

Melanoma biomarkers: *Vox clamantis in deserto* (Review)

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Abstract. Detecting malignant melanoma at an early stage, monitoring therapy, predicting recurrence and identifying patients at risk for metastasis continue to be a challenging and demanding objective. The last two decades have witnessed innovations in the field of melanoma biomarkers. However, global agreement concerning monitoring and early detection has yet to be reached. This is a review of the current literature regarding melanoma biomarkers including demographic, clinical, pathological and molecular biomarkers that are produced by melanoma or non-melanoma cells. A number of these biomarkers demonstrate promising results as possible methods for early detection, predicting recurrence and monitoring therapy. Other biomarkers appear to be promising for identifying patients at risk for metastasis. We reviewed the most pertinent information in the field thus far and how this knowledge can impact, or not, the management of melanoma patients prognostically and therapeutically.

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1. Introduction

According to the National Institute of Health (NIH) definition, a biomarker is 'a characteristic that is objectively measured and

evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to therapeutic intervention' (1). Tumor biomarkers can be clinical or statistical features, pathological findings or molecular markers that are produced by tumor or non-tumor cells (such as immune cells) (2). The detection of tumor markers has significant prognostic implications for a number of solid tumors, such as breast, prostate, ovarian and testicular. Malignant melanoma is trailing behind in spite of the discoveries achieved in the last decade. Lactate dehydrogenase (LDH) is the only molecular biomarker used in the current staging system (3), although it has low sensitivity and specificity (4). The prognosis of stage I and II melanoma according to the current staging system developed by the American Joint Committee on Cancer (AJCC) classification depends on the thickness of the lesion (3) as well as the presence of microscopic or macroscopic melanoma cells in the lymph nodes (4). The presence of such cells upstages the melanoma to stage III. For stage III and IV melanomas, the outcome is variable and the determination of prognostic factors is more difficult. No single biomarker has yet been identified that can determine the prognosis of these patients. Thus, at the present time, various biomarkers are used in an attempt to estimate the risk of recurrence. These indicators include demographic and clinical factors (5), primary tumor characteristics and pathological features (3), in addition to novel molecular biomarkers. Recurrent melanoma may be amenable to surgical resection if detected early, but there is no agreement regarding monitoring and early detection. Chest X-ray, computed tomography, positron emission tomography and magnetic resonance imaging are often utilized.

Melanoma biomarkers are promising factors for identifying patients at risk for melanoma, early detection, confirming diagnosis, monitoring therapies and predicting recurrence. This review reported on current demographic, clinical and pathological features as well as numerous key discoveries regarding serological and molecular biomarkers of malignant melanoma.

2. Demographic and clinical factors

Several demographic factors that affect the survival of patients with melanoma have been identified. In a study by White *et al*, AJCC stage III melanoma patients <50 years of age were found to have a 5-year survival of 48%, while for patients >50 years survival was ~38% (5). Female gender was found

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to be associated with a more favorable prognosis (6,7), while patients with an axial primary tumor had a worse prognosis than those with a peripheral tumor (5). Barth *et al* (6) found that patients with tumors that required >72 months to progress from stage II to stage IV melanoma had a median overall survival of 9.6 months. However, patients with tumors that required <72 months had an overall survival of 7.6 months (6). Karakousis *et al* (9) found that the number of metastases had a considerable effect on the 5-year survival of patients with stage IV melanoma. Patients with one metastasis had a 5-year survival rate of 33%, while those who had >4 metastases had a 5-year survival of 13% (9). It has been demonstrated that sites of metastatic foci appear to impact 5-year survival rates (3). For example, a cutaneous metastasis was associated with the most favorable survival rate of 27%, while lymph node and visceral metastases had survival rates of 15 and 2%, respectively (3).

Therefore, patients with distant metastasis in the skin, subcutaneous tissue or distant lymph nodes are categorized as M1a (3). Such patients have a relatively better prognosis compared to those with metastases located in any other anatomic site (3,6,8). Patients with metastasis to the lung are categorized as M1b and have an intermediate prognosis when 1-year survival rates are compared. Those patients with metastases to any other visceral sites have a relatively worse prognosis and are designated as M1c (3).

Eton *et al* (10) and Allen *et al* (11) demonstrated that i) patients bearing tumors with a doubling time of >60 days had a 5-year survival of 20.7%, whereas patients bearing tumors with <60-day doubling time had a 0% survival; ii) patients who received complete resection of their tumors had a 5-year survival of 24%, while patients who had incomplete resection had ~0% survival rate; and iii) responders to systemic therapy had a better survival (10,11). In a recent retrospective study, Bedikian *et al* (12) identified treatment with bio-chemotherapy as one of the favorable prognostic factors that affect long-term survival in patients with advanced melanoma.

3. Primary tumor characteristics

For stage I and II melanoma, thickness of the primary tumor is a significant prognostic factor (1). For stage III melanoma, overall 5-year survival rates based on thickness are as follows: thickness <1 mm, 53%; thickness 1-2 mm, 47%; thickness 2-4 mm, 40%; and 34% for tumors >4-mm thick (1). The thickness of the primary tumor was not found to have a significant effect on prognosis after tumor metastasis.

The presence of ulceration, which is defined as the absence of an intact epidermis overlying a significant portion of the primary melanoma based on microscopic examination of the histological sections (3,13,14) of the primary tumor site, is also one of the main prognostic factors for stage III disease. The presence or absence of ulceration is the third most significant prognostic factor in determining the pathological stage (3) in that it independently predicts outcome in stage I, II and III melanoma (3,13-16). Survival among patients with an ulcerated primary tumor is more severe than among those with no ulceration (3).

According to AJCC, the second most significant prognostic factor for patients with nodal metastasis is the tumor

burden in the lymph nodes (3). Lymph nodes without clinical or radiologic evidence of metastasis, but with pathological documentation of metastasis are considered to be micrometastases. Those with clinical or radiologic evidence of metastasis are considered macrometastases. For patients with micrometastases, documenting the number of lymph node metastases after lymphadenectomy is important (3,4,17). Survival rates for patients with micrometastases are significantly higher than for those with macrometastases (3).

The number of involved lymph nodes has been a key determinant of survival for stage III melanoma; AJCC used lymph node involvement as the determining criterion for the N category (3). N1 designates the presence of the involvement of one lymph node, N2, 2-3, while N3 indicates the involvement of four or more lymph nodes (3). According to White *et al*, the 5-year survival rate declined from 53% in patients with involvement of one lymph node to 25% in patients with four or more lymph nodes (5). Furthermore, in patients with lymph nodes positive for metastasis, microscopic vs. macroscopic involvement significantly impacts the survival rates and prognosis in that patients with macroscopic involvement have a worse prognosis than those with microscopic lymph node metastasis.

The presence of lymphocytes infiltrating the primary tumor site was found to have a favorable prognostic effect on the survival of stage III patients since lymphocytes play a role in the immune defense against melanoma. For example, patients with efficient infiltrate had a 40-month disease-free survival of 73%, compared to a 40-month disease-free survival of 23% (18,19).

On the other hand, aneuploidy was found to have an unfavorable prognostic effect on the survival of patients (20).

4. Serological factors

Lactate dehydrogenase (LDH). LDH is an enzyme that is present in a wide variety of organisms, including plants and animals. It catalyzes the interconversion of pyruvate and lactate with the concomitant interconversion of NADH and NAD⁺. It converts pyruvate, the final product of glycolysis, to lactic acid when oxygen is absent or in short supply, and it performs the reverse reaction during the Cori cycle in the liver. It is used for the assessment of tissue breakdown, in general, a process that is possible when there are no other indicators of hemolysis. LDH is used to follow up cancer (especially lymphoma) patients, as cancer cells have a high rate of turnover with destroyed cells leading to an elevated LDH activity. Serum LDH is an independent prognostic factor in patients with stage IV melanoma (21-23). It is the only biomarker that is used by the AJCC staging system of melanoma (3). When serum LDH is elevated above the upper limits of normal at the point of staging, patients with distant metastases are assigned to M1c regardless of the site of their distant metastases (3). Elevated serum LDH should be used only when there are two or more determinations obtained >24 h apart (3), since results of a single determination can be falsely positive due to hemolysis or other factors unrelated to melanoma metastases.

S100 β. S100 β is a low molecular weight dimeric acidic calcium-binding protein. The name is derived from its solubility in 100% saturated ammonium sulfate at a neutral pH (24). S100 protein is expressed by a wide range of cells derived from

the neural crest (Schwann cells, melanocytes and glial cells), chondrocytes, adipocytes, myoepithelial cells, macrophages, Langerhans cells, dendritic cells and keratinocytes. The protein consists of α and β subunits, and the form that is found in melanoma is $\alpha\beta$ dimer (24). The $\alpha\beta$ protein is also present in glial, Schwann and Langerhans cells. The serum level of S100 β is not specific, as abnormal levels are noted in liver, brain and renal injury along with many inflammatory and infectious processes (25). The β subunit is the form related to tumor invasiveness. S100 protein is thought to interact with tumor suppressor p53 protein, thus affecting its function in cellular growth (24). S100 levels are detected in the serum of melanoma patients using immunoassays of monoclonal antibodies directed against the β subunit of the protein (24). An increased S100 β level is specific to melanoma when brain disease is excluded (26). Patients with the highest level of S100 β are those with liver and skeletal muscle metastases (27). Several studies have shown conflicting results. In a recent study of 3,393 patients, S100 β was found to be a prognostic factor independent of the TNM staging system (25,29). In a study of 296 melanoma patients (29), comparisons were made between S100, melanoma inhibitory activity, LDH, alkaline phosphatase (AP) and reverse transcriptase (RTC). S100 sensitivity was found to be 29%, which was higher than all of the above-mentioned biomarkers. The diagnostic accuracy was higher than LDH, AP and RTC. The elevated values of S100 β were associated with a decreased survival rate and worse disease (30). Although its sensitivity is low, the prognostic value is high (31). The level of the protein was shown to be related to the stage of melanoma (32-35), the degree of metastasis and disease activity (26), and tumor mass. It has also shown promise in monitoring therapeutic interventions (34,37,38), predicting relapse (28), as well as survival (26-28).

Melanoma inhibitory activity (MIA). MIA is an autocrine growth regulatory factor secreted by melanoma cells. It is strongly expressed by malignant melanoma cells, moderately expressed by nevi and not significantly expressed by normal skin melanocytes, keratinocytes or other skin cells (39-41). It was found to be expressed in cartilage (42,43) and, therefore, mildly elevated in patients with arthritis. MIA was also observed in breast cancer, colon cancer and chondrosarcoma (43,44). The postulated theory is that MIA decreases melanoma cell adhesion to the extracellular matrix and promotes metastasis (40). The MIA gene was assigned to the 19q13 locus by fluorescent *in situ* hybridization (45) in 1996. Since then, this protein has been extensively studied. ELISA is used to measure the MIA serum levels of melanoma patients (39). MIA was evaluated by several studies as one of the most specific and sensitive biomarkers available for malignant melanoma (31,39,43). The level of MIA in the serum of patients with malignant melanoma was found to correlate with tumor stage. It was also shown to be helpful in detecting the progression from localized to metastatic disease (46) and in monitoring the treatment of melanoma (39,41).

TA90 antigen. TA90 antigen is a 90-kDa tumor-associated antigen that is a free antigen and an IgG-bound immune complex (TA90-IC) in the serum of melanoma patients (47). Patients with stage IV melanoma and extensive tumor burden

were found to express high levels of free TA90 and low levels of TA90-IC, whereas patients with early stage disease and low tumor burden rarely had free antigen, but exhibited TA90-IC instead. The expression of the TA90 antigen in non-melanoma tissues is limited to certain inflammatory states, such as cirrhosis associated with hepatitis (48). ELISA is used to detect TA90-IC in the serum of melanoma patients. Studies have found this marker to be highly prognostic in patients with various stages of melanoma (49). It was found to be highly correlated with an improved 5-year disease-free and overall survival (50). Several analysis studies identified anti-TA90 IgM as an independent prognostic factor for melanoma (50). IgG and IgM antibody titers were found to be the basis for monitoring immune response to polyvalent vaccines. Administration of antibodies may play a significant role in modulating survival in melanoma patients (50). Additionally, elevation in anti-TA90 IgM antibody but not IgG titers was associated with prolonged patient survival (50-52).

Tyrosinase. Tyrosinase is a monooxygenase copper-containing enzyme that catalyzes the oxidation of tyrosine to dopa and then to dopaquinone, and is a specific marker for melanocytes and Schwann cells (53). Melanocytes and Schwann cells do not normally circulate in peripheral blood (54), thus the detection of tyrosinase mRNA in blood is assumed to indicate the presence of metastasis. Reverse transcriptase-polymerase chain reaction (RT-PCR) is used to analyze peripheral blood for tyrosinase mRNA expression. Several studies reported a correlation between tyrosinase RT-PCR and melanoma relapse progression (12,55-57). Positive tyrosinase in peripheral venous blood was found to be statistically significant and, more importantly, an independent negative predictor of survival (12,55). RT-PCR for tyrosinase mRNA was found to be a significant prognostic factor for progression-free and overall survival in melanoma (12).

Melanoma antigen gene A3 protein (MAGE-A3). MAGE-A3 is a member of the MAGE-A family. This family encodes proteins with a 50-80% identity to each other. The variability resides in the promoters and the first exon, indicating that this family plays the same role that is expressed under different transcriptional controls. The family is expressed by chromosomal location Xq28.

The genes are silent in normal cells, except male germ-line cells. However, the genes are expressed in melanoma, as well as in various other tumors (58,59). MAGE-A3 protein is detected in the blood using RT-PCR. Elevated blood levels of MAGE-A3 protein were found to be associated with early stages of melanoma (58). However, Reynolds *et al* found that MAGE-A3 is not an independent prognostic factor for melanoma (60).

Glycoprotein YKL-40. Glycoprotein YKL-40, also known as Chondrex or Chitinase 3-like (CHI3L1), cartilage glycoprotein-39 or chondrocyte protein YKL-40, is secreted by a wide range of cells, including articular chondrocytes, synovial cells, macrophages and mature neutrophils. High levels of YKL-40 were found in synovial fluid from patients with active rheumatoid arthritis (RA) and in the hippocampus of schizophrenic patients. YKL-40 belongs to the 18-glycosyl hydrolase

(mammalian chitinase), and the term YKL represents its N-terminal tripeptide sequence. YKL-40 is thought to play a role in the migration of endothelial cells and/or formation of branching tubules that promote angiogenesis (61,62). Serum levels are determined using ELISA. Although the serum level is neither specific nor sensitive, it was correlated with the stage of melanoma (63) and poor survival (61).

Cytoplasmic melanoma-associated antigen (CYT-MAA). CYT-MAA is a cytoplasmic protein comprising four polypeptides and is synthesized by many tumor and normal cells. This protein was found in melanoma at higher levels than in normal cells (64). The protein is neither sensitive nor specific, and does not correlate with the stage of melanoma. However, it was linked to disease recurrence and progression (64,65). Serum levels are measured using double-sandwich ELISA. Reynolds *et al* conducted the first study to measure CYT-MAA levels in melanoma patients receiving immunotherapy (65). The results indicated that it is a potential marker that can be used to monitor response to immunotherapy.

Melanotransferrin (MTF). MTF or melanoma-associated tumor antigen p97 is a membrane-bound glycoprotein that is a homolog of the transferrin (Tf) family of non-heme Fe-binding proteins (66). It is expressed in normal adult tissues (67), fetal tissues, as well as in various tumor cells (68). A larger amount of MTF is expressed in the salivary glands, pancreas and epididymis at the tissue level (70). The exact function of MTF in melanoma has yet to be elucidated. However, MTF was implicated in a diverse range of biological processes, and emerging data indicate that it plays a role in angiogenesis (70,71), cellular proliferation (72) and tumorigenesis. MTF is detected in the blood using RT-PCR. The test, however, lacks both specificity and sensitivity.

Microphthalmia-associated transcription factor (MITF). MITF is a basic helix-loop-helix leucine zipper transcription factor for tyrosinase and tyrosinase-related proteins. It plays a critical role in the differentiation and development of melanocytes and osteoclasts (73). MITF is implicated in the proliferation of malignant melanoma (74). Two methods of detection have been identified against MITF, including RT-PCR (reverse transcriptase-PCR) and monoclonal antibody. MITF detection after melanoma treatment was shown to be an independent prognostic factor for patient survival (75). MITF indicates subclinical metastatic disease and predicts treatment outcome in melanoma patients (75). Its concentration was also found to correlate with the stage of melanoma (76).

Glycoprotein 100 (gp100). gp100, also known as SILV, ME20 or Pmel17, is a 100-kDa type I transmembrane protein. The processing of this protein results in the formation of melanosomal striation (77). This protein is expressed at low levels in adult melanocytes, but is overexpressed by proliferating neonatal melanocytes and in melanoma. The protein is present in the membranous form, ME20-M, which secretes a soluble glycoprotein, ME20-S. The latter protects melanoma cells from antibody-mediated immunity (77). gp100 is determined using monoclonal antibody HMB-45. The expression of gp100 varies within the tumor and between patients (78).

No correlation was found between the expression of gp100 and responsiveness to immunotherapy (78). Furthermore, the protein lacked specificity (79).

C-reactive protein (CRP). CRP is a protein synthesized by hepatocytes in response to cytokines, such as interleukin (IL)-6, IL-1 and tumor necrosis factor α (80,81). This protein is produced as a non-specific acute phase response to almost all forms of inflammation (80). In a study of melanoma, CRP was assayed in human blood using an immunonephelometric technique on a protein system analyzer (82). Elevated serum CRP levels along with IL-6 were associated with reduced survival in metastatic melanoma and resistance to IL-2 therapy (82-86). CRP was found to be an independent prognostic factor for survival (86). In one study, CRP was found to be superior to LDH in discriminating melanoma patients entering AJCC stage IV from patients remaining in AJCC stages I, II or III (82). CRP was detected with a sensitivity of 77% and specificity of 90% in determining the progression of disease into stage IV (82).

5. Circulating tumor cells

Circulating tumor cells (CTCs) are defined as the leukemic phase of solid tumors and are required for the establishment of distant metastasis for a certain cancer type (87). In this model of metastasis, CTCs migrate from the tumor site, travel through lymphatics and blood, form micrometastases at distant organ sites and ultimately lead to overt metastatic disease (87). For patients with stage II and III melanoma, Mocellini *et al* showed that the detection of CTCs in peripheral blood is used to predict tumor relapse, assess treatment failure and select patients for adjuvant therapy (88). However, conflicting evidence exists in the literature regarding the correlation between CTC detection, stage of disease and clinical outcomes in melanoma. The discovery of melanoma CTCs in peripheral blood has been widely inferred, with the RT-PCR demonstration of tyrosinase and MAGE-A3 mRNA markers thus far (88,89). This method is limited by false-positive and false-negative results and low reproducibility (87). Discrepancies in results are influenced by genomic instability of melanoma cells, discontinuous shedding of CTCs into the bloodstream and the low numbers of CTCs compared to normal blood components (87). Alternative detection methods are essential for the ultimate use of CTCs in the clinical setting. Promising techniques that provide genetic and molecular characterization of tumor metastases include transcriptional studies, comparative genomic hybridization and single nucleotide polymorphism genotyping (87). Ulmer *et al* described the detection of intact circulating melanoma cells by immunomagnetic cell sorting with subsequent genomic analysis (89). The statistical significance found between the detection of CTCs and tumor stage is promising for the elucidation of the molecular-genetic basis of the establishment of malignant melanoma (80).

6. Current practice

Current management of advanced stage melanoma does not involve the use of molecular biomarkers to assess tumor

response to cytotoxic or immune-modulating chemotherapy. Clinical history, physical examination, pathology and various imaging modalities are presently used to gauge disease progression. Serum LDH levels, although used as an independent prognostic factor, are not explicitly employed to assess response to treatment (3). Pre-treatment serum LDH is used as a significant predictor of survival (90). It remains unclear whether change in serum LDH during treatment of advanced disease is associated with survival (91). This review has identified several experimental biomarkers as potential indicators of disease progression, treatment response and recurrence. The data warrant further investigation into the detection of tyrosinase mRNA during interferon therapy as a marker of therapeutic response (92). An analysis of additional serum melanogenic markers pre- and post-therapy may potentially be used to guide treatment.

7. Conclusion

The AJCC staging system lays the present framework for determining the stage and prognosis of malignant melanoma using various clinical and pathological biomarkers. The only molecular biomarker presently included is LDH. Several studies involving other molecular markers have been conducted and various markers have been identified as useful future methods for the detection and evaluation of therapeutic response and risk of metastasis. Despite these advances, a specific molecular marker or method of detection has yet to be applied to the clinical management of melanoma patients. Studies confirming these biomarkers as valuable prognostic factors require validation using more specific and sensitive detection methods, as well as large-scale patient cohorts. Forging ahead with studies that combine the use of different biomarkers is essential in the identification of a valuable long-awaited method that can aid in the prediction of prognosis, therapeutic response and risk of metastasis in patients with malignant melanoma.

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