



Review

Many ways to resistance: How melanoma cells evade targeted therapies

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ABSTRACT

Melanoma is an aggressive malignancy originating from pigment-producing melanocytes. The development of targeted therapies (MAPK pathway inhibitors) and immunotherapies (immune checkpoint inhibitors) led to a substantial improvement in overall survival of patients. However, the long-term efficacy of such treatments is limited by side effects, lack of clinical effects and the rapidly emerging resistance to treatment. A number of molecular mechanisms underlying this resistant phenotype have already been elucidated.

In this review, we summarise currently available treatment options for metastatic melanoma and the known resistance mechanisms to targeted therapies. A focus will be placed on “phenotype switching” as a mechanism and driver of drug resistance, together with an overview of novel approaches to circumvent resistance. A large body of recent data and literature suggests that tumour progression and phenotype switching could be better controlled and development of resistance prevented or at least delayed, by combining drugs targeting fast- and slow-proliferating cells.

1. Introduction

Melanoma is a malignancy that develops from melanocytes, the melanin-producing cells [1]. Despite being a rare type of skin cancer, it is responsible for the vast majority of skin cancer-related deaths [2]. Additionally, metastatic melanoma is one of the most highly mutated, heterogeneous and lethal types of cancer [3]. The most prominent mutations in melanoma affect the serine/threonine kinase BRAF (50%), the small GTPase NRAS (25%), or the tumour suppressor and negative regulator of RAS, neurofibromin 1 (NF1) (14%), which all lead to an increased proliferation and survival [3]. Until recently, the treatment options for advanced stage melanoma patients were limited to conventional chemotherapeutic drugs with an overall low efficacy and limited response rate (RR) [4]. Only in the past few years, the progression-free (PFS) and overall survival (OS) of melanoma patients has markedly improved by the introduction of targeted and immunotherapies [5]. Despite the substantial progress that has been made in the clinical management of advanced melanoma, treatment failures, severe side effects and intrinsic as well as acquired resistances against all forms of current therapies warrant continued research efforts to find more efficient, durable and potentially personalised treatment options. The identification of mechanisms underlying the switch from a drug sensitive to a drug resistant phenotype has been the focus of melanoma research in the past few years. This review provides an overview of past and present treatments and summarises mechanisms of resistance to targeted therapies and highlights new potential drug targets that have

emerged in recent studies on metabolic effects, slow cycling tumour cells, phenotypic switching, as well as ER-stress, autophagy and miRNA-mediated resistance mechanisms.

2. Therapeutic options for melanoma patients

Current therapeutic options for melanoma patients mainly consist of surgical excision, chemotherapy, immunotherapy, and targeted therapy [6]. These therapies can be administered as single agents or in combination depending on the stage of the disease, location and genetic profile of the tumour, as well as the general health and the age of the patient (Fig. 1).

The main curative treatment for accessible and early stage cutaneous melanoma tumours is **surgery**. Metastatic melanoma, however, is very unlikely to be cured by surgery due to the often high number of metastases, low accessibility and the difficult detection of small metastatic lesions by commonly used imaging tools [7]. **Chemotherapy**, consisting of temozolomide (TMZ) and dacarbazine (DTIC), is commonly used in late stage melanoma patients with progressive, refractory, or relapsed disease [8] (Fig. 1). In addition, high doses of interferon α -2b (IFN α -2b) and interleukin-2 (IL-2), which have been FDA-approved as single agents in 2011 and 1998, respectively, are applied to resected stage II/III patients, and in some cases to stage IV melanoma patients albeit with limited success [9,10]. A major progress in treatment of several solid cancers was made by monoclonal antibodies that function as **immune checkpoint inhibitors** [11–13]

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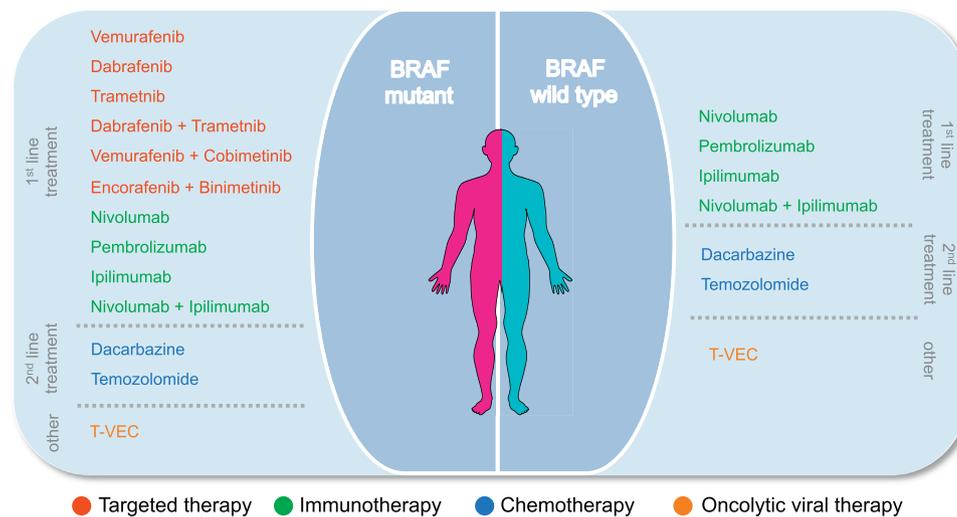
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treatment using chemotherapeutic agents such as dacarbazine or temozolomide. Additionally, melanoma tumours can be treated locally using oncolytic virus-based therapy (T-VEC).

(Fig. 1). The cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) inhibitor (ipilimumab) and programmed cell death protein 1 (PD-1) inhibitors (pembrolizumab and nivolumab) show an increased overall response rate (ORR), median PFS and OS in melanoma patients. Albeit the convincing results in about 30% of advanced stage patients, most patients either do not respond to immunotherapies or have severe side effects, which make a cessation of treatment necessary [14]. **Oncolytic viruses** have recently been integrated in anti-tumour therapies due to their capacity of directly lysing tumour cells, leading to the release of soluble antigens and interferons that drive antitumor immunity [15]. Currently, the attenuated herpes simplex virus-based oncolytic virus talimogene laherparepvec (T-VEC) is the only approved oncolytic virus for cancer treatment and has been FDA-approved in 2015 as local treatment of unresectable advanced stage melanoma [5,15] (Fig. 1). Although the use of oncolytic viruses in combination with targeted therapies or immunotherapies has shown promising results, only limited data on optimal dosing and scheduling of the different therapies exists, and an appropriate patient eligibility for T-VEC-based mono- or combination therapy is still lacking [16].

The identification of mutations in the serine/threonine kinase BRAF, which result in the constitutive activation of the MAPK pathway in > 50% of melanoma patients, has fuelled the generation of **targeted therapies** with small molecule inhibitors acting on mutated BRAF [17] (Fig. 1). Vemurafenib was the first FDA approved BRAF inhibitor (BRAFi) to be administered in patients with advanced stage melanoma from 2011 onwards, as it showed improved PFS and OS and significant tumour reduction compared to chemotherapy [18]. Two years later, another BRAF-specific inhibitor, dabrafenib was FDA-approved, which had fewer side effects and higher potency than vemurafenib [4,19]. Although these inhibitors initially showed an excellent response with significant reduction of tumour burden, long-term success is still scarce because of the development of drug resistance, which will be further discussed below [20].

Due to the frequent BRAFi-induced reactivation of the MAPK pathway, MEK inhibitors (MEKi) have been developed (Fig. 1). Trametinib, which blocks MEK1/2, was the first MEKi for metastatic melanoma to receive FDA approval in 2013 [21]. The combined administration of BRAFi and MEKi (Dabrafenib/Trametinib or Vemurafenib/Cobimetinib) extends the PFS compared to BRAFi monotherapy [22,23]. However, once again and similar to BRAFi monotherapy, patients also become irresponsive to the combined treatment within several months of treatment [20,24]. The most recent FDA-approved targeted therapy for advanced stage un-resectable melanoma is the

Fig. 1. Approved treatment options for patients with unresectable metastatic melanoma. The first-line treatment for melanoma patients highly depends on the BRAF mutation status as well as how quickly the disease progresses. BRAF-mutant melanoma patients can receive targeted therapies or immunotherapies as first-line treatment. As treatment responses to single-agent immunotherapy may take longer, targeted therapies with BRAFi monotherapy or in combination with MEKi are preferred if an early response is needed in BRAF-mutant patients. On the other hand, melanoma patients with BRAF wild type tumours usually receive immunotherapies as first-line treatment. Patients that do not respond to BRAFi as first-line treatment can receive immunotherapies as second line treatment, and vice-versa. Additionally, non-targeted therapies can be administered as second line

combination of Encorafenib (BRAFi)/Binimetinib (MEKi), that appears to efficiently delay resistance. BRAF-mutant patients showed a further improvement in PFS and OS compared to Vemurafenib monotherapy [25]. Moreover, recent clinical data show promising results in stage III melanoma patients treated with adjuvant immune- or targeted therapies. For instance, adjuvant pembrolizumab as well as adjuvant dabrafenib/trametinib combination therapy led to a significantly lower risk of recurrence in stage III BRAF^{mutant} melanoma patients [26,27].

The benefits of MAPKi and immune checkpoint inhibitor-based therapies fuelled the interest in combining these two therapeutic regimens to achieve more durable therapy responses in melanoma patients. Although checkpoint inhibitors have shown promising effects in BRAFi-resistant tumours [5], treating patients with immunotherapy after targeted therapy and thus after development of resistance is rather inefficient, as the tumours appear to be less responsive to immunotherapy due to the depletion of intra-tumoral T cells, CD8 T cell exhaustion, as well as lack of antigen presentation [28,29]. Consequently, the benefits of starting patient treatment with targeted therapy followed by immunotherapy or vice versa, as well as the emerging irresponsiveness, remain to be determined and are currently being investigated in ongoing clinical trials [5].

3. Mechanisms of resistance to targeted therapy

3.1. Re-activation of the MAPK pathway

Although BRAFi and MEKi efficiently inhibit the MAPK pathway by reducing ERK activation and thus stalling cell proliferation in cells harbouring mutated BRAF, MAPK pathway reactivation occurs in up to 80% of BRAFi-resistant tumours, indicating that tumour cells highly depend on the MAPK pathway and rapidly adapt to its inhibition [30]. The main mechanisms leading to MAPK reactivation and sustained ERK signalling involve alterations in BRAF, NRAS, MEK, and neurofibromin 1 (NF1) [31,32] (Fig. 2). Additionally, the expression of the RAF isoform, CRAF (RAF1), can reduce the sensitivity to BRAFi and drive resistance via direct MEK activation or via paradoxical transactivation of RAF dimers and subsequent ERK signalling [33]. Also, the kinase COT, also known as TPL2 or MAP3K8, which directly activates MEK/ERK signalling in a RAF-independent manner, is often elevated in BRAFi-resistant tumours [34]. As BRAF depletion leads to an increase in COT protein levels, it was suggested that BRAF might antagonize COT expression levels by altering COT protein stability [35]. Subsequently, COT expression is sufficient to re-activate MAPK signalling by directly

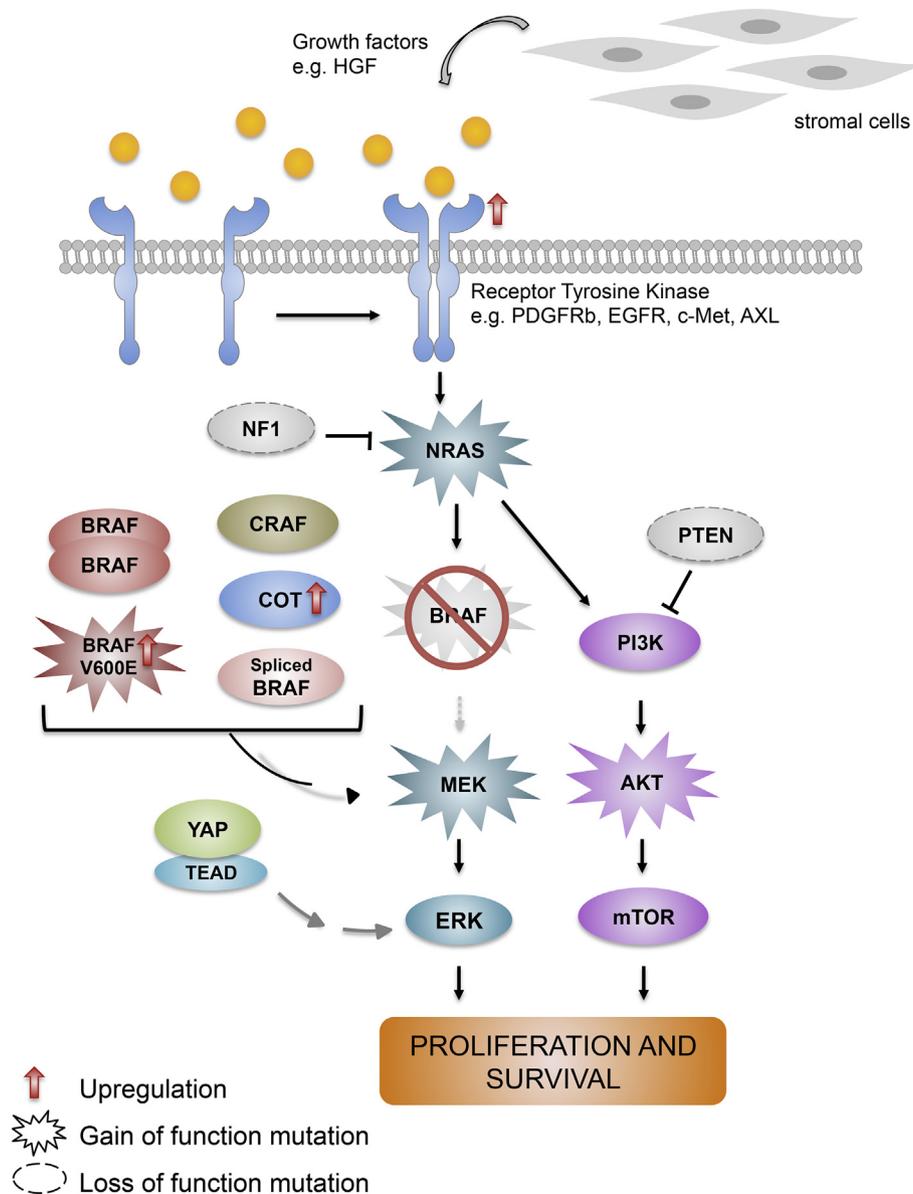


Fig. 2. Common mechanisms of resistance to targeted therapies. Reactivation of the MAPK pathway (80% of resistance cases) and alternative pathways most commonly occurs via mechanisms involving activating mutations in genes involved in proliferation and survival, RAF-mediated resistance mechanisms, loss of tumour suppressor genes, as well as the tumour microenvironment.

activating MEK and is most likely responsible for the de-novo resistance to BRAFi in ~10% of BRAF^{mutant} melanomas [35]. Furthermore, BRAF allele amplification or splice variants, present in up to 30% of patients with BRAFi resistant tumours, were shown to lead to enhanced RAF dimerisation and MEK association due to increased BRAF S729 phosphorylation [36]. In order to reduce the paradoxical BRAFi-induced ERK activation, a new generation of so-called paradox-breaking BRAFi is currently being tested [37].

3.2. Activation of substitutive pathways

Apart from MAPK signalling, the PI3K-mTOR pathway is most commonly activated in drug resistant melanomas (Fig. 2). Increased PI3K signalling can be due to loss of function via gene mutation or deletion of PTEN in 10% of melanomas, or the activation of receptor tyrosine kinases (RTKs) [38–41]. Remarkably, the increased RTK levels can also originate from reduced proteolytic shedding of cell surface receptors in MAPKi-treated cells [42]. The reduced levels of circulating

RTKs can result in an accumulation of RTKs on the cell surface, which increases the flux through proliferation and survival pathways, allowing the cell to bypass the inhibition of ERK signalling [42].

3.3. Tumour microenvironment

The tumour microenvironment is another important factor in drug resistance, as stromal cells have been shown to promote intrinsic resistance to BRAFi through secretion of growth factors and subsequently activating the MAPK or PI3K pathways [43–45] (Fig. 2). Besides, melanoma progression has been linked to an increased abundance of the extracellular matrix (ECM) proteins such as collagen, which can confer stiffer and more rigid properties to the ECM that favour tumour cell proliferation [46]. The overall density of melanocytes throughout life appears to be controlled, among others, by the Hippo signalling pathway, which plays a role in controlling organ size in animals by negatively regulating YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) activity [47,48].

Interestingly, collagen stiffness appears to be regulated by fibroblast-secreted TGF- β , which in turn regulates nuclear YAP localisation as well as melanoma cell adhesion [46,49]. Consequently, YAP/TAZ and their transcriptional binding partner TEAD (TEF transcription factors/TEA domain) are connected to the de-differentiated and invasive phenotype and have further been shown to initiate tumour progression and metastasis, as well as conferring drug resistance to targeted therapies in melanoma [50–52]. The involvement of this important signalling pathway in evading targeted therapies will be discussed in more detail in the section on “phenotype switching”.

3.4. Autophagy and ER stress

Tumour cells can adapt to drug-induced stress by upregulating autophagy, which was increased in 74% of patients treated either with BRAFi monotherapy or in combination with a MEKi, resulting in lower RR and PFS [53–55]. The mechanisms leading to a BRAFi-mediated autophagy induction include ER stress and TAM (TYRO3, AXL, MER) receptor pathway activation [56–58]. Thus, the application of the autophagy inhibitor hydrochloroquine (HCQ) was able to re-sensitize resistant cells to BRAFi [53,59]. Furthermore, an excessive increase in ER stress-mediated autophagy can lead to cancer cell death [60]. HA15, a thiazole benzenesulfonamide-based compound that specifically targets the chaperone BiP/GRP78/HSPA5 caused increased ER stress and subsequently apoptosis and autophagy, which was shown to trigger cell death of both BRAFi-sensitive and -resistant cells [60]. Compounds triggering autophagy and/or apoptosis alone or in combination with targeted therapies might therefore constitute a promising group of new treatment approaches.

3.5. miRNA-mediated resistance mechanisms

MicroRNAs (miRNAs) are ~22 nucleotide short non-coding RNA molecules known to regulate the expression of genes and proteins involved in the MAPK as well as other resistance-associated pathways [61,62]. In this context, miR-509-3p, miR-204-5p, and miR-211-5p are rapidly upregulated in response to short-term BRAFi treatment [63,64]. miR-204-5p and miR-211-5p whose expression is induced by the transcription factors signal transducer and activator of transcription 3 (STAT3) and Microphthalmia-associated transcription factor (MITF), respectively, appear to confer BRAFi resistance by reactivating the MAPK or PI3K/AKT pathway, while the exact effect of drug-induced and resistance-associated miR-509-3p upregulation remains to be elucidated [63–65]. Additionally, miR-550a-3-5p, which acts as a tumour suppressor in several different cancers, is downregulated in resistant melanomas, and it has the potential to reverse BRAF-mediated resistance by directly targeting YAP, which can mediate drug resistance [50]. A more exhaustive list of miRNAs involved in drug resistance can be found elsewhere [65,66].

3.6. Therapy-mediated selection of resistant tumour cell subpopulations

Intra-tumour heterogeneity, which mostly results from genetic and epigenetic variations, is considered to impact on disease evolution and progression [67]. Consequently, drug resistance can arise by a “Darwinian-type” selection of pre-existing subclones with cancer stem cell-like properties that are able to withstand drug treatment [68]. Alternatively, cancer cells can become resistant by acquiring genetic mutations or by rewiring the epigenome or metabolome under drug treatment-mediated selection pressure, in a “Lamarckian-type process” [69,70]. It is unclear whether the observed adaptations of cancer cells arise from selection of pre-existing subclones or rather by tumour cell plasticity or both. Schaffer et al. have observed that cells do not accumulate mutations that would provide a selective advantage in presence of the BRAFi, but rather that cells develop resistance to BRAFi by temporary and reversible adaptations to selective pressure (cell

plasticity), a concept that is also supported by previous studies [28,68]. High levels of i.e. AXL, EGFR, and WNT5A have been associated with the resistant phenotype in melanoma and could be a potential mechanism of resistance [68]. These genes are expressed sporadically on single cell level prior to drug exposure, thus the cells that are capable of temporarily upregulating these genes in presence of BRAFi are more likely to become resistant [68,71,72]. The transient transcriptional state is converted to a stably resistant state upon drug-induced epigenetic reprogramming, which is initiated by the SOX10-mediated de-differentiation, and thereby activating several transcription factors, including TEAD [68]. SOX10 is known to regulate neural crest development in melanocytes [73], whereas TEADs play a role in regulating invasion in melanoma [74]. These data suggest that the transition to the stably resistant state is characterised by a generalised de-differentiation followed by activation of several different new signalling pathways, which confer survival advantages in the presence of drugs.

4. Phenotype switching

Melanoma cells are not only capable of rapidly adapting to therapies by acquiring mutations, but they also tend to switch their molecular and cellular phenotype in an epithelial-to-mesenchymal transition (EMT)-like manner, in order to bypass drug treatment. The most common phenotypic changes that melanoma cells undergo to escape inhibition are linked to the expression of the master transcription factor MITF and the RTK AXL and implicate, among others, differentiation/de-differentiation, changes in proliferation rates, and metabolic rewiring (Fig. 3). MITF is a melanocyte lineage-specific transcription factor that is required for melanoblast survival, it plays important roles in melanocyte development from neural crest precursors, and it regulates the expression of pigment-producing enzymes and proteins participating in melanosome export in response to environmental triggers (e.g. UV) and extracellular signals (e.g. melanocyte stimulating hormone, MSH) [75]. AXL, on the other hand, belongs to the TAM (TYRO3, AXL, MERTK) family of RTKs, which are commonly expressed on macrophages and which are activated in response to the Growth arrest-specific 6 (GAS6), thus playing a role in inflammatory responses [57]. Several recent studies have attributed an important role to AXL in melanoma, as its level are often elevated and inversely correlate with MITF expression

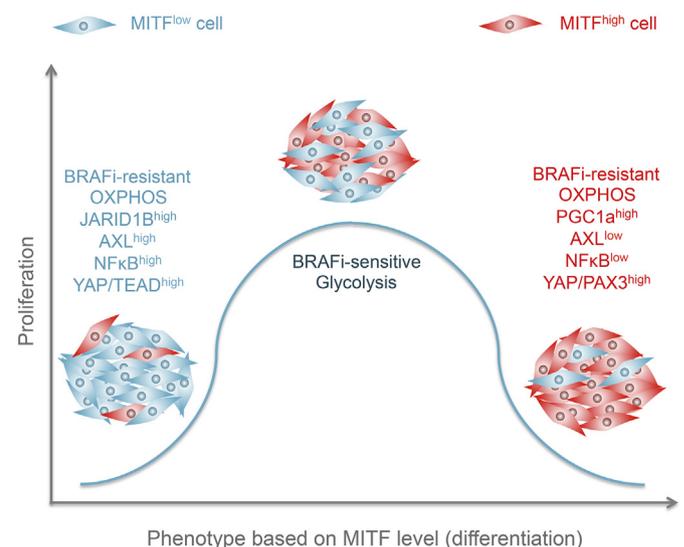


Fig. 3. MITF-linked phenotype switching. Proliferation rate based on the molecular phenotype and MITF levels (differentiation). Melanoma tumours are highly heterogeneous with subpopulations of cells expressing high or low MITF levels, which usually respond well to targeted therapies. However, during drug treatment, tumours can switch their phenotype to an MITF^{high} or AXL^{high} slow proliferating state in order to bypass BRAFi.

patterns in BRAFi-resistant melanomas [71,72,76,77]. In the following paragraphs, we provide an overview of most important phenotypic switches in melanoma that are often associated with MITF and AXL expression patterns.

4.1. Differentiation/De-differentiation

The invasive and de-differentiated phenotype is a prerequisite of cancer metastasis. It has been demonstrated that melanoma cells have a de-differentiated phenotype during the process of invasion and metastasis formation, which is characterised by low pigmentation and reduced proliferation. Once the cells reach the secondary site where the metastatic growth is formed, cells switch back to a differentiated, highly pigmented and proliferative phenotype [78]. These observations indicate that the switch to a differentiated phenotype is most probably induced by factors from the microenvironment (e.g. endothelin 3 (EDN3)) [79]. MITF expression heterogeneity is a commonly observed phenomenon with high and low MITF expressing subpopulations of cells, which have been suggested to confer different phenotypes, as well as modulate sensitivity to drug treatment [80,81] (Fig. 3). Recently, Tsoi and colleagues have identified four distinct differentiation states in melanoma thus providing evidence for the development of drug resistance through a stepwise de-differentiation process with intermediate transcriptional programs, further highlighting the plasticity of melanoma cells. The four phenotypes (undifferentiated, neural crest like, transitory, melanocytic) have overlapping characteristics and can be defined by the expression of a defined set of genes (e.g. MITF, AXL) [80]. Subsequently, melanoma cells can bypass targeted therapies by transitioning from one phenotype to another, accompanied by differential MITF expression. While the role of MITF in differentiation is well described, more and more studies link MITF expression levels to the cell proliferation rate.

4.2. Proliferation rate

Current cancer therapies mainly target fast proliferating cells, leaving slow-proliferating cells largely undamaged. Consequently, these slowly proliferating cells often become enriched during treatment and gain proliferative characteristics, causing tumour relapse [82] (Fig. 3). The switch to a slow proliferating phenotype is often mediated via histone demethylase-mediated chromatin remodelling factors, such as Jumonji/ARID domain-containing protein 1B (JARID1B) that can be regulated by hypoxia and several cytokines, and which lead to an increased flux through the PI3K/AKT pathway [83,84]. Although a recent study has reported that inducible MITF downregulation reflects, rather than causes EMT-like changes, and that low MITF levels result in de-differentiation but not necessarily in reduced proliferation or phenotype switching [85], several other studies have linked MITF levels to phenotype switching [71,72] (Fig. 3). These slow cycling melanoma cells tend to be de-differentiated and treatment-resistant, which is characterised by a MITF^{low}/JARID1B^{high} gene expression profile [84]. Additionally, MITF levels show inverse correlation with other markers of the slow cycling, invasive and drug-resistant phenotype, such as AXL or NF-κB [86]. In this context, combinatorial targeting of fast and slow cycling melanoma cells using a combination of MAPKi and an AXL antibody-drug conjugate (AXL-107-MMAE) has shown promising results [76]. Taken together, as slow-cycling cells display invasive properties and play a role in drug resistance as well as in early tumour relapse in early stage melanoma patients, their enrichment during treatment could be prevented by combining therapies that target both fast and slowly proliferating cells [76,82] (Fig. 3).

4.3. Metabolic rewiring

MITF and JARID1B, do not only have an impact on the cell proliferation rate, but together with PPARC coactivator 1 alpha (PGC1α),

they are important mediators of metabolic switches in response to drug resistance (Fig. 3). While de-differentiated, slow-cycling and drug-resistant melanoma cells often display a MITF^{low}/JARID1B^{high} ratio, differentiated, slow cycling and therapy-resistant melanoma cells often have a MITF^{high}/PGC1α^{high} ratio [87,88]. Also, upon BRAFi treatment, oxidative phosphorylation (OXPHOS) and reactive oxygen species (ROS) are increased in a PGC1α-mediated manner, which is directly triggered by MITF [89]. Usually, cells with high OXPHOS also have high amounts of ROS, thus slow cycling cells are more sensitive to drugs that promote oxidative stress [90]. Hence, a combination of BRAFi with drugs promoting oxidative stress might combat resistance in these slow-cycling cells. In contrast, reduced OXPHOS levels were observed as an immediate response to BRAFi, leading to the phosphorylation of the pyruvate dehydrogenase (PDH) complex as well as to upregulation of ROS in BRAF^{V600E} and BRAF^{wt}/NRAS^{mutant} cells [91]. This BRAFi-induced increase in ROS production could be impaired when using ROS scavenger compounds [91]. As PDH is only phosphorylated upon short-term BRAFi and not in BRAFi-resistant melanoma cells, adaptive metabolic rewiring might occur during prolonged drug treatment. Also, BRAFi-resistant cells with high ROS levels have acquired vulnerability towards histone deacetylase inhibitors (HDACi), which are known to further increase ROS levels [92]. Therefore, a sequential treatment with BRAFi that induce increased ROS, followed by HDACi (Vorinostat), which further increase ROS levels, might more efficiently induce cell death and eradication of resistant melanoma cells [92].

4.4. Roles of MITF and AXL in phenotype switching

Single cell sequencing has revealed that melanoma tumours display high intra-tumour heterogeneity and contain both MITF^{high} and MITF^{low} cells [71,72]. The switch from a MITF^{high}/AXL^{low} to MITF^{low}/AXL^{high} has been described as a mechanism of resistance to targeted therapy in a subset of melanoma patients as well as in in vitro cell culture systems [86,93].

In order to explain the heterogeneous effects of MITF, the so-called rheostat model was introduced, which among others, describes the different MITF-driven phenotypes [94] (Fig. 3). Subsequently, some studies reported that a MITF^{high} state is associated with MAPKi therapy resistance and poor prognosis [95,96], whereas others show that a MITF^{low} state in combination with high expression levels of several RTKs (e.g. AXL) is responsible for therapy resistance [76,93]. MITF^{high} tumours were shown to be responsive to MAPKi, however, tumours that were initially MITF^{low} upregulate MITF upon treatment, causing the development of resistance [96].

The paired-box transcription factor (PAX3)-mediated overexpression of MITF is implicated in reversible early drug resistance [96] (Fig. 4). Very recently, the same group showed that BRAF regulates MITF levels via the transcription factors PAX3 and BRN2, providing an explanation for the dynamic MITF levels in patients in response to targeted therapy [97]. Targeting the MITF “build-up” via PAX3 depletion could postpone the development of resistance, and re-sensitize melanoma cells to MAPKi, which inhibit PAX3 via SMAD2/4 and the salt-inducible kinase (SKI) [96]. SMAD2/4 phosphorylation is induced by TGF-β, and leads to the formation of SMAD2/4/SKI repressor complex, which suppresses the expression of PAX3 and thus the expression of MITF [96]. On the other hand, drug-resistant melanoma cells and patient biopsies are rather characterised by a MITF^{low}, AXL^{high}, and NFκB^{high} phenotype [86]. Short-term overexpression of mutant BRAF in melanocytes induced an MITF^{low}/NFκB^{high}/AXL^{high} phenotype, which was partially reversed using an IκBα super-repressor, suggesting that AXL is induced by NF-κB signalling [86]. Overexpressing MITF interferes with the BRAF and MEK mutation-induced AXL expression and TNFα-mediated NFκB induction led to reduced MITF expression and activity, further supporting the role of MITF and AXL in phenotype switching [86].

BRAFi resistance can also be mediated via the AXL/AKT axis in

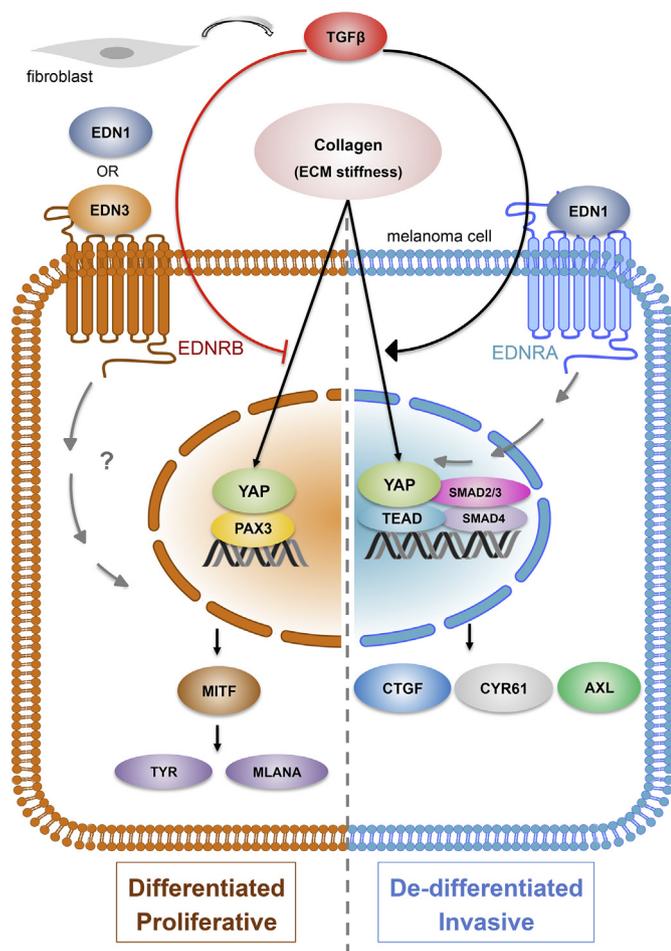


Fig. 4. Potential mechanisms regulating the MITF/AXL ratio and phenotype switching. A low MITF/AXL ratio has been linked to BRAFi-resistance in melanoma, however, the exact mechanisms leading to a switch from a differentiated, proliferative, MITF^{high}/AXL^{low} to a de-differentiated, invasive, MITF^{low}/AXL^{high} phenotype are not fully understood. The scheme summarises promising recent data obtained in studies on melanoma and other cancers (e.g. HCC and CRC) that give insight into the potential regulation of this phenotype switch including the role of the tumour microenvironment (e.g. collagen stiffness), YAP and EDN signalling.

PTEN^{WT} melanoma cells or via ERK signalling in PTEN^{mutant} melanoma cells [38]. MAPK signalling appears to be reactivated in all BRAFi resistant cell lines regardless of PTEN status, whereas AKT signalling is only reactivated in PTEN^{WT} cells. Additionally, the resistant melanoma cell models with PTEN^{WT} exhibited significantly higher AXL-driven AKT activity compared to their corresponding parental cells. On the other hand, resistant melanoma cell lines with PTEN deficiency showed low AKT activity, which suggests that AKT-mediated BRAFi resistance is only occurring in melanoma cells with PTEN^{WT} [38].

Although, the exact mechanism of how cells switch from one phenotype to the other is not yet fully understood, a low MITF/AXL ratio predicts early resistance to several targeted therapies. In this context, targeting AXL^{high} subpopulations of cells using an AXL antibody drug conjugate (AXL-107-MMAE) might be beneficial for MITF-low, BRAF- and NRAS-mutant tumours and a combination of MAPKi and AXLi could support the elimination of resistant melanoma cells [76]. Although it is generally accepted that either way of the inverse correlation of MITF and AXL expression levels is an important denominator of cell plasticity, recent studies in melanoma and other cancers also suggest YAP-mediated mechanisms to drive switching from one phenotype to another.

4.5. YAP-mediated phenotype switch

Hippo signalling is often reduced in cancer, leading to an accumulation of YAP/TAZ complexes in the nucleus and augmented cell proliferation and survival via increased ERK1/2 activity [51,79]. YAP-mediated BRAFi resistance mechanisms have been reported in melanoma as the overexpression of YAP can restore BRAFi-mediated ERK inhibition in drug-sensitive melanoma cell lines [51] (Fig. 4). YAP-induced BRAFi-resistance is mediated via the YAP/TAZ/TEAD axis, favoring an invasive, de-differentiated and slow cycling phenotype [46,51]. Verteporfin is a drug that blocks YAP function in BRAFi-resistant melanoma cancer stem cells by inhibiting the interaction between YAP and TEAD, and thereby reducing nuclear YAP/TAZ levels, ERK1/2 signalling and finally tumour growth [51,98]. Combination of BRAF and YAP inhibition could be another promising approach to overcome drug resistance mechanisms in melanoma (Fig. 4, Fig. 5).

Additionally, fibroblast-secreted TGF- β can induce a switch from a YAP/PAX3/MITF to a YAP/SMAD/TEAD signalling cascade, which is supported by the fact that TGF- β can inhibit PAX3, hence reduce the binding of YAP to the MITF promoter and favoring a MITF^{low}-TEAD-regulated invasive phenotype [46,74,99,100] (Fig. 4). While YAP binding to the MITF promoter decreases in presence of TGF- β , it increases at the promoters of the TEAD target genes, connective tissue growth factor (CTGF), Cysteine rich protein 61 (CYR61), and AXL, also establishing a differentiated slow cycling phenotype [46,101]. A study in hepatocellular carcinoma (HCC) cells revealed AXL mediated YAP-dependent oncogenic functions, e.g. increased anchorage-independent growth, tumour formation, invasion and migration [101]. The AXL promoter region contains four putative TEAD-binding sites, 1200 bp upstream of the AXL transcription start site (TSS), and the binding of YAP and TEAD to the AXL promoter was confirmed by ChIP and luciferase assays in HCC cells [101]. AXL has also been described to activate the PI3K/AKT and MAPK/ERK pathways, hence high YAP levels induce AXL expression that in turn induces ERK1/2 and AKT activity [101] (Fig. 5).

Moreover, the vasoconstrictor peptide endothelin 1 (EDN1) was shown to promote colorectal cancer (CRC) growth by activating YAP/TAZ signalling through the G-protein coupled receptors endothelin receptor A and B (EDNRA and EDNRB), which have an impact on cell growth [93,102]. EDNRA activation via EDN1 has been reported to initiate the expression of TEAD-targets, CTGF and CYR61 [103]. Additionally, endothelial cells can promote pigmentation through EDNRB activation [104], and EDN1 has been demonstrated to induce melanogenesis by activating MITF [105]. Interestingly, the expression of EDN1 maintains MITF^{high} as well as AXL^{high} cell populations through EDNRB and EDNRA respectively, and thereby regulates phenotype heterogeneity in melanoma [93] (Fig. 4).

Taken together, phenotype switching is of critical importance in emergence of resistance to BRAFi. Despite the high heterogeneity in resistance mechanisms, current data obtained from studies on melanoma and other cancers suggest that YAP signalling mediates the switch from one phenotype to another. Several groups have linked MITF and AXL to different molecular phenotypes [71,72], however, the regulation of these distinct phenotypes has been poorly understood. Recent studies in melanoma have linked EDN1 signalling to MITF and AXL expression levels [93], as well as tumour microenvironment-mediated YAP/PAX3 and YAP/TEAD signalling to the MITF^{high} and MITF^{low} phenotypes, respectively [46]. Studies performed in other cancers (e.g. HCC and CRC), highly suggest a link between EDN1, AXL, and YAP signalling, which could also be an important regulatory network in melanoma worth exploring further (Fig. 4). Fig. 5 summarises the key molecular pathways that are involved in melanoma drug resistance mediated by phenotype switching and other mechanisms covered in this review (Fig. 5).

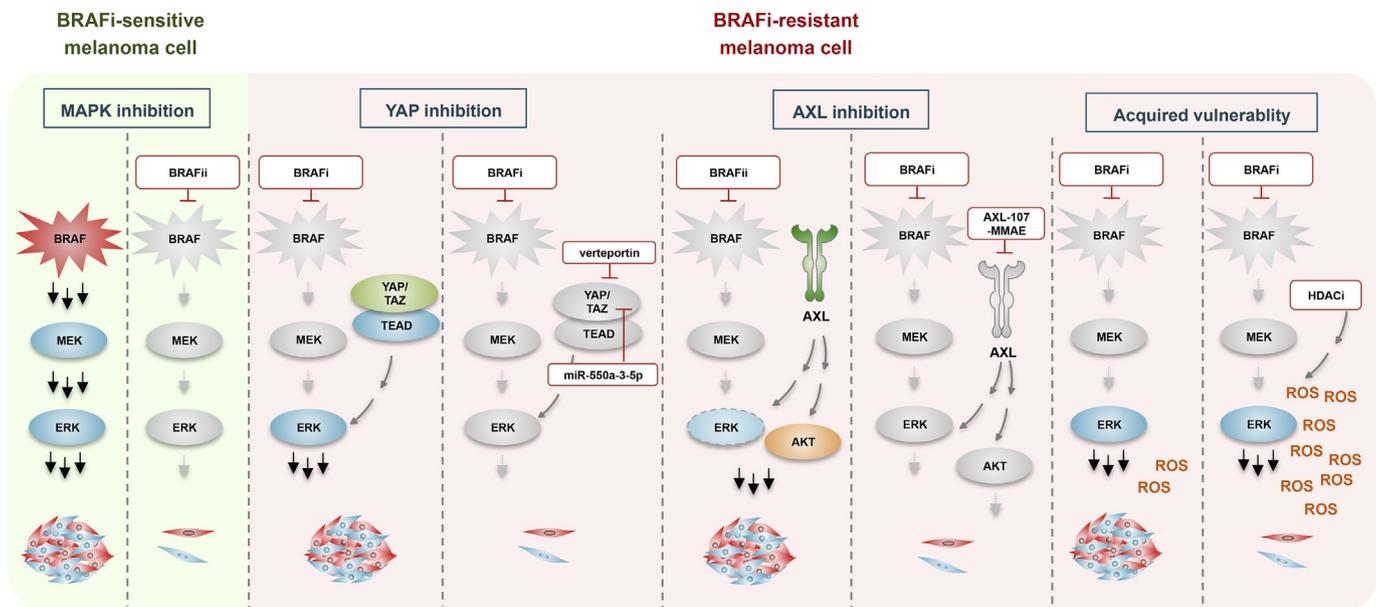


Fig. 5. Novel treatment options for melanoma patients. The potential phenotype switch-mediator, YAP, is often upregulated in BRAFi-resistant melanomas, and could be inhibited using drugs such as verteporin, or miRNAs such as miR-550a-3-5p. Similarly, the receptor tyrosine kinase AXL, whose expression inversely correlates with MITF, is not only involved in phenotype switching but is also upregulated in BRAFi-resistant cells. The AXL antibody-drug conjugate AXL-107-MMAE in combination with BRAFi/MEKi showed promising results in BRAFi-resistant tumours, thus it might be used as a novel targeted therapy for melanoma tumours. Additionally, increasing cancer cell lethality in BRAFi-resistant cells by increasing ROS levels using HDACi represents another novel therapeutic approach.

5. Conclusion

Melanoma is the posterchild of modern cancer treatment efforts. The development of BRAFi and MEKi as targeted treatment options for patients with BRAF-mutant tumours as well as the introduction of immune checkpoint inhibitors contributed profoundly to an increased overall survival of patients with metastatic melanoma. Nevertheless, development of resistance to treatment in most patients remains a major clinical issue. To this date, a myriad of resistance mechanisms to targeted therapies have been described, ranging from acquired activating mutations to adaptive processes of cell plasticity in response to treatment-induced pressure. Interestingly, the dynamic phenotype switches involving various fundamental cellular processes (cell proliferation rate, differentiation, and metabolic rewiring) allow melanoma cells to robustly resist current therapies. These processes are generally linked to the master regulator MITF, whose expression levels inversely correlate with the RTK AXL. Here, we summarised insights into potential mechanisms regulating phenotype switching. Current data suggest that targeting de-differentiated and slowly proliferating AXL^{high} cells in combination with BRAFi could have a significant impact on the overall survival of melanoma patients. Also, the inhibition of the ECM stiffness-induced and YAP-mediated switch between a MITF^{high} and AXL^{high} phenotype could be prevented by modulating YAP signaling.

Drugs targeting the phenotypic switch will require thorough and elaborate clinical testing before more personalised and efficient treatments can be offered to melanoma patients. Nevertheless, before such drugs come into clinical practice, other kinase inhibitors that are currently in clinical use in different cancers will be further tested and might induce more durable treatment outcomes in patients with advanced stage melanoma when combined with current targeted therapies.

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