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Review

Resistance mechanisms to targeted therapy in BRAF-mutant melanoma - A mini review

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ABSTRACT

Background: The introduction of targeted therapies for the treatment of BRAF-mutant melanomas have improved survival rates in a significant proportion of patients. Nonetheless, the emergence of resistance to treatment remains inevitable in most patients.

Scope of review: Here, we review known and emerging molecular mechanisms that underlay the development of resistance to MAPK inhibition in melanoma cells and the potential strategies to overcome these mechanisms.

Major conclusions: Multiple genetic and non-genetic mechanisms contribute to treatment failure, commonly leading to the reactivation of the MAPK pathway. A variety of resistance mechanisms are enabled by the underlying heterogeneity and plasticity of melanoma cells. Moreover, it has become apparent that resistance to targeted therapy is underpinned by early functional adaptations involving the rewiring of cell states and metabolic pathways.

General significance: The evidence presented suggest that the use of a combinatorial treatment approach would delay the emergence of resistance and improve patient outcomes.

1. Background

Nearly half of cutaneous melanomas carry a genetic mutation that leads to a substitution in position 600 of the serine/threonine kinase BRAF [1]. BRAF is a key protein in the Mitogen-Activated Protein Kinase (MAPK) pathway, which regulates several important cellular functions, including proliferation, differentiation, migration and apoptosis. The demonstration of the mutant *BRAF* oncogene-addiction of melanomas led to the development of mutant specific BRAF inhibitors (BRAFis), such as vemurafenib (Zelboraf), dabrafenib (Tafinlar), and encorafenib (Braftovi) [2–4]. FDA approval and broad clinical use of vemurafenib and dabrafenib was astoundingly achieved within 10 years of the *BRAF* oncogene discovery. Further clinical benefits were gained from combining BRAFis with MEK inhibitors (MEKis) such as cobimetinib (Cotellic), trametinib (Mekinist), and binimetinib (Mektovi), significantly improving response rates and overall survival [5–7]. Interestingly, some serious adverse effects, including cutaneous squamous-cell carcinoma, were attenuated by the combination therapy. However, nearly all patients treated with single-agent BRAFis, or in combination with MEKis, eventually developed resistance and relapsed on therapy.

2. Scope of review

In this review, we discuss our current understanding of the mechanisms that mediate drug resistance in melanomas treated with targeted therapy. Tumour relapse is driven by a subpopulation of drug-tolerant cells that persist treatment and remain viable, while the rest of the population gets killed.

Drug resistance is a multifaceted phenomenon and a complex scenario involving genetic, epigenetic and metabolic changes within the tumour cells as well as in the tumour microenvironment. Here, we categorised the various mechanisms underlying treatment failure into those conferring intrinsic, adaptive and acquired resistance (Fig. 1), and highlight potential strategies to overcome these mechanisms of resistance.

3. Intrinsic resistance

Around 1 in 5 melanoma patients treated with BRAFis show disease progression on their first assessment during treatment, despite carrying a $BRAF^{V600E}$ mutation, indicating the presence of intrinsic resistance in a substantial proportion of cells within these tumours rendering drug resistance [8]. Pre-existing genetic alterations and endogenously secreted factors from stromal or tumour cells have been identified as

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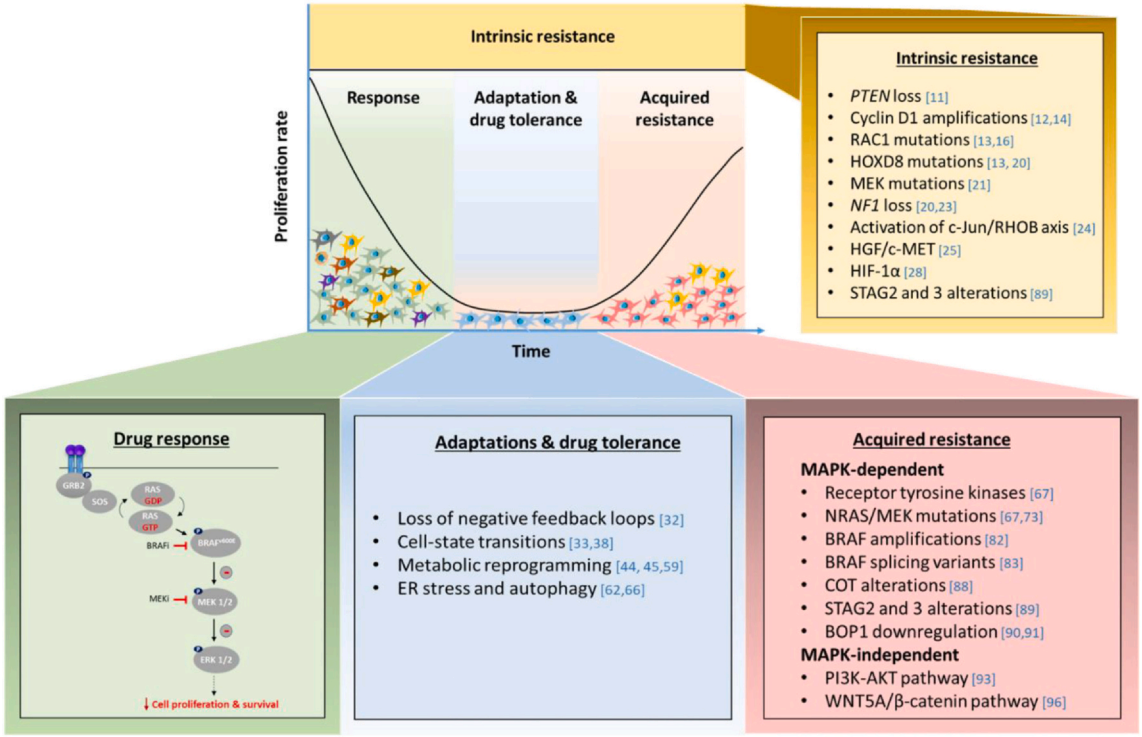

Fig. 1. Mechanisms conferring intrinsic, adaptive and acquired resistance in BRAF-mutant melanomas. The numbers within the square brackets represent the references for each mechanism.

drivers of intrinsic resistance to BRAF inhibition in some $BRAF^{V600}$ mutant melanoma cell lines and tumours.

3.1. PTEN loss

Phosphatase and tensin homolog (*PTEN*) is a tumour suppressor gene, and a major regulator of the phosphatidylinositol-3-kinase (PI3K) pathway [9]. Endogenous *PTEN* mutations and deletions are frequently identified in *BRAF*-mutant melanomas [10]. *In vitro* studies demonstrated that *PTEN* loss activates MAPK and PI3K/AKT pathways in *BRAF*-mutant melanomas, and confer intrinsic resistance by supressing the BIM-mediated apoptosis [11]. Moreover, melanoma patients with a *PTEN* loss of function alterations/mutations treated with BRAFi monotherapy or BRAFi plus MEKi were found to have shorter median progression-free survival (PFS) and overall survival [9,12], but interestingly *PTEN* loss did not affect overall response rates. In fact, only 10% of *PTEN*-null BRAF mutant melanomas exhibit *intrinsic* resistance to BRAFis [9,13]. The above suggests that *PTEN* loss contribution to resistance to BRAFis in melanoma may be contextual, but overall can reduce treatment effectiveness.

3.2. Amplifications of cyclin D1

In *BRAF*-mutant melanomas, the activation of MAPK/PI3K pathways drives uncontrolled cell proliferation *via* dysregulation of the RB pathway ($p16^{INK4A}$: cyclin D-CDK4/6: RB) [14]. Loss of *p16* in melanoma cells can occur *via* deletions (50–80%), inactivating mutations (16%) and epigenetic silencing (20%) of *CDKN2A* [14]. In addition, the *CCND1* gene, encoding for cyclin D1, is amplified in 11% of melanomas, including 17% of $BRAF^{V600E}$ melanomas [15]. Overexpression of cyclin D1 was linked to resistance to BRAFis in an earlier *in vitro* study [15]. In line with this observation, a clinical study found *CDKN2A* deletion and *CCND1* amplification to be associated with shorted PFS in patients treated with dabrafenib [12].

3.3. RAC1 mutations

RAC1 is a member of the Rho family of small GTPase that regulates cell proliferation, cytoskeletal reorganization and cell migration [16]. The $RAC1^{P29S}$ is the third most common hot-spot mutation (4%) in melanoma after $BRAF^{V600}$ (50%) and $NRAS^{Q61}$ (20%) [16,17]. The $RAC1^{P29S}$ is an activating mutation leading to perpetual RAC1 phosphorylation resulting in increased cell proliferation and migration [16]. Endogenous $RAC1^{P29S}$ in tumours correlated with early resistance and lack of response to BRAF inhibition [13]. Silencing the $RAC1^{P29S}$ in $BRAF^{V600E}$ mutant melanomas restored sensitivity to BRAFis, indicating the $RAC1^{P29S}$ could be a predictive biomarker to RAF resistance in melanoma patients [13,18].

3.4. HOXD8 mutations

HOXD8 is a homeobox transcription factor that plays a crucial role in cell division, adhesion, proliferation, apoptosis and differentiation [19]. Dysregulated *HOX* expression has been reported in multiple malignancies [19]. A *HOXD8* mutation was observed in the tumour from one patient in a comprehensive whole-exome sequencing study of 45 patients with early resistance to BRAFis [13]. In addition, an *in vitro* RNAi screening implicated *HOXD8* suppression in resistance to a broad RAF inhibitor [20]. However, the significance of *HOXD8* mutations for BRAFis remain unclear, as no other case has been reported to date.

3.5. MEK mutations

MEK1/2 proteins are downstream components of RAF protein that promote ERK phosphorylation and MAPK signalling [21]. Pre-existing *MEK1* mutations are present in 5% of the melanomas [10,17]. Pre-existing $MEK1^{P124}$ mutations have been attributed to shorter PFS in *BRAF*-mutant melanomas treated with BRAFis [22]. *In vitro* studies identified the *MEK1* mutation, $MEK1^{C121S}$, confer resistance to BRAFis and MEKis [21].

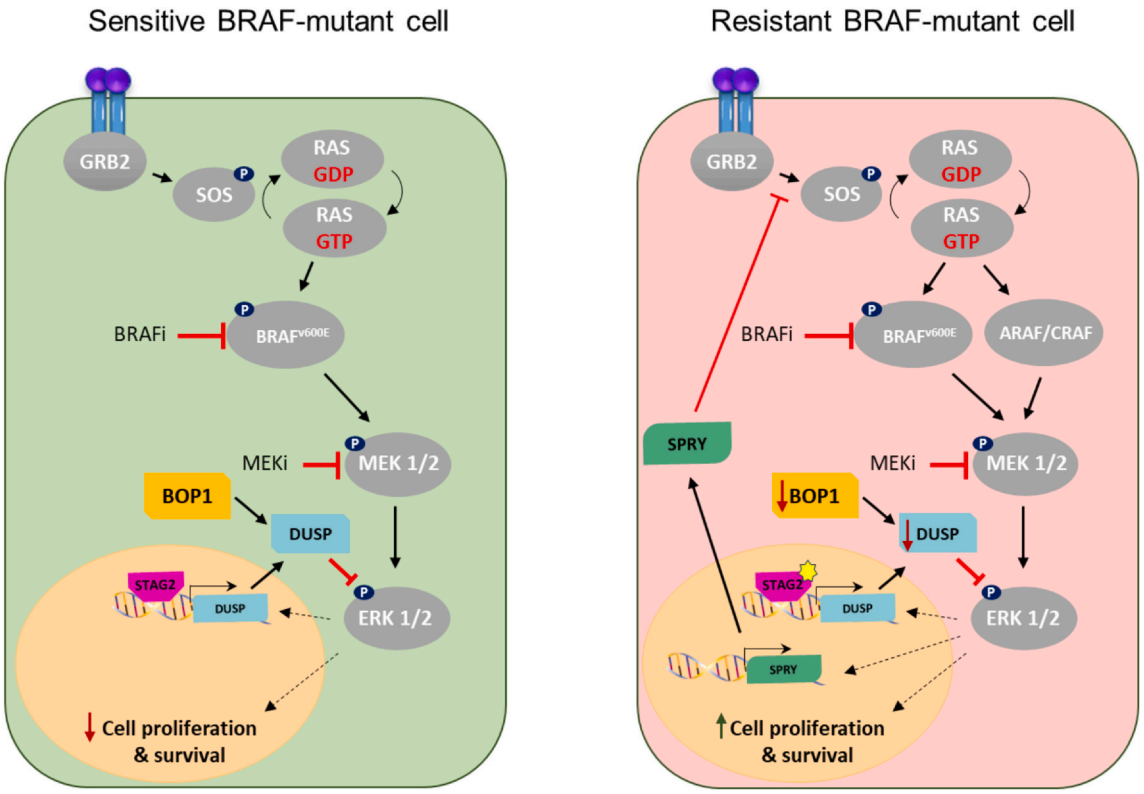

Fig. 2. Loss of negative feedback regulation of ERK. ERK activation controls MAPK pathway activation through negative feedback loops through the upregulation of *SPRY* and *DUSP*. SPRY inhibits SOS phosphorylation, dampening MAKP activation. BOP1 and STAG are both regulators of DUSP, which inhibits ERK phosphorylation and maintain control of the MAPK pathway. Loss of function mutations in STAG2, and downregulation of BOP1 results in reduced control of phosphorylated ERK, allowing cell survival and resistance to BRAFis.

3.6. *NF1* loss

Neurofibromin 1 (NF1) was discovered to play an important role in the inhibition and suppression of RAS, constraining the MAPK pathway [20]. Genome scale screening using RNAi and CRISPR-Cas9 have implicated *NF1* loss in the resistance to BRAFi and *NF1* mutations were observed in *BRAF*-mutant tumour cells intrinsically resistant to RAF inhibition [20,23]. Importantly, endogenous *NF1* alterations were found in pre-treatment tumours of patients who were refractory to vemurafenib treatment demonstrating the clinical significance of *NF1*-driven resistance [20].

3.7. Activation of c-Jun/RHOB axis

Ras homolog gene family, member B (RHOB) GTPase expression are induced by treatment of *BRAF*-mutant cell lines with BRAFis, *via* transcription factor c-Jun [24]. Low basal RHOB expression in melanoma cells lines correlated with BRAFi sensitivity, while depletion of RHOB restored sensitivity to MAPK inhibition [24]. Analysis of biopsies from patients treated with vemurafenib, indicated significantly shorter PFS in patients whose tumour samples displayed a positive RHOB staining before treatment compared to those with negative RHOB staining. It is thought that activation of the c-Jun/RHOB axis affects response to BRAFis through the activation of AKT pathway [24].

3.8. Factors secreted by tumour microenvironment

3.8.1. Hepatocyte growth factor /c-MET

Increased hepatocyte growth factor (HGF)/c-MET signalling mediates melanoma cell proliferation, invasion and offer protection from apoptosis [25]. *In vitro* studies demonstrated increased cell proliferation driven by the stromal HGF/c-MET signalling resulting in the reactivation of the MAPK and PI3K/AKT pathways, thereby rescuing melanoma cell lines from RAF and MEK inhibition [25,26]. Treatment with BRAFis plus HGF/c-MET inhibitors restored the sensitivity of these cells to BRAF inhibition, supporting the role of HGF signalling in melanoma resistance [25]. A recent study indicated that MAPK inhibition induced rapid increase in MET and GAB1 levels, priming the tumour cells for HGF-mediated rescue [27]. In contrast with previous studies implicating stromal cells in HGF secretion [25], tumour derived HGF was reported to convey resistance to BRAFis [27]. Nevertheless, treatment with a selective MET inhibitor completely attenuated HGF-mediated rescue, underscoring the role of the HGF/c-MET axis in mediating cell survival and resistance.

3.8.2. Hypoxia inducible factor

Hypoxia inducible factor 1α (HIF-1α) is a component of the HIF transcriptional factor which acts as an oxygen sensing machinery and a key modulator of the transcriptional responses under hypoxic stress and excess reactive oxygen species [28]. Under hypoxic conditions, melanoma cells exhibited decreased sensitivity to vemurafenib, trending towards resistance [28]. Proliferative melanoma cell lines exposed to hypoxic conditions transitioned into an invasive phenotype through a HIF-1α dependent transcriptional mechanism, indicating the role of HIF-1α in phenotype switching [29,30]. HIF-1α induced transcriptional programming has a profound effect on the central carbon metabolism, increasing glycolytic rates and decreasing mitochondrial respiration [31].

4. Adaptive response and drug tolerance

Despite the initial tumour reduction observed in most melanoma patients treated with BRAFis, complete tumour regression occurs rarely. This is due to the emergence of BRAFi induced compensatory

mechanisms, referred here as adaptive responses, that enhance the pro-survival and pro-proliferative capacity of a proportion of the original tumour population. These adaptive responses are temporary responses that are reversible, and non-transferrable to progenies. Some of the adaptive mechanisms identified to date include, phenotypic switching or cell-state transitions, secretion of factors by the tumour micro-environment, and the more recently trending metabolic reprogramming.

4.1. Loss of negative feedback loops

BRAFi sensitive $BRAF^{V600E}$ mutant cells exhibit low expression of RAS-GTP before treatment, due to ERK-dependent feedback suppression of the receptor tyrosine kinases (RTK) signalling [32]. Active ERK can directly regulate signalling intermediates, such as EGFR and SOS, or indirectly activate the expression of negative feedback regulators such as SPROUTY (SPRY) and DUSP proteins [32] (Fig. 2). Inhibition of the MAPK pathway by BRAFis relieves this feedback, resulting in the re-activation of multiple pathways and attenuation of the antitumor effects of the targeted inhibitors [33]. This adaptation occurs within hours, thus diminishing the effectiveness of the therapy.

From the perspective of BRAFi resistance, it is important to note that the loss of these kinase-dependent negative feedback loops is unlikely to solely drive resistance, but they rather facilitate a subpopulation of tumour cells to survive in an adapted drug-tolerant state.

4.2. Cell-state transitions

The identification of distinctively high and low microphthalmia-associated transcription factor (MITF) levels within a melanoma tumour population [34] marked the conceptualization of the MITF-rheostat model [35]. MITF is a melanocytic-lineage transcriptional factor crucial for early melanogenesis and differentiation in melanocytes, and identified a master regulator of several biological processes in melanoma cells such as invasion, survival, cell cycle regulation and autophagy [30,36,37].

The $MITF^{high}$ population expressed increased levels of MITF downstream targets such as *MLANA*, *PMEL*, *TYRP-1* and *TYRP-2* genes, and are more proliferative and retained sensitivity to BRAFis [34,38,39]. On the other hand, the $MITF^{low}$ population expressed genes associated with invasiveness such as high WNT ligand WNT5A, receptor tyrosine kinase AXL, TGFβ, TNFα/NF-κβ activation, JUN and TEAD, and conferred resistance to targeted therapy [35,38,39]. Numerous studies confirmed the intrinsic resistance conferred by the AXL^{high} and $MITF^{low}$ phenotype in response to MAPK inhibition in melanoma cells [38–41]. Activation of markers of invasiveness in the sensitive population reduced the MITF expression, with cells transitioning into a resistant phenotype.

In notable contrast with the above observations, upregulation of MITF has been identified as a driver of drug tolerance state and suppression of MITF pharmacologically sensitised the cells to MAPK inhibitors (MAPKi) [42]. High expression of MITF and its target genes (*MLANA*, *PMEL*, *TYRP-1* and *TYRP-2)* with BRAFi treatment in BRAF-mutant melanomas was linked to resistance [43]. Upregulation in *MITF* expression as an early driver of non-mutational drug tolerant state in melanoma cells and linked to intrinsic resistance [13,39,42,44–48], and PAX3, an upstream regulator of MITF, was identified as a regulator of MITF expression [42]. Thus, both $MITF^{high}$ and $MITF^{low}$ phenotypes have been linked to innate resistance in melanoma cells (Fig. 3).

Multiple studies have reported in a phenotypic switch between the “proliferative” and the “invasive” state in melanoma cells upon BRAFi treatment and associated with resistance [34,35,49–51]. Interestingly, such a phenotypic switch was activated by factors secreted by the tumour microenvironment and stress factors contributing to tumour plasticity [40,52]. Bulk sequencing and, more recently, single-cell RNA sequencing of melanoma tumours have confirmed the presence of these different states in individual cell within tumours [51,53]. This underlying tumour heterogeneity allows for rapid adaptation and survival of tumour cells early during treatment leading the emergence of resistance.

4.3. Metabolic reprogramming

Metabolic reprogramming is a hallmark of cancer, driven by oncogenic signalling pathways or poorly vascularized tumour microenvironment to meet the increasing cellular biomass needs [54]. *BRAF*-mutant melanoma cells exhibit high glycolytic activity and decreased mitochondrial respiration, to meet the increasing biomass and ATP needs of high proliferative cells [55]. The Warburg phenotype in melanomas is partially driven by the MAPK or PI3K pathway by increasing the production of HIF-1α and MYC and promoting glycolysis or inhibiting MITF, a key regulator and promoter of oxidative phosphorylation (OXPHOS) in tumour cells [31]. Treatment with BRAFis and MEKis triggers metabolic programming in the BRAF-mutant cells to reduce glycolysis and increase mitochondrial respiration by activating MITF-PGC1α-OXPHOS pathway. Evidence of increased peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) levels, a marker of elevated OXPHOS, was exhibited by melanoma patients treated with single-agent BRAFi and in combination with MEKi [44,45]. *KDM5B* (JARID1B) expression has been indicated as a marker of these slow-cycling BRAFi resistant cells with increased oxidative phosphorylation [56]. Endogenous JARID1B and PGC1α overexpressing cells in patient tumours demark a subset of cells with increased mitochondrial capacity and resistance to oxidative stress that survive BRAFi [57,58].

In addition to the alteration in the glycolysis pathway, MAPK inhibition alters the fatty acid oxidation (FAO) in *BRAF*-mutant melanoma cells, which supply the structural material for cell and organelle membranes [59]. Short-term treatment with MAPK inhibition led to upregulation in fatty acid transporter CD36 in BRAF-mutant melanoma cells and PPARα-mediated and carnitine palmitoyl transferase 1A (CPT1A)-dependent FAO [59]. In parallel, fatty acid synthase, a key enzyme in endogenous fatty acid synthesis, is indirectly activated by the MAPK and PI3K pathways [60]. Thus, the continuous signal for cell proliferation is supported by multiple metabolic pathways.

4.4. ER stress and autophagy

The nuclear translocation and reactivation of ERK drive a non-canonical ER stress response *via* ATF4 phosphorylation, to induce cytoprotective autophagy. Dephosphorylation of ERK activates the translocation of MAPK components from the cytoplasm to the ER by GRP78 (molecular chaperone), KSR2 (scaffolding protein), early endosomes and SEC6 (ER translocase) [61,62]. This translocation is required for the re-phosphorylation of ERK in the cytoplasm, which is carried out by the cytoplasmic lipid kinase domain of pERK [62].

Autophagy can be promoted by the activation of the MAPK pathway [63,64] or by the LBK1-AMPK pathway to rescue the cells from glucose starvation [65]. BRAF inhibition also induces autophagy by the activation of the transcriptional factor, TFEB [66]. The “BRAF-TFEB-autophagy-lysosome” axis constitutes an intrinsic regulatory pathway in BRAF-mutant melanoma, coupling BRAF signalling with TGF-β signalling to drive tumour progression and chemoresistance [66].

5. Acquired resistance

Around 50% of patients treated with BRAFis alone or in combination with MEKis experience an initial significant shrinking of the tumour followed by tumour outgrowth, due to the emergence of acquire resistance. This resistance often spawns from the acquisition of a mutation that either reactivates the MAPK pathway or circumventing the MAPK pathway altogether through the utilisation of alternative pathways to support cellular growth.

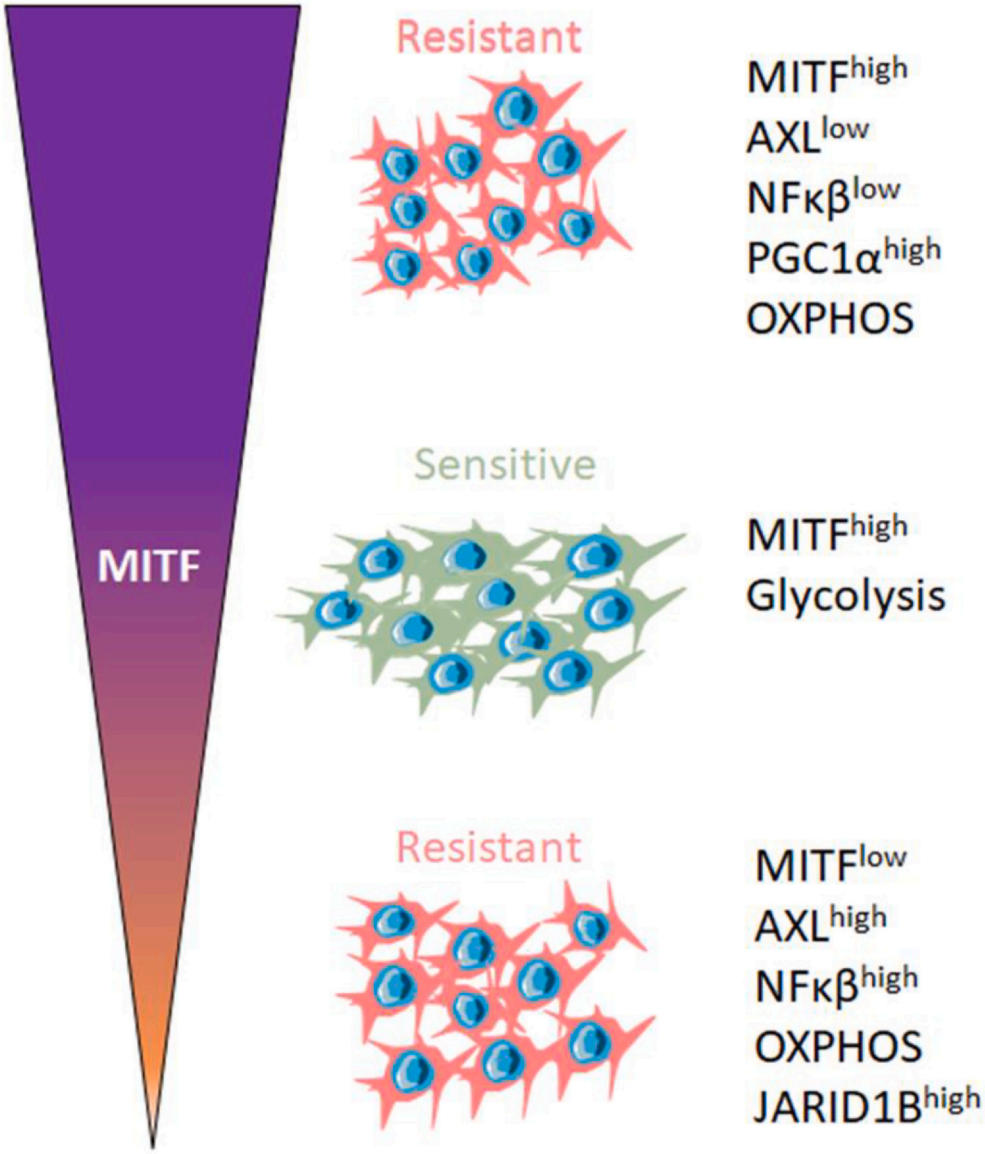

Fig. 3. Distinctive phenotypic states and metabolic signatures in BRAFi treated melanoma cells. Highly proliferative BRAFi sensitive cells exhibit high glycolysis rate and high MITF. BRAFi resistant cells are characterised by OXPHOS activation but can exhibit two distinct cellular states with either high or low MITF. In the $MITF^{high}$ state high OXPHOS is driven PGC1α activation, while the $MITF^{low}$ state is characterised by high AXL and NFKβ, with a high OXPHOS signature driven by JARID1B expression.

5.1. MAPK reactivation-based survival

5.1.1. Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) act as upstream activators of MAPK signalling. Nazarian et al. first demonstrated that increased expression of PDGFRβ conferred resistance to BRAFis [67], which was further demonstrated by others using different cell lines [68,69]. In contrast, another study showed increased expression of EGFR, KIT and MET with decreased expression of PDGFRβ in resistant M249 cells [70]. Supra-physiologic levels of c-MET transcripts have been found BRAFi resistant melanomas [71]. A study by Shaffer et al. suggested that multiple RTKs, such as *AXL, EGFR, PDGFRβ* and *JUN* are expressed in small subpopulation of melanoma cells prior treatment by non-heritable, transient expression [41]. BRAFi treatment selects for an increased proportion cells expressing these RTKs, which mediate resistance through the activation of the MAPK pathway or alternative PI3K/AKT pathway.

5.1.2. NRAS and MEK mutations

NRAS serves as an activating mutation within melanoma encompassing around 28% of melanomas, with Q61R being the most common [10]. NRAS mutants preside within both combinational and monotherapy cohorts [67,72]. *NRAS* mutations as a resistance mechanism have an occurrence of 5–18% [73–75]. Resistant cells with secondary $NRAS^{Q61K}$ mutation require CRAF expression and SHOC2 scaffold protein to re-activate MAPK [76]. BRAF inhibition specifically, not drug binding, drives wild-type BRAF binding to CRAF and activation of MEK [77]. Despite the theoretical and preclinical support for CRAF overexpression to mediate BRAFi resistance it has yet to be reported within clinical samples [78].

MEK1 mutations are rare in melanoma and are often associated with either *BRAF* or *NRAS* mutations [10]. *MEK1* mutations within either exon 3 or 6 were found to confer resistance to BRAF inhibition [79]. Further studies also supported that MEK1 mutations in $BRAF^{V600E}$ melanomas are linked to both intrinsic and acquired resistance to BRAFis [22,80]. Various meta-analysis studies have described an

overall incidence of 7–8% for *MEK1/2* mutations in BRAFi monotherapy and BRAFi plus MEKi -resistant melanomas [72,73]. In contrast, Shi et al. showed that pre-existing exon 3 mutations, *MEKP*^{124S} and *MEK*^{111S}, do to not confer resistance to vemurafenib [81]. It has been postulated *MEK1* exon 3 mutations are not constitutively activating but render MEK1 more readily activated. Further studies are required to disentangle the role of MEK mutations in pathogenesis and treatment resistance of melanoma.

5.1.3. BRAF amplification

The overproduction of BRAF^{V600E} due to the genetic amplification of the mutant gene has been established as a common mechanism of resistance to both BRAFi or BRAFi plus MEKi [72,82], confirmed by various large studies of clinical specimens [72–75]. *BRAF*^{V600E} amplification drives resistance through the excess generation of activated MEK, which in turn activates downstream constitutes of the MAPK pathway. *BRAF* amplification and alternative splicing were observed most frequently followed by *NRAS* mutations and *MEK1/2* mutations [73].

5.1.4. BRAF splicing variants

Resistance to the BRAFis can also be conferred through the production of aberrantly spliced *BRAF*^{V600E} isoforms that lack the RAS binding domain (RBD) encoded by exons 3–5 [83]. These splicing variants lacking the RBD, can dimerize in the presence of low levels of RAS and confer drug resistance [83]. Four *BRAF* splicing variants have been described, referred as p61, p55, p48 and p41 based on their predicted molecular weight. Alternative *BRAF* spliced isoforms have been identified in patients progressing on BRAFi alone and in combination with MEKis and as in preclinical models [83–86]. In fact, expression of aberrantly spliced *BRAF* ^{V600E} isoforms mediates resistance in 13–30% of melanoma patients [73–75]. Although *BRAF* splicing variants are capable of conferring resistance to BRAFi, cell line studies have shown that melanoma cells carrying splicing variant remained susceptible to MEK inhibition [72]. Moreover, enhanced association between *BRAF* splicing variants and their substrate, MEK, that is required for resistance to BRAFis [87].

5.1.5. COT alterations

The Ser/Thr MAP kinase *MAP3K8* (or COT) has the potential to directly phosphorylate MEK to trigger downstream cascades. Johannessen et al. reported that COT expression was associated with acquired resistance to BRAFi in melanoma cell lines and tissue obtained from relapsing patients following treatment with MEKis or RAF inhibitors [88]. Over activation of MEK within the cell line A375 was established to occur through COT signalling, also generating resistance to the MEKis, selumetinib and CI-1040 [88].

5.1.6. STAG 2 and 3 alterations

Loss-of-function mutations in STAG2 and decreased expression of STAG2 and STAG3 proteins in several tumour samples from patients with acquired resistance to BRAFi and in BRAFi-resistant melanoma cell lines [89]. Furthermore, STAG3 mutations were found 3/14 pre-treatment samples of patients who developed resistance vemurafenib within 12 weeks of treatment and the post-relapse sample of another 6 cases [89], suggesting STAG2 mutations to mediate intrinsic as well as acquired BRAFi resistance (Fig. 2). STAG2 knockdown let to decreased dual-specificity phosphatase 6 (DUSP6). DUSP6 acts as a negative regulator of ERK activation [90]. Thus, STAG2/3 alterations result in ERK activation, by limiting dephosphorylation.

5.1.7. BOP1 downregulation

The ribosome biogenesis protein Block of Proliferation 1 (BOP1) acts as a regulator of DUSP4 and DUSP6 [90,91]. Unlike STAG knockdown, which saw a reduction in DUSP6 but not DUSP4, loss of BOP1 generated a reduction in both, leading to an increase in MAPK signalling [89,91] (Fig. 2). A small scale investigation into patient samples both pre and post BRAFi alone or in combination with MEKi revealed a reduced protein expression of BOP1 within 7 of the 11 cases that relapsed [91]. The results of this study, though initial highlight another escape mechanism that can be utilised by melanomas.

5.2. MAPK independent based survival

5.2.1. PI3K-AKT pathway

Another established pathway is the PI3K/AKT pathway [92]. The induction of the PI3K-AKT by insulin could protect BRAF^{V600E} cells from vemurafenib [93]. Cross-talk between the PI3K and MAPK pathways has been established, with BRAFi resistant cell lines utilising AKT to trigger ERK within MAPK for cell survival, circumventing both BRAFis and MEKis [94]. *AKT1* mutant based resistance to BRAFis has been identified previously in progressive patient samples to monotherapy [74]. Recent work demonstrated that PI3K activity is capable of promoting survival but not proliferation of cell lines when challenged with BRAFis plus MEKis [95].

5.2.2. WNT5A/β-catenin pathway

Continuous BRAFi in *BRAF*-mutant cell lines results in elevated WNT5A transcripts. Furthermore, 7 out of 11 tumours from patients who progressed in BRAFis presented increased WNT5A expression compared to pre-treatment samples [96]. *In vitro* studies demonstrated that a loss of WNT5A reduced the viability of the cells in the presence of BRAFis [96]. WNT5A overcomes BRAF inhibition through the increased phosphorylation of AKT and activation of RYK and FZD7 receptors supporting non-canonical WNT signalling [96].

6. Potential strategies to overcome resistance

As discussed above, intrinsic and acquired resistance primarily involve the reactivation of the MAPK pathway. Concomitant inhibition of the BRAF and MEK nodes to overcome the MAPK signalling from hyperactivation of MEK has shown increase PFS, but still emerge similar resistance mechanisms [72]. Reactivation of ERK is central to most acquired resistance mechanisms. Thus, next generation ERK inhibitors have been suggested as a suitable target for effective MAPK inhibition, with molecules such as LY3214996 (NCT02857270) and BVD-523 (NCT02465060) progressing into clinical trial [97]. However, the success of this approach remains uncertain considering the role of feedback loops such as DUSP/SPRY which could lead to reactivation of the MAPK pathway [32].

To address the upregulation of RTK as mechanism of resistance, combination of BRAF/MEK blockade with RTK inhibitors (*e.g.*, LY3022855, lapatinib and foretinib) are currently in trials to prevent the MAPK reactivation (NCT03101254, NCT03455764) [98]. Increased RTK signalling was reported to activate SRC/FAK/STAT3 signalling leading to invasive phenotype [99]. Thus, combination of BRAF/MEK blockade with SRC, FAK or STAT3 inhibitors (SAB298, saracatinib) have been suggested to target the BRAFi-resistant melanoma population with high dedifferentiated state or invasive phenotype [99].

Combination of BRAFi/MEKi with PI3K inhibitors have been trialled to block the activation of parallel signalling by the AKT/PI3K pathway, but found associated with increased toxicities or unmet therapeutic goals due to rapid clearance [100]. Alternatively, combination of mTOR inhibitors with BRAFi was thought to be effective in tumours with PI3K/AKT pathway activation, by inducing apoptosis. Preclinical evidence also demonstrated that the combination of mTOR inhibitors with MAPKi to desensitise the high MITF expressing cells with high OXPHOS and mitochondrial activity [45]. Initial phase I trials showed positive outcome with limited toxicity (NCT01596140) [101], but treatment efficiency evaluation in larger cohort of molecularly matched patients is still needed. Another approach trialled in overcoming intrinsic and acquired resistance is the combination of MAPK inhibition and

apoptosis induction by targeting molecules such as BCL-2 family protein (NCT01989585), CDK4/6 (NCT02645149) [102], histone deacetylase (NCT02836548) and heat shock protein 90 (NCT02097225) [103].

Acknowledging the understanding that cell plasticity enables the survival of a dormant population of MAPK-inhibited melanoma cells, multiple approaches have been tested to overcome adaptive resistance. For example, the combination of BRAFi with hydroxychloroquine is able to re-sensitise resistant cells by inhibiting cytoprotective autophagy induced by the ER stress response (NCT03754179) [61]. Other strategies have involved the inhibition of PAX3 mediated MITF upregulation using the HIV protease inhibitor nelfinavir [42], or by targeting AXL with the antibody conjugate AXL-107-MMAE [104]. On the other hand, nicotinamide phosphoribosyl transferase inhibitors and the combination of FAO and glycolysis inhibitors, may avoid the early metabolic reprogramming induced by MAPKi that underscores adaptive resistance [59,105].

Despite the multiple alternatives being tested, the benefits of these approaches might not be apparent in a clinical setting when patients harbour multiple active resistance mechanisms [106]. Clinical and preclinical evidence has demonstrated diverse resistance mechanisms within the same tumour, underscoring the contribution of genomic heterogeneity to BRAFi treatment failure. Hence, early treatment in the adjuvant setting may be the best strategy to avoid the emergence of resistance. In line with this, the introduction of combination MAPKi therapy into stage III melanoma has had a significant impact in the prevention of relapse post intervention [107].

7. Major conclusions

Overall, acquired resistance to BRAF inhibition depends on oncogenic signalling through reactivation of MAPK or activation of alternative pathway such as the PI3K/AKT pathway. Resistance can be acquired by upregulation of receptor tyrosine kinases signalling, by directly affecting genes in these pathways, or by enhancing downstream signalling. However, the mechanisms underlying acquired drug resistance are hugely diverse, with evidence of high inter- and intra-tumour heterogeneity [75]. These resistance mechanisms are distinct to those observed following targeted therapy treatment in other cancer types. For example, no secondary “gatekeeper” threonine mutations in BRAF have been observed, which is a common resistance mechanism to other kinase inhibitors [108,109].

As reviewed above, multiple studies have identified numerous mechanisms of resistance in patients failing single-agent BRAFis or in combination with MEKis. Interestingly, the vast majority lead to the reactivation of the MAPK pathway underscoring its importance for melanoma cell maintenance [110]. In addition, to those events observed only in treated tumours (acquired resistance), several resistance effectors already exist in pre-treatment samples (intrinsic resistance), in some cases constituting a tumour cell subpopulation. However, cumulative they still failed to explain all the observed clinical relapse cases. Thus, further studies are needed to elucidate the complete landscape of resistance mechanisms.

The picture is less clear for cellular processes conferring adaptive resistance. Prior to relapse, targeted therapy induces cellular adaptations to survive treatment, and within these cells acquire mechanism of resistance develops. Now it is better understood the role of tumour plasticity and metabolic reprogramming in these adaptation processes [51,111–113]. This has led to new treatment paradigms suggesting the combination of oncogenic inhibitors with metabolic targets. This may provide effective control of tumour growth, by avoiding the survival of cell subpopulations from which acquired resistance may emerge.

Finally, the emergence of effective immunotherapies provides alternative treatments with the potential to deliver long term control of melanoma growth [114]. The identification of increased tumour immune infiltrate in BRAFi treated melanomas, combination with immunotherapies were thought to enhance tumour control (NCT02902042, NCT02858921). However, these combinations have significant associated toxicities [115]. Sequential administration of targeted and immunotherapy also has its limitations, with translational studies suggesting that an immuno-resistant phenotype emerges on progression after BRAF inhibition [116]. Moreover, innate and adaptive BRAFi resistance mechanisms overlap with that found in tumours failing immunotherapy such as MITF/AXL programming, WNT pathway activation and PTEN loss [117]. In this context, the rationale for treatment selection may need to be based on the phenotypic and molecular characteristics of the pre-treatment tumours, which requires a clear and comprehensive understanding of the mechanism mediating resistance.

8. General significance

Although treatment with BRAFis provides rapid response in most melanoma patients, at present the emergence of resistance remains unavoidable. Numerous studies have addressed the mechanisms underlying the rapid emergence of resistance, but only half of these cases can be explained by the known mediators. Preclinical studies support the mechanisms observed in patients, indicating that the development of resistance is more complex than a single mutation. Further studies are required to better understand BRAFi resistance and to aid the developing strategies that can retain long-term durable responses of combination therapy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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