accumulation of $p16^{INK4A}$ [52]. Mutation of $p16^{INK4A}$ in addition to BRAF leads to transformation of cells *in vitro*, and deletion of PTEN or $p16^{INK4A}$ results in the formation of invasive melanoma in BRAF(V600E) mice [53]. In zebrafish, concomitant BRAF(V600E) mutation and deletion of TP53 leads to the formation of invasive and metastatic melanoma [54].

Given the high incidence of BRAF mutations in nevi, mutation of BRAF was traditionally thought to be a founder event that preceded all other oncogenic events in BRAF mutant melanoma [46]. In this model, senescence induced by BRAF activation is overcome by cooperating genetic lesions such as loss of $p16^{INK4A}$ or PTEN. However, other evidence suggests that the order of melanocytic lesions and relationship between nevi and tumor may be more complex. Although BRAF mutations are found in most nevi and half of vertical growth and metastatic melanomas, they are rare in initial malignant lesions: only 10 % of radial growth phase melanomas and 6 % of in situ melanomas have mutant BRAF. In addition, many nevi and primary melanomas are polyclonal (contain both BRAF wild type and BRAF mutant cells) while metastatic melanomas are not polyclonal, suggesting that BRAF mutation might occur at later stages of melanomagenesis [25, 46, 55, 56]. In addition, recent data have suggested that a stereotypical mutation in the promoter of the enzyme telomerase reverse transcriptase (TERT) is found in both BRAF mutant or NRAS mutant melanomas, suggesting that it may be an earlier mutation event [57]. This mutation in the TERT promoter occurs at a frequency of approximately 70 % in melanomas and is also found in many non-melanoma cancers [57]. Regardless of when BRAF lesions occur, activation of BRAF in invasive melanoma promotes cell growth and dependence on the MAPK signaling pathway [58].

Dysregulation of MAPK signaling in melanoma can alternatively be caused by overexpression or hyperactivation of growth factor receptors such as c-Met, KIT, and epidermal growth factor receptor (EGFR) [59–61]. Mutations in the tumor suppressor neurofibromatosis 1 (NF1), a negative regulator of Ras, were identified in 5 out of 21 tumors without BRAF or NRAS mutations [62]. In the context of BRAF(V600E), NF1 mutations dysregulate the MAPK and PI3K pathways, ultimately suppressing mutant BRAF-induced senescence and promoting melanoma development and proliferation [63]. In some melanomas, inactivating mutations have also been identified in the tumor suppressor neurofibromatosis 2 (NF2) [64]. Germline mutations in NF1 and NF2 are associated with hereditary neurofibromatosis. Recent whole-exome sequencing approaches have identified somatic mutations in downstream MAPK effectors such as MAP3K5, MAP3K9, MEK1, and MEK2 in melanomas [65, 66].

2.4 RB and p53 Pathways

The retinoblastoma (RB) and TP53 tumor suppressor pathways are dysregulated in many sporadic and familial melanomas, and all known inherited high-risk melanoma susceptibility loci are genes in the RB pathway. However, loss or lesions of RB and TP53 occur much less frequently in sporadic melanomas than in most other solid tumors. Instead, genetic alterations in CDKN2A can eliminate upstream signaling in these pathways in melanoma. The CDKN2A locus at chromosome 9p21 encodes four exons, and alternative splicing yields two distinct tumor suppressors that share a common second exon: $p16^{INK4a}$ and $p14^{ARF}$ [67]. Mutations in $p16^{INK4A}$ functionally inactivate the RB pathway while mutations in $p14^{ARF}$ functionally inactivate the p53 pathway. The most common CDKN2A lesions in melanoma are point mutations, which are found as germline lesions in 25–40 % of familial melanomas and as sporadic alterations in 10 % of non-familial melanomas [67]. CDKN2A point mutations are also associated with dyplastic nevi. As with PTEN loss, CDKN2A mutation tends to coincide with BRAF mutation [21].

The RB pathway regulates the G1/S cell cycle checkpoint. During normal cell cycle progression, the RB tumor suppressor is phosphorylated by mammalian G1 cyclin-CDK complexes. Hyperphosphorylation of RB triggers release of E2F family members, transcription factors that activate expression of genes important for entry into S phase and DNA synthesis. $p16^{INK4a}$ binds and inhibits cyclin-dependent kinases 4 and 6 (CDK4/6) from inappropriately phosphorylating the RB protein [68]. Thus, loss of $p16^{INK4a}$ facilitates RB phosphorylation and subsequent re-entry into the cell cycle. Point mutations or transcriptional silencing are responsible for loss of $p16^{INK4a}$ expression in 30–70 % of melanomas, leading to increased cellular proliferation and escape from oncogene-induced senescence.

Activating mutations in CDK4 are found in a small number of melanomas. CDK4 germline mutations always occur at a conserved arginine residue, R24, that is necessary for regulatory inhibition of CDK4 by $p16^{INK4a}$ [8, 69]. 5 % of melanomas

contain somatic CDK4 point mutation or amplification [70]. $p16^{INK4a}$ and CDK4 mutations are mutually exclusive [29, 71, 72].

While TP53 mutations are found in 5% of melanomas [73], the p53 apoptotic pathway is more often deficient due to loss of $p14^{ARF}$ function in melanomas [74]. $p14^{ARF}$ binds and inhibits the mouse double minute 2 homolog (MDM2). MDM2 encodes an E3 ubiquitin ligase that inhibits p53 transcriptional activity and targets p53 for proteasomal degradation. Inactivating $p14^{ARF}$ mutations permit the p53-antagonizing activity of MDM2 and subsequent genomic instability [75–77]. In rare cases, amplification of MDM2 without alterations in CDKN2A sequence or expression has been observed in melanoma [70].

2.5 MITF

MITF is the master lineage regulator of melanocyte development and survival. It serves as the transcription factor for differentiation and pigmentation genes such as TYR, tyrosinase-related protein 1 (TYRP1), dopachrome tautomerase (DCT), melanoma antigen recognized by T-cells 1 (MART1, also known as gp100), and premelanosome protein (PMEL17, also known as SILV). Although MITF is essential for melanocyte differentiation, it can alternatively promote malignant behavior in some melanomas. The most common genetic alteration of MITF is amplification, which occurs in 15–20% of melanomas with a higher prevalence among metastatic melanomas [78]. MITF amplification is thought to usually occur as a late event in melanoma progression and was associated with poorer 5 year survival in the pre-vemurafenib and ipilimumab era [79]. Many melanomas continue to depend on MITF expression for survival, and suppression of MITF *in vitro* is lethal to most melanoma cell lines [80, 81].

The transcriptional targets of MITF that mediate its oncogenic activity as distinct from its regulation of pigmentation and differentiation are not fully characterized. However, MITF is known to enhance expression of genes involved in cell cycle progression, cell proliferation, and cell survival. For example, MITF is a transcription factor for cell cycle kinase CDK2 [81], CDK inhibitors $p16^{INK4a}$ [82] and p21 [83], and anti-apoptotic mitochondrial membrane protein B-cell lymphoma 2 (BCL-2) [84] as well as its related family member BCL2A1 [85]. In melanomas with elevated MITF activity, increased expression of these MITF targets likely contributes to growth, invasion, and survival of melanoma cells.

MITF is known to cooperate with BRAF in melanoma transformation *in vitro* [78] and *in vivo* [86]. MAPK pathway activation, which is found in the majority of melanomas, results in MITF phosphorylation at Ser73 by ERK2 [87]. Phosphorylation at Ser73 affects MITF regulation in two ways: enhanced recruitment of p300, an MITF transcriptional coactivator and histone acetyltransferase, and increased ubiquitination of MITF [88, 89]. Because Ser73 phosphorylation ultimately accelerates proteasomal degradation of MITF, MAPK signaling in melanomas can reduce

expression of many MITF targets. BRAF inhibitors may enhance immunotherapy by stabilizing MITF and upregulating transcription of targets like MART1 and other antigens that are recognized by the immune response to melanoma [90].

Other post-translational modifications of MITF include phosphorylation by ribosomal S6 kinase (RSK), glycogen synthase kinase-3β (GSK-3), and p38 and sumoylation by protein inhibitor of the activated STAT3 (PIAS3) [91–94]. MITF is also a substrate for proteolytic degradation by caspase 3 [95]. Protein kinase C interacting protein 1 and PIAS3, which preferentially binds Ser73-phosphorylated MITF, inhibit MITF binding to DNA [14, 96–98]. Sumoylation of MITF reduces transcription of a subset of MITF targets whose promoters contain multiple MITF binding sites [99, 100]. In light of this observation and the complexity of MITF regulation, it is tempting to speculate that post-translational modifications determine MITF target gene specificity in response to cell context. By such mechanisms, MITF may be able to switch between its two recognized functions of regulating melanocytic differentiation/pigmentation and modulating survival/proliferation effects capable of producing an oncogenic transcriptional program in melanoma.

Germline loss-of-function mutation of MITF in humans causes Waardenburg syndrome type IIA, an autosomal dominant inherited condition characterized by lack of melanocytes in the eye, forelock, and inner ear [101]. Melanocyte deficiencies in individuals with Waardenburg syndrome result in deafness, white forelock (unpigmented hair in the midline), and eye color variability [102]. In contrast, increased numbers of nevi and darker eye colors are associated with the gain-of-function mutation conferred by a germline missense mutation in codon 318 of MITF. As previously discussed, this mutation abrogates a sumoylation site, resulting in altered transcription of some MITF targets and elevated melanoma susceptibility [12, 13] (Fig. 2.2).

2.6 Acral and Mucosal Melanomas

KIT mutations and amplifications are the most common genetic alterations in melanomas arising in acral, mucosal, and chronically sun-damaged skin. Although KIT mutations are found in only 1 % of all melanomas, they are reported in 10 % of acral and 10 % of mucosal melanomas [103]. Less than 10 % of KIT mutant melanomas contain BRAF or NRAS mutations.

c-KIT encodes the receptor tyrosine kinase for stem cell factor. In response to ligand binding, KIT activates signaling of pathways such as RAS. The most commonly observed KIT variant in melanoma, found in a third of KIT-mutant melanomas, is L576P [55]. Activating mutations such as L576P promote melanocyte growth and survival by causing constitutive stimulation of MAPK and PI3K/AKT signaling. KIT mutations are associated with poorer clinical outcomes in acral and mucosal melanoma [119].

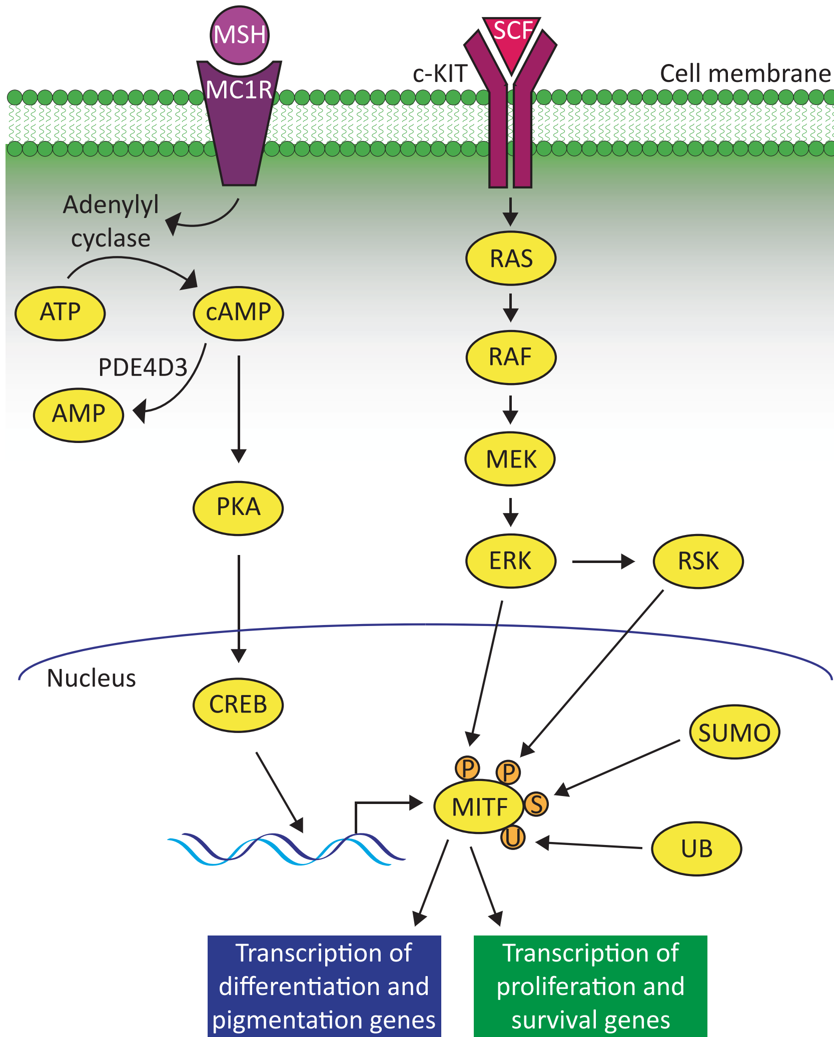

Fig. 2.2 The MITFaxis. In melanocytes, microphthalmia-associated transcription factor (*MITF*) is expressed in response to melanocortin 1 receptor (*MC1R*) signaling upon binding of melanocyte-stimulating hormone (*MSH*). Non-signaling variants of *MC1R* are associated with the red hair/fair skin phenotype and increased melanoma susceptibility. *MITF* activity is modulated by phosphorylation, sumoylation, and ubiquitination. *MITF* target genes include regulators of differentiation and pigmentation as well as proliferation and survival. *c-KIT* signaling is essential for melanocyte development. *c-KIT*, *NRAS*, *BRAF*, and *MITF* are known melanoma oncogenes in the *c-KIT* pathway. *SCF* stem cell factor, *cAMP* cyclic AMP, *PKA* protein kinase A; *CREB* cAMP-responsive element-binding protein

2.7 Uveal Melanoma

Unlike other clinical subtypes of melanoma, uveal melanomas rarely if ever involve mutations in BRAF, NRAS, or KIT. The dominant genetic alterations observed in uveal melanomas are somatic activating mutations in one of two heterotrimeric G protein α-subunits: GNAQ and GNA11. These mutations are almost never concomitant and are exclusively found in 80 % of uveal melanomas, with GNAQ and GNA11 each affected in 40 % of uveal melanomas. GNAQ and GNA11 mutations are also commonly found in proliferations of dermal melanocytes called blue nevi. In contrast, GNAQ and GNA11 mutations were only found in 1 of 273 (0.4 %) of extraocular melanomas [104, 105].

G protein α-subunits are GTPases that serve as molecular switches for the G protein, which is active in its GTP-bound state and inactive in its GDP-bound state. In uveal melanoma, GNAQ and GNA11 mutations are restricted to codon R183 in exon 4 and codon Q209 in exon 5 [105], and their effect is to trap GNAQ and GNA11 in their active, GTP-bound states [106, 107]. As a result, GNAQ and GNA11 mutations contribute to uveal melanomagenesis by activating signaling of numerous pathways regulated by GPCRs including the MAPK pathway [105].

Interestingly, when taken together, the incidence of GNAQ and GNA11 mutations is not higher in uveal melanoma metastases than in primary uveal melanomas. However, in one study of 187 patients GNAQ mutations were proportionally more common in primary uveal melanomas while GNA11 mutations were found in a greater fraction of metastases, suggesting that stratifying by affected G protein α-subunit may be clinically useful [105].

Loss of the tumor suppressor BRCA1-associated protein (BAP1) on chromosome 3 is associated with metastatic uveal melanoma. BAP1 encodes a deubiquitinase that is a component of Polycomb-repressive complexes. Loss of BAP1 in uveal melanoma is thought to most often result from loss of one chromosome 3 allele combined with somatic mutation in the other BAP1 allele. Complete or partial monosomy of chromosome 3 occurs in about 25 % of uveal melanomas [108].

While uveal melanoma may be diagnosed at relatively early stages due to visual symptoms, the disease has a striking propensity to metastasize to the liver. BAP1 mutation predicts poor clinical outcome and is particularly associated with risk of metastatic disease: in one study BAP1 was mutated in 84 % of uveal melanomas from patients at high risk for metastasis but only 4 % of tumors from patients at low risk for metastasis [109].

Germline BAP1 mutation or loss predisposes individuals to malignancy, with familial uveal melanoma accounting for 2–5 % of all uveal melanoma cases. However, penetrance of disease is relatively low in these families, perhaps because inactivation of BAP1 occurs as a late event in melanoma progression [110].

2.8 Melanoma Genomics

Recently, improving technologies, robust bioinformatics platforms, and declining costs of sequencing have made comprehensive analysis of melanoma mutations accessible. These analyses are complicated by tumor heterogeneity and the high mutation rate associated with melanoma. Genome sequencing has revealed that the rates of base mutation are higher in melanoma than in other solid tumors [111]. The elevated mutational load is almost entirely attributable to cytidine to thymidine (C>T) transitions, which can be induced by UVR exposure. Traditionally, C>T mutations at dipyrimidine sequences in the context of melanoma are considered UVB signature mutations while G>T mutations are attributed to oxidative damage mediated by UVA. However, many recurrent mutations in melanoma, including oncogenic BRAF and NRAS lesions, do not involve C>T or G>T base changes, suggesting that alternate mutagenic mechanisms may be involved.

The high somatic mutation rate in melanoma is an important challenge when discriminating between true driver mutations, which confer a fitness advantage to the tumor cell during melanomagenesis, and passenger mutations. A recent statistical approach to sequence analysis refined the predicted background passenger mutation rate to be heterogeneous rather than genome-uniform by allowing for variations associated with transcriptional status and location relative to exons. This approach infers positive selection at each locus based on the exon/intron distribution of mutations and predicted functional consequences of mutations. By this analysis, 46 and 9% of melanoma driver mutations can be attributed to C>T or G>T mutations, respectively, accounting for two-thirds of all non-BRAF or NRAS driver mutations [62].

Since the first genome of a melanoma cell line was published in 2010 [111], exome and whole-genome sequencing of patient tumors has identified multiple novel melanoma genes. In studies sampling up to 25 tumors, recurrent somatic mutations were identified in the downstream MAPK pathway components MAP3K5, MAP3K9, MEK1, and MEK2 [65, 66], ionotropic glutamate receptor GRIN2A [112], and the phosphatidylinositol 3,4,5-trisphosphate RAC exchange factor PREX2 [113].

In one report, GRIN2A mutations were found in one quarter of melanomas [112]. Although GRIN2A has not been functionally validated as an oncogene, glutamate receptor pathway dysregulation was previously implicated in melanoma in studies of another glutamate receptor, GRM3 [114]. Activated GRM3 is an accessory to MAPK signaling and can itself be mutated in melanomas [115]. PREX2 has been shown to negatively regulate PTEN in breast cancer and was mutated in 23 out of 107 melanomas in another study [113].

Whole-exome sequencing of larger melanoma cohorts, including 147 and 121 tumors respectively, identified novel melanoma genes including RAC1 and PPP6C [62, 116]. Recurrent mutations in both RAC1 and PPP6C result from C>T transitions. Somatic gain-of-function mutations in RAC1 were found in 5–10% of melanomas. These mutations destabilize Rac1's inactive GDP-bound state and result in

increased Rac1 activation, promoting cell proliferation and migration [116]. PPP6C encodes a serine/threonine phosphatase that was mutated in approximately 10% of melanomas. PPP6C acts as a tumor suppressor by negatively regulating levels of cyclin D1 (CCND1) during the G1 phase of the cell cycle. Thus, PPP6C loss-of-function mutations likely dysregulate cell cycle and mitosis in some melanomas.

2.9 Conclusion

Although melanoma is a highly heterogeneous disease with respect to clinical behavior, histology, and underlying genomic aberrations and mutations, several themes have emerged in our understanding of its molecular pathogenesis. The MAPK pathway is the key signaling pathway, with activating mutations in BRAF, NRAS, KIT, GNAQ, or GNA11 found in almost all melanomas. The RB and p53 pathways are also frequently dysregulated in melanoma and are implicated in many familial cases. Given the important role of MAPK, RB, and p53 signaling in other malignancies, understanding abnormalities of these pathways may have broad implications for research and treatment of many cancers.

Lineage-specific activity is known to contribute to melanomagenesis as well, with amplification and dysregulation of MITF found in 20% of melanomas. Other genes, which are less commonly affected, have been identified by analysis of large exome sequence datasets and other methods. In the future, intron and UTR sequence data from whole-genome sequencing will allow further refinement of algorithms and increased statistical power to find low frequency driver mutations in melanoma.

Despite substantial progress in our understanding of melanoma pathogenesis, several important observations remain unexplained. Sun exposure is the leading environmental risk factor for melanoma, but the most common oncogenic mutations (in BRAF and NRAS) are not caused by known UV-related mechanisms. Sunscreens confer protection against cutaneous squamous cell carcinoma and have been shown to diminish melanoma incidence in certain contexts, but less so (or not at all) in other studies, suggesting a complexity that is poorly understood [117, 118]. Moreover, a recent study reported that the red hair/fair skin pigmentation phenotype is associated with elevated melanoma risk independent of UV exposure [18]. Elucidating the molecular basis for UV-independent melanoma susceptibility and genetic lesions will provide the framework for progress in melanoma prevention.

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Chapter 3
Molecular Diagnostics and Tumor Mutational Analysis

Melissa A. Wilson and Katherine L. Nathanson

Abstract Genetic and genomic analysis of melanoma tumor samples has identified a number of somatic mutations integral to melanoma pathogenesis, with the most prevalent mutation being the *BRAF* V600 mutation. Targeted inhibitors directed against this mutation have produced improved overall survival compared to chemotherapy. Multiple additional somatic mutations have been identified, and some also have prompted the development of therapy targeted against them. In this chapter, we review common techniques used to identify gene mutations and genomic aberrations, and briefly describe mutations important in melanoma pathogenesis. We also describe massively parallel sequencing and discuss advances that have been made in the identification of novel driver mutations in melanoma tumors. Finally, the application of these techniques with respect to clinical testing is addressed, specifically as they pertain to the development and advancement of personalized medicine.

Keywords BRAF · Melanoma · Molecular diagnostics · Molecular testing · Mutational analysis · Massively parallel sequencing · Somatic mutations · Tumor analysis

3.1 Introduction

Genetic mutations and genomic aberrations have been identified in all tumor types and implicated in multiple aspects of pathogenesis, including examples such as in *EGFR* in lung cancer, *KRAS* in colon cancer, and *BRAF* in melanoma [1–5].

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Identification of these genetic events has been integral in guiding the development of targeted therapies, which have improved progression free or overall survival of patients in some cases, as compared to prior standard of care therapies. Cutaneous melanoma is the most aggressive form of skin cancer and its incidence continues to increase, in contrast to some other cancers such as breast, colon, and lung [6]. Genetic and molecular studies have detected a number of somatic mutations in melanoma cell lines and tumor samples integral to the pathogenesis of melanoma (reviewed in [7–10]). The discovery of mutant *BRAF* as a driver mutation in melanoma led to the development of targeted inhibitors, which have demonstrated an increased overall survival in patients with advanced melanomas containing mutant *BRAF* [1, 3, 4, 11, 12]. In addition, mixed responses have been observed in *KIT* mutant melanomas, treated with the tyrosine kinase inhibitors imatinib or dasatinib [13–19]. Moreover, new driver mutations are continually being identified in melanoma tumor samples [20, 21] which may serve as future targets for therapeutic intervention.

Recently, genetic and genomic profiling of tumors has moved from research to clinical laboratories as targeted therapies have become the new standard of care for the treatment of a sub-set of malignancies, including melanoma, and as techniques have been optimized for high-throughput analysis of somatic mutations. Moreover, tumor mutational analysis is advancing beyond single gene mutation testing; new sequencing methods allow for the simultaneous analysis of multiple genetic mutations within an individual tumor sample. These advances in sequencing techniques continue to have a large impact on the clinical testing of patient tumor samples, with the results having implications for therapeutic interventions such as inclusion or exclusion from clinical trials and initial therapy choices. This chapter will focus on techniques used in molecular diagnostics and mutational analysis of melanoma tumor samples, review sequencing methods, and discuss current and future technologies integral to the field of genetic sequencing. In addition, we will highlight relevant somatic mutations in melanoma tumors, which may be important in the development of future targeted therapies.

3.2 Somatic Mutations in Melanoma

3.2.1 UV Damage-Induced Mutations

Somatic driver mutations identified in patient tumors, both in melanoma and other cancers, tend to be recurrent single nucleotide changes in oncogenes, with mutations leading to stop codons and frameshift mutations, and insertion and deletions as observed in tumor suppressor genes. Likewise, critical genomic aberrations also exist and include loss of heterozygosity or amplification at specific loci, splice variants, and epigenetic dysregulation. As such, the molecular diagnostic testing of melanoma tumor samples needs to reliably detect these diverse somatic mutations and genomic aberrations, since identification of these mutations in patient tumors is critical for the determination of appropriate therapy.

Notably, samples from melanoma cell lines and tumor samples have demonstrated mutations consistent with UV exposure [22], which is a known risk factor for the development of melanoma [23–25]. Pyrimidine dimers are characteristic of UV-induced DNA damage and mutations are predominantly C to T/G to A transitions, along with CC to TT transitions [26, 27]. These mutations are frequently observed in adjacent pyrimidine sequences and at higher frequencies of CpG dinucleotides. Indeed, massively parallel sequencing of a melanoma cell line derived from a metastatic tumor sample demonstrated increased C to T transitions in bases at pyrimidine dinucleotides (92 %, as compared to predicted 53 % due to chance) and at CpG dinucleotides (10 %, as compared to predicted 4.4 % due to chance) [22]. Whole exome sequencing of larger numbers of melanoma tumor samples, 121 melanoma tumor/normal pairs and 147 melanoma tumor samples, confirmed these observations of UV-induced damage [20, 21] along with identification of different mutation patterns in tumors from sun-exposed and sun-shielded sites [21]. Cells have a number of mechanisms for repairing DNA damage. In UV-induced DNA damage, nucleotide excision repair (NER) is the predominant mechanism for DNA damage repair [22, 28, 29], with preferential repair of actively transcribed strands [22, 27, 30, 31]. Results from massively parallel sequencing of melanoma tumor samples demonstrate that fewer somatic mutations are identified on transcribed DNA strands within genes than non-transcribed strands, consistent with transcription coupled repair [21, 22]. UV-induced DNA damage is highly prevalent in melanoma tumor samples from sun-exposed areas. However, somatic mutations due to UV-induced DNA damage are under-represented as causative driver mutations in melanoma, as cells have developed mechanisms to repair DNA damage through NER. The converse is observed in tumors derived from xeroderma pigmentosum (XP), a hereditary syndrome characterized by deficient nucleotide excision repair. In these tumor samples, patterns of somatic mutations in key tumor suppressor genes, including *TP53*, are a result of deficient NER, with significant UV-induced DNA damage, although preferential transcription-coupled repair is preserved [26, 27].

3.2.2 **BRAF** ***Mutations***

BRAF is the most common driver mutation identified in melanoma tumor samples and is mutated in approximately 50 % of melanomas [32–34]. Within *BRAF*, the most prevalent mutation is a glutamic acid substitution for valine at codon 600 (*BRAF* V600E) which occurs in the kinase domain and results in a constitutively active protein [32–34]. Additional *BRAF* V600 and proximate mutations are observed in melanoma cell lines and tumor samples, as well as in the loop domain (exon 11) [34–36]. The *BRAF* V600E mutation is associated with younger age of diagnosis and truncal site of primary lesion [36, 37]. The *BRAF* V600K mutation is a result of a two base change within codon 600; it has been observed in 9–19 % of melanomas and is associated with increased age and higher cumulative sun damage [37, 38]. Improved clinical response to targeted BRAF inhibition compared to chemotherapy

has been observed in patients whose melanomas carry *BRAF* V600E and V600K mutations [3, 11, 12, 36]. However, lower response rates to targeted BRAF inhibition are observed in patients with *BRAF* V600K mutant melanoma [39, 40]. The BRAF V600 inhibitors were developed to target the mutated protein. Thus, it is not entirely clear whether patients with melanomas harboring the non-V600 *BRAF* mutations will respond similarly to *BRAF* inhibition. Dahlman et al. [41] demonstrate both preclinical and clinical data supporting the use of targeted inhibition of the MAPK pathway in *BRAF* L597 mutated melanoma. A patient with *BRAF* L597S mutated metastatic melanoma responded to treatment with the MEK inhibitor, TAK-733 [41]. In addition, preclinical data suggest that *BRAF* K601 mutant melanomas may respond to treatment with MEK inhibitors; as expression of *BRAF* K601E induced signaling through the MAPK pathway was abrogated with MEK inhibition [41]. Further studies are needed to determine the role of BRAF and/or MEK inhibition in non-*BRAF* V600 mutant melanoma.

3.2.3 NRAS *Mutations*

NRAS mutations are the second most prevalent mutations, and are found in 15–20 % of melanomas [42–44]. The predominant mutations in *NRAS* occur in exon 2 at codon 61 with substitution of glutamine with several different amino acids (Q61) [45, 46], resulting in activation leading to uncontrolled cell proliferation. In addition, somatic mutations have been identified in exon 1 at codons G12 and G13 [47]. *NRAS* Q61 mutations are associated with the nodular subtype of melanoma, increased tumor thickness, and worsened clinical outcome, demonstrating shorter melanoma specific survival time [38, 42, 48, 49]. It has been challenging to target RAS mutations in tumors generally, however, current clinical trials are underway investigating the use of MEK inhibitors (*MAP2K1* and *MAP2K2*, mitogen-activated protein kinase 1 and 2) either as single agents or in combination with parallel intracellular signaling pathway inhibitors, such as PI3K/mammalian target of rapamycin (mTOR) inhibitors to treat *NRAS*-mutant melanomas [50–53] (www.clinicaltrials.gov).

3.2.4 KIT *Mutations*

KIT is a receptor tyrosine kinase and is mutated in a small percentage of cutaneous melanomas. However, mucosal and acral lentiginous melanomas, along with melanomas arising in chronic sun-damaged skin, have an increased prevalence of *KIT* mutations; mutations and increased copy number have been identified in approximately 30 % of these specific melanoma subtypes [54, 55]. *S*omatic mutations in *KIT* have been observed in a number of different exons including 9, 11, 13, and 17. As there is no single predominant mutation in *KIT*, molecular testing must evaluate multiple exons within the gene. Variable responses to treatment

with imatinib, a KIT and PDGFR tyrosine kinase inhibitor, have been observed in patients with melanomas with *KIT* mutations [13–18]. Several studies have found that the maximal response to imatinib is seen in patients whose melanomas have *KIT* mutations in exons 11 and 13 [13, 14]. Responses have also been observed upon treatment with dasatinib, a tyrosine kinase inhibitor similar to imatinib, in melanoma [19].

Approximately 30 % of melanoma tumors do not contain mutations in *BRAF*, *NRAS*, or *KIT* genes, and therefore do not currently have mutations that can be therapeutically targeted. However, additional driver mutations in melanomas have been identified, which may lead to the eventual development of appropriate targeted therapies. In particular, massively parallel sequencing has delineated mutations in *ARID2*, *NF1*, *PPP6C, RAC1*, *SNX31*, *STK19*, and *TACC1* [20, 21]. PPP6C is a component of the PP6 protein phosphatase complex and a proposed tumor suppressor; it functions to regulate cyclin D1 during cell cycle progression [56, 57]. *STK19* is thought to encode a kinase of unknown function and mutations within this gene are identified within hotspot regions in melanoma tumor samples [20]. RAC1 is a member of the Rho family of GTPases and functions in melanocyte proliferation and cell migration through its role in cell adhesion, migration, and invasion [20, 21, 58]. With the data from several published studies using whole exome and genome massively parallel sequencing in melanoma [20–22,59–62], as well as the on-going Cancer Genome Atlas effort, the spectrum of genetic mutations and genomic aberrations in untreated cutaneous melanoma is likely to be well described in the near future. With the routine use of targeted therapies in the treatment of *BRAF* mutated melanoma, clinicians have observed resistance to therapy. Discovery of additional or acquired mutations in these tumor samples is important for identification of resistance mechanisms, which may fall outside the spectrum of mutations observed in untreated melanomas, with the eventual goal of preventing and overcoming these mechanisms of resistance.

3.3 Somatic Mutation Testing—Technology

Understanding of the genetic underpinnings of melanoma has led to current treatment advances for advanced stage melanoma and will continue to aid in the development of future therapies. Therefore, it is important to identify known mutations in melanoma tumors in order to stratify patients for therapeutic options, as well as identify mechanisms and mutations involved in treatment resistance. A number of techniques have been used to identify somatic mutations and genomic aberrations providing clinicians with tools to genotype melanoma tumor samples from patients, at all stages of disease.

For many years, molecular diagnostic techniques have evaluated single gene mutations individually or a small number of genes through reaction multiplexing. Massively parallel sequencing allows for the simultaneous testing and identification of multiple mutations and genomic aberrations within tumor samples concurrently.

Although knowledge of all mutations and genomic aberrations within tumor samples would appear on the surface to be most helpful, currently there are limited gene mutations that are clinically actionable. Thus, assaying individual genes and/or mutations is still appropriate in many circumstances. It is important to note that although full profiling of tumors may shed light on future research and clinical trial endeavors, it is very possible that mutations will be identified for which no therapeutic intervention is currently available.

Tumor samples are heterogeneous, which may result in only a fraction of tumor cells harboring a specific mutation, and also may contain surrounding normal tissue resulting in decreased amount of mutated DNA in tumor samples (admixture). Thus, assay sensitivity is important so that mutations can be detected even when they represent a small portion of the DNA extracted from the tumor sample. Advances in several technologies have allowed for the detection of mutations in samples with as little as 5 % mutant DNA in the total DNA sample. Different sources of tumor samples are available for testing including fresh frozen tumor samples and formalin fixed paraffin-embedded (FFPE) tumor samples. FFPE tumor samples, which are commonly used for clinical mutation detection, can have DNA which is degraded and fragmented [63]. A specimen of large enough size for DNA extraction also needs to be available, which can be particularly an issue for primary melanomas. However, in the vast majority of cases, FFPE specimens from metastatic melanomas can be used for mutation identification, even for alleles at relatively low frequency.

When evaluating tumor samples with gene specific mutation testing, consideration must be given to the type of mutation being sought. Some somatic point mutations occur at specific sites in a given gene, known as hotspot mutations (which can be seen in oncogenes), whereas other mutations can occur anywhere within a gene (which can be seen in tumor suppressor genes). Mutational patterns will dictate the type of analysis optimal for mutational detection, as does the number of samples being analyzed, as some methods are better suited for processing of multiple samples, and others more appropriate for limited numbers of samples. We will review techniques used in clinical laboratories focusing on individual gene testing, along with newer sequencing technologies used to identify mutations within melanoma tumor samples.

3.3.1 Direct Sequencing

DNA isolated from tumor samples can undergo direct sequencing to identify point mutations in a specific stretch of DNA. Sanger sequencing, or chain terminating method, can be performed on DNA from tumor samples using a variety of dye-terminators, but is relatively insensitive with a mutation detection rate of ~25 % allele frequency [64, 65]. Pyrosequencing™ (Qiagen, Inc., Alameda, CA) is another direct sequencing technique [66] and can be used to sequence specific short regions of DNA up to 50 bases. Somatic mutations can be identified when clustered within a small region of interest providing for the identification of mutations within a given

DNA locus. Pyrosequencing™ is used by many molecular pathology laboratories to evaluate somatic mutations located within mutation hotspots, and has the advantage of detecting mutant DNA alleles at frequencies as low as 5–15% of the total, depending on the gene being investigated [63, 66]. This method is useful for sequencing *BRAF* mutations in tumor samples, as mutations have been identified in several different nucleotides within and around *BRAF* V600 [32–36].

Allele-specific primers are used to detect single nucleotide changes in tumor samples. For single mutations, Taqman® mutation detection assays (Life Technologies, Carlsbad, CA) is a popular choice. Single nucleotide extension assays also can be used to identify specific point mutations in a given gene, as the technique evaluates changes at an individual nucleotide. Two commonly used platforms for multiplexed single nucleotide extension assays include iPlex™ (Sequenom, Inc, San Diego, CA) [67, 68] and SNaPshot™ (Applied Biosystems, Inc, Foster City, CA) [69]. These techniques make use of primer sets to amplify the DNA and detect the mutated base, along with specific tags, which results in amplification and multiplexing [70]; the tags vary depending upon the platform that is employed. For the iPlex™ platform, nucleotides are detected by matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF) analysis [71]. For the SNaPshot™ platform, nucleotides are fluorescently labeled and nucleotide incorporation into extension products are detected [70].

The iPlex™ [72] and SNaPshot™ [70] technologies can detect mutant DNA with a sensitivity of 5–10% of DNA, thus demonstrating a higher sensitivity for mutation detection than direct sequencing. Additionally, these platforms are effective in genotyping DNA from FFPE tumor samples, allowing for mutation detection in lower quality DNA. Given the use of primer tags, multiple single nucleotide extension assays can be multiplexed, allowing for the interrogation of a number of different mutations within a given reaction. Multiple mutations or single nucleotide polymorphisms within a given region can be assessed in specific tumor samples, albeit not within the same multiplex. These platforms are also commonly used by molecular pathology laboratories and are well suited to assess genes which demonstrate mutational hotspots, such as *NRAS*, *BRAF*, and *GNA11/GNAQ*.

In 2011, the United States Food and Drug Administration (FDA) approved the targeted mutant BRAF inhibitor, vemurafenib, for the treatment of advanced melanoma in patients with the *BRAF* V600E mutation [3]. As this therapy is targeted to a specific somatic mutation identified in patient tumor samples, tumor samples must undergo molecular testing to detect this mutation prior to initiation of therapy. Simultaneously, with the approval of vemurafenib, the FDA also approved a commercially available test for the *BRAF* V600E mutation, the cobas® 4800 BRAF V600 Mutation Test, in order to determine the presence of the *BRAF* mutation and to receive treatment with vemurafenib. The cobas® test probes are specific for and bind to wild-type and mutant V600E *BRAF* sequences and are detected when the probes bind to their correct sequence. However, the cobas® test is limited in its ability to detect *BRAF* non-V600E mutations, with a 66% cross-sensitivity for *BRAF* V600K, and V600E mutations that are a result of a two base pair mutation (package insert). Evidence

suggests that patients with non-V600E *BRAF* mutations, such as V600K, also respond to therapy with targeted BRAF inhibitors, such as vemurafenib and dabrafenib [3, 11, 12]. As such, it is important that somatic mutations in patient tumor samples are accurately detected, thus using the cobas® test alone may not be adequate.

3.3.2 Genomic Aberrations

DNA copy number alterations have been shown to be involved in the pathogenesis of a number of cancers [73] and may have predictive value relating to disease progression or clinical outcome in different tumor types [74–78]. Copy gains or losses are structural variants of segments of DNA, and thought to exert their effects through dysregulation of gene expression. Techniques to determine cancer copy number profiles have improved over the years. Initially copy number determination was performed with probe sequences derived from bacterial artificial chromosomes. Single nucleotide polymorphism (SNP)-based arrays and array-based Comparative Genomic Hybridization (aCGH) are used currently, with massively parallel sequencing the next step for copy number determination, with the development of precise analytical techniques needed. SNP arrays also provide increased identification of loss of heterozygosity and allele copy number, however, these arrays are less suitable for analysis of FFPE tumor samples given the concerns of DNA quality. DNA degradation is common in FFPE tumor samples. As such, shorter DNA fragments are present and limit accurate detection of copy gains and losses in tumor samples. These array techniques rely on the presence of longer DNA fragment sizes to map regions of copy gain and loss, thus, these shorter fragments can result in increased background signal in assay data and could contribute to imprecise DNA copy gains and losses in tumor samples.

Copy number alterations have been analyzed in melanoma cell lines and tumor samples in order to detect genomic aberrations and distinct genomic changes involved in melanoma pathogenesis [79]. A number of genetic regions have been found to be altered in melanoma tumor samples, including gain of chromosomes 5 and 7 and loss of chromosomes 4, 10, 11, 12, 17, and 22 [35,80]. Amplifications of *BRAF*, *NRAS*, *MITF*, *CCND1*, *MDM2*, and *NOTCH2*, and homozygous deletions of *CDKN2A* and *PTEN* have been identified as driver aberrations [35, 81–83]. Moreover, specific patterns of chromosomal gains and losses have been associated with *BRAF* and *NRAS* mutation status [35, 80], suggesting that additional genetic alterations or aberrations cooperate in the pathogenesis of these melanomas.

Other techniques evaluating genomic aberrations have been used to provide supplemental information which, in different tumor types, can be used in risk stratification and prognostic implications in clinical settings. Larger genomic alterations, deletions and rearrangements (over 100,000 base pairs) can be detected using Fluorescence in Situ Hybridization (FISH). FISH is routinely used in hematologic malignancies to delineate cytogenetic characteristics with direct impact on disease stratification and treatment decisions. FISH is gaining popularity to evaluate solid malignancies as well [84, 85]. FISH is being used to

detect *ALK* rearrangement in lung cancers, the presence of which provides the rationale for treatment with the ALK inhibitor crizotinib [86]. FISH based assays are emerging as tools to assist in the diagnosis of histologically indeterminate melanoma. Using three specific probes for *RREB1*, *MYB*, and *CCND1* genes and a centromere specific control probe, Senetta et al. [87]. assessed their use in distinguishing between benign nevi and melanoma. Although specific probe patterns were established in benign nevi vs. melanoma in the validation samples, results were ambiguous in the indeterminate samples in their sample set. Hossain et al. [88] evaluated the use chromosome specific probes to categorize benign lesions vs. melanoma. Results from these studies established chromosomal abnormalities in 94 % melanoma samples, 6 % compound nevi, and 0 % normal skin. Moreover, the most frequent abnormality was gain of chromosomal 11, along with observed gains in chromosomes 6, 7, and 20 [88]. Clinicians can use results from FISH analysis, due to these characteristic genetic events, to guide clinical decisions in the setting of indeterminate histology.

Multiplex probe ligation amplification (MLPA, MRC-Holland, Amsterdam, Netherlands) is used to perform targeted analysis in tumor samples in order to evaluate specific, localized amplifications and deletions [89, 90]. Probes are annealed adjacent to the genomic region of interest, ligated together, and amplified. Quantification and determination of copy number is determined by normalization to controls. MLPA provides copy number profiles for specific genes of interest, requiring less tumor DNA as starting material relative to aCGH. In addition, MLPA can be multiplexed to evaluate a number of genes within the same reaction. MLPA had been successfully used to identify genetic rearrangements in genes associated with inherited syndromes, contiguous gene deletion syndromes, and somatic copy number alterations [91–94]. Moreover, evaluation of specific chromosomal loci, including chr 9p21 (*CDKN2A*), for genetic changes by MLPA has been used to evaluate genetic heterogeneity of uveal melanomas [95] and to distinguish between Sptiz nevi and atypical spitzoid melanocytic tumors [96], as it can be difficult to distinguish these two lesion based on histology alone.

Identification of genetic alterations in melanoma tumor samples and cell lines provides investigators with pertinent information regarding genetic alterations which may contribute to melanoma pathogenesis, which also has the potential to lead to development of novel targeted therapeutics. Additionally, detection of genetic events known to be associated with melanoma can help to guide clinical decisions and treatment plans in the setting of indeterminate lesions.

3.3.3 Massively Parallel Sequencing

The development of massively parallel sequencing (MPS), also referred to as next-generation sequencing, has revolutionized the way in which DNA from tumor samples is analyzed. MPS allows for the analysis of whole genomes, exomes, or targeted regions (i.e. select genes) in individual tumor samples providing simultaneous information regarding mutational analysis of a wide range of genes

and identified mutations, genetic alterations (including deletion and insertions), and copy number gains and losses. A number of different platforms are available to perform massively parallel sequencing such as the HiSeq™2000, HiSeq™2500 and miSeq (Illumina, San Diego, CA) and IonTorrent™ and IonProton™ (Life Technologies, Grand Island, NY), which are reviewed in detail by Ross and Cronin [97]. To perform MPS, DNA libraries are prepared from individual samples. In brief, genomic DNA is sheared to 150–200 bp fragments, blunt ended, and ligated with tagged adaptors and indexes (bar codes), which allow for sample identification. Optimal fragment sizes of DNA within these libraries depend on the length of the sequence reads. DNA libraries are combined with capture baits for targeted sequencing and whole exome sequencing (WES) or remain uncaptured for whole genome sequencing (WGS). The DNA then is sequenced, undergoing amplification and repetitive cycles of sequencing and detection. With improving technology, an increasing number of samples can be multiplexed while retaining mutation detection capability. The ideal read depth, which is the number of sequence reads of a particular nucleotide, varies depending upon whether whole genome, whole exome, or targeted MPS is being done [98].

Somatic mutations in tumor DNA can be challenging to identify given possible admixture of surrounding normal cells and tumor heterogeneity. Identification of low frequency mutations is crucial in the characterization of all tumor samples, including melanoma tumor samples, as it has implications for treatment options, including targeted therapies. Initial platforms for MPS were higher in cost per sample compared to traditional sequencing techniques, which was prohibitive for running large number of samples. However, over time, as technology has advanced, the cost per sample has decreased making it more attractive to use these methodologies to analyze multiple tumor DNA samples. Initially, the source DNA was restricted to fresh frozen tumor samples; however, several studies have demonstrated that adequate results can be achieved using FFPE tumor samples [98–101]. Despite these advances, DNA quality remains a crucial determinant of MPS success.

In addition to whole genome and whole exome analysis, massively parallel sequencing with targeted capture is also used to evaluate tumor samples focusing on specific genes of interest. A number of targeted capture platforms are commercially available to test for common cancer somatic mutations, including such examples as TruSeq Amplicon Cancer Panel (Illumina, San Diego, CA) and Somatic Mutation Analysis (SOMA) panel (Ambry Genetics, Aliso Viejo, CA). These targeted captures provide the advantage of deep sequencing of select, known genes. Of note, whole exome captures generally only select for ~85 % of the complete exome [102–105], so if there is poor coverage over your gene of choice, it will provide limited information.

An immense amount of data is generated from massively parallel sequencing and analysis remains a challenge. The softwares available for data analysis are constantly evolving. Moreover, methodologies used for analysis also depend upon whether germline or somatic genomes are being sequenced. Mutations are first identified and then annotated in order to best assess their potential function. Briefly, sequence data is aligned to the human genome most commonly with the Burrows-Wheeler Aligner (BWA) [106, 107]. Variants are found using programs which detect single

nucleotide variants (SNVs), as compared to the reference sequence, as well as insertions and deletions (indels), though programs such as the Genome Analysis Toolkit (GATK) [108] and Pindel [109]. The analysis of genomic rearrangements and copy number alterations for targeted massively parallel sequencing lags behind that of SNVs and small indels, but are evaluated using programs specific to these types of genetic aberrations, such as VarScan2 [110, 111]. Annotation with programs, such as ANNOVAR, provides information regarding the potential function of identified genetic variants [112]. ANNOVAR calls variants as frameshift indel, non-frameshift indel, stopgain, stoploss, synonymous, non-synonymous and splicing (intronic and exonic). ANNOVAR automatically identifies variants previously reported in pubic databases, including EVS6500, 1000 Genome (1000G), dbSNP (Flagged/Nonflagged) and COSMIC [113]. ANNOVAR also annotates SNVs using SIFT, Polyphen2, MutationTaster and PhyloP to make predictions about function [113–118]. Mutation information obtained using ANNOVAR can be used to filter variants based on specific score cutoffs for the different software programs. The pipeline for mutation identification and annotation will differ depending on input DNA, that is, germline DNA versus tumor DNA. Software has been developed specifically for the analysis of somatic genomes and mutations including BreakPointer, Indelocator, and MuTect (www.broadinstitute.org/cancer/cga/). MuTect is a sequence analysis program that uses the sequence of both normal and tumor to identify somatic point mutations [119]. Despite technology and software advances, the pathogenicity of a number of the detected genetic variants, both germline and somatic, will have unknown significance. These variants of unknown significance pose challenges for clinicians as these variants are not clinically actionable and it is not clear whether these variants are involved in tumor pathogenesis.

3.3.4 Results of Whole Exome Sequencing/Whole Genome Sequencing in Melanoma

Melanoma tumor samples have been evaluated using whole genome and whole exome sequencing. An initial whole genome sequencing study identified 33, 345 somatic mutations, 680 deletions, 303 insertions, and 51 rearrangements in a single melanoma cell line derived from metastatic melanoma when compared to matched germline DNA [22]. Whole genome sequencing detected known somatic mutations involved in melanoma pathogenesis including *BRAF* V600E, *PTEN* deletion, and a two base pair deletion within *CDKN2A*. Potential driver mutations were also identified in transcription factors, including *SPDEF*; genes thought to be involved in metastasis, including *MMP28*; and proposed tumor suppressor genes, including *UVRAG* [22]. Wei et al. [62] described the identification of recurrent mutations within *TRRAP* in 4 % (6/167) metastatic tumor samples with functional studies of TRRAP suggesting it functions as an oncogene. Additional somatic mutations were identified in *GRIN2A*, which was mutated in 33 % (17/52) of melanoma samples [62]. Somatic mutations in *GRIN2A* also were identified by whole genome sequencing of a melanoma tumor sample/normal DNA pair [41], but other

somatic mutations suggested by the study have not been validated in subsequent massively parallel sequencing analyses. In addition to these novel genes, whole exome sequencing detected known somatic mutations including *BRAF* mutations in 50 % of samples, consistent with previously published observations. However, no *NRAS* mutations were identified in these melanoma samples, in contrast to the multiple publications showing a frequency of mutations in 15–20 % of melanomas [42–44]. Additional whole exome studies also identified gain of function mutations in genes found in pathways known to be involved in melanoma pathogenesis, such as *MAP2K1* and *MAP2K2* [61]. Evaluation of an expanded panel of melanoma samples identified mutations within these two genes in 8 % (10/127) samples. Additional previously unidentified somatic mutations were observed in *FAT4*, *DSC1*, and *LRP1B*, but their role in melanoma pathogenesis in unknown [61]. However, it is important to note that subsequent studies have not validated the *FAT4*, *DSC1*, and *LRP1B* mutations in independent analysis of multiple melanoma tumor samples.

Two recent studies using whole exome sequencing of a large number of samples generated a more comprehensive understanding of the genetic landscape of somatic mutations in melanoma [20, 21]. Hodis et al. [20] reported on the results from whole exome sequencing analysis of 121 melanoma/normal DNA pairs. In this study, the authors used a statistical approach comparing the frequency of mutations in intron sequences adjacent to exon sequences to identify novel driver mutations in melanoma. Six genes demonstrated recurrent somatic mutations novel in melanoma. Activating mutations were described in *PPP6C*, catalytic subunit of PP6 protein phosphatase and potential tumor suppressor [20, 57]; *RAC1*, member of Rho family of GTPases [58, 120]; *SNX31*, protein sorting nexin 31, a possible Ras effector protein [121]; *TACC1*, transforming acidic coiled-coil protein 1 which potentially stimulates Ras and PI3K pathways [122]; and *STK19*, a predicted kinase, generally clustered around hotspot regions. Loss of function mutations were observed in *ARID2*, component of the SWI/SNF chromatin remodeling complex [123]. In addition to these novel somatic mutations, mutations were identified in known genes, such as *BRAF*, *NRAS*, *PTEN*, *TP53*, *CDKN2A*, and *MAP2K1* [20]. All mutations were identified in over 4 % of melanoma samples. Whole exome sequencing also was done by Halaban and colleagues at Yale University, in 147 melanoma samples, either primary melanomas or metastases. They also identified novel somatic mutations at higher rates in *NF1*, *PPP6C*, *RAC1*, and *ARID2*. In addition, Krauthammer et al. [21] also identified additional novel somatic mutations in melanoma samples in *PTPRK*, protein tyrosine phosphatase, receptor type K; *PTPRD*, protein tyrosine phosphatase receptor type D; and *DYNC1I1*, dynein, cytoplasmic 1, intermediate chain 1, which may be involved in chromosomal segregation [124]. Some of these newly identified driver mutations are associated with *BRAF*/*NRAS* mutations, but others have been specifically identified in melanomas lacking these mutations.

In addition to the individual examination of melanoma tumor samples by investigators, mutational data on a large number of melanoma tumor samples, from tumor metastases, are publically available through the Cancer Genome Atlas (TCGA) (http.//www.cbioportal.org/public-portal/; http.//gdac.broadinstitute.org/; and https.//tcga-data.nci.nih.gov/tcga/), and are continuing to be collected.

Currently, the skin cutaneous melanoma (SKCM) TCGA dataset reports the results of available mutational analysis of 337 biospecimens from metastatic melanoma tumor samples including somatic mutations, copy number, methylation clustering, protein activities, and gene expression analyses. Within the skin cutaneous melanoma (metastatic) dataset, specific copy number changes can be identified as well as somatic mutations and the types are available, highlighting those that are UV-induced. Co-mutation plots provide information regarding simultaneous mutations in different samples, allowing for grouping of melanoma tumor samples. Moreover, the available dataset can be queried to investigate specific genes of interest, either singly or for pathway analysis, and results provide information regarding copy number alterations and somatic mutations. Detailed information regarding specific types of somatic mutations are available, and provide insight into types of mutations commonly identified within a particular gene. mRNA and protein expression data is provided along with methylation profiling. The TCGA endeavor undertaken by a number of collaborators provides a large dataset of metastatic melanoma tumor samples and subsequent analysis in one central repository, making it available to all investigators to use this information for research and clinical purposes.

3.4 Conclusion

Treatment options for advanced stage cancers, especially metastatic melanoma, have advanced with the advent of effective targeted therapy, necessitating the use of molecular diagnostics for clinical decision making. Standard mutation detection techniques may still remain the optimal choice in somatic testing, in particular when evaluating an individual mutation or gene (e.g. *BRAF* or *KIT*), as these tests have been validated and are cost effective with relative quick turnaround time. The information garnered with next generation sequencing provides clinicians with a large amount of information, extending past the presence or absence of a particular mutation (e.g. V600E in *BRAF*). However, many of the mutations identified beyond those available with targeted mutation screening may not be clinically actionable. As MPS strategies are used to profile tumors, they have become available in Clinical Laboratory Improvement Amendments (CLIA) certified laboratories, so the data generated will be usable in the clinical setting. As concurrent mutations are evaluated and detected, it is possible to ascertain the presence of mutations which would predict for resistance to a specific targeted therapy (e.g. additional pathway mutations, such as in *MAP2K1*). Thus, MPS provides valuable information with potential implications regarding treatment options for patients at the time of initial evaluation. More importantly, the use of MPS at the time of disease relapse, progression, or development of resistance to therapy to classify the genetic landscape within an individual's tumor will provide information regarding potential therapeutic options. From a research perspective, the identification of somatic mutations and mechanisms of resistance will further guide research endeavors and clinical trial development as clinicians seek out improved therapeutic options.

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Chapter 4
Clinical Utility of BRAF-Targeted Therapy in Melanoma

Jeffrey A. Sosman and Douglas B. Johnson

Abstract The identification of BRAF^{V600} mutations in melanoma rapidly translated into a search for strategies to exploit this recurrent genetic alteration. The selective BRAF inhibitors, vemurafenib and dabrafenib have demonstrated impressive antitumor activity with objective response rates of approximately 50 % and improved progression-free and/or overall survival compared to cytotoxic chemotherapy. The MEK inhibitor trametinib also subsequently demonstrated improved survival compared to chemotherapy. Acquired resistance, however, has limited the long-term antitumor efficacy of these therapies. Combined BRAF and MEK inhibition represents one strategy to delay the onset of resistance and potentially extend survival. Additional BRAF and MEK inhibitors and combinations are being developed with a goal of improving outcomes further. In this chapter, we review the development of approved BRAF and MEK inhibitors, the experience with combination therapy, and special clinical situations for BRAF-targeted therapy.

Keywords BRAF inhibitor · MEK inhibitor · Melanoma · BRAF-mutant · Vemurafenib · Dabrafenib · Trametinib

4.1 Introduction

Constitutive activation of the mitogen activated protein-kinase (MAPK) pathway drives growth and progression in most melanomas of which 40–50 % harbor BRAF^{V600} mutations. The discovery of small molecule inhibitors which suppress MAPK signaling has represented a major step forward in melanoma therapeutics. Pathway inhibition has now been achieved by targeting different levels of the pathway and has efficacy in advanced melanoma through direct targeting of mutant BRAF and blockade of its downstream signaling partner, MEK. Two selective

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inhibitors of BRAF (vemurafenib, dabrafenib) and one MEK inhibitor (trametinib) are now approved for clinical use and several other agents are advancing in the developmental pipeline. These targeted therapies induce rapid tumor regressions in many patients and improve clinical outcomes in comparison to cytotoxic chemotherapy based on progression-free and overall survival. Acquired resistance remains the significant problem, although progression can be forestalled by combination therapy. In this chapter, we will review the clinical utility of these small molecule inhibitors in BRAF^{V600} mutant melanoma, focusing on approved agents but also briefly discussing an early BRAF inhibitor and newer, experimental agents.

4.2 BRAF Inhibitors

4.2.1 Sorafenib

The identification of recurrent mutations in the 600th codon of BRAF in nearly half of melanomas in 2002 by Davies et al. represented a major therapeutic opportunity [1]. Sorafenib (Nexavar, Bayer), a putative BRAF inhibitor, was the first agent to show pre-clinical activity in BRAF mutant melanomas, partially inhibiting ERK signaling and inducing cell death [2]. The clinical experience with this agent, however, was disappointing. In an early study, 37 unselected patients with advanced melanoma received sorafenib, with only one patient experiencing a partial response and 19 % achieving temporary stable disease [3]. Moreover, there was no correlation between disease stability and BRAF mutation status. Subsequent trials combined sorafenib with cytotoxic chemotherapy but demonstrated no advantage over chemotherapy alone and no genotype-specific effect for those patients with BRAF mutant melanoma was observed [4, 5]. The modest activity of sorafenib is now generally attributed to its anti-angiogenic properties rather than to specific inhibition of mutant BRAF. Additional clinical development of sorafenib in melanoma is not ongoing since more effective BRAF inhibitors have now been approved.

4.2.2 Vemurafenib

4.2.2.1 Early Phase Studies

Vemurafenib (Zelboraf®, PLX4032, RG7204, Roche/Genentech, Basel) was the first selective inhibitor of mutant BRAF developed. Pre-clinical studies demonstrated exquisite sensitivity of most cell lines harboring BRAF^{V600E} mutations [6], leading to further clinical development. In the phase I trial, patients were initially treated with a crystalline formulation of vemurafenib, which was found to have minimal efficacy and poor bioavailability. The drug was reformulated to a microprecipitated bulk-powder formulation and dose escalation was performed, with a recommended phase two dose (RP2D) of 960 mg twice daily. An expansion cohort

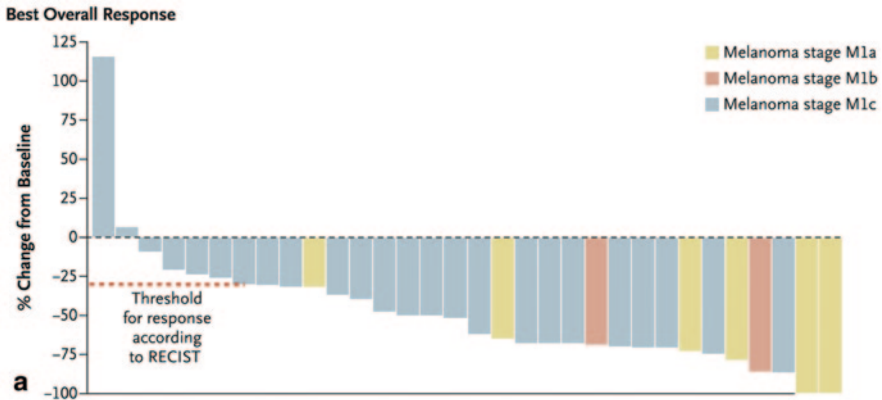

Fig. 4.1 Best overall response for 32 patients treated at the recommended phase II dose of vemurafenib in the phase I study (960 mg twice daily), measured as the change from baseline in the sum of the largest diameter of each target lesion

of 32 patients with BRAF mutant melanoma were treated at this dose, of which 24 (80 %) experienced a partial response (investigator assessed, including both confirmed and unconfirmed), often with rapid and dramatic regression of disease. This trial demonstrated that almost all patients experience at least some disease regression with vemurafenib with the exception of two patients with primary disease progression (Fig. 4.1) [7]. The median progression-free survival (PFS) was approximately 7 months; nearly all patients eventually developed disease progression. The drug was relatively well tolerated although 41 % of patients required a dose reduction to 720 mg twice daily for chronic toxicity.

4.2.2.2 Phase II/III Studies and Subsequent Experience

The follow-up non-randomized phase II study, BRIM-2, enrolled 132 patients with $BRAF^{V600E}$ mutant melanoma ($BRAF^{V600K}$ mutations were excluded). The overall response rate was 53 % (6 % with complete responses) with a median PFS of 6.7 months and a median overall survival (OS) of 15.9 months [8]. These results were observed despite unfavorable baseline patient characteristics: 61 % had AJCC stage M1c disease and nearly half had elevated lactate dehydrogenase (LDH). The most common toxicities observed were cutaneous, including rash (52 %), pruritis (29 %), skin papilloma (29 %), skin cutaneous squamous cell carcinoma (cSCC; 26 %), and palmar-plantar erythrodysesthesia (10 %). Cutaneous SCCs were generally limited with one or two lesions managed with surgical resection although a few patients had multiple and recurrent SCCs eventually limiting therapy. Arthralgia was common but not severe (78 %); elevated liver function tests were also observed (17 %) and managed with dose reduction.

BRIM-3 was a randomized trial comparing vemurafenib with dacarbazine, enrolling 675 patients with a 1:1 randomization between arms. At the first interim analysis (3.8 months median follow up for the vemurafenib arm, performed soon after

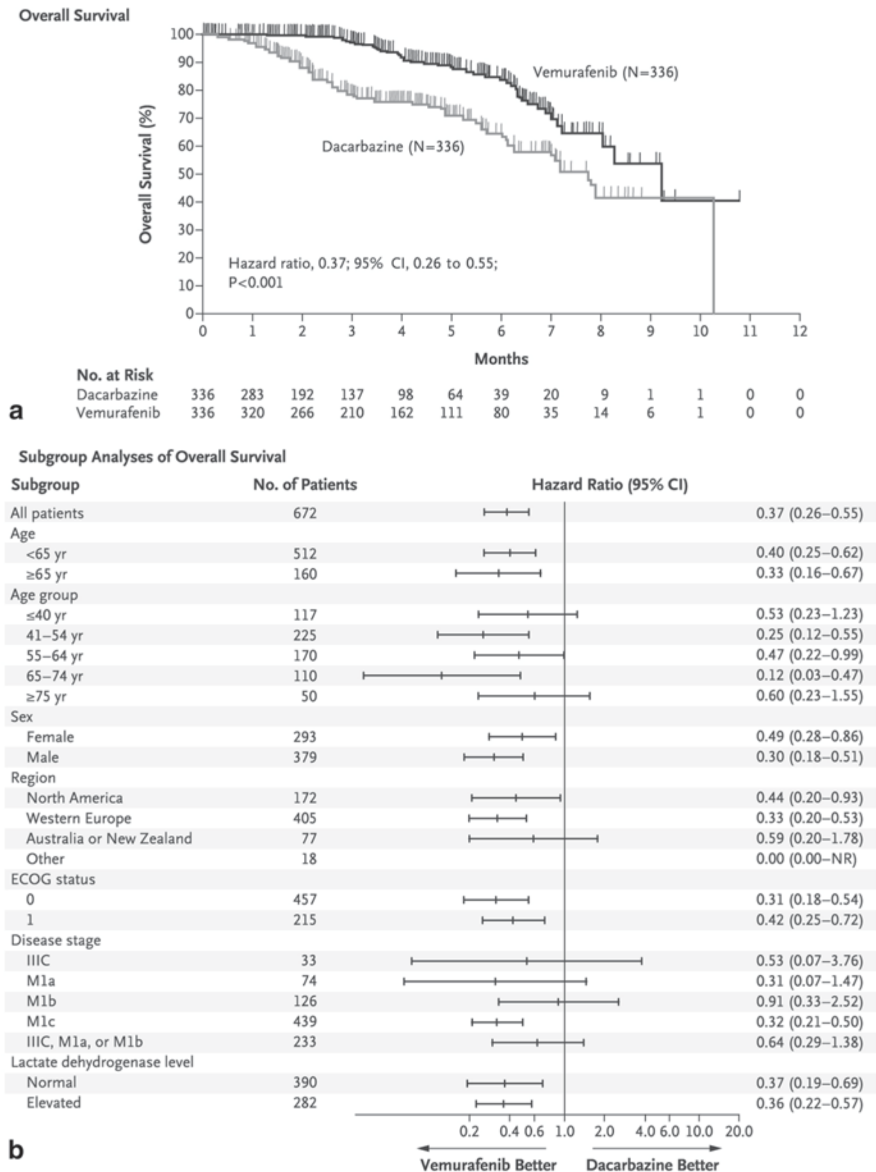

Fig. 4.2 Kaplan-Meier estimates of survival in patients with $BRAF^{V600E}$ mutant melanoma treated with vemurafenib or dacarbazine in the phase III study of vemurafenib

enrollment was completed) the OS and PFS endpoints had been met and patients on dacarbazine were allowed to cross over [9]. Vemurafenib-treated patients had a decreased hazard of death (hazard ratio 0.37, 95 % CI 0.26–0.55, $p<0.01$), and progression (hazard ratio 0.26, 95 % CI, 0.20–0.33; $p<0.001$), and an overall response rate was 48 % (Fig. 4.2). Toxicities were observed in similar incidence to

BRIM-2; photosensitivity was also described in this trial which could be prevented with sunblock in many cases. Notably, 2.4 % of patients also developed a second primary melanoma. Vemurafenib received regulatory approval in the United States for treatment of advanced melanoma in August of 2011 and is now widely used in first-line and previously treated advanced or metastatic melanoma.

Following approval, pre-clinical studies suggested that the intermittent administration of BRAF inhibitors delay the onset of acquired resistance [10]. This has not yet been verified in the clinical setting, therefore this strategy should not be recommended for patients. However, when patients develop intolerable chronic toxicities, we prefer a strategy of intermittent dosing (i.e. 2 weeks on and 1 week off) over dose reduction below 720 mg twice daily. This approach has not yet been evaluated in a clinical trial.

4.2.3 Dabrafenib

4.2.3.1 Early Phase Trials

Dabrafenib (Tafinlar®, GSK2118436, GlaxoSmithKline, London) is a selective BRAF inhibitor developed after vemurafenib. A phase I/II study of dabrafenib was conducted in Australia and the United States between May 2009 and March of 2011 [11]. The phase I component initially permitted entry regardless of BRAF mutation status but subsequently restricted enrollment to $BRAF^{V600E}$ or $BRAF^{V600K}$ mutant melanoma after several patients lacking BRAF mutations failed to respond. In contrast to the vemurafenib trials, patients with $BRAF^{V600K}$ mutant melanoma were allowed to enroll, including nine patients in this study. While dose-limiting toxicity was not found, the phase II recommended dose (RP2D) was determined to be 150 mg twice daily; 46 patients received this dose. Disease characteristics were unfavorable including 91 % with AJCC stage M1c melanoma and 22 % with brain metastases (see "Special Clinical Situations" below). Of the 36 patients with only extracranial metastases treated with the RP2D, 69 % experienced partial or complete responses (50 % confirmed) with median PFS of 5.5 months. Elevated LDH and worse baseline performance status predicted for more rapid disease progression. Cutaneous squamous cell carcinomas developed in 11 % of patients who received at least 50 mg twice daily. Additional cutaneous toxicities were similar to vemurafenib, although photosensitivity was not observed. The most prevalent distinct non-cutaneous toxicity was pyrexia (20 %, grade 3–4 in 4 %).

4.2.3.2 Phase III Clinical Trial

This encouraging clinical activity led to the initiation of a multicenter, phase III trial of dabrafenib compared to cytotoxic chemotherapy [12]. Two-hundred fifty patients were randomized 3:1 to dabrafenib 150 mg twice daily or dacarbazine; enrollment in this trial was limited to $BRAF^{V600E}$ mutant melanoma and to patients

without brain metastases. The primary endpoint was PFS; crossover was allowed from dacarbazine to dabrafenib at disease progression. Baseline characteristics included 67 % with ECOG performance status of 0, 65 % with AJCC stage IVc disease, 34 % with elevated LDH, and 98 % with any previous therapy. Median PFS was 5.1 months with dabrafenib compared to 2.7 months with dacarbazine (hazard ratio for progression 0.30, 95 % CI 0.18–0.51; $p<0.0001$). An independent review determined median PFS durations of 6.7 months and 2.9 months for dabrafenib and dacarbazine, respectively. This trial was not powered for overall survival, although a trend to improved OS with dabrafenib was observed (hazard ratio 0.61, 95 % CI 0.25–1.48). Confirmed objective responses were seen in 53 % of patients on dabrafenib (3 % CR). As with vemurafenib, the vast majority of patients had some degree of tumor shrinkage with primary disease progression occurring only rarely. Adverse events were similar and included cSCCs/keratocanthomas (6 %) and 11 % with pyrexia (3 % with ≥ grade 3). Arthralgias, asthenia, headaches, and fatigue also occurred in > 5 % of patients but were rarely severe. Notably, two patients developed second primary melanomas and four developed basal cell carcinomas. Dabrafenib received regulatory approval in the United States in May of 2013.

4.2.4 Encorafenib (LGX818)

Encorafenib (LGX818; Novartis) is a selective BRAF inhibitor currently undergoing phase II and III testing. This agent has a longer dissociation time compared to available BRAF inhibitors which may confer additional activity. In a phase I trial, 16 of 24 (67 %) BRAF inhibitor naïve patients experienced partial responses [13]. Among patients pre-treated with other BRAF inhibitors, the response rate was < 10 %. Toxicities were similar to vemurafenib and dabrafenib although palmar-plantar erythrodysesthesia was observed more frequently and only two patients developed cSCCs. Ongoing development is focused on monotherapy across malignancies harboring BRAF mutations and on combination therapy with other agents in advanced melanoma.

4.2.5 Secondary Malignancies and Rare Toxicities

The incidence of cSCCs is strongly increased with both vemurafenib and dabrafenib (6–26 %). This led to concerns that selective BRAF inhibitors may induce secondary cancers. After further study, it appears that these agents promote progression of existing cancers (or pre-malignant conditions) by paradoxically promoting MAPK pathway activity. This effect appears to primarily occur in neoplasia with RAS mutations. For example, 60 % of secondary cSCCs harbor activating mutations in RAS [14]. Additional primary melanomas appear to occur more frequently in patients previously diagnosed with melanoma although it has not been determined whether BRAF inhibitors contribute to this increased incidence. Patients receiving BRAF

inhibitors should be evaluated by a dermatologist if suspicious lesions occur during therapy.

Diagnoses of new non-cutaneous malignancies have been uncommon during BRAF inhibitor therapy. A case of chronic myelomonocytic leukemia (CMML) was diagnosed by rapidly rising white blood cell (WBC) count in a patient on vemurafenib [15]. Periodic drug cessation and rechallenge induced clear regression and progression of the CMML as measured by fluctuating WBC counts. The development of colon adenomas and gastric polyps have also been identified during BRAF inhibitor therapy [16]. The incidence of visceral, RAS-mutant carcinomas (e.g. lung, pancreas, colon etc.) appears to be rare, although these remain a potential concern.

Other severe toxicities are relatively rare with these agents. Bilateral peripheral facial nerve palsy has been observed with vemurafenib (in a patient who achieved a complete remission) [17]. Also in two patients who had previously received agents targeting the programmed cell death-1 (PD-1) receptor, a syndrome of rash, hepatic and renal injury, and hypotension occurred when they received vemurafenib [18]. Fevers were the most common severe toxicity with dabrafenib, and were occasionally associated with hypotension requiring temporary drug cessation and intravenous hydration. Hypoglycemia was also observed.

4.2.6 Summary

Although clinical activity cannot be directly compared across trials, vemurafenib and dabrafenib provide fairly equivalent benefit for patients with $BRAF^{V600E}$ mutant melanoma [12, 19]. Median PFS and response rates were comparable. Side effect profiles were also similar with phototoxicity and elevated liver function tests occurring more frequently with vemurafenib and pyrexia more commonly observed with dabrafenib. A suggestion of fewer cutaneous SCCs was also considered with dabrafenib in the phase III trial but this was called into question in a subsequent trial [20]. See Sect 5.5 for the discussion of BRAF inhibitor therapy in brain metastases and in alternative BRAF mutations (non-V600E).

4.3 MEK Inhibitors

4.3.1 Selumetinib

Selumetinib (AZD-6244, AstraZeneca) is a selective MEK1/2 inhibitor which demonstrated pre-clinical efficacy against BRAF mutant melanoma cell lines. In an unselected melanoma population, selumetinib was compared with temozolomide and did not demonstrate any improvement in PFS [21]. In a randomized phase II trial, selumetinib combined with dacarbazine was compared to dacarbazine alone in BRAF mutant mel-

anoma. The combination arm demonstrated improved PFS (5.6 vs. 3 months) but no change in OS [22]. Although this agent is undergoing further development in other malignancies (lung adenocarcinoma, thyroid carcinoma, leukemias), it is not likely selumetinib will be used in BRAF mutant melanoma in the future.

4.3.2 Trametinib

4.3.2.1 Early Phase Trials

Trametinib (Mekinist, GSK1120212, GlaxoSmithKline, London) is a newer generation selective MEK1/2 inhibitor which has been widely tested in melanoma. A phase I trial was conducted which included 30 patient with BRAF mutant melanoma not previously treated with a BRAF inhibitor and 39 BRAF wild type patients [23]. The response rate was 40 % in the untreated BRAF mutant group with a median PFS of 5.7 months. Notably, 10 % of patients in the BRAF wild type group also demonstrated an objective response. Within this BRAF wild type cohort, a patient later found to have $BRAF^{L597V}$ mutant melanoma also experienced a response. Side effects were relatively minor and commonly included acneiform rash (38 %), diarrhea (35 %), and peripheral edema (31 %). No cSCCs were identified, and no episodes of retinal vein occlusion (RVO) occurred (complications of early generation MEK inhibitors) in patients receiving the RP2D of 2 mg daily.

4.3.2.2 Phase III Trial

A phase III trial (METRIC) was then conducted 322 patients with advanced $BRAF^{V600E/K}$ mutant melanoma, randomized in a 2:1 fashion to trametinib or investigator's choice of cytotoxic chemotherapy (dacarbazine or carboplatin/paclitaxel). Improved overall survival was demonstrated (hazard ratio for death 0.54, $p=0.01$), despite 47 % of patients on the chemotherapy arm crossing over and receiving trametinib. Other key clinical outcomes favored trametinib including median PFS (4.8 months vs. 1.5 months, $p<0.001$), and objective response rate (22 vs. 8 %, $p=0.01$). Although only 22 % of patients met criteria for RECIST partial responses, >70 % experienced at least some disease regression. Toxicity profile was similar to the phase I trial although one case of reversible chorioretinopathy occurred. Cardiotoxicity was seen in 7 % who developed decreased ejection fraction and two patients who experienced grade 3 cardiac events requiring drug cessation. Based on the results of this trial, trametinib received FDA approval in May 2013.

4.3.2.3 Trametinib in BRAF Inhibitor-Resistance

Since many of the mechanisms of acquired resistance to BRAF inhibitors could be hypothesized to confer sensitivity to MEK inhibition, a phase II trial was conducted to assess the efficacy of trametinib in this setting. A total of 40 patients received

trametinib following progression with a BRAF inhibitor (either vemurafenib or dabrafenib). Of the patients truly refractory to BRAF inhibitors, no patients had objective responses with 11 patients (28 %) experiencing temporary stable disease. Two patients who had developed BRAF inhibitor toxicity but had not progressed on BRAF inhibitor therapy before receiving trametinib did experience a partial response. Median PFS was 1.8 months in this cohort. A sequential strategy of BRAF inhibitors followed by MEK inhibitors is thus not of clinical benefit.

4.3.3 Binimetinib (MEK162)

Binimetinib (MEK162; Novartis) is an experimental small molecule inhibitor of MEK1/2 with a recently completed phase II study. The response rate in the BRAF V600 mutant group was 23 % with a median PFS of 3.6 months. In contrast to other MEK inhibitors, binimetinib induced responses in NRAS mutant melanoma (response rate 20 %). Clinical development for this agent as monotherapy has largely focused on the 15–20 % of melanomas harboring NRAS mutations. However, trials in combination with encorafenib are also ongoing for patients with BRAF mutant melanoma.

4.3.4 Conclusions

Trametinib, the only currently approved MEK inhibitor, is an active therapy and is superior to cytotoxic chemotherapy. The single agent activity appears to be somewhat inferior to vemurafenib or dabrafenib with a lower response rate and lower median PFS (although no direct comparison has been performed). Trametinib has minimal benefit following progression on BRAF inhibitors and is not used widely as monotherapy currently except in patients with contraindications to vemurafenib and dabrafenib. Its role in combination therapy, however, may be much more significant. Binimetinib and other experimental MEK inhibitors may also have clinical utility in the future.

4.4 Combination Therapy

4.4.1 Rationale and Efficacy

The inevitable onset of acquired resistance and disease progression in patients treated with BRAF or MEK inhibitor monotherapy led to significant interest in combining these agents. Pre-clinical rationale for combination therapy was strong, as many mechanisms of acquired resistance involve reactivation of the MAPK pathway, including acquired NRAS mutations, [24] MEK1 mutations [25], COT overexpression [26], BRAF amplification [27], alternate splicing of BRAF [28] and

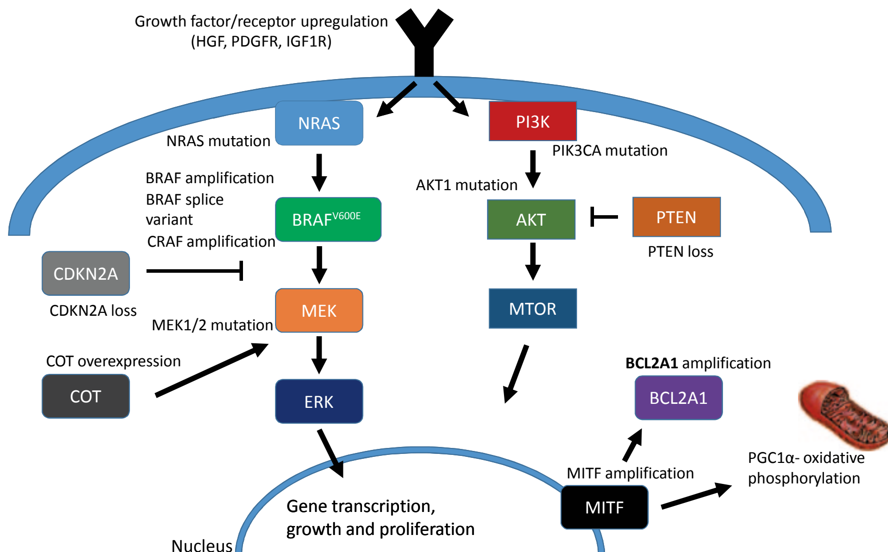

Fig. 4.3 Mechanisms of acquired resistance to BRAF inhibitors. Resistance arises reactivation of the MAPK pathway, growth factor upregulation, dysregulation of the PI3K-AKT pathway, and decreased apoptosis

loss of CDKN2A (through indirect dysregulation of MAPK signaling) [29]. Growth factor upregulation, alterations in the PI3K-AKT-MTOR pathway and decreased apoptosis, also appear to play a role in acquired resistance which may not respond to combined BRAF/MEK inhibition [29–34] (Fig. 4.3).

A phase I/II trial evaluating dabrafenib and trametinib was conducted with rapid dose escalation to a recommended phase II dosing [20]. A randomized comparison of combination therapy (dabrafenib 150 mg twice daily and trametinib 1 mg or 2 mg daily) to dabrafenib monotherapy was then performed in 162 patients. Patients on the 150/2 mg dosing arm had improved median PFS of 9.4 months compared to 5.6 months with dabrafenib alone, with 41 % of patients remaining progression-free at 12 months (hazard ratio for death or progression of 0.39; $p<0.001$). Improvements were demonstrated regardless of BRAF mutation (V600E or K) and across metastatic stages (M1a/b and M1c). Objective responses occurred more frequently in the combination therapy arm (76 vs. 54 %, 9 % vs. 4 % CR rate). For responding patients, the median duration of response was also superior (10.5 months vs. 5.6 months). Despite the clear improvement in PFS and response rate, an improvement in OS has not yet been demonstrated. An OS benefit may be observed with continued follow-up.

4.4.2 Toxicity

The toxicity profile was significantly altered by combining BRAF and MEK inhibition compared with monotherapy. The incidence of cutaneous squamous cell

carcinoma was decreased (19 vs. 7 %) as was the classic BRAF inhibitor-associated dermatitis (36 vs. 27 %). The addition of a MEK inhibitor appears to attenuate the risk of secondary malignancies by preventing paradoxical MAPK activation. The acneiform dermatitis induced by MEK inhibitors also appeared to occur less frequently than in the METRIC trial [35]. Other class toxicities characteristic of trametinib were observed in the combination group including decreased ejection fraction (9 %) and ocular events (one patient with retinopathy). Also significantly, 25 % of patients on the combination 150/2 mg arm developed severe pyrexia (defined as associated with severe chills, hypotension, or requiring hospitalization). Anti-pyretics and temporary interruption of therapy are generally sufficient in mild cases although severe cases may necessitate intravenous fluid repletion and oral steroids. The onset of pyrexia is not predictable and may occur even after prolonged therapy. Neutropenia (11 %), fatigue, nausea, and diarrhea also occurred more commonly in the combination arms.

4.4.3 Crossover

Patients initially assigned to dabrafenib monotherapy were eligible for crossover to combination therapy. In this BRAF inhibitor resistant population, the combination was much less effective (median PFS 3.6 months, objective response rate 9 %) compared to BRAF inhibitor naïve patients [36]. A long duration of PFS on dabrafenib monotherapy appeared to predict a longer benefit from crossover although this was not universal. Patients who rapidly progressed on dabrafenib generally received minimal or no benefit from the combination. In our opinion, BRAF inhibitor resistant patients could be considered for crossover if they derive a long term benefit from monotherapy (> 12 months).

4.4.4 Current Status of Combination Therapies

The combination of dabrafenib and trametinib has advanced through the developmental pipeline and received regulatory approval in January 2014. We strongly consider this combination for patients as first-line treatment or following progression with an immune-based regimen. Clinical trials are also ongoing for vemurafenib plus the MEK inhibitor cobimetinib (GDC-0973, Roche/Genentech) and encorafenib plus binimetinib.

A large variety of trials assessing other combinations in the BRAF inhibitor naïve and refractory populations are also ongoing. These include BRAF inhibitors plus agents inhibiting one of the following: the PI3K-AKT pathway, colony-stimulating factor-1 receptor (CSF-1R), cyclin dependant kinase signaling (CDK4/6), heat-shock protein-90 (HSP90), hepatocyte growth factor, fibroblast growth factor receptor (FGFR) and angiogenesis. Table 4.1 is a non-comprehensive list of currently accruing trials combining BRAF inhibitors with other agents [37]. Although each combination has pre-clinical rationale, it is not clear whether one combination

Table 4.1 Currently accruing combination therapy trials including BRAF inhibitors as of January 27, 2014

Investigational agents	Mechanism of action	National clinical trials identification number	Phase of development
Trials only including BRAFi naïve patients			
Vemurafenib and Cobimetinib vs. Vemurafenib alone	BRAFi	NCT01689519	III
	MEKi		
Dabrafenib and Trametinib vs. Vemurafenib alone	BRAFi	NCT01597908	III
	MEKi		
LGX818 and MEK162 or LGX818 alone vs. Vemurafenib alone	BRAFi	NCT01909453	III
	MEKi		
Vemurafenib Interleukin-2	BRAFi	NCT01683188	IV
	Immune therapy		
Dabrafenib Trametinib	BRAFi	NCT01726738	II
	MEKi		
Vemurafenib Bevacizumab	BRAFi	NCT01495988	II
	Anti-angiogenic		
Vemurafenib	BRAFi	NCT01603212	I/II
Interleukin-2	Immune therapy		
Interferon-alpha	Immune therapy		
Dabrafenib	BRAFi	NCT02027961	I/II
Trametinib	MEKi		
MEDI4736	Anti-PD-L1		
Vemurafenib Metformin	BRAFi	NCT01638676	I/II
	Anti-diabetic		
Vemurafenib BKM120	BRAFi	NCT01512251	I/II
	PI3K inhibitor		
Vemurafenib PLX3397	BRAFi	NCT01826448	Ib
	CSF1R inhibitor		
Vemurafenib MPDL3280A	BRAFi	NCT01656642	Ib
	Anti-PD-L1		
Vemurafenib Hydroxychrolorquine	BRAFi	NCT01897116	I
	Unknown		
Dabrafenib	BRAFi	NCT01767454	I
Trametinib	MEKi		
Ipilimumab	Anti-CTLA4		
Vemurafenib XL888	BRAFi	NCT01657591	I
	HSP inhibitor		
Dabrafenib	BRAFi	NCT01940809	I
Trametinib	MEKi		
Ipilimumab	Anti-CTLA4		

Table 4.1 (continued)

Investigational agents	Mechanism of action	National clinical trials identification number	Phase of development
Vemurafenib Cabozantinib	BRAFi	NCT01835184	I
	MET inhibitor		
Trials allowing for BRAFi resistant patients			
Dabrafenib Trametinib	BRAFi	NCT01619774	II
	MEKi		
LGX818 and[a] MEK162 or	BRAFi	NCT01820364	II
	MEKi		
LEE011 or	CDK4/6 inhibitor		
BGJ398 or	FGFR inhibitor		
BKM120 or	PI3K inhibitor		
INC280	MET inhibitor		
Vemurafenib P1446A-05	BRAFi	NCT01841463	I/II
	CDK4/6 inhibitor		
Vemurafenib Decitabine	BRAFi	NCT01876641	I/II
	Hypomethylating agent		
LGX818	BRAFi	NCT01777776	I/II
LEE011	CDK4/6 inhibitor		

All trials are evaluating combination therapy with agents listed in "Investigational agents" column
BRAFi BRAF inhibitor, *MEKi* MEK inhibitor, *NCT* National Clinical Trials
[a] Choice of combination therapy is determined by molecular testing at the time of progression

will emerge as clearly superior. Likely, a personalized approach will be needed and will be assessed in a planned trial (LOGIC 2, Novartis).

4.4.5 Summary

The combination of BRAF and MEK inhibitors appears to represent a step forward in therapy for $BRAF^{V600}$ mutant melanoma, leading to improved outcomes via enhanced blockade of the MAPK pathway. However, acquired resistance and disease progression still occurs in less than one year for most patients, suggesting that blockade of additional signaling pathways and alternate treatment strategies may be necessary to achieve more durable clinical benefit. Clinicians should note that the toxicity profile is distinct from BRAF inhibitor monotherapy, with decreased incidence of cSCCs and less theoretical concern of promotion of other RAS mutated malignancies [15]. However, systemic side effects including pyrexia, hypotension, and neutropenia occur more frequently with this regimen and patients should be monitored closely.

4.5 Special Clinical Situations

4.5.1 Targeted Therapy in Brain Metastases

Targeted therapies may play a key role in the multidisciplinary management of patients with brain metastases. Although BRAF inhibitors do not appear to cross an intact blood brain barrier in pre-clinical studies, multiple trials have demonstrated their efficacy in brain metastases [38]. Evidence of activity is limited to vemurafenib and dabrafenib; no clinical trials evaluating MEK inhibitors in brain metastases have been performed.

Dabrafenib has been studied most extensively in this setting. The phase I trial of dabrafenib led by Falchook and colleagues initially suggested activity. Ten patients with untreated brain metastases were included, and eight experienced a decrease in size of their intracranial disease [11]. A phase II trial (BREAK-MB) was then conducted exclusively for patients with BRAF V600E ($n=139$) or V600K ($n=33$) mutant melanoma with brain metastases [39]. Two cohorts were evaluated; cohort A with untreated brain metastases ($n=89$) and cohort B with previously treated but progressing brain metastases ($n=83$). Clinical activity was similar in both cohorts for patients with $BRAF^{V600E}$ mutant melanoma; the objective intracranial response rate was 39% and 31% with a durable intracranial disease control rate of 81 and 89% respectively. Response rates appeared to be lower in the $BRAF^{V600K}$ mutant group in both cohort A (intracranial responses in 1 of 15 patients) and cohort B (4 of 18). Median PFS for both groups was similar at approximately 4 months and median OS was nearly 8 months.

In a single center subset of patients from the BREAK-MB trial, intracranial tumor regression correlated well with extracranial responses although exceptions did occur [40]. At the time of disease progression, several patterns of tumor growth were noted. These included systemic progression with intracranial disease control, isolated intracranial progression, or commonly, multiple foci of intracranial progression. The median time to intracranial progression in this subset was 16–20 weeks. Dabrafenib has also been reported to have intracranial activity for patients with $BRAF^{V600R}$ melanoma [41].

Vemurafenib also appears to have activity in patients with brain metastases. A pilot study was performed in heavily pre-treated patients [42]. Of 19 evaluable patients, seven had intracranial tumor shrinkage with three meeting criteria for partial response; median PFS was 3.9 months. Functional outcomes were also improved, with 25% of patients reporting a reduction in pain, 83% of patients with improvement in performance status, and 67% of patients with decreased corticosteroid requirements.

No clinical trials have been performed to evaluate the role of BRAF inhibitors in conjunction with local therapies. For patients with significant neurologic deficits on presentation, radiation therapy or surgery should be considered prior to initiating a BRAF inhibitor. However, for patients with asymptomatic brain metastases or when symptoms are controlled with steroids, BRAF inhibitors can be considered

prior to or instead of local therapies, particularly when rapidly progressing extra-cranial disease is present [43]. Mixed responses may be observed in some patients, necessitating local treatment to enlarging lesions. Additionally, a recent case report demonstrated the feasibility and potentially durable benefit of neoadjuvant vemurafenib followed by resection [44]. This approach can be considered particularly for patients with borderline resectable melanoma or a metastasis that is too large for stereotactic radiosurgery. Also, the combination of vemurafenib and radiation has been described to cause skin toxicity; we therefore hold BRAF inhibitors for 2–3 days around radiation [45]. The complexity of management in some cases highlights the need for multidisciplinary input into treatment decisions.

4.5.2 Treatment Beyond Progression

Selected patients develop progression at isolated disease sites while being treated with BRAF inhibitors which can be managed with local therapies (surgery, stereotactic radiosurgery). A retrospective analysis suggests that continuation of BRAF inhibitor therapy following local treatment for a solitary site of progression may be beneficial in this group of patients [46]. Patients in the initial phase I trial who continued vemurafenib following local therapy had a further progression-free interval of 3.6 months with a median OS which had not been reached at 6 month follow up. By contrast, patients who discontinued vemurafenib had a median overall survival of only 1.4 months. This finding may be a surrogate for the pace of disease progression (e.g. BRAF inhibitors are discontinued when there is obvious, rapid progression) or may be a genuine effect of continuing therapy.

Additionally, there have been case reports of objective responses occurring with re-treatment following a treatment-free interval. Two patients who developed disease progression (on vemurafenib and dabrafenib, respectively) had a treatment-free interval of 8 and 4 months [47]. Upon BRAF inhibitor rechallenge, both patients experienced dramatic regression in their melanoma (qualifying as mixed response and partial response by RECIST criteria). This strategy can be considered in selected patients.

4.5.3 Non-V600E Melanoma

The most common oncogenic point mutation in BRAF mutant melanoma results in substitution of a valine for a glutamic acid at codon 600 (V600E) which comprises 80–90% of BRAF V600 mutations [48]. Pre-clinical experiments and clinical experience suggest that alternate V600 mutations also confer sensitivity to BRAF inhibitors [49]. These genetic alterations do confer sensitivity to approved therapies and may be missed on standard $BRAF^{V600E}$ mutational testing. The second most common BRAF mutation is $BRAF^{V600K}$ which also appears to be quite sensitive to BRAF and MEK inhibition. $BRAF^{V600R}$ mutations also occur infrequently, although

in one small series five of six patients with $BRAF^{V600R}$ mutant melanoma experienced an objective response to dabrafenib [41]. Additionally, a patient with melanoma harboring both BRAF V600E and V600M mutations experienced a dramatic response to dabrafenib [50].

Mutations in BRAF at locations other than codon 600 may also occur, most commonly at codon 597. These genetic alterations may occur with a frequency of up to 5% in presumed BRAF wild-type melanoma. Based on pre-clinical and limited clinical experience, these mutations appear to confer sensitivity to MEK inhibitors, including one patient with $BRAF^{L597S}$ mutant melanoma who experienced a partial response to TAK-733, an experimental MEK inhibitor [51]. Pre-clinical data does not clearly define whether these melanomas should be sensitive to BRAF inhibitors although one patient with a $BRAF^{L597R}$ mutation experienced a major response to vemurafenib [52]. Additionally, BRAF fusions have been recently described in melanoma and seem to confer sensitivity to MEK inhibitors in pre-clinical studies. A clinical trial of trametinib for patients with these uncommon BRAF alterations is planned.

4.6 Conclusion and Future Directions

In conclusion, targeted therapy with BRAF and MEK inhibitors as monotherapy or in combination represents a major step forward in the management of patients with BRAF mutant melanoma. Although dramatic and rapid responses occur in the majority of patients, acquired resistance limits the duration of benefit for these patients. Improved combinations of targeted therapies to forestall acquired resistance are urgently needed. Currently, ongoing clinical trials are evaluating BRAF inhibitors in conjunction with MEK inhibitors as well as a large variety of other targeted agents. Agents targeting ERK, the final common signaling partner in the MAPK pathway are also ongoing. Additionally, the combination of immune therapies with these agents is an intriguing avenue to pursue (see Chap. 9).

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Chapter 5
The Ethics of Randomized Trials in Oncology

Pallavi Kumar and Ryan J. Sullivan

Abstract This chapter focuses on ethical issues involved in randomized clinical trials (RCTs) in oncology. It includes a brief review of the history of ethical issues in human research and selected historical examples, as well as an overview of existing ethical guidelines and codes for clinical research. It then discusses clinical trial design, including the benefits of randomized controlled trials, as well as the ethical issues surrounding RCT design including value, scientific validity, fair selection, randomization, the concept of equipoise, and control groups. Also covered briefly are the ethical issues involved in the conduct of clinical trials including regulation and oversight of trials, informed consent, and respect for participants. Special issues in oncology trials, such as phase I trials, and early stopping rules are also discussed. The chapter concludes with a case study: Ethical Issues in the BRIM-3 Trial, illustrating key points from this section.

Keywords Phase III trials · Equipoise · Crossover design · Early stopping rules

5.1 Introduction

Ethical issues have been a focus of controversy since the advent of clinical research in humans. More recently, much has been written about the ethics of clinical trials in oncology. The past few years have borne witness to much debate over key aspects of randomized study design involving targeted therapies for advanced melanoma; equipoise, randomization, crossover, and early stopping rules. In this chapter, we will summarize the key ethical issues in clinical research, with a focus on the topics relevant to randomized clinical trials of novel therapies for melanoma.

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5.2 A Brief History of Clinical Research in Humans: Selected Examples

The earliest recorded clinical trial is often cited as that of a special diet of legumes and water followed by Daniel in the Old Testament (Daniel 1:11, 15). During the 1530s Renaissance-era French barber-surgeon Ambroise Parè used a mix of turpentine, rose oil and egg yolk as an antiseptic for battle wounds, and enjoyed improved efficacy compared to the traditional treatments used at that time [1]. James Lind, often thought of as the father of controlled experimental design, conducted therapeutic trials of several agents (cider, seawater, elixir of vitriol, vinegar, a purgative tonic, and oranges and lemons) in the treatment of scurvy in British sailors and found that the sailors who ate oranges and lemons fared better than those who were given alternative treatments [2]. Here we have reviewed selected cases of clinical research in the last several decades that are notable either for their innovation, or more commonly for marking a period of controversy and moral outrage in the field of ethics of human research.

The Randomized Trial of Streptomycin in Pulmonary Tuberculosis Sir Austin Bradford Hill and the Medical Research Council (MRC) of the United Kingdom are often credited for carrying out the first published randomized controlled trial testing streptomycin as a treatment for pulmonary tuberculosis, reported in the British Medical Journal (BMJ) in October 1948 [3]. Hill advocated the use of random assignment of participants in a blinded manner to a treatment arm [3]. The justifications for the use of a control arm were: (1) streptomycin had not yet been established as effective for the treatment of tuberculosis and (2) due to a national shortage of streptomycin owing to the simultaneous tuberculous meningitis and military tuberculosis epidemics, the drug was not readily available for patients with pulmonary tuberculosis [4]. 109 patients were randomized to receive either standard care (bed rest) or streptomycin for four months. Results showed that mortality in the control arm was nearly four times that of the streptomycin group, establishing streptomycin as efficacious in the treatment of pulmonary tuberculosis [4]. Since these landmark findings were published in 1948, there have been several high profile human research experiments highlighting ethical controversies in clinical research conducted over the last eight decades.

The Nazi Medical Experiments During the rise of Adolf Hitler in the 1930s, civil rights and ethics suffered at the expense of the Nazi values of racial purity and Germanic/Aryan superiority. As part of the movement to purge Germany of unfit members of society, a series of horrific medical experiments in addition to euthanasia and murder were undertaken to advance Nazi medicine in areas like racial-anthropological research, brain research and neurology, military medical research, and genetics experiments [5]. The subjects were made to endure experiments involving a wide range of toxic exposures including high-altitude pressure chambers, immersion in freezing water, typhus, malaria, and mustard gas, among others [5]. These experiments are considered by many as acts of torture and murder, barely veiled under the pretense of medical research.

The Nuremberg Code is a legal and ethical framework arising from the trial of Nazi physicians at the Nuremberg war crimes trial conducted by the Allies at the close of World War II. The evidence presented at the trial, which was conducted over nearly 5 months in 1946–47, demonstrated that Nazi physicians not only conducted medical experiments under dangerous and cruel conditions, but that the experiments themselves were designed with the purpose of developing methods of extermination and ethnic cleansing [6]. In August 1947, the Nuremberg Code, a 10-item guideline written to protect the rights of research participants, was published along with the final verdicts [7]. The code emphasizes the primacy of voluntary informed consent in any research endeavor [6]. It also highlights the requirements of minimizing harm to subjects, freedom to withdraw consent at any time, and scientific design aimed at achieving results for the good of society [7, 8].

The Tuskegee Syphilis Experiment From 1930 to the early 1970s, the United States Public Health Service (PHS) carried out a series of observational studies on 400 African-American men affected by syphilis. By the early 1930s, the medical community had acquired a clear understanding of the natural history of untreated syphilis in its three stages, including the devastating cardiovascular and central nervous system manifestations observed in tertiary syphilis. However, there was a commonly held belief, even among physicians, that the syphilis epidemic was a particularly serious problem in African-Americans owing to their "inherently promiscuous nature", and that poverty and lack of education prevented them from seeking and completing treatment once infected [9].

Over 4 decades, the Tuskegee experimenters observed the natural arc of illness resulting from untreated syphilis. Among the many violations of research ethics committed in the study, the most egregious by any standards include: (1) misleading the participants about the nature of their illness, instead referring to it as "bad blood," (2) failure to obtain informed consent, (3) offering burial stipends to the families of the deceased to secure permission for autopsies, (4) offering aspirin and iron tonic as purported "treatments," (5) preventing men who were offered treatment by the military during the WWII draft from receiving it [9]. It was not until Peter Buxtun, a psychiatric social worker-turned-lawyer, raised protests against these violations in the mid-1960s, that there was a bona fide investigation of the study [9]. Even after such an inquiry at the Centers for Disease Control (CDC) in 1969, the study was deemed worthy of continuation with some modifications to improve its scientific integrity [9]. Finally, in 1972, an Associated Press reporter, Jean Heller, with the help of Peter Buxtun, published a series of articles [10] exposing the heinous violations of human rights and the deaths of over 100 men from the Tuskegee Study, and it was finally brought to an end. A class action lawsuit brought against the U.S. government resulted in a settlement consisting of little more than penicillin treatment for the survivors and nominal payments to the survivors and families of the deceased [11]. In 1974, after hearings on Tuskegee were held, the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research was founded when the National Research Act was signed into law [12], and in turn drafted the guiding principles comprising the Belmont Report [13], one of the most recognized moral frameworks governing medical research.

The Jesse Gelsinger Case The death of teenager Jesse Gelsinger after enrolling on a gene transfer protocol at the University of Pennsylvania is a recent memorable case. Gelsinger had a rare recessive X-linked genetic deficiency in the hepatic enzyme ornithine transcarbamylase (OTC), which leads to abnormal metabolism of ammonia [14]. He was diagnosed with partial OTC deficiency in his childhood, and despite adherence to the appropriate low protein diet and drug therapy, he developed many severe episodes of hyperammonemia requiring admission [15]. The University of Pennsylvania initiated a phase I trial of a functional OTC gene contained in an adenoviral vector, with the United States Food and Drug Association (FDA) approval as well as approval from the Recombinant DNA Advisory Committee (RAC), a regulatory body within the National Institutes of Health (NIH) [16, 17]. Jesse Gelsinger was the 18th patient enrolled on the phase I trial, which employed successively higher doses of adenoviral vector. He developed jaundice and confusion within 24 h of gene transfer, which rapidly progressed to multisystem organ failure and death within 4 days of receiving the therapy [15]. Autopsy studies revealed that the cause of death was an overwhelming cytokine storm and inflammatory response to the adenoviral vector [14, 15].

The case spawned a great deal of ethical controversy due to several lapses in the conduct and monitoring of the study. Previous participants had experienced liver function abnormalities which were not reported, raising the question of whether the requirement for informed consent was fulfilled [14]. Gelsinger also had baseline abnormalities in his liver enzymes, which should have disqualified him from the study at the outset [15]. Furthermore, several changes were made to the protocol that were not reported to the FDA [15], highlighting deficiencies in the conduct of clinical trials at the University of Pennsylvania. Inadequate research staff training and absent or poorly implemented procedures for clinical trial monitoring were yet other examples of misconduct in the Gelsinger case [14].

5.3 Ethical Guidelines for Clinical Research

Throughout history, there have been periods of ethical controversy surrounding clinical research in human subjects. From the atrocities committed by Nazi physicians and medical researchers during the Holocaust to the experiments carried out in African-American patients in the Tuskegee Syphilis Experiment, clinical research has had a complex and sordid past.

Over the past several decades, there have been numerous ethical frameworks proposed by both U.S. and international ethics organizations. Among the most widely cited are the Nuremberg Code [8], the Declaration of Helsinki [18], Belmont Report [13], the International Ethical Guidelines for Biomedical Research Involving Human Subjects [19], and the Common Rule (45 CFR 46) [20]. A detailed exploration of the historical evolution and a critique of the strength and weaknesses of each framework are beyond the scope of this chapter.

The majority of these codes and declarations were created as a response to scandal or controversy, and therefore each can be criticized for a narrowed focus on the

issues from which it arose, and most existing frameworks overlook important ethical considerations. Many of the existing guidelines also fail to provide a systematic overview of ethical principles that can be applied to all clinical research involving human participants.

In 2000, Emanuel, Wendler and Grady published a framework of seven core ethical principles that are widely considered a comprehensive guide to both theoretical and practical aspects of clinical research [21]. The 7 requirements proposed by Emanuel et al. are:

1. Social or scientific value
2. Scientific validity
3. Fair subject selection
4. Favorable risk-benefit ratio
5. Independent review
6. Informed consent
7. Respect for potential and enrolled subjects

This list, with the addition of "collaborative partnership" as an eighth requirement, appears in The Oxford Textbook of Clinical Research Ethics, the most comprehensive research ethics textbook published in the last two decades [22]. Readers are advised to use the Oxford text as a reference for ethical issues in clinical research.

5.4 Clinical Trials: Research Questions and Study Design

Clinical research specifically applies to research involving human participants. The goal of clinical research is often thought of as that of advancing the goals of medicine, either by development of new therapies or by deepening understanding of disease mechanisms [22]. An individual physician undoubtedly has a fiduciary duty to her patient, which entails upholding widely accepted principles such as beneficence, non-malfeasance, and autonomy [23]. Though the ultimate goal of clinical research is to move the field forward, these endeavors sometimes put the physician-researcher at odds with this highly individualized therapeutic contract.

In this section we will review the ethical aspects of clinical research in therapeutic interventions, those that pertain to the conception, design, and implementation of clinical trials in oncology, with a focus on the issues relevant to randomized, controlled study design and special issues for early phase trials in oncology.

5.4.1 Advantages of Randomized Controlled Trials (RCTs)

RCTs represent the cleanest study design, capable of minimizing the sources of systematic error due to confounding or bias which would otherwise lead to either a) erroneous rejection of the null hypothesis when in fact it is true (the false-positive case) or b) failure to reject the null hypothesis despite sufficient evidence to do so

(the false-negative case). RCTs, as compared to other epidemiologic study designs (i.e. case-control studies, cohort studies, and non-randomized single-arm trials) inherently contain several features that help to minimize these potential errors.

First, the principle of random assignment helps to minimize or eliminate differences in the study arms that would otherwise be present due to selection bias or certain disease or clinical characteristics that would predispose a participant to be more or less likely to receive the treatment or control. That is, sicker patients may be more likely to be enrolled on a treatment arm if not randomly assigned [24]. Second, the use of concurrent control groups enables timely comparison of treatment and non-treatment groups as opposed to the confounding that arises when historical controls are used [25, 26]. Third, the use of power and sample size calculations to minimize type I and type II errors are valuable features of statistical analyses of RCTs [27]. Some RCTs also use blinding (of participants, investigators, or both) to further eliminate unconscious sources of bias [24].

Although it is hard to argue that statistical rigor and the goal of reducing systematic error inherently pose ethical issues, the practice of randomization, the use of control groups (particularly those employing placebo controls), and even blinding have been debated in the ethics community for many years. In this next section we will explore the ethical considerations surrounding the concept and design of randomized controlled trials, the role of equipoise in assessing appropriateness of an investigation, and the use of control groups in clinical trials.

5.4.2 Ethical Considerations in the Design of RCTs

Value (Value to Participants Versus to Society) One of the primary requirements of ethical clinical research is arguably that it involves a test or treatment of inherent value by either (a) improving the health of trial participants and future patients or (b) contributing to the body of knowledge encompassing pathophysiology, diagnosis, or treatment of a particular disease [21, 28]. Emanuel et al. argue that research that is likely to produce nongeneralizable results, results that cannot be translated into clinical practice, or knowledge that cannot be disseminated to the scientific community would not meet the minimum ethical standard of value [21]. As we move forward into an increasingly strained research funding environment, identifying questions that provide high scientific or social value will become ever more important.

Scientific Validity Research lacking scientific rigor has a questionable ethical basis. Scientific validity is comprised of: a sound hypothesis, established methods, rational statistical analyses, adequately powered studies, and a feasible study plan [19, 21] The importance of scientific validity arises from the principles of judicious use of limited resources and minimizing the risk of exploitation of research subjects [29]. In the case of molecularly targeted therapies, one can argue that a study examining a novel agent targeting a known pathway or oncogene, reasonable efficacy in preclinical or animal studies, and one that has a measurable biomarker would

constitute sound biological rationale. These requirements would ensure that participants are exposed to a favorable risk-benefit ratio, and that the results of such research will be useful to future patients.

Fair Participant Selection There are numerous historical examples of exploitation of vulnerable populations. Out of these controversies, fair selection of participants has arisen as a key component of clinical research ethics. The major elements of fair selection are (a) a sound scientific basis upon which to define a study population, (b) appropriate exclusion of participants who would incur an unreasonably high risk of harm from study participation, (c) enrichment of a study population with individuals most affected by a particular condition, and (d) appropriate safeguards to ensure the protection of vulnerable populations [29].

Randomization and the Concept of Equipoise The notion of uncertainty regarding the superiority of a novel treatment as compared to the standard of care (which may be either no treatment or a previously established and accepted therapy) is considered essential to the justification for an RCT. When inferiority in any treatment arm is identified, whether at the outset or in the midst of the trial, the investigation should be halted and access to the superior therapy for participants provided [30].

The definition of equipoise becomes more complicated when we consider whether equipoise exists on a personal or community level, also known as "theoretical" or "individual equipoise" and "clinical equipoise," respectively. The former, first defined by Charles Fried, can be thought of in terms of the individual doctor-patient relationship, and implies that a physician may (only) recommend a clinical trial to a patient if the physician does not deem one treatment superior [31]. Arthur Schafer, however, suggests that even if a physician does not remain in a true state of equipoise throughout a study, a full explanation of risks and benefits to the participants allows them to make an informed choice, thereby preserving and ensuring equipoise [32]. This rationale neglects to account for the inherent trust that patients place in their physicians, and the potential for subtle coercion or even suggestion of a particular course to a patient who may be in a vulnerable state owing to advanced illness. Although medicine has been moving towards a more patient-centered and shared approach to decision-making [33], shifting the burden of determining equipoise to patients appears morally questionable.

These individualized and imperfect definitions of equipoise exclude the broader and perhaps more utilitarian implications of "clinical equipoise," which Freedman argues exists when "there is no consensus within the expert clinical community about the comparative merits of the alternatives to be tested," thereby removing the personal biases and preferences of individual physicians from the decision to move forward with investigation [30]. Freedman asserts that when true clinical equipoise exists among a medical community regarding a treatment or intervention, only then is a randomized controlled trial ethical.

In reality, it is likely that equipoise needs to exist on both a personal and community level. At the patient level, individual or personal equipoise would be requisite in order for a physician to recommend a trial to a particular patient, but clinical equipoise within the medical community may be necessary to justify initiation of a

trial in the first place [34]. There are certainly situations where one of these may be met in absence of the other, or neither of the conditions is fulfilled.

Despite the popularity of clinical equipoise among the scientific community as the justification for conducting RCTs, there are several problems with relying solely on this concept. Miller and Joffe recently proposed that equipoise fails to offer resolution to five key problems in determining the appropriateness of RCTs [35]:

1. *The ill-defined nature of equipoise itself*. Even with an expanded view of equipoise (that of "clinical equipoise" as proposed by Freedman), it remains a difficult entity to clearly define. For example, what level of agreement must there be in a medical community to infer true uncertainty? What is the quality of evidence on which "expert opinions" are based must be present in order to assume that the consensus is a valid one?
2. *Emphasis on expert opinion*. Seeking the expertise of experienced members of a medical community does not necessarily guarantee a balanced, unbiased review of the therapies under investigation, nor does it ensure that the opinions themselves are based on prior evidence from other RCTs.
3. *Pitfalls of defining therapeutic efficacy based on surrogate outcomes.* This is particularly relevant to the conception of oncology clinical trials, where equipoise is often considered lost due to encouraging results of phase I and II trials, which typically use response rates as a surrogate endpoint. Miller and Joffe argue that we must be careful not to conflate tumor response with improvements in overall survival or even quality of life [35, 36, 37, 38]. An example illustrating this is that of bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF), in metastatic breast cancer. While it initially garnered FDA approval on the basis of improvements in tumor response and progression-free survival, a meta-analysis revealed that there was no overall survival benefit, prompting the FDA to remove breast cancer as an indication for this agent [39].
4. *High costs of novel therapies.* Given the rising costs of cancer care, at least partially attributable to novel agents [40], there is an argument to be made for using an RCT *even when true equipoise does not exist* to be certain that a treatment provides sufficient benefit to justify its often high cost. This calls into question the physician's ethical duty to the patient versus the medical community's obligation to society at large to help curb healthcare costs.
5. *Early termination of RCTs based on interim analyses.* The concept of equipoise mandates that a trial be brought to a close if the balance tips, even minutely, in favor of one treatment over another [30, 41]. This has the potential of overstating the benefits of a particular therapy, and underreporting of adverse events [35].

Control Groups Assignment of the appropriate concurrent control group is a central issue to the design of any clinical trial, especially that of an RCT. The basic purpose of any control group is to give the experimenter a basis for comparison and a way to measure efficacy of the treatment under investigation. Miller makes the distinction between *absolute* and *relative* efficacy, where the former is a pure measure of the effect of the intervention in a given clinical scenario, whereas the latter measures if and to what degree the treatment being studied is better (or equal/worse)

to a standard therapy [42]. Similarly, the concepts of superiority and equivalence (non-inferiority) trials are pertinent to the discussion of control groups. Superiority trials are designed to demonstrate that the treatment under study results in a better clinical outcome than the control, whereas non-inferiority trials are designed to show that the new treatment is not worse than the standard.

Control groups are classified by the FDA into five major categories: placebo concurrent controls, active treatment concurrent controls, no treatment concurrent controls, dose-comparison concurrent controls, and external controls [43]. In this section we will discuss placebo and active treatment controls as the two most commonly encountered types of controls in oncology RCTs.

1. Placebo concurrent controls. A placebo is an intervention (usually pharmacologic) which appears indistinguishable from the intervention under investigation, but is inactive and not expected to impact the natural course of the disease. In a RCT setting, it is the closest approximation of the "counterfactual," or what would have happened to the intervention group in the absence of the intervention [24, 44]. Placebo controls used in a double-blinded, randomized setting are also the best measure of *absolute* efficacy of a particular intervention, and are used to demonstrate superiority [42]. In cases where no established therapy exists to treat the disease under investigation, it would be ethically sound to use placebo controls.

However, the controversy arises when there are known therapies for a disease state. Proponents of placebo controls assert that even if a previously established treatment is available, there may be a methodological rationale for the use of placebo controls; cases where an established therapy may behave different in a subset of patients, have previously demonstrated historically marginal benefits, or have a significant side effect profile [42]. Additionally, disease for which the natural history is indolent may be more amenable to a placebo-control design.

Miller proposes three areas that should be addressed in order to accurately assess the burden of a placebo control: severity of illness, likelihood of harm, and duration of harm. It is widely accepted that placebo-controlled trials are ethically suspect for severe, life-threatening conditions that will likely result in death or serious disability, or in cases where withholding any form of treatment has a significant chance of long-lasting harm to participants [25, 26]. Critics of placebo controls also argue that intolerable suffering, even if self-limited and non-life threatening ought not to be allowed to be studied in this manner. Emanuel and Miller refer to placebo-controlled trials of ondansetron in the 1990s, when other anti-emetics (such as metoclopramide) had already been proven superior to placebo for chemotherapy-induced nausea [45, 46]. Trials of this nature, where participants are denied symptomatic relief with an established and effective nature, are not ethical. Oversight by external and institutional review boards is essential to ensure the appropriateness of RCTs that use placebo controls, and rigorous review of informed consent procedures are particularly important in this scenario. Provisions should be in place to ensure that specific criteria are established to quickly remove participants from trials if they suffer significant harm or suffering, and placebo administration should be kept as limited as possible [45].

2. Active treatment concurrent controls: These studies are designed to compare a new treatment to an established, typically standard treatment. Active treatment controls are a test of *relative* efficacy, and can be used in either superiority of non-inferiority trials. A methodological weakness of active or positive controls is that although the new treatment may or may not be better than the standard of care, the difference between the two is likely of lesser magnitude than the difference between the new treatment and placebo. Power and effect size calculations usually dictate, then, that a larger sample size is needed to detect an effect size in the case of an active controls trial [42]. Proponents of active control trials assert that placebo-control design is inferior because the theoretical counterfactual in the latter case is not one of clinical interest. This is particularly true in oncology clinical trials for stage IV disease, where participants receiving standard therapy as an active control are more representative of a non-protocol patient, who would likely not be receiving "no treatment."

5.5 Ethical Issues in the Conduct of Clinical Trials

Regulation and Oversight The independent review process in clinical research is an integral part of minimizing financial and academic conflicts of interest [21, 47, 48], and in the United States these functions are carried out by a diverse group of regulatory bodies; institutional review boards (IRBs), data and safety monitoring boards (DSMBs), the FDA, as well as independent and private review boards.

The major role of the IRB is the determination of the ethical acceptability of a proposed study. The board is typically comprised of at least five members consisting of both men and women without conflicts of interest, at least one of whom has scientific training, one whose focus is on nonscientific issues, and one member from the community (not affiliated with the institution) [49]. The IRB typically determines whether a submitted proposal is exempt for IRB oversight or may undergo expedited review. The main functions of the IRB are: initial and ongoing review of research activities, determination of informed consent, records retention, communication with investigators, and expedited reviews [50].

Ongoing monitoring of clinical trials is typically carried out by DSMBs. The nature of the research study (i.e. phase of drug development, blinded vs. unblinded, single center vs. multicenter) often determine the need for and extent of involvement by the DSMB [51]. The DSMB members must be independent of the investigators and have no competing financial conflicts of interests. Perhaps inevitably, there may be situations in which board members have intellectual conflicts of interest, which are more difficult to avoid.

The primary duties of the DSMB are to: (1) ensure the ongoing safety of clinical trial participants and (2) assess the integrity of the trial design and data analysis in order to increase the chance of valid results [51, 52]. At the conclusion of each DSMB session, recommendations are made regarding continuation, amendment,

or cessation of the study. Trials are typically terminated for one of several reasons: unanticipated effectiveness of therapy, unacceptable toxicities, poor accrual, and the low likelihood that collecting additional data would alter a negative result. Additionally, the DSMB may recommend halting a trial if the study question has been answered by evidence that has become available during the course of the trial [51, 53, 54].

Informed consent Due to the many historical abuses perpetrated against clinical research participants throughout history, informed consent has been incorporated into the existing major ethical guidelines for research. In the United States, the Common Rule (45CFR 46) mandates that research studies obtain informed consent from study participants, and that this process is overseen by the IRB [28], Code of Federal Regulations, (Title 45, part 46). The three concepts that are central to valid consent are generally accepted to be: (1) information, (2) voluntariness, and (3) competence. Informed consent has been written about extensively in the research ethics literature, and an exhaustive review of its history and components exceeds the scope of this chapter. Readers are encouraged to refer to the Oxford Textbook of Clinical Research Ethics [55, 56] for an exploration of the philosophical justifications and regulatory standards of informed consent in research.

Respect for Participants Despite the emphasis on enrollment and consent procedures, ongoing attention to the treatment of study subjects is needed to meet the requirements for respect for participants. Emanuel et al list five areas that must be satisfied [21]:

1. Protection of privacy and confidentiality of research participants
2. Revocability of consent (ability to withdraw informed consent and disenroll from the trial at any time, without fear of redress
3. Access to new information available throughout the course of a study
4. Proper measures to record and prevent adverse events and undue harm
5. A means of communicating results from the study to participants

5.6 Special Issues in Oncology Clinical Trials

Phase One Trials Early phase clinical trials in oncology have long been assailed as promoting enrollment in an endeavor that inherently has an unfavorable risk-benefit ratio, because the chance for therapeutic benefit is low yet there are significant risks of toxicity and even death from adverse events related to a novel therapy. Given that the primary objective of a phase I clinical trial is to establish safety, short-term toxicities, and maximum tolerated doses of agents in preparation for a phase II trial which would then study efficacy, one can see why phase I trials have a “bad name.” While the concern for real harm exists around phase I trials involving new cytotoxic chemotherapies, the landscape for novel agents has changed considerably in the era of targeted therapies [57]. Another point worth considering is that participation

in active treatment, possibly via a phase I trial, may have potential psychosocial benefits that arise from improvements in quality of life [58] as well as the notion of helping future patients suffering from cancer [59]. Another major criticism of phase I oncology trials is the assumption that there may be glaring omissions in the informed consent process and possibly a low quality of disclosure on the part of researchers, given the low potential for benefit and the vulnerability of a patients with incurable cancers.

Interim Analyses and Early Stopping Rules As previously discussed, in phase III RCTs, ongoing monitoring of trial activities is typically overseen by the DSMB, whose functions are to review trial data, including interim analyses, with the goal of determining whether a trial is considered appropriate for continuation based on early stopping rules that involve definitions of unacceptable toxicities or unacceptable differences in certain pre-specified endpoints (typically overall survival). If the DSMB determines that one group of participants has suffered excess toxicity or that there is a significant survival advantage to one treatment over another, the trial is usually halted and participants are offered a chance to receive the more efficacious therapy. This is, in theory, one way to preserve equipoise throughout the life of an RCT, and to protect participants for unnecessary harm while ensuring that they have access to the maximum amount of benefit.

The ethical concerns pertaining to early stopping rules arise from three major areas: the validity of informed consent in light of interim findings, risks and benefits to current and future patients and the broader implications of the trial under question on the field [60]. In the case of informed consent, one can argue it is an ongoing responsibility of investigators (as overseen by an IRB) to fully disclose interim results to patients, who may then exercise their right to terminate participation in a trial. One solution that has been proposed to this dilemma is that informed consent may explicitly state that preliminary analyses will not be disclosed prior to trial completion, which would address the purely regulatory concern [5252, 61]. However, others would argue that asking a participant to relinquish his or her rights to information jeopardizes informed consent, and in some cases may even approach fraud [62].

With respect to the impact of early study termination on reporting of benefits of an investigational regimen, stopping a trial due to an improvement in a surrogate endpoint such as progression free survival (PFS) without an improvement in overall survival (OS) may lead to incomplete data collection on the adverse events and toxicities associated with therapy. It is conceivable that a new regimen may lead to tumor regression initially, but that toxicity may be dose-limiting or even necessitate that patients discontinue therapy, which may in turn negate any potential overall survival benefit. Stephen Cannistra cites an illustrative example in the Journal of Clinical Oncology in 2004 [63]:

- A phase III RCT of 12 months of maintenance paclitaxel versus 3 months of paclitaxel was performed in patients with advanced ovarian cancer [64]. The primary co-endpoints were PFS and OS. The trial was halted early after interim analysis demonstrated that there was a statistically significant improvement in

PFS of 7 months in the 12 month maintenance group without any evidence of an OS benefit [64, 65]. However, a significantly higher proportion of participants in the 12 month arm compared to the 3 month arm had grade 2 or 3 neuropathy (23% vs. 15%), and no quality of life measures were assessed [64]. Thus, it is conceivable that participants on the 12-month arm suffered a higher burden of side effects for several months of progression-free survival that may or may not have translated into an OS benefit (had the trial been followed to completion).

Cannistra proposes the following recommendations as a way to frame the discussion about the role of early stopping rules in oncology RCTs [63].

1. Early stopping rules should be limited to and based on either: unacceptable toxicity or improvements in overall survival or quality of life.
2. Informed consents should include clear statements regarding criteria for early trial termination in the interest of the most complete disclosure
3. Investigators *and* participants should have access to interim analyses reporting on either toxicity or overall survival.
4. There should be a greater effort to invite patient representation and advocates to serve on DSMBs.

5.7 Case Study: Ethical Issues in the BRIM-3 Trial

Advanced melanoma is a uniformly lethal disease, with a 5 year survival of less than 10% [66]. Prior to 2011, dacarbazine, which has a response rate of 5–15% [67], and high-dose interleukin 2 (response rate 16%) were the only agents FDA-approved for the systemic therapy for advanced melanoma. While numerous other agents or combination strategies showed promising phase 2 data, all failed to show an overall survival benefit when tested in phase 3 studies against dacarbazine or other routinely used, and similarly effective/ineffective, chemotherapies [68].

In 2002, mutations in the gene encoding BRAF, a serine-threonine kinase of the mitogen-activated protein kinase (MAPK) pathway, were first reported to be present in over half of malignant melanomas, marking the discovery of a critical potential therapeutic target [38]. In June 2009, the results of a phase I clinical trial testing vemurafenib (PLX4032, RG7204, RO5185426) in patients with metastatic melanoma and other solid tumors were presented at the annual American Society of Clinical Oncology (ASCO) meeting. In the seven patients harboring *BRAF* mutations, five had tumor regression, with disease control lasting up to 14 months [69].

In August 2010, updated results from the phase I trial of vemurafenib were reported in the New England Journal of Medicine; of the 49 patients with melanoma enrolled, 16 harbored the BRAF V600E mutation, and an additional 32 metastatic melanoma patients found to have the V600E mutation were later enrolled in the expansion phase. Of the initial 16 patients who enrolled on the dose-escalation phase, 10 had a partial response and 1 had a complete response, while 24 of the 32 patients enrolled in the expansion had a partial response and 2 had a complete response

[70]. Symptom improvement was observed within 1–2 weeks of starting therapy. Of equal importance, no responses were seen in any of the patients treated whose tumors did not harbor a BRAF mutation.

Soon after, the New York Times published a series of articles notably detailing the story of two cousins with metastatic, BRAF-mutant melanoma [71]. One cousin enrolled on the phase II trial of vemurafenib (BRIM2) and responded well to treatment for 9 months at the time of the publication. In contrast, the other cousin was enrolled onto the phase III trial of vemurafenib (BRIM3) and was randomized to the control arm (dacarbazine). Unfortunately, the cousin who was randomized to dacarbazine had rapidly progressing disease which led to clinical decline, and was denied access to vemurafenib according to the protocol, which did not allow for crossover to the treatment arm. He died of metastatic melanoma just over 6 months after diagnosis. While the story of the two cousins did not account for confounding factors that may have explained why one did more poorly than the other, it made a powerful point and sparked a debate as to the ethical nature of the BRIM3 study.

The differing views of the debate were described as part of these series of articles and presented the views of prominent oncologists and researchers in the melanoma community regarding the BRIM3 study. While some argued against extrapolating a survival benefit from a phase I trial showing response, and one in which OS was not a primary endpoint, others argued that the phase I results were convincing proof that vemurafenib should not be withheld from a subset of patients who stand to benefit the most from it. The controversy over the ethics of withholding a non-approved drug from patients with BRAF-mutant melanoma continued for months. Following the first interim analysis, it was clear that the patients randomized to vemurafenib were doing much better than those treated with dacarbazine; results which led to a meeting with the FDA, study sponsor, and study investigators. As a result of this meeting, the end-point of the study was changed from OS to a composite end-point of OS and PFS. Additionally, cross-over to vemurafenib was then allowed.

The results of the BRIM3 study were published in the New England Journal of Medicine on June 30, 2011 [72]. At the time of the interim analysis conducted after 98 deaths, a relative reduction of 63 % in the risk of death was seen with vemurafenib therapy among the 675 previously untreated BRAF-mutant melanoma patients randomly assigned to receive either vemurafenib or dacarbazine. In the analysis at 6 months, overall survival with vemurafenib was significantly improved compared to dacarbazine (84 % vs. 64 %, $p < 0.001$) [72]. In June 2012, an updated analysis of the BRIM3 study was presented. Importantly, while treatment with vemurafenib was still associated with an improvement in overall survival, the risk reduction of death was 30 %, as opposed to the initial presented 63 %, and in subgroup analysis, the bulk of this could be accounted for in patients with the most aggressive disease (AJCC M1c) [73].

Ethical Considerations

1. Was there equipoise between the two treatments tested in the BRIM-3 randomized clinical trial? What element(s) of trial design could have been modified to address clinicians' concerns that dying patients were denied a potentially life-prolonging therapy?

As Freedman defines equipoise as the state in which “there is no consensus within the expert clinical community about the comparative merits of the alternatives to be tested” [30], one might question whether equipoise existed for this study. Specifically, nearly every investigator and researcher in the medical community was certain that the BRIM3 study was going to find that vemurafenib was a superior agent to dacarbazine and would improve survival of patients. Still, the melanoma community (and the FDA) had seen a number of promising agents fail in phase 3 studies following compelling phase 2 data; thus the burden of proof was on the sponsor and the investigators to show that vemurafenib was indeed better. The implementation of an interim analysis was used appropriately and action was taken to functionally end the study as originally composed. With the benefit of hindsight it is clear that patients with rapidly growing disease had worse outcomes with dacarbazine than with vemurafenib, and patients with less aggressive disease (unresectable Stage III, Stage IV M1a and M1b) did not. While the first finding would have been predicted from the initial clinical studies with BRAF inhibitors in general and vemurafenib specifically, the latter conclusion would not have been. In our opinion, this information would still likely have been discovered if crossover from dacarbazine to vemurafenib was allowed from the beginning of the study. The FDA typically considers overall survival as the benchmark for oncology drug approvals, although accelerated approval may be granted based on surrogate endpoints such as PFS or objective response rate (ORR) [74]. Still, in the case of bevacizumab in metastatic breast cancer, the FDA revoked its accelerated approval in 2011 [75], stating that the time-to-progression benefit upon which the drug was initially approved failed to translate into a significant improvement in overall survival [76, 77, 78]. OS is an ideal end-point, but one that is difficult to achieve in crossover studies. In the end, the sponsor and investigators carried out the BRIM3 study in good faith to define the effectiveness of a promising agent on the hardest end-point, built in early analysis, and ultimately changed the protocol to allow crossover based on the results of this interim analysis.

2. Does dacarbazine represent a reference standard against which novel therapies should be measured? Would the use of a placebo as the standard arm be more or less ethical?

When the BRIM3 study was being designed, the frontline setting was chosen for evaluation given the lack of a truly accepted standard of care for this disease. Based on the lethality of this disease and the well defined, though quite modest, activity of chemotherapy, an active control was chosen; as it has been in nearly every randomized, phase III study in the field. Dacarbazine has been used most often in these studies, though other agents/regimens such as single-agent temozolomide, single-agent paclitaxel, and the combination of carboplatin and paclitaxel have also been implemented. The use of placebo would not have been ethical based on the possibility of rapidly progressing, fatal disease in the absence of effective therapy [68].

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Chapter 6
Parallel and Serial Blockade Strategies in BRAF-Mutant Melanoma

Michael A. Davies

Abstract Activating mutations in *BRAF* are the most common somatic aberration in cutaneous melanomas. These mutations result in constitutive activation of BRAF's catalytic activity and its downstream effectors in the RAS-RAF-MEK-ERK signaling pathway. Both selective BRAF and MEK inhibitors have demonstrated high clinical response rates in metastatic melanoma patients with activating *BRAF* mutations. These successes have illustrated several keys to the successful development of targeted therapies, and the potential for personalized therapeutic strategies for cancer. However, the ultimate clinical benefit of BRAF and MEK inhibitors has been limited by both de novo and secondary resistance mechanisms. Initial preclinical and clinical studies support that these resistance mechanisms may broadly be characterized as those that result in (1) re-activation of the RAS-RAF-MEK-ERK signaling pathway, or (2) activation of other pro-survival mediators. These findings are now leading to the development of new combinatorial approaches that involve serial and/or parallel blockade strategies in order to overcome resistance mechanisms, and ultimately to improve outcomes in melanoma patients with activating *BRAF* mutations. Further, these concepts are also being explored and tested in melanoma patients with other oncogenic mutations.

Keywords BRAF · Mutation · Amplification · Splicing · Targeted therapy · Resistance · MEK · NRAS · PI3K · AKT · mTOR · IGF1R · Immunotherapy · Combinatorial approaches

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6.1 Introduction

Melanoma is the most aggressive of the common forms of skin cancer. Although melanoma represents only ~5% of the skin cancers that are diagnosed each year, it is responsible for more than 70% of skin cancer-related deaths. While the incidence of many cancers has declined over the last few decades, the number of new cases of melanoma diagnosed every year continues to rise. Overall, the annual incidence of melanoma has increased over 600% since 1950. Unfortunately, many of the patients who are diagnosed with melanoma, and who ultimately succumb to the disease, are young, particularly women. Thus, melanoma has one of the highest life-years lost per cancer-related death among all malignancies. For these many reasons, melanoma is a significant disease which is likely to become an increasingly important public health issue in the future if current trends are not reversed [1].

Multiple treatment modalities are utilized in the care of melanoma patients. Surgery is the mainstay of treatment for patients with both clinically localized (i.e. cutaneous primary tumor) and regionally metastatic (i.e. regional lymph nodes or in-transit disease) disease, and may also be utilized for palliation in patients with distant metastases. Radiation therapy has a clear role for palliation of painful metastases, but its benefits in earlier, potentially curable stages of disease are less clear [2]. Systemic therapies are used in some patients to reduce the risk of relapse after surgical treatment of regional metastases [3], and they are generally the primary treatment modality for patients with distant metastases or unresectable regional tumors.

Although cytotoxic chemotherapies represent the backbone of systemic therapy for most cancers, historically these agents have demonstrated minimal benefit in patients with metastatic melanoma [4]. For example, dacarbazine (DTIC) was approved for use in metastatic melanoma in the mid-1970s despite achieving clinical responses in ≤10% of patients and having no demonstrated (or appreciable) impact on median progression-free (PFS) or overall survival (OS). Combining chemotherapy agents together in various regimens resulted in increased toxicity, but no proven impact on survival [1]. With these disappointing results, other therapeutic strategies have been investigated extensively in melanoma. Much of this effort has focused on the development of agents that stimulate the immune system to attack or control the cancer, which as a class have been termed immunotherapies. High-dose bolus interleukin-2 (HD IL-2) therapy was the first such agent to gain approval in patients with metastatic melanoma, in 1998. Non-randomized studies of metastatic melanoma patients treated with HD IL-2 demonstrated that this therapy was able to achieve durable (>10 year) disease control in metastatic melanoma patients, leading to it regulatory approval [5, 6]. However, this was only achieved in the patients who had complete responses to treatment, which only occurred in ~5% of patients. Overall, only 15% of patients achieved even transient clinical responses. Further, HD IL-2 therapy is extremely toxic, requiring ICU-level care to manage the many side effects of the treatment, and resulting in treatment related deaths in ~1% of

patients in early phase clinical trials. More recently, a number of new strategies and agents have been identified to stimulate anti-tumor immune responses. Most notably, ipilimumab, an antibody that blocks the inhibitory CTLA-4 receptor on the surface of T cells, was granted regulatory approval for patients with metastatic melanoma in 2011. While ipilimumab has a moderate clinical response rate of only ~10 %, in randomized clinical trials treatment with this agent resulted in statistically significant improvements in PFS and OS compared to controls, and a three year survival rate of ~25 % [7, 8]. In contrast to HD IL-2, ipilimumab has very few acute side effects and can be given in the outpatient setting. However, ipilimumab can produce significant autoimmune toxicities in some patients, including colitis, hepatitis, and endocrinopathies.

A relatively new systemic therapy modality to be explored in melanoma is targeted therapy. Conceptually, targeted therapies inhibit the molecules and/or pathways that are specifically dysregulated in cancer cells. Targeted therapies have demonstrated efficacy in a number of diseases, including those that are generally refractory to chemotherapy [9]. One of the earliest examples of the potential of targeted therapy was the development of imatinib for chronic myelogenous leukemia (CML). Almost all CML cells are characterized genetically by a translocation event between chromosomes 9 and 22, resulting in the characteristic Philadelphia chromosome that is the hallmark of this disease. This genetic event produces a novel fusion protein (BCR-ALB) that includes the kinase domain of the *ABL* gene. Imatinib, a small molecule inhibitor of ABL and other kinases, produced marked improvements in clinical outcomes even in very early phase clinical trials in CML, and rapidly became the standard of care of patients with this disease [10]. Targeted therapies have also become the standard of care for specific, molecularly-defined subpopulations of other cancers, including breast cancers with amplification of the *HER2/neu* gene (trastuzumab) and lung cancers with *EGFR* mutations (erlotinib) [11–14]. While targeted therapies have proven clinical benefit in these populations, efficacy is frequently limited by the rapid development of resistance. An improved understanding of the mechanisms of resistance is now leading to the development of new inhibitors and/or combinatorial strategies that aim to achieve a greater degree or duration of cancer control across multiple tumor types.

Perhaps more than any other cancer, the recent history of the development of targeted therapy for melanoma demonstrates both the promise and challenges of this therapeutic strategy. Specifically, the development of targeted therapies for melanomas with activating mutations in the *BRAF* gene has illustrated a number of key factors in this area of research. Further, both clinical and preclinical studies have now set in motion the development of various combinatorial strategies for this disease. The following is a summary of the foundation that had led to this new era of combinatorial therapies, and the rationale behind several of the leading combinations that are being pursued.

6.2 Molecular Biology of Melanoma

The sentinel event in the development of targeted therapy for melanoma was the discovery of point mutations in the *BRAF* gene [15]. These mutations were identified as part of a screen for mutations in the genes that encode the RAF kinases, which are part of the RAS-RAF-MEK-ERK signaling cascade (Fig. 6.1). This initial screen of cell lines and tumors identified recurrent point mutations in exon 15 of the *BRAF* gene, most frequently in the melanomas that were included in the study, but also in colorectal, primary brain, lung, liver, ovarian, and other cancer types. Subsequent studies have demonstrated that more than 90 % of the *BRAF* mutations that are detected in melanoma occur in exon 15 and result in substitutions for the valine at the 600 position (V600) [16]. The most common mutation results in substitution of a glutamic acid (V600E), which in multiple series has been shown to represent ≥70 % of the detected *BRAF* mutations [17, 18]. The catalytic activity of the BRAF V600E mutant protein is increased more than 400-fold in comparison to the wild-type BRAF protein and results in constitutive activation of MEK and ERK. Other substitutions at the V600 site, including V600K and V600D, also markedly (more than 100–200-fold) increase the catalytic activity of BRAF. A variety of other

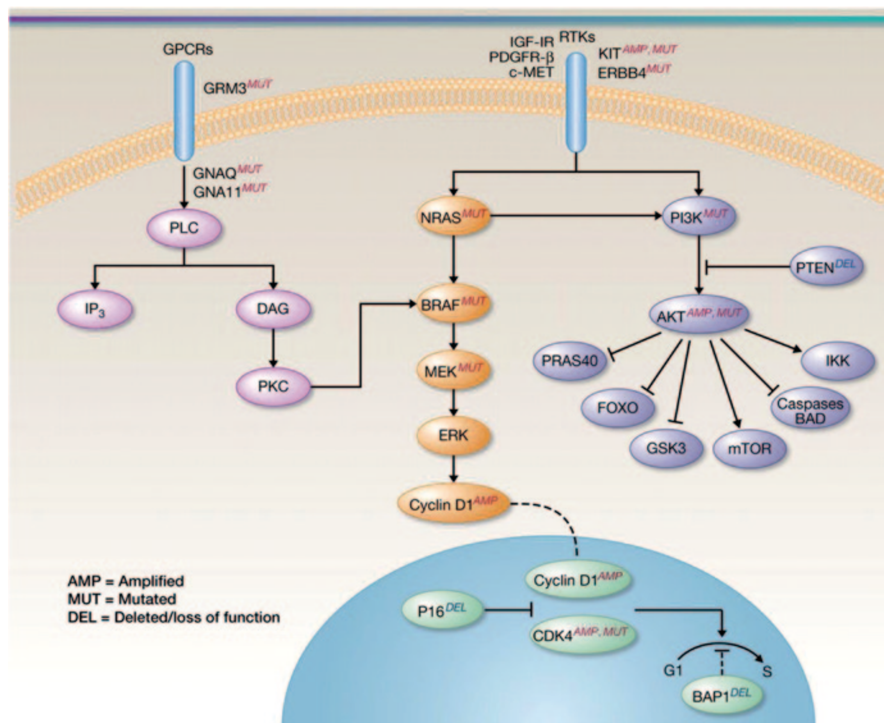

Fig. 6.1 Frequent somatic mutations in signaling pathways in melanoma. (Used with permission from [22])

rare point mutations in BRAF have also been detected, both in exon 15 (i.e. K601E, L597V) and exon 11 (i.e. G469E, G464E). Interestingly, these mutations are quite variable in their effects on the catalytic activity of BRAF, with some mutations actually resulting in decreased kinase activity (i.e. G466E, D594V, and G596R) [19]. However, expression of essentially any of these mutations results in increased activation of MEK and ERK, as the kinase-inactivating mutations promote the formation of BRAF-CRAF heterodimers that activate the pathway through CRAF's catalytic activity [20].

Meta-analyses of large cohorts of melanoma clinical samples have demonstrated that substitutions of the V600 residue of BRAF occur in 40–50 % of cutaneous melanomas [16] (Table 6.1). These mutations are most frequent in cutaneous melanomas arising in areas with intermittent sun exposure, but are less common in tumors that arise in areas of chronic sun exposure and have histologic evidence of chronic sun damage (CSD) [21, 22]. The mutations are less prevalent (10–15 %) in acral melanomas, which arise on the relatively sun-protected palms of the hands, soles of the feet, and nailbeds. Mucosal melanomas, which arise from mucosal surfaces throughout the body, have a *BRAF* mutation rate of < 5 %. Finally, *BRAF* mutations have not been detected in uveal melanomas that arise from melanocytes in the eye.

Activating mutations in *NRAS,* which also activate the RAS-RAF-MEK-ERK signaling pathway, are the second most common somatic activating mutations detected in melanoma. These mutations occur in 15–20 % of cutaneous melanomas, most commonly resulting in substitutions at the Q61 residue of exon 2 (~ 80 % of mutations) or the G12/13 residues of exon 1 (~ 20 %) [16]. *NRAS* mutations are also detected in acral and mucosal melanomas, but are not found in uveal melanomas (Table 6.1). In treatment-naïve patients, hotspot *NRAS* mutations and *BRAF V600* mutations are essentially mutually exclusive, with both mutations found in less than 1 % of tumors [17]. However, *NRAS* mutations are frequently detected in melano-

Table 6.1 Prevalence and pattern of common somatic mutations in different melanoma subtypes. "CSD", chronic sun damaged. "–", insignificant number reported. "?", not yet reported. (Adapted with permission from [22])

	Mutations				
	BRAF	NRAS	KIT	GNαQ/11	BAP1
Cutaneous (Non-CSD)	45 %	15–20 %	~ 1 %	–	?
Cutaneous (CSD)	5–30 %	10–15 %	2–17 %	–	?
Acral	10–15 %	10–15 %	15–20 %	–	?
Mucosal	5 %	5–10 %	15–20 %	–	?
Uveal	–	–	–	80 %	50 % (85 % of monosomy 3)
Melanoma from an Unknown Primary	50 %	20 %	–	–	–

mas with non-V600 BRAF mutations, particularly those that fail to increase the catalytic activity of BRAF [20]. Similar to NRAS, strong genetic interaction has also been identified for loss of function mutations of the *PTEN* tumor suppressor [23]. PTEN is a phosphatase that dephosphorylates phospho-lipids in the cell membrane, thereby antagonizing signaling by the oncogenic lipid kinase PI3K. Loss of PTEN results in constitutive signaling through the PI3K-AKT pathway. A number of analyses have demonstrated that loss of function and/or expression of PTEN in melanomas are mutually exclusive with the presence of *NRAS* mutations [24–26]. In contrast, PTEN can occur in melanomas with activating BRAF mutations, and is detected in 20–30 % of BRAF V600-mutant melanomas.

Focused sequencing studies have identified a number of other somatic changes in oncogenes in melanoma in, or downstream of, the canonical RAS-RAF-MEK-ERK and PI3K-AKT pathway, such as rare activating point mutations in *AKT1, AKT3, MEK1,* and amplifications of cyclin D1 [27–29] (Fig. 6.1). In addition, deletions and inactivating mutations of the *CDKN2A* gene that cause loss of expression/function of the P16 protein are germline mutations in many cases of familial melanoma, and may also occur somatically [30]. Activating mutations and amplifications of the *CDK4* gene are also detected in melanomas as germline or somatic events [31]. In addition to these events in cutaneous tumors, studies have revealed a number of mutations in other melanoma subtypes. Somatic mutations and gene amplifications of the *KIT* gene on chromosome 4 have been identified as frequent events (10–30 %) in acral and mucosal melanomas [32]. Some studies have also suggested that these mutations are also common in cutaneous melanomas with evidence of chronic sun damage (CSD), but this has not been observed in other studies [33]. Molecular characterization of uveal melanomas demonstrated a lack of *BRAF, NRAS,* or *KIT* mutations in these tumors, but loss of expression of PTEN has been observed [34, 35]. Uveal melanomas instead have a high prevalence of activating point mutations in the *GNαQ* (35 %) and *GNα11* (45) genes, which encode regulatory subunits of G-protein coupled receptors [36–38]. As these mutations are mutually exclusive, altogether they are present in ~80 % of uveal melanomas, and preclinical studies suggest that they can cause activation of multiple signaling pathways. Approximately 80 % of uveal melanomas that have monosomy 3, which correlates with poor prognosis, have inactivating mutations of the *BAP1* gene, which is located at 3p21 [39]. Germline mutations in *BAP1* have also been identified in families with an increased risk of developing uveal melanoma [40, 41].

Recently, broad sequencing efforts that characterize the entire exome or genome have been initiated melanoma [42–45]. These studies have demonstrated that cutaneous melanomas have an extremely high somatic mutation rate. The majority of the observed somatic mutations were C → T or G → A transitions, which are associated with DNA damage from ultraviolet radiation (UVR) [46]. This data is consistent with multiple functional and epidemiological studies implicating UVR in the development of melanoma [47]. These broad sequencing studies have demonstrated the molecular complexity and heterogeneity of melanomas (Fig. 6.2) [43]. The studies have identified many additional somatic events that occur in melanomas with activating *BRAF* or *NRAS* mutations, as well as candidate drivers in melanomas that

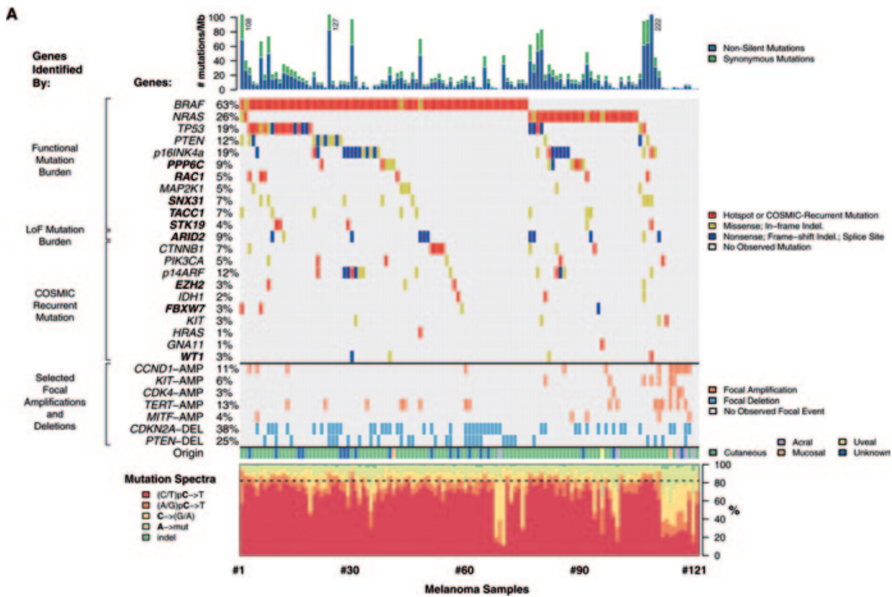

Fig 6.2 Pattern of novel and known somatic alterations in a cohort of 121 melanomas. (Adapted with permission from [43])

do not have a hotspot mutation in either of those genes. While these studies have already provided significant insight into the molecular pathogenesis of melanoma, it has also illuminated that a critical challenge to researchers will be to determine which mutations are clinically significant. In addition to being therapeutic targets, mutations may have clinical utility if they add to risk prediction models that are used to guide the selection of treatments for patients, or to inform the appropriate design of clinical trials [17, 18, 48]. While the large number of alterations observed in melanoma makes this overall appear to be a daunting challenge, the clinical experience with BRAF V600-mutant melanomas has already demonstrated the tremendous clinical impact such findings can have.

6.3 Development of BRAF Inhibitors

Early preclinical studies demonstrated that inhibition of BRAF in melanoma cell lines and xenografts with V600 BRAF mutations significantly slowed growth both in vitro and in vivo [49–51]. Based on this promising data, the effects of BRAF inhibition were tested in melanoma patients in clinical trials. Initial clinical trials mainly were performed with sorafenib. Sorafenib is a small molecule inhibitor of many kinases, including BRAF, although it actually binds to other targets (i.e. CRAF) with greater affinity. The first clinical trial of sorafenib in metastatic melanoma

patients demonstrated that less than 5 % of patients achieved a clinical response with this agent [52]. Another trial in which patients were treated with paclitaxel and carboplatin, and then were randomized to receive sorafenib or a placebo, again demonstrated that sorafenib had minimal impact on clinical response rates or progression-free survival (PFS) [53].

While these results were disappointing, a second wave of testing was precipitated by the development of drugs that were designed to be highly selective inhibitors of BRAF- and specifically of the BRAF V600E mutant protein encoded by the most common mutation of this gene. The first such agent to undergo testing was vemurafenib (also called PLX4032) [54]. Preclinical studies demonstrated that vemurafenib potently inhibited the MAPK signaling pathway, growth, and survival of BRAF V600-mutant human melanoma cell lines, but almost no effect was seen in cell lines without such a mutation [55]. Treatment of xenografts of these cell lines in mouse models demonstrated that the vemurafenib treatment caused tumor regression. This impressive activity accurately predicted the results seen in patients. In the phase I clinical trial of vemurafenib, approximately 80 % of the patients with BRAF V600E-metastatic melanoma had significant tumor shrinkage; in contrast, none of the 5 patients who did not have this mutation responded [56]. Subsequent preclinical studies in melanoma and other cancers by multiple groups found that treatment of cancer cells that did not have a BRAF V600 mutation, and particularly those with activation of RAS proteins, with vemurafenib and other compounds in this class caused increased tumor growth in vitro and in vivo [20, 57–59]. These studies showed that selective inhibitors of the BRAF V600-mutant protein actually caused increased activation of the MAPK pathway in these cell lines, as measured by increased phosphorylation of activation-specific sites on both MEK and ERK. This effect appears to be due to inhibitor-induced changes in the structure of the wild-type BRAF protein which results in a conformation that facilitates the formation of heterodimers with CRAF proteins. These BRAF-CRAF heterodimers activate MEK and ERK, and subsequently increase the growth of the tumor cells. Interestingly, this paradoxical activation of the MAPK pathway appears to be largely responsible for an interesting toxicity seen with vemurafenib: the development of cutaneous squamous cell carcinomas (SCCs) and/or keratoacanthomas (KAs). These lesions are observed in 20–25 % of patients treated with vemurafenib, and are generally treated successfully with surgery [56]. Molecular analyses demonstrated that these lesions frequently have mutations in *RAS* genes, and they demonstrate increased MAPK pathway activation following treatment with the mutant-selective BRAF inhibitors [60, 61]. This mechanism was recapitulated in animal models. Importantly, these studies demonstrated that adding a MEK inhibitor to the mutant-selective BRAF inhibitor blocked the formation of these hyperproliferative cutaneous lesions [60].

In addition to the critical importance of selecting patients with BRAF V600 mutations for treatment with vemurafenib, the phase I trial also demonstrated the specific relevance of MAPK pathway inhibition to the observed clinical benefit. A series of patients enrolled in the phase I trial underwent biopsies of their tumors before the start of treatment, and after 1 to 2 weeks of therapy. Analysis of P-ERK expression by immunohistochemistry (IHC) demonstrated that variable degrees

of MAPK pathway inhibition were achieved in these patients with vemurafenib treatment. When the changes in P-ERK were compared to the maximal changes in tumor size, a nearly linear relationship between these two factors was observed [62]. Greater inhibition of the pathway correlated with greater inhibition of tumor growth. This finding reinforced the importance of this pathway that was implied by the high prevalence of mutations observed in melanoma.

Subsequent clinical testing of vemurafenib was limited to patients with metastatic melanoma with V600E BRAF mutations. In the pivotal BRIM-3 phase 3 trial, such patients were randomized to treatment with vemurafenib or dacarbazine [63]. This trial was halted at its first analysis, and it was the shortest phase III clinical trial ever conducted in oncology. Treatment with vemurafenib produced significant improvements in response rate (48 versus 5 %, $p<0.001$), PFS (median 5.3 versus 1.6 months, Hazard ratio [HR] 0.26, $p<0.001$), and OS (6 month OS 84 versus 64 %, HR 0.37, $p<0.001$). Based on this data vemurafenib received regulatory approval for the treatment of metastatic melanoma patients with BRAF V600E mutations in 2011.

Dabrafenib is a structurally unrelated small molecule that also is a highly potent and selective inhibitor of V600-mutant BRAF proteins [64, 65]. In a randomized phase III trial comparing dabrafenib to dacarbazine in metastatic melanoma patients with BRAF V600E mutations, dabrafenib treatment resulted in significant improvements in response rate (50 versus 6 %) and PFS (5.1 versus 2.7 months, HR 0.30, $p<0.0001$) [66]. The effects on OS did not reach statistical significance (HR 0.61, 95 % confidence interval [CI] 0.25–1.48). However, in this trial patients who progressed on dacarbazine were allowed to cross-over to the dabrafenib treatment, which was not allowed in the BRIM-3 trial of vemurafenib. A third mutant-selective BRAF inhibitor, LGX818, is currently in early phase clinical testing[1].

The relatively short time that elapsed from the discovery of activating BRAF mutations to the regulatory approval of vemurafenib and dabrafenib stands as a powerful example of the speed and potential impact of genomics and translational research. It is clear that the selective BRAF inhibitors have delivered tremendous clinical benefit to patients with this highly aggressive disease. Indeed, symptomatic improvement is often observed within days of starting treatment. Frustratingly, however, the clinical benefit of the BRAF inhibitors is variable, and often short-lived. For example, in the BRIM-3 trial, only ~3 % of patients had disease progression as their best response, reinforcing that almost all patients experienced some degree of disease control. However, only 2 out of 219 patients achieved a complete response, and ~50 % of patients achieved only minor clinical responses (<30 % reduction in tumor size) [63]. This tremendous variability in the degree of tumor response likely reflects pre-existing heterogeneity among patients and/or tumor cells with activating BRAF mutations. Furthermore, the median duration of the responses with the BRAF inhibitors has generally been only 5–7 months in the various clinical trials with vemurafenib and dabrafenib [56, 63, 66, 67]. Approximately 90 % of patients develop resistance within 1 year of starting treatment. This resumption of growth

[1] www.clinicaltrials.gov.

after initial responsiveness to the BRAF inhibitors reflects the development of acquired, also called secondary, resistance.

Research has now identified a variety of mechanisms that may mediate resistance to the selective BRAF inhibitors. In general terms, these mechanisms either (1) cause re-activation of MAPK pathway effectors, or (2) result in activation of other pro-survival pathways. Similar to the selective benefit of vemurafenib and dabrafenib in patients with V600 BRAF mutations, these findings support the rationale to develop personalized approaches that will overcome these various mechanisms.

6.4 Rationale for Dual Inhibition of the MAPK Pathway

The strong correlation between MAPK pathway inhibition and clinical benefit observed in the clinical development of the selective BRAF inhibitors led to the hypothesis that resistance could be due to reactivation of signaling by the pathway. Due to the highly selective effects of the BRAF inhibitors in melanoma cells with V600 BRAF mutations, and the paradoxical pathway activation and growth observed in cells without these mutations, one explanation for the emergence of resistance could be the selective depletion of BRAF-mutant cells from molecularly heterogeneous tumors. Indeed, some studies have suggested that different regions of individual tumors vary in the relative frequency of cells with and without BRAF mutations [68]. However, sequencing analyses of melanoma samples collected at the time of resistance in multiple studies have demonstrated in all cases the continued presence of the same activating BRAF mutation that was present before the start of therapy [69, 70]. Similar results were also observed in cell lines that were selected in vitro for secondary resistance through chronic exposure to increasing doses of the BRAF inhibitors.

A second potential mechanism that could potentially cause resistance to the BRAF inhibitors would be the acquisition of secondary mutations in the BRAF gene. Secondary mutations in drug targets are a common finding in CML and gastrointestinal stromal tumors (GISTs) that have developed resistance to imatinib. Preclinical studies demonstrated that artificially introducing mutations at the Thr529 gatekeeper residue of BRAF could negate the inhibitory effects of vemurafenib and other selective BRAF inhibitors in melanoma cell lines [71]. However, despite this demonstration, and the experience with other targeted therapies, to date no secondary mutations in the BRAF gene have been identified in resistant melanoma tumors or cell lines [69].

While new mutations in BRAF have not been identified as a mechanism of resistance, two other alterations have: copy number gain and alternative splicing. Copy number gain of the mutant BRAF allele was identified in 4 of 20 (20%) progression samples by whole exome sequencing, with corresponding increased BRAF protein expression [72]. Resistance in cell lines with BRAF copy number gain could be overcome by treating the cells with increased doses of the selective

BRAF inhibitors, suggesting a therapeutic strategy for patients with resistance due to this mechanism. However, this strategy will likely not be effective in patients with resistance due to aberrant splicing of BRAF. This phenomenon was identified in 6 of 19 (32 %) progression samples from patients, as well as in several cell lines selected for resistance, which demonstrated expression of a smaller (61 kDa) form of the BRAF protein [73]. This truncated form of the protein efficiently forms heterodimers with CRAF, which subsequently activates MEK and ERK. This interaction between CRAF and the truncated BRAF was not prevented by treatment with increased doses of the selective inhibitors of BRAF. However, the continued dependence on MAPK pathway signaling was demonstrated by the fact that the cells remained sensitive to MEK inhibitors. The utilization of heterodimers by BRAF with other RAF isoforms at the time of resistance was also identified by another group of investigators, although the mechanism underlying the switch to this capability was not identified [74]. Those studies demonstrated that treatment of the studied resistant cell lines with MEK inhibitors was able to block activation of the pathway and induce growth inhibition. However, in contrast to the parental (sensitive) cells from which the resistant clones were selected, MAPK pathway inhibition alone was not sufficient to induce apoptosis, suggesting the potential for additional aberrations to be driving resistance concurrently.

In addition to alterations in BRAF, alterations in other members of the MAPK pathway produce reactivation of MEK and ERK signaling in spite of continued exposure to BRAF inhibitors. As mentioned previously, co-occurrence of BRAF V600E and activating NRAS mutations is detected in less than 1 % of newly diagnosed melanomas. However, this overlap is more common after exposure to selective BRAF inhibitors. The presence of activating NRAS mutations was initially described in 2 progressing tumors derived from the same patient; interestingly, the tumors actually had different NRAS mutations (Q61K and Q61R), implying that they had arisen from independent clones [69]. NRAS mutations were also identified in 4 of 19 (21 %) progressing lesions in another study, and were mutually exclusive with aberrant splicing of BRAF [73]. In vitro studies demonstrated that the presence of a concurrent NRAS mutation results in re-activation of ERK via CRAF and remains sensitive to MEK inhibitors. Whole exome sequencing of a single patient with acquired resistance to a BRAF inhibitor identified acquisition of a somatic mutation that resulted in a C121S substitution in MEK1 as a cause of resistance [75]. A subsequent sequencing analysis of *MEK* in clinical samples obtained before the start of treatment with vemurafenib and at the time of progression identified several mutations in the gene. Interestingly, some of the mutations (i.e. P124L substitution) were identified in the pre-treatment samples in patients who achieved clinical responses, suggesting that they were not sufficient to cause resistance. However, other mutations (i.e. Q56P) were identified only in progressing lesions, and thus likely causative of disease progression [70]. This heterogeneity implies that additional studies will be needed over time to classify the functionality and clinical significance of various MEK mutations [76]. Finally, overexpression of COT, a serine-threonine kinase that is capable of activating downstream components of the MAPK pathway, was observed following BRAF inhibition in 2 of 3 patients

samples obtained early in their treatment with vemurafenib, and in 1 patient was highest at the time of disease progression [77]. While in vitro studies suggested that COT may be able to phosphorylate ERK directly, BRAF inhibitor-resistant cells with enforced COT expression remained sensitive to MEK inhibitors.

The identification of multiple molecular aberrations that cause reactivation of MAPK pathway signaling supports the rationale to target this pathway at multiple levels (Fig. 6.3) [78]. Analysis of tumor biopsies obtained after 2 weeks of treatment in the phase II clinical trial of vemurafenib demonstrated that patients who did not achieve clinical responses had significantly less inhibition of ERK activation than patients who responded [70]. This demonstration of early, incomplete inhibition of the pathway in some patients suggested that combined inhibition may not only be an effective strategy to use after acquired resistance develops, but also potentially as a way to improve the magnitude, and hopefully duration, of the initial responses to therapy. These hypotheses are now supported by the clinical experience with combinatorial therapy with BRAF and MEK inhibitors.

Trametinib is an orally available potent inhibitor of MEK1/2 [79]. Clinical testing has demonstrated that trametinib has activity as a single agent in metastatic melanoma patients with BRAF V600 mutations who have not previously been treated with BRAF inhibitors. In a randomized phase III trial of trametinib versus chemotherapy that allowed cross-over at the time of progression, trametinib treatment

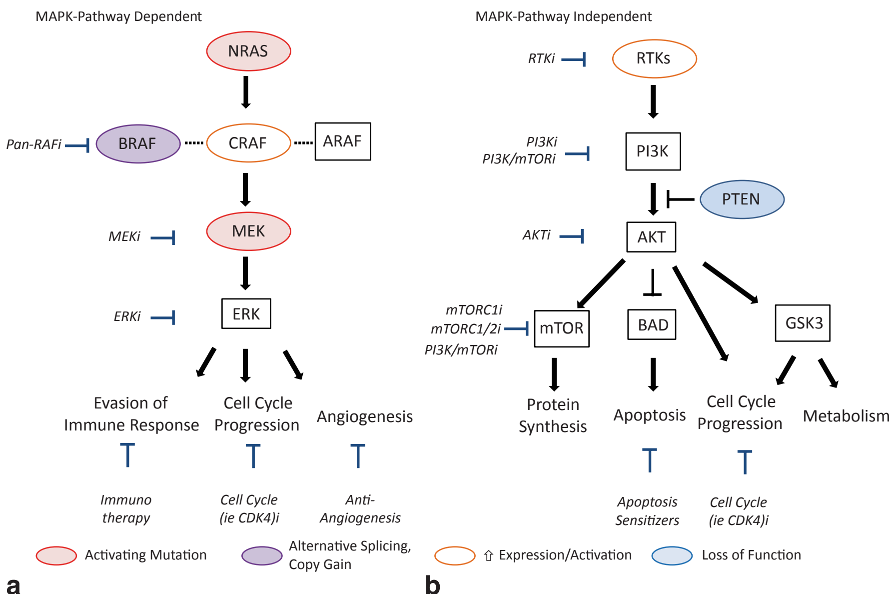

Fig 6.3 Resistance mechanisms and combinatorial strategies for BRAF-mutant melanomas. Schema of described mechanisms of resistance to selective inhibitors of mutant BRAF in melanoma. **a** MAPK-pathway dependent mechanisms. **b** MAPK-pathway independent mechanisms. Classes of agents that may be used to target components of the pathways are indicated to the side in each panel. (Used with permission from [78])

produced significant improvements in response rate (22 versus 8%, $p = 0.01$), PFS (4.8 versus 1.5 months, HR 0.45, $p < 0.0001$), and OS (6 month OS 81 versus 67%, HR 0.54, $p = 0.01$) [80]. While the enthusiasm about these results were dampened specifically in the melanoma field in light of the parallel development and results of selective BRAF inhibitors, this trial represents the first positive phase III trial for a MEK inhibitor in any cancer type, thus confirming the potential for clinical utility for these agents. However, even more impressive results were observed when trametinib was combined with the selective BRAF inhibitor dabrafenib. A randomized phase II study was conducted in BRAF inhibitor-naïve metastatic melanoma patients with BRAF V600 mutations (V600E or V600K) [81]. All patients received the standard dose of vemurafenib (150 mg twice daily), and then were randomized to receive placebo, half-dose (1 mg per day; referred to as "150/1" treatment) or full-dose (2 mg per day; "150/2") trametinib after these combinations were demonstrated to be safe and well-tolerated. Consistent with preclinical studies implicating paradoxical activation of the MAPK pathway as the mechanism of cutaneous SCCs and KAs from BRAF inhibitor therapy, the incidence of these lesions was markedly reduced in patients who received MEK combination therapy (2% with 150/1, 7% with 150/2) compared to those who received dabrafenib alone (19%). Patients who received the combination were also less likely to develop rashes, although other toxicities (i.e. acneiform dermatitis, fevers/chills, nausea/vomiting, diarrhea, neutropenia) were more frequent. However, these toxicities were generally manageable with supportive care or interruption of treatment. More importantly, the combination demonstrated significant improvements in multiple clinical outcomes. The clinical response rates were 54% for dabrafenib monotherapy, 50% for the 150/1 combination, and 76% (including 9% complete responses) for the 150/2 combination. The median PFS was 5.8 months for dabrafenib monotherapy, 9.2 months for 150/1 (HR 0.56, $p = 0.006$), and 9.4 months for 150/2 (HR 0.39, $p < 0.001$). At 12 months only 9% of patients treated with dabrafenib alone remained progression-free, compared to 26% with 150/1 and 41% with 150/2.

Trametinib has also undergone early evaluation in patients who have progressed on BRAF inhibitors. Despite preclinical evidence that cells with acquired resistance to BRAF inhibitors often remain sensitive to MEK inhibition, to date these results have been relatively disappointing. Treatment with single agent trametinib in failed to result in a clinical response in 37 patients who had developed resistance to a selective BRAF inhibitor, although 2 of 3 patients who had stopped BRAF inhibitor therapy due to toxicities did respond [82]. The median PFS of the patients overall was only 1.8 months. Combined treatment (150/2) with dabrafenib and trametinib achieved clinical responses in 4 out of 21 (20%) patients who had previously progressed on a BRAF inhibitor, and in 1 of 5 (20%) patients who had progressed on a MEK inhibitor [83]. Although full interpretation of the results will require additional follow-up to allow for meaningful assessment of time-dependent outcomes, one implication of the results is that it may be more effective to continue BRAF inhibitors and add other agents to this therapy in patients who progress on BRAF inhibitors than to simply change them to different targeted agents. This finding, if confirmed, would be similar the clinical experience in HER2/neu-positive breast cancer patients following progression on trastuzumab.

While the slight improvement observed with the combination of dabrafenib and trametinib treatment compared to trametinib alone in the progressing patients is interesting, overall the relatively low activity has been disappointing in the face of the results observed in BRAF inhibitor naïve patients, and the multiple studies supporting reactivation of MEK at the time of resistance. The evidence of significant benefit in some patients, however, does suggest that it may be possible to predict which patients this regimen is effective in by comparison of the clinical outcomes to the underlying resistance mechanisms. Alternatively, early assessment of the regimen's ability to inhibit ERK activation may predict benefit. However, no data has been presented to date testing either possibility. In turn, while combined inhibition of BRAF and MEK as front-line therapy has been very impressive, it is clear that many patients are still developing resistance in a relatively short period of time, and it is unclear how many, if any, of the patients treated with the combination are achieving the durable disease control that is seen in some patients treated with immunotherapies.

Despite these limitations, the rapid advances in outcomes that have been achieved again demonstrate the dramatic potential clinical benefit for rational combinatorial treatment approaches. Additional testing is currently ongoing with other BRAF and MEK inhibitors to determine whether differences in pharmacological properties may result in greater efficacy. Alternative dosing regimens have also been proposed as another strategy to prevent or delay resistance in preclinical models, but there is no clinical data yet addressing this hypothesis [84]. Evaluation of inhibitors of other targets in the MAPK pathway, including ERK, is also ongoing [85]. However, multiple lines of evidence also suggest that strategies that combine MAPK pathway inhibition with targeting of other pathways may be an effective clinical strategy for some patients.

6.5 The PI3K-AKT Pathway as Combinatorial Target

Although the activating BRAF mutation is the most frequent somatic event in melanoma, and a valuable therapeutic target, several lines of evidence support that other pathways likely play a critical role in this disease. For example, benign nevi have a rate of BRAF V600 mutations that is similar to or higher than the rate observed in melanomas [86]. As benign nevi have an extremely low rate of malignant transformation, this finding demonstrates that other events must complement the BRAF mutation to fully explain the aggressive biology of this cancer. Of note, mutations in NRAS are also common in benign nevi [87]. In addition to this observation, functional studies in zebrafish, mice, and human cells have demonstrated that introducing expression of V600-mutant BRAF proteins alone in normal melanocytes fails to induce malignant transformation [88–90]. These model systems have provided a way to functionally interrogate candidates that may contribute to melanomagenesis. Finally, while many clinical specimens and cell lines with acquired resistance to selective BRAF inhibitor exhibit re-activation of the MAPK pathway, this has not

been a universal finding [69, 77, 91]. While many pathways remain to be interrogated, a number of studies support that the PI3K-AKT pathway can play an important role in this disease.

The PI3K-AKT pathway is a critical regulator of many cellular processes, including growth, survival, anchorage independence, motility/invasion, angiogenesis and metabolism, among others. The significance of the PI3K-AKT pathway in cancer is supported by the finding of a high rate of somatic alterations, including mutations, amplifications, and deletions, in multiple components of this pathway in many tumor types [92–94]. As described previously, activation of the PI3K-AKT pathway was initially implicated in melanoma by the identification of activating NRAS mutations and loss of function of the PTEN tumor suppressor. Interestingly, similar to previous comparison of PTEN loss and PI3K mutations, quantitative analysis demonstrated that PTEN loss correlates with significantly greater activation of AKT than NRAS mutations, as measured by expression of phosphorylated (activated) AKT protein, in melanoma cell lines and clinical specimens [95, 96]. While mutations in the catalytic subunit of PI3K, *PIK3CA,* are common in several tumor types, they are detected in only 1–2 % of melanomas [97]. Point mutations in the regulatory pleckstrin homology (PH) domain of AKT1 have been identified as rare events in several tumor types, including melanoma (~ 1 %) [98]. In addition, the analagous mutation in *AKT3* has been identified uniquely in melanoma [28]. This finding builds upon several other studies specifically implicating AKT3 as an important AKT isoform in this disease, whereas most research in other cancers implicates AKT1 and/or AKT2 [99–101]. Finally, mutations and amplifications of oncogenic receptor tyrosine kinases (RTKs) that activate signaling through the PI3K-AKT pathway in other cancers, such as HER2/neu and the epidermal growth factor receptor (EGFR), have not been detected as significant events in cutaneous melanomas, although aberrations of the KIT RTK have been implicated in other subtypes [32]. One report indicated that mutations throughout the sequence of the ERBB4 (HER4) gene were detected in ~ 20 % of melanomas [102]. Although this pattern of mutations was curious for a proposed oncogene, functional studies in cell lines with enforced expression of several of the variants detected in patients did suggest that the mutations were activating. However, recent whole exome sequencing efforts have not identified somatic mutations of ERBB4 as a significant event [42, 43].

A role for activation of the PI3K-AKT pathway in the transformation of melanocytes has been suggested primarily in preclinical models. In a genetically engineered mouse model (GEMM) in which inducible loss of PTEN in melanocytes was achieved with topical treatment with 4-hydroxytamoxifen, no melanocytic lesions were observed. In the same model, induction of the BRAF V600E mutation in newborn mice resulted in melanocyte hyperplasia, but no invasive lesions (melanomas) were observed. However, crossing of the two strains of mice to generate targeted expression of the BRAF V600E mutation with loss of PTEN expression in melanocytes resulted in invasive melanomas in all mice within 7–10 days of 4-hydroxtamoxifen treatment. In addition to being 100 % penetrant, the tumors formed spontaneous metastases in all of the mice. All mice required euthana-

sia within 25–50 days of induction [88]. Expression of an activated form of AKT3 (myr-AKT3) also transforms human melanocytes that express the BRAF V600E protein [100]. Interestingly, although NRAS mutations and genetic loss of PTEN are mutually exclusive in patients, loss of PTEN increased the metastatic potential and invasive behavior of NRAS-mutant melanomas in another GEMM [103].

Studies in advanced melanomas also support that PTEN loss is important functionally. In particular, a number of studies have compared BRAF-mutant human melanoma cell lines that lack PTEN to those that have normal PTEN function. Loss of PTEN correlates with increased activation of AKT in BRAF-mutant cell lines and tumors, and is also observed after knockdown of PTEN expression with RNAi [104]. Treatment of BRAF-mutant, PTEN-null human melanoma cell lines with BRAF or MEK inhibitors generally results in cytostatic effects, although one study identify a subset of resistant lines that also had loss of *Rb* [105]. In contrast to other BRAF-mutant cell lines, most of the cell lines with loss of PTEN fail to undergo apoptosis following treatment with BRAF or MEK inhibitors [104–107]. Resistance to apoptosis can also be induced in BRAF-mutant cell lines by inhibiting PTEN expression with RNAi [104]. These findings support that BRAF-mutant melanomas with loss of PTEN may exhibit at least some degree of de novo resistance to MAPK pathway inhibitors. Sequencing and copy number analysis of 34 patients enrolled in the phase I and phase II studies of dabrafenib detected aberrations in the PTEN gene in 11 (32 %) of the patients [108]. Patients with PTEN loss had a similar rate of clinical response (36 %) as those with genetically intact PTEN (43 %). However, PTEN loss showed a very strong trend, even in this relatively small set of patients, for shorter PFS (18 weeks versus 32 weeks, $p = 0.06$). Overall, analysis of samples collected at the time of disease progression found that homozygous deletion of PTEN was observed more frequently (4/10) than in the pre-treatment samples (2/34, $p = 0.017$). A previous analysis of 5 patient that had matching pre-treatment and disease progression samples found discordance in 1 sample, which exhibited homozygous loss of PTEN at disease progression [74].

In addition to constitutive activation in melanomas with PTEN loss, it appears that activation of the PI3K-AKT pathway through growth factor receptors can mediate resistance to BRAF and MEK inhibitors. Characterization of two BRAF-mutant, PTEN-expressing human melanoma cell lines with de novo cell resistance to apoptosis induction demonstrated that these cell lines had similar degree and duration of inhibition of the MAPK pathway as cell lines destined to undergo apoptosis, but they were unique in that they developed marked activation of AKT after MEK inhibitor treatment [104]. Similar results were also observed subsequently with selective BRAF inhibitors [107]. Inhibition of the insulin-like growth factor 1 receptor (IGF1R), which both of the resistant cell lines expressed at high levels, abrogated the compensatory activation of AKT. Inhibition of IGF1R alone did not induce apoptosis in the cells, but marked cell death was observed when that was combined with MEK inhibition. This synergistic effect on apoptosis induction was recapitulated by knocking down AKT, or by inhibiting AKT activation with a dual TORC1/2 inhibitor, demonstrating that PI3K-AKT activation was mediating IGF1R-induced resistance.

Overexpression of IGF1R was also observed independently by investigators characterizing cell lines selected in vitro for secondary resistance to selective BRAF inhibitors [74]. These cell lines also demonstrated resistance to MEK inhibition by BRAF inhibitors through utilization of multiple RAF isoforms. While the MAPK pathway activation could be blocked in these cells by treatment with a MEK inhibitor, this failed to induce apoptosis in the resistant clones. Apoptosis was only seen with the MEK inhibitor when it was combined with a small molecule inhibitor of either IGF1R or PI3K. Analysis of matching samples from 5 patients treated with a selective BRAF inhibitor detected increased IGF1R expression in 2 patients at the time of disease progression (a third tumor had loss of PTEN). Resistant cell lines developed and characterized by another group of investigators also identified multiple RTKs that were upregulated at the time of resistance [69]. Although multiple RTKs were overexpressed (i.e. KIT, MET, EGFR), only the PDGFRβ was found to be activated by antibody array analysis. Increased activation of PDGFRβ was also identified in 4 (36 %) of 11 patients with matching pre-treatment and progression samples following BRAF inhibitors. Functional testing demonstrated that the cell lines did not undergo apoptosis with MEK inhibitors alone, but did when MEK inhibitors were combined with either AKT or dual PI3K-mTOR inhibitors [109]. It is interesting to note that the two groups of investigators found completely non-overlapping RTKs mediating resistance in their different experimental systems. In addition, investigations by both groups failed to identify any mutations or amplifications of the genes encoding the implicated receptors [69, 74]. Thus, the induction of the RTKs appears to reflect an epigenetically-mediated mechanism of resistance.

While these studies identified resistance mechanisms that are intrinsic to the tumor cells, there is also evidence that activation of the PI3K-AKT pathway may be mediated in part by extrinsic factors. Two groups independently demonstrated that production of the growth factor HGF by stromal cells was capable of mediating resistance to BRAF inhibitors in BRAF-mutant human melanoma cells in co-culture systems [110, 111]. Supporting the clinical relevance of this finding, analysis of pre-treatment samples of patients treated with BRAF inhibitors demonstrated that increased expression of HGF in stromal cells correlated with a decreased chance of achieving a clinical response [110]. While not evaluated in patients, analysis of BRAF-mutant human melanoma cell lines showed that HGF did not rescue the cells from inhibition of MAPK signaling by BRAF inhibitors, but it induced PI3K-AKT pathway activation. The resistance mediated by exogenous HGF could be overcome by treating the cells with inhibitors of c-MET, the receptor for HGF, or with PI3K inhibitors.

The data implicating PTEN loss, RTK overexpression, and stromal growth factors together provide a strong rationale targeting the PI3K-AKT pathway in BRAF-mutant melanomas. Of note, data from these preclinical models suggests that only inhibiting the PI3K-AKT pathway is unlikely to be effective, due to both constitutive and compensatory activation of MAPK pathway signaling. In contrast, multiple studies have demonstrated that inhibition of the PI3K-AKT pathway can specifically sensitize cells to apoptosis induction by BRAF or MEK inhibitors [104–107,

109, 112]. In addition to increasing the degree of apoptosis, it appears that the timing of apoptosis induction is also shorter than what is observed with MAPK pathway inhibition alone. This suggests that intermittent dosing of PI3K-AKT pathway inhibitors may be an effective therapeutic strategy, which is supported by xenograft studies [113]. Examination of various dosing schedules may be critical to clinical development in this area, as the important role of the PI3K-AKT pathway in many basic physiological processes will likely make achieving an acceptable therapeutic index challenging. In contrast to the opportunity to target a tumor-specific activating mutation afforded by the BRAF V600 mutations in the MAPK pathway, activating mutations in the PI3K-AKT pathway are rare in melanoma. One possible route to improved therapeutic indices may be the use of isoform-specific inhibitors. For example, data in melanoma supports that the AKT3 isoform may be selectively important in melanoma progression, whereas its expression and function in most normal tissues appears to be rather limited [99, 114]. While inactivating mutations in PTEN are not directly targetable, two different studies have shown that PTEN loss may result in selective dependence on the β-isoform of the catalytic unit of PI3K (P110β, or *PIK3CB*) [115, 116]. As P110β appears to have a much more limited role in normal physiology, this may again allow for selective targeting of PTEN-null tumor cells, and thus an acceptable therapeutic index.

The clinical development of combinatorial strategies against the PI3K-AKT pathway is also complicated by several other factors [117, 118]. First, there are multiple different classes of inhibitors available to target the pathway, and generally multiple agents in each class undergoing clinical evaluation (Table 6.2). These classes include PI3K inhibitors (pan-PI3K and isoform-specific), dual PI3K-mTOR inhibitors, AKT inhibitors, TORC1 inhibitors (rapamycin-like agents), and dual TORC1/2 inhibitors. Previous studies support that different mechanisms of PI3K-AKT pathway activation can result in functional dependence on different effectors [96]. Thus, optimal clinical testing of the pathway may need to match the choice of therapeutic agent to the mechanism of pathway activation that is present in the patient. As the development of vemurafenib demonstrated, the rational testing and assessment of PI3K-AKT pathway inhibitors for melanoma would be facilitated by the identification of a reliable biomarker that correlates with efficacy/clinical benefit. However, while pharmacodynamic markers that do exist to determine if targets in the pathway have been inhibited, it still is unclear which targets, and what degree of target inhibition, are required for efficacy/synergy. Finally, studies in both patients and cell lines have demonstrated that the PI3K-AKT pathway is regulated by multiple feedback loops [119]. As a result, inhibition of a single target in the pathway may rapidly lead to a compensatory signaling mechanism that reactivates itself and/or other pathway effectors. Such feedback compensatory mechanisms have been observed with AKT, TORC1, and dual TORC1/2 inhibitors [120–122]. Thus, meaningful analysis of the effects of PI3K-AKT pathway inhibitors will likely require broad analysis of pathway markers in additional to pharmacodynamic evaluation of on-target effects.

Table 6.2 Classes of PI3K-AKT pathway inhibitors. GSK=GlaxoSmithKline. (Adapted with permission from [118])

Category	Examples
PI3K Inhibitors	BAY 80-6946 (Bayer) BKM120 (Novartis) GDC-0941 (Genentech) PX-866 (Oncothyreon) XL-147 (Exelixis) ZSTK474 (Zenyaku Kogyo)
PI3K: isoform-specific inhibitors	p110α-specific: BYL719 (Novartis) INK1117 (Intellikine) P110β-specific: GSK2636771 (GSK) SAR260301 (Sanofi) p110δ-specific: AMG 319 (Amgen) CAL-101 (Calistoga)
Dual PI3K/mTOR inhibitors	BEZ235, BGT226 (Novartis) GDC-0980 (Genentech) GSK2126458 (GSK) PF-4691502, PF-5212384 (Pfizer) SF-1126 (Semafore) XL765 (Exelixis)
AKT inhibitors	GDC-0068 (Genentech) GSK2110183 (GSK) MK-2206 (Merck) Perifosine (Keryx)
mTORC1 inhibitors	Everolimus (Novartis) Sirolimus (Pfizer) Ridaforolimus (Merck) Temsirolimus (Pfizer)
Dual mTORC1/2 inhibitors	AZD8055 (AstraZeneca) OSI-027 (Astellas)

6.6 Other Targets and Oncogenes

The clinical development of combinatorial approaches utilizing selective inhibitors of the V600-mutant BRAF protein is progressing rapidly as described. As these inhibitors may increase the growth of melanomas with a wild-type BRAF gene, these approaches are not likely to be applicable to patients without activating *BRAF* mutations. The non-V600 BRAF mutant population includes more than half of cutaneous melanoma patients, and even higher percentages of patients with other types of melanoma (i.e. acral, mucosal, and uveal). Thus, combinatorial strategies are also being developed for other targets that have been identified in this disease.

Activating mutations of *NRAS* are the second most common oncogenic somatic mutation detected in cutaneous melanomas. In addition to their prevalence, studies in both early- and late-stage melanoma patients support that melanoma patients with *NRAS* mutations have a worse prognosis than patients with activating *BRAF* mutations or wild-type *BRAF* and *NRAS* [17, 48]. Thus, the development of effec-

tive therapies for this subset of patients is a high priority. Direct targeting of RAS proteins is difficult to achieve due to the high affinity of the mutant RAS for GTP. Targeting RAS activation by inhibiting post-translational modifications that are required for its activation has been attempted in multiple tumor types, but to date this strategy has failed to produce clinical benefit [123]. As targeting RAS itself is challenging, multiple strategies have been developed to inhibit the multiple effector pathways that mediate its oncogenic effects [124, 125]. As activation of the RAS-RAF-MEK-ERK signaling cascade appears to be central to its effects, MEK inhibitors have been explored extensively as single agents and in combinations. A clinical trial with the MEK inhibitor binimetinib (MEK162) reported that clinical responses were observed in 28 % of patients with activating *NRAS* mutations, while an additional 46 % achieved disease stabilization [126]. However, the duration of disease control was quite short, and the overall median PFS was only 3.65 months. Multiple preclinical studies support that combined inhibition of MEK with targets in the PI3K-AKT pathway may be an effective strategy in RAS-mutant cancers, including melanoma [127–129]. Multiple clinical trials are currently ongoing testing this strategy. Recently, a GEMM of doxycycline-inducible mutant NRAS-expressing melanoma was used to compare the effects of MEK inhibitor treatment to complete extinction of NRAS signaling (doxycycline withdrawal) [130]. Surprisingly, the experiments demonstrated that MEK inhibition had similar efficacy to NRAS withdrawal in terms of apoptosis induction, but it was inferior at blocking cellular proliferation. Pathway analysis identified the cell cycle regulator CDK4 as a targetable node that correlated with this difference, and combined treatment with small molecule CDK4 inhibitors induced complete tumor regression in both the GEMM and in xenografts of NRAS-mutant human melanoma cells. Clinical trials will test the safety and efficacy of this strategy in patients. CDK4 is also an attractive combinatorial target in melanomas with activating BRAF mutations, as these tumors can have loss of P16, as well as activation of CDK4 (mutation or amplification) [42, 43]. Both loss of P16 and increased gene copy number of cyclin D1, another cell cycle regulator, correlated with shorter PFS in patients treated with dabrafenib in phase I/II clinical trials, providing further support for the clinical testing of this approach [108].

Activating mutations in *GNαQ* or *GNα11* are present in the majority of uveal melanomas, particularly those that have metastasized [36]. The most common mutations in these genes occur at the residue that is analogous to the Q61 residue of RAS proteins. Thus, similar to RAS, therapeutic development is generally focusing on effector pathways that are downstream of these mutations [35]. The initial characterization of *GNαQ* mutations demonstrated that this event activates signaling through the RAS-RAF-MEK-ERK signaling pathway. Preliminary results suggest that MEK inhibitors may be clinically effective in these patients. However, in vitro studies demonstrated that the efficacy of MEK inhibition may be compromised by compensatory activation of the PI3K-AKT pathway [131]. Combined treatment with MEK and PI3K inhibitors induced synergistic growth inhibition and apoptosis, supporting the rationale for testing of this combination in uveal melanoma. Testing is also ongoing with other effectors, including inhibitors of protein kinase C (PKC).

Alternatively, strategies to target growth factors and/or their receptors that are critical to growth in the liver, which is the most common metastatic site for uveal melanoma, are being evaluated clinically and preclinically [132].

6.7 Summary and Future Directions

The development of targeted therapy strategies for metastatic melanoma is evolving rapidly due to the improving understanding of molecular biology, new insights into the key determinants of clinical efficacy of targeted therapies, and the availability of multiple new agents against targets of interest. Based on emerging clinical and preclinical data, testing is rapidly moving from evaluation of single agents to rational combinatorial approaches. While this discussion has focused on the development specifically of combinations of multiple targeted therapies, the clinical management of melanoma patients generally utilizes multiple different therapeutic modalities. Experimental data supports that targeted therapies may synergize with many of these modalities, including chemotherapy, immunotherapy, and radiation [133–137]. In turn, the use of targeted therapy in combination with surgery, either in the adjuvant or neoadjuvant setting, has a strong rationale for development to see if this can improve cure rates in patients with clinically localized or regional disease. Thus, while the initial development of targeted therapy for melanoma has been highlighted by both successes and disappointments, the potential and future for this therapeutic approach remains bright.

Conflicts of Interest M.A.D. has served on advisory boards for GlaxoSmithKline, Genentech, Sanofi-Aventis, and Novartis, and has received research funding from GlaxoSmithKline, Genentech, AstraZeneca, Merck, Myriad, Sanofi-Aventis, and Oncothyreon.

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Chapter 7
Targeting the Cell Cycle and p53 in Combination with *BRAF*-Directed Therapy

Dale Han and Keiran SM Smalley

Abstract The incidence of melanoma continues to increase with the vast majority of skin cancer-related deaths attributable to melanoma. Historically, response rates for systemic treatments for metastatic melanoma were only 5–20 % for chemotherapy, and the prognosis of patients with metastatic disease was extremely poor. The discovery of *BRAF* mutations in melanoma led to the development of *BRAF*-directed therapy which dramatically increased response rates. However, most patients treated with *BRAF* inhibitors eventually show disease recurrence, and it is now believed that combination therapies based upon a *BRAF* inhibitor backbone is the therapeutic future. Appropriate regulation of the cell cycle is critical for preventing progression to cancer, however the vast majority of melanomas harbor alterations in cell cycle and p53 regulatory pathways such as loss of CDKN2A and overexpression of CDK4, cyclin D1, MDM2 and MDM4. The alterations in these pathways appear to play critical roles in the development of melanoma and may represent potential therapeutic targets. Furthermore, some studies suggest that there is interaction between *BRAF*, key cell cycle proteins and the p53 pathway and that *BRAF* inhibitors may synergize with treatments that either enhance p53 function or inhibit CDK activity. Preclinical studies in melanoma have shown the potential efficacy of enhancing p53 function through inhibition of MDM2 or MDM4. Other studies have shown potential benefit in antagonizing CDK activity through use of small molecule inhibitors. However, targeting p53 and CDK function in melanoma is at an early stage and additional studies are needed particularly to understand the effects of combining these therapies with *BRAF* inhibition. Furthermore, clinical trials testing these therapeutic combinations specifically in melanoma patients are also needed to determine if the results of preclinical studies can be translated into beneficial effects in humans.

Keywords Melanoma · *BRAF0* · Cell cycle · Cyclin dependent kinase · p53 · MDM2 · MDM4 · CDKN2A · p14 · p16

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7.1 Introduction

The incidence of melanoma continues to increase at a rate of 2–5% per year with an estimated 76,250 new cases diagnosed in 2012 [120]. The magnitude of this disease is further exacerbated by the fact that although melanoma represents 5% of skin cancers, it is the cause of 80% of skin cancer-related deaths [93]. The majority (70%) of newly diagnosed patients present with thin melanoma (≤1 mm) and the prognosis for patients with only local disease is excellent [87, 46, 7]. However, approximately 4% of patients present with distant metastatic melanoma and a subset of patients with localized melanoma eventually develop systemic metastases and have significantly worsened survival [7, 32].

In the past, systemic treatments for distant metastatic melanoma were generally ineffective with response rates of 5–20% for chemotherapy, and the prognosis of patients with distant disease is dismal with median survival rates of less than 1 year [48, 7]. However, in 2011 a turning point was achieved in the fight against metastatic melanoma with the FDA approval of both anti-CTLA-4 antibody immunotherapy and *BRAF* targeted therapy. In patients treated with the anti-CTLA-4 antibody ipilimumab, an objective response was seen in 10.9% of patients and median overall survival was significantly increased to 10 months [59]. Even more impressive were the results seen with targeted therapy using vemurafenib in *BRAF* mutated melanoma. In the pivotal phase III trial, 48% of treated patients with metastatic melanoma had an objective response as determined in an interim analysis, although the vast majority of patients had some decrease in tumor size, and the median progression-free survival was significantly extended to 5.3 months compared with 1.6 months for patients treated with dacarbazine [26]. Despite the impressive results and dramatic response rates seen after vemurafenib therapy, nearly all of these patients eventually developed recurrent disease. The mechanisms by which melanomas acquire resistance to *BRAF* inhibitors is an active area of research, and it is now apparent that combination therapy based upon a *BRAF* inhibitor backbone is the therapeutic future for disseminated disease [124, 127, 122, 123]. At this juncture, the cellular pathways that need to be targeted in conjunction with mutant *BRAF* are still being determined.

Acquisition of a *BRAF* mutation is believed to be an early event in melanoma development as evidenced by the fact that over 80% of nevi harbor a *BRAF* mutation [104, 75]. As a single hit, oncogenic *BRAF* drives melanocytes into senescence and it is known that additional genetic insults are required for melanomagenesis. Among the hits identified so far that contribute to melanoma development are alterations in regulatory pathways for p53 and the cell cycle [28, 33; 16, 63, 135]. In this chapter, we will review the important alterations in the cell cycle and p53 regulatory pathways implicated in melanoma initiation and progression and will discuss the potential for targeting these alterations in combination with oncogenic *BRAF*.

7.2 Cell Cycle

Cells divide through a systematic and precisely regulated process with the ultimate goal of producing viable daughter cells that each possesses a set of faithfully duplicated chromosomes (Fig. 7.1). The majority of cells exist in G0 phase of the cell cycle, which is also known as quiescence or senescence. In the quiescent state, cells no longer replicate but have the potential to re-enter the cell cycle, whereas senescence refers to a cellular response to various types of stress (e.g. DNA damage, oncogene activation, oxidative stress, etc.) in which a cell is primarily arrested in G1 phase and has irreversibly lost the capability to replicate [48, 4]. The ability of cells to enter senescence in response to oncogene activation is believed to be a potential barrier to tumorigenesis [4]. Upon receiving mitogenic signals, a cell leaves G0 phase and enters G1 phase in which there is growth in preparation for S phase. In S phase, DNA is replicated with high fidelity, and is followed by G2 phase where cells continue to grow and make final preparations for M phase where mitosis and later cytokinesis occur. Depending on the cellular and signaling milieu, cells may either return to G1 phase to continue dividing or enter G0 phase [49, 83, 112].

7.2.1 Regulation of the Cell Cycle: Cyclin Dependent Kinases

The cell cycle is tightly regulated by a series of serine/threonine kinases known as cyclin-dependent kinases (CDKs) that form heterodimers with regulatory cyclins [83, 112]. According to the "classical" model, each phase of the cell cycle is controlled by the cyclic expression and activation of specific cyclins and CDKs

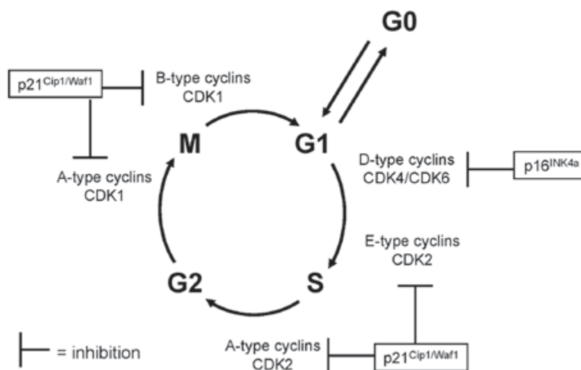

Fig. 7.1 Phases of the cell cycle. *G0* represents quiescent or senescent cells. Upon receiving mitogenic signals, cells enter *G1* phase and proceed through *S*, *G2* and *M* phases. Cells may then either re-enter *G1* phase to continue dividing or enter *G0* phase. Based on the "classical" model of the cell cycle, each phase is controlled by the expression of specific cyclins and cyclin-dependent kinases. In addition, cyclin-dependent kinase inhibitors such as $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ play important roles in helping to regulate the cell cycle. *CDK* cyclin-dependent kinase

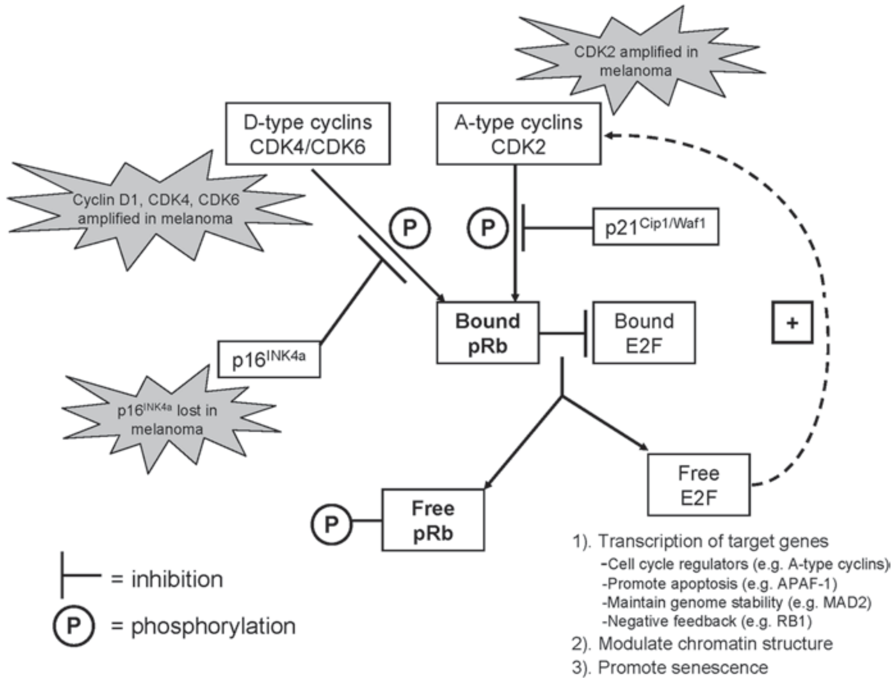

Fig. 7.2 The retinoblastoma protein (*pRb*) pathway. The transcription factor *E2F* is normally bound to and repressed by *pRb*. *E2F* plays a critical role in controlling the transcription of numerous genes involved in cell cycle regulation, apoptosis and maintaining genome stability. In addition, *E2F* is also involved in regulating chromatin structure and in promoting senescence. *CDK4* and *CDK6* that have been activated by *D-type cyclins* phosphorylate *pRb* which causes the release of *E2F* and transcription of *E2F* target genes. A positive feedback loop exists where *E2F*-mediated transcription leads to increased levels of *A-type cyclins* and eventual activation of *CDK2*. Activated *CDK2* then further phosphorylates *pRb* leading to release of additional *E2F* and passage through the "restriction point" of the cell cycle. In contrast, a negative feedback loop also exists where *E2F* activation leads to increased *pRb* levels, via transcription of the *RB1* gene, and sequestration of *E2F*. *CDK* inhibitors $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ play pivotal roles in regulating the *pRb* pathway by inhibiting *CDK4/CDK6* and cyclin-*CDK2/CDK1* complexes, respectively. In melanoma, prominent alterations in the *pRb* pathway are seen and include loss of $p16^{INK4a}$ and amplification of *cyclin D1*, *CDK2*, *CDK4* and *CDK6*. *CDK* cyclin-dependent kinase, *P* phosphorylation, *pRb* retinoblastoma protein

(Fig. 7.1). In response to mitogenic signals, D-type cyclins are expressed in early G1 phase and activate CDK4 and CDK6. Activated CDK4 and CDK6 then phosphorylate retinoblastoma protein (pRb) causing the release of transcription factor E2F, which is normally bound to and repressed by pRb (Fig. 7.2). This allows E2F to proceed with transcription of target genes including E-type and A-type cyclins. Expression of E-type cyclins during G1 phase activates CDK2, which then further phosphorylates pRb leading to amplification of E2F-mediated transcription. These steps ultimately result in G1 to S phase transition and passage through the "restriction point" at which point the cell has committed to cellular division. During S

phase, CDK2 associates with A-type cyclins to allow for progression from S to G2 phase. Eventually, CDK1 binds to A-type cyclins to initiate mitosis (G2 to M phase). A-type cyclins are degraded during mitosis and CDK1 then binds to B-type cyclins to complete mitosis.

7.2.2 Regulation of the Cell Cycle: Cyclin-Dependent Kinase Inhibitors

In addition to its regulation by cyclins, CDK activity is also regulated by two families of specific CDK inhibitors [22, 83, 112, 144]. The first family consists of the INK4 proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$) that inhibit CDK4 and CDK6 during G1 phase and therefore primarily affect the pRb pathway [22]. In addition to its role in promoting cell cycle arrest, $p16^{INK4a}$ has also been associated with cellular aging and senescence particularly in melanocytes, however the exact role of $p16^{INK4a}$ in promoting cellular senescence is still debated [91, 53, 22, 46, 121]. The second family consists of the Cip/Kip family of proteins ($p21^{Cip1/Waf1}$, $p27^{Kip1}$, $p57^{Kip2}$) which inhibit CDK2 and CDK1 when complexed with E-type, A-type and/or B-type cyclins [144]. Inhibition of CDK2 leads to decreased pRb phosphorylation and sequestration of E2F. In addition, $p21^{Cip1/Waf1}$ further antagonizes pRb function by promoting proteosomal degradation of pRb [17]. Of note, levels of $p21^{Cip1/Waf1}$ are under the transcriptional control of activated p53 that utilizes $p21^{Cip1/Waf1}$ to arrest the cell cycle and to activate senescence pathways [140, 121, 94].

7.2.3 Regulation of the Cell Cycle: Retinoblastoma Protein Pathway

The retinoblastoma gene family consists of three members and encodes for the proteins pRb, p107 and p130 [56, 20, 29, 51]. Of these three proteins, pRb (encoded by the RB1 gene) has been extensively studied due to its key role in regulating the cell cycle and in functioning as a tumor suppressor gene. It is a 928 amino acid protein that consists of tandem cyclin fold regions separated by spacers and a C-terminal domain. These domains form a "pocket" which is the basis of pRb function. Targets that interact with the pRb pocket include E2F transcription factors and regulators of pRb, such as CDK-cyclin complexes. The affinity of the binding pocket is regulated by post-translational modifications, most commonly phosphorylation of serine and threonine residues in N-terminal and C-terminal domains and in spacer regions, which alter the conformation of the pocket and the binding affinity for specific targets.

CDK inhibitors such as $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ also play critical roles in regulating pRb function by directly inhibiting CDK4/CDK6 or inhibiting cyclin-CDK2/CDK1 complexes, respectively (Fig. 7.2) [56, 22, 20, 1, 29, 51, 144]. Feedback loops exist that also regulate pRb function [29]. Phosphorylation of pRb releases

E2F and allows for transcription of E-type and A-type cyclins that leads to further phosphorylation of pRb via CDK2. This positive feedback loop allows the cell to progress through the “restriction point” of the cell cycle. However, E2F that has been freed of pRb repression also initiates a negative feedback loop by promoting RB1 gene transcription. This results in an increase in pRb levels, sequestration of E2F and concomitant downregulation of E2F. Epigenetic signaling may also play a role in regulating pRb activity specifically through promoter hypermethylation and silencing of the RB1 gene [51].

Despite the key role played by pRb in regulating the G1 phase of the cell cycle, it is interesting to note that control of cell cycle arrest requires cooperation between pRb and p53 as shown by the fact that RB null mouse embryonic fibroblasts still transition from G1 to S phase but arrest in G2 phase under conditions of serum starvation due to upregulation of $p21^{Cip1/Waf1}$ via p53 [42]. Other studies have shown that combined heterozygous loss of pRb and p53 result in the development of a wider range of tumors compared with mice with heterozygous pRb loss alone [145]. Small cell lung cancer (SCLC) is induced in the lung epithelium of mice deficient in pRb and p53, while mice with CDKN2A loss and functional inactivation of pRb and p53 via loss of $p16^{INK4a}$ and $p19^{ARF}$ (mouse homologue of $p14^{ARF}$ in humans) respectively, develop sarcomas and lymphomas [115, 90]. Another study showed that over 75 % of melanoma cell lines had defects in both the p53 and pRb pathways [146]. These results highlight the importance of the interaction between the pRb and p53 pathways and the potential role of pRb in tumor initiation and the critical role played by p53 in acting as a failsafe cell cycle checkpoint [79, 82].

In addition to regulation of the cell cycle, pRb plays a role in several other related cellular functions. pRb is known to bind factors that regulate chromatin structure such as DNA methyltransferases, histone methyltransferases, histone demethylases and histone deacetylases [29, 51]. Through chromatin modification and interaction with E2F, pRb plays a fundamental role in regulating the transcription of an array of genes. In addition, pRb may play a role in promoting cellular senescence, through chromatin remodeling and formation of senescence associated heterochromatin foci, and in regulating apoptosis through E2F-1 which can transcribe genes necessary for apoptosis such as APAF-1 [29, 51, 121]. Another essential role of pRb that has been recently brought to light is its ability to help maintain genomic stability and to prevent aneuploidy [85]. Loss of pRb is associated with accumulation of DNA damage and with defects in the mitotic spindle, kinetochores and centrosomes. It is believed that dysregulation of E2F and its target genes such as MAD2 may help explain some of these mitotic defects.

7.3 p53 Pathway

Progression through the cell cycle is very tightly controlled with regulation mediated through a number of important checkpoints. Specifically, these checkpoints ensure that a cell is ready for the subsequent phases of the cell cycle by preventing the

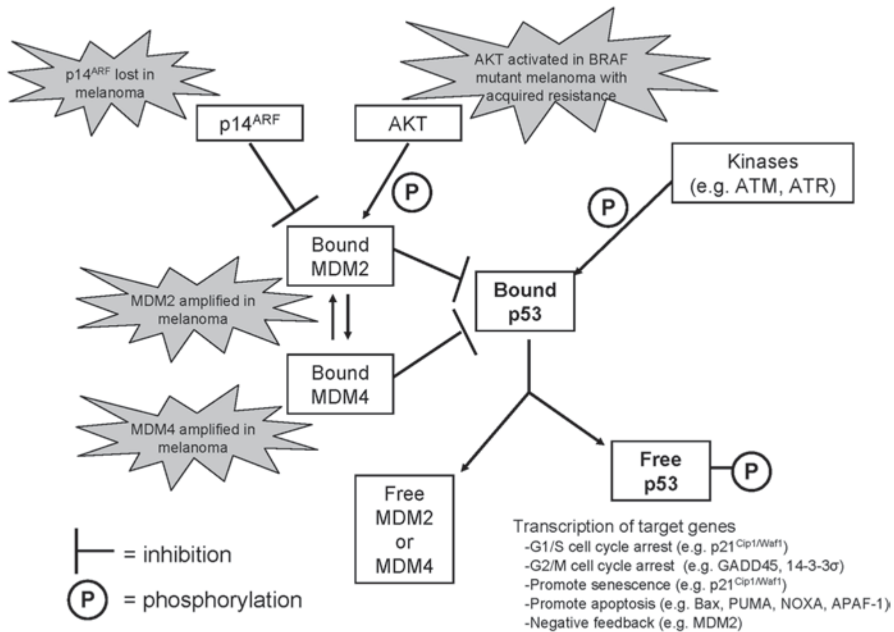

Fig. 7.3 The *p53* pathway. The cell cycle checkpoint protein *p53* mediates the transcription of a wide array of genes that are involved in senescence (*p21*$^{Cip1/Waf1}$), apoptosis (*Bax, PUMA, NOXA* and *APAF-1*) and cell cycle arrest, both at *G1/S* and at *G2/M* via the actions of *p21*$^{Cip1/Waf1}$, *GADD45* and *14-3-3σ*. A negative feedback loop also exists where activated *p53* induces the transcription of its negative regulator *MDM2*. Regulation of *p53* involves several proteins. Normally, *p53* levels are kept low through binding to *MDM2*. In addition, *MDM4* can also bind either to *p53* and directly inhibit the activity of *p53* or to *MDM2* and modify the inhibitory effects of *MDM2* on *p53*. In response to cellular stresses, kinases such as *ATM* or *ATR* phosphorylate *p53* and activate *p53* through release of *MDM2* or *MDM4*. Furthermore, *MDM2* activity is regulated by *p14*^{ARF} and by other kinases such as *AKT*. Binding of *p14*^{ARF} to *MDM2* results in decreased binding of *MDM2* with *p53* and ultimately in *p53* activation. In contrast, *AKT* phosphorylates *MDM2* leading to increased binding of *MDM2* with *p53* and downregulation of *p53* activity. In *melanoma*, prominent alterations in the *p53* pathway are seen and include loss of *p14*^{ARF} and amplification of *MDM2* and *MDM4*. Furthermore, activation of AKT is also seen in *BRAF* mutant melanoma that has acquired resistance to *BRAF*-directed therapy. *CDK* cyclin-dependent kinase, *P* phosphorylation

propagation of DNA damage. One of the most recognized and well-known checkpoint is the p53 pathway. In response to DNA damage and/or cellular stress, p53 can either arrest cells at the G1/S phase and G2/M phase transitions to allow time for repair or alternatively activate senescence or apoptotic pathways in response to irreversible damage.

The TP53 gene encodes for the 393 amino acid protein known as p53 [132, 140, 94]. Structurally, p53 consists of an N-terminal transactivation domain, followed by a proline-rich domain, a DNA binding domain, which binds to sequence-specific p53 response elements, a tetramerization domain and a C-terminal domain. The activity of p53 is regulated through several types of post-translational modifications such as phosphorylation of serine and threonine residues in the N-terminal

transactivation domain and acetylation, methylation, ubiquitination, neddylation and sumoylation of C-terminal lysine residues.

In addition, protein-protein interactions are critical in regulating p53 activity (Fig. 7.3). Several important p53 regulators include MDM2 (also known as HDM2 in humans), MDM4 (also known as MDMX or HDMX in humans) and p14^{ARF} [132]. MDM2 is part of the RING finger family of E3 ubiquitin-ligases. MDM2 binds to p53 and functions to inhibit the transcriptional activity of p53, to export p53 to the cytoplasm and to target p53 for proteosomal degradation through the action of an E2 ubiquitin-conjugating enzyme which is bound to the C-terminal RING finger domain of MDM2 [86, 61]. The importance of MDM2 in normal cellular function is highlighted by the fact that MDM2 knockouts are lethal in mouse embryos [97]. Normally, p53 is kept at low levels through binding to MDM2. However, cellular stress induces kinases to phosphorylate p53 at its N-terminal transactivation domain, leading to release of MDM2 and activation of p53. In contrast, p14^{ARF} binds to MDM2 and prevents its binding to p53 with the net result of also increasing p53 activity. Other kinases, such as AKT, can upregulate MDM2 through phosphorylation, thereby decreasing p53 function [5]. There is also a negative feedback loop in which p53 promotes the transcription of MDM2, resulting in increased MDM2 levels and in downregulation of p53 [132].

Another negative regulator of p53 is MDM4. Although MDM4 and MDM2 are similar, MDM4 lacks ubiquitin-ligase activity [86, 84]. However, MDM4 does form heterodimers with MDM2 and is able to modify the ubiquitin-ligase activity of MDM2. MDM4 functions similarly to MDM2 and inhibits p53 activity by binding to the transactivation domain of p53. MDM4 null mice also die *in utero* but at a different time point from MDM2 knockout mice [101]. These results and additional studies suggest that the functions of MDM2 and MDM4 are not overlapping and are instead complementary [132]. It is believed that MDM2 primarily functions by degrading p53 while MDM4 inhibits p53 activity, however these hypotheses are still a matter of debate.

The activity of p53 is enhanced in response to various cellular stresses, such as DNA damage, hypoxia, metabolic stress, heat shock, and oncogene activation [132, 140]. This occurs due to increased stability and post-translational modifications of p53, ultimately leading to a cascade of potential responses such as cell-cycle arrest, DNA repair, apoptosis and senescence. Specifically in the setting of DNA damage, p53 halts progression of the cell cycle to either allow time for DNA repair or activate apoptotic or senescence pathways in the setting of irreparable damage. ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) kinases are activated in response to specific types of DNA damage and activate Chk2 and Chk1 kinases, respectively [94]. All four of these activated proteins can then phosphorylate and activate p53 leading to cell cycle arrest or apoptosis.

Many of p53's functions extend from its ability to regulate the expression of a wide array of genes. For G1 cell cycle arrest, p53 induces the expression of p21$^{Cip1/Waf1}$ resulting in inhibition of cyclin/CDK complexes and sequestration of E2F by pRb [1]. The induction of p21$^{Cip1/Waf1}$ also can stimulate senescence pathways leading to irreversible cell arrest [121, 144]. The product of other genes that are

regulated by p53 can induce a G2 arrest and include GADD45 and 14-3-3σ [57, 149]. In response to irreparable stress, p53 can also promote cell death through the induction of various proapoptotic factors such as Bax, PUMA, NOXA and APAF-1 [132, 140].

7.4 Abnormal p53 and Cell Cycle Regulation in Melanoma

Over the past several decades, it has become evident that most melanomas harbor alterations in regulatory pathways of the cell cycle and p53 and that these alterations play a prominent role in the development of melanoma.

7.4.1 Alterations in CDKN2A

The CDKN2A locus comprises 4 exons (1α, 1β, 2 and 3) and through alternative splicing creates two different proteins, $p16^{INK4a}$ (exons 1α, 2 and 3) and $p14^{ARF}$ (exons 1β and 2) [106]. Both of these proteins play important roles in regulating the cell cycle, and in addition, both $p16^{INK4a}$ and $p14^{ARF}$ have been implicated in promoting senescence [22, 54, 121]. The tumor suppressive effects of CDKN2A are manifested by *in vivo* studies where homozygous CDKN2A knockout mice develop spontaneous tumors particularly sarcomas and lymphomas, and by the finding of CDKN2A mutations and deletions in various cancers [67, 99,115, 111]. Most alterations of CDKN2A appear to predominantly affect $p16^{INK4a}$ with either preservation or inactivation of $p14^{ARF}$ [116]. Inactivation of $p16^{INK4a}$ has been found in many human cancers, and $p16^{INK4a}$ specific knockout mice readily developed tumors, including sarcoma, lymphoma and melanoma [117, 116].

The discovery of germline CDKN2A mutations in cases of familial melanoma highlighted the prominent role of cell cycle dysregulation in the development of melanoma [62]. In approximately 10% of melanoma cases, a family history of melanoma is seen, and from these familial cases, several high penetrance genetic loci have been determined that confer a high-risk for the development of melanoma [103]. Two of these loci map to CDKN2A on chromosome 9p21 with approximately 20–40% of familial melanoma cases having germline mutations in CDKN2A. Most of these CDKN2A germline mutations occur in exons 1α and 2 which encode for $p16^{INK4a}$, but in most cases deletions were found that also affected $p14^{ARF}$ [135]. Taken together, all of these studies suggest that $p16^{INK4a}$ is a true melanoma susceptibility gene. However, although much rarer than $p16^{INK4a}$ mutations, germline mutations specifically affecting $p14^{ARF}$ have also been found suggesting a separate tumor suppressor role [108, 58].

Melanomas also appear prone to somatic alterations in CDKN2A with the vast majority of melanoma cell lines demonstrating loss of CDKN2A [10, 25, 143]. It has

been further shown that CDKN2A knockout mice with activating *HRAS* mutations develop melanomas while murine cell lines cultured from spontaneous melanomas demonstrate loss of p16^{INK4a} and p19^{ARF} [118, 88]. Furthermore, approximately 70 % of melanoma cell lines and melanoma samples from 44 % of patients with metastatic disease were found to harbor mutations in CDKN2A with inactivating deletions representing the most common type of mutation [95, 143, 129, 52, 146]. The vast majority of CDKN2A mutations primarily affect p16^{INK4a} with promoter silencing via methylation and deletions in p16^{INK4a} representing most of these defects [129, 116, 11, 22]. Mice with specific knockout of p16^{INK4a} develop melanoma although at a lower frequency compared with other tumor types [117]. Melanoma was also seen in mice with activating *KRAS* mutation and loss of p16^{INK4a}, particularly when this occurred in mice that showed a concurrent loss of p53 expression [96]. Transgenic mice with activating *HRAS* mutation in conjunction with p16^{INK4a} deficiency readily develop melanomas, while mice with activating *NRAS* mutations and INK4a deficiency developed melanomas in > 90 % of cases [118, 3].

Alterations in CDKN2A that preserve p14^{ARF} function but specifically inactivate p16^{INK4a} (exon 1α) are rare in cancers and are most commonly due to promoter hypermethylation [11, 100]. However, studies suggest that p14^{ARF} has a distinct role in tumor suppression. Knockout studies in mice show that p19^{ARF} null phenotypes are prone to the development of various tumors including sarcoma, lymphoma and lung cancer [119]. However, a role for melanomagenesis was suggested by the finding in familial melanoma cases of germline mutations in CDKN2A that specifically affected p14^{ARF} [108, 58]. Furthermore, various types of transgenic mice with knockout of p19^{ARF} were shown to develop melanoma [70, 68, 118, 54). In another study, 2 of 5 human melanoma cell lines demonstrated CDKN2A deletions that specifically affected p14^{ARF} while preserving p16^{INK4a} [73]. These lines of evidence suggest that in melanoma, p14^{ARF} has a separate and important tumor suppressor role that is separate from p16^{INK4a}.

7.4.2 Alterations in the p53 Axis

Germline mutations in TP53 are seen in patients with Li-Fraumeni syndrome; a group of individuals with increased susceptibility to sarcomas, breast cancers, brain and adrenal tumors [50]. Somatic mutations in TP53 are very common in cancers with up to 50 % of solid tumors found to harbor TP53 mutations [18] In the remaining half of tumors without TP53 mutations, alterations in other elements of the p53 pathway are often seen. For instance, approximately 10 % of cancers have amplification of MDM2 while MDM4 is amplified in approximately 10–20 % of cancers [132].

The important role of the p53 pathway in the development of melanoma has been demonstrated through animal modeling studies. Transgenic mice with activating *HRAS* mutation and either heterozygous or homozygous p53 loss developed melanomas at higher rates (two of 17 Tyr-RAS p53$^{+/-}$ and seven of 27 Tyr-RAS p53$^{-/-}$ mice) and at shorter latency (65 and 17 weeks, respectively) compared with

mice homozygous for wild-type p53 (one of 49 Tyr-RAS p532009$^{-/-}$ mice after 1 year) [9]. In another model, zebrafish with activating *NRAS* mutations developed hyperpigmentation, but melanoma developed in zebrafish that had both activating *NRAS* mutations and loss of p53 [37]. In addition, the tumor suppressive function of p53 in melanoma may be particularly dependent upon p21$^{Cip1/Waf1}$ [131].

However, in sharp contrast to what is seen in other cancers, TP53 mutations in melanoma are relatively uncommon and seen in <15 % of primary tumors [28, 33, 63, 132]. Instead functional loss of the p53 pathway is relatively common and achieved not by actual loss or mutation in p53 itself, but instead by dysregulation of other components of the p53 pathway (Fig. 7.3). Studies have reported transcriptional inactivation of p53 or of its target genes in melanoma cell lines that have wild-type p53 and in tumor samples from melanoma metastases [6, 60]. In addition, inactivating mutations affecting p14^{ARF} are seen in familial melanoma cases and in some melanoma cell lines, and studies in transgenic mice with activating RAS mutations and p19^{ARF} loss also highlight the dysfunction of the p53 pathway in melanoma.

Other alterations in p53 regulators have also been found in melanoma. Overexpression of MDM2 protein was seen in 50 % of human melanoma tumors in one study, although MDM2 gene amplification was seen in only one of 100 cases (1 %), while a second study showed that two of 53 (3.8 %) human melanoma samples had MDM2 gene amplification although increased MDM2 protein expression was again seen in several cases without gene amplification [105, 98]. Furthermore, in contrast to what is seen in melanocytes, melanoma cells appear to rely on MDM2 in order to suppress p53 activity and escape senescence [139]. Another negative regulator of p53 is MDM4 which has been shown to be overexpressed in 65 % of melanoma specimens [43]. In this same study, MDM4 overexpression in transgenic mice with activating *NRAS* mutation and wild-type p53 was associated with the development of melanomas in all cases. In another mouse model study using activating *HRAS* mutation and the carcinogen 7,12-dimethylbenz-alpha-anthracene (DMBA), mice that were heterozygous for functional MDM4 showed increased survival and decreased melanoma growth [131]. *In vitro*, MDM4 knockdown inhibited melanoma cell growth while MDM4 expression protected melanoma cells from p53 mediated apoptosis [43]. These studies further exemplify how the p53 pathway is dysregulated in melanoma and also shed light into potential targets for therapy.

7.4.3 Alterations in the Retinoblastoma Protein Axis

Mutations in pRb play a prominent role in the initiation of retinoblastoma, osteosarcoma and SCLC [20, 29, 51]. Germline mutations in the RB1 gene are associated with hereditary retinoblastoma which is inherited in an autosomal dominant fashion [69]. Hereditary retinoblastoma manifests primarily as an ocular tumor, and although many of these patients achieve long-term cures with 5-year survival rates of over 90 %, these patients have a 20-fold increased risk of developing secondary tumors. Most often (40–60 %) the secondary tumors are sarcomas although much of the sarcoma risk is attributable to radiation exposure during treatment. However,

long-term survivors with germline RB1 mutations also appear susceptible to the development of melanoma which is likely due to loss of heterozygosity [132].

In melanoma, somatic mutations in pRb have not been well studied but appear to be infrequently found in melanoma cell lines [10, 147]. However, alterations in other parts of the pRb pathway are seen in greater frequency in melanoma (Fig. 7.2). The majority of melanomas have alterations in CDKN2A, which usually affect the $p16^{INK4a}$ locus [41, 72]. Knockout of $p16^{INK4a}$ in mice is associated with the development of melanoma, although at a lower frequency compared with other tumor types [117, 118, 3, 96].

Another component of the pRb pathway that is often altered in melanoma is the cell cycle kinase CDK4. Several melanoma prone families have been found to harbor autosomal dominant germline mutations in the CDK4 gene on chromosome 12q14 [103]. These families all have mutations at codon 24 in which arginine is changed to either cysteine or histadine. This amino acid change abrogates the ability of $p16^{INK4a}$ to bind to and inhibit the function of CDK4. CDK4 mutations can occur in the absence of $p16^{INK4a}$ mutations in these familial melanoma cases suggesting that these mutations are mutually exclusive. Amplification of CDK4 with preservation of CDKN2A expression has also been found in a small subset of melanoma tissue samples [98]. Mouse models also support a role for CDK4 in the development of melanoma. Transgenic mice with an activating *HRAS* mutation, wild-type $p16^{INK4a}$ and an R24C *CDK4* mutation developed melanoma in 58 % of cases, with the incidence increasing to 83 % following ultraviolet (UV) radiation treatment [55]. A second study showed that mice with wild-type $p16^{INK4a}$ and R24C *CDK4* mutation also developed melanomas after topical treatment with DMBA [128].

Amplification of other elements of the pRb pathway has also been reported in melanoma. In mice with activating *HRAS* mutation and $p19^{ARF}$ loss, UV radiation promoted the development of melanoma, and CDK6 amplification was found in approximately half of these tumors [68]. CDK2 amplification has also been found in melanoma in some studies, and melanoma cells seem particularly dependent on CDK2 for proliferation [130, 44, 38]. Amplification of cyclin D1, which binds to and activates CDK4 and CDK6, has been found in approximately 10 % of melanomas particularly in the acral lentiginous histologic subtype (44 %) and in tumors with *BRAF* mutation [114, 14, 15, 126]. Knockdown of cyclin D1 in melanoma cells reduced cell proliferation by 97 % *in vitro*, decreased tumor growth in a mouse xenograft model and was associated with apoptosis [114]. Taken together, all of these studies demonstrate that multiple areas in the pRb pathway can become dysfunctional in melanoma and that these alterations could serve as potential targets for therapy.

7.5 Interaction of BRAF with p53 and Cell Cycle Pathways in Melanoma

Approximately 50 % of melanoma have activating *BRAF* mutations, most commonly the V600E mutation [71]. Interestingly, *BRAF* mutations are also seen in approximately 80 % of benign nevi suggesting that mutations in *BRAF* are an early event in

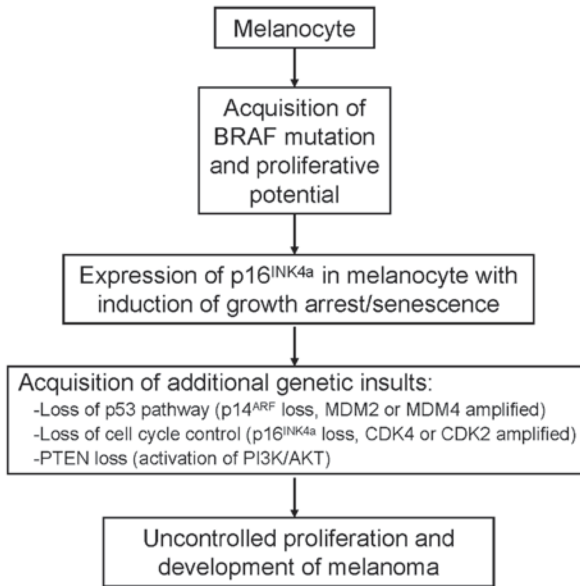

Fig. 7.4 A model for melanomagenesis and potential therapeutic targets. The development of melanoma requires multiple sequential genetic insults. *BRAF* mutation is an early event and found in the vast majority of nevi and melanocytes. Furthermore, co-expression of *p16*^{INK4a} and markers of senescence are also found in melanocytes with *BRAF* mutations. It is believed that the proliferative influences of *BRAF* mutations in melanocytes are countered by *p16*^{INK4a} expression and induction of senescence. Additional mutations are eventually acquired such as loss of *p16*^{INK4a} and/or *p14*^{ARF}, *CDK* amplification, *MDM2* or *MDM4* amplification, *RAS* mutation or *PTEN* loss. These additional genetic insults lead to uncontrolled proliferation and the development of melanoma. However, these additional alterations and the interaction of *BRAF* with cell cycle and *p53* pathways provide potential therapeutic targets in conjunction with *BRAF*-directed therapy

the development of melanoma [104, 74]. Nevi appear to represent growth-arrested melanocytes since congenital nevi stain positively for the senescence-associated marker acidic β-galactosidase [91, 53, 92]. *In vitro* studies have demonstrated that the expression of *BRAF*^{V600E} in melanocytes causes cell cycle arrest that is associated with p16^{INK4a} expression [91]. It is believed that the expression of p16^{INK4a} may protect the melanocyte from the proliferative influences of *BRAF* mutations by promoting senescence (Fig. 7.4) [33].

However, the presence of *BRAF* mutations alone is insufficient to transform melanocytes into melanoma and additional genetic alterations are required (Fig. 7.4). Specifically, alterations in components of the p53 pathway (p14^{ARF}, MDM2 and MDM4) and/or in components of the pRb pathway (p16^{INK4a}, CDK4 and CDK2) may cooperate with *BRAF* mutations to allow for the development of melanoma. Furthermore, activation of other pathways, as exemplified by increased PI3K/AKT activity through PTEN loss, may also interact with mutated *BRAF* to prevent senescence in melanocytes and to promote tumor growth [141]. This interaction was highlighted in a zebrafish study where the combination of

BRAF mutation in the background of p53 deficiency induced the development of melanoma while the presence of *BRAF* mutation alone was associated with the development of only benign nevi [102]. In another study, melanocytes within human skin tissue were transfected and then xenografted onto immunodeficient mice [30]. Melanoma was seen when either activating RAS or PI3K mutations were combined with hTERT expression and inhibition of the p53 or pRb pathways, whereas only benign nevi were seen with *BRAF* mutation. The loss of CDKN2A ($p16^{INK4a}$ and $p14^{ARF}$) in association with *BRAF* mutation appears to be the most frequent combination of mutations in melanoma and can promote the formation of tumors [34, 52]. This was shown in a study where mice that had $BRAF^{V600E}$ mutations and were deficient in $p16^{INK4a}$ showed a higher percentage of cases that developed melanoma, more cases of multiple tumors and shorter latency when compared with mice with wild-type $p16^{INK4a}$ [35]. In human melanocytes with $BRAF^{V600E}$ mutation, knockout of p53 enhanced proliferation, created lesions that resembled melanoma in situ and was associated with loss of the RB1 locus [148].

It is evident that the development of melanoma is associated with derangements in regulation of the cell cycle and p53 pathways. Loss of $p16^{INK4a}$ appears especially important in melanomagenesis by circumventing the senescence response in melanocytes that develop activating *BRAF* mutations. Furthermore, alterations in cell cycle pathways may also diminish therapeutic responses. For instance, melanomas that overexpress CDK4 and cyclin D1 show intrinsic resistant to *BRAF* inhibition [14, 126]. One alternative cellular pathway utilized by melanomas to overcome *BRAF* inhibition therapy is activation of the PI3K/AKT pathway [39]. MDM2 is upregulated via phosphorylation by activated AKT thereby inhibiting p53 function, and it is conceivable that increased PI3K/AKT activity in melanoma cells resistant to *BRAF* inhibition may play a role in promoting proliferation in these resistant cells [141]. In melanoma, there is obvious interaction amongst these pathways providing the potential for combining *BRAF* inhibitor treatment with therapies aimed at targeting these alterations in cell cycle and p53 regulation.

7.6 Therapeutic Potential of BRAF Inhibition in Combination with Modulation of p53 or Cell Cycle Pathways

BRAF mutations in melanoma have been successfully targeted through single agent treatment, but despite the dramatic responses initially seen, recurrences inevitably develop. *BRAF* mutant melanoma readily develops resistance and utilizes alternative cellular pathways to overcome *BRAF* inhibition, highlighting the need for additional therapeutic targets for combination therapy. The ubiquitous alterations in cell cycle and p53 pathways in melanoma and the interaction of *BRAF* with these pathways provide new potential therapeutic targets that can be combined with *BRAF* inhibition.

One promising area of cancer therapy research is looking into ways to reactive p53 function in tumors with inactivating p53 mutations [18]. However, p53 mutations are only seen in 10–15 % of melanoma, but for this small subset, agents such as PRIMA-1, which help to stabilize the protein folding of p53, may help to reactivate mutated p53 and restore its function. *In vitro*, PRIMA-1 has been shown to promote p53-dependent apoptosis and inhibit growth in melanoma cells [8]. Much more common in melanoma is the functional loss of p53 caused by alterations in p53 regulatory pathways, and ongoing research is looking into ways to enhance the function of wild-type p53. MDM2 is overexpressed in many melanomas and serves as an attractive therapeutic target to increase p53 activity [142]. The nutlin class of inhibitors is derived from cis-imidazoline compounds and functions to displace MDM2 from p53 thereby increasing p53 activity [137]. In one study, melanocytes and melanoma cells were treated with the MDM-2 specific antagonist nutlin-3, and at doses < 20 μM, cell cycle arrest was seen while doses > 20 μM promoted apoptosis [131]. Based on these results, it was proposed that nutlin-3 primarily functioned to decrease melanoma growth by promoting cell cycle arrest. A second study demonstrated that restoration of p53 function in melanoma cells through use of nutlin-3 decreased cell viability in a dose-dependent manner [64]. More importantly, 27 of 51 (53 %) melanoma cell lines tested had a *BRAF* mutation and wild-type p53, and the combination of nutlin-3 and MEK inhibition (U0126) appeared to synergistically decrease growth in 60 % of melanomas. The effects of MDM2 antagonism are dependent upon the presence of functional p53, and the greatest effect was seen in cells that had *BRAF* mutation and wild-type p53.

Whereas some studies have demonstrated that melanoma cells treated with nutlin-3 exhibited either minimal apoptosis or favored cell cycle arrest, work from our lab demonstrated that treatment of melanoma cells with nutlin-3 induced p53-dependent apoptosis while Ji et al. showed that inhibition of both MDM2 (nutlin-3) and MEK (U0126 and AZD6244) in *BRAF* mutated melanoma promoted apoptosis [125, 131, 136, 64]. It could be extrapolated based on these studies that inhibition of mutated *BRAF* instead of MEK in combination with nutlin-3 therapy would produce similar effects, however this would need to be validated. A major potential issue in MDM2 inhibition therapy is the negative feedback loop between MDM2 and p53 [133, 142]. Activation of p53 via nutlin-3 would theoretically later increase MDM2 levels and diminish p53 activity. Despite this potential caveat, the MDM2 antagonist RO5045337 (RG7112) has been used in patients with MDM2-amplified liposarcoma and based on best RECIST response, resulted in a partial response in 1 patient, stable disease in14 patients and progressive disease in five patients [107]. Furthermore, this preliminary study demonstrated that after treatment with RG7112, there was an increase in p53 levels and a decrease in cell proliferation as determined by Ki-67 staining in tumor samples. RO5045337 has also been tested in clinical trials for both solid and hematologic malignancies (NCT00559533, NCT00623870)* and is currently being tested in soft tissue sarcoma in conjunction with doxorubicin (NCT01605526) and as an extension study (NCT01677780) in patients previously treated with RO5045337 [64]. Other MDM2 antagonists such as RO5503781 and thioureidobutyronitrile

* NCT: National Clinical Trial Identifier. Please refer to www.clinicaltrials.gov for additional information on individual clinical trials.

(Kevetrin) are also being tested in clinical trials (NCT01462175, NCT01664000), but the results of all of the aforementioned trials are still pending [75].

Other studies have shown that MDM2 overexpression was seen infrequently in melanoma, however MDM4 protein levels were upregulated in the majority of melanoma specimens regardless of *BRAF* mutational status, thus making MDM4 an appealing therapeutic target [43]. Gembarska et al. demonstrated the key role played by MDM4 in promoting the development of melanoma *in vivo* and in allowing for cell proliferation and protection against apoptosis *in vitr*o. Importantly, inhibition of MDM4 using the α-helical peptide SAH-p53-8, a compound that has high specificity for MDM4 and disrupts the binding of p53 with MDM4, appeared to synergize with *BRAF* inhibition in melanoma cells and also decreased cell viability in melanoma cells that had developed resistance to *BRAF* inhibition [12, 13]. SAH-p53-8 was also tested in uveal melanoma cell lines and inhibited growth in cells that overexpressed MDM4 and to a lesser extent in cells that overexpressed MDM2 [76]. Although MDM2 targeted therapy appears to inhibit melanomas that overexpress MDM2, MDM2-specific agents such as nutlin-3 appear to have little effect on melanomas that overexpress MDM4. This highlights the fact that tumor genotyping will play a critical role for determining specific oncogenic alterations (e.g. overexpression of MDM2 versus MDM4) so that targeted therapies can be fashioned on a case by case basis.

Inhibitors of several other targets that interact with p53 have also been described. Glycogen synthase kinase-3β (GSK-3β) regulates glycogen metabolism but is also involved in cell migration, proliferation, apoptosis and regulation of p53 [45, 65]. In one study, GSK-3β inhibition using the organometallic inhibitor DW1/2 promoted apoptosis in melanoma cells through a p53-dependent mechanism that involved downregulation of MDM2 and MDM4 [125]. The combination of the MDM2 inhibitor MI-319 and sorafenib appeared to have a GSK-3β-dependent cytotoxic effect in some melanoma cells lines [81]. Other studies have looked at BH3 mimetics in combination with MEK inhibition (U0126) and have shown a p53-dependent synergistic cytotoxicity in melanoma cells [138]. Theoretically, upregulating the function of $p14^{ARF}$ and $p21^{Cip1/Waf1}$ could also enhance p53 function. In one study, B16 mouse melanoma cells were transfected with retrovirus containing $p19^{ARF}$ and were subsequently treated with nutlin-3 [89]. The combination of direct MDM2 inhibition via nutlin-3 and indirect MDM2 inhibition through $p19^{ARF}$ expression resulted in enhanced p53 activity and decreased B16 cell viability *in vitro* and *in vivo*. TBX2 downregulates the expression of both $p14^{ARF}$ and $p21^{Cip1/Waf1}$, and interestingly it is overexpressed in melanoma [47]. TBX2 inhibition appears to promote senescence and may serve as a potential therapeutic target to augment p53 function. However, the role of the above-mentioned targets in relation to *BRAF* status has not been fully assessed and requires further study.

Alterations in the pRb axis, primarily due to upregulation of CDK4 either through loss of $p16^{INK4a}$ or amplification of either CDK4 or cyclin D1, are readily seen in melanoma. Furthermore, melanoma cells appear especially dependent on CDK2 for growth [38]. Taken together, these results suggest that direct small molecule CDK inhibition would serve as an attractive therapeutic option. Numerous types of CDK

inhibitors have been developed and used in clinical trials including broad-range first generation inhibitors such as flavopiridol and roscovitine and second generation specific inhibitors such as PD-0332991 and PHA-848125 [36, 78, 31, 21]. Although preclinical results appeared promising, the results of clinical trials using small-molecule CDK inhibitors for the treatment of various solid tumors have generally been disappointing. One of the issues was that first generation compounds lacked specificity and inhibited several CDKs thereby limiting efficacy and causing off-target effects and toxicity. For instance, flavopiridol inhibits CDK1, 2, 4, and 7 while roscovitine inhibits CDK1, 2, 5 and 7 [31]. New second generation compounds have been developed that are more selective and potent as exemplified by PD-0332991 which inhibits CDK4 and 6 and by PHA-848125 which is a potent CDK2 inhibitor although it also is capable of inhibiting CDK1, 4 and 7 [21]. Clinical experience with the second-generation CDK inhibitors has been relatively limited and the results of most trials are pending. However, the preliminary results of one phase II clinical trial were recently reported (NCT00721409) and showed promising results [40]. Post-menopausal women with estrogen receptor-positive/HER2 negative advanced breast cancer who were treated with PD-0332991 and aromatase inhibitor letrozole had a significant increase in progression-free survival to 26.2 months compared with 7.5 months for patients treated with letrozole alone. Another potential issue is that studies have shown that only CDK1 is essential for cell cycle progression, which is contrary to the "classical" model of the cell cycle where sequential expression of several CDKs is required [83, 112]. Specifically, the loss of other CDKs can either be compensated by CDK1 or the loss of a specific CDK is detrimental only in specialized cells types such as hematopoietic cells and cardiomyocytes. Therefore, inhibition of CDKs outside of CDK1 may have limited effect or benefit.

Studies evaluating CDK inhibition for melanoma are very limited and are even more limited in evaluating the combination of *BRAF* and CDK inhibition. In two studies, CDK activity was inhibited in *BRAF* mutant melanoma by upregulating $p16^{INK4a}$ activity. In both of these studies, siRNA knockdown of *BRAF* along with expression of $p16^{INK4a}$ in melanoma cells harboring *BRAF* mutations significantly inhibited cell growth, and in one study there was also a significant increase in apoptosis [110, 150]. In another set of studies, CDK activity was inhibited by antagonizing cyclin D1 [113, 114]. *In vitro* cyclin D1 antisense treatment in melanoma cells induced apoptosis, while *in vivo* cyclin D1 antisense therapy along with transfection with wild-type p53 led to tumor shrinkage and to a complete response in 57% of cases. Flavopiridol was tested in $p16^{INK4a}$ positive and $p16^{INK4a}$ negative melanoma cells and caused a dose-dependent growth inhibition, although the IC_{50} for $p16^{INK4a}$ positive melanoma was higher [109]. Furthermore, a dose-dependent increase in apoptosis was seen in both $p16^{INK4a}$ positive and $p16^{INK4a}$ negative melanoma cells treated with flavopiridol. A study utilizing melanoma cultures in a 3D skin reconstruction model demonstrated that treatment with roscovitine was associated with decreased cell growth and survival and increased apoptosis in melanoma cells but not in melanocytes [95]. In another study, melanoma cells were treated with the CDK inhibitor SCH 727965 which decreased cell proliferation at a dose as low as 0.5 μM, promoted apoptosis and slowed tumor growth in a mouse xenograft

model [2]. Caporali et al. tested the second-generation CDK inhibitor PHA-848125 in melanoma cells and demonstrated G1 arrest and growth inhibition with IC_{50} values ranging from 0.123 to 0.680 μM [23]. Treatment with PHA-848125 was also associated with increased $p21^{Cip1/Waf1}$ expression, decreased pRb phosphorylation at the CDK2 and CDK4 sites and differential expression of genes involved in cell cycle control [23, 24]. Another CDK inhibitor, P276-00, has shown efficacy *in vitro* in decreasing cell proliferation and colony formation in several cancer cell lines including melanoma [66]. The CDK inhibitors roscovitine and DRB were used in combination with nutlin-3 to treat melanoma cells [27]. This combination appeared to show additive effects on inhibiting cell growth and synergy in inducing p53-dependent apoptosis. Recently, a study was presented in which a *BRAF* mutant melanoma cell line (SKMel 28) was treated with *BRAF* inhibitor PLX-4720 and CDK inhibitors roscovitine and olomoucine [134]. The combination of CDK inhibitor and *BRAF* inhibition led to downregulation of MAP3K8 and PRKD3 survival pathways, decreased pRb phosphorylation and ultimately to decreased cell viability. Li et al. treated melanoma cells with MEK inhibitor PD98059 and CDK4 inhibitor 219476 and demonstrated a decrease in cell viability and a significant increase in apoptosis in cells treated with both agents [80].

Based on these encouraging preclinical results, several clinical trials were developed to evaluate CDK inhibitor therapy in patients with melanoma. SCH 727965 is being evaluated in stage IV and unresectable stage III melanoma patients (NCT01026324, NCT00937937), while PD-0332991 is being tested against various solid tumors, including recurrent and stage IV melanomas (NCT01037790). P276-00 is being evaluated as treatment for stage IV and unresectable stage III melanomas that express cyclin D1 (NCT00835419). The results of most of these trials are pending, however preliminary results were reported for NCT00937937 (SWOG S0826) in which no responses were seen in 65 evaluable patients [77]. Stable disease was seen in 22% of patients, and the 1-year overall survival rate was 36%. Similar results from a phase II study using flavopiridol to treat metastatic melanoma patients were also reported with no objective responses seen in 16 evaluable patients [19]. However, no definitive conclusions can be made since these results are either preliminary or were based on a small number of patients, and the therapeutic effect of CDK inhibition requires more rigorous evaluation. In addition, the effects of *BRAF* mutation status and use of *BRAF* inhibition along with CDK inhibition also need further study and the first of these combinations is underway with the BRAF/MEK inhibitor doublet, encorafenib and binimetinib, in combination with LEE011 (NCT01543698).

7.7 Conclusions

Melanoma represents a prime model for developing targeted therapy due to the well-validated identification of oncogenic “drivers” that promote tumor proliferation. However, despite the initial success of single agent *BRAF* targeted therapy, it

is evident that single agent treatment ultimately fails due to cellular adaptation and the development of resistance. It is now believed that combination therapy, which targets multiple cellular pathways, is the key to overcoming or sidestepping the development of treatment resistance. The fact that the vast majority of melanomas harbor alterations in regulators of the cell cycle and p53 makes these pathways extremely attractive targets for therapeutic intervention. Furthermore, preclinical studies suggest that therapies aimed at melanoma with *BRAF* mutation and wild-type p53, which represents the most common genotype, may have synergistic results when *BRAF* directed therapy is combined with treatments that either enhance p53 function or inhibit CDK activity. However, the use of agents that modify p53 and CDK function in melanoma is at an early stage and further studies are needed. In particular, melanoma specific clinical trials testing *BRAF* inhibition in combination with MDM2/MDM4 inhibitors and/or CDK inhibitors are needed to determine the efficacy of this treatment combination in humans. These questions highlight the fact that it is an extraordinarily exciting time in the field of melanoma research as our knowledge about melanoma biology continues to expand. Additional work in this area will undoubtedly lead to the development of new agents for the treatment of metastatic melanoma thereby further improving the prospects for melanoma patients.

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Chapter 8
Combination BRAF-Directed Therapy and Immunotherapy

Zachary A. Cooper, Zain Ahmed and Jennifer A. Wargo

Abstract There have been two major advances in the treatment of metastatic melanoma within the past several years, including immunotherapy and BRAF-directed therapy. Both of these classes of therapy demonstrate survival benefit, but also have limitations as monotherapy with regard to overall response rate and/or durability of response. We have gained significant insight into mechanisms of response to BRAF-directed therapy and to potential synergy between these two treatment modalities. This chapter focuses on the limitations of each of these strategies as monotherapy, and provides the rationale for combining these therapies. Importantly, ongoing clinical trials of combined BRAF-directed therapy and immunotherapy are discussed, as well as considerations and future directions for therapy.

Keywords Melanoma · BRAF · Immunotherapy · Immune checkpoint · Targeted therapy

8.1 Introduction

There have been significant advances in the past few years with regard to BRAF-directed therapy. Despite these advances, resistance to BRAF monotherapy develops in the majority of patients with most patients progressing within 6 to 7 months [1–3]. A better understanding of resistance mechanisms has led to therapeutic strategies that improve responses and enhance survival, including additional MAPK

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blockade via combination BRAF and MEK inhibition. Thus far, such combinations (e.g. BRAF+MEK inhibition) have yielded significant improvements in the durability of response, though most patients still progress within 10 months and only a small fraction of patients achieve a CR or prolonged PR [4]. More sustained responses are clearly needed, and other combinations are currently being tested in preclinical studies and in clinical trials.

In addition to advances in targeted therapy, significant headway has been made with regard to immunotherapy for melanoma. Several immunotherapy agents are currently approved by the US Food and Drug Administration (FDA) for the treatment of metastatic melanoma, including cytokine-based therapy with interleukin-2 (Aldesleukin) and the immune checkpoint inhibitor targeting Cytotoxic T-lymphocyte antigen 4 (CTLA-4) called ipilimumab. Several other agents are currently under investigation in the context of clinical trials (immune checkpoint inhibitors targeting programmed death receptor 1 [PD1] and its ligand [PDL1]), and have shown promise in early phase studies [5, 6]. The advantage of immunotherapy over BRAF-directed therapy is that responses are often durable, however the drawback is that overall response rates remain low (10–15 % in the case of Ipilimumab), with a minority of patients obtaining an objective response [7].

There is increasing evidence that BRAF-directed therapy may synergize with immunotherapy [8–13], with the potential to maintain high response rates while extending the durability of responses. Evidence regarding potential synergy is presented herein, and ongoing clinical trials combining these strategies are discussed. Finally, important questions are posed with regard to potential issues of toxicity, timing and sequence of the different strategies, and the duration of therapy.

8.2 Rationale for Combination BRAF-Directed Therapy and Immunotherapy

8.2.1 Limitations of BRAF-directed Therapy

Functional redundancy and compensatory activity through alternate signaling pathways might explain the emergence of resistance seen in patients treated with selective BRAF inhibitors. Intense research efforts are focused on resistance mechanisms, and several mechanisms have been identified [14–21]. To address these issues, combination of BRAF/MAPK-targeted therapy with other signal transduction inhibitors, or with conventional chemotherapy has been proposed.

Combination strategies to overcome resistance have gained traction, and the combination of dabrafenib (a BRAF inhibitor) with tremetinib (a MEK inhibitor) has been FDA-approved based on an improved progression free survival (PFS) benefit in comparison to either BRAF inhibitor alone. Specifically, median PFS was extended from under 6 months for BRAF inhibitor monotherapy to over 10 months with combination BRAF+MEK inhibition [4]. Perhaps more impressive is the percentage of patients alive without disease progression at 1 year, increasing from 10 %

for BRAF inhibitor monotherapy to 40 % in the setting of combined BRAF inhibition and MEK inhibition [4]. Other strategies combining MAPK inhibition with blockade of additional signaling pathways are currently in clinical trials, however data regarding response rates and durability of response are not yet available.

Despite these advances, most patients progress within a year even with the best of these combination strategies [4]. Nonetheless this incremental benefit in survival provides a window of opportunity to offer novel agents and combination strategies, including combinations with immunotherapy. This strategy can be used on a backbone of BRAF inhibitor monotherapy or with combined BRAF and MEK inhibition, though there are important considerations with each which will be discussed herein.

8.2.2 Limitations of Immunotherapy

Several forms of immunotherapy are either FDA-approved or in clinical trials for the treatment of metastatic melanoma. High dose IL-2 was FDA-approved in 1998 based on its ability to produce durable responses in 6–10 % of patients [22]. However, its application has been limited to a select group of patients treated in specialized centers due to its severe and unique acute toxicity [23].

Another form of immunotherapy that is currently FDA-approved for melanoma involves the use of a blocking antibody against the Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) molecule on the surface of T lymphocytes. CTLA4 is an immunomodulatory molecule that functions to down-regulate an immune response [24]. Treatment with a monoclonal antibody that blocks this interaction (Ipilimumab) relieves cytotoxic T-lymphocytes from the inhibitory effects of CTLA4, resulting in an enhanced immune response. Treatment with ipilimumab has shown an overall survival advantage in patients with advanced melanoma in a randomized, placebo controlled trial [7] and received approval by the FDA in 2011. In this trial, patients with previously treated advanced melanoma were randomly assigned in a 3:1:1 ratio to ipilimumab plus a gp 100 vaccine, ipilimumab alone, or gp 100 alone. A significant improvement in median overall survival for patients receiving either ipilimumab containing regimen (median 10 months) relative to patients receiving the vaccine alone (6.4 months) was shown as well as a reduction of the risk of death (ipilimumab + vaccine or ipilimumab alone vs gp 100 vaccine; HR 0.68 or 0.66, respectively). Overall survival rates for the three groups were 44, 46 and 25 % at 12 months and 22, 24 and 14 % at 24 months, respectively [7].

Other forms of immunotherapy are in clinical trials and have shown promising results. Blockade of the immune-modulatory molecule PD1 on the surface of T lymphocytes has shown significant promise in the treatment of metastatic melanoma with response rates approaching 40 % in a phase II clinical trial [5]. Interestingly, responses were also seen in other solid tumors, including renal cell carcinoma and non small cell lung cancer [5]. Monoclonal antibodies blocking the immunosuppressive ligand PDL1 are also in clinical trials, though data regarding responses and durability are not yet mature [6].

Another area of great promise in immunotherapy involves the use of adoptive cell transfer, and includes the administration of autologous tumor infiltrating lymphocytes (TIL) or genetically-modified peripheral blood lymphocytes (PBL) to mediate an anti-tumor response. TIL-based approaches have been quite successful in expert hands [25–27], with response rates ranging from 30 to over 70 % depending on the pre-conditioning regimen used [28]. However this therapy is still considered experimental and to date its use is limited to expert centers given the complexity and cost of generating this individualized form of treatment. Nonetheless, strategies are under development to optimize and standardize preparation of this type of product so that its use may be more generalizable. In addition, approaches using transduction of PBL with antigen-specific T cell receptors [29] and chimeric antigen receptors [30] are also underway and have shown some promising results.

The field of immunotherapy has certainly advanced the treatment of patients with metastatic melanoma, and treatment responses are often long-lasting. Unfortunately, only a minority of patients will ultimately benefit from these treatments. Thus a critical question is whether or not we can increase the durability of responses and/ or complete response rate by the addition of BRAF-directed therapy to immunotherapy regimens.

8.2.3 Effects of BRAF Inhibition on the Tumor Microenvironment and Immune System

8.2.3.1 Pre-Clinical Studies

Preliminary evidence suggests that oncogenic BRAF ($BRAF^{V600E}$) may contribute to immune escape in melanoma [31], and that blocking its activity via MAPK pathway inhibition leads to increased expression of melanocyte differentiation antigens (MDAs) [32]. We studied this extensively in the laboratory, and demonstrated that targeted inhibition of the MAPK pathway leads to up to a 100-fold increase in expression of MDAs in melanoma cell lines and fresh tumor digests (Fig. 8.1a) which is associated with significantly enhanced recognition by antigen-specific T lymphocytes (Fig. 8.1b) [9]. This appears to be mediated through microphthalmia-associated transcription factor (MITF), a master transcriptional regulator of melanocytes [9].

Importantly, BRAF-directed therapy does not appear to have deleterious effects on T lymphocytes [8, 9]. This is in contrast to MEK inhibitors, which demonstrate dose-dependent inhibition on T cell function *in vitro* [9]. This has relevance when contemplating combinations of BRAF-directed therapy with immunotherapy, as combination therapy including a MEK inhibitor may potentially have deleterious effects on T cells, which may abrogate any potential synergy.

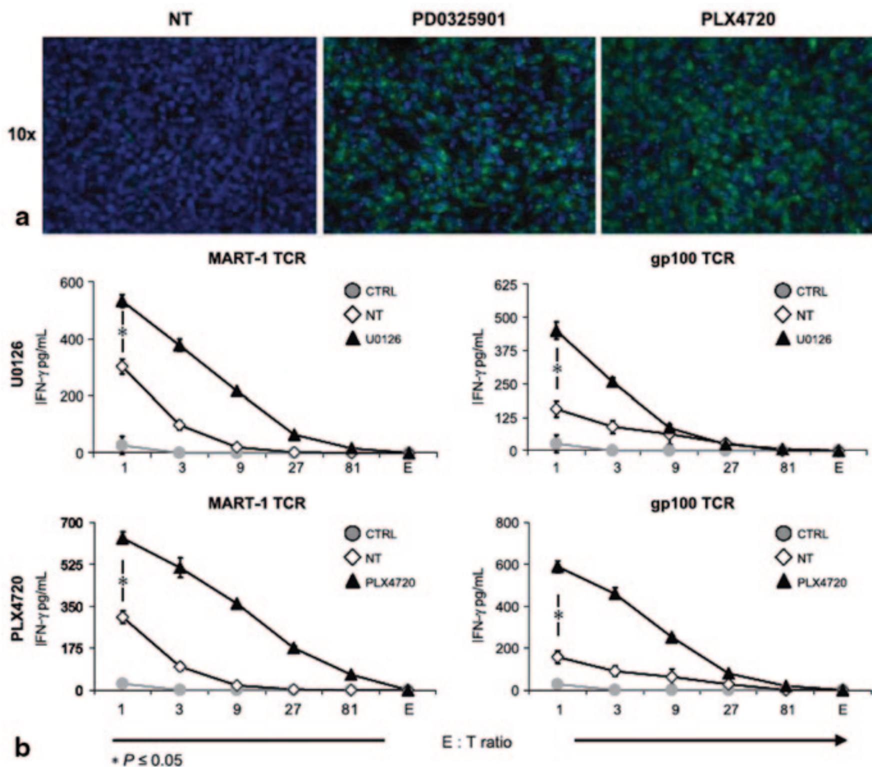

Fig. 8.1 MAPK pathway inhibition increases melanoma antigen expression. Expression of *MART-1* is increased with MEK inhibition and BRAF inhibition (**a**), which is associated with enhanced recognition by antigen-specific T lymphocytes (**b**), HLA-A2+UACC903 melanoma cells were treated as above with a MEK (*U0126*) or BRAF (*PLX4720*) inhibitor and cultured with CTL specific for *MART1* or gp100 versus control lymphocytes (GFP-transduced) at various E:T ratios. *IFN*γ release was measured by ELISA. (Adapted from Boni et al. [9])

8.2.3.2 Clinical Evidence

The first evidence that BRAF inhibition could result in increased immunogenicity in patients with metastatic melanoma was presented and published by several groups in 2012, demonstrating enhanced T cell infiltrates in tumors of patients with metastatic melanoma treated with BRAF inhibitors [33, 34] (Fig. 8.2a). Since these original reports, evidence regarding the immune effects of BRAF inhibition has mounted. In addition to an increase in CD8 T cell infiltrate, treatment with BRAF inhibitors is associated with a decrease in immunosuppressive cytokines IL-6, IL-8 [8] (Fig. 8.2b) and a decrease in vascular endothelial growth factor (VEGF) [12] (Fig. 8.2c). The tumor stroma appears to play a critical role, as stromal cell-mediated immunosuppression via interleukin 1 (IL-1) is induced by oncogenic BRAF and blocked with BRAF inhibitors [13].

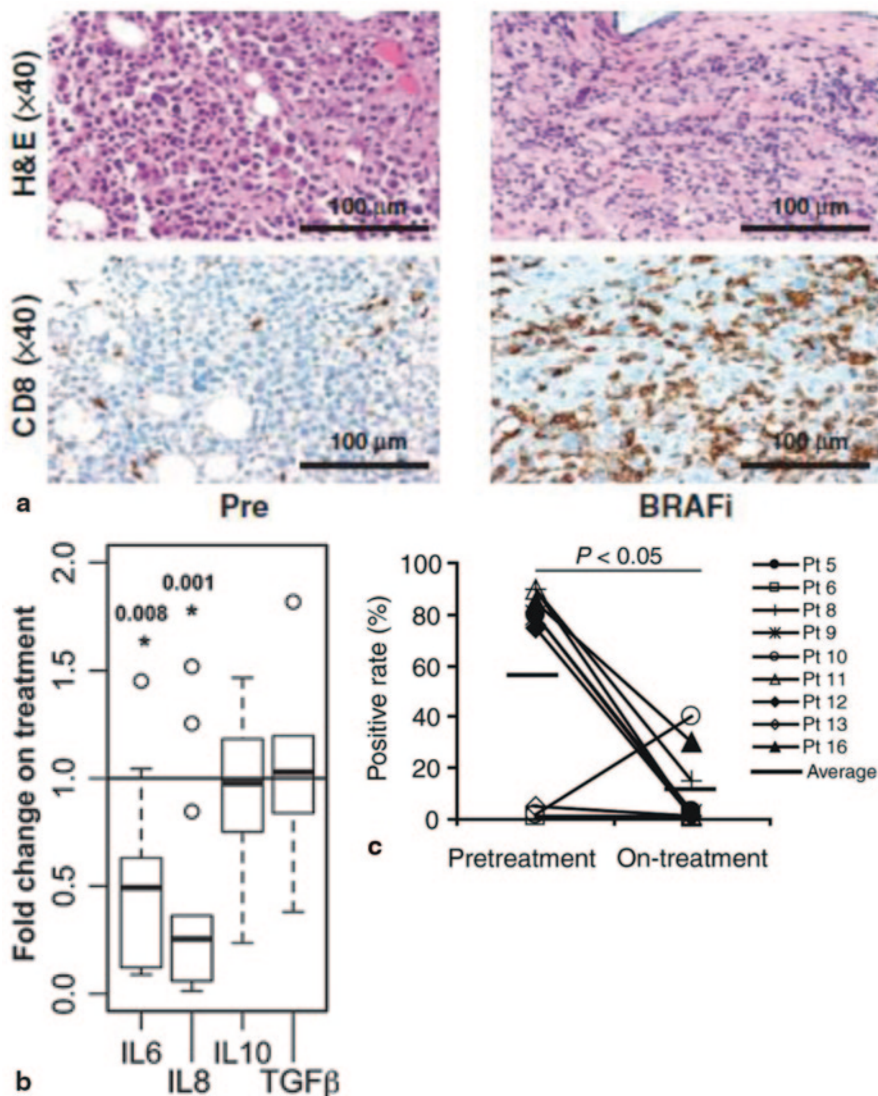

Fig. 8.2 *BRAF* inhibition is associated with increased CD8+ T-cell infiltrate, decreased immunosuppressive cytokines and VEGF in tumors of patients with metastatic melanoma. Patients with metastatic melanoma were treated with *BRAF* inhibitor +/− MEK inhibitor and tumor biopsies were performed before treatment and within 1–2 weeks of initiation of therapy. *CD8+* T cell infiltrate was assayed via immunohistochemistry (IHC) showing a significant increase of *CD8+* T cells on therapy (**a**), This was associated with a decrease in IL-6 and IL-8 (**b**), as well as a decrease in VEGF (**c**). (Adapted from Frederick et al. [8] and Liu et al. [12])

An additional piece of evidence supporting the hypothesis that T cells play an important role in response to BRAF-targeted therapy and that BRAF-directed therapy may synergize with immunotherapy comes from analysis of melanoma antigen expression and CD8+ T cell infiltrate in lesions of patients who have progressed on

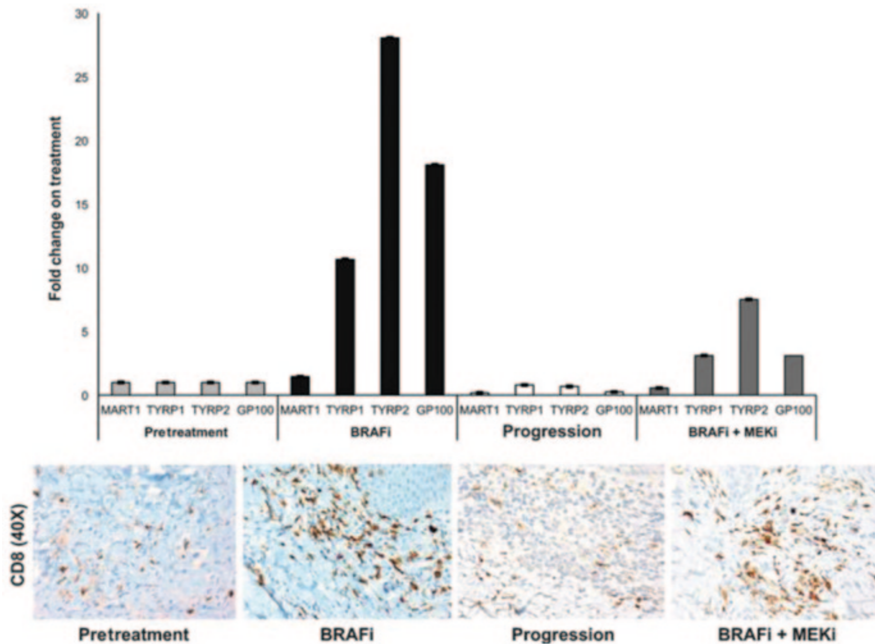

Fig. 8.3 Melanoma antigen expression and CD8+ T-cell infiltrate are decreased at time of progression and restored through MEK inhibition. Tumors were harvested pre-treatment, 10–14 days after *BRAFi* initiation, at time of progression and at time of treatment with combined BRAF inhibition and MEK inhibition for a patient. mRNA levels of the melanoma antigens gp100, MART-1, TYRP-1, and TYRP-2 were assayed. Immunohistochemistry (IHC) was conducted for CD8+ T cells on patient tumor samples. (Adapted from Frederick et al. [8])

BRAF-directed therapy [8]. Based on our initial data, we would expect that resistance to therapy would be associated with a decrease in melanoma antigen expression and a decrease in CD8 T cell infiltrate. We tested this by analyzing melanoma antigen expression and CD8+ T cells in lesions of patients who progressed on therapy and we found exactly what we expected (Fig. 8.3), namely reduced melanoma antigen expression and CD8+ T cell infiltrate at time of progression. Interestingly, if you treat with additional MAPK blockade you can potentially restore antigen expression and T cell infiltrate (Fig. 8.3) [8].

Another insight into tumor—stromal—T cell interactions came with the observation that the infiltrating T cells in tumors of patients treated with BRAF inhibitors demonstrate an activated phenotype and express high levels of PD-1 (Fig. 8.4a) [8]. The PD-1 molecule is an immunomodulatory molecule that serves to down-regulate an immune response after an initial period of activation, functioning normally to prevent autoimmunity. However another critical finding in patients treated with BRAF inhibitors is that the tumor cells themselves express high levels of PD-L1 within 2 weeks of initiation of BRAF inhibitor therapy (Fig. 8.4b) [8]. This may represent a mechanism of resistance, and is corroborated by in vitro work demonstrating high PD-L1 expression in melanoma cell lines resistant to BRAF inhibition [35]. Inter-

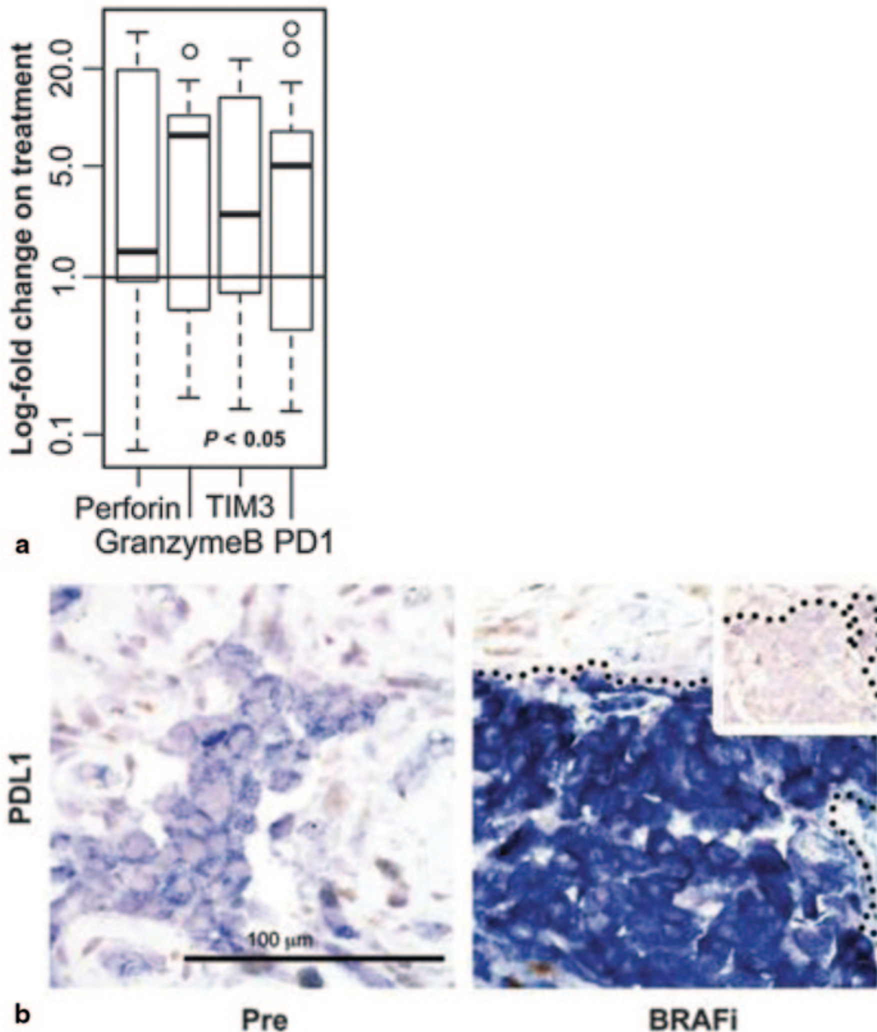

Fig. 8.4 BRAF inhibition is associated with decreased markers of T-cell cytotoxicity but increased T-cell exhaustion markers and PDL1 in tumors of patients with metastatic melanoma. Tumors were harvested and mRNA levels perforin ($n=11$), Granzyme B ($n=11$), TIM-3 ($n=14$) and PD1 ($n=14$; (**a**), in patients with metastatic melanoma undergoing treatment with a selective inhibitor of BRAF^{V600E} were assayed. All patients are expressed in a box and whiskers plot. *Open circles* represent data points greater than 1.5 times the interquartile range. P values indicated are from a 2-tailed Student t test with a μ of 1, which represents no change in mRNA value with respect to the pretreatment value. *, $P \leq 0.05$. Immunohistochemistry (×40 magnification) for PDL1 in a representative pretreatment and on-treatment biopsy (**b**). *The dotted line* = tumor–stroma interface and the inset is the isotype-specific control. (Adapted from Frederick et al. [8])

estingly, the addition of MEK inhibition may abrogate the up-regulation of PD-L1 in these cell lines in vitro, which has significant translational implications [35]. Taken together, these data suggest that addition of an immune checkpoint inhibitor to a regimen of BRAF inhibition may augment responses to therapy (Fig. 8.5) [36].

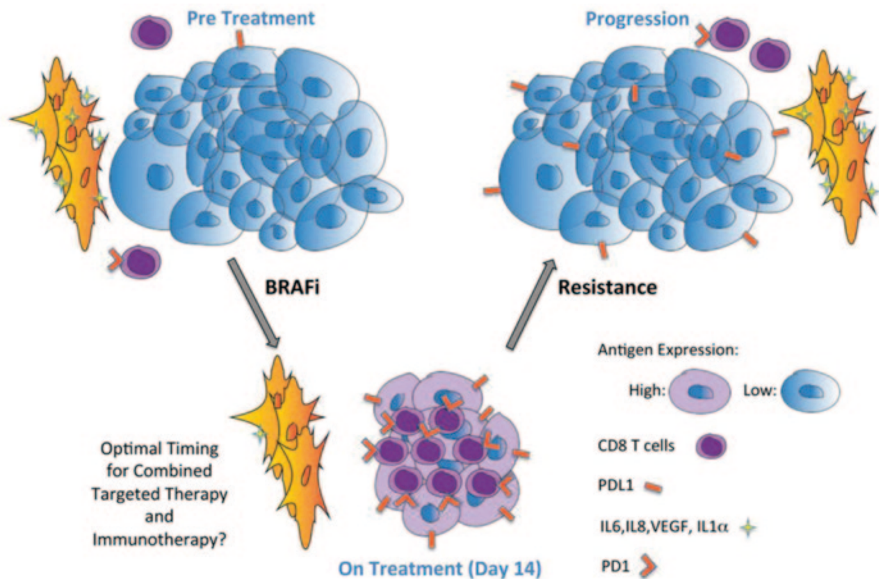

Fig. 8.5 Oncogenic *BRAF* contributes to immune escape through the down-regulation of melanoma-differentiation antigens and by establishing an immunosuppressive tumor microenvironment. The administration of a *BRAF* inhibitor promotes clinical responses along with an increased expression of melanoma-differentiation antigens by malignant cells, an increased tumor infiltration by CD8+T cells, and a decreased production of immunosuppressive cytokines such as *IL-6*, *IL*-8 and *IL-1* as well as of the angiogenic mediator vascular endothelial growth factor (VEGF). This phenotype is reverted at time of disease progression. Importantly, the expression of immunomodulatory molecules on T cells (e.g., *PD1*) and on tumor cells (e.g., *PDL1*) is also increased within 14 d of BRAF-targeted therapy initiation. Taken together, these data suggest that the therapeutic potential of BRAF-targeted agents may be significantly improved by the early blockade of immune checkpoints. (Adapted from Cooper et al. [36])

8.2.3.3 Murine Models

Mouse models have provided important insights into cancer development, progression, therapy, and resistance. Recent melanoma models have incorporated interactions of several signature mutations found in human melanoma, enabling the generation of a mouse that recapitulates hallmark features of the disease. To date, several studies have been published showing synergy of BRAF-directed therapy in murine models [10–12, 37] and one study has shown no synergy [38].

The first model demonstrating synergy was published by Koya, et al. and utilized a $BRAF^{V600E}$-driven murine model of melanoma, SM1, which is syngeneic to fully immunocompetent mice. In this mouse model of $BRAF^{V600E}$ melanoma, Koya et al. showed improved anti-tumor activity, in vivo cytotoxic activity, and intratumoral cytokine secretion by adoptively transferred cells in combination with a BRAF inhibitor [10]. However, T cell analysis also showed that BRAF inhibition did not alter the expansion, distribution or tumor accumulation of adoptively transferred T cells [10].

Another model demonstrating synergy between BRAF-directed therapy and immunotherapy was published by Liu, et al. In this manuscript, the authors used melanoma cells transduced with gp100 and H-2Db in a xenograft model on pmel-1 TCR transgenic mice on a C57BL/6 background and found an increase in tumor infiltrate and anti-tumor activity of adoptively transferred cells after BRAF inhibition (Fig. 8.6a) [12]. In this model, BRAF inhibition induced T-cell infiltration that was associated with a decrease in VEGF (Fig. 8.6b). In this paper they also found that VEGF overexpression in melanoma cells abrogates T cell infiltration [12]. This corroborates what is seen in patients treated with BRAF-directed therapy, as down regulation of intratumoral VEGF correlates with increased T-cell infiltration when melanoma patients are treated with a BRAF inhibitor [12].

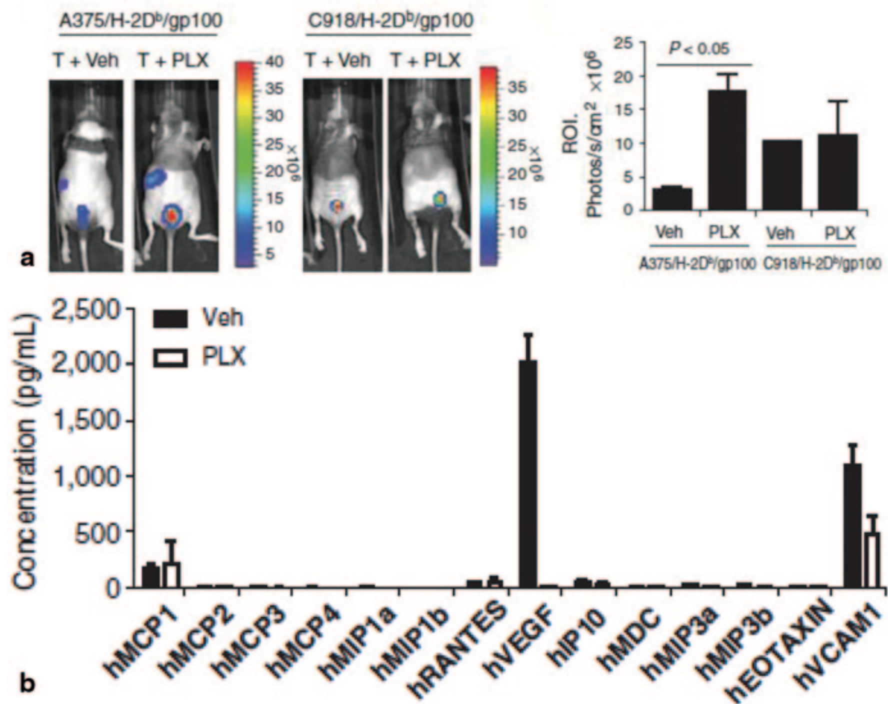

Fig. 8.6 PLX4720 increases infiltration of adoptively transferred T cells only in tumors containing $BRAF^{V600E}$. B6 nude mice (5 mice/group) bearing $BRAF^{V600E}$ A375/H-$2D^b$/gp 100 and BRAFT WT C918/H-$2D^b$/gp 100 tumors were treated with OFL-expressing pmel-1 T cells, along with gp100 peptide-pulsed dendritic cells, by intravenous injection on day seven after tumor inoculation. 2 days after T-cell transfer, PLX4720 or vehicle alone was administered by oral gavage daily for 3 days. Luciferase imaging showing *in vivo* trafficking of OFL-expressing pmel-1 T cells on day five after T-cell transfer. Quantitative imaging analysis of transferred T cells at the tumor sites is summarized and expressed as the average of photon flux within ROI (**a**), Data shown are expressed as mean + SEM and are representative of two independent experiments with similar results. In addition, BRAF mutant A375 tumor-bearing mice were sacrificed 3 days after oral gavage of PLX4720, and tumors were resected and weighed. Tumors were homogenized and sonicated in lysis buffer containing protease inhibitors. Cleared tumor lysates after centrifugation were tested using protein array analysis (**b**). (Adapted from Liu et al. [12])

Additionaly, Knight et al. utilized two relatively resistant syngeneic variants of $BRAF^{V600E}$-driven mouse melanoma, SM1 and SM1WT1, and a transgenic mouse model of melanoma to illustrate the ability of the BRAF inhibitor, PLX4720, to reduce melanoma CCL2 production Interestingly, host CCR2 was demonstrated in the antitumor activity of PLX4720. While there was no obvious target molecules influenced with in the SM1WT1 tumor, there was an increase in the CD8/Treg ratio in the TILs with PLX4720 treatment. In addition, depleting CD8+T cells, but not NK cells, were partially required for the therapeutic activity of PLX4720. Combination therapy of BRAF-directed therapies and anti-CCL2 or anti-CD137 antibodies demonstrated significant antitumor activity in these models supporting the therapeutic potential of combining BRAF inhibitors with immunotherapy [11].

Recently, a BRAF(V600E)/$Pten^{-/-}$ syngeneic tumor graft immunocompetent mouse model showed synergy of adding immune checkpoint blockade to BRAF inhibition [37]. In this model, BRAF inhibition leads to a significant increase in intratumoral CD8+T cell density and cytokine production, similar to effects of BRAF inhibition in patients. Furthermore, administration of anti-PD-1 or anti-PD-L1 blockade together with BRAF inhibitor led to an enhanced response, significantly prolonging survival and slowing tumor growth, as well as significantly increasing the number and activity of tumor infiltrating lymphocytes [37].

One manuscript has been published disputing possible synergy between BRAF-directed therapy and immunotherapy [38]. This manuscript described work utilizing a murine model with conditional melanocyte-specific expression of $BRAF^{V600E}$ combined with Pten gene silencing which leads to development of melanoma with 100% penetrance, short latency, and lung and lymph node metastases. The mice are responsive to BRAF and MEK inhibition. In this paper, primary melanoma tumors were induced via topical Tamoxifen and were then treated with BRAF-directed therapy alone or in combination with immune checkpoint blockade. Of note, the induced melanomas showed histological and immune cell compartment similarities to human melanomas [38]. However, unlike in humans [8, 34], there is a decrease in tumor resident lymphocytes in the setting of BRAF-directed therapy [38]. Furthermore, the addition of CTLA4 blockade did not improve tumor growth control [38].

It is important to note that tumors generated in this model may be implanted into syngeneic C57BL/6 mice, suggesting a potential for a syngeneic subcutaneous tumor model [38]. This is relevant as other groups (including our own) have used this approach with syngeneic subcutaneously implanted tumors and have demonstrated synergy with BRAF-directed therapy and immunotherapy [37]. The syngeneic subcutaneously implanted tumor model in C57BL/6 may better recapitulate metastatic disease, though this is a hypothesis that clearly needs to be tested.

8.3 Ongoing Clinical Trials

Based on promising results from pre-clinical and clinical studies demonstrating potential synergy between immunotherapy and targeted therapy for melanoma, clinical trials are underway to investigate the efficacy and safety of combining targeted therapy and immunotherapy in patients with melanoma positive for BRAF mutations (Table 8.1).

There are several clinical trials studying the combination of BRAF-directed therapy with the FDA-approved agent aldesleukin (interleukin-2). The first of these trials was developed at the Massachusetts General Hospital (Boston, Massachusetts) and is a phase II trial (NCT01754376) of BRAF-directed therapy (vemurafenib) and immunotherapy using aldesleukin (IL-2) in patients with metastatic melanoma harboring a BRAFV600E mutation. In this trial, patients receive a 2 week "lead-in" with vemurafenib and then receive high dose IL-2. The primary endpoints for this trial include efficacy (as measured by progression-free survival and durable response rate) and toxicity and comparisons will be made to historic controls of vemurafenib alone and aldesleukin alone. Importantly, this trial also includes correlative studies to assess for treatment response and immunologic parameters. The target accrual for this clinical trial is 42 patients over a 2 year time period.

A similar study is being run by the cytokine working group (CWG) (NCT01683188), examining the complete response rate to combination therapy of vemurafenib and high dose IL-2 in two cohorts: (1) BRAF V600 mutation-positive metastatic melanoma patients ($n=135$) who receive vemurafenib < 7 weeks before treatment with high dose IL-2 and (2) BRAF V600 mutation-positive metastatic melanoma patients ($n=50$) who receive vemurafenib > 7–18 weeks before treatment with IL-2.

In addition to IL-2 and vemurafenib combination strategies, clinical trials are also assessing the investigational use of adoptive cell therapy (ACT) in patients with metastatic melanoma. A pilot trial (NCT01585415) at the National Cancer Institute (Bethesda, Maryland) is investigating the safety of vemurafenib in combination with the investigational use of ACT of autologous tumor-infiltrating lymphocytes (TILs) in patients with metastatic melanoma. In this interventional study, investigators will first biopsy or resect melanoma tumors from patients ($n=25$) in order to generate and expand autologous TILs ex vivo. Patients will first undergo non-myeloablative lymphocyte depletion by chemotherapeutic agents: cyclophosphamide (60 mg/kg/day IV) on days seven and six and fludarabine (25 mg/day IV) on days five until one. On day zero, patients will receive up to 10^{11} TILs and aldesluekin (a total of 15 doses of 720,000 IU/kg IV every 8 hours). Patients will then start vemurafenib (960 mg) regimen on day one. Similarly, a single-center, Phase II Trial (NCT01659151) has commenced at H. Lee Moffitt Cancer Center and Research Institute (Tampa, Florida) to improve: (1) drop-out rates from ACT and (2) 12 month- PR and CR in patients with metastatic melanoma ($n=60$) that receive a combination of vemurafenib, lymphodepletion using cyclophosphamide and fludarabine plus adoptive cell transfer and high dose IL-2.

Table 8.1 Clinical trials investigating the combination of targeted therapy and immunotherapy

Official title of study	Clinical trials. gov identifier	Study type	Cohorts	Intervention	End-points	Institution/ sponsor
COMBAT 1: A Phase II Trial of Combined BRAF-Targeted Therapy and Immunotherapy for Melanoma	NCT01754376	Phase II clinical trial	*N*=49 BRAFV600 mutation positive metastatic melanoma patients	Vemurafenib + aldesleukin	Primary end-point: Efficacy of combination of vemurafenib and aldesleukin as measured by progression-free survival compared to historic controls Secondary end-points: (1) complete, partial and durable response rates (2) overall survival (3) toxicity and safety (4) confirm pre-clinical data that BRAF inhibitors increase immunogenicity of melanoma (5) exploration of biomarkers	Massachusetts General Hospital
A Multi-Center Study of High Dose Aldesleukin (Interleukin-2) + Vemurafenib Therapy in Patients With BRAFV600 Mutation Positive Metastatic Melanoma	NCT01683188	Open-label, uncontrolled, nonrandomized two-arm, multi-center, Phase IV clinical trial	Arm 1: BRAFV600 mutation positive metastatic melanoma patients ($n=135$) who receive vemurafenib < 7 weeks before treatment with IL-2 Arm 2: BRAFV600 mutation positive metastatic melanoma patients ($n=50$) who receive vemurafenib > 7–18 weeks before treatment with IL-2	BRAF Inhibitor: Vemurafenib High Dose Interleukin-2: Aldesleukin	Primary end-point: Complete response (CR) rate in BRAFV600 mutation positive metastatic melanoma patients who receive vemurafenib plus IL-2 at 10 (±3) weeks and 26 (±3) weeks from the start of IL-2	Prometheus Laboratories

Table 8.1 (continued)

Official title of study	Clinical trials.gov identifier	Study type	Cohorts	Intervention	End-points	Institution/sponsor
A Pilot Trial of the Combination of Vemurafenib With Adoptive Cell Therapy in Patients With Metastatic Melanoma	NCT01585415	Open label, single group, Phase I clinical trial	*N*=26 BRAFV600 mutation positive metastatic melanoma patients	Combination of non-myeloablative lymphodepletion, vemurafenib, ACT of autologous TIL, and high dose aldesleukin	Primary end-point: safety of combination of non-myeloablative lymphodepletion, vemurafenib, ACT of autologous TIL, and high dose aldesleukin Secondary: Tumor regression and effect of vemurafenib on tumor infiltrating lymphocytes	National Cancer Institute
A Phase II Clinical Trial of Vemurafenib With Lymphodepletion Plus Adoptive Cell Transfer and High Dose IL-2 in Patients With Metastatic Melanoma	NCT01659151	Open-label, Phase II, single-center clinical trial	*N*=60 BRAFV600 mutation positive metastatic melanoma patients	Vemurafenib followed by lymphodepletion with fludarabine and cyclophosphamide chemotherapy, adoptive cell therapy (ACT) with tumor infiltrating lymphocytes (TIL) infusion, and high dose IL-2	Primary end-point: Overall response & drop-out rate Secondary end-point: progression free survival	H. Lee Moffitt Cancer Center and Research Institute
A Phase I/II Trial of Vemurafenib and Ipilimumab in Subjects With V600 BRAF Mutation-positive Metastatic Melanoma	NCT01400451	Phase I clinical trial Phase II clinical trial	*N*=50 BRAFV600 mutation positive metastatic melanoma patients	Ipilimumab (CTLA-4 inhibitor)+vemurafenib	Primary end-point of Phase I trial: Safety and tolerability of ipilimumab and vemurafenib combination Primary end-point of Phase II trial: Overall survival	Bristol-Myers Squibb and Roche-Genentech

Table 8.1 (continued)

Official title of study	Clinical trials. gov identifier	Study type	Cohorts	Intervention	End-points	Institution/ sponsor
A Single Arm Open-Label Phase II Study of Vemurafenib Followed by Ipilimumab in Subjects With Previously Untreated V600 BRAF Mutated Advanced Melanoma	NCT01673854	Phase II clinical trial	$N=45$ BRAFV600E mutation positive previously untreated metastatic melanoma patients	Vemurafenib followed by Ipilimumab (CTLA-4 inhibitor)		Bristol-Myers Squibb
A Phase Ib, Open-Label Study of The Safety and Pharmacology of MPDL3280A Administered in Combination With Vemurafenib (Zelboraf®) in Patients With Previously Untreated BRAFV600-Mutation Positive Metastatic Melanoma	NCT01656642	Phase 1 clinical trial	$N=44$ BRAFV600E mutation positive previously untreated metastatic melanoma patients	Concomitant MPDL3280A (PDL1 Blockade) + Vemurafenib	Primary end-point of Phase I trial: Safety and tolerability of MPDL3280A and vemurafenib combination	Genentech
A Sequential Safety and Biomarker Study of BRAF-MEK Inhibition on the Immune Response in the Context of CTLA-4 Blockade for BRAF Mutant Melanoma	NCT01940809	Phase 1 clinical trial	$N=40$ BRAFV600E mutation positive metastatic melanoma that is surgically unresectable	A 25 day lead in of dabrafenib, trametinib or both followed by ipilimumab or ipilimumab as monotherapy	Primary endpoint: Safety and tolerability of ipilimumab following lead-in of BRAF or MEK inhibitors, either alone or in combination, in patients with BRAFV600 mutant melanoma Secondary end-point: Disease-control rate and response rate for the total treatment period (4 weeks after completion of study treatment)	National Cancer Institute

There are also several clinical trials of BRAF-directed therapy in combination with immune checkpoint inhibitors. The first of these trials was a phase I/II trial of vemurafenib and ipilimumab given concurrently in patients with BRAF mutant melanoma (NCT01400451). This trial involved a run-in of 1 month of BRAF-directed therapy (vemurafenib) alone followed by four infusions of ipilimumab. The primary goal of this trial was to assess safety and to define a schedule that could be used for further clinical trials. The target accrual for this trial was 50 patients, though the trial was stopped early due to toxicity (see discussion below). After the trial was stopped, another trial was opened with sequential (i.e. non-overlapping) administration of these agents. The target accrual for this trial is 45 patients.

Another trial is currently underway investigating the combination of BRAF-directed therapy with immune checkpoint blockade using anti-PD-L1 (NCT01656642). This trial aims to enroll 44 patients with BRAF-mutant melanoma with the primary endpoint of safety and tolerability.

Given the encouraging findings of combined BRAF-directed therapy with MEK inhibition, efforts are also underway to use combined BRAF + MEK inhibition with immune checkpoint blockade using ipilimumab (anti-CTLA-4). A phase I trial is currently underway and involves a 25 day lead-in of dabrafenib, trametinib, or both followed by ipilimumab (NCT01940809). The primary endpoint of this study is safety and tolerability, with a secondary endpoint of disease control rate and response rate. Importantly, biomarkers will also be studied with the goal of identifying potential predictors of response.

8.4 Summary of Responses and Toxicity to Date

Clinical trials investigating combination BRAF inhibitors and immunotherapeutic strategies to address metastatic melanoma remain in the early stages of patient accrual, and mature response and toxicity data are not yet available. However, some interesting data has emerged regarding toxicity with the combination of BRAF-directed therapy (Vemurafenib) and anti-CTLA-4 (Ipilimumab). Specifically, hepatotoxicity was observed in a phase 1 study of the concurrent administration of these two agents leading to closure of the trial to further accrual. Of note, the grade two or three elevations in liver function tests were completely asymptomatic, and resolved after the therapy was discontinued or with the systemic steroid administration [39]. Nonetheless this highlights the potential for unexpected toxicity in these trials, and suggests the need for well-controlled clinical trials, even when combining FDA-approved agents.

8.5 Future Directions

As a classic example of a bedside-to-bench-to-bedside paradigm, results from these trials will set the foundation for future clinical and translational studies to elucidate potential synergistic effects of combined BRAF-directed therapy and immunotherapy in patients with BRAF-mutant melanoma. Important questions remain and need to be answered. Will there be synergy between these two strategies? Namely, will the combination increase durable response rates and lead to more complete responses? Will there be increased toxicity with these combinations?

Additional questions regarding timing of therapy and duration of therapy also remain. What is the appropriate sequence and timing, and does therapy need to be continued even in the setting of a complete response or prolonged partial response?

There is some question as to whether or not other not synergy will be seen when immunotherapy is combined with other forms of MAPK pathway blockade (e.g. MEK inhibitors), as MAPK pathway activity is critical to T cell activation and may abrogate T cell responses [9]. These questions all beg answers, which will be provided in the context of translational research and carefully planned clinical trials with appropriate correlative studies.

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Chapter 9
Moving Forward: Making BRAF-Targeted Therapy Better

Keith T. Flaherty

Abstract For half of the advanced melanoma population, selective BRAF inhibitor therapy has transformed the natural history of disease and provided a platform for developing molecularly targeted therapy combinations. The clinical utility of vemurafenib, FDA approved BRAF inhibitor, has been validated by another potent and selective agent, dabrafenib. However, two clinical limitations of BRAF inhibitor therapy frame the problem for the melanoma field: *de novo* and acquired resistance. Insights into the mechanisms underlying both of these phenomena have set the stage for clinical investigation of several novel BRAF inhibitor based combination therapies. Foremost among them is the combination of a MEK inhibitor with BRAF inhibitor. Preliminary clinical evidence suggests that this combination may supplant single agent BRAF inhibitor therapy in the near future as the standard approach for metastatic patients. Yet resistance remains a challenge and strategies to target non-MAP kinase pathway dependent mechanisms are needed. This chapter will outline the preclinical evidence that supports the categorization of resistance mechanisms and the framework for clinical investigation of novel combination therapies.

Keywords Melanoma · BRAF · Resistance · Receptor tyrosine kinases · PI3K pathway · Cyclin dependent kinases · Apoptosis

9.1 Introduction

Selective BRAF inhibitors induce tumor regression in approximately 90 % in patients with activating *BRAF* mutations that harbor the V600 position, with complete responses in 5 % [1, 2]. Disease control is achieved for 6–7 months, on average. However, responding patients relapse as quickly as 2 months after the first evi-

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dence of tumor regression and a small subset of patients remain progression-free for more than 2 years [3]. Early clinical studies that incorporated early assessment of metabolic response (fluorodeoxyglucose positron emission tomography) suggest that all patients have at least metabolic responses to therapy within the first several weeks [4]. Based on this data, it would appear that BRAF inhibitor therapy is able to impact all tumors, but to a highly variable degree with regard to magnitude of initial effect. Similarly, the time to emergence of resistance is highly variable. These clinical observations give rise to two question that chapter will attempt to address:

1. what cell survival mechanisms underlie survival of some *BRAF* mutant tumors?
2. how do melanoma cells restore proliferation in the face of ongoing BRAF inhibition?

Addressing these questions will explain the rationale for the BRAF inhibitor-based combination therapy regimens that are currently being pursued clinically.

9.2 Genetic Complexity

BRAF mutant melanomas vary significantly with regard to the number of somatic genetic alterations that co-occur with *BRAF* mutations [5]. Typically arising on intermittently sun-unexposed skin, many of these tumors lack the very large number of cytosine to thymidine mutations, thought to derive from ultraviolet radiation, that can be found in melanomas that arise on chronically sun-exposed skin. Additionally, several of the oncogenic pathways that are known to contribute to melanoma formation in some instances are genetically normal in a distinct subset of *BRAF* mutant melanomas. Two examples of tumor suppressor genes that are commonly inactivated through mutation or deletion are *CDKN2A* and *PTEN*. Amongst *BRAF* mutant melanoma cell lines, those that harbor *PTEN* loss are more resistant to BRAF inhibitors than cell lines that lack of these abnormalities [6, 7]. Preliminary analysis of patient tumor samples from a subset of participants in phase II trials of vemurafenib and dabrafenib appears to confirm this association, and suggests that CDKN2A loss is also associated with worse outcome [8]. Conversely, those patients in whom *CDKN2A* and *PTEN* are wild-type, are amongst those who achieve the most long-lasting responses. These observations raise a very simple hypothesis: lesser degree of genetic complexity is associated with greater response and duration of response. With relatively long-term follow-up of patients treated on phase II and phase III trials with vemurafenib and dabrafenib and pretreatment tumor samples being available for the vast majority of patients, this can be readily confirmed using deep sequencing methods to characterize genetic alterations across all expressed regions of the genome. A corollary to this hypothesis that patients receive BRAF inhibitor therapy in the metastatic setting would be more likely to achieve initial and long-lasting responses if were treated when there is less, rather than more burden of disease. Subset analyses from vemurafenib and dabrafenib trials support this hypothesis [1, 2].

9.3 MAP Kinase Pathway-Dependent Resistance

Following 2 weeks of treatment with vemurafenib, analysis of tumor biopsy specimens reveals profound but incomplete inhibition of ERK activation/phosphorylation [9]. In vitro, exposure of *BRAF* mutant melanoma cell lines to a selective BRAF inhibitor at concentrations that are comparable to those achieved in human plasma similarly results in incomplete inhibition of ERK [10]. The addition of a MEK inhibitor to these same concentrations of BRAF inhibitor results in greater ERK suppression and significantly more cell death, thus validating that melanomas depend on the residual amount of MAP kinase pathway activity to survive.

At the time of disease progression on single agent BRAF inhibitor therapy, analysis of tumor biopsies patients treated with vemurafenib revealed that most tumors demonstrate reactivation of MAP kinase pathway, measured by immunohistochemistry for phosphorylated ERK [9]. As reviewed elsewhere in this volume, significant insight has been gained into the molecular mechanisms that account for this mechanism (Fig. 9.1). Taken together with the evidence of low level, persistent ERK activation early in the course of therapy, these finding suggests that *BRAF* mutant melanomas can survive with markedly reduced ERK signaling, but need to restore ERK activation to near-normal levels in order to proliferate. Both lines of evidence supported the clinical evaluation of BRAF/MEK combination therapy.

In a phase I/II clinical trial, dabrafenib and trametinib (a potent and selective MEK 1/2 inhibitor) were combined at a range of doses including the full single-agent

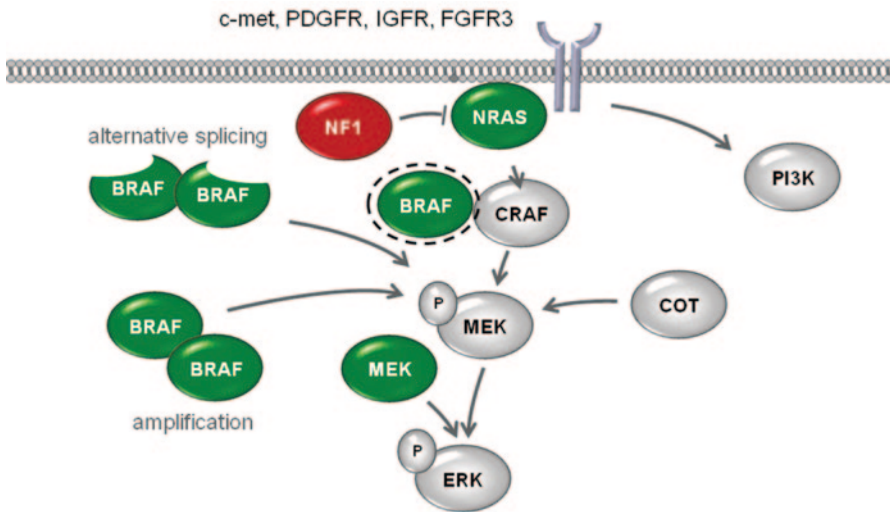

Fig. 9.1 BRAF inhibitor acquired resistance mechanisms c-met, PDGFR, IGFR, FGFR3

doses of both drugs [11]. Remarkably, the combination of both drugs at full doses produced a lower rate of dose limiting toxicity than either agent alone previously conducted phase I trials. This is thought to be a consequence of the MEK inhibitor counteracting inhibitor associated paradoxical activation, and BRAF inhibitor associated paradoxical activation attenuating MEK inhibitor related toxicities. With regard to efficacy, the dabrafenib/trametinib combination was associated with a significantly higher response rate, including a complete response rate of 10 %, compared to single agent dabrafenib which was evaluated concurrently in a randomized phase II component of this trial. These results support the preclinical observation that suppression of residual ERK activation by co-administering a MEK inhibitor results in cell death. A similar outcome was observed when vemurafenib was combined with another experimental MEK inhibitor [12].

Duration of response was also significantly improved with the dabrafenib/trametinib combination compared to single agent dabrafenib, with a near doubling of median response duration from 5.6 to 10.5 months [11]. This confirmed that reactivation of ERK following BRAF inhibitor monotherapy was clinically relevant and that some mechanisms of restored MAP kinase pathway signaling can be successfully suppressed, if not prevented, with a MEK inhibitor. It is not currently known which *BRAF* mutant tumors are most susceptible to BRAF/MEK combination therapy compared to BRAF inhibitor monotherapy. A small subset of patients with disease progression on single-agent BRAF inhibitor therapy have persistently suppressed ERK phosphorylation and are presumed to depend on mechanisms outside of the MAP kinase pathway to drive tumor proliferation at that time [9]. For this group, BRAF/MEK combination therapy may add little to efficacy of single-agent BRAF inhibitor therapy. Additionally, there are no methods available to predict which tumors will emerge with *NRAS* mutations, splice variants of BRAF, *BRAF* amplification, or activating *MEK* mutations. And therefore, there is no basis by which one can tailor the use of BRAF/MEK combination therapy at the present time.

9.4 BRAF/MEK Resistance and ERK Inhibition

With most *BRAF* mutant tumors demonstrating evidence of restored ERK activation at the time of resistance to single agent BRAF inhibitor therapy, there is increased interest in exploring agents that block MAP kinase pathway signaling further downstream. As discussed above, MEK inhibition has been explored extensively preclinically and clinically. While the preclinical evidence suggests that MEK inhibitors can inhibit cell growth and induce cell death comparably to selective BRAF inhibitors in vitro and in vivo, the clinical evidence suggests that the antitumor effects achieved at tolerable doses are slightly less robust compared to BRAF inhibitors [13, 14]. This raises the issue of therapeutic index for each point of intervention in the pathway with regard to normal tissue dependencies and effects in tumor tissue relative to normal tissue.

BRAF and MEK inhibitors have a starkly different profile with regard to their impact on the MAP kinase pathway in *BRAF* mutant tumor tissue versus normal tissue as discussed previously [15]. Specifically, the BRAF inhibitors for which the

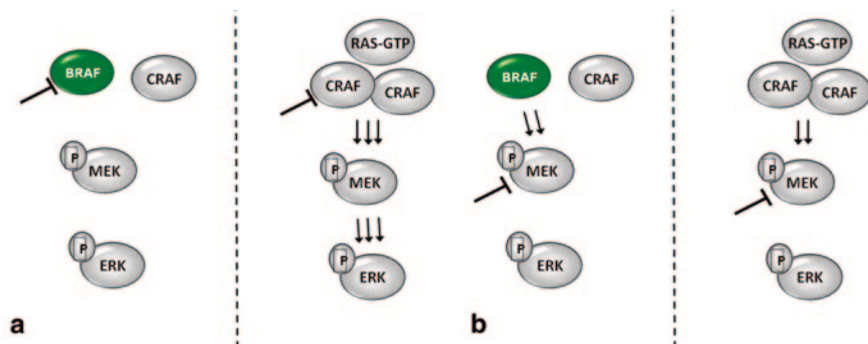

Fig. 9.2 Inhibition of MEK/ERK in the setting of BRAF mutation. **a** Activation of MEK/ERK in the setting of activated RAS. **b** Inhibition of MEK/ERK in the setting of activated RAS

most clinical experience exists do not appear capable of overcoming paradoxical activation and having a net inhibitory effect on the MAP kinase pathway at doses/exposures that can be safely administered to patients (Fig. 9.2a). Therefore, BRAF inhibitor related toxicities appear to occur as a consequence of paradoxical activation or via inhibition of kinases other than BRAF and CRAF [16]. MEK inhibitors, on the other hand, are associated with ERK inhibition in nearly all cell types analyzed to date, including cancer cell lines with a variety of oncogenic drivers as well as normal cell lines (Fig. 9.2b) [13, 17]. Therefore, MEK inhibitors are thought to mediate toxicity via inhibition of the MAP kinase pathway inhibition in normal tissues [10, 16]. Co-administration of BRAF and MEK inhibitors results in greater degrees of MAP kinase pathway suppression in tumor suppression, but less activation or inhibition of the pathway in normal tissues. The reduction in the rate of cutaneous squamous cell carcinomas and keratoacanthoma when BRAF and MEK inhibitors are co-administered is taken as clinical validation of these biochemical observations [18].

ERK inhibition represents a novel strategy that has not been fully explored (Fig. 9.3). Perhaps the most compelling current evidence in support of development of ERK inhibitors is the presence of activating *MEK* mutations at baseline and, in a larger subset of patients, following exposure to BRAF inhibitor therapy in some patients [9]. Preclinically, these mutations appear to confer resistance to the currently available allosteric MEK 1/2 inhibitors [19]. But, the known differences in feedback regulation of BRAF and MEK and the absence of such feedback loops that effect ERK, provides another rationale for considering this point of intervention in hopes that compensatory feedback mechanisms would not erode the pharmacodynamic effects of an ERK inhibitor as they would BRAF or MEK inhibitors [20].

Two ATP competitive, selective ERK 1/2 inhibitors have recently entered clinical development and extensive preclinical data is now available for one of these agents (SCH772984) [NCT01781429 & NCT01358331]. Like MEK inhibitors, SCH772984 is able to inhibit MAP kinase pathway signaling in both *BRAF* mutant and *RAS* mutant models [21]. But, more relevant to the issue of BRAF inhibitor resistance, this agent inhibits the MAP kinase pathway and cell proliferation in BRAF mutant melanoma cell lines with acquired or engineered resistance to BRAF inhibitors. Specifically, cell lines into which activating *RAS* mutation, the truncating

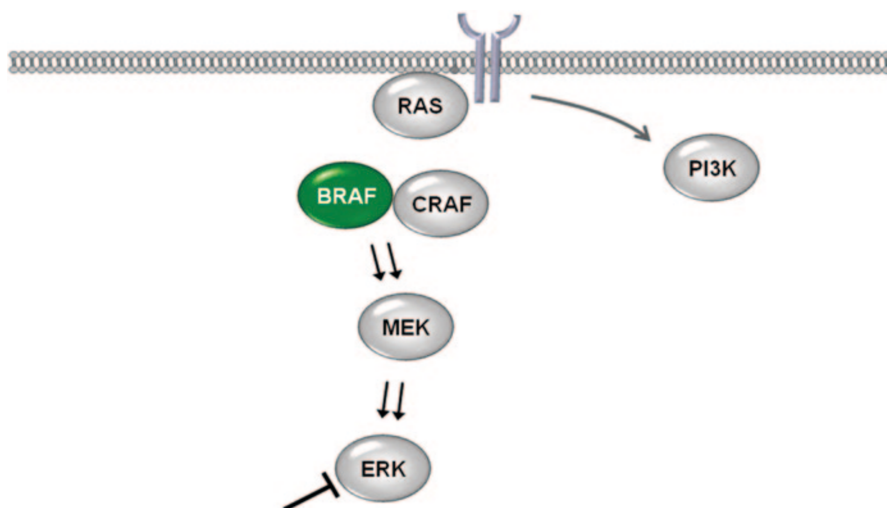

Fig. 9.3 Blocking downstream in the MAPK pathway: ERK inhibitors

BRAF splice variants, forced overexpression of BRAF, or activating *MEK* mutation are sensitive to single agent SCH772984, but not a BRAF or MEK inhibitor. And, in a xenograft established with a melanoma cell line with acquired resistance to concomitant BRAF and MEK inhibitor exposure, SCH772984 produces growth control as a single agent and in combination with continued BRAF/MEK combination therapy. These data point to a very clear potential application for selective ERK inhibitors in *BRAF* mutant/BRAF inhibitor refractory patients. But, it remains possible that ERK inhibition could have greater single-agent efficacy than either BRAF or MEK inhibitors in the BRAF inhibitor naïve setting. Or, an ERK inhibitor could be a more optimal component of a BRAF inhibitor-based combination approach, supplanting MEK inhibition. This possibility is particularly intriguing in light of the observation that concomitant administration of a BRAF inhibitor with a MEK inhibitor attenuates the frequency and severity of the typical MEK inhibitor associated toxicities: acneiform rash and diarrhea [11, 14]. Presumed to be a consequence of paradoxical activation associated with selected BRAF inhibitors, ERK inhibitors could benefit from this compensatory signaling effect in normal tissues as well.

9.5 Alternative Schedules

Alternative strategies to continuous suppression of the MAP kinase pathway with either BRAF inhibitor monotherapy or BRAF/MEK inhibitor combination therapy are scheduled interruption of therapy and pulsatile dosing.

The concept of introducing interruptions in the dosing of BRAF inhibitor therapy stems from the observed mechanisms of resistance that have been described in patient tumor specimens procured and characterized at the time of disease progression following initial response to BRAF inhibitor therapy. Knowing that over activation of oncogenic pathways has been previously demonstrated to induce senescence

(oncogene-induced senescence) or cell death in other contexts, investigators explored the consequences of withdrawal of vemurafenib following development of acquired resistance in vitro [21, 22]. They observed the hypothesized effect: in cells that restored MAP kinase pathway signaling in the face of chronic BRAF inhibition, withdrawal of the BRAF inhibitor resulted in hyperactivation of ERK, cell cycle arrest and cell death. They demonstrated that *BRAF* mutant melanoma cells require ERK activation within a certain range to survive and proliferate. Too little MAP kinase pathway output (in the setting of initial BRAF inhibitor therapy) impacts proliferation and survival and, based on these recent findings, too much pathway output is similarly toxic. This suggests the possibility of exposing cells to a BRAF inhibitor long enough for them to reset their ability to survive in the face of decreased MAP kinase pathway output, followed by withdrawal of the BRAF inhibitor, and then reinstitution of the BRAF inhibitor after MAP kinase pathway output reequilibrates. This strategy successfully postponed the outgrowth of resistant clones in vitro. Using a patient-derived xenograft from a *BRAF* mutant melanoma patient whose tumor acquired high-level *BRAF* amplification, these investigators demonstrated potential clinical relevance of this strategy by showing that interrupted schedule of administration resulted in longer duration of tumor control compared to continuous dosing. While this results support the clinical investigation of interrupted schedule of vemurafenib, dabrafenib, or other selected BRAF inhibitors, one wonders whether greater clinical impact could be achieved by investigating an interrupted schedule of administration for BRAF/MEK combination therapy.

Pulsatile dosing refers to the strategy of administering higher doses of therapy than can be safely administer continuously for a short duration of time. This is not a new concept in cancer therapeutics, as nearly all conventional cytotoxic chemotherapy are administered in this way. However, this strategy has not yet been explored in a widespread fashion with molecularly targeted therapies. Promising preclinical evidence has been generated for small molecule EGFR inhibitor therapy given in a pulsatile fashion in combination with conventional cytotoxic chemotherapy to patients with metastatic non-small cell lung cancer [23]. One might hypothesize that an even greater incremental benefit could be observed if one were to this strategy in an oncogene-defined subpopulation receiving the relevant oncogene targeted therapy. Preclinical evidence has been generated in support of this concept for abl kinase inhibitors in chronic myelogenous leukemia harboring BCR-ABL translocations [24]. This strategy has not yet been explored for BRAF inhibitor-based therapy in *BRAF* mutant melanoma, but certainly warrants consideration.

9.6 More Potent and Selective BRAF Inhibitors

Based on the evidence supporting greater initial antitumor effect in vitro, in vivo, and in patients when a MEK inhibitor is combined with a BRAF inhibitor, it remains possible that further optimization in the properties of a selected BRAF inhibitor could result in greater efficacy than is observed with vemurafenib or dabrafenib. With this motivation, LGX818 was selected for further development as a more potent and more selective BRAF inhibitor than the currently available agents [25]. Other than allowing for lower doses of drug, greater potency it is not intuitively

expected to produce improvement in therapeutic effect. However, careful analysis of several BRAF inhibitors and their capacity for inducing paradoxical activation has shown that, for most agents, activation can be overcome with sufficiently high concentrations of drug [15]. Some BRAF inhibitors are associated with a narrow range of concentrations at which initial activation is observed and then overcome. Vemurafenib, for example has a particularly broad range of doses over which these phenomena are observed, likely making it impossible to achieve sufficient drug concentrations in patients to overcome paradoxical activation LGX818, on the other hand, is several-fold more potent for V600E BRAF, and has a relatively narrow range of concentrations over which paradoxical activation can be induced and then overcome. Thus, it is possible that this type of BRAF inhibitor could be dosed in a fashion that produces not only greater MAP kinase pathway inhibition in *BRAF* mutant tumors, but is not associated with paradoxical activation and the toxicities that appear to be a consequence. Increased selectivity raises the possibility of producing a greater impact on BRAF signaling without perturbing signaling mediated by the next most potently inhibited kinases. To date, it is not clear what BRAF inhibitor toxicities are a consequence of effects on non-RAF kinases. But, photosensitivity, for vemurafenib, and fever, with dabrafenib, appear to be compound specific effects and may not relate to RAF inhibition [26, 27].

9.7 Degrading BRAF

The appearance of BRAF splice variants at the time of disease progression on a BRAF inhibitor as well as the smaller number of cases associated with high-level *BRAF* amplification point to the possibility that targeting BRAF in ways other than ATP competitive kinase inhibition may be useful [28]. It has been known for several years that oncogenic BRAF is a client protein for the chaperone heat shock protein 90 (HSP90). Disruption of the HSP90/BRAF interaction would be hypothesized to lead to accelerated BRAF degradation, lower expression, and decreased oncogenic potential (Fig. 9.4).

Various HSP90 inhibitors have been developed over the past decade and represent an opportunity for exploring this mechanism of action. In vitro, it is clear that both geldanamycin-derivative and novel chemical classes of HSP90 inhibitors produce this effect [29, 30]. However, the immediate concern with regard to clinical application is that HSP90 has a large number of client proteins not all of which are uniquely relevant to cancer pathophysiology. Assuaging this concern regarding potential low therapeutic index are data supporting tumor growth control in xenograft experiments at doses that do not produce overt toxicity [30]. However, it is clear from single agent phase I and phase II clinical trials with HSP90 inhibitors that toxicity does occur at doses that produce drug exposures that are not high above the threshold for antitumor effects in preclinical models [31, 32]. Specifically, severe fatigue is a common, class-effect toxicity that is not well appreciated in preclinical toxicology or in vivo efficacy experiments, yet is commonly observed in patients receiving potentially therapeutic doses of HSP90 inhibitor therapy. In one clinical study amongst patients with metastatic melanoma, evidence of decreased BRAF

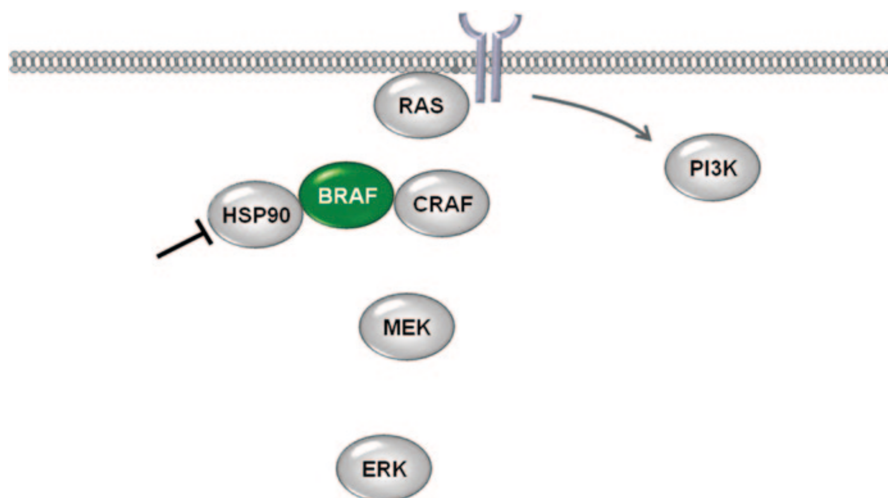

Fig. 9.4 Targeting BRAF protein stability with HSP90 inhibitors

expression was documented in patient tumor biopsies obtained on therapy and compared to biopsies from immediately before initiation of treatment [31]. However, the lack of significant single-agent efficacy in the same trial suggested that the magnitude of effect on BRAF expression is insufficient and higher doses/exposures not possible due to dose limiting toxicities.

While single agent HSP90 inhibition may have limited clinical application at least in the BRAF inhibitor naïve setting, it is possible that these agents would serve as compelling agents to investigate in combination with BRAF or BRAF/MEK dual inhibitor strategies. By decreasing expression of oncogenic BRAF, the pool of V600 mutated BRAF molecules would be diminished and presumably could be occupied with a lower concentration of a selective BRAF inhibitor. This potential interaction is supported by preclinical evidence in vitro, demonstrating not only decreased BRAF expression, but greater inhibition of proliferation and induction of cell death with combined BRAF/HSP90 inhibition [30]. And, in vivo, this combination produces more durable tumor control then BRAF inhibitor monotherapy. This strategy is currently being explored in a phase I/II clinical trial in which XL888 is combined with vemurafenib[NCT01657591]. But, given the apparently greater efficacy and attenuated toxicity of BRAF/MEK combination therapy, this may be the preferred MAP kinase pathway targeting strategy with which to combine an HSP90 inhibitor.

9.8 CRAF Dependent Resistance and RAF Dimer Blockers

The canonical MAP kinase pathway signaling cascade is comprised of RAS, RAF, MEK and ERK. In the setting of oncogenic V600 mutated BRAF, RAS activation is not required and, in fact, low concentrations of RAS-GTP (activated RAS) are

observed [15]. Notably, this creates a state in which oncogenic BRAF is responsible for nearly all RAF related signaling as low RAS-GTP results in decreased CRAF and ARAF activation. However, in the setting of acute and chronic BRAF inhibition, RAS-GTP levels are increased in vitro. This causes CRAF activation and is thought to be responsible for the rapid rebound in MEK and ERK activation observed after just 48–72 hours of exposure to a selected BRAF inhibitor [10]. At the time of acquired resistance to BRAF inhibitor therapy in patients, a notable minority are found to have activating *NRAS* mutations along with persistence of BRAF V600 mutations [33]. In the absence of a *BRAF* mutation, activating *NRAS* mutations have been shown to drive MAP kinase pathway signaling primarily through CRAF, not BRAF [34]. These clinical and preclinical observations lend support to the hypothesis that restoration of CRAF signaling is a potentially important component of BRAF inhibitor resistance.

Independent investigations outside of the context of *BRAF* mutant cancers have shown that CRAF, but not BRAF, has other activities beyond direct phosphorylation of MEK. In the setting of elevated RAS-GTP, CRAF is recruited to the plasma membrane and complexes with several scaffolding proteins in association with MEK, which CRAF directly phosphorylates. However, activated CRAF can localize to two additional intracellular compartments: the outer membrane of mitochondria and the mitotic spindle (Fig. 9.5) [35, 36]. When localized to the mitochondria, activated CRAF complexes directly with proapoptotic BAD tipping the balance of apoptosis-related proteins toward cell survival. At the mitotic spindle, CRAF co-localizes with polo-like kinase 1 and contributes to cell cycle progression through mitosis [37]. Therefore, in the setting of BRAF inhibitor therapy mechanisms that restore RAS activation could, indirectly, lead to CRAF-mediated cell survival and cell cycle progression in the face of ongoing BRAF inhibition. Additionally, acti-

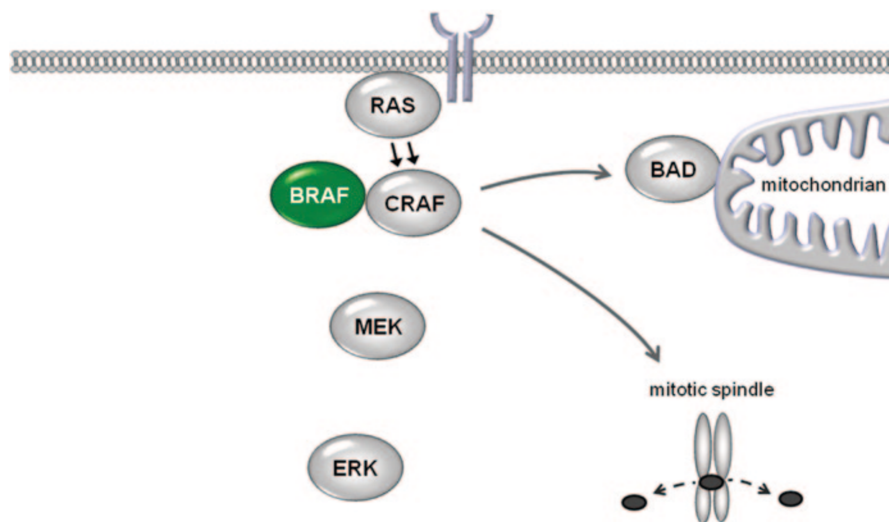

Fig. 9.5 The MEK-independent functions of activated CRAF

vated CRAF would be expected to phosphorylate MEK. The addition of a MEK inhibitor to the BRAF inhibitor would be anticipated to overcome only this last component of CRAF-dependent signaling. To overcome CRAF-mediated effects on cell survival and cell cycle that are MEK/ERK independent, CRAF itself could targeted or the downstream effectors of the pro-survival or mitotic progression effects. There is considerable interest in the possibility of developing potent and selective CRAF inhibitors, with relative selectivity for CRAF as opposed to BRAF. This is a critical design feature as both vemurafenib and dabrafenib are equipotent for BRAF and CRAF in isolated kinase assays, but ultimately have the net effect of contributing to paradoxical activation of the MAP kinase pathway in *BRAF* wild-type cells [15, 38, 39]. So, to function as an inhibitor of the MAP kinase pathway via CRAF inhibition, more potent and selective activity is needed against CRAF. Such an agent has not yet been described. With regard to downstream effectors, BH3 or SMAC mimetics may overcome the CRAF/BAD mediated pro-survival signal. And, aurora kinase inhibitors may intercept the effect of activated CRAF at the mitotic spindle.

An alternative strategy for disrupting both CRAF mediated resistance to BRAF inhibitors and paradoxical activation as a consequence of RAF dimer formation is to develop agents that inhibit the dimer interface. This region has been well-characterized from the crystal structure of BRAF and CRAF [40]. However, designing drugs that would selectively disrupt this protein-protein interaction without having widespread interactions with other intracellular proteins may be a far greater challenge than developing ATP competitive RAF inhibitors. Early attempts to identify such compounds suggest that it is feasible to identify small molecules with drug-like properties that could serve as the basis for further developing clinical candidates [41].

9.9 MITF Dependent Resistance

MITF, the transcription factor considered the master regulator of the melanocyte lineage, is suppressed by oncogenic BRAF and constitutively active MAP kinase pathway signaling [42, 43]. It is not surprising, then, that BRAF inhibitor therapy is associated with significant increases in MITF expression in vitro and in human tumors [44]. While this has the potentially positive consequence of upregulating the expression of melanocyte lineage antigens that could allow for more effective immune surveillance [45], MITF itself is a known oncogene [46]. The potential adverse consequences of increased MITF expression have only recently been elucidated. MITF directly regulates the expression of the BCL-2 family member, BCL2A1 [21]. Following exposure to a BRAF inhibitor, BCL2A1 expression is significantly increased. The pro-survival effects of BCL2A1 expression are supported by evidence that greater cell death can be induced by genetically silencing BCL2A1 expression in conjunction with BRAF inhibitor therapy. In patient tumor specimens, induction of BCL2A1 expression early in the course of BRAF inhibitor therapy is associated with lesser response to therapy compared to those patients in whom BCL2A1 expression is not induced. Overcoming this pro-survival impact of BRAF

inhibitor therapy would require either an agent that can suppress MITF expression or antagonize BCL2A1. Only agents that nonspecifically impact MITF or BCL2A1 currently exist. HDAC inhibitors appear to cause degradation of MITF [47], but have far-reaching effects on gene transcription and protein stability beyond MITF, and the BH3 mimetic, obatoclax, is able to bind BCL2A1 in addition to other BCL-2 family members [48]. More direct or selective pharmacologic inhibitors of MITF or BCL2A1 may be to address this particular mechanism of resistance.

9.10 BCL-2

Independent of the connection between MITF and BCL2A1, elevated expression of BCL-2 has been documented in melanoma [49]. The functional relevance of BCL-2 in terms of contributing to melanoma cell survival is supported by genetic silencing experiments in cell culture. Attempts to modulate BCL-2 clinically with an anti-sense oligonucleotide (oblimersen) were ultimately unsuccessful. Early clinical investigations with this agent in metastatic melanoma patients suggested only moderate impact on BCL-2 expression in patient tumor specimens assayed after treatment with oblimersen compared to pretreatment tumor specimens [50]. More recently, small molecule, BH3 mimetics have been developed to antagonize BCL-2 in, perhaps, a more effective fashion. In preclinical models, two BH3 mimetics (ABT-737 & ABT-263) results and down regulation of BCL-2 and potentiates BRAF inhibitor induced cell death and produces more durable tumor regression in vivo [51, 52]. This agent has been explored clinically in chronic lymphocytic leukemia and small cell lung cancer, both of which are associated with nearly ubiquitous high-level expression of BCL-2 [53, 54]. In those settings ABT-737 appears to augment the effect of conventional cytotoxic chemotherapy to some extent. It is hoped that in melanoma, where BRAF inhibitor therapy is a more active cytotoxic backbone, ABT-737 may contribute to an even greater therapeutic impact.

9.11 FOXO/ERBB3

Analogous to the relationship between oncogenic BRAF and MITF, inhibition of mutated BRAF appears to have more widespread consequences with regard to transcription factor expression and activity. Specifically, FOXD3 has been identified as another transcription factor for which expression is suppressed by oncogenic BRAF and upregulated as a consequence of BRAF inhibition [55, 56]. Combining gene expression profiling with chromatin immunoprecipitation assays, several FOXD3 regulated genes were identified that might relate to counterproductive downstream consequences of BRAF inhibition. The epidermal growth factor receptor family member, ERBB3, was identified as one of the genes whose expression was restored when FOXD3 was overexpressed in melanoma cells [57]. Genetic silencing of

FOXD3 or ERBB3 potentiated the efficacy of BRAF inhibition. Given that ERBB3 is a far more tractable potential therapeutic target than FOXD3, the therapeutic value of targeting ERBB3 has been further explored preclinically. ERBB3 is unique in comparison to ERBB1, ERBB2, and ERBB4 in that it lacks intrinsic kinase activity. It is thought that ERBB3 activates downstream signal transduction through heterodimer formation with these other ERBB family members. Therapeutic strategies that are currently being explored clinically in other tumors for which ERBB3 is thought to be a potential target include monoclonal antibodies that block ligand-dependent activation, both ligand-dependent and ligand-independent activation, or the kinase activity of ERBB1, ERBB2, and ERBB4. To date, evidence has been generated with lapatinib, a small molecule inhibitor of of ERBB1, ERBB2, and ERBB4 in combination with BRAF inhibition in both *BRAF* mutant melanoma and thyroid cancer [58, 59]. The availability of lapatinib for further clinical investigation in this setting as well as an increasing number of ERBB3 monoclonal antibodies in clinical development provides the opportunity to rapidly conduct clinical trials in combination with BRAF inhibition. A challenge that remains is that there are not currently predictive biomarkers that can be used to restrict the investigation of these combinations to those patients whose tumors will ultimately manifiest FOXD3/ERBB3 upregulation.

9.12 PI3K/pS6

The PI3 kinase pathway has been associated with melanoma pathophysiology for many years. Specifically, *BRAF* mutations are commonly accompanied by deletion or inactivating mutations in *PTEN* or *AKT3* amplification in melanoma, supporting their role of this pathway in contributing to melanocytic transformation [60, 61]. In advanced melanoma, there is compelling evidence that the PI3K pathway can confer resistance to BRAF inhibitor therapy, particularly in melanomas that have loss of PTEN expression [6, 7]. In the same models, synergy is observed when a BRAF inhibitor is combined with a selective PI3K inhibitor and points toward one combination targeted therapy approach which may be particularly well-suited for the *BRAF* mutant/*PTEN* deleted subset of patients.

Emerging evidence suggests that downstream elements of the PI3K pathway can be differentially regulated in *BRAF* mutant melanoma cells. Outside of the cancer context, crosstalk between downstream elements of the MAP kinase pathway and PI3K/AKT pathway has been demonstrated. Specifically, activated RSK, a substrate for ERK, directly phosphorylates S6 kinase, which is otherwise known to be regulated by mTOR(). In some *BRAF* mutant melanoma cell lines, S6 kinase is under the control of the MAP kinase pathway whereas, in others, it is not. This has been documented in vitro and inhibition of S6 kinase is strongly associated with robust induction of cell death in comparison to cells with persistent S6 kinase phosphorylation which do not undergo apoptosis [62]. Combined inhibition of the MAP kinase pathway and mTOR results in inhibition of S6 kinase in these refrac-

tory cells and induces a comparable degree of cell death compared to MAP kinase pathway inhibition alone in S6 kinase responsive tumors. In patient tumor samples obtained immediately before and soon after initiation of BRAF inhibitor therapy, the inhibition of S6 kinase versus persistent phosphorylation correlates with improved progression free survival.

This observation points to the possibility that some *BRAF* mutant melanomas have an adequate signaling response to single agent BRAF inhibitor therapy and that monitoring S6 kinase phosphorylation early in the course of therapy could identify patients who should continue on single agent therapy and those who should pursue combination therapy. The challenge of testing this hypothesis in the clinic is that real-time molecular monitoring of an activated phosphoprotein has not been previously attempted. The research methods described above would need to be developed into a robust and reproducible pathology assay in the proper, quality-controlled environment. The absence of a predictive biomarker that identifies which patients will have an adequate versus inadequate signal transduction inhibitory effect forces consideration of this cutting-edge approach in an attempt to personalize BRAF inhibitor-based single agent in combination therapy. The same can be said for monitoring the upregulation of ERBB3 as discussed above.

9.13 Microenvironment-Mediated Resistance

As discussed extensively thus far, much of the focus of the melanoma field has been to elucidate mechanisms of acquired resistance to single agent BRAF inhibitor therapy and to understand the role of concomitant somatic genetic alterations in de novo resistance. Undoubtedly, these tumor cell autonomous factors are promising therapeutic co-targets with BRAF inhibition, or BRAF/MEK combination therapy. However, an unanswered question in the field is how growth factor receptor tyrosine kinases become activated and contribute to BRAF inhibitor resistance in the absence of activating mutations or amplification (Fig. 9.6).

Two seminal preclinical investigations have shed light into the potential interaction of the tumor microenvironment with *BRAF* mutant cells under the selective pressure a BRAF inhibitor therapy [63, 64]. In one set of experiments, cell types known to exist in the tumor microenvironment were individually co-cultured with *BRAF* mutant melanoma cell lines (in parallel with other oncogene defined tumor models), including fibroblasts, endothelial cells, pericytes, and others [63]. Fibroblasts were uniquely capable of conferring resistance in *BRAF* mutant melanoma cell lines exposed to a selective BRAF inhibitor. It was subsequently shown that conditioned media from fibroblasts was similarly able to induce resistance. And a large-scale screen of all known secreted growth factors and cytokines identified hepatocyte growth factor (HGF) as the molecule that was able to mimic this effect. In an independent laboratory-based investigation, this same approach of exposing *BRAF* mutant melanoma cell lines in the context of BRAF inhibition to a large panel of growth factors, again identified HGF as

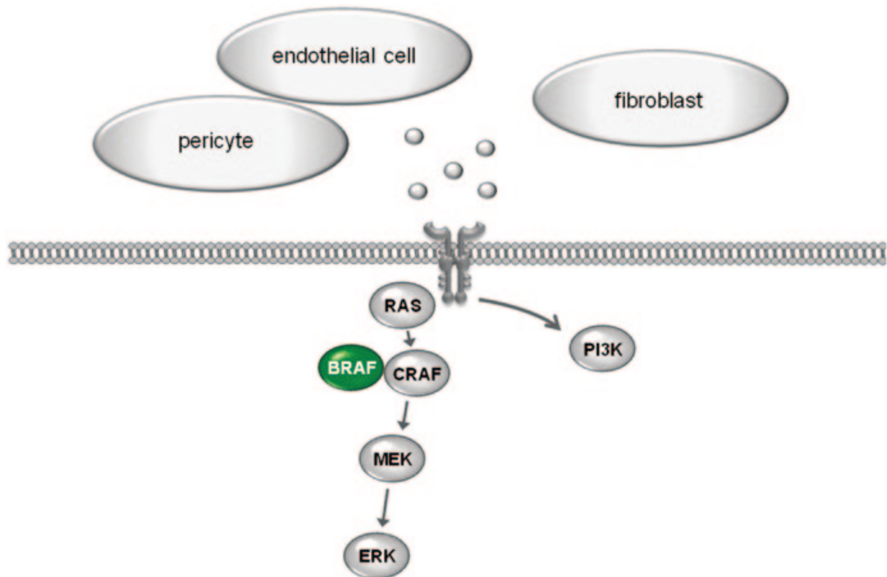

Fig. 9.6 Tumor microenvironment mediated BRAF inhibitor resistance

the most capable of protecting *BRAF* mutant melanoma cells from apoptosis following exposure to a BRAF inhibitor [64].

Two lines of clinical evidence support the potential relevance of stroma-derived HGF to de novo resistance to BRAF inhibitor therapy. First, HGF can be readily detected at the periphery of metastatic melanomas, where fibroblasts typically reside [63]. And, the presence of stromal HGF in and around the tumors of patients who receive BRAF or BRAF/MEK inhibitor therapy predicts lesser degrees of tumor regression compared to patients with no detectable stromal HGF. Second, elevated levels of serum HGF prior to treatment with a BRAF inhibitor predict shorter progression-free survival to BRAF inhibitor therapy compared to lower than average HGF levels [64]. A large number of HGF targeted monoclonal antibodies and small molecule c-met inhibitors are currently in clinical trials and potentially available to investigate in combination with BRAF or BRAF/MEK combination therapy. Experimentally, c-met inhibitors appear capable of overcoming this mechanism of resistance.

9.14 Summary and Conclusions

BRAF inhibitor therapy has changed the landscape of treatment options and the ability to rapidly ascertain common mechanisms of acquired resistance has led to a further clinical advance in BRAF/MEK combination therapy. As the field now focuses on mechanisms of *de novo* and acquired resistance to BRAF/MEK com-

bination therapy a number of additional questions rise to the top of the research agenda. Optimal schedule of administration has not yet been explored clinically and represents an opportunity to maximize the impact of already available agents. Opportunities to further exploit the profound dependence that BRAF mutant tumors have on the MAP kinase pathway are evident with emerging preclinical data with HSP90 and ERK inhibitors. Intercepting pathways that are activated as a consequence of BRAF inhibitor therapy, such as up regulation of BCL2A1 and ERBB3, represent tractable strategies for improving on the early impact of therapy. And, blocking compensatory pathways not impacted by BRAF inhibitor therapy such as the PI3K pathway (in some cases) and growth factor receptor activation derived from the tumor microenvironment provide further opportunities for improving on a backbone of optimal MAP kinase pathway inhibition. As this broad array of novel therapeutic strategies are investigated clinically, an immediate need arises for the development of predictive biomarkers that allow for the novel combinations to be deployed in as personalized a fashion as BRAF inhibitor therapy was itself.

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