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Emerging Drug Combination Approaches in Melanoma Therapy

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1. Introduction

The FDA approvals of ipilimumab targeting the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), pembrolizumab targeting the programmed cell death protein 1 (PD-1), BRAF inhibitors vemurafenib and dabrafenib, and MEK inhibitor trametinib represent significant milestones in more effective treatment of advanced melanoma. However, it is clear that the use of these single-agent therapies have limitation clinically. For example, ipilimumab only showed 4.5% objective response rate when used alone in a Phase II clinical trial [1]. The efficacy of vemurafenib lasts only 6.7 months before the disease relapses especially in patients with metastatic melanoma [2]. Therefore, rational combination approaches are strongly preferred in order to improve the overall patient progression-free survival (PFS), overcome or delay the development of multi-drug resistance and reduce the incidents of side effects [3-6].

In this chapter, we will summarize the emerging combination therapy approaches from both clinical trial and preclinical research in the past five years.

2. Combination of kinase inhibitors for melanoma treatment

2.1. Combined inhibitions targeting components within the Mitogen-Activated Protein Kinase (MAPK) signaling pathway

2.1.1. Targeting BRAF: Mechanism of action, toxicity and drug resistance

BRAF is a serine/threonine growth signal transduction protein kinase from RAF family which plays important roles in the RAS/RAF/MEK/ERK pathway and directs cell division, prolifer-

ation and secretion [7]. BRAF inhibitors (BRAFi) are ATP-competitive ligands which inactivate the function of BRAF protein by either stabilizing the inactive form of kinase domain (sorafenib) or preferentially inhibit the active form of the kinase (vemurafenib, dabrafenib) [8, 9]. Various mutations of *BRAF* gene have been identified in cancers including melanoma, colorectal and ovarian cancer. Around 60% of human melanoma adopted the T1799A transversion in exon 15, which lead to $BRAF^{V600E}$ mutation and the over-activated monomer phosphorylation for $BRAF^{V600E}$ [9, 10]. The two FDA approved BRAFi (Vemurafenib and dabrafenib) selectively and potently block the activation of $BRAF^{V600E}$ and thus inhibit the MAPK signaling pathway. These drugs show very high clinical efficacy in metastatic melanoma patients harboring the $BRAF^{V600E}$ mutation [11-13]. Interestingly, in a clinical study which treated 43 patients with any V600 BRAF mutation including the rare V600R variant, five out of the six melanoma patients having V600R mutation had clinical response to the therapy of vemurafenib or dabrafenib (response rate 86%) [14].
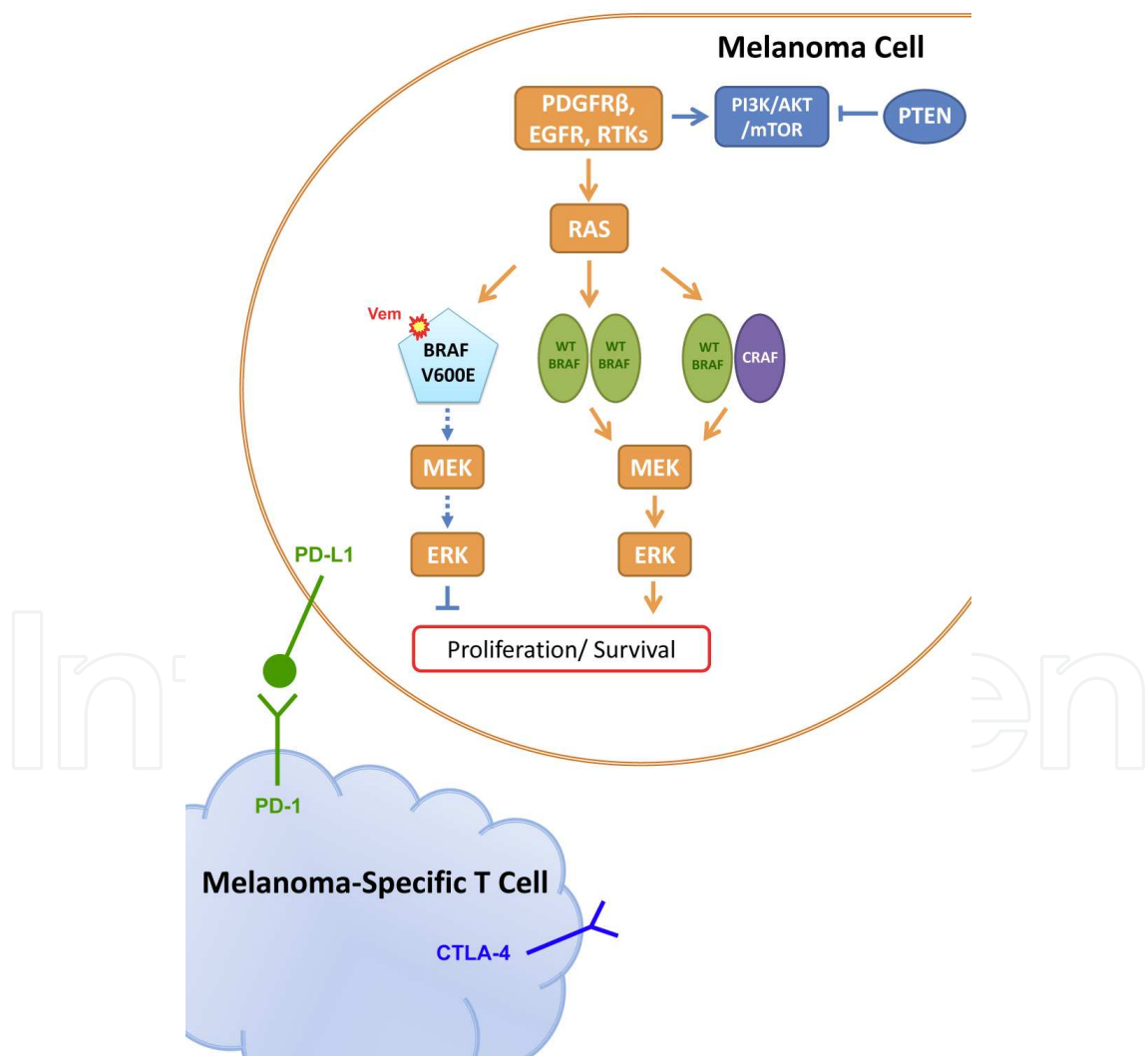

Figure 1. The mechanisms of BRAF inhibitor vemurafenib (Vem) action, toxicity and the interaction between melanoma cells with T lymphocytes.

However, wide type BRAF melanoma tumors do not respond to vemurafenib or dabrafenib inhibition, although they are sensitive to the MEK inhibitors [9]. Paradoxically, in cells with RAS mutation and wild-type BRAF, treatment with vemurafenib or dabrafenib will promote the formation of BRAF-CRAF heterodimer and lead to the activation of subsequent MEK/ERK signaling and cell proliferation as shown in Figure 1 [5]. This mechanism is used to explain the observation of typical clinical side effects associated with the use of vemurafenib: nearly 25% of patients developed skin lesions and even cutaneous squamous cell carcinoma (CSCC). In addition, *in vitro* study has revealed that vemurafenib inhibits multiple off-target kinases including c-Jun N-terminal kinase (JNK), suppresses JNK-dependent apoptosis, and generates CSCC toxicity [15].

2.1.2. Mechanisms of resistance to BRAF inhibition

In general, due to alternative pathway activations and inter-and intra-patients melanoma genetic heterogeneity, various mechanisms of resistance to BRAF inhibition have been identified [10, 16-19]. As we mentioned before, melanoma tumors bearing wide type BRAF are intrinsically resistant to vemurafenib and dabrafenib. Tumor micro-environment also contributes to the innate resistance to BRAF inhibition in melanoma. For example, stromal cells secrete hepatocyte growth factor (HGF), which activates the HGF-receptor MET, MAPK and PI3K-AKT pathways [20].

Eventually, nearly all BRAF mutated melanoma tumors develop acquired drug resistance upon treatment with BRAF inhibitors. The disease progression arises as early as two-month continuous treatment [18, 19]. The mechanisms of acquired resistance to BRAF inhibition can be generalized into two categories: $BRAF^{V600E}$-bypass mechanisms and MAPK-bypass mechanisms.

First, the $BRAF^{V600E}$-bypass mechanisms reactivate MAPK signaling and lead to ERK-dependent tumor cell survival and proliferation (Figure 2A). COT, which is coded by gene *MAP3K8*, is a MEK kinase. The overexpression of COT or amplification of *MAP3K8* directly activates MEK signaling without the participation of RAF protein [21]. The mutant of $MEK1^{C121S}$ increases catalytic capability and circumvents BRAF to activate basal level of ERK phosphorylation [22]. Before the treatment of vemurafenib or dabrafenib, melanoma cells with $BRAF^{V600E}$ mutation have over-activated monomer BRAF/MEK/ERK cascade which forms an ERK-dependent negative feedback loop. This negative feedback loop reduces the expression of the active RAS-GTP. In the presence of vemurafenib or dabrafenib, ERK phosphorylation level is rapidly reduced and the feed-back suppression on RAS activation is abolished (Figure 1). Therefore, eventually the ERK cascade level is restored through RAS over-activation. NRAS mutants including $NRAS^{Q61K}$ and $NRAS^{Q61R}$ can drive ERK activation through ARAF or CRAF homo-or hetero-dimers which are alternative MEK activators [23]. The combinations of BRAF inhibition plus MEK or ERK inhibition have showed efficacy of overcoming the resistance through these $BRAF^{V600E}$-bypass mechanisms [24-26], leading to the recent FDA approval of dabrafenib plus trametinib combination therapy for advanced melanoma.

Second, the MAPK-bypass mechanisms allow melanoma cells to escape from the cytotoxicity of BRAF or MEK inhibition through the activation of ERK-independent survival pathways

(Figure 2B). The PI3K-AKT signaling pathway can be activated through the overexpression of receptor tyrosine kinases (RTKs), for example, insulin-like growth factor 1 (IGF-1) receptor (IGF-1R) and platelet-derived growth factor receptor beta (PDGFRβ) [27]. The elevated levels of IGF-1R, PDGFRβ or HGF can also stimulate another receptor tyrosine kinase, MET, and increase the activity of PI3K. Phosphatase tensin (PTEN) is a negative regulator of PI3K. The PTEN loss-of-function mutation induces the resistance of BRAF inhibition and reduces the PFS of dabrafenib therapy in melanoma patients due to the PI3K activation [28]. Moreover, the upregulation of cyclin D1 can activate cyclin-dependent kinase 4 (CDK4) and 6 (CDK6) and make melanoma cells less dependent on MAPK signaling in cell cycle progressing [29].
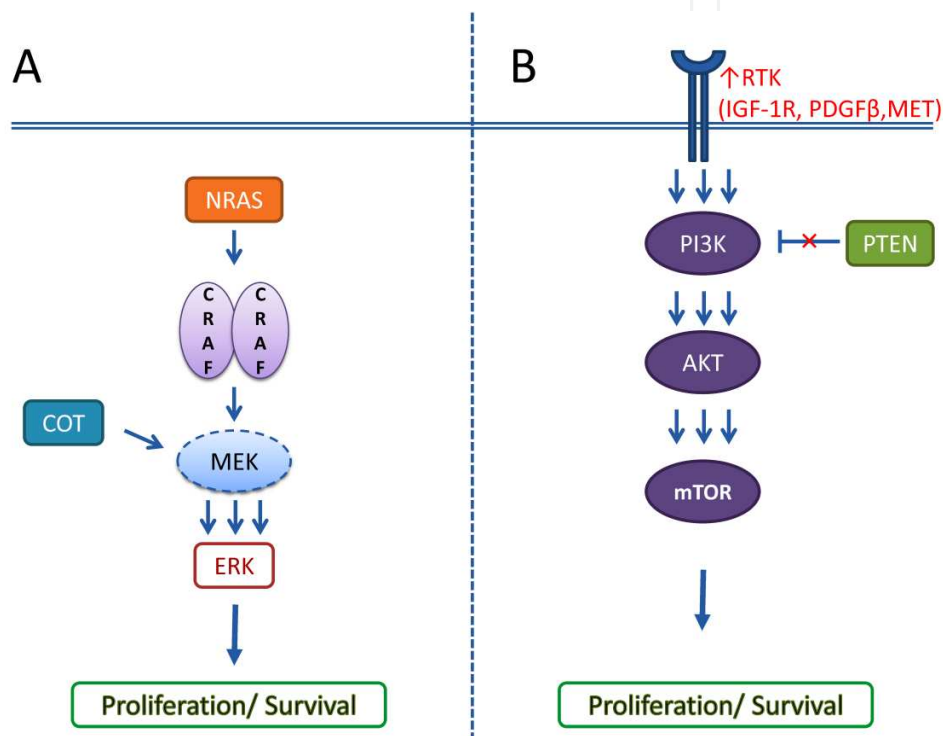

Figure 2. The mechanisms of acquired resistance to BRAF inhibition.

Additionally, Jaehyuk Choi *et al* has reported a BRAF^{L505H} mutation which changes an amino acid residue in BRAF-vemurafenib interface and causes the resistance to vemurafenib treatment *in vitro* [30]. Since vemurafenib is a substrate of the ATP-binding cassette sub-family G member 2 (ABCG2), the overexpression of ABCG2 in BRAF^{V600E} melanoma cell lines has caused the increasing of vemurafenib efflux *in vitro* [31]. The elucidation on the mechanism of acquired-resistance to BRAFi opens a door to rationally design and explore the proper combination strategies to overcome or delay the development of BRAFi resistance.

2.1.3. Targeting MEK: Mechanism of action, toxicity and resistance

Trametinib, which is approved by FDA in May 2013 as a monotherapy agent against advanced melanoma with BRAF^{V600E} and BRAF^{V600K} mutations, is a first-in-class, orally available, allosteric (non-ATP-competitive) MEK1/MEK2 inhibitor (MEKi) [32, 33]. It selectively inhibits

MEK, the down-stream kinase protein of RAF in the RAS-RAF-MEK-ERK pathway. As a result, melanoma cells with acquired resistance to BRAFi are commonly cross-resistant to MEKi such as trametinib or selumetinib, another selective allosteric MEKi [24, 34]. This mechanism explains the clinical trial results in which trametinib monotherapy fails to significantly benefit patients who have already developed acquired BRAFi resistance [35]. In contrast to the use of a BRAFi, no CSCC side effects are observed among the patients received trametinib treatment in clinical trials [13, 32]. However, similar to the use of vemurafenib, disease progression occurs within 6-7 months in patients receiving single-agent trametinib treatment [36]. Nevertheless, a retrospective analysis of 23 patients, who were first treated with MEKi and upon progression with a selective BRAFi, shows that the median time to progression (TTP) has been prolonged to 8.9 months from 4.8 months using a single-agent MEKi or 4.4 months for a single-agent BRAFi treatment, respectively [37]. However, a recent clinical trial indicated that if melanoma patients were treated with a BRAFi first then MEKi therapy, no confirmed response was observed [35]. This indicates that optimal treatment schedule and sequence is important for the melanoma therapy targeting the MAPK pathway.

2.1.4. Drug combination targeting MAPK pathway: From lab bench to clinical practice

Given that the mechanisms of tumor cells develop resistance to BRAFi partially by reactivating the ERK cascade and side effects such as CSCC are RAF-dependent, combining BRAFi with MEKi has attracted lots of research interest in order to further block the MAPK signaling pathway. *In vitro* and murine models first show the synergistic anti-proliferation and anti-tumor growth effects using the combined BRAFi and MEKi treatment [9, 27, 38, 39]. Further, this combination overcomes the acquired resistance to BRAFi [27, 38] in both cellular based assay and mouse xenograft models. In addition, the combined inhibition of BRAF-MEK suppresses the paradoxical BRAFi-induced MAPK signal elevation in melanoma cells and reduces the incidences of skin lesions in a rat model [9].

When it comes to the clinical trial data, the combined inhibition of BRAF-MEK has presented significant improvements of major patient benefits (PFS and overall survival). A phase I/II trial (ClinicalTrials.gov, NCT1072175) investigated the combination of oral dabrafenib (150 mg twice per day) plus oral trametinib (1 or 2 mg daily) (combination 150/1 and 150/2) versus monotherapy of dabrafenib (150 mg twice per day) over 108 metastatic melanoma patients bearing either V600E (92 patients) or V600K (16 patients) BRAF mutation [12, 36]. Median PFS in combination 150/2 group reached 9.4 months, compared to 5.8 months in the dabrafenib monotherapy group (hazard ratio 0.39, 95% confidence interval 0.25 to 0.62). The incidence of CSCC adverse events among combination 150/2 group is non-significantly lower than that among monotherapy group (7% versus 19%, *P*=0.09). But more frequent cases of pyrexia which is not common in trametinib single treatment have been reported in combination 150/2 group (71%, with recurrent rate 79%), as compared with dabrafenib monotherapy group (26%) [40]. These promising data lead to an accelerated FDA approval of the combination of dabrafenib (BRAFi) and trametinib (MEKi) for the treatment of unresectable or metastatic melanoma patients with BRAF V600E or V600K mutation, although further phase III studies with recruitment of more patients comparing the combination therapy with dabrafenib or vemur-

afenib single treatment (ClinicalTrials.gov, NCT01584648, NCT01597908) are still being assessed.

In addition, several ongoing phase I/II clinical trials now have shown that generally the combination of other BRAFi and MEKi is well tolerated in patients with or without receiving BRAFi treatment before (ClinicalTrials.gov, NCT01271803 vemurafenib (BRAFi)+cobimetinib (MEKi), NCT01543698 LGX818 (BRAFi)+MEK162 (MEKi)) [41-43] and overall response rate has increased comparing to the monotherapy groups, although the anti-tumor efficacy data haven't been released.

2.2. Combination targeted therapy using Phosphatidylinositol 3-Kinase (PI3K)/AKT/ mammalian Target of Rapamycin (mTOR) inhibitors

The activation of PI3K/AKT/mTOR pathway have been widely proved to be one of the major mechanisms of intrinsic or acquired resistance to both DNA-methylation agents (e.g. dacarbazine) and targeted BRAF inhibitor therapy (Figure 2). Some cell lines that are cross-resistant to both BRAFi and MEKi, are still sensitive to the inhibition of AKT/mTOR [34]. On the other hand, mechanistic study revealed evidences of a negative crosstalk between RAF/MEK/ERK and PI3K/AKT/mTOR pathways through RAS kinase. Therefore, when the downstream mTOR function is blocked, PI3K will be able to activate MAPK pathway via a switch of RAS [44, 45]. These investigations suggest a promising combination strategy of targeting MAPK pathway together with PI3K/AKT/mTOR cascade. Several preclinical studies widely proved that in MAPK inhibition sensitive melanoma cell lines, co-targeting PI3K/AKT/mTOR effectively induces cancer cell apoptosis with down-regulated anti-apoptotic BCL-2 family proteins [34, 46-48]. Such a co-targeting strategy can also postpone the emergence of acquired resistance to BRAFi dabrafenib mediated by PTEN mutation or disruption [49, 50]. Further, the dual inhibition of two pathways has successfully overcome NRAS mutation mediated resistance to MAPK blockade *in vitro* and induced xenograft tumor regression *in vivo* [34, 38, 51]. Finally, the combination of vemurafenib (BRAFi) or selumetinib (MEKi) with BEZ235 (dual PI3K and mTOR1/2 inhibitor) has been shown to overcome the PDGFRβ-driven resistance to MAPK pathway inhibition [52].

A series of Phase I studies have evaluated the clinical relevance of the combination therapy which co-targets PI3K/AKT/mTOR and RAF/MEK/ERK pathways in terms of the incidence on severe side effect and anti-tumor efficacy in 236 patients. These patients have advanced cancers including melanoma, colorectal, pancreatic and non-small cell lung cancers. Results from three combination groups (AKTi MK2206+MEKi selumetinib, NCT01021748; AKTi GSK2141795+MEKi trametinib, NCT01138085; mTOR inhibitor everolimus+MEKi trametinib, NCT 00955773) are compared to the single treatment groups [53]. Overall, the combination therapy did not provide significant increase of tumor control rate (64.6% for combination, 52.7% for monotherapy, P=0.16), although all five colorectal patients with co-activation of both pathways in combination group achieved tumor regression to varied extent between 2% and 64%. However, this combination strategy causes significant higher rates of drug-related grade III and above side effects (53.9% for combination, 18.1% for monotherapy, $P < 0.001$). Furthermore, two clinical trials which involve the combination therapy of BRAFi or MEKi with AKTi

DNE3 recently have been terminated due to the safety concerns of the toxic properties of DNE3 (ClinicalTrials.gov, NCT02087254 and NCT02095652). Nevertheless, in another ongoing phase I/II trial which measures the safety and efficacy of a well-tolerated pan-PI3K inhibitor BKM120 combined with vemurafenib therapy, preliminary data reveals that a vemurafenib-refractory melanoma patient with PTEN expression achieved a 35.9% reduction in target tumor (ClinicalTrials.gov, NCT01512251) [54]. In general, drug-related toxicity is one of the major issues for this cross-pathway targeted combination therapy and patients genetic profiling is very important to achieve the maximum objective response.

2.3. Combining targeted therapy with anti-angiogenic agents

Melanoma is a vascular tumor. The abnormal expression of the epidermal growth factor (EGF) family protein and the up-regulation of EGFR-mediated alternative survival pathway have critically shaped the response of melanoma to the current chemotherapy agents [55-58]. In a recent study by Sun *et al*, six out of sixteen melanoma cell lines display acquired EGFR expression after the development of resistance to BRAFi and MEKi [59]. Even before the FDA approval of BRAFi and MEKi, the combination of bevacizumab, a recombinant human monoclonal antibody VEGF inhibitor, with a specific chemotherapy agent (for example, fluorouracil [60] or fotemustine[61]), has become a first-line treatment for metastatic melanoma patients. Clinical trials that study the combination of anti-angiogenic agents with cytotoxic agents have achieved promising anti-tumor activity, although tolerability issues exist [62]. VEGF blockage has been shown to enhance the efficacy of a GM-CSF-secreting immunotherapy *in vitro* [63]. In addition, a VEGF receptor-2 inhibitor, semaxanib, prolonged both the complete and partial response time of an immunomodulatory drug, thalidomide, over 10 recurrent metastasis melanoma patients without showing significant drug-drug interaction toxicity in a phase II trial [64].

Along with the rapid development of targeted melanoma therapeutics, the combined inhibition of VEGFR plus PDGFR or mTOR has shown synergy anti-tumor effects on mouse models of B16 metastatic melanoma without increasing toxicity [65, 66]. A large-scale, unbiased drug screening study, which aims to discover effective genotype selective combinatorial therapeutics of vemurafenib-resistant BRAF and RAS mutant melanoma, identifies a triple BRAF+EGFR +AKT inhibition as highly effective approach [3]. In the year of 2010, combination of bevacizumab with an mTOR inhibitor, everolimus, was evaluated in a phase II trial for patients with metastatic melanoma [67]. The treatment was well tolerated in most patients. Seven out of fifty-seven patients (12%) receiving combination therapy have shown major responses, although the median PFS was only 4 months. This year (2014), in a phase II trial that combines bevacizumab and sorafenib, which is an inhibitor of both RAF kinase and VEGFR-2/PDGFRβ signaling, no objective tumor responses are seen in all the fourteen patients receiving treatment [68, 69]. Interestingly, the median TTP of patients with low VEGF (<300 pg/ml) was longer than that of patients with high VEGF (50 weeks versus 15 weeks, P=0.02). Therefore, the levels of VEGF in patients do influence the tumor progression profile (ClinicalTrials.gov, NCT00387751).

2.4. Combination therapy using targeted therapy with versatile chemotherapy agents

Since the abnormally activated (phosphorylation) of ERK and AKT constitutively exist in melanoma cells and promote the disease progression especially metastasis, blocking ERK or AKT pathway can sensitize the metastatic melanoma to the apoptosis induced by chemotherapeutic agents including cisplatin, temozolomide, DTIC and arsenite [70-72]. With the understanding of tumor biology about the programmed cell apoptosis and the rapid development of agents that can trigger the cell death process in melanoma, the combination of a MAPK inhibitor with a BCL-2 inhibitor (ABT-737 [73] or navitoclax [74]), or a MDM2 antagonist nutlin-3 [75], has synergistically induced apoptosis of melanoma *in vitro* and suppressed xenograft tumor growth *in vivo*. A comparative analysis on the samples collected from patients receiving vemurafenib or dabrafenib/trametinib combination treatment showed that BCL-2 expression level is closely related to the onset of MAPK inhibition resistance [74]. Clinical trials are being conducted to investigate the combination of BCL-2 inhibitor (BH3 mimetics) navitoclax and vemurafenib [74].

Due to the heterogenetic characteristics of melanoma disease, Vultur A *et al* [76] recently report that MEK or BRAF inhibition can potentially strengthen the invasion property of human melanoma cells by about 20%. As a result, co-inhibiting kinases that are actively involved in cell invasion process, such as RTK, STAT3 and Src, together with MEK inhibition has effectively abolished the invasive phenotype and further caused the tumor cell death in a 3D matrix model.

Metformin, a biguanide oral anti-diabetic drug, has been discovered with antitumor activity in various cancer types including melanoma. Although the exact mechanisms remain to be elucidated, accumulating data suggest that metformin can activate AMP-activated protein kinase (AMPK) and thus increase the activities of VEGF and ERK in BRAF^{V600E} mutated melanoma cells [77]. AMPK negatively regulates malignant cell proliferation and viability [78]. The combination of vemurafenib and metformin has shown synergistic anti-proliferative effects on six out of eleven tested BRAF^{V600E} melanoma cell lines [79]. Pilot clinical studies that evaluate the safety and efficacy of metformin combination therapies (plus dabrafenib or trametinib) are now recruiting patients (ClinicalTrials.gov, NCT0184000, NCT02143050).

Unlike the cutaneous melanoma, over-activation of MAPK pathway in uveal melanoma is associated GNAQ or GNA11 mutations instead of BRAF or RAS mutations [80]. Protein kinase C (PKC) inhibitors such as enzastaurin or AEB071 induce apoptosis in GNAQ-mutant but not in GNAQ wild type uveal melanoma cells [81]. The level of ERK phosphorylation also decreases in these cells when they are treated using PKC inhibitors [81]. Chen *et al.* has recently confirmed the synergy of the combination using a PKC inhibitor with a MEKi (PD0325901 or MEK162) in GNAQ/11 mutant uveal melanoma cells [82].

Understanding the mechanisms of resistance to MAPK inhibition in melanoma can lead to rational combination designs in order to overcome acquired drug resistance to BRAF inhibitors. For example, our lab recently identified a synergistic combination in which a novel tubulin inhibitor ABI-274 combined with vemurafenib could overcome the acquired vemurafenib-resistance [83]. This combination treatment effectively arrested the vemurafe-

nib-resistant melanoma cells in both G_0/G_1 and G_2/M phases and induced strong apoptosis through the down-regulation of AKT phosphorylation. In addition, the combination of a MEKi (TAK-733) with an Hsp90 inhibitor (ganetespib) induces tumor regressions in vemurafenib-resistant xenograft models also through the depletion of AKT signaling [84]. With the finding that up-regulated cyclin D1 expression is critical for the survival of vemurafenib-resistant cells, a selective inhibitor of cyclin dependent kinase (CDK) 4/6, LY2835219, has been reported to overcome the reactivation of MAPK signaling in vemurafenib-resistant BRAF[V600E] melanoma [85].

3. Combinations involving immunotherapy in melanoma treatment

3.1. Combined blockade of immuno-checkpoints

Given the unsatisfactory results of cytokine-based melanoma immunotherapy (recombinant interferon-α 2b and high dose interleukin-2) in the past decade, the development and approval of ipilimumab (anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) monoclonal antibody) in 2013 have marked a breakthrough of immune-checkpoints blockade therapy [86]. CTLA-4 (CD152) expresses on the surface of active T-lymphocytes and inhibits the initial T-cell proliferation and migration to the tumor tissue [87]. CTLA-4 antibodies preferentially target the suppressive regulatory T cells and prevent them from being hijacked by tumors [88]. In a double-blinded phase III study in 676 patients with pretreated and refractory metastatic melanoma, ipilimumab at the dose of 3 mg/kg achieved a median OS of 10 months [86]. In a meta-Kaplan-Meier-analysis of data collected from 1,861 melanoma patients in a clinical trial, a plateau of survival curve starts from around 3 years after ipilimumab treatment with follow-up extends as long as ten years, indicating a long-term survival benefits of ipilimumab therapy (ClinicalTrials.gov, NCT01844505). In addition, ipilimumab showed good tolerance and efficacy in several other clinical trials in which it was combined with a standard chemotherapy agent such as dacarbazine, fotemustine or temozolomide [89].

Another success of immune-check point blockade strategy is the development of anti-programmed death-1 (PD-1) antibodies, represented by pembrolizumab (MK-3475) and nivolumab [90, 91]. Pembrolizumab, as the first-in-class PD-1 inhibitor, has obtained FDA approval in September 2014 for patients with advanced or unresectable melanoma. The cDNA of PD-1 (CD279) is first cloned in programmed death T cells although PD-1 itself does not directly induce apoptosis. PD-1 is over-expressed on the surface of dysfunctional activated T-cells and contributes to the maintenance of T cell dysfunction (exhaust) phenotype and proliferation disability in the tumor site [92]. Two counter receptors of PD-1 have been identified: PD-L1 and PD-L2. PD-L1 is more frequently and exclusively expressed in various tumor cells; therefore, antibodies targeting PD-L1 (MPDL3280A and BMS-936559) also have anti-tumor activity in advanced cancer including melanoma [91, 93]. The PD-1-PD-L1 ligation retards the recognition and destroying of tumor cells by CD8+cytotoxic T-lymphocytes [87]. As a result, blocking PD-1 or PD-L1 will reverse the cancer cell immune escape. Because both CTLA-4 and PD-1 are key negative receptors that cooperatively modulate the adaptive

immune response in tumor progression, their combination has been shown to be synergistic in B16 melanoma tumors without overt toxicity [94].

In a cohort phase I trial that studied the concurrent administration of ipilimumab and nivolumab to 53 patients with advanced, treatment-resistant melanoma, more than 80% tumor reduction was observed in 30% patients after 12 weeks treatment at the maximum tolerated dose. Twenty-one out of fifty-three patients had objective responses and over 80% of these patients had tumor regression. Grade 3/4 adverse events are diagnosed in 53% patients but the toxicities are manageable with immune-suppressants [95]. Consequential trials with more enrollment number of patients are necessary to further evaluate the safety and efficacy of this promising double immune-checkpoints blockage therapy comparing with each of its mono-therapy regiments.

Finally, combinatorial clinical trials using ipilimumab with other immunotherapy agents have shown some favorable therapeutic benefits. For example, combination of ipilimumab with peginterferon α-2b (pegylated interferon α-2b) in patients with unresectable melanoma both demonstrated significant increase of response rate and OS comparing with the monotherapy arm [96, 97] in recent phase I trials.

3.2. Combined therapy inhibiting both immuno-checkpoint and MAPK signaling pathway

Checkpoint blockade immunotherapy and MAPK targeted chemotherapy have distinct clinical profiles. For example, targeted therapy has relative higher initial response rate (~60% for BRAFi) with rapid onset of effect, but its efficacy restrictively rely on the continuous treatment and the therapeutic response is usually not durable due to the quick development of acquired drug resistance. In contrast, immunotherapy has much a lower response rate (4.5% for ipilimumab), delayed onset of effect and difficulty in predicting patient outcome, but it has shown potentially durable responses and long-term survival benefit even off treatment. In addition, since the MAPK pathway is not required in the process of anti-tumor immune response, blocking MAPK signaling should not interfere with the efficacy of checkpoint blockade immunotherapy. Therefore, it seems very rational that the combination of a MAPKi and an immunotherapy agent such as ipilimumab or pembrolizumab can maximize the therapeutic benefits in advance melanoma.

Interestingly, BRAF and MEK inhibition displayed an "endogenous vaccine-like" effects in melanoma cells [98]. Cytotoxic agents like BRAFi induce tumor cell death and promote the uptake and presentation of tumor antigens to the effector immune cells (T cells and B cells) through antigen-presenting cells [54]. MEK inhibition, BRAF^{V600E} RNA silencing or BRAF inhibition by PLX4720 increases the CD4+ and CD8+ lymphocytes mediated T-cell infiltration and reduce the level of immune-suppressants including IL-6, IL-10 or VEGF [99-101] in mice. The expression of PD-L1 is found to be elevated in BRAFi-resistant melanoma cells and it is mediated through the off-target activity of BRAFi in JUN and STAT3 signaling [102]. However, Vella *et al* has published a paper in 2014 and stated that they have not found any impact of dabrafenib treatment on T lymphocytes. trametinib alone or in combination with dabrafenib has suppressed T lymphocyte proliferation, cytokine secretion and antigen-specific expansion

in their isolated T lymphocyte and monocyte-derived dendritic cells. These findings should be carefully tested *in vivo* to evaluate the clinical relevance [103].

As for the clinical practice, dose-limiting hepatotoxicity issues have led to the premature termination of the first phase I study on combination of ipilimumab with vemurafenib (ClinicalTrials.gov, NCT01400451). This signified the complexity of adverse effect in combined therapy of immune-regulating agents and kinase inhibitors. Another phase I study of ipilimumab plus dabrafenib, or ipilimumab plus the combination of dabrafenib with trametinib is still active and a phase II study is exploring the safety and efficacy of sequential administration of vemurafenib followed by ipilimumab (ClinicalTrials.gov, NCT01767454, NCT01673854). The data of these most recent trials will be released in the near future.

4. Conclusions

Extensive efforts and remarkable progresses have been made to discover and investigate rational approaches in combination melanoma therapy since the recent approval of MAPKi and immune checkpoints blockade antibodies. A number of new targeted or immune drugs for metastatic melanoma are currently under commercial development or late stage clinical trials, some of which will likely be approved in the next few years. Quality of life for many melanoma patients has been dramatically increased. However, significant challenges still remain. While some clinical evidence has really raised the expectation of survivals for patients with advanced melanoma, the benefits of combination therapy are usually accompanied by limitations. Comprehensive genetic profile and tailored patient matching is essential for targeted therapy, while biomarkers are critical to predict the patient immunotherapy response. Drug-related toxicity for combination treatment usually is not a simple one-plus-one situation, and potential drug-drug interactions, especially the combination of a targeted agent with an immunotherapeutic agent must be carefully evaluated in order to achieve both fast and durable responses. Adverse effects should be closely monitored and potential alternative dosing regiments is worth further exploration. Optimized dose schedule may help to delay the resistance development and reduce the frequency of adverse effect. For example, intermittent doses of BRAFi was able to enhance the tolerance in combination with immunotherapy, decrease the paradoxical MAPK activation, which might be the main cause of severe toxicity in clinical trial [104]. Solid evidence of synergistic combination in preclinical research must be established before clinical trial conduction. In fact, with the relatively large number of available targeted agents and immunotherapeutic agents for metastatic melanoma, the huge number of possible drug combinations coupled with dosing sequences or schedules already presents a significant challenge in designing proper clinical trials. To test all the possible drug combinations along with different dosing sequences clinically will not only have low benefits to patients, but is also a huge financial burden to the society. Carefully designed, predictive preclinical studies will be essential to provide critical supports for rational prioritization of clinical trials using drug combinations. Finally, clear understandings of various combination mechanisms and patient genetic profiles are critically important for the development of new combination approaches, prediction of expected therapy response and potential side effects.

With the rapid advances in this field, it is likely that optimal combination treatments will great improve the management of advanced melanoma in cancer patients.

Abbreviations

AMPK: 5' adenosine monophosphate-activated protein kinase

BRAF: B-Raf protein

BRAFi: BRAF inhibitor

CDK: Cyclin dependent kinase

CR: Complete response

CTLA-4: Cytotoxic T lymphocyte-associated antigen 4

ERK: Extracellular signal-regulated kinase

HR: Hazard ratio

JNK: c-Jun N-terminal kinase

MAPK: Mitogen-activated protein kinase

MEKi: MEK inhibitor

MHC: Membrane histocompatibility complex

mTOR: Mammalian target of rapamycin

ORR: Overall response rate

OS: Overall survival

PD-1: Programmed cell death 1

PD-L1: Programmed cell death 1 ligand 1

PDGFR: Platete-derived growth factor receptor

PFS: Progression-free survival

PI3K: Phosphoinositide 3-kinase

PKC: Protein kinase C

PR: Partial response

RTKs: Receptor tyrosine kinases

TCR: T cell receptor

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

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