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Review

New Frontiers in Melanoma Epigenetics—The More We Know, the More We Don't Know

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Academic Editors: Ernesto Guccione and Gaetano Verde
Received: 31 December 2016; Accepted: 25 January 2017; Published: 30 January 2017

Abstract: Skin cancer is one of the most common neoplasms worldwide, with a surprising tendency to increase its incidence. As with many cancer types nowadays, early diagnosis and proper management carries an excellent prognosis, up to 5-year survival rate of above 95% for most skin cancers, even though the long-term survival rate among metastatic melanoma patients remains only 5%. This review aims to summarize recent discoveries in epigenetic changes connected with cutaneous malignant melanoma (CMM), comprising of DNA methylation, histone modifications, miRNA regulation, nucleosome positioning and chromatin remodelling. Undoubtedly, personalised medicine based on both genetic and epigenetic changes of cancer is the future, the question remains: how long will it take to transport this treatment from the bench to the bedside?

Keywords: Epigenetics; melanoma; miRNA; epigenetic changes in melanoma; skin cancer

1. Introduction

Skin cancer is one of the most common neoplasms worldwide, with a surprising tendency to increase its incidence [1,2]. Some preliminary data suggest that this alarming tendency could be at least partially attributed to a change in the holidaying style: as we travel more frequently, choosing sunny countries for holidays, our skin remains unadapted to the more intensive ultra violet (UV) radiation [3]. As with many cancer types nowadays, early diagnosis and proper management carries an excellent prognosis, up to 5-year survival rate of above 95% for most skin cancers, even though the long-term survival rate among conventionally-treated metastatic melanoma patients remains only 5% [1,4,5]. In fact, at early stages melanoma is easily curable using surgical procedures (the average 5-years survival rate after a surgical resection is as high as 99%), but once it metastasizes, it remains an intractable therapeutic task, currently challenged by emerging immunotherapies [1]. Until recently, most metastatic melanoma cases were almost inevitably fatal, with rather fast and turbulent course. Today, better understanding of aetiology and molecular pathways in melanoma has led to significant scientific and therapeutic progress, resulting in new and effective therapeutic advancements.

Recently, the molecular landscape of melanoma has been gradually revealed. It has been studied extensively due to its relatively simple laboratory management, especially its cell cultures, and, because of its highly diverse genetic landscape, it has the highest number of somatic mutations among all sequenced cancers to date, shown by the results of The 1000 Genomes Project, among others [6]. Even though there are many ongoing studies on melanoma genetics and epigenetics, it still remains largely undiscovered, especially with regard to epigenetic changes and their dynamics.

This review summarizes recent discoveries in epigenetic changes connected with cutaneous malignant melanoma (CMM), as a solid tumour, comprising of DNA methylation, histone modifications, miRNA regulation, nucleosome positioning and chromatin remodelling. Even though impaired DNA methylation and modifications of histones are the best investigated epigenetic mechanisms of carcinogenesis to date [1], there are several other important mechanisms which should be mentioned while discussing melanoma. In particular, miRNA studies no longer linger, providing interesting insight into various melanoma biology aspects and therefore being widely represented in this review. Moreover, the presence of some genetic and epigenetic changes prior to the clinical manifestation and diagnosis of cancer offer a promising target for further diagnostic and therapeutic approaches, especially in the light of reversibility of epigenetic changes.

Although clinical aspects of melanoma are not in the main scope of this review, some background information assembled might be helpful for better understanding the complexity of this type of cancer.

2. Melanoma: Clinical Background and Aetiology Hypotheses

Cutaneous malignant melanoma is a tumour originated from epidermal melanocytes— pigment-producing cells [1,4]. Thus, most melanomas arise from the skin melanocytes and are therefore easy to notice on the skin surface and more amenable to early medical intervention [4]. Unfortunately, sometimes melanoma may occur in a few other places, significantly less accessible, such as the anus, vagina, oral mucosa and eye, as all those places contain melanocytes [4]. That is because melanocytes are derived from the neural crest structure during embryogenesis, so melanoma can potentially arise in any site of the body to which neural crest cells migrate [1]. Moreover, melanoma may appear de novo or arise from a pre-existing mole, nevi [1]. It is estimated that around one third of all melanomas arise from previously existing nevi, and the remaining two thirds arise de novo [4].

The unique location of the skin, unlike any other organ, makes it more accessible for any treatment therapies, including potential epigenetic therapy, the possible side effects and complications of which might be less intense than those of oral treatments affecting the entire system [1,7,8].

Not only the location but also the clinical presentation of melanoma may vary appreciably, impeding early diagnosis and treatment implementation. In order to expedite melanoma diagnosis, ABCDE criteria were developed [4], as highlighted in Table 1.

Table 1. The ABCDE criteria of melanoma; based on [4,9,10].

A	asymmetry; benign moles are usually symmetrical, while malignant moles tend to have irregular shape
B	border and surface irregularity; benign moles usually have smooth, even borders, whereas malignancies are very irregular
C	colour variegation; benign moles are usually of the same colour, whereas different colours in the same mole may indicate malignancy
D	diameter; malignant lesions are usually greater than 6mm in diameter (approximately pencil eraser size)
E	evolution; while benign moles look similar over time, malignant lesions may change in shape, colour or even start bleeding, itching, etc.

It is widely accepted that exposure to UV light remains the most important risk factor contributing to melanoma development [4]. Both natural environmental exposure, recreational exposure, using tanning beds, workplace-based exposure, sunburn history as well as sunscreen use are being intensively investigated in terms of cancer prevention, development and progression. It has been also suggested that the Earth's ozone layer is thinning, thus more intensive UV radiation reaching the Earth's surface is an exacerbating factor explaining a global increase in melanoma incidence [4].

Since melanoma may occur in many different places around the body, or even originate from melanocytes never exposed to sunlight, it becomes clear that melanoma cannot be attributed solely to

UV radiation, there must be at least one more agent contributing to development of this malignancy. Other factors known to increase the risk of cutaneous malignant melanoma comprise history of nonmelanoma or melanoma skin cancer, both in patient and among family members, atypical nevi, numerous nevi, giant melanocytic nevi, a history of sunburns, freckling, as well as blond hair and light skin colour [3,4].

Inheritance of melanoma remains ambiguous, recent studies denote several candidate genes for further investigations (*CDKN2A*, *CDK4*, *CCND1*, *MITF*); however, family history is positive in 6%–12% of melanoma patients [1,4]. Besides, in so-called "B-K mole syndrome", also known as Familial Dysplastic Nevus Syndrome, members of affected families may, in a lifetime, share up to a 50% cumulative risk for melanoma malignancy [4]. The latest study among twins from Nordic Countries shows that melanoma heritability may be as high as 58% (familial risk; 95% Cl) [11]. It has also been suggested that familial melanoma might be connected with mutations in *INK4a* gene, the product of which is involved in stabilisation of p53 protein complex [12], or/and with *POT1* and other genes of the Shelterin Complex [3,13]. It has been proposed that *TP53* mutations occur very early during melanoma development, which can be either due to inherited alleles per se, environmental factors, or epigenetic interactions [14].

The currently adopted model of melanoma development is multifactorial, it consists of a close interplay between genetic and epigenetic factors, environmental contribution (UV light being the most important one) and several other determinants (the immune system is the most important) [1]. Since the genetic and epigenetic background of melanoma remains unclear, further studies are necessary in order to illuminate biological mechanisms behind this complex disease.

3. Melanoma Genetics and UV Light

Even though most of the well-studied changes induced by UV light are not connected with epigenetics directly, they are crucial for many further molecular events. Skin exposure to this radiation induces DNA damage, oxidative stress, inflammatory responses and sometimes suppression of immune reactions, all of which may significantly change epigenetic status, especially DNA methylation and modifications of histone proteins [15]. It has been noted that chronic inflammation itself may lead to acquisition of epigenetic alterations [16]. UV-induced DNA damage inside skin cells cannot be more clearly demonstrated than in Xeroderma Pigmentosum patients, where DNA repair mechanism called nucleotide excision repair (NER) is impaired due to congenital mutation [4,12]. Those patients are hypersensitive to the sun, having 1000× increased risk of developing any skin cancer, including melanoma [12].

A study conducted in the UK proved that no less than 86% of all melanomas on record have been connected with the patients being exposed to UV radiation stemming from sunrays [17]. Ultraviolet UVB light (wavelengths between 315 and 280 nm) from the sun is absorbed by skin cell DNA and results in a type of direct DNA damage called cyclobutane pyrimidine dimers (CPDs). Thymine-thymine, cytosine-cytosine or cytosine-thymine dimers are formed by the joining of two adjacent pyrimidine bases within a DNA strand. Somewhat similarly to UVB, UVA light (longer wavelengths between 400 and 315 nm) from the sun or from tanning beds can also be directly absorbed by skin DNA (at about 100 to 1000 fold lower efficiency than UVB is absorbed) [18]. It is suggested that everyday usage of SPF 15 or a higher sunscreen reduces the risk of melanoma by about 50% [19].

Some results suggest that UVB radiation might induce not only TP53 mutations, but also alterations of methylation in keratinocytes and other skin cell types at a relatively early stage in areas of human skin which were chronically exposed to sunlight [15]. Similar results were obtained on mice exposed to UV light, where DNA hypomethylation patches were observed [15,20].

Worthwhile studies by Katiyar et al. have also shown a connection between regional DNA hypermethylation and hypoacetylation of histones with downregulation of several tumour suppressor genes [15]. Especially, UV-induced hypoacetylation of histones H3 and H4 in the transcriptionally-silenced tumour-suppressor genes *RASSF1A* and $p16^{INKa}$ was discovered. Furthermore, the same study has also

revealed a UV-induced increase in the recruitment of methyl-CpG-binding domain 1 and MeCP2 to the *RASSF1A* and $p16^{INKa}$ methylated heterochromatin [15]. Another study also suggested existence of a strong connection between DNA methylation and acetylation of histones on chronic UVB skin exposure in mouse models [15,21].

One of the first successes of the Cancer Genome Project was identification of acquired *BRAF* mutations as crucial for melanoma cancer and present in 50%–66% of all malignant melanomas, whereas major pathway affected in melanomas, MAPK-pathway, is mutated in over 90% of all cases [1,3,12,22]. Interestingly, the most common mutation (T>A) identified in the BRAF kinase domain region (as BRAF is a Ser/Thr kinase) is not typical for UV-light induced mutation signatures (CC>TT) [12]. Nevertheless, this discovery led to the development of a new drug, Vemurafenib (Zelboraf) dedicated tomelanoma patients carrying V600E mutation in BRAF [23]. Such a highly personalised treatment resulted in partial or complete tumour remission (81% of patients) in early clinical trial [12,24]. This magnificent success shows the potential of personalised medicine based on the genetic makeup, even if today just a small fraction of melanoma patients could be treated this way. Further effort is certainly needed first in order to investigate biological pathways shaped by both genetic and epigenetic factors, and to invent new therapeutic agents and approaches later.

Going back to the epigenetics, several mechanisms and components of the epigenetic machinery would be unveiled, stressing their further potential in melanoma early diagnosis, management, personalised treatment and even potential prevention. It is clear that some of those epigenetic events can serve not only as targets for pharmacological treatment, but also as cancer biomarkers, having the utility of minimally-invasive detection and monitoring during treatment and progression. Most epigenetic events are known to be reversible, thus, they potentially remain an excellent target for further investigations regarding new treatment strategies in melanoma management. Moreover, epigenetic changes serving as biomarkers might be detected in liquid biopsies based on circulating tumour DNA (ctDNA) or circulating tumour cells (CTCs), obtained in a non-invasive manner [25].

4. Drivers and Passengers Theory

Cancer develops because of the acquisition of somatic mutations. For many years it has been clear that every cancer is a genetic disease, but it took many years to conclude that an individual patient's prognosis and treatment strategy cannot be based only on a single biopsy [26,27]. Recent studies shed new light on the molecular landscape of cancer as a highly heterogeneous disease, composed of many clones with distinct mutational pattern and mutational signatures, both genetic and epigenetic, therefore also presenting differential susceptibilities to treatment [6].

NGS techniques have revolutionized cancer research enabling fast and precise detection of tumour-specific mutations, both genetic and epigenetic [28]. In most cases, such mutations are located in coding sequence of a gene, leading to inactivation or hyperactivation of this gene and aberrations in that particular signalling pathway [29].

Cancer initiation and further progression occur due to positive selection of some "driver" mutations, which can be either genetic or epigenetic, or possibly both. Such mutations promote tumorigenesis—they inactivate tumour suppressor genes or activate oncogenes [30–32]. Average cancer contains 2–8 drivers and the total number of genetic drivers seems to be definite, since all of those genes affect such pathways like apoptosis, proliferation, chromatin regulation and genome stability [33,34]. Other mutations, which were not positively selected during cancer evolution, are called "passengers" [31]. Globally-expressed genes have more important functions such as housekeeping genes. Since most of the positively-selected cancer mutations are localised within globally-expressed genes, development of therapeutic agents targeting them without causing harm to healthy cells, could be even more challenging [31]. Identification of drivers and passengers remains a straitened task, requiring a multidisciplinary approach with a particular contribution of bioinformatic tools for data processing and analysis [1].

To facilitate the hunt for drivers, new treatment strategies and prevention methods, a catalogue of genetic changes in cancer was created: The Cancer Genome Atlas [35,36] and The Human Epigenome Project [37]. The information from this project is used in online genomic databases, e.g., Cosmic [34,36].

In this review, drivers and passengers are evoked in terms of both genetic and epigenetic events occurring in melanoma, therefore understanding this concept remains of utmost importance.

5. DNA Methylation

Disrupted methylation and demethylation processes have already been reported in several melanoma cases. Nowadays, it is well-accepted that DNA methylation does not comprise a simple "on and off" switch, as it was believed for a long time. Instead, it is connected with an abundant amount of additional processes of an epigenetic nature. It is exactly these processes that lead to activating or silencing a specific site or gene [38].

Overall, DNA methylation can be commonly encountered in the area of the genome densely filled with CpG (cytosine residues preceding guanine)—these regions are referred to as "CpG islands" [39]. DNA methylation in mammalian genomes occurs mostly in CpG islands, sometimes also in CpN dinucleotides [15,40]. In 40% of all cases, they have a close proximity to or are situated within the gene promoter regions [41], DNA sequences located in the 5′ region adjacent to the transcriptional start site, and they regulate gene activity by activating or repressing transcription [42]. Therefore, it can be inferred that these CpG islands are areas with frequent regulation through methylation [43]. There are two distinct situations connected with how methylation affects DNA: hypermethylation and hypomethylation. If the regular level of methylation, found in normal healthy tissue, is seen as the baseline, hypomethylation and hypermethylation of DNA means less and more methylation respectively. This terminology is common in the field of cancer epigenetics [44]. Genome-wide methylation is known to decrease in the earliest neoplastic stages of carcinogenesis, leading some researchers to hypothesize that hypomethylation could potentially allow the cells that were previously benign to "start experimenting" with novel gene products, in order to have a survival advantage [45]. Chromosomal instability is exacerbated by DNA hypomethylation mainly if it occurs inside or around centromeric repeats and other repetitive sequences [46]. The extent of how much all those situations influence melanoma is still being researched.

In the case of melanoma, the first reported genes known to be regulated by DNA methylation were *PTEN* (found to be methylated in approximately 60% of melanoma cases), *LOX* (50%), *COL1A2* (80%), *TNFSF10D* (80%), *SYK* (30%), and also *HOXA9, CDH11*, *CLDN11*, *MAPK13* [25]. Epigenome-wide methylation studies very soon showed a global hypomethylation, which activates several oncogenes, such as reactivation of *MITF* (microphthalmia-associated transcription factor)—the master regulator of melanin production. It has been claimed to be a melanoma lineage-specific oncogene, which stays under the control of *BRAF* [25]. Surprisingly, neither *MITF* nor *BRAF* mutations are sufficient for melanoma progression, even though their role in the pathogenesis of melanoma remains unquestionable [25].

Also, reactivation of *EGFR* (epidermal growth factor receptor) due to demethylation of its regulatory elements located on the DNA both upstream and downstream of the transcription start site, has recently been reported [25].

Furthermore, there is an association between *DSS1* gene overexpression and the upregulation of mRNA levels in early TPA-treated hyperplastic skin samples. A positive correlation of *DSS1* expression was observed in pre-neoplastic epidermal cells, meaning that *DSS1* gene could have oncogenic properties, if overexpressed [47]. In addition, it is also known that *DSS1* being overexpressed is associated with metastasised tumour cells and a reduced survival rate, so it is possible that it could potentially serve as a biomarker of poor prognosis [48].

Meanwhile, the expression of certain oncogenes is believed to be induced by the DNA hypomethylation [49]. Among the genes found to be aberrantly methylated in melanoma cells, there are cancer testis antigens, such as the MAGE, GAGE, NY-ESO, and SSX families [50].

Normally, in non-affected cells, these would be repressed through the hypermethylation of the promoter [51]. Nonetheless, it remains unknown whether the fact that they become re-expressed is a sufficient activating factor in the tumour formation. One possible alternative explanation is that it might simply be a secondary effect of the global chromosomal instability, known to be induced by genome-wide DNA demethylation in some forms of cancer [52]. Moreover, more attention is being paid to what connects melanoma and TSPY, the testis-specific protein, located on the Y chromosome. The full potential of its role as a putative oncogene is now being researched in animal studies [53]. In melanoma, this TSPY becomes activated due to the loss of the aforementioned promoter hypermethylation, causing the cell to be pushed faster through its cell cycle, thereby increasing cell proliferation within the affected area [54,55].

Moreover, it has been proposed that hypomethylation may also play a role in melanoma by allowing the re-expression of disadvantageous cancer germ line genes, due to the behaviour of the transcription factor BORIS when it becomes upregulated, yet it has also been established that such activation of cancer germ line genes cannot be caused solely because of BORIS [56].

In addition to global hypomethylation, cancer cells concurrently and paradoxically display localized hypermethylation of CpG islands of multiple tumour suppressor genes, early in carcinogenesis [1,57]. However, it must be emphasized that this is not limited just to melanoma cells. Hypermethylation has been found in a multitude of human cancers, so that the expression "CpG island methylator phenotype" (CIMP) has been coined [1,58,59]. The CIMP phenomenon has been showed in many human cancers to date, including melanoma, confirming a relationship between clinical outcome and region-specific inappropriate DNA methylation [1,59]. In several cancer types, such colorectal cancer, an aberrant DNA methylation in many loci coexisted with the inactivation of several tumour-suppressor genes, DNA repair genes as well as microsatellite instability [1,60]. Recently it was suggested that such global DNA hypermethylation has the potential to elicit tumorigenesis by changing the expression of some genes, including tumour suppressor genes [1]. On the other hand, DNA hypomethylation in human cancers might contribute to tumorigenesis by reactivation of some latent elements of the genome, for example retrotransposons or by activation of previously silenced proto-oncogenes [1,61].

In several cancer cells it has been noted that such CpG islands can acquire aberrant DNA methylation [43]. Whenever this happens, transcription factors lose their ability to bind, leading either to a reduced or complete inhibition of gene expression. This has been explained by the expression upregulation of the de novo DNA methyltransferases (DNMTs) 3A and 3B in CMM progression [62]. Tanemura et al. analysed the methylation status of the CpG islands in several tumour-related genes and methylation loci, as they pertain to the progression of malignant melanoma, and identified significant increase in methylation of *WIF1*, *SOCS1*, *RASSF1A*, *TFPI2*, *MINT17* and *MINT31* genes and loci in advanced clinical stage tumours, and significant hypermethylation of *RAR-b2* and *GATA4* in early-stage tumours [1,11]. Moreover, the methylation status of *MINT31* was proposed as an important predictor (biomarker) of survival for stage III melanoma patients [1,59].

Several other genes have been proposed as good biomarkers for melanoma, such as *CLDN11*, *CDH11*, *MAPK13*, whereas genes such as *PTEN* or *HOXD9* were correlated with overall melanoma patient survival [25].

In another study conducted by Hoon et al., seven tumour suppressor genes were assessed for aberrant methylation in 15 melanoma cell lines and 130 primary and metastatic tumours [63]. Four most frequently hypermethylated genes were found: *RAR-b2*, *RASSF1A*, *MGMT* and *DAPK*. This elegant work has also demonstrated similar *RAR-b2* hypermethylation rates among primary and metastatic tumours, but a higher rate of methylation of *RASSF1A* in metastatic versus primary tumours, suggesting that *RAR-b2* might play a role in initiation, and *RASSF1A* is likely to be associated with tumour progression [14]. Actually, hypermethylation of *RASSF1A* gene (which is a tumour suppressor gene) was investigated profoundly in one of the earliest studies on improper DNA methylation in melanoma [1]. Soon after this, Spugnardi et al. (2003) treated melanoma cell lines with

5-aza-2′-deoxycytidine (DNA demethylating agent) in order to prove that re-expression of *RASSF1A* gene is possible. Indeed, those studies not only showed the reversibility of DNA methylation using adequate pharmacological agents, but also potentially predictive value of the gene's promoter's hypermethylation, thus gene silencing, for melanoma development [1].

An interesting study on melanoma global hypomethylation and promoter CpG islands hypermethylation of selected gene panel was performed by Tellez et al. (2009). This team has constructed a panel of 15 genes known to be involved in important tumour-related processes, such as cell cycle, DNA repair mechanisms, tumour suppression, apoptosis and cell adhesion. With only one exception (*MLH1* gene) all promoter regions were hypermethylated in selected genes. Furthermore, the same study also checked the methylation status of two major repetitive elements of the human genome, *LINE-1* and *Alu*, showing their global hypomethylation in melanoma cell lines [1,64].

Other studies were focused on differentiation between malignant melanoma lesions and benign nevi, when methylation status of selected genes was compared. Work of Conway at al. (2011) led to the identification of 19 loci that are hypomethylated and 7 loci that are hypermethylated in melanoma, comparing with benign nevi. Those genes were involved in proliferation, apoptosis, cell cycle, cell adhesion, cell signalling and even immune system response [1,65].

Among survival prediction studies based on methylation status at least one research is worth mentioning. Sigalotti et al. (2012) designed a huge panel of genes for methylation analysis of their CpG sites and identified a 17-gene prognostic set for patients with stage IIIC melanoma [66]. A methylation score was calculated and on this basis a significant survival advantage was observed among patients from the low-methylation cohort, compared with the high-methylation group of patients [1,66]. This study illustrated the prognostic value of DNA methylation pattern in human melanoma, also potentially helpful in monitoring treatment progress, relapse or qualifying patients into different possible therapeutic pathways. It remains amazing how a subset of differentially methylated genes can explain survival inequalities among patients having the same stage tumour [1].

Finally, some studies on melanoma epigenetics have been focused on finding new biomarkers of this complex cancer. One interesting study has demonstrated that loss of 5-hmC (5-hydroxymethylcytosine; it is a conversion product of 5-methylcytosine, 5-mC) through downregulation of IDH2 (isocitrate dehydrogenase 2) and TET family enzymes (ten-eleven translocation family enzymes) might be an epigenetic hallmark of malignant melanoma progression [1,67].

6. Chromatin Structure and Histone Modifications

Conformational changes of the chromatin through various post-translational modifications remain the best studied mechanisms of carcinogenesis among known epigenetic processes [1,68,69]. The role of chromatin was seen mainly to serve as a foundation containing DNA, although today it is clear that chromatin structure is able to affect the activity of DNA in a variety of potential ways. While it has long been known that modifications of histones are possible also post-translation, the exact ways of such modifications are still being studied.

Histones can be modified by a variety of post-translational modifications affecting N-terminal tails of histone proteins, such as methylation, phosphorylation, acetylation, ubiquitylation, glycosylation, carbonylation, ADP-ribosylation and sumoylation [1]. Most of the abovementioned types of histone modifications are poorly understood and deeper investigation is required before we will apprehend the complexity of those changes, biochemical pathways and their possibilities for therapeutic targets. It is clear, however, that histone modifications possess the ability to both activate or silence gene transcription, since DNA is wrapped around histone octamers [70]. Such processes are dependent on the accessibility of DNA combined with either excluding or recruiting certain protein complexes [71]. In other words, histone modifications regulate transcription through changing the chromatin structure, for example, condensation of chromatin makes it less accessible for transcription factors, thus, transcription is lower or inhibited [1,72,73].

Methylation remains just one among at least 130 other possible modifications which have yet to be studied in depth [74]. Considering histone methylation, until the discovery of the first histone demethylase (KDM1A) just 12 years ago [75], it was believed that the process of methylation could not be reversed. Now we also know that certain modifiers determine the exact physical association of the genome's anatomical segments with corresponding histone modifications [76].

Many components of the methylation machinery (such as DNMTs, MBDs or MeCP2—DNA methyltransferases, methyl-CpG-binding domains and methyl-CpG-binding protein 2, respectively) crosstalk with other machinery's components, for example, histone modification systems, to enhance transcription regulation processes [1,73,77]. Even though it is not clear which components and processes are the cause and which are the consequences, it is currently accepted that histone modifications might be the first step of epigenetic silencing, causing the recruitment of DNA methylation machinery molecules [1,78]. Future studies are crucial to determine the proper sequence of events and discover all the components of those pathways.

In order to take another look at this matter, the intercorrelation between aberrant DNA methylation and histone modifications in melanoma was investigated using melanoma cell lines [1,79]. Eleven melanoma cell lines were cultured with the addition of TSA drug (trichostatin A, which is a histone deacetylase inhibitor) alone or as a combination therapy with 5-aza-CdR (demethylating agent). During the entire experiment, gene expression analysis was performed in order to check the transcriptional changes in two selected genes: *CCR7* and *CXCR4*. Melanoma cells treated with either of two agents, TSA or 5-aza-CdR, showed significantly higher levels of mRNA for both genes, whereas combination therapy including both agents synergistically induced gene expression [1,79].

A more recent study was focused on the impact of aberrant DNA methylation and histone modifications crosstalk on the regulation of transcription of the germline specific gene *MAGEA1*. This particular gene is known to be activated in a multitude of human cancers, including melanoma [1,80]. Those melanoma cell lines with low or zero levels of the *MAGEA1* expression showed a preferential enrichment of H3K9me2 (a repressive histone mark) as opposed to those melanoma cell lines, which had significantly higher levels of *MAGEA1* expression, and high enrichment of H3ac and H3K4me (active histone marks) [80]. In the next step of the experiment, TSA (histone deacetylase inhibitor) was used to treat non-expressing melanoma cell lines. This resulted in a transient and barely detectable increase in expression of *MAGEA1* without causing any change to the promotor methylation status. Treating the same melanoma cell lines with 5-aza-CdR (demethylating agent) led to significantly higher and more stable expression of *MAGEA1* gene, as a result of its promoter hypomethylation [80]. The aforementioned study of Cannuyer et al. (2013) confirmed the action of chromatin modifying drugs in treating epigenetic aberrations in cancer, as shown in melanoma example [80]. Reversibility of epigenetic changes paves the path for pharmacogenomics and pharmacogenetics to develop new therapeutic agents, acting selectively and having minimal possible side effects.

Another example of chromatin structures being modified which needs to be mentioned is the SWI/SNF complex, an evolutionarily conserved multi-subunit complex with the specific function of remodelling chromatin, using the energy of ATP hydrolysis to mobilize nucleosomes. In humans, this complex regulates chromatin organization at regulatory elements both before and after the recruitment of regulators [81]. There is now increasing evidence that points to how this complex plays an active role in the development of various cancers, as several subunits of the SWI/SNF complex exhibit intrinsic tumour-suppressor activity, while other subunits are necessary to bolster other tumour-suppressor genes [82]. Within the complex, we can find suppressor genes such as *BRG1* and *BRM*, both of which can be downregulated in melanoma, however, combined downregulation of both at the same time has not been observed yet [83]. Investigating melanoma cells, there is evidence that the SWI/SNF complex can be found upstream of *MITF*, meaning the stronger expression of this gene, and a part of the effect of *MITF* is to recruit this complex to important melanocyte genes such as *TYRP1*, since chromatin remodelling is a necessity for these genes to become activated [84,85]. Finally, there is

also evidence suggesting that another component of the SWI/SNF complex, the *SMARCB1* gene, tends to be downregulated in melanoma, thereby working against the apoptosis inducing effect provided by $BRAF^{V600E}$ [86].

Finally, it is worth mentioning the study of Kapoor et al. from 2010, where researchers observed that the melanoma aggressiveness increases with the presence of histone variant called macroH2A decreases. The lowering or removal of the macroH2A level during early-stage melanoma cells led to a fast progression of the malignancy, aggressiveness and metastasis. Intuitively, when the level of macroH2A was increased in the advanced stage melanoma cells, the effect was opposite. Further studies of the same team were focused on finding whether macroH2A is a driver or a passenger of melanoma. Using functional analysis, it has been determined that macroH2A can suppress cancer progression through CDK8 expression regulation [87].

7. The Role of Non-Coding RNAs (ncRNAs)

What has to be considered especially important is how ncRNAs are involved in the creation of CMM affected cells. Noncoding RNA (ncRNA)—a heterogenous group of RNA—is what approximately 90% of the human genome is transcribed into [88]. The function of ncRNA is to regulate various cellular processes: DNA imprinting, demethylation, gene silencing, RNA interference, gene transcription and chromatin structure dynamics. Their sizes vary in length and they are divided into subgroups accordingly. The shorter ncRNAs, containing no more than 24 nucleotides, have been named microRNA (miRNA) [89,90]. miRNAs bind to miRNA response elements located within their target mRNA transcripts and subsequently recruit the RNA-induced silencing complex, which alters mRNA stability and/or interferes with translation [91]. Another crucial group is comprised of the longest ncRNAs, starting from 200 nucleotides, the long noncoding RNAs, commonly abbreviated to lncRNA. Their main role is to bind other protein complexes, nevertheless, they are also able to form secondary structures. Although these have been known for a while, they are not yet completely understood. The fact that we do not know the machinery operating their primary sequence or any molecular factors affecting their dynamics is of particular interest [92]. Because of their versatile functions, both groups will be described separately in relation to the melanoma molecular landscape, starting with the less researched lncRNAs.

8. Long Noncoding RNAs (lncRNAs)

Early evidence suggests that a certain lncRNA, known as SPRY4-IT1, might be linked to pathobiology of melanoma [93]. Compared to the normal healthy melanocytes, melanoma cells have highly expressed SPRY4-IT1and its knockdown results in defects in invasion, higher rates of apoptosis as well as an extensive cell growth [94]. Another important lncRNA is HOTAIR (HOX Transcript Antisense RNA), a gene located on chromosome 12 within the Homeobox C gene cluster [95], one which has gained notoriety as the first example of an RNA molecule expressed on one chromosome and influencing the transcription of another chromosome [95]. Due to the analysis by Tang et al., it has been discovered that HOTAIR is significantly overexpressed in the lymph node melanoma metastasis tissue, especially in comparison with primary melanoma; meanwhile HOTAIR has also been identified as the lncRNA associated with the invasion and motility of melanoma cells [96]. This is in addition to the extracellular matrix degradation that it also causes. Another two important lncRNAs have started being researched: Malat-1, a large and infrequently spliced non-coding RNA, and UCA-1, an lncRNA also known as "Urothelial cancer associated 1". A recent study has showed that they are overexpressed in melanoma tissue when they are compared with melanocytes, affecting the ability of cell migration [97], meaning that a higher amount of these two lncRNAs seems to be present in metastatic melanoma. Finally, it should be mentioned that lncRNAs are starting to be seen as a potential prognostic indicator of metastasis in various types of cancer, including, but not limited to malignant melanoma [96,98,99].

9. The Influence of miRNAs

According to the currently available research, the miRNAs responsible for negative regulation of gene expression at the post-transcriptional level might give us much more insight into the exact mechanisms governing the formation of melanoma [100].

By controlling the expression of differing genes and regulating such important processes as apoptosis, miRNAs have been increasingly seen as considerable and even crucial factors in the formation of cancer. Hundreds of such miRNAs have already been identified and linked with various cancer types. Hence, an attempt has been made to create a miRNA registry [101]. It is known as miRBase and there are 28,645 entries available, providing scientists with a valuable tool for research [102].

CMM is no exception as pertains to the importance of miRNAs, as more research into them could hold the clue to understanding this particular malignancy. One such miRNA is miR-221/222. Its overexpression has been linked with reducing the expression of p27, a tumour suppression protein (which in turn increases the proliferation of melanoma cells) [103,104]. Owing to this fact, miR-221/-222 could potentially be used as a new tumour marker to detect malignant CMM in patients [105]. Another example is downregulated miR-193b, targeting cyclin D1, a protein necessary to progress through the G1 phase of the cell cycle under normal circumstances [106]. Interfering with cyclin D1 can cause the suppression of melanoma cell proliferation [107]. Similarly, a different miRNA known as miR-205 is downregulated in melanoma, disrupting the expression of some genes, as it targets the transcription factors E2F1 and E2F5 [108]. The former one is known to be able to mediate both cell proliferation and TP53/p53-dependent apoptosis [109] while the latter one has the function of controlling tumour suppressor proteins p130 and p107 [110], therefore the disruption of either one may cause malignancies to occur. One other miRNA is implicated in the metastasis of melanoma. A study identified the overexpression of miR-21 in melanoma, as compared to benign nevi [111]. Further research confirmed that this miRNA becomes significantly upregulated during the growth of primary melanoma tumours [112]. This in turn boosts the invasiveness of melanoma cell lines because of the inhibition effect of TIMP3 (tissue inhibitor of metalloproteinases) [113], binding to metalloproteinases and other proteolytic enzymes, thereby reducing their activity. Moreover, upregulation of miR-21 was also shown to decrease apoptosis in the melanocyte preparation M1, yet there was no alteration of either proliferation and apoptosis in the MEWO melanoma cell line [114].

Research on the let-7 family of miRNAs shows that these miRNA are implicated in melanoma tumourigenesis [115]. It was demonstrated that let-7a regulates the expression of integrin beta3 by a direct interaction with a binding site in its 3′UTR [115]. If there is a loss of let-7a expression, it is actually the main mechanism that leads to increased integrin beta3 expression in melanoma cells, with a much lesser role played by promoter-dependent mechanisms [115]. Heightened levels of integrin beta3 have been linked with melanoma progression, giving the melanoma cells enhanced migratory and invasive potential [115]. Through transfection of let-7a mimics into melanoma cells, it was possible to reduce their invasiveness by approximately 75% [115]. Meanwhile, using let-7a anti-miRs induced the migratory potential in normal melanocytes, proving the potent connection existing between this miRNA and the progression of melanoma cells [115]. It can be said that its effects are twofold: firstly, any loss of let-7a will lead to enhanced cell proliferation, because of the Ras oncogene becoming unregulated [115]. Secondly, this newfound connection of let-7a and integrin beta3 shows another way how the loss of expression of this miRNA causes adverse effects [115].

There is also promising research into the effects of let-7b [116]. It was shown that in melanoma let-7b targets cell cycle regulators in melanoma, both directly and indirectly [116]. The team verified that let-7b interacts directly with the cyclin D1 3′UTR [116]. Most importantly, a transfection of artificial let-7b was proved to decelerate cell cycle in melanoma cells, by way of significantly reducing the number of melanoma cells in the S-phase and increasing the number of cells in the G1 phase [116]. This transfection also weakened the ability of the cells for anchorage-independent growth [116].

Research conducted at the chromosomal region 1p22, due to its documented connection with melanoma susceptibility (source), revealed that miR-137, encoded in this region, possibly targets the important *MITF* gene [117]. It was further shown that melanoma cell lines expressing *MITF* contain a larger number of a 15-base pair variable number tandem repeat (VNTR) in the 5′UTR of themiR-137 primary transcript [117]. This sheds a new light on the puzzle of variable expression of *MITF* in melanoma [117]. Furthermore, it was also shown that miR-182 is a negative regulator of *MITF* expression [118]. The overexpression of this miRNA was linked to increased survival and invasive potential in melanoma cells [118]. It represses MITF and FOXO3 (a transcription factor of the Forkhead family), thereby stimulating the oncogenic properties of established melanoma cells, such as their ability for the anchorage-independent growth and to move through the extracellular matrix [118]. Experiments done in vivo in a mouse model confirmed that miR-182 bolsters the ability of melanoma cells to build metastases in distant organs [118]. Taking the above into account, a hypothesis was made that MITF is upregulated in early melanoma development and then downregulated when the tumour becomes invasive [118]. Therefore, the whole process could potentially come down to the interplay between miR-137 and miR-182 [118]. The most important miRNA with relation to their function in melanoma context, are presented in a Table 2.

Table 2. Main miRNAs involved in the melanoma progression.

miRNA	Target	Expression in Melanoma	Functions in Melanoma	Reference
Let-7a	*ITGB3*	Down	Invasion, Migration	[115]
Let-7b	*CDK4*, *CCND1*	Down	Proliferation, Differentiation, Metastasis formation, Cell cycle progression, Anchorage-independent growth	[116]
miR-9	*NFKB1*	Down	Proliferation, Invasion	[119]
miR-15b	*BIM1*	Up	Invasion, Survival, Apoptosis	[120]
miR-18b	*MDM2*	Down	Proliferation	[121]
miR-21	*PTEN*, *BCL-2*	Up	Proliferation, Apoptosis	[122]
miR-30b/d	*GALNT7*, *GALNT1*, *GNAI2*, *SEMA3A*	Up	Invasion, Immune Response	[123]
miR-34a/c	*c-MET*, *ULBP2*	Down	Proliferation, Immune Response	[124,125]
miR-125b	*c-Jun*	Down	Proliferation, Migration	[126–128]
miR-126	*ADAM9*, *MMP7*	Down	Proliferation	[129]
miR-137	*MET*, *MITF*, *YB1*, *EZH2*	Up	Invasion	[130]
miR-145	*c-MYC*, *FSCN1*	Down	Proliferation, Invasion	[131]
miR-149	*GSK3a*	Up	Proliferation	[132]
miR-155	*SKI*	Down	Proliferation, Apoptosis	[133]
miR-182	*MITF*, *FOXO3*	Up	Migration, Invasion, Survival	[118]
miR-193b	*Cyclin D1*	Down	Proliferation	[134]
miR-196a	*HOX-C8*, *HOX-B7*, *BMP4*	Down	Invasion	[135,136]
miR-199a	*MET*	Down	Migration, Invasion, Survival	[137]
miR-200a/c	*MARCKS*	Down	Invasion	[138]
miR-203	*E2F3*, *ZBP-89*	Down	Proliferation	[139]
miR-205	*E2F1*, *E2F15*	Down	Proliferation, Apoptosis	[108]
miR-206	*CDK4*, *cyclin D1*, C	Down	Proliferation	[140]
miR-210	*PTPN1*, *HOXA1*, *TP53I11*	Up	Proliferation, Immune response	[141]

Table 2. *Cont.*

miRNA	Target	Expression in Melanoma	Functions in Melanoma	Reference
miR-211	*BRN2*, *KCNMA1*, *NUAK1*, *IGF2R*, *TGFBR2*, *NFAT5*	Down	Invasion	[142]
miR-214	*TFAP2C*	Up	Invasion	[143]
miR-221/222	*KIT*, *p27*	Up and Down	Proliferation, Invasion (Up), Differentiation (Down)	[144]
miR-328	*TGFB2*	Down	Proliferation	[145]
miR-376a/c	*IGF-1R*	Down	Migration	[146]
miR-532-5p	*RUNX3*	Up	Invasion	[147]

10. Immunoediting and Immunotherapy in Melanoma—The Future Is Now

Undoubtedly, the contribution of the host immune system to the cancer development and progression remains significant. Several attempts have been made to use patient immune forces to literally fight against cancer cells, also through vaccine development trials against melanoma cancer [12,148]. It has not been long since immunoediting emerged, capturing the attention of cancer-related scientists all around the globe. Instantaneously it became clear that this could be the future of cancer treatment and possibly highest amount of resources should be allocated to this kind of research.

The term immunoediting was coined to describe the way our immune system copes with the tumour, how it shapes tumour immunogenicity and evolution, how they "talk" to each other [12]. The strongest early evidence of the immunoediting phenomenon in human cancer comes from the studies that show a correlation between patient prognosis and survival with the profile of tumour-infiltrating lymphocytes [12]. To exemplify this, among melanoma patients those with high level of lymphocytes CD8+ cell infiltration have significantly longer survival compared to patients whose tumours have lower numbers of those immune cells [12].

In recent years, the resistance of melanoma to chemotherapy [149,150] has spurred the research and development of alternative methods of treatment, facing the problem of immune evasion. Among the most promising are checkpoint inhibitors, for example, the monoclonal antibody targeting the programmed cell death receptor PD-1 or its ligand, PD-L1 (pembrolizumab and nivolumab, respectively) [151,152]. Apart from promising results and relatively good tolerance, the treatment lead to a 3-year survival rate as high as 30%–41% [152,153]. Ipilimumab, an inhibitor of CTLA-4, has similarly positive effects among melanoma patients, with a sizeable group of patients treated with it surviving even up to 10 years [154], even though only 10%–15% patients show durable response [153].

Apart from molecules mentioned above, there are plenty of other receptors and their ligands under investigation, such as TIM-3, LAG-3, B7-H3 and B7-H4 to mention just a few [153]. Other incoming techniques involving genetically modified T cells to recognize antigens associated with tumours by chimeric antigen receptors (CAR) or transgenic high-affinity T-cell receptors (TCRs) have been recently applied in clinical trials in melanoma and few other cancer types [155–157]. Emerging immunotherapies in melanoma flagged here, even though thrilling and promising, require separate review papers, unfortunately remaining far beyond the scope of this work.

11. Epigenetic Causes of Drug Resistance in Melanoma

Resistance to any existing cancer therapy is a common process, although barely understood, due to its complexity and multifactorial aetiology. Not only the type and stage of the tumour matters, but also such factors as location of the tumour, its genetical heterogeneity, ways of drug uptake and metabolisation, genetic and epigenetic makeup of the tumour and finally, patient him/herself, both physically (e.g., lifestyle factors before and during the cancer treatment) and

psychically (e.g., whether the patient sticks to the prescription regime). All those factors may affect the effectiveness and eventual toxicity of the medicines, and that is why all those factors need further research in the wake of personalised medicine development.

To exemplify the epigenetic mechanisms of resistance acquisition, alkylating agents issue will be introduced. They are a group of cytotoxic drugs triggering apoptosis by binding to the DNA and leading to formation of base-pair cross-links [12]. This cell damage is immediately recognised by DNA repair mechanisms, such as mismatch-repair machinery, to restore the proper DNA sequence and salvage the cell [12,25]. One of the DNA-repair enzymes called MGMT (O^6-methyl-guanine-DNA methyltransferase) is known to be able to reverse the alkylation, thus, avoiding the formation of base-pair cross-links [25]. Hypermethylation of its promoter region, therefore inactivation, has already been related with better survival among some patients with gliomas, and recently also among other cancer patients, including melanoma patients [25,158].

Although general mechanisms of drug resistance development, together with environmental and genetic factors, are extremely important for understanding the concept of multidrug resistance in melanoma, they are beyond the scope of this paper.

12. Epigenetic Treatment

The necessity of new drugs development, especially in the light of latest discoveries has been mentioned in this review several times. Epigenetic treatment has just been approved by several regulatory agencies; however, only for certain for haematological malignancies until now [159]. The success observed in treatment of such cancers like cutaneous lymphoma may represent a proof of the principle that comparable results might be obtained in some solid tumours, including melanoma [159]. Several chemicals that interfere with DNA methylation or demethylation, and a few more drugs that cause histone acetylation or deacetylation have been already studied even in preclinical or early clinical trials, and some of them (such as decitabine, azacytidine, vorinostat, valproic acid) are already in use [159]. Some researchers try to combine novel epigenetic therapies with currently used chemotherapy and personalised medicine innovations, especially in case of resistant metastatic melanoma [160]. Over a decade ago, the identification of molecules and specific, highly recurrent mutations involved in the altered pathway of apoptosis in melanoma, such as *BCL-2*, *EZH2* and $BRAF^{V600E}$, provided an insight into molecular basis of chemoresistance in this cancer type, and led to the discovery of personalised medicine drugs, with the most spectacular success of vemurafenib [161–163].

It is worth mentioning that finding epigenetic treatment is not a simple task. As easy as it might look, in reality it is very challenging, particularly because the biological pathways behind epigenetics remain only partially unrevealed. Furthermore, several known enzymes have many subclasses (like histone deacetylases) [1] with new ones still being discovered. Finally, cancer itself, and melanoma in particular, is an extremely complex phenomenon, comprised of both epigenetic and genetic aberrations, which first must be rapidly and accurately diagnosed.

A study conducted by Segura et al. reveals that a member of the BET family of proteins, BRD4, is notably overexpressed in melanoma, primarily in its metastatic tissues, as compared with melanocytes. Furthermore, it has been reported that BET inhibition weakens melanoma proliferation in vitro and its growth in vivo, giving promising results that treatment with BET inhibitors might be the future of epigenetic therapies [164].

Some opportunities for treatment could be offered by physiological levels of vitamin C, discovered to boost the levels of the crucial 5-hydroxymethylcytosine (5hmC), an epigenetic hallmark of melanoma [67]. Research into vitamin C in A2058 melanoma cell lines seems to indicate that TET enzymes are constantly prepared to generate more 5hmC, needing the hydroxylation reaction to be assisted by a catalyst such as vitamin C [165]. Nevertheless, using pharmacological levels, exceeding 0.5 mM, adversely affected healthy melanocytes, while not offering any greater benefits in 5hmC generation [165].

Finally, some environmental factors and bioactive food components were shown to affect selected epigenetic alterations in cells exposed to UV light, thus, the result is skin cancer prevention [15]. Scanty, but interesting research suggests that some dietary phytochemicals might have the ability to restore the expression of silenced genes in human skin cancer, like hypermethyated p16INK4a and Cip1/p21, by downregulation of the activity of such enzymes like histone deacetylase (HDAC) and DNMT [15].

One of the most promising new treatments of melanoma comes from a concomitant use of HDAC inhibitors together with the TRAIL cytokinase [166]. The reason this works so well is that both kinds of approach focus on achieving the desired goal of the apoptosis of tumorous cells. The class of HDACIs is now being described as potent inducers of apoptosis, due to the high sensitivity shown by tumour cells to their activity, such as the upregulation of the apoptosis supporting BMF gene combined with a downregulation in the pro-survival *BCL2A1* gene [167]. Meanwhile, the recent discovery of a TNF apoptosis inducing ligand, known simply as TRAIL, means that its effects are still not fully evaluated and riddled with complexity [168]. It has been shown that TRAIL might actually promote both invasion and metastasis in cells resistant to it [169]. Nevertheless, when the TRAIL treatment was combined with the silencing of c-FLIP-short and normal expression of c-FLIP-long, the result was a drastic increase in tumour cell apoptosis [169]. The usefulness of such cooperation between TRAIL and HDACIs in treating melanoma was shown in different studies. Melanoma cells were induced to apoptosis when TRAIL was applied together with a HDACI known as SBHA, while other research points to a higher apoptosis rate due to the interactive effects of applying TRAIL together with the inhibitors known as MS-275 and MC1575 [170,171].

Moreover, some well-established carcinogens, such as lead and cadmium, have been recently considered as having an impact on the epigenome [172], even though the relation with melanoma was pointed outover 10 years ago [173,174]. Despite the inability to change the gene structure directly, heavy metals, such as cadmium, could interfere with gene expression, probably by inducing epi-mutations, modifying the DNA methylation patterns [172].

13. Summary

Melanoma remains the fifth most common type of cancer in women [175] and the sixth most common among men worldwide [176]. While CMM occurs in less than 1% of all reported cases of skin cancer, it can be held responsible for the vast majority of skin cancer deaths—up to 65% of all skin cancer deaths [4,177]. Even though the prognosis for patients with metastatic melanoma remains poor, with a long-term survival rate around 5%, early detection results in better prognosis and treatment possibilities [4]. The way that the presence of epigenetic changes influence cancer diagnosis together with the reversibility of epigenetic changes using pharmacological or genetic manipulations, open a wide range of possibilities for further research on melanoma. In fact, we are only at the very beginning of our path, barely knowing even which pathways are disrupted in melanoma, both genetically and epigenetically, and how they are interconnected, for example, gene-gene interactions. In order to find a drug targeting such aberrations, we must comprehend highly complex biological pathways both in melanoma and in healthy tissues. Moreover, inter- and intra-patient heterogeneity must be taken into account and fused with our knowledge about cancer diversity.

Undoubtedly, personalised medicine based on both genetic and epigenetic changes of cancer is the future, the question remains: how long will it take to transport this treatment from the bench to the bedside? For now, the acquaintance of risk factors and clinical manifestation of very early malignancy should be a matter of action for public health providers globally. Thus, improving public awareness and rapid diagnostic methods remain of utmost importance for melanoma management.

Acknowledgments: Authors would like to thank Raya Leibowitz-Amit for her support and inspiration in melanoma cancer research.

Author Contributions: Both authors Marzena Nguyen and Paula Dobosz contributed equally to this review paper.

Conflicts of Interest: The authors declare no conflict of interest.

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