New Strategies in Melanoma: Molecular Testing in Advanced Disease

Running Title: Molecular testing in melanoma

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Abstract

Melanoma is one of the most aggressive forms of skin cancer. The management of melanoma is evolving rapidly due to an improved understanding of the molecular heterogeneity of this disease, and the development of effective, personalized targeted therapy strategies. While previous classification systems have been based predominantly on clinical and histologic criteria, there is now a strong rationale to add molecular markers to the diagnostic evaluation of these tumors. Research has demonstrated that the types and prevalence of genetic alterations varies between melanoma subtypes. Thus, rational molecular testing should be based on an understanding of the events likely to be present in a given tumor, and the clinical implications of testing results. This review summarizes the existing data supporting the rationale for molecular testing in clinically-defined melanoma subtypes. Emerging challenges and controversies in the use of various molecular testing platforms, and the implications of these differences for clinical testing, will also be discussed.
Background

Melanoma, the most aggressive of the common skin cancers, will be diagnosed in an estimated 166,900 patients in developed countries in 2011 (1). Melanomas are currently classified based on clinical and histologic characteristics of the primary tumors. Cutaneous melanomas, the most common subtype, arise from melanocytes in the skin, and are strongly linked to ultraviolet (UV) radiation exposure. Acral melanomas also arise from melanocytes in the skin, but specifically on the palms, soles, and subungal surfaces, which have less UV exposure. Mucosal melanomas arise in mucosal tissues in the respiratory, digestive, and reproductive tracts. Finally, uveal melanomas arise from melanocytes in the choroid, ciliary body, or iris of the eye. Whole-genomic approaches have demonstrated that these different subtypes are characterized by distinct patterns of DNA copy number alterations (2, 3). These subtypes are also characterized by distinct oncogenic mutations, some of which are critical determinants of the efficacy of targeted therapies, while others have potential diagnostic and/or prognostic value.

BRAF

The BRAF gene encodes a serine-threonine kinase that is a key effector of the RAS-RAF-MEK-ERK pathway. The overall rate of BRAF mutations in melanoma patients is approximately 45% (4). While this accurately reflects the rate in the common cutaneous melanomas that develop in areas with intermittent sun exposure, the rate of BRAF mutations is lower in acral, mucosal, and cutaneous melanomas with evidence of chronic sun damage (CSD), and are essentially absent in uveal melanomas (2, 4-6) [Table 1]. The mutations in BRAF overwhelmingly occur in exon 15, which encodes the catalytic domain of the BRAF protein (4). Substitutions at the valine at
position 600 (V600) represent ~95% of the reported point mutations in melanoma, most commonly V600E (~75% of V600 mutations), followed by V600K (20%) (6, 7). The V600 mutations increase the kinase activity of BRAF 130- to >700-fold (8, 9). Other patient-derived mutations (G464E, G466V, D594V) decrease BRAF catalytic activity but promote dimerization with CRAF proteins, and thus still increase activation of MEK and ERK (10).

Several studies have identified a significant association of BRAF mutations with younger age, but there is no reproducible association with relapse-free survival (RFS) or overall survival (OS) from melanoma diagnosis (11-13). Recently, two large retrospective studies identified significantly shorter OS from the diagnosis of stage IV disease for patients with an activating BRAF mutation (6, 7). While the stage-specific correlation with worse outcomes is intriguing, this finding must now be viewed in the context of mutant-selective BRAF inhibitors. Vemurafenib (also known as RG7204, or PLX4032; Roche) and GSK2118436 (GlaxoSmithKline) are potent BRAF inhibitors with increased affinity for V600-mutant over wild-type BRAF (14, 15). Both agents achieved an overall response rate (ORR) of >50% in early-phase clinical testing in patients with BRAF V600E mutations (15, 16). No patients with a wild-type BRAF responded, which is consistent with pre-clinical studies showing that selective BRAF inhibitors actually increase the growth of such melanomas (10, 17-19). In the BRIM3 phase III trial, vemurafenib treatment resulted in significant increases in ORR, progression-free survival (PFS), and OS as compared to dacarbazine in BRAF V600E-positive metastatic melanoma patients (20). Clinical responses have also been reported with both vemurafenib and GSK2118436 in patients with BRAF V600K mutations, and with GSK2118436 with V600G mutations (15, 20, 21). No clinical responses with GSK2118436 were achieved in two patients with BRAF K601E mutations (15). Retrospective studies of outcomes in stage IV melanoma
patients demonstrated that \textit{BRAF}-mutant melanoma patients treated with selective BRAF inhibitors had longer OS from stage IV compared to patients with a wild-type \textit{BRAF} gene, while \textit{BRAF}-mutant patients who did not receive such treatment had worse outcomes (6, 7).

\textit{NRAS}

The RAS-RAF-MEK-ERK pathway is also activated in melanoma by point mutations in \textit{NRAS} [Figure 1]. Like \textit{BRAF}, the prevalence of \textit{NRAS} mutations is highest in cutaneous tumors without CSD, and they are not detected in uveal melanomas [Table 1]. The majority of mutations affect residues Q60 and Q61 in exon 2 (~80%), with most other mutations affecting G12 and G13 in exon 1 (~20%) (4, 6). Activating mutations in \textit{NRAS} are generally mutually exclusive with activating \textit{BRAF V600} mutations, with co-occurrence observed in <1% of treatment-naive cutaneous melanomas (6). Some melanoma clinical specimens and cell lines with secondary resistance to selective BRAF inhibitors have recently been reported with concurrent \textit{BRAF V600} and \textit{NRAS} mutations; only the \textit{BRAF} mutations were detectable prior to treatment (22). Of note, all reports to date have shown retention of the same \textit{BRAF V600} mutations at disease progression with no secondary mutations, but the number of cases analyzed is relatively small (22-24).

\textit{NRAS} mutations have been associated with increased Breslow (BT) thickness of primary melanomas in two independent series of >200 consecutive melanoma patients (11, 25). One series also reported significantly shorter RFS and OS from primary melanoma diagnosis among patients with an \textit{NRAS} mutation (25). A retrospective study of metastatic melanoma patients also identified shorter OS from stage IV diagnosis for patients with \textit{NRAS} melanomas as
compared to patients with wild-type \textit{BRAF} and \textit{NRAS} genes, with similar outcomes to patients with \textit{BRAF} mutations who were not treated with BRAF inhibitors (6).

\textit{KIT}

Detailed analysis of the 4q12 chromosomal region in mucosal, acral, and CSD-cutaneous melanomas identified frequent increased copy number (~25\%) and mutations (10-20\%) of the \textit{KIT} receptor tyrosine kinase gene in these subtypes [Table 1] (26). \textit{KIT} copy number changes and mutations are very rare in non-CSD cutaneous melanomas, but there are mixed data about the prevalence in tumors with CSD (5, 26). \textit{KIT} genetic aberrations in melanoma differ from other cancers (i.e. gastrointestinal stromal tumors [GIST]) in the frequent occurrence of copy number gain, the preponderance of substitution mutations (deletions or insertions are rare), and the distribution of mutations (increased \textit{de novo} prevalence of mutations in exons 13, 17, and 18) (27).

Reports on the association of \textit{KIT} mutations with survival differ. Kong et al., reported a cohort of 502 Chinese patients, which included a high frequency of acral (38.4\%) and mucosal (33.3\%) melanomas (28). In this cohort, \textit{KIT} mutations correlated with shorter overall survival, but survival data specific to mucosal or acral melanomas was not reported. Omholt et al., reported the results of a Swedish cohort of 71 mucosal melanoma patients characterized for \textit{KIT} (35\%), \textit{NRAS} (10\%) and \textit{BRAF} (6\%) mutations (29). \textit{KIT} mutation status did not correlate with survival.
Multiple reports have demonstrated impressive responses with FDA-approved KIT inhibitors in individual melanoma patients with KIT mutations (30, 31). However, two recent prospective clinical trials treating patients with KIT genetic aberrations with imatinib have reported relatively disappointing clinical response rates of 23% and 16% (32, 33). Although these were relatively small trials, the accompanying molecular studies suggest that clinical responses occurred at higher frequency in tumors with recurrent mutations in exons 11 and 13 (i.e. L576P, K642E), are more likely with increased mutant: wild-type allelic ratios, and tend not to occur with KIT increased copy number without mutation. Studies are ongoing with other KIT inhibitors.

GNAQ/GNA11 and BAP1

Uveal melanomas have frequent mutations in the alpha subunits of the G-proteins GNAQ (~35%) and GNA11 (~45%) (34, 35). Mutations in GNAQ and GNA11 are essentially absent in cutaneous and mucosal melanomas, but have also been detected in dermal melanocytic proliferations (e.g., blue nevi) and primary meningeal melanoma (34, 36). Mutations in GNAQ and GNA11, which are mutually exclusive, involve the hotspot residues R183 and Q209 and, like RAS mutations, inactivate their intrinsic GTPase activity. Mutations in GNAQ and GNA11 activate the MEK/ERK pathway, although other pathways may also be affected. There is no significant correlation of GNAQ or GNA11 mutations with clinical outcomes, pathological, or cytogenetic features. (34, 35, 37, 38).

While GNAQ/11 mutations are not prognostic, a gene expression profiling assay has been developed and commercialized that separates primary uveal melanomas into low (class 1) and
high (class 2) metastatic risk groups (39). The risk of metastasis is also elevated in primary uveal melanoma tumors that demonstrate monosomy 3 (40). The recent identification of inactivating mutations in uveal melanomas in \textit{BAP1} on chromosome 3p21 in \textasciitilde80% of tumors with monosomy 3 implicates it as a tumor suppressor (41). \textit{BAP1} mutations were present in 84% of tumors with a class 2 gene expression profile, and only 4% of tumors with a class 1 profile. Somatic mutations in \textit{BAP1} have also been identified in 23% of sporadic mesothelioma tumors (42), and germline mutations in have been observed in families with a high propensity for uveal melanoma and other cancers (43, 44).

\textbf{On the Horizon}\n
There is ample evidence to support molecular testing of patients diagnosed with melanoma. Generally, preference is given to performing testing on metastases to most accurately reflect tumor biology at the time of systemic treatment. Nonetheless, existing data has demonstrated high concordance (\textasciitilde95%) for \textit{BRAF} and \textit{NRAS} mutation status between primary tumors and regional metastases (45). Thus, it is reasonable to perform \textit{BRAF/NRAS} mutation testing on primary tumors if metastatic disease is not available; very little data is available at this time about the concordance of other mutations. The discovery of multiple new genetic events after development of resistance to \textit{BRAF} inhibitors, many of which have implications for rational therapeutic approaches, strongly supports the testing of enlarging or new metastases in those patients. Reliable mutation testing also depends upon the prevalence and viability of tumor cells in a given specimen, although specific criteria are dependent upon the testing platform being used.
Sanger sequencing, which was used in the initial identification of most melanoma-prevalent mutations, is an open method that detects virtually any genomic mutational event occurring between the sequencing primer pairs, including substitutions, deletions, and insertions. It is clear that many of the genes of interest in melanoma (i.e. $BRAF$, $NRAS$, $GNAQ$, $GNA11$) are altered by a limited number of mutations clustered at particular codons. The pattern of recurrent hotspots enables the use of more focused techniques that detect these specific alterations. Such focused approaches include allele-specific real-time PCR, pyrosequencing, SNaPshot® and mass spectrometry-based methods (Sequenom®) (46, 47). These methods have the general advantages of increased sensitivity and speed. SNaPshot® and Sequenom® also allow detection of hotspot mutations in multiple genes simultaneously; however, these platforms only detect predetermined hotspots and will not discover new mutations for which probes have not been designed. Thus these techniques are generally not effective for screening genes affected by non-recurrent or multi-nucleotide substitutions, insertions, or deletions (i.e. $KIT$, $BAP1$). The rapid development of microfluidic PCR amplification linked to next generation sequencing technologies will likely allow for virtually unlimited mutation detection and multiplexing for molecular testing in the near future.

One of the most important and rapidly evolving areas in molecular testing centers around $BRAF$ mutations. In 2011 the US FDA approved vemurafenib for the treatment of metastatic melanoma patients with a $BRAF V600$ mutation, along with the Cobas 4800 companion diagnostic test, both from Roche. This real time PCR test is focused on the qualitative detection of the $BRAF V600E$ mutation. While full characterization of this test is forthcoming, data presented at research meetings suggest very high sensitivity (>99%) and specificity (88%) for the detection of the $BRAF V600E$ mutation (48, 49). However, certain two nucleotide ("E2")
mutations that result in a BRAF V600E mutation are not detected, and there is reduced sensitivity for other amino acid substitutions at the V600 site (i.e. estimated 66% sensitivity for BRAF V600K). Currently, there is insufficient data to define the true response rates, but at least some patients with BRAF V600K mutations achieve clinical responses with BRAF inhibitors. Thus, there is a rationale to identify highly accurate methods that detect all BRAF V600 mutations at this time to determine which genotypes should be treated with these inhibitors.

How the FDA approval of the Cobas 4800 diagnostic test will impact the use of the current portfolio of validated BRAF tests performed in CLIA-certified laboratories around the country is uncertain. Although the high sensitivity of the Cobas 4800 could lead to the detection of BRAF mutations lacking clinical relevance, the high rate of clinical benefit observed in the BRIM-3 trial, which used this platform, suggests that this may not be a significant problem (20). More globally, requiring a specific platform for a test goes against prevailing currents for work flow management in molecular diagnostics. As more results are demanded from ever smaller samples, platforms that robustly multiplex molecular assays are becoming essential for patient management. The availability of multiple validated platforms is also important to resolve testing anomalies, as all platforms have situational advantages and disadvantages in both sensitivity and specificity. The relative cost of testing by different platforms is also an important economic issue.

While multiplexed testing with next generation sequencing technologies may soon make such considerations obsolete, current practice efficiency and economic considerations may demand that molecular testing in newly diagnosed advanced melanoma patients be done in a triaged fashion. In this setting the virtual mutual exclusivity of BRAF, NRAS and KIT mutations is very useful. As both BRAF and NRAS are hotspot mutation tests, they can easily fit into
panels, and the identification of an \textit{NRAS} mutation in the setting of a \textit{BRAF} wild-type result provides added confidence of the technical quality of the result. Such information is particularly helpful in the analysis of clinical samples in which the proportion of neoplastic cells in the tumor mass is in the minority (sometimes as low as 10\%), a common scenario in which there is a critical need to rule out false-negative results. Thus, in non-acral cutaneous melanomas, \textit{BRAF} can be tested first, preferably with \textit{NRAS} in a panel, with \textit{KIT} only being offered if these two are negative. This can reduce the number of the more labor-intense \textit{KIT} testing by up to 70\%. If validated in additional studies, documentation of \textit{KIT} locus copy gain may also have utility in tumors with \textit{KIT} mutations (33). In acral and mucosal melanomas, mutation frequencies support the simultaneous testing of \textit{BRAF} and \textit{KIT} mutations as a first step, preferably with \textit{NRAS} testing. Testing for \textit{BRAF}, \textit{NRAS}, and \textit{KIT} mutations in uveal melanomas is not recommended, but testing for \textit{GNAQ}, \textit{GNA11}, and/or \textit{BAP1} may become important in the future. For patients with metastases from an unknown primary lesion, testing for \textit{BRAF} and \textit{NRAS} is a reasonable first step based on analyses showing a mutational profile similar to cutaneous melanomas (6). Subsequent testing for other melanoma-prevalent mutations, including \textit{GNAQ/11} for diagnostic purposes, can then be performed if they are negative (Table 1).

These recommendations are largely based on existing studies in advanced melanoma patients. Planned clinical trials in patients with early-stage melanoma will provide additional data regarding the clinical value of these tests in that setting. Ongoing research about clinical outcomes, and resistance mechanisms to targeted therapies, may also support the rationale to test other molecular aberrations known to occur in melanoma (i.e. \textit{PTEN} loss, \textit{MEK} mutation, \textit{CDK4} mutation, \textit{CYCLIN D1} amplification) [Figure 1] in the future. This field will also likely evolve
over the next few years with more data about, and increased availability of, additional molecular testing platforms.
REFERENCES


Figure Legends

Figure 1. Molecular alterations in melanoma. MUT, mutated; AMP, amplified; DEL, deletion or other alteration that results in loss of expression/function.
Table 1. Gene Mutation Prevalence and Molecular Testing Recommendations for Melanoma Subtypes

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Testing Recommendations</th>
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<tbody>
<tr>
<td>BRAF</td>
<td>NRAS</td>
</tr>
<tr>
<td>Cutaneous (Non-CSD)</td>
<td>45%</td>
</tr>
<tr>
<td>Cutaneous (CSD)</td>
<td>5-30%</td>
</tr>
<tr>
<td>Acral</td>
<td>10-15%</td>
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<tr>
<td>Mucosal</td>
<td>5%</td>
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<tr>
<td>Uveal</td>
<td>-</td>
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<tr>
<td>Melanoma from an Unknown Primary</td>
<td>50%</td>
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*Gene Expression Profiling and Monosomy 3 Analysis of primary uveal melanomas have been used as prognostic tests for metastatic risk<sup>41</sup>; they do not have a defined role in patients with metastatic disease.

Non-CSD = Non-chronic sun-damaged
CSD = Chronic sun-damaged
- Insignificant number reported
? Not yet reported