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Epigenetic Biomarkers in Skin Cancer

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Abstract

Epigenetic aberrations have been associated with cutaneous melanoma tumorigenesis and progression including dysregulated DNA gene promoter region methylation, histone modification, and microRNA. Several of these major epigenetic aberrations have been developed into biomarkers. Epigenetic (methylation) biomarkers can be detected in tissue and in blood as circulating DNA in melanoma patients. There is strong evidence that biomarkers in cutaneous melanoma will have an important role as companions to therapeutics and overall patient management. Important progress has been made in epigenetic melanoma biomarker development and verification of clinical utility, and this review discusses some of the key current developments and existing challenges.

Keywords

epigenetics; biomarker; methylation; microRNA; melanoma; prognosis

1. Introduction

Major skin cancers worldwide predominantly include basal cell carcinoma (BCC), cutaneous melanoma and cutaneous T-cell lymphoma (CTCL). This review will focus specifically on cutaneous melanoma, the most malignant form of skin cancer with the most extensive epigenetic analysis on biomarkers (BMs) conducted to date. Cutaneous malignant melanoma is a highly aggressive disease, comprising less than 5% of skin cancers but accounting for a majority of the deaths from skin malignancies [1]. Overall, the incidence rates of melanoma have been rising in the United States over the past 10 years, with patient survival dependent on early detection and diagnosis. Patients with metastatic melanoma have a poor prognosis, with 5-year overall survival (OS) for patients with regional or distant metastasis less than 70% and 20% respectively, as compared to over 95% for those with localized disease [2]. In patients with advanced melanoma, although some promising new therapies have recently emerged, a better understanding of the molecular alterations such as genomic and epigenomic aberrations involved in melanoma progression, particularly from

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localized tumors to metastasis, will aid in early detection and development of BMs and future targeted treatment strategies.

Melanoma, like other solid tumors, is thought to arise from a series of genetic and epigenetic events. Genetic aberrations have been identified in the past decade and have potential utility as BMs [3–9]. Recently, multiple studies have revealed that epigenetic events, such as genomic promoter region methylation of CpG islands, histone modification, and microRNA (miRNA) expression, have been shown to be important regulators of melanoma progression, and that these epigenetic changes can potentially serve as molecular BMs in tumor tissues and in blood as circulating DNA, for diagnosing disease and predicting disease outcome and progression (Figure 1) [10–13].

2. DNA methylation

Epigenetics refers to heritable changes in gene expression that are not caused by changes in the genomic DNA sequence. DNA methylation is one of the hallmark epigenetic events most studied in cancers. DNA methylation involves the addition of a methyl group to the 5’ carbon of a cytosine ring located 5’ to a guanosine base in a CpG dinucleotide and is catalyzed by DNA methyltransferases (DNMTs) [1]. These CpGs are often clustered in short CpG-rich DNA stretches; deemed CpG islands, and the majority are found in the promoter region of genes [14]. Methylation events of promoter regions have been strongly implicated in cutaneous melanoma progression [10,12]. Hypermethylation of CpG islands in the promoter region leads to gene silencing through the inhibition of transcription or via recruitment of chromatin remodeling co-repressor complexes [15]. Silencing of tumor suppressor genes or tumor-related genes (TRGs) can occur during melanoma development or later in advanced stage melanoma [12,16]. Epigenetic inactivation of multiple TRGs has been implicated in the establishment of malignancy and throughout stages of melanoma progression and metastasis [1,14]. Many of these genes are involved in cell cycle control, cell signaling, migration and invasion, apoptosis, angiogenesis, and metastasis [14,17]. At the same time there are TRGs that are activated in melanoma that are silenced in melanocytes. This transcription activation can be attributed to hypomethylation of the CpG islands in the promoter region and histone modifications.

2.1 DNA methylation detection techniques

One of the challenges in evaluating the DNA methylation status of genes is the fact that several techniques exist for evaluation of CpG island methylation. A recent review by Laird provides an excellent overview of the main principles of DNA methylation analysis, dividing these techniques into various types of pretreatment (enzyme digestion, affinity enrichment, sodium bisulfite) followed by different analytical steps (locus-specific analysis, gel-based analysis, array-based analysis, and next-generation sequencing-based analysis) [18]. Technique selection depends on the quality and quantity of input DNA needed, purity and type of tissue or fluid DNA is being extracted from, extent of genome coverage, and overall assay reproducibility, sensitivity, specificity, accuracy, and quantification. Moreover, despite advances in this field and development of multiple platforms for studying genomic methylation, uniformity and standardization remain significant issues in evaluating and comparing results.

Sodium bisulfite modification (SBM) of genomic DNA is one of the most well-utilized techniques for assessing CpG methylation status, based on the modification of genomic unmethylated cytosines to uracil [19,20]. Bisulfite conversion requires DNA denaturation before treatment and subsequent purification to remove the sodium bisulfite, thus causing substantial DNA degradation and often times requiring a large amount of high-purity input DNA. Other limitations of SBM include incomplete bisulfite conversion and differential

PCR efficiency for methylated versus unmethylated sequences [18]. Bisulfite-based DNA methylation analysis is currently regarded as the “gold standard” and offers the advantage of quantitative assessment, detection sensitivity and the ability to analyze a wide variety of samples, though it is limited by the amount of DNA isolated from SBM as described above. The most commonly used SBM assays for assessing epigenomic BMs include: bisulfite sequencing, bisulfite pyrosequencing, combined bisulfite restriction analysis (CoBRA), methylation-specific realtime PCR (MSP), and gel electrophoresis [15,21]. The advantages of MSP include that it can be performed on very small quantities of DNA, such as that from paraffin-embedded tissues or free-circulating DNA in blood, and its products can be assessed by various platforms including gel electrophoresis, capillary array electrophoresis (CAE), MassARRAY, or real-time quantitative MSP as BMs [22]. CAE, MassARRAY and real-time MSP offers the ability to quantitatively evaluate a promoter region; moreover, with the MassARRAY method, one can determine the specific CpG island that best correlates with gene expression [22].

A newer approach of BM assessment is methylation-sensitive restriction enzyme digestion assays, based on the sensitivity of sequence-specific restriction enzymes that can recognize methylated cytosine within their cleavage recognition site. Although a cost effective and sensitive approach when coupled with PCR following enzyme digestion, this technique is limited to the analysis of CpG sites located within the enzyme recognition site(s) and is prone to false-positive results secondary to incomplete enzyme digestion. Affinity enrichment assays use antibodies specific for methylated CpGs or methyl-binding proteins with affinity for methylated genomic DNA. These methods allow for genome-wide assessment of DNA methylation but are limited by lack of specificity in areas of low CpG density and cannot be used to obtain information on individual CpG sites [16,18].

Another major challenge is identifying the key regulator(s) CpG islands in the promoter region. There can be more than one gene or a specific repetitive sequence region that controls mRNA transcription. It is also possible that no CpG regulatory site exists in a gene promoter region. The majority of promoter region regulatory CpG sites are near the open-reading frame. It takes methodical analysis of CpG region sequencing in conjunction with mRNA expression to determine key regulatory sites. Studies may often report hyper- or hypo-methylation in the gene promoter region that plays little role in affecting respective gene transcription.

2.2 DNA methylation BMs for diagnosis and prognosis

Aberrantly methylated melanoma TRGs can serve as BMs for early diagnosis of cancer, evaluation of cancer progression, and as prognostic indicators in melanoma patients. Our group identified and verified the inactivation of RAS association domain family protein 1A (RASSF1A), a tumor suppressor gene, in melanoma [23]. RASSF1A is involved in the regulation of apoptosis, migration, and metastasis [1]. An increase in RASSF1A methylation positively correlates with advancing tumor stage, suggesting that RASSF1A may be a useful BM of melanoma progression [12]. Separately, RASSF1A methylation has also been reported in approximately 50% of Merkel cell carcinoma (MCC) cases in a study of 83 tumors [24].

Tanemura *et al.* examined the methylation status of CpG islands in the promoter region of six TRGs involved in melanoma progression (WIF1, TFPI2, RASSF1A, RAR-β2, SOCS1, and GATA4) and a panel of methylated-in-tumor (MINT) non-coding genomic repeat sequences (MINT1, MINT2, MINT3, MINT12, MINT17, MINT25, and MINT31) to determine whether there exists a clinically significant CpG island methylator phenotype (CIMP), or a distinct methylation pattern of TRGs, related to melanoma progression [12]. MINT loci are hypermethylated CpG sites located in non-coding DNA regions that have

been reported in gastrointestinal cancer and correlated with hypermethylation of TRGs with a defined CIMP [25,26]. Comparing the methylation status of melanoma primary and metastasis, they found that an increase in hypermethylation of WIF1, TFPI2, RASSF1A, and SOCS1 was seen with increasing clinical tumor stage [12]. Moreover, there was a significant association between the methylation status of MINT17 and MINT31 and TRGs, supporting the existence of a CIMP that is associated with advancing clinical stage in melanoma patients and suggests a worse prognosis in patients with hypermethylation of these genes.

DNA promoter methylation analysis can be successfully performed in both tissue and fluids. The assessment of these BMs as cell-free circulating nucleic acids (cf-CNAs) in blood provide a non-invasive and clinically useful way to repetitively monitor patients compared to tissue biopsy. Our group was one of the first to report the prognostic utility of combining detection of circulating tumor cells (CTCs) with assessment of methylated blood DNA BMs [27]. Matched pairs of nucleated cells from whole blood and serum specimens from 50 AJCC stage IV melanoma patients were assessed for three mRNA CTC BMs (MART-1, GalNAc-T, and MAGE-A3) and two methylated DNA BMs (RASSF1A and RAR-β2). The CTC BMs were significantly associated with the presence of methylated cf-CNA and the presence of both was an indicator of poor OS under biochemotherapy (BC) [27]. This suggested that CTC may be a significant source of circulating methylated DNA. We have demonstrated that cf-CNA as methylated TRGs such as RASSF1A, MGMT, RARβ2, and ERα can be used as epigenomic BMs for monitoring cutaneous melanoma and have prognostic utility (Figure 2) [28]

Epigenomic BMs can also potentially be used to monitor patient treatment response. Mori *et al.* demonstrated, in serum DNA of stage IV melanoma, that circulating methylated RASSF1A was significantly less frequent for BC responders than nonresponders, indicating that increased methylation correlates not only with a worse prognosis but also can be used to monitor development of resistance to therapy. In a study of stage IV melanoma patients undergoing BC with tamoxifen, serum estrogen receptor alpha (ER-α) methylation was an unfavorable prognostic factor and a negative predictor of overall and progression-free survival in patients treated with BC (Figure 3) [10].

2.3 DNA global hypomethylation

Global DNA hypomethylation can lead to chromosomal instability, activation of endogenous retroviral elements, and reactivation of genes with oncogenic activity, such as cancer testis genes, for example, the MAGE (melanoma antigen) family [17]. In malignant melanoma, aberrant expression of MAGE genes occurs secondary to promoter hypomethylation [29]. It has also been observed that methylation levels of genomic repetitive sequences such as long interspersed nuclear element-1 (LINE-1) are representative of global methylation status [16]. In patients with Stage IIIC cutaneous melanoma, Sigalotti *et al*., identified LINE-1 methylation as a molecular marker of prognosis with patients demonstrating LINE-1 hypomethylation having a significantly better OS compared to those with hypermethylated LINE-1 sequences [30]. However, discordance between blood LINE-1 status and melanoma tumors was reported, and the studies will need to be verified.

2.4 Mechanisms of melanoma TRG methylation

DNMT1 is associated with the maintenance of established DNA methylation patterns; whereas DNMT3A and DNMT3B have been implicated in the generation of *de novo* methylation patterns at previously unmethylated CpGs [15]. A recent study by Nguyen *et al.* demonstrated that DNMT3A and DNMT3B protein expression are significantly correlated with increasing AJCC stage and that high expression of DNMT3B by immunohistochemistry (IHC) staining was significantly correlated with worse OS in AJCC

stage III melanoma patients by multivariate analysis (p=0.004) [11]. DNMT3 can be a potential BM for melanoma progression. Significant correlation of these events was associated with RASSF1A promoter methylation. This indicated that significant changes in mechanisms involved in epigenomic regulation of melanoma are occurring during clinical progression. Several DNMT inhibitors have been investigated in clinical trials, most notably 5-aza-2'-deoxycytidine, also known as decitabine [1]. However, there is concern for drug toxicity as the lack of specificity of these demethylating agents may lead to global hypomethylation and potentially activation of tumor proto-oncogenes [17]. This area still remains of major interest to pharmaceuticals for development of less toxic drugs with greater specificity towards cancer.

Temozolomide (TMZ), an orally available drug, has been used to treat melanoma patients with metastatic disease [31]. TMZ depletes levels of methylguanine methyltransferase (MGMT), a DNA repair enzyme, and TMZ sensitivity has been correlated with methylation of the MGMT promoter [32]. In a phase II study, Rietschel *et al*., treated unresectable stage III and stage IV melanoma patients with extended-dose TMZ and assessed their clinical response in conjunction with MGMT promoter methylation status. They found that MGMT promoter methylation level more than 25% positively correlated with a partial clinical response.

Overall, these epigenetic changes in melanoma can serve as potential BMs for prognosis and prediction. Development of clinically efficient tests will be important in assisting with decision making in treatment management. Epigenomics in melanoma offer new potential BMs for tumor and blood while at the same time offering potential targets for therapy.

3. microRNA

miRNAs are evolutionarily conserved, endogenous, non-coding RNA transcripts of ~22 nucleotides in length that serve to temporally and spatially regulate biological function [33]. miRNA are considered an epigenomic mechanism that can have normal regulatory function but also can have negative influence when dysregulated, particularly in cancer progression as in melanoma. They are derived from non-coding intergenic or intronic regions of DNA that, once in their mature form, interfere with protein translation from mRNA transcripts. miRNA can preferentially bind with the 3' untranslated region (UTR) of mRNA transcripts to inhibit translation or degrade the mRNA transcript before translation can take place [34].

miRNA can modulate biological functions, e.g., cell cycle, proliferation, apoptosis, and angiogenesis, which, if aberrantly regulated, can lead to malignancy [35]. For example, miR-221/222 was found to interfere with c-KIT and p27, causing dysregulation of the cell cycle during the progression of melanoma [36]. miRNA regulation may influence the microenvironment, and can contribute to tumor cell invasion, migration and metastasis [37]. miRNA regulation of protein coding genes is, therefore, an essential regulatory element in normal biological development and function.

Deregulated miRNA expression may serve as diagnostic or prognostic BM in cutaneous melanoma (see Table 1 for a list of recent miRNA melanoma studies). In the previously mentioned Nguyen *et al*. study, DNA methyltransferases DNMT3A and DNMT3B were shown to be regulated by miR-29c in melanoma [11]. This mechanism was first demonstrated earlier in lung malignancy [38], thus implying that the epigenetic pathways currently associated with other cancers may be applicable in skin cancers as well. In addition to the demonstration of functional association in melanoma, both miR-29c and DNMT3B were found to be significantly independent prognostic predictors of OS in Stage III melanoma patients through multivariate analysis including common melanoma prognostic

factors. This also shows that multiple epigenetic factors within a single pathway can function as independent BMs, supporting both the functional and prognostic value of the discovery of epigenetic pathways.

As a potential BM of progression and outcome, Gaziel-Sovran *et al*. [37] showed that increased expression of miR-30b/30d downregulated GALNT7 leading to enhanced migration and lowered immunosuppressive features. They were able to identify a novel immunosuppressive role for GALNT7 as a direct regulator of immunosuppressive cytokine IL-10. Interestingly, this immunosuppressive effect was also found at the site of metastasis, suggesting a possible long range of deregulation and control of immunosuppression in cancer that extends beyond the microenvironment. In terms of prognostic BM potential, expression levels of these miRNAs have been correlated with transition from primary to metastasis, and to recurrence and OS. However, the specificity and significance of these miRNAs have yet to be verified in large sample sizes and multicenter studies. One of the major drawbacks of miRNA is that they target many types of genes and can have multiple functions in both normal and tumor tissues [see www.miRwalk.com website]. Other major problems in miRNA are that miRNA assays are not always controlled, properly quantified, or uniformly reported.

Grignol, *et al*. characterized deregulated miRNA expression with a change in tissue from benign nevi status to borderline melanocytic lesions. The authors demonstrated a relationship between upregulation of *miR-21* and *miR-155* and malignant phenotypical changes (increased mitotic activity and lesion depth, respectively). A significant number of patients whose tissue was shown to overexpress these two miRNAs progressed to develop sentinel lymph node metastasis. These miRNAs may therefore serve to supplement and further characterize traditional methods of melanoma analysis such as Breslow depth and the mitotic index [39].

Interestingly, CpG island methylation has been shown to regulate expression of miR-375 in Stage III melanoma cell lines [40] through treatment of cell lines with demethylating agents 5AzadC and 4-PBA. The methylation state of the miR-375 CpG islands was significantly greater in Stage II or III melanoma cell lines when compared to Stage I melanoma or benign melanocytes, which demonstrates a potential utility for miR-375 methylation status as a BM for progression. The study additionally demonstrated a functional association of miR-375 with inhibition of proliferation and invasion of melanoma cell lines, which provides rationale for its role in progression.

Other studies have demonstrated regulation of multiple miRNAs by methylation [41], with a group recently demonstrating CpG island methylation regulation of miR-34b [42]. Increased methylation frequency was shown in Stage III and IV melanoma cell lines when compared with normal melanocytes, keratinocytes, and Stage I and II melanoma cell lines, indicating miR-34b expression as a possible marker for metastatic disease.

3.1 Assay for circulating miRNA

Assays for circulating miRNA have added a promising new platform in the search for BMs. Circulating assays offer advantages over other BM assessment procedures in the clinical setting. Tissue biopsy can impose the burden of morbidity risks that accompany any invasive procedure [43], failure rate in small tumors [44], relative expense, and the inherent limitation of available or accessible sample. Further, circulating miRNA appear to exhibit superior stability over tissue derived species [45,46]. Serial bleeds can serve as the source of samples used to monitor changes in miRNA expression throughout a patient's treatment course. miRNA circulating in blood derivatives, including whole blood, plasma, and serum, have been shown to be differentially expressed in a variety of cancers versus normal patients

[47–51]. A recent study of *miR-221* in the circulation [52], using serum from 90 clinically well characterized patients along with 8 postoperative recurrence sera, demonstrated its potential as a non-invasive detection BM capable of distinguishing between *in situ* and invasive melanoma, and confirming prior miR- 221/222 studies in tissue [36].

3.2 Challenges

Overall challenges to the BM use of miRNA remain, nevertheless. Conflicting results are widely reported and study variations make comparisons somewhat difficult. Limitations of miRNA BM studies include the fact that individual study samples may be limited and not always well characterized in terms of clinicopathological factors, microdissection is not always used for precise tissue sample acquisition, and interpretation of PCR results and choice in reference markers vary [53]. The diversity of assay platforms available also presents an additional opportunity for discordance [54]. Several detailed reviews of the more general issues that surround the interpretation of study results have recently been published [55–57].

Challenges to the circulating miRNA assay remain as well. Unlike the direct tissue assay, other tissues besides tumor are known to release miRNA into the circulation and may influence circulating miRNA assay results [51]. As with tissue assays, platforms, methods, and results are variable in publication [58]. Further, controls in circulating miRNA as BMs remain a critical problem [56].

While the promise of a non-invasive bodily fluid assay for circulating miRNA is appealing, additional challenges arise from the unique nature of the assay and the source of sample, such as differences in expression that are related to the particular blood fraction and the method of miRNA secretion [59]. Yet despite these obstacles, in melanoma these miRNA in tissues and blood are potential epigenomic BMs that should be further exploited.

4. Histones

Histones are paired core protein octamers around which DNA chromatin are organized in a plastic architecture of ~147 bp nucleosomes. Third dimensional conformation of the nucleosome can be altered by a variety of catalytic enzymes that can deposit or remove covalent molecules on the n-terminal histone tail residues that extends from each histone, in order to repress RNA transcription through a closed formation (heterochromatin) or to allow transcription through an open formation (euchromatin). Molecule depositions can also interact with other direct DNA methylating agents, a form of epigenetic crosstalk, to further mediate transcription status in relation to other epigenetic and biological expression programs. In melanoma these relations may become deranged, leading to tumorigenesis [60].

4.1 Histone Modification in Melanoma

The study of histone modification (methylation, acetylation, phosphorylation, sumoylation, uquitinylation) [61,62] is a form of epigenomics. This is a field still in its infancy particularly as BMs. However, preliminary studies suggest histone types and modifications may be potential BMs of gene regulation. The lysine tail of each of the four main histones (H2A, H2B, H3 and H4) can accommodate many different arrangements of the same set of covalent molecules, with different regulatory outcomes depending on the particular modification pattern [60]. In addition, entire histones may be substituted with variants that come with preconfigured histone tail patterns [63,64].

Histone changes have been primarily linked to the ability for malignant melanoma to escape the senescent phenotype that characterizes benign nevi and normal tissue. Melanoma cells

are able to escape the senescent phenotype ordinarily imposed on DNA damaged cells by the p53/p14ARF pathway. Studies have shown that histone marks and the expression level of histone modifying enzymes are correlated with uncontrolled proliferation in melanoma [15,65].

Bachmann IM *et al.* reported that higher EZH2 expression was associated with thicker primary melanomas and Clark's invasion level V. Interestingly, higher EZH2 expression was associated with loss of p16 expression and strong expression of Cyclin D1 [66]. They also reported that 5-year survival in patients with high EZH2 expression was 48%, compared with 71% ($p = .032$) among the cases with low EZH2 expression, suggesting prognostic BM potential. EZH2 has been shown to downregulate expression of tumor suppressor Rap1GAP [67] and p21 in melanoma [68].

Histone acetylation has been closely studied in tissue, cell line and animal models of melanoma where it has been shown to correlate with senescence [69,70]. This has led to multiple therapeutic trials for HDACi, including in melanoma (Vorinostat, MS-275, Pivanex, Valproic Acid, and Panobinostat), though many have shown limited benefit entailing significant side effects. However, clinical trials are still ongoing. Completion of a multicenter phase II trial of MS-275, an HDAC1/3 inhibitor, in patients with unresectable metastatic melanoma who have received at least one other systemic treatment [71] showed well-tolerated, long-term tumor stabilizations, but no objective responses in pretreated metastatic melanoma. Despite the development and pursuit of HDACi in clinical trials, expression of acetylation or acetylation enzymes has yet to be tied to diagnostic or prognostic outcomes in a robust assay.

Development of histone BMs in melanoma has been challenged by the absence of an efficient, robust assay method. Indirect measurement of histone status by methods such as IHC and chromatin immunoprecipitation (ChIP) are less than quantitative and, in the case of ChIP, are subject to wide procedural variation between labs, with multiple steps that are prone to error and requiring several days at the bench per assay [72]. Mass spectrometry, though potentially quantitative, likewise contains many processing steps that have seen difficulty in replication between labs [73]. No platform as yet provides a direct measure of histone enzyme activity at the lysine residue. These represent challenges yet to be resolved in the effort to establish epigenetic histone BMs in melanoma [74].

5. Epigenetic Studies in Non-melanoma Skin Cancers

We will briefly mention other skin cancers as there have been limited studies on epigenetic BMs for other skin cancer types. BCC and squamous cell carcinoma (SCC) represent the majority of skin cancer cases, with over 2 million cases treated in 2006 [75]. However, these skin cancer types are less aggressive than melanoma and are often curable by surgical excision. As a result, BM work on BCC and SCC has been minimal. In BCC, methylation of the FHIT promoter has been demonstrated [76], while methylation in the PTCH gene was shown to likely play only a minor role in carcinogenesis [77]. Other rarer forms of skin cancer, such as CTCL and its variants [78] have studies that confirmed repression of BCL7a, PTPRG, TP73, and FAS through methylation [79–81]. MCC [82] demonstrated p14ARK (encoding tumor suppressor p14) methylation in 42% of 19 tumor samples [83]. Studies regarding application of these epigenetic events as BMs, however, have seen limited use due to the rare nature of CTCL and MCC.

6. Conclusions

The development of epigenetic BMs for the diagnosis and prognosis of cutaneous melanoma continues to be promising, yet challenging. BM validation is founded upon robust, accurate

assays that, in turn, depend on transparency and detailed standard operating procedures abiding by state and national regulatory guidelines that deliver well-characterized qualitative and quantitative results that are meaningful to clinical outcomes. REMARK guidelines [84], were developed to help researchers deliver these objectives, facilitating the comparison of results between publications that study the same candidate BM.

Melanoma BM investigations do not always meet REMARK criteria, and results often appear to conflict between studies and even within the same study. This is evident in all assay platforms and within all areas of epigenetic BM research. Omissions of BM study-critical information include patient treatment prior to sample acquisition, sample preparation and handling, quantitative confirmation for qualitative assays, hazard ratios, confidence intervals and receiver operating characteristic (ROC) curves for specificity and sensitivity of the BM.

Challenges that accompany specific histone, methylation, and miRNA assays have been cited above. Quantitative realtime PCR may generally be expected to deliver the accuracy, robustness, and precision that can validate miRNA and methylation qPCR assays in large-scale studies, yet one must be careful in result interpretations. MIQE guidelines for a consistent qPCR assay have also been developed that, along with REMARK guidelines, should serve to facilitate the reporting, discovery and validation of clinically useful BMs [85]. As assays and techniques for epigenomic BMs become standardized, the development of new and reliable melanoma BMs will be made possible in the near future.

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Abbreviations

BC	Biochemotherapy
BCC	Basal cell carcinoma
BM	Biomarker
SBM	Sodium bisulfite modification
CAE	Capillary Array Electrophoresis
cf-CNA	cell-free circulating nucleic acid
ChIP	chromatin immunoprecipitation
CIMP	CpG Island Methylator Phenotype
CoBRA	Combined Bisulfite Restriction Analysis
CTC	Circulating Tumor Cell
CTCL	Cutaneous T-cell Lymphoma
DNMT	DNA Methyltransferase
IHC	immunohistochemistry
LINE-1	Long Interspersed Nuclear Element-1
MCC	Merkel cell carcinoma

MINT	Methylated-in-tumor
miRNA	micro-RNAs
MSP	Methylation-specific realtime PCR
OS	Overall Survival
PCR	Polymerase Chain Reaction
ROC	Receiver Operating Characteristics
SCC	Squamous cell carcinoma
TMZ	Temozolomide
TRG	Tumor-related Genes
UTR	Untranslated Region

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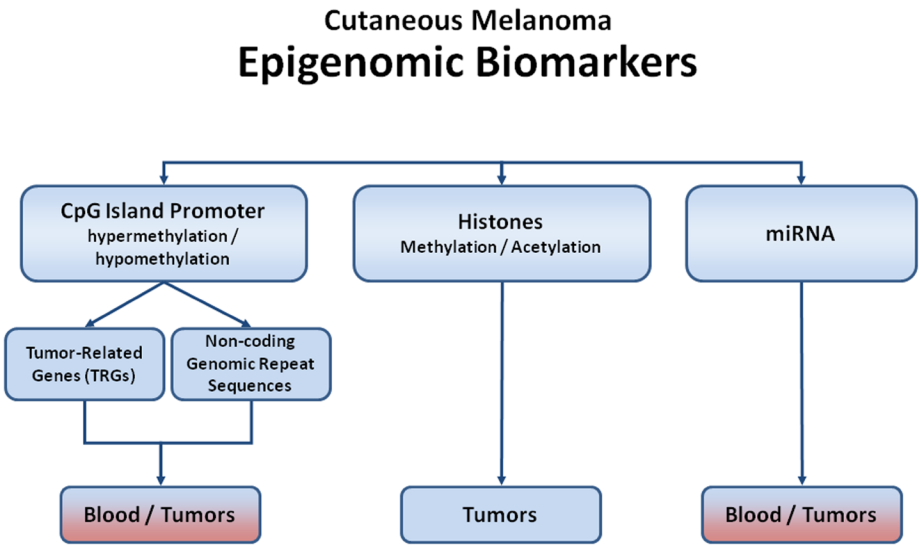

Figure 1.
Epigenomic BMs and studied applications.

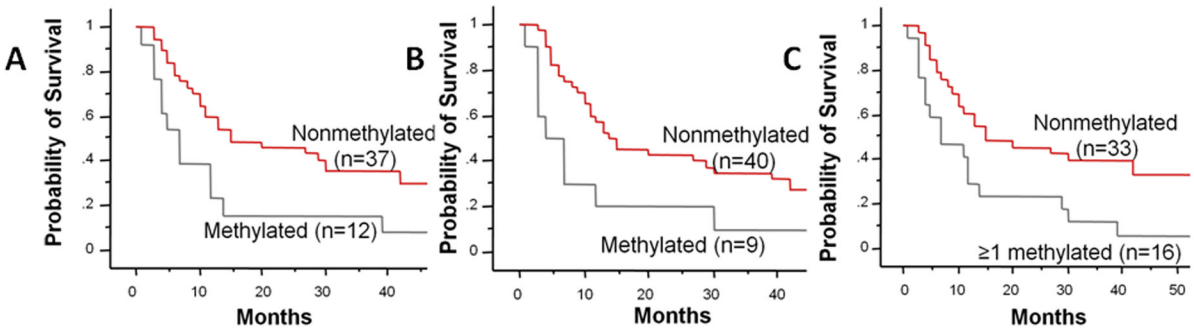

Figure 2.

(**A**) Kaplan-Meier survival curves of biochemotherapy (BC) patients: Correlation of pre-BC serum RASSF1A methylation BM with overall survival (log-rank test, P = .013). Methylated: Patients with serum methylation of RASSF1A. Nonmethylated: Patients with no serum methylation of RASSF1A. (**B**) Correlation of pre-BC serum RARβ-2 methylation status with overall survival (log-rank test, P = .02). Methylated: Patients with serum methylation of RARβ-2. Nonmethylated: Patients with no serum methylation of RARβ-2. (**C**) Correlation of pre-BC serum methylation of at least one BM with overall survival (log-rank test, P = .01). ≥ 1 methylated: Patients with serum methylation of at least one BM. Nonmethylated: Patients with no serum methylation of genes. Figure reproduced with permission from Mori et al. 2005 [28].

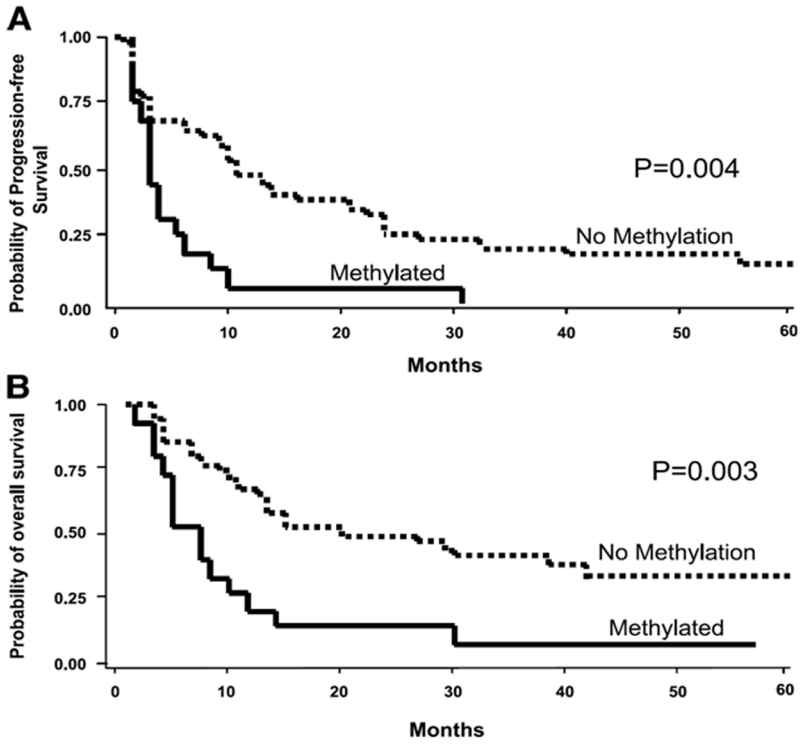

Figure 3.

(A) Kaplan-Meier curves showing the correlation of pre-BC serum ER-α methylation status with progression-free survival (Cox proportional hazard, P = 0.004). Methylated, patients with serum methylated ER-α DNA. No methylation, patients with no detectable serum methylated ER-α. (**B**) Kaplan-Meier curves showing the correlation of pre-biochemotherapy serum ER-α methylation status with OS (Cox proportional hazard, P = 0.003). Figure reproduced with permission from Mori *et al*. 2006 [10].

Table 1

Recent Melanoma Epigenetic Studies

Epigenetic Element	Publication Year	Possible Biomarker Use	Sample	Target/Pathway	Epigenetic melanoma Expression	Function
miR-29c[11]	2011	Progression, Prognosis	Tissue	DNMT3	Down	Tumor Suppressor
miR-214[86]	2011	Diagnosis, Progression	Cell, Tissue	TFAP2C	Up	Oncogene
miR-205[87]	2011	Detection, Progression	Cell, Tissue	E2F1, E2F5	Down	Tumor
miR-30b/ miR-30d [37]	2011	Progression, Prognosis	Cell, Tissue	GALNT7	Up	Oncogene
miR-34b[42]	2011	Progression	Cell, Tissue	Cell Adhesion, Migration, motility	Down	Tumor Suppressor
miR-506/ miR-514[88]	2011	Detection, Progression	Cell, Tissue	Growth, Apoptosis, Invasion	Up	Oncogene
miR-21/ miR-155 [39]	2011	Detection, Prognosis	Tissue	Proliferation, Metastasis	Up	Oncogene
miR-221 [52]	2011	Progression	Serum	P27Kip1/CDKN1B and C-Kit (from a prior study18417445)	Up	Oncogene
miR-375[40]	2011	Detection, Progression	Cell, Tissue	Proliferation, Invasion, Migration	Down	Tumor Suppressor
DNA Methylation LINE-1[30]	2011	Prognosis	Cell	Surrogate for hypomethylated TSGs	Up	Marker for inactive Tumor Suppressor
DNA Histone Modifier EZH2[68]	2011	Detection, Progression	Cell	Proliferation	Up	Oncogene

This table reflects recent significant studies published in 2011. The reader is strongly encouraged to refer to earlier reviews that offer insightful and comprehensive coverage of studies published prior to 2011 [15,17,89–93].