

Review

Current Status of Biological Therapies for the Treatment of Metastatic Melanoma

TIANYI TANG¹, ROBERT EL DABAJE¹ and LIXI YANG²

¹Department of Internal Medicine, St. Mary's Medical Center, San Francisco, CA, U.S.A.;

²Radiobiology Laboratory, California Pacific Medical Center, Research Institute, San Francisco, CA, U.S.A.

Abstract. Compared to early-stage melanoma when surgical excision is possible, metastatic disease continues to offer a much grimmer prognosis as traditional chemotherapy treatment regimens offer relatively little survival benefit. This has led to changes in treatment approaches over the preceding two decades as contemporary methods for the treatment of advanced or metastatic melanoma now involve a number of biological modalities, which include immunotherapeutic approaches, targeted therapies and epigenetic modification therapies. Clinically available immunotherapeutic agents include interleukin 2 (IL-2), as well as drugs targeting the important immune checkpoint molecules, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1). The targeted therapeutic agents modulate specific pro-oncogenic mutations such as *v-Raf murine sarcoma viral oncogene homolog B (BRAF)*, receptor tyrosine kinases, *MEK inhibitors* and potential future therapeutic targets, such as the *CDK4/CDK6*, *PTEN* and *GNAQ/GNA11* genes. Additionally, an increasing understanding of the role of epigenetic alterations in the development and progression of melanoma now offers a new potential drug target. Several of these agents have shown promising results; however, in many

investigations, combinations of different therapeutic approaches, each with different mechanisms of action, have yielded improved outcomes as treatment regimens continue to be further optimized by active research and patient disease sub-group analyses. This review summarizes the novel biological agents and new treatments, directly contributing to the significant improvement of biological therapies and markedly advancing knowledge of clinical application of newly approved and developed therapies in treatment of patients with metastatic melanoma.

Melanoma is a highly aggressive skin cancer arising from the malignant transformation of melanocytes residing in the basal layer of the epidermis. Although early stage surgical excision can reach a 5-year survival rate over 95%, the prognosis for advanced stage, metastatic disease remains extremely poor as the overall incidence and mortality associated with melanoma continues to rise on an epidemiological scale. Traditional chemotherapies, generally aimed at inhibiting cell division, have historically shown little survival benefit. Over the past decade, the institution of new biologic therapies, including immunotherapy and targeted therapy, have shown some improvement in the setting of metastatic melanoma.

It has long been observed that there is an increased prevalence of melanoma among immunosuppressed transplant patients (1). In addition, the infiltration of melanoma tumors by T-cells may be positively correlated with longer length of patient survival (2). Along these lines, immunotherapy aims to use immune system components, such as cytokines and antibodies, to enhance response to the malignancy. Multiple different growth factors and downstream components of their intracellular signaling pathways, such as MAPK/ERK and PI3K/AKT, have also been associated with melanoma tumor growth and metastasis (3-5). Additionally, a number of mutated oncogenes with constitutive kinase activity in the aforementioned pathways, such as BRAF, c-KIT and RAS, have been identified and associated with different subtypes of

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Correspondence to: Tianyi Tang, MD, Ph.D., Department of Internal Medicine, St. Mary's Medical Center, 450 Stanyan Street, San Francisco, CA 94117, U.S.A. Tel: +1 2255710109, Fax: +1 4157508149, e-mail: Tianyi.Tang@DignityHealth.org and Li-Xi Yang, MD, PhD, Radiobiology Laboratory, California Pacific Medical Center Research Institute, #602, OPR Bldg, 3801 Sacramento Street, San Francisco, CA 94118, U.S.A. Tel: +1 4156006296, Fax: +1 4156006215, e-mail: yangl@cpmcri.org

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metastatic melanoma (6), thus providing new antibody targets for the treatment of melanoma in a field where investigation remains ongoing. The inhibition of mutated kinases aims to block the growth, progression and spread of malignancy. Though there has been excitement regarding advancements in targeted therapy showing some survival benefit, patients often relapse and show evidence of disease progression after several months of monotherapy with acquired resistance thought to usually rise from the activation of alternative pathways. Thus, combination therapy targeting different molecules or modulators at different points in cellular pathways are now being intensively researched. An additional pathogenic mechanism that is being targeted presently is epigenetic regulation, such as at the level of DNA or histone modification or RNA transcription. Herein, we will review each of these potential diagnostic biomarkers and therapeutic targets for the treatment of melanoma, exploring the current therapeutic and long-term implications of each.

Immunotherapy

Melanomas are frequently associated with a large number of individual somatic mutations. Amino acid residues resulting from such mutations yield neopeptides, eliciting an antitumor response *via* innate and adaptive immune mechanisms that aims to eliminate the early lesion (7). Tumor cells that are not eradicated may be further suppressed by the immune system. A disruption in the equilibrium between the immune system and tumor growth and activity can result in the rise of tumor cells that are able to avoid, resist or, even, suppress the natural immune response (8). Tumor cells may escape the immune response by any of a number of major categories of mechanisms, including impaired antigen presentation, expression of factors with immunosuppressive properties, such as transforming growth factor-beta (TGF- β), vascular endothelial growth factor (VEGF) and interleukin-2 (IL-2), induction of tolerance and resistance to apoptosis (9). More specifically, melanoma cells are known to express CTLA-4 and PD-1, cell surface protein receptors, that normally function as immune checkpoints down-regulating the immune system response. Thus, major goals of immunotherapy have become the activation of an immune response through the immunostimulant IL-2, up-regulation of tumor-inhibiting T-cells and the inhibition of the above referenced immune checkpoints.

IL-2

Interleukin-2 is a critical T-cell growth factor that promotes differentiation of T-cells into regulatory T-cells and memory T-cells for example *via* tightly regulated feedback loops. In 1998, the FDA approved aldesleukin, high-dose recombinant intravenous IL-2, for the treatment of metastatic melanoma. In early studies involving 270 patients across 8 trials, the overall

response rate (ORR) was 16% (43 patients; 95% confidence interval (CI)=12-21%), with a complete response (CR) in 17 of those patients (6%). Responses to treatment were observed in all sites of disease and the median duration of response (MDR) was 5.9 months for those with partial response (PR) and not yet reached for those with CR, with 10 of the 17 patients with CR still ongoing at 24 to 106 months at the time of the referenced publication (10). More specifically, analysis of the efficacy of intralesional IL-2 for the treatment of skin and soft tissue melanoma metastases in one trial of 72 patients yielded CR of treated metastases in 62.5% of patients treated, with CR in 85% of all individual metastatic tumors treated (11). Long-term outcomes have proven quite favorable in stage IIIB patients (cutaneous metastases only without lymph node involvement) and stage IVM1a patients (soft tissue metastases without visceral involvement), with 2- and 5-year overall survival (OS) for stage IIIB of 95.5% and 86.8%, respectively, and 2- and 5-year OS of 66.7% and 16.7%, respectively, for stage IVM1a (12). Though treatment alone with high-dose IL-2 has been approved for the treatment of metastatic melanoma for nearly two decades, promising data continue to be collected regarding its concomitant use with other therapies and further optimization of these synergistic outcomes.

Glycoprotein 100 (Gp100) Peptide Vaccine

To be considered with the above results are data indicating that the first cycle high-dose IL-2 injection induces the expansion of inducible T-cell costimulatory (ICOS)-positive regulatory T-cells, a shift that is immunosuppressive in nature, with the observation that this expansion is associated with worse clinical outcomes relative to those patients with fewer ICOS+ regulatory T-cells (13). Thus, an additional approach given this consideration has yielded a phase III trial combining IL-2 with the gp100:209-217(210M) peptide vaccine, with the aim of using the immunostimulant IL-2 to increase the efficacy of the gp100 peptide vaccine, which itself had been previously found to result in high levels of circulating T-cells capable of recognizing and killing melanoma cells *in vitro*. This gp100 peptide vaccine plus high-dose IL-2, compared to a control group treated with high-dose IL-2 alone, resulted in significantly improved OS (16% *versus* 6%; $p=0.03$), longer progression-free survival (PFS) (2.2 months; 95% CI=1.7-3.9 months *versus* 1.6 months; 95% CI=1.5-1.8 months; $p=0.008$) and a non-significant trend towards longer median overall survival (mOS) (17.8 months; 95% CI=11.9-25.8 months *versus* 11.1 months; 95% CI=8.7-16.3 months; $p=0.06$) (14).

CTLA-4, PD-1 and PD-L1 Inhibitors

Melanoma cells are known to express the cell receptor proteins CTLA-4 and PD-1, which are normally found on the surface of T-cells and function as immune checkpoints,

inhibiting further T-cell activation and down-regulating the immune response. More specifically, CTLA-4, found on activated CD4+ and CD8+ T-cells, outcompetes CD28 (a T-cell surface protein that provides costimulatory signals for T-cell activation and continued survival) for the binding of B7 on antigen presenting cells. Similarly, PD-1 is a cell surface receptor expressed on T-cells that interacts with PD-L1, a cell surface ligand expressed on macrophages and other antigen presenting cells, the interaction of which results in the inhibition of T-cell receptor medication activation of further T-cell proliferation and IL-2 production (15). Thus, inappropriate expression of the immune checkpoints by tumor cells is one suggested tumor escape method by which melanoma may evade the physiologic immune response (16). These potential escape mechanisms are targeted by a number of agents and therapeutic approach strategies.

Ipilimumab

Ipilimumab, FDA approved in 2011 for the treatment of metastatic melanoma, is an anti-CTLA-4 monoclonal antibody that has been studied with the concomitant administration of a number of therapies. Promising results include a phase III study involving 676 patients with unresectable stage III and IV metastatic melanoma whose disease had progressed despite treatment for metastatic disease. Patients were randomized into three treatment arms: ipilimumab plus gp100 peptide vaccine, gp100 peptide vaccine monotherapy or ipilimumab monotherapy. The mOS in the ipilimumab plus gp100 peptide vaccine combined therapy group was 10.0 months compared to 6.4 months among patients receiving gp100 peptide vaccine monotherapy (hazard ratio (HR) for death=0.68; $p<0.001$). Additionally, the mOS of patients treated with ipilimumab alone was 10.1 months (HR compared to gp100 peptide vaccine alone=0.66; $p=0.003$; HR compared to ipilimumab plus gp100 peptide vaccine combination therapy=1.04; $p=0.76$). Important to note was an incidence of grade 3/4 immune-related adverse events (AEs) in 10-15% of patients treated with ipilimumab, including 7 deaths (17). This rate of grade 3/4 immune-related AEs holds at 10-20% of patients treated across multiple clinical trials, though the majority of these involve the integumentary and gastrointestinal systems and may be generally reversible with the administration of corticosteroids (17-19).

In a different approach to ipilimumab combination therapy, a phase III trial randomized 502 patients with previously untreated stage III or IV metastatic melanoma to ipilimumab plus dacarbazine *versus* dacarbazine monotherapy. The combination therapy arm again showed improved outcomes with a mOS of 11.2 months (95% CI=9.4-13.6 months) *versus* 9.1 months (95% CI=7.8-10.5 months) with dacarbazine monotherapy. Estimated survival in these two groups were 47.3% *versus* 36.3% at 1 year, 28.5% *versus* 17.9% at 2 years

and 20.8% *versus* 12.2% at 3 years, respectively (HR for death with ipilimumab plus dacarbazine combination therapy=0.72; $p<0.001$) (20).

Additionally important is an understanding of long-term outcomes with ipilimumab therapy. In a study citing long-term follow-up of 177 patients across three different clinical trials, different treatment arms were isolated, including ipilimumab plus gp100 peptide vaccine and ipilimumab plus IL-2. The former group had an average follow-up of 92 months with a 5-year OS of 13% and CR in 7% of patients treated. The latter group had a 5-year OS of 25% and CR in 17% of patients treated with an average length of follow-up of up to 84 months. Importantly, survival curves seemed to plateau in these ipilimumab treatment groups by 48 months, suggesting that there could be durable response and potentially curative tumor regression in a small percentage of metastatic melanoma patients treated with ipilimumab combination therapy strategies (21).

Nivolumab

Similar in mechanism to ipilimumab, the monoclonal antibody nivolumab is directed against the PD-1 receptor, thus blocking the inhibitory ligand's suppression of immune response. This example of immune checkpoint blockade has translated into clinically significant improved outcomes for patients with metastatic melanoma. The initial phase I clinical trial of nivolumab enrolled patients with advanced non-small cell cancer, melanoma and renal cell cancer. Of the 94 patients enrolled with melanoma, the cumulative response rate was 28%, comparable to the other malignancies enrolled in parallel. Immunohistochemical analysis of pretreatment specimens revealed that none of the 17 patients whose tumors were PD-L1-negative had any response, while 36% of the 25 patients with PD-L1-positive tumors had an objective response ($p=0.006$) (22). Other studies have provided long-term follow-up data on nivolumab-treated patients advanced melanoma, with 1- and 2-year OS of 62% (95% CI=53-72%) and 43% (95% CI=32-53%) respectively, a mOS of 16.8 months (95% CI=12.5-31.6 months) and tumor regression in 31% of patients with a Kaplan-Meier estimated MDR in that population of 2 years (23). In a trial randomizing 418 patients with advanced metastatic melanoma without a BRAF mutation to nivolumab *versus* dacarbazine, the nivolumab group enjoyed significant improvements in OS and PFS, with an objective response rate of 40.0% (95% CI=33.3-47.0%) *versus* 13.9% (95% CI=9.5-19.4%) in the dacarbazine group (odds ratio (OR)=4.06; $p<0.001$) (24).

More recent data show similar efficacy in patients with melanoma refractory to anti-CTLA-4 therapy with ipilimumab, suggesting a role for future investigation of potential combination therapy between agents targeting the different mechanisms (25). Upon this suggestion, an important phase III

trial was recently published randomizing 945 previously untreated patients with stage III and IV melanoma to nivolumab plus ipilimumab combination therapy, nivolumab monotherapy or ipilimumab monotherapy. The median PFS was 11.5 months (95% CI=8.9-16.7 months) for the combination-therapy-treatment group relative to 2.9 months (95% CI=2.8-3.4 months) with ipilimumab alone (HR for death or disease progression=0.42; 95% CI=0.31-0.57; $p<0.001$) and 6.9 months (95% CI=4.3-9.5) with nivolumab alone (HR for death or disease progression=0.57; 95% CI=0.43-0.76; $p<0.001$). Interestingly, PFS was similar in the combination-therapy group and nivolumab group (14.0 months) in patients with tumors positive for PD-L1; however, in patients with PD-L1-negative tumors, PFS was potentially longer in the patients receiving combination therapy with nivolumab and ipilimumab (11.2 months *versus* 5.3 months) (26). Tumor target profiling is, thus, clearly important for future treatment algorithms, as those with PD-L1-negative tumors appear to have greater efficacy with anti-PD-1 and anti-CTLA-4 immune checkpoint blockade than with either agent alone.

Pembrolizumab

Pembrolizumab, another anti-PD-1 monoclonal antibody, was FDA approved in 2014 for the treatment of advanced melanoma. A recent phase III trial randomized 834 patients with advanced melanoma to pembrolizumab every 2 weeks or every 3 weeks and to ipilimumab. The estimated 6-month PFS rates were 47.3% and 46.4% for patients treated pembrolizumab every 2 and 3 weeks, respectively, and 26.5% for patients treated with ipilimumab (HR for disease progression=0.58; $p<0.001$ for both pembrolizumab regimens *versus* ipilimumab; 95% CI=0.46-0.72 and 0.47-0.72, respectively). Median estimates of PFS were 5.5 months (95% CI=3.4-6.9 months), 4.1 months (95% CI=2.9-6.9 months) and 2.8 months (95% CI=2.8-2.9 months), respectively. The 1-year estimates of survival were 74.1% for patients receiving pembrolizumab every 2 weeks (HR for death *versus* ipilimumab group=0.63; 95% CI=0.47-0.83; $p<0.0005$), 68.4% for patients receiving pembrolizumab every 3 weeks (HR for death *versus* ipilimumab group $p=0.69$; 95% CI=0.52-0.90; $p=0.0036$) and 58.2% for those receiving ipilimumab. Importantly, the rates of grade 3/4 immune-related AEs were lower in the pembrolizumab group (13.3% and 10.1% respectively) than in the ipilimumab group (19.9%) (27). These data suggest comprehensively improved outcomes with the treatment of advanced metastatic melanoma with pembrolizumab over ipilimumab in head-to-head monotherapy.

Potential Future Agents

Newer agents currently under investigation for efficacy in the treatment of melanoma and other malignancies, but not yet

FDA approved, include pidilizumab (an anti-PD-1 monoclonal antibody) and atezolizumab (an anti-PD-L1 monoclonal antibody); however, trials remain in early phases.

Toll-like Receptor (TLR) Activation Imiquimod

The exact role and mechanism of imiquimod are unknown but it is thought to activate immune cells by acting as a toll-like receptor 7 (TLR7) agonist, inducing the production of a number of proinflammatory cytokines, including interferon alpha (IFN- α), IL-6, IL-12 and tumor necrosis factor alpha (TNF- α) in addition to increasing the local and regional number of T-cells (28-30). Topical imiquimod is used in the treatment of genital warts, actinic keratosis and superficial basal cell carcinoma. There has been some suggestion that imiquimod could be used to effectively treat patients with high-risk primary melanoma or cutaneous melanoma metastases when surgical resection is not an option (30-32). The effectiveness of this therapy, however, remains in question as there is evidence of drug resistance development (33). Imiquimod has been suggested as a possible synergistic agent in a retrospective case series of 11 patients with cutaneous metastatic melanoma treated with intralesional IL-2 combined with topical imiquimod and retinoid, reporting an observed complete local response rate of 100% at an average long-term follow-up of 24 months (34).

Adoptive T-cell Therapy (ACT)

Adoptive T-cell therapy involves the isolation of tumor-infiltrating lymphocytes (TILs) from a malignant tumor, the generation of large numbers of TILs *in vitro* and reintroduction of these TILs into the tumor in combination with IL-2. The TILs are transduced with high-affinity T-cell receptors against major melanoma tumor antigens and T-cells transduced with chimeric antigen receptors composed of hybrid immunoglobulin light chains hybridized with major T-cell signaling molecules (35). Prior to infusion of TILs and IL-2, patients undergo chemotherapy to deplete native lymphocytes, namely regulatory T-cells that could potentially suppress anti-tumor immune activity (36). A recent review analyzed 3 sequential clinical trials employing ACT in 93 total patients with metastatic melanoma, 95% of which had had progressive disease despite systemic therapy. The noted response rates of each individual trial ranged from 49-72%. The 3- and 5-year OS were 36% and 29%, respectively. Complete tumor regression was achieved in 22% of total patients, with 20% having ongoing complete regression beyond 3 years (37). These findings indicate that there may be a role in the future for ACT in the treatment of advanced melanoma as there is evidence of the potential for durable complete response (DCR) in some patients, independent of prior treatment.

In trying to understand how to optimize ACT, it is important to note that the initial TIL outgrowth has variable success with several factors associated with improved success: female gender (71% success *versus* 57% for males; $p=0.04$) and age ≤ 30 years (94% success; $p=0.01$). Factors associated with a negative impact on initial TIL outgrowth include systemic therapy in the 30 days prior to tumor harvest (47% *versus* 71% in those who had not received any such systemic therapy; $p=0.02$) and biochemotherapy at any point during the 60 days prior to tumor harvest (16% success compared to an overall success rate of 62%; $p<0.0001$) (38). Furthermore, it is important to understand that TILs initially represent a heterogeneous population of lymphocytes within the tumor, including lymphocytes that are not tumor reactive or selective. Understanding of the identification and expansion of the ideal sub-population of TILs for eventual ACT continues to improve, for example with regards to the identification of the co-stimulatory 4-1BB receptor as having capacity for activation and proinflammatory polarization of antitumor lymphocytes (39).

The efficacy of concurrent administration of IL-2 as part of ACT remains under debate. The lymphoproliferative effects of IL-2 could potentially increase numbers of regulatory T-cells, which would theoretically result in a poorer response to TILs in some patients. Additionally, AEs and toxicity associated with high-dose IL-2 are well documented, causing investigators to attempt to administer moderate- to low-dose IL-2 during ACT, hoping for comparable efficacy. A recent pilot study involving 6 patients demonstrated that DCR could be induced by autologous TILs with the co-administration of low-dose IL-2 (40). The currently ongoing trial TILTherapy in Metastatic Melanoma and IL2 Dose Assessment (METIILDA trial) in the United Kingdom is a two-arm, open labeled phase I randomized trial of TIL therapy in metastatic melanoma, randomizing patients originally receiving preconditioning chemotherapy with cyclophosphamide to either high- or low-dose IL-2 for up to 12 doses after autologous transfusion of TILs. This trial should provide more substantive data to further evaluate previous inferences from smaller studies or cases.

Finally, in animal models it had been observed that BRAF inhibitors (vemurafenib and dabrafenib) and MEK inhibitors (trametinib) showed an increase in intratumoral cytotoxic activity and cytokine secretion of re-infused TILs when co-administered during ACT, leading to clinical trials analyzing the combination of these targeted signaling pathway drugs with TILs (41, 42). The ongoing trial Vemurafenib and White Blood Cell Therapy for Advanced Melanoma has completed enrollment at the time of this publication but has not yet released any study results.

Oncolytic Viral Therapy

An oncolytic virus is an attenuated, tumor-selective, replicable agent that preferentially infects and kills cancer cells *via* direct

oncolysis (43). Such viruses, designed to preferentially infect cancer cells, can be further designed to encode antibodies that inhibit angiogenesis in infected tumors (*e.g.* GLAF-1 or inhibitor of growth protein 4) to further increase potential clinical yield (44, 45). Other engineering theories have induced increased local and systemic immune responses in addition to direct oncolytic effects through expression of ligands for TLRs (46). The only oncolytic virus presently approved by the FDA is talimogene laherparepvec (T-VEC), approved for the treatment of advanced inoperable melanoma. The oncolytic virus T-VEC has been engineered to replicate selectively within tumor cells and to express granulocyte-macrophage colony-stimulating factor (GM-CSF) (47). In an open-label phase III trial, 436 patients with unresected stage IIIB to IV melanoma were randomized to intralesional T-VEC or subcutaneous GM-CSF. The durable response rate was significantly higher in patients treated with T-VEC than those treated with GM-CSF (16.3%; 95% CI=12.1-20.5% *versus* 2.1%; 95% CI=0.0-4.5%; OR=8.9; $p<0.001$). Furthermore, the ORR was also higher in the T-VEC arm (26.4%; 95% CI=21.4-31.5% *versus* 5.7%; 95% CI=1.9-9.5%) as was the mOS (23.3 months; 95% CI=19.5-29.6 months *versus* 18.9 months; 95% CI=16.0-23.7 months; HR=0.79; 95% CI=0.62-1.00; $p=0.51$). Additionally, T-VEC was well tolerated in this trial, with cellulitis being the most frequent grade 3/4 AE at a frequency of 2.1% (48). Present investigations aim to study the efficacy of combining T-VEC with other immunotherapy regimens for the treatment of advanced melanoma.

Targeted Therapies

In addition to immunologic approaches for the treatment of advanced or metastatic melanoma, targeted therapies have isolated a number of oncogenic DNA mutations and potential therapeutic targets. Agents that have already been FDA-approved include BRAF inhibitors and MEK inhibitors, while receptor tyrosine kinases are actively being investigated for potential therapeutic benefit. Other potential targets have been isolated as well and are at varying stages of investigation, including *CDK4/CDK6*, *PTEN* and *GNAQ/GNA11*.

BRAF Inhibitors

The *BRAF* gene encodes the B-raf protein, a signal transduction serine/threonine-specific protein kinase. The B-raf protein participates in the regulation of the MAPK/ERK signaling pathway, which regulates cell proliferation, differentiation and progression through the cell cycle. Up to 66% of malignant melanomas may have detectable *BRAF* somatic missense mutations, all of which are within the kinase domain (49). The substitution of valine with glutamic acid at amino acid 600 is the single most common *BRAF* mutation accounting for more than 95% of all such *BRAF* mutations.

This V600E substitution makes the B-raf protein constitutively active, with a 500-fold increase in kinase activity (50).

Vemurafenib

Vemurafenib is a selective BRAF inhibitor, 10-fold more selective for B-raf kinase than wild-type B-raf (51). Vemurafenib has efficacy in melanoma patients with the V600E mutation of the *BRAF* gene (as well as the much less common V600K mutation). In patients without these mutations, vemurafenib can actually activate normal BRAF and promote tumor growth (52). In a defining phase III trial randomizing 675 patients with previously untreated, metastatic melanoma with the *BRAF* V600E mutation to receive either vemurafenib or dacarbazine, the OS at 6 months was 84% (95% CI=78-89%) in the vemurafenib arm compared to 64% (95% CI=56-73%) in patients treated with dacarbazine. There was such a marked difference in positive clinical outcomes in patients treated with vemurafenib that the safety and monitoring board, after interim review and analysis, recommended discontinuation of the dacarbazine arm with patients switching to vemurafenib. Response rates for vemurafenib were 48% compared to 5% for dacarbazine. However, it is important to note that 18% of patients in the vemurafenib arm developed either a cutaneous squamous-cell carcinoma or a keratoacanthoma or both, all of which were treated by simple excision (53).

A factor limiting BRAF inhibitors' present utility is the development of resistance, often occurring within 6 months of treatment initiation. One proposed mechanism of resistance development is the ability of tumor cells to continue through the MAPK/ERK signaling pathway in spite of BRAF inhibition through the up-regulation and increased expression of the CRAF isoform (54). Additionally, the RAF signaling component of the above mentioned signaling cascaded can be bypassed by COT kinase activation of extracellular signal-regulated kinases (ERKs), thus eliminating any effectiveness of BRAF inhibition providing, however, a new potential therapeutic target for future investigation (55). As a result of trials attempting to overcome BRAF inhibitor resistance with the addition of an MEK inhibitor, a recent phase III trial randomized 495 patients from the same patient population randomized to vemurafenib plus cobimetinib *versus* vemurafenib plus placebo. The combination therapy arm had a median PFS of 9.9 months *versus* 6.2 months in the vemurafenib monotherapy arm (HR for death or disease progression=0.51; 95% CI=0.39-0.68; $p<0.001$), with CR or PR rates of 68% and 45%, respectively ($p<0.001$) (56).

Dabrafenib

Similar to vemurafenib, dabrafenib is another FDA-approved selective BRAF inhibitor that showed improved outcomes

relative to dacarbazine. In a phase III trial randomizing 733 patients with previously untreated, unresectable stage III or IV *BRAF* V600E mutation-positive melanoma to either dabrafenib or dacarbazine, the median PFS was 5.1 months for the dabrafenib arm relative to 2.7 months for patients treated with dacarbazine (HR=0.30; 95% CI=0.18-0.51; $p<0.0001$) (57).

The issue of BRAF inhibitor resistance development holds true for dabrafenib therapy as well, leading to investigations of combination therapy regimens. Confirming results from a concurrent phase III trial randomizing the above patient population to dabrafenib plus trametinib *versus* dabrafenib monotherapy (58) was a phase III trial randomizing this same patient population to receive dabrafenib plus trametinib or vemurafenib monotherapy, revealing significant clinical benefit to BRAF inhibitor/MEK inhibitor combination therapy as the study was terminated early for efficacy. The OS at 12 months was 72% (95% CI=67-77%) for the combination therapy group compared to 65% (95% CI=59-70%) in the vemurafenib group (HR for death in the combination therapy group=0.69; 95% CI=0.53-0.89; $p=0.005$). The median PFS was 11.4 months in the combination therapy group *versus* 7.3 months in the vemurafenib group (HR=0.56; 95% CI=0.46-0.69; $p<0.001$) with an objective response rate of 64% *versus* 51%, respectively ($p<0.001$). Most importantly, there was no observation of increased severe AEs with combination drug therapy and, as previously described, the vemurafenib treatment group was again noted to have a high rate of cutaneous squamous-cell carcinoma and keratoacanthoma (18% with vemurafenib therapy *versus* 1% in the combination therapy group) (59). At present, the FDA has approved the combination of dabrafenib and trametinib for the treatment of unresectable or metastatic melanoma with a *BRAF* V600E or V600K mutation.

In summary, it has become clear over the previous two years that there is a clinical benefit to combination BRAF inhibitor/MEK inhibitor therapy as more therapeutic agents are developed and treatment regimens are further optimized. Nevertheless, there is clear evidence of the development of further resistance mechanisms. Mutations in MEK inhibitor targets, MEK1 and MEK2, have now been associated with acquired resistance to RAF inhibition (60, 61). BRAF inhibitor-resistant cells also have been noted to have increased PI3K/AKT signaling, indicating a role in tumor resistance and another potential route of combination therapy investigation (62). Such investigations are in their early stages but research in this area remains aggressive for further understanding of combination therapy.

Receptor Tyrosine Kinase Inhibitors

Tyrosine kinases, constitutively well-conserved, are important mediators of a multitude of cellular processes and regulation.

Receptor tyrosine kinase dysregulation has been associated with malignancy development and progression, in addition to being implicated as another potential mechanism of BRAF inhibitor resistance development (63). The *KIT* gene encodes for the tyrosine kinase, c-Kit, which is a cell-surface receptor that interacts with dozens of proteins, most notably stem cell factor (SCF). Mutations or amplifications of the *KIT* gene have been identified in an important proportion of melanomas, up to 39% in some settings (64, 65).

Imatinib

Imatinib, a tyrosine kinase inhibitor, is specific for the tyrosine kinase domains of c-Kit, Abl and platelet-derived growth factor receptor (PDGF-R) and has been investigated for the treatment of several malignancies. Multiple trials have considered the use of imatinib to treat *KIT* mutation-positive advanced or metastatic melanoma, with two notable trials: one achieving median PFS of 12 weeks and a mOS of 46.3 weeks and the other achieving a comparable median PFS of 3.5 months with a 1-year OS of 51% (66, 67).

Nilotinib

Another tyrosine kinase inhibitor, nilotinib, was recently administered in a small phase II trial evaluating the same patient population, achieving similar results with a median time to progression of 3.3 months (90% CI=2.1-3.9 months) (68). Further evaluation of nilotinib's efficacy in this patient population remains ongoing.

Potential Future Targets

Another receptor tyrosine kinase, epidermal growth factor receptor (EGFR), has shown increased expression associated with melanocyte tumor progression (69) and likewise been found to have increased expression in cases of BRAF inhibitor resistance (vemurafenib) and MEK inhibitor resistance (trametinib) (70, 71). There is presently limited clinical data supporting the use of EGFR inhibitor monotherapy for the treatment of melanoma; however, the above mechanisms of resistance again suggest a role in the investigation of possible combination targeted therapy.

MEK Inhibitors

The Ras family of proteins is involved in cellular signal transduction *via* GTPase activity and can trigger a number of important intracellular pathways, including the MAPK/ERK pathway (also known as the Ras-Raf-MEK-ERK pathway) and the PI3K/AKT pathway (72). A significant percentage of melanoma tumors have evidence of *RAS* mutations (most commonly *N-ras*) (73), a factor that is associated with poorer

prognosis with thicker tumors and higher rates of mitosis (74). Interestingly, in the overwhelming majority of melanoma cases, *BRAF* and *N-RAS* mutations found are mutually exclusive (75, 76). Nonetheless, enhanced N-RAS expression has been explained as a potential mechanism for the development of resistance to BRAF inhibitors, vemurafenib more specifically (77). With regards to the treatment of tumors with *N-RAS* mutations, the downstream signaling pathways of MAPK/ERK and PI3K/AKT pathways provide therapeutic targets. Binimetinib (MEK162), a MEK inhibitor, is currently being investigated in combination with a CDK4/CDK6 inhibitor (LEE011) and, in other trials, with experimental PI3K/AKT pathway inhibitors. Much work remains to develop therapy targeting this pathway and to determine its efficacy and role in the setting of the multitude of different therapeutic targets for the treatment of advanced melanoma.

CDK4/CDK6 Inhibitors

Cyclin-dependent kinases 4 and 6 (*CDK4* and *CDK6*), encoded by the *CDK4* and *CDK6* genes, are members of the serine/threonine-specific protein kinase family and are important for cell cycle progression. The cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene encodes for 2 proteins, p16 and p14arf, the former of which inhibits CDK4 (and CDK6), with both proteins acting as tumor suppressors. In one study, 96% of melanoma cell lines analyzed had deletion, mutation or methylation of *CDKN2A* or mutation or amplification of *CDK4* (78). Despite this high prevalence, melanomas with wild-type *BRAF* or *N-RAS* are frequently noted to have an increased number of copies of the genes for CDK4 and *CCND1* (a protein that forms a complex with *CDK4*, functioning as a regulatory subunit), thus implicating CDK4 as an independent oncogene in such cases (79). The CDK4/CDK6 inhibitor LEE011 is undergoing multiple combination therapy trials, such as its coupling with binimetinib, the MEK inhibitor described above, as well as with the drug candidate encorafenib (LGX818), a BRAF inhibitor.

PTEN

The tumor suppressor gene *PTEN* encodes for phosphatidylinositol-3,4,5-triphosphate 3-phosphate (PTEN), an enzyme that regulates the PI3K/AKT signaling pathway by catalyzing the hydrolysis of phosphatidylinositol-3,4,5-triphosphate (PIP3) into phosphatidylinositol-3,4,5-biphosphate (PIP2), thus influencing cellular proliferation, metabolism, transcription and apoptosis. Inactivating deletions or mutations of the *PTEN* gene are frequently notable in many different malignancies, including melanoma. Alterations in *PTEN* were found to be prevalent in 7.3% of primary melanomas and 15.2% of metastatic melanomas (80). Allelic loss of *PTEN*

comprises 20% of all melanoma tumors, while altered expression of *PTEN* comprises 40% of all melanoma tumors (81). The combination of *BRAF* V600E and *PTEN* loss has been observed to induce metastatic melanoma in mouse models (82). Important treatment considerations will take into account a better understanding of the role *PTEN* mutations in patients with melanoma.

GNAQ/GNAI1

Uveal melanomas generally show an absence of *BRAF* or *RAS* mutations (83-85). Frequent somatic mutations occur, however, in either the *GNAQ* gene, which encodes for guanine nucleotide-binding protein G(q) subunit alpha or the *GNAI1* gene, which encodes for guanine nucleotide-binding protein subunit alpha-11. Mutations in *GNAQ* and *GNAI1* are associated with activation of the MAPK/ERK pathway, thus providing a potential therapeutic target in cases of uveal melanoma (86, 87).

Epigenetic Modulations in Melanoma

Epigenetic mechanisms that affect gene expression without changing the underlying DNA sequence include hypo- and hypermethylation of DNA, histone modifications (such as acetylation, methylation and phosphorylation) and posttranslational modifications, such as RNA silencing. Epigenetic changes have been implicated over the past decade in association with melanoma, thus providing a possible therapeutic target that is potentially more easily reversible than genetic mutations.

Aberrant DNA Methylation

DNA methylation is the covalent binding of a methyl group to the C5 position of cytosine by DNA-methyltransferases (DNMT), usually in areas of CpG islands, cytosine-phosphate-guanine dinucleotide rich regions. Gene silencing takes place in hypermethylated regions of DNA, a potentially important oncogenic mechanism (88). The methylation of promoter regions of DNA encoding tumor suppressor genes has been associated with the development and worsened prognosis of several different malignancies, including melanomas, more specifically *via* the *CDKN2A* (89), *RASSF1* (90) and *PTEN* genes (91). Evidence has shown an increase in hypermethylation of tumor suppressor genes with advancing clinical tumor stage (92). Conversely, DNA hypomethylation and, thus, activation of oncogenes has also been suggested as a mechanism promoting malignancies. Decitabine, a DNMT inhibitor, has been investigated regarding its ability to induce demethylation of DNA at 5' CpG islands, thus up-regulating gene expression. Understanding this pattern of DNA demethylation is complex, however, as it appears that both the

level of methylation and the level of promoter CpG content are important determining factors of decitabine demethylation activity, more specifically being less effective in areas of high CpG content and most effective in areas with high methylation levels and intermediate CpG content (93). Current early-phase clinical trials are investigating the combination of DNMT with other agents for the treatment of metastatic melanoma, including temozolomide plus panobinostat and vemurafenib plus cobimetinib.

Histone Modification and Microphthalmia-associated transcription factor (MITF)

With regards to histones, methylation generally decreases gene transcription while histone acetylation is associated with activation of gene transcription. The inhibition of histone deacetylase (HDAC) has been observed in melanomas to induce cell cycle blockade and subsequently apoptosis in the setting of increasing expression of CDK inhibitor p21 and modulation of Bcl2 family proteins, suggesting the role of aberrant histone deacetylation or hypoacetylation in the pathogenesis of melanoma (94, 95).

MITF, encoded by the *MITF* gene, is known to regulate numerous processes within melanoma cells, such as differentiation, proliferation and migration, playing a survival oncogene role by activating expression of Bcl2 and the melanoma inhibitor of apoptosis (ML-IAP) (96). *MITF* amplification may be associated with decreased survival and increased chemoresistance, as this amplification is interestingly associated with *CDKN2A* inactivation (97). *BRAF* mutations may actually be promoting melanoma cell proliferation *via* the up-regulation of *MITF* transcription (98). Expression of MITF was repressed in melanocytes, melanoma and clear cell sarcoma cells when HDAC inhibitors were administered (99). Multiple phase II trials are currently under investigation, analyzing the efficacy of treating advanced or metastatic melanoma with the HDAC inhibitor vorinostat; however, more data are required before recommendation of their use.

MicroRNA-based Gene Regulation

MicroRNAs (miRNAs) regulate cellular processes, such as differentiation, proliferation and apoptosis by functioning in post-transcriptional gene expression regulation and by silencing RNA translation into particular proteins. Both oncogenic and tumor suppressor genes in the setting of melanoma have been found to be regulated by miRNAs (100-103). At present, the majority of melanoma-associated miRNAs identified thus far can only be used for tumor detection. However, recent investigation has revealed that the miRNA strands miR-205 and miR-18b may suppress melanoma cell proliferation through different individual

mechanisms (104, 105). The suggestion of potential therapeutic role for miRNAs can be easily imagined but much further investigation is required at this time.

Conclusion

Discoveries regarding the various molecular subtypes of melanoma mutations have elicited radical change in approach to treatment, therapeutic targets and clinical outcomes. While many trials have already proven the improved prognosis and outcomes with biological treatments, refractory cases and resistance development remain a significant challenge as multiple ongoing trials analyze further new therapeutic targets and the costs and benefits of new combination drug therapies. In conjunction with these treatment strategies, epigenetics represents a new category of field for improved understanding and potentially efficacious treatment target. Continued improvements appear inevitable as the understanding of this treatment field continues to advance at incredible pace.

Conflicts of Interest

The Authors declare that they have no conflict of interest.

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