European Journal of Pharmacology 862 (2019) 172621

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Drug resistance of BRAF-mutant melanoma: Review of up-to-date mechanisms of action and promising targeted agents

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ARTICLE INFO

Keywords:
Target therapy
BRAF
MEK
Immunotherapy
Melanoma
Mechanisms of resistance

ABSTRACT

Melanoma onset and progression are associated with a high variety of activating mutations in the MAPK-pathway, most frequently involving BRAF (35–45%) and NRAS (15–25%) genes, but also c-KIT and PTEN. Targeted therapies with BRAF and MEK inhibitors showed promising results over the past years, but it is known that most responses are temporary, and almost all of patients develop a tumor relapse within one year. Different drug-resistance mechanisms underlie the progression of disease and activation of both MAPK and PI3K/AKT/mTOR pathways. Therefore, in this article we reviewed the main studies about clinical effects of several target inhibitors, describing properly the most prominent mechanisms of both intrinsic and acquired resistance. Furthermore, suggestive strategies for overcoming drug resistance and the most recent alternative combination therapies to optimize the use of MAPK pathway inhibitors were also discussed.

1. Introduction

Melanoma is associated with a high variety of somatic mutations (Hodis et al., 2012), most frequently involving BRAF (35–45%) and NRAS (15–25% of melanoma patients) genes, but also c-KIT and PTEN (Devitt et al., 2011; Ekedahl et al., 2013; Jakob et al., 2012; Long et al., 2011; Siroy et al., 2015).

Several studies suggested that NRAS mutations are significantly related with poorer prognoses (Bucheit et al., 2013; Ekedahl et al., 2013; Kong et al., 2011). Commonly they involve exon 2 (82%) and less often exon 1 (18%) (Lyle and Long, 2013).

Also PTEN mutations (loss) are predictive of shorter overall survival (OS) and shorter time between primary diagnosis and brain metastases (Lito et al., 2013); furthermore, they can be associated with BRAF V600E mutations or wild-type BRAF/NRAS, but not with NRAS mutations, because they are mutually exclusive.

C-KIT mutations are common in mucosal and acral melanomas (20%), especially in Asian patients (10%, compared to 2–4% of Caucasians) (Jakob et al., 2012; Lito et al., 2013; Thomas et al., 2007).

BRAF is a serine-threonine kinase involved in the RAF-MEK-MAPK pathway, which is activated once by extracellular signals bound to their membrane receptor, usually a receptor tyrosine kinase. Firstly, this leads to the activation of RAS, that downstream activates BRAF. Then, BRAF can phosphorylate and activate MEK1/2 kinases, which in turn phosphorylates and activates ERK1/2 (known as MAPK-activated protein kinase), leading to cellular proliferation, survival and differentiation (Murphy et al., 2010).

Point mutations of BRAF account for 33–47% of primary and 41–55% of metastatic melanomas (Jakob et al., 2012; Lito et al., 2012; Siroy et al., 2015). The most common mutations are V600E (80%) and V600K (14%), which lead respectively to the substitution of a valine (V) with glutamate (E) or with lysine (K) at codon 600, resulting in constitutive kinase activity and unregulated cell growth. Other V600 mutations (e.g.V600D or V600R) are rare (6%). BRAF mutations appear to have some prognostic value. While there is no difference in the interval from first-ever melanoma to distant metastasis between BRAF-mutant and wild-type patients, those with V600K mutations have a shorter disease-free interval compared with V600E melanoma patients (17.4 vs 39.2 months, $P = 0.048$). However, specific BRAF isoform mutation does not appear to be predictive of different therapeutic responses, though there are limited data regarding the rarer isotypes in large clinical trials (Wagle et al., 2011). Despite the promising results obtained with the combination of BRAF and MEK inhibitors, all of the patients affected from metastatic melanoma will develop a tumor

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https://doi.org/10.1016/j.ejphar.2019.172621
Received 6 March 2019; Received in revised form 9 August 2019; Accepted 19 August 2019
Available online 22 August 2019

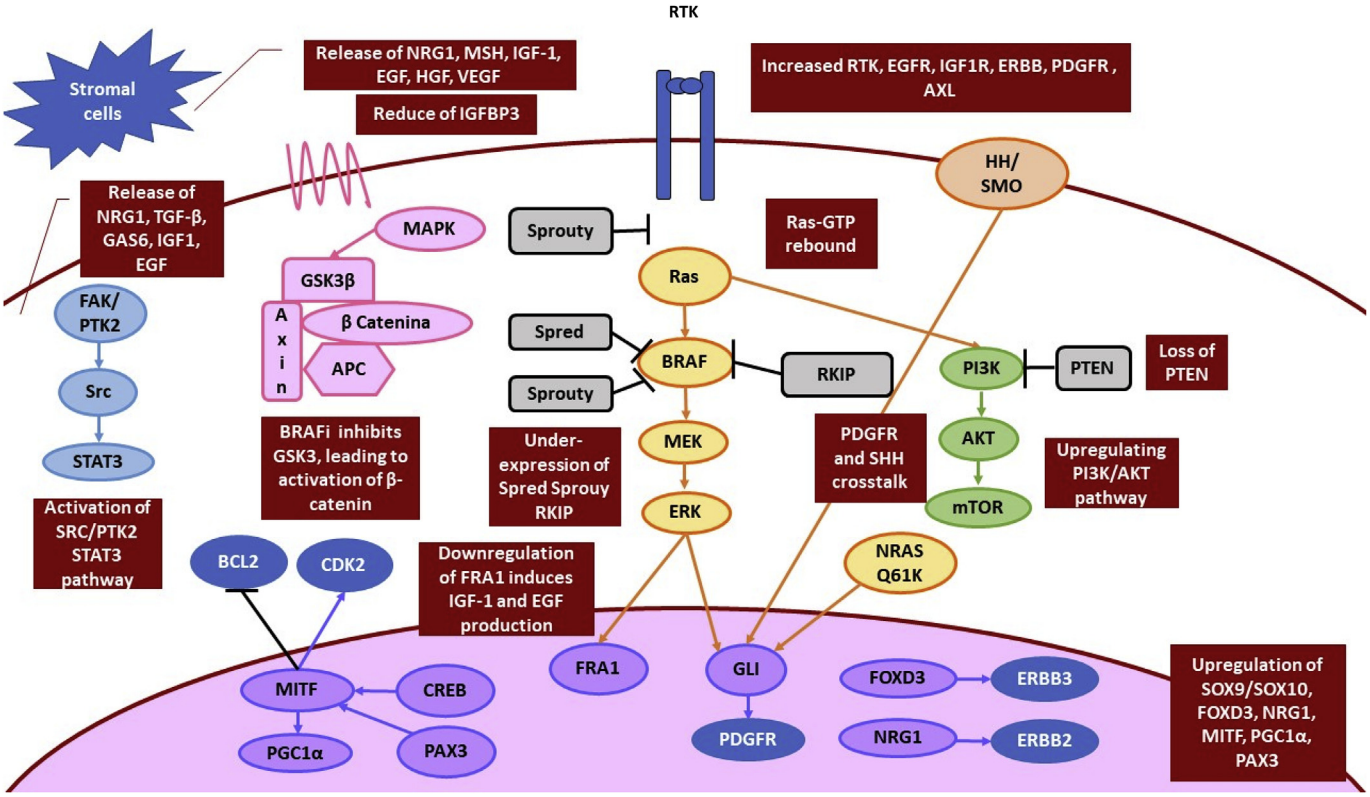

Fig. 1. Epigenetic, transcriptomic and paracrine causes of BRAF inhibitor resistance in melanoma (coloured)
Abbreviations: SPRED, sprouty-related EVH1 domain-containing; EGFR, epidermal growth factor receptor; PI3K/AKT/mTOR, phosphatidylinositol-4,5-biphosphate 3-kinase/v-Akt murine thymoma viral oncogene homolog/mammalian target of rapamicin; SOX10, SRY-Box 10; FOXD3, forkhead fox D3; RTK ERBB-3 or HER3, human epidermal growth factor receptor 3; NRG1, Neuregulin 1; IGFR1, insulin-like growth factor receptor 1; PTK2, protein tyrosine kinase 2; FAK, focal adhesion kinase; STAT3, signal transducer and activator of transcription-3; PDGFR, platelet-derived growth factor feceptors; HH, hedgehog; SMO, smoothened; SHH, sonic sedgehog; GLI1, GLI family zinc finger 1; MITF, microphtalmia-associated transcription factor; MSH, melanocyte-stimulating hormone; PGC1α, PPARG coactivator 1 alpha; PAX3, paired box 3; GAS6, growth arrest-specific 6; FRA1, fos-related antigen 1; IGFBP3; IGF-binding protein; TGF-β; transforming growth factor-β; HGF, hepatocyte-growth factor; VEGF, vascular-endothelial growth factor; RTK: receptor tyrosine kinase.

relapse within several months (Wagle et al., 2011). Thus, understanding the events behind intrinsic and acquired resistances, as well as the potential of future drug combination strategies in BRAF-mutant melanoma could be really important to improve patients' outcome.

This review summarizes the complex scenario that characterizes this dynamic process, and the relationship between melanoma cells and tumor microenvironment. It also analyzes possible future drug combinations to find out how we can strike specific targets that are critically involved in the development of the disease. In order to better understand the biology underlying the principle mechanisms involved in BRAF inhibitor resistance we divided them into epigenetic, transcriptomic and paracrine causes (Fig. 1) and genetic and microRNA-based causes (Fig. 2).

2. Epigenetic or transcriptomic causes of resistance to BRAF inhibitors

Epigenetic and transcriptomic changes account for 39–42% of cases in BRAF-mutant melanoma treated with BRAF inhibitors (Kakadia et al., 2018) (Table 1).

The RAS/RAF/MEK/ERK pathway is activated when receptor tyrosine kinases (RTKs) bind to their extracellular ligands, and this activation is strictly controlled by feedback mechanisms under physiological conditions. There are several negative feedback proteins, like sprouty (SPRY) and sprouty-related EVH1 domain-containing (SPRED), which are induced by ERK1 and ERK2 MAP kinases which act mostly as repressors of RAS and RAF MAP kinases (Wagle et al., 2011). When the MAPK pathway is permanently activated, like in BRAF V600E-mutated cells, the negative feedback by SPRY and SPRED is also upregulated to repress the PTKs signaling (Amaral et al., 2017). When BRAF and/or MEK are inhibited by targeted drugs, this negative ERK-dependent feedback is intuitively decreased. Moreover, in BRAF V600E mutated cells, the activation of MAPK pathway is RAS-independent, and the protein signals as a BRAF-sensitive monomer. BRAF target therapy strongly inhibits RAF monomers and ERK signaling, causing the relief of negative feedback, the reactivation of ligand-dependent signal transduction, the increase of Ras-GTP bound, and generation of BRAF-resistant RAF dimers (Amaral et al., 2017; Rizos et al., 2014).

Easty et al. (2011) have shown that melanoma cells can express several members of the epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) families. Then, Haydn et al. (2014) revealed that SPRED1 and SPRED2 proteins can negatively regulate the EGFR-dependent activation of RAS gene in BRAF V600E-mutated cells; as explained before, inhibition of BRAF leads to an enhanced RAS activity, which in turn can downstream activate phosphatidylinositol-4,5-bisphosphate 3-kinase/v-Akt murine thymoma viral oncogene homolog/mammalian target of rapamicin (PI3K/AKT/mTOR) pathway signaling, that is strictly related to cell proliferation and protection from apoptosis, with negative consequences. Furthermore, many authors demonstrated that the loss of PTEN, a suppressor of PI3K activity, brings to lower apoptotic response rates to BRAF inhibitors and the inhibition of PI3K/AKT/mTOR pathway reduces tumor growth and improves sensitization to chemotherapeutics, such as cisplatin and temozolomide (Paraiso et al., 2011; Sinnberg et al., 2009; Werzowa et al., 2011). Moreover, it has been observed that patients with melanoma carrying PTEN mutation/loss treated with dabrafenib,

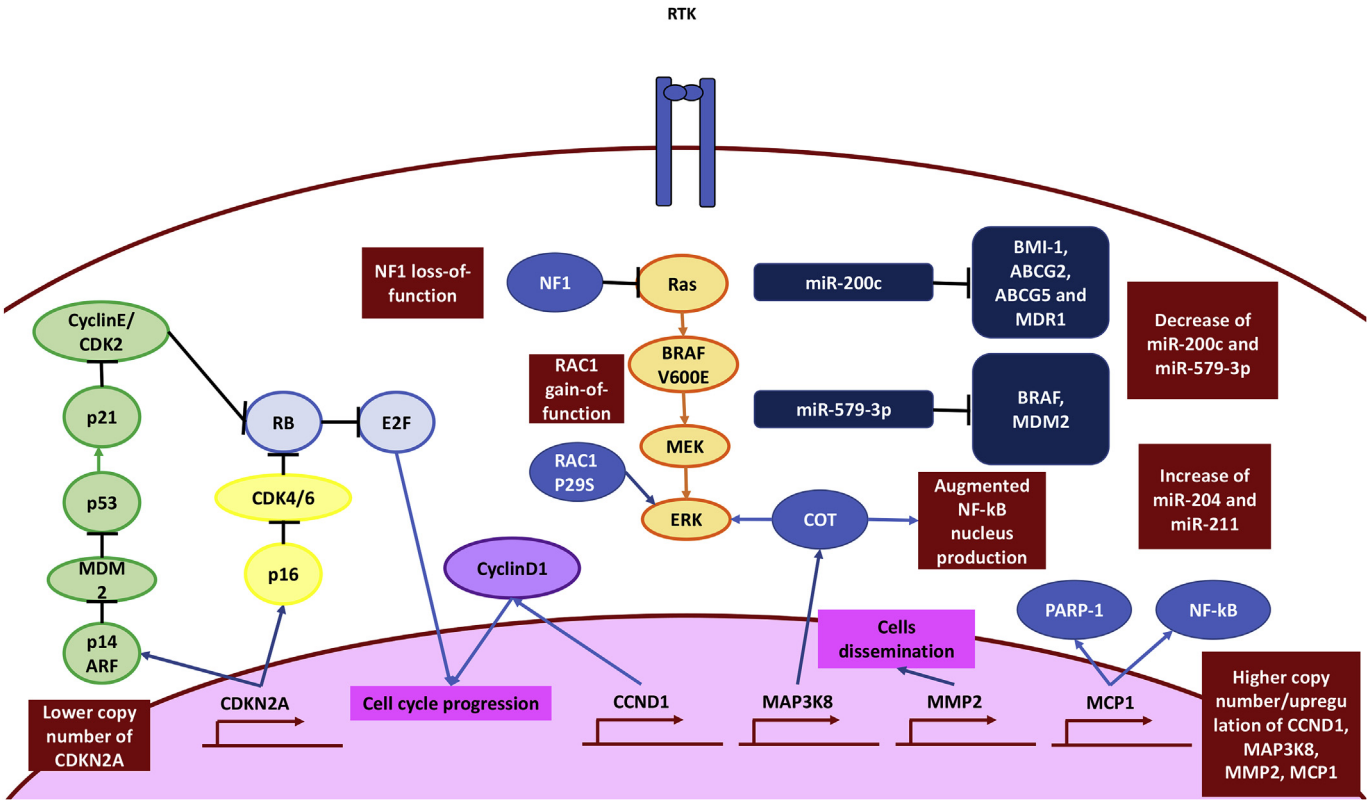

Fig. 2. MicroRNA and Genetic causes of BRAF inhibitor resistance in melanoma (coloured)
Abbreviations: RAC1, ras-related C3 botulinum toxin substrate 1; NF-1, neurofibromatosis-related protein 1; CDKN2A, cyclin-dependent kinase Inhibitor 2A; Rb, retinoblastoma; MMP2, matrix metalloprotease 2; MCP1, monocyte chemoattractant protein 1; PARP-1, poly(ADP-ribose) polymerase-1; miR, microRNAs; RTK: receptor tyrosine kinase.

have shorter median progression-free survival (PFS) (Nathanson et al., 2013) as well as shorter median OS and an increased risk of brain metastasis (Bucheit et al., 2014).

There is another mechanism that can lead to EGFR activation, related to the transcription factor SRY-box 10 (SOX10). Briefly, SOX10 acts as a negative mediator on EGFR expression. Indeed, as demonstrated by Sun et al. (2014), those melanoma cells harboring low-SOX10 levels (and consequently high EGFR levels) presented an increased resistance when exposed to BRAF inhibitor. Interestingly, many studies revealed that microenvironmental factors and epigenetic programming were responsible of SOX9 or SOX10 expression, which is mutually exclusive; SOX10 seems to be related with a “proliferative” signature, while SOX9 seems to be associated with an “invasive” signature. This is called “phenotype switching” (Cheng et al., 2015; Hoek et al., 2008; Levesque et al., 2017; Shakhova et al., 2015; Verfaillie et al., 2015; Widmer et al., 2012).

The relationship between an augmented EGFR expression and RAF inhibition was also reported by several studies on BRAF V600E-positive colorectal cancers (5–8% of all colorectal cancers) treated with BRAF inhibitors, that likewise lead to a decreased efficacy or therapy failure (Corcoran et al., 2012; Davies et al., 2002; Prahallad et al., 2012). However, it seems that only up to one third of melanomas that express EGFR present the MAPK-EGFR crosstalk (Haydn et al., 2014; Sun et al., 2014).

A phosphorylation-dependent regulatory mechanism of SOX10 transcription activity is recently proposed as another possible mechanism of adaptive resistance to the BRAF inhibitor (Han et al., 2018). Indeed, in BRAF-mutant melanoma cells, forkhead box D3 (FOXD3) is induced by the inhibition of ERK1/2 signaling, whose phosphorylation regulates SOX10 sumoylation, which in turn upregulates transcription of another member of the EGFR family, namely RTK ERBB-3, also known as human epidermal growth factor receptor 3 (HER3). ERBB-3 lacks several important residues, including the catalytic base aspartate, so it has to dimerize with other members of the ERBB family to be fully active. ERBB-2 is the preferred dimerization partner of all the ERBB family members and ERBB-3 preferentially dimerizes with ERBB-2 (Tzahar et al., 1996). Many authors observed that ERBB-3 is phosphorylated when BRAF V600E-mutated cells are treated with BRAF or MEK inhibitors. In detail, a specific phosphorylation at position Y1289 of ERBB-3 gene provides a docking site for the PI3K, leading to the activation of PI3K/AKT/mTOR pathway (Fattore et al., 2013; Kim et al., 1994). The study of Abel et al. (2013) showed a reduced tumor burden and an extended latency of tumor regrowth in cell lines treated properly with a dual tyrosine kinase inhibitor which interrupts both ERBB-2 and EGFR signaling (lapatinib) plus a BRAF inhibitor (PLX4720) compared to the BRAF inhibitor alone.

Fattore et al. (2013) noted that enhanced ERBB-3 expression can bring to an upregulation of neuregulin 1 (NRG1), which binds preferentially ERBB-2 to increase its phosphorylation on tyrosine residues. Furthermore, Capparelli et al. (2015) provided evidence that NRG1 is highly expressed by dermal fibroblasts and cancer-associated fibroblasts isolated from mutant BRAF melanomas, stimulating the cell growth and promoting resistance to BRAF inhibitor therapy.

Moreover, lungs are rich in NRG1, and this could explain the increased risk of melanoma metastases in pulmonary tissues (Tiwary et al., 2014; Ueno et al., 2008).

All these studies suggest that targeting the ERBB-3/ERBB-2 pathway will likely improve the efficacy of BRAF inhibitors and might help overcoming resistance.

However, not only RTKs from the ERBB family can mediate resistance after BRAF or MEK inhibition. Also insulin-like growth factor receptor 1 (IGFR1) seems to be involved, mainly through activation of

Table 1
Epigenetic or transcriptomic causes of BRAF inhibitor resistance in melanoma.

Study	Mechanisms of resistance	Comment
Murphy et al. (2010) Wagle et al. (2011) Amaral et al. (2017)	Alterations in expression of RKIP1, MKP, SPRY and SPRED families.	Negative ERK-dependent feedback
Lito et al. (2012) Rizos et al. (2014); Amaral et al. (2017)	BRAF target therapy increases Ras-GTP bound and resistant RAF dimer	
Haydn et al. (2014)	Increase RAS and P-AKT levels due to MEK inhibitors	MEK inhibition could protect tumor cells from apoptosis
Paraiso et al. (2011) Werzowa et al. (2011) Sinnberg et al. (2009)	Loss of PTEN	The loss of PTEN leads to the activation of PI3K/AKT/mTOR pathway
Sun et al. (2014)	Underexpression of SOX10	This leads to an increase of EGFR levels
Widmer et al. (2012) Hoek et al. (2008) Verfaillie et al. (2015) Shakhova et al. (2015) Cheng et al. (2015) Levesque et al. (2017)	Overexpression of SOX9 or SOX10	SOX9 promotes an “invasive” signature; SOX10 is related to “proliferative” signature
Corcoran et al. (2012) Prahallad et al. (2012)	Overexpression of EGFR	
Han et al. (2018)	Phosphorylation of SOX10	ERK-mediated phosphorylation regulates SOX10 sumoylation, and further ERBB-3 expression
Tzahar et al. (1996) Fattore et al. (2013) Kim et al. (1994)	Augmented FOXD3 protein levels	FOXD3 upregulates ERBB-3 and activates PI3K/AKT/mTOR pathway
Fattore et al. (2013)	Upregulating of NRG1	NRG1 leads to increase of ERBB-2 phosphorylation
Capparelli et al. (2015) Ueno et al. (2008) Tiwary et al. (2014)	NRG1 is overexpressed by dermal and cancer-associated fibroblasts	NRG1 acts in a paracrine manner
Karasic et al. (2010) Villanueva et al. (2010)	Overexpression of IGF1R	
Vultur et al. (2014)	Increased activation of SRC/PTK2 and of STAT3 signaling	Determinates a 20% more of invasive potential
Nazarian et al. (2010) Stecca et al. (2007) Sabbatino et al. (2014)	Crosstalk between PDGFR A and B and SHH pathway	BRAF inhibition induces Gli1 transcription factor which promotes PDFR expression Mutated NRAS (Q61K) can promote the expression of Gli1
Wellbrock and Marais, 2005 McGill et al. (2002) Du et al. (2004) Rodríguez and Setaluri, 2014 Huber et al. (2003) Johannessen et al. (2013)	Increase of MITF levels	MITF keep melanoma cells alive possibly by regulating of BCL2 and CDK2 Also MSH leads to an increase of MITF
Gopal et al. (2014) Haq et al. (2013)	Overexpression of PGC1α	This leads to an increase oxidative phosphorylation and mitochondrial activity
Smith et al. (2016)	Overexpression of PAX3 due to MEKi therapy	
Sensi et al. (2011) Konieczkowski et al. (2014)	Overexpression of AXL	This receptor could lead to a positive feedback by promoting GAS6
Alver et al. (2016)	Upregulation of FOXD3, NRG2 and ERBB3 (due to downregulation of MITF)	
Meierjohann, 2017	Connection between MAPK and WNT/β-catenin pathways	

MAPK and PI3K/AKT/mTOR pathway. Karasic et al. (2010) demonstrated that cyclolignan picropodophyllin, a specific inhibitor of IGFR1 kinase activity, down-regulates the basal levels of AKT and ERK1/ERK2 activity in several melanoma cell lines, including those harboring a BRAF V600E mutation, with increased apoptotic rates. Interestingly, Villanueva et al. (2010) have likewise found that while parental cells are highly sensitive to BRAF inhibition by SB-590885 (a selective BRAF inhibitor, IC50: 0.01–0.1 μM), melanoma cells which had been chronically treated with SB-590885 needed higher doses of the drug for partial growth inhibition (IC50: 5–10 μM), and that only treatment with cyclolignan picropodophyllin led to decreased viability of melanomas resistance to BRAF inhibitors.

In another study by Vultur et al. (2014) MEK inhibition was associated with an enhanced receptor tyrosine kinase activity and increased activation of the SRC/FAK/STAT3 signaling axis, with a 20% more of invasive potential. Authors also noted that combining BRAF and MEK inhibitors with SRC, FAK or STAT3 inhibitors prevented this invasive phenotype and led to higher apoptotic rates, suggesting a new feasible treatment option for melanoma. The combination of MEK and SRC inhibitor (Selumetinib + Saracatinib), for example, abolished the MEK inhibitor-induced invasion and efficiently reduced melanoma cells growth (Ferguson et al., 2013).

Also platelet-derived growth factor receptors (PDGFR) A and B appear to be involved in BRAF-resistance, with a crosstalk mechanism between these receptors and the sonic hedgehog (SHH) pathway (Nazarian et al., 2010; Sabbatino et al., 2014; Stecca et al., 2007). Interestingly, BRAF inhibition induces Gli1 transcription factor, that is a downstream effector of the SHH pathway; at the same time, BRAF V600E seems to downregulate the expression of Gli1 when not exposed to BRAF inhibition. Gli1 affects and promotes PDGFR A expression, and this results in an enhanced signaling toward the RTK, with shorter time to disease progression and less tumor regression. Nazarian et al. (2010) and Stecca et al. (2007) revealed that mutated NRAS (Q61K) can promote the expression of Gli1, while Sabbatino et al. (2014) demonstrated that inhibition of the SHH pathway with LDE-255 (a smoothened antagonist) or inhibition of PDGFR A with Sunitinib restores and increases melanoma cells' sensitivity to BRAF inhibitors, providing evidence of another possible combination therapy for melanomas. Furthermore an innovative inhibitor of smoothened NVP-LDE225 acts to reduce proliferation in both BRAF V600E and BRAF(wild type) melanoma cells,

Table 2
Genetic causes of BRAF inhibitor resistance in melanoma.

Study	Mechanisms of resistance	Comments
Atefi M et al., 2011	Mutations in NRAS and MAP2K	MAPK-dependent resistance
Krauthammer et al. (2012)	RAC1 gain-of-function (P29S)	It was found in 9.2% of sun-exposed melanoma
Whittaker et al. (2013) Gibney and Smalley, 2013 Nissan et al. (2014) Shalem et al. (2014) Maertens et al. (2013)	NF-1 loss-of-function	It determines continuous RAS activation
Nathanson et al. (2013)	Lower copy number of CDKN2A Higher copy number of CCND1	
Amaral et al. (2017)	MAP3K8 expression Nonsense mutation in HOXD8	

representing a potential therapeutic option in melanoma even in the absence of BRAF V600E mutation (Jalili et al., 2013).

In the last few years many efforts are made to clarify the role of microphtalmia-associated transcription factor (MITF), a lineage-specific marker of melanocytes, in the complex biological scenario of this tumor. In the early 2000s, Wellbrock and Marais (2005) noted that MITF expression was decreased in melanoma cells harboring BRAF V600E mutation, and that high levels of MITF inhibited proliferation in mutant clones, so their hypothesis was that this transcription factor's activity might be regulated by MAPK pathway activation and strictly related to melanocytes transformation. However, authors also found that MITF expression in BRAF V600E melanomas was diminished, but not abolished, suggesting that its function was to keep melanoma cells alive, possibly by regulating BCL2 and CDK2 gene expression (Du et al., 2004; McGill et al., 2002). It was supposed that MITF levels were adequate for cell growth but too low to inhibit tumor progression.

Based on these findings, the use of a BRAF inhibitor should lead to increase the MITF levels and activity.

Recently, many authors revealed that MITF regulation is influenced by various other proteins and mechanisms, and also that there might not be such a linear correlation between MITF levels and BRAF/MEK inhibition. For example, when melanocyte-stimulating hormone (MSH) binds to its receptor, cAMP response element binding protein, namely CREB, is activated. CREB promotes MITF transcription, leading to an increase of its levels; curiously, also SOX10 can increase MITF production (Huber et al., 2003; Johannessen et al., 2013; Rodríguez and Setaluri, 2014). Johannessen et al. (2013) revealed that treatment with a BRAF inhibitor for 10–14 days induced CREB reduction in melanoma cells, and this likely caused a decrease in MITF levels. Authors also found that higher expression of MITF was related to BRAF inhibitor resistance, making this scenario even more complex.

Moreover, Gopal et al. (2014) demonstrated an increase in MITF levels when BRAF or NRAS-mutated melanoma cells were treated with a MEK inhibitor, called selumetinib. MITF in turn promoted the transcription of PPARG coactivator 1 alpha (PGC1α), which led to an increased oxidative phosphorylation and mitochondrial activity (Haq et al., 2013). This resulted in a BRAF-therapy resistance. Authors also noted that AZD8055, an mTORC1/2 inhibitor, was able to resensitize cells that displayed increased oxidative phosphorylation but not those with low mitochondrial activity.

Also paired box 3 (PAX3), a protein which binds to DNA sequences to control gene transcription that is highly active in neural crest cells, is induced after target therapy with MEK inhibitor; this is important because PAX3 can upregulate MITF expression and consequently lead to acquired resistance. Smith et al. (2016) demonstrated that Nelfinavir, an HIV protease inhibitor, was able to suppress PAX3 expression and resensitize BRAF or NRAS-mutated melanoma cells to MAPK pathway inhibitor.

The correlation between MITF and the receptor tyrosine-kinase AXL is also very interesting. Briefly, AXL protein is involved in cell proliferation, motility and invasion (Sensi et al., 2011). Konieczkowski et al. (2014) noted that inhibitor-resistant BRAF V600E cell lines displayed low MITF expression and high AXL expression. At the same time, Sensi et al. (2011) revealed that melanomas, presenting higher AXL levels, exhibited both low MITF and melanocyte differentiation antigen (like MART-1 and gp100) expression, with greater invasive potential when compared to AXL-low cell lines. Authors also noted that the receptor could lead to a positive feedback by promoting its ligand growth arrest-specific 6 (GAS6) protein, with an autocrine loop. Therefore, several AXL inhibitors are under investigation (Wu et al., 2014).

However, MITF probably acts in a context-dependent manner, and evaluation of its specific role needs further investigation.

Alver et al. (2016) treated melanoma cells with Vemurafenib for two weeks, and this led to a downregulation of MITF. As a consequence, FOXD3, NRG1 and ERBB3 were upregulated with a feedback mechanism, because they all contain MITF-binding sites; as explained before FOXD3 increased ERBB3 expression, which in turn stimulated NRG1 production, confirming the close connection between MITF and ERBB3 pathways (Capparelli et al., 2015).

There are also pieces of evidence for connections between the MAPK and the WNT/β-catenin pathways, which are described in detail by Meierjohann, 2017.

Thus, target therapy with MAPK inhibitor could lead to resistance in many different ways, and further studies are needed to confirm which compound is the best when associated with BRAF or MEK inhibitors therapy to help overcoming acquired resistances.

3. Genetic causes of resistance to BRAF inhibitors in melanoma

Many specific gene signatures are involved in melanoma intrinsic resistance to BRAF inhibitor (Table 2).

Among the acquired resistance mechanisms, mutations in NRAS and MAP2K genes determine the dependence on the MAPK pathway (Atefi et al., 2011). Mutations in RAS and BRAFV600 are mutually exclusive and represent 25% and 22% of genetic alterations leading to resistance, respectively (Goel et al., 2006; Hugo et al., 2015; Moriceau et al., 2015; Shi et al., 2014). Moreover, due to redundant feedback mechanisms and toxicities, complete inhibition of NRAS is difficult to obtain pharmacologically. For these reasons there is still no effective therapy in mutated NRAS melanoma (Kwong et al., 2012).

Ras-related C3 botulinum toxin substrate 1 (RAC1), which belongs to Rho subfamily, is a GTPase that plays an important role in different cellular processes, like signal transduction, differentiation, protein biosynthesis, migration, adhesion and cytoskeletal rearrangement (Gastonguay et al., 2012; Michaelson et al., 2008). A specific RAC1 gain-of-function mutation (P29S) is related to enhanced proliferation and migration and was found in 9.2% of sun-exposed melanomas (Krauthammer et al., 2012). Thus, combination of MAPK and RAC1 downstream effector inhibitors could be considered a feasible treatment option in those melanoma patients harboring both mutations.

Neurofibromatosis-related protein 1 (NF-1) inhibits RAS activity under physiological conditions, so its role in resistance to BRAF

targeted therapy was widely studied (Gibney and Smalley, 2013; Maertens et al., 2013; Nissan et al., 2014; Shalem et al., 2014; Whittaker et al., 2013). It was demonstrated that NF-1 loss-of-function mutations can lead to continuous RAS activation, which can downstream activate both MAPK and PI3K/AKT/mTOR signaling pathways and confer resistance to target therapy. This is intuitively related to worse outcomes.

Cyclin-dependent kinase inhibitor 2A (CDKN2A) is a gene coding for p16 and p14ARF proteins, both related to tumor suppression; the first one inhibits CDK4 and CDK6 and promotes retinoblastoma protein expression, while the second one activates the protein p53. Nathanson et al. (2013) demonstrated that a lower copy number of CDKN2A was significantly associated with decreased PFS ($P = 0.012$) in patients treated with dabrafenib. Furthermore, authors noted that a higher copy number of CCND1, a gene coding for cyclin-D1 protein (important for cell cycle G1/S transition), was also significantly related with poorer prognosis ($P = 0.009$).

Recently other two genes, namely MAP3K8 (encodes for a protein kinase that can activate ERK1/2 and stimulate nuclear production of NF-kB) and HOXD8 (an homeobox gene), were related to BRAF inhibitor resistance, but further studies are needed to clarify their role (Amaral et al., 2017).

4. Microenvironment and BRAFi resistance

Treatment with BRAF and/or MEK inhibitors also seems to be associated with autocrine and paracrine effects as well as with microenvironment modifications (Table 3).

Many authors noted that target therapy led not only to BRAF inhibitor-sensitive cells apoptosis, but also to a stress-induced senescence phenotype in the remaining cell population, confirmed by heterochromatin formation, changes in cell shape and augmented β-galactosidase activity (Haferkamp et al., 2013; Li et al., 2016). “Senescent phenotype”- melanoma cells also upregulated two genes, namely matrix metalloprotease 2 and monocyte chemoattractant protein 1; the first one promotes cell invasion and dissemination, while the second one is associated with activation of the poly(ADP-ribose) polymerase-1 (PARP-1)/NF-kB signaling cascade, which can confer protumoral and prometastatic properties to melanoma cell (Ferguson et al., 2013; Hanna et al., 2013; Ohanna et al., 2011; Shaverdashvili et al., 2014).

It was demonstrated that BRAF inhibitor-sensitive cells could react with MAPK pathway inhibition in many different ways (Table 3). One of the more interesting is the acquisition of a “therapy-induced secretome” phenotype, which can lead to the outgrowth of drug-resistant cells. When exposed to vemurafenib, sensitive cells downregulated the protein fos-related antigen 1 (FRA1, encoded by the FOSL1 gene), a member of the activator protein 1 transcription factor complex and effector of the MAPK pathway. Briefly, FRA1 downregulation induced IGF-1 and EGF production by BRAFi-sensitive cell, and these stimulated BRAFi-resistant cell in a paracrine manner, with activation of PI3K/AKT/mTOR and NF-Kb pathways. Notably, treatment with BRAF inhibitor did not affect FOSL1 expression in drug-resistant cells (Obenauf et al., 2015). Moreover, levels of insulin-like growth factor binding protein 3 (IGFBP3), which physiologically binds to IGF1 to prevent its interaction with IGF receptor, were strongly reduced in the tumor microenvironment. In an interesting work by Fedorenko et al. (2015), authors noted that treatment with vemurafenib was associated with an enhanced transforming growth factor-β release from BRAF inhibitor-sensitive melanoma cells. This induced fibroblast differentiation, confirmed by fibronectin deposition, increased α-smooth muscle actin expression and NRG1 production. As explained before, NRG1 binds to ERBB-2, while fibronectin enhances RTK activity towards its interaction with β1 and β3 integrins on cell surface, creating a local clustering of signaling platforms. A study (Fedorenko et al., 2016) demonstrated that fibronectin inhibition with siRNA limited EGFR and ERBB3 receptor phosphorylation after ligand engagement, with decreased PI3K/AKT/mTOR pathway signaling. Fibroblast also produces hepatocyte-growth factor (HGF), which can bind to its receptor MET. HGF production by the tumor niche has been widely associated with BRAF inhibitor therapy resistance (Straussman et al., 2012; Wilson et al., 2012). Hirata et al. (2015) noted that treatment with vemurafenib was followed by paradoxical activation of MAPK pathway even in the cancer-associated fibroblast population, with enhanced production of all the already mentioned mediators.

Also cancer-associated macrophages (CAMs) seem to have tumor-promoting features. Historically, CAMs were considered the most represented cell population in melanoma microenvironment (Bröcker et al., 1988), and many studies highlighted the correlation between levels of macrophage-produced factors and poorer prognosis both in early and late stages of the tumor (Gazzaniga et al., 2007; Jensen et al., 2009; Torisu et al., 2000). Wang et al. (2015) revealed that treatment with vemurafenib led to paradoxical MAPK pathway activation in these cells, with production of vascular-endothelial growth factor (VEGF); this in turn can stimulate its receptors, namely FMS-related tyrosine kinase 1 and kinase insert domain receptor, on melanoma cells surfaces, with reactivation of MAPK pathway and decreased BRAF inhibitor efficacy. Thus, macrophages protect melanoma from vemurafenib-induced apoptosis and necrosis. Further studies confirmed that combining BRAF inhibitor with macrophage colony-stimulating factor receptor (MCSFR) inhibitors resensitize melanoma cells to vemurafenib (Mok et al., 2015; Ngiow et al., 2016), suggesting a new promising therapy for melanomas. Notably, melanoma cell kept in monoculture were much more sensitive to BRAF inhibition when compared to those co-cultured with fibroblasts or macrophages, strengthening the results of these studies and confirming the primary role of tumor microenvironment.

Table 3
Microenvironment and BRAF inhibitor resistance.

Study	Mechanisms of resistance	Comments
Haferkamp et al. (2013) Li et al. (2016)	Senescence phenotype	Heterochromatin formation, augmented β-galactosidase activity
Ohanna et al. (2011) Hanna et al. (2013) Shaverdashvili et al., 2014	MMP2 and MCP1 upregulation	Correlated with PARP-1/NF-kB activation
Obenauf et al. (2015)	Downregulation of FRA1 (FOSL1 gene) induces IGF-1 and EGF production of BRAF inhibitor sensitive cells Reduce of IGFBP3	Acquisition of “therapy-induced secretome” phenotype
Fedorenko et al. (2015) Fedorenko et al. (2016)	Enhanced TGF-β release	This induces fibroblasts differentiation and NRG1 production
Straussman et al. (2012) Wilson et al. (2012)	HGF produced by fibroblast	
Hirata et al. (2015)	Activation of MAPK pathway even in cancer-associated fibroblasts	
Wang et al. (2015)	Activation of MAPK pathway in Cancer-associated macrophages (CAMs)	Production of VEGF

Table 4
Cancer Stem Cells (CSCs) and microRNA (miR) causes of BRAF inhibitor resistance in melanoma.

Study	Mechanisms of resistance	Comments
Moitra, 2015 Bao et al. (2006)	Increased level of ABC (ATP-binding cassette) efflux pump and DNA repair ability	
Fattore et al. (2017) Hayes et al. (2014) Acunzo et al. (2015)	Destabilization and degradation of target miRs	
Liu et al. (2012) Liu et al. (2015)	Decrease of miR-200c levels	Upregulation of BMI-1, ABCG2, ABCG5 and MDR1
Fattore et al. (2016)	Decrease of miR-579–3p levels	
Vitiello et al. (2017)	Increase of miR-204 and miR-211	

5. Cancer stem cells and microRNAs

In the last few years the role of both cancer stem cells (CSCs) and microRNAs in BRAF inhibitor mechanisms of resistance has been evaluated (Table 4).

CSCs are a particular population which maintains most of the normal stem cell characteristics, such as self-renewal and high proliferation ability. Furthermore, they are considered radio- and chemo-resistant due to their increased levels of ATP-binding cassette (ABC) efflux pumps and DNA repair ability (Bao et al., 2006; Moitra, 2015). It is important to note that these CSCs might govern tumor recurrence and metastasis, because currently available treatments often fail to affect them. Thus, understanding their nature and determining their specific antigens could help us to provide new target therapies that might lead to complete tumor eradication. In the complex melanoma scenario, five specific markers have been associated with CSCs: CD133,CD20,ABC B5,CD271 and aldehyde dehydrogenase 1; targeting these with monoclonal antibodies in addition to standard therapies might be a feasible treatment option for melanoma patients. For example, Rappa et al. (2008) demonstrated that downregulation of CD133 with short hairpin RNAs led to decreased melanoma cell growth, motility and metastatic potential in a dose-dependent manner. Furthermore, Calvani et al. (2016) revealed that treatment with etoposide + bevacizumab was able to significantly reduce melanoma sphere-forming ability in a CD133 + /VEGF receptor 2 + subset of melanoma cells. All therapeutic implications and new possibilities of targeted therapies against CSCs are reviewed in the work provided by Kumar et al. (2017).

At the same time, the role of microRNAs has been increasingly evaluated in tumors, and recently many authors revealed interesting results about their possible role in melanoma development and resistance to target therapy (Fattore et al., 2017). MicroRNAs mainly act as post-transcriptional modulators of gene expression. With a few nucleotide sequences at their 5′ regions they can bind to the 3' untranslated regions of target microRNAs with an imperfect match; this leads to destabilization and degradation of target microRNAs. Moreover, a single microRNA is able to bind several microRNAs, with pleiotropic effects (Acunzo et al., 2015; Hayes et al., 2014). With this mechanism, microRNAs could control several key signaling pathways. For example, Liu et al. (2012) demonstrated that microRNA-200c levels were diminished in both primary and metastatic melanomas. When microRNA-200c levels are decreased, there is an upregulation of those genes involved in proliferation, self-renewal and drug resistance, such as B lymphoma Mo-MLV insertion region 1 homolog, ABC G2, ABC G5, and p-glycoprotein 1 (Liu et al., 2015). At the same time, increased levels are related to lower expression of those above, with an anti-tumorigenic effect. Thus, authors suggested that microRNA-200c may help increasing melanoma cells sensitivity to currently available therapies. Furthermore, Fattore et al. (2016) showed that microRNA-579–3p acts as a tumor suppressor by targeting the 3′ untranslated region of two proteins: BRAF and mouse double minute 2 homolog. Vitiello et al. (2017) revealed that microRNA-204 and mcroRNA-211 are the most induced under vemurafenib therapy, and that they could act in a context-dependent manner. Many efforts have been made in the last few years to design specific nano-carriers, polymers, lipoproteins or exosomes for microRNAs delivery (Muthiah et al., 2013; Valadi et al., 2007; Vickers et al., 2011), but further studies are needed to refine these techniques. Notably, deregulated microRNAs could also be used as new specific biomarkers to predict resistance to target therapies in the next future (Margue et al., 2015; Ono et al., 2015; Schwarzenbach et al., 2014).

6. Immunomodulatory effect of target therapy and immunologic mechanism of resistance

Several preclinical and clinical data (Boni et al., 2010; Ebert et al., 2016; C. Liu et al., 2013; Ott et al., 2013; Sapkota et al., 2013; Steinberg et al., 2014) highlighted the role of BRAF and MEK inhibitors in modulating melanoma microenvironment and favoring tumor immunogenicity.

In BRAF-mutated melanoma microenvironment, low T-cell infiltrates as well as high levels of immunosuppressive cells might protect tumor cells from immune system activity (Bradley et al., 2015; Frederick et al., 2013; Sumimoto et al., 2006). However, BRAF and MEK inhibitors combination is able to modify these unfavorable characteristics, overcoming the immunological resistance.

In fact, Wilmott et al. (2012) showed an increased level of tumor-infiltrating lymphocytes in patients' biopsy specimens after 7 days of treatment with dabrafenib or vemurafenib, compared to those samples collected before BRAF inhibitor treatment. Furthermore, the level of $CD8^+$ in the tumor niche appeared to be strictly related to reduction in tumor size and increase in neoplastic tissue necrosis. Similar results were reported in several studies (Cooper et al., 2013; Frederick et al., 2013; Kakavand et al., 2015).

Target therapy also promoted an increase in melanoma-related antigensi, such as MART-1, glycoprotein-100, tyrosinase related protein 1, and tyrosinase related protein 2 (Frederick et al., 2013), a rise in serum levels of immune-stimulatory cytokines like interferon-gamma, tumor necrosis factor (TNF)-α, and CCL4 antigene and lower levels of IL-8 and IL-6, which have an immunosuppressive effect (Frederick et al., 2013; Wilmott et al., 2014).

Also serum levels of myeloid-derived suppressor cells, that protect melanoma from immune system, appear to be decreased during treatment with vemurafenib (Schilling and Paschen, 2013).

However, the immune-stimulatory effect of target therapy with BRAF and MEK inhibitors reduces over time. It was observed that, at the time of disease progression, patients treated with combined target therapy had lower $CD8^+$ T-cell infiltrate in biopsy samples, compared to samples of patients treated with pembrolizumab (Cooper et al., 2016). This is probably due to a decrease in melanoma differentiation antigen expression (Pieper et al., 2018).

Also the increased expression of PD-L1 in melanoma cells, mediated by c-Jun and STAT3 activation, may be another mechanism of immune-modulated resistance to BRAF inhibitor therapy (X. Jiang et al., 2013) (See Table 5).

Table 5
Immunologic mechanism of BRAF inhibitor resistance in melanoma.

Study	Mechanisms of resistance
Frederick et al. (2013) Bradley et al. (2015) Pieper et al. (2018)	Low $CD8^+$ T-cell infiltrates; under-expression of melanocyte differentiation antigens and immune exhaustion
Sumimoto et al. (2006)	Increased production of immune-suppressive cytokines (IL-6, IL-10, and VEGF)
Jiang et al. (2013)	Increased expression of PD-L1

Table 6
Phase III clinical trials of BRAFi alone or in combination with MEKi in metastatic melanoma.

Trial name	Comparison	Primary endpoint	Treatment arms (number of patients)	PFS in months	OS in months	Overall response rate
BRIM-3	BRAFi vs	OS + PFS	Vemurafenib (338)	5.3	13.6	48%
	Chemotherapy		Dacarbazine (337)	1.6	9.7	5%
BREAK III	BRAFi vs	PFS	Dabrafenib (187)	6.9	20	50%
	Chemotherapy		Dacarbazine (63)	2.7	15.6	6%
METRIC	MEKi vs	PFS	Trametinib (214)	4.9	15.6	19%
	Chemotherapy		Dacarbazine or Paclitaxel (108)	1.6	11.3	5%
COMBI-V	BRAFi + MEKi vs	OS	Dabrafenib +	11.4	72%[a]	64%
	MEKi		Trametinib (352)	7.3	65%[a]	51%
			Vemurafenib (352)			
COMBI-D	BRAFi + MEKi vs	PFS	Dabrafenib + Trametinib (211)	22%[b]	44%[b]	68%
	BRAFi		Dabrafenib + Placebo (212)	12%[b]	32%[b]	55%
COBRIM	BRAFi + MEKi vs	PFS	Vemurafenib + Cobimetinib (247)	12.3	22.3	68%
	BRAFi		Vemurafenib+	7.2	17.4	45%
			Placebo (248)			
COLUMBUS	BRAFi + MEKi vs	PFS	Encorafenib + Binimetinib (192)	14.9	33.6	64%
	BRAFi vs		Encorafenib (194)	9.6	23.5	52%
	BRAFi		Vemurafenib (191)	6.3	16.9	41%

Abbreviations: BRAFi: BRAF inhibitor; MEKi: MEK inhibitor, OS, Overall survival; PFS, Progression-free survival.
[a] Data at 1 year long-term surbival
[b] Data at 3 years long-term survival and safety analysis.

7. Possible mechanisms to overcome resistance and ongoing clinical trials

Historically, the prognosis of patients affected by advanced melanoma patients has been poor, with 5-year survival of only ~6% and median overall survival (OS) of 7.5 months (Barth et al., 1995; Long et al., 2017).

The introduction of new drugs, such as immunotherapy, BRAF and MEK inhibitors, has significantly changed the therapeutic landscape in melanoma (Table 6).

In fact, the use of immune checkpoint inhibitors such as ipilimumab, nivolumab and pembrolizumab, which inhibit the activity of CTLA4, PD1 and PD-L1 respectively, as well as BRAF inhibitors (vemurafenib, dabrafenib and encorafenib), used as monotherapy or in combination with MEK inhibitors (trametinib, cobimetinib and binimetinib), has been shown to perform an important antitumor activity.

Vemurafenib and dabrafenib demonstrated an advantage in PFS, OS, and overall response rate when compared to conventional chemotherapy (dacarbazine) in the BRIM3 and BREAK3 studies, respectively (Chapman et al., 2011; Hauschild et al., 2012). According to BRIM3 data, patients treated with vemurafenib had an OS and PFS of 13.6 and 5.3 months, whereas those treated with dacarbazine had 9.7 and 1.6 months, respectively (Chapman et al., 2011). On the same way, according to the BREAK3 study, patients treated with dabrafenib had an OS and PFS of 20 and 6.9 months, whereas those treated with standard chemotherapy had 15.6 and 2.7 months, respectively (Hauschild et al., 2012).

Based on these findings, vemurafenib and dabrafenib were approved by FDA in the treatment of unresectable or metastatic melanomas carrying the BRAF V600E mutation (Dummer et al., 2015).

Moreover, preclinical data showed that BRAF inhibitor-resistant cells were sensitive to MEK inhibitor (Solit et al., 2006; Tsai et al., 2008). Among MEK inhibitors, trametinib, a selective inhibitor of MEK1 and MEK2 molecules, is one of the most studied. METRIC trial demonstrated that trametinib was significantly superior compared to chemotherapy with dacarbazine or paclitaxel in the treatment of advanced melanoma (Flaherty et al., 2012).

According to the results of COMBI-D and COMBI-V trials, the combination of dabrafenib and trametinib has become the standard of care for BRAF mutant advanced melanoma (Long et al., 2017; Robert et al., 2015). COMBI-V is an open label trial that compared combined therapy with dabrafenib plus trametinib and vemurafenib monotherapy as first line treatment in patients with metastatic mutated melanoma. The interim OS rate at 12 months was 72% and 65%, whereas median PFS was 11.4 and 7.3 months in the combined-versus vemurafenib group respectively (Robert et al., 2015). In the COMB-D trial patients with unresectable (stage IIIC) or metastatic BRAF V600E mutated melanoma (stage IV) were randomized 1:1 to receive dabrafenib + trametinib or dabrafenib alone. Three-years PFS and OS with combined treatment were 22% and 44% compared to 12% (HR 0.71) and 32% (HR 0.75) with monotherapy, respectively (Long et al., 2017).

Several studies have investigated other combination treatments: COBRIM trial demonstrated an advantage in the use of combination therapy with vemurafenib and cobimetinib compared to vemurafenib monotherapy (Ascierto et al., 2016); COLUMBUS open label trials showed improvements in both PFS and OS parameters in group of patients treated with encorafenib plus binimetinib when compared to vemurafenib (Dummer et al., 2018b).

Despite these promising results, almost all of the patients affected from BRAF-mutaed metastatic melanoma will develop a tumor relapse within several months.

Different drug-resistance mechanisms underlie the progression of disease and activation of both MAPK and PI3K/AKT/mTOR pathways. Therefore, approaches aiming to inhibit both MAPK and PI3K–Akt pathways have been proposed. In particular, preclinical data support the possible introduction of lapatinib in the treatment of melanoma:

Abel et al. (2013) have shown a reduced tumor burden and an extended latency of tumor regrowth in cell lines treated with lapatinib plus PLX4720 (a BRAF inhibitor), when compared to PLX4720 alone; Dratkiewicz et al., 2018 observed synergistic cytotoxic effect with lapatinib and foretinib, a potent inhibitor of MET, VEGF receptor, RON, AXL, HGF (Dufies et al., 2011); Thakur et al. (2017) demonstrated a 60% reduction in melanoma tumor growth over controls with lapatinib alone. Also insulin-like growth factor receptor 1 (IGFR1), through activation of MAPK and PI3K/AKT/mTOR pathways, is involved in the early response to BRAF inhibitors. Therefore, cyclolignan picropodophyllin, a specific inhibitor of IGFR1 kinase activity, could represent in the future another therapeutic alternative (Karasic et al., 2010; Villanueva et al., 2010).

BRAF inhibitor resistant cell might also present MEK-independent survival mechanisms that could be arrested by PI3K–Akt–mTOR pathway inhibitors (Jiang et al., 2011). Atefi et al. (2011) showed that, in presence of a disease progression to vemurafenib, the shift to a combination therapy with vemurafenib or a MEK inhibitor plus a specific Akt or mTOR inhibitor can potentially overcome the resistance.

Combined therapies of BRAF and MEK inhibitor with SRC (i.e selumetinib, saracatinib), FAK or STAT3 inhibitors also prevented the progression of disease, by suppressing the so called “invasive phenotype”, and efficiently reduced melanoma cells growth (Ferguson et al., 2013; Vultur et al., 2014). Also sunitinib, that targets PDGFR A, can restore melanoma cells sensitivity to BRAF inhibitors (Sabbatino et al., 2014).

As previously described, among all the cells that characterize tumor microenvironment, CAMs can contribute to BRAF inhibitor resistance. Preclinical studies confirmed that combination of BRAF inhibitor with a MCSFR inhibitor could resensitize melanoma cells to vemurafenib (Mok et al., 2015; Ngiow et al., 2016), suggesting a new promising target therapy. Based on these preclinical data, several clinical trials are currently ongoing, such as the phase 1b study (NCT01826448), that aims to test the safety of an investigational new drug called PLX3397, a colony-stimulating factor 1 receptor (CSF1R) inhibitors (Cannarile et al., 2017) when used in combination with vemurafenib at different dose levels. NCT03101254, and an ongoing Phase I/II clinical trial, that is studying the combination of LY3022855, a CSF-1R inhibitor, with vemurafenib and cobimetinib («A Phase 1b Open Label, 2019, Dose Escalation Study of PLX3397 in Combination With Vemurafenib in V600-Mutated BRAF Melanoma - Full Text View - ClinicalTrials.Gov » s.d.).

Moreover, targeting specific CSCs markers with monoclonal antibodies in addition to standard therapies might be a feasible treatment option for melanoma patients. In fact, small molecule inhibitors (HNK, ABT-737, ABT-263), nanoparticles conjugated drugs (HA-SLNs-PTX), signaling antagonist (Cyclopamine, Gant61), monoclonal receptor antibodies (anti-CD20, anti-CD133, anti-ABCB5) and microRNAs (miR-200c, miR-33b) may, in the future, represent new therapeutic alternatives in the management of metastatic melanoma (Kumar et al., 2017). In particular, among the CSCs pathways that promote tumor development, Notch1, through an increase in CD133 expression, promotes VEGF and matrix metalloproteinase synthesis, leading to melanoma growth, angiogenesis, and lung metastasis. Recent data showed that andrographolide, inhibiting Notch1 pathway in CSCs, can suppress melanoma growth (Kumar et al., 2016).

It was shown that BRAF inhibition with target therapies determines the paradoxical activation of MAPK pathway in wild type BRAF cells, favoring the activation of T cells (Holderfield et al., 2014). On this rationale it is possible to hypothesize that the combination of target therapy and immunotherapy may overcome the resistance mechanisms that develop individual therapeutic approaches (Table 7).

An initial phase I trial aimed to establish a schedule of administration of ipilimumab and vemurafenib in patients with mutated BRAF metastatic melanoma. The trial studied 2 patients' cohorts: a) the first (6 patients) received vemurafenib (960 mg orally twice daily) as monotherapy, with the addition of ipilimumab in combination with the target therapy at a dosage of 3 mg/kg after one month; b) the second (6 patients) received a lower dose of vemurafenib (720 mg twice daily) in combination with a full dose of ipilimumab. Both groups of patients developed important liver toxicity (Ribas et al., 2013). Another early Phase 1/2 study investigated the safety of doublet therapy with dabrafenib and ipilimumab and of triplet therapy with dabrafenib, trametinib, and ipilimumab in metastatic BRAF V600E/K-mutated melanoma. 2 out of 7 patients that received triplet therapy developed colitis with intestinal perforation (Minor et al., 2015). These two early studies underline the difficulty of combining immunotherapy and target therapy adequately in order to reduce toxicity profile.

Among the ongoing trials, as reported in Table 7, the design of phase II, single-arm, open-label study with BRAF inhibitor plus anti-CTLA4 (CA184-240, ClinicalTrials.gov identifier: NCT01673854) involve 2 phases. In Part 1 all 46 enrolled patients received vemurafenib (960 mg twice daily) for 6 weeks followed by 4 induction doses of ipilimumab (10 mg/kg every 3 weeks), and after maintenance dose ipilimumab every 12 weeks until disease progression or unacceptable toxicity. Patients who progressed after IPI received vemurafenib at their previously tolerated dose (Part 2). The study showed that the aforementioned administration schedule is much more manageable (Amin et al., 2016).

Finally, we summarized more recent ongoing studies that have shown lower toxicity rates related to the combination treatment, when anti-PD-1 antibodies were used in place of ipilimumab (Ascierto et al., 2017, 2018; Dummer et al., 2018a; Miller et al., 2017; Puzanov 2015; Ribas et al., 2012, 2015, 2017; Ryan et al., 2017; Sullivan et al., 2017; Tawbi et al., 2018; A study of atezolizumab plus cobimetinib and vemurafenib versus placebo plus cobimetinib and vemurafenib, 2019).

8. Discussion

To date, a lot of promising compounds seems to be useful to overcome melanoma's resistance to the standard therapy with BRAF/MEK inhibitors. One of the principle mechanisms is that related to the activation of EGFR pathway. Indeed, BRAF/MEK inhibition can cause elevation of FOXD3 protein levels, which in turn can upregulate transcription of ERBB-3. Therefore, a combination of BRAF inhibitor with lapatinib, as demonstrated in cell lines by Abel et al. (2013), could be a great strategy to reduce tumor burden and to extend latency of tumor regrowth.

Combined therapies of BRAF and MEK inhibitors with SRC (i.e selumetinib, saracatinib), FAK or STAT3 inhibitors also prevented progression of the disease, by suppressing the so called “invasive phenotype”, and efficiently reduced melanoma cells growth (Ferguson et al., 2013).

However, an increase in MITF levels, which is involved in the increased oxidative phosphorylation and mitochondrial activity that lead to a BRAF inhibitor resistance, occurred when BRAF or NRAS-mutated melanoma cells were treated with selumetinib (Haq et al., 2013). Therefore, a combination with a mTORC1/2 inhibitor (AZD8055) seem to be able to desensitize cells that displayed increased oxidative phosphorylation, but not cells with low mitochondrial activity.

Also PDGFR A and B appear to be involved in BRAF inhibitor resistance, with a crosstalk mechanism between these receptors and SHH pathway (Nazarian et al., 2010; Sabbatino et al., 2014; Stecca et al., 2007). As demonstrated by Sabbatino et al. (2014), the inhibition of the SHH pathway with LDE-255 (a smoothened antagonist) or inhibition of PDGFR A with sunitinib restores and increases melanoma cells' sensitivity to BRAF inhibitors, providing evidence for another possible combination therapy for melanomas.

The MITF expression is also upregulate by PAX3, a protein that controls gene transcription, that is induced after target therapy with MEK inhibitors. This mechanism can lead to acquired resistance. Therefore, as showed by Smith et al. (2016), the use of nelfinavir, an

Table 7
Ongoing and recently completed clinical trials with BRAF ± MEK inhibitors and Immunotherapy.

Trial	Phase	Treatment arms	Status	Preliminary results (if available)
NCT01673854 1	II	V → I Restart V if progression after I	Enrollment completed	m OS 18.5 mo[a] m PFS 4.5 mo[a]
NCT02130466 2 (KEYNOTE-022) 3	I/II	Phase I → BRAF-mut: D + T + P Phase II → BRAF-mut: D + T + P vs BRAF-mut: D + T + Placebo	Recruiting	ORR 67% m PFS 16.3 vs 10.3[b] m DOR 18.7 vs 12.5[b] OS at 12 mo 80% vs 73%
NCT02910700 4	II	D + T + N	Recruiting	ORR 91%
NCT02027961 5	I	BRAF-mut: D + T + Du BRAF-wt: T + Du BRAF-wt: T → Du	Enrollment completed	ORR 76% ORR 21% ORR 50%
NCT01656642 6	Ib	V + C for 28 days, then V + C + A	Enrollment completed	ORR 85.3%
NCT01988896 7	Ib	BRAF-mut and BRAF-wt: C + A	Enrollment completed	m PFS 12 mo ORR 45%
NCT02967692 8 (COMBI-I)	III, 1st part	Arm A: D + T + S Arm B: D + T + Placebo	1st part completed 3rd part recruiting	ORR 100%
NCT02631447 9 (SECOMBIT)	II	Arm A: E + B until progression, then N + I Arm B: N + I until progression, then E + B Arm C: E + B for 8 weeks → N + I until progression, then E + B	Recruiting	Not available
NCT01767454 10	I	Arm A: D + I Arm B: D + T + I	Enrollment completed	Not available
NCT01400451 11	I	I + V	Terminated due to dose-limiting toxicities	Not available
NCT02908672 12 (TRILOGY, IMspire150)	III	Arm A: V + C + A Arm B: V + C + Placebo	Enrollment completed	Not available

Abbreviations: A, atezolizumab; B, binimetinib; D, dabrafenib; E, encorafenib; DOR, duration of response; Du, durvalumab; N, nivolumab; P, pembrolizumab; PFS, Progression-free survival; ORR, Overall response rate; OS, Overall survival; S, spartalizumab; T, trametinib; V, vemurafenib; mo, months.
[a] At a median follow up of 15.3 months.
[b] At a median follow up of 9.6 months.

HIV protease inhibitor, which is able to suppress PAX3 expression, could resensitize BRAF or NRAS-mutated melanoma cells, overcoming resistance.

However, MITF probably acts in a context-dependent manner, and its evaluation needs further investigation.

Treatment with BRAF and/or MEK inhibitors also seems to be associated with changes in tumor microenvironment, but confirmatory studies about its primary role is still under investigation.

Moreover, the role of CSCs is very interesting: targeting one of the five specific markers which have been associated with CSCs (CD133,CD20,ABCB5,CD271 and ALDH1) with monoclonal antibodies in addition to standard therapies might be a feasible treatment option for melanoma patients (Calvani et al., 2016; Kumar et al., 2017; Rappa et al., 2008). However, the published studies are still on cell culture, so human trials are needed to confirm their role.

Interesting results have also been shown about the role of microRNA in melanoma development and resistance to target therapy (Fattore et al., 2017). It has been demonstrated that microRNA-200c levels were diminished in both primary and metastatic melanomas, with a consequent upregulation of several genes involved in proliferation, self-renewal and drug resistance. In the last few years the design of specific nano-carriers, polymers, lipoproteins or exosomes for microRNAs delivery (Muthiah et al., 2013; Valadi et al., 2007; Vickers et al., 2011) is having a great increase, but further studies are needed to refine these techniques.

Furthermore, the possible immune-modulating role of BRAF inhibitors and preclinical data about the enhancing efficacy of checkpoint inhibitors (Kuske et al., 2018), even if with some differences in accordance with the gender (Botticelli et al., 2017), are gaining big interest.

8.1. Conclusion

Despite this higher evidence of both new mechanism and drugs discovered to overcome melanoma's resistance, unfortunately disease progression seems to be still an inevitable passage, so further studies and efforts are needed to fight against this negatively prognostic disease.

However, as widely described by this review, we are on the right path to develop new treatment strategies that can help overcoming melanoma's BRAF inhibitor resistance mechanisms and improving patient's overall survival.

Authors' individual contributions: AR: Conceptualization; MR, FM, AB and PM: Supervision; PM: Validation and Visualization; AR, MR: Writing - original draft; AR, MR, MP: Writing - review & editing.

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