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Biomarkers in Malignant Melanoma: Recent Trends and Critical Perspective

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Abstract: The worldwide incidence of malignant melanoma is steadily increasing, suggesting a probable melanoma "epidemic." From a clinical point of view, malignant melanoma still is an unpredictable disease and, once in the advanced stage, allows only scarce therapeutic options. There is an urgent need to identify, characterize, and validate informative biomarkers, biomarker patterns, or surrogate markers in order to not only improve early diagnosis of melanoma but also for differential diagnosis, staging, prognosis, therapy selection, and therapy monitoring. In this chapter, an update on the ongoing debate on serologic and histologic markers such as lactate dehydrogenase, tyrosinase, S100 family of calcium-binding proteins, cyclooxygenase-2, matrix metalloproteinases, and stem and/or progenitor cell markers are presented, and novel, innovative, and promising trends currently being explored are discussed.

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Introduction

Melanoma is the most common malignant type of all skin neoplasms. Although current clinical, biochemical, and histological methods provide insights into disease behavior and outcome, melanoma is still an unpredictable disease. Once metastasized, it remains a fatal neoplasm with scarce therapeutic options, despite current progress in immunomodulatory therapy. Therefore, significant efforts still need to be made in finding suitable biomarkers that could aid or improve its early diagnosis, its correct staging, the discrimination of other pathological conditions, as well as indicate patients' prognosis or the most appropriate personalized therapeutic regimes. On the other hand, well-defined diagnostic markers are strictly necessary to avoid the apparent overdiagnosis of melanoma. This chapter provides an overview of the literature on recent efforts in cutaneous malignant melanoma biomarker research. A PubMed database search was performed in March 2017 using key words and phrases such as "biomarker," "serum/plasma/tissue biomarker," "biomarker analysis," "immunohistochemistry," linked to the key words "melanoma," "malignant melanoma," and "metastatic melanoma". Regarding earlier literature, the authors refer to two very comprehensive review articles on protein and nonprotein biomarkers in melanoma published in 2012 by our group (1) and, more recently, in 2015 by Karagiannis et al. (2), with the latter, however, also mostly referring to the literature before 2013 (113 out of 130 citations).

Biomarkers in Malignant Melanoma: A Current Status

Melanoma incidence and mortality have been steadily increasing in almost all countries, especially in fair-skinned populations. Exemplarily, 2013 German incidence rates (mortality rates) of cutaneous melanoma were 19.1 (3.0) per 100,000 males and 17.4 (1.7) per 100,000 females, with cutaneous melanoma responsible for about 1.3% of all cancer deaths (Association of Population-based Cancer Registries in Germany, GEKID; http://www.gekid.de). Considering variations between countries, 5-year survival for people of all races diagnosed with primary cutaneous melanoma <1.5 mm in depth is about 90%, amounting to 99% for local disease. The 5-year survival for people diagnosed with mucosal and intraocular melanoma is about 70%. However, 5-year survival is only 60–65% if the disease is spread within the region of the primary melanoma, dramatically dropping to below 10% if widespread. Albeit screening campaigns and intensive public health programs resulting in decreasing incidence rates, especially in younger age groups, incidence and burden of melanoma continue to rise. This is mainly due to the aging population, continued high recreational sun exposure habits, changing climate

patterns, and increasing environmental contamination with carcinogenic agents (1, 3). Thus, sensitive screening, early detection of high-risk groups and personalization of therapy are the major principles of melanoma control. In this regard, biomarkers represent molecular attributes of the individual patient that will not only allow for detection and diagnosis but also answer questions about the biologic behavior of the tumor and metastases, mechanisms of resistance, and/or sensitivity to therapy. Prospectively, melanoma therapy will substantially be improved by the use of biomarkers that will (i) offer the potential to identify and treat melanoma before it is clearly visible or symptomatic, (ii) facilitate easy detection without even minimal surgical procedure, and (iii) serve as candidates for population-based screenings. In this regard, this chapter summarizes and critically discusses the current trends and perspectives in malignant melanoma biomarker research.

Melanoma biomarkers can be divided into different categories. Most of them show higher expression in melanoma cells than in normal tissue and, therefore, are used as diagnostic markers. Other biomarkers may serve as prognostic or predictive markers because of their increased expression in advanced stages of disease, as indicators of treatment response or of disease recurrence during follow-up (4). Moreover, melanoma progenitor and/or stem cell markers are of potential use for identification of cell subpopulations that exhibit critical properties like high carcinogenicity, metastatic potency, and treatment resistance. The ideal biomarker should be a metabolically and analytically stable molecule detectable and/or quantifiable in the blood or other body fluid compartments, which are accessible through minimally invasive procedures. This biomarker should allow for the diagnosis of a growing tumor in a patient or for the prediction of the likely response of a patient to a certain treatment, even earlier or better than by applying clinical imaging modalities. Thereby, the biomarker must exhibit sufficient sensitivity and specificity in order to minimize false-negative as well as false-positive results (1, 4).

At this moment, no ideal biomarker exists in the field of melanoma. Pathological characteristics of the primary melanoma, for example, tumor thickness (*Breslow index, Breslow thickness*), mitotic rate, and ulceration are important prognostic factors (5). However, these characteristics can only be determined after localization and biopsy or surgical resection of the tumor. Regarding the points mentioned above, either circulating melanoma cells or melanoma-associated extracellular molecules provide suitable noninvasive analytical access. Melanoma cells release many proteins and other molecules into the extracellular fluid. Some of these molecules can end up in the bloodstream and hence serve as potential serum biomarkers. From a pathobiochemical point of view, these biomarkers comprise molecules, including enzymes, soluble proteins and/or antigens, melanin-related metabolites, and circulating cell-free nucleic acids, released by (i) necrosis, (ii) active secretion, and (iii) ectodomain membrane shedding (1) (Table 1). These molecules exhibit different prognostic and predictive values in melanoma diagnosis, staging, and treatment monitoring (1, 4, 6, 7). On the other hand, biomarkers obtained from histological and immunohistochemical analyses of biopsy material play a very important role in melanoma management. Therefore, novel results and promising trends in this field also have been considered in this chapter.

TABLE 1 Potential Biomarkers in Malignant Melanoma

	Biomarker	Correlation with	Major laboratory methodologies	References[§]
Enzymes	LDH	prognosis, tumor stage, survival rate	photometric assay, meta-analysis[#]	(1)
	Tyrosinase	poor prognosis, survival rate, overall survival	RT-PCR, nested RT-PCR	(1)
	Cox-2	*Breslow index*, tumor progression	IHC	(1)
	MMP-1, MMP-3	disease-free survival	IHC	(1)
	MMP-9	disease, poor prognosis	ELISA	(1, 26)
	MMP-2	tumor progression	TMA, IHC	(27, 28)
	MMP-12	overall survival	IHC	(31)
	MMP-23	progression-free survival	IHC	(32)
	MT1-MMP	tumor progression	IHC	(30)
	TIMP-1	disease-free and overall survival	ELISA	(26)
	IDO		HPLC	(79)
	Cathepsin K	overall survival	IHC	(34)
	CD10	disease	cytomorphology, IHC	(33, 35)
	Legumain	overall survival		(36)
		overall survival	IHC	
Secreted proteins/ antigens	VEGF	tumor stage, survival, tumor progression	ELISA, RT-PCR	(1)
	VEGF-C, VEGFR-3	tumor burden	ELISA	(1)
	Osteopontin	*Breslow index*, survival, poor prognosis	IHC, TMA	(1)
	Galectin-3	poor prognosis, tumor progression	IHC, ELISA	(1)
	YKL-40	tumor stage, tumor progression, poor prognosis	ELISA	(1)
	MIA	survival poor prognosis	ELISA	(1)
	C-reactive protein	survival tumor progression	IP	(1)
	sICAM, sVCAM	survival	ELISA	(1)
	CEACAM	tumor stage, tumor progression, overall survival	IHC, ELISA	(1)
	CYT-MAA	tumor progression	ELISA	(1)
	MAGE	tumor progression	RT-PCR	(1)
	MART-1	tumor stage	RT-PCR	(1)
	TA90	survival, recurrence	ELISA	(1)

Table continued on following page

TABLE 1 Potential Biomarkers in Malignant Melanoma (Continued)

	Biomarker	Correlation with	Major laboratory methodologies	References§
S100 Proteins	S100B	tumor stage, survival, recurrence	ELISA, LIA	(47, 50)
	S100A2	tumor progression (negative correlation)	Northern blot	(1)
	S100A4	tumor progression	IHC	(1)
	S100A6	survival	Northern blot	(1)
	S100A8/A9	tumor progression	IHC, ELISA, FC	(67, 70)
	S100A13	tumor progression	MS, IHC	(65, 66)
	S100P	tumor progression	IHC	(1, 80)
Progenitor/ stem cell-like markers	SOX protein family	disease	IHC	(74, 75)
Metabolites*	5-S-cysteinyl-DOPA	poor prognosis, response to treatment	HPLC	(1)
	L-DOPA/L-tyrosine	tumor burden, tumor progression	HPLC	(1)
	6H5MI2C	*Breslow index*	HPLC	(1)
Nucleic acids	miRNA-221	*Breslow index*	RT-PCR	(1)
	miRNA-29c	overall survival	RT-PCR	(1)

The table was modified according to Ref. (1) (*cf. references therein*).

Biomarker abbreviations: 6H5MI2C, 6-hydroxy-5-methoxyindole-2-carboxylic acid; CEACAM, carcinoembryonic antigen-related cell adhesion molecule 1; Cox-2, cyclooxygenase-2; CYT-MAA, cytoplasmic melanoma-associated antigen; L-DOPA, L-3,4-dihydroxyphenylalanine; IDO, indoleamine-2,3-dioxygenase; LDH, lactate dehydrogenase; MAGE, melanoma-associated antigen-1; MART-1, melanoma antigen recognized by T-cells 1; MIA, melanoma inhibitory activity; MMP, matrix metalloproteinase; sICAM, soluble intercellular adhesion molecule 1; sVCAM, soluble vascular cell adhesion molecule 1; TA90, tumor-associated antigen 90; VEGF, vascular endothelial growth factor; YKL-40, heparin- and chitin-binding lectin YKL-40 (*syn.* human cartilage glycoprotein-39).

Method abbreviations: ELISA, enzyme-linked immunosorbent assay; FC, flow cytometry; HPLC, high performance liquid chromatography; IHC, immunohistochemistry; IP, immunoprecipitation; LIA, luminescence immunoassay; MS, mass spectrometry; RT-PCR, reverse transcription polymerase chain reaction; TMA, tissue microarray.

*Metabolites of melanin synthesis pathways, #meta-analysis based on AJCC melanoma staging database (5).

§The references given in the table refer to original articles, which describe novel biomarkers, and were published between 2012 and 2017. The original articles on melanoma biomarkers that have been described before 2012 were discussed in detail in Refs. (1) and (2).

LACTATE DEHYDROGENASE

Lactate dehydrogenase (LDH, EC 1.1.1.27) is a ubiquitous enzyme catalyzing the conversion of pyruvate to lactate. This reaction is essential when oxidative phosphorylation is disrupted, for instance, in anaerobic conditions and in hypoxia (8), and the latter is quite common in fast-growing tumors with high consumption of nutrients and oxygen. In the American Joint Committee on Cancer (AJCC)

staging system, serum LDH is the only serum biomarker that was accepted as a strong prognostic parameter in clinical routine for melanoma, classifying those patients with elevated serum levels in Stage IV M1C (4, 5). In the recent past, the role of LDH as a prognostic factor and as a marker for treatment response has been confirmed further. In a meta-analysis of 76 studies on the prognostic role of LDH in solid tumors, including 12 melanoma studies from 1998 to 2014, Petrelli and colleagues confirmed that high serum LDH concentration is associated with lower overall survival in melanoma patients (9). Recent studies analyzed the suitability of serum LDH as marker for outcome of advanced melanoma patients after treatment with immunomodulatory drugs. In this regard, baseline serum LDH was demonstrated to be a strong predictive factor for overall survival after ipilimumab treatment in metastatic melanoma (10). The authors further concluded that long-term benefit of ipilimumab treatment was unlikely for patients with baseline serum LDH greater than twice the upper limit of normal. An independent study showed that low baseline serum LDH is associated with favorable outcome of late-stage melanoma patients treated with ipilimumab and, therefore, confirmed that baseline serum LDH is a strong marker for prognosis in advanced melanoma (11). The suitability of serum LDH as a predictive factor was also demonstrated for therapy with further immunomodulatory drugs, anti-programmed death receptor-1 (anti-PD-1) antibodies pembrolizumab and nivolumab (12). The authors documented that anti-PD-1-treated patients with a relative reduction of serum LDH compared with their baseline LDH achieved partial remission. On the other hand, patients with an increased serum LDH level compared with the baseline LDH showed progressive disease. They conclude that serum LDH is a useful marker not only at baseline but also during treatment in patients treated with anti-PD-1 antibodies in advanced melanoma. Despite many promising results, there are also some limitations in measuring LDH as a melanoma biomarker. First of all, LDH is not an actively secreted enzyme. Thus, LDH is only released through cell damage and cell death, which occur more frequently in malignant neoplasms. However, there are also false-positive values through hemolysis; hepatocellular injuries like hepatitis, myocardial infarction, and muscle diseases; and other infectious diseases with high amounts of necrotic cells (4). Moreover, LDH is nonspecific for melanoma and elevated levels are also found in many other benign and malignant diseases.

TYROSINASE

An indicator for the presence of circulating melanoma cells and increased probability of the occurrence of metastases is the detection of tyrosinase (EC 1.14.18.1) mRNA in peripheral blood. Although the serological analyte actually is a nucleic acid isolated from circulating melanoma cells, in the literature tyrosinase often is considered as an enzyme biomarker in melanoma (1, 4). The enzyme itself is constitutively expressed in melanocytes and melanoma cells and is involved in the biosynthesis of melanin catalyzing the oxidation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and of L-DOPA to DOPAquinone. Due to the fact that tyrosinase mRNA is detected through nested reverse transcription polymerase chain reaction (RT-PCR), the analytical sensitivity is very high. It is possible to detect one melanoma cell among 10^6 normal blood cells. In the last decades, however, tyrosinase mRNA expression was determined in

many different studies, resulting in a wide range of variability (30–100%). One reason might be the transient presence of tumor cells in the bloodstream. On the other hand, nonstandardized protocols for PCR-based techniques contribute to the observed variability, lower sensitivity, and different thresholds for melanoma cell detection. In order to overcome these limitations, complementary analysis of other nucleic acid–based markers should be considered. Salvianti et al. assessed the diagnostic value of a tumor-related, methylated, cell-free DNA marker, the hypermethylated *Ras association domain family 1 isoform A* promoter, in melanoma patients (13). This marker showed good predictive capability in discriminating melanoma patients (*in situ*, invasive, and metastatic) and healthy controls. Particularly, when jointly considered with circulating tumor cells analyzed both for size and tyrosinase mRNA expression, a higher sensitivity of the detection of positive cases in invasive and metastatic melanomas was obtained. Alternatively, determination of tyrosinase as a tissue biomarker also has been taken into account. In this regard, Lin et al. very recently presented a novel methodology using scanning electrochemical microscopy for mapping expression and distribution of the Type 3 copper protein tyrosinase in tissue microarrays of skin biopsies taken from melanoma patients (14). Interestingly, the progression from a homogeneous tyrosinase distribution in Stage II to a more heterogeneous pattern in Stage III was clearly visualized. Of note, the scanning electrochemical microscopy is not limited by the presence of optically interfering species, such as melanin. The authors conclude that this methodology might be implemented as a complementary prognostic technique for diagnosing metastatic and non-metastatic melanoma stages.

CYCLOOXYGENASE-2

Another enzyme marker of interest is cyclooxygenase-2, which, in theory, should be analytically accessible by measurement of certain circulating or urinary eicosanoid products of the enzyme reaction (1). Cyclooxygenase-2 is the inducible isoenzyme of cyclooxygenases (prostaglandin-H-synthases, EC 1.14.99.1) whose overexpression is implicated in a number of inflammatory or inflammation-associated processes, including tumor inflammogenesis, angiogenesis, metastasis, and radiosensitivity (15). The enzyme catalyzes the conversion of arachidonic acid into prostaglandin H_2 (PGH_2). PGH_2 afterward is converted to a multitude of eicosanoids, for example, other prostaglandins like PGE_2, prostacyclin, and thromboxanes, depending on definite downstream synthase and/or isomerase pathways present in various cell types. These eicosanoids act as potent paracrine and endocrine mediators of metabolic processes via G-protein-coupled receptors not only in homeostasis but also in inflammatory and neoplastic processes. Regarding those cyclooxygenase downstream enzymes, special attention was paid to microsomal PGE_2 synthase-1 (EC 5.3.99.3). Very recently, Kim et al. suggested a prognostic and predictive value of this enzyme in melanoma (16). However, although eicosanoid analytics made an enormous leap, particularly by progress of liquid and/or gas chromatography and mass spectrometry, a quantitative melanoma-specific profiling of plasma or urinary eicosanoids seems remote. Therefore, recent research focuses on analysis of intracellular expression of cyclooxygenase-2 in melanoma tissue specimens. In this regard, Kuźbicki et al. established an immunohistochemical scoring algorithm showing some value of

cyclooxygenase-2 as negative prognostic marker, directly correlated with other negative prognostic factors in melanoma such as tumor thickness, ulceration, and lymph node metastasis (17). In a retrospective analysis in metastatic lymph node samples obtained from melanoma patients, Panza et al. demonstrated that when cyclooxygenase-2 expression rises above a certain threshold level, it is a negative prognostic factor for human metastatic melanoma (18). They conclude that differentiation of cyclooxygenase-2 expression in more detail would help to delineate when cyclooxygenase can be defined a negative prognostic factor. Others demonstrated cyclooxygenase-2 to be a useful immunohistochemical marker for the differentiation of melanoma from benign melanocytic lesions in the oral cavity (19). Among others, these observations substantiate findings that suggest cyclooxygenase-2 expression and/or activity as both a pathogenic key player and a promising molecular target in melanoma (20). The latter, besides pharmacological targeting, offers a rationale for developing novel radiotracers for noninvasive imaging and functional characterization of cyclooxygenase-2 in melanoma. Particularly, the development of an appropriate radiotracer for positron emission tomography would provide substantial impact to the melanoma biomarker approach (21). It should be mentioned here that the development of imaging biomarkers and quantitative imaging techniques has been identified as a major and auspicious approach to move toward personalized treatment strategies. To remain with cyclooxygenase-2 as one example, quantitation of this enzyme's functional expression by imaging is assumed to be a predictive marker for radioresistance and chemoresistance and, in turn, for therapy response, particularly under hypoxic conditions (21). In the case of other target molecules, such as membrane receptors or melanin, functional imaging of molecular markers can be combined directly with targeted therapies (22).

MATRIX METALLOPROTEINASES

The human matrix metalloproteinase (MMP) family comprises 25 members in five groups: collagenases, gelatinases, stromelysins, membrane type MMPs (MT-MMP), and others. MMPs are directly implicated in almost every biological process involving matrix degradation and remodeling, for instance, in embryogenesis, normal tissue maintenance (angiogenesis, wound healing), and in pathologies such as chronic inflammatory diseases and cancer. MMPs not only degrade and process components of the ECM but also mobilize the release of growth factors from degraded matrix and cleave proteins that block growth factors (23, 24). Melanomas express a number of MMPs that are often associated with disease progression, and key roles are mostly (25) assigned to MMP-2 and MMP-9 (26). Indeed, findings differ in some ways. In a tissue microarray and immunohistochemistry study comprising 482 melanoma tumor and 149 nevi biopsies, Rotte et al. found that strong MMP-2 (EC 3.4.24.24) expression is associated with significantly poorer survival of melanoma patients but is independent of tumor thickness and ulceration (27). In contrast, Kamyab-Hesari et al. immunohistochemically analyzed 24 consecutive primary melanoma samples and found that MMP-2 expression correlates with tumor thickness in melanoma and is an independent predictive factor for lymph node involvement (28). However, in a different study, MMP-2 was found to be expressed in 96% of the analyzed uveal melanoma patients but showed no significant difference between

metastatic and nonmetastatic groups (29). In a study with patients in Stages I–III versus controls, MMP-2 expression in blood samples was similar in both groups. On the other hand, serum MMP-9 (EC 3.4.24.35) was higher in melanoma patients than in controls. However, the authors found no association between MMP-9 concentration and clinicopathological parameters, such as disease-free survival and overall survival (26). Recently, the potential of further MMPs as melanoma biomarkers and possible immunotherapeutic targets was investigated. MT1-MMP (EC 3.4.24.80; *syn.* matrix metalloproteinase-14), an activator of MMP-2, was found to be higher expressed in primary melanoma than in nevi, and its expression continues to increase during melanoma progression and portends poorer patient outcome (30). MMP-12 (EC 3.4.24.65; *syn.* macrophage metalloelastase) was also found to be increased in cutaneous melanoma compared to normal skin and was significantly associated with invasion and metastasis. Furthermore, patients with high MMP-12 level had unfavorable overall survival (31). Finally, increased MMP-23 (EC 3.4.24.-) expression in primary melanomas is inversely associated with the presence of tumor infiltrating lymphocytes, suggesting a role for tumor-derived MMP-23 in the suppression of antitumor immune responses (32).

OTHER ENZYME MARKERS

Other potential enzyme markers of melanoma currently under research, accessible mostly via immunohistochemical approaches of tissue specimens, comprise the proteases cathepsin K (EC 3.4.22.28), CD10 (EC 3.4.24.11; *syn.* neutral endopeptidase and/or neprilysin), and legumain (EC 3.4.22.34; *syn.* asparaginyl endopeptidase) (33–36). However, on the basis of only few current data, their usefulness as biomarkers still is difficult to estimate. Caution also should be considered for the enzyme aldehyde dehydrogenase 1 (ALDH-1, EC 1.2.1.'3'), which has been proposed not only as a promising therapeutic target but also as a biomarker of stem cell–like cells for certain human cancers, including melanoma (37, 38). Of interest, very recently, Taylor et al. demonstrated ALDH-1 to be an independent prognostic factor in melanoma, with results based on a score derived from immunohistochemical staining (39).

ENDOGENOUS ENZYME INHIBITORS

Tissue inhibitor of metalloproteinases (TIMPs), which are natural endogenous inhibitors of MMPs, including TIMP-1, also play a significant role in tumor development. TIMPs participate in the degradation of extracellular matrix, angiogenesis, apoptosis, differentiation, as well as in proliferation of normal and tumor cells (40). In this regard, patients with melanoma at Stages I–III in comparison with the control group had significantly higher median concentrations of serum TIMP-1, and this increase had an effect on disease-free survival and overall survival. Regarding MMP-9, the authors did not observe significant correlation between concentration of TIMP-1 and depth of invasion, clinical stage, or nodal status (26). Some attention also has been paid to other protease inhibitors, namely, maspin (serpinB5) and serpinB1, which both are members of the serine protease inhibitor superfamily. Loss of melanoma maspin has been suggested to contribute to disease progression and metastatic dissemination, but this subject is of

controversial debate (41, 42). SerpinB1 has been suggested as an indicator of chemotherapy response. Willmes et al. reported experimental and clinical data on serpinB1 expression, demonstrating that melanoma Stage IV patients showing strong serpinB1 protein expression in tumor tissue are likely to benefit from cisplatin-containing chemotherapy regimens. Moreover, serpinB1 protein expression was proved to be predictive for the outcome of cisplatin-based chemotherapy in melanoma (43).

S100 PROTEINS

The S100 family of calcium-binding proteins gained importance as both potential molecular key players and biomarkers in the etiology, progression, manifestation, and therapy of neoplastic disorders, including malignant melanoma. Twelve S100 family members are expressed in melanoma: four exhibit no change in expression (S100A8, S100A9, S100A10, and S100A11); one is downregulated (S100A2); and seven are upregulated (S100A1, S100A4, S100A6, S100A13, S100B, and S100P) (44). So far, different S100 tumor markers have been tested as prognostic factors (1, 45–47), and *in vivo* studies have confirmed that S100B, S100A4, and S100A9 contribute to melanoma progression and may be therapeutic targets (44). S100B protein is highly specific and increased levels are registered in 74–100% of patients with Stage IV melanoma (48, 49). Several studies confirmed a positive correlation between advanced stage of disease and disease-free survival (48, 50, 51). Wevers et al. showed that S100B level in Stages IIIB–IIIC patients also has a strong association with melanoma prognosis. Here, preoperative measurements of S100B and S100B measured on postoperative day 2 showed the strongest association with disease-free survival. For disease-specific survival, the preoperative S100B level seems to be the strongest independent predictor (52). S100B is further suggested to be a useful marker to monitor response to chemo- and immune-chemotherapy in metastatic malignant melanoma (53). Abusaif et al. were interested in determining whether S100B is able to monitor and predict objective tumor responses and tumor progression in vemurafenib-treated patients (54). Here, the S100B level during treatment with vemurafenib showed an initial response, but repeated measurements of S100B did not seem to be sufficient for detecting tumor progression and is thus not an alternative compared to computed tomography. Another prospective study demonstrated that S100B level during response to dabrafenib or vemurafenib treatment is of prognostic value. Here, patients with high S100B levels showed a shorter progression-free disease (55). In patients with lesions of Breslow thickness >1 mm, Swiss and German guidelines recommend S100B quantification every 3–6 months for the first 1–5 years, and every 6–12 months for years 6–10. Serum concentration appears to correlate with Breslow thickness and tumor burden measured under RECIST *(Response Evaluation Criteria In Solid Tumors)* 1.1 (8). Reports show that all Stages IIIB–IV patients with S100B higher than 0.13 µg/L had metastases, and all had distant metastases if S100B was higher than 1.6 µg/L (8). Stages of malignant melanoma and the relative hazard of death increased 5-fold when circulating S100B exceeded 0.6 µg/l (48). Only the European Society of Medical Oncology (ESMO), German and Swiss guidelines recommend serum S100B as the most accurate serologic test for follow-up having better specificity for

progressive disease versus LDH (8, 56–58). In the United States, serum S100B is not used routinely because the prognostic value is limited to advanced and/or disseminated melanoma, and LDH is the predominant serum marker (58). S100A4, also called metastasis-associated protein, is universally overexpressed in a variety of tumor entities and is an independent marker for tumor progression, invasion, metastasis, poor survival, and prognosis (1). S100A4 influences cell motility, inflammation, angiogenesis, and apoptosis due to interaction between tumor cells and their microenvironment (59–64). However, extracellular S100A4 seems to be of major importance in this context and, therefore, may possibly serve as a blood marker. Besides some initially promising results on the use of S100A4 serum levels as a prognostic marker in melanoma, the greatest problem might be that of low serum protein concentration which impedes clinical relevance (1). An attractive approach for the treatment of cancer seems to be the blocking of extracellular S100A4 with a neutralizing monoclonal antibody, leading to abolished endothelial cell migration, tumor growth, and angiogenesis *in vivo* in a melanoma subcutaneous xenograft model (60). S100A13, another promising prognostic marker for melanoma, is proposed to be an indicator of the angiogenic switch that facilitates disease progression. Massi and colleagues found expression in dysplastic nevi and in primary and metastatic melanoma with increasingly higher correlation in more aggressive and/or advanced tumors (Breslow thickness and Clark's level) (64). A proteomics study reported S100A13 to be elevated in cisplatin-resistant melanoma cell lines (65). There is also a correlation between S100A13 expression and chemotherapy resistance vis-à-vis dacarbazine and temozolomide in human melanoma tumors (66). Here, low or no expression of S100A13 could be a valuable marker to identify melanoma patients responding to chemotherapy. The calcium-binding proteins S100A8 and S100A9 can dimerize to form calprotectin, the release of which during tissue damage has been implicated in inflammation and metastasis (67). The calprotectin is one of the many proinflammatory mediators released from UVR-exposed keratinocytes. S100A8/A9 stimulates cell proliferation and migration via the pattern recognition receptor RAGE (receptor for advanced glycation end products) (68). Because of the RAGE expression in melanocytes and melanoma cells, calprotectin seems to be an activator in these cells and it is a potential target for intervention in melanomagenesis (69). The latter should also be considered regarding interaction of S100A4 with RAGE (61, 62). Another study presented evidence for S100A8/A9 as a novel predictive marker for ipilimumab treatment of metastatic Stage IV melanoma patients. A pronounced upregulation of S100A8/A9 serum levels could be detected in nonresponding patients already after the first ipilimumab infusion, and a decrease as compared with baseline levels in responding melanoma patients (70).

PROGENITOR AND/OR STEM CELL–LIKE MARKERS

Animal models have demonstrated that, aside from the aforementioned markers, other proteins can be detected in circulating melanoma cells. Some of them possibly represent melanoma progenitor and/or stem cell–like markers. This includes ATP-binding cassette (ABC) multidrug transporters and the neuroepithelial intermediate filament nestin (1, 6). In this regard, immunohistochemical analysis of

nestin performed by Akiyama et al. in various melanoma specimens revealed a positive association of nestin expression with advanced disease (71). However, in this study, compound nevi also showed high expression of nestin. Among progenitor cell markers of interest are also SOX (*Sry-related HMG-Box gene*) proteins. Some represent nuclear transcription factors in the differentiation of neural crest progenitor cells to melanocytes, while others are more versatile regulators of stem and progenitor cell fate (72, 73). The immunohistochemical profile of SOX10 was used to detect metastatic melanoma in sentinel lymph nodes with high sensitivity and specificity and is supposed to be a reliable marker for supplementing other immunohistochemical stains, like S100B or melan-A (74). On the other hand, SOX10 staining cannot discriminate melanoma metastasis from nodal nevi (74). In contrast, there is evidence that suggests that SOX2, besides nestin, can effectively differentiate nodal melanocytic nevi from metastatic melanomas and, thus, may serve as a powerful diagnostic adjunct in melanoma staging (75). The differing value of these SOX protein family members as markers well reflects the excessive heterogeneity of melanoma. The same is applicable for many other melanoma biomarkers. These results, in part, in conflicting observations, essentially complicate a final evaluation. As an example, the value of two other stem cell–like markers, CD271 (nerve growth factor receptor) and CD133 (*syn.* prominin-1), both of which have been recognized recently as crucial molecules driving melanoma initiation and metastasis, has not been clarified (76–78). Other proteins considered as melanoma biomarker candidates are given in Table 1. Furthermore, various nonprotein biomarkers are potential targets for melanoma biomarker research. Those comprise metabolites of the melanin synthesis pathways, originating from the amino acid L-tyrosine, and cell-free nucleic acids (1).

NONPROTEIN BIOMARKERS

MicroRNAs (miRNAs) are small, single-stranded noncoding RNAs that regulate gene expression in normal cellular processes, and alterations in miRNAs are involved in several pathologies such as cancer ((1, 81), *cf. references therein*). Due to their stability, detectability in serum, and easy analytical accessibility, they may also be considered as useful biomarkers in malignant melanoma (81). Alterations in the expression of miRNAs and their targets are discussed as risk factors and prognostic factors in malignant melanoma (81). In serum samples of melanoma patients, Kanemaru et al. found significantly higher miR-221 levels than in healthy controls and miR-221 levels were correlated with tumor thickness. Moreover, a longitudinal study revealed a tendency for the miR-221 levels to decrease after surgical removal of the primary tumor, and to increase again at recurrence (82). In another study, Nguyen et al. analyzed paraffin-embedded archival tissue and found miR-29c expression significantly downregulated in Stage IV melanoma compared to early-stage melanoma. Furthermore, in lymph nodes from Stage III melanoma patients, higher expression of miR-29c was found to be a significant predictor of improved overall survival (83). However, further data concerning miRNAs in patient samples are needed to better assess the potential of miRNAs as biomarkers in melanoma genesis and progression.

Conclusion

All these markers offer the potential to predict the risk of progression to metastatic disease states, treatment resistance, and disease relapse. Lack of sufficient sensitivity, specificity, and accuracy are the most relevant limitations of blood-based melanoma biomarker in clinical use. Given the heterogeneity of malignant melanoma, this is taking on a special significance. In contrast, a cluster of biomarkers for one disease would be a better diagnostic tool with much higher sensitivity, specificity, and clinical accuracy. Therefore, new investigations called "proteomic profiling" or "multimarker profiling" focus on the identification of multiple co-expressed biomarkers or signature biomarker patterns which allow early detection, staging, therapeutic monitoring, and prognostic predictions (7, 84–88). This approach can be adopted for both serum and tissue specimens. In addition, multimarker analyses of circulating tumor cells could be more useful for monitoring therapy response in melanoma patients and for providing prognostic information relating to overall survival (89, 90). Identification, establishment, and validation of the optimal combination of biomarkers for multimarker profiling is a challenge and the subject of currrent research in the melanoma field.

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