

Molecular genetic and immunotherapeutic targets in metastatic melanoma

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Abstract In recent years, melanoma treatment has radically changed with the emergence of targeted therapies and immunotherapies. Both have led to improved survival for patients with advanced or unresectable melanoma. Targeted therapies with BRAF inhibitors in the lead use the presence of activating driver mutations to inhibit tumour growth. Forty to 60% of melanomas harbour *BRAF* mutations, which makes them susceptible to treatment with BRAF and/or MEK inhibitors. In parallel, the development of immunotherapeutic agents has also expanded. These agents stimulate the endogenous immune system of the patient to eradicate cancer cells. Immune checkpoint inhibitors targeting cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed death 1 (PD-1) resulted in durable responses in a subset of patients. An important issue with immunotherapy lies in the identification of patients who will benefit from treatment. In this review, we will discuss these recent developments in melanoma therapy and highlight the role of the pathologist in both types of treatment.

Keywords Melanoma · BRAF · Targeted therapy · Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) · Programmed death 1 (PD-1) · Immunotherapy

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Introduction

Melanoma has an increasing incidence worldwide [1, 2]. In early stages, complete surgical resection is often curative, but once metastases have occurred, the prognosis looks grim [1, 3]. Melanoma is remarkably resistant to various types of chemotherapy with few responders and very few durable responses [2]. Historically, the median survival for patients with stage IV disease was ≤ 1 year [2]. The limited effectiveness of chemotherapy has prompted the search for new therapeutic agents.

In recent years, two therapeutic strategies have changed the landscape of melanoma treatment and have led to improved survival for patients with advanced or unresectable melanoma. First, the targeted therapies have emerged as the result of the ever-advancing knowledge of underlying molecular pathways and the identification of activating driver mutations. Unfortunately, these therapies are nearly exclusively suitable for patients with certain genetic aberrations in their melanoma and resistance to targeted therapy develops in the majority of patients. Second, immunotherapy in melanoma enhances the patients' own immune system in order to eradicate the cancer cells. Theoretically, this treatment is applicable to all patients with metastatic or unresectable melanoma, irrespective of mutation status.

In this review, we will discuss these recent developments in melanoma therapy and we will highlight the role of the pathologist in both types of treatment.

Targeted therapies

Unravelling the molecular genetics of melanoma

Primary melanoma of the skin is characterised by an enormous number of mutations, partly due to the carcinogenic effects of ultraviolet (UV) light [4–6]. Although most of these

mutations are ‘passenger’ mutations, some of them are ‘driver’ mutations affecting critical genes involved in the cell cycle. These driver mutations constitute unique targets for treatment.

The mitogen-activated protein kinase (MAPK) pathway has been the main target of research into the genetic mechanisms underlying melanoma development. Important members of this pathway are RAS, BRAF, MEK and ERK [7]. Activation of the MAPK pathway leads to increased cell proliferation and survival. When RAS is activated, it drives BRAF dimerization and activation. Activated BRAF phosphorylates and hence activates MEK, which subsequently phosphorylates and activates ERK (Fig. 1). Within the MAPK pathway, we encounter two proteins whose genes frequently harbour mutations in melanoma, namely RAS and BRAF.

Mutations in *BRAF*, a serine/threonine kinase, occur in approximately 40–60% of cutaneous melanomas [7–11]. Hereby, BRAF is activated independently of upstream stimulation and of RAS activity, resulting in an increased kinase activity and a constitutively active cascade [10, 12]. The mutations in BRAF are clustered in two regions of the protein: the P-loop and the activation segment [10, 12]. Interactions between these two regions regulate the active and inactive conformation of BRAF. Oncogenic mutations destabilise the inactive conformation of BRAF, as a result of which the active state of BRAF is greatly promoted [12]. The most frequent *BRAF* mutation, V600E, is a valine to glutamic acid substitution within the activation segment [7, 10, 11]. The V600E

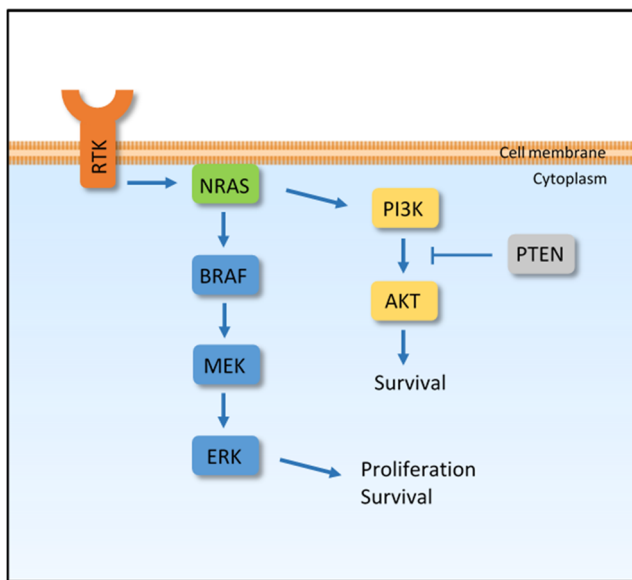


Fig. 1 The mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3' kinase (PI3K) pathway. Activated receptor tyrosine kinases can transmit signals to NRAS. This signal can be transduced via the MAPK pathway consisting of BRAF, MEK and ERK, leading to proliferation and survival as well as via the PI3K pathway consisting of PI3K and AKT, leading to survival (RTK receptor tyrosine kinase)

mutation accounts for approximately 80% of the *BRAF* mutations encountered in melanoma [9–11]. The V600K mutation (a valine to lysine substitution) is the second most common [9, 13, 14]. In comparison to V600E mutations, V600K mutations are more common in older patients and occur more frequently in skin with chronic sun-induced damage (CSD) [14].

BRAF mutations in general occur more often in melanomas originating in skin, intermittently exposed to UV. They are relatively rare in skin with CSD and are more frequent in younger patients [4, 9, 15–17]. The effect of *BRAF* mutations on prognosis and survival of patients is biased by the use of BRAF inhibitors (cf. infra). Long et al. found no significant impact on the disease-free interval from diagnosis of the primary melanoma to first distant metastasis, but there was a trend towards poorer outcome of *BRAF* mutant (not treated with a BRAF inhibitor) versus *BRAF* wild-type metastatic melanoma [9]. Houben et al. reported similar data [11]. The presence of a *BRAF/NRAS* mutation was associated with a poorer prognosis in metastatic, but not in primary, lesions. However, not all series confirm these findings [9]. *BRAF* mutant melanomas have a tendency to metastasise to regional lymph nodes, while *BRAF* wild-type melanomas more often show in transit or systemic metastases [17].

Melanomas harbouring *BRAF* mutations show some distinct morphological features [16–18]. They show more pagetoid spread and nest formation of intraepidermal melanocytes. The involved epidermis is thickened, and the demarcation with the surrounding skin is sharper. The cells are usually larger, more epithelioid and more pigmented (Fig. 2).

RAS is another protein of the MAPK pathway, in which frequent oncogenic mutations occur. In melanomas, almost all mutations in *RAS* concern *NRAS* [5, 15]. They occur in roughly 20% of melanomas and are almost exclusively present in melanomas without *BRAF* mutation [11, 15, 19, 20]. The oncogenic mutations in *NRAS* lead to constitutive activation of

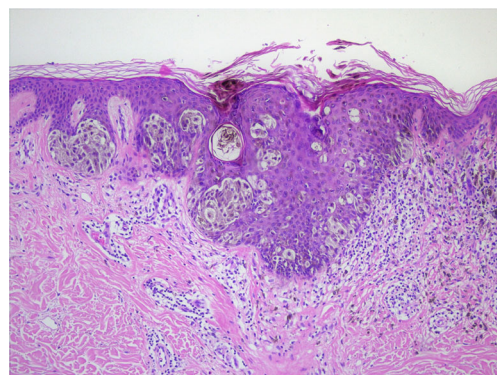


Fig. 2 Morphological features of a *BRAF*-mutated melanoma. Superficial spreading melanoma harbouring a *BRAF*^{V600E} mutation. Presence of pagetoid spread and nest formation of intraepidermal melanocytes, thickened epidermis and sharp demarcation with the surrounding skin. The cells are large, epithelioid and pigmented (original magnification $\times 100$)

the NRAS protein due to decreased GTP-ase activity and accumulation of the active, GTP-bound protein [7]. NRAS activates both the MAPK pathway and the phosphatidylinositol 3' kinase (PI3K) pathway (Fig. 1) [7, 15]. In contrast to *BRAF* mutant melanomas, melanomas with *NRAS* mutations do not seem to possess distinct morphological features [16]. However, Broekaert et al. found an association between low or absent scatter of intraepidermal melanocytes and better circumscription on the one hand and *NRAS* mutant status on the other hand [17]. *NRAS* mutant tumours are also associated with thicker primary tumours and increased mitotic rate [20]. Most of the *NRAS* mutations in melanoma have been linked to a poorer overall survival (OS) although a mutation in *NRAS*, protecting melanoma patients from metastasis, has also been described [20, 21].

Multiple other genetic aberrations are present in melanomas. Mutations and amplification of *KIT* occur in a relatively low proportion of melanomas but are more frequent in mucosal and acral melanomas (15–40%) and in melanomas arising in skin with CSD [4, 22, 23]. They are associated with worse prognosis [24]. *KIT* is a receptor tyrosine kinase with a number of different effector pathways, including the MAPK and the PI3K pathway [22–24]. In melanoma, mutations in *KIT* are widely distributed over the coding region [23]. This makes it harder to separate driver from passenger mutations.

Inactivating mutations of *NF1* are associated with a high mutational burden and occur in older patients [5]. They arise in 14% of melanomas and more often in desmoplastic/neurotropic melanomas and in melanomas arising in skin with CSD [4, 5, 25]. *NF1* normally downregulates RAS activity, hence its inactivation leads to increased RAS signalling [5].

PTEN is an inhibitory protein of the PI3K pathway. Loss of functional PTEN leads to upregulation of the PI3K pathway and increased survival of the melanoma cell (Fig. 1) [15]. This occurs more frequently in *BRAF*-mutated melanomas [5].

Uveal melanomas have a different genetic background [4, 26, 27]. In comparison with cutaneous melanomas, uveal melanomas display a different pattern of mutated driver genes, among which *GNAQ* and *GNAI1* [27]. Mutations in *GNAQ* and *GNAI1* arise early in the development of uveal melanoma. These can be found in 83% of uveal melanomas in a mutually exclusive manner [26]. The mutations in both genes cause an upregulation of the MAPK pathway and have the same effect as the V600E mutation in *BRAF* [26, 27].

The insights into the underlying molecular mechanisms of melanoma have inspired some researchers to propose another classification scheme of melanoma. Nowadays, we are used to the classification scheme of Clark on which the current WHO classification is based [4, 28]. On morphological grounds, one can distinguish four main subtypes: superficial spreading melanoma (SSM), lentigo maligna melanoma (LMM), nodular melanoma (NM) and acral lentiginous melanoma (ALM). These subtypes have very little impact on clinical decision

making on optimal treatment [15, 16]. The OS or response to treatment does not differ significantly between these morphological groups when tumours of equivalent microstaging (encompassing thickness, presence or absence of ulceration and mitotic count) were compared [16].

An alternative type of classification subdivides the melanomas on non-glabrous skin in lesions occurring in skin without chronic sun-induced damage (non-CSD) and lesions in skin with CSD. The first group has frequent *BRAF* mutations and no *KIT* mutations. The second group has less frequent *BRAF* mutations but more *NF1* and *KIT* mutations. Melanomas on glabrous skin and the nail apparatus are termed acral melanomas, those on mucosal membranes, mucosal melanomas. Finally, uveal melanomas and intradermal melanocytic proliferations (blue nevus spectrum) are discerned. They show frequent mutations in the same genes (*GNAQ* and *GNAI1*) [4, 17].

Yet another option is to create a genomic classification with subtypes based on the presence or absence of mutated genes. The Cancer Genome Atlas Network proposes four genomic subtypes: mutant *BRAF*, mutant *RAS*, mutant *NF1* and Triple-WT (wild type) [5]. The Triple-WT subtype is enriched for *KIT* mutations and amplification. Although there is no significant correlation between outcome and genomic group, this classification might be of help in guiding treatment choices, since it is based on the type of driver mutation [5].

Therapeutic agents

Not all oncogenic mutations in melanoma are targetable. At the moment, only two *BRAF* inhibitors (vemurafenib and dabrafenib) and two MEK inhibitors (trametinib and cobimetinib) are European Medicines Agency (EMA)- and FDA-approved to be used in metastatic or unresectable melanoma with *BRAF*^{V600} mutations [29, 30]. Many more agents are under evaluation in clinical trials. For example, encorafenib (*BRAF* inhibitor) and binimetinib (MEK inhibitor) are currently assessed in a phase 3 trial with encouraging results in line with those of other combinations (NCT01909453) [31].

Vemurafenib and dabrafenib are potent and highly specific inhibitors of the *BRAF*^{V600} mutant protein [8]. They produce objective responses (ORs) in roughly half of the patients with metastatic melanoma harbouring a *BRAF*^{V600} mutation, and they have showed survival benefit in these patients [32, 33]. In spite of this success, most patients develop resistance to these small molecule inhibitors and disease progression after 6 to 7 months [7, 8, 34].

The mechanisms of resistance are diverse. Consistent with their temporal occurrence, they can be divided into intrinsic and acquired resistance. In intrinsic resistance, the tumour virtually does not respond to therapy. Upon administration of a *BRAF* inhibitor, there are rapid re-adjustments of signalling pathways, which render the drug ineffective [7, 8]. This is also the case in many other malignancies with *BRAF*

mutations such as colorectal adenocarcinoma and papillary thyroid cancer [8]. In most cases of acquired resistance, the tumour cell manages to reactivate the MAPK pathway in the presence of the BRAF inhibitor. The most frequent mechanisms are *NRAS* or *KRAS* mutations, *BRAF* splice variants, *BRAF*^{V600E/K} amplification and *MEK* mutations [7, 8, 35]. Another mechanism of resistance is the upregulation of the PI3K pathway, for instance by loss of PTEN function [8, 36]. Table 1 gives an overview of different mechanisms of resistance to *BRAF* inhibitors in melanoma [7, 8, 35, 36]. Much research is directed towards finding solutions to overcome these different patterns of resistance [1, 7, 8].

Frequent adverse events of *BRAF* inhibitors are arthralgia, pyrexia, rash, fatigue, keratoacanthoma or squamous cell carcinoma, photosensitivity, nausea, diarrhoea and liver function abnormalities [32, 33]. Rarely, these drugs can also cause QT prolongations, pericarditis and potentially pneumonitis. Specific attention should be paid to the emergence of cutaneous malignancies, which is related to the paradoxical activation of the MAPK pathway in cells without the *BRAF*^{V600} mutation (via RAF dimerization) [7, 8, 37]. Work is done to create ‘paradox breaking’ *BRAF* inhibitors that do not cause this paradoxical MAPK activation in non-mutant cells [1, 7, 8].

MEK inhibitors use a downstream target of *BRAF* [7]. As a single agent, their activity is rather modest [7, 38]. However, the combination of MEK and BRAF inhibition has the potential to achieve a more robust inhibition of the MAPK pathway than BRAF inhibition alone. Due to the good rationale for combining BRAF and MEK inhibitors, this combination was tested in clinical trials. Besides a higher efficacy, these

studies also aimed at overcoming or delaying resistance to BRAF inhibition and blocking paradoxical activation of the MAPK pathway [7, 39]. Two MEK inhibitors are approved by the EMA and FDA: trametinib in combination with dabrafenib and cobimetinib in combination with vemurafenib. These combinations yielded a significantly improved response rate, a significantly better progression-free survival and OS compared to BRAF inhibition alone [39–41]. Patients treated with the combination regime developed fewer cutaneous malignancies, a finding consistent with suppression of paradoxical MAPK pathway activation [8, 39, 41]. This is achieved, however, at the expense of adding MEK inhibitor-specific adverse events such as ophthalmological complications, decreased left ventricular ejection fraction and fluid retention [38, 41]. MEK inhibition has shown very modest activity in uveal melanomas, where more active drugs or combinations are necessary [1, 20].

AKT, PI3K and ERK inhibitors are under development [1, 7, 8]. Direct pharmacological inhibition of NRAS has proven to be difficult [7, 20]. A part of the efforts is targeting post-translational modifications (farnesylation, prenylation) of NRAS to prevent its attachment to the cell membrane, a step essential for NRAS activation [7]. *NRAS* mutant tumours are resistant to BRAF inhibition [19, 20, 37]. Trials are ongoing to test the efficacy of MEK inhibition and combination of MEK and CDK4/6 inhibition (a downstream target of ERK) in patients with *NRAS*-mutated melanomas [20, 37]. The results of a phase 3 trial comparing binimetinib to chemotherapy (dacarbazine) in unresectable or metastatic *NRAS* mutant melanoma indicate a small but statistically significant improvement in progression-free survival with no significant difference in OS [42]. Currently, there are no approved therapeutic agents specifically for *NRAS* mutant melanoma [20].

Inhibitors of KIT, such as imatinib, dasatinib, nilotinib and sunitinib, have shown activity in patients whose melanomas harbour mutations or amplification of *KIT* [22–24]. Although trials in unselected patients with advanced melanoma were negative, studies focussing on selected patients with melanomas harbouring *KIT* alterations could identify responders, albeit in a relatively small number of patients compared to MAPK pathway inhibition in *BRAF*-mutated melanoma [23, 24]. Activating mutations in *KIT* were therapeutically relevant, i.e. associated with an objective response to KIT inhibitors, whereas an increased copy number of wild-type *KIT* seems to be associated with a lower clinical activity [23, 24]. In most studies, clinical benefit was largely transient [22]. Targeting downstream components of the MAPK and PI3K pathways may be an attractive, alternative approach [22].

Table 1 Mechanisms of resistance to BRAF inhibitors in melanoma [7, 8, 35, 36]

Intrinsic resistance	
Rapid re-adjustments of signalling pathways, for instance relief of feedback inhibition	
Downregulation/loss of NF1	
Acquired resistance	
Reactivation of MAPK pathway	<i>NRAS</i> or <i>KRAS</i> mutation <i>BRAF</i> splice variants <i>BRAF</i> ^{V600E/K} amplification <i>MEK</i> mutation Overexpression of CRAF or COT
Upregulation of PI3K pathway	Loss of PTEN function Overexpression or activation of receptor tyrosine kinases (PDGFR β , IGF1R) HGF signalling through MET <i>PIK3CA</i> mutation
<i>MITF</i> amplification	

Abbreviations: *COT* MAP3K8, *PDGFR β* platelet-derived growth factor receptor- β , *IGF1R* insulin-like growth factor 1 receptor, *HGF* hepatocyte growth factor, *MITF* microphthalmia-associated transcription factor

Techniques to discover the presence of molecular targets

Next to giving the appropriate diagnosis, the pathologist has an important role in the detection of oncogenic mutations. The

pathologist is frequently regarded as ‘the guardian’ of the patient’s tissue. He or she is obliged to the rational use of tissue, especially when the available amount is limited. To investigate the mutation status of a molecular target, tissue of the most recent metastasis is preferred, because metastases can acquire additional mutations; subclones can be selected or generated. Heterogeneity between the primary tumour and the metastases has been reported in literature, although at a relatively low frequency [11, 18, 43]. Colombino et al. found that the distribution of *BRAF/NRAS* mutations was highly consistent between primary melanomas and lymph node or visceral metastases, whereas rates of consistency between primary tumour and brain or skin metastases were significantly lower [43]. This heterogeneity poses a challenge for targeted therapies since it can influence treatment choices. Retesting patients with initially *BRAF* wild-type melanoma can be considered when new metastases appear, since there is a small chance that a *BRAF* mutation becomes detectable making patients eligible for MAPK pathway inhibition [44, 45]. Apart from tissue, tumour-derived circulating cell-free DNA (cfDNA) from patient plasma is a potential alternative source to assess mutation status. This is of special interest in patients with lesions not easily accessible for repeated biopsy like patients with brain metastases [46].

Several methods exist to detect *BRAF*, *NRAS* and/or *KIT* mutations: High-resolution-melting PCR, real-time (allele specific amplification) PCR including competitive amplification of differentially melting amplicons, Sanger sequencing, pyrosequencing, mismatch ligation assay, ligase detection reaction, denaturing high-performance liquid chromatography, SNAPshot and mass spectrometry [13, 18, 47–49]. In recent years, next-generation sequencing (NGS) has become more widely available. All these assays show differences in sensitivity, specificity, costs and time to result. When tumour cell fraction is low, some tests require macro-/microdissection to improve sensitivity [48, 49]. It is important to bear in mind that not all assays detect all possible mutations of a certain gene. For example in *BRAF*, some tests do not detect all possible mutations in V600 or other non-V600 mutations. Table 2 gives a summary of sensitivities, advantages and disadvantages of various techniques, including immunohistochemistry (IHC) [48–51].

For certain molecular targets, IHC can be an alternative to molecular testing. This is certainly the case for the detection of the *BRAF*^{V600E}-mutated protein. VE1 is a *BRAF*^{V600E} mutant-specific monoclonal antibody (Fig. 3). In a meta-analysis conducted by Anwar et al., pooled sensitivity of IHC for detection of the *BRAF*^{V600E}-mutated protein was 96%, and pooled specificity was 100% [47]. The VE1 antibody is very specific for the V600E mutation but occasional cross reactivity with other V600 mutations, for example V600K, has been variably reported [13, 49, 52]. Staining with the VE1 antibody is in most cases strong, diffuse and

cytoplasmic [18, 37, 53]. However, several authors have reported difficulty in interpreting weak staining in a subset of samples [13, 37, 53]. Different ways to report IHC results are used. Some use a dichotomous positive versus negative system [18, 37, 49, 52]. Others use a four-tier system to report staining results from 0 (no staining) to 3+ (strong staining) [13, 53].

Most of the molecular assays are costly and take several days to produce results, especially when the tissue has to be transferred to another laboratory [47, 52]. In comparison, IHC is cheaper and more widely available in most pathology laboratories [37, 49, 52, 53]. Results can be obtained the next day. IHC is particularly suitable for very small samples or samples with a very low tumour cell content [47, 52]. For example, in a lymph node with single or small clusters of tumour cells, IHC shows single cell-resolution images [52]. In samples with low-quality or non-amplifiable DNA, proteins may be better preserved [47, 53].

IHC can be used as a first-line method to yield rapid results and direct patients to appropriate treatment, for instance in the case of symptomatic brain metastasis. This has to be followed by molecular confirmation and detection of other V600 mutations [37, 47, 49, 52, 53]. Another option is to perform molecular testing only on samples with negative or equivocal IHC results. To produce clinically applicable results, stringent antibody optimisation and quality control is required [47].

Concerning *NRAS*, antibodies are available for some of the most prevalent mutations (*NRAS*^{Q61R} and *NRAS*^{Q61L}) [37]. The anti-*NRAS*^{Q61R} IHC has been reported to be highly sensitive and specific [19, 37].

KIT expression or *KIT* copy number does not correlate with the presence of activating *KIT* mutations, and *KIT* expression does not always predict response to *KIT* inhibitors in melanoma. Therefore, IHC has little use in molecular profiling for *KIT* aberrations [54].

Immunotherapy

How does it work?

Melanoma is an immunogenic cancer with a high mutational burden that triggers the adaptive immune system. This can even result in spontaneous partial or complete regression [4, 6, 36, 55]. The presence of tumour-infiltrating lymphocytes (TILs) in cutaneous melanomas with a vertical growth phase is a significant and independent prognostic factor [56]. Also, immune infiltration of metastatic melanoma has been correlated with a more favourable prognosis [5]. Unfortunately, due to immunomodulation and immunoediting, this immune response is often abrogated during tumour progression [55]. Immunotherapy tries to overcome this abrogation.

Table 2 Summary of sensitivities and advantages and disadvantages of various techniques used to detect driver mutations in melanoma [48–51]

Technique	Relative sensitivity [48–51]	Advantage	Disadvantage
Sanger sequencing	Low	Can detect other rare <i>BRAF</i> mutations	Macro-/microdissection needed
High-resolution melting PCR analysis	Low (47.5%)		Macro-/microdissection needed
Pyrosequencing	Medium	Quantitative method; can detect other rare <i>BRAF</i> codon 600 mutations	
Allele-specific PCR methods			False-positive results if false amplification of WT-DNA in early PCR rounds
COBAS 4800 BRAF V600 Mutation Test	High (90%)		Results are binary; detects only V600E, V600D and V600K mutations without distinction; macro-/microdissection needed if low tumour cell content
TaqMan based	High (93%)	Quantitative method	Less need of macro-/ microdissection; can detect other V600 mutations
Competitive amplification of differentially melting amplicons (CADMA)	Very high (>98%)		May detect other V600 mutations; less need of macro-/microdissection
Co-amplification at lower denaturation temperature-PCR (COLD-PCR)	Very high (>98%)		Less need of macro-/microdissection
Next-generation sequencing	Reference	Fast, quantitative technique	Macro-/microdissection needed
Immunohistochemistry [49–51]	High sensitivity (can detect and localise single mutant cells)	Fast, cost-effective, very specific; works in small and heterogeneous samples; role as first screening tool?	No detection of non-V600E and of V600E2-mutated melanomas; false positives (nuclear; macrophages)

A central role in cancer immunotherapy is reserved for the T cell (Fig. 4). Activation of the T cell requires stimulation via the T cell receptor and a co-stimulatory interaction between CD28 and its ligand B7-1 or B7-2 [36, 55, 57]. To avoid overactivation of the T cell with the risk of developing autoimmune reactions and to promote self-tolerance, also activation of co-inhibitory molecules comes into play [58, 59]. These are known as immune checkpoints and include cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed death 1 (PD-1) [36, 58, 59]. CTLA-4 binds to B7-1

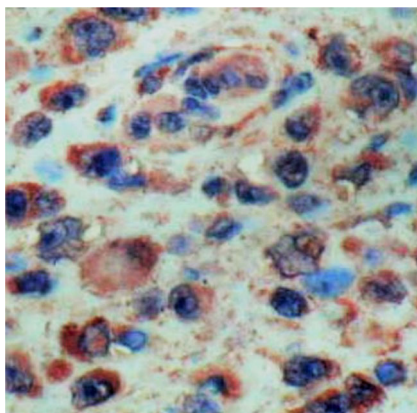


Fig. 3 Immunohistochemical demonstration of mutated BRAF protein in scattered melanoma cells (Immunoperoxidase, VE1 antibody, counterstained with haematoxylin, original magnification $\times 400$)

or B7-2 with greater affinity than CD28 and downregulates T cell activation. Also, binding of PD-1 and its ligands programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2) limits T cell activation [58–60]. Interferon gamma (IFN γ), produced by activated T cells and natural killer cells, is an important stimulator of PD-L1 expression [61]. Since PD-L1, among others, may be expressed on the tumour cell membrane, these inhibitory signals are obstacles to T cell antitumour activity and make it possible for tumour cells to evade immune reaction [36, 58]. Hence, antibodies, referred to as immune checkpoint inhibitors, which block these co-inhibitory signals, are expected to augment T cell antitumour activity and may enhance pre-existing immune responses to tumour antigens [36, 58].

Other treatments deploying the immunogenicity of cutaneous melanoma include vaccination strategies, adoptive transfer of autologous T cells directed against melanoma antigens, T cell engineering therapy and oncolytic virotherapy [36]. The latter is approved by the EMA and FDA [29, 30]. Further discussion on these therapeutic principles is beyond the scope of this review.

Therapeutic agents

One of the first immunotherapeutic drugs was high-dose interleukin-2 (IL-2). It induces responses in 15–20% of patients

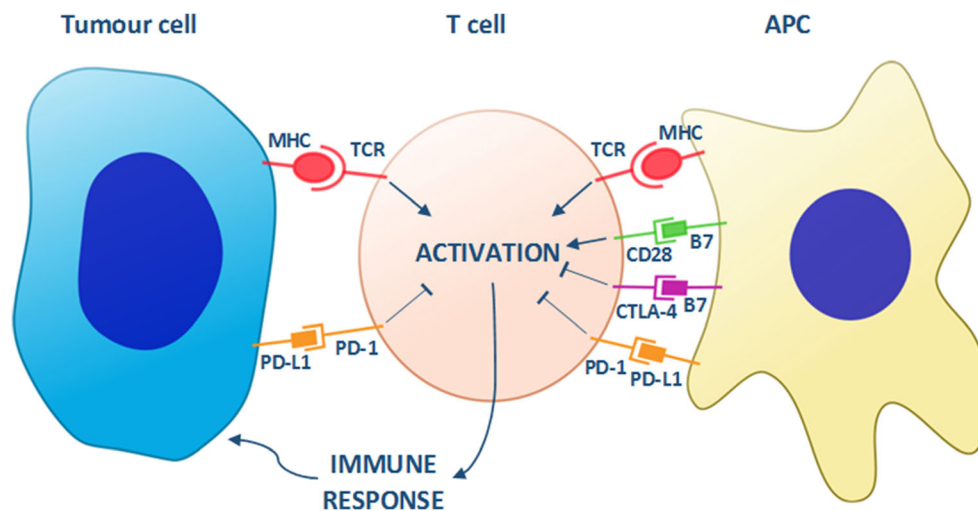


Fig. 4 Mechanism of action of immune checkpoints. For activation of the immune response, T cells require stimulation by the major histocompatibility complex (MHC) presenting antigens. Interaction between CD28 and B7 provides a co-stimulatory signal, predominantly during interactions with the antigen-presenting cells (APCs) (the afferent arm of the T cell response). To avoid uncontrolled stimulation, immune checkpoints, including cytotoxic T-lymphocyte-associated antigen-4

(CTLA-4) and programmed death 1 (PD-1), provide co-inhibitory interactions. CTLA-4 acts mainly in the afferent arm of the T cell response, while PD-1 operates primarily during the effector phase of the T cell response. These interactions lead to a decreased activation of the T cell and consequently a decreased immune response (*TCR* T cell receptor, *PD-L1* programmed death ligand 1)

with durable responses in 5–8% of patients. Toxicities are severe and often life threatening. However, it established a role for immunotherapy in the treatment of advanced melanoma [1, 3, 20, 36]. Also, interferon alpha ($\text{IFN}\alpha$) is an immunotherapeutic agent that can be used in the treatment of melanoma. $\text{IFN}\alpha$ adjuvant therapy, in patients with high-risk cutaneous melanoma, showed improvement in both disease-free survival and OS, although the gain in OS was relatively small and most of the studies failed to establish a significant impact of adjuvant cytokine treatment on OS [62].

With the immune checkpoint inhibitors, a new type of immunotherapeutic agent has emerged. To date, three such antibodies are approved by the EMA and FDA: ipilimumab, nivolumab and pembrolizumab [29, 30]. Many more agents are under evaluation in clinical trials.

Ipilimumab is a monoclonal antibody that blocks CTLA-4. Next to a decreased inhibition of T cell activation, it also promotes cytotoxic T lymphocyte trafficking to the tumour and a decline in the number of (inhibitory) regulatory T cells in the tumour microenvironment (TME) [1, 63]. In two phase 3 trials, ipilimumab significantly improved OS in previously treated and untreated patients with metastatic or unresectable melanoma [64, 65]. ORs are achieved in 11% of patients with a disease control rate (the proportion of patients with a partial or complete response or stable disease) of 29% [64]. Treatment with ipilimumab has shown durable tumour regression despite relatively brief therapy durations. An analysis of long-term survival in melanoma patients treated with ipilimumab showed a 3-year survival rate of 22%. Interestingly, at this time, the survival curve reaches a plateau which extended up to 10 years in some patients [66].

Ipilimumab toxicities are frequent and include immune-related adverse events. These consist of rash, pruritus, fatigue, diarrhoea, endocrinopathy, colitis, hepatitis, pancreatitis and other rare autoimmune phenomena [64, 65]. These adverse events require patient education, a close follow-up and prompt medical intervention [64, 65].

Nivolumab and pembrolizumab are monoclonal antibodies that block PD-1. They have shown improved OS in patients with metastatic or unresectable melanoma in comparison to chemotherapy or ipilimumab. They have shown OR in 30–40% of patients with many appearing durable [67–69].

Immune-related adverse events occurred less commonly than with ipilimumab [20, 69]. The most common adverse events include fatigue, pruritus, nausea and diarrhoea [67–69]. PD-1 antibodies have gained broad approvals, including use as first-line therapy regardless of *BRAF* mutation status [1].

Responses to immunotherapy show sometimes an unconventional pattern. A portion of patients on immunotherapy achieves an objective response after initial progression or after a period with stable disease [58, 66, 68, 70]. Conventional response criteria like RECIST 1.1 may therefore be less adequate to accurately assess the activity of immunotherapeutic agents.

Immunotherapies can be associated with intrinsic resistance and the emergence of acquired resistance too. Sharma et al. wrote an excellent review on this topic [71]. Low tumour immunogenicity and an immunosuppressive TME are associated with intrinsic resistance. Mutations affecting the sensitivity of tumour cells to T cell-derived interferons and mutations

which limit tumour-cell antigen presentation have been reported [72, 73]. For example, loss of function mutations in Janus kinase 1 (*JAK1*) or Janus kinase 2 (*JAK2*) lead to a lack of response to IFN γ and have been associated with both intrinsic and acquired resistance to PD-1 blockade [74, 75]. Another route to resistance consists of the upregulation of alternative immune checkpoints or vascular endothelial growth factor (VEGF) [73, 76]. Table 3 gives an overview of different mechanisms of resistance to immune checkpoint inhibitors [61, 71–76].

Ipilimumab and nivolumab or pembrolizumab act via different signalling pathways, suggesting that they could have a synergistic effect [60]. Clinical trials using the combination of ipilimumab and nivolumab have shown a higher activity than either agent alone. In a recently published phase 3 trial, the combination of ipilimumab and nivolumab yielded an objective response in 58% of patients [77]. However, there was also an increase in the number and severity of adverse events seen in patients treated with the combination regime [77].

Many other antibodies are currently under evaluation in clinical trials. Besides anti-CTLA-4 and anti-PD-1 antibodies, these include also anti-PD-L1 and anti-PD-L2 antibodies. Anti-PD-L1 has gained much attention in other diseases and is currently evaluated in melanoma in several ongoing trials [57, 63, 68]. Next to these and other co-inhibitory receptors and ligands, also co-stimulatory targets are the subject of active research [60]. Different combinations of immunotherapeutic agents are evaluated with the success of the ipilimumab-nivolumab combination in mind [60, 63].

Predictive biomarkers?

Theoretically, every patient with metastatic melanoma could benefit from immunotherapy. There is no need for the presence of a certain genetic aberration to respond to this type of therapy, unlike, for example, therapy with a BRAF inhibitor. This offers a treatment opportunity even for patients not eligible for targeted therapy due to absence of a targetable genetic aberration. Even patients who developed resistance to targeted therapies can benefit from immunotherapy. Unfortunately, only a subset of patients does respond to immunotherapy. It would therefore be useful to identify those patients who are most likely to benefit from immunotherapy. Such an approach avoids treatment-related adverse events in non-responders and would also be more cost-effective.

Finding predictive markers has proven difficult. It is a hot research area. Studies have shown that a high mutational load and a high neo-antigen load are associated with a better response to immune checkpoint inhibitors [55, 78, 79]. This association could be an explanation for the lower responses to immunotherapies in uveal melanomas as compared to cutaneous melanomas, since they have a lower mutational burden [27]. However, mutational burden and neo-antigen load are not sufficient to predict clinical benefit [55, 78, 79].

There is increasing evidence supporting the hypothesis that an immune-active TME correlates with clinical benefit from immune checkpoint inhibitors. An immune gene expression signature, reflecting a cellular and humoral immune response, for example expression of cytolytic markers, is associated

Table 3 Mechanisms of resistance to immune checkpoint inhibitors [61, 71–76]

Intrinsic resistance	
Low tumour immunogenicity	Low mutational burden and low neo-antigen load Impaired tumour-cell antigen presentation
Immunosuppressive TME	Low T cell infiltration Alternative immune checkpoints Immunosuppressive cells: Myeloid-derived suppressor cells: inhibition of T cell replication and function Cancer-associated fibroblasts: preventing T cells from reaching cancer cells Regulatory T cells
Lack of sensitivity to T cell-derived interferons (among which IFN γ)	Loss of function mutations in <i>JAK1</i> or <i>JAK2</i>
Alteration of signalling pathways	MAPK, PI3K, WNT/ β -catenin
Acquired resistance	
Mutations limiting tumour-cell antigen presentation	Loss of function mutation in β -2-microglobulin
Immunosuppressive TME	Upregulation of alternative immune checkpoints Upregulation of vascular endothelial growth factor (VEGF)
Lack of sensitivity to T cell-derived interferons (among which IFN γ)	Loss of function mutations in <i>JAK1</i> or <i>JAK2</i>

with clinical benefit from CTLA-4 or PD-1 inhibition [76, 78, 80–82]. Also an inflammatory TME, (CD8+) TILs, CD8+ T cells at the invasive tumour margin, PD-1+ cells, expression of forkhead box P3 (FOXP3) (a marker of regulatory T cells) and indoleamine 2,3-dioxygenase (IDO) (associated with an immunosuppressive TME) have been claimed to predict clinical benefit from immune checkpoint inhibitors [76, 80–85]. However, none of these is perfectly predictive and biomarker profiles between patients with or without clinical benefit are often overlapping [76].

In a study of Chen et al., adaptive immune signatures in early on-treatment tumour biopsies were predictive of response to immune checkpoint blockade [76]. Similar to this, Hamid et al. found that an increase in TILs between baseline and 3 weeks after start of treatment with ipilimumab was associated with clinical efficacy [81]. Tumeh et al. reported a proliferation of CD8+ TILs in regressing tumours under PD-1 blockade [83]. These findings are illustrated in Fig. 5 showing melanoma metastases before and during successful treatment with an anti-PD-1 antibody (Fig. 5).

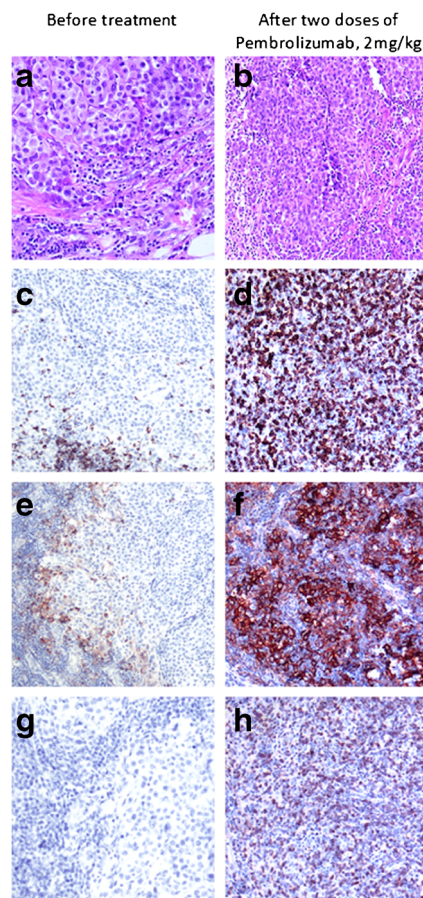


Fig. 5 Cutaneous melanoma metastasis before (a, c, e, g) and after two doses of Pembrolizumab 2 mg/kg (b, d, f, h), stained for H&E (a, b), CD8 (c, d), PD-L1 (e, f) and PD-1 (g, h). During treatment, there is a sharp increase in the number of T cells (b) and expression of CD8 (d) and PD-1 (h) with a concomitant increased expression of PD-L1 on the melanoma cells (f)

For treatment with anti-PD-1 or anti-PD-L1 antibodies, multiple studies examined the association of PD-L1 expression by the TME (assessed with an immunohistochemical assay) and objective response rate (ORR). These studies are difficult to compare since they use different methodologies such as different antibodies and thresholds to determine PD-L1 positivity [1]. PD-L1 can be expressed on tumour cells and on the immune infiltrate [84, 85]. Also, the dynamic nature and possible heterogeneity of PD-L1 expression within a patient adds complexity to its use as a predictive marker [63, 69]. Several studies have shown an association between PD-L1 expression and ORR to monotherapy with PD-1 pathway blockade in patients with metastatic melanoma [3, 63, 84]. But, ORs were not limited to the PD-L1-positive tumours and also patients with PD-L1-negative tumours benefited from treatment [67, 68]. In a phase 3 trial, nivolumab-treated patients had improved OS in comparison with dacarbazine-treated patients, regardless of PD-L1 status [67]. The ORR in nivolumab-treated patients was 52.7% in the subgroup with positive PD-L1 status versus 33.1% in the subgroup with negative or undetermined PD-L1 status [67]. Hence, PD-L1 status alone does not seem suitable for selection of patients for treatment with an anti-PD-1 antibody in monotherapy. Larkin et al. suggested that patients with PD-L1-negative tumours may benefit more from combination therapies [63, 77]. More clinical data are needed to fully evaluate the potential of PD-L1 status in making treatment decisions. Currently, PD-L1 status should not be used as guidance for directing treatment choices in patients with metastatic or unresectable melanoma.

It is possible that in the near future, pathologists will be asked to make a certain assessment of the immune TME in combination with PD-L1 expression or evaluate on-treatment tumour biopsies in order to predict response to immunotherapy. However, the ideal predictive biomarker has yet to be found.

Conclusion

Targeted therapy and immunotherapy have both become first-line treatments in patients with metastatic or unresectable melanoma. For patients without targetable genetic aberrations, it is mainly immunotherapy that can be applied. Combinations of both strategies are under evaluation. Most likely, they generate a synergistic effect [60, 63]. Although these therapies have greatly improved the prognosis of patients with metastatic or unresectable melanoma, challenges still remain. The costs, the increasing pressure on healthcare systems and the adverse events necessitate the identification of better predictive biomarkers. A tool allowing distinguishing responding from non-responding patients as well as patients at risk for developing early treatment resistance is of utmost importance. Strategies to increase the proportion of responders and to

overcome resistance would be of great value. Treatment options are evolving rapidly and clinicians have to select the most appropriate therapy for individual patients. In this selection process, the pathologist will play a crucial role. He or she is undoubtedly a part of the collaborated effort to further advance and improve precision medicine.

Compliance with ethical standards

Conflict of interest C. Melis, J.J. van den Oord and O. Bechter declare that they have no conflict of interest. A. Rogiers received travel support from BMS and Novartis.

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