

Received: 2019.10.25

Accepted: 2020.01.06

Available online: 2020.02.14

Published: 2020.04.10

# A Review of the Molecular Pathways Involved in Resistance to BRAF Inhibitors in Patients with Advanced-Stage Melanoma

Authors' Contribution:

Study Design A

Data Collection B

Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

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**Source of support:** The National Natural Science Foundation of China (No. 81625020 and No. 81602906)

Melanoma is an aggressive malignancy of melanocytes and most commonly arises in the skin. In 2002, *BRAF* gene mutations were identified in melanoma, and this finding resulted in the development of several small-molecule molecular inhibitors that specifically targeted the *BRAF* V600E mutation. The development of targeted therapies for advanced-stage melanoma, including tyrosine kinase inhibitors (TKIs) of the *BRAF* (V600E) kinase, vemurafenib and dabrafenib, have been approved for the treatment of advanced melanoma leading to improved clinical outcomes. However, the development of *BRAF* inhibitor (BRAFi) resistance has significantly reduced the therapeutic efficacy after prolonged treatment. Recent studies have identified the molecular mechanisms for BRAFi resistance. This review aims to describe the impact of BRAFi resistance on the pathogenesis of melanoma, the current status of molecular pathways involved in BRAFi resistance, including intrinsic resistance, adaptive resistance, and acquired resistance. This review will discuss how an understanding of the mechanisms associated with BRAFi resistance may aid the identification of useful strategies for overcoming the resistance to *BRAF*-targeted therapy in patients with advanced-stage melanoma.

**MeSH Keywords:** **Drug Resistance • Melanoma • Molecular Targeted Therapy • Proto-Oncogene Proteins B-raf**

**Full-text PDF:** <https://www.medscimonit.com/abstract/index/idArt/920957>

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## Background

Melanoma is an aggressive malignancy derived from melanocytes and most commonly arises in the skin. Risk factors for the development of melanoma include the presence of multiple dysplastic nevi, a familial history of melanoma, racial skin phenotype, and exposure to ultraviolet (UV) radiation [1]. It was estimated that in 2019, there were 96,480 new cases of melanoma diagnosed in the US and 7,230 deaths due to melanoma. The survival rate of melanoma is highly dependent on the clinical stage. Patients with localized or early-stage melanoma of the skin can be treated successfully by surgical excision. However, patients with advanced-stage melanoma that has metastasized still face many challenges. A recent study has shown that for patients with advanced-stage melanoma, combined treatment with the monoclonal antibody checkpoint inhibitors, nivolumab and ipilimumab, has significantly prolonged the 3-year survival rate to 58% [2].

In 2002, the *BRAF* gene mutation was identified in more than 60% of all patients with melanoma, which prompted the investigation of the effect of *BRAF* mutation on melanoma pathogenesis [3]. The most commonly-occurred type of *BRAF* mutation is the transition from valine to glutamic acid at position 600 (V600E), while other variants like V600K, V600D and V600R occupy around 12%, 5%, and 1%, respectively [4,5]. These mutations can constantly activate the kinase domain and result in MAPK pathway hyperactivation [6], thus driving the development of melanoma. Since the progress of high-throughput sequencing technologies in recent years, a series of new gene mutations in melanoma like *NRAS*, *NF1*, *GNAQ*, *KIT*, and *FBW7* [7–10], have been found to regulate the MAPK pathway and other signaling pathways.

This review aims to describe the impact of BRAFi resistance on the pathogenesis of melanoma, the current status of molecular pathways involved in BRAFi resistance, including intrinsic resistance, adaptive resistance, and acquired resistance. This review will discuss how an understanding of the mechanisms associated with BRAFi resistance may aid the identification of useful strategies for overcoming the resistance to BRAF-targeted therapy in advanced-stage melanoma.

## The Impact of *BRAF* Gene Mutation on the Pathogenesis of Melanoma and BRAF-Targeted Therapy

BRAF belongs to the RAF family and acts as a protein kinase [11]. Through the direct activation of downstream MEK1/2 that is the kinase of ERK1/2, RAF can activate MAPK signaling pathway, which activates their target proteins in the cytoplasm or nucleus and subsequently potentiates downstream transcriptional

factors that can regulate the genes related to cell proliferation, differentiation or survival [12]. While the expression of RAF1 (commonly known as CRAF) is much more ubiquitous in different tissues than other isoforms of the RAF family, the pathogenic mutations of *RAF1* are very rare. Nevertheless, with high expression in melanocytes, neuronal tissues, hematopoietic cells as well as testis, the *BRAF* mutation is much more common in the pathogenesis of cancer [13].

The V600E mutation in the *BRAF* gene significantly potentiates its kinase activity [14,15], which activates the downstream MAPK pathway that contributes to tumor development through the potentiation of the cell cycle and the suppression of cell apoptosis [16,17]. The *BRAF* V600E mutation induces the activation of the MAPK pathway even without the stimulation of cytokines, hormones, or growth factors, and contributes to increased cell proliferation and tumorigenesis. Therefore, activation of the MAPK pathway is responsible for the pathogenesis of *BRAF* mutation in the initiation and growth of melanoma.

Apart from the effect on tumor growth, *BRAF* mutation is also involved in melanoma metastasis. Oncogenic BRAF facilitates tumor invasion by activating the Rho family of GTPases [18], the down-regulation of phosphodiesterase 5A (PDE5A) [19], and actin cytoskeleton reorganization [20]. Also, the inhibition of *BRAF*V600E reduces the number of cortactin foci in both a mouse melanoma model and in tumor biopsies from patients with melanoma [21]. Mechanistically, *BRAF*V600E induced the phosphorylation of both cortactin and Exo70 in an ERK-dependent manner, which then promoted MMP secretion and the assembly of actin. Also, *BRAF*V600E was also able to mediate many invadopodia-related genes, which were highly associated with cancer metastasis. These findings provide sufficient evidence that *BRAF*V600E contributes to melanoma metastasis through multiple pathways [21].

Reprogramming of cell metabolism is a characteristic of tumor cells, which is characterized by potentiated glucose uptake and enhanced aerobic glycolysis to support tumor development. In melanoma, BRAF is an important regulator of metabolic reprogramming due to its significant effect on glucose metabolism in multiple ways [22]. Activated BRAF could induce metabolic rewiring through the suppression of oxidative phosphorylation gene program by reducing the expression of melanocyte lineage-specific transcriptional factor MITF and the mitochondrial master regulator PGC1 $\alpha$ . Specifically, in a subgroup of melanomas with relatively high MITF expression, PGC1 $\alpha$  expression was consistently upregulated and was correlated with enhanced mitochondrial biogenesis and oxidative phosphorylation. The knockdown of MITF could result in impaired PGC1 $\alpha$  transcription and thereby suppressed transcription of mitochondrial genes of oxidative metabolism, leading to reduced mitochondrial function and compensated activation of glycolysis.

Knockdown of PGC1 $\alpha$  induced similar metabolic rewiring as that of MITF deficiency in melanoma [23]. Following BRAFi treatment, the expression and activity of MITF could be augmented to promote mitochondrial biogenesis via the transcriptional activation of its direct target PGC1 $\alpha$  [23]. Therefore, the transcriptional MITF-PGC1 $\alpha$  axis connected BRAF mutation to mitochondrial dysfunction in melanoma. However, BRAF mutations have been shown to activate glycolysis by promoting the expression of the target molecules of hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) and are involved in glucose utilization as well as uptake along the pathway [24,25]. Also, recent phosphoproteomic studies in BRAF-mutated melanoma cells have identified the glycolytic molecular PFKFB2 as the downstream phosphorylation substrate of RSK [22].

PFKFB2 controls the generation of fructose-2,6-bisphosphate to potentiate the activity of PFK-1, which is a key regulatory enzyme that controls glycolytic flux. The knockdown of PFKFB2 alone could significantly suppress the glycolytic capacity of melanoma cells, resulting in tumor regression. Importantly, in BRAF-mutant melanoma, RSK could directly phosphorylate the regulatory domain of PFKFB2 to enhance its activity and potentiate tumor cell glycolysis. Therefore, RSK could promote glycolysis and the development of BRAF-mutated melanoma through the phosphorylation modification of an important glycolytic enzyme, PFKFB2. Therefore, BRAF mutation governs the metabolic reprogramming of melanoma cells to support tumor development.

These findings support the potential role of targeted therapeutic strategies in melanoma harboring BRAF mutation. Currently, vemurafenib and dabrafenib have been approved by the US Food and Drug Administration (FDA) for the treatment of advanced-stage melanoma with BRAF mutations [26,27]. The response rate to vemurafenib is more than 50%, and some patients with melanoma have shown a complete response [28]. Similar results were obtained for dabrafenib, with an overall response rate of 50% [29].

Although inhibitors to BRAF have shown significant effectiveness in patients with advanced-stage melanoma, the efficacy of these therapies is limited to a subgroup of patients. Also, due to the resistance to targeted therapy, the recurrence of melanoma is inevitable, which limits the duration of survival. Therefore, frequent occurrence of treatment resistance to BRAFi significantly reduces the effect of targeted therapy. The mechanisms for BRAFi resistance include three main factors: primary or intrinsic resistance with the characteristic of no response to therapy; adaptive resistance with an initial response or non-mutational drug tolerance, which occurs early and is reversible; and acquired resistance with mutational drug tolerance, which occurs late and is irreversible (Table 1) [7,30–32]. Improvements in clinical outcome for patients with advanced-stage melanoma

require further studies to identify the mechanisms underlying BRAFi resistance and to identify novel treatment strategies.

## Mechanisms of Intrinsic Resistance

Intrinsic resistance is defined as the innate capacity of melanoma cells to resist a target inhibitor with no clinical benefit. Approximately 20% of patients with melanoma harboring BRAF mutations have intrinsic resistance to MAPK inhibition therapy [8]. Studies have identified the molecular mechanisms responsible for the intrinsic resistance include loss of PTEN, loss of NF1, CCND1 amplification, COT overexpression, mutations in RAC1, and loss of the USP28-FBW7 complex [8,33–37].

### Loss of PTEN expression

PTEN has been identified as a highly effective tumor inhibitor gene by homozygous deletion mapping in 1997 [38,39]. The lipid phosphatase function of PTEN is responsible for converting PIP3 to PIP2, resulting in reduced AKT activity, which promotes stress-induced apoptosis through the down-regulation of anti-apoptotic protein BCL2 and the upregulation of pro-apoptotic machinery including caspases. PTEN can also dephosphorylate FAK and Shc, resulting in the inhibition of tumor proliferation, invasion, migration, and focal adhesion formation and the suppression of MAPK signaling stimulated by growth factors.

In 1998, the involvement of PTEN in melanoma was identified using *in vitro* loss of heterozygosity (LOH) studies [40]. PTEN is recognized as a candidate melanoma inhibitor gene, which was confirmed by ectopic gene expression studies in melanoma cells [40]. Screening of melanoma cell lines has shown that approximately 20% of human melanoma cell lines contain homozygous deletions [40]. *In vitro* studies and tumor xenograft studies showed that the combination of the BRAFV600E mutation and silencing of the PTEN gene resulted in the development of melanoma with short latency, 100% penetrance, and metastases in the lungs and lymph nodes [41].

Deletions or mutations in PTEN were partially responsible for the intrinsic resistance to BRAF-targeted treatment in melanoma, and cell lines with loss of function of PTEN were more resistant to BRAF inhibitors [33,41–43]. Also, patients with wild-type (WT) PTEN showed better survival after treatment with BRAF inhibitors [42]. The activation of AKT after PTEN deficiency is necessary for intrinsic resistance to BRAF suppression, and suppression of BRAF and PI3K were identified as a method to overcome this resistance and promote cell apoptosis of BRAF-mutant melanomas with PTEN deletion [42].

**Table 1.** The mechanisms of BRAF inhibitor resistance in patients with advanced-stage melanoma.

| Gene mutation                                | Mechanisms of resistance  |
|--|---|
| <b>Intrinsic mechanisms</b>                  |   |
| Loss of PTEN                                 | <i>PTEN</i> is a melanoma growth inhibitor of the PI3K-AKT pathway. Loss of <i>PTEN</i> leads to AKT activation   |
| Amplification of <i>CCND1</i>                | <i>CCND1</i> gene encodes cyclin D1 and regulates proliferation through binding to CDK4 and CDK6, which activate retinoblastoma protein and lead to cell cycle progression  |
| COT (MAP3K8) overexpression                  | COT can independently activate the MAPK/ERK pathway, and increased COT promotes cellular proliferation despite BRAF inhibition  |
| Loss of NF1                                  | <i>NF1</i> is a tumor suppressor of RAS. Loss of <i>NF1</i> leads to activation of RAS, PI3K-AKT-mTOR, and MAPK pathways  |
| RAC1 mutation                                | RAC1 is a key regulator of motility and proliferation cells and a GTPase effector of RAS  |
| Loss of the USP28-FBW7 complex               | The loss of the USP28-FBW7 complex could stabilize BRAF to potentiate downstream MAPK pathway activation  |
| <b>Adaptive mechanisms</b>                   |   |
| Gene mutation                                | Mechanisms of resistance  |
| Resetting of ERK1/2 pathway activation       | Adaptive resetting of ERK1/2 flux occurs in some mutant- <i>BRAF</i> melanoma lines following RAF inhibition due to the reduction of negative feedback regulators   |
| Upregulation of RTKs                         | Upregulation and activation of RTKs, including ERBB3, PDGFR, EGFR, and FGFR contribute to cell proliferation and impairs cell apoptosis in response to BRAF inhibition  |
| MITF upregulation                            | BRAF/MEK inhibitor treatment upregulates MITF through a MAPK-dependent rewiring of the transcriptional activation of MITF expression, which suppresses cell apoptosis   |
| The paradoxical role of SOX10                | The increased transcriptional activity of SOX10 could impair the sensitivity of <i>BRAF</i> -mutant melanomas to targeted therapy during the early phase. However, the suppression of SOX10 can upregulate RTKs, which driven the acquired resistance to MAPK inhibition in melanoma  |
| Metabolic rewiring                           | BRAF inhibition leads to the metabolic rewiring characterized by suppressed glycolysis and activates mitochondrial oxidative phosphorylation to ensure cell viability and proliferation   |
| <b>Acquired mechanisms</b>                   |   |
| Gene mutation                                | Mechanisms of resistance  |
| RAS mutations                                | Constitutively active <i>RAS</i> mutants enhance <i>BRAFV600E</i> dimerization, reactivate the ERK pathway, and confers resistance to BRAF inhibitors, which only block monomeric <i>BRAFV600E</i>  |
| RAF paradox and dimerization of RAF proteins | BRAF <sub>i</sub> can paradoxically activate the WT-BRAF kinase through the induction of dimerization and CRAF activation, resulting in MEK/ERK phosphorylation and eventually promoting cell proliferation   |
| BRAF gene amplification and splicing         | The amplification of the <i>BRAF</i> gene led to significant upregulation of BRAF protein expression, contributing to the reactivation of ERK in the presence of BRAF inhibitors. Alternative splicing can lead to the expression of truncated BRAF proteins that lack the N-terminal RAS-binding domain but retain the kinase domain, which can form homodimers that are resistant to BRAF inhibitor |
| MEK1/2 mutations                             | MEK1/2 mutations could reactivate downstream ERK signaling without the need for BRAF stimulation  |
| Hyperactivation of RTKs                      | Overexpression of hyperactivation of RTKs could promote acquired resistance through the activation of parallel pathways or by direct induction of the RAS pathway   |
| Aberrations in the PI3K -AKT pathway         | PI3K and AKT-activating mutations enhance AKT signaling, which promotes anti-apoptotic signals and upregulates expression of essential proliferative genes, allowing survival signals independently of BRAF   |

**Table 1 continued.** The mechanisms of BRAF inhibitor resistance in patients with advanced-stage melanoma.

| Gene mutation                                | Mechanisms of resistance  |
|--|---|
| Down-regulation of STAG2 or STAG3 expression | Down-regulation of STAG2 or STAG3 expression suppressed CTCF-mediated expression of dual-specificity phosphatase 6 (DUSP6), resulting in the reactivation of ERK  |
| Activation of the YAP/TAZ pathway            | The activation of YAP/TAZ pathway after actin remodeling renders resistance to BRAF targeted therapy  |
| Down-regulation of expression of DUSPs       | DUSPs are the largest group of phosphatases for dephosphorylating ERK1/2 kinase, DUSPs are considered to be the negative feedback loop of MAPK signaling in response to BRAF targeted therapy                   |
| Down-regulation of expression of RNF125      | Deficiency of RNF125 suppressed the ubiquitination and degradation of JAK1, thereby promoting the expression of EGFR that activated downstream ERK signaling and conferring resistance to BRAF targeted therapy |

### Amplification of the CCND1 gene

Dysregulated cell cycle progression is one of the key hallmarks of the pathogenesis of malignancy. *CCND1* is amplified in around 11% of all patients with melanoma [34,44]. Activated *BRAF* mutation may drive uncontrolled cell proliferation through the MAPK-induced expression of cyclin D1, which promoted the progression of the cell cycle by binding to CDK4 as well as CDK 6, and regulates the cell cycle through the retinoblastoma tumor suppressor protein. Also, *BRAF*-stimulated tumorigenesis was significantly increased following dysregulation of cyclin D1/CDK4 activity [45,46]. These findings indicate that the dysregulation of cyclin D1 is involved in melanoma tumorigenesis.

A recent study using an micro-array comparative genomic analysis showed that 17% of patients with melanoma harbored *BRAF* V600E mutations with concurrent *Cyclin D1* amplification [34]. Melanoma cell lines with *Cyclin D1* amplification had upregulated cyclin D1 expression and were intrinsically resistant to BRAF inhibition. Also, the re-introduction of *Cyclin D1* could facilitate cell cycle progression when BRAF was inhibited in previously drug-sensitive cells [34]. Cyclin D1 overexpression alone may mediate resistance, and this could be potentiated when cyclin D1 and CDK4 are both overexpressed, suggesting that upregulated cyclin D1 expression contributed to the resistance to BRAFi in a subgroup of patients with melanoma who had the *BRAF*V600E mutation.

### COT (MAP3K8) gene overexpression

*MAP3K8*, which encodes the COT/TPL2 protein, is a protein kinase contributing to the constant ERK1/2 activation through the phosphorylation of its direct substrate MEK [47]. In 2010, nine kinases that conferred resistance to BRAF treatment were identified, where CRAF and COT/TPL2 emerged as the top gene candidates and were shown to activate ERK signaling [35].

COT is not only involved in the regulation of ERK activation but is also inversely associated with *BRAF*V600E. Mutant *BRAF* suppressed the expression of COT and the dysregulated expression of COT conferred resistance to BRAF inhibitors. To be specific, two *BRAF*-mutant cell lines contained the gains of chromosomal copy spanning the *MAP3K8/COT* locus [35]. These cells expressed relatively high COT expression to empower intrinsic resistance to BRAF inhibition, which could be reduced using a COT inhibitor. COT overexpression is significantly involved in the intrinsic resistance to treatment for patients with melanoma that harbors *BRAF* mutations.

### Loss of NF1 gene activity

The *NF1* gene encodes the protein neurofibromin, which is a protein activating GTPase that can suppress Ras by augmenting intrinsic GTPase activity [48]. In 1993, it was found that the loss of *NF1* frequently occurred in both melanoma cells and tissues [49]. Then, *NF1* loss was shown to mediate the escape from *RAF*-induced OIS in melanoma, which was further confirmed by employing a transgenic mouse model where mutant *BRAF* cooperated with *NF1* loss to promote the process of melanoma [36].

The loss of *NF1* is associated with the activation of other genes in the MAPK pathway, especially with *BRAF* mutations that have reduced kinase activity and induces ERK signaling through the dimerization and activation with CRAF [50]. Activated and phosphorylated ERK regulates the transcription of a network of genes, including dual-specificity phosphatase and the *SPRY* gene families that negatively influenced RTKs, RAS, and RAF [50]. Importantly, *NF1* deficiency may inhibit ERK-dependent feedback inhibition of RAS activity in melanoma cells harboring *BRAF* mutations [50].

Since the loss of *NF1* can result in RAS resistance to negative feedback, *NF1* inactivation in melanoma harboring *BRAF* mutation results in a selective advantage by reversing

oncogene-mediated suppression of RAS, which is driven by ERK-induced negative feedback [10]. These tumors were intrinsically resistant to MAPK inhibition, leading to a reduced drug response and could be adequate to facilitate the establishment of clones with drug resistance [10]. Therefore, *NF1* loss is regarded as a critical mediator of intrinsic BRAFi resistance [10].

### **RAC1 gene mutation**

*RAC1* is a member of the Rho family that has a role in tumorigenesis and tumor metastasis [51], and affects the re-arrangement of the cytoskeleton. Apart from frequent mutations in *RAS* and *BRAF*, the *RAC1* P29S mutation was found in approximately 4–9% of cases of recurrent melanoma [37]. The P29S mutation is capable of activating *RAC1* by facilitating the exchange of a guanine nucleotide to promote the ratio of active GTP-bound *RAC1* to inactive GDP-bound *RAC1* [52].

In a previous clinical investigation that enrolled 45 patients treated with BRAF inhibitors, three patients harboring *RAC1* mutations showed no significant response [37]. This clinical correlation may predict those patients who were intrinsically resistant to targeted therapy. In 2014, Watson et al. showed that cells harboring the *RAC1* P29S mutation were more resistant to MAPK pathway inhibition [37]. The overexpression of the P29S mutation contributed to the intrinsic resistance to MAPK inhibition *in vitro*, and deficiency of the *RAC1*P29S mutant amplified the effect on promoting cell death following *BRAF* inhibition [37]. Also, *RAC1* P29S upregulation resulted in reduced drug effects on tumor growth *in vivo*. Therefore, *RAC1* mutations are involved in the development of melanoma and also intrinsic resistance to BRAF-targeted therapy [53].

### **Loss of the USP28-FBW7 complex**

Modification of MAPK pathway molecules by ubiquitination is a critical regulatory mechanism of MAPK signaling [8,9]. Recent studies have shown that loss of function genetic screening using an RNA inhibitor (RNAi) targeting 94 forecasted or known deubiquitinating enzymes (DUBs), USP28 was identified based on a feedback loop to make RAF family members in melanoma unstable [8,9]. While BRAF activation resulted in the down-regulation of USP28 expression, USP28 could interact with and de-ubiquitinated FBW7 to maintain the stability of FBW7, and then USP28 acted in conjunction with FBW7 to form a complex for targeting BRAF for degradation [8,9]. Therefore, the loss of the USP28-FBW7 complex could stabilize BRAF to potentiate activating downstream MAPK pathway, promoting the therapeutic resistance to BRAF-targeted therapy. More importantly, *USP28* was deleted in a subset of patients suffering from melanoma, which acted as a potential biomarker for the prediction of the efficacy of BRAF-targeted therapy. Collectively, the loss of USP28-FBW7 complex-mediated BRAF

degradation stimulates the intrinsic resistance to MAPK inhibition targeted therapy in melanoma [8,9].

## **Mechanisms of Adaptive Resistance**

Intrinsic resistance is characterized by the innate capacity to resist the toxicity of a specific agent. However, during the early phase of targeted inhibitors, especially during the first 24 to 48 hours, the adaptive response can occur, which is rapidly activated to enable cell survival and the establishment of acquired resistance through offering sufficient time to develop alternative mutations in melanoma cells. The occurrence of the adaptive response to *BRAF* inhibitors significantly hinders the treatment outcome of targeted therapy, resulting in a complete response rate ranging only 3–6% after the treatment of vemurafenib and dabrafenib [54,55]. Therefore, antagonization of the adaptive response can increase the effects of the drug to impair the occurrence of acquired resistance. Understanding the regulatory mechanisms underlying the establishment of adaptive resistance will contribute to finding novel potential treatment methods that can effectively suppress tumor progression and prolong the survival of patients.

### **Resetting the activity of the ERK1/2 pathway**

Resetting of the activity of the ERK1/2 pathway was first documented to contribute to the adaptive response to MAPK inhibition [50]. Through microarray analysis, BRAF or MEK inhibitors were shown to be capable of reducing the expression of SPRY2, SPRY4, and DUSPs 4 and 6 in *BRAF*-mutant melanoma cells [50]. Although the existence of *BRAF*V600E was associated with low expression of active GTP-loaded RAS, the reduction of SPRY2 expression after vemurafenib treatment was responsible for RAS activation [56]. The increase of RAS activity potentiated the activation of ERK1/2 signaling via BRAF/CRAF heterodimers. Therefore, the reduction of DUSP and SPRY proteins by vemurafenib resulted in RAS activation more efficiently to reset the activity of the ERK1/2 pathway [57].

### **Upregulation of receptor tyrosine kinases (RTKs)**

Recent studies have shown that the upregulated expression of RTKs (receptor tyrosine kinases) contribute to the adaptive resistance to MAPK suppression in *BRAF*-mutant melanoma [58]. Tyrosine phosphorylation of ERBB3 is an important upstream activator of the AKT pathway for resistance to the pro-apoptotic effect of MAPK inhibition. ERBB3 expression was induced rapidly following exposure to Vemurafenib and was mediated by FOXD3 [59]. Also, ERBB3 upregulation was correlated to increased sensitivity to its ligand NRG1, as shown by the potentiated phosphorylation of ERBB3 after the treatment with exogenous NRG1. In addition to ERBB3, platelet-derived growth

factor receptor (PDGFR) and EGFR were also significantly involved in adaptive resistance to BRAF suppression. Following treatment with BRAFi, the suppression of SOX10 could potentiate transforming growth factor (TGF) signal, resulting in the upregulation of PDGFR and EGFR. Treatment with TGF- $\beta$  in melanoma cells that expressed EGFR resulted in a slow-growing phenotype with characteristics of cell senescence and MAPK inhibition [60]. PDGFR $\beta$  was significantly upregulated in melanoma cells with resistance to BRAFi, leading to cell proliferation and tumor growth [60]. Therefore, targeting PDGFR and EGFR could be promising in overcoming the adaptive resistance to BRAF inhibition.

It has also been shown that BRAF inhibition led to a secretome with promotive influences on both nearby fibroblasts as well as melanoma cells. FGFs are senescence-associated factors secreted by melanoma cells [61]. After treatment with MAPK pathway inhibitors, FGF1 impairs the toxicity of drugs and activate fibroblasts to promote the secretion of HGF, which has been shown to induce cell proliferation [61]. Therefore, when both FGFR inhibitors and BRAF inhibitors were used simultaneously, the adaptive resistance of melanoma cells was reduced, indicating that the suppression of the FGF/FGFR pathway could be a potential approach to increase the efficacy and sensitivity of BRAF-targeted therapy [58].

### Upregulation of MITF

MITF is a melanocytic lineage-specific transcriptional factor that is essential for the development from the neural crest to melanocyte. The shortest isoform *MITF-M* is uniquely expressed in melanocytes and governs melanin synthesis, cell differentiation, and cell survival. As an oncogene, *MITF* is increased in 10–20% of patients with melanoma [62] and is asked for maintaining the survival and the proliferation of melanoma cells, to facilitate the progression of melanoma.

MITF is significantly upregulated by BRAF/MEK inhibitors through MAPK-dependent transcriptional regulation [31]. This regulation occurs in the initial period of BRAF/MEK inhibition and enabled melanoma cells to progress to a drug-tolerance phase [31]. Importantly, the HIV protease inhibitor, nelfinavir, is a potent inhibitor of MITF, and nelfinavir increases the efficacy of BRAF and suppresses MEK, indicating that this combination as a potential treatment strategy to augment the efficacy of MAPK suppression in *BRAF*-mutant melanoma cells [31]. These findings demonstrate that MITF upregulation is important for adaptive resistance to targeted therapy in melanoma.

### The paradoxical role of SOX10

As an important member of the SOX family of transcriptional factors, sex deciding area Y-box 10 (SOX10) promotes the

proliferation, melanogenesis, and the survival of melanocytes through the activation of its targets, DCT, MITF, TYRP1 and TYR. Also, SOX10 is involved in the initiation, invasion, and cell migration in melanoma [63–65]. The paradoxical role of SOX10 in the adaptive resistance to vemurafenib treatment in melanoma has recently been demonstrated [65].

Increased transcriptional activity of SOX10 impairs the sensitivity of *BRAF*-mutant melanomas to targeted therapy. *BRAF* mutation induced the hyperactivation of ERK, which phosphorylated SOX10 to suppress its transcription capacity toward various target genes via regulating the sumoylation of SOX10 at K55. Therefore, on the inhibition of BRAF, the transcriptional function of SOX10 was revived to potentiate the expression of its target FOXD3, which was essential for cell survival in melanoma. More importantly, the deficiency of SOX10 could sensitize *BRAF*-mutant melanoma cells to MAPK inhibition. Increased transcriptional activity of SOX10 confers adaptive resistance to BRAF-targeted treatment [66,67].

Also, a study has shown that the inhibition of SOX10 induced the activation of TGF- $\beta$ , resulting in the upregulation of EGFR and PDGFR $\beta$ , which drove acquired resistance to MAPK inhibition in melanoma [66]. Also, in a heterogeneous population of melanoma cells, those with low SOX10 expression and high EGFR expression could be increased quickly in response to the treatment with drugs long-term, suggesting that low SOX10 expression was correlated with the occurrence of acquired resistance [66]. Therefore, treatment interventions involving SOX10 should be based on the phase and status of drug resistance.

### Metabolic rewiring

Malignant cells are also characterized by changes in cell metabolism when compared with normal cells, which can include dependency on fatty acid and nucleotide synthesis, glutaminolysis, and aerobic glycolysis. However, following treatment with MAPK pathway inhibitors in *BRAF*-mutant melanoma, the metabolic phenotype was rewired by suppressing glycolysis and reactivating mitochondrial oxidative phosphorylation [68]. Also, the inhibition of the ERK1/2 pathway could significantly upregulate the expression of transcriptional coactivator peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1 $\alpha$ ), which was directly mediated by the upregulation of MITF expression [68]. Therefore, the application of oxidative phosphorylation inhibitors could suppress the adaptive resistance to MAPK inhibition. The findings from a study reported by Herlyn et al. identified a group of treatment-resistant cells with high expression of JARID1B, which were highly dependent on mitochondrial oxidative phosphorylation [69]. Transcription factor A, mitochondrial (TFAM) rather than PGC1 $\alpha$ , facilitated oxidative phosphorylation in this cell population after the establishment of treatment resistance, indicating that adopted

high oxidative phosphorylation could be independent of the MITF-PGC1 $\alpha$  axis [7,71].

## Mechanisms of Acquired Resistance

Most patients with melanoma who undergo long-term BRAFi treatment eventually develop acquired resistance due to the establishment of additional mutations. The most common mechanisms underlying acquired resistance is reactivation of the MAPK/ERK downstream pathway or at the BRAF level. Also, the activation of the AKT pathway may be involved in this process.

### NRAS mutations

*NRAS* mutations are common oncogenic alterations that occur in 20% of cases of melanoma and are significantly associated with an aggressive tumor phenotype and shorter patient survival [72–74]. *NRAS* mutations were shown by Nazarian et al. to promote the acquired resistance to BRAF inhibition [60]. A deficiency of *NRAS* reduced the growth of melanomas with resistance to BRAF inhibitors and overexpression of *NRAS*Q61K resulted in tumor cell resistance in sensitive parental cell lines, suggesting that the reactivation of the MAPK pathway stimulated by *NRAS*, contributes to the acquired resistance to BRAF inhibition [75]. Mutation of *NRAS* could activate the signaling pathway through CRAF when BRAF was blocked by its inhibitor, inducing the dimerization of BRAF and CRAF and subsequent trans-activation of MAPK [76,77]. Also, mutant *RAS* has been shown to promote *BRAF*V600E dimerization, which subsequently reactivated the ERK pathway and induced the resistance to BRAF inhibitors, as BRAFi only blocked monomeric *BRAF*V600E [78]. In preclinical studies, MEK inhibitors suppressed the increase of cells resistant to BRAFi that harbored the *NRAS* mutation, which indicated that the reactivation of the MAPK pathway was involved in *RAS* signaling-mediated resistance to BRAFi. Also, the mutations in *KRAS*, part of the *RAS* gene family, occurred in 7% of tumors resistant by BRAFi. These results demonstrate the involvement of *RAS* mutations in regulating the adaptive resistance to BRAF inhibitors in melanoma.

### The RAF paradox and dimerization of RAF proteins

Current RAF inhibitors can suppress the activity of RAF and downstream ERK signaling selectively in cells incorporating *BRAF* mutation. However, in cells expressing WT-BRAF, RAF inhibitors do not inhibit but paradoxically promote the activity of RAF and downstream MEK-ERK pathway, which is named RAF inhibitor paradox. To be specific, the inhibitors binding to the WT-BRAF monomer were shown to induce homodimerization and heterodimerization with a second BRAF protomer (BRAF/BRAF, BRAF/CRAF) in the presence of GTP-loaded *KRAS*

at the membrane. Recent studies have shown that the dimerization of BRAF had a lower affinity for the BRAF inhibitors. Since that Ras-GTP levels were relatively lower in melanoma cells harboring *BRAF* mutation, dimerization-mediated MAPK pathway activation was disfavored. Therefore, in cells with WT-BRAF, the binding of these inhibitors could paradoxically activate the WT-BRAF kinase through the induction of conformational changes and dimerization and further CRAF activation, resulting in MEK/ERK phosphorylation and eventually promoting cell proliferation [79].

Recent studies have focused on the development of BRAF inhibitors, termed ‘paradox breakers,’ which could prevent the activation of the MAPK pathway in cells with WT-BRAF. These paradox breakers were capable of inhibiting the MAPK pathway in melanoma harboring *BRAF* mutations and did not result in MAPK pathway activation in melanoma with WT-BRAF. For example, one paradox breaker, PLX7904, significantly suppressed ERK1/2 activation in *BRAF*-mutant melanoma cells and inhibited the dimerization of BRAF with paradoxical potentiation of the downstream MAPK pathway [80]. The further development of paradox breakers could improve the efficacy of BRAF targeted therapy and reduce the side effects on cells with WT-BRAF.

### BRAF gene amplification and splicing

The reactivation of the MAPK pathway at the level of *BRAF* could occur in several ways, including the increase of gene copy number, or by *BRAF* gene amplification and alternative *BRAF* gene splicing [81,82]. The increase of gene copy number of *BRAF* could result in overexpression and induce ERK signaling reactivation [82,83]. Whole exome sequencing (WES) of melanoma with acquired resistance to BRAF inhibitors showed the occurrence of *BRAF* gene amplification, which has been identified in around 20% of patients with melanoma after treatment with BRAF inhibitors. *BRAF* gene amplification resulted in significant upregulation of the expression of BRAF protein, contributing to the reactivation of ERK in response to BRAF inhibition. A small amplification of the *BRAF* gene and BRAF protein overexpression could confer sufficient acquired resistance to vemurafenib. In contrast, even high amplitude *BRAF* gene amplification could be saturable by the treatment with micromolar concentrations of vemurafenib, mediating similar degrees of vemurafenib resistance to that of modest amplification of *BRAF* gene [82].

Apart from the implications for resistance to BRAFi, the amplification of the *BRAF* gene also participated in the regulation of resistance to the MEK inhibitor in *BRAF*-mutant melanoma [81]. Fluorescence *in situ* hybridization (FISH) analysis of drug-naïve cell cultures has shown that a high copy number of *BRAF* was identified in a small number of cells [81].



However, on stimulation with the MEK inhibitor, AZD6244, the *BRAF*-amplified cell population was significantly expanded [81]. *BRAF* amplification in these cells promoted the phosphorylation of MEK and reduced the capacity of AZD6244 to suppress ERK phosphorylation [81]. The capacity of AZD6244 to suppress ERK phosphorylation in AZD6244-resistant cells was recovered by a BRAF inhibitor that could reduce the phosphorylation of MEK in parental cells. *BRAF* copy number amplification induced the reactivation of ERK, which could be suppressed by higher doses of BRAF inhibitors, or by combined treatment with BRAF and MEK inhibitors.

*BRAF* splicing occurs in up to 32% of cases of melanoma [83,84]. The p61*BRAF*V600E splice variant was identified in a subgroup of patients with acquired resistance to BRAFi [85]. Alternative splicing could lead to the expression of truncated BRAF proteins that lacked the N-terminal RAS-binding domain but maintain the kinase domain, which could form homodimers that were resistant to BRAF inhibitors [85]. The combined suppression of both BRAF and MEK may be useful to prevent this, while *BRAF* splicing could also occur in patients with combined treatment with BRAF and MEK inhibitors [86].

### MEK1/2 mutations

Mutations in *MEK1/2* have been detected in about 7% of cases of BRAFi-resistant melanoma, and the degree of resistance was significantly associated with the location and type of *MEK1/2* mutations [87]. The *MEK1* point mutation (C121S) was identified as conferring acquired resistance to BRAF inhibitors in a post-relapse biopsy from a patient with melanoma [87]. Also, *MEK1* mutations in E203K, Q56P, and K57E showed high intrinsic RAF-independent kinase activity and were associated with acquired resistance to BRAF inhibitors [88–90]. However, *MEK1* P124L/S/Q mutations that have been identified in 8% of untreated *BRAF*-mutant melanoma, had moderate RAF-independent kinase activity without reduced response to BRAF inhibition, but the response rates were lower than in patients with melanoma who had WT-*MEK1* [91]. Apart from *MEK1*, *MEK2* point mutations, including E207K and Q60P, were also involved in acquired resistance to MAPK inhibition [92–94]. These mutations could reactivate downstream ERK signaling without the need of BRAF stimulation.

### Hyperactivation of receptor tyrosine kinases (RTKs)

The overexpression or increased activation of RTKs could promote acquired resistance by activating parallel pathways or the direct activation of the RAS pathway [95]. The most commonly involved receptors are PDGFR $\beta$ , EGFR, and IGF-1R [96,97]. A study showed that several RTKs were over-expressed in resistant cells, like MET, KIT, PDGFR $\beta$ , and EGFR. Among the four RTKs, PDGFR $\beta$  as well as EGFR demonstrated promoted protein

expression in the resistant cell lines, and merely PDGFR $\beta$  exhibited promoted activation-correlated tyrosine phosphorylation in resistant cells. The role of upregulated PDGFR $\beta$  expression in acquired resistance was forwardly proved by that the introduction of PDGFR $\beta$  into treatment-naïve cells significantly decreased the efficacy of vemurafenib treatment in melanoma cells. Also, the knockdown of PDGFR $\beta$  was capable of reducing cell survival as well as the growth of the vemurafenib-resistant cell lines [60]. Also, a previous study reported that 6 out of 16 melanomas had acquired upregulation of EGFR after the establishment of acquired resistance to MAPK suppression. It was the down-regulation of SOX10 in melanoma that activated TGF- $\beta$  signaling to induce the upregulation of EGFR and PDGFR $\beta$ , which thereby conferred resistance to MAPK inhibition. The investigations highlight the involvement of EGFR and PDGFR $\beta$  in rendering acquired resistance to targeted therapy [66].

Apart from PDGFR $\beta$  and EGFR, phosphor-receptor tyrosine kinase arrays had identified that IGF1R was constantly activated in the resistant cells [98]. Mechanistic studies showed that IGF1R signaling significantly promoted the activation of PI3K/AKT signaling in the resistant cells, which was able to be inhibited through the combined use of a PI3K inhibitor and a MEK inhibitor or an IGF1R inhibitor and a MEK inhibitor [98]. Therefore, IGF1R could be another target to overcome the adaptive resistance via the suppression of hyperactivation of downstream PI3K/AKT signaling [99].

### Aberrations in the PI3K/AKT pathway

The suppression of ERK signaling can result in hyperactivation of the PI3K/AKT pathway that contributes to acquired resistance [100,101]. The cells with high PI3K/AKT pathway activity could have a survival advantage, as they would not be influenced by BRAF inhibitors. Melanoma cells with high PI3K/AKT pathway activity may be present in patients with melanoma who initially responded to BRAF inhibitors, but who subsequently developed acquired resistance. Specifically, the potentiation of the PI3K/AKT pathway may be induced by several mechanisms. IGF-1R expression results in persistent PI3K/AKT signaling activation that has been shown to suppress apoptosis and facilitate cell survival [101]. However, the mutations that are correlated with the PI3K/AKT activation pathway have been detected in up to 22% of patients with melanoma who have acquired resistance [102,103]. PI3K and AKT activating mutations enhanced AKT signaling, leading to the activation of anti-apoptotic signals and the upregulation of the expression of pro-proliferative genes [102,103]. These changes make it possible for cancer cells to proliferate independently of BRAF and are significantly involved in adaptive resistance of MAPK inhibition.

### Down-regulation of *STAG2* or *STAG3* expression

In 2016, Shen et al. identified the loss of function of *STAG2* by sequencing the entire exome from melanoma tissues from a patient treated vemurafenib and following relapse with disease progression [104]. Also, in patients with melanoma with acquired resistance to BRAF-targeted therapy, the expression of *STAG2* and *STAG3* were significantly down-regulated, which reduced the sensitivity of BRAF-mutant melanoma cells to MAPK inhibition both *in vitro* and *in vivo* [104]. Specifically, the knockdown of *STAG2* or *STAG3* could suppress CTCF-regulated expression of dual-specificity phosphatase 6 (DUSP6), leading to the reactivation of ERK. Therefore, down-regulation of *STAG2* or *STAG3* expression and the loss of function mutations in *STAG2* mediate the acquired resistance to BRAF-targeted therapy [104].

### Activation of the YAP/TAZ pathway

The Hippo pathway is regulated by the homologous proteins Yes-associated protein (YAP) and the transcriptional coactivator with PDZ-binding motif (TAZ). The Hippo pathway is involved in the regulation of organ size, cellular differentiation, and stem cell homeostasis. YAP and TAZ are two Hippo pathway transducers that also act as the coactivators of transcriptional factors that include TEADs, SMADs, and RUNX. The oncogenic role of the YAP/TAZ pathway has been well documented in several cancers not only for the malignant transformation and initiation of cancer but also for the growth, metastasis, and survival of cancer stem cells [105,106]. In melanoma cells that are resistant to BRAF-targeted therapy, the occurrence of actin cytoskeleton remodeling increased the nuclear translocation of both YAP and TAZ and potentiated its transcriptional activity to promote the expression of cell cycle molecules [105,106]. The knockdown of YAP or TAZ suppressed the viability of melanoma cells with acquired resistance to BRAF inhibitors [105,106]. Therefore, the activation of the YAP/TAZ pathway after actin remodeling rendered resistance to BRAF-targeted therapy [105]. The expression of YAP and TAZ were promoted in BRAF inhibitor-resistant melanoma stem cells, which was correlated with continuous ERK1/2 activity not inhibited by BRAF inhibitors [106]. The inhibition of either YAP or TAZ could restore the inhibitory effect of BRAFi on ERK1/2 signaling and melanoma progression. Therefore, YAP/TAZ activation may also be involved in regulating cancer stem cells that develop BRAFi resistance in melanoma [106].

### Down-regulation of the expression of dual-specificity MAPK phosphatases (DUSPs)

Dual-specificity MAPK phosphatases (DUSPs or MKPs) are the largest group of phosphatases involved in dephosphorylating ERK1/2 kinase. DUSPs can be transcriptionally induced

to remove phosphor groups from phosphorylated residues on ERK kinase, resulting in reduced activation of ERK, with DUSPs involved in the negative feedback loop of MAPK signaling [107]. The expression of some DUSPs, including DUSP4 and DUSP6, were directly regulated by oncogenic BRAF mutation. In comparison with the melanoma cells with wild-type BRAF, DUSP6 expression was higher in melanoma cells with BRAF mutation [108,109]. Therefore, DUSP6 could be used for the prediction of the efficacy of MAPK inhibition in melanoma [106,110]. DUSP6 was found to be down-regulated following treatment with BRAF-targeted agents, which indicates that the loss of negative feedback inhibition of ERK signaling is important for the development of resistance to MAPK inhibition therapy [109].

### Down-regulation of the expression of ring finger protein 125 (RNF125)

The RING domain protein TRAC-1 (T cell RING protein in activation), RNF125, was first recognized from a retroviral vector-based T cell surface activation marker screen [111]. Through an unbiased screen of a siRNA library in melanoma cells with acquired resistance and parental melanoma cells, RNF125 was found to be expressed at low levels in melanoma cells with resistance to BRAF inhibitors and conferred acquired resistance. Subsequent liquid chromatography-tandem mass spectrometry assay identified JAK1 as the novel substrate of RNF125 in BRAFi-resistant cells. The deficiency of RNF125 suppressed the ubiquitination and subsequent degradation of JAK1 and promoted the expression of EGFR that activated downstream ERK signaling. More importantly, the inhibition of JAK1 and EGFR signaling reduces the acquired resistance to BRAF inhibitors in melanoma with low RNF125 expression. Therefore, JAK1 and EGFR could be promising therapeutic targets in melanoma with acquired resistance to BRAF inhibitors and with low expression of RNF125 [112].

### Future Approaches to Overcome Resistance to BRAF-Targeted Therapy in Melanoma

Melanoma a primary malignancy of the skin that is associated with a poor prognosis in the advanced stage. Multiple genetic mutations, including BRAF mutations, drive the progression of melanoma. BRAF mutations occur in up to 60% of patients with melanoma, which triggers the persistent activation of the MAPK pathway to promote the growth of melanoma. The BRAF mutation potentiates the invasive and capacity of melanoma by metabolic reprogramming. These findings provide a molecular basis for targeted therapeutic strategies in melanoma that harbors the BRAF mutation.

Currently, several small-molecule inhibitors have been developed that target *BRAFV600E*, which have resulted in improved patient survival rates, but resistance to treatment hinders the long-term efficacy. Specifically, intrinsic resistance is the innate capacity of melanoma cells to resist the effects of BRAF-targeted drugs. During the early phase of targeted inhibitors, especially within the first 24 to 48 hours, the adaptive response occurs, which can be rapidly activated to enable cell survival and may be responsible for the subsequent establishment of acquired resistance by allowing sufficient time to develop additional mutations. Genetic alterations, including loss of *PTEN*, amplification of *CCND1*, loss of *NF1*, *RAC* mutations, and loss of the *USP28-FBW7* complex, have been identified in the development of intrinsic resistance. Also, the rewiring of several molecular pathways, including the ERK1/2 pathway, RTKs signaling, the MITF pathway, and SOX10 signaling, are significantly associated with adaptive resistance. Reactivating the MAPK pathway downstream and upstream or at the level of BRAF and the activation of the AKT pathway are responsible for adaptive resistance. Therefore, the molecular mechanisms underlying these three mechanisms for the development of resistance in distinct treatment periods are differentially characterized. Therefore, future strategies used to overcome BRAFi resistance should be based on the context of melanoma treatment for each patient. More importantly, the combined inhibition of the activated molecular pathways in resistant melanoma may be beneficial to increase the sensitivity and efficacy of targeted therapy. Continued molecular studies are required to refine current knowledge on the mechanisms underlying BRAFi resistance to improve the clinical outcome for patients with advanced-stage melanoma.

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## Conclusions

This review has described the impact of BRAF inhibitor (BRAFi) resistance on the pathogenesis of advanced-stage melanoma, the current status of molecular pathways involved in BRAFi resistance, including intrinsic resistance, adaptive resistance, and acquired resistance. Although the clinical effect of BRAF inhibitors, such as tyrosine kinase inhibitors (TKIs) of BRAF (V600E) kinase, vemurafenib and dabrafenib, is restricted by drug resistance. However, the use of BRAF inhibitors for melanoma has become a landmark in targeted therapy and personalized medicine. The importance of the long-term response to BRAF inhibitors in patients with *BRAF* mutations and the presence of long-term treatment responders highlight their success. Also, targeted therapy in advanced-stage melanoma using BRAF inhibitors reflect the core role of the MAPK pathway in melanoma. Although the mechanisms of the early emergence of drug resistance are challenging, new combination therapies, such as immunotherapy, other pathway inhibitors with intermittent BRAFi schedules, may result in novel treatment approaches for patients with melanoma. Remaining challenges include a lack of understanding of resistance mechanisms for novel treatments and their combinations and the current lack of predictive clinical trials for personalized therapy in melanoma. These challenges and the recognition of the importance of improving the prognosis for patients with advanced-stage melanoma motivate continued study on the mechanisms of BRAFi resistance with the aim of developing new treatment strategies.

## Conflict of interest

None.

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