New Strategies in Melanoma: Molecular Testing in Advanced Disease

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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. Although previous classification systems were based predominantly on clinical and histologic criteria, there is now a strong rationale for adding molecular markers to the diagnostic evaluation of these tumors. Research has shown that the types and prevalence of genetic alterations vary among melanoma subtypes. Thus, rational molecular testing should be based on an understanding of the events that are likely to occur in a given tumor and the clinical implications of test results. This review summarizes the existing data that support the rationale for molecular testing in clinically defined melanoma subtypes. Emerging challenges and controversies regarding the use of various molecular testing platforms, and their implications for clinical testing, are also discussed. \textit{Clin Cancer Res; 18(5); 1195–200.} \textcopyright 2012 AACR.

Background

Melanoma, the most aggressive of the common skin cancers, was 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 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 shown 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 these mutations are critical determinants of the efficacy of targeted therapies, and others have potential diagnostic and/or prognostic value.

**BRAF**

The \textit{BRAF} gene encodes a serine–threonine kinase that is a key effector of the RAS–RAF–mitogen-activated protein kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway. The overall rate of \textit{BRAF} mutations in melanoma patients is \textasciitilde 45\% (4). Although this accurately reflects the rate in the common cutaneous melanomas that develop in areas with intermittent sun exposure, the rate of \textit{BRAF} mutations is lower in acral, mucosal, and cutaneous melanomas with evidence of chronic sun damage (CSD), and they are essentially absent in uveal melanomas (Table 1; refs. 2, 4–6). The mutations in \textit{BRAF} overwhelmingly occur in exon 15, which encodes the catalytic domain of the \textit{BRAF} protein (4). Substitutions at the valine at position 600 (V600) represent \textasciitilde 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 \textit{BRAF} from 130- to greater than 700-fold (8, 9). Other patient-derived mutations (G464E, G466V, and D594V) decrease \textit{BRAF} catalytic activity but promote dimerization with CRAF proteins and thus still increase activation of MEK and ERK (10).

Several studies identified a significant association of \textit{BRAF} mutations with younger age but found no reproducible association with relapse-free survival or overall survival from melanoma diagnosis (11–13). Recently, 2 large retrospective studies identified significantly shorter overall survival from the diagnosis of stage IV disease for patients with an activating \textit{BRAF} mutation (6, 7). Although the stage-specific correlation with worse outcomes is intriguing, this finding must now be viewed in the context of mutant-selective \textit{BRAF} inhibitors. Vemurafenib (also known as RG7204 or PLX4032; Roche) and dabrafenib (also known as GSK2118436; GlaxoSmithKline) are potent \textit{BRAF} inhibitors with increased affinity for V600-mutant over wild-type \textit{BRAF} (14, 15). Both agents achieved an overall response rate of greater than 50\% in early-phase clinical...
Activating mutations in \( \text{NRAS} \) residues Q60 and Q61 in exon 2 are exclusive with activating \( \text{BRAF} \) mutations at disease progression with no secondary mutations; only the \( \text{BRAF} \) mutations at \( V600 \) were detectable prior to treatment (22). Of note, all reports to date have shown retention of the same \( \text{BRAF} \) mutations at disease progression with no secondary mutations; however, the number of cases analyzed is relatively small (22–24).

In 2 independent series of more than 200 consecutive melanoma patients, \( \text{NRAS} \) mutations were associated with an increased Breslow thickness of primary melanomas (11, 25). One series also reported significantly shorter relapse-free survival and overall survival from primary melanoma diagnosis among patients with an \( \text{NRAS} \) mutation (25). A retrospective study of metastatic melanoma patients also identified shorter overall survival from stage IV diagnosis for patients with \( \text{NRAS} \) mutations compared with patients with wild-type \( \text{BRAF} \) and \( \text{NRAS} \) genes, with similar outcomes in patients with \( \text{BRAF} \) mutations who were not treated with \( \text{BRAF} \) inhibitors (6).

\textbf{KIT}

A 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 \( \text{KIT} \) receptor tyrosine kinase gene in these subtypes (Table 1; ref. 26). \( \text{KIT} \) copy number changes and mutations are very rare in non-CSD cutaneous melanomas; however, data regarding the prevalence in tumors with CSD are mixed (5, 26). \( \text{KIT} \) genetic aberrations in melanoma differ from those in other cancers (i.e., gastrointestinal stromal tumors) 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 KIT mutations with survival differ. Kong and colleagues (28) examined a cohort of 502 Chinese patients, which included a high frequency of acral (38.4%) and mucosal (33.3%) melanomas. In this cohort, KIT mutations correlated with shorter overall survival, but survival data specific to mucosal or acral melanomas were not reported. Omholt and colleagues (29) reported the results of a Swedish cohort of 71 mucosal melanoma patients characterized for KIT (35%), NRAS (10%), and BRAF (6%) mutations. KIT mutation status did not correlate with survival.

Multiple studies have reported impressive responses with U.S. Food and Drug Administration (FDA)–approved KIT inhibitors in individual melanoma patients with KIT mutations (30, 31). However, 2 recent prospective clinical trials that treated patients with KIT genetic aberrations with imatinib reported relatively disappointing clinical response rates of 23% and 16% (32, 33). Although these were relatively small trials, the accompanying molecular studies suggest clinical responses that occur at higher frequency in tumors with recurrent mutations in exons 11 and 13 (i.e., L576P and K642E), are more likely to occur 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 α 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 they have 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. No significant correlation of GNAQ or GNA11 mutations with clinical outcomes, pathologic features, or cytogenetic features has been reported (34, 35, 37, 38).

Although GNAQ/11 mutations are not prognostic, researchers have developed and commercialized a gene expression profiling assay 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 show monosomy 3 (40). The recent identification of inactivating mutations in uveal melanomas in BAP1 on chromosome 3p21 in ~80% of tumors with monosomy 3 implicates it as a tumor suppressor (41). BAP1 mutations were shown to be 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 BAP1 were also identified in 23% of sporadic mesothelioma tumors (42), and germline mutations were observed in families with a high propensity for uveal melanoma and other cancers (43, 44).

On the Horizon

There is ample evidence to support molecular testing of patients who have been diagnosed with melanoma. Generally, preference is given to testing metastases to most accurately reflect the tumor biology at the time of systemic treatment. Nonetheless, existing data show a high concordance (>95%) for BRAF and NRAS mutation status between primary tumors and regional metastases (45). Thus, it is reasonable to perform BRAF/NRAS mutation testing on primary tumors if metastatic disease is not available; at present, very few data regarding the concordance of other mutations are available. The discovery of multiple new genetic events that occur after patients develop resistance to BRAF inhibitors, many of which have implications for rational therapeutic approaches, strongly supports the testing of enlarging or new metastases in such patients. Reliable mutation testing also depends on the prevalence and viability of tumor cells in a given specimen, although specific criteria are dependent on the testing platform 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, and 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 can detect these specific alterations. Such focused approaches include allele-specific real-time PCR, pyrosequencing, SNaPshot, and mass spectrometry–based methods [e.g., Sequenom (46, 47)]. These methods have the general advantages of increased sensitivity and speed. SNaPshot and Sequenom also allow the simultaneous detection of hotspot mutations in multiple genes; however, these platforms only detect predetermined hotspots and cannot discover new mutations for which probes have not been designed. Thus, these techniques are generally not effective for screening genes that are affected by nonrecurrant or multinucleotide substitutions, insertions, or deletions (i.e., KIT and 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 is centered on BRAF mutations. In 2011, the FDA approved vemurafenib for the treatment of metastatic melanoma patients with a BRAF V600 mutation, along with the cobas 4800 companion diagnostic test (both Roche). This real-time PCR test is focused on qualitative detection of the BRAF V600E mutation. Although a full characterization of this test is forthcoming, data that have been presented at research meetings suggest a very high sensitivity (>99%) and specificity (88%) for detection of the BRAF V600E mutation (48, 49). However, certain 2-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., an estimated 66% sensitivity for BRAF V600K). Currently, there are insufficient data to define the true response rates, but it is clear that at least some patients with BRAF V600K mutations achieve clinical responses with BRAF inhibitors. Thus, to determine which genotypes should be treated with these inhibitors, we need highly accurate methods that can detect all BRAF V600 mutations.

How the FDA approval of the cobas 4800 diagnostic test will influence the use of the current portfolio of validated BRAF tests performed in Clinical Laboratory Improvement Amendments–certified laboratories around the country is uncertain. Although the high sensitivity of 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 workflow management in molecular diagnostics. As more results are being 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.
Although 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 patients with advanced melanoma be done in a triaged fashion. In this setting, the virtual mutual exclusivity of BRAF, NRAS, and KIT mutations is very useful. Because both BRAF and NRAS are hotspot mutation tests, they can easily fit into panels, and the identification of an NRAS mutation in the setting of a BRAF wild-type result provides added confidence in the technical quality of the result. Such information is particularly helpful for analyzing 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, BRAF can be tested first, preferably with NRAS in a panel, and KIT can be offered if these 2 tests are negative. This can reduce the number of KIT tests, which are more labor intensive, by up to 70%. If it is validated in additional studies, documentation of KIT locus copy gain may also have utility in tumors with KIT mutations (33). In acral and mucosal melanomas, mutation frequencies support the simultaneous testing of BRAF and KIT mutations as a first step, preferably with NRAS testing. Testing for BRAF, NRAS, and KIT mutations in uveal melanomas is not recommended, but testing for GNAQ, GNA11, and/or BAP1 may become important in the future. For patients with metastases from an unknown primary lesion, testing for BRAF and NRAS is a reasonable first step, based on analyses showing a mutational profile similar to that of cutaneous melanomas (6). Subsequent testing for other melanoma-prevalent mutations (e.g., GNAQ/11) for diagnostic purposes can then be performed if the BRAF and NRAS tests are negative (Table 1).

These recommendations are largely based on recent studies in patients with advanced melanoma. 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 mechanisms of resistance to targeted therapies may also support the rationale to test other molecular aberrations known to occur in melanoma [i.e., PTEN loss, MEK mutation, CDK4 mutation, and CYCLIN D1 amplification (Fig. 1)] in the future. This field will also likely evolve over the next few years as we acquire more data about additional molecular testing platforms and such platforms become more widely available.

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S. Woodman received commercial research grants from Bristol-Myers Squibb and GlaxoSmithKline, and honoraria from Quintiles. A. Lazar received commercial research grants from Myriad, Roche, GlaxoSmithKline, and Merck; has an ownership interest in Novartis, is a consultant to Pfizer; and received honoraria from AstraZeneca and Novartis. K. Adalpe is a consultant to Genentech, AstraZeneca, and Bristol-Myers Squibb. M. Davies received a commercial research grant from GlaxoSmithKline, Roche, AstraZeneca, Merck, and Myriad, and is a consultant to GlaxoSmithKline and Genentech.

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