DNA Methylation and Melanoma

Subjects: Oncology

Contributor: Cristina Poggiana

Malignant melanoma is the most lethal form of skin cancer. While new therapeutic approaches have improved survival in patients with metastatic melanoma, responses are rarely sustained due to the high degree of heterogeneity at the inter- and intra-metastatic levels. The development of reliable biomarkers to monitor therapeutic response and disease progression is critical. Many aberrantly methylated genes play a role in cell cycle control, apoptosis, and cell invasion, as well as in melanoma progression. Longitudinal monitoring of DNA methylation via liquid biopsy can provide real-time information on the behavior and stage of melanoma.

melanoma liquid biopsy DNA methylation biomarkers circulating melanoma cells cell-free circulating tumor DNA tumor extracellular vesicles

1. DNA Methylation at a Glance

DNA methylation, along with histone modifications and non-coding RNAs (ncRNAs), is classified as an epigenetic modification. Epigenetic modifications are defined as heritable alterations in gene expression that occur without modifying the DNA sequence [1][2][3]. As is the case with several other physiological mechanisms, when improperly activated or silenced, it can affect the expression of oncogenes or tumor suppressor genes (TSGs), resulting in tumor initiation, development, and progression [4][5]. DNA methylation consists of the covalent addition of a methyl group (-CH3) to the fifth position of the pyrimidine ring of the cytosine nucleotide (5-methylcytosine, 5-mC) [2][3]. DNA methylation can putatively occur at any cytosine nucleotide in the genome, but its distribution is not random and is mostly restricted to the so-called CpG dinucleotides. CpGs are more abundant at gene promoters, where they tend to congregate and form CGIs [2][3][4]. Two main enzyme families are involved in a balanced and regulated manner in DNA methylation and demethylation processes [2]. DNA methyltransferases (DNMTs), which establish and maintain methylation patterns [4], and ten-eleven translocation (TET) methylcytosine dioxygenases, which are involved in the demethylation pathway (**Figure 1**) [6][7]. Under normal conditions, DNA methylation encompasses the entire genome, with the exception of short unmethylated regions within the CGIs [8]. The deregulation of DNA methylation mechanisms, characterized mainly by hypermethylation of CGIs in TSG promoters or global loss of DNA methylation, is common in cancer. CGI hypermethylation in promoter regions has been shown to affect genes involved in the regulatory circuits that control cell proliferation and homeostasis, enabling malignant cells to sustain their abnormal growth [3]. Cancer-associated promoter hypermethylation may affect between 5–10% of promoters containing CGIs [9]. Global loss of DNA methylation leads to molecular consequences that are advantageous to tumor development, including the generation of chromosomal instability [10][11], the loss of genomic imprinting [12], and the reactivation of transposable elements such as *LINE-1* [3][13][14].

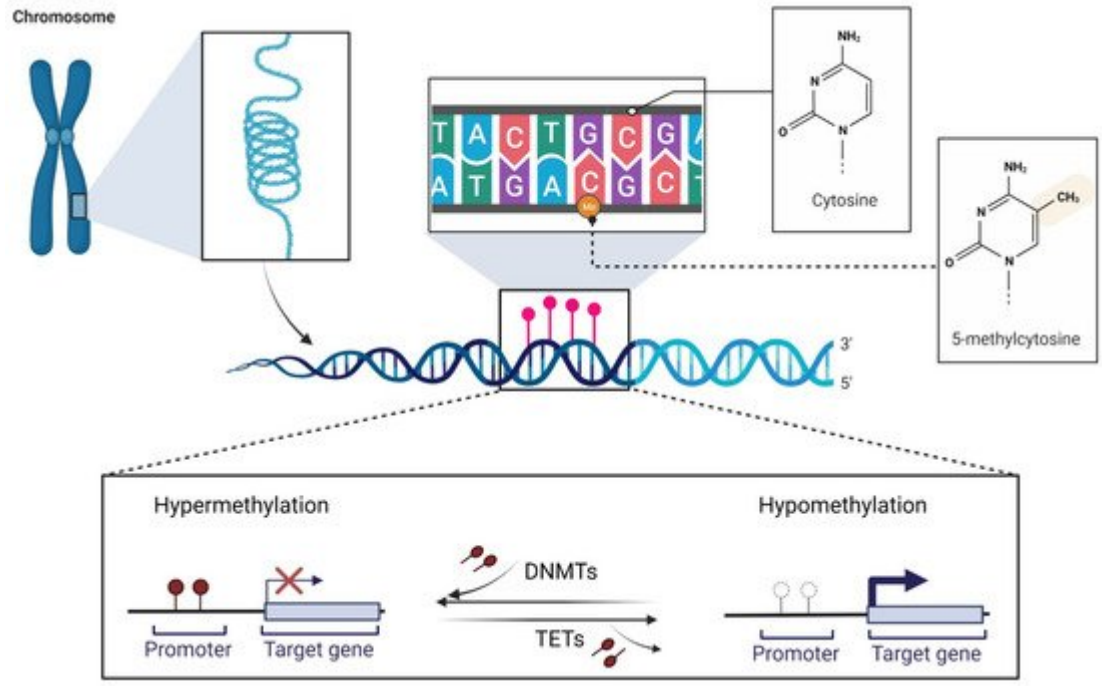

Figure 1. Schematic mechanism of DNA methylation and demethylation. In the DNA sequence, unmethylated cytosines are converted to 5'-methylcytosines (addition of the -CH3 group) by DNMTs. This event encompasses the CpG islands enriched in gene promoters and is generally associated with gene silencing. This reaction is potentially reversible due to the activity of TET enzymes, resulting in the loss of DNA methylation (hypomethylation). The white circles indicate unmethylated CpG sites, and the red circles denote methylated CpG sites. The crossed arrow on the left indicates the absence of transcription after DNA promoter methylation. The thick arrow on the right indicates the start of gene transcription as a consequence of promoter demethylation. Abbreviations: DNMTs, DNA methyltransferases; TETs, ten-eleven translocation methylcytosine dioxygenases. The above diagram was created using BioRender (https://biorender.com/ (accession date 8 October 2021)).

2. DNA Methylation in Melanoma Development

Confined hypermethylation at CpG islands and global hypomethylation are epigenetic hallmarks of melanoma, both of which influence tumor behavior [15][16][17]. Melanoma initiation and progression have been associated with loss of tumor suppressors and oncogene activation [17]. Indeed, many TSGs are known to be aberrantly regulated via inactivation caused by specific methylation in the promoter region [18]. These genes appear to be involved in various signaling pathways, which are frequently altered during melanoma development and evolution. These pathways encompass the protein kinase activated by mitogen (MAPK), the phosphoinositide 3-kinase (PI3K), the tumor suppressor retinoblastoma (pRb), and the p53 protein pathways [15][17][19][20] (**Figure 2**). Since they can act synergistically or independently to control growth and apoptosis, their prolonged and uncontrolled activation is linked to proliferation, invasion, and metastasis. A gradual gain in DNA hypermethylation has been observed to increase in parallel with tumor aggressiveness and is known as the CpG island methylator phenotype (CIMP). It has been suggested that the genes involved in this increasing hypermethylation pattern form the melanoma CIMP [21]. Although less studied, DNA hypomethylation is equally important in the initiation and progression of melanoma.

As previously stated, hypomethylation promotes tumor progression by causing genome instability via the demethylation of transposons and pericentromeric repeats, as well as the activation of specific oncogenes [17][22].

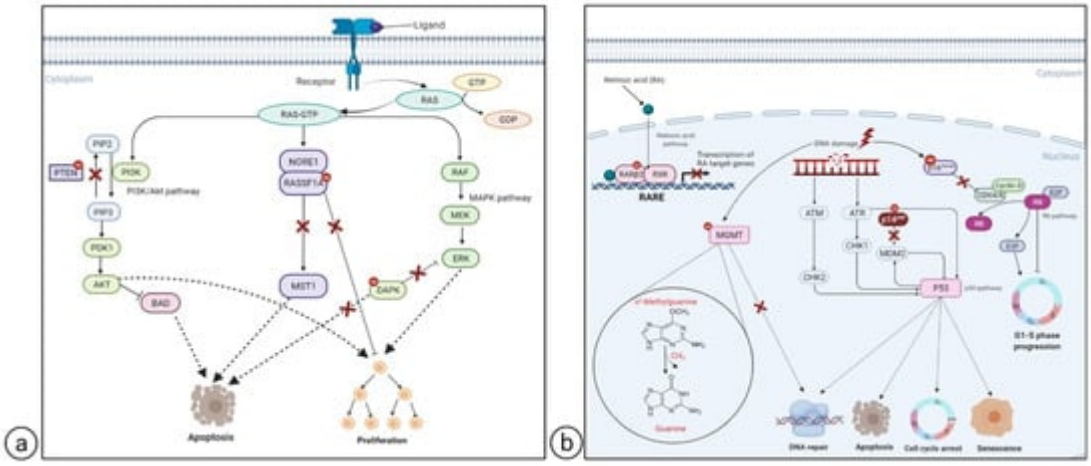

Figure 2. Pathways and genes involved in melanoma development. Aberrantly methylated genes and signal transduction pathways frequently altered during melanoma development and/or progression. (**a**) Shows the MAPK and PI3K-Akt pathways; while (**b**) depicts the p53, Rb, retinoic acid signaling, and DNA repair pathways. Hypermethylated TSGs are marked with a red dot; the alteration induced in the downstream pathway by TSG hypermethylation is represented by a red cross. The diagrams were created by means of https://biorender.com/ (accession date 8 October 2021).

3. DNA Methylation in Melanoma Liquid Biopsies

In this era of "personalized medicine," much attention is being paid to different approaches that provide repeatable and safer insights into tumor evolution and heterogeneity. Since cutaneous melanoma is characterized by extreme heterogeneity and high tumor mutation burden (TMB) [23], the early detection of tumor-related changes such as chromosomal rearrangements, copy number variation, or mutations in oncogenes and tumor suppressor genes, is mandatory for the choice and adjustment of targeted therapy, treatment monitoring, and detection resistance [24]. In the last decade, the so-called "liquid biopsy" [25], which refers to a test of body fluids (i.e., blood, urine, and saliva), has emerged as a novel biomarker with significant application in translational research due to its ability to provide comparable (or more detailed) information than the conventional tissue biopsy [24]. Indeed, the analysis of circulating tumor cells (CTCs), cell-free circulating nucleic acids (cfDNA and cfRNA), and extracellular vesicles (EVs) via a minimally invasive blood draw has opened new avenues for cancer diagnostics, enhancing risk assessment, real-time monitoring of therapeutic efficacy, early detection of relapse, and monitoring of tumor evolution (**Figure 3**) [24][26][27][28]. Like all circulating tumor cells, Circulating Melanoma Cells (CMCs) are released into the bloodstream by the primary tumor and/or metastases [24][29]. Due to their high heterogeneity and rarity in the bloodstreams of metastatic melanoma patients [30][31], the continuous improvement of even more sensitive methods to detect and characterize them is of paramount importance. On the other hand, cell-free circulating DNA is produced from physiological functions such as apoptosis, necrosis, or secretion. Moreover, cancer patients have higher amounts of cfDNA than healthy controls [28][32]. Thus, the fraction of cfDNA that originates from tumor cells,

called circulating tumor DNA (ctDNA), has been extensively studied as a putative disease marker, both in terms of quantity and composition. Indeed, the identification and monitoring of ctDNA using mutation-detection techniques (i.e., droplet digital PCR (ddPCR) and targeted next-generation sequencing (NGS) panels) has been well documented, and its potential as a promising biomarker for diagnosis, evaluation of treatment effectiveness, and as a tracker of tumor evolution has been well assessed in many cancers, including melanoma [28][33][34][35][36][37][38]. Finally, EVs actively participate in intercellular communication by taking part in the transfer of lipids, proteins, and RNA, thus suggesting a putative active role in cancer development [39][40]. Moreover, they have enormous potential as biomarkers, given that their tumor-derived cargo may be used for different applications, i.e., tumor burden estimation and survival prediction [24][41]. All things considered, the search for reliable biomarkers capable of tracking the disease evolution and heterogeneity in real-time may be successful in liquid biopsy, which is the sum of systemic disease. Since the use of targeted therapies and immunotherapy has significantly altered the natural history of melanoma, it is critical to closely monitor its genetic landscape to ensure its success. As previously stated, aberrant methylation of gene promoters can be a characteristic of cancer [42], and, as a result, the analysis of methylated DNA in liquid biopsy is an emerging field of interest [43][44][45]. It has already been demonstrated that hypermethylation of the promoters of certain selected genes can be used to distinguish melanoma patients from healthy individuals [43], demonstrating its utility as a diagnostic marker. Notably, DNA methylation patterns can change during melanoma progression; thus, longitudinal monitoring of DNA methylation in a non-invasive manner via liquid biopsy can provide real-time information about the behavior and stage of melanoma [46].

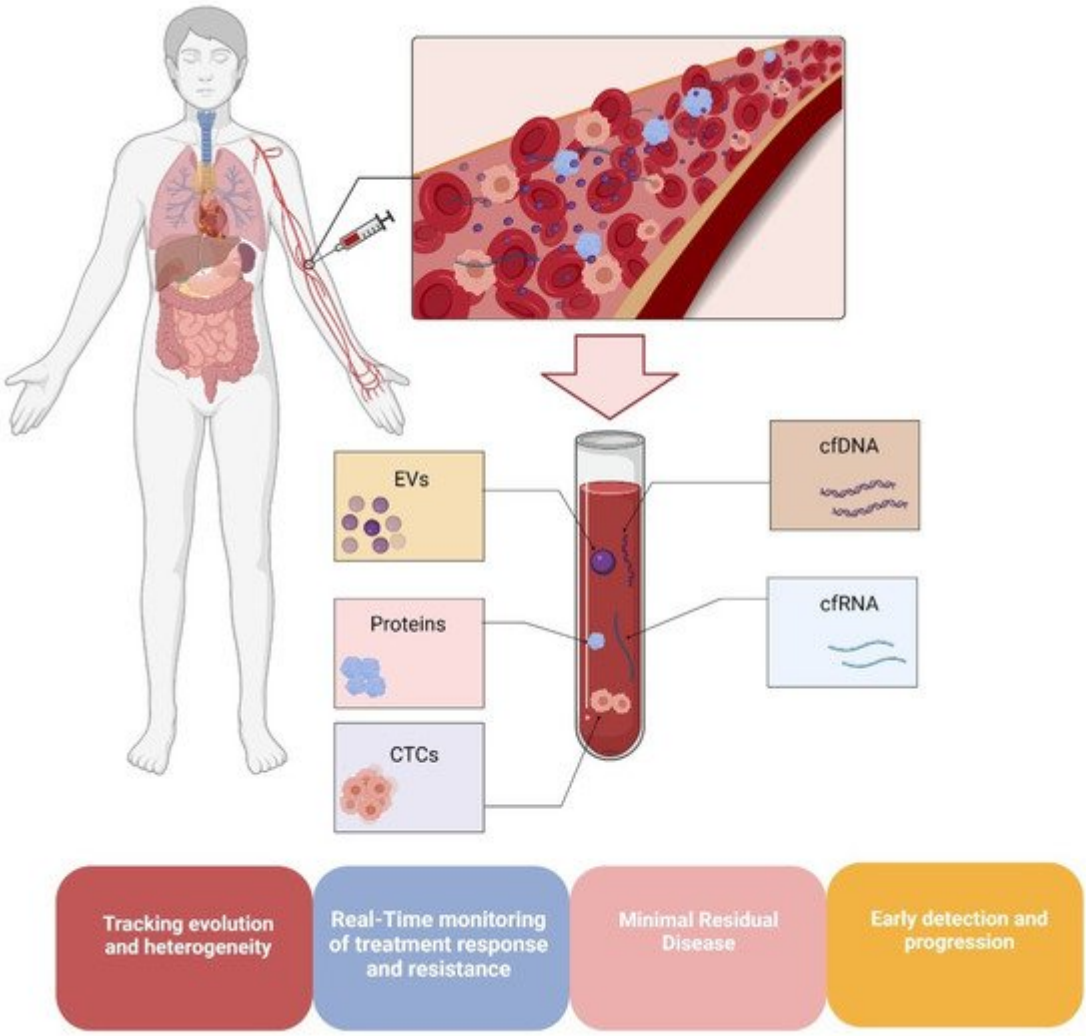

Figure 3. Liquid biopsy in cancer patients. Circulating tumor cells (CTCs), circulating cell-free tumor DNA (ctDNA), circulating cell-free RNA (cfRNA), and extracellular vesicles (EVs) can be isolated simultaneously from the same blood sample. Their analysis provides real-time information on tumor progression, minimal residual disease, treatment response, and resistance. Abbreviations: CTCs, circulating tumor cells; ctDNA, circulating cell-free tumor DNA; cfRNA, cell-free circulating RNA; EVs, extracellular vesicles. Diagram created by means of Biorender (https://biorender.com/ (accession date 8 October 2021)).

3.1. DNA Methylation in Circulating Melanoma Cells

To date, the CTC count as a prognostic/predictive marker is well established for several malignancies for which the cut-off has already been validated [47][48][49]. For others, melanoma included, the process is still ongoing. In any case, this biomarker has garnered considerable attention due to the type of information that its genetic characterization could offer, and more recently, the DNA methylation profile has attracted significantly more attention. However, the study of CTC methylome remains largely unexplored due to the lack of adequate investigation techniques. One of the first studies examined the simultaneous detection of CMCs, inferred by the identification of specific mRNAs in the blood and specific methylated genes. More in detail, Koyanagi and colleagues identified a correlation between the number of melanoma markers (MART-1, GalNAc-T, and MAGE-A3

mRNAs), detected in the blood through quantitative real-time reverse transcription-PCR assay, the presence of circulating methylated *RASSF1A* and *RAR-β2* genes (MSP detection), and patient outcome. Patients with both CMCs and methylated genes showed a significantly poorer response to biochemotherapy, as well as a shorter PFS and OS [50]. Salvianti and colleagues conducted a similar study, focusing on methylated cfDNA and CMCs as two complementary liquid biopsy biomarkers that can be combined to enhance the possibility of disease monitoring [51]. Indeed, they compared *RASSF1A* promoter methylation tracked in cfDNA by real-time qPCR with the presence of CMCs in both healthy controls and patients at different melanoma stages (in situ, invasive, and metastatic). They found that the percentage of cases with methylated *RASSF1A* promoter was higher in melanoma patients than in healthy subjects (46% vs. 10%), thus indicating *RASSF1A* promoter methylation as a good predictor of disease (AUC of 0.905). However, when they checked the presence of CMCs in the three different patient categories, they found no significant association with methylated *RASSF1A* tracked in cfDNA [51].

Currently, no studies have been conducted to determine the methylation status of CMCs. However, the few studies that have investigated CTC methylation in other cancers are very interesting. Recent publications have focused on finding the optimal technique for studying CTC methylome in lung [52], breast [53][54], and colon cancer [53]. More in detail, Zhao and colleagues developed an approach called LCM-μWGBS that combines laser capture microdissection (LCM) CTC capture and whole-genome bisulfite sequencing (μWGBS), enabling the analysis of a small number of CTCs to obtain information on their DNA methylation landscape [52]. They defined the DNA methylome of CTCs from lung cancer patients and compared it to the global DNA methylation of normal tissues. Interestingly, they found a progressive decrease in global DNA methylation from normal tissue to primary tumors and CTCs, suggesting a gradual loss of DNA methylation during tumorigenesis. Moreover, they observed a tendency toward the increased methylation of several TSG promoters in CTC-DNA compared to the primary tumor.

In a very recent study, Chen and colleagues, by means of single-cell bisulfite sequencing (scBS-seq), provided an important analysis of the single-cell DNA methylome in CTCs, characterizing tumor heterogeneity and the evolution of the tumor cell methylome during cancer progression [53]. Seventeen cancer patients covering six different types of cancers (lung adenocarcinoma, small-cell lung cancer, breast, colon, gastric, and prostate cancer) were tested to assess the methylation level in single CTCs [53]. Firstly, they observed that CTCs, similar to primary tumors, exhibit lower methylation levels than those in normal cells. They also observed that these cells show inter- and intra-patient heterogeneity in terms of promoter methylation. Interestingly, they also investigated the dynamic methylome changes, which occur during cancer metastasis, in the promoter regions of 20 known tumor-associated genes of primary, metastatic tissues, CTCs, and white blood cells of gastric cancer patients accompanied by abdominal ovarian metastasis. For instance, they observed that the methylation level of the *LTF* gene, often reported down-regulated in tumors, increased with cancer progression. The methylation level of several genes expressed in the ovaries, such as *1orf35*, *DENND6A*, and *ZNF285*, decreased transiently. Furthermore, the methylation level of genes involved in oncogenic transformation- and cell adhesion-associated pathways (*FBP2*, *HIVEP3*, *PTPN21*, and *CEACAM5* genes) decreased, suggesting a role for these pathways in tumor progression.

Since CTCs can be found in patient blood as single CTCs or CTC clusters, Gkountela and colleagues found that CTC clusters are distinguishable from single CTCs based on their methylation status, assessed by single-cell whole-genome bisulfite sequencing. They observed that in CTC clusters, hypomethylated regions are associated with key regulators of stemness and metastasis (*OCT4*, *NANOG*, *SOX2*, and *SIN3A*), while hypomethylated regions in single CTCs are independent of the pluripotency network [54].

3.2. Methylation in Circulating Melanoma DNA

Several recent studies have focused on ctDNA methylation screening and its potential clinical application, in addition to genomic and/or expression analyses [55][56]. Furthermore, most recent studies have focused on the analysis of ctDNA rather than FFPE samples in an attempt to overcome the limitations associated with the poor quality of the FFPE genetic material. In one of the first studies, based on real-time MSP detection, Hoon and colleagues examined the presence of hypermethylated TSG promoters in the serum of melanoma patients [57]. They observed that the incidence of TSG hypermethylation increased during tumor progression and that *MGMT*, *RASSF1A*, and *DAPK* hypermethylation were significantly lower in primary melanomas compared to metastatic ones. On the other hand, the frequency of hypermethylated RAR-ß2 was similar in both primary and metastatic melanomas [57].

A subsequent study by Mori and colleagues highlighted the utility of detecting circulating methylated tumor-related genes in serum (MSP analysis) as a predictive marker of response to biochemotherapy and OS. Among the most frequently hypermethylated genes in melanoma, they discovered a significant correlation between the hypermethylation of *RASSF1A* and RAR-ß2, the response to biochemotherapy, and OS [58]. Interestingly, this correlation with treatment response was not observed when analyzing the *MGMT* methylation status [58].

A recent study by Liu and colleagues focused on assessing the feasibility of using cfDNA methylation profiles in advanced cancer patients for the detection of metastatic disease [55]. They developed an NGS targeted methylation sequencing assay to measure the methylation status of more than 9000 CpG sites, selected according to TCGA data, and parallel classify the presence of advanced cancer, being also able to predict tumor origin (i.e., melanoma, colorectal cancer, NSCLC, and breast cancer). The authors demonstrated that plasma cfDNA methylation scores detected the presence of cancer in 83.8% of cancer patients with 100% specificity and predicted cancer type in 78.9% of cases. Focusing on melanoma, Diefenbach and colleagues developed an efficient ctDNA methylation analysis workflow using an amplicon-based NGS panel performed on bisulfite-treated DNA. They confirmed the hypermethylation of seven genes (*GJB2*, *HOXA9*, *MEOX2*, *OLIG3*, *PON3*, *RASSF1*, and *TFAP2B*), known to be hypermethylated in metastatic melanoma patients but not in healthy individuals [43]. Moreover, in contrast to previous studies on cfDNA methylation, they were able to examine the methylation of symmetrical CpG sites in both DNA strands to accurately quantify the level of gene methylation [43].

Some studies have explored the potential of methylation pattern tracking in early-stage tumors. In this regard, the clinical validity of a targeted methylation-based Multi-Cancer Early Detection (MCED) test using cfDNA sequencing has been investigated in several cancer types, including melanoma [56]. This new approach aims to provide the

tools to detect tumors at an earlier stage, thus reducing cancer mortality by identifying the cancer signal origin (CSO) [56][59]. The MCED test demonstrated a specificity of 99.5% (false-positive rate of 0.5%) and an overall sensitivity of 51.5% for cancer signal detection. The authors suggested to use this test either in combination with other single-cancer screening tests (for the detection of breast, colorectal, cervical, lung, and prostate cancers) or as a screening for those cancers for which tests are not yet available in the United States. However, the high costs of this type of analysis could represent an obstacle for its application in the clinical setting [56].

In summary, the role of DNA methylation in melanoma deserves deeper investigation, as its role in tumorigenesis is only partially understood. Its usefulness as a biomarker could be helpful in defining a precision medicine workflow. In particular, a suitable and optimal analysis technique should be sought, which is currently lacking. In addition, a cost-benefit analysis should be conducted. While whole-genome bisulfite sequencing can provide a complete methylation profile at a very high cost, a PCR-based approach is cheaper but assesses only a small number of CpG sites [55]. The use of NGS panels, which allows simultaneous analysis of the methylation status of several tens to hundreds of genes, could be an alternative and, if customized and restricted to a limited number of regions of interest, might be an excellent compromise to limit costs.

3.3. Methylation in Melanoma Extracellular Vesicle-Derived DNA (evDNA)

In recent years, there has been growing interest in understanding the role of extracellular vesicles (EVs) in melanoma progression since they are considered to be an alternative means of intercellular communication. EVs are composed of a heterogeneous group of membrane-delimited nanoparticles, usually classified into exosomes (30–120 nm), microvesicles (100–1000 nm), and apoptotic bodies (500–4000 nm) based on their size [60]. They play a key role in the delivery of active cargoes, including DNA fragments, coding and non-coding RNA, proteins, and lipids, from donor to distal cells, and they are released by normal and cancerous cells into the external microenvironment [61]. Tumor-derived EVs have been shown to affect the pathophysiology of recipient cells, modulating a variety of processes involved in cancer progression (increased invasiveness, proliferation rate, and chemoresistance) [62].

The study of EV content is emerging as an innovative and helpful strategy to understand tumor processes due to the fact that the bioactive molecules are packaged and protected within the phospholipid bilayer of EVs. Although research on EV-derived RNA and proteins has been largely explored, only a few studies have been conducted on EV-associated DNA (evDNA). Recent evidence suggests that most of the DNA associated with tumor-derived EVs is double-stranded (dsDNA), representing the entire genome and informing on the mutational status of parental tumor cells [63]. However, the mechanism of DNA loading onto EVs remains unclear. The main hypothesis is the encapsulation of cytosolic DNA during EV biogenesis [39]. In recent years, the methylation analysis of evDNA has attracted attention as a biomarker for the detection of various cancers. The benefit of using evDNA is its stability and protection from digestive enzymes due to encapsulation in the lipid bilayer of EVs. However, major steps forward are needed to overcome issues related to the source of evDNA, sample collection, and DNA extraction methods that appear to affect methylation detection. For example, it is necessary to develop a better strategy for isolating evDNA without cfDNA contamination because it has been observed that DNA tends to stick to any

surface, including the lipid envelope of EVs, resulting in the possible co-isolation of cfDNA with the EV during the purification protocol [64]. In this regard, the digestion of the EV pellet by DNAses may be an optimal strategy to overcome the co-isolation of nucleic acids with EVs. The DNA extraction efficiency largely depends on which method of EV isolation and evDNA extraction is used. Moreover, the presence of DNA in exosomes continues to be controversial. In 2019, while Coffey and colleagues concluded that exosomes do not contain DNA, Yokoi and colleagues observed genomic DNA and nucleoprotein in them. Perhaps it may depend on an over-strict exosome isolation strategy that can lead to the loss of evDNA, the levels of which are too low to be detected. Because the most effective approach for EV isolation has not yet been well established, and different methods of EV isolation and DNA extraction are currently used, it is mandatory to develop an optimal common strategy for downstream applications. For example, García-Romero and colleagues compared the most common EV-isolation methods and found that polyethylene glycol precipitation (PEG) seems to be the most feasible and affordable EV isolation technique [65]. Moreover, in a study by Kamyabi and colleagues, a microfluidic platform was described as a method for the rapid isolation of EVs from the plasma of pancreatic cancer patients [66].

Although data on melanoma are still limited, and most of the data are restricted to in vitro experiments, overall findings suggest that the methylation profile of evDNA shows similarities in methylation profile with that of genomic DNA (gDNA) in murine melanoma cells [63]. This evidence has also been confirmed in other cancer types, such as metastatic castration-resistant prostate cancer [67], diffuse large B-cell lymphoma [68], and glioblastoma [69]. A new and interesting approach was recently developed using an electrochemical detection method based on the differential absorption capacities of different methylation levels of DNA on a gold surface [61]. Using this highly sensitive microdevice, Sina and colleagues performed exploratory research to develop a method to isolate evDNA from ctDNA based on methylation-dependent physicochemical properties. They found that evDNA has surface-based properties similar to cellular gDNA, but not to cfDNA, probably due to the longer size of gDNA. Moreover, they demonstrated that this method was able to discriminate cancer and normal evDNA, suggesting a potential use of this device for clinical applications (the adsorption level on the gold surface of patient evDNAs was 20–40%, whereas that of the normal evDNAs was below 20%) [61].

In addition, the promoter regions of specific genes in metastatic castration-resistant prostate cancer (GSTP1, *RASSF1A*, and *SLFN11*) and in diffuse large B-cell lymphoma (*CDKN2A* and *CDKN2B*) are found to be methylated in both evDNA and primary tumor tissue or CTCs, respectively [67][68]. These findings not only highlight the potential use of evDNA methylation analysis as a biomarker for the detection of cancer but also point toward a better understanding of the evDNA methylation profile in melanoma cancer, which is poorly understood.

4. Circulating Methylated DNA Biomarkers for Tracking Response to Therapy and Resistance Onset

The development of treatment resistance is the main limitation to many anti-cancer therapeutic approaches. DNA methylation has the potential to be used as a biomarker in many clinical situations, including for the prediction of response to therapies and the monitoring of recurrence [15]. As previously discussed, positive signals for a putative prognostic role for methylation status, tracked through liquid biopsy, were found in the early 2000s by Koyanagi

and colleagues, who hypothesized the clinical utility of two different molecular variables (CMC-mRNA and serum methylated DNA) in a cohort of patients undergoing biochemotherapy [50]. Similarly, Mori and colleagues assessed the prognostic and predictive significance of detecting specific methylated genes in the serum of melanoma patients undergoing biochemotherapy [58]: they found that hypermethylation of *RASSF1A* was the best predictor of response and OS, thus corroborating previous evidence found in other malignancies of resistance to cisplatin and tamoxifen associated with *RASSF1A* hypermethylation [70][71]. Moreover, the methylated status of *RAR-β2* was significantly related to survival. As *RAR-β2* is involved in the control of cell growth and apoptosis, its expression in tumor cells susceptible to its mediated apoptosis could be an important biomarker of response to biochemotherapy [58]. A recent study by de Vos and colleagues involved a large patient cohort with different malignancies from the Cancer Genome Atlas (TCGA) Research Network to test the value of two hypermethylated genes, *SHOX2*, and *SEPT9*, as pan-cancer biomarkers [72]. The quantitative methylation analysis of these genes has shown a correlation with treatment response: when compared to conventional monitoring, cfDNA longitudinal screening revealed an association between an increase in the methylation score (CMS, cumulative cfDNA methylation score) and non-responsiveness to treatment with an 80-day advantage.

Nowadays, chemotherapy with two alkylating agents, DTIC or TMZ, is still an alternative in the event of the development of resistance to immune checkpoint inhibitors (ICI) and/or targeted therapy, or in the case of mutation-negative melanomas [17][73][74]. Therefore, biomarkers capable of identifying the minority of patients who may benefit from this type of approach would be critical, as they are currently lacking. The effects of methylating agents have been putatively related to the expression of *MGMT*. Its overexpression protects against cell death induced by alkylation. On the other hand, low expression is correlated with a higher possibility of responding to methylating agents [75][76]. As melanomas are likely to express low levels of *MGMT* [77][78], this could explain why they respond to methylating drugs, including DTIC and TMZ, but not to other anti-cancer drugs. The *MGMT* methylation status can thus determine the outcome in melanoma patients treated with methylating drugs. In fact, *MGMT* promoter methylation has been associated with response to single-agent DTIC/TMZ and longer PFS in disseminated cutaneous melanoma [79], even if conflicting data have been reported [74][80]. Melanoma cells appear to be intrinsically resistant to drugs and/or acquire resistance through different strategies involving multiple other players [80]. Ultimately, the simultaneous tracking of the *MGMT* methylation status and other players could be of great interest for determining the success of TMZ therapy, and liquid biopsy, as a sum of systemic disease, may be the optimal source for performing this type of longitudinal screening.

References

1. Waterland, R.A. Epigenetic mechanisms and gastrointestinal development. J. Pediatr. 2006, 149, S137–S142.
2. Yang, Y.; Wang, Y. Role of Epigenetic Regulation in Plasticity of Tumor Immune Microenvironment. Front. Immunol. 2021, 12, 640369.

3. Ortiz-Barahona, V.; Joshi, R.S.; Esteller, M. Use of DNA methylation profiling in translational oncology. Semin. Cancer Biol. 2020.
4. Kulis, M.; Esteller, M. 2-DNA Methylation and Cancer. In Advances in Genetics; Herceg, Z., Ushijima, T., Eds.; Epigenetics and Cancer, Part A; Academic Press: Cambridge, MA, USA, 2010; Volume 70, pp. 27–56.
5. Witte, T.; Plass, C.; Gerhauser, C. Pan-cancer patterns of DNA methylation. Genome Med. 2014, 6, 66.
6. Ito, S.; Shen, L.; Dai, Q.; Wu, S.C.; Collins, L.B.; Swenberg, J.A.; He, C.; Zhang, Y. Tet Proteins Can Convert 5-Methylcytosine to 5-Formylcytosine and 5-Carboxylcytosine. Science 2011, 333, 1300–1303.
7. Tahiliani, M.; Koh, K.P.; Shen, Y.; Pastor, W.A.; Bandukwala, H.; Brudno, Y.; Agarwal, S.; Iyer, L.M.; Liu, D.R.; Aravind, L.; et al. Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1. Science 2009, 324, 930–935.
8. Suzuki, M.M.; Bird, A. DNA methylation landscapes: Provocative insights from epigenomics. Nat. Rev. Genet. 2008, 9, 465–476.
9. Baylin, S.B.; Jones, P.A. Epigenetic Determinants of Cancer. Cold Spring Harb. Perspect. Biol. 2016, 8, a019505.
10. Karpf, A.R.; Matsui, S.-I. Genetic Disruption of Cytosine DNA Methyltransferase Enzymes Induces Chromosomal Instability in Human Cancer Cells. Cancer Res. 2005, 65, 8635–8639.
11. Sheaffer, K.L.; Elliott, E.N.; Kaestner, K.H. DNA Hypomethylation Contributes to Genomic Instability and Intestinal Cancer Initiation. Cancer Prev. Res. 2016, 9, 534–546.
12. Holm, T.M.; Jackson-Grusby, L.; Brambrink, T.; Yamada, Y.; Rideout, W.M.; Jaenisch, R. Global loss of imprinting leads to widespread tumorigenesis in adult mice. Cancer Cell 2005, 8, 275–285.
13. Lee, E.; Iskow, R.; Yang, L.; Gokcumen, O.; Haseley, P.; Luquette, L.J.; Lohr, J.G.; Harris, C.C.; Ding, L.; Wilson, R.K.; et al. Landscape of Somatic Retrotransposition in Human Cancers. Science 2012, 337, 967–971.
14. Tubio, J.M.; Li, Y.; Ju, Y.S.; Martincorena, I.; Cooke, S.L.; Tojo, M.; Gundem, G.; Pipinikas, C.P.; Zamora, J.; Raine, K.; et al. Extensive transduction of nonrepetitive DNA mediated by L1 retrotransposition in cancer genomes. Science 2014, 345, 1251343.
15. Micevic, G.; Theodosakis, N.; Bosenberg, M. Aberrant DNA methylation in melanoma: Biomarker and therapeutic opportunities. Clin. Epigenet. 2017, 9, 1–15.
16. Schinke, C.; Mo, Y.; Yu, Y.; Amiri, K.; Sosman, J.; Greally, J.; Verma, A. Aberrant DNA methylation in malignant melanoma. Melanoma Res. 2010, 20, 253–265.

17. Dobre, E.-G.; Constantin, C.; Costache, M.; Neagu, M. Interrogating Epigenome toward Personalized Approach in Cutaneous Melanoma. J. Pers. Med. 2021, 11, 901.
18. Howell, J.P.M.; Liu, S.; Ren, S.; Behlen, C.; Fodstad, O.; Riker, A.I. Epigenetics in Human Melanoma. Cancer Control 2009, 16, 200–218.
19. Gutiérrez-Castañeda, L.D.; Nova, J.A.; Tovar-Parra, J.D. Frequency of mutations in BRAF, NRAS, and KIT in different populations and histological subtypes of melanoma: A systemic review. Melanoma Res. 2020, 30, 62–70.
20. Xia, J.; Jia, P.; Hutchinson, K.E.; Dahlman, K.B.; Johnson, D.; Sosman, J.; Pao, W.; Zhao, Z. A Meta-analysis of Somatic Mutations from Next Generation Sequencing of 241 Melanomas: A Road Map for the Study of Genes with Potential Clinical Relevance. Mol. Cancer Ther. 2014, 13, 1918–1928.
21. Tanemura, A.; Terando, A.M.; Sim, M.-S.; Van Hoesel, A.Q.; De Maat, M.F.; Morton, D.L.; Hoon, D.S. CpG Island Methylator Phenotype Predicts Progression of Malignant Melanoma. Clin. Cancer Res. 2009, 15, 1801–1807.
22. Ehrlich, M. DNA hypomethylation in cancer cells. Epigenomics 2009, 1, 239–259.
23. Alexandrov, L.B.; Nik-Zainal, S.; Wedge, D.C.; Aparicio, S.A.J.R.; Behjati, S.; Biankin, A.V.; Bignell, G.R.; Bolli, N.; Borg, A.; Børresen-Dale, A.-L.; et al. Signatures of mutational processes in human cancer. Nature 2013, 500, 415–421.
24. Kamińska, P.; Buszka, K.; Zabel, M.; Nowicki, M.; Alix-Panabières, C.; Budna-Tukan, J. Liquid Biopsy in Melanoma: Significance in Diagnostics, Prediction and Treatment Monitoring. Int. J. Mol. Sci. 2021, 22, 9714.
25. Pantel, K.; Alix-Panabières, C. Circulating tumour cells in cancer patients: Challenges and perspectives. Trends Mol. Med. 2010, 16, 398–406.
26. Alix-Panabières, C.; Pantel, K. Liquid Biopsy: From Discovery to Clinical Application. Cancer Discov. 2021, 11, 858–873.
27. Cabel, L.; Proudhon, C.; Gortais, H.; Loirat, D.; Coussy, F.; Pierga, J.-Y.; Bidard, F.-C. Circulating tumor cells: Clinical validity and utility. Int. J. Clin. Oncol. 2017, 22, 421–430.
28. Alix-Panabières, C.; Schwarzenbach, H.; Pantel, K. Circulating Tumor Cells and Circulating Tumor DNA. Annu. Rev. Med. 2012, 63, 199–215.
29. Siravegna, G.; Marsoni, S.; Siena, S.; Bardelli, A. Integrating liquid biopsies into the management of cancer. Nat. Rev. Clin. Oncol. 2017, 14, 531–548.
30. Marsavela, G.; Aya-Bonilla, C.A.; Warkiani, M.E.; Gray, E.S.; Ziman, M. Melanoma circulating tumor cells: Benefits and challenges required for clinical application. Cancer Lett. 2018, 424, 1–8.

31. Rapanotti, M.C.; Campione, E.; Spallone, G.; Orlandi, A.; Bernardini, S.; Bianchi, L. Minimal residual disease in melanoma: Circulating melanoma cells and predictive role of MCAM/MUC18/MelCAM/CD146. Cell Death Discov. 2017, 3, 17005.
32. Phallen, J.; Sausen, M.; Adleff, V.; Leal, A.; Hruban, C.; White, J.; Anagnostou, V.; Fiksel, J.; Cristiano, S.; Papp, E.; et al. Direct detection of early-stage cancers using circulating tumor DNA. Sci. Transl. Med. 2017, 9, 2415.
33. Gray, E.S.; Witkowski, T.; Pereira, M.; Calapre, L.; Herron, K.; Irwin, D.; Chapman, B.; Khattak, M.A.; Raleigh, J.; Hatzimihalis, A.; et al. Genomic Analysis of Circulating Tumor DNA Using a Melanoma-Specific UltraSEEK Oncogene Panel. J. Mol. Diagn. JMD 2019, 21, 418–426.
34. Cheng, F.; Su, L.; Qian, C. Circulating tumor DNA: A promising biomarker in the liquid biopsy of cancer. Oncotarget 2016, 7, 48832–48841.
35. Cree, I.A.; Uttley, L.; Woods, H.B.; Kikuchi, H.; Reiman, A.; Harnan, S.; Whiteman, B.L.; Philips, S.T.; Messenger, M. The Evidence Base for Circulating Tumour DNA Blood-Based Biomarkers for the Early Detection of Cancer: A Systematic Mapping Review. BMC Cancer 2017, 17, 697.
36. Gray, E.S.; Rizos, H.; Reid, A.L.; Boyd, S.C.; Pereira, M.R.; Lo, J.; Tembe, V.; Freeman, J.; Lee, J.H.; Scolyer, R.A.; et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget 2015, 6, 42008–42018.
37. Santiago-Walker, A.; Gagnon, R.; Mazumdar, J.; Casey, M.; Long, G.; Schadendorf, D.; Flaherty, K.T.; Kefford, R.; Hauschild, A.; Hwu, P.; et al. Correlation of BRAF Mutation Status in Circulating-Free DNA and Tumor and Association with Clinical Outcome across Four BRAFi and MEKi Clinical Trials. Clin. Cancer Res. 2016, 22, 567–574.
38. Bai, Y.; Wang, Z.; Liu, Z.; Liang, G.; Gu, W.; Ge, Q. Technical progress in circulating tumor DNA analysis using next generation sequencing. Mol. Cell. Probes 2020, 49, 101480.
39. Kalluri, R. The biology and function of exosomes in cancer. J. Clin. Investig. 2016, 126, 1208–1215.
40. Lin, J.; Li, J.; Huang, B.; Liu, J.; Chen, X.; Chen, X.-M.; Xu, Y.-M.; Huang, L.-F.; Wang, X.-Z. Exosomes: Novel Biomarkers for Clinical Diagnosis. Sci. World J. 2015, 2015, 1–8.
41. Alegre, E.; Zubiri, L.; Perez-Gracia, J.L.; González-Cao, M.; Soria, L.; Martín-Algarra, S.; González, A. Circulating melanoma exosomes as diagnostic and prognosis biomarkers. Clin. Chim. Acta Int. J. Clin. Chem. 2016, 454, 28–32.
42. Moran, B.; Silva, R.; Perry, A.; Gallagher, W.M. Epigenetics of malignant melanoma. Semin. Cancer Biol. 2018, 51, 80–88.
43. Diefenbach, R.J.; Lee, J.H.; Chandler, D.; Wang, Y.; Pflueger, C.; Long, G.V.; Scolyer, R.A.; Carlino, M.S.; Menzies, A.M.; Kefford, R.F.; et al. Hypermethylation of Circulating Free DNA in

Cutaneous Melanoma. Appl. Sci. 2019, 9, 5074.

44. Diefenbach, R.J.; Lee, J.H.; Rizos, H. Methylated circulating tumor DNA as a biomarker in cutaneous melanoma. Melanoma Manag. 2020, 7, MMT46.
45. Constâncio, V.; Nunes, S.P.; Henrique, R.; Jerónimo, C. DNA Methylation-Based Testing in Liquid Biopsies as Detection and Prognostic Biomarkers for the Four Major Cancer Types. Cells 2020, 9, 624.
46. Wouters, J.; Vizoso, M.; Martinez-Cardus, A.; Carmona, F.J.; Govaere, O.; Laguna, T.; Joseph, J.; Dynoodt, P.; Aura, C.; Foth, M.; et al. Comprehensive DNA methylation study identifies novel progression-related and prognostic markers for cutaneous melanoma. BMC Med. 2017, 15, 101.
47. Cristofanilli, M.; Budd, G.T.; Ellis, M.J.; Stopeck, A.; Matera, J.; Miller, M.C.; Reuben, J.M.; Doyle, G.V.; Allard, W.J.; Terstappen, L.W.; et al. Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer. N. Engl. J. Med. 2004, 351, 781–791.
48. De Bono, J.S.; Scher, H.I.; Montgomery, R.B.; Parker, C.; Miller, M.C.; Tissing, H.; Doyle, G.V.; Terstappen, L.; Pienta, K.; Raghavan, D. Circulating Tumor Cells Predict Survival Benefit from Treatment in Metastatic Castration-Resistant Prostate Cancer. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 2008, 14, 6302–6309.
49. Cohen, S.J.; Punt, C.J.A.; Iannotti, N.; Saidman, B.H.; Sabbath, K.D.; Gabrail, N.Y.; Picus, J.; Morse, M.; Mitchell, E.; Miller, M.C.; et al. Relationship of Circulating Tumor Cells to Tumor Response, Progression-Free Survival, and Overall Survival in Patients with Metastatic Colorectal Cancer. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 2008, 26, 3213–3221.
50. Koyanagi, K.; Mori, T.; O'Day, S.J.; Martinez, S.R.; Wang, H.-J.; Hoon, D.S. Association of Circulating Tumor Cells with Serum Tumor-Related Methylated DNA in Peripheral Blood of Melanoma Patients. Cancer Res. 2006, 66, 6111–6117.
51. Salvianti, F.; Orlando, C.; Massi, D.; de Giorgi, V.; Grazzini, M.; Pazzagli, M.; Pinzani, P. Tumor-Related Methylated Cell-Free DNA and Circulating Tumor Cells in Melanoma. Front Mol. Biosci. 2016, 2, 76.
52. Zhao, L.; Wu, X.; Zheng, J.; Dong, D. DNA methylome profiling of circulating tumor cells in lung cancer at single base-pair resolution. Oncogene 2021, 40, 1884–1895.
53. Chen, H.; Su, Z.; Li, R.; Zhang, N.; Guo, H.; Bai, F. Single-cell DNA methylome analysis of circulating tumor cells. Chin. J. Cancer Res. 2021, 33, 391–404.
54. Gkountela, S.; Castro-Giner, F.; Szczerba, B.M.; Vetter, M.; Landin, J.; Scherrer, R.; Krol, I.; Scheidmann, M.C.; Beisel, C.; Stirnimann, C.; et al. Circulating Tumor Cell Clustering Shapes DNA Methylation to Enable Metastasis Seeding. Cell 2019, 176, 98–112.e14.

55. Liu, L.; Toung, J.; Jassowicz, A.; Vijayaraghavan, R.; Kang, H.; Zhang, R.; Kruglyak, K.; Huang, H.; Hinoue, T.; Shen, H.; et al. Targeted methylation sequencing of plasma cell-free DNA for cancer detection and classification. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 2018, 29, 1445–1453.

56. Klein, E.; Richards, D.; Cohn, A.; Tummala, M.; Lapham, R.; Cosgrove, D.; Chung, G.; Clement, J.; Gao, J.; Hunkapiller, N.; et al. Clinical validation of a targeted methylation-based multi-cancer early detection test using an independent validation set. Ann. Oncol. 2021, 32, 1167–1177.

57. Hoon, D.S.B.; Spugnardi, M.; Kuo, C.; Huang, S.K.; Morton, D.L.; Taback, B. Profiling epigenetic inactivation of tumor suppressor genes in tumors and plasma from cutaneous melanoma patients. Oncogene 2004, 23, 4014–4022.

58. Mori, T.; O'Day, S.J.; Umetani, N.; Martinez, S.R.; Kitago, M.; Koyanagi, K.; Kuo, C.; Takeshima, T.-L.; Milford, R.; Wang, H.-J.; et al. Predictive Utility of Circulating Methylated DNA in Serum of Melanoma Patients Receiving Biochemotherapy. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 2005, 23, 9351–9358.

59. Liu, M.C.; Oxnard, G.R.; Klein, E.A.; Swanton, C.; Seiden, M.V. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. Ann. Oncol. 2020, 31, 745–759.

60. Catoni, C.; Di Paolo, V.; Rossi, E.; Quintieri, L.; Zamarchi, R. Cell-Secreted Vesicles: Novel Opportunities in Cancer Diagnosis, Monitoring and Treatment. Diagnostics 2021, 11, 1118.

61. Ibn Sina, A.A.; Lin, T.-Y.; Vaidyanathan, R.; Wang, Z.; Dey, S.; Wang, J.; Behren, A.; Wuethrich, A.; Carrascosa, L.G.; Trau, M. Methylation dependent gold adsorption behaviour identifies cancer derived extracellular vesicular DNA. Nanoscale Horiz. 2020, 5, 1317–1323.

62. Sadovska, L.; Santos, C.B.; Kalniņa, Z.; Linē, A. Biodistribution, Uptake and Effects Caused by Cancer-Derived Extracellular Vesicles. J. Circ. Biomark. 2015, 4, 4.

63. Thakur, B.K.; Zhang, H.; Becker, A.; Matei, I.; Huang, Y.; Costa-Silva, B.; Zheng, Y.; Hoshino, A.; Brazier, H.; Xiang, J.; et al. Double-stranded DNA in exosomes: A novel biomarker in cancer detection. Cell Res. 2014, 24, 766–769.

64. Miranda, K.C.; Bond, D.T.; McKee, M.; Skog, J.; Paunescu, T.; Da Silva, N.; Brown, D.; Russo, L.M. Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease. Kidney Int. 2010, 78, 191–199.

65. Garcia-Romero, N.; Madurga, R.; Rackov, G.; Aliana, I.P.; Núñez-Torres, R.; Asensi-Puig, A.; Carrión-Navarro, J.; Esteban-Rubio, S.; Peinado, H.; Gonzalez-Neira, A.; et al. Polyethylene glycol improves current methods for circulating extracellular vesicle-derived DNA isolation. J. Transl. Med. 2019, 17, 75.

66. Kamyabi, N.; Abbasgholizadeh, R.; Maitra, A.; Ardekani, A.; Biswal, S.L.; Grande-Allen, K.J. Isolation and mutational assessment of pancreatic cancer extracellular vesicles using a microfluidic platform. Biomed. Microdevices 2020, 22, 1–11.

67. Zavridou, M.; Strati, A.; Bournakis, E.; Smilkou, S.; Tserpeli, V.; Lianidou, E. Prognostic Significance of Gene Expression and DNA Methylation Markers in Circulating Tumor Cells and Paired Plasma Derived Exosomes in Metastatic Castration Resistant Prostate Cancer. Cancers 2021, 13, 780.

68. Barıs, I.C.; Hacıoglu, S.; Turk, N.S.; Cetın, G.O.; Zencır, S.; Bagcı, G.; Caner, V. Expression and DNA methylation profiles of EZH2-target genes in plasma exosomes and matched primary tumor tissues of the patients with diffuse large B-cell lymphoma. Clin. Transl. Oncol. 2021, 23, 1152–1166.

69. Maire, C.L.; Fuh, M.M.; Kaulich, K.; Fita, K.D.; Stevic, I.; Heiland, D.H.; A Welsh, J.; Jones, J.C.; Görgens, A.; Ricklefs, T.; et al. Genome-wide methylation profiling of glioblastoma cell-derived extracellular vesicle DNA allows tumor classification. Neuro-Oncology 2021, 23, 1087–1099.

70. Koul, S.; McKiernan, J.M.; Narayan, G.; Houldsworth, J.; Bacik, J.; Dobrzynski, D.L.; Assaad, A.M.; Mansukhani, M.; Reuter, V.E.; Bosl, G.J.; et al. Role of promoter hypermethylation in Cisplatin treatment response of male germ cell tumors. Mol. Cancer 2004, 3, 16.

71. Fiegl, H.; Millinger, S.; Mueller-Holzner, E.; Marth, C.; Ensinger, C.; Berger, A.; Klocker, H.; Goebel, G.; Widschwendter, M. Circulating Tumor-Specific DNA: A Marker for Monitoring Efficacy of Adjuvant Therapy in Cancer Patients. Cancer Res. 2005, 65, 1141–1145.

72. De Vos, L.; Jung, M.; Koerber, R.-M.; Bawden, E.G.; Holderried, T.A.; Dietrich, J.; Bootz, F.; Brossart, P.; Kristiansen, G.; Dietrich, D. Treatment Response Monitoring in Patients with Advanced Malignancies Using Cell-Free SHOX2 and SEPT9 DNA Methylation in Blood. J. Mol. Diagn. JMD 2020, 22, 920–933.

73. Bouchereau, S.; Chaplain, L.; Fort, M.; Beauchet, A.; Sidibé, T.; Chapalain, M.; Gonzalez-Lara, L.; Longvert, C.; Blom, A.; Saiag, P.; et al. Impact of prior treatment with immune checkpoint inhibitors on dacarbazine efficacy in metastatic melanoma. Br. J. Cancer 2021, 125, 948–954.

74. Makita, K.; Hara, H.; Sano, E.; Okamoto, Y.; Ochiai, Y.; Harada, T.; Ueda, T.; Nakayama, T.; Aizawa, S.; Yoshino, A. Interferon-β sensitizes human malignant melanoma cells to temozolomide-induced apoptosis and autophagy. Int. J. Oncol. 2019, 54, 1864–1874.

75. Zuhur, S.S.; Tanik, C.; Karaman, Ö.; Velet, S.; Çil, E.; Öztürk, F.Y.; Özkayalar, H.; Müslüman, A.M.; Altuntaş, Y. MGMT immunoexpression in growth hormone-secreting pituitary adenomas and its correlation with Ki-67 labeling index and cytokeratin distribution pattern. Endocrine 2011, 40, 222–227.

76. Kaina, B.; Fritz, G.; Mitra, S.; Coquerelle, T.; Bernd, K. Transfection and expression of human O6-methylguanine-DNA methyltransferase (MGMT) cDNA in Chinese hamster cells: The role of MGMT in protection against the genotoxic effects of alkylating agents. Carcinogenesis 1991, 12, 1857–1867.
77. Kaina, B.; Ziouta, A.; Ochs, K.; Coquerelle, T. Chromosomal instability, reproductive cell death and apoptosis induced by O6-methylguanine in Mex−, Mex+ and methylation-tolerant mismatch repair compromised cells: Facts and models. Mutat. Res. Mol. Mech. Mutagen. 1997, 381, 227–241.
78. Jian-Min, C.; Yang-Pei, Z.; Wang, C.; Sun, Y.; Fujimoto, J.; Ikenaga, M. O6-Methylguanine-DNA methyltransferase activity in human tumors. Carcinogenesis 1992, 13, 1503–1507.
79. Tuominen, R.; Jewell, R.; Oord, J.J.V.D.; Wolter, P.; Stierner, U.; Lindholm, C.; Johansson, C.H.; Lindén, D.; Johansson, H.; Stolt, M.F.; et al. MGMT promoter methylation is associated with temozolomide response and prolonged progression-free survival in disseminated cutaneous melanoma. Int. J. Cancer 2014, 136, 2844–2853.
80. Naumann, S.C.; Roos, W.; Jöst, E.; Belohlavek, C.; Lennerz, V.; Schmidt, C.; Christmann, M.; Kaina, B. Temozolomide- and fotemustine-induced apoptosis in human malignant melanoma cells: Response related to MGMT, MMR, DSBs, and P53. Br. J. Cancer 2009, 100, 322–333.