Biomarkers for Melanoma

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Abstract

Biomarkers of melanoma may eventually be used in diagnostics, prognostics, as well as prediction of clinical outcome and/or treatment response. These biomarkers may be either clinical characteristics or molecular patterns or profiles, mainly derived from tumor tissue or from the peripheral blood. The molecular biomarkers can be categorized into genetic alterations, epigenetics, proteins, or other types of molecules, which provide a "signature" of risk, prognosis, and/or treatment response. Because of the potential of biomarkers to improve the prognostication and hereby the outcome of patients with melanoma, research is underway to identify and validate melanoma biomarkers from numerous sources, including tumor cells (cultured, freshly biopsied, and paraffin-embedded), draining regional lymph nodes, serum/plasma, tumor environment, and cellular compartments of the peripheral blood. The biomarkers reviewed here comprise tumor tissue-based biomarkers, tumor environment-based biomarkers, soluble biomarkers of the peripheral blood, as well as treatment-associated biomarkers. These biomarkers might not only be useful for diagnostics, prognostication, and prediction of treatment outcomes but in particular for the continuous monitoring of a patient's course of disease over time.

Biomarker Definition and Use

Definition of Cancer Biomarkers

The Biomarkers Definitions Working Group of the National Institutes of Health (NIH) gives the definition of a biomarker as a cellular, biochemical, and/or molecular (including genetic and epigenetic) characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (The Biomarkers Definitions Working Group 2001). Specifically for cancers, a biomarker is defined as a biological molecule, either produced by the tumor cells themselves or by normal tissues in response to cancer, which is objectively measured and evaluated as an indicator of cancerous processes within the body (Fuzery et al. 2013). Thus, a cancer or tumor

biomarker may be defined as a "molecule that indicates the presence of cancer or provide information about the likely future behavior of a cancer, i.e., likelihood of progression or response to therapy" (Duffy 2013).

Ideally, a tumor biomarker should be (a) produced only by the tumor cells; (b) correlated with tumor burden and endowed with a sufficient lead time (i.e., the time between asymptomatic cancer still localized to the organ of origin and clinical diagnosis; e.g., aggressive cancers have shorter lead times than indolent cancers); (c) present in measurable quantities (or in concentrations significantly higher than normal) in the blood (or other human biological fluids) of cancer patients at early or preclinical stages (preferably in one cancer type only); (d) undetectable (or present at a very low levels) in the blood (or other biological fluids) of healthy individuals or with benign disease; and (e) easy to measure even in small amounts and with little preparation, by means of a reliable test, cost-effective and associated to high analytical sensitivity (the percentage of individuals with cancer who test positive for the biomarker) and to specificity (the percentage of individuals without cancer who test negative for the biomarker) (Mordente et al. 2015; Duffy 2013; Kulasingam and Diamandis 2008). Thus, an ideal biomarker test would have 100% sensitivity and specificity (i.e., everyone with cancer would have a positive test, while everyone without cancer would present a negative test) (Mordente et al. 2015; Duffy 2013; Kulasingam and Diamandis 2008). No more than 19 protein cancer biomarkers have been approved by the US Food and Drug Administration (FDA) to date (Mordente et al. 2015). These comprise serum biomarkers like prostate-specific antigen (PSA) for prostate cancer, carbohydrate antigen 19–9 (CA 19–9) for pancreatic cancer, and human chorionic gonadotropin-beta (beta-HCG) for testicular cancer, as well as tissue biomarkers like epidermal growth factor receptor (EGFR) for colorectal cancer and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (HER2-neu) for breast cancer. However, even these officially approved biomarkers are far from ideal, and it is assumed that ideal biomarkers fulfilling all abovementioned quality criteria do not exist in the real-world situation.

Differential Utilization of Cancer Biomarkers

Biomarkers may have differential utilizations in cancer patients. They may be suitable for diagnosis, prognosis, prediction of treatment response and outcome, disease monitoring, and early detection of relapse (Mordente et al. 2015). Hereof, prognosis and prediction of treatment response are the most important functions. *Prognostic biomarkers* are factors that predict the natural disease outcome in the absence of systemic therapy or despite empiric (not targeted to the marker) systemic therapy (Sargent et al. 2005; Duffy and Crown 2008; Mordente et al. 2015). Prognostic biomarkers therefore are of particular interest at the time of initial diagnosis of cancer, providing a probability estimate of aggressiveness, metastasis and overall disease outcome. In contrast, *predictive biomarkers* are molecules that provide information as to whether or not a patient is likely to benefit from a specific treatment before that treatment has started (Duffy et al. 2011). Hereby, predictive biomarkers help to categorize patients into probable responders and non-responders upfront to therapy start, which ideally allows to choose the best suitable treatment for each individual patient and to spare patients from treatment with low probability of response but potentially harmful side effects.

Biomarker Use in Melanoma

In melanoma, biomarkers may eventually be used in diagnostics, prognostics, as well as prediction of clinical outcome and/or treatment response. These biomarkers may be either clinical characteristics or molecular patterns or profiles, mainly derived from tumor tissue or from the peripheral blood. The molecular biomarkers can be categorized into genetic alterations, epigenetics, proteins, or other types of molecules, which provide a "signature" of risk, prognosis, and/or treatment response. Because of the potential of biomarkers to improve the prognostication and hereby the outcome of patients with melanoma, research is underway to identify and validate melanoma

biomarkers from numerous sources, including tumor cells (cultured, freshly biopsied, and paraffin-embedded), draining regional lymph nodes, serum/plasma, tumor environment, and cellular compartments of the peripheral blood (Eisenstein et al. 2018; Huang and Hoon 2016; Lim et al. 2018).

There are four major histomorphologic subtypes of cutaneous melanoma: superficial spreading, lentigo maligna, mucosal, acral lentiginous, and nodular. However, this histopathologic classification alone has no independent prognostic value. Breslow thickness and ulceration currently are the most widely used histopathologic prognostic markers in cutaneous melanoma, also as part of the American Joint Committee on Cancer (AJCC) clinicopathological staging system of melanoma (Balch et al. 2001a; Gershenwald et al. 2017). In the recent years, major progress has been made in the molecular classification of melanoma subtypes, mainly based on the recognition of distinct patterns of genetic alterations. Hitherto, the largest cohort of melanoma tissues and cell lines has been analyzed within The Cancer Genome Atlas project (TCGA) (The Cancer Genome Atlas Network 2015; Sanchez-Vega et al. 2018; Bailey et al. 2018; Liu et al. 2018). This project identified four distinct genetic melanoma subtypes by their profile of activating driver gene mutations, BRAF-mutant, N/K/HRAS-mutant, NF1-mutant, and triple-wild-type, representing approximately 50%, 25%, 15%, and 10% of melanomas, respectively. This genetically based classification of melanomas also has major impact on a patient's prognosis, since some of these gene mutations are targetable by selective kinase inhibitors. This option is currently implemented primarily for BRAF-mutated melanomas, but other molecular targeted inhibitors are hopefully to be identified in the near future.

The amount of research being dedicated to the identification of biomarkers for melanoma is illustrated by the results of a PubMed search done in mid-2018 using the search terms "melanoma" and "biomarker," which yielded 9959 publications. Unfortunately, despite these thousands of documented efforts, to date, only one molecular marker, the serum level of lactate dehydrogenase (LDH), has been deemed robust enough to be approved as part of the AJCC staging system of melanoma (Balch et al. 2001a,b; Gershenwald et al. 2017). Furthermore, this detection of serum LDH levels is limited to prognostication purposes in patients with stage IV melanoma disease. However, new biomarkers from the patient's peripheral blood are under extensive testing and validation, including microRNAs, circulating tumor DNA, and others (Eisenstein et al. 2018; Huang and Hoon 2016; Lim et al. 2018). These biomarkers might not only be useful for prognostication and prediction of treatment outcomes but in particular for the monitoring of a patient's course of disease over time.

Biomarker Discovery and Validation

As outlined above, the number of reports on "biomarkers" is tremendously high which is in part caused by an unclear definition (Fuentes-Arderiu 2013). The process of discovering and developing molecular cancer biomarkers is a work in progress and is evolving, representing an "integral component of contemporary cancer research" (Mordente et al. 2015). In 2001, the National Cancer Institute's Early Detection Research Network (EDRN), to promote efficiency and scientific rigor in biomarkers research, introduced guidelines "to guide the process of biomarker development" consisting of five "phases that are generally ordered according to the strength of evidence that each phase provides in favour of the biomarker, from weakest to strongest and the results of earlier phases are generally necessary to design later phases" (Pepe et al. 2001). Based on these guidelines, the phase structure of biomarker development pipeline includes phase 1 (preclinical exploratory studies), phase 2 (clinical assay development), phase 3 (retrospective longitudinal studies), phase 4 (prospective screening studies), and phase 5 (cancer control studies). These phases are not rigorously distinct from each other, and to proceed from one phase to another, a candidate biomarker needs to overcome pre-analytical, analytical, and post-analytical challenges at different levels. Only biomarkers that will reach the last

step successfully will be implemented into the clinical use.

In a recent meta-analysis of known blood-based biomarkers in metastatic breast cancer regarding their clinical utility and state-of-the-art position in the validation process, the authors reported that most studies focused on identifying new biomarkers and in almost 70% (of 320 studies included) of the investigated studies, a biomarker was investigated in only one study (Berghuis et al. 2017). Only 9.8% of all biomarkers was investigated in more than five studies, and the authors concluded "that there is a lack of studies focusing on identifying the clinical utility of these biomarkers," which certainly is also true for the melanoma field. The rate of successful translation of biomarkers into the clinical use is still very low (estimated around 0.1%) (Poste 2011). Unfortunately, in melanoma, the situation is even worse with no clear biomarker development plan anywhere obvious.

Biomarker Discovery

The biomarker developmental process is visualized in Fig. 1. Biomarker development always starts with its "discovery" and should typically include a validation in parallel. Validation is based on predefined prediction rules and should be performed ideally on an independent patient cohort. Similar to the successful CONSORT initiative for randomized trials and the STARD statement for diagnostic studies, the REMARK guidelines have been proposed to provide relevant information about the biomarker study design, preplanned hypotheses, patient and specimen characteristics, assay methods, and statistical analysis methods (McShane et al. 2005a). In addition, these REMARK guidelines suggest helpful presentations of data and important elements to include in discussions (see Table 1). With the current availability of high-throughput "omics" technologies where several thousand individual

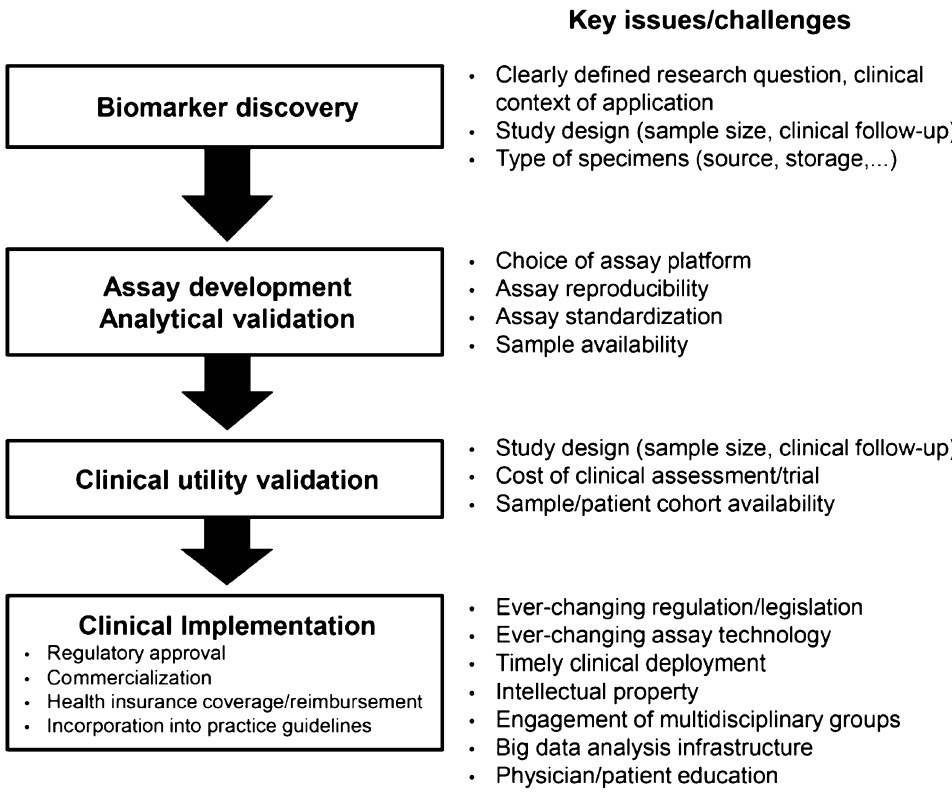

Fig. 1 Schematic overview of the processes of cancer biomarker development. (From Goossens et al. 2015)

Table 1 REporting recommendations for tumor MARKer prognostic studies (REMARK) (From McShane et al. 2005b)

Introduction
1. State the marker examined, the study objectives, and any prespecified hypotheses
Materials and Methods
Patients
2. Describe the characteristics (e.g., disease stage or comorbidities) of the study patients, including their source and inclusion and exclusion criteria
3. Describe treatments received and how chosen (e.g., randomized or rule-based)
Specimen characteristics
4. Describe type of biological material used (including control samples) and methods of preservation and storage
Assay methods
5. Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study end point
Study design
6. State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g., by stage of disease or age) was employed. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time
7. Precisely define all clinical end points examined
8. List all candidate variables initially examined or considered for inclusion in models
9. Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size
Statistical analysis methods
10. Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled
11. Clarify how marker values were handled in the analyses; if relevant, describe methods used for cut point determination
Results
Data
12. Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events
13. Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values
A*nalysis and presentation*
14. Show the relation of the marker to standard prognostic variables
15. Present univariate analyses showing the relation between the marker and outcome, with the estimated effect (e.g., hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan-Meier plot is recommended
16. For key multivariable analyses, report estimated effects (e.g., hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model
17. Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their significance
18. If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation
Discussion
19. Interpret the results in the context of the prespecified hypotheses and other relevant studies; include a discussion of limitations of the study
20. Discuss implications for future research and clinical value

molecules can be easily interrogated without a priori assumptions, research hypotheses are often generated in a post hoc manner, often followed by serendipitous discovery from unbiased mining of available data (data-driven hypothesis generation) (Goossens et al. 2015).

Biomarker Validation

Following the biomarker discovery process, an analytical and clinical validation is mandatory. The analytical validation process includes questions like how accurately and reliably does the test assay detect and quantify the biomarker in the patient specimen. This includes obviously pre-analytical conditions and parameters such as storage and handling as well as assay parameters including standardization and reproducibility. The clinical validation describes how robust and reliable the test result correlates with the desired phenotype and/or clinical outcome. Challenges in biomarker work are numerous. This includes complex mixtures of components such as DNA, RNA, proteins, metabolites, lipids, etc., various physical and chemical properties relevant for stability, and specimen handling as well as storage – all having a tremendous impact on pre-analytical generation of artifacts. Furthermore, (tumor) heterogeneity within and between individual patients additionally has profound impact on biomarker research. Likewise, detection of candidate biomarkers with a low expression profile remains a significant challenge. Together with "background noise" created by impurity of cell populations to be analyzed or the number of cells needed for precise marker measurement, the ease of assessability is also a practical limitation of the clinical utility of biomarker implementation in routine use. A meta-analysis of biomarkers in primary melanomas (stage I/II) published in 2010 described more than 100 proteins from more than 500 manuscripts (Gould Rothberg and Rimm 2010); however, an additional future assessment of these proteins in methodologically robust prognostic studies as requested by the authors to evaluate their clinical potential as independent predictors of outcome among patients with localized melanomas is still missing today.

Tumor Tissue-Based Markers

Diagnostic Markers for Primary Melanoma

The histopathologic diagnosis of melanoma can represent one of the most challenging tasks in surgical pathology, given melanoma's many mimics, including dysplastic and Spitz nevi. While numerous histological attributes have been defined to differentiate between melanoma and benign nevi, including circumscription; symmetry; maturation with descent, cytologic atypia; and mitotic figures, the rank order to be assigned to these different attributes has not been firmly established and can (and does) differ among different pathologists. In addition, there is a subset of lesions that are highly atypical, in which a firm diagnosis may be difficult to assign. As a result, several studies have shown a high degree of interobserver variability in the diagnosis of melanocytic neoplasms, even when evaluated by a panel of expert dermatopathologists (Farmer et al. 1996; Corona et al. 1996; Scolyer et al. 2003). This discordance can be greatest in the diagnosis of benign versus malignant lesions, setting aside ambiguous lesions, in which one would expect a high degree of concordance (Shoo et al. 2010).

These issues highlight the need for molecular adjuncts to the histological diagnosis of melanocytic neoplasms. In recent years, there have been a number of attempts in order to develop molecular diagnostic assays to distinguish melanomas from benign nevi. These are distinct from markers to determine melanocytic lineage that distinguish between melanoma and other cancers, such as S100, SOX-10, and Melan-A/MART-1. To date, three different platforms have been utilized to develop diagnostic assays

for melanoma and include fluorescence in situ hybridization (FISH), gene expression profiling using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and immunohistochemical analysis (IHC).

FISH-Based Diagnostic Assays for Melanoma

Initial studies using comparative genomic hybridization identified a number of chromosomal alterations in primary cutaneous melanoma, involving either gains or losses in various chromosomal loci. Subsequent studies using FISH validated several of these aberrant loci and pinpointed specific genes whose copy number was altered within these loci. In 2009, a FISH-based diagnostic assay was described to distinguish between melanoma and benign nevi (Gerami et al. 2009). This four-probe assay targeted 6p25 (encompassing the RREB1 gene), 6q23 (encompassing the MYB gene), 11q13 (encompassing the CCND1 gene), and Cep6 (encompassing the centromere of chromosome 6). Application of this assay to a cohort of 301 melanocytic neoplasms initially yielded a sensitivity of 86.7% and specificity of 95% in the diagnosis of melanoma. This assay became commercially available in the USA, offered by NeoGenomics. Subsequent studies using this four-probe assay showed a markedly reduced sensitivity in the diagnosis of Spitzoid lesions, reducing its clinical utility (Gaiser et al. 2010). In addition, 5% of Spitz nevi had sufficient tetraploid cells to potentially result in a false-positive test.

More recently, this assay was modified to incorporate a probe targeting 9p21, which encompasses the CDKN2A gene. Addition of this marker to the original assay appeared to increase the sensitivity in a small validation cohort of 51 nevi and 51 melanomas. A version of this assay is now offered by NeoGenomics as the NeoSITE assay, including the following probes, 6p25 (RREB1), 6q23 (MYB), 11q13 (CCND1), 9p21 (CDKN2A), and Cep9 (encompassing the centromere of chromosome 9), with a reported sensitivity of 86% and specificity of 90% (Gerami et al. 2012). One of the practical issues with the version of the assay offered clinically is the reporting of different algorithms and cut points, potentially resulting in different diagnoses, resulting in confusion regarding the ultimate diagnosis rendered for practicing physicians and patients.

Gene Expression Profiling of Melanocytic Neoplasms

Beyond FISH, additional platforms have been evaluated for their utility in the diagnostic assessment of melanoma. Early studies using cDNA microanalysis identified numerous differentially expressed genes in the comparison between nevi and melanomas, suggesting the potential utility of transcriptomic analysis to assist in this differential diagnosis. In 2013, Myriad Genetics launched the myPath assay, consisting of a 23-gene expression signature, as an adjunctive diagnostic test. The assay consists of 14 genes involved in melanoma pathogenesis (including a number of genes involved in regulating the immune response) (Table 2) and 9 housekeeping genes assessed by qRT-PCR, with an algorithm that applies a diagnostic score which includes benign, malignant, and indeterminate. The assay was initially applied to a validation cohort of 437 neoplasms, with a reported sensitivity of 90% and specificity of 91% (Clarke et al. 2015). More recently, application of the assay to 993 unambiguous cases determined following a consensus review by three different pathologists resulted in a reported sensitivity of 91.5% and specificity of 92.5% (Clarke et al. 2017). However, this does not include an additional 14% of cases that were excluded due to lack of pathologist agreement, and 13% of the cases in which an indeterminate test result was rendered, reducing the sample set from an initial cohort of 1400 cases. Additional analysis suggested the presence of false-positive cases in dysplastic nevi and false-negative cases in lentigo maligna.

Separately, a two-gene classification method has been launched by DermTech that uses an adhesive patch that is applied to the melanocytic neoplasm. In a recent study, the patch was applied to 555 pigmented lesions, including a training set of 157 and validation set of 398 cases, which then underwent surgical biopsy and routine pathologic evaluation (Gerami et al. 2017). Following RNA extraction, the level of expression of two genes, PRAME and LINC00518, was assessed using

Table 2 Molecular diagnostic markers for melanoma by testing platform

Gene symbol	Gene name	Reference(s)
FISH markers		
RREB1 (6p25)	Ras-responsive element binding protein	Gerami et al. (2009, 2012)
MYB (6q23)	V-myb avian myeloblastosis oncogene homolog	Gerami et al. (2009, 2012)
CCND1 (11q13)	Cyclin D1	Gerami et al. (2009, 2012)
CDKN2A (9p21)	Cyclin-dependent kinase inhibitor 2A (aka p16)	Gerami et al. (2012)
qRT-PCR markers		
PRAME	Preferentially expressed antigen in melanoma	Clarke et al. (2015, 2017)
S100A7	S100 calcium-binding protein A7	Clarke et al. (2015, 2017)
S100A8	S100 calcium-binding protein A8	Clarke et al. (2015, 2017)
S100A9	S100 calcium-binding protein A9	Clarke et al. (2015, 2017)
S100A12	S100 calcium-binding protein A12	Clarke et al. (2015, 2017)
PI3	Proteinase inhibitor 3	Clarke et al. (2015, 2017)
CCL5	Chemokine, C-C motif, ligand 5	Clarke et al. (2015, 2017)
CD38	CD38 antigen	Clarke et al. (2015, 2017)
CXCL9	Chemokine, C-X-C motif, ligand 9	Clarke et al. (2015, 2017)
CXCL10	Chemokine, C-X-C motif, ligand 10	Clarke et al. (2015, 2017)
IRF1	Interferon regulatory factor 1	Clarke et al. (2015, 2017)
LCP2	Lymphocyte cytosolic protein 2	Clarke et al. (2015, 2017)
PTPRC	Protein-tyrosine phosphatase, receptor-type, C	Clarke et al. (2015, 2017)
SELL	Selectin L	Clarke et al. (2015, 2017)
IHC markers		
ARPC2	Actin-related protein 2/3 complex, subunit 2	Kashani-Sabet et al. (2009)
FN1	Fibronectin 1	Kashani-Sabet et al. (2009)
RGS1	Regulator of G signaling protein 1	Kashani-Sabet et al. (2009)
SPP1	Secreted phosphoprotein 1 (aka osteopontin)	Kashani-Sabet et al. (2009)
WNT2	Wingless-type MMTV integration site family, member 2	Kashani-Sabet et al. (2009)
CDKN2A	Cyclin-dependent kinase inhibitor 2A (aka p16)	Kashani-Sabet et al. (2009)

qRT-PCR. The two-gene assay yielded a sensitivity of 91% and specificity of 69% in the validation set. Specifically, in 203 serially collected samples, the assay achieved a sensitivity of 79% and specificity of 80%.

Immunohistochemical Analysis in the Diagnosis of Melanocytic Neoplasms

IHC analysis has been a mainstay in the assessment of melanocytic neoplasms, but largely in the setting of distinguishing melanomas from tumors of other lineage. However, in 2009, the utility of IHC analysis was reported in the differential diagnosis of nevus versus melanoma (Kashani-Sabet et al. 2009). A five-marker IHC assay (consisting of ARPC2, FN1, RGS1, SPP1, and WNT2) was developed and applied to a training set of 534 melanocytic neoplasms. IHC analysis revealed a distinct pattern of marker expression in nevi versus melanomas, with decreasing marker expression in the base of nevi. An algorithm taking advantage of marker expression levels as well as a gradient score (from the top to the bottom of the lesion) achieved a sensitivity of 91% and specificity of 95% in melanoma diagnosis. The diagnostic algorithm was also assessed and validated in separate cohorts of dysplastic and Spitz nevi, in melanomas arising in a nevus, and a prospectively collected cohort of misdiagnosed melanocytic lesions.

Separately, a number of centers have been using IHC analysis of p16/CDKN2A as an adjunct in the diagnosis of difficult to classify cases. This is supported by the presence of biallelic CDKN2A loss in a majority of melanomas versus nevi identified by FISH, and by some

studies suggesting differential p16 expression in Spitz nevi versus melanomas (George et al. 2010), which was not reproduced by other studies (Garrido-Ruiz et al. 2010). Some pathology reports include a description p16 immunostaining, without scoring marker expression, without use of a diagnostic algorithm, and with no documented sensitivity or specificity of this approach. Importantly, dysplastic nevi and Spitz nevi may have heterozygous loss in 9p21, further complicating the use of this marker.

In conclusion, significant progress has been made in the molecular assessment of melanocytic neoplasms, resulting in the development of several diagnostic assays to assist in the differential diagnosis of melanoma versus nevus. Each assay has its own advantages and disadvantages. In situ assays (such as FISH and IHC) have the advantage of enabling assessment of the entire lesion by an observer, but are operator-dependent (with its inherent variability) and can practically assay a limited number of markers. qRT-PCR-based assays have the advantage of assaying a larger number of genes in a consistent, observer-independent fashion, but are limited by the small tumor volume that can be available in a significant proportion of lesions and by the lack of assessment of key histological aspects of the lesion (i.e., in situ versus invasive component) or by the contamination of benign nevus cells in the setting of melanoma arising in a nevus. In addition, there are few (if any) markers shared between these different assays. There are few studies that have compared the performance of these assays in the same tissue set. In one such study, a high degree of discordance was found between the assays and with the consensus pathologic diagnosis (Minca et al. 2016). Thus, at the current time, none of these assays is perfect, and additional advancements in this setting are clearly warranted.

Prognostic Markers for Primary Melanoma

Melanoma exhibits an unpredictable clinical behavior, with the potential to metastasize to virtually any distant site, either early or late in the course of the disease. As a result, prognostic marker research to define subsets of melanoma patients with differential risk of metastasis and death has been an area of active investigation. Historically, this has relied on the identification of histological features that can refine the risk for an individual risk.

Molecular markers represent the next frontier in melanoma prognosis research. Numerous individual prognostic markers have been defined with the potential ability to predict melanoma prognosis, and their inclusion is beyond the scope of this chapter, but the reader is directed to a recent review of these individual markers (Mandala and Massi 2014). In addition, a meta-analysis of melanoma prognostic marker research performed in 2009 identified certain individual markers as worthy of further validation (Gould Rothberg et al. 2009b). In this section, we will focus our attention on markers that have undergone a significant degree of analysis and validation, which is primarily the case with a number of multi-marker signatures using qRT-PCR and IHC platforms.

Gene Expression Profiling of Melanoma Prognostic Markers

The advent of microarray analysis resulted in a number of studies that attempted to identify molecular markers of aggressive melanoma. One salient example of such a study was a pangenomic oligonucleotide array analysis of 58 patients, identifying the following markers that were predictive of melanoma prognosis using IHC analysis: MCM3, MCM4, MCM6, KPNA2, and geminin (Winnepenninckx et al. 2006).

More recently, a 31 gene profiling assay using qRT-PCR was commercially launched as the DecisionDx test by Castle Biosciences to predict melanoma prognosis (Table 3). To date, a number of retrospective analyses have been performed and reported using this assay. One analysis included tissues from 217 patients with primary melanoma who underwent sentinel lymph node biopsy (SLNB). Radial basis machine modeling was performed to predict the risk of melanoma metastasis, with the identification of low-risk

Table 3 Molecular prognostic markers for melanoma by testing platform

Gene symbol	Gene name	Reference(s)
qRT-PCR markers		
BAP1	BRCA1-associated protein 1	Gerami et al. (2015a,b) and Zager et al. (2018)
MGP	Matrix G1a protein	Gerami et al. (2015a,b) and Zager et al. (2018)
CXCL14	Chemokine, C-X-C motif, ligand 14	Gerami et al. (2015a,b) and Zager et al. (2018)
SPP1	Secreted phosphoprotein 1 (aka osteopontin)	Gerami et al. (2015a,b) and Zager et al. (2018)
CLCA2	Chloride channel accessory 2	Gerami et al. (2015a,b) and Zager et al. (2018)
S100A8	S100 calcium-binding protein A8	Gerami et al. (2015a,b) and Zager et al. (2018)
BTG1	B-cell translocation gene 1	Gerami et al. (2015a,b) and Zager et al. (2018)
SAP130	Sin3A-associated protein, 130 kDa	Gerami et al. (2015a,b) and Zager et al. (2018)
ARG1	Arginase 1	Gerami et al. (2015a,b) and Zager et al. (2018)
KRT6B	Keratin 6B	Gerami et al. (2015a,b) and Zager et al. (2018)
GJA1	Gap junction protein, alpha 1, 43 kDa	Gerami et al. (2015a,b) and Zager et al. (2018)
ID2	Inhibitor of DNA binding 2	Gerami et al. (2015a,b) and Zager et al. (2018)
EIF1B	Eukaryotic translation initiation factor 1B	Gerami et al. (2015a,b) and Zager et al. (2018)
S100A9	S100 calcium-binding protein A9	Gerami et al. (2015a,b) and Zager et al. (2018)
CRABP2	Cellular retinoic acid-binding protein 2	Gerami et al. (2015a,b) and Zager et al. (2018)
KRT14	Keratin 14	Gerami et al. (2015a,b) and Zager et al. (2018)
ROBO1	Roundabout, *Drosophila*, homolog of, 1	Gerami et al. (2015a,b) and Zager et al. (2018)
RBM23	RNA-binding motif protein 23	Gerami et al. (2015a,b) and Zager et al. (2018)
TACSTD2	Tumor-associated calcium signal transducer 2	Gerami et al. (2015a,b) and Zager et al. (2018)
DSC1	Desmocollin 1	Gerami et al. (2015a,b) and Zager et al. (2018)
SPRR1B	Small proline-rich protein 1B	Gerami et al. (2015a,b) and Zager et al. (2018)
TRIM29	Tripartite motif containing 29	Gerami et al. (2015a,b) and Zager et al. (2018)
AQP3	Aquaporin 3	Gerami et al. (2015a,b) and Zager et al. (2018)
TYRP1	Tyrosinase-related protein 1	Gerami et al. (2015a,b) and Zager et al. (2018)
PPL	Periplakin	Gerami et al. (2015a,b) and Zager et al. (2018)
LTA4H	Leukotriene A4 hydrolase	Gerami et al. (2015a,b) and Zager et al. (2018)
CST6	Cystatin 6	Gerami et al. (2015a,b) and Zager et al. (2018)
KRT9	Keratin 9	Brunner et al. (2013)
DCD	Dermicidin	Brunner et al. (2013)
PIP	Prolactin-induced protein	Brunner et al. (2013)
SCGBID2	Secretoglobin family 1D member 2	Brunner et al. (2013)
SCGB2A2	Secretoglobin family 2A member 2	Brunner et al. (2013)
COL6A6	Collagen alpha 6 (VI)	Brunner et al. (2013)
KBTBD10	Kelch repeat and BTB (POZ) domain containing 10	Brunner et al. (2013)
ECRG2	Esophageal cancer-related gene 2	Brunner et al. (2013)
HES6	Hairy and enhancer of split 6	Brunner et al. (2013)
IHC markers		
ATF2	Activating transcription factor 2	Gould Rothberg et al. (2009a)
WAF1	Cyclin-dependent kinase inhibitor 1A (aka p21)	Gould Rothberg et al. (2009a)
CDKN2A	Cyclin-dependent kinase inhibitor 2A (aka p16)	Gould Rothberg et al. (2009a)
FN1	Fibronectin 1	Gould Rothberg et al. (2009a)
CTNNB1	β-Catenin	Gould Rothberg et al. (2009a)
RGS1	Regulator of G signaling protein 1	Kashani-Sabet et al. (2009, 2017)
NCOA3	Nuclear receptor coactivator 3 (aka SRC-3, AIB1)	Kashani-Sabet et al. (2009, 2017)
SPP1	Secreted phosphoprotein 1 (aka osteopontin)	Kashani-Sabet et al. (2009, 2017)

(termed class 1) and high-risk (termed class 2) subgroups. By Kaplan-Meier analysis, the gene expression profile (GEP) was predictive of disease-free survival (DFS), distant metastasis-free survival (DMFS), and overall survival (OS) (Gerami et al. 2012). Multivariate Cox regression analysis including GEP and SLNB, but apparently no other factors, revealed a significant impact of GEP and SLN status on DFS and DMFS, but only of GEP on OS. In a different report, the GEP was assessed in a training cohort of 107 cases and a validation cohort of 104 cases (Gerami et al. 2009). In the validation set, the GEP was significantly predictive of DFS, OS, and melanoma-specific survival (MSS) by Kaplan-Meier analysis. Multivariate Cox regression performed on stage I/II cases incorporating GEP, AJCC stage (IIB/IIC), tumor thickness, ulceration, mitotic rate ($>1/mm^2$), and age revealed an independent impact of GEP and AJCC stage on DFS. In the most recently reported analysis to date (Zager et al. 2018), the GEP was assessed in an independent cohort of 523 primary melanoma patients with at least 5 years of follow-up or documented relapse. Class 1 patients had a 98% 5-year MSS rate, compared with 78% for class 2 patients. Multivariate Cox regression analysis of RFS and DMFS examining GEP, tumor thickness, mitotic rate ($>1/mm^2$), ulceration, and SLN status revealed an independent impact of tumor thickness, SLN status, followed by GEP, for DFS and DMFS. One major limitation of this assay is its strong dependency on tumor cell content making it rather unlikely of help particularly for the large number of thin melanomas which eventually progress.

In addition, a separate nine-gene qRT-PCR-based test termed MelaGenix has been offered by NeraCare to predict melanoma prognosis. The assay was developed on fresh-frozen melanomas and tested on a training subset of 38 cases, a training cohort of 91 cases, and a validation cohort of 44 cases (Brunner et al. 2013). A dichotomized risk score was developed using this gene signature and was significantly predictive of OS in the training cohort. Multivariate Cox regression analysis of molecular risk score, AJCC stage, Clark level, age, and sex, revealed AJCC stage, followed by the risk score, as independently predictive of OS. The signature-based GEP score was recently confirmed on formalin-fixed paraffin-embedded (FFPE) melanoma and was shown to be independent of hospital-specific tissue fixation procedures and highly stable even in aged FFPE samples. Interestingly, the MelaGenix GEP score is determined in whole FFPE tissue sections and does not require microdissection of tumor tissue. Out of the GEP score, seven genes originate from tumor stroma making this assay particularly suitable for analyzing the prognosis of primary cutaneous melanomas.

Taken together, these GEP assays await evaluation in prospectively collected cohorts with defined eligibility. In addition, to date, a predictive signal for either of the assays in identifying benefit to any adjuvant therapy regimen for melanoma (e.g., interferon alpha, ipilimumab, anti-PD-1 antibody, or targeted therapy) has not been demonstrated. As a result, at this time, use of GEP assays has not been recommended by the National Comprehensive Cancer Network (NCCN) guidelines for melanoma patients outside of the setting of a clinical trial.

IHC Analysis of Melanoma Prognostic Markers

Beyond gene expression profiles, several putative prognostic factors for melanoma have been assessed for their predictive impact using IHC analysis. Specifically, two multi-marker signatures have been developed and undergone more extensive analysis and will be discussed in detail here.

In 2009, Rimm and colleagues reported the development and performance of a melanoma prognostic model following an analysis of 38 candidate markers using the automated quantitative analysis (AQUA) method (Gould Rothberg et al. 2009a). Assessment of these markers in a tissue microarray (TMA) cohort, including a training sample of 192 cases, identified a consistent prognostic signal for five markers (ATF2, p21/WAF1, p16/CDKN2A, β-catenin, and fibronectin). An algorithm was developed to combine marker expression scores, and a dichotomized analysis of low- versus high-risk subgroups based on

marker expression showed a significant difference in MSS between the two subgroups in the training set. This differentiation was also observed in the validation set, with a 10-year survival of greater than 90% in the low-risk group versus 60% in the high-risk group, albeit with a trend toward statistical significance ($P = 0.09$). Multivariate analysis of MSS that included the multi-marker score, tumor thickness, age, anatomical site, sentinel lymph node status, and receipt of nonsurgical therapy revealed (in an order of descending statistical significance) receipt of nonsurgical therapy, age, sentinel lymph node status, the multi-marker score, and tumor thickness to be significantly predictive of MSS.

Separately, also in 2009, Kashani-Sabet and colleagues reported the performance of a three-marker IHC assay incorporating the following markers: NCOA3, SPP1, and RGS1 (Kashani-Sabet, Venna, et al. 2009). This assay was assessed initially in a TMA cohort of 395 primary melanoma patients from the USA and separately in tissue sections from a cohort of 141 patients from two German centers. Marker expression was assessed both by pathologist scoring and using a digital imaging platform. An index was developed in each cohort to combine marker expression levels. In the US cohort, by multivariate logistic regression analysis, the multi-marker expression score was independently predictive of SLN status, following age, but with an impact greater than tumor thickness. Multivariate Cox regression analysis of tumor thickness, SLN status, ulceration, Clark level, age, gender, anatomical location, and multi-marker score revealed the multi-marker score as the top factor predicting disease-specific survival (DSS). A dichotomization of multi-marker scores revealed a 5-year survival of 96% in the low-risk group versus 60% in the high-risk group. Separately, the multi-marker score was also independently predictive of DSS in the German cohort, surpassing tumor thickness and other available factors.

More recently, this three-marker IHC assay was assessed on tissues collected as part of the Eastern Cooperative Oncology Group trial E1690 examining the utility of two doses of interferon alpha versus observation in patients with resected, high-risk melanoma, including eligible patients with a tumor thickness of greater than 4 mm or node-positive disease (Kashani-Sabet et al. 2017). The tissue cohort from the E1690 trial included both primary melanoma specimens and lymph node metastases. IHC analysis was performed to determine expression of NCOA3, SPP1, and RGS1, and marker analysis was assessed using a digital imaging platform. Once again, an index was developed to combine marker analysis and was dichotomized to split the cohort into low-risk and high-risk subgroups. By Kaplan-Meier analysis, the multi-marker score was significantly predictive of relapse-free survival (RFS) and OS in the entire cohort. By stepwise multivariate Cox regression analysis, multi-marker score was the only factor significantly predictive of RFS and was followed by tumor thickness as the only factors significantly predictive of OS in the entire cohort. When a potential interaction between marker expression and treatment assignment was analyzed, the interferon-treated arms (combining both low-dose and high-dose cohorts) had a significantly improved RFS versus the observation arm in the molecularly defined low-risk subgroup. These results demonstrated the independent prognostic significance of this assay in a prospectively collected cohort amassed in a cooperative group-led clinical trial and identified a potential subset of patients that could benefit from systemic therapy with IFN. Additional validation studies of this IHC assay are currently planned in other clinical trial cohorts.

In conclusion, significant progress has also been made in the development of molecular prognostic markers for primary melanoma. This effort has been facilitated by genome-wide profiling efforts of distinct stages of melanoma progression that have identified a plethora of putative prognostic markers. However, the development of molecular prognostic markers for melanoma has been hampered by the lack of large, well-annotated tissue cohorts of primary melanoma patients with sufficient follow-up. For the promising multi-marker assays developed, further development has been hampered by the lack of sufficient prospective validation of these assays. Lastly, while accurately predicting melanoma prognosis

would be useful, current treatment guidelines do not recommend aggressive radiographic surveillance in high-risk patient cohorts. As a result, identifying a high-risk cohort may not be clinically actionable. However, given the recent development of landmark, effective adjuvant therapies for melanoma (both immunotherapies and targeted therapies), these assays would have their greatest clinical utility in identifying subsets of patients that derive either the maximum benefit or, alternatively, no benefit from routinely performed clinical interventions.

Tumor Environment-Based Non-soluble Biomarkers

Melanomagenesis and tumor progression are a complex and dynamic process that is manifested by tumor heterogeneity and a myriad of yet to be fully understood interactions within the tumor microenvironment. Historically, the most robust diagnostic and prognostic parameters in cutaneous melanoma have been primary tumor characteristics detected histopathologically on routinely processed hematoxylin and eosin-stained tissue sections. These factors include tumor thickness, ulceration, proliferation activity, lymphovascular invasion, and the presence of micrometastases. In the last two decades, tumor-associated molecular biomarkers have been identified, individually and in combination, that correlate with diagnosis and prognosis. Most recently, attention has been given to non-tumor cell markers in the primary tumor environment that facilitate local tumor progression and metastasis. In addition to tumor-infiltrating lymphocytes and other immune factors, biomarkers in the tumor stromal environment may aid in diagnosis, predict prognosis, and even serve as therapeutic targets (Jacobs et al. 2012). It is clear that the tumor microenvironment is shaped by the cross talk between mesenchymal stromal cells and immune cells (English 2013). This realm will likely serve as the next frontier of effective melanoma therapy.

Melanoma tumor cell and immune cell interactions have been known to correlate with prognosis since the late 1980s when Clark et al. identified an improved prognosis in patients with primary cutaneous melanomas that had robust "brisk" lymphocytic infiltrates (Clark et al. 1989). Further refinements of this histopathological factor revealed that there is a complex interplay between the distribution and density of the infiltrates and potential for tumor immune escape or tumor suppression (Clemente et al. 1996). Most recently, chemokines and their receptors have been described to orchestrate melanoma cell and immune cell dynamics (Neagu et al. 2015). Chemoattractant cytokines were initially identified as factors that recruited leukocytes in inflammatory and immune responses (Cyster 1999). It is now known that chemokines play a role in a broad range of melanoma pathways to tumor growth and metastasis including maintenance of tumor-initiating cells, cell proliferation, epithelial-mesenchymal transition-like processes, angiogenesis, senescence, epigenetic responses to oxidative stress, and immune evasion (Sarvaiya et al. 2013).

Tumor-Initiating Cells

A relationship between melanoma-initiating cells and antitumor immunity has been identified (Schatton et al. 2010). Stem cells, also known as tumor-initiating cells, are capable of self-renewal and differentiation and are responsible for tumor development and therapeutic resistance (Schatton et al. 2008). The ATP-binding cassette (ABC) efflux transporter ABCB5 is a marker of tumor-initiating cells that has been shown to maintain slow cycling chemoresistant cells through a complex cytokine signaling pathway that includes IL1-beta, IL-8, and CXCR1, thus playing a role in stem cell maintenance and tumor growth (Wilson et al. 2014). Another marker of melanoma-initiating cells, CD133 (human prominin 1), is a transmembrane pentaspan glycoprotein that plays a role in vasculogenic mimicry and formation of a vascular niche (Lai et al. 2012). It is likely that a restricted number of tumor cells may possess the capacity to modulate tumor-directed immune responses; clearer understanding of these processes will aid in the development of future therapeutic strategies.

Epithelial-Mesenchymal Transition

Melanoma has been shown to progress through a distinct epithelial-mesenchymal transition (EMT)-like process (Caramel et al. 2013). This transition to cells with enhanced migration, invasiveness, resistance to apoptosis, and production of extracellular matrix components is a plastic phenomenon. Tumor cells may cycle between a differentiated state (associated with increased ZEB2 and Slug) and an oncogenic state (with high levels of ZEB1 and twist) (Li et al. 2015). Additionally, tumor cells may alter the environmental niche through release of miRNA containing melanosomes into fibroblasts (Dror et al. 2016). It is likely that the plasticity of this transition between differentiated and oncogenic states contributes to the tumor heterogeneity characteristic of melanoma. Several factors involved in this transition have been explored as potential biomarkers, including the transcription factor SNAI1 (snail1) which may lead to reduced E-cadherin expression and induction of N-cadherin (Miller and Mihm 2006). Indeed, expressions of the EMT-associated proteins N-cadherin, osteopontin, and SPARPC/osteonectin are significantly associated with the risk of metastasis (Alonso et al. 2004). Additionally, twist1 and twist2 are regulatory proteins that induce EMT and may also have a role in limiting oncogene-induced senescence (Ansieau et al. 2008). On the other hand, mechanisms of senescence induction include activation of DNA damage signaling by oncogenes and short telomeres (Bennett 2008). The irreversible arrest of proliferation associated with senescence occurs through the p53 and p16-pRB tumor suppressor pathways (Campisi and d'Adda di Fagagna 2007). In addition to adhesion marker interactions and interference with senescence, stromal-derived proteases may modulate antitumor immune responses. Melanoma-associated fibroblast production of metalloproteinases including MMP-7 decreases tumor cell susceptibility to natural killer cell-mediated tumor necrosis (Ziani et al. 2017). Immunohistochemical analysis of MMP-7 reveals increased expression in melanoma that correlates with tumor thickness and adverse prognosis (Kawasaki et al. 2007). These EMT-like processes likely advance melanoma progression through promoting invasion and a proliferative advantage (Li et al. 2015).

Epigenetic Changes

Epigenetics is an important mechanism by which gene expression may be modified in cancer. DNA methylation is one of the epigenetic hallmarks that is most studied. Hypermethylation of CpG islands in the promoter region leads to gene silencing and has been described for genes throughout melanoma progression and metastasis (Rothhammer and Bosserhoff 2007; Schinke et al. 2010; Tanemura et al. 2009). Loss of 5-hydroxymethylcytosine (5-hmC) in melanoma has been described as a fundamental epigenetic event that correlates with tumor progression and is associated with decreased expression of the enzyme ten-eleven translocase (TET) (Lian et al. 2012). Epigenetic regulation of CD73 has also been described (Wang et al. 2012). CD73 is an ectonucleotidase expressed on Tregs that along with adenosine (ADO) has been implicated in tumor-associated immunosuppression. ADO levels in the extracellular microenvironment are usually low; however, high levels have been identified at the tumor stromal interface. CD73+ is associated with increased ADO production and has been correlated with poor prognosis (Wang et al. 2012). These examples of epigenetic modifications in melanoma are but a few of those that have been recently described. As techniques to detect epigenetic biomarkers evolve, this field may provide important novel information regarding prognosis and potential response to therapy (Greenberg et al. 2014).

Immune Escape Mechanisms

Recruitment of suppressor immune cells, including Tregs and tumor-associated macrophages, may facilitate tumor cell evasion of the immune system (Buchbinder and Hodi 2015). Insufficient co-stimulation of the immune system by tumor cells limits antitumor immunity. For example,

CTLA-4 is a key inhibitory receptor that blocks co-stimulation. CTLA-4 blockade with ipilimumab is an effective therapy that increases antitumor immune responses through enhanced effector T-cell function and inhibition of Treg activity. The impressive clinical responses seen with immunotherapy, in particular anti-CTLA-4 and anti-PD-1, are based on the critical role the immune system plays in melanoma tumor progression. However, many patients' tumors are refractory to these therapies. Dendritic cell-mediated tumor evasion mechanisms may contribute to this; tolerized dendritic cells drive Treg differentiation and may establish a milieu of immune privilege. Tumor and stromal cell-derived cytokines, including TGF-beta and prostaglandin-E2, may exert immunosuppressive effects by contributing to the establishment of immune tolerance (Balsamo et al. 2009; Pietra et al. 2012). Similarly, indoleamine (2,3)-deoxygenate (IDO) generates an immunosuppressive tumor microenvironment by suppressing effector T cells and actively tolerizing the tumor microenvironment by promoting Treg development (Holtzhausen et al. 2015). The Wnt-beta-catenin signaling pathway promotes dendritic cell tolerization through induction of IDO. In sentinel lymph nodes, a decrease in interdigitating dendritic cells, antigen-presenting cells involved in T-cell activation, has been associated with a poor prognosis (Cochran et al. 2004). Melanoma metastasis in sentinel lymph nodes is associated with a higher frequency of Foxp3+ CD4+ CD25 high Tregs and IDO-expressing dendritic cells (Lee et al. 2011). Increased IDO expression by dendritic cells in sentinel lymph nodes has been shown to correlate with adverse prognosis (Speeckaert et al. 2012). This cytokine microenvironment is likely to determine the functional immune status of the sentinel lymph nodes. As such, these complex stromal immune interactions remain a topic of intense investigation.

There are many other pathways that are being elucidated in the immune evasive mechanisms employed by melanoma. For example, the surface glycoprotein CD47 is a regulator of melanoma immune evasion. This transmembrane integrin-associated protein is present on all normal cells and upregulated in some melanoma cells; ligands include thrombospondin-1 (TSP1) and signal-regulatory protein alpha (SIRP-alpha) (Brown and Frazier 2001). Increased expression of CD47 in melanoma has been associated with increased risk of metastasis and poor survival (Fu et al. 2017). SIRP-alpha is expressed on myeloid cells including dendritic cells; binding of CD47 to SIRP-alpha leads to reduced macrophage phagocytosis allowing melanoma to evade elimination by innate immunity. Indeed, CD47 expression on tumor cells has been coined a "don't eat me" signal enhancing tumor cell survival by inhibiting phagocytosis by macrophages (Jaiswal et al. 2009; Willingham et al. 2012). TSP1 is highly expressed in tumor stroma and has many functional interactions other than with CD47. CD47-TSP1 interactions have been associated with modulation of nitric oxide (NO) signaling and vascular responses (Isenberg et al. 2006). Functional CD47-TSP1 interactions have also been associated with an increased capacity for self-renewal when CD47 is highly expressed on tumor-initiating cells (cancer stem cells) (Kaur and Roberts 2016). Initially identified as an integrin-associated protein, CD47 also is necessary for ligand recognition by a variety of integrins including alphavbeta3, alpha2beta1, and alpha4beta1 (Brown and Frazier 2001). These studies and many others have identified the CD47-SIRP-alpha interaction as a promising innate immune checkpoint; however, there are important mechanistic issues still to be resolved. This is a rapidly expanding field which will likely have a major impact on melanoma therapy (Matlung et al. 2017).

In summary, the process of melanoma tumorigenesis, tumor progression, and metastasis is a dynamic interplay between the tumor and the surrounding microenvironment including stromal and immune elements. As we learn more about these interactions, it is likely that melanoma microenvironment biomarkers will be clinically deployed. Future investigations of melanoma microenvironment biomarkers will face the same challenges posed in the past: it is critical to the understanding of these pathways that tissue-based studies are performed that identify cell subsets. This will require an ever-diligent attention

to tumor sample management and annotation. Additionally, the tissue-based technologies that provide for simultaneous detection of multiple biomarkers localized to cell subsets and individual cells will be required for a complete understanding of the complex tumor, immune, stromal interactions in melanoma tumor progression.

Soluble Biomarkers

Lactate Dehydrogenase (LDH)

At present, the most widely used prognostic serum biomarker in the clinical care of melanoma patients is lactate dehydrogenase (LDH). LDH is an unspecific biomarker indicating high metabolism and/or high tumor load in a variety of tumor entities including melanoma (Manola et al. 2000; Egberts et al. 2012). Studies comparing different serological markers including LDH, S100B, and MIA in multivariate analysis showed LDH as the strongest independent prognostic factor in stage IV melanoma patients (Deichmann et al. 1999). Due to its high prognostic significance together with easy, cost-efficient, and widely distributed detection techniques, serum LDH is the only molecular marker so far that has been incorporated into the melanoma staging and classification system of the AJCC, beginning with the edition of 2001 (Balch et al. 2001a). The implementation of serum LDH into this classification system took place after LDH was demonstrated as an independent predictor of overall survival in a very large cohort of nearly 8000 patients with advanced metastatic melanoma (Balch et al. 2001b). This cohort showed a 1-year survival rate of 65% for patients with normal serum LDH, whereas patients with elevated LDH levels had a significantly reduced 1-year survival rate of 32% only. Serum LDH is therefore commonly used in the clinical routine of melanoma patient care, in particular in patients with advanced metastatic disease. It serves as a reliable prognostic marker before the start of a new systemic therapy and in regular intervals thereafter in order to monitor treatment response. It is moreover used as a common stratification parameter in randomized clinical trials testing therapeutic interventions in advanced inoperable disease. Due to its low specificity, false-positive results of elevated LDH levels are common and originate from conditions like hemolysis, muscle or liver disease, injuries, or other pathologies. In patients with low or clinically undetectable tumor burden, LDH serum levels are generally normal; thus, serum LDH cannot be recommended as a marker of minimal residual disease or early relapse (Eisenstein et al. 2018).

S100B

The S100 protein is a 21-kd thermolabile acidic dimeric protein which was originally isolated from tissue of the central nervous system. It consists of two subunits, alpha and beta, in different pairings. S100 is of functional importance for the assembly of microtubules and interacts in a calcium-dependent manner with the tumor suppressor gene p53. The beta subunit (S100B) is expressed in cells of the central nervous system as well as in cells of the melanocytic lineage. Therefore, the serum concentration of S100B has been described as a biomarker of central nervous system damage (Persson et al. 1987) as well as of the presence of melanoma metastasis (Guo et al. 1995). The serum level of S100B is an indicator of tumor burden and therefore correlates with the clinical stage of melanoma patients. With regard to prognosis, S100B is a useful marker in melanoma patients with presence of metastases (Schultz et al. 1998; Hauschild et al. 1999a), but fails to provide prognostic significance in patients with microscopic disease, as well as in patients who are clinically tumor-free after surgery (Guo et al. 1995; Acland et al. 2002; Egberts et al. 2010). However, a meta-analysis of 22 studies including a total of 3393 melanoma patients revealed S100B as a significant prognostic factor in all clinical stages of melanoma, even in stages I–III (Mocellin 2008). Despite S100B is a melanoma serum marker of higher specificity than LDH, it still has limitations not only by elevated levels due to central nervous system damage but also by liver or cardiovascular diseases (Vaquero et al. 2003; Li et al. 2011). The stringent correlation of serum

S100B concentrations with tumor burden, however, renders it a useful marker for the monitoring of treatment response in patients with advanced metastatic melanoma (Hauschild et al. 1999b; Egberts et al. 2012); see Fig. 2. Its use in the routine clinical care of melanoma patients is still mainly restricted to European countries.

Other Serum Biomarkers (CRP, FGF, IL-8, MIA, SAA, VEGF, YKL-40)

Worldwide, the serum biomarker which is most widely implemented into the clinical routine of melanoma patient care is LDH. S100B is the second often used serum biomarker and is of similar prognostic significance as LDH with higher specificity for melanoma versus other cancer entities.

Various other serum factors have been investigated for their prognostic significance in melanoma. Up to now, none of them revealed a higher sensitivity-specificity profile than LDH or S100B. An extensively studied serum protein named melanoma inhibitory activity (MIA) was originally detected in melanoma cell culture supernatants (Bogdahn et al. 1989) and was shown to exert an important role in cell-matrix interaction, invasion, and metastasis (Blesch et al. 1994). Studies comparing MIA and S100B demonstrated that S100B is superior to MIA in its value as an early indicator of tumor progression, relapse, or metastasis (Deichmann et al. 1999; Krähn et al. 2001). Proangiogenic factors like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and interleukin-8 (IL-8) have been demonstrated to reveal prognostic significance in case of elevated serum levels (Ugurel et al. 2001a; Sanmamed et al. 2014; Yuan et al. 2014). Besides, proteins associated with antigen presentation and recognition like HLA molecules, as well as receptor or ligand molecules associated with anticancer immune response like NKG2D, CEACAM, and others, have been described as soluble variants detectable in sera from melanoma patients and correlating to the patients' prognosis (Ugurel et al. 2001b; Rebmann et al. 2002; Paschen et al. 2009; Sivan et al. 2012). Members of the acute phase proteins, like C-reactive protein (CRP) and serum amyloid

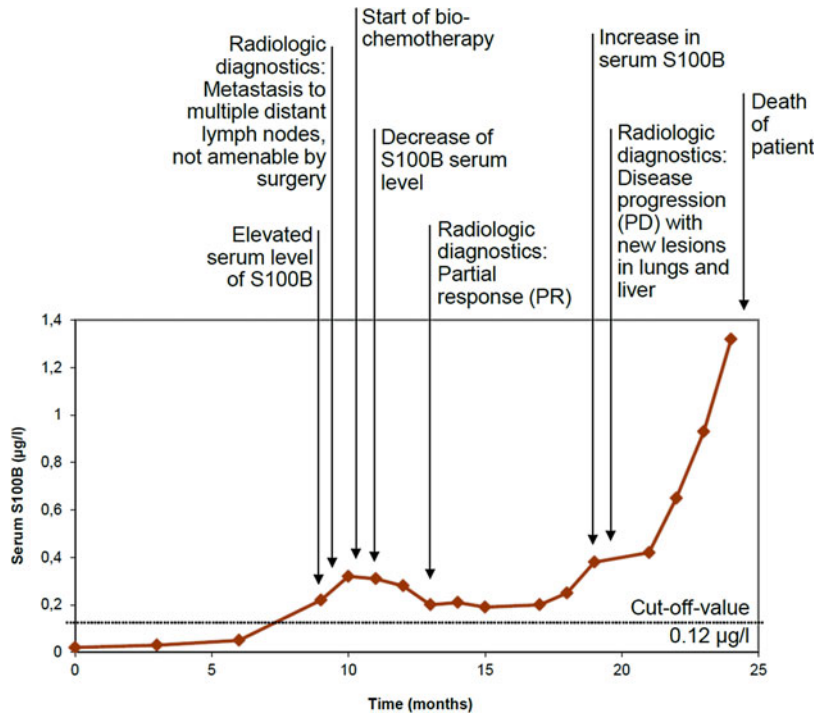

Fig. 2 Monitoring of the course of melanoma disease by the serum marker S100B. (From Ugurel 2005)

A (SAA), are associated with inflammatory processes and have also been described as prognostically significant serum factors in melanoma (Deichmann et al. 2004; Findeisen et al. 2009). YKL-40, a chitinase-like glycoprotein produced by cancer cells as well as by inflammatory cells, has recently been described as a prognostic marker in melanoma, giving promising results even in tumor-free early-stage patients (Schmidt et al. 2006). However, in comparative studies, YKL-40 showed an inferior prognostic value as compared to S100B (Egberts et al. 2012). Moreover, YKL-40 was reported to lose its prognostic significance in patients treated with interferons (Krogh et al. 2016). None of the abovementioned serum markers succeeded in reproducible superiority compared to LDH and/or S100B, resulting in no further development of these biomarkers into clinically applicable test systems.

Circulating Tumor Cells (CTCs)

Like cells of other cancer entities, melanoma cells are known for their ability to leave their tissue of origin and enter the blood stream as free floating cells. These circulating tumor cells (CTCs) become detectable in blood draws and may serve as biomarkers of tumor burden, prognosis, and treatment response. The detection and capture of CTCs in the blood stream mainly rely on surface protein or antigen expression. However, in melanoma, this approach is limited due to the inter- as well as intraindividual heterogeneity of the antigen profiles presented by the patients (Khoja et al. 2014; De Souza et al. 2017). Thus, various antigens like tyrosinase, Melan-A/MART-1, gp100, MAGE-3, and EpCAM have been used alone or in different panel combinations to identify CTCs in melanoma patients. Moreover, various techniques have been used to capture and quantify the identified CTCs including magnetic or electrophoretic separation systems, microfluidics-based techniques, filtration approaches, and cell exclusion systems (Khoja et al. 2015; De Souza et al. 2017; Lim et al. 2018). This inconsistency in methodologies resulted in a high variation of results reported of CTC numbers in melanoma patients of different disease stages and led to a questionable clinical applicability of CTC detection and quantification as a biomarker in melanoma (Nezos et al. 2011). However, CTCs have been demonstrated in multiple studies using non-comparable methodologies to be of prognostic value in melanoma patients; see Fig. 3. A meta-analysis of 53 studies describes a correlation of the presence of CTCs in a patient's blood stream with advanced disease stage and impaired progression-free as well as overall survival (Mocellin et al. 2006). One study described that melanoma patients with a positive detection of CTCs after completion lymph node dissection revealed a higher risk of disease recurrence (Mocellin et al. 2004). Moreover, CTC numbers were shown to be of use in the monitoring of systemic therapies (Khoja et al. 2013). Despite these promising results, the clinical use of CTC detection and quantification as a reliable biomarker will remain limited until techniques will be found providing valid and reproducible results applicable to a majority of patients.

Circulating Tumor DNA (ctDNA)

The abovementioned problems in the capture and quantification of CTCs could be overcome by the indirect detection of tumor cells via DNA sequences which are present in tumor cells only and not in benign cells and tissues. In melanoma, the common driver gene mutations like in BRAF or NRAS are perfectly suited for this purpose. The circulating tumor DNAs (ctDNA) mainly originate from apoptotic or necrotic circulating tumor cells and have been shown to be detected and quantified in peripheral blood samples (Schwarzenbach et al. 2011). To this end, plasma samples are superior to serum samples due to a greater extent of cell lysis during the clotting process of serum (Sorber et al. 2017). Due to the low abundance of ctDNA, its detection requires highly sensitive and specific techniques. Thus, modern detection technologies like digital droplet PCR or allele-specific ligation PCR have significantly improved the detection rate of ctDNA in

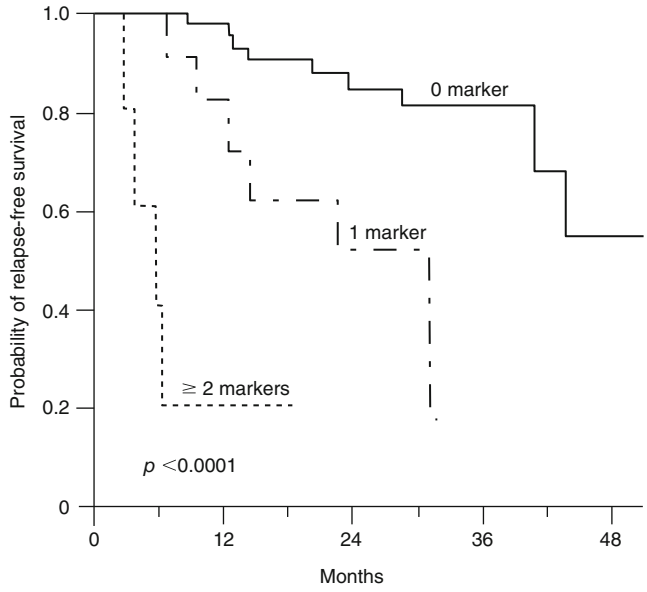

Fig. 3 Prognostic utility of CTCs in blood of patients with melanoma. Kaplan-Meier curves of relapse-free survival of CTC monitoring in patients with AJCC stage III melanoma in a multicenter trial of biochemotherapy treatment. After treatment, relapse-free survival decreased significantly when blood specimens were qRT (real-time RT-PCR)–positive for MART-1, GalNAc-T, and/or MAGE-A3 ($p = 0.0003$, $p < 0.0001$, and $p < 0.0001$, respectively). The level of decrease was directly correlated with the number of positive markers ($p < 0.0001$). (From Koyanagi et al. 2005)

different tumor entities. Additionally, targeted sequencing techniques like amplicon sequencing or hybrid-capture sequencing have been used, with the disadvantage of high data volumes to be processed after each analysis (Newman et al. 2014).

Both the presence and the quantity (copy number) of ctDNA may serve as prognostic markers in cancer patients. In melanoma, peripheral blood samples from patients with known tumor tissue BRAF and NRAS mutational status have been analyzed for these mutations in ctDNA in various studies, revealing an association of positive results with impaired prognosis and reduced survival (Huang and Hoon 2016). ctDNA frequencies were associated with tumor burden, location of metastasis, and tumor cell metabolism (Lim et al. 2018). In melanoma, patients in early disease stages are often negative for ctDNA detection (Daniotti et al. 2004). Nevertheless, in patients in advanced disease stages and clinically detectable tumor burden, ctDNA has been shown to be a useful marker in the monitoring of treatment response and outcome. Patients starting with a blood draw testing mutation-positive into a treatment with BRAF/MEK inhibition or anti-PD1 immunotherapy revealed impaired treatment outcomes compared to patients who were mutation-negative at baseline (Gray et al. 2015). Moreover, patients starting with mutation-positive ctDNA results but turning mutation-negative during treatment were superior in their treatment outcomes compared to patients who stayed mutation-positive during the continuous treatment course (Lee et al. 2017). However, the concordance between peripheral blood and tumor tissue is not yet satisfactory in all cases. Thus, matched plasma and tumor tissue samples from melanoma patients showed a 68% concordance only for the detection of TERT promoter mutations in ctDNA versus DNA extracted from tissue (McEvoy et al. 2017).

Besides of mutations, tumor-specific gene methylation patterns of ctDNA are detectable in the peripheral blood and have been shown to serve as biomarkers in cancer patients (Warton and Samimi 2015). Tumor suppressor genes are known to be frequently inactivated by methylation as an early event in the etiopathogenesis

of multiple cancer entities including melanoma (Calapre et al. 2017). Thus, it has been shown that the methylation of tumor suppressor genes like ras association domain family 1 isoform A (RASSF1A), retinoic acid receptor beta 2 (RAR-b2), or O-6-methylguanine-DNA methyltransferase (MGMT) is associated with an inferior survival and therapy outcome in melanoma patients (Hoon et al. 2004; Mori et al. 2005, 2006); see Fig. 4.

Circulating MicroRNA (miRNA)

MicroRNAs (miRNAs) are short, noncoding RNA molecules which are functional in the regulation and modulation of gene transcription, post-transcription, and epigenetic expression. miRNAs are mainly actively secreted by their cells of origin and not passively released after cell apoptosis or necrosis like ctDNAs (Chen et al. 2012). Additionally, miRNAs in comparison to ctDNAs are relatively stable in the blood stream since they are commonly packed in vesicles or bound to proteins or lipoproteins (Vickers et al. 2011) and therefore are interesting biomarker candidates (Mitchell et al. 2008). Circulating miRNA expression in blood samples from melanoma patients has been shown to exert diagnostic, prognostic, and predictive relevance (Aftab et al. 2014; Fattore et al. 2017). In many studies, this correlation was not found for only one single miRNA (Kanemaru et al. 2011) but for distinct miRNA profiles (Friedman et al. 2012). A recent study described a seven-miRNA panel (MELmiR-7) out of 17 miRNAs to correctly discriminate between melanoma patients of all disease stages and healthy controls with a sensitivity of 93% and a specificity of 82% (Stark et al. 2015). Moreover, the authors reported that this miRNA panel characterized the patients' overall survival with higher accuracy than the serum markers LDH and S100B. Notably, the majority of miRNAs are not tumor-specific, but may also be expressed in inflammation, immune activation, and other conditions (Cortez et al. 2011). As another disadvantage, miRNAs in serum or plasma have to be quantified in relation to housekeeping miRNAs like U6, miR-451, or miR-16, which might be deregulated in cancer patients (Aftab et al. 2014). For translating the promising results of miRNA detection and quantification into clinical use in the routine care of melanoma patients, validation studies in large patient

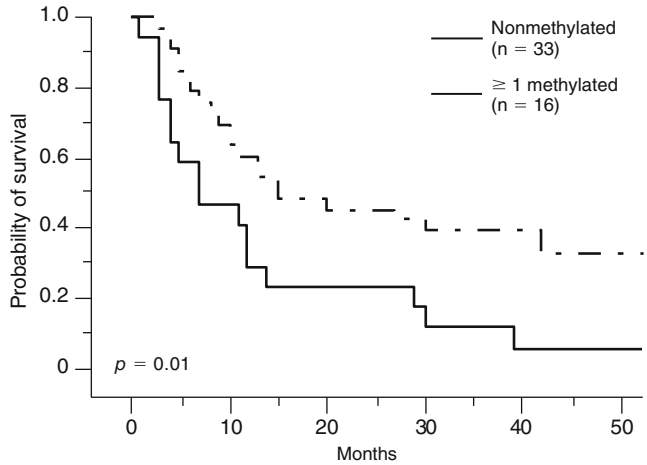

Fig. 4 Prognostic utility of circulating methylated DNA in blood of patients with melanoma. Kaplan-Meier survival curves of patients with stage IV melanoma undergoing biochemotherapy. Correlation of prebiochemotherapy serum methylation of at least one marker with overall survival (logrank test $p = 0.01$). Patients with serum methylation of only RAR-β2, methylation of only RASSF1A, or methylation of at least one marker had significantly worse overall survival compared with patients who had no methylated markers (logrank test $p = 0.010$). *≥ 1 methylated marker*, patients with serum methylation of at least one marker; *nonmethylated*, patients with no serum methylation of genes. (From Mori et al. 2005)

cohorts, preferably accompanying a clinical trial, are needed.

Circulating Immune Cells

The frequency of immunologically active cells in the blood stream has been analyzed for an association with the course of disease and outcome of cancer patients for a long time. Recent advances in suitable technologies like multiparametric flow cytometry allow the quantification of multiple subsets of circulating immune cells in one single blood sample. In melanoma, immune cell frequencies are particularly appealing as biomarkers for immunotherapeutic strategies. Thus, it has been shown for patients treated with the anti-CTLA-4 mAb ipilimumab that low peripheral blood baseline counts of monocytes and myeloid-derived suppressor cells (MDSCs), as well as high counts of eosinophils, lymphocytes, and regulatory T cells (Tregs), were associated with a favorable survival of the corresponding patients (Martens et al. 2016). For anti-PD-1 treatment with pembrolizumab, it has been shown that high numbers of eosinophils and lymphocytes are strongly correlated with a favorable treatment outcome and prolonged survival (Weide et al. 2016). Another recent study of T-cell subsets in the peripheral blood of melanoma patients before and after treatment with pembrolizumab revealed a change in the subset of exhausted CD8+ T cells, which in relation to the patients' tumor burden was correlated with treatment response (Huang et al. 2017). Since the analysis and quantification of immune cell subsets are a straightforward methodology and results are quickly achieved, it might gain more attention in the near future as a useful biomarker in melanoma patients, particularly in association with immunotherapy.

Treatment-Associated Biomarkers

As large bodies of evidence have been generated supporting the use of BRAF inhibitor-based therapy and immune checkpoint antibody therapy in metastatic melanoma, extensive retrospective analyses have been undertaken to understand which patient subpopulations are more or less likely to derive benefit. One theme that has emerged from this research is that previously known prognostic factors have even more significance as predictors of clinical benefit from therapy. As this has borne out to be true for both targeted therapy and immunotherapy in melanoma, we will consider this evidence as it pertains to both types of therapy in this chapter. Molecular features that predict response or resistance to immune checkpoint antibody therapy are discussed in detail in the chapters dealing with this treatment modality. Therefore, molecular predictors of treatment outcome to BRAF inhibitor-based therapy will be further developed here.

Serum Lactate Dehydrogenase (LDH)

Serum LDH has been long recognized as a prognostic factor in metastatic melanoma and a component of the AJCC staging system. In the 2009 AJCC analysis, patients with elevated serum LDH had a median overall survival of less than 1 year compared to approximately 2 years in the patients with normal serum LDH (Balch et al. 2009). And, whereas only 10% of patients with elevated serum LDH survive to 5 years, 25% of patients with normal LDH survived to that landmark. Notably, this analysis did not just for sites of metastatic disease, also known to have prognostic significance, with comparable differences in survival time when comparing patients with skin, subcutaneous, distant lymph node metastases versus those with visceral organ involvement beyond the lung. Considering baseline serum LDH values in relation to outcome on BRAF inhibitor-based targeted therapy and immune checkpoint antibody therapy, there is a similar magnitude of difference in intermediate and long-term outcomes. With long-term follow-up of the vemurafenib single-agent phase III trial, the median overall survival in the normal LDH subpopulation was 18.1 months compared to 9.6 months in those with elevated LDH (Chapman et al. 2017). And with mature follow-up data out to 4 years, 22.8% of patients with normal LDH were still alive

versus 9.0% of patients with elevated LDH at baseline.

For BRAF/MEK combination therapy, the largest available database exists for dabrafenib/trametinib with regard to outcomes in patient subpopulations. Among 563 patients treated with dabrafenib/trametinib across to phase III trials, the 2-year progression-free survival rate was 39% for those with normal baseline serum LDH and 14% for those with elevated LDH (Long et al. 2016). The rates of overall survival at 2 years for these two groups were 66% and 27%, respectively. This difference was sustained with 3-year outcomes: 55% overall survival likelihood for normal LDH patients versus 22% in elevated LDH patients. In the vemurafenib/cobimetinib phase III trial, similarly disparate outcomes were seen based on serum LDH. Patients with normal serum LDH at baseline had a median progression-free survival of 13.4 months versus 8.2 months in those with elevated LDH. Median overall survival in the normal LDH subgroup had not been defined as of the most recent, updated analysis but was clearly superior to the 14.8-month median overall survival in the elevated LDH subgroup.

In the three-arm randomized trial of ipilimumab/nivolumab versus nivolumab versus ipilimumab, 3-year outcomes varied markedly by baseline serum LDH value (Wolchok et al. 2017). For combination ipilimumab/nivolumab therapy, the 3-year progression-free survival rate for patients with normal serum LDH was 45%, 28% for those with elevated serum LDH, and 17% for those with a baseline LDH that was two times the upper limit of normal. For nivolumab monotherapy, the rates of 3-year progression-free survival were 37%, 21%, and 11% for these same subgroups. And, for ipilimumab monotherapy, they were 14%, 3%, and 0%, respectively. For overall survival, ipilimumab/nivolumab combination therapy produced a 66% 3-year overall survival rate in normal LDH patients compared to 44% and 31% in those with elevated LDH or two times the upper limit of normal. Nivolumab monotherapy was associated with a 61% likelihood of survival at 3 years versus 34% in those with elevated LDH and 14% in the two times upper limit of normal subgroup. Lastly, ipilimumab monotherapy yielded survival rates of 42%, 20%, and 7% across these three subgroups.

Burden of Metastatic Disease

As is the case for prognostication in the metastatic melanoma population, various measures of extent of metastatic disease have prognostic value independent of serum LDH in the setting of both BRAF inhibitor-based therapy and immune checkpoint therapy. The first such published analysis came from the pooled phase 3 populations treated with dabrafenib/trametinib (Long et al. 2016). Patient demographic and disease characteristics were all entered into a classification and regression trees analysis which selects outcome discriminating features in an unbiased and hierarchical fashion. While serum LDH was the most powerful discriminator of both progression-free and overall survival outcomes, disease burden as described by aggregate size of measured lesions at baseline and number of involved organs/sites were the second and third and only additional discriminating feature for progression-free survival. And, these subgroups only had predictive value within the population with normal serum LDH. The metastatic site classification was not determined based on number of lesions within or across metastatic sites, but rather on the number of discrete tissue or organ sites involved. For example, the lung and liver were each considered as discrete sites of involvement, as were the skin and lymph nodes. Significant stratification in outcomes was observed for those patients with one or two sites of metastatic disease versus those with three or more. This analysis yielded a particularly striking difference in progression-free survival at 3 years, with 42% of those patients with only one or two sites of metastatic disease measuring less than 6.6 cm in aggregate remaining progression-free versus 0% of the two times upper limit of normal serum LDH population.

In a subgroup analysis of the three-arm randomized trial of ipilimumab/nivolumab versus nivolumab versus ipilimumab, two unique measures of disease burden were applied (Wolchok

et al. 2017). The entire patient population was divided into quartiles in one subgroup analysis, and, for the other, the number of metastatic sites was so grouped into one, two to three, and greater than three. There was a threefold difference in the aggregate measure of lesions at baseline in the highest quartile versus lowest quartile. The likelihood of 3-year progression-free survival for a nivolumab/nivolumab, nivolumab, and ipilimumab was 45%, 38%, and 15%, respectively, in the lowest tumor burden quartile. This compares to 33%, 29%, and 5% rates of 3-year PFS in the highest tumor burden quartile. More subtle differences were seen for 3-year PFS rate across subgroups defined by number of metastatic sites. Comparing the greater than three-site subgroup to the single-site subgroup, ipilimumab/nivolumab was associated with a 47% 3-year PFS rate versus 27%, and nivolumab produced very similar rates of 37% and 36%, while ipilimumab yielded similar rates of 12% and 11%. Interestingly, more striking differences were noted in 3-year overall survival outcome across these disease site subgroups: 70% versus 42%, 65% versus 44%, and 48% versus 28% for ipilimumab/nivolumab, nivolumab, and implement, respectively.

Body Mass Index (BMI)

A recent, striking analysis has found that elevated body mass index has positive predictive value in the setting of both BRAF inhibitor-based therapy and immune checkpoint antibody therapy (McQuade et al. 2018). Unlike serum LDH and various measures of metastatic disease burden, BMI had a statistically significant predictive value, but no prognostic significance. Very similar improvements in progression-free survival and overall survival were seen between targeted therapy and immunotherapy-treated populations. A 28% improvement in PFS outcomes was observed for obese patients compared to those with normal BMI and a 40% improvement in overall survival among those receiving BRAF/MEK combination therapy. Among those treated with immune checkpoint antibody therapy, 25% improvement in PFS and 36% improvement in overall survival were seen. Nearly all of this apparently beneficial effect of obesity was observed in men, in whom there was a highly statistically significant 47% advantage in overall survival for men versus a nonsignificant 15% better outcome for obese women. Notably, there was no difference in outcome across the subpopulations when treated with chemotherapy. These findings have led to hypotheses regarding hormonal and metabolic effects on melanoma biology, therapeutic vulnerability, and resistance that are being pursued in ongoing translational studies.

Molecular Features Associated with Outcome on BRAF Inhibitor-Based Therapy

Only preliminary data are available regarding co-occurring somatic alterations accompanying BRAF V600 mutations and their association with outcome on BRAF inhibitor-based therapy. Some studies have provided both preclinical and clinical evidence that MAP kinase pathway intrinsic components can confer relative resistance to BRAF inhibitor-based therapy. Among 124 BRAF V600-mutant melanoma patients treated with BRAF inhibitor monotherapy, 10% harbored coexisting P124L/Q/S substitutions in MEK1 (Carlino et al. 2015). The likelihood of response was substantially lower in these patients compared to the rest of the cohort (33% vs. 72% in MEK1P124Q/S vs. MEK1P124 wild-type, $p = 0.018$) as well as shorter PFS. An analysis of BRAF allele copy number among the cohort of 46 BRAF-mutant melanoma patients treated with MAP kinase pathway inhibitors demonstrated a beneficial association between elevated BRAF copy number and outcome (Stagni et al. 2018). This finding raises the hypothesis that melanoma with both activities mutations and copy number increases of BRAF is most “addicted” to the oncogenic function of this gene. While there are extensive preclinical data supporting the relevance of genetic alterations in PTEN, p53, Rb, and CDKN2A in relation to BRAF inhibitor sensitivity, systemic interrogation of clinical cohorts is still awaited.

Multiple groups have identified markers of BRAF inhibitor resistance that align with a dedifferentiated, neural crest cell-like phenotype (Konieczkowski et al. 2014; Muller et al. 2014; Zuo et al. 2018). These studies leveraged the availability of large numbers of immortalized melanoma cell lines with variable sensitivity/resistance to BRAF inhibitors to nominate transcription factors, receptor tyrosine kinases, and activated components of the PI3 kinase pathway as designators of the cell state that is intrinsically resistant to therapy. Fewer studies have included analysis of patient tumor samples but those that have found statistically significant differences in clinical outcome even among small number of samples. In one analysis of just 12 V600 mutant BRAF melanoma patients treated with BRAF/MEK combination therapy, there was a threefold difference in median progression-free survival when comparing those tumors that had markers of dedifferentiation (low MITF, high AXL) compared to more differentiated tumors (high MITF, low AXL) (Konieczkowski et al. 2014). In another analysis of BRAF inhibitor-treated patients, supportive evidence of this low MITF expressing phenotype conferring resistance was demonstrated by showing the emergence of the low MITF melanoma cells at the time of clinical progression (Muller et al. 2014). But noting that numerous melanoma cell lines and untreated melanoma patient tumor samples feature the low MITF/high AXL state, this appears to be a cell state that can emerge during the evolution of melanoma before the application of therapy. Notably, some of these same molecular features (notably high AXL expression) have been associated with intrinsic resistance to PD-1 antibody therapy (Hugo et al. 2017). This raises the concerning possibility that a significant proportion of melanomas have adopted a cell state that is resistant to either therapeutic modality.

A last line of evidence suggests that markers of immune recognition and activated effector T cells in sites of metastatic melanoma positively associate with outcomes on BRAF inhibitor therapy (Massi et al. 2017). Among 39 patients treated with BRAF inhibitor monotherapy and 25 patients treated with BRAF/MEK inhibitor combination therapy, presence of CD8+ T cells strongly associated with likelihood of response and superior overall survival. Interestingly, co-expression of markers of beta-catenin pathway activation additionally informed BRAF inhibitor treatment outcome. Previously published data link beta-catenin pathway signaling and exclusion of T cell from the melanoma tumor microenvironment (Spranger et al. 2015). So, it would be anticipated that activity in this pathway would overlap with the CD8-negative subpopulation. Yet, those patients with the highest level of CD8 T-cell infiltration and lack of beta-catenin pathway activation had a 75% superior PFS and overall survival outcome that was significant even after adjusting for other disease characteristics known to impact likelihood of benefit from BRAF inhibitor therapy. Long known to be associated with favorable prognosis in both early and advanced melanoma, these preliminary findings suggest that greater degrees of immune recognition have even bigger impact on the outcome of patients treated with BRAF inhibitor-based therapy.

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