**Introduction**

Cancer is caused by the acquisition of genetic alterations which transforms a normal cell into a malignant one. The Sanger Institute’s Cancer Genome Project has identified several genes that when mutated may facilitate this cellular transformation, including the initial discovery that a somatic mutation of the BRAF gene plays a key role in oncogenesis [1]. The BRAF gene encodes a protein in the Mitogen-Activated Protein Kinase (MAPK) pathway which regulates several important cellular functions, including proliferation, differentiation, migration, and apoptosis [2]. Under normal circumstances, the MAPK pathway is triggered when extracellular signals activate receptor tyrosine kinases and G-protein-coupled receptors. Davies et al. [3] discovered that approximately 80% of BRAF mutations involved an amino-acid change in the region of the enzyme that catalyzes phosphorylation and thereby activation of the MAPK cascade. The most common BRAF mutation is a Valine to Glutamic Acid substitution at position 600 (V600E) within the activation loop [2]. This modification causes constitutive activation, independent of extracellular signals, of the catalytic activity of the Raf protein leading to deregulation of the MAPK pathway. Once activated, Raf forms either a homo- or heterodimer that phosphorylates MEK which then phosphorylates ERK [4-6]. As a result, ERK interacts with several downstream molecules leading to a transforming signal and a pro-growth state that ultimately leads to malignancy.

The BRAF mutation was originally identified by examining the genomic DNA from 15 cancer cell lines (6 breast, 1 small cell lung, 6 non-small cell lung, 1 mesothelioma, 1 melanoma) along with matched lymphoblastoid cell lines from the corresponding patients [3]. Three single base substitutions were found in BRAF exons 11 and 15 in three of the cancer cell lines that were not present in the corresponding lymphoblastoid cells, indicating that the mutations were somatic in nature. Subsequently, an additional 530 cell lines of varying cancer types were sequenced in an attempt to identify any patterns of somatic mutations in BRAF. Forty-three of these cancer cell lines were found to harbor a somatic BRAF mutation, all of which were located in exons 11 and 15.

Currently, 8% of all cancers are estimated to harbor a mutation in the BRAF gene [7]. Papillary thyroid cancer (40%), serous ovarian cancer (30%), colorectal cancers (10%), lung cancer (2-3%) [7] and a variety of brain tumors [8]. However, the highest frequency occurs in melanoma with 40-60% of these tumors containing a BRAF mutation [9-11]. The majority of BRAF mutations in melanoma are V600E mutations as described above, the remainder involve the substitution of a lysine for a valine known as V600K. Melanoma is the fifth most common cancer in men and the seventh most common cancer in women making the discovery of mutant BRAF in 40-60% of melanoma tumors decidedly noteworthy [12]. A more detailed summary of BRAF mutations in different cancer subtypes is provided in Table 1.

**BRAF Inhibitors**

Metastatic melanoma is an aggressive disease with no known cure. High-dose interleukin-2 with objective response rates of 16% and dacarbazine with a median overall survival of approximately 7 months were the only FDA approved therapies from 1976 through 2011 [13-15]. More recently,
Frequency of BRAF point mutation in different tumor types.

Table 1: Frequency of BRAF point mutation in different tumor types.

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>Frequency of BRAF mutation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid</td>
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</tr>
<tr>
<td>Melanoma</td>
<td>40-60</td>
</tr>
<tr>
<td>Ovarian</td>
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<td>Colorectal</td>
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<tr>
<td>Lung</td>
<td>3-Feb</td>
</tr>
<tr>
<td>CNS</td>
<td>7</td>
</tr>
<tr>
<td>Ocular</td>
<td>10</td>
</tr>
<tr>
<td>Biliary tract</td>
<td>6</td>
</tr>
</tbody>
</table>

Likewise, Dabrafenib (GSK2118436) is a selective BRAF V600E kinase inhibitor with demonstrated activity against melanoma cells containing a BRAF mutation. In a phase I study involving 182 patients with BRAF mutant metastatic melanoma, overall response rate was 69% with activity also demonstrated in brain metastasis (response rate of 90%). The most frequent grade 2 or higher adverse events observed were cutaneous squamous cell carcinoma or keratoacanthoma (11%), fatigue (8%) and pyrexia (6%) [19]. A subsequent phase 2 study showed a confirmed response rate of 59% (95% CI, 48.2 to 70.3). After median follow-up of 11.9 months, median OS was 13.1 months and 12.9 months for BRAF V600E and BRAF V600K, respectively [20].

To further assess the efficacy of dabrafenib, a phase 3 clinical trial involving 250 patients with treatment naive BRAF V600E mutant melanoma were randomized (ratio 3:1) to either receive oral dabrafenib 150 mg twice daily (n = 187) or intravenous dacarbazine 100 mg/m² every three weeks (n = 63). Independent reviewer estimated median progression free survival was 6.7 months for the dabrafenib group compared to 2.9 months for the dacarbazine group [21]. After median-follow up of 16.9 months, median overall survival was 20 months for the dabrafenib group and 15.5 months for the dacarbazine group (HR for death 0.77). The most common grade 2 or higher adverse events associated with dabrafenib were hyperkeratosis (41%), arthropalgia (37%), headache (36%), pyrexia (33%) and alopecia (29%). Serious adverse events in more than 5% of dabrafenib treated patients included cutaneous squamous-cell carcinoma/keratoacanthoma (10%) and pyrexia (5%) [22].

Given the profound success of BRAF inhibition, it rationally followed to study MEK inhibition in the same patient population. Trametinib, an allosteric inhibitor of MEK1/MEK2 activation and kinase activity, has shown activity against BRAF V600E mutated melanoma cells in vitro [23] and substantial activity against advanced melanoma in a phase 1 clinical trial [24]. A subsequent phase 2 study showed that clinical response was observed in BRAF-inhibitor -naive patients previously treated with chemotherapy and/or immunotherapy [25]. In a phase 1/2 study involving 247 patients with BRAF mutant metastatic melanoma, the safety and efficacy of combination of dabrafenib and trametinib was compared to dabrafenib alone. The response rate was 76% vs 54%, favoring the combination (p=0.03) and median progression-free survival was 9.4 vs 5.8 months also favoring the combination group. Furthermore, dose limiting toxicities did not differ significantly when combining dabrafenib with trametinib versus dabrafenib alone. The most common grade 3 or higher adverse events in the full dose combination group were pyrexia (5%), fatigue (4%), and cutaneous squamous-cell carcinoma (7%) [26].

The improved efficacy with combination of BRAF and MEK inhibitor compared to BRAF inhibitor alone was shown in two phase 3 clinical trials with comparable adverse events and lower risk of cutaneous squamous cell carcinoma. The combination of dabrafenib and trametinib was found to have a better progression free survival of 9.3 months compared to 8.8 months with dabrafenib alone (HR of progression or death 0.75; p 0.03). The rate of cutaneous squamous cell carcinoma was 2% in the combination group vs 9% in the dabrafenib group [27]. Similarly, another phase 3 trial involving 703 patients with BRAF V600 mutant metastatic melanoma, combination of dabrafenib and trametinib showed improved median overall survival of 72% at 12 months compared to 65% in the vemurafenib alone group (HR for death 0.69, 95% p = 0.005). The median progression free survival was 11.4 months.
months in the combination group and 7.3 months in the vemurafenib group (HR for disease progression 0.56, P < 0.001). The rate of cutaneous squamous cell carcinoma and keratoacanthoma was 1% in the combination group and 18% in the vemurafenib alone group. The rate of any grade pyrexia was 53% in the combination group and 21% in the vemurafenib group [28]. Based on these two trials, the combination of dabrafenib and trametinib is now considered a standard of care for patients with BRAF mutant metastatic melanoma. More recently, another combination has also been approved utilizing vemurafenib and cobimetinib [29].

Iplimumab is a human anti-CTLA-4 monoclonal antibody that promotes T-lymphocyte antitumor activity and has demonstrated a median Overall Survival (OS) of 10 months [30]. Additionally, combination of ipilimumab and dacarbazine has shown improved OS of 11.2 months compared to 9.1 months with dacarbazine alone (HR for death 0.72, p<0.001) [31]. Nivolumab is a human IgG4 anti-programmed death 1 (PD-1) monoclonal antibody that has also demonstrated a survival benefit over chemotherapy [32]. The combination of nivolumab and ipilimumab has shown a prolonged objective response rate and Progression Free Survival (PFS) among patients with metastatic melanoma compared to ipilimumab alone [16].

Pembrolizumab is an anti-PD-1 antibody that prevents PD-1 from binding to its ligands, PD-L1 and PD-L2 and was first evaluated in the large, phase 1 KEYNOTE-001 study [33]. In a pooled analysis of 411 patients with advanced melanoma enrolled in the study and after a median follow-up duration of 18 months, the response rate was 34%, the response was maintained in 81% of those patients, and median overall survival was 25.9 months. In the updated analysis of the phase 3 KEYNOTE-006 study [34]; 37.3% of patients in the overall study population were still alive at 4 years and median Overall Survival (OS) was 23.8 months (95% CI, 20.2-30.4). In ipilimumab-naive and ipilimumab-treated patients, median OS was 29.1 months (95% CI, 22.8-39.0) and 20.2 months (95% CI, 17.8-27.1), respectively; estimated 4-year OS rates were 41.7% and 32.7%, respectively.

Mechanisms of BRAF Resistance

Despite the excellent initial response rates, durable responses to BRAF inhibitors are challenged by the eventual development of either intrinsic or acquired resistance. Less than 10% of patients with BRAF mutant melanoma have intrinsic resistance and do not respond to BRAF inhibitors from the onset of therapy. Alternatively, acquired resistance to BRAF inhibitors generally develops within the first year of treatment for the majority of patients with BRAF mutant melanoma [35,36]. Resistance to single agent MEK inhibitors has also been documented, with 70% of patients with BRAF-mutant melanomas experiencing disease progression within a year of beginning trametinib monotherapy [16]. Interestingly, more than 80% of patients who fail BRAF therapy also fail to respond to MEK inhibition, suggesting cross-resistant mechanisms between the two therapies [25].

BRAF resistance mechanisms are quite heterogeneous. Mechanisms of acquired resistance to BRAF inhibitor therapy can be summarized into three general categories: a secondary reactivation of BRAF, development of a mutation in an associated gene that bypasses the need for BRAF activation, or activation of another growth pathway [37]. A comprehensive evaluation of both genetic and clinical data from 100 patients who progressed on BRAF inhibitor therapy demonstrated 95% of patients had distinct inter- and intra-tumor resistant mechanisms [38]. 70-80% of melanomas which progressed on BRAF inhibitor monotherapy demonstrated reactivation of the MAPK pathway, making this the most common mechanism of resistance [36]. This reactivation occurs via several mechanisms, including mutations in NRAS or KRAS, BRAF splice variants, BRAF amplification and/or MEK 1/2 activation [36,38].

NRAS mutations are present in 10-30% of melanomas and are mutually exclusive to BRAF mutations, suggesting that either gene alteration is sufficient to activate the MAPK pathway 36. De novo activating NRAS mutations have been identified in approximately 30% of BRAF mutant melanomas with acquired resistance to BRAF inhibitors [36,38]. Mutant NRAS activates CRAF, another member of the Raf protein family, which in turn can stimulate downstream MAPK signaling [36,39], thereby bypassing the need for BRAF. Elevated levels of both CRAF and ARAF, both isoforms of RAF which can activate MAPK signaling, have been reported in melanoma cells resistant to BRAF inhibitors in vitro, suggesting that these cells can switch between isoforms to activate MAPK [38]. Activation mutations in NRAS can also result in enhanced dimerization of RAF or RAF-CRAF with subsequent ERK activation, thereby reactivating the MAPK pathway.

Activation of other MAPKs has also been shown to result in resistance to BRAF inhibitors. Over expression of Cancer Osaka Thyroid (COT), a kinase identified by DNA library screening of BRAF mutant cell lines and found to be over expressed in melanoma tissue from patients who failed BRAF inhibitor therapy, is thought to confer resistance to BRAF inhibitors by direct activation of MEK and ERK [36,40].

BRAF amplification and truncated BRAF variants have also been reported to play a role in resistance to BRAF inhibitor therapy. Shi and colleagues reported an increase in genomic copy number in tumors of melanoma patients whose cancer became resistant after initially responding to BRAF inhibitors [41]. Furthermore, alterations in BRAF splicing result in a truncated form of BRAF that lacks the RAS-binding domain, promoting RAF dimerization and activation of MAPK even in the presence of BRAF inhibitors [42]. Activating mutations in MEK1/2 in the absence of RAF activation have been shown to occur in approximately 7% of BRAF inhibitor resistant melanomas [38]; however, the degree of resistance to BRAF inhibitors appears to be dependent on the location and type of mutation in MEK1/2 [36].

Finally, loss of the NF1 gene that encodes the tumor suppressor neurofibromin, an inhibitor of RAS activity, has been suggested as a potentially novel mechanism of resistance [43]. Loss of NF1 results in continuous MAPK activation and may be able to override the action of RAF/MEK inhibitors.

In addition to reactivation of the MAPK cascade, compensatory activity of other signaling pathways can result in BRAF inhibitor resistance. Constitutively active mutated NRAS protein can stimulate the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, resulting in an alternative growth pathway for BRAF mutant resistant melanomas. The PI3K/AKT cascade can

also be triggered by the loss of function in Phosphatase and Tensin Homolog (PTEN). Mutations or deletions of PTEN have been reported in 44% of BRAF mutant melanomas [35,44]. Moreover, activation of the Notch 1 pathway and other Receptor Tyrosine Kinases (RTKs) such as Platelet-Derived Growth Factor Beta (PDGFB), Epidermal Growth Factor Receptor (EGFR), and Insulin-Like Growth Factor IR (IGF-IR) have been associated with the acquisition of BRAF inhibitor resistance in melanoma independent of MAPK reactivation [45].

Interactions between melanoma and its microenvironment can also play a role in BRAF inhibitor resistance. Work reported by Strausmann and colleagues demonstrated that stromal cells secrete Hepatocyte Growth Factor (HGF) which in turn activates the HGF receptor MET on the surface of melanoma cells, leading to reactivation of both the MAPK and PI3K pathways [46,47].

Tirosh et al. demonstrated heterogeneity in tumor cells and cells from the tumor microenvironment (endothelial cells, immune infiltrating cells) [48]. Their data suggests that there was a high level of transcription factors and AXL kinase in metastatic melanoma specimens which may be involved in metastasis. Berger et al 49 performed whole-genome sequence analysis of melanoma sample and identified Phosphatidylinositol Triphosphate Dependent Rac Exchange Factor 2 (PREX2), a PTEN-interacting protein and negative regulator of PTEN, as a frequently mutated (14% of cases) gene in human melanomas. The frequency of PREX2 mutations was markedly higher in metastatic melanomas (about 45%) [49].

**Future Horizons**

While the development of BRAF inhibitors is a substantial advancement in the treatment of BRAF-mutant melanomas, responses are not sustained as disease progression is seen within 12 months. Mechanisms of extrinsic resistance to BRAF inhibitor therapy described above can be summarized into three general categories: a secondary reactivation of BRAF, development of a mutation in an associated gene that bypasses the need for BRAF activation, or activation of another growth pathway [37]. In an attempt to overcome resistance to monotherapy, current first-line treatment for metastatic melanoma involves the combination of BRAF inhibitors with MEK inhibitors or immunotherapy. Combined targeted therapy demonstrated significant improvement in progression-free survival, higher response rates, and improved overall survival with less cutaneous toxicities when compared to BRAF inhibitor monotherapy [26,27]. Unfortunately, resistance to BRAF and MEK inhibitor combinations often still develops; thus, other avenues must be explored for treatment of the BRAF-mutant melanoma population.

Encorafenib is a novel oral small molecule kinase inhibitor with potent and selective inhibitory activity against mutant BRAF kinase. Results from Part 1 of the COLUMBUS study comparing combination of encorafenib plus binimetinib versus Vemurafenib and encorafenib monotherapy in BRAF-mutant melanoma (NCT01909453) showed that there was an increase in PFS in patients with advanced BRAF-mutant melanoma, 14.9 months compared with 7.3 months observed with vemurafenib [Hazard Ratio (HR) 0.54, (95% CI 0.41-0.71, P<0.001)]. These results were encouraging and led to a Phase 3 trial with encorafenib in advanced cancer patients that are currently ongoing: COLUMBUS (encorafenib in combination with binimetinib in BRAF-mutant melanoma).

Another target of interest is the direct inactivation of the ERK protein as this is the terminally activated protein of the MAPK pathway. Several ERK inhibitors are currently in development and three have entered phase I clinical trials: CC-90003 [50], BVD-523 [51] and GDC-0994 [52]. Preliminary reports using BVD-523 suggest that this agent has an acceptable toxicity profile and resulted in durable responses in patients with NRAS mutant and/or BRAF mutant melanomas [51]. Further evaluation of ERK inhibitors, either as monotherapy or in combination therapy with other agents, is warranted.

The focus of several alternative treatment strategies for the BRAF-mutant melanoma population is the use of BRAF inhibitors in combination with other agents that target alternative growth pathways. The combination of PI3K/AKT inhibitors with MAPK inhibitors (either BRAF or MEK) has been evaluated in several trials based on the positive pre-clinical data reported. Shimizu et al. [53] reviewed the records of 76 patients treated with a combination of a PI3K/AKT/mTOR inhibitor and a RAS/MEK/ERK inhibitor enrolled in the Phase I Clinical Trials Program at the South Texas Accelerated Research Therapeutics Center. The patient population included all-comers regardless of cancer type or mutation status. Overall analyses of this patient population suggested that dual inhibition of both pathways exhibited a better efficacy profile compared to inhibition of either pathway alone; however, toxicity was much greater in those patients who received dual therapy 53. More recent data with SWOG S1221 evaluating the combination of the AKT inhibitor GSK2141795 with either dabrafenib alone or in combination with trametinib in patients with BRAF-mutant tumors suggest this combination was well tolerated and led to a durable response, albeit in a small patient population with a short reporting time frame [54]. Several additional phase I trials are currently ongoing. The results of these trials, as well as further phase 2 or 3 trials with larger patient cohorts, will be pivotal in guiding future treatment decisions.

The clinical development of immune checkpoint inhibitors has resulted in a paradigm shift in the treatment of many cancers, including melanoma. Durable responses and prolonged survival rates have been observed in patients with melanoma that far exceed what historically was seen with interleukin-2 (IL-2) immunotherapy [13]. Despite these advances, there are still patients who do not respond to this therapy. The combination of an immune checkpoint inhibitor with a BRAF/MEK inhibitor has been shown to be more effective than the use of either inhibitor alone in preclinical models [55,56]. A recent report by Sullivan and colleagues suggest this combination is reasonably tolerated with promising anti-tumor effects [57]. Various clinical trials combining MAPK inhibitors with immune checkpoint inhibitors are currently ongoing [36].

Cesi et al. [58] demonstrated that inhibition of the RAS/RAF/MEK/ERK pathway induces phosphorylation of the pyruvate dehydrogenase PDH-E1α subunit and use of a BRAF inhibitor induces the up-regulation of Reactive Oxygen Species (ROS). Their data suggests that ROS mediate the activation of Pyruvate Dehydrogenase Kinases (PDKs). In vitro studies demonstrate that inhibition of PDK1 with AZD7545 specifically suppresses growth of BRAF-mutant and BRAF inhibitor resistant melanoma cells. Small
molecule PDK inhibitors such as AZD7545 may be promising drugs for combination treatment in melanoma patients who have developed resistance to BRAF inhibitors.

In addition to the above combinations, other agents with novel mechanisms are being explored in the BRAF-mutant melanoma patient population. Vorinostat is a Histone Deacetylase Inhibitor (HDACi) which has been used in hematologic malignancies. The HDACi in mouse models with BRAF-inhibitor resistant melanoma led to complete regression of the tumor after two months of treatment. HDAC is cause accumulation of ROS leading to apoptosis and up regulation of the MAPK-pathway. Vorinostat is currently in a phase 1 study in resistant BRAF V600 mutated advanced melanoma (NCT02836548) (Figure 1).

Another potential target is adenosine signaling, particularly via the A2A Adenosine Receptor (A2AR). Targeting this pathway may potentially down-regulate cytotoxic lymphocytes (CD8+ T cells and NK cells) while promoting Myeloid-Derived Suppressor Cells (MDSC) and T regulatory cells (Tregs), which could increase immune policing of the tumor. Similarly, inhibiting conditions that initiate adenosine production, such as hypoxia, provide protection against tumor formation. Importantly, targeting adenosine in solid tumors, by using anti-CD73 (NCT02503774) or small molecule A2AR antagonism (NCT02403193 and NCT02655822), has entered clinical trials. Therapeutic approaches targeting the adenosinergic pathway alongside immune checkpoint blockade and chemotherapies is currently in trials, though concrete results are not yet available. Table 2 lists few selected drug combinations currently under trial.

**Table 2: Selected drug combinations currently under trials.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>ClinicalTrials.gov Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vemurafenib+Cobimetinib</td>
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<td>NCT03224208</td>
</tr>
<tr>
<td>Vemurafenib+Fotemustine</td>
<td>Nitrosurea + BRAF inhibitor</td>
<td>NCT01983124</td>
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<td>Combination of HSP90 and BRAF inhibitors</td>
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<tr>
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<tr>
<td>MED4736+Dabrafenib+Trametinib</td>
<td>Combination of PD-1, BRAF, MEK inhibitor</td>
<td>NCT02027961</td>
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<tr>
<td>Nivolumab+ Dabrafenib and/or Trametinib</td>
<td>Combination of PD-1, BRAF, MEK inhibitor</td>
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<tr>
<td>Pembrolizumab+ Trametinib+ Dabrafenib</td>
<td>Combination of PD-1, BRAF, MEK inhibitor</td>
<td>NCT02130466</td>
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<tr>
<td>Onalespib+ Dabrafenib+ Trametinib</td>
<td>Combination HSP90, PD-1 and BRAF inhibitors</td>
<td>NCT02097225</td>
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</table>

**Conclusion**

The discovery of the BRAF mutation has led to improved survival rates in a significant portion of the melanoma patient population. Patients are living longer while taking oral therapies with reasonable safety and toxicity profiles. The improvement in patient quality of life cannot be overstated with the use of these newer agents. Nonetheless, resistance to treatment ultimately develops in nearly all of these patients. To date, nine mechanisms of resistance have been described including the most frequent: mutant BRAF allele amplification, BRAF splice variants, and MEK1/2 activation.
Several new combination strategies are being employed in clinical trials in an attempt to overcome or delay resistance. Unacceptable toxicities can be a significant barrier to combination therapies as seen in studies combining PI3K inhibitors with MEK inhibitors. Additionally, given the overall diversity of resistance mechanisms, more than one new drug combination will likely be required.

Prior to 2011, metastatic melanoma patients had a life expectancy of 6-9 months. The identification of mutant BRAF, RAF inhibitors, MEK inhibitors, and immunotherapy now offer these patients the hope of living for years or even decades. This degree of benefit was unlikely to have been predicted in 2002 at the time of the Sanger Institute’s Cancer Genome Project.

References


