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Journal

Journal of Molecular Cell Biology, 6(5)

ISSN

1674-2788

Authors

Besaratinia, Ahmad

Tommasi, Stella

Publication Date

2014-10-01

DOI

10.1093/jmcb/mju027

Peer reviewed

Review

Epigenetics of human melanoma: promises and challenges

Ahmad Besaratinia* and Stella Tommasi

Department of Preventive Medicine, Keck School of Medicine, University of Southern California, M/C 9603, Los Angeles, CA 90033, USA

* Correspondence to: Ahmad Besaratinia, E-mail: besarati@med.usc.edu

Melanoma is the deadliest form of skin cancer with rising incidence and mortality rates. Although early-stage melanoma is highly curable, advanced-stage melanoma is refractory to treatment. This underscores the importance of prevention and early detection as well as the need to improve treatment and prognostication of human melanoma. Elucidating the underlying mechanisms of the initiation and progression of human melanoma can help identify potential targets of intervention for prevention, diagnosis, therapy, and prognosis of this disease. Aberrant DNA methylation and histone modifications are the best-established epigenetic mechanisms of carcinogenesis. The occurrence of epigenetic changes prior to clinical diagnosis of cancer and their reversibility through pharmacologic/genetic approaches offer a promising avenue for basic and translational research on human melanoma. Candidate gene(s) or genome-wide aberrant DNA methylation and histone modifications have been observed in human melanoma tumor tissues and cell lines, and correlated to cellular and functional characteristics and/or clinicopathological features of this malignancy. The present review summarizes the published researches on aberrant DNA methylation and histone modifications in connection with human melanoma. Representative studies are highlighted to set forth the current state of knowledge, gaps in the knowledgebase, and future directions in these epigenetic fields of research. Examples of epigenetic therapy applied for human melanoma *in vitro*, and the challenges of its *in vivo* application for clinical treatment of solid tumors are discussed.

Keywords: aberrant DNA methylation, epigenetic therapy, histone modifications, melanoma cell lines, skin cancer

Introduction

Cutaneous malignant melanoma (hereinafter referred to as ‘melanoma’) is a tumor that originates from the malignant transformation of the pigment-producing cells of the epidermis, melanocytes (Miller and Mihm, 2006; Gray-Schopfer et al., 2007). Melanoma is the most aggressive and virulent form of skin cancer (Miller and Mihm, 2006; Fecher et al., 2007; Gray-Schopfer et al., 2007). Nearly 75% of all deaths from skin malignancies are attributable to melanoma (American Cancer Society, 2013). For the past several decades, the incidence of melanoma has continued to increase in the USA and many parts of the world, a trend that is in stark contrast to the steady decline in the overall cancer incidence (Little and Eide, 2012; American Cancer Society, 2013). Melanoma is a curable disease when detected at early stages; however, around 20% of melanoma cases are diagnosed at advanced stages that are refractory to treatment (Miller and Mihm, 2006; Gray-Schopfer et al., 2007; American Cancer Society, 2013). This underscores the importance of prevention and early detection as well as the need to improve treatment and

prognostication of human melanoma. Elucidating the underlying mechanisms of the initiation and progression of human melanoma can help identify molecular targets of intervention for prevention, early detection, treatment, and prognosis of this malignancy (Ko and Fisher, 2011; La Porta, 2012; Tsao et al., 2012).

Aberrant DNA methylation and conformational changes in chromatin through post-translational modification of histones are the best-studied epigenetic mechanisms of carcinogenesis (Kulis and Esteller, 2010; Laird, 2010; Portela and Esteller, 2010; Baylin and Jones, 2011; Rodriguez-Paredes and Esteller, 2011). The occurrence of epigenetic changes prior to clinical diagnosis of cancer and the reversibility of these changes through pharmacologic/genetic manipulations (Rhee et al., 2002; Gius et al., 2004; Gronbaek et al., 2007; Jacinto et al., 2009; Rodriguez-Paredes and Esteller, 2011) offer a great avenue for basic and translational research on human melanoma (Howell et al., 2009; Sigalotti et al., 2010; Tanaka et al., 2011; van den Hurk et al., 2012; Griewank et al., 2013). Candidate gene(s) or genome-wide aberrant DNA methylation and histone modifications have been observed in human melanoma tumor tissues and cell lines, and correlated to cellular and functional characteristics and/or clinicopathological features of this disease (Patino and Susa, 2008; Howell et al., 2009; Rodriguez-Cerdeira and Molares-Vila, 2011; van den Hurk

Received March 27, 2014. Revised April 16, 2014. Accepted April 23, 2014.

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et al., 2012; Griewank et al., 2013; Pimiento et al., 2013). However, the mechanistic roles of these epigenetic aberrancies in the initiation and progression of human melanoma have not been fully determined. Uncovering the roles played by epigenetic abnormalities in the genesis and progression of human melanoma can help identify specific pathways that are disrupted during the evolution of this disease (Ko and Fisher, 2011; La Porta, 2012; Tsao et al., 2012). This mechanistic knowledge will be critical to developing biological markers that can best predict the initiation and progression of human melanoma. Such biomarkers will have utility for prevention, early detection, treatment, and monitoring of the progression of human melanoma. From a therapeutic perspective, these biomarkers will highlight the reversible and ‘drugable’ epigenetic changes that can be used for personalized medicine and molecular-targeted therapy of human melanoma (Ko and Fisher, 2011; La Porta, 2012; Tsao et al., 2012).

The present review summarizes the published researches on aberrant DNA methylation and histone modifications in connection with human melanoma. Representative studies are highlighted to set forth the current state of knowledge, gaps in the knowledge-base, and future directions in this rapidly evolving field of research. Examples of epigenetic therapy applied for human melanoma *in vitro*, as well as the challenges of its application for clinical treatment of solid tumors *in vivo*, are discussed. Moreover, our proposed model of human melanoma genesis and progression is outlined, in which the interplays between epigenetic and genetic determinants, environmental factors (sunlight ultraviolet radiation (UV) being the most important one) (Lucas et al., 2006; Besaratinia and Pfeifer, 2008; Garibyan and Fisher, 2010), and other determinants (e.g. immune response) govern the development of this disease. Figure 1 is a schematic representation of our proposed model of human melanoma genesis and progression. We will refer to recent comprehensive reviews on other epigenetic mechanisms of carcinogenesis, e.g. microRNA (miRNA)-, nucleosome positioning-, and chromatin remodeling-mediated gene deregulation, and genetic and environmental determinants of human melanoma (Garibyan and Fisher, 2010; Kulis and Esteller, 2010; Portela and Esteller, 2010; Baylin and Jones, 2011; Ko and Fisher, 2011; Rodriguez-Paredes and Esteller, 2011; Flaherty et al., 2012; La Porta, 2012; Tsao et al., 2012).

General features of human melanoma

Melanoma is characterized by the uncontrolled growth of melanocytes that are specialized pigment-producing cells (Miller and Mihm, 2006; Gray-Schopfer et al., 2007). Because melanocytes are derived from the neural crest, melanomas can arise in any anatomical sites to which neural crest cells migrate, including the eye, gastrointestinal tract, and brain, although they mostly occur in the skin (Miller and Mihm, 2006; Gray-Schopfer et al., 2007; American Cancer Society, 2013). Cutaneous melanomas may appear suddenly without warning in unmarked skin, but they can also originate from or near a mole, i.e. melanocytic nevus (Miller and Mihm, 2006; Gray-Schopfer et al., 2007). Often, melanomas are found on the trunk of men and women (i.e. on the chest and upper back) or on the legs of women, although they may also occur

elsewhere in the body (American Cancer Society, 2013). Melanomas are usually detected at localized stages before spreading beyond the outer layers of the skin; however, ~20% of melanomas are diagnosed at advanced stages with metastasis to lymph nodes and/or internal organs, such as the lung, liver, or brain (Miller and Mihm, 2006; Gray-Schopfer et al., 2007; American Cancer Society, 2013). When detected at early stages, melanoma is highly curable; however, advanced-stage melanoma has an unfavorable prognosis (American Cancer Society, 2013). From the standpoint of public health, both prevention and early detection are high priorities for melanoma control, although improving the therapeutic and prognostic approaches for melanoma remains equally important (Ko and Fisher, 2011; La Porta, 2012; Tsao et al., 2012).

Melanocyte biology

Epidermal melanocytes are derived from the neural crest, and migrate to the epidermis during embryogenesis, where they reside in the basal layer in contact with keratinocytes (Miller and Mihm, 2006; Gray-Schopfer et al., 2007). Melanocyte homeostasis is finely regulated by keratinocytes that upon UV irradiation, secrete α -melanocyte stimulating hormone (α -MSH) and other related proopiomelanocortin-derived peptides, which bind to the melanocortin 1 receptor (MC1R) on melanocytes (Tsao et al., 2012). This G protein-coupled receptor activates the cyclic adenosine mono-phosphate (cAMP) pathway in a protein kinase A-dependent fashion leading to melanocyte proliferation and melanogenesis (Fecher et al., 2007; Flaherty et al., 2012). Stimulation of melanogenesis results in increased production of melanins, a family of closely related molecules derived from tyrosine, at a high ratio of dark/black ‘eumelanins’ to yellowish ‘pheomelanins’, a process otherwise known as ‘tanning’. Whereas eumelanins provide protection from the damaging effects of sunlight UV, pheomelanins trigger UV-induced photosensitization reactions through generation of reactive oxygen species, thus, constituting a risk factor for sunlight-associated macromolecular damage (Garibyan and Fisher, 2010; Tsao et al., 2012). Once produced, these melanin pigments are packaged into melanosomes, and subsequently delivered to keratinocytes by way of dendritic projections (Gray-Schopfer et al., 2007). In keratinocytes, melanosomes are carefully distributed according to the UV irradiation profile, and strategically positioned over the sun-exposed side of the nuclei, thus, forming cap-like structures that shield the genetic materials (Gray-Schopfer et al., 2007; Garibyan and Fisher, 2010; Tsao et al., 2012).

Environmental and genetic determinants of human melanoma

Environmental factors are implicated in the development of human diseases, including cancer (Garibyan and Fisher, 2010; Flaherty et al., 2012; Tsao et al., 2012). Sunlight UV is the best-known environmental determinant of human melanoma (Lucas et al., 2006; Besaratinia and Pfeifer, 2008; Garibyan and Fisher, 2010). Early age, intermittent, and recreational-type sun exposure are associated with human melanoma development (Chin et al., 2006; Tsao et al., 2012). Also, exposure to blasts of sunlight,

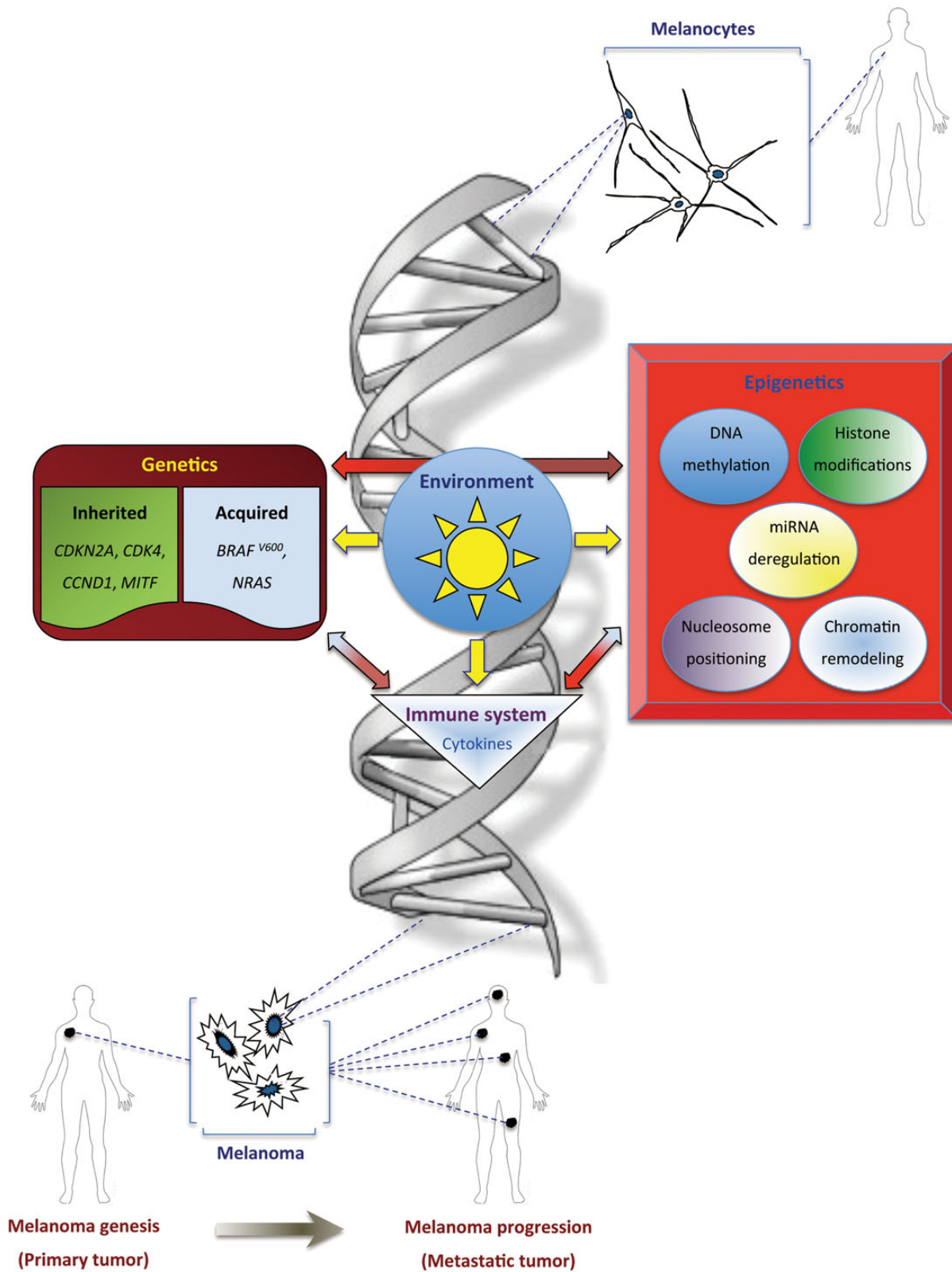


Figure 1 Proposed model of human melanoma genesis and progression. Our proposed model of human melanoma genesis and progression centers on the interplays between genetic alterations and epigenetic modifications influenced by environmental factors (e.g. sunlight UV) and other determinants, e.g. immunologic responses (e.g. cytokine-dependent), which ultimately disrupt crucial molecular pathways leading to human melanoma development. The *CDKN2A* and *CDK4* mutation/deletion, *CCND1* and *MITF* amplifications, and *BRAF^{V600}* and *NRAS* mutations are known genetic alterations in human melanoma. Aberrant DNA methylation and histone modifications are well-established epigenetic changes occurring in human melanoma, which in conjunction with the above determinants and other epigenetic mechanisms (e.g. microRNA-, nucleosome positioning-, and chromatin remodeling-mediated gene deregulation) drive the initiation and progression of human melanoma.

often received during leisurely sunbathing or sun tanning, is linked to *BRAF* mutagenesis, which is a common genetic alteration in human melanoma. Oncogenic *BRAF* mutations are prevalent at anatomical sites that have undergone episodes of sunburn but less frequently found at sites protected from the sun or chronically exposed to solar UV (Miller and Mihm, 2006; Devitt et al., 2011; Tsao et al., 2012). To date, the involvement of sunlight UV in the genesis of human melanoma has mostly been ascribed to its genotoxic and immunogenic effects, although an epigenetic mode of action for solar UV is also beginning to emerge (Besaratina and Pfeifer, 2008). Notwithstanding, the mechanistic aspects of sunlight UV-associated human melanoma are far from being fully understood, and await further delineation (Besaratina and Pfeifer, 2008; Garibyan and Fisher, 2010).

Genetic alterations in various components of the mitogen-activated protein kinase (MAPK: RAS-RAF-MEK-ERK) pathway occur frequently in human melanoma (Garibyan and Fisher, 2010; Flaherty et al., 2012; Tsao et al., 2012). Somatic mutations in two crucial genes of the MAPK pathway, including *BRAF* and *NRAS*, are highly significant for human melanoma development because up to 70% of all melanomas harbor an oncogenic mutation in one of these two genes, with the former being mutated in the vast majority of cases (Chin et al., 2006; Miller and Mihm, 2006; Tsao et al., 2012). Specifically, single/tandem base substitutions in codon 600 of *BRAF* gene (*BRAF*^{V600}) comprise up to 92% of all mutations occurring in this proto-oncogene in human melanoma (Chin et al., 2006; Flaherty et al., 2012; Tsao et al., 2012). However, *BRAF*^{V600} mutagenesis *per se* is not sufficient to produce melanoma *in vivo* because benign melanocytic nevi, which rarely progress to melanoma, also carry *BRAF* mutations at high frequencies (Chin et al., 2006; Flaherty et al., 2012; Tsao et al., 2012). In addition, activating *BRAF* mutation in transgenic zebrafish induces benign nevi formation only, whereas progression to frank- and serially transplantable melanoma requires additional loss of *Trp53* expression (Patton et al., 2005). Also, whereas induction of *BRAF*^{V600} expression in a conditional mouse model promotes benign melanocytic hyperplasia, additional silencing of the phosphatase and tensin homolog gene results in invasive and metastatic melanoma (Dankort et al., 2009). Altogether, it is likely that genetic factors in cooperation with additional determinants (e.g. environmental factors and epigenetic modifications) contribute to human melanoma development. In support of this theory, work in our laboratory (Besaratina and Pfeifer, 2008; Besaratina et al., 2008) has shown that sunlight UV irradiation of human and mouse cells *in vitro* induces specific types of DNA lesions that are capable of producing similar types of mutations as those found in *BRAF*^{V600} variants in human melanoma (see Figure 2 for our proposed model of UV-associated *BRAF*^{V600} mutagenesis). Most recently, we have demonstrated that exposure to a prototype environmental carcinogen in a mouse model *in vivo* leaves a unique signature on the epigenome, as reflected in the patterns of DNA methylation and concomitant gene deregulation, which is predictive of the initiation and progression of tumorigenesis (Tommasi et al., 2014). Furthermore, an elegant study by Lian et al. (2012) has shown that loss of 5-hydroxymethylcytosine (5-hmC), the conversion

product of 5-methylcytosine (5-mC), through downregulation of isocitrate dehydrogenase 2 (IDH2) and the ten-eleven translocation (TET) family enzymes is an epigenetic hallmark of melanoma progression.

Convergence of cancer epidemiology and epigenetics: a new era in melanoma research

Melanoma incidence rates continue to increase in the USA and many parts of the world, despite the steady decline in the overall cancer incidence rates (Little and Eide, 2012; American Cancer Society, 2013). It is estimated that 137990 new cases of melanoma will be diagnosed in the USA in 2013—61300 non-invasive and 76690 invasive (45060 in men and 31630 in women), of which 9480 cases (6280 men and 3200 women) will die from the disease. Under this trend of mortality, one American dies of melanoma almost every hour (American Cancer Society, 2013). The World Health Organization estimates that over 65000 people die from melanoma each year, worldwide (Lucas et al., 2006). Early diagnosed melanoma patients who undergo surgical resection of a localized tumor have an average 5-year survival rate of ~99% (Miller and Mihm, 2006; Gray-Schopfer et al., 2007; American Cancer Society, 2013). However, patients with advanced-stage melanomas metastasized to lymph nodes and/or viscera have a median survival of 6 months and 5-year survival rate of <5% (American Cancer Society, 2013). The socio-economic costs of melanoma are also immense; for example, in the USA, this malignancy imposes an estimated burden of 3 billion dollars annually on the health-care system (Grant et al., 2005). The disease causes disproportionately high life-loss in young- and productive-age groups, averaging 19 years of life-loss, which is one of the highest for adult-onset cancers (Ries et al., 2004; American Cancer Society, 2013). The combination of rising incidence, high mortality for late-stage disease, and adverse socio-economic impact underscores the need to improve preventive, diagnostic, therapeutic, and prognostic approaches for human melanoma (Chin et al., 2006; Miller and Mihm, 2006; Fecher et al., 2007).

Epigenetics is a fast growing field in cancer biology, and has enormous potential for clinical and translational research (Portela and Esteller, 2010; Baylin and Jones, 2011; Shen and Laird, 2013). Cancer epigenetics refers to inheritable, yet, reversible changes associated with gene deregulation, which manifest in a (pre)malignant phenotype where the sequence of the genome remains unaltered (Gronbaek et al., 2007; Portela and Esteller, 2010; Tsai and Baylin, 2011). Epigenetic changes include aberrant DNA methylation, histone modifications and variants, miRNAs deregulation, chromatin remodeling, and nucleosome positioning (Kulis and Esteller, 2010; Portela and Esteller, 2010; Baylin and Jones, 2011; Rodriguez-Paredes and Esteller, 2011). Of these, aberrant DNA methylation and histone modifications are the best-studied epigenetic alterations in human carcinogenesis (Kulis and Esteller, 2010; Portela and Esteller, 2010; Baylin and Jones, 2011; Rodriguez-Paredes and Esteller, 2011). The occurrence of epigenetic changes prior to malignant transformation, which frequently manifests in target- and non-invasively obtainable surrogate organs, and the reversibility of these changes

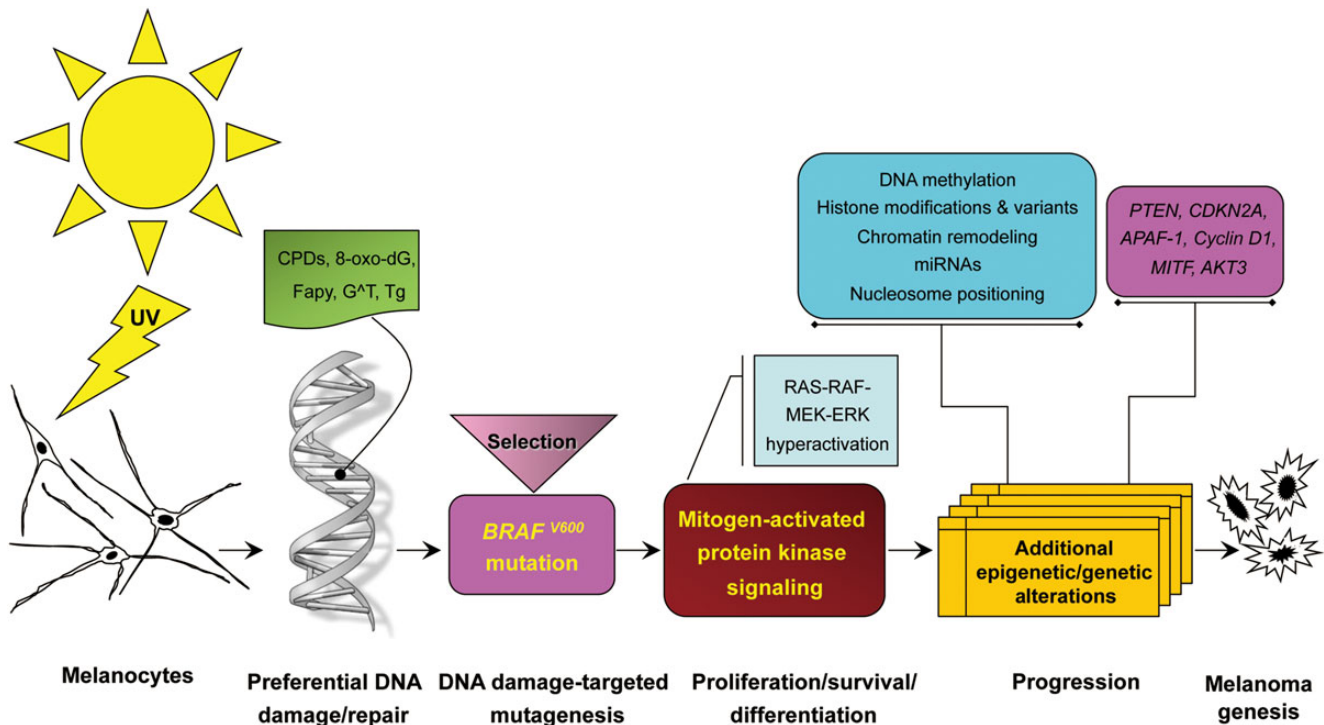


Figure 2 Proposed model of $BRAF^{V600}$ mutagenesis in human melanoma. Sunlight UV-induced DNA lesions can potentially produce mutations similar to those found in $BRAF^{V600}$ variants in human melanoma, which in conjunction with other genetic and epigenetic alterations contribute to melanomagenesis. CPDs, cyclobutane pyrimidine dimers; 8-oxo-dG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; Fapy, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; G^T, guanine–thymine intrastrand cross-link; Tg, thymine glycol.

through pharmacologic/genetic interventions (Rhee et al., 2002; Gius et al., 2004; Gronbaek et al., 2007; Jacinto et al., 2009; Wolff et al., 2010; Rodriguez-Paredes and Esteller, 2011) provide a unique opportunity for cancer research, ultimately leading to the discovery of non-intrusive preventive, diagnostic, therapeutic, and prognostic approaches for human malignancies (Besaratinia et al., 2013). Investigating the epigenetic changes that occur during the initiation and progression of human cancer can help uncover the underlying mechanisms of carcinogenesis and the chronology and sequence of events therein (Besaratinia et al., 2013). Characterizing the epigenetic changes that initiate and promote human melanoma development can help identify biological markers that can be used for prevention, early detection, treatment, and monitoring of the progression of this malignancy (Howell et al., 2009; Sigalotti et al., 2010; Tanaka et al., 2011; van den Hurk et al., 2012; Griewank et al., 2013). From a diagnostic perspective, the accessibility of the skin, wherein 'primary' melanoma arises, is highly advantageous for minimally invasive sampling of the tissue(s) that can be used for surveillance of the epigenetic landscape (Tanaka et al., 2011; Flaherty et al., 2012). From a therapeutic standpoint, the unique anatomy of the skin makes this organ amenable to localized epigenetic therapy whose complications and side effects should be fewer and less severe than those of systemic treatments (Momparker, 2005; Sigalotti et al., 2010; Yang et al., 2010; Rodriguez-Paredes and Esteller, 2011; Tsai and Baylin, 2011; Flaherty et al., 2012; La Porta, 2012). It should, however, be noted that although epigenetic therapy may show great

promise for 'primary' melanomas, epigenetic targeting of tumors that have metastasized to distant organs might still prove challenging.

Aberrant DNA methylation and human cancer

In mammalian genomes, DNA methylation occurs almost exclusively in the context of 5'-CpG dinucleotides (CpGs) (Kulis and Esteller, 2010; Baylin and Jones, 2011). A family of DNA methyl transferases (DNMTs) catalyzes this reaction by transferring a methyl group from the donor *S*-adenosyl methionine (SAM) to cytosine at the fifth carbon position of the pyrimidine ring (Bird and Macleod, 2004; Feinberg and Tycko, 2004; Kulis and Esteller, 2010; Shen and Laird, 2013). The maintenance DNMT1 preferentially catalyzes methylation of the unmethylated strand of hemimethylated DNA during DNA replication (Bird and Macleod, 2004; Feinberg and Tycko, 2004; Kulis and Esteller, 2010; Shen and Laird, 2013). Conversely, the DNMT3A and DNMT3B serve as *de novo* DNA methyl transferases to establish the methylation patterns on both DNA strands during embryogenesis or differentiation processes in adult cells, although both enzymes also possess the maintenance DNA methyl-transferase activities (Bird and Macleod, 2004; Feinberg and Tycko, 2004; Kulis and Esteller, 2010; Shen and Laird, 2013). Aberrant DNA methylation is the most-studied epigenetic mechanism of carcinogenesis (Kulis and Esteller, 2010; Laird, 2010; Baylin and Jones, 2011; Rodriguez-Paredes and Esteller, 2011). Gain of methylation (hypermethylation) at CpG islands, clustered at the promoter, untranslated

5'-region and exon 1 of known genes (promoter CpG islands) or localized within gene bodies is a common event in human carcinogenesis (Maunakea et al., 2010; Deaton and Bird, 2011; Jones, 2012). Also, global loss of methylation (hypomethylation) at repetitive DNA elements, such as long- and short-interspersed nuclear elements (LINE and SINE, respectively), and long terminal repeat retrotransposons (LTR), as well as at single-copy genes is a frequent occurrence in human cancer (Wilson et al., 2007; Ehrlich, 2009; Nagarajan and Costello, 2009). DNA hypermethylation is believed to elicit tumorigenesis by deregulation of gene expression, e.g. through transcriptional silencing of tumor suppressor genes (Esteller, 2008; Suzuki and Bird, 2008; Jones and Liang, 2009; Portela and Esteller, 2010), whereas DNA hypomethylation is thought to contribute to oncogenesis by reactivation of latent retrotransposons, induction of genomic instability, and activation of proto-oncogenes (Feinberg and Vogelstein, 1983; Wilson et al., 2007).

Histone modifications and human cancer

Post-translational modifications of histones are a common epigenetic change in human carcinogenesis (Feinberg and Tycko, 2004; Gronbaek et al., 2007; Kondo et al., 2008; Portela and Esteller, 2010; Shen and Laird, 2013). The nucleosome, the fundamental unit of chromatin, is composed of two copies of each of the four core histones (i.e. H2A, H2B, H3, and H4), around which is coiled ~147 bp of DNA (Portela and Esteller, 2010; Baylin and Jones, 2011). The N-terminal tails of histone polypeptides can be altered by a variety of post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitylation, glycosylation, ADP-ribosylation, carbonylation, and SUMOylation (collectively known as histone modifications) (Feinberg and Tycko, 2004; Gronbaek et al., 2007; Gal-Yam et al., 2008; Rodriguez-Paredes and Esteller, 2011). Histone modifications can regulate transcription through modulation of chromatin structure or through chromatin condensation (Portela and Esteller, 2010; Tsai and Baylin, 2011; Shen and Laird, 2013). Although the role, function, and transcriptional regulatory effects of many histone modifications are poorly understood, significant progress has been made in recent years through studies of Chromatin immunoprecipitation coupled to microarray analysis (ChIP-chip) and ChIP-sequencing analysis (ChIP-Seq). For instance, methylation of histone 3 at lysine 4 (H3K4me) or at lysine 36 (H3K36me), and acetylation of histone 3 at lysine 9 (H3K9ac) or at lysine 27 (H3K27ac) are associated with transcription activation. Conversely, methylation of histone 3 at lysine 9 (H3K9me), at lysine 27 (H3K27me), or at lysine 79 (H3K79me), and of histone 4 at lysine 20 (H4K20me) are correlated with repression of transcription (Gronbaek et al., 2007; Schones and Zhao, 2008; Jacinto et al., 2009; Izzo and Schneider, 2010; Portela and Esteller, 2010; Thomson et al., 2010; Zhou et al., 2011; Shen and Laird, 2013).

The interplay between aberrant DNA methylation and histone modifications in human cancer

It is well established that various components of the DNA methylation machinery, e.g. DNMTs and methyl-CpG-binding domain

proteins (MBDs and MeCP2) crosstalk with histone modification machinery to reinforce the transcriptional regulation of genes (Bird and Macleod, 2004; Jones and Liang, 2009; Portela and Esteller, 2010; Clements et al., 2012). For example, DNA methylation can recruit or repel different histone-modifying enzymes and *vice versa*, thus, influencing the chromatin architecture and (de-)regulating gene expression (see Figure 3) (Portela and Esteller, 2010; Clements et al., 2012). Notwithstanding the interconnection between aberrant DNA methylation and histone modifications, it is not, however, known which one of these two epigenetic alterations is the cause and which one is the consequence (Portela and Esteller, 2010; Deaton and Bird, 2011; Jin et al., 2011; Rodriguez-Paredes and Esteller, 2011). The classic view is that histone modifications constitute the first layer of epigenetic silencing, which dictates the recruitment of key components of the DNA methylation machinery. In this scenario, aberrant DNA methylation serves merely as a lock-off mechanism to bolster the transcriptional silencing of already turned off genes (Bird, 2002). An alternative view is that aberrant DNA methylation plays a dominant role in reversing the epigenetic state at certain genomic loci, thereby, activating the normally silent genes (Jin et al., 2011; Cannuyer et al., 2013). The latter scenario is best exemplified by the concomitant hypomethylation and activation of germline-specific genes in somatic tissues/tumors in specific types of human cancer (De Smet and Loriot, 2013). Future studies will determine the sequence of epigenetic events that occur during the initiation and progression of human carcinogenesis.

Synopsis of research on DNA methylation and human melanoma

The evolution of DNA methylation detection technologies, from the single-gene assays to genome-wide microarray based analysis and next-generation sequencing platforms, has offered a unique avenue of research for profiling the patterns of DNA methylation in human carcinogenesis (Estecio and Issa, 2009; Laird, 2010; Jones, 2012). Thus far, candidate gene(s) and genome-wide aberrant DNA methylation have been observed in human melanoma tumor tissues and cell lines, and correlated to cellular and functional characteristics and/or clinicopathological features of this disease (Patino and Susa, 2008; Howell et al., 2009; van den Hurk et al., 2012; Griewank et al., 2013). The following is a synopsis of research on aberrant DNA methylation in connection with human melanoma. Representative studies are highlighted to showcase the advancement of this field of research, and the potential utility of DNA methylation marks for clinical diagnosis, treatment, and prognosis of human melanoma.

In one of the earlier studies on aberrant DNA methylation and human melanoma, the methylation-specific polymerase chain reaction (MSP) assay was used to investigate the methylation status of two regions of the tumor suppressor gene, *RASSF1A*, in 11 melanoma cell lines and 44 human melanoma tumors (stage: III–IV) (Spugnardi et al., 2003). Whereas region 2 is located within the first exon (1 α) of the open reading frame of the *RASSF1A* transcript, region 1 is located upstream of the transcription start codon and contains three Sp1 consensus binding sites. Hypermethylation of *RASSF1A* region 1 and 2 was observed in

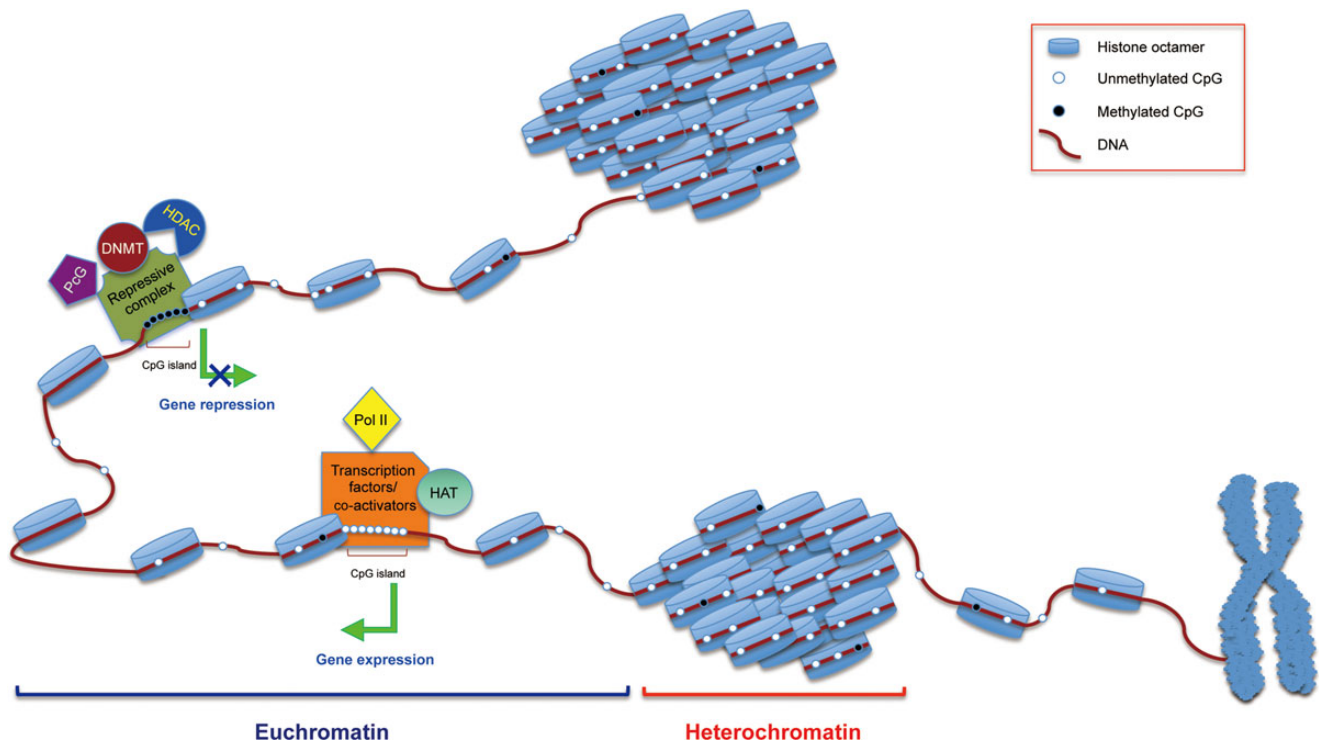


Figure 3 Schematic representation of aberrant DNA methylation and histone modifications. Key components of the DNA methylation and histone modification machineries are indicated.

64% (7/11) and 82% (9/11), respectively, of all cell lines, with 64% of the cell lines being hypermethylated in both regions. No *RASSF1A* gene expression was detectable in cell lines that exhibited complete methylation in both regions 1 and 2. However, *RASSF1A* gene expression was detectable in cell lines that were unmethylated in both regions or partially methylated in region 2 only. Likewise, hypermethylation of *RASSF1A* regions 1 and 2 was observed in 41% (18/44) and 50% (22/44), respectively, of melanoma tumors, with 36% (16/44) of all tumors being hypermethylated in both regions. Treatment of melanoma cell lines with the DNA demethylating agent, 5-aza-2'-deoxycytidine (5-Aza-CdR), resulted in re-expression of the *RASSF1A* gene, as determined by real-time reverse transcription-polymerase chain reaction (RT-PCR) of the mRNA (Spugnardi et al., 2003). This study showed the predictive value of promoter CpG island hypermethylation and concomitant gene silencing for human melanoma development. It also demonstrated the reversibility of aberrant DNA methylation through a pharmacological approach, thus, laying the ground for future drug-based strategies for treating human melanoma.

Pyrosequencing was used to analyze promoter CpG island hypermethylation in 15 cancer-related genes, global hypomethylation in two major repetitive DNA elements (*LINE-1* and *Alu*), and loss of methylation in two single-copy genes (*MAGEA1* and *maspin*) in 16 melanoma cell lines compared with normal epidermal melanocytes (Tellez et al., 2009). The panel of 15 cancer-related genes included *ER α* , *MGMT*, *RAR β 2*, *RIL*, *RASSF1A*, *PAX7*, *PGR β* , *PAX2*, *NKX2-3*, *OLIG2*, *HAND1*, *ECAD*, *CDH13*, *MLH1*, and *p16* that are

known to be involved in tumor suppression, cell cycle, apoptosis, cell adhesion, or DNA repair. Except for the *MLH1* gene, promoter CpG island hypermethylation was observed in all the cancer-related genes in melanoma cell lines. The methylation frequencies of the hypermethylated genes, in decreasing order, were as follows: *RIL* and *ECAD* (88%), *RASSF1A* (69%), *NKX2-3*, *HAND1*, and *OLIG2* (63%), *PGR β* (56%), *ER α* and *MGMT* (50%), *RAR β 2* and *CDH13* (44%), *PAX2* (38%), *PAX7* (31%), and *p16* (6%). Furthermore, global hypomethylation of the *LINE-1* and *Alu* sequences was found in all the melanoma cell lines, with mean methylation levels being 36% (vs. 65% in normal melanocytes) and 40% (vs. 44% in normal melanocytes) for the respective repeat elements. Of note, the methylation levels of *LINE-1* and *Alu* sequences in the melanoma cell lines were positively correlated. There was also a direct relationship between the number of hypermethylated genes and global hypomethylation in the *LINE-1* and *Alu* sequences. Moreover, loss of methylation in the *MAGEA1* and *maspin* genes was observed at frequencies of 44% and 25%, respectively, in the melanoma cell lines tested. Gene expression analysis of six hypermethylated genes in the melanoma cell lines confirmed that transcriptional gene silencing was associated with promoter CpG island methylation in the respective genes, as determined by RT-PCR (Tellez et al., 2009). This study verified that hypermethylation of the promoter regions of cancer-related genes concurrent with transcriptional deregulation are frequent events in human melanoma. In addition, hypomethylation of repetitive DNA elements and demethylation of cancer-specific genes were determined as hallmarks of this malignancy.

To study the clinical aspects of human melanoma in relation to aberrant DNA methylation, the methylation status of CpGs in the promoter region of six tumor-related genes (TRG), including *WIF1*, *TFPI2*, *RASSF1A*, *RARβ2*, *SOCS1*, and *GATA4*, and seven methylated-in-tumor loci (MINT), including *MINT1*, *MINT2*, *MINT3*, *MINT12*, *MINT17*, *MINT25*, and *MINT31*, was investigated in 122 primary and metastatic melanomas of diverse clinical stages (I–IV) (Tanemura et al., 2009). The methylation status of TRG and MINT was determined by the MSP assay and absolute quantitative analysis of methylated alleles, respectively. Hypermethylation of four TRG and two MINT was significantly associated with advancing clinical tumor stage, including *WIF1*, *TFPI2*, *RASSF1A*, *SOCS1*, *MINT17*, and *MINT31*. In metachronous tumors from patients with paired lesions (early stage vs. advanced stage), a progressively increasing hypermethylation was found in the *WIF1* gene. The methylation status of *MINT31* was a significant predictor of improved overall survival in stage III melanoma patients. There was a positive association between the methylation status of *MINT17* and *MINT31* and that of TRG, which supports the existence of the CpG island methylator phenotype (CIMP) in human melanoma. The CIMP phenomenon, which is frequently observed in specific types of human tumors, including gastrointestinal/colorectal cancers (Tanemura et al., 2009), manifests as aberrant DNA methylation in multiple genes/loci that is concomitant with the inactivation of tumor suppressor and mismatch-repair genes and microsatellite instability (Issa, 2004; Hinoue et al., 2012). The overall findings of this study confirmed a relationship between region-specific aberrant DNA methylation and clinical outcomes of human melanoma. It will be important to further verify the presence of CIMP in melanoma patients with different clinical outcomes. This information will be critical to developing stratification strategies for prognosis and response to therapy in different populations of melanoma patients.

With the advent of high-throughput DNA methylation microarrays, the Illumina GoldenGate methylation Cancer Panel I array was used to profile the DNA methylation patterns in 22 primary invasive melanomas of different histologic subtypes and Breslow thicknesses and 27 benign melanocytic nevi (Conway et al., 2011). This array platform is designed to interrogate the methylation status of 1505 CpG sites in the promoter and regulatory regions of 807 cancer-related genes. Twenty-six CpG loci in 22 genes showed significant differential methylation in melanoma tumors when compared with benign nevi (adjusted for age, sex, and Bonferroni correction for multiple comparisons). The differentially methylated CpG sites included 19 hypomethylated and 7 hypermethylated loci. Functional annotation analysis revealed that the differentially methylated genes were associated with apoptosis, cell cycle, proliferation, cell adhesion, cell communication and signaling, and immune system response. Validation of the 22 differentially methylated genes in an independent set of 25 melanomas of different histologic subtypes and Breslow thicknesses and 29 dysplastic nevi confirmed that 14 genes (at 16 CpG loci) had significant aberrant DNA methylation, after adjustment for sex, age, and correction for multiple comparisons (Conway et al., 2011). The overall findings of this study favor the utility of

comprehensive DNA methylation analysis for investigating the functional impact of aberrant DNA methylation on gene regulation during human melanoma development. The study also reveals the potential challenges of (semi-) epigenome-wide DNA methylation analysis in sorting through high volume of data generated. Although microarray- or next-generation sequencing-based DNA methylation analysis offers a significantly increased coverage of the methylome relative to classic single-gene assays, these technologies are most informative if complemented with cutting-edge bioinformatics approaches in order to allow meaningful interpretation of the results. A challenging aspect of the bioinformatics data processing and analysis is the identification of ‘driver’ and ‘passenger’ DNA methylation events. Whereas driver DNA methylation events are thought to be pathologic (e.g. cancer-specific and initiator/promoter of tumorigenesis), passenger DNA methylation events are believed to merely accompany the driver DNA methylation events without having any effect *per se* on the pathogenesis of the disease (Illingworth et al., 2008; Doi et al., 2009; Estecio and Issa, 2009; Irizarry et al., 2009; Hansen et al., 2011; De Carvalho et al., 2012). Although driver DNA methylation events are of most relevance for carcinogenesis, passenger DNA methylation events—if sensitive and specific for the disease process—may also prove useful as biomarkers for diagnostic, therapeutic, or prognostic purposes, especially when detectable in non-invasively obtainable tissues.

More recently, the Illumina Human Methylation 27 BeadChip assay was used to catalogue the patterns of DNA methylation in short-term autologous cell cultures prepared from 45 stage IIIC melanoma patients (Sigalotti et al., 2012). This array-based assay enables the interrogation of 27578 CpG sites in 14495 genes of the human genome. Methylation score, representing the overall density of methylation in the genome, was calculated for each cell culture established from every individual melanoma tumor. Using the unsupervised k-means partitioning clustering, two subgroups were identified, including low- and high-methylation groups, with population’s mean methylation score serving as the cut-off point. Kaplan–Meier analysis showed a significant survival advantage for patients from the low-methylation group when compared with those from the high-methylation group. The overall survival medians for patients from the low- and high-methylation groups, respectively, were 31.5 and 10.4 months. The 5-year overall survivals in the respective groups were 41.2% and 0%. Cox proportional hazard regression analysis revealed that DNA methylation profile was the most robust predictor of overall survival. Using the nearest shrunken centroid classification algorithm, a 17-gene panel methylation signature was identified that could accurately ascertain prognosis in this population of stage IIIC melanoma patients (Sigalotti et al., 2012). This study showed the prognostic value of aberrant DNA methylation for human melanoma, and its potential utility for monitoring the progression of this malignancy. Future mechanistic studies are needed to elucidate how aberrant DNA methylation of a subset of genes can explain differential survival of melanoma patients. The mechanistic knowledge will expedite the development of effective therapeutic strategies for human melanoma.

Synopsis of research on histone modifications and human melanoma

The high-throughput genome-wide technologies for the detection of histone modifications (e.g. ChIP-chip and ChIP-Seq) are yet to bring to fruition a global mapping of histone marks in human melanoma. Notwithstanding the abundance of research on DNA methylation and human melanoma, scarce literature and limited data are available on the patterns of histone modifications in this malignancy (Patino and Susa, 2008; Howell et al., 2009; Pan et al., 2010; Molognoni et al., 2011). Given the interdependence of these two epigenetic events, however, it is anticipated that investigations of histone modification in human melanoma will gain momentum in the coming years to put the available DNA methylation data in a more mechanistic context. In the following part, we highlight the published research on histone modifications in connection with human melanoma development.

To study the interrelation between histone modifications and aberrant DNA methylation in human melanoma development, eleven melanoma cell lines were treated with the histone deacetylase inhibitor, trichostatin A (TSA), alone or in combination with the demethylating agent, 5-Aza-CdR, and subsequently, gene expression analysis was performed to investigate the transcriptional changes in two metastasis-associated chemokine receptors, including *CCR7* and *CXCR4* (Mori et al., 2005). Quantitative RT-PCR, immunohistochemistry, and western blot analysis were used to assess changes in the mRNA and protein expression levels of *CCR7* and *CXCR4* genes in drug(s)-treated melanoma cell lines. In addition, cell migration and proliferation assays were utilized to find the interdependence of the *CCR7* and *CXCR4* gene expression and cell function and cell growth, respectively, in cells treated with chromatin modifying drugs, individually or combined. Melanoma cell lines treated with TSA or 5-Aza-CdR showed increased levels of mRNA for both *CCR7* and *CXCR4* genes, with TSA having a more robust effect than 5-Aza-CdR, whilst combination treatment synergistically inducing gene expression, as determined by quantitative RT-PCR. In confirmation, immunohistochemistry analysis of both *CXCR4* and *CCR7* genes and Western blot analysis of the *CXCR4* gene showed similar results to those obtained by the quantitative RT-PCR. In cell proliferation experiments, select melanoma cell lines treated with TSA and/or 5-Aza-CdR showed that the combination treatment had the highest suppressive effects on cell growth. To determine the functional activities of the *CCR7* and *CXCR4* receptors-positive melanoma cells toward their respective ligands, CCL21 and CXCL12, cell migration experiments were performed on a select number of melanoma cell lines pre-treated with TSA and/or 5-Aza-CdR. A significant increase in the number of migrating cells bound to CCL21 or CXCL12 was observed in melanoma cell lines treated with a combination of TSA and 5-Aza-CdR, which correlated with the elevated levels of *CCR7* or *CXCR4* mRNA expression, respectively, in the same cell lines, as detected by quantitative RT-PCR (Mori et al., 2005). Given the fact that *CCR7* and *CXCR4* genes are not differentially methylated in melanoma, the overall findings of this study indicate that chromatin modifying drugs may impact pathways that are deregulated through non-epigenetic mechanisms—in addition

to reversing epigenetically disrupted pathways—in human melanoma. Future studies are needed to uncover how epigenetic drugs can modulate network of genes that are deregulated through different mechanisms in human melanoma.

More recently, the relative contribution of histone modifications and aberrant DNA methylation to transcriptional regulation of the *MAGEA1* germline-specific gene that is commonly activated in a variety of human tumors, including melanoma, was investigated (Cannuyer et al., 2013). ChIP experiments examining the 5' region of the *MAGEA1* gene in two melanoma cell lines with no detectable level of *MAGEA1* expression showed a preferential enrichment of the repressive histone mark, H3K9me2. Conversely, high enrichment of the active histone marks, H3ac and H3K4me2, was observed in three melanoma cell lines with known *MAGEA1* gene expression activity. Treatment of the non-expressing melanoma cell lines with the histone deacetylase inhibitor, TSA, showed a slight and transient increase in the mRNA expression level of the *MAGEA1* gene, without affecting the methylation status of its promoter region, as determined by quantitative RT-PCR and MS-PCR analyses, respectively. However, treatment of the melanoma cell lines with the demethylating agent, 5-Aza-CdR, resulted in an extensive and durable increase in the *MAGEA1* mRNA levels concomitant with its promoter hypomethylation. Validation experiments were conducted in a transgenic cell system derived from a melanoma cell line with demonstrated *MAGEA1* expression activity (Loriot et al., 2006). This cell line harbors a selectable *MAGEA1* construct that contains a large portion of the gene (including its promoter) and a sequence encoding resistance to hygromycin (*MAGEA1/hph*). As the transgene is methylated *in vitro* before transfection and remains silent upon integration into the genome, this transgenic cell line confers sensitivity to hygromycin (Loriot et al., 2006). ChIP experiments identifying the histone modifications associated with the endogenous/exogenous *MAGEA1* gene revealed that there was strong depletion of the H3ac and H3K4me2 active marks and high enrichment of the repressive H3K9me2 in the silent *MAGEA1/hph* transgene when compared with the endogenous *MAGEA1* gene in this melanoma cell line. Treatment of the transgenic cells with TSA induced a transient de-repression of the *MAGEA1/hph* without causing demethylation in the promoter region of this transgene or conferring resistance to hygromycin. However, 5-Aza-CdR treatment of these cells resulted in re-activation of the transgene concomitant with its promoter hypomethylation and reversal of its associated histone marks toward an active configuration, as well as the emergence of hygromycin-resistant cell population (Cannuyer et al., 2013). This study confirms the utility of chromatin modifying drugs in reversing the epigenetic abnormalities in human melanoma, as well as the importance of selecting the right drug or combination of drugs for exerting sustained effects. The ideal pharmacological approach would target the specific pathways that are disrupted during human melanoma development without disturbing the normal regulation of the unaffected pathways.

Epigenetic therapy for human cancer: promises and challenges

Today, the only Food and Drug Administration (FDA)-approved drugs for epigenetic therapy are the DNA demethylating agents

and histone deacetylase inhibitors (Gal-Yam et al., 2008; Kelly et al., 2010; Rodriguez-Paredes and Esteller, 2011; Popovic and Licht, 2012). The cytosine analogues Decitabine (5-Aza-CdR) and Vidaza (5-Azacytidine; 5-Aza-CR) function to demethylate DNA by first being incorporated into DNA, followed by irreversible binding of DNMTs to DNA containing the cytosine analogues. The binding results in depletion of DNMTs and global demethylation upon cell divisions (Mompalmer, 2005; Yang et al., 2010; Baylin and Jones, 2011; Rodriguez-Paredes and Esteller, 2011). Targeting histone deacetylases for epigenetic therapy is complicated because this group of enzymes has multiple subclasses with mechanisms of action still under dispute (Portela and Esteller, 2010; Cherblanc et al., 2012; Popovic and Licht, 2012). More than a dozen histone deacetylase inhibitors are currently undergoing preclinical and clinical investigations for treatment of hematological malignancies and/or solid tumors (Gal-Yam et al., 2008; Kelly et al., 2010; Cherblanc et al., 2012; Popovic and Licht, 2012). The common mechanism of action of histone deacetylase inhibitors is the chelation of Zn^{2+} ions, which is fundamental to the activity of these enzymes (Gal-Yam et al., 2008; Cherblanc et al., 2012; Dawson and Kouzarides, 2012; Popovic and Licht, 2012). As single agents, two histone deacetylase inhibitors, including Vorinostat and Romidepsin have FDA approval for the treatment of cutaneous T-cell lymphoma (Gal-Yam et al., 2008; Kelly et al., 2010; Cherblanc et al., 2012; Popovic and Licht, 2012). Notwithstanding the encouraging results of epigenetic therapy for hematological malignancies, pharmacological targeting of epigenetic aberrancies in solid tumors (e.g. melanoma) has not been straightforward (Gal-Yam et al., 2008; Guil and Esteller, 2009; Kelly et al., 2010). Major obstacles include the delivery of epigenetic drugs, maintenance of a pharmacodynamics response, and achievement of a therapeutic index (Cherblanc et al., 2012; Popovic and Licht, 2012). Obviously, a better understanding of the mechanisms of action of epigenetic drugs and gaining more insights into tumor biology will help improve the efficacy of epigenetic therapy and adjuvant therapeutic approaches for human cancer. The ultimate goal is to combine epigenetic therapy with other chemotherapeutic approaches, such as hormonal therapies, immunomodulatory therapies, and standard chemotherapy, to help sensitize tumor cells to cytotoxic effects of targeted/systemic therapies or to durably slow or reverse resistance to these therapies (Besaratina et al., 2013). The availability of sensitive and specific epigenetic biomarkers, such as DNA methylation and histone modification marks, will be critical to predicting the response to such therapeutic modalities. For more detailed information on the promises and challenges of epigenetic therapy for solid tumors, we refer the readers to recent comprehensive reviews on this topic (Hanahan and Weinberg, 2011; Dawson and Kouzarides, 2012; Azad et al., 2013).

Concluding remarks: potential challenges and future directions

Melanoma is a highly complex disease with multi-faceted etiology. Until the past decade, melanoma research has been dominated by the investigations of the genetic and environmental determinants of this malignancy. Both inherited- and acquired-

genetic components and environmental factors have been identified for human melanoma development (Garibyan and Fisher, 2010; Ko and Fisher, 2011; Flaherty et al., 2012; Tsao et al., 2012). For example, the *CDKN2A* and *CDK4* mutation/deletion, *CCND1* and *MITF* amplifications, and *BRAF*^{V600} and *NRAS* mutations are frequently occurring genetic alterations in human melanoma (see Figure 1) (Ko and Fisher, 2011; Flaherty et al., 2012; Tsao et al., 2012). Also, sunlight UV is the best-established environmental determinant of human melanoma (Lucas et al., 2006; Garibyan and Fisher, 2010). More recently, however, epigenetic mechanisms of carcinogenesis are gaining momentous attention in the field of melanoma research (Patino and Susa, 2008; Howell et al., 2009; Rodriguez-Cerdeira and Molares-Vila, 2011; van den Hurk et al., 2012; Griewank et al., 2013; Pimiento et al., 2013). In recent years, the less-than-satisfactory results of the genetically or environmentally based intervention strategies for melanoma prevention, early detection, treatment, or prognosis have brought epigenetics to the forefront of melanoma research. Elucidating the epigenetic mechanisms of melanoma genesis and progression holds great promise for prevention, diagnosis, therapy, and prognostication of this malignancy because epigenetic alterations occur in the early stages of carcinogenesis, and are reversible through pharmacologic/genetic manipulations (Rhee et al., 2002; Gius et al., 2004; Gronbaek et al., 2007; Jacinto et al., 2009; Wolff et al., 2010; Chung et al., 2011; Rodriguez-Paredes and Esteller, 2011). The promise of epigenetic therapy for human melanoma can be realized by establishing the epigenome of this tumor type as it evolves from an early-stage neoplasia to an advanced and invasive malignancy. It is likely that the interplays between genetic and epigenetic determinants and environmental factors govern the genesis and progression of human melanoma (see Figure 1). Thus, a multi-disciplinary approach based on genetic manipulation, epigenetic therapy, and mitigation of exposure to environmental risk factors will best represent future strategies for prevention, early detection, treatment, and monitoring of the progression of this disease. We will conclude by highlighting the outstanding questions in the field of melanoma epigenetics, which were touched upon in this review:

- What molecular pathways are epigenetically disrupted during the evolution of human melanoma?
- What is the sequence of epigenetic events occurring during the initiation and progression of human melanoma?
- Does aberrant DNA methylation precede or follow histone modifications in human melanoma development?
- What are the roles, functions, and transcriptional regulatory effects of epigenetic aberrancies in the initiation and progression of human melanoma?
- How do the global patterns of histone modifications change during the initiation and progression of human melanoma?
- Can global mapping of histone modifications in human melanoma put the corresponding DNA methylation data in a more mechanistic context?

- How can we improve the efficacy of epigenetic therapy in solid tumors, including melanoma?
- What are the interplays between genetic and epigenetic determinants and environmental factors that govern the genesis and progression of human melanoma?

Acknowledgements

The authors thank all of their colleagues in the field of melanoma research and cancer epigenetics, and apologize to all those whose work could not be cited due to space constraints.

Funding

This work was supported by grants from the American Cancer Society (RSG-11-083-01-CNE) and the University of California Tobacco Related Disease Research Program (20XT-0116) to A.B.

Conflict of interest: none declared.

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