Well-Defined Melanoma Antigens as Progression Markers for Melanoma: Insights into Differential Expression and Host Response Based on Stage

□□ *Commentary on Barrow et al. p. 764*

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Decades of investigation have sought the identification of melanoma antigens associated with immune-mediated tumor destruction. These efforts arose from a need for effective therapies as limited options for patients with advanced disease exist. Compelling evidence showed that melanoma stimulates endogenous immune responses that play a critical role in combating this disease and such can be manipulated in some individuals to result in clinically significant effects. The progression of melanoma from *in situ* to metastatic disease has been well described in clinical as well as pathologic terms. The molecular mechanisms responsible for this development, however, are only beginning to be appreciated. Technological advances in profiling cancer have brought attention to new genetic alterations involved with melanoma. These occur and are influenced by complex interactions with the host. As the result of these interactions, the changes that result with melanoma progression are likely selected, with the immune system playing a prominent role in this process.

Antigen Expression According to a Particular Stage of Melanoma

In the current issue of *Clinical Cancer Research*, Barrow et al. (1) examined the expression pattern of two categories of melanoma antigens, the differentiation antigens represented by gp100, Melan-A, and tyrosinase and the cancer-testis antigens MAGE-A1, MAGE A4, and NY-ESO-1 in a large series of primary and metastatic samples. They discovered that the differentiation antigens were strongly expressed in the vast majority of both primary and metastatic melanomas. In comparison, two of three of the cancer-testis antigens revealed greater expression in metastatic deposits. Specifically, MAGE-A1 was more prevalent in ulcerated primaries, a significant adverse prognostic sign as exemplified by its important addition to the 2002 American Joint Committee on Cancer melanoma staging system.

Previous attempts to correlate antigen expression patterns and tumor progression in melanoma have resulted in varying conclusions (2–5). Consistently reported is higher expression of the cancer-testis antigens in metastatic deposits compared with primary lesions. Expression patterns, however, were previously determined by differing methods that included both PCR and immunohistochemical staining. The significance of the current report is the large numbers of samples analyzed for protein expression by immunohistochemical staining and the ability to contrast expression patterns with disease progression in the same individual. These results may now be applied within the context of our current understanding to improve critical insights involving melanoma progression.

Malignant Melanoma Is Immunogenic

Most primary melanoma begins with a radial growth phase represented by the horizontal extension of abnormal cells along the epidermis with minimal invasion of the papillary dermis (Fig. 1). During the radial growth phase, a dermal lymphocytic infiltrate resulting in partial tumor destruction is commonly seen on histologic examination (6) and is one criterion used to distinguish melanoma from a benign nevus. Following this initial radial growth phase, the vertical growth phase proceeds with penetration of malignant cells to deeper levels of skin accompanied by further potential to metastasize (Fig. 1). In contrast to the radial growth phase, a brisk lymphocytic infiltrate during the vertical growth phase occurs relatively infrequently (10-20% of cases), but when present is tightly correlated with prolonged survival and a reduced incidence of developing metastatic disease (7, 8). Furthermore, the presence of CD4, CD8, CD68 macrophage, and HLA-DR cells independently offers a favorable outcome. Even when melanoma metastasizes to local regional lymph nodes, a lymphocytic infiltrate may occasionally be witnessed that is also highly associated with improved survival (9). In rare cases of disseminated melanoma undergoing spontaneous regression, pathologic examination strongly implicates immunomediated tumor destruction with disease sites diffusely infiltrated by lymphocytes, plasma cells, and macrophages (10).

Melanoma Antigens Have Been Identified

Identified through autologous T cell– and antibody-based approaches, human melanoma antigens can be grouped into several classes (Table 1). The melanocyte lineage proteins, or normal differentiation antigens, function to produce melanin pigment present in both melanoma and normal melanocytes (11–14). Those antigens pose important questions regarding immune tolerance and cancer, specifically whether the epidermis where normal melanocytes are found provides a site of

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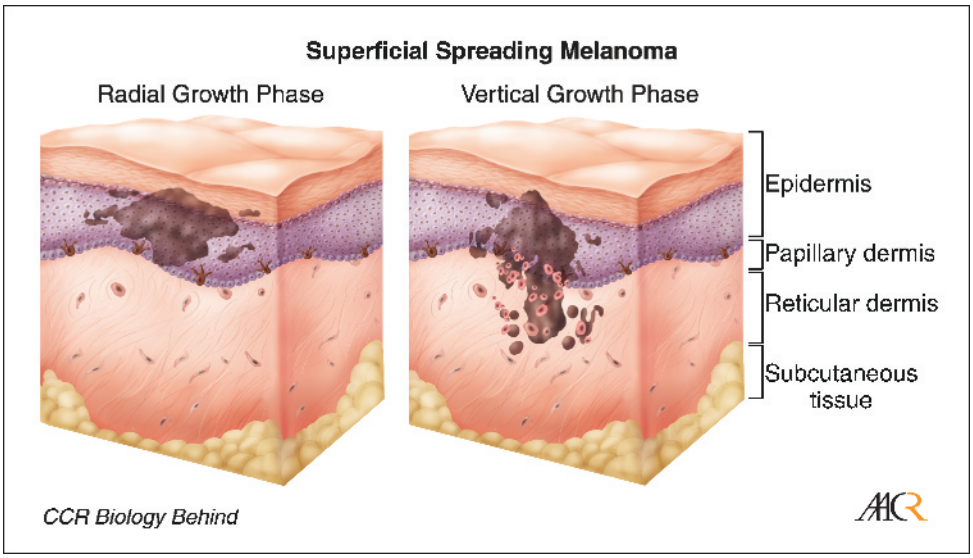

Fig. 1. Pathologic progression of a primary superficial spreading melanoma. A majority of primary melanomas begin with a radial growth phase involving the spread of melanoma cells horizontally along the surface of the skin within the epidermis (*left*). A reactive lymphocytic response during the radial growth phase is common. With subsequent progression to the vertical growth phase, malignant cells gain additional metastatic potential and invade into deeper layers of the skin (*right*). During the vertical growth phase, the presence of a brisk lymphocytic infiltrate within the nests of tumor cells is uncommon but when present offers a prognostic benefit.

immune privilege. The cancer-testis antigens are highly expressed in normal tissues during development, but in adults expression is limited to the testis and placenta (15–19). Interestingly, they are also found aberrantly expressed in a variety of cancers. Because they are principally expressed in the testis, the cancer-testis antigens may also exist in a site of immune privilege further supported by the blood-testis barrier. Other antigen classes comprise subtle mutations of normal proteins (20, 21) or intronic sequences expressed as a result of cellular transformation (22–24). Finally, antibody-based strategies identified a variety of antigens, including the melanoma gangliosides (25–31). Patient sera used to screen melanoma cDNA expression libraries initially identified a novel 37 kDa protein D-1, MAGE-1, tyrosinase, and SSX-2 (32–35). This serologic-based cloning strategy has identified hundreds of antigens, representing a wide array of targets.

Antigen-Based Vaccination Strategies for the Treatment of Melanoma

Although melanoma cells express multiple antigenic targets that result in innate immune responses, this is insufficient to prevent disease progression in patients that develop clinically significant disease. With the cloning of *MAGE-1*, the first gene product identified to be the target of T cells, antigen-specific immunization has been investigated (36). The most common antigens tested are MAGE 1, MAGE 3, MART-1, tyrosinase, gp100, and NY-ESO-1. Vaccination with MAGE-1 and MAGE-3 HLA-A1-restricted peptides (37) resulted in ~30% responses, but MAGE-3-specific T cells were not detected in patients. NY-ESO-1 peptides with binding affinity for HLA-A2 have also been tested (38), revealing T-cell responses associated with disease stabilization or regression in a subset of patients. Newly defined class I and class II peptide epitopes for NY-ESO-1 have led to additional clinical trials using a variety of vaccination strategies.

Several epitopes of the melanocyte differentiation antigens found to be CTL targets were initially tested in vaccination trials and resulted in objective tumor responses in some patients (39). The most frequent differentiation antigens targeted in HLA-A2-specific vaccination strategies have been MART-1, tyrosinase, and gp100 (40). These efforts included the use of both native peptide motifs as well as peptide sequences modified for high-affinity binding to HLA-A2. These melanoma antigens have been loaded directly onto dendritic cells *ex vivo* from hematogenous progenitors followed by inoculation into skin, blood, or lymph node of melanoma patients (39–41). A final antigen-specific treatment approach involves the generation and expansion of tumor-reactive T cells *ex vivo* followed by their infusion into unmanipulated of lymphopenic hosts. This showed disease stabilization or responses in a number of patients, but limited ability to assess antitumor T-cell responses. Recently, HLA-A2-specific peptides have been combined with an antibody antagonist to cytotoxic T lymphocyte antigen 4 (CTLA-4) to augment specific T-cell effector activity and impede regulatory T-cell function.

Molecular Implications and Susceptibility Genes in Melanoma Development

The transformation from benign melanocytes to metastatic melanoma is the result of a compilation of genetic alterations crucial to cell division, differentiation, antiapoptosis, invasion, angiogenesis, and sustenance in a microenvironment distant from the point of origin of the cell. The complexity of genetic alterations involved in melanoma development is just beginning to be appreciated. Analyses of family kindreds via loss of heterozygosity (42) discovered tumor suppressor genes involving the *cyclin-dependent kinase inhibitor 2A* (*CDKN2A*) locus, encoding the proteins $p16^{INK4A}$ and $p14^{ARF}$ by alternative reading frames. This is believed to be one of the earliest events in melanoma development. Other familial genetic alterations include xeroderma pigmentosa, retinoblastoma, RECQL2, BRCA2, and TP53 (43).

Table 1. Human melanoma antigens

- Melanocyte lineage/differentiation antigens: abundant proteins function in melanin production
  - Tyrosinase: human homologue of the mouse *albino* locus
  - gp 75: tyrosinase related protein-1 (TRP-1); human homologue of the mouse *brown* locus
  - gp100: Pmel17; target for monoclonal antibody HMB45; the human homologue of the mouse *silver* locus
  - Melan A/MART-1
  - TRP-2
- Oncofetal/cancer-testis antigens: normally expressed testis and placenta
  - MAGE family
  - BAGE family
  - GAGE family
  - NY-ESO-1
- Tumor-specific antigens: subtle mutations of normal cellular proteins, examples of coding region mutations
  - CDK4
  - β-catenin
- Other mutated peptides: activated as a result of cellular transformation
  - Mutated introns
  - *N*-acetylglucosaminyltransferase V gene product
  - MUM-1
  - p15
- Antigens identified by monoclonal antibodies
  - Gangliosides (GM2, GD2, GM3, and GD3)
  - High molecular weight chondroitin sulfate proteoglycan
  - p97 melanotransferrin
- SEREX antigens
  - D-1
  - SSX-2

In addition to mutations in relevant tumor suppressor genes, activation of members of cell signaling pathways have also proved important (44). Activating NRAS mutations believed to drive the progression from radial growth phase to vertical growth phase lead to activation of both the RAF and phosphatidylinositol 3-kinase (PI3K) pathways (Fig. 2). Subsequent identification of genetic alterations in members of both of these pathways further confirms their importance. BRAF mutations are found in a significant proportion of both primary melanomas and benign nevi, ranging in incidence from 30% to 70%, strongly suggesting that BRAF plays a key role in the process of melanocyte transformation. In the PI3K pathway, the frequent loss of function of phosphatase and tensin homologue (PTEN) is believed to be involved in late phases of melanoma progression (Fig. 2; ref. 43), whereas Akt is constitutively activated in >50% of melanomas, with expression directly related to progression and inversely to survival. These signaling pathways converge on the microphthalamia-associated transcription factor (MITF) promotor that leads to transcription of a number of genes under its control, including the melanosomal differentiation antigens and INK4A (45). MITF recently has been found to be amplified in metastatic melanoma and seems to work in conjunction with BRAF and p16 in melanoma progression (46).

Progression Markers for Melanoma

Progression markers reveal preferential expression in defined stages of melanoma development. As a result, they are useful to distinguish benign and malignant stages as well as offer prognostic value. Examples of prognostic markers include growth factors, signaling molecules, adhesion molecules, proteases, and immunomodulatory factors (Table 2).

Recent efforts to identify genes associated with aggressive melanoma phenotypes used differential cDNA display. First identified in the murine B16 melanoma model, melastatin, a putative calcium channel protein, is expressed at high levels in melanoma with low risk for metastases, whereas it is expressed at reduced levels in variants with high risk for metastases (47). In human pigmented lesions, benign nevi express high levels of melastatin, primary melanomas have variable expression, whereas melastatin mRNA expression is down-regulated in melanoma metastases. Within primary melanomas, melastatin expression correlates inversely with tumor thickness, suggesting that loss of melastatin expression augments the aggressiveness of primary melanoma. Evaluation of melastatin expression by *in situ* hybridization in stage I and II primary melanomas revealed that down-regulation of melastatin mRNA was an independent prognostic marker for the development of metastases (48). Melastatin expression as a prognostic marker in primary melanomas is currently being examined prospectively in a Cancer and Leukemia Group B trial correlating expression in stage I and II primary melanomas with metastases to local regional lymph nodes.

A variety of other molecules are also implicated in prognostic expression patterns. Microtubule-associated protein 2, present in primary melanomas, is absent in metastatic deposits. Activating transcription factor-2 (ATF-2) is important for melanoma progression as reduction of ATF-2 inhibits proliferation and invasive potential while making melanoma cells more susceptible to chemotherapy and radiation (49). Nuclear expression of ATF-2 is associated with the development of metastatic disease and a poor prognosis (50). Relevant to cell cycle progression, increase of S-phase kinase-associated protein-2 (Skp-2) and reduction of the cyclin-dependent kinase (CDK) inhibitor $p27^{kip1}$ are associated with progressive melanoma, further linking important pathways of altering cell cycle regulation.

Progression of melanoma from an early pigmented lesion to metastatic deposit requires the orchestrated modifications of molecules responsible for cell adhesion, migration, and invasion. Early lymph node and vascular access are important pathways to melanoma progression. Strong associations between the transcription factor nuclear factor-κB and melanoma vascularity have been made (51). Both vascular endothelial growth factor and lymphatic endothelial hyaluron receptor-1 are implicated in melanoma progression (52). Differential expression of pleitrophin, a growth factor for many tissue types and angiogenic factor, revealed expression in primary melanomas at risk for developing metastases (53). Activated leukocyte cell adhesion molecule is critical for melanoma progression, being induced at progression to vertical growth phase (54). In murine models, c-kit expression is lost during melanoma transformation whereas endothelin receptor B is strongly enhanced, suggesting a marker for melanoma progression (55). Expression of the CXCR4 receptor, implicated in the metastatic potential for a number of cancers, provides an unfavorable prognosis in primary

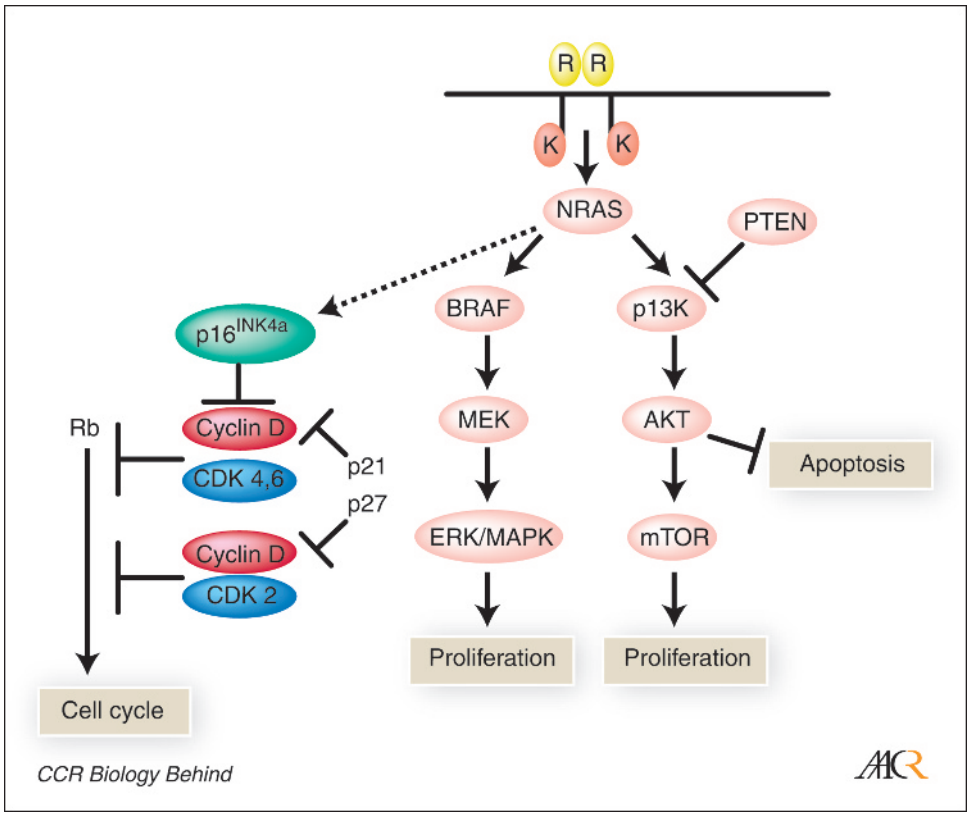

Fig. 2. Signaling pathways implicated in melanoma development. NRAS activation can lead to effects of both the mitogen-activated protein kinase (*MAPK*) and PI3K pathways resulting in cellular proliferation and antiapoptosis. Several members of these pathways are strongly implicated in melanoma development and progression (see text). Mutations involving key regulators of CDKs contribute to progression through the cell cycle via effects on the retinoblastoma gene product (*Rb*). MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; mTOR, mammalian target of rapamycin.

melanomas (56) as does the expression of $\alpha_v\beta_3$ integrin (57). Recently, dysadherin, a novel cell membrane glycoprotein that down regulates E-cadherin and inhibits cell adhesion, was found overexpressed in poor-prognosis melanomas (58). Finally, matrix metalloproteinases (MMP) are proteolytic enzymes that play important roles in cellular invasion of the extracellular matrix. MMP-2, MMP-9, and MMP-13 have been associated with different phases of melanoma progression, with MMP-2 specifically implicated with progressive invasiveness of melanoma (59, 60).

Additional important determinants of melanoma progression are regulated by DNA methylation and are implicated specifically in the transition of a primary melanoma from radial growth phase to vertical growth phase (61). The role of DNA methylation in melanoma progression, however, is complex. Hypermethylation at CpG islands correlates with silencing tumor suppressor genes while also resulting in the overexpression of a number of gene products, including the cancer-testis antigens. Demethylation studies confirm the effects of cancer-testis antigen expression, suggesting a clustering phenomenon based on a common genetic mechanism (62).

Finally, the decision point of apoptosis is critical in cellular transformation. Increased expression of bcl-2 and inhibitors of apoptosis, such as survivin (63), a key responder to UV radiation–induced apoptosis, as well as the melanoma-associated inhibitor of apoptosis, have been implicated in melanoma progression. Furthermore, immune responses to these antiapoptotic proteins have been discovered, with specific antibody and T-cell responses against melanoma witnessed (64). This provides additional evidence for a host's immune response to key components of malignant progression.

Host Response to Melanoma Progression

Besides endogenous host immune responses, melanoma cells express stress-related genes as they gain functional capacity to metastasize. MICA and MICB, for example, are ligands for NKG2D receptors found on natural killer (NK) cells, phagocytes, and other cytotoxic lymphocytes (65). NK cells react to loss of MHC class I molecules on the surface of melanoma cells, which frequently occurs with disease progression (66). Further surveillance by the host immune response involves subsequent phagocytosis of apoptotic melanoma cells. Antigen presentation is further enhanced by heat shock proteins released from necrotic melanoma cells that chaperone peptides for dendritic cell and macrophage processing (67).

Additional correlations between pathologic and genetic changes associated with melanoma progression and host immune response already exist. CDK4 is not only a prevalent mutated locus but its mutated protein is recognized by T cells. In addition, BRAF-specific antibody responses have been found in melanoma patients (68) with mutant-specific T-cell responses to BRAF found in HLA-B*2705 individuals (69). In this context, the differential expression of the melanocyte differentiation and cancer-testis antigens greatly influence our future study of endogenous host immune responses via better understanding of mechanisms involved with melanoma progression.

Table 2. Melanoma progression markers

	Increased expression with progression	Decreased expression with progression
Tumor suppressors		$p16^{INK4A}$ p53 PTEN pRb
Signal transducers	BRAF Akt EGFR receptor	c-kit
Oncogenes	MITF NRAS	MITF AP-2 transcription factor
Cell cycle	MIB-1 Cyclin A, B, D, E CDK2	$p27^{kip1}$
Growth factors	VEGF bFGF TGF-β Transferrin receptor	
Cell adhesion and motility	N-cadherin Integrin $\alpha_v\beta_3$ MMPs	E-cadherin APAF-1 β-catenin
Antiapoptosis	Survivin ML-IAP Bcl-2	Bcl-2
Immunoregulators	Cancer-testis antigens HMB-45	HLA class I molecules HMB-45
Others	Telomerase	Melastatin

This Is Just the Beginning

Each stage in the development of melanoma has genetic alterations with corresponding pathologic features. Comprehending these steps in malignant transformation and changes in protein expression are critical to the future development of effective therapies. Genetic and biochemical techniques to characterize melanoma antigens revealed the surprising innate antigen-specific recognition of melanoma cells by its host. The correlation of immune responses to clinical outcome and improved understanding of the molecular targets involved in melanoma progression provide the foundation to define mechanisms of immune escape and novel therapeutic options.

The fundamentals outlined in the current article by Barrow et al. (1) offer important insight into this process. It suggests a need to target appropriately relevant gene products that are dependent on stage and pattern of antigen expression. From this principle, much work still needs to be done. Current technologies permit further correlation of the host immune response with genetic alterations that occur, for example, between the radial growth phase and vertical growth phase. Differential display analyses of genetic and protein expression continue to delineate the complex interactions between host and cancer. Rational therapeutics based on understanding changes in the cancer cell and host responses during development will permit rigorous evaluation of melanoma treatments and maximize the potential for their success.

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