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REVIEW ARTICLE

Protein and non-protein biomarkers in melanoma: a critical update

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Abstract Melanoma is the most malignant type of all skin neoplasms. Its worldwide incidence has steadily increased during the past decades, suggesting a probable melanoma 'epidemic'. Although current clinical, morphologic, and histopathologic methods provide insights into disease behavior and outcome, melanoma is still an unpredictable disease. Once in an advanced stage, it remains a disastrous affliction with scarce therapeutic options. Therefore, significant efforts need to be made in finding informative biomarkers or surrogate markers that could aid or improve early diagnosis of melanoma, its correct staging, the discrimination of other pathological conditions as well as indicate patients' prognosis or the most appropriate therapeutic regimes. Ideally these markers are secreted into body fluids and easily amenable to the design of non-invasive clinical tests. A critical view on the current debate on serologic protein markers, e.g., lactate dehydrogenase, tyrosinase, and melanoma inhibiting activity, and some selected non-protein markers, e.g., 5-*S*-cysteinyl-dopa and circulating nucleic acids, will be offered and novel innovative approaches currently being explored will be discussed. Special emphasis is put on the S100 family of calcium binding proteins that is more and more emerging as a potentially important group of both molecular key players and biomarkers in the etiology, progression, manifestation, and therapy of neoplastic disorders, including malignant melanoma. Notably, S100B and, possibly, other S100 proteins like S100A4 are assumed to fulfill requirements which make them strong biomarker candidates in melanoma. Moreover, S100 proteins receive attention as possible targets of therapeutic intervention moving closer to clinical impact.

Keywords Diagnostic markers · Melanomata · Molecular targets · Pigment cells · Prognostic markers · Serological markers · Skin cancer · Stem cell-like markers · S100 proteins · Therapy monitoring

Abbreviations

5-SCD	5-*S*-cysteinyl-dopa
CAM	Cell adhesion molecule
COX	Cyclooxygenase
CRP	C-reactive protein
Gal-3	Galectin 3
LDH	Lactate dehydrogenase
L-dopa	3,4-dihydroxyphenylalanine
MAA	Melanoma-associated antigen
MART-1	Melanoma antigen recognized by T cells 1
MIA	Melanoma inhibitory activity
MMP	Matrix metalloproteinase
OPN	Osteopontin
VEGF	Vascular endothelial growth factor

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Malignant melanoma—an introduction

Malignant cancers involving the skin comprise basal cell carcinomata (basalioma), squamous cell carcinomata, and melanomata, as well as some rare neoplasms, such as Merkel cell carcinoma and Dermatofibrosarcoma

protuberans. Melanoma only accounts for about 5 % of all invasive skin cancer cases, but it is by far the deadliest, responsible for up to 90 % of skin cancer deaths. Melanomata are tumors arising from melanocytes. Melanocytes are pigmented dendritic cells of neuroectodermal origin that migrate to reside at the basal layer of the epidermis and other epithelial sites, including the eye, gastrointestinal tract, and vagina (Boissy 1988; Kefford 2009; Mintz 1971). Melanomata can arise at any of these sites, but most commonly arise in deeper layers of the epidermis (cutaneous melanomata), where the function of melanocytes is normally to produce melanin, a protective skin pigment, in response to solar ultraviolet (UV) radiation. Another prominent melanoma entity, ocular melanoma, the most common form of intraocular tumors, arises from melanocytes in the uveal stroma (Kefford 2009, and references therein). Melanomata are strictly speaking not epithelial cancers or carcinomata, because melanocytes ontogenetically are derived from the neural crest in the developing embryo (Uong and Zon 2010; Weber 2007, and references therein).

There is epidemiologic evidence that multiple exposures to solar UV radiation are the key etiological factors in melanoma pathogenesis (Whiteman et al. 2011). Primarily, the energy of UV in the 290–320 nm wavelength range, UVB, is absorbed by DNA resulting in the formation of cyclobutane dipyrimidine photoproducts and (6–4) photoproducts (Pfeifer and Besaratinia 2012). Under conditions of slow or insufficient repair these DNA lesions are precursors to C→T and tandem CC→TT transitions that are the classic molecular signatures of mutagenesis by solar UV radiation. The *p53* tumor suppressor gene is among the major targets for multiple mutations, resulting in its inactivation (Hodis et al. 2012). Of interest, the most common oncogenic mutations in melanoma affecting the *raf* (V600E) and *ras* (Q61L/R) genes, do not appear attributable to UV-induced C→T/CC→TT transitions. Very recently, Hodis et al. (2012) discovered an activating mutation in the *rac1* (P29S) gene, encoding for a RAS-related member of the Rho subfamily of GTPases, as a first example of a common hot-spot mutation in melanoma attributable to direct UVB-mediated damage. This finding and, furthermore, the observation of hot-spot mutations in two other genes (*stk19* and *ppp6c*), provide definitive evidence for UV mutagenesis in pathogenesis of melanoma (Hodis et al. 2012). Another recent study indicates that noncoding RNA damaged by UVB radiation stimulates Toll-like receptor 3 (TLR-3)-mediated production of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) from non-irradiated keratinocytes (Bernard et al. 2012). In addition, UV radiation also results in oxidative stress, inflammation, and immunosuppression (Kanavy and Gerstenblith 2011; Pfeifer and Besaratinia 2012; Robertson 2009; Weber 2007). Finally, chronic exposure to UVB results in the disruption of the epithelial structure and the expansion of premalignant clones, which undergo further genomic changes leading to full malignancy. There is experimental evidence that the more abundant UVA (320–400 nm) not only results in indirect effects on DNA, e.g., by oxidative stress, but also causes direct DNA changes, such as formation of cyclobutane dipyrimidine photoproducts (Mouret et al. 2006). Other models of melanoma pathogenesis refer to chemical carcinogenesis (Robertson 2009).

Melanoma is uncommon in non-caucasians. The incidence of melanoma is highest in fair-skinned and blond or red-haired individuals living in areas of high sunlight exposure. Propensity to freckle is also a pigmentary trait at high risk for melanoma when combined with sunlight exposure. Episodes of sunburn, especially during childhood, enhance the risk for melanoma (Green et al. 2011; Mancini 2004). Consequently, the incidences for cutaneous melanoma vary between high levels in countries like Australia and New Zealand (>25/100,000), intermediate levels in northern Europe and mainland USA (5–25/100,000), and low levels in Africa and South-East Asia (<5/100,000). Exemplary, in Germany incidence rates (mortality rates) of cutaneous melanoma were 17.4 (2.6) per 100,000 males and 16.0 (1.7) per 100,000 females in 2009, 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). The world-wide incidence of ocular melanoma is less than 0.7/100,000. Recently, intensive public health sun protection programs resulted in decreasing incidence rates in, particularly, younger age groups. However, the world-wide incidence and burden of melanoma continue to rise, mainly due to the aging population, continued high recreational sun exposure habits, changing climate patterns which may increase ambient UV radiation, and increasing environmental contamination with carcinogenic chemical compounds (Kefford 2009; Riker et al. 2010; Singh et al. 2011; Yuspa 1986).

The majority of melanomata fall into four groups: (a) melanoma of the superficial-spreading type, (b) melanoma of the lentigo-maligna type, (c) melanoma of the nodular type, and (d) melanoma of the eye. Primary malignant melanomata of the superficial-spreading type and of the lentigo-maligna type develop through a characteristic biphasic growth pattern (Weber 2007, and references therein). The initial radial growth phase of these melanomata is only rarely associated with the development of metastases, while the ensuing vertical growth phase is commonly associated with subsequent metastatic disease (Laga and Murphy 2010; Massi et al. 1999; Weber 2007). Melanomata in the radial growth phase sometimes may

regress. However, once cells penetrate the dermis, invasion of blood and lymphatic vessels leads to dissemination. Metastases may occur early on or after a disease-free period of many years (Saleh and Peach 2011). Often, the first clinical signs of melanoma are generated by the metastases. The patterns of dissemination are highly varied and unpredictable (Kefford 2009). In typical cases of cutaneous melanoma, the draining lymph nodes are the first sites of apparent metastasis. In other cases, hematogenous dissemination occurs early. The skin, lungs, liver, brain, and spinal cord are particularly common sites for metastases, but in later stages nearly every organ and tissue can be involved, including those that are uncommonly affected by other tumors, such as the heart, intestines, and spleen (Mooi and Krausz 2009, and references therein). Metastases in the central nervous system are present in over 80 % of autopsy cases. If the brain is colonized, there is a rapid decline in the quality of life and ensuing death. Uveal melanoma, on the other hand, metastasizes hematogenously, with a particular affinity for the liver, which may remain the only site of indolent metastatic disease until preterminal stage, when lung, brain, and distant lymph node sites are also involved (Gaudi and Messina 2011; Kefford 2009).

According to the criteria of various national committees on cancer, e.g., the American Joint Committee on Cancer (AJCC), for melanoma staging and classification, patients can be divided in five stages, from local disease (stages 0, I, and II) to locoregional disease (stage III) and distant metastatic disease (stage IV) (Warner and Cockerell 2011). In stage 0, melanoma involves the epidermis but has not reached the underlying dermis. This stage is sometimes called melanoma in situ. In stage I, melanoma is characterized by tumor thickness, presence and number of mitoses, and ulceration status. There is no evidence of regional lymph node or distant metastases. In stage II, melanoma is also characterized by tumor thickness and ulceration status. There is still no evidence of regional lymph node or distant metastases. In stage III, melanoma is characterized by the level of lymph node metastases. There is still no evidence of distant metastases. Stage IV melanoma is characterized by the location of distant metastases and the level of serum lactate dehydrogenase (LDH). Of note, by now LDH is the only serum marker that has been included in clinical melanoma staging systems (Balch et al. 2011; Dickson and Gershenwald 2011).

Melanomata show some prominent molecular abnormalities in transforming signaling pathways that are associated with etiology and progression of the primary tumor (Gaudi and Messina 2011). In this regard, melanomata show activating somatic mutations in signaling pathways that mediate proliferation in melanocyte development (Bloethner et al. 2009; Weber 2007). Most important, somatic mutations in *raf* and *ras* genes have been observed in about 70 % of melanomata. These mutations initiate a proliferative signal through B-RAF/MEK/ERK signaling cascade and at least represent a gain of function in a pathway that is physiologically engaged by α-melanocyte stimulation hormone (Dumaz 2011; Hodis et al. 2012). In melanomagenesis, the B-RAF/MEK/ERK signaling cascade is further influenced by activating mutations of upstream receptor tyrosine kinases, such as KIT (Woodman and Davies 2010). Epigenetic inactivation by hypermethylation of the RAS effector and potential tumor suppressor RAS-association domain family protein 1 contributes to this abnormal signaling and occurs in about 50 % of melanomata (Spugnardi et al. 2003). Recent evidence links RAC1, a RAS-related member of the Rho subfamily of GTPases, and an activated PAK (p21-activated protein kinases) signaling pathway to these processes (Hodis et al. 2012). Furthermore, melanomagenesis frequently is associated with abnormalities in the APC/β-catenin (Wnt/β-catenin) pathway, characterized by activating mutations in the *ctnnb1* (β-catenin) gene or hypermethylation of the APC promoter 1A (Larue and Delmas 2006; Worm et al. 2004). Alterations in apoptosis pathways also are common characteristics of transformed melanocytes (Hussein et al. 2003). In this regard, mutations affecting the RAS-triggered phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which conducts antiapoptotic signals, and the phosphatase and tensin homolog (PTEN), which antagonizes PI3K/AKT pathway, are of increasing importance (Haluska et al. 2007; Tsao et al. 2004). In uveal melanoma, which differs from cutaneous melanoma, overexpression of insulin-like growth factor 1 receptor (IGF1R) often contributes to cell growth (All-Ericsson et al. 2002). Recently, mutations in G protein alpha subunits, encoded by *gnaq* and *gna11*, have been assumed to be involved in malignant transformation in uveal melanoma (Van Raamsdonk et al. 2010). Moreover, mutations in the breast cancer type 1 and 2 susceptibility proteins (BRCA1 and BRCA2) as well as BAP1 (BRCA1-associated protein-1) have been reported that possibly demarcate a molecular edge beyond which metastasis becomes highly likely (Cruz et al. 2011; Harbour et al. 2010; Leyvraz and Keilholz 2012). Of interest, BAP1 mutations can also occur in the germline, leading to a distinctive cancer predisposition syndrome (Abdel-Rahman et al. 2011).

Approximately 10 % of melanoma patients have a positive family history of the disease (Yeh and Bastian 2009). Genetic predisposition of melanoma susceptibility in about 55 % of multiple case families (≥3 cases) is associated with inherited mutations of the *cdkn2A* (cyclin-dependent kinase inhibitor 2A) gene, which codes for the cell-cycle regulating tumor suppressor proteins $p16^{INK4A}$ and $p14^{ARF}$ (Gaudi and Messina 2011). The location of

other melanoma susceptibility genes is the subject of intense research by the international Melanoma Genetics Consortium (GenoMel), and genome-wide screening has revealed several promising new melanoma susceptibility loci (Barrett et al. 2011; Bishop et al. 2009). Potential modifier genes include the *mc1r* (melanocortin-1 receptor). This receptor is a key determinant of the pigmentation process and genetically it is highly polymorphic. Various MC1R alleles are associated with increased risk for all skin cancers, but also correlate with varying penetrance and age of onset in familial melanoma. Another potential melanoma susceptibility gene is *cdk4* (cyclin-dependent kinase 4) (Gaudi and Messina 2011). Importantly, there are some heritable malignant and premalignant conditions, like xeroderma pigmentosum, retinoma, retinoblastoma, oculodermal melanocytosis, nevoid basal cell carcinoma, familial atypical multiple mole melanoma syndrome, and dysplastic nevus syndrome, all of them characterized by an increased risk of developing primary melanomata, a higher incidence of multiple primary melanomata, and an earlier age of onset of the disease (Weber 2007, and references therein).

Melanoma metastasis is also associated with various prominent molecular abnormalities. For instance, transition of melanoma cells from the radial growth phase to the vertical growth phase, which is a marker of progression, is associated with overexpression of transcription factors CREB (cAMP response element binding protein) and ATF-1 (activating transcription factor), both of which act as survival factors during dissemination (Nyormoi and Bar-Eli 2003). Also *raf* and *ras* mutation frequency increases with melanoma progression (Greene et al. 2009). On the other hand, progression of melanoma is associated with a loss of expression of transcription factor and tumor suppressor AP-2 (activator protein-2), which results in overexpression, e.g., of matrix metalloproteinase-2 that is associated with tumor invasiveness (Nyormoi and Bar-Eli 2003). Another regular finding in melanoma probes is the down-regulation of metastasis suppressor genes, such as *kiss1* (kisspeptin-1 or malignant melanoma metastasis-suppressor) and *brms1* (breast cancer metastasis-suppressor 1). Loss of *kiss1* expression occurs in more than 80 % of metastatic melanomata (Nash and Welch 2006; Li et al. 2011). Other mechanisms in melanoma progression and metastasis comprise the HGF/c-MET signaling pathway and various integrins (Pinon and Wehrle-Haller 2011; Ye et al. 2008).

An important feature in melanoma metastasis is the involvement of specific homing receptors. These receptors are suggested to modify organ-specific melanoma metastasis, e.g., mannose receptors on hepatic sinusoidal endothelial cells or neurotrophin receptors/neurotrophins in brain tissue. In melanoma cells metastasizing to the brain, neurotrophins promote invasion by enhancing the production of extracellular matrix-degrading enzymes, such as the endo-β-D-glucuronidase heparanase, type IV collagenases, and cathepsins. The outcome of homing receptors is influenced by proinflammatory conditions. In the liver, the production of interleukin-1 mediates an increase in mannose receptor expression on the endothelium. In the brain, astrocytes may provide paracrine signals that attract melanoma cells (Marchetti et al. 2003; Mendoza et al. 1998; Quintanilla-Dieck et al. 2008; Roy and Marchetti 2009; Truzzi et al. 2008; Weber 2007).

Primary melanoma is treated by wide local excision, usually with a margin of 1 cm of apparently uninvolved skin, to minimize the risk of local recurrence. Regional lymph node metastases, as manifest clinically or detected by sentinel lymph node biopsy, are generally treated by regional lymph node dissection. This procedure still offers a significant chance of cure. Distant hematogenous metastasis is not amenable to curative surgery. However, a few patients benefit from surgical removal of solitary metastases. The response of melanomata to radiation therapy and chemotherapy is usually modest to marginal. The reason for this is probably the evolution within the tumor of robust and redundant mechanisms of inhibition of apoptotic pathways (Dallaglio et al. 2012). However, there are some chemotherapeutic approaches showing great promise for therapy of malignant melanoma, e.g., using the alkylating agent dacarbazine both alone and in combination with other drugs or, more recently, the BRAF kinase inhibitor vemurafenib (Chapman et al. 2011). Biochemical pathways of particular interest for future rational and specific approaches for chemotherapy or adjuvant therapy of melanoma include receptor signaling pathways, cell cycle regulation, angiogenesis, inflammation, integrin expression, and regulation of apoptosis (Khan et al. 2011).

Moreover, there continues to be keen interest in novel immunotherapeutic strategies in melanoma treatment (Slingluff et al. 2006). These strategies aim to exploit the antitumor immune response that is often evident in primary melanoma, and which substantially affects or effaces the primary tumor in some patients. Of importance, spontaneous regression, a phenomenon likely mediated by the immune system, is more common in melanoma than in most other cancers. Melanoma may regress completely, and in few cases even regression of metastases has been reported (Kalialis et al. 2009). The role of the host immune system in malignant melanoma is evidenced by the association of autoantibodies and autoimmune reactions with an improved prognosis in patients treated with interleukin-2 and/or interferon-α therapy (Chianese-Bullock et al. 2005; Gogas et al. 2006). Recent immunotherapeutic approaches focus on administration of peptide-primed autologous dendritic cells, immunotoxins, regulatory T cell function blocking antibodies, and vaccination against

melanoma cell-surface antigens (Mansh 2011; Marquez-Rodas et al. 2011; Steele et al. 2011). Melanoma cell-surface antigens are mainly derived from lineage differentiation tumor antigens, such as tyrosinase, gp100 (melanocytes lineage-specific antigen gp100) and MART-1/MelanA (melanoma antigen recognized by T cells 1), or from cancer-testis antigens, such as MAGE (melanoma associated antigen-1), BAGE (B melanoma antigen-1) and GAGE (G antigen). The latter are expressed normally only in the testes, but become expressed in melanoma because of demethylation of the corresponding genes (Engelhard et al. 2002; Ferrucci et al. 2012; Sang et al. 2011). Moreover, individually mutated tumor antigens, such as CDK4 or β-catenin contribute to the presentation of melanoma cell-surface antigens (Kirkin et al. 1998). Of interest, these melanoma cell-surface antigens, when secreted or proteolytically released from cells are promising biomarker candidates in malignant melanoma.

Once in an advanced stage, malignant melanoma remains a fatal neoplasm with scarce therapeutic options. 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 % (Eisemann et al. 2012). For local disease it amounts to 99 %. Five-year survival for people diagnosed with mucosal and uveal melanoma is about 70 %. If spread is within the region of the primary melanoma, the 5-year survival is 60–65 %, dramatically dropping to below 10 % if the disease is widespread (Hiripi et al. 2012; Howell et al. 2010; Mallone et al. 2012). In this regard, sensitive screening and early detection of high risk groups are the major principles of melanoma control. Therefore, much more efforts need to be made in finding informative biomarkers or surrogate markers in melanoma. This could aid or improve early diagnosis of melanoma, but also its correct staging, the discrimination of other pathological conditions as well as indicate patients' prognosis or the most appropriate therapeutic regimes. Ideally these markers are secreted into body fluids and easily amenable to the design of non-invasive clinical tests. Moreover, well-defined sensitive diagnostic markers are necessary to avoid potential overdiagnosis of melanoma (Norgaard et al. 2011; Weyers 2012; Wisco and Sober 2012).

In this review article, a critical view on the current debate on non-invasive, serologic protein markers and some selected non-protein markers in melanoma will be offered and novel innovative approaches currently being explored will be discussed.

Serological protein biomarkers in melanoma: a current status

Biomarkers can be divided into different categories. Most tumor markers show higher expression in tumor cells than in normal tissue and, therefore, are used as diagnostic markers. Furthermore, some biomarkers may serve as prognostic or predictive markers because of their increased expression in advanced disease or as indicators of treatment response and/or of recurrence during follow-up (Brochez and Naeyaert 2000; Vereecken et al. 2012). Moreover, stem cell-like markers are of potential use for identification of cancer progenitor/stem cell subpopulations that exhibit specifically critical properties like high tumorigenicity, metastatic potency, and treatment resistance (Ma et al. 2010).

The ideal biomarker should be a molecule detectable and/or quantifiable in the blood or other body fluid compartments, which are accessible minimal invasively. This biomarker should allow for the diagnosis of a growing tumor in a patient or for prediction of the likely response of a patient to a certain treatment. Thereby, the biomarker must exhibit sufficient sensitivity and specificity to minimize false negative as well as false positive results (Vereecken et al. 2012). At this moment no ideal biomarker exists in the melanoma field. Histological characteristics of the primary melanoma, e.g., tumor thickness (Breslow index), mitotic rate, and ulceration are important prognostic factors (Balch et al. 2009). However, these characteristics can only be determined after localization and biopsy or surgical resection of the tumor. The aim of the investigations in the field of biomarkers is to evaluate markers for the early detection of a growing tumor, detection of metastases, and therapy monitoring through non-invasive methods even earlier or better, respectively, than by applying computed tomography (CT), positron emission tomography (PET), single photon emission computed tomography (SPECT) and other imaging modalities (Bronstein et al. 2012; McArthur et al. 2012; Patel and Finger 2012; Ulrich et al. 2011; Vermeeren et al. 2011).

Although screening for primary tumors and metastases through tracer technologies like ^{18}F-fluorodeoxyglucose uptake reveals a sensitive and specific tool for staging of melanoma patients, especially, in those cases where no biomarker is detectable, some false negative results of small tumors and metastases reduce the clinical accuracy (Mirk et al. 2011).

As potential non-invasive biomarkers, particularly, in cutaneous melanoma various proteins and other molecules are under investigation, which comprise enzymes, soluble proteins or antigens, and S100 proteins, and, on the other hand, melanin-related metabolites and circulating cell-free nucleic acids (Fig. 1). These potential biomarkers are discussed concerning their prognostic and predictive value in melanoma diagnosis, staging, and treatment monitoring. The results of different key publications which reported a correlation between any biomarker level and melanoma

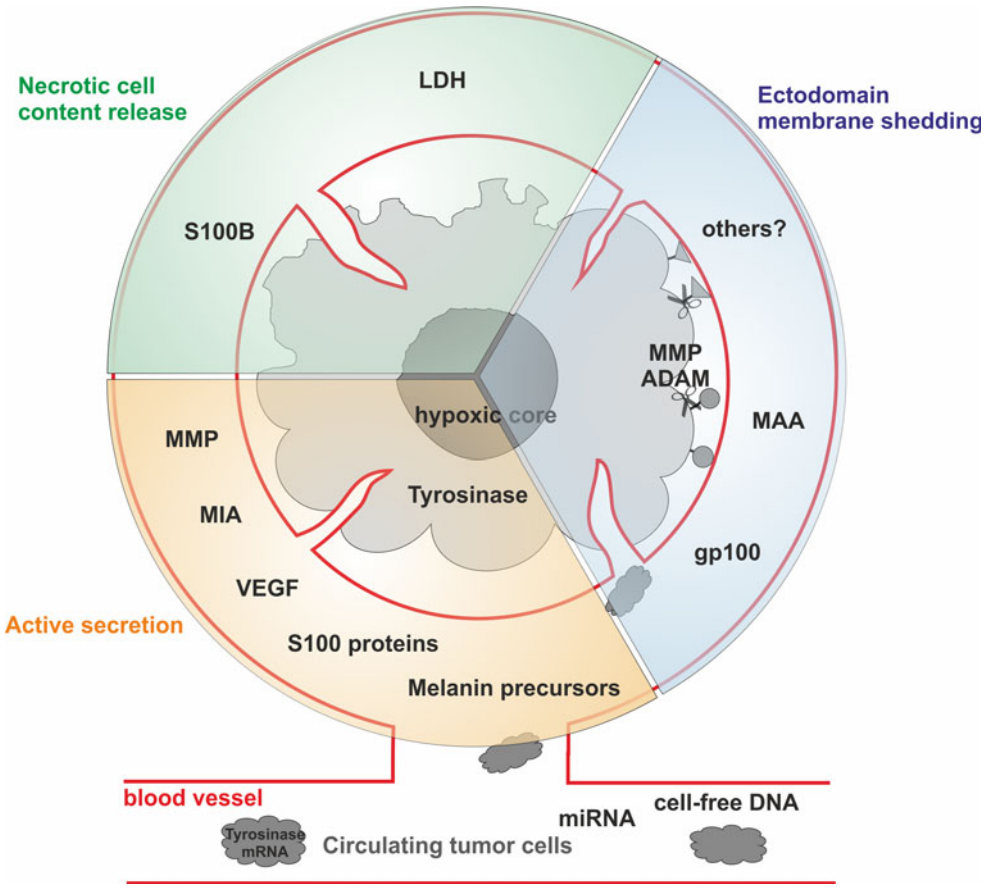

Fig. 1 Origin of protein and non-protein biomarkers in melanoma. The scheme illustrates major mechanisms of formation and release into the blood stream of proteins and non-protein molecules with predictive, diagnostic, and prognostic potential in cutaneous malignant melanoma

staging, tumor progression, or survival are summarized in Table 1.

Enzymes

Lactate dehydrogenase

Lactate dehydrogenase (LDH, EC 1.1.1.27) is an enzyme encoded by two genes, the LDH-A (the M subunit-muscle type) and LDH-B (the H subunit-heart type). They form two polypeptide chains which in turn build up five different isoenzymes (LDH1-5) (Perrotta et al. 2010). As LDH is involved in the energy production of cells, its overexpression is associated with anaerobic metabolism. Because of frequently occurring hypoxic areas in tumors, it is not possible to generate ATP through oxidative phosphorylation of glucose. The alternative pathway of converting pyruvate to lactate under conditions of oxygen deficiency is catalyzed by LDH (Palmer et al. 2011). LDH-A (also known as LDH-5) was shown to be upregulated in hypoxic environments (Perrotta et al. 2010). Until now, serum LDH is the strongest prognostic biomarker in melanoma being used in clinical routine that increases as a function of tumor burden in various tumor entities including malignant melanoma (Solassol et al. 2011). In the new American Joint Committee on Cancer (AJCC) staging system, LDH is the only serum biomarker that was accepted as a prognostic parameter for melanoma classifying those patients with elevated serum levels in stage IV M1C (Kluger et al. 2011; Vereecken et al. 2012). Serum values of LDH correlate with prognosis, in which changes in concentration are associated with progression or regression of metastatic diseases (Egberts et al. 2010; Deichmann et al. 1999). Egberts et al. (2012) found a significant correlation between tumor stage and serum values of LDH; increasing levels accompany with tumor stage. Furthermore, elevated levels are an adverse prognostic parameter in advanced stages. In the study from Balch and others they showed that 1- or 2-year overall survival rates for stage IV patients with normal LDH values are 65 and 40 %, respectively, compared with 32 and 18 %, respectively, when serum LDH was elevated at the time of staging (Balch et al. 2009). Despite all these promising results, there are also some limitations in measuring LDH as biomarker for melanoma. 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,

Table 1 Summary of biomarker analyses in melanoma patients

Biomarker	Patient cohort/samples	Correlation with	Methodology	Reference[a]
Enzymes				
LDH	50 patients stages I/II and 61 patients stage IV before and after treatment	Tumor stage, prognosis	Photometric assay	Egberts et al. (2012)
	30,946 patients stages I–III and 7,972 patients stage IV	Survival rate	Meta-analysis[b]	Balch et al. (2009)
Tyrosinase	200 patients stage IV	Poor prognosis	Nested RT-PCR	Quaglino et al. (2007)
	85 patients stage IV	Survival rate	RT-PCR	Schmidt et al. (2005)
	114 patients stages I–IV and 20 healthy controls	Survival rate	RT-PCR	Visus et al. (2007)
	201 patients stages I–IV and 40 healthy controls	Overall survival	RT-PCR	Samija et al. (2010)
COX-2	64 human melanocytic skin tumors (17 nevi, 36 primary cutaneous melanomas and 11 lymph node metastases)	Tumor progression	IHC	Kuzbicki et al. (2006)
	101 primary malignant melanomas and 28 metastases	Breslow index	IHC	Becker et al. (2009)
MMP-1, MMP-3	70 melanoma metastases	Disease-free survival	IHC	Nikkola et al. (2002)
MMP-9	71 patients stage IV and 8 healthy controls	Poor prognosis	ELISA	Nikkola et al. (2005)
MMP-2	482 melanoma (330 primary and 152 metastatic) tumor biopsies and 149 nevi biopsies (49 normal and 100 dysplastic nevi)	Tumor progression	TMA, IHC	Rotte et al. (2012)
Secreted proteins/antigens				
VEGF	125 patients stages I–IV and 30 healthy controls	Tumor stage, survival	ELISA	Ugurel et al. (2001)
	155 patients stages I–IV	Tumor progression	RT-PCR	Osella-Abate et al. (2002)
	324 patients stages I–IV	Tumor stage	ELISA	Pelletier et al. (2005)
VEGF-C, VEGFR-3	75 patients stage IV and 30 healthy controls	Tumor burden	ELISA	Mouawad et al. (2005)
Osteopontin	345 patients stages I–IV	Breslow index, survival	IHC	Rangel et al. (2008)
	34 vertical growth phase melanoma	Poor prognosis	TMA, IHC	Alonso et al. (2007)
Gal-3	21 cases of melanoma and 20 benign pigmented nevi	Poor prognosis	IHC	Abdou et al. (2010)
	104 melanoma samples (71 superficial spreading and 33 nodular melanomas)	Tumor progression	IHC	Buljan et al. (2011)
	53 cases of benign nevi, 31 cases of dysplastic nevi, 59 in situ melanomas, 314 cases of primary melanoma and 69 metastatic melanomas	Tumor progression	IHC	Brown et al. (2012)
	83 patients stages III–IV	Poor prognosis	ELISA	Vereecken et al. (2009)
YKL-40	110 patients stage IV and 245 healthy controls	Tumor progression	ELISA	Schmidt et al. (2006a)
	234 patients with stages I–II	Poor prognosis	ELISA	Schmidt et al. (2006b)
	50 patients stages I/II and 61 patients stage IV before and after treatment	Tumor stage	ELISA	Egberts et al. (2012)
MIA	110 patients with advanced melanoma stages IIIB/C-IV, 66 disease-free patients, and 65 healthy controls	Survival	ELISA	Diaz-Lagares et al. (2011)
	125 patients stages II–IV	Poor prognosis	ELISA	Essler et al. (2011)
CRP	30 patients stage IV	Survival	IP	Tartour et al. (1994)
	216 patients stages I-IV	Tumor progression	IP	Deichmann et al. (2004)

Table 1 continued

Biomarker	Patient cohort/samples	Correlation with	Methodology	Reference[a]
sICAM, sVCAM	50 patients with advanced melanoma	Survival	ELISA	Vuoristo et al. (2001)
CEACAM	100 primary melanomas, 11 distant metastases, and 6 sentinel lymph node metastases	Tumor progression	IHC	Thies et al. (2002)
	49 patients stages III–IV	Tumor stage, overall survival	ELISA	Sivan et al. (2012)
CYT-MAA	117 patients stages II–IV	Tumor progression	ELISA	Reynolds et al. (2006)
MAGE	65 patients stages IIB–III	Tumor progression	RT-PCR	Arenberger et al. (2008)
MART-1	94 patients stages I–IV	Tumor stage	RT-PCR	Koyanagi et al. (2005)
TA90	70 patients stage IV;	Survival	ELISA	(Chung et al. 2002;
	166 patients stages I–III			Kelley et al. 2001)
	75 patients stage III	Recurrence	ELISA	Faries et al. (2007)
S100 proteins				
S100B	50 patients stages I/II and 61 patients stage IV before and after treatment	Tumor stage	ELISA	Egberts et al. (2012)
	56 patients stages I–IV	Survival	LIA	Kruijff et al. (2009)
	211 patients stages II–III	Survival	LIA	Bouwhuis et al. (2011)
	97 patients stages II–III; 670 patients stages II–III	Recurrence	LIA	Domingo-Domenech et al. (2007);
				Tarhini et al. (2009)
S100A2	45 melanoma metastases and 20 benign nevi	Tumor progression (negative correlation)	Northern Blot	Maelandsmo et al. (1997)
S100A4	99 superficial spreading and 60 nodular primary melanomas	Tumor progression	IHC	Andersen et al. (2004)
S100A6	45 melanoma metastases and 20 benign nevi	Survival	Northern Blot	Maelandsmo et al. (1997)
Metabolites of the melanin synthesis pathway				
5-SCD	478 patients stages I–IV	Poor prognosis	HPLC	Banfalvi et al. (2003)
	11 patients stage IV	Response to treatment	HPLC	Wimmer et al. (1997)
L-Dopa/L-tyrosine	90 patients stages I–IV	Tumor burden	HPLC	Letellier et al. (1999)
	170 patients stages I–IV	Tumor progression	HPLC	Garnier et al. (2007)
6H5MI2C	47 patients stages I–IV and 14 healthy controls	Breslow index	HPLC	Hara et al. (1994)
Nucleic acids				
miRNA-221	94 patients stages I–IV and 20 healthy controls	Breslow index	RT-PCR	Kanemaru et al. (2011)
miRNA-29c	149 patients stages I–IV	Overall survival	RT-PCR	Nguyen et al. (2011)

ELISA enzyme-linked immunosorbent assay, *LIA* luminescence immunoassay, *RT-PCR* reverse transcription polymerase chain reaction, *HPLC* high performance liquid chromatography, *IHC* immunohistochemistry, *IP* immunoprecipitation, *TMA* tissue microarray

[a] Table shows key publication which demonstrated a correlation between the appropriate biomarker and tumor disease. Controversial data are discussed in the text

[b] Based on AJCC melanoma staging database

muscle diseases, and other infectious diseases with high amounts of necrotic cells (Vereecken et al. 2012). Moreover, LDH is non-specific for melanoma and elevated levels are also found in many other benign and malignant diseases. In early stages, LDH is less sensitive in comparison with other biomarkers (Hofmann et al. 2011) and also the specificity for LDH as a predictor of metastatic relapse is low (Kluger et al. 2011). Although LDH was presented as the most predictive independent factor in many studies, there also are some controversial results. Hamberg et al. (2003) only found elevated LDH values in 38 % of stage IV patients and Hauschild et al. (1999) even failed to demonstrate the independent prognostic value of LDH. In the study from Egberts et al. (2012), LDH was not useful to differentiate between healthy controls and melanoma patients in early stages of the disease and they further

could not demonstrate LDH as a significant independent prognostic parameter with respect to progression-free and overall survival. Nevertheless, LDH is still used in clinical routine because of its cost-effectiveness and easy measurement. In contrast to other markers, there are standardized analyzing protocols available for detecting LDH which make it possible to generate comparable results (Diaz-Lagares et al. 2011).

Tyrosinase

Tyrosinase (EC 1.14.18.1) is constitutively expressed in melanocytes and melanoma cells and is involved in the biosynthesis of melanin catalyzing the oxidation of tyrosine to dopa and of dopa to dopaquinone. The detection of tyrosinase mRNA in peripheral blood is an indicator for the presence of circulating melanoma cells and increased probability of the occurrence of metastases. Of importance, although the serological analyte is a nucleic acid isolated from circulating cells tyrosinase is considered as a protein biomarker in melanoma. Advanced metastatic melanoma patients expectedly show a substantial tyrosinase mRNA level in their peripheral blood (Quaglino et al. 2007). Due to the fact that tyrosinase mRNA is detected through nested RT-PCR the analytical sensitivity is very high. It is possible to detect 1 melanoma cell among 10^6 of normal blood cells (Visus et al. 2007). 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 (Quaglino et al. 2007). The analyzing method of PCR exhibits another discrepancy between different studies. The major technical differences among the published studies are the sample processing, RNA extraction, or PCR amplification, resulting in lower sensitivity and different thresholds for melanoma cell detection (Visus et al. 2007). Some groups reported tyrosinase mRNA to be an independent prognostic parameter for tumor progression (Quaglino et al. 2007; Schmidt et al. 2005; Visus et al. 2007). They demonstrated that tyrosinase is a reliable factor associated with response to treatment, development of metastases, progression, and overall survival. Samija et al. (2010) showed that positive values for tyrosinase were associated with shorter overall survival, but were not significantly correlated with progression-free survival. However, Garbe et al. (2003) reported that detection of circulating tumor cells through RT-PCR for tyrosinase has no significant influence on the prognosis at all and Tsukamoto et al. (2000) could even not detect tyrosinase mRNA in peripheral blood of Japanese melanoma patients. More recently, in a study with 67 melanoma patients (stages II–IV) the tyrosinase maintained normal levels compared to healthy controls (Zhang et al. 2011). Of importance, the, in part, controversial results strongly lead to the necessity of evaluation of standardized protocols for PCR-based techniques, comparable to those available for standardized measurement of enzyme activities or (specific) protein concentration (Santonocito et al. 2005; Vendittelli et al. 2009). Furthermore, independent studies with blood samples of melanoma patients at different stages are required.

Cyclooxygenase-2

The cyclooxygenase (COX, EC 1.14.99.1) catalyzes the first step in the conversion of arachidonic acid to prostaglandins. It exists in two isoforms—the constitutively expressed COX-1 and the inducible COX-2. COX-1 is expressed in many tissues and mediates the synthesis of prostaglandins under normal physiological conditions, whereas COX-2 is also expressed in tumors and is induced by different stimuli like inflammatory reactions (Meyer et al. 2009; Bosserhoff 2006). COX-2 expression was reported in different melanoma cell lines (Denkert et al. 2001). In contrast, Goulet et al. (2003) did not detect COX-2 in any primary melanoma cell. In a study of Becker et al. (2009), COX-2 was found in 95 % of melanoma revealing a significant correlation between immunohistochemical staining intensity and Breslow index. Kuzbicki et al. (2006) reported a coherency between COX-2 expression and development as well as progression of human melanoma. Thus, COX-2 was suggested to be a potential prognostic and predictive marker. Very recently, the same group showed that COX-2 expression was significantly higher in melanomata compared to nevi. Their test allowed for differentiation of early skin melanomata and nevi with high sensitivity and specificity (Kuzbicki et al. 2012). However, direct COX-2 determination is only possible in tissue biopsies through immunohistochemical staining. Thus, COX-2 does not represent a non-invasive serologic biomarker. On the other hand, the COX-2 reactions and consecutive enzyme reactions result in the formation of certain lipid mediators comprising various eicosanoids/prostanoids, which can be detected as a measure of cyclooxygenase activity non-invasively in blood serum (Müller-Decker and Fürstenberger 2007). In this regard, there is experimental evidence on overexpression of COX-2 in human malignant melanoma cell lines detected by specific and sensitive analysis of secreted lipid mediators, e.g., prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Kniess et al. 2012; Nicolaou et al. 2004). This might be indicative for the potential use of increased levels of prostanoids for detection of melanoma progression. However, COX-2 overexpression, and, accordingly, high eicosanoid/prostanoid levels are present in various metabolic and inflammatory processes presumably resulting in false-positive results.

Matrix metalloproteinases

The matrix metalloproteinases (MMPs, EC 3.4.24.X) are a family of 24 structurally related zinc-dependent endopeptidases that degrade the extracellular matrix during neoplastic growth and metastasis through lysis of its compounds (Vereecken et al. 2012). Some of them are actively secreted by cells while others are membrane-associated enzymes. There are some studies reporting an association between MMP expression and/or secretion and both tumor progression and angiogenesis. Vihinen and Kähäri (2002) demonstrated that MMP-1 (interstitial collagenase) correlated with favorable treatment response in human melanoma. Furthermore, high expression of MMP-1 and MMP-3 (stromelysin 1) was associated with shorter disease-free survival (Nikkola et al. 2002). Although MMP-1 was lower in melanoma patients, it did not correlate with overall survival (Nikkola et al. 2005). Higher plasma values of MMP-9 (gelatinase-B, 92 kDa gelatinase) correlated with many tumor sites and were reported as an independent prognostic factor for overall survival (Nikkola et al. 2005). In the same study, MMP-13 (collagenase 3) was detected in melanoma patients but also in healthy controls being not suitable as a biomarker for melanoma. However, Vuoristo et al. (2000) found no association between serum MMP-2 (gelatinase-A, 72 kDa gelatinase) and the presences of metastases or survival in advanced melanoma just as Wollina et al. (2001), who showed no difference between MMP-2 level and tumor stage. Although the activity of MMP-2 is low in normal tissue and elevated in different malignancies, it is not tumor specific and not a reliable marker. In the study of Redondo et al. (2005) neither MMP-2 nor MMP-9 plasma levels showed a statistical significant change between different melanoma groups with disease progression or good clinical treatment response. Moreover, MMP-3 is not associated with any typical prognostic factor like histology, localization or Breslow index. Therefore, it is not an indicator for invasion and metastasis (Tas et al. 2005). More recently, Rotte and coworkers demonstrated a significantly increased expression of MMP-2 in primary (25 %) and metastatic melanoma (43 %) in comparison with normal (5 %) and dysplastic nevi (10 %). Furthermore, MMP-2 expression correlated with tumor progression and might predict patient survival independent of thickness and ulceration. However, these investigations were performed with tissue samples from 482 melanoma tumor biopsies and 149 nevi biopsies (Rotte et al. 2012).

Soluble proteins/antigens

Soluble proteins, detectable in the serum, can be actively secreted from melanoma cells. On the other hand, membrane proteins might be released from the cells by ectodomain membrane shedding through degrading enzymes like MMPs or ADAMs (A Disintegrin And Metalloproteinases) (Anderegg et al. 2009; Ii et al. 2006). Moreover, circulating melanoma cells, as already discussed above, are a potential target for protein biomarker determination. In the following the use of, particularly, secreted proteins and antigens as serological melanoma biomarkers is discussed.

Vascular endothelial growth factor

Angiogenesis represents a parameter of potential prognostic value in solid tumors because of its contribution to tumor growth and metastasis. The vascular endothelial growth factor (VEGF) is a central figure in the regulation of proliferation, differentiation, and survival of the microvascular endothelium (Pelletier et al. 2005; Mouawad et al.2010). This glycoprotein exists in seven isoforms in order to the different splice variants. Different investigations showed elevated VEGF serum levels in melanoma patients compared to healthy controls as well as an association between increased VEGF levels and tumor stage and/or prognosis in melanoma patients (Mouawad et al. 2005; Osella-Abate et al. 2002; Palmer et al. 2011; Tas et al. 2008; Ugurel et al. 2001). Ugurel et al. (2001) demonstrated that serum levels of VEGF, βFGF, and IL-8 correlated with tumor stage and tumor burden. Increased values were strongly correlated with poor survival and higher probability of progression. Furthermore, Mouawad et al. (2005) reported that the soluble forms of VEGF-A and VEFG-C and their receptor, VEGFR-3, were elevated in sera of melanoma patients. A significant increase of serum levels and a correlation of high VEGF-C and VEGFR-3 levels with high tumor burden led to the supposition that VEGF-A and VEGFR-3 pretreatment levels may identify high-risk melanoma patients with a worse prognosis. Likewise, VEGFR-1 level may be a predictive factor of time to progression and overall survival. However, there are also some studies revealing controversial results. Osella-Abate et al. (2002) found a significant elevation of VEGF in melanoma patients, especially in those with metastases but VEGF did not correlate independently with overall survival and time to progression. In a further study, VEGF-C levels were higher in patients with distant metastases than in those with subcutaneous metastases. Although VEGF levels were significantly related to deep lymph node involvement, they found no association between tumor burden and survival (Vihinen et al. 2007). Pelletier et al. (2005) found elevated VEGF levels in sera of patients in every stage compared to healthy controls. Furthermore, VEGF levels in stages I, II and III were significantly different from those in stage IV. However,

there was no association between baseline VEGF and disease progression. Moreover, the sensitivity (57 %) and specificity (78 %) were low compared to other markers. Activation of angiogenesis seems to be dependent on the interaction between VEGF and other fibroblast growth factors (FGFs) (Presta et al. 2005). Until now, this mechanism is only poorly understood. Furthermore, VEGF is non-specific for melanoma because it is also secreted by other cancer cells. However, several studies, as discussed above, demonstrated the prognostic potential of VEGF especially in advanced melanoma. Indeed, more studies will be necessary for further evaluation of VEGF as prognostic marker.

Osteopontin

Osteopontin (OPN) is a secreted, integrin-binding glycophosphoprotein which initiates different signal transduction pathways through activation of kinases and transcription factors. OPN is involved in many cellular functions like adhesion, migration, immune and inflammatory responses because of its inhibition of apoptosis and activation of MMP-2 and MMP-9 (Perrotta et al. 2010). OPN is strongly expressed and upregulated during progression in different tumor entities, including melanoma (Zhou et al. 2005). Zhou and coworkers could detect higher OPN expression via immunostaining in melanoma cells compared with benign nevi. However, there was no correlation to other melanoma markers (Zhou et al. 2005). In contrast to this, Rangel and colleagues showed an association between OPN and an increased Breslow index, Clark level of invasion, and mitotic index. Furthermore, they found a correlation between OPN expression and relapse-free survival and disease-specific survival. Multivariate analysis revealed that OPN expression level is an independent predictor for disease-specific survival (Rangel et al. 2008). Alonso and coworkers demonstrated a significant association of OPN expression in primary tumors with increased incidence of metastases during disease progression. Thus, they confirmed its potential prognostic value (Alonso et al. 2007). A more recent study revealed elevated plasma levels in melanoma patients with metastatic disease with a specificity of 97.2 %. However, the sensitivity was too low for clinical application (68.2 %), which is more important for screening tests due to the risk of metastasis. Therefore, Maier et al. (2011) supposed that OPN might represent an additional biomarker in a multiple screening test. Because of the diverse functions of OPN, it is not a specific tumor marker being also elevated in other medical conditions, such as autoimmune diseases or infections (Maier et al. 2011). In addition to the inconsistent results of the studies, retrospective studies with higher numbers of patients are needed to further validate the usefulness of OPN as a biomarker for melanoma.

Galectin-3

Already in 2006, Vereecken and colleagues demonstrated a high expression of Gal-3 in primary melanoma lesions. Therefore, they supposed that Gal-3 might play a possible role as a soluble marker for the metastatic process (Vereecken and Heenen 2006). Gal-3 belongs to the galectin gene family of carbohydrate-binding proteins. The 31-kDa multifunctional protein is secreted by melanoma and inflammatory cells being associated with protumorigenic and prometastatic activity (Abdou et al. 2010; Buljan et al. 2011). It interacts with several serum proteins, surrounding cells, and extracellular matrix resulting in an influence on cell growth, adhesion, proliferation, transformation, and metastasis (Forgber et al. 2009). Further immunohistochemical studies confirmed a higher expression of Gal-3 in melanoma cases compared to nevi. Abdou et al. (2010) observed that nucleocytoplastic pattern of Gal-3 expression revealed a higher probability of a malignant phenotype and poor prognostic impact on patients' outcome. Furthermore, an increased expression was correlated with tumor characteristics associated with a more aggressive phenotype (Buljan et al. 2011). Brown and others published a study with 481 patients with cutaneous melanoma, also showing a higher Gal-3 expression in primary melanomas compared to nevi and a strong decrease in expression between thin melanomas, thicker melanomas and metastases. A multifactorial Cox regression analysis revealed an association of Gal-3 expression with an improved overall and melanoma-specific survival (Brown et al. 2012). In a recent study, Kaplan–Meier analysis revealed a worse prognosis for patients with elevated Gal-3 serum levels. Moreover, a multivariate analysis with higher cutoff-values clarified a strong independent prognostic value for Gal-3 in advanced melanoma patients (Vereecken et al. 2009). The correlation with other established biomarkers confirmed the promising prognostic significance of Gal-3 being determined by further experiments.

YKL-40

The phylogenetically highly conserved heparin- and chitin-binding lectin YKL-40, also called human cartilage glycoprotein-39, is a member of the mammalian chitinase-like proteins and is expressed as well as secreted by many cell types, including cancer cells, macrophages, and activated neutrophils (reviewed in Johansen et al. 2006). Johansen et al. (1992) identified the YKL-40 being secreted by the human osteosarcoma cell line MG63. They named this protein YKL-40 because of its amino acids tyrosine (Y),

lysine (K), and leucine (L) at the N-terminus and its molecular weight 40 kDa. The cellular function of YKL-40 still remains unknown. However, it may have a role in cancer cell proliferation, survival, protection from apoptosis, angiogenesis, stimulation of fibroblasts around the tumor, and extracellular tissue remodeling (Johansen et al. 2006). Schmidt and colleagues demonstrated that patients with metastatic melanoma had significant higher YKL-40 serum levels compared to healthy controls. During follow-up in 9 of 11 patients a significant increase in serum YKL-40 was observed together with disease progression. Furthermore, in another study of the same group they confirmed these results with 234 patients (stages I and II), whereby YKL-40 was shown as an independent prognostic factor of relapse-free survival and overall survival (Schmidt et al. 2006a, b). More recently, Egberts and others demonstrated a significant correlation between tumor stage and YKL-40 serum levels. However, there was no statistically significant difference between stage I and stage II melanoma patients and, in contrast to the study of Schmidt and colleagues, YKL-40 was not an independent prognostic factor (Egberts et al. 2012). In contrast to this, Diaz-Lagares et al. (2011) did not demonstrate a difference between YKL-40 serum levels neither in different stages nor between patients and healthy controls. Despite some promising studies on the prognostic potential of YKL-40, it has to be mentioned that YKL-40 is neither organ- nor tumor-specific and has a limited sensitivity. Not all tumors secrete YKL-40 or only at a low level because of the different phenotype of cancer cells. Furthermore, YKL-40 values were influenced after treatment with immunotherapy such as interleukin-2 and interferon α (Krogh et al. 2010; Schmidt et al. 2006a). Therefore, false-negative results due to a variety of other cellular molecules limit the use of YKL-40 as a routine marker.

Melanoma inhibitory activity

Melanoma inhibitory activity (MIA) is a soluble, 11 kDa protein being strongly expressed and secreted from melanoma cells and acting as an autocrine growth factor. From the present point of view, the name is a misnomer because high levels are associated with an increased invasion, extravasation, progression, and metastasis (Palmer et al. 2011). MIA influences cell–cell contacts between melanoma cells and extracellular matrix leading to a decreased adhesion combined with increased migration and metastatic potential. Studies revealed that clinically elevated values correlated with a more advanced stage and a poorer prognosis (Perrotta et al. 2010). In stage IV patients MIA serum levels correlated with response to chemotherapy and relapse during follow-up period (Juergensen et al. 2001). Diaz-Lagares and coworkers published a study with 110 patients with advanced melanoma showing elevated MIA levels in stage IIIc and IV compared to disease-free patients and healthy controls. The sensitivity of MIA (51.8 %) was even higher than the one of LDH (38.2 %) (Diaz-Lagares et al. 2011). Further studies demonstrated higher sensitivities of about 67.6 % in stage I patients and 65.6 % in stage II patients with high specificities of 76.9 and 66.7 %, respectively (Hofmann et al. 2009). In addition, Garbe et al. (2003) also reported a higher sensitivity, specificity and diagnostic accuracy for MIA than for LDH. In a more recent study of Essler and coworkers, metastases were detected by measuring MIA levels with clinical valuable sensitivity and specificity. Subgroups of metastasis could be established with poorer prognosis (Essler et al. 2011). However, a multivariate analysis revealed significantly more frequent false-positive values in elderly women (Hofmann et al. 2009). Bosserhoff et al. (2004) recognized elevated MIA serum levels also in children and pregnant women. Thus, further investigation should be performed to reveal other tumor-unspecific elevations of MIA for a reliable evaluation of MIA as a predictive or prognostic biomarker.

C-reactive protein

In contrast to these secreted proteins some other soluble proteins might also be useful for melanoma detection, e.g., the acute phase C-reactive protein (CRP) which is produced by hepatocytes and stimulated by inflammatory cytokines such as interleukin-6 (IL-6). CRP was first discovered in 1930 during a study with patients with *Streptococcus pneumoniae* infection (Tillett and Francis 1930) and consists of five identical, non-associated 23 kDa protomers (Black et al. 2004). In a study with 30 melanoma patients stage IV elevated IL-6 levels were detected and were associated with a shorter survival (Tartour et al. 1994). Therefore, CRP serum levels might also have a prognostic potential. In the same study, patients, treated with IL-2, having elevated IL-6 and/or CRP serum levels, showed a poor clinical response to therapy. In a further study, CRP was measured in the serum of 91 consecutive melanoma patients of stage IV in comparison with 125 melanoma patients stages I, II or III (Deichmann et al. 2004). High levels of CRP were associated with tumor progression and presence of distant metastases. CRP yielded a sensitivity of 77 % and a specificity of 90 % and was even superior to LDH in discriminating patients of different stages. Deichmann et al. (2004) recommended the routine measurement of CRP during follow-up period for earlier detection of distant metastases. Because of its stimulation through different interleukins, CRP is a non-specific marker for inflammation and, therefore, elevated levels are found in many other inflammatory and infectious

diseases. Nevertheless, ongoing studies will resolve whether CRP may have a significant role in staging and follow-up of patients with melanoma.

Cell adhesion molecules

Cell adhesion molecules (CAMs) are integral membrane proteins that mediate cell–cell or cell–matrix contacts through interaction with other cell surface molecules on adjacent cells. The cell–cell adhesion molecule carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) is expressed in many different cell types and acts on the one hand as a tumor suppressor in colorectal, liver, and breast cancer, and on the other hand it stimulates angiogenesis and adhesion and protects tumor cells from immune system-mediated attack, for instance in malignant melanoma (reviewed in Sapoznik et al. 2012). The intercellular adhesion molecule 1 (ICAM-1) is also expressed on the surface of melanoma cells and interacts with leukocytes stimulating the extravasation of melanoma cells. The vascular cell adhesion molecule 1 (VCAM-1) is mainly expressed on lymphocytes and monocytes. However, melanoma cells also express VCAM to adhere to the endothelium. Many cell adhesion molecules are secreted into cell culture medium (sCEACAM-1, sICAM-1 or sVCAM-1), and therefore might exhibit a prognostic potential. Former studies already revealed that expression and serum levels of cell–cell and cell–matrix adhesion molecules are significantly associated with the development of metastases in malignant melanoma (Ebrahimnejad et al. 2004; Franzke et al. 1998; Hirai et al. 1997). Patients with liver and/or bone metastases had significantly higher levels of sICAM-1 compared to those with soft tissue or lung involvement. Univariate analysis levels of sICAM-1 and sVCAM-1 were found to be statistically significant prognostic factors for survival (Vuoristo et al. 2001). Furthermore, Thies et al. (2002) demonstrated that CEACAM1 was an independent factor for the risk of metastasis with a predictive value even superior to Breslow index. In more recent studies, Kluger and coworkers analyzed levels of seven markers, including CEACAM, ICAM-1, OPN, and MIA. This combination of markers was elevated in unresected stage IV melanoma compared to resected stages I and II disease. No marker elevation was found in 81 % of the stages I and II patients and 69 % of stage IV patients had elevation in at least one marker (Kluger et al. 2011). Sivan and colleagues confirmed in their study that sCEACAM-1 correlated with disease state and overall survival. According to this, decreased sCEACAM-1 levels after treatment were a dominant predictor for an increased survival (Sivan et al. 2012). These promising results imply further evidences for using especially sCEACAM-1 in monitoring tumor progression in advanced melanoma patients.

Melanoma-associated antigens

Melanoma-associated antigens (MAAs) are not considered to be classical serological biomarkers. These antigens can be located in the cytoplasm and on the cell membrane, respectively. They are neither secreted nor soluble proteins but might be released from cells by ectodomain membrane shedding through MMPs. MAAs are produced according to genomic mutations or changed gene expression profiles during malignant transformation of melanocytes and are able to excite immune response. Melanoma-specific antigens are only expressed in tumor tissue, whereas MAAs are synthesized in average levels by normal melanocytes and can be overexpressed in melanoma cells (Vereecken et al. 2012). Those antigens were recognized by specific cytotoxic T cells and might also trigger immune response. Of note, tyrosinase and MIA are also considered as MAAs, but have been discussed above in detail. The high-molecular weight MAA (HMW-MAA), a membrane proteoglycan, is highly expressed in melanomas and the cytoplasmic MAA (CYT-MAA), consisting of four polypeptides, is expressed in normal cells and elevated in melanoma cells and other malignant cell types. Vergilis et al. (2005) showed that both MAAs are significantly increased in sera of melanoma patients compared to healthy controls with equally high levels in all stages. CYT-MAA was significantly correlated with the recurrence or progression of melanoma, being an independent predictor. Elevated HMW-MAA levels were not associated with progression. Reynolds et al. (2006) reported that CYT-MAA serum levels were elevated in patient stages IIb–IV. Furthermore, during immunotherapy the level decreased by at least 1 U in 90 % of the patients. Thus, they supposed that CYT-MAA might serve as an early marker of prognosis in patients with stages IIb–IV melanoma and might reveal an intermediate marker of response to therapy. MAGE, GAGE, LAGE, and BAGE family genes are expressed in different tumor entities, including melanoma. Their expression is associated with the occurrence, development, and prognosis of cancer (Castelli et al. 2000; Sang et al. 2011). For example, in peripheral blood MAGE-A3 was correlated with early stage melanomas but not with advanced melanomas (Koyanagi et al. 2005). In the study of Arenberger et al. (2008), MAGE-A3 became the most sensitive progression marker. Furthermore, melanoma antigen recognized by T cells-1 (MART-1), also known as MelanA, has been shown to be associated with advanced stage melanoma and is commonly detected in the peripheral blood of patients with stages III and IV disease (Koyanagi et al. 2005). Schmidt and coworkers demonstrated that MART-1 had no

independent prognostic impact on relapse-free survival. Further studies with controversial results made MART-1 not a potential useful marker (Medic et al. 2007).

Other soluble proteins

Although apparently of minor importance, there are other soluble proteins that have been considered as potential markers in melanoma. Paired box 3 (PAX3), a transcription factor, is involved in proliferation, migration and differentiation of melanoblasts and regulates the microphthalmia-associated transcription factor (MITF) expression also stimulating melanoma proliferation (Medic et al. 2007). Matsuzaki et al. (2005) identified PAX3d as a melanoma-specific antigen in the peripheral blood. The premelanosomal protein gp100 (Pmel17, Me20, silver, melanocytes lineage-specific antigen gp100) is a transmembrane protein with amyloid characteristics and is suggested to be involved in the biosynthesis of melanin intermediates. Its release from melanoma cells also is supposed to be the result of ectodomain shedding (Hoashi et al. 2010). The gp100 is preferentially expressed in melanocytes/melanomata and is not found in normal tissues or in carcinomas. It is normally expressed at early stages and overexpressed by proliferation of melanoma cells and during tumor growth. However, it does not show significant correlations between immunohistochemical staining and tumor progression (de Vries et al. 2001). Furthermore, the group of Morton and colleagues investigated the prognostic potential of the tumor-associated antigen 90 (TA90). They demonstrated that TA90 could be detected in serum and urine of more than 70 % of melanoma patients. Patients with distant metastases had high levels of free antigen TA90 and low levels of circulating IgG-bound immune complex (TA90-IC). Furthermore, TA90-IC status in early postoperative periods was strongly correlated with survival. Five-year overall survival rates were 84 and 36 % for TA90-IC-negative and TA90-IC-positive patients, respectively (Chung et al. 2002; Kelley et al. 2001). They reasoned that TA90-IC level might differentiate patients, who need further adjuvant therapy even if their sentinel node specimens have no evidence of tumor. In stage-III melanoma patients TA90-IC was the first marker being elevated in 57 % recurrences (Faries et al. 2007). However, this study only investigated patients receiving vaccines. Thus, the prognostic potential of TA90-IC should be evaluated in further studies with different patient cohorts. Although MAA were discovered several years ago, their function still remains unclear. Thus, further investigations are needed to identify their functional role in pathogenic mechanisms, tumors, and immunotherapy (Sang et al. 2011). Problems in clinical use of MAA are mostly related to T-cell tolerance or other immunoregulatory mechanisms and should be considered in clinical trials (Castelli et al. 2000).

S100 proteins

In the last years, the family of S100 proteins was getting more and more into the focus of investigations in the field of cancer research. S100 proteins regulate protein phosphorylation, dynamics of cytoskeleton constituents, activate enzymes and transcription factors, influence cell growth and differentiation as well as inflammatory processes (Donato 2007; Pietzsch 2011). The term 'S100' is closely related to the specific characteristic of these proteins being soluble in 100 % saturated ammonium sulfate solution. S100 proteins, mostly acting as homo- or heterodimers, consist of two EF-hands which are able to bind calcium and other ions. Therefore, S100 proteins are also involved in the intracellular calcium homeostasis (Donato 2007). Some S100 proteins such as S100A2, S100A4, S100A6, S100A7, S100A9, S100A10, and S100A11, as well as S100B and S100P, are specifically up-regulated in aggressive, advanced, metastatic tumors relative to non-invasive, non-metastatic tumors (Arumugam and Logsdon 2011; Logsdon et al. 2007; Xie et al. 2009). Further studies also revealed that some S100 proteins are expressed and secreted in a cell- and tissue-specific manner (Davey et al. 2000; Hsieh et al. 2002; Petersson et al. 2009; Rammes et al. 1997). Therefore, S100 proteins, in particular, those that are actively secreted by tumor cells, are proposed to exhibit a promising concept for prognostic biomarkers in various malignancies. However, there are only a few studies on serum levels of S100 proteins in melanoma patients reporting an association of elevated S100 serum levels with advanced melanoma stage and poor prognosis (Beyeler et al. 2006; Jury et al. 2000; Oberholzer et al. 2008). In this regard, the by now best-studied S100 protein is S100B being already applied in clinical investigations of tumor tissue (Gaynor et al. 1981; Kruijff et al. 2012; Kruijff and Hoekstra 2012).

S100B

Transcription levels of S100B were significantly increased 100- to 200-fold in melanoma stages III and IV compared to normal tissue (Leclerc et al. 2009). Neuss et al. (2011) found a significant correlation between Breslow index and S100 in tumor stage III. Patients with deeper infiltrated tumors had higher levels of S100. Based on this positive results concerning intracellular S100, blood serum of tumor patients was investigated. For instance, higher levels of melanoma-positive lymph nodes were associated with elevated serum levels of S100. In the following, we will take a closer examination of some S100 proteins and their

potential as biomarkers for cutaneous melanoma. S100B was first investigated in brain tissue (Moore 1965) and is mainly secreted by astrocytes and adipocytes (Donato et al. 2009). Intracellular S100B influences many cellular processes like cell proliferation, survival, cell locomotion, protein phosphorylation and degradation, calcium homeostasis, and is involved in cytoskeleton interactions and enzyme activity regulation (Donato et al. 2009). S100B likely contributes to cancer progression by down-regulating the tumor suppressor p53 (Lin et al. 2010). The role of extracellular S100B is still poorly understood. Actively secreted S100B might serve as an autocrine or paracrine activator of cell surface receptors like the receptor for advanced glycation endproducts (RAGE), resulting in stimulation of pro-proliferative as well as pro-differentiative mechanisms (Donato et al. 2009; Leclerc 2011). Secretion of S100B via an endoplasmatic reticulum–Golgi independent secretion pathway has been reported for glioblastoma cells (Davey et al. 2001). On the other hand, S100B seems not to be considerably overexpressed in melanoma cells and also not to be substantially secreted by them. However, these findings by Ghanem et al. (2001) have been demonstrated only in one melanoma cell line. Increased S100B serum levels in melanoma patients are chiefly attributed to the loss of cell integrity and proteolytic degradation as a result of apoptosis and necrosis of tumor cells. Serum concentrations of S100B correlated with clinical melanoma stage, with low levels in stages I and II, elevated levels in stage III, and highest levels in stage IV (Egberts et al. 2012; Kruijff et al. 2009; Sedaghat and Notopoulos 2008). The study of Egberts and coworkers revealed that S100B significantly correlated with treatment outcome and was also significantly associated with progression-free and overall survival in stage IV melanoma patients. In a former study, S100B was even superior to LDH (Egberts et al. 2008). However, S100B serum levels are not suitable for early detection of melanoma because discrimination between healthy controls and melanoma patient stages I and II was not possible. Kruijff et al. (2009) investigated S100B serum levels in stage III melanoma patients and found a correlation between preoperative elevated serum levels and decreased disease-free survival. They remarked that S100B might be used to select patients for adjuvant therapy as well as to provide prognostic information for stage III patients. Another trial with 211 patients serial determinations of serum levels showed that S100B was a strong prognostic marker for disease-free survival and overall survival (Bouwhuis et al. 2011). A former study revealed that S100B serum levels correlated with unfavorable clinicopathologic prognostic factors and identified patients, showing high S100B levels, with a worse clinical outcome. S100B levels could serve as a strong predictor of disease relapse during therapy (Domingo-Domenech et al. 2007). Furthermore, measurement of S100B serum levels during follow-up of melanoma patients was a useful tool for discovering tumor progression in asymptomatic patients. A followed whole body PET-CT was even able to increase the value (Peric et al. 2011). In a former study of Tarhini et al. (2009), high S100B serum levels have been associated with higher risk of relapse during follow-up period and significantly correlated with reduced overall survival. In contrast to this, Egberts et al. (2010) demonstrated no correlation between preoperative S100B level and the histopathologic status of sentinel lymph node (SLN). Therefore, S100B might neither replace SLN dissection, nor provide prognostic information. They pointed out that the characterization of SLN remains an important staging criterion because there are no standardized methods for non-invasive investigations of serum levels available.

However, false positive results can be caused by unfortunately abnormal circulating levels in liver and renal injury, neuroinflammatory/neurodegenerative disorders, cardiovascular pathologies, as well as in inflammatory and infectious diseases (Harpio and Einarsson 2004; Molina et al. 2002; Tsoporis et al. 2011). Elevated S100B serum levels are also caused brain-related pathologies like perinatal brain distress, acute brain injury, brain tumors, psychiatric disorders, and in this regard, also melanoma brain metastases (Michetti et al. 2012). Of importance, S100B is not a melanoma-specific marker and might also be elevated in non-melanoma skin cancers, central nervous system tumors, and various gastrointestinal cancers (Vereecken et al. 2012). Furthermore, results of S100B measurement are especially controversial in early stages of melanoma. Essler et al. (2011) presumed that small tumors might be too small to produce enough tumor markers to significantly elevate the serum level. Therefore, S100B serum level was less specific and in their study PET/CT had a higher prognostic power in the assessment of tumor-related mortality. Furthermore, small sample sizes of some studies, heterogeneity of disease stages, and the lack of evidence that S100B might serve as an independent prognostic factor led to controversial reports in the past (Mocellin et al. 2008). Moreover, the missing standardized protocols for analysis of serum samples and S100B measurement impede comparison of results of different studies and the pooling of their analysis for a significant conclusion. Therefore, the application of S100B as a routine clinical serum marker of melanoma is still not established.

S100A2

S100A2, firstly isolated from bovine lung tissue, is discussed controversially. On the one hand, it acts as tumor suppressor in some tumor entities, and on the other hand it

promotes tumorigenesis (reviewed in Wolf et al. 2011). In contrast to other S100 proteins, S100A2 expression is down-regulated in malignant melanoma during tumor progression. Due to the fact, that S100A2 was not detected in metastases and was highly expressed in nevi, Maelandsmo et al. (1997) supposed loss of S100A2 gene expression to be an early event in melanoma development. In this regard, S100A2 expression seemed to be correlated with a poorer prognosis and shorter survival. Contrary to this, Andersen et al. (1996) reported that S100A2 expression is very low in normal melanocytes and malignant melanoma cells. However, there are no further studies on S100A2 and melanoma progression available giving any evidence for the prognostic value of S100A2.

S100A4

Another member of the S100 family, the metastasis-associated protein, S100A4 influences cell motility, angiogenesis, and apoptosis. The mechanism how S100A4 stimulates metastasis is still under investigation, however, extracellular S100A4 seems to be of major importance in this context and, therefore, possibly might serve as a blood marker. In this regard, S100A4 became a candidate as a suitable molecular biomarker for metastatic potential with high prognostic significance being already shown for breast, colorectal, gallbladder, pancreatic, and other cancers (reviewed in Helfman et al. 2005). Maelandsmo et al. (1997) examined 45 melanoma metastases and 20 benign nevi concerning their S100A4 content. Although S100A4 was found in 78 % of biopsies, there was no correlation with clinical parameters. In another study, S100A4 was analyzed by immunohistochemistry in 99 superficial spreading and 60 nodular primary melanomas (Andersen et al. 2004). Although S100A4 was expressed highest in the nodular, S100A4 had a more significant influence on patient outcome in early superficial spreading melanomas, in agreement with the tendency of decreased disease-free survival in patients expressing high levels of S100A4. Besides some promising results on the use of S100A4 serum levels as prognostic marker, the greatest problem might be the low protein concentration in the blood which impedes clinical relevance.

S100A6

Another S100 protein, S100A6 or calcyclin, also is found in cell culture media and physiological fluids (reviewed in Lesniak et al. 2009). Weterman et al. (1992) found expression of S100A6 in human melanoma cell lines to be well correlated with metastatic behavior in nude mice. Fullen et al. (2001) demonstrated S100A6 to be found in a variety of cutaneous and extracutaneous lesions including: melanocytic nevi, melanoma, some salivary gland and epithelial tumors, and malignant fibrous histiocytoma. Nonaka et al. (2008) reported S100A6 to be diffusely expressed in melanomata. On the other hand, Maelandsmo et al. (1997) reported that the expression of S100A6 was significantly correlated with the survival time and the thickness of the primary melanoma. Of interest, the finding on association and co-localization of S100A6 and S100B in various melanoma cell lines supports the possibility that S100A6 plays a functional role in these cells (Yang et al. 1999). However, it has to be further elucidated whether S100A6, like S100A4, might be involved in melanoma metastasis and possibly provides a biomarker for tumor progression in advanced stages.

S100A13

Very recently, Massi et al. (2010) demonstrated the expression of S100A13 in melanocytic lesions. Furthermore, it was significantly upregulated in melanoma compared to benign nevi and correlated positively with another potential biomarker, VEGF-A, as discussed above. S100A13 expression also significantly increased during disease progression. Due to the cooperation of S100A13 with other angiogenic molecules it may serve as an additional prognostic marker.

S100P

Another member of the S100 family, S100P, was first purified from placenta ("P") by (Becker et al. (1992). Recent studies showed that S100P plays a key role in tumor development, progression, and metastasis (Gibadulinova et al. 2011). S100P gene expression was found in several tumor entities including pancreas, breast, colon, prostate, and lung cancer (reviewed in Arumugam and Logsdon 2011). S100P is specifically and highly expressed in pancreatic cancer cells. Being secreted from pancreatic cancer cell lines, extracellular S100P was found to interact with RAGE (Arumugam et al. 2004, 2005). Thus, S100P might serve as a useful blood biomarker in early stages of pancreatic cancer. In breast cancer the presence of S100P was shown in early stages, and suggests that S100P could serve as a marker to discriminate lesions at higher risk of developing metastatic phenotype (Arumugam and Logsdon 2011). There is also a correlation between intensity of immunohistochemical staining of S100P of carcinoma cells and reduced survival in breast cancer patients (Wang et al. 2006). Because S100P is expressed in many different tumor entities and might also interact with RAGE, there is the suggestion that it also might play a role in melanoma progression. However, there is no investigation concerning S100P expression and melanoma published until now.

Non-protein biomarkers

Besides the reported protein biomarkers there are also some non-protein biomarkers, which significantly correlate with tumor progression in malignant melanoma. On the one hand, there are melanin-related metabolites that originate from the amino acid L-tyrosine and, on the other hand, some cell-free nucleic acids should also be considered as biomarkers in melanoma.

Metabolites of the melanin synthesis pathway

5-S-cysteinyl-dopa

Melanocytes and melanoma cells synthesize two different types of melanin pigments, pheomelanin and eumelanin (Fig. 2). The metabolite 5-*S*-cysteinyl-dopa (5-SCD) is produced during the biosynthesis of pheomelanin. Small amounts of 5-SCD are secreted into blood stream, methylated in the liver and excreted with urine. In general, measurement of metabolites of the melanin synthesis pathway, in particular, 5-SCD, L-dopa, and 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI2C), is suggested a complementary method of monitoring activity, growth, and even malignant transformation of melanin-producing cells. Considering varying reference values for fair-skinned and dark-skinned populations, it is assumed that increased serum and/or urine levels of these metabolites are associated with metastasis and progression of melanoma (Meyerhoffer et al. 1998, and references therein). More recently, Banfalvi and coworkers reported that 5-SCD serum levels were elevated in stage IV melanoma patients. The observed 50 % sensitivity, 100 % specificity and 86 % positive predictive values turn 5-SCD into an appropriate marker for advanced melanoma and an independent significant prognostic factor in this study (Banfalvi et al. 2003). Furthermore, Wimmer et al. (1997) measured elevated serum levels of 5-SCD in melanoma patients compared to healthy controls. The increase in concentration ranged from 2.3-fold in early stages to 50-fold in advanced stages of the disease. However, this study only determined serum levels from 11 patients with metastatic malignant melanoma before and after each cycle of immunotherapy. Furthermore, they observed that 5-SCD was a good marker for monitoring the clinical course, for discriminating between responders and non-responders to immunotherapy, and as a prognostic factor for survival time and death risk. A comparative study of the prognostic significance of 5-SCD, LDH, and S100B revealed a significant difference between serum levels of 5-SCD in melanoma patients and healthy controls as well as between stages III and IV. Thus, they assumed that it is a useful marker correlating well with the prognosis of stage IV patients, although S100B had the highest sensitivity (Banfalvi et al. 2002).

L-Dopa

L-Tyrosine is a precursor of 3,4-dihydroxyphenylalanine (L-dopa), which is the first intermediate of melanogenesis. Serum levels of L-dopa and the L-dopa/L-tyrosine ratio have been found to correlate with tumor burden in metastatic melanoma patients. The L-dopa/L-tyrosine ratio was significantly higher in stages II and IV than in stage I patients (Letellier et al. 1999). In a further study with 60 melanoma patients with progressive disease, an increase in one or both markers was detected. Stage IV patients with a high L-dopa/L-tyrosine ratio had shorter survival times. Consequently, melanoma patients with lower levels had a longer survival and a better prognosis (Stoitchkov et al. 2003). More recently, they reported that the combination of L-dopa/L-tyrosine ratio with S100B revealed the highest sensitivity and specificity (73 and 70 %) compared to LDH and MIA. The L-dopa/L-tyrosine ratio was the only marker being significantly increased during progression from stages I–III to advanced stages (Garnier et al. 2007).

6-Hydroxy-5-methoxyindole-2-carboxylic acid

Another marker being part of the melanogenesis is the 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI2C), a metabolite in eumelanin synthesis (Fig. 2). Hara and coworkers reported that all melanoma patients in their study with positive metastases showed a higher plasma 6H5MI2C level. Furthermore, the serum level correlated with Breslow index and was more sensitive than 5-SCD (Hara et al. 1994). Kärnell and coworkers compared the predictive potential of 5-SCD and 6H5MI2C in 91 patients with histopathologically verified malignant melanoma. The overall survival rate was significantly correlated to urinary levels of 5-SCD but not to 6H5MI2C, which was only found in few patients with extremely elevated levels (Kärnell et al. 1997). However, the general problem in working with melanin metabolites is related to the influence of exposure to UV radiation. Due to the fact that melanocytes produce melanin as a protection against UV-induced DNA-damage, the levels of melanin-related metabolites can be changed by this. Therefore, false-positive results might occur and decrease the specificity and sensitivity of those markers.

Other non-protein biomarkers

Intact circulating tumor-related, cell-free DNA in serum mostly derives from apoptotic or necrotic cells and is also

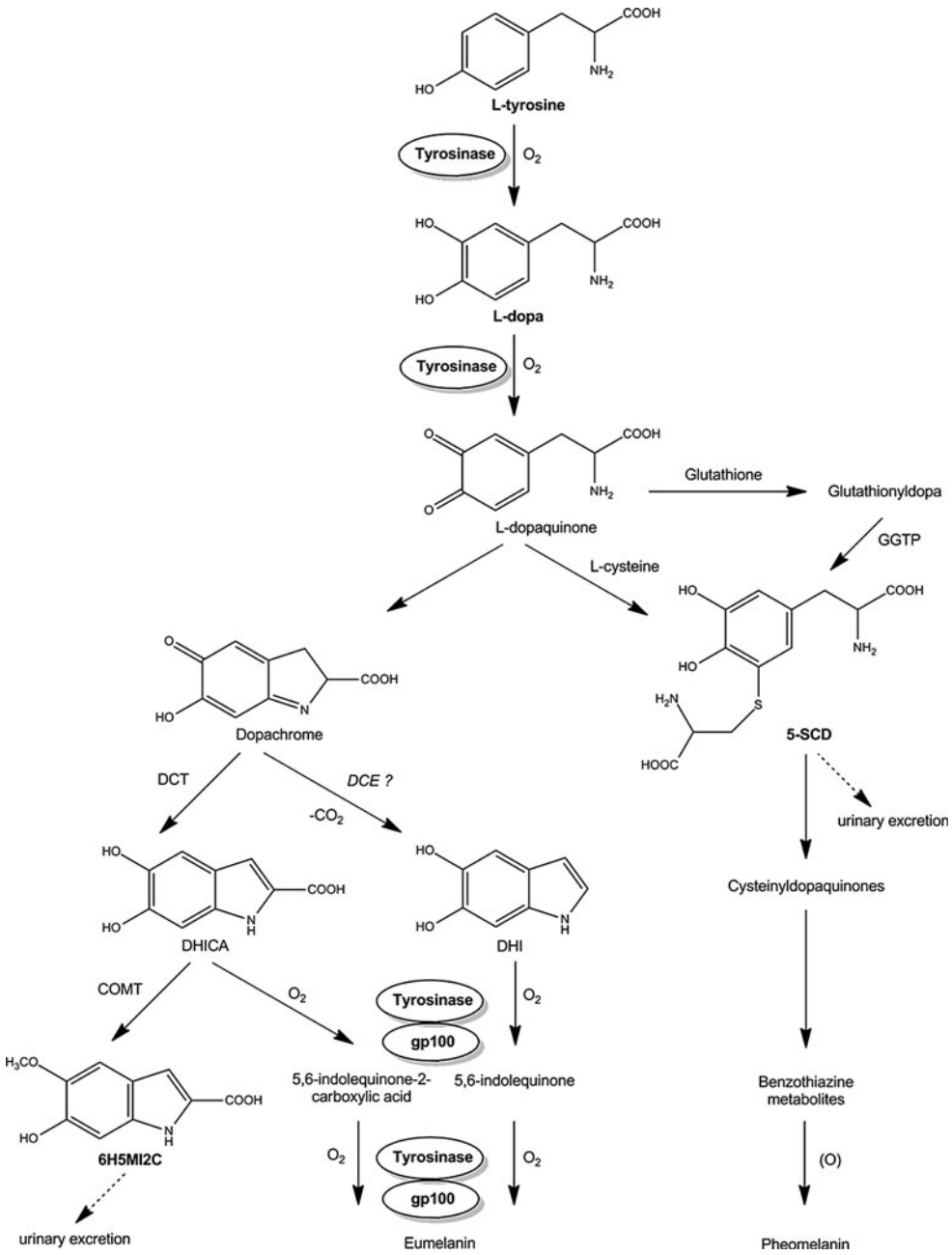

Fig. 2 Biomarkers associated with the melanin synthesis pathway. The scheme illustrates major steps of the pathway resulting in synthesis of both eumelanin and pheomelanin. Molecules that are considered as potential biomarkers in melanoma are given in *bold letters*. The rate-limiting enzyme is tyrosinase, a copper-containing oxidase. Tyrosinase catalyzes the two-step oxidation of L-tyrosine to dopaquinone using dioxygen. Furthermore, tyrosinase plays a key role in the final oxidation steps of eumelanin synthesis, presumably supported by gp100. This pathway also comprises various non-enzymatic conversions and rearrangements, e.g., the formation of 5-*S*-cysteinyldopa (5-SCD) by conjugation of dopaquinone with L-cysteine or the spontaneous cyclization of dopaquinone forming dopachrome. Dopachrome is a substrate of dopachrome tautomerase (DCT) that catalyses the formation of 5,6-dihydroxy-*1H*-indole-2-carboxylic acid (DHICA). The dopachrome conversion to 5,6-dihydroxy-*1H*-indole by decarboxylation also occurs spontaneously, but presumably is supported by a dopachrome decarboxylase activity (DCE). Of note, the subsequent conversion of DHICA by the enzyme catechol-*O*-methyltransferase (COMT) results in the formation of two *O*-methyl derivatives, 6-hydroxy-5-methoxy-*1H*-indole-2-carboxylic acid (6H5MI2C) and 5-hydroxy-6-methoxy-*1H*-indole-2-carboxylic acid (5H6MI2C). However, this article exclusively refers to publications demonstrating 6H5MI2C as potential biomarker in melanoma. Finally, both eumelanin and pheomelanin form mixed type melanins (not shown). *L-dopa* 3,4-dihydroxyphenylalanine, *5-SCD* 5-*S*-cysteinyldopa, *6H5MI2C* 6-hydroxy-5-methoxy-*1H*-indole-2-carboxylic acid, *COMT* catechol-*O*-methyltransferase (EC 2.1.1.6), *DCT* dopachrome tautomerase (L-dopachrome isomerase, tyrosine-related protein-2; EC 5.3.3.12), *DCE* dopachrome conversion enzyme (L-dopachrome carboxylyase/decarboxylase activity), *DHI* 5,6-dihydroxy-*1H*-indole, *DHICA* 5,6-dihydroxy-*1H*-indole-2-carboxylic acid, *GGTP* gammaglutamyl transpeptidase (EC 2.3.2.2), *gp100* melanocytes lineage-specific antigen GP100

actively secreted by living cells. Three percent of tumor DNA was released into the blood per day (Schwarzenbach et al. 2011). Through necrosis, which is typical for solid tumors, a variable spectrum of DNA fragments due to random digestion by DNases can be detected in serum of cancer patients. The programmed cell death, apoptosis, reveals shorter and uniform DNA fragments. Experiments confirmed that cell-free DNA (cfDNA) could be distinguished between originating from cancer patients and healthy controls with higher levels in patients with advanced diseases (Pinzani et al. 2011). Blood-based assays are used to detect melanoma progression by monitoring levels of circulating tumor cells (CTC) and circulating DNA, serving as a 'liquid biopsy'. The analysis enables the detection of tumor-related genetic and epigenetic alterations without invasive surgery (Schwarzenbach et al. 2011). The main challenge is to find an appropriate DNA marker, which could reliably differentiate between normal and tumor cell DNA, for example, the point mutation in Raf (Kounalakis and Goydos 2005). The B-RAF/MEK/ERK signaling pathway regulates cell growth through activation due to ligand binding (VEGF, EGF). After that, phosphorylation cascades activate RAS, RAF, and finally ERK 1/2 leading to a regulation of gene transcription and cytoskeleton alterations due to phosphorylated substrates. A constitutive activation is caused by mutations or overexpression, for example a mutation of one RAF isoform, BRAF. This mutation occurs in 50–60 % of all melanoma cell lines whereby the substitution of valine through glutamic acid in position 600 is most frequent (V600E), and leads to an 500-fold activation of ERK pathway in a growth-dependent manner (Vallacchi et al. 2011). However, circulating cfDNA is also released by other physiological and pathological processes which are not tumor specific such as benign lesions, inflammatory diseases or tissue trauma (Schwarzenbach et al. 2011). Daniotti and coworkers found elevated levels of circulating cfDNA in melanoma patients. Detection of BRAFV600E is useful in monitoring the disease in stage IV but it appears to be unsatisfactory for the early detection of melanoma caused by low sensitivity of analysis technique (Daniotti et al. 2007). In contrast to further studies with promising results, Shinozaki and others found BRAF mutations in only 31 % of their samples. BRAF-levels did neither correlate with Breslow index nor had an effect on overall disease-free survival. They describe the BRAF mutation as being no major genetic prognostic factor. It may be acquired during development of metastases, but it is not a significant factor for primary tumor development and disease outcome (Shinozaki et al. 2004). Kim et al. 2010 tested the prognostic potential of four metastasis-associated gene transcripts circulating in patient blood. Among them, only the lymphoid-specific helicase (*HELLS*) and the non-SMC condensin I complex, subunit H (*NCAPH*) were found to be significantly increased in serum of patients with distant organ metastases than in those with localized tumors whereby *HELLS* was identified as a statistically significant independent marker for metastasis with a better potential than LDH.

Epigenetic aberrations were strongly correlated with the development of a malignant phenotype and with tumorigenesis in cutaneous melanoma including dysregulated DNA gene promoter methylation, histone modification, and microRNA (miRNA). MiRNAs are endogenous, small, evolutionary conserved, non-coding RNA transcripts that are involved in regulation of several cellular processes including proliferation, differentiation, stress response, and apoptosis (Greenberg et al. 2012). The 22 nucleotide long fragments derive from non-coding intergenic and intronic regions of the DNA and interact with protein translation of mRNA transcripts through binding to the 3′ end. Therefore, protein translation is inhibited or the degradation of the mRNA transcript is caused before it could be translated into the appropriate amino acid sequence (Stark et al. 2010). In normal cell development, miRNA regulation of protein-coding genes is an essential regulatory element. However, abnormalities and dysregulation of their expression might have an impact on cell cycle, proliferation, apoptosis, and angiogenesis and is strongly correlated with tumorigenesis (Palmer et al. 2011). Specificity and significance are investigated in several studies through histological and serological analysis. The availability of powerful approaches for miRNA identification and the existence of simple, general applicable analyzing methods (e.g., qRT-PCR) turn the short RNA fragments into promising and useful biomarkers (Mitchell et al. 2008). Many genes are possible targets for miRNA resulting in various functions in normal and tumor tissue. Aberrations of miRNA expression due to deletions and amplifications or mutations lead to specific miRNA profiles for different tumor entities (e.g., miR-141 is specific for prostate cancer, Mitchell et al. 2008). The ubiquitous expression of miRNA influences the biological function and clinical phenotype of tumors. They are often involved in the up-regulation of oncogenes or the down-regulation of tumor suppressor genes. Some of the novel candidate miRNAs may be specific for melanoma progression and might be useful for early detection of distant metastases by measuring circulating levels (Stark et al. 2010). Especially circulating miRNAs are very stable even under harvest conditions (low and high pH), and are resistant against RNAse (Chen et al. 2008). In human melanoma cell lines, the promyelocytic leukemia zinc finger transcription factor (PLFZ) inhibits the transcription of miRNA-221 and miRNA-222. In contrast, through the lack of PLFZ in melanoma miRNA-221 and miRNA-222 are unblocked and increasingly expressed

in tumor progression. They in turn inhibit c-Kit and p27 translation, which are associated with the development of a malignant phenotype (Felicetti et al. 2008). MiRNA-221 levels ware also significantly increased in serum of patients with malignant melanoma compared to healthy controls correlating with Breslow index (Kanemaru et al. 2011). Kanemaru and coworkers assumed that miRNA-221 is useful not only for diagnosis, but also for discrimination between malignant melanoma in situ and stages I–IV, as well as for monitoring during follow-up period. Leidinger and others demonstrated that blood samples from patients with malignant melanoma could be differentiated from healthy controls by analyzing 16 deregulated miRNAs. Through microarray analysis and qRT-PCR they achieved an accuracy of 97.4 %, a specificity of 95 % and a sensitivity of 98.9 % (Leidinger et al. 2010). MiRNA-29c expression was found to be decreased in advanced melanoma. Since a more frequent methylation was observed in advanced stages, miRNA-29c was said to influence the expression of DNA methyltransferases. Thus, leading to the suggestion that miRNA-29c could serve as a potential biomarker for differentiation of melanoma stages. Its down-regulation might reveal an indicator for a more aggressive disease. Nguyen and colleagues showed a correlation between miRNA-29c methylation and advanced stages in melanoma. Furthermore, expression of DNA methyltransferase DNMT3A and miRNA-29c was significantly associated with overall survival (Nguyen et al. 2011). Heneghan et al. (2010) analyzed the level of miRNA-145, a tumor suppressor, in blood samples from different tumor patients. In melanoma patients the expression was decreased compared to healthy controls. In addition, they reported specific miRNA profiles for different tumor entities like breast, colon, and prostate cancer. Recently, many studies have attempted profiling of miRNA expression in different tumor entities. However, only few miRNAs have been identified as being significant prognostic and predictive markers for tumor development and progression (Friedman et al. 2012). Further evaluation of blood-based miRNAs with a larger cohort of melanoma patients is needed to better define the specific expression profiles and to further elucidate the practicability of developing circulating miRNA assays specific for individual cancers as clinically useful tools. Follow-up studies of the functional roles of pigment cell-specific miRNAs and the further identification of targets might clarify the mechanism of developing melanoma.

Future directions in melanoma biomarker discovery

In the last years, various serological biomarkers were intensively investigated to establish appropriate analysis methods to estimate melanoma patients' prognosis, to discover tumor progression as well as recurrence during follow-up periods, and to refine treatment response. Several melanoma- and non-specific biomarkers were tested and investigated concerning their independent prognostic and predictive value. Among these, S100B seems to be the most promising serum marker in advanced melanoma, even more specific and sensitive than LDH but still not applied in clinical routine. Different intrinsic properties of the tumor itself and the development of a specific molecular signature due to molecular modifications of melanoma tumors lead to large variations between different samples (Leclerc et al. 2009). Therefore, comparison of different studies is impeded. Alternative methods were used to identify proteins of interest in tumor tissue and analyzing them using more conventional immunoassays (Solassol et al. 2011). Other promising targets for biomarker analysis are circulating melanoma cells. In particular, highly tumorigenic and metastatic melanoma cells should be present in peripheral circulation. Besides markers discussed above, other proteins, some of them possibly representing melanoma progenitor/stem cell-like markers, can be detected in circulating melanoma cells, at least demonstrated in animal models. This includes the ABCB5 multidrug transporter and neuroepithelial intermediate filament nestin (Fusi et al. 2011; Ma et al. 2010).

Lack of sufficient sensitivity, specificity, and accuracy is the most relevant limitation of a single blood-based biomarker in clinical use. By contrast, a cluster of biomarkers for one disease would be a better diagnostic tool with much higher sensitivity, specificity, and clinical accuracy (Chen et al. 2008). With the help of genomic and proteomic approaches, new molecules are detected and defined according to their potential of being useful biomarkers. Based on this, new investigations focused on the identification of multiple coexpressed biomarkers called 'proteomic profiling' (Solassol et al. 2011). This analysis of serum proteins may lead to identification of signature biomarker patterns which are specific for different tumor entities and allow early detection, staging, therapeutic monitoring, and prognostic predictions (Palmer et al. 2011). Mian et al. (2005) were able to discriminate patients of different clinical stages using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and identified the correct disease stage in 84 of 96 samples. In another study, serum amyloid A (SAA), unnoticed as potential biomarker up to now, was detected in serum of melanoma patients also using MALDI-TOF-MS. Multivariate analysis revealed SAA among others as independent prognostic factor. In patients stages I–III, the combination of SAA and CRP was even superior to S100B in predicting progression-free and overall survival, and discriminating low-risk from high-risk patients (Findeisen

et al. 2009). Furthermore, Takikawa et al. (2009) identified nine proteins in plasma of melanoma patients being not expressed in plasma of healthy controls. Via surface-enhanced laser desorption/ionization TOF-MS (SELDI-TOF-MS), Caron et al. (2009) discriminated serum samples of 30 melanoma and 24 non-cancer patients with a good diagnostic accuracy of 98.1 %. In contrast to MALDI, where all proteins are mixed with the matrix solution and are co-crystallized on a surface, SELDI only analyzes those proteins that bound on a chemical functionalized surface. Although protein expression patterns reveal new approaches for novel biomarkers, there are also several obstacles. Proteins are not as robust as DNA and tend to denature and, therefore, a considerable greater methodological effort is required. Furthermore, proteins are more difficult to attach to surfaces and, especially, the identification of low-abundant proteins is limited (Sabel et al. 2011).

Concluding remarks

Biomarkers that could aid or improve the diagnosis and correct staging of melanoma as well as indicate patients' prognosis or the most appropriate therapeutic regimes would fit into the frequently discussed model of personalized medicine. In this regard, the clarification of the adequacy to the intended use of proteins and non-protein molecules, ideally those secreted into body fluids, as biomarkers or surrogate markers of melanoma, which are amenable to the design of noninvasive clinical tests, is of utmost importance. Until now, LDH is the only accepted biomarker in clinical use in melanoma but has demonstrably some failings and, especially in early stages of disease, sensitivity and specificity are too low. On the other hand, there is increasing evidence indicating the calcium-binding EF-hand protein S100B to be a reliable biomarker in melanoma not only for immunohistochemical investigations but also for non-invasive, serological detection. Of interest, other S100 proteins also are suggested to be biomarker candidates of melanoma. As more specific reagents for individual S100 proteins are being generated, their potential diagnostic and prognostic usage will increase substantially. Furthermore, the use of tyrosinase, MIA, and Gal-3 discretely or in combination with other markers showed some promising results. However, greater efforts and more clinical studies with larger patient populations are needed to gain the reliable biomarkers or biomarker patterns providing sufficient sensitivity and specificity in early diagnosis of melanoma, melanoma staging, monitoring treatment response, and identifying high-risk melanoma patients for adjuvant targeted and adjuvant therapies.

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