Epigenetic mechanisms involved in melanoma pathogenesis and chemoresistance

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Contributions: (I) Conception and design: A Martinez-Cardús, M Vizoso; (II) Data analysis and interpretation: All authors; (III) Manuscript writing: All authors; (IV) Final approval of manuscript: All authors.

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Abstract: The discovery of highly recurrent mutations in melanoma, such as BRAF^{V600E}, completely changed the clinical management including therapy of melanoma patients. In the era of Personalized Medicine targeted melanoma therapies showed high response rates, currently evidenced by BRAF inhibitors or immune-stimulating therapies. In addition to genetic biomarkers, epigenetic knowledge in melanoma has undergone a major step forward in recent years. In particular, epigenetics is unveiling new perspectives to fight this disease, providing an encouraging number of DNA methylation based biomarkers that will likely improve patient stratification for prognosis and treatment. In this regard, putative targetable biomarkers or those with predictive value for the outcome of currently applied therapies are promising tools for future precision oncology strategies. In addition, the progress made in genetic and epigenetic profiling technologies and their reconfiguration to real-time clinical screening approaches makes personalized medicine in melanoma an achievable objective in upcoming years.

Keywords: Melanoma; DNA methylation; biomarkers; target therapy; chemoresistance; dynamic rewiring; translational science

Epigenetics in melanoma: new biomarkers based on DNA methylation

Melanoma is one of the solid tumors less epigenetically interrogated. However, huge efforts have been made in the last decade to improve the knowledge we had regarding this aggressive and multi-resistant tumor type. Clinical outcome of melanoma patients justifies this scientific involvement given the pronounce survival difference between localized and metastatic disease (98% and 16-62% 5-year survival, respectively) (1). In addition, approximately 15% of diagnostic melanomas are in fact benign lesions (false positive rate) and 17% of diagnostic benign lesions are malignant melanomas (false negative rate). Hence, this setting provided enough stimuli to derive resources to improve the clinical management and diagnosis of melanoma patients.

Epigenetic inactivation of particular tumor suppressor genes has been extensively implicated in tumor initiation, promotion and progression (2-6). It was the first epigenetic alteration studied in melanoma occurring preferentially by the specific DNA methylation of promoter regions. Promoters are CG enriched portions of the genes involved in the transcription and susceptible to be silenced by the deposition of methyl groups in the cytosines closed by guanines. *Figure 1* summarized first reported genes regulated by DNA methylation in melanoma: *PTEN* (methylated in ~60% of melanoma) (7,8), *SYK* (methylated in 30% of melanomas) (9), *TNFSF10D* and *LOX* (methylated in 80% and 50% of melanomas) (10), or *COL1A2* (methylated in 80% of melanomas) (9). Later on, genome-wide screenings
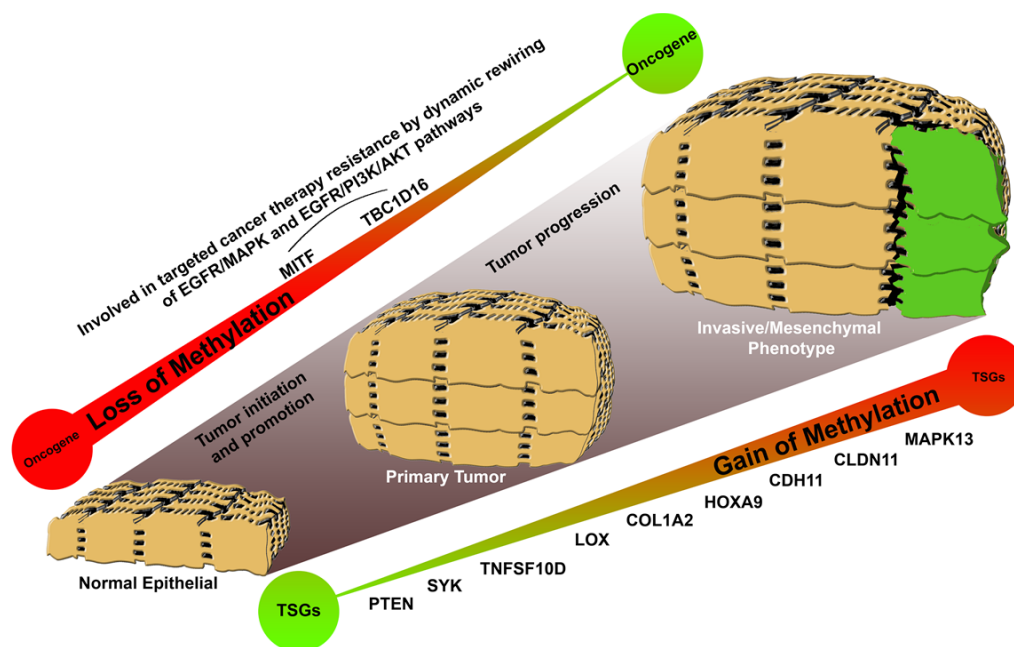
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Figure 1 Illustrative image of melanoma evolution from an epigenetic point of view, highlighting the aberrant DNA methylation events occurring during the malignization of melanocytes. TSGs, tumor suppressor genes.

led to the discovery of multi-locus DNA-methylation signatures and new target genes associated with melanoma malignancy: MeDIP assay (11), Illumina GoldenGate Methylation Cancer Panel I (12), Illumina Infinium HumanMethylation27 BeadChips (13-16), or the Illumina Infinium HumanMethylation450 Beadchips (17-19).

Even though the first discoveries were made on disrupted genes by DNA hypermethylation, epigenome-wide DNA methylation landscape of melanoma revealed a global wave of hypomethylation throughout intergenic regions, gene bodies and locations away from CpG islands (genome regions with enriched CpG sites). Actually, Guo and coworkers comparing the epigenomes of a set of 34 primary cutaneous melanoma tumors harboring BRAF^{V600E} mutation with those of 27 BRAFWT samples, found that the 98% of the disrupted methylated CpG sites were events of loss of methylation whilst only a 2% were events of methylation gain (20). Interestingly, the authors related this particular mutation (BRAF^{V600E}) with an event of global hypomethylation and a decrease in DNMT3A expression, one of *de novo* DNA methyltransferases in cells. Regarding to the effects that a global wave of hypomethylation can produce at transcriptional level, it is noteworthy the reactivation of microphthalmia-associated transcription factor (MITF) which is the master regulator of melanin

production and identified as a lineage-specific oncogene in melanoma. Guo and colleagues defined the reactivation of MITF as one of the most significant up-regulated genes under the control of BRAF^{V600E} mutation (20) and Lauss and colleagues demonstrated its regulation by DNA methylation in melanoma tumors (17). Interestingly, neither the BRAF^{V600E} mutation, which is also present in at least 20% of normal nevi, or the MITF expression are sufficient to drive the melanoma progression, but their roles in melanoma tumorigenesis are undisputed. Firstly, MITF introduces complex transcriptional changes in cells, triggering new gene pathways affecting cell proliferation, pigmentation synthesis, endosome trafficking or drug resistance (21,22). Secondly, BRAF^{V600E} mutation throughout its hyperactivity reprograms the proliferative cell machinery but the bright side is that patients become susceptible to specific BRAF-inhibitor treatments (23). Responsiveness to BRAF-inhibitors has been one of the quickest and most potent feedbacks found into the clinics in the last decade but the patients also acquired resistance to the treatment in short-term which makes more difficult to deal with the malignancy. Interestingly, new mechanisms of resistance depending on epigenetic aberrations have been already described. Among them, we highlight the reactivation of the epidermal growth factor receptor (EGFR)

by the demethylation of its own regulatory DNA elements located upstream and downstream of the transcription start site or the reactivation of a cryptic variant of the TBC1D16 gene (19,24,25). Particularly, Vizoso and colleagues describe in melanoma the modulation of EGFR receptor by an imbalance of the endosome/lysosome cell machinery. They explain how the epigenetic modulation of EGFR by TBC1D16 contributes to the dynamic rewiring of survival pathways in melanoma to evade target therapy-associated cell death (19).

Systematic characterization of epigenetically disrupted genes in melanoma has provided new insights regarding to the progression of the malignancy or the acquisition of resistance in melanoma patients. In this line, some genes have been reported as good candidates to be used as melanoma biomarkers (e.g., CDH11, CLDN11, MAPK13) and others have shown correlation with disease-free survival and overall survival (e.g., *PTEN* and *HOXD9* genes). However, it would be desirable to identify novel target genes epigenetically silenced or reactivated with an extra clinical load, improving the stratification capacity of known histopathological parameters such as the Breslow index, the mitotic index, or ulceration. At this respect, the most valuable DNA methylation-associated biomarkers would be (I) those intimately related to the metastatic stage but being also present in low grade primary melanomas with bad prognosis; (II) those able to re-stratify traditional markers such as tumor thickness and ulceration; and (III) those that provide information about targeted cancer therapy-associated response in melanoma patients. Thus, melanoma could be molecularly stratified and personalized treatments could be applied improving the melanoma patients survival. For this purpose, it is essential to do a right selection of the patients' cohort according with all these clinical prognostic factors. Finally, the best biomarkers would be those also susceptible to be directly targetable by small drug molecules.

Resistance to antineoplastic agents

Resistance to cancer therapy is a multifactorial process related not only to the kind of neoplasia and the tumor genotype and heterogeneity but also to own patients' features. Actually, drugs are uptaken, processed and metabolized in a different way for the same tumour type in patients with different genetic and epigenetic background, altering both effectiveness and toxicity of treatments. Moreover, some neoplasias, such as melanoma,

are refractory to particular therapies due to molecular characteristics that are decisive in their carcinogenesis process (26,27). Antineoplastic agent's administration exerts selective pressure on those clones with more appropriate alterations to survive, making them refractory to treatment (intrinsic or primary resistance). In addition, cancer cells frequently can adapt to and evade the drug effects, either by developing new favourable molecular mechanisms or by activating alternative compensatory pathways that can bypass the effects of the therapy (acquired or secondary resistance) (28,29).

General mechanisms of cell chemoresistance

In order to comprehend mechanisms implicated in chemoresistance is important to know pathways triggered by anticancer drugs (30,31). Mechanisms of resistance to chemotherapy (*Figure 2*) have widely been associated with alterations in drug uptake, not only at level of cell capacity to assimilate the drug but also in the detoxification processes (32). It is important noteworthy the influence of tumour intrinsic factors such as size or low vascularisation, and pharmacokinetic drug specific parameters. As well, therapeutic target interaction is also crucial for its effectiveness. This interface activates damage recognition machinery and cell gatekeepers that triggered cell cycle arrest and DNA repair systems and, subsequently, mechanisms of cell death. Alterations that benefit cell survival have been related with lack of drug efficacy. Hence, resistance phenomenon appears associated with alterations that led to the cell bypass this blockage and remain viable (33). Apart from the cell kinetic parameters, tumour microenvironment factors have to be taken into account. Angiogenesis, lymphangiogenesis, tumour-associated fibroblasts (TAFs) and immune system exert a significant influence on the anticancer activity of these agents (34-36).

Chemoresistance in melanoma

Multidrug-resistance

The impact of vesicle trafficking in melanocytes

Melanoma is a highly aggressive form of skin cancer, with an average survival of 6-10 months in advanced cases who receives conventional treatment (37,38). Despite the unquestionable clinical benefit of the new recently approved targeted therapies, conventional chemotherapy continues being an option of treatment, both combined
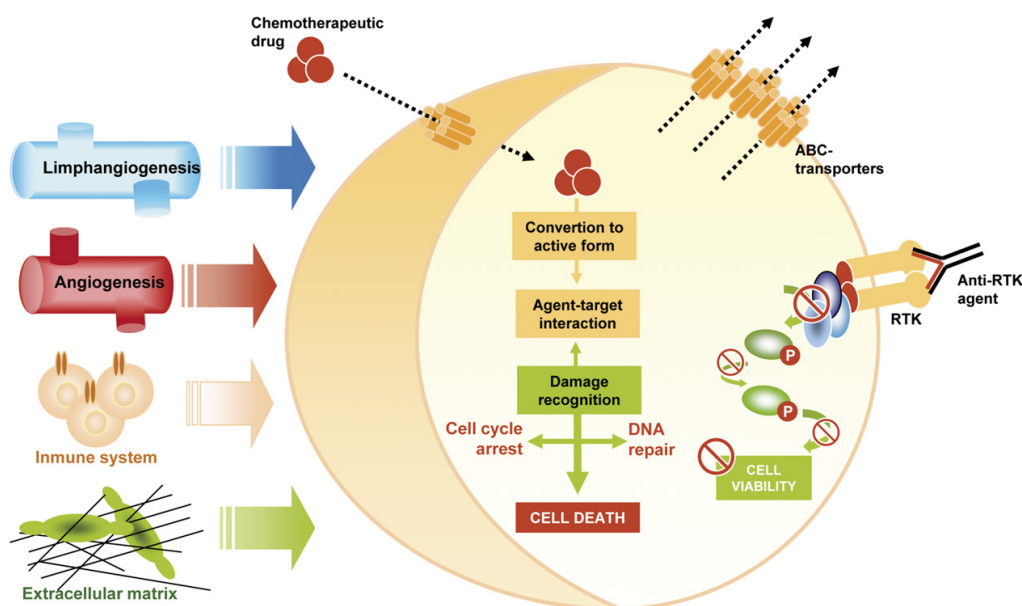
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Figure 2 Representation of drug resistance associated cell mechanisms that can take place against the anticancer therapy. Besides, tumor microenvironment associated processes also affects tumor cell drug responsiveness.

with immunotherapy strategies at first-line treatment and after targeted therapy progression (27,39). The notorious unresponsiveness of melanoma cells to current chemotherapy has been widely observed, presenting an intrinsic multi-drug resistance (MDR) phenotype, often related to over-expression of ABC-transporters family of proteins, responsible for extrusion of lots of substances chemically different (32,40). Particularly, some studies described the influence of ATP-binding cassette sub-family B member 5 (ABCB5) over-expressions in melanoma chemoresistance, proposing it as potential therapeutic target, even in clinical trials (40-43). Apart from this, other mechanisms involved in chemotherapy uptake have been associated with melanoma multi-drug refractoriness. Vesicle trafficking are crucial during melanogenesis and processes of autophagy. In this sense, Chen *et al.* described an *in vitro* association between drug accumulation and melanogenesis phases, suggesting the importance of this process in chemoresistance (40). Autophagy and endoplasmatic-reticulum (ER) stress both also play roles although their real impact on melanoma is still unclear. Corazzari *et al.* reported a chronic ER stress status directly increasing basal cell autophagy, which finally results in higher levels of drug resistance in melanomas BRAF^{V600E} (44). Likewise, the effect of vesicle trafficking in chemoresistance has also been comprehensively demonstrated using *in vitro* models. Huang

et al. showed the effect of the vacuolar protein sorting 33 homologs A (VPS33A) and the cappuccino homolog protein (CNO) down-regulation, implicated in vesicles formation during melanosomes maturation, increasing sensitivity to cisplatin and dacarbazine. The authors also suggested the inhibition of receptor-mediated signalling for melanosome formation as potential therapeutic strategy of chemoresistance reversion (45).

Novel pathways

Combination treatments between conventional chemotherapy and new agents that targeted downstream drug-associated pathways could be a good strategy to alleviate chemoresistance in melanoma cells (46,47). In this sense, Kreiseder *et al.* suggested the cytoskeletal linker protein a-catulin (CTNNAL1) as a potential targetable candidate, demonstrating its role in the upregulation of epithelial-to-mesenchymal (EMT) genes, nuclear factor kappa (NF-κB) and MAPK pathway, and chemosensitization of melanoma cells with elevated basal-expression of the gene upon CTNNAL1 depletion (48). Elevated levels of other proteins involved in EMT process, such as thrombospondin 1 (THBS1), has been related to chemoresistance, giving support to its inhibition as a potential therapeutic strategy (49). A comparable effect was observed by other authors when it was targeted particular proteins, direct or indirectly implicated in apoptosis
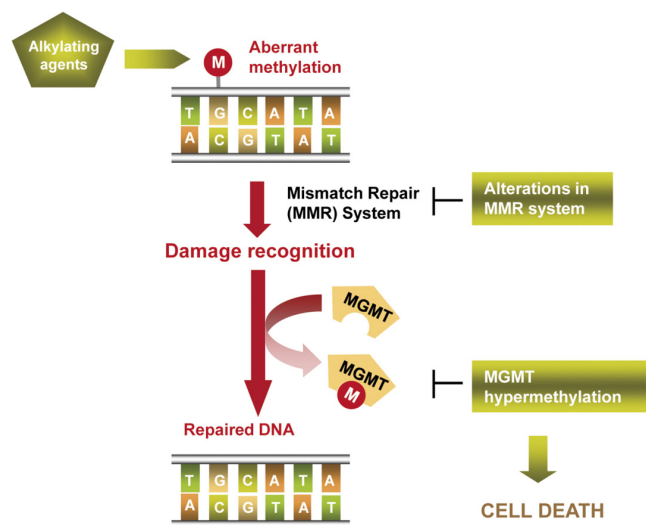
Figure 3 Scheme of alkylating agents' mechanism of action and the role of MGMT in repairing drug-associated DNA damage. MGMT inactivating alterations enhance drug activity avoiding tumor cell viability by cell death activation. MGMT, methyl-guanine-DNA methyltransferase.

(47,50,51). Hence, Liu *et al*. reported the therapeutic potential of targeting myeloid cell leukemia 1 (Mcl-1), an anti-apoptotic protein belonging to the Bcl-2 family, widely expressed in melanoma, which contributes to chemoresistance (50). Additionally, it has been observed a decreased cell apoptosis against cisplatin caused by the overexpression of aldolase A (ALDOA) and angiopoietin-like 4 (ANGPTL4) (51). At this regard, TBC1D16 could also be another good candidate as mentioned before.

Epigenetic mechanisms of resistance acquisition to alkylating agents

Alkylating agents have been the unique family of cytotoxic drugs that have demonstrated some efficacy in melanoma patients, being only effective in around 10-20% of cases in monotherapy (37,38). They trigger cell death by binding to DNA, mainly introducing in O^6 position of guanine aberrant alkylations (methylation or chloroethylation). Cell damage is recognized by cell mechanisms such as the mismatch repair (MMR) machinery, activating DNA repair to counteract the drug effects (52). DNA-repair enzyme O^6-methyl-guanine-DNA methyltransferase (MGMT) is responsible for reversing the alkylation and avoiding the formation of lethal base-pair cross-links in DNA (*Figure 3*).

Its inactivation by hypermethylation in the promoter of the gene in relation with a better overall survival and disease-free survival in gliomas treated by alkylating agents was described at first time by Esteller *et al*. The authors observed that this alteration was present in a 40% of gliomas and that it was an independent prognostic factor (2). Later, this finding was observed in other tumour types, as glioblastoma, melanoma and colorectal cancer (52-54). In melanoma context, it was described an *in vitro* reactivation of MGMT previous to fotemustine resistance acquisition and a better tolerance to temozolamide treatment in MGMT-methylated tumours from melanoma patients (56). Apart from methylation, SNPs in the sequence of the gene could also affect to MGMT expression levels and, consequently, to response to the treatment (56,57). Additionally, the levels of other proteins involved in damage recognition and DNA repair have been linked with resistance to alkylating agents. Naumann *et al*. observed an *in vitro* refractory MGMT-independent effect of methylating agents in melanoma cell lines caused by a loss of expression of MSH2 and MSH6, responsible for the DNA damage recognition. However, these MMR proteins downregulation did not affect cell sensitivity to chloroethylating agents, indicating a partial cross-resistance between alkylating agents subfamilies (58).

Epigenetic perspectives in tailored oncology

The implementation of personalized medicine has revolutionized the current strategies in oncology. Human tissue samples remain the gold standard resource to identify biomarkers that could be of utility in cancer clinical care (59). Paraffin-embedded tissue (FFPE) is the most widely used method for tissue preparation after surgical excision to preserve histology, whereas the application of diagnostic assays based on frozen tissue is and will stay behind limited due to its unwieldy collection process in the hospital setting. Tumor nucleic acids can be degraded upon formalin fixation and this can limit researchers' ability to perform high-throughput analyses. However, advances in technology mean that the analyses are becoming more feasible (59). In the last years, a substantial number of publications have reported the study of DNA methylation in FFPE and other kind of stored samples of readily accessible in the hospital environment (52,60-62). Moreover, DNA methylation analyses can be performed using a wide range of technical approaches, not only at small-scale, as MSP and pyrosequencing, but also new-generation strategies, such

as microarray platforms or WBGS (63,64). Even though most of them are ready to use into the clinical setting, the ground-breaking technology of NGS has just been adapted to diagnostic requirements. Interestingly, Moran *et al.* demonstrated the suitability of Infinium 450K DNA methylation microarray platform to analyze DNA samples coming from FFPE tissue, obtaining similar results as comparing with frozen tissue counterparts (62). As well, it has been reported the opportunity to perform epigenetic studies in a considerable range of biological samples obtained at non-invasive manner. For instance, based on the rational approach that tumour cells are continuously shed into the colonic lumen and mixed with stool, Carmona *et al.* confirmed the recognition of epigenetic biomarkers in this kind of samples (60). In the same way, it has been detected DNA methylation in urine, specially highlighting GSTP1 methylation as a potential predictive factor of disease aggressiveness in prostate cancer (61). Indeed, epigenetic biomarkers are capable to be detected in liquid biopsies, based on detection of DNA methylation in circulating tumour-DNA (ctDNA) from tumour or circulating tumour cells (CTC) (65,66). It is worth to emphasize the relevance of studying molecular biomarkers in liquid biopsies, since ctDNA approach let to monitor genetic and epigenetic markers, in easy-getting samples at non-invasive manner, along disease follow-up and treatment exposure, in order to notice cancer recurrence and secondary resistance phenomenon (67,68). An example is the use of hypomethylation in ctDNA for monitoring the tumours follow-up in hepatocellular carcinoma (69). Furthermore, deciphering the precise spatial orientation of stored FFPE tumor blocks using the routine clinical annotation to reconstruct intratumour heterogeneity can be difficult (70,71). ctDNA is more amenable to serial sampling and presumably represents cancer genomes and epigenomes from multiple metastatic sites (72,73). Nevertheless and despite the introduction of more suitable and optimized tools as regards histopathologic and molecular analyses, the supply of appropriate samples is still a limiting issue.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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