REVIEW

BRAF-mutant melanoma: treatment approaches, resistance mechanisms, and diagnostic strategies

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Abstract: BRAF inhibitors vemurafenib and dabrafenib achieved improved overall survival over chemotherapy and have been approved for the treatment of BRAF-mutated metastatic melanoma. More recently, the combination of BRAF inhibitor dabrafenib with MEK inhibitor trametinib has shown improved progression-free survival, compared to dabrafenib monotherapy, in a Phase II study and has received approval by the US Food and Drug Administration. However, even when treated with the combination, most patients develop mechanisms of acquired resistance, and some of them do not achieve tumor regression at all, because of intrinsic resistance to therapy. Along with the development of BRAF inhibitors, immunotherapy made an important step forward: ipilimumab, an anti-CTLA-4 monoclonal antibody, was approved for the treatment of metastatic melanoma; anti-PD-1 agents achieved promising results in Phase I/II trials, and data from Phase III studies will be ready soon. The availability of such drugs, which are effective regardless of *BRAF* status, has made the therapeutic approach more complex, as first-line treatment with BRAF inhibitors may not be the best choice for all BRAF-mutated patients. The aim of this paper is to review the systemic therapeutic options available today for patients affected by BRAF V600-mutated metastatic melanoma, as well as to summarize the mechanisms of resistance to BRAF inhibitors and discuss the possible strategies to overcome them. Moreover, since the molecular analysis of tumor specimens is now a pivotal and decisional factor in the treatment strategy of metastatic melanoma patients, the advances in the molecular detection techniques for the *BRAF* V600 mutation will be reported.

Keywords: melanoma, BRAF, vemurafenib, dabrafenib, resistance, BRAF inhibitor

Background

Cutaneous melanoma is the most aggressive form of skin cancer, with a global incidence of about 200,000 new cases per year, likely to increase over the next years. Although melanoma represents only 4% of all types of skin cancers, it is correlated with about 80% of skin cancer-related deaths (about 65,000 per year). Survival rates depend on the clinical stage at the diagnosis, with 5-year survival ranging from 15% to 60% in patients with distant and local metastases, respectively.[1]

The prognosis of metastatic melanoma has recently changed substantially thanks to the approval of kinase inhibitors vemurafenib,[2] dabrafenib,[3] and trametinib,[4] and the immune checkpoint inhibitor ipilimumab.[5,6] More recently, immune checkpoint inhibitors nivolumab[7] and pembrolizumab[8] have achieved promising results in clinical trials, which will probably lead to the approval of these drugs by the regulatory agencies.

Vemurafenib and dabrafenib are selective inhibitors of BRAF V600, a mutation carried by almost half of melanomas,[9] and are approved by the US Food and Drug

Administration (FDA) and European Medicines Agency for the treatment of unresectable or metastatic melanoma with mutant BRAF V600. In the Phase III trial of vemurafenib,[2] median overall survival (OS) was 13.6 months in the vemurafenib group, compared with 9.7 months in the dacarbazine group; median progression-free survival (PFS) was 6.9 months in the vemurafenib group and only 1.6 months in the dacarbazine group; response rate for vemurafenib was 57%, compared with 9% for dacarbazine. Dabrafenib achieved similar results, with a PFS of 5.1 months in the Phase III study.[3]

Trametinib is an MEK inhibitor that achieved improved PFS (4.8 versus 1.5 months) compared to chemotherapy in a randomized Phase III study[4] and, more importantly, was investigated as combination therapy with dabrafenib. In the Phase II trial comparing trametinib plus dabrafenib with dabrafenib alone,[10] the median PFS were 9.4 months and 5.8 months for patients treated with dabrafenib 150 mg twice daily plus trametinib 2 mg daily and dabrafenib monotherapy, respectively. On the basis of these results, at the beginning of 2014 the FDA approved the combination of dabrafenib plus trametinib for the treatment of unresectable or metastatic melanoma with a BRAF V600E or V600K mutation.

Other kinase inhibitors are in late stages of clinical development. LGX818 is a potent and selective BRAF inhibitor with a dissociation half-time about 10 times longer than other BRAF inhibitors; in the Phase I trial a response rate as high as 67% was achieved among BRAF inhibitors-naïve patients.[11] A three-arm Phase III trial is currently recruiting participants in order to compare the efficacy and safety of LGX818 monotherapy and LGX818 in combination with MEK inhibitor MEK162 as compared to vemurafenib in patients with locally advanced unresectable or metastatic melanoma with BRAF V600 mutation (NCT01909453). Moreover, a Phase III clinical trial comparing vemurafenib in combination with MEK inhibitor cobimetinib versus vemurafenib monotherapy (NCT01689519) met its primary endpoint (PFS), and these data are planned to be submitted to health authorities.[12]

Along with the development of BRAF and MEK inhibitors, immunotherapy made some steps forward as well: ipilimumab, a fully human IgG1 monoclonal antibody that blocks cytotoxic T-lymphocyte antigen (CTLA)-4 to elicit antitumor T-cell-mediated responses, was approved for the treatment of metastatic melanoma as it achieved a statistically significant improvement in OS in two different randomized Phase III trials in pretreated[5] and in treatment-naïve[6] patients with metastatic melanoma; nivolumab and pembrolizumab, monoclonal antibodies targeting the programmed cell death-1

(PD-1) receptor on infiltrating T-cells, which otherwise produces an inhibition of T-cells directed against melanoma antigens, showed promising clinical activity and efficacy, and pembrolizumab was recently approved by the FDA for the treatment of patients with unresectable or metastatic melanoma and disease progression following ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor. A compassionate use of both drugs is available in several countries all over the world.

Signaling pathways in BRAF-mutated melanoma

The mitogen-activated protein kinase (MAPK) pathway plays an important role in the pathogenesis of melanoma. This pathway is physiologically activated when extracellular signals bind to their cognate membrane receptor, typically a receptor tyrosine kinase (RTK). RAF kinases are components of the pathway: their activity requires the formation of dimers, which is promoted by RAS activation. Activated RAF kinases phosphorylate and activate MEK1/2, which in turn phosphorylate and activate ERK1/2, leading to cellular proliferation, survival, and differentiation, and to an inhibitory feedback toward upstream components of the pathway.[13]

About 50% of melanomas harbor an activating mutation in BRAF, the most common being $BRAF^{V600E}$,[9] which renders the kinase constitutively active. In BRAF-mutated melanomas, RAS is negatively regulated by ERK-dependent feedback and $BRAF^{V600E}$ exists mainly as a monomer.[13]

BRAF inhibitors inhibit ERK signaling only in BRAF-mutated tumors. In wild-type (WT) cells, BRAF and CRAF form homo- and heterodimers on RAS activation; BRAF inhibitors binding to one member of the dimer causes an allosteric transactivation of the drug-free protomer, leading to ERK activation. This is enhanced when RAS, which promotes RAF dimerization, is overexpressed:[14] in fact, most cutaneous tumors developing in patients treated with BRAF inhibitors harbor RAS mutations.[15]

The PI3K/AKT/mTOR pathway is a key regulator of cellular growth and protein synthesis.[16,17] The MAPK pathway interacts with the PI3K/AKT/mTOR pathway at multiple points: for example, RAS directly activates PI3K, and the inhibition of a pathway may upregulate the other one.[16,18]

Diagnostic strategies

Detection of BRAF V600 mutations has recently become mandatory to treat patients with advanced or metastatic melanoma. Several methods have been used to detect BRAF mutations, including Sanger sequencing, mismatch ligation assay,

ligase detection reaction, denaturing high-performance liquid chromatography, SNAPshot®, high-resolution melting, mutation-specific polymerase chain reaction (PCR) and mutation-specific real-time (RT) PCR, including EntroGen molecular probes (CE-IVD), pyrosequencing, and mass spectrometry.[19–24] Each method has its own sensitivity, specificity, cost, and response delay. Several studies have been performed to compare the methods for detection of these mutations.[24–29]

Recently, detection of *BRAF* p.V600E mutation was also performed by immunohistochemistry (IHC) with VE1 antibody, and next-generation sequencing (NGS) technology is currently being used to analyze melanoma specimens in many research institutions. Below, we compare the most commonly used methods for the detection of *BRAF* mutations in melanomas to determine the method or combination of methods that should be used in diagnostic daily practice (Table 1). Our suggestion is that sequential analysis, with initial detection of p.V600E-positive cases by IHC and/or Sanger sequencing, followed by pyrosequencing or RT-PCR-based tests in negative or uninterpretable cases, is the most efficient method to use in daily practice, in certified laboratories, with validated techniques.

Immunohistochemistry

IHC with VE1 monoclonal antibody was found to be efficient to detect p.V600E mutations.[30] The advantages of this technique are that only two slides are needed, no specialized equipment is needed, it is cheaper than other techniques, and results are easily obtained within 48 hours. Interestingly, IHC with VE1 monoclonal antibody was reported to have a 100% sensitivity and specificity for detection of p.V600E, even when compared with methods more sensitive than Sanger sequencing.[31] However, important disadvantages are that staining interpretation is easy in most, but not all, cases, and that the VE1 antibody is highly specific for the V600E mutation, but other clinically relevant *BRAF* mutations are missed. For these reasons, it could be used as a cost-effective first-line method for *BRAF* V600E detection in a daily practice sequential combination of methods.[32]

Sanger sequencing

For many years, Sanger sequencing has been considered the reference method for identification of acquired mutations in tumors. However, because of its low sensitivity (direct sequencing cannot detect the presence of mutant alleles when the mutant/WT ratio is less than 1:5),[28] detection of mutations from tumor DNA requires a high percentage of tumor cells within the samples, a requirement that cannot always be met in routine diagnostic testing of human samples. A recent study compared four methods for the detection of *BRAF* mutations in metastatic melanomas.[31] Microdissection of metastatic melanomas was performed to increase the number of tumor cells to at least 60%. However, 3 of the 40 melanomas with p.V600E mutation detected by other methods were negative with Sanger sequencing. The sensitivity of Sanger sequencing was 92.5% (95% confidence interval [CI], 78.5%–98.0%) for p.V600E detection. Thus, 7.5% of patients eligible for treatment with BRAF inhibitors would have been excluded. In line with this, another recently published series found a false-negative rate of Sanger detection of *BRAF* mutations of 9.2%.[24] Interestingly, false-negative cases with Sanger sequencing corresponded to tumors with a high proportion of tumor cells within the samples. This finding may be related to tumor heterogeneity, with only some subclones containing p.V600E mutation and others being WT. These data indicate that Sanger sequencing failed to detect *BRAF* mutations in melanomas and therefore should no longer be considered as the reference test, but as a first screening or a confirmation test. Alternatively, in samples negative with Sanger sequencing, one of the tests described below should be performed.

Pyrosequencing

Pyrosequencing is an RT sequencing by synthesis approach which allows the quantification of mutated alleles. It is a rapid and more sensitive method compared with direct sequencing for quantifying the *BRAF* V600 mutation. In a study comparing different techniques, pyrosequencing has been shown to have 100% sensitivity (95% CI, 92.4%–100%) and specificity (95% CI, 91.6%–100%) for p.V600 detection.[31] Another recent study comparing different detection methods

Table 1 Sensitivity and specificity of the described methods

Diagnostic method	Sensitivity (%)	Specificity (%)	Reference
IHC	93–97	92–98	28–31
Sanger sequencing	80–93.4	100	28,29
Pyrosequencing	95	90–94	29,34
HRM	93.7	100	28,29
Cobas®	93	98	26,29
PNA-clamping real-time PCR	99.5	100	33,34
NGS	98	100	29

Notes: Data were obtained either from the manufacturer (CE-marked tests, Cobas® and PNA clamp) or from the literature, when comparing different techniques in the same samples. A range of sensitivity and specificity has been reported when different data were obtained in different studies.
Abbreviations: IHC, immunohistochemistry; HRM, high-resolution melting; PNA, peptide nucleic acid; NGS, next-generation sequencing.

found that there was no difference in sensitivity between the high-resolution melting analysis and Sanger sequencing (98%). All mutations down to 6.6% allele frequency could be detected with 100% specificity. In contrast, pyrosequencing detected 100% of the mutations down to 5% allele frequency but exhibited only 90% specificity, being prone to errors without using a customer-designed setup to identify all *BRAF* mutations at codon V600.[29] Overall, pyrosequencing could be performed only on cases not interpretable or negative, corresponding to approximately 50%–60% of samples analyzed by IHC and Sanger sequencing.

RT-PCR-based tests

Several RT-PCR-based tests, certified or not, have been developed, with an overall sensitivity of 97.5% (higher than that of Sanger sequencing) and variable specificity (depending on their design specific for V600E mutation or not, and thus ranging from 87.8% to 100%). The two FDA/CE-IVD-approved tests for *BRAF* mutations (cobas® 4800 *BRAF V600* mutation test and THxID®-BRAF) are both RT-PCR-based assays. Major disadvantages are that they are optimized for the most common *BRAF* mutation, and less common *BRAF* mutations that may still be responsive to BRAF inhibitor therapy may be missed. Further, although these assays require only a small amount of DNA, if the specimen contains <10% of tumor cells, these assays may fail to detect the mutation (the cobas® test guidelines recommend a tumor content of 50% in samples).

Recently, the cobas® test was found to detect only 70% of p.V600K, whereas 100% of p.V600E was detected.[24] Interestingly, in a study comparing different techniques, RT-PCR detected 39 of the 40 p.V600E mutations and all WT cases. The sensitivity of RT PCR for p.V600E detection was 97.5% (95% CI, 87.1% to 99.6%), and specificity was 87.8% (95% CI, 75.8%–94.3%). Surprisingly, the six cases with p.V600K mutation were also positive, although with a lower signal. The four other *BRAF* mutations, including p.V600R, were not detected by RT-PCR. The RT-PCR detection of p.V600K was possible only after inhibition of WT allele amplification. This may be the cause of a lower sensitivity.[31] Inhibition of the WT allele amplification, as performed with peptide nucleic acids (PNAs) to analyze the mutated allele, is in fact an added value to these techniques.

Recently, CE-IVD RT-PCR tests based on PNA inhibition have been developed to detect all the mutations at codon V600, with a sensitivity comparable to that of pyrosequencing.[33] PNA-mediated clamping PCR (PNA-clamping PCR) is based on the principle that PNA inhibits WT by hybridizing normal sequences, and therefore mutant DNA is preferentially amplified.[34] Indeed, PNA clamp RT-PCR detected a 0.5% *BRAF* V600E mutant in the background of the WT with high sensitivity. PNA-clamping PCR may offer a sensitive and reliable alternative method to pyrosequencing, particularly for the detection of a small amount of mutant.

Overall, RT-PCR-based assays, however, provide qualitative information only on *BRAF* at codon 600; no other genes are characterized, and the results are not quantitative (how much mutated *BRAF* is present). Further, when they are optimized for the most common *BRAF* mutation (V600E), less common *BRAF* mutations that may still be responsive to inhibitor therapy may be missed with the exception of PNA-clamp PCR, which detects all mutations at p.V600 with a sensitivity comparable to that of pyrosequencing.

A recent study comparing different methods for detecting *BRAF* mutations concluded that in their present setup, the cobas® 4800 *BRAF* V600 test as well as the therascreen® *BRAF* Pyro Kit (Qiagen NV, Venlo, the Netherlands) are not sufficient for the European approval of vemurafenib because there is a therapeutic option for melanoma patients with any mutation in codon 600 of the *BRAF* gene.[35] The authors suggest a combination of VE1 antibody staining and high-resolution melting or sequencing for p.V600E mutation analysis, combining the lowest detection limit with a fast, reliable method with 100% sensitivity for routine diagnostics at the moment.

Next-generation sequencing

NGS is currently being used to analyze melanoma specimens in many research institutions. Many platforms are available, but what they have in common is that they are massively parallel sequencing techniques in which relatively small stretches (which may cover an exon, a gene, or the whole genome) of DNA are sequenced many times (typically 20 to several hundred times). NGS has several advantages over RT-PCR techniques, the most important being that it provides far more genetic information: besides mutations, NGS can detect rearrangements, amplifications, and deletions, and can analyze many genes. This will become increasingly important as we discover other actionable mutations/mechanisms of resistance to BRAF inhibition. NGS is also more sensitive than many RT-PCR assays, and can detect mutations even when tumor DNA represents less than 10% of the total DNA. NGS could analyze 100% of the cases with 100% specificity and exhibited 98.6% sensitivity in a recent study comparing different methods.[29] Among disadvantages, NGS generally requires more tumor material, has a longer turn-around time,

and requires a higher expertise in computational biology than any other established methods. Most of the information obtained is not yet clinically relevant, and the assays are not FDA approved. International validation is ongoing. NGS requires specialized equipment, computers, and bioinformatics, making it more expensive. This makes NGS largely a research tool at this time. In the near future and with growing experiences, it is an inevitable fact that NGS will replace all established methods for molecular diagnostics, in view of the high sensitivity and multiplexing options of this method allowing generation of a molecular profile of each tumor sample analyzed.[29] A recent study performing whole cancer genome sequencing by NGS methods states that almost 75% of cancer gene variations may be missed by an approach analyzing only hotspot mutations.[36]

Treatment approaches

The presence of a *BRAF* V600 mutation is an important factor to decide the treatment approach that is the best for each patient, but it is not the only one. In fact, first-line treatment with BRAF inhibitors or BRAF inhibitors in combination with MEK inhibitors may not be the best therapeutic strategy for all patients, and the possibility to start with immunotherapy must be considered. FDA approval of ipilimumab includes the first-line treatment of metastatic melanoma, and even if in Europe, initially, the indication was in pretreated patients only, since October 2013 it has been broadened to first-line as well. Moreover, anti-PD-1 agents achieved promising results in clinical trials[7,8,37] and may be recommended soon as a first-line treatment.

The role of chemotherapy as a frontline approach for *BRAF*-mutated metastatic melanoma has been limited by the introduction of targeted therapies. Currently, dacarbazine is the only FDA-approved chemotherapeutic drug for the treatment of metastatic melanoma, and it has not been shown to improve PFS or OS. BRAF inhibitors and BRAF inhibitors in combination with MEK inhibitors achieve tumor regression in a high rate of patients (50%–76%),[2,3,10] PFS[2,3] and OS[2] are improved compared to those seen with chemotherapy, and the onset of tumor regression is early,[41] allowing to treat successfully even symptomatic patients with low performance status and a rapidly evolving disease. Even if mechanisms of resistance, which will be discussed in the "Resistance mechanisms" section, arise in most patients, leading to tumor regrowth, there is the chance for long-term survival at least for a subset of patients, with 26% of patients from the Phase I study being alive at 3 years.[38] Emerging clinical evidence suggests that extended BRAF inhibition after progression

on BRAF inhibitors may prolong survival.[42,43] In a series of 114 patients treated with vemurafenib and dabrafenib within clinical trials, continued therapy with BRAF inhibitors after progressive disease was associated with prolonged survival;[42] similar results were observed in the Phase I study of vemurafenib.[38] However, these data may be biased by patient selection, and prospective randomized trials are needed to investigate the role that prolonged BRAF inhibition may have in the treatment strategies for BRAF-mutated patients. A prospective, single-arm Phase II study was designed to evaluate the activity of treatment after progression during the therapy with vemurafenib with the combination of vemurafenib and fotemustine (NCT01983124).

Preliminary results of the Phase III trial of the combination of dabrafenib and trametinib versus dabrafenib monotherapy were presented at ASCO 2014 annual meeting:[39] even if PFS was only slightly better in the combination arm (9.3 versus 8.8 months), dabrafenib and trametinib achieved 29% improvement in response rate and 37% reduction in risk of death over the monotherapy. In addition, the analysis ad interim of the other Phase III trial comparing the combination of dabrafenib and trametinib with vemurafenib monotherapy showed an OS benefit in the combination arm, allowing the crossover to the combination arm for the patients in treatment with vemurafenib.[40]

In contrast, the response rate and PFS with ipilimumab are lower than those with BRAF/MEK inhibitors and the onset of tumor regression is slow, as it may take time to build an immune antitumor response. However, even if the number of objective responses is relatively low, ipilimumab can induce long-lasting disease control and long-term survival: 18.2% of patients treated within Phase II studies with the approved dose of ipilimumab were alive after 4 years.[44]

Retrospective clinical data seem to indicate that the activity of ipilimumab is not influenced by *BRAF* mutational status,[45] but the administration of ipilimumab after the failure of BRAF inhibitors may have suboptimal results;[46–48] however, no prospective data are available to date. In the ECOG E1612 trial, patients with *BRAF*-mutated metastatic melanoma were randomized to receive either ipilimumab followed by vemurafenib at progression or vice versa; patients were stratified based on ECOG performance status (0 or 1), stage (III and M1a/b or M1c), and prior treatment (yes or no). ECOG trial E4613 will similarly investigate sequential treatment with the combinations ipilimumab and nivolumab versus dabrafenib and trametinib. Until the availability of prospective data from these studies, the choice of first-line treatment for *BRAF*-mutated metastatic melanoma patients

relies on retrospective data and expert opinion. Patients with poor performance status and rapidly evolving disease, whose estimated life expectancy is less than 3 months, may not benefit from front-line treatment with ipilimumab, as may patients with high LDH levels.[49]

The presence of brain metastasis is historically associated with lack of efficacy of systemic therapies and poor prognosis.[50] Even if ipilimumab showed clinical activity in patients with pretreated, asymptomatic, and not steroid-dependent brain metastasis,[51,52] only BRAF inhibitors have evidence of activity in case of active brain metastasis.[53–55]

The identification of new biomarkers may also help selecting patients who are likely to respond to ipilimumab: for example, CTLA-4 gene polymorphisms seem to influence the response to anti-CTLA-4 antibodies,[56] and some immunological signature may predict response to immunotherapy in general.[57]

Anti-PD-1 agents nivolumab and pembrolizumab achieved higher response rates in Phase I–II studies than did ipilimumab (25%–38%),[7,8] and the onset of response was shorter. The duration of response is also impressive, with most responses lasting more than 12 months. Their availability in daily clinical practice may influence the therapeutic strategies, as also patients unfit to be treated with ipilimumab may benefit from treatment with anti-PD-1 drugs. Until then, patients with short life expectancy or who are unlikely to respond to ipilimumab should be treated with BRAF inhibitors or BRAF and MEK inhibitors in combination as a first-line treatment. Two BRAF inhibitors are currently available: vemurafenib and dabrafenib. Data from clinical trials showed that they have substantially the same clinical activity in patients with the *BRAF* V600E mutation, but they slightly differ in toxicity. Both treatments are well-tolerated, and dose reductions were needed in the range of 28% for dabrafenib[3] and 38% for vemurafenib[2] in the Phase III studies. Cutaneous side effects, fatigue, arthralgia, and nausea are the most common adverse events shared by the two drugs;[2,3] however, vemurafenib causes a higher rate of hepatic transaminitis, photosensitivity, and cutaneous hyperproliferative lesions (including squamous cell carcinomas and keratoacanthomas), whereas pyrexia is more commonly seen with dabrafenib.[2,3] As for the *BRAF* V600K mutation, which is the second most common *BRAF* V600 mutation in melanoma (19% as compared with 73% for *BRAF* V600E),[58] in the Phase III study, vemurafenib achieved similar PFS and OS irrespective of the mutation;[2] in the Phase III trial of dabrafenib,[3] patients whose melanoma harbored a V600K mutation were excluded from the study, but some data are

available from the Phase II trial:[59] only 2 patients with the V600K mutation had a response (13%), compared with 45 patients (59%) harboring V600E. However, median PFS (4.5 versus 6.3 months) and OS (12.9 versus 13.1 months) were similar in the two groups. About 8% of melanomas harbor other genotypes than V600E and V600K,[58] such as V600R, and some clinical evidence suggest that vemurafenib and dabrafenib may have clinical activity in this setting.[60] The clinical activity of BRAF inhibitors in patients whose melanomas harbor a non-V600E mutation underlines the importance of using a diagnostic tool that detects all *BRAF* V600 mutations.

The role of trametinib monotherapy may be limited as a first-line treatment of *BRAF*-mutated melanoma, as well as after the failure of therapy with BRAF inhibitors.[61] In the Phase II study of MEK inhibitor trametinib in *BRAF*-mutated patients,[61] there were no confirmed objective responses in the cohort of patients previously treated with a BRAF inhibitor. On the contrary, combination therapy with trametinib and dabrafenib achieved improved clinical activity over dabrafenib monotherapy, but adverse events leading to treatment discontinuation (9% versus 5%), dose reduction (24% versus 13%), and dose interruption (45% versus 30%) were more frequent in patients treated with the combination than in those receiving only dabrafenib.[43] Pyrexia was the most notable risk for the combination compared with dabrafenib, while fewer cutaneous hyperproliferative events were observed in the combination arm, consistent with the identification of activating RAS mutations in most skin tumors developing during therapy with BRAF inhibitors.[13]

Resistance mechanisms

About 15% of patients treated with BRAF inhibitors do not achieve tumor regression, because of intrinsic/primary mechanisms of resistance, and most patients who respond to therapy ultimately develop a mechanism of acquired/secondary resistance, leading to progressive disease.

Mechanisms of primary resistance include *RAC1*[P29S] mutations,[62] *COT* overexpression,[63] alterations in RTK signaling,[64,65] loss of function of *NF1*,[66–69] alterations in the RB1 pathway,[70,71] and alterations in the PI3K-AKT-mTOR pathway (loss of function of *PTEN*) (Table 2).[71–74]

The reactivation of the MAPK pathway is the most frequent cause of acquired/secondary resistance; it may be driven by events that occur upstream (upregulation and activation of the RTKs,[75–79] *NRAS* activating mutations[75]), downstream (activating *MEK1/2* mutations[62,74,80–83]), or at

Table 2 Mechanisms of intrinsic/primary resistance

Aberration	Mechanism of resistance	Reference
RAC1[P29S] mutations	RAC1 regulates cell proliferation and migration	62
COT overexpression	COT activates ERK through mechanism that does not depend on RAF signaling	63
Alterations in RTK signaling	RTK activation can signal either through CRAF or through the PI3K pathway	64,65
Loss of *NF1*	NF1 is a tumor suppressor that inhibits RAS; inactivation of NF1 leads to activation of the signaling pathways downstream of RAS, including PI3K/AKT and MAPK	66–69
Dysregulation of CDK4 and/or cyclin D1	Cyclin D1 binds CDK4 and CDK6, which in turn phosphorylate the retinoblastoma protein and lead to cell cycle progression	70,71
Loss of *PTEN*	PTEN is a tumor suppressor of the PI3K-AKT pathway; loss of function of PTEN leads to AKT activation	71–74

Abbreviation: RTK, receptor tyrosine kinase.

the level of *BRAF* (alternative splicing of V600E BRAF,[84] *BRAF*[V600E] copy number amplification,[85] elevated CRAF levels[86]) (Table 3). The PI3K-PTEN-AKT pathway is a second core resistance pathway: *AKT1/3* mutations and mutations in PI3K-AKT positive-regulatory and negative-regulatory genes may upregulate this pathway,[62,80,87] driving resistance to BRAF inhibitor.

No association was observed between clinical outcome (best response and PFS) and specific mechanisms of resistance.[80] Some tumors develop multiple mechanisms of

resistance simultaneously in the same patient (intrapatient heterogeneity) or even in the same lesion (intratumor heterogeneity).[60,87]

In addition to intrinsic and acquired resistance, mechanisms of adaptive response to BRAF inhibition limit the efficacy of treatment with BRAF inhibitors, leading mostly to partial responses, with complete response rate being in the range of only 3%–6% in the Phase III studies of vemurafenib and dabrafenib.[2,3] In *BRAF*-mutated cells, *ERK* transcriptional products are upregulated, including negative-feedback components, which suppress RAS activation. As a result, RAS does not promote RAF dimerization and BRAF exists predominantly as an active monomer. Treatment with BRAF inhibitors, in addition to arresting tumor growth, relieves ERK negative feedback, partially restoring the sensitivity to extracellular signaling and the activity of RAS, promoting the formation of RAF dimers. BRAF inhibitors bind to one component of the dimer and cause an allosteric activation of the other one.[14] ERK is reactivated and negative-feedback pathways are partially restored over time, leading to the formation of a new steady state of reactivated ERK signaling, which is different among different cell lines.[13] The PI3K-AKT-mTOR pathway is also involved in the mechanisms of adaptive resistance: in fact, the inhibition of the MAPK pathway leads to early, adaptive AKT signaling, unleashing a rebound activation of PI3K-AKT pathway.[88]

Discussion and conclusion

Intrapatient and intratumor heterogeneity of resistance,[62,87,89,90] cross-resistance,[17,91] and alternative pathways activation[88] are

Table 3 Mechanisms of acquired/secondary resistance

Aberration	Mechanism of resistance	Reference
RTKs upregulation	RTK activation can signal either through CRAF or through the PI3K pathway	75–79
Mutations in *NRAS*	*NRAS*-activating mutations (*NRAS*[Q61], *NRAS*[T58], *NRAS*[G13R]) promote enhanced RAF dimerization; RAF inhibitors binding of one member of the dimer results in allosteric transactivation of the drug-free protomer and activation of MEK/ERK	75
Activating *MEK1/2* mutations	MEK1 is situated immediately downstream of RAF proteins in the MAPK pathway and promotes ERK phosphorylation; MEK2 forms heterodimers with MEK1 which activate ERK. Only some mutations have been associated with resistance (*MEK1*[C121S], *MEK1*[Q56P], *MEK1*[K57E], *MEK1*[E203K], *MEK1*[V60E], *MEK1*[G128V], *MEK2*[F57C], *MEK2*[C125S], *MEK2*[V35M], *MEK2*[L46F], *MEK2*[N126D])	62,74,80–83
Alternative splicing of V600E BRAF	Owing to high dimerization property irrespective of RAS status, strongly activates MEK and ERK1/2 in the presence of an RAF inhibitor	84
V600E *BRAF* copy number amplification	MEK/ERK reactivation in an RAS and CRAF-independent manner due to an increased expression of BRAF	85
Elevated CRAF	Elevated CRAF protein levels have been associated with increased levels of phosphorylated ERK1/2 levels and may account for the acquisition of resistance to BRAFi due to increased RAF dimerization	86
Alterations of PI3K-AKT pathway	*AKT1/3* mutations (Q79K and E17K), mutations in PI3K–AKT positive-regulatory genes (*PIK3CA* and *PIK3CG*) and in negative-regulatory genes (*PIK3R2* and *PHLPP1*) upregulate the PI3K-AKT pathway; the missense mutation *AKT1*[A102V] has not been associated with AKT1 activation.	62,80,87

Abbreviation: RTK, receptor tyrosine kinase.

Table 4 Ongoing Phase I/II studies investigating multitargeted combinations

Combination regimen	Phase	Status	ClinicalTrials.gov identifier
MEK inhibitor MEK162			
PI3K inhibitor BKM120	I	Active, not recruiting	NCT01363232
PI3K/mTOR inhibitor BEZ235	I	Completed	NCT01337765
MEK inhibitor MSC1936369B			
PI3K/mTOR inhibitor SAR245409	I	Completed	NCT01390818
Trametinib			
PI3K inhibitor GSK2126458	I	Terminated because of lack of efficacy	NCT01248858
MEK inhibitor GDC-0973			
PI3K inhibitor GDC-0941	I	Completed	NCT00996892
Vemurafenib			
HSP90 inhibitor XL888	I	Recruiting	NCT01657591
Cabozantinib-S-malate	I	Recruiting	NCT01835184
PI3Kβ inhibitor SAR260301	I	Recruiting	NCT01673737
mTOR inhibitors everolimus and temsirolimus	I	Recruiting	NCT01596140
Dabrafenib + trametinib			
Hsp90 inhibitor AT13387	I	Recruiting	NCT02097225
Vemurafenib			
Metformin	I/II	Recruiting	NCT01638676
CDK inhibitor P1446A-05	I/II	Recruiting	NCT01841463
PI3K inhibitor BKM120	I/II	Recruiting	NCT01512251
PI3K inhibitor PX-866	I/II	Active, not recruiting	NCT01616199
Dabrafenib			
AKT inhibitor GSK2141795	I/II	Recruiting	NCT01902173
Dabrafenib + trametinib			
Bcl-2 inhibitor navitoclax	I/II	Recruiting	NCT01989585
MDM2-p53 inhibitor AMG 232	I/II	Not yet recruiting	NCT02110355
MEK inhibitor selumetinib			
AKT inhibitor MK2206	II	Terminated because of slow accrual	NCT01519427
Vemurafenib			
Bevacizumab	II	Recruiting	NCT01495988

Table 5 Ongoing Phase I/II studies investigating the combination of MAPK inhibitors with immunotherapy

Combination regimen	Phase	Status	ClinicalTrials.gov identifier
Vemurafenib			
Adoptive cell therapy	I	Recruiting	NCT01585415
Anti-PDL1 antibody MPDL3280A	I	Recruiting	NCT01656642
Dabrafenib ± trametinib			
Ipilimumab	I	Recruiting	NCT01767454
Vemurafenib			
High-dose interferon alfa-2b	I/II	Recruiting	NCT01943422
PEG-interferon	I/II	Recruiting	NCT01959633
Interleukin 2 + interferon alfa-2b	I/II	Recruiting	NCT01603212
Dabrafenib + trametinib			
Anti-PDL1 MEDI4736	I/II	Recruiting	NCT02027961
Pembrolizumab	I/II	Recruiting	NCT02130466
Vemurafenib			
Adoptive cell transfer and high-dose interleukin 2	II	Recruiting	NCT01659151
Interleukin 2	II	Recruiting	NCT01754376

trials are evaluating the safety and clinical activity of such combination regimens in patients (Table 4).

An alternative to combination strategies may be an adaptive sequential approach based on the biopsy of progressing tumors during therapy with BRAF inhibitors. A Phase II study (NCT01820364) is currently recruiting metastatic melanoma patients who progress on treatment with BRAF inhibitor LGX818: resistant tumors will be biopsied and compared with a pretreatment biopsy to identify the mechanism of resistance. On the basis of the alterations identified in the tumor samples, a second agent from a list of MEK, CDK4/6, FGFR, PI3K, and c-MET inhibitors will be added to the regimen. A limitation of this study is that single biopsy may underestimate the tumor genomics landscape due to tumor heterogeneity. In the future, the analysis of circulating tumor cells or circulating tumor-derived DNA may provide a complete genetic profile compared to single tumor biopsies,[92] but no standard method for their detection and molecular analysis is currently available.[93,94]

In addition to inhibiting multiple molecular targets, either in combination or in sequence, the combination of BRAF inhibitors and immunotherapy may be a strategy to provide durable responses in a high rate of metastatic melanoma patients. BRAF inhibitors do not seem to impair the immune system[95,96] and, on the contrary, may enhance immune activation.[97–102] Combined BRAF inhibitor vemurafenib and anti-CTLA-4 antibody ipilimumab are not tolerated.[103] Nevertheless, anti-PD-1 antibodies pembrolizumab and nivolumab seem to be more tolerated than ipilimumab: their

a challenge for personalized targeted therapies. Preclinical and clinical evidence suggest that one strategy to overcome resistance to BRAF inhibition may be the combination of multiple inhibitors. The combination of BRAF and MEK inhibitors dabrafenib and trametinib achieved a slightly better PFS over dabrafenib monotherapy in the Phase III study. Nevertheless, most mechanisms of resistance may confer cross-resistance to MEK inhibition and may lead to the activation of additional pathways, such as PI3K-AKT-mTOR, suggesting that multiple pathways may be needed to be targeted to achieve durable responses.[17,91] Phase I/II

safety in combination with BRAFi and BRAFi + MEKi is under investigation in Phase I–II clinical studies. In addition, clinical studies are underway to determine the safety and clinical activity of the combination of BRAFi with other immunotherapeutic agents such as anti-PDL-1 antibodies, interleukin, adoptive cell therapy, and interferon (Table 5).

As mentioned in the "Treatment approaches" section, clinical evidence suggests that prolonged BRAF inhibition after progression on BRAF inhibitors may prolong survival in a subset of patients.[42,43] In contrast with these clinical observations, Das Thakur et al demonstrated that cessation of BRAF inhibition may lead to regression of tumors expressing BRAF alternative splicing variants or amplified BRAF and that, in vivo, a discontinuous dosing strategy delayed the onset of resistance over continuous administration.[104–106] The safety and efficacy of an intermittent regimen with BRAF inhibitors will be prospectively evaluated in a Phase II trial (NCT01894672): LGX818 will be administered on a daily schedule dosing for the first 6 weeks; this will be followed by a 2-week break and, thereafter, patients will resume LGX818 on a 2-weeks-on/2-weeks-off schedule.

Until further investigations and availability of new drugs, in clinical practice patients with rapid and extensive progressive disease during treatment with BRAF inhibitors, alone or in combination with MEK inhibitors, are unlikely to benefit from extended MAPK inhibition and should switch to another treatment; patients with isolated progression, on the other hand, could continue treatment with BRAF inhibitors after local treatment of resistant lesions, as resistance mechanisms are not always shared by all metastases.[89]

Disclosure

The authors have no financial interests in any of the products, devices, or drugs mentioned in this paper. Paola Queirolo has received lecture fees from Bristol-Myers Squibb and Roche and served on advisory boards for Bristol-Myers Squibb, Roche, and GSK; Francesco Spagnolo has received lecture fees from Bristol-Myers Squibb, GSK, and Roche. The authors declare no other conflicts of interest.

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