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Prognostic Molecular Biomarkers for Cutaneous Malignant Melanoma

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Abstract

Molecular signatures of melanoma have propelled new approaches to early diagnosis, monitoring of treatment response, and targeted therapy. This review discusses messenger RNA (mRNA), genomic and epigenomic melanoma biomarkers in blood and tissue specimens. The major focus is on tissue-based molecular assays to upstage sentinel lymph nodes and blood-based assays to detect melanoma progression by monitoring levels of circulating tumor cells and circulating DNA.

Keywords

biomarkers; melanoma; circulating tumor cells; microRNA; lymph node; epigenetics

INTRODUCTION

Although molecular alterations have been investigated as potential biomarkers of cancer progression or outcome, only a handful of prognostic biomarkers have been validated in cutaneous melanoma. Here, we report some of the biomarker technical platforms and assays that have been developed by our group for diagnostic and/or prognostic assessment of patients with cutaneous melanoma. When validated in the phase III setting, these biomarker assays may facilitate accurate diagnosis, stratification for treatment, and monitoring of treatment response.

Molecular biomarkers can be categorized based on the molecular component that is assessed for the tumor-related alterations. The first biomarker category comprises messenger RNA (mRNA) biomarkers, which are being validated for clinical use in multiple studies. The detection of these mRNA biomarkers is sensitive, specific, and robust using available molecular technology. Several clinical trials have investigated the use of mRNA biomarkers for upstaging melanoma-draining sentinel lymph nodes; other studies are using mRNA biomarkers to detect circulating tumor cells (CTC) in patients receiving systemic treatment for advanced melanoma.

The other biomarker categories comprise genomic and epigenomic biomarkers. Genomic biomarkers such as mutation (mt), single nucleotide polymorphism (SNP), and loss of heterozygosity (LOH) have been found in high frequency in melanoma. Most recently, epigenetic aberrations such as gene promoter region methylation of CpG islands and microRNA(miR) have taken center stage in the hunt for biomarkers. Several of these

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genomic and epigenomic biomarkers show promise for prognostic assessment of primary cutaneous melanoma. These different types of molecular biomarkers (Table 1) will be discussed in more detail along with important studies that demonstrate potential use in clinical settings.

mRNA BIOMARKERS

Specific genes with functional roles in tumor progression are of particular interest as mRNA biomarkers in both tissue and blood. These include genes from the melanogenesis pathway, such as tyrosinase, gp100, TRP-1, TRP-2, and MART-1 [1,2]. However, some mRNA biomarkers also can be expressed in low levels in normal melanocytes and nevi, producing false-positives [3,4]. As described below, our group has extensively investigated many prognostic mRNA biomarkers for upstaging lymph node status and identifying circulating tumor cells (CTC) [1,5-12].

Candidate mRNA Biomarkers

Some of the candidate mRNA biomarkers we have extensively investigated are described below. We have divided them into melanoma-related, apoptosis-related, and chemokine receptor biomarkers (Table 1).

MAGE-A3 is a melanoma-associated antigen that is not found in normal tissues except testis and placenta [13,14]. This gene is a member of the MAGE family of testis-related antigens that are highly specific in cancer tissues including melanoma. Their function is not well understood, limiting their potential utility. These so-called testis-related genes may be just non-specific activation of genes of limited function in cancer.

Cell surface tumor-related gangliosides of melanoma such as GM2 and GD2 are well defined oncofetal melanoma-related antigens usually found in aggressive melanomas [15,16]. β1→4-N-acetylgalactosaminyltransferase (GalNAc-T) is a key enzyme involved in synthesis of gangliosides GM2 and GD2 from GM3 and GD3, respectively [9,15,16]. GM2 and GD2 are not expressed in melanocytes or nevi. Our data indicate that elevated GalNAc-T mRNA expression in melanoma cells appears to be a biomarker for aggressive melanoma [9].

PAX3 (paired-box homeotic gene transcription factor 3) is involved in the regulation of melanin synthesis, migration and anti-apoptosis [17,18]. PAX3 has been considered as a stem cell marker and is well-expressed in melanomas and not in normal skin melanocytes or benign nevi by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and *in situ* hybridization [18]. Microphthalmia-associated transcription factor (MITF) plays an important role in melanocyte biology and in melanoma progression. PAX3 is considered as a regulator of MITF, which has demonstrated utility for detecting CTC [19].

HMW-MAA, also known as melanoma chondroitin sulfate proteoglycan [20], is a sensitive mRNA biomarker for primary desmoplastic melanoma (DM) [21]. Because metastatic DM is very difficult to diagnose by immunohistochemical staining (IHC) such as with anti-MART-1 and anti-HMB-45 antibodies, we used HMW-MAA RT-qPCR to assess 40 primaries and 23 metastases of DM. Results showed that 25 (63%) DM primaries and 16 (70%) DM metastases expressed HMW-MAA mRNA, whereas MART-1 was expressed in 9 (23%) primaries and 5 (22%) metastases in the same melanoma specimens. HMW-MAA mRNA was expressed in 8 (57%) of 14 nodal metastases, whereas MART-1 mRNA was expressed in 3 (21%) of 14 nodal metastases.

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A new biomarker recently found in melanoma is FABP7 (Fatty acid binding protein 7). FABP7 was reported to have lipid-metabolizing capacity associated with fatty acids [22,23]. In the brain, it has been linked to cell proliferation and tissue differentiation [24]. FABP7 can be regulated by protein kinase C (PKC) and the MAPK/ERK1/2 pathway through independent mechanisms in melanoma cell lines. Furthermore, *in vitro* studies demonstrated that FABP7 is involved in cell proliferation and invasion [25]. We reported that FABP7 mRNA was detected in 60 of 87 (69%) AJCC Stage I-III melanomas. However, it was detected only in 13 of 68(19%) AJCC Stage III-IV metastatic melanomas. Analyzing of 37 paired primary and metastatic melanomas by IHC assessment, FABP7 was detected in 27 of 37 (73%) primary melanomas and in 10 of 37(27%) metastatic melanomas[26]. FABP7 detection in metastatic tissues was inversely correlated with disease-free and overall survival and was a significant independent prognostic factor for survival (Figure 1).

Survivin is a member of anti-apoptosis molecular inhibitors(IAP), which often promote various cancers. Survivin is a protein known to be significantly expressed in highly malignant cutaneous melanomas. Lower expression of survivin was correlated with good prognosis among stage IV patients who received postoperative vaccine immunotherapy [27].

Chemokines are small secreted chemotactic cytokines involved in cell trafficking and specific organ site methylation [28]. Chemokines and their receptors have been identified as key factors that control the migration of tumor cells to specific organ sites [29]. The expression of several chemokines and their receptors is upregulated during melanoma progression. In melanoma, several chemokines and chemokine receptors have been linked to tumor growth and specific organ metastasis [30-34]. Two of these chemokine receptors expressed in melanoma are CXCR4 and CCR7 [35-37]. In 2005, Scala et al. reported that CXCR4 expression was detected in 31 of 71(43.6%) primary cutaneous melanomas and associated with poor prognosis [36]. We assessed CXCR4 expression in resected tumor tissue from patients who underwent hepatic surgery for melanoma liver metastasis [38]. We identified CXCR4 as the most common chemokine receptor expressed in paraffin-embedded liver metastases. RT-qPCR demonstrated CXCR4 expression in 24 of 27 (89%) metastases. *In vitro* treatment of melanoma cells with CXCL12 (CXCR4-specific ligand) significantly increased cell migration ($P<0.001$). CXCL12 is highly expressed by liver cells and supports the attraction of CXCR4-positive melanoma cells.

CCR9-CCL25 interaction has been implicated as being critical for the migration of peripheral T-cells to inflammatory small intestine [39]. We demonstrated CCR9 expression in 88 of 102 small intestine metastatic melanomas, in 8 of 8 melanoma lines derived from small intestine metastases, and in 0 of 96 metastatic melanomas to other organ sites [40]. *In vitro* migration and invasion studies on CCR9(+) melanoma lines showed migration in response to CCL25. CCR9 expression by small intestine metastases and concomitant $\alpha 4$ and $\beta 1$ integrin expression were confirmed by flow cytometry. These findings demonstrate the importance of the CCR9-CCL25 axis in preferential metastasis to small intestine. This is a significant metastasis event because metastasis of any cancer type to the small intestine is uncommon. This demonstrated that chemokine-receptor axis can promote site-specific metastasis to the small intestine from a primary tumor located at any anatomical location, without a direct anatomical blood drainage pattern.

Recently, using human melanoma xenografted in athymic nude SCID mice for melanoma brain metastasis, Izraely et al. [41] showed that CCR4 expression was significantly higher in brain metastatic variants than in corresponding local variants. CCR4 is suggested to be associated with brain metastasis in human melanoma and may be an important factor for identifying melanoma cells likely to metastasize to the brain [41]. In general, chemokine

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receptors are not diagnostic but provide valuable information of potential metastasis ability and site of metastasis. These need to be further explored.

Molecular Upstaging of Sentinel Lymph Nodes

Lymph node metastasis of melanoma is one of the most significant prognostic determinants. Sentinel lymphadenectomy is standard for surgical staging of clinically localized melanoma [42]. Accurate assessment of the sentinel lymph node (SLN) is important in determining disease staging and prognosis. However, the detection of micrometastasis in SLNs is not always accurate based on hematoxylin and eosin (H&E) and IHC staining. Although IHC using anti-S100p, HMB45, and MART-1 antibodies is standard, a significant number of patients with histopathology-negative SLNs subsequently develop recurrent disease. Improved techniques for detecting clinically significant micrometastases in melanoma-draining SLNs are needed to reduce the subjectivity of current detection methods. Also, better biomarkers are needed for prognosis.

Our group developed multimarker RT-PCR assays to detect occult metastasis in SLNs. These assays, initially applied to frozen specimens, have since been validated for paraffin-embedded (PE) tissues [11,12,43-49]. We performed RT-PCR on archived PE SLNs from 215 clinically SLN(-) patients who underwent lymphatic mapping and SLND for melanoma and were followed up for >8 yrs. PE SLNs (n=308) from these patients were sectioned and assessed by qPCR for four melanoma-associated biomarkers: MART-1, MAGE-A3, GalNAcT, and PAX3. Fifty three (25%) patients had histopathology-positive SLNs by H&E and/or IHC [10]. Forty-eight (30%) of the 162 patients with histopathology-negative SLNs had SLNs which expressed >1 mRNA biomarker, and Cox proportional hazards model analysis showed a significantly increased risk of disease recurrence of these 48 patients ($P<0.0001$). The presence of >1 biomarker in histopathology-negative SLNs was significantly associated with lower survival rate by multivariate analysis ($P<0.0002$). This study was recently updated with longer median follow-up (>11 yrs) and results remain highly significant (Figure 2; [50]). The study demonstrates the prognostic utility of molecular upstaging of SLNs with specific mRNA biomarkers. It is very clear that selection of mRNA biomarkers, sampling of SLN, and molecular assays used are highly important. The study demonstrates that specific molecular upstaging of SLNs has more prognostic value than IHC positivity in longer-term follow-up. Previous studies reporting no correlation between RT-PCR upstaging of SLN and clinical outcome had flaws in specimen sampling, mRNA biomarkers, assay sensitivity, assay specificity, and/or patient cohorts.

A recent systematic meta-analysis [51] assessed multimarker RT-qPCR upstaging of SLNs in 22 studies that were conducted in years 1998-2006 and enrolled 4,019 patients with clinical stage I/II cutaneous melanoma. RT-qPCR status was associated with TNM stage and with overall and disease-free survival. Currently, RT-qPCR upstaging of histopathology-negative SLNs is being investigated in the phase III Multicenter Selective Lymphadenectomy Trial-II (MSLT-II). Over 1900 patients have been entered into this multicenter international trial to date. Results of the molecular studies are expected to validate results of phase II RT-qPCR studies and determine the clinical relevance of molecular upstaging in SLN specimens.

Landmark Blood Molecular Biomarker Studies

During tumor progression, malignant melanoma cells invade lymphatic and blood vessels and shed cells that circulate in the peripheral bloodstream. Thus molecular biomarkers in blood are promising surrogates for monitoring tumor progression [44,52-55]. RT-qPCR detection of CTC in blood of melanoma patients has been associated with disease stage and clinical outcome [53]. RT-qPCR assay can detect a few CTC among millions of peripheral

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blood leukocytes (PBL) [56]. Investigations have used a single-biomarker assay for detection of CTC, but single-biomarker assays are limited by known heterogeneous expression, particularly in advanced disease [1,3,56]. Hoon et al. [56] previously reported that a combination of CTC mRNA biomarkers is necessary to compensate for heterogeneous biomarker expression in melanoma patients; multiple biomarkers can increase the sensitivity of CTC detection and reduce false-negative results.

The efficacy of a multimarker assay depends on the careful selection of CTC biomarkers [57], serial rather than single-point assessment [7,8], and quantification of biomarker expression to compensate for ectopic and background mRNA [58]. Based on these strategies, Koyanagi et al. [5,19] have developed a multimarker RT-qPCR assay to detect CTC in blood of melanoma patients. Koyanagi et al. demonstrated the utility of the RT-qPCR CTC assays in monitoring patients treated with immuno and chemotherapy [6-8]. Recently, Kitago et al. described monoclonal antibody and immunomagnetic bead capture assay to isolate melanoma CTC expressing HMW-MAA; isolated CTC were assessed by multimarker RT-qPCR assay (mRNA biomarkers) and by qPCR assay (BRAFmt) [59]. The bead assay validated the direct RT-qPCR, demonstrating presence of CTC. The direct assay is logistically more feasible and can be easily performed in a multi-institutional clinical trial setting.

Tissue-based prognostic assessment is a static (single-point) measurement that does not directly reflect ongoing disease progression, particularly subclinical systemic metastasis. By contrast, blood-based assessment can be performed as serial measurements, which may be particularly valuable to manage melanoma patients receiving systemic chemo- and/or immunotherapy [60]. Koyanagi et al. assessed serial blood specimens from patients enrolled in a prospective phase II multicenter neoadjuvant clinical trial of biochemotherapy before and after surgical treatment of AJCC stage III melanoma. They found that changes in CTC detection were significantly correlated with disease progression and overall survival (Figure 3A) [8]. Separately, they also assessed serial blood specimens collected from 87 patients before and during induction biochemotherapy and maintenance biotherapy for stage IV melanoma; changes in CTC detection were significantly correlated with treatment response, progression-free survival, and overall survival (Figure 3B) [7]. Recently, a large prospective multicenter clinical trial demonstrated the prognostic utility of CTC in stage IV melanoma patients [61] and stage III patients (D. Hoon, unpublished data).

CTC detection in serial blood specimens in melanoma may similarly be employed to determine which component of the treatment is most effective and which needs to be improved. As treatment regimens for advanced melanoma become multimodal and multiphasic, the development of a tool to identify high-risk patients and to monitor response to systemic therapy is urgently needed. Serial RT-qPCR assay can assess CTC changes during different phases of treatment, and this makes RT-qPCR detection of CTC a promising method to evaluate treatment efficacy. Identification of CTC with specific gene expression allows us to better screen high-risk CTC likely to establish distant metastasis. Individual CTC do not have equal metastasis potential. The measurement of numbers of CTC may not be prognostic particularly in early stages of cancer.

GENOMIC AND EPIGENOMIC BIOMARKERS

Sequential genetic aberrations have been correlated with the development and progression of various cancers. Several somatic gene alterations in melanoma have been reported, such as *CDKN2A* and *CDK4* [62]. Recently, epigenetic alterations have become a hot topic in melanoma. In 2001, Wu et al [63] defined epigenetics as “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in

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DNA sequence”. Today this definition is globally accepted. The major players in epigenetic gene regulation to date are gene promoter methylation, histone modifications, and short non-coding mRNAs. It will be important to investigate genomic, transcriptome, and epigenetic biomarkers together in relation to tumor progression and disease outcome.

Genomic biomarkers

BRAF kinase has become a key target of interest in melanoma because of its high frequency of mutation. BRAF kinase is a component of the Ras-MAPK-ERK pathway; *BRAF* mutation occurs frequently in exons 11 V600E. We found *BRAF V600E* mutation (*BRAFmt*) frequency was significantly ($P<0.0024$) higher in metastatic tumors (n=68) than in primary melanomas (n=59) [64]. This suggested that the *BRAFmt* may be acquired during melanoma progression to distant metastasis. This also suggested *BRAFmt* does not always occur at initiation of melanoma, supporting the concept that it is not always the cause of melanoma. In reviewing the literature, there is variability in reporting the actual frequency of *BRAFmt* in primary melanoma. Assessment of melanoma *BRAFmt* has gained importance because of the effectiveness of PLX4032 [65] and GSK2118436 [66], new agents that target *BRAFmt*.

RET (rearranged during transfection) proto-oncogene encodes a receptor tyrosine kinase [67-69] containing four cadherin-related motifs and a cysteine-rich region in the extracellular domain [67,70]. Glial cell line-derived neurotrophic factor (GDNF) family members bind the extracellular domain of RET through a complex formed with glycosyl-phosphatidylinositol-anchored coreceptor (GFRα1–4), a member of the GDNF receptor family [71]. Activated RET induces signaling through RAS-BRAF-ERK, phosphatidylinositol 3-kinase (PI3K)-Akt and p38 mitogen-activated protein kinase (MAPK) pathways [70]. Activation of both RET-RAS-BRAF-MEK-ERK and RET-PI3K-Akt pathways leads to cell proliferation and survival, whereas the RET-PI3K pathway is frequently involved in cell motility [70,71]. Several germline mutations of *RET* play an important role in development of multiple endocrine neoplasia (MEN) syndromes MEN2A, MEN2B, and familial medullary thyroid carcinoma [71,72].

G691S *RET* polymorphism (*RETp*) is a single nucleotide germline polymorphism in exon 11 of the juxtamembrane region of *RET*, which enhances the response of *RET* to GDNF in pancreatic cancer [73]. In cutaneous malignant melanomas, particularly desmoplastic cutaneous melanomas, which are highly neurotropic, *RETp* reportedly plays an important role in enhancing malignant behavior [74]. *RETp* was shown to enhance and prolong phosphorylation of RET signaling pathways including ERK1/2 and AKT after GDNF stimulation, leading to vigorous cell proliferation and migration in melanoma cells (Figure 4). *RETp*-induced enhancement of cell proliferation and migration was not affected by BRAFmt, which is found frequently in metastatic melanomas. We demonstrated that *RETp* could be acquired during melanoma progression. This would be a significant advantage for melanoma survival and invasion. If *RETp* plays a central role in inducing primary tumor neurotropism in melanomas, then targeting tyrosine kinase inhibitors against *RETp* might be a useful approach to melanoma therapy.

The frequency of loss of heterozygosity (LOH) in tumors, along with specific gene function in tumor cells, suggests that LOH may play a significant role in regulating tumor-suppressor genes and oncogenes. Frequent LOH of DNA microsatellites on specific chromosomal regions has been reported in cutaneous melanoma [75,76]. These LOH biomarkers have prognostic potential. For example, apoptotic protease activating factor-1 (*APAF-1*) is a tumor-suppressor gene that mediates apoptosis [77]. We found that LOH of microsatellites covering the *APAF-1* locus (12q22-23) was significantly more common in metastatic tumors (36 of 98 specimens; 37%) than in primary melanomas (10 of 54 specimens; 19%). In

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metastatic melanomas, *APAF-1* loss significantly correlated with a worse prognosis. More recently, we reported the frequency of LOH in the region covering *FABP7* [26], which is a tumor-related gene involved in proliferation and invasion of melanoma cells [25]. LOH was identified in 10 of 20 (50%) metastatic melanomas at the 6q22.31 region containing the *FABP7* gene, and 0 of 14 primary melanomas. FABP7 expression is significantly decreased in metastases of melanoma due to LOH, and its decrease is associated with significantly poorer disease outcome.

Overall, there are many genomic biomarkers identified in melanoma, but few have high frequency and functional utility with the exception of *BRAF* V600Emt. Continued genomic deep-sequencing will probably reveal genomic aberrations that are functionally important in specific melanoma subsets.

Epigenomic biomarkers

DNA methylation plays one of the most important roles in regulating gene expression and chromatin architecture. CpG island methylation can result in suppression of gene expression, and contribute to tumorigenesis and cancer progression [78]. Epigenetic suppression can occur by methylation of specific CpG islands in the promoter region, histone methylation or acetylation and/or miR activation [79-81]. In melanoma, more than 50 genes have been reported to demonstrate aberrant hypermethylation of promoter CpG islands [82].

We were the first group to identify and verify the inactivation of RAS association domain family protein 1A, *RASSF1A*, which is a human tumor suppressor gene in melanoma [83]. Hypermethylation of two regions in the RASSF1A CpG island was investigated in metastatic cutaneous melanomas. Methylation of the RASSF1A CpG island was detected in 57% of tumors. No methylation was detected in normal skin tissues or lymphocytes. Hypermethylation of CpG regions correlated with no expression of the *RASSF1A* gene. *RASSF1A* is key gene in regulating mitosis and methylation; thus, it is highly important in controlling tumor invasion and metastasis. *RASSF1A* gene suppression by promoter methylation is strongly correlated with melanoma progression and outcome [79,80].

The CpG island methylator phenotype (CIMP) may be associated with development of malignancy through coordinated inactivation of tumor suppressor and tumor-related genes (TRGs) and methylation of multiple noncoding, methylated-in-tumor (MINT) loci. These epigenetic changes create a distinct CIMP pattern that has been linked to progression and disease outcome in gastrointestinal cancers [84]. The existence of a clinically significant CIMP in cutaneous melanoma progression was recently demonstrated by our group [79]. We showed an increase in hypermethylation of several TRGs (WIF1, TFPI2, RASSF1A, and SOCS1) with advancing clinical tumor stage. Furthermore, we reported a significant positive association between the methylation status of MINT17, MINT31, and specific TRGs. These findings demonstrated that a CIMP pattern is significant for melanoma progression. We recently demonstrated that DNMT3(a/b), which plays a significant role in methylation of TRGs, was significantly upregulated during melanoma progression [80]. The regulation of DNMT3 was controlled by another epigenetic factor, miR29c [80]. These events strongly indicate that specific epigenetic aberrations in melanoma progression are very significant, thus new potential targets.

Recently, we investigated the expression of RUNX3 and its regulatory factor, microRNA miR-532-5p [85]. *RUNX3* is a tumor-suppressor gene [86,87]. Expression of RUNX3 mRNA and human miR-532-5p was assessed in cell lines and in primary and metastatic melanomas. RUNX3 expression was down-regulated in all 11 (100%) melanoma lines relative to normal melanocytes. In primary and metastatic melanomas, RUNX3 had reduced expression relative to normal skin. Evidence of RUNX3 promoter region methylation was

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demonstrated in 5 of 17 (29%) melanoma cell lines, 2 of 52 (4%) primary melanomas, and 5 of 30 (17%) metastatic melanomas. miR-532-5p expression was upregulated in melanoma lines and metastatic melanomas relative to normal melanocytes and primary melanomas, respectively. To investigate the relationship between RUNX3 and miR-532-5p, we transfected anti-miR-532-5p into melanoma lines and evaluated RUNX3 expression. Inhibition of miR-532-5p activated RUNX3 mRNA and protein expression. miR-532-5p may contribute to melanoma progression by downregulation of RUNX3 expression. In the future, we will likely identify miR that play a significant role in melanoma progression as seen on other cancer systems.

Landmark Studies Using Serum Specimens

Cell-free tumor-specific DNA has been detected in plasma and serum from cancer patients [88-90]. This observation has been expanded to develop blood biomarkers. We have demonstrated prognostic utility for circulating DNA microsatellites, mutation, and methylation. In one study, we assessed nine microsatellites to examine allelic instability (AI) in serum specimens obtained from 41 stage IV melanoma patients before the initiation of biochemotherapy [91]. AI was detected in 12 of 41 (29%) patients. The response rate of these 12 patients was 17%, whereas that of the 29 patients without AI was 72%. The presence of AI was statistically significant and independently associated with disease progression.

AI encompassing the *APAF-1* locus (12q22-23) is found frequently in metastatic melanoma [77]. When we evaluated 12q22-23 AI status as a surrogate biomarker to predict response to biochemotherapy, we found that AI of the 12q22-23 region was significantly lower in responders (5 of 24, 21%) compared with nonresponders (11 of 20, 55%). As expected, nonresponders to biochemotherapy (AI-positive group) had a significantly worse survival than responders (AI-negative group) [92].

Also, we showed the presence and predictive utility of circulating *BRAFmt* in DNA from melanoma patients [93]. Overall survival was significantly lower in 20 patients with the *BRAF*mt before biochemotherapy compared with those that did not have the *BRAF*mt. After biochemotherapy, *BRAFmt* was lower in the responder group (1 of 10, 10%) than in the nonresponder group (7 of 10, 70%). The *BRAF* V600Emt can be useful for monitoring melanoma patients receiving biochemotherapy.

Methylation detected in serum DNA can predict disease outcome and therapeutic response in patients receiving concurrent biochemotherapy for metastatic melanoma [94,95]. We developed multiple methylated serum biomarkers based on frequently methylated tumor-related genes in melanoma tissues. In a study of RASSF1A, RAR-β2 and MGMT, we found that circulating methylated RASSF1A was significantly less frequent in biochemotherapy responders (3 of 23, 13%) than nonresponders (10 of 24, 42%), and it was significantly correlated with overall survival [95] (Figure 5). Patients with RASSF1A, RAR-β2, or at least one of the three biomarkers had significantly worse overall survival than patients with no biomarkers. In a separate study, we demonstrated serum estrogen receptor α hypermethylation was detected more frequently in advanced melanomas than localized melanomas and was the only factor predicting progression-free and overall survival in patients receiving biochemotherapy [94].

Our group was among the first to report the prognostic potential for combined assessment of CTC and methylated blood DNA biomarkers [6]. We assessed matched pairs of PBL and serum specimens from 50 AJCC stage IV melanoma patients before administration of biochemotherapy. PBL were analyzed for three mRNA CTC biomarkers: MART-1, GalNAc-T, and MAGE-A3. Sera were analyzed for two methylated DNA biomarkers:

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RASSF1A and RAR-β2. CTC were detected in 13 of 15 (86%) patients with serum tumor-related methylated DNA, and in 13 of 35 (37%) patients without methylated DNA. The number of CTC biomarkers detected was significantly associated with methylated DNA. Patients with both CTC and methylated DNA showed significantly poorer response to biochemotherapy and poorer progression. Findings indicate that these two assays in combination may be a very useful determinant of disease status and may improve efficacy of monitoring melanoma progression.

CONCLUSION

The above review covers only some of the highlights of our approaches in molecular biomarkers applied to both tissues and blood for diagnosis and prognosis. The technology available for development and validation of molecular biomarkers has significantly improved in the last decade; as a result, a number of molecular biomarkers have been investigated in a multitude of platforms. In the near future molecular biomarkers will play a significant role in the diagnosis and management of melanoma. Also, a validated molecular signature for melanoma may eventually allow highly efficient, tailored treatments for this cancer. Molecular oncology has and will continue to impact clinical practice and be at the forefront of melanoma translational research. As molecular technology and sensitivity towards specific targets improves, it is inevitable that melanoma will be better classified based on molecular characteristics.

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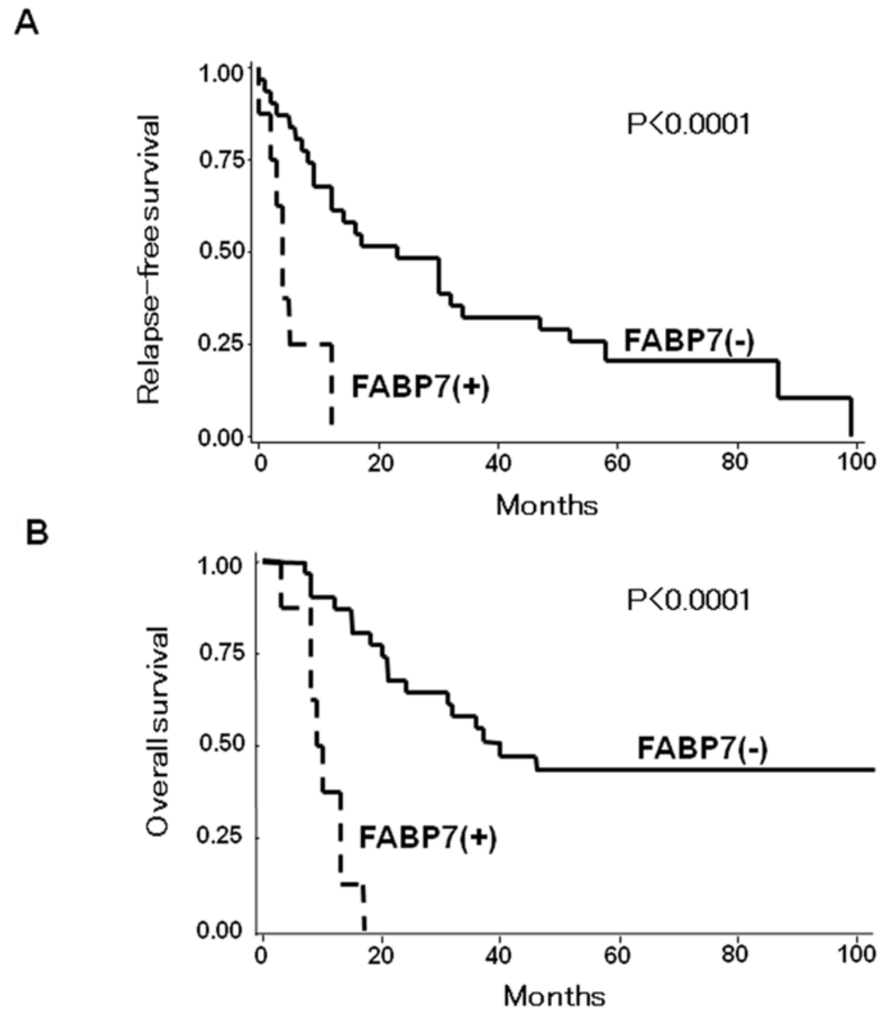

Figure 1.

Kaplan–Meier curves of relapse-free survival (**A**) and overall survival (**B**) based on FABP7 mRNA detection in melanoma metastases. Reprinted with permission from *Goto Y et al., Aberrant Fatty Acid-Binding Protein 7 Gene Expression In Cutaneous Malignant Melanoma, J Invest Dermatol, 130:221-9, 2010.*

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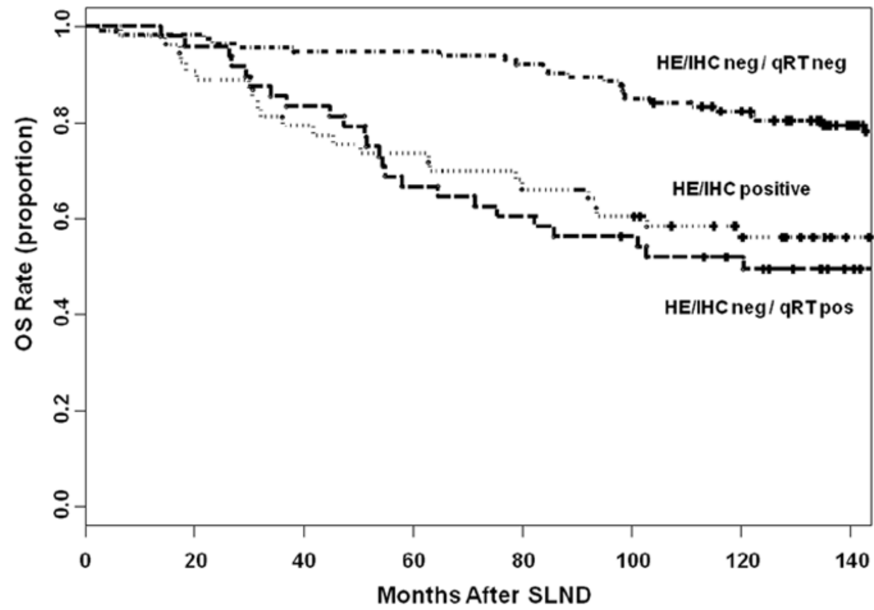

Figure 2.

Kaplan–Meier curves of overall survival based on histopathologic (H&E/IHC) and molecular (qRT) status of the sentinel lymph node (SLN). Reprinted with permission from *Nicholl MB et al., Molecular upstaging based on paraffin-embedded sentinel lymph nodes: ten-year follow-up confirms prognostic utility in melanoma patients, Ann Surg, 253:116-22, 2011.*

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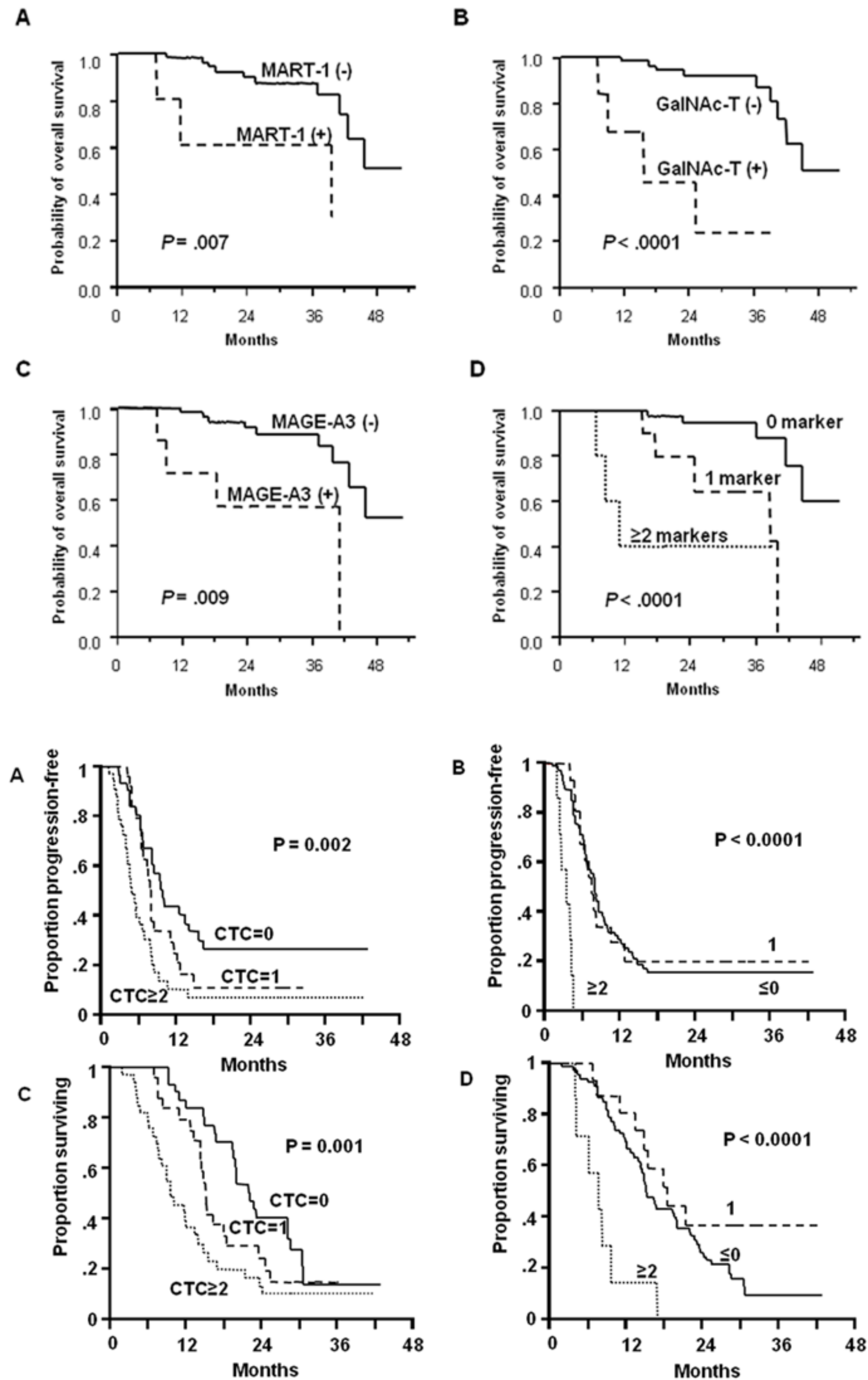

Figure 3.

A. (**A**) Kaplan-Meier curves of OS based on MART-1 detection after treatment. (**B**) Kaplan-Meier curves of OS based on GalNAc-T detection after treatment. (**C**) Kaplan-Meier curves of OS based on MAGE-A3 detection after treatment. (**D**) Kaplan-Meier curves of OS based on CTC BM detection after treatment. Reprinted with permission from *Koyanagi K et al., Serial monitoring of circulating melanoma cells during neoadjuvant biochemotherapy for*

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B. (**A**) Kaplan-Meier curves of PFS based on CTC BM detection after two cycles of induction BC. (**B**) Kaplan-Meier curves of PFS based on changes in CTC BM detection during two cycles of induction BC. (**C**) Kaplan-Meier curves of OS based on CTC BM detection after two cycles of induction BCT. (**D**) Kaplan-Meier curves of OS based on changes in CTC BM detection during two cycles of induction BC. In each panel, the solid line corresponds to no CTC BMs, the broken line is 1 CTC BM, and the dotted line is 2 CTC BMs. Reprinted with permission from *Koyanagi K et al., Serial monitoring of circulating melanoma cells during neoadjuvant biochemotherapy for stage III melanoma: outcome prediction in a multicenter trial, J Clin Oncol. 23:8057-64, 2005. License Number: 2510280678967.*

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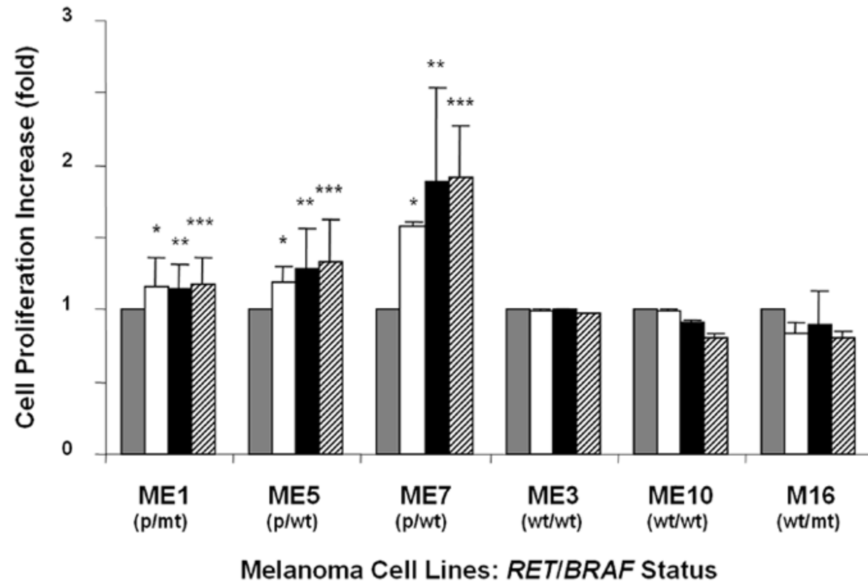

Figure 4.

GDNF-induced cell proliferation and migration in melanoma cells bearing G691S RET polymorphism and/or V600E BRAF mutation. p; G691S RET polymorphism, mt; V600E BRAF mutation, wt; wild type. Proliferation of melanoma cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 48h after GDNF stimulation. GDNF □ 5 ng/ml, ■ 25 ng/ml or ▨ 50 ng/ml. ■ Delivery agent-treated control. Bars±s.d. show fold increase over each control cell. *,**,*** P<0.05 versus each control. Bars±s.d. without asterisks show no significant change compared to each control. Reprinted with permission from *Narita N et al., Functional RET G691S Polymorphism in Cutaneous Malignant Melanoma, Oncogene, 28:3058-3068, 2009.*

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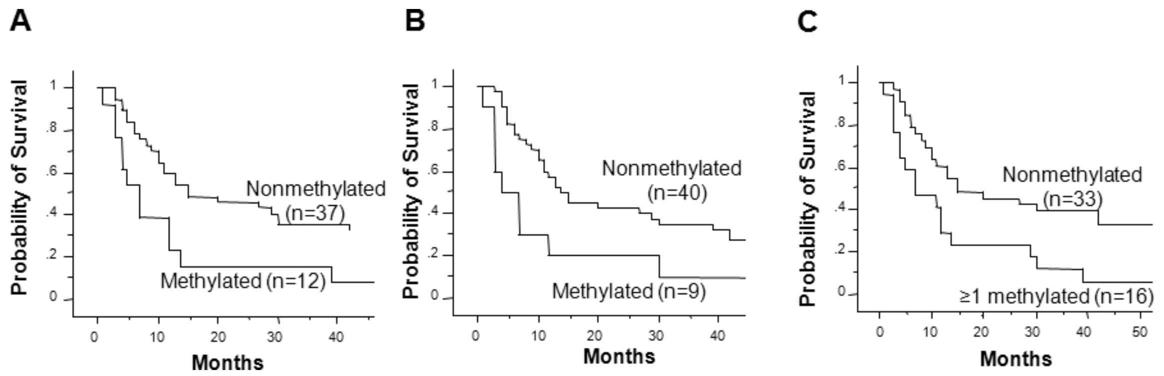

Figure 5.

Kaplan-Meier curves of overall survival based on pre-biochemotherapy methylation status of three serum biomarkers: RASSF1A, RAR-β2 and MGMT. (A) Survival probability according to RASSF1A methylation status (log-rank test, P=.013). (**B**) Survival probability according to RAR-β2 methylation status (log-rank test, P= .02). (**C**) Survival probability according to methylation of at least one of three markers (log-rank test, P= .01). Reprinted with permission from *Mori T et al., Predictive Utility of Circulating Methylated DNA in Serumof Melanoma Patients Receiving Biochemotherapy, J Clin Oncol. 23:9351-58, 2005. License Number: 2587200686801.*

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Table 1

Melanoma Molecular Biomarkers

MARKER	CHARACTERISTICS	CLINICAL RELEVANCE	REF
mRNA BM			
MART-1	a frequent melanoma associated antigen specific for melanoma	Diagnostic/Prognostic: Combination of these biomarkers can be used in the diagnosis of SLN to upstage melanoma patients and for detection of CTC during treatment or follow-up.	1, 5-8, 10, 11, 46, 48-50, 59
MAGE-A3	cancer-testis antigen not found in normal tissues except testis and placenta		5-8, 10, 14, 49, 50, 53, 56, 59
GalNac-T	key enzyme involved in gangliosides GM2 and GD2 synthesis		5-10, 15, 16, 50
PAX3	involved in the regulation of melanin synthesis, migration and anti-apoptosis well-expressed in melanomas		5, 7, 8, 10, 50
MITF	essential for the development and postnatal survival of melanocytes		7, 19, 48, 59
HMW-MAA	melanoma chondroitin sulfate proteoglycan	Diagnostic: Improve desmoplastic melanoma diagnosis	21
FABP-7	involved in lipid-metabolism	Prognostic: independent poor-prognostic factor for DFS and OS if found in tumor	25, 26
Survivin	inhibitor of apoptosis protein family	Prognostic: expression in tumor is correlated to good prognosis among stage IV patients who received postoperative immunotherapy	27
CXCR4	chemokine receptor	Tumor characterization: the most common chemokine receptor expressed in PE liver melanoma metastases	38
CCR-9	chemokine receptor	Tumor characterization: Expression in tumor may facilitate metastasis to the small intestine	40
Genomic BM			
BRAF	a component of the Ras-MAPK-ERK pathway	Diagnostic/Prognostic: V600E mutation detected in patient serum can predict disease outcome and therapeutic response	59, 64, 91
RET	receptor tyrosine kinase	Diagnostic: G691S polymorphism improves desmoplastic melanoma diagnosis	74
Apaf-1	tumor-suppressor gene mediating p53-induced apoptosis	Prognostic: LOH detection in tumor and serum associated with poor prognosis in patient	77, 92
FABP7	Lipid-metabolizing capacity associated with fatty acids	Prognostic: LOH detection in tumor is associated with poor prognosis in patient	26
RASSF1A/RARb(beta)	tumor suppressor gene	Prognostic: Detection of hypermethylated RASSF1A in patient serum is associated with worse survival in patients receiving biochemotherapy	6, 79, 83, 95
RARβ	tumor suppressor gene		6, 79, 83, 95
estrogen recepter-α	sex hormone receptor	Prognostic: Hypermethylation of ER-αfound in serum is associated with poor prognosis	94
MINT31	multiple noncoding, methylated-in-tumor loci	Prognostic: Hypermethylation of MINT31 found in tumor is linked to good prognosis in stage III melanoma	79
DNMT3	DNA methyltransferase	Prognostic: high level of DNMT3 in LN metastatic tumor is associated with poor prognosis in patients	80
miR-29c	microRNA	Prognostic: high level of miR-29c in LN metastatic tumor is associated with better prognosis in patients	80
miR-532-5p	microRNA	Tumor characterization: may contribute to melanoma progression by downregulation of RUNX3 expression	85