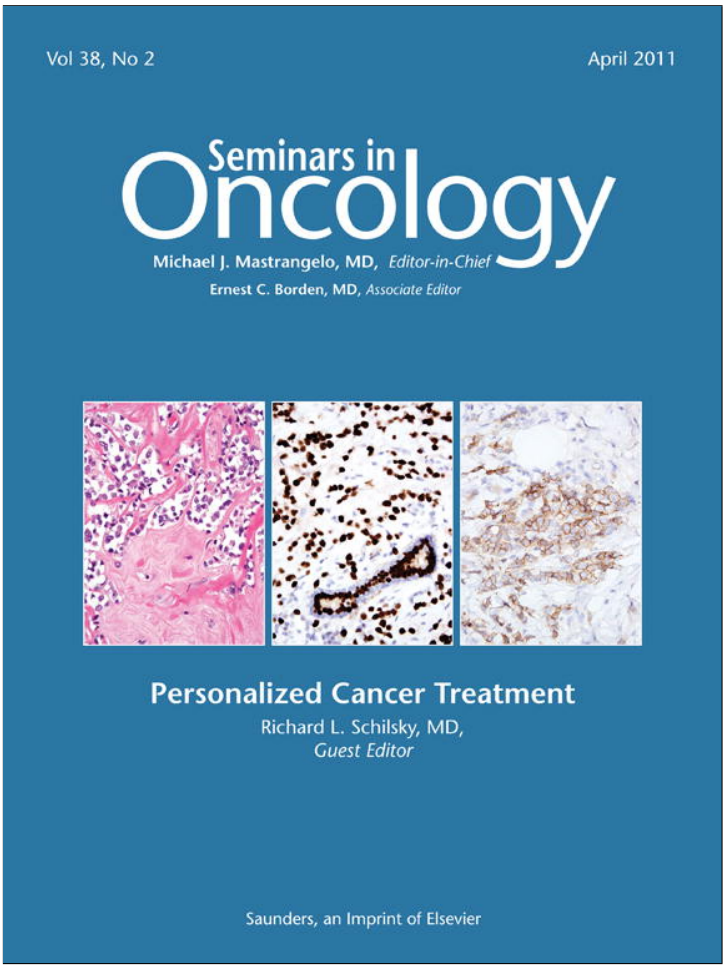

Molecular Profiling of Melanoma and the Evolution of Patient-Specific Therapy

Thomas F. Gajewski

It recently has become clear that multiple molecular subtypes of melanoma likely exist that may be associated with clinical response to defined therapeutic modalities. Gene expression profiling has revealed a signature that is associated with clinical benefit to melanoma vaccines, with preliminary work suggesting a correlation with response to other immunotherapy agents as well. Activating mutations in B-Raf and c-kit are associated with clinical response to the specific kinase inhibitors PLX4032 and imatinib, respectively. Several other signal transduction pathways have been found to be constitutively active or mutated in other subsets of melanoma tumors that are potentially targetable with new agents. Together, these emerging data suggest the evolution of a new paradigm in melanoma therapy in which molecular analysis of the tumor will be used to assign the most appropriate therapeutic modality for each individual patient, to maximize therapeutic success.
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EVIDENCE FOR EXISTENCE OF BIOLOGIC SUBSETS OF MELANOMA

Only two US Food and Drug Administration–approved drugs are available to treat patients with metastatic melanoma, the chemotherapeutic agent dacarbazine (approved in 1976) and the immunomodulatory cytokine interleukin-2 (IL-2; approved in 1998). Each produces response rates of less than 15% in unselected patients. While it has been argued that melanoma is simply an aggressive tumor generally resistant to therapies, it is of interest that there is not cross-resistance between dacarbazine and IL-2.[1] Some patients who progress after treatment with dacarbazine clearly can respond to IL-2, and vice versa. In addition, combination regimens of chemotherapy and cytokines (biochemotherapy designs) appear to show additive but not synergistic activity, with increased response rates but no improvement in overall survival observed in randomized trials.[2] Together, these observations have suggested that there may be subsets of melanoma patients with biologic characteristics that render them susceptible to the therapeutic effect of one modality (chemotherapy) versus another (immunotherapy). However, all histological subtypes of melanoma (superficial spreading, acral lentiginous, nodular, lentigo maligna, and mucosal) have traditionally been clinically managed identically.

Molecular evidence pointing to the existence of clinically meaningful subsets of melanoma came from the comparative genomic hybridization and systematic oncogene mutation analyses studies of Bastian and colleagues.[3] In those studies, melanomas arising in a context of sun-damaged skin, non–sun-damaged skin, acral surfaces, or mucosal regions were found to have distinct major molecular aberrations. In particular, mutations in B-Raf were most frequently found in lesions from non–sun-damaged skin. Amplifications in CCND1 and CDK4 (downstream mediators of cell cycle progression in the Ras pathway) were found in lesions that lacked upstream mutations in N-Ras or B-Raf. In addition, amplifications in the *c-kit* gene locus were found in a significant proportion of acral and mucosal lesions. Thus, rather than classical histological subcategorization of melanoma lesions, these comprehensive results suggested that molecular subtyping of melanoma may have greater practical utility, as drugs that specifically target receptor tyrosine kinases, downstream kinases, and other signaling proteins become available.

GENE EXPRESSION PROFILING AS A PREDICTIVE BIOMARKER FOR RESPONSE TO MELANOMA VACCINES

The best clinical responses of metastatic melanoma to immunotherapeutic agents such as IL-2, interferon

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(IFN)-α2b, and experimental cancer vaccines are around 10% to 15%. In the case of vaccines, it has been possible to investigate potential correlations between clinical response in terms of tumor regression and the magnitude of the specific T-cell response induced by the vaccine as measured in the peripheral blood. While such correlations have been observed in some studies, clinical responses clearly have been seen in patients with frequencies of such T cells below the limit of detection using standard assays,[4] and, conversely, complete lack of clinical benefit has been seen in patients with very high T-cell frequencies.[5] This apparent paradox led investigators to perform a systematic analysis of the tumor site to probe for factors in the tumor microenvironment that may determine clinical outcome to melanoma vaccines. Using Affymetrix (Santa Clara, CA) gene expression profiling, clinical benefit was seen in a subset of patients who showed an "inflamed" tumor microenvironment at baseline.[6,7] Metastases of this type show expression of an array of chemokines predicted to be capable of recruiting activated T cells into the tumor site; differential expression of these chemokines has been confirmed at the protein level and/or by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR). Using an in vivo xenograft model, preferential recruitment of $CD8^+$ effector T cells into those tumors producing high levels of chemokines was confirmed.[8] These results suggest that one major determining factor for clinical response to melanoma vaccines is whether T-cell trafficking into the tumor microenvironment can be supported in individual cases, thus allowing T-cell access to tumor cells and an opportunity for immune-mediated tumor cell killing. A similar immune signature suggesting an "inflamed" phenotype has been seen in two additional vaccine trials in association with favorable clinical benefit.[9,10]

It may seem on the surface paradoxical that a subset of tumors shows spontaneous inflammation that includes activated $CD8^+$ T cells. In a limited number of patients with sufficient fresh tissue for analysis, peptide/human leukocyte antigen (HLA)-A2 tetramer staining has confirmed that a subset of these T cells in fact recognizes tumor antigens.[11–14] Why these tumors are not rejected spontaneously may be explained by T-cell dysfunction in the tumor microenvironment. Ex vivo analysis of $CD8^+$ T cells from melanoma metastases has shown minimal expression of cytotoxic granule proteins and defective cytokine production in response to specific antigen in vitro. The T-cell–rich tumors also express the highest level of defined immune inhibitory factors.[15] These include the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO), which has been implicated in maternal/fetal tolerance[16]; the ligand PD-L1/B7–H1, which engages an inhibitory receptor on activated T cells called PD-1[17]; and the presence of regulatory T cells expressing the $CD4^+CD25^+FoxP3^+$ phenotype, which have been shown to mediate extrinsic suppression of activated T cells in the tumor setting, as well as other immune responses in vivo.[18] In addition, these tumors lack meaningful levels of expression of the T-cell costimulatory ligands B7–1 or B7–2, arguing that classical T-cell anergy also may be operational.[19]

Together, these observations are beginning to have several important implications. First, the presence of the "inflamed" tumor phenotype might serve as a viable predictive biomarker for clinical benefit to melanoma vaccines. This notion is currently being explored in prospective multicenter studies of the MAGE-3 protein vaccine developed by GSK-Bio (Rixensart, Belgium).[20] Second, the presence of defined immune inhibitory mechanisms in those inflamed tumors suggests that, as an alternative to vaccine approaches aiming to increase the number of functional T cells that enter the tumor, it may be possible to interfere with the function of immune suppressive pathways. To this end, blockade of IDO with small molecule inhibitors,[21,22] interference with PD-1/PD-L1 interactions with specific monoclonal antibodies (mAbs),[23,24] depletion of T-regulatory cells (Tregs) by targeting CD25,[25] and reversal of T-cell anergy through forced homeostatic proliferation[26] all have shown efficacy in preclinical in vivo tumor models. Moreover, recent clinical experience has begun to be generated with several of these approaches. The IDO inhibitor 1-methyltryptophan is currently undergoing phase I clinical testing, and a newer more potent IDO inhibitor has just entered the clinic.[27] Impressive phase II results with an anti–PD-1 monoclonal antibody were presented at the 2010 American Society of Clinical Oncology (ASCO) annual meeting, in which approximately 30% of patients with advanced melanoma, renal cell carcinoma, and non-small cell lung cancer showed clinical responses.[28] Preliminary biomarker studies in a subset of patients with available tissue for analysis has suggested that clinical benefit may be enriched in the patients with tumors showing high cell surface staining for PD-L1. Depletion of Tregs has been pursued with denileukin diftitox,[29] an IL-2–diptheria toxin fusion protein, and with daclizumab, an anti-CD25 mAb.[30] Interestingly, clinical responses with denileukin diftitox as a single agent have been reported in melanoma.[31] Finally, homeostatic proliferation of T cells, driven by their transfer into lymphopenic hosts, has been found to markedly increase the clinical efficacy of adoptively transferred autologous tumor-infiltrating lymphocytes in melanoma.[32] Together, these observations firmly support the continued study of the tumor microenvironment for clues to improve the effector phase of the anti-tumor T-cell response toward improved tumor rejection in patients.

In terms of the predictive biomarker implications of these studies, one obvious open question is whether clinical benefit from other immunotherapeutic ap-

proaches beyond vaccines also might be associated with the "inflamed" melanoma tumor microenvironment phenotype. In fact, preliminary results presented at the ASCO 2009 annual meeting are consistent with this notion. Atkins and colleagues reported that tumors with expression of a set of chemokines and cytokines were more likely to respond after treatment with IL-2.[33] In addition, Hamid et al reported that clinical response to the anti–CTLA-4 mAb ipilimumab were more likely to occur in patients with tumors expressing several immunoregulatory molecules.[34] Together, these observations suggest that an ongoing dialogue between the tumor and the host immune response might be a prerequisite for clinical benefit to several classes of immunotherapeutic interventions, a concept that should be evaluated in prospective clinical studies of those agents.

KINASE MUTATIONS AND CLINICAL RESPONSE TO KINASE INHIBITORS

B-Raf

The identification of activating mutations in *B-Raf* as a common genetic alteration in melanoma[35] rapidly led to the hypothesis that kinase inhibitors that target *B-Raf* might have therapeutic utility. More than 90% of *B-Raf* mutations in melanoma involve a substitution of glutamate for valine at position 600 (the V600E mutation), and more than 50% of melanomas carry such a mutation. A wave of enthusiasm embraced the first agent with potential inhibitory activity against Raf family kinases, sorafenib. Despite the lack of meaningful clinical responses among melanoma patients treated on the phase I/II studies of sorafenib as a single agent,[36] an unusually high response rate to carboplatin and paclitaxel combined with sorafenib sustained interest in the drug for melanoma.[37] These observations led to two phase III trials of carboplatin and paclitaxel with or without sorafenib in patients with metastatic melanoma, either in the first-line or second-line setting. Unfortunately, there were no significant differences in clinical outcome in either study,[38] which temporarily dampened enthusiasm for the concept of Raf blockade in this disease. The clinical activity of sorafenib in other cancers, such as renal cell carcinoma, is presumed to be mediated through inhibition of other kinases, including the vascular endothelial growth factor (VEGF) receptor tyrosine kinase. Other clinical trials aiming to block Ras pathway signaling at other levels, including farnesyltransferase inhibitors (aiming to target Ras proteins directly) and MEK inhibitors (targeting the kinase downstream from Raf), also showed disappointing clinical activity in melanoma.[39]

However, other small molecule inhibitors with more potent activity against mutant *B-Raf* had continued in development. PLX4032 was reported to have eightfold greater activity against mutant *B-Raf* over wild-type Raf, showing inhibition in vitro at nanomolar concentrations. A phase I study with an expansion cohort in melanoma was conducted and recently reported.[40] Of the 48 V600E *B-Raf*–mutated melanoma patients treated at the recommended phase II dose, 34 partial and three complete responses were observed. In contrast, the five patients with melanomas expressing wild-type B-Raf had no clinical response. These impressive results have provided the first evidence that an agent targeting a commonly mutated signaling protein in melanoma can exert meaningful clinical activity. A phase III study of PLX4032 is ongoing to determine whether an overall survival benefit can be observed.

c-kit

Along with the report by Bastian and colleagues that the *c-kit* gene is amplified in a subset of melanomas, activating mutations in *c-kit* have been observed. Interestingly, these have been seen in around 30% of tumors from mucosal and acral sites, as well as a minority of patients with melanomas arising out of sun-damaged skin.[41,42] The spectrum of mutations identified to date parallel those reported in gastrointestinal stromal tumors, which are associated with clinical response to c-kit inhibitors such as imatinib.[43] Based on these observations, imatinib has been investigated clinically in melanoma patients bearing *c-kit* mutations, and studies with nilotinib and other kinase inhibitors have recently been initiated. Several case reports have now been published,[44–47] revealing at least 10 clinical responders in total. Although clinical testing of imatinib in unselected melanoma patients revealed minimal clinical activity,[48,49] it is very likely that a clinically relevant response rate will be observed in the subset of melanoma patients with tumors that have specific activating mutations in *c-kit*. This notion is currently being examined systematically in a series of prospective phase II and phase III clinical trials.

MOLECULAR FEATURES ASSOCIATED WITH RESPONSE TO CHEMOTHERAPY

The fact that clinical response to kinase inhibitors might be predicted based on the presence of activating mutations in the relevant kinase has raised the question of whether the activity of traditional chemotherapeutic agents also might be associated with a predictive biomarker. Sensitivity versus resistance to alkylating agents such as dacarbazine or temozolomide might be predicted to be inversely correlated with expression of DNA repair enzymes, such as O^6-methylguanine methyltransferase (MGMT). Indeed, methylation and presumed silencing of the *MGMT* gene has been shown to be associated with a favorable clinical response of glioblastoma to temozolomide plus radiation.[50] With this

premise as a foundation, Tawbi et al recently reported on a study of molecular profiling in melanoma and the association with outcome in 21 patients treated with dacarbazine. Using a combination of gene expression profiling and analysis of gene locus methylation status, they identified a nine-gene predictor.[51] Interestingly, some of these genes encode signaling proteins (RasSF4) or immunoregulatory molecules (NKG7). It is noteworthy that MGMT did not emerge as a candidate gene in this study. This is consistent with the lack of added clinical benefit with the addition of the MGMT inhibitor O^6-benzylguanine in melanoma,[52] and suggests that alternative resistance mechanisms of melanoma to alkylating agents likely are dominant. While still early in development, these initial observations support continued investigation of potential predictive biomarkers for clinical benefit to standard chemotherapeutic agents in this disease.

NEW PATHWAYS SHOWING HETEROGENEITY AMONG INDIVIDUAL MELANOMA PATIENTS

In addition to mutations that lead to activation of the Ras pathway, other parallel signaling pathways have been found to be constitutively activated in subsets of melanoma tumors and could lead to new therapeutic approaches in select groups of patients. The Notch pathway has been reported to be activated in many melanomas, apparently via ligand and receptor overexpression rather than through mutation.[53] Notch signaling is mediated, in part, through proteolytic cleavage by an enzyme called gamma secretase, which liberates the intracellular domain of Notch to participate in transcriptional regulation. Gamma secretase inhibitors (GSIs) have been developed for clinical exploration as a strategy to inhibit Notch pathway signaling in patients. Results of a phase I study of a GSI were presented at the ASCO 2010 annual meeting, with two melanoma patients showing clinical responses.[54] Phase II studies of this agent in melanoma are in the planning stages. Activating mutations in PI3 kinase have been reported in a minor subset of melanomas,[55] and the critical negative regulator of PI3 kinase activity, the lipid phosphatase PTEN, is mutated or epigenetically silenced in many melanomas.[56,57] The recent development of PI3 kinase inhibitors for clinical testing makes it attractive to consider targeting this pathway in this disease. A total kinome sequencing study in melanoma has recently been published, which suggested for the first time that activating mutations in ErbB4 might be present in a subset of melanomas.[58] As functional activity of ErbB4 can be inhibited by the already available kinase inhibitor lapatinib,[59] it is attractive to consider testing of lapatinib in melanoma patients bearing ErbB4 mutant tumors. Mutations in *c-met* also have been reported in a series of melanoma cell lines,[60] and the recent development of agents with inhibitory activity against *c-met* for clinical testing makes a similar hypothesis attractive for this molecule.

Table 1. Emerging Molecular Markers That May Facilitate Patient-Specific Therapy in Melanoma

Molecular Biomarker	Therapeutic Modality
Ongoing in development	
B-Raf V600E	PLX4032
Mutant *c-kit*	Imatinib, nilotinib
MAGE-3$^+$	MAGE-3 protein vaccine
"Inflamed" tumor microenvironment	Various immunotherapeutics
Future potential	
Active Notch	GSIs
Mutant PI3K/PTEN loss	PI3K inhibitors
Mutant *c-met*	c-met inhibitors
Mutant ErbB4	Lapatinib
Stabilized β-catenin	?
Active Stat3	?

Several additional signaling pathways have been reported to be active in subsets of melanomas, and functionally important for melanoma biology, but without pharmacologic agents yet available for pathway inhibition. Expression of stabilized β-catenin has been observed in a major subset of melanomas, and β-catenin has been shown to contribute to melanoma development in mouse models.[61] Constitutive phosphorylation of the transcription factor Stat3 also has been identified in a subset of melanomas.[62] In vitro, knockdown of Stat3 has direct anti-tumor activity but also induces expression of important immunoregulatory genes, including a subset of chemokines that might mediate lymphocyte trafficking.[63] Therefore, Stat3 inhibitors, if developed, might have two complementary mechanisms of action, and synergy of such agents with other immunotherapeutic agents might be anticipated. Developing novel strategies to inhibit the β-catenin and Stat3 pathways in melanoma therefore should receive significant attention.

THE VERY NEAR FUTURE OF MELANOMA THERAPY

Melanoma recently has earned the designation as "an unlikely poster child for personalized cancer therapy."[64] It is not difficult to envision that within the next several years, melanoma tumors will be routinely screened for the presence of a panel of specific markers to determine assignment of individual patients to the most appropriate therapeutic approaches (Table 1). Indeed, analysis of these markers is now frequently

used in academic centers for the majority of new patients presenting with metastatic disease and is on the verge of becoming standard practice. Mutations in *B-Raf* or *c-kit* will determine eligibility for treatment with the respective specific kinase inhibitors. The activation status of other signaling pathways may be used to predict benefit to other new targeted agents. Expression of selected tumor antigens (eg, MAGE-3) will be used to identify patients for treatment with antigen-specific vaccines. The presence of the "inflamed" tumor microenvironment, anticipated to be characterized using a small gene set analyzed by qRT-PCR much like the OncotypeDx (Genomic Health, Redwood City, CA) in breast cancer[65] might be used to consider patients for a range of immunotherapeutic interventions. Having these predictive biomarkers in hand will affect the care of melanoma patients in multiple ways. First, it should lead to a greater likelihood of clinical response with the first therapeutic modality selected for a given patient. This should lead, in turn, to improved overall survival of this traditionally difficult to treat population. Second, having a specific molecular pathway that is being targeted should enable identification of resistance or escape mechanisms that, when studied, may lead to the more rapid identification of new therapeutic interventions to pursue in order to maximize clinical benefit. Third, characterization of tumors that lack any of these potential predictive biomarkers (eg, *B-Raf* and *c-kit* wild-type, absence of inflammatory signature) should proceed at an accelerated pace, to identify new pathways that might be amenable to novel therapeutic approaches. Finally, an infrastructure will need to be developed for rapid assessment of patients' tumors for expression of these predictive biomarkers, using quality-controlled and validated assays and with a rapid turnaround time performed in appropriately credentialed laboratories. This will likely involve a combination of molecular diagnostic laboratories at academic oncology centers and commercial laboratories with expertise in specific assay systems, as well as educational programs for updating community oncologists on the rapidly evolving standard practice. Indeed, the future of melanoma therapy has never looked brighter.

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