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Revolutionizing Non-Invasive Biomarker Discoveries: The Power of Methylation Screening Analysis in Cell-Free DNA Liquid Biopsy

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Abstract

Epigenetic changes of DNA, including methylation, have long been recognized as key indicators of various diseases, including aging, cancer, and neurological disorders. Biomarker discoveries based on distinct methylation patterns for both hypermethylation and hypomethylation lead the way in discovery of novel diagnosis and treatment targets. Many different approaches are present to detect the level of methylation in whole genome (whole genome bisulfite sequencing, microarray) as well as at specific loci (methylation specific PCR). Cell-free DNA (cf-DNA) found in body fluids like blood provides information about DNA methylation and serves as a less invasive approach for genetic screening. Cell-free DNA and methylation screening technologies, when combined, have the potential to transform the way we approach genetic screening and personalized therapy. These technologies can help enhance disease diagnostic accuracy and inform the development of targeted therapeutics by providing a non-invasive way for acquiring genomic information and identifying disease-associated methylation patterns. We highlight the clinical benefits of using cell-free DNA (cf-DNA) liquid biopsy analysis and available methylation screening technologies that have been crucial in identifying biomarkers for disease from patients using a non-invasive way. Powering such biomarker discoveries are various methods of cf-DNA methylation analysis such as Bisulfite Sequencing and most recently, Methylation-Specific Restriction Enzyme (MSRE-seq) Analysis, paving the way for novel epigenetic biomarker discoveries for more robust diagnosis such as early disease detection, prognosis, monitoring of disease progression and treatment response as well as discovery of novel drug targets.

Keywords

Epigenetics, Biomarkers, Cell-Free DNA (cf-DNA), Methylation, Liquid Biopsy, Drug Target, Methylation-Specific Restriction Enzyme (MSRE), Cancer, Epigenetic Drugs, Hypermethylation, Hypomethylation

1. Introduction

Epigenetic biomarker discovery is the identification of changes in epigenetic marks, such as DNA methylation and histone modifications, that are associated with a particular disease or condition [1]. These biomarkers can be used for early detection [2] [3], diagnosis [4], prognosis [5], and to monitor the response to treatment [6] as well as therapeutic targets for drug discovery [7].

One of the most common approaches for epigenetic biomarker discovery is through the use of high-throughput screening techniques such as genome-wide epigenetic arrays [8] and next generation sequencing (NGS) of bisulfite treated samples for methylation changes [9], and ChIP-Seq and ATAC-Seq for histone modifications [10]. These techniques allow for the simultaneous analysis of thousands of genes and targets, making it possible to identify novel biomarkers that would have been difficult to detect through traditional methods.

Methylation is a process by which a methyl group (CH_3) is added to the DNA molecule [11]. This modification can affect the function of the gene to which it is attached [12]. In recent years, researchers have discovered that methylation patterns in the DNA can be used as biomarkers for various diseases [13]. One of the most well-known examples of methylation as a biomarker is in cancer [14]. Tumors often have distinct methylation patterns compared to normal tissue, and these patterns can be used to diagnose and classify different types of cancer. For example, in colorectal cancer, methylation of the APC gene is a common event [15] [16]. In breast cancer, methylation of the BRCA1 gene is a frequent event [17]. These biomarkers are useful in the diagnosis of cancer, as well as in determining the prognosis and treatment options for patients. Methylation biomarkers can also be used in the early detection of cancer [18]. Hypermethylation of certain genes such as tumor suppressor proteins [19], and hypomethylation of oncogene [20] is a common event in the early stages of cancer development, and it can be detected in blood or other bodily fluids. This makes it possible to detect cancer at an early stage, when it is more treatable.

In addition to cancer, methylation biomarkers are also being studied for their use in other diseases. For example, methylation patterns in the DNA have been linked to several neurological disorders, including Alzheimer's disease [21] and multiple sclerosis [22]. Other studies have shown that methylation patterns in the DNA can be used to predict the risk of developing certain diseases, such as heart disease [23] and diabetes [24].

Epigenetic drugs are a class of therapeutics that target the epigenetic mechan-

isms that regulate gene expression [25]. Examples of epigenetic drugs include DNA methyltransferase inhibitors (DNMTi) [26], Histone deacetylase inhibitors (HDACi) [27]. Histone methyltransferase inhibitors (HMTi) [28]. Bromodomain and extra-terminal domain inhibitors (BETi), Histone lysine methyltrasferase inhibitors (HLMi), and Epigenetic modulation of miRNA (miRNA based therapies) [29]. These drugs are currently being studied in clinical trials for a variety of diseases, including cancer, neurodegenerative diseases, autoimmune diseases, cardiovascular disease, and others.

A large amount of research has been conducted in the field of DNA methylation and the identification of biomarkers for early detection but, discovery of these biomarkers from cell free DNA is very challenging. However, the discovery of methylation biomarkers in cfDNA has revolutionized the field of cancer diagnostics, as it provides a non-invasive method for detecting and diagnosing cancer effectively even at a very early stage. Methylation patterns in cfDNA can be used to identify specific cancer types, provide customized treatment plan, monitor treatment response, and predict disease recurrence. Cell-free DNA liquid biopsy is among those highly effective approaches that can help achieve this goal. The aim of epigenetic biomarker discovery from cell-free DNA liquid biopsy is to identify molecular changes in the epigenome that can be used to detect, diagnose, and monitor diseases, particularly cancers. By analyzing cell-free DNA, which is DNA that is circulating in the bloodstream, scientists can gain a non-invasive window into the changes that occur in cancer cells. Methylation, a process that changes the way genes are expressed, is one of the key epigenetic changes that can be studied using cell-free DNA. By identifying specific patterns of methylation that are associated with cancer, scientists can develop new diagnostic and prognostic tools, as well as new targets for therapeutic intervention. The discovery of epigenetic biomarkers from cell-free DNA liquid biopsy is a promising new approach for improving the early detection, management, and treatment of cancer and other diseases. This approach holds great potential for transforming the field of cancer and other diseases and improving patient outcomes.

2. Methylation Analysis for Biomarker Discovery

Methylation analysis has been used to identify a wide range of novel biomarkers for various diseases and conditions. Some examples of epigenetic biomarkers that have been discovered through methylation analysis include cancer [30], neurological disorders [31], cardiovascular [32], and other disease including diabetes (**Table 1**). The methylation of various genes, including APC, RASSF1A [33], and BRCA1 [34], has been observed in different types of cancer such as colon, breast, and ovarian cancer. These biomarkers can be used for early detection, diagnosis, prognosis and treatment of cancer. Methylation analysis has also been used to identify biomarkers for neurological disorders such as Alzheimer's disease, where genes such as BDNF have been found to be methylated [35].

Table 1. Methylation types associated with diseases and biomarkers. The study of methylation patterns and their association with disease is a rapidly growing field that holds great promise for improving our understanding of disease biology and the development of new diagnostics and therapeutic strategies.

Methylation Type	Diseases Associated	Biomarkers	References
DNA Hypermethylation	Different Cancer Types	APC, RASSF1A, CDH13, p16INK4a, MGMT, p73, BRCA1	[33] [39] [40]
DNA Hypomethylation	Different Cancer Types	TERT, Dlk1/Gtl2	[41] [42]
Imprinted Gene Methylation	Imprinted Disorders	H19, Igf2,	[43]
X-Chromosome Inactivation	X-linked Disorders	XIST, MEG3, SNRPN	[44] [45]
LINE-1 Methylation	Cancer, Alzheimer's Disease	LINE-1 Repeat Elements	[46] [47]
Repeat Element Methylation	Autism disorder	Alu, LINE-1 repeat Element	[48]

These biomarkers can be used to monitor the progression of the disease and to develop new therapies. Methylation of the promoter of the NOS3 gene has been found to be associated with an increased risk of coronary artery disease. These biomarkers can be used to identify individuals at high risk for cardiovascular disease and to develop new therapies [36] [37]. It has also been used to identify biomarkers for other diseases, such as diabetes, where genes such as HNF4A have been found to be methylated [38].

One example of a novel epigenetic biomarker discovered through this approach is the methylation of the O6-methylguanine DNA methyltransferase (MGMT) promoter in glioblastoma, a type of brain cancer [39]. The MGMT gene is involved in DNA repair, and its inactivation through methylation has been associated with poor prognosis and resistance to therapy [49]. Therefore, the MGMT methylation status can be used as a biomarker for predicting the response to therapy and patient outcome in glioblastoma. Another example is the use of histone modifications such as H3K27me3 as a biomarker for cancer diagnosis [50]. The Polycomb repressive complex 2 (PRC2) is an enzyme that catalyzes the trimethylation of histone H3 lysine 27 (H3K27me3) and its overexpression is associated with cancer progression [51]. Therefore, H3K27me3 has been proposed as a biomarker for cancer diagnosis and therapeutic target.

3. Disease with Hypermethylation and Hypomethylation

Both hypermethylation and hypomethylation are considered as a hallmark of cancer, aging [52] and other diseases that leads to the development and progression of disease [53]. Hypermethylation and hypomethylation refer to the increase or decrease, respectively, of methyl groups on the DNA. Hypermethyla-

tion refers to the increase in methyl groups on the DNA, particularly in the promoter regions of genes. This mostly leads to the silencing of genes and the inhibition of their expression [55]. Hypermethylation is commonly observed in cancer, where it leads to the silencing of tumor suppressor genes, which are responsible for controlling cell growth and division [54]. Both the phenomenon of hypomethylation and hypermethylation can be represented in a diagram as shown below in **Figure 1**.

Hypomethylation refers to the decrease in methyl groups on the DNA, particularly in the promoter regions of genes. This leads to the activation of genes and the increase in their expression [55]. Hypomethylation is commonly observed in aging and certain diseases, such as Alzheimer's disease, where it leads to the activation of transposable elements, which can cause genomic instability [56]. Hypomethylation has been associated with a wide range of diseases, including cancer, neurological disorders, and developmental disorders. Hypomethylation can lead to the activation of oncogenes or the silencing of tumor suppressor genes, which can contribute to the development and progression of cancer. For example, loss of methylation in the promoter of the c-myc oncogene has been observed in various types of cancer, such as breast [57], and colon cancer [58]. Hypomethylation has been also observed in the brain of individuals with neurological disorders such as Alzheimer's disease [59], Huntington's disease [60], and schizophrenia [61]. Hypomethylation in specific genes, such as BDNF and S100B, has been associated with these disorders [62] [63]. It has been associated with several developmental disorders such as Rett syndrome, a neurodevelopmental disorder caused by mutations in the MECP2 gene. Hypomethylation of the MECP2 gene has been observed in individuals with Rett syndrome [64]. Hypomethylation has also been observed in other diseases such as diabetes and autoimmune disorders, where genes such as HNF4A have been found to be hypomethylated.

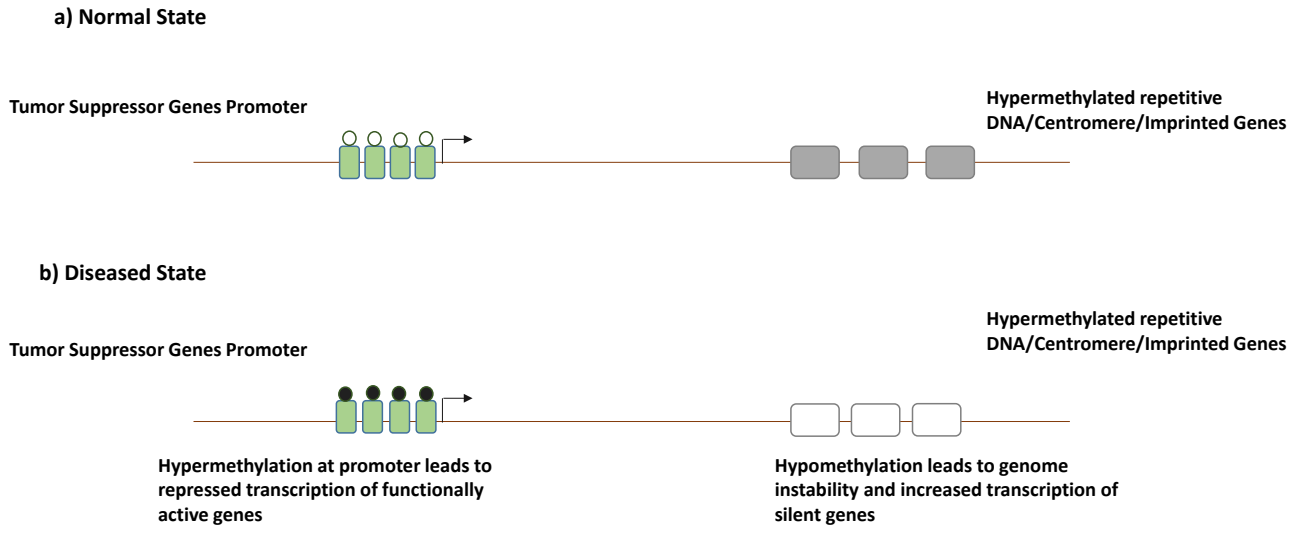

Figure 1. Representation of hypermethylation and hypomethylation in normal and diseased states.

Hypermethylation is commonly observed in cancer, where it leads to the silencing of tumor suppressor genes, which are responsible for controlling cell growth and division. This results in the development and progression of cancer [65]. Examples of hypermethylated genes in cancer include p16INK4a and BRCA1. Hypermethylation of genes involved in the immune response has been observed in autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [66] [67]. It is also involved in the development and function of the brain has been observed in neuropsychiatric disorders such as schizophrenia and bipolar disorder. The cardiovascular diseases such as hypertension [68], atherosclerosis and heart failure have been linked to the genes for hypermethylation change [69]. Hypermethylation has been observed in various diseases such as obesity, diabetes and certain respiratory diseases.

4. Cell Free DNA (cfDNA) Liquid Biopsy Analysis for Biomarker Discovery

Cell-free DNA (cfDNA) is a novel biomarker discovery molecule that can be used to detect and analyze DNA that is present in the blood, but not associated with cells [70]. The ability to detect and analyze cfDNA in blood samples as a liquid biopsy makes it a non-invasive method of biomarker discovery [70]. It is highly sensitive allowing for the detection of small amounts of DNA that makes it useful for early detection of cancer and other diseases. The analysis highly specific, allowing for the detection of specific genetic changes or mutations. However, there are also some disadvantages of cell-free DNA analysis that requires a relatively large amount of blood to be collected to obtain enough DNA for analysis. The DNA molecules are highly degraded, and make it difficult to do analysis using conventional methods. Furthermore, the analysis of cfDNA requires sophisticated laboratory techniques and requires trained personnel to perform the analysis [71].

The greatest advantage of using cfDNA analysis for biomarker discovery is its non-invasive nature as it involves liquid biopsy testing. Blood samples can be easily obtained and analyzed, without the need for invasive procedures. Additionally, cfDNA analysis is highly sensitive and specific, allowing for the detection of small amounts of DNA and specific genetic changes or mutations [72]. The cfDNA analysis has been used to detect a variety of biomarkers associated with cancer, including mutations in genes such as EGFR, KRAS, and ALK. These biomarkers can be used to predict the response to specific therapies, monitor treatment response, and detect the recurrence of cancer [73].

One of the most promising applications of cfDNA analysis in cancer is in the early detection of the disease [73]. In many types of cancer, cancer cells die and release their DNA into the bloodstream. This can be detected through cfDNA analysis, even before the cancer has formed a tumor that can be detected by imaging or other methods. This makes it possible to detect cancer at an early stage, when it is more treatable [74]. Another application of cfDNA analysis in cancer

is in the monitoring of cancer treatment. Cancer treatments, such as chemotherapy and radiation, can cause cancer cells to die, releasing their DNA into the bloodstream. By analyzing cfDNA, it is possible to detect changes in the amount of cancer-specific DNA in the blood, which can indicate whether the treatment is working or not. The cfDNA analysis can also be used to monitor the recurrence of cancer after treatment [75]. Cancer cells that have spread to other parts of the body can release DNA into the bloodstream, which can be detected through cfDNA analysis. This makes it possible to detect recurrence of cancer at an early stage. It has the potential to revolutionize cancer diagnosis and treatment, by enabling early detection, monitoring treatment response and recurrence of cancer [76].

5. Cell Free DNA Methylation Analysis

Methylation analysis in cell-free DNA (cfDNA) is a technique used to detect and analyze patterns of methylation in DNA that is circulating in the bloodstream [77]. This can include DNA from both normal cells as well as cancer cells. The ability to detect and analyze methylation patterns in cfDNA has led to the development of several new diagnostic and prognostic tools as well as potential targets [78] for treatment of the cancer [79], (**Figure 2**).

Methylation analysis in cfDNA can be used to detect methylation patterns in specific genes, such as tumor suppressor genes and oncogenes, that are associated with the development and progression of cancer [80]. For example, methylation of the promoter regions of genes such as BRCA1, p16INK4a, and RASSF1A have been observed in several types of cancer, including breast [81], colon [82], and

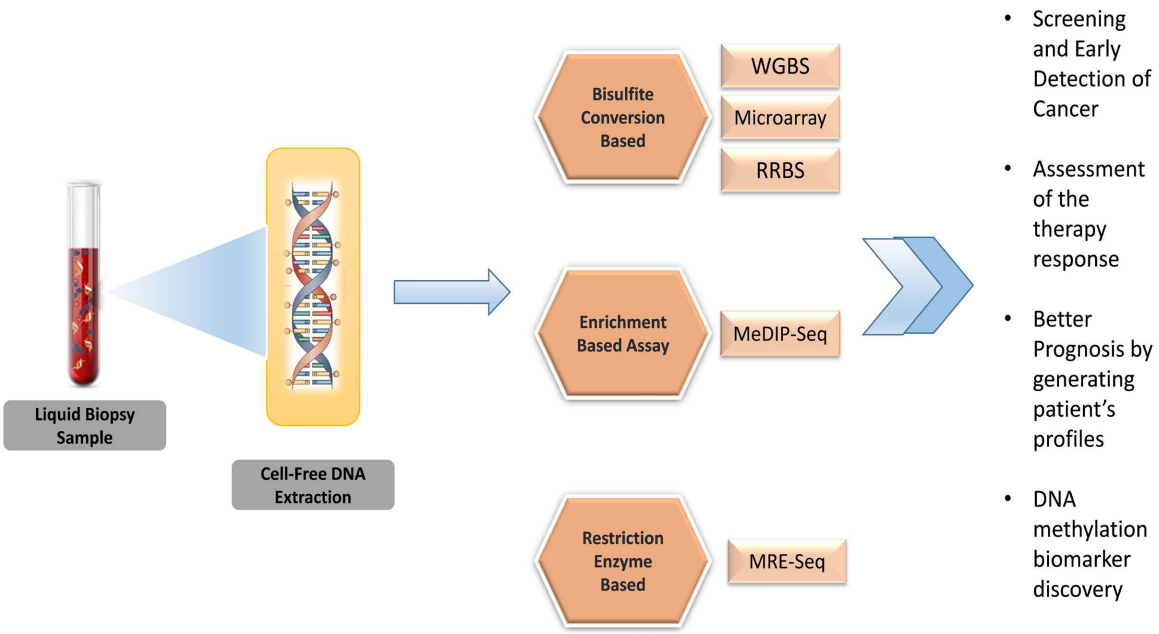

Figure 2. Use of cell-free DNA to discover novel epigenomic biomarkers for early detection of cancer. The use of cell-free DNA to discover novel epigenomic biomarkers for early detection of cancer is a promising new approach that holds great potential for transforming the field of cancer care.

lung cancer [83], respectively. Methylation of these genes can be used as biomarkers for early cancer screening [84].

Several methods are currently used to analyze methylation patterns in cfDNA. These methods allow for the detection of methylation patterns at specific sites in the genome and can identify individuals at high risk of developing certain types of cancer. Additionally, cfDNA methylation analysis can be used in liquid biopsy, which is a non-invasive method of detecting and monitoring cancer [85]. It allows the detection of cancer-associated mutations in cfDNA and can be used to monitor treatment response, detect recurrence and monitor minimal residual disease.

6. Case and Control Methylation Study

A case-control methylation study is a type of study that compares the methylation patterns of DNA from individuals with a particular disease (cases) to those without the disease (controls) in order to identify biomarkers for the disease. The goal of a case-control methylation study is to identify differentially methylated regions (DMRs) or genes that are associated with the disease of interest and could potentially serve as biomarkers for diagnosis, prognosis, or treatment [86] [87]. In a case-control methylation study, DNA samples are collected from individuals with the disease (cases) and individuals without the disease (controls) and analyzed for methylation patterns using techniques such as Methylation-specific PCR (MSP) [88], Bisulfite sequencing [89], methylation-specific restriction enzyme analysis (MSRE) [90] or Infinium methylation arrays [91]. The methylation patterns of the cases are then compared to the methylation patterns of the controls to identify DMRs or genes that are associated with the disease. Once DMRs or genes are identified, further validation studies can be conducted to confirm the association and to determine the clinical utility of the identified biomarkers. For example, the biomarkers can be used in a larger cohort of patients to evaluate their diagnostic or prognostic potential or used to monitor the response to treatment [92]. It's worth noting that case-control methylation study is a powerful tool for biomarker discovery, but it has some limitations. It relies on the availability of well-matched case and control samples, and the difference in methylation levels between cases and controls should be statistically significant to be considered as a biomarker.

7. Therapeutic Targets for Methylation

Methylation markers are specific regions of the genome that have been found to be methylated in certain diseases or conditions, and they can be used as therapeutic targets for the development of new drugs and therapies [93]. Targeting these methylation markers with drugs or other therapies that can reverse or inhibit the methylation of these genes may have therapeutic potential for treating cancer [94] and other diseases, such as neurological disorders and cardiovascular diseases [87].

In cancer, methylation markers have been identified in several genes that are

associated with tumor suppression, such as BRCA1, APC, and RASSF1A. Another example of methylation markers as therapeutic targets is in the development of drugs for neurological disorders. For example, the gene for the brain-derived neurotrophic factor (BDNF) is methylated in Alzheimer's disease [95] [96]. Therefore, drugs that can target and reverse this methylation may have therapeutic potential for treating Alzheimer's disease.

8. Epigenetic Drugs Targets

Drugs that target methylation are a class of drugs that specifically target the process of methylation in the DNA, which can affect the function of the gene to which it is attached [97] [98] [99]. These drugs can be used to modulate methylation patterns in the DNA and potentially restore normal gene expression. One class of drugs that targets methylation are epigenetic drugs, which focus on enzymes involved in regulating methylation patterns in DNA. These enzymes include DNA methyltransferases (DNMTs) [26] and histone deacetylases (HDACs) [27]. By inhibiting these enzymes, these drugs can modulate methylation patterns and potentially restore normal gene expression. Another class of drugs that target methylation are small molecules that can bind to and inhibit specific methyltransferases. This can lead to the reversal of gene silencing caused by hypermethylation and the reactivation of tumor suppressor genes. Class of methylation inhibitors are DNA methyltransferase inhibitors (DNMTi), Histone deacetylase inhibitors (HDACi) and Histone methyltransferase inhibitors (HMTi). DNA methyltransferase inhibitors (DNMTi) inhibit the activity of enzymes called DNA methyltransferases, which are responsible for adding methyl groups to the DNA (**Table 2**). Examples of DNMTi include Azacytidine and Decitabine [100]. They are approved for the treatment of myelodysplastic syndromes and acute myeloid leukemia [101] [102]. Histone deacetylase inhibitors (HDACi) [27] impede the activity of enzymes called histone deacetylases, which are responsible for removing acetyl groups from histones. Vorinostat (Zolinza), Romidepsin [103], Belinostat (Beleodaq) and Panobinostat [104] are Epigenetic modulators such as the HDAC inhibitors [105] [106]. Histone methyltransferase inhibitors (HMTi) [28] deter the enzymes called histone methyltransferases, which are responsible for adding methyl groups to histones. Histone methyltransferase inhibitors (HMTi) include 3-deazaneplanocin A (DZNep) [107] and Chaetocin [108]. Bromodomain and extra-terminal domain (BET) inhibitors responsible for recognizing and binding to acetylated lysine residues on histones [109]. Examples of BET inhibitors include JQ1, I-BET762 and OTX015. Several candidate molecules that targets G9a, EZH2 and GLP genes are Histone lysine methyltrasferase inhibitors (HLMi) in early stages of development and clinical trials as cancer therapeutics [110] [111]. Epigenetic modulation of miRNA (miRNA based therapies) has been developed to modulate the expression of specific miRNA and target the epigenetic mechanisms that regulate gene expression [29]. Several antisense oligonucleotides are being studied in clinical trials.

Table 2. Epigenetic drug targets. The development of drugs targeting epigenetic modifications is a promising field that holds great potential for the treatment of a wide range of diseases, including cancer. The increasing understanding of the molecular mechanisms underlying epigenetic modulation of gene expression and the development of new technologies are expected to drive further progress in this field.

Mechanisms	Drugs	References
DNA Methyltransferase Inhibitors (DNMTi)	Azacytidine, Decitabine	[26] [100]
Histone deacetylase inhibitors (HDACi)	Vorinostat, Belinostat Romidepsin, Panobinostat	[27] [103] [104]
Histone methyltransferase inhibitors (HMTi)	3-deazaneplanocin A (DZNep), Chaetocin	[28] [107] [108]
Histone lysine methyltrasferase inhibitors (HLMi)	G9a, EZH2, GLP	[110] [111]
Bromodomain and extra-terminal domain inhibitors (BETi)	JQ1, I-BET762 and OTX015	[109]
Epigenetic modulation of miRNA	Antisense Oligonucleotides (ASOs)	[29]

9. Methylation Changes in Cancers

In the context of cancer, methylation analysis can be used to detect methylation patterns in specific genes that are associated with the development and progression of cancer [112] [113]. Methylation of certain genes, such as tumor suppressor genes, can lead to the silencing of these genes and contribute to the development of cancer. For example, methylation of the promoter regions of genes such as p16INK4a, BRCA1, and RASSF1A have been observed in several types of cancer, including breast [81], colon [82], and lung cancer [83], respectively. Several methods are currently used to analyze methylation patterns in cancer, and these methods allow for the detection of methylation patterns at specific sites in the genome and can identify individuals at high risk of developing certain types of cancer. Methylation analysis can also be used to monitor treatment response and detect recurrence by monitoring the changes in methylation patterns over time. This can provide a non-invasive method of monitoring cancer progression and treatment response, which can be useful for patients who cannot undergo invasive procedures.

Methylation-based cancer screening aims to identify individuals at high risk of developing certain types of cancer by analyzing methylation patterns in DNA samples obtained from blood, urine, or other bodily fluids [114] [115] [116]. The goal of early cancer screening is to detect cancer at an early stage, when it is most treatable, and can reduce the mortality rate.

Colon Cancer

Colon cancer is a type of cancer that starts in the colon or rectum and is caused by the abnormal growth of cells in the lining of the colon [117]. In colon cancer, methylation of certain genes has been found to play a role in the development and progression of the disease. One of the most commonly methylated

genes in colon cancer is APC [118]. The APC gene is a tumor suppressor gene, and its inactivation through hypermethylation is a key event in the development of colon cancer. Methylation of this gene leads to the loss of its function and allows the accumulation of mutations in other genes that promote cancer growth [119]. Another gene that is frequently methylated in colon cancer is MLH1 [120] [121]. This gene plays a role in DNA mismatch repair, and its inactivation through methylation can lead to the accumulation of mutations in other genes, which promote the development of colon cancer. Promoter methylation of other genes such as RASSF1A, CDKN2A, and NDRG4 have also been observed in colon cancer, and they are considered as tumor suppressor genes, their inactivation through methylation can contribute to cancer progression [122].

Lung and Breast Cancers

In lung cancer, the methylation of the tumor suppressor gene p16INK4a has been observed in lung cancer and is associated with poor prognosis [123]. The loss of methylation in the p16INK4a promoter results in the inactivation of the gene and the loss of its tumor suppressor function. Another example is the activation of oncogenes through the loss of methylation in the promoter regions. Methylation can also occur in repetitive elements such as transposable elements, leading to genomic instability, which is a hallmark of cancer. In addition, methylation-based therapies are being developed to target specific genes in lung cancer.

In breast cancer, the methylation of the tumor suppressor gene BRCA1 has been observed in breast cancer, which is associated with poor prognosis. The loss of methylation in the BRCA1 promoter results in the inactivation of the gene and the loss of its tumor suppressor function. Hypomethylation in the promoter of the c-myc oncogene has been also observed in breast cancer. Methylation-based therapies that target RASSF1A gene in breast cancer are being developed [124].

10. Methylation Analysis Technologies

There are several methods used to analyze methylation patterns in the DNA, each with their own advantages and limitations. Some of the technologies can be also used in the analysis of methylation in cf DNA.

Methylation-specific PCR (MSP) uses PCR amplification to specifically target methylated or unmethylated regions of the DNA. The amplified DNA is then analyzed by gel electrophoresis to determine the presence or absence of methylation [88] [125]. MSP is a sensitive and specific method, but it can only be used to analyze a limited number of specific regions of the DNA. Bisulfite sequencing method uses bisulfite treatment to convert unmethylated cytosines to uracils, while methylated cytosines remain unchanged. The treated DNA is then sequenced, and the presence or absence of methylation can be determined by comparing the sequence of the treated DNA to the original DNA sequence. Bisulfite sequencing is a highly sensitive and specific method, but it is also time-consuming and expensive [125]. Methylation-specific restriction enzymes (MSREs) are a class of enzymes that spe-

cifically recognize and cut methylated DNA sequences. These enzymes can be used to analyze methylation patterns in the DNA by cutting only the methylated regions of the DNA and leaving the unmethylated regions intact. One of the most commonly used MSREs is HpaII, which recognizes and cuts the sequence CCGG, only when it is methylated. Another commonly used MSRE is MspI, which recognizes and cuts the sequence CGG, only when it is methylated. By cutting only the methylated regions of the DNA, these enzymes can be used to analyze the methylation status of specific genes or regions of the genome. The use of MSREs in combination with other techniques, such as gel electrophoresis or PCR, Sequencing allows for the detection and quantification of methylation patterns in the DNA [126].

Illumina Infinium Methylation Array methodology used in this technique involves bisulfite conversion of the genomic DNA followed by its amplification and hybridization of the bisulfite converted DNA to the already designed probes of arrays. The probes can differentiate between the methylated and unmethylated cytosines based on the sequence of provided DNA. The most potentially used array is Illumina's Infinium HumanMethylation450 BeadChip which has the capacity of identifying more than 480,000 methylated sites covering about 96% islands, 92% shores, and 86% shelves [91]. This widely used array data of about 19 different cancer types is present in the public repository Genomic Data commons (GDC) data portal. This data is a part of The Cancer Genome Atlas (TCGA) and is utilized to discover many cancer biomarkers for early detection and diagnosis of cancer.

Table 3. Summarized experimental techniques for methylation analysis.

Methylation Analysis technique	Advantages	Limitations	Example	References
Whole genome bisulfite sequencing (WGBS)	Investigate almost all the CpG sites in the genome	Costly and might cause degradation of DNA after bisulfite treatment	WGBS revealed Breast cancer biomarkers [127]	[128]
Methylation DNA Immunoprecipitation (MeDIP)	Specific to methylation of cytosine based on the antibody	Cannot identify individual CpG site and mostly biased towards DNA hypermethylation	Methylation analysis using MeDIP technique	[129]
Comprehensive High Throughput arrays for relative methylation (CHARM)	Examine genome-wide CpG sites regardless of their closeness to the gene or CGIs	Restricted to regions near to recognition sites of enzymes	Altered DNA methylation is found in CGI shore in Colon cancer [130]	[131]
Illumina Infimium Methylation Assay	A cost-efficient method and require less DNA quantity	Limited because of array designs and degradation of DNA might be possible due to bisulfite treatment	Breast cancer biomarkers identified using microarray [132]	[133]
TET-assisted Bisulfite Sequencing (TAB)			DNA demethylation levels identified in prenatal germline [134]	[134]
Reduced Representation Bisulfite Sequencing (RRBS)	High coverage and sensitivity of CGIs	Limited coverage at distal regions and intergenic regions	Liver methylation patterns found [135]	[135]

Table 3 summarizes some experimental approaches that are widely used in the discovery of Genome-wide methylation analysis to identify epigenomic biomarkers of cancer and many other disorders. These biomarkers can be utilized as indicators for early detection and diagnosis of many diseases.

Bisulfite Sequencing for Methylation Analysis

Bisulfite sequencing is a technique used to analyze the methylation status of specific regions of DNA, such as promoter regions of genes [136]. The method relies on the conversion of unmethylated cytosine residues to uracil by treatment with bisulfite, while methylated cytosine residues remain unchanged. The resulting DNA is then sequenced and analyzed to determine the methylation status of specific regions [136].

The process of bisulfite sequencing can be divided into several steps:

1) Bisulfite treatment: The DNA sample is treated with bisulfite, which converts unmethylated cytosine residues to uracil while leaving methylated cytosine residues unchanged [137].

2) PCR amplification: The bisulfite-treated DNA is then amplified using PCR with primers specific to the region of interest.

3) Sequencing: The amplified DNA is then sequenced using a high-throughput sequencing platform, such as Illumina or PacBio.

4) Data Analysis: The resulting sequencing data is analyzed to determine the methylation status of specific regions. The methylation status of each cytosine residue is determined by comparing the ratio of C's to T's at each position in the sequence [138].

Bisulfite sequencing is a powerful and widely used method for methylation analysis, as it allows for the high-resolution analysis of methylation patterns, including single-base resolution. However, it has some limitations, it can only be used for the analysis of CpG dinucleotides, and it may not detect other types of methylation, like non-CpG methylation.

In bisulfite DNA sequencing, genomic DNA is first treated with sodium bisulfite, which converts unmethylated cytosine residues into uracil, but leaves methylated cytosines unchanged. The converted DNA is then amplified using polymerase chain reaction (PCR) and sequenced. The resulting sequencing data can be used to identify methylated cytosine residues and infer the pattern of DNA methylation across the genome. The whole methodology and the steps involved in this technique are shown in Figure 3.

Bisulfite sequencing is a powerful and widely used method for methylation analysis, but it also has some limitations [139]. Some of the main limitations of bisulfite sequencing include:

Limited to CpG dinucleotides: Bisulfite sequencing is only able to detect methylation at CpG dinucleotides, which make up a small portion of the genome. Therefore, it is not able to detect other types of methylation, such as non-CpG methylation.

Low sensitivity: Bisulfite sequencing has a relatively low sensitivity, meaning it may not detect all methylated sites in the genome. The sensitivity of the method

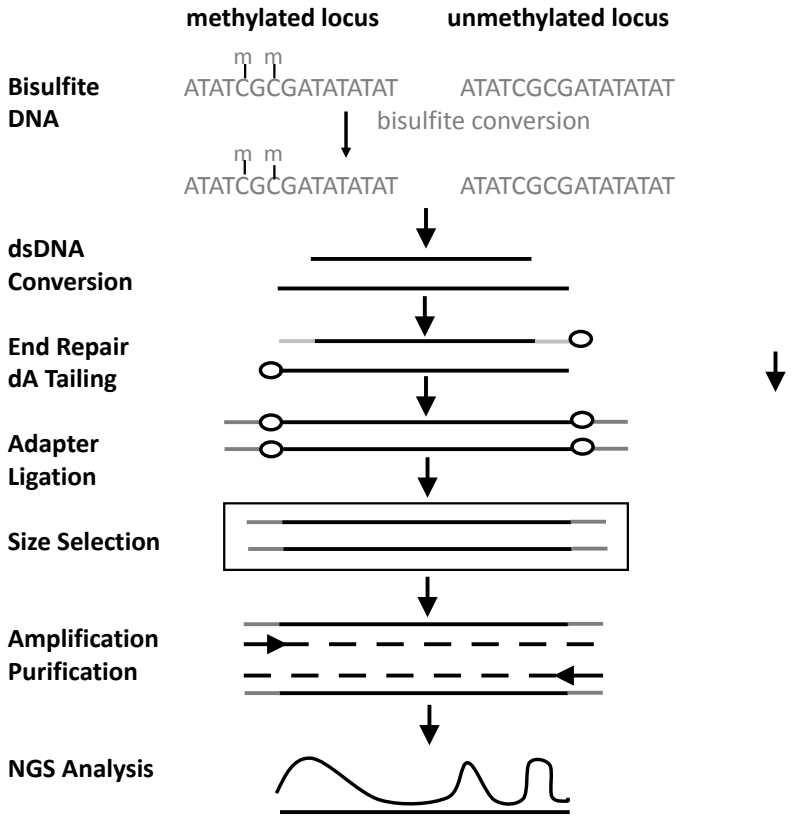

Figure 3. Steps involved in the bisulfite DNA sequencing. In bisulfite DNA sequencing, genomic DNA is first treated with sodium bisulfite, which converts unmethylated cytosine residues into uracil, but leaves methylated cytosines unchanged. The converted DNA is then amplified using polymerase chain reaction (PCR) and sequenced. The resulting sequencing data can be used to identify methylated cytosine residues and infer the pattern of DNA methylation across the genome.

is also affected by factors such as the quality and quantity of the DNA sample, the sequencing platform used, and the data analysis method.

High cost: Bisulfite sequencing is a relatively expensive method, as it requires the use of high-throughput sequencing platforms, which are costly to use.

Complexity: Bisulfite sequencing requires a relatively complex protocol that involves multiple steps, including bisulfite treatment, PCR amplification, and sequencing.

False-positive and False-Negative: The bisulfite sequencing method can lead to false-positive and false-negative results, especially when the bisulfite treatment is not thorough enough, or when the PCR amplification is not efficient [6].

Limited to a specific region: Bisulfite sequencing is limited to a specific region of interest and therefore it doesn't provide a comprehensive view of the methylation patterns of the genome [139].

Methylation-Specific Restriction Enzyme Sequencing (MSRE-seq) Analysis

Methylation-specific restriction enzyme analysis (MSRE) is a technique used to detect and analyze DNA methylation patterns [126]. The method relies on the recognition of specific sequences by restriction enzymes that are sensitive to

methylation. By cutting or not cutting the DNA at specific sites, the method allows for the identification of methylated or unmethylated regions of the genome. The process of MSRE can be divided into several steps including end-repair and A-tailing, adapter ligation, PCR amplification, size selection, and next generation sequencing. The DNA sample is digested with a restriction enzyme that recognizes a specific sequence and is sensitive to methylation (**Table 4**). For example, the restriction enzyme HpaII recognizes the sequence CCGG, and SacII identify the sequence of GCGG and cuts the DNA only when the cytosine residues are unmethylated [140]. The human genome has 63,266 target sites for the SacII enzyme, and only cuts for hypomethylated sites (**Figure 4**).

Table 4. The table lists MSREs that may be useful for epigenetics analyses. These enzymes are commonly used in the study of epigenetic modifications, such as DNA methylation, and can be used for various applications, including DNA methylation analysis, genomic bisulfite sequencing, and ChIP-seq (chromatin immunoprecipitation followed by next-generation sequencing).

Restriction Enzyme	Recognition Sequence
Aat II	GACGT↓C
Acc II	CG↓CG
Aor13H I	T↓CCGGA
Aor51H I	AGC↓GCT
BspT104 I	TT↓CGAA
BssH II	G↓CGCGC
Cfr10 I	R↓CCGGY
Cla I	AT↓CGAT
Cpo I	CG↓GWCCG
Eco52 I	C↓GGCCG
Hae II	RGCGC↓Y
Hap II	C↓CGG
Hha I	GCG↓C
Mlu I	A↓CGCGT
Nae I	GCC↓GGC
Not I	GC↓GGCCGC
Nru I	TCG↓CGA
Nsb I	TGC↓GCA
PmaC I	CAC↓GTG
Psp1406 I	AA↓CGTT
Pvu I	CGAT↓CG
Sac II	CCGC↓GG
Sal I	G↓TCGAC
Sma I	CCC↓GGG
SnaB I	TAC↓GTA

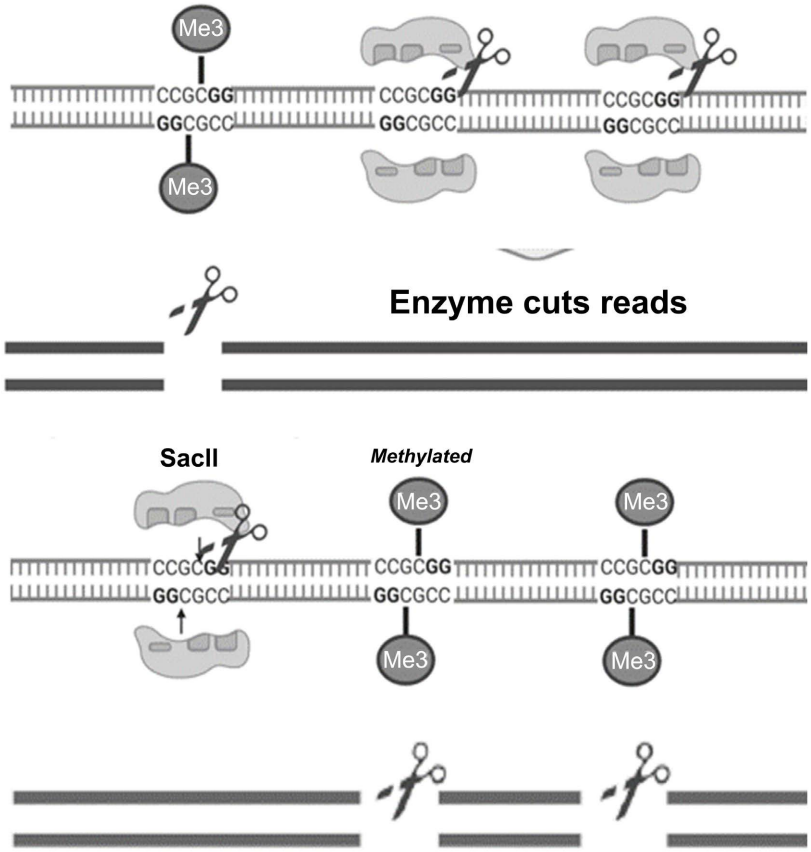

Figure 4. Analysis of differentially methylated regions (DMRs) by Methylation-sensitive restriction enzyme sequencing (MSRE-seq) techniques. The method takes advantage of the specificity of methylation-sensitive restriction enzymes, which recognize and cleave specific DNA sequences only when they are methylated. In MSRE-seq, DNA is first treated with a methylation-sensitive restriction enzyme, which cleaves the DNA at specific sites based on the presence or absence of methylation. The resulting DNA fragments are then amplified using polymerase chain reaction (PCR) and sequenced.

MSRE is a relatively simple and cost-effective method for methylation analysis, as it allows for the detection of methylation patterns at specific sites in the genome. It requires small number of samples to do the analysis compare to bisulfite treated samples. However, it has some limitations, it can only be used to analyze a limited number of sites at a time and it may not provide a comprehensive view of the methylation patterns of the genome [141].

11. Conclusions

In conclusion, methylation analysis in cell-free DNA (cfDNA) samples is a powerful tool for biomarker discovery in various diseases. Methylation, the process by which methyl groups are added to the DNA, can lead to changes in gene expression that can be used as biomarkers to indicate the presence or progression of a disease. cfDNA methylation analysis can be used to detect methylation patterns in specific genes that are associated with the development and progression of diseases such as cancer. It is non-invasive method of detecting biomarkers

that can provide early detection and monitoring of the disease.

Methods such as Methylation specific PCR (MSP), bisulfite sequencing and methylation-specific restriction enzyme analysis (MSRE) can be used to detect methylation patterns in the genome. Methylation analysis in cfDNA can offer revolutionary way of monitor treatment response and detect recurrence by monitoring changes in methylation patterns over time. Overall, methylation analysis in cfDNA samples has enormous potential as a biomarker discovery tool in various diseases, including cancer, neurodegenerative disease, cardiovascular disease, aging and others. It has the possibility to improve early diagnosis, treatment monitoring, and recurrence detection, providing a non-invasive method of monitoring disease progression and treatment response as well as discoveries of targets for novel disease treatment. The discovery of methylation biomarkers in cfDNA is a new and exciting development in the field of epigenetics. The potential of cfDNA methylation biomarker analysis for disease diagnosis, treatment monitoring, and prognosis prediction is still being explored and is expected to lead to new and innovative strategies for disease care and patient treatment.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Millan, J., Lesarri, A., Fernández, J.A. and Martínez, R. (2021) Exploring Epigenetic Marks by Analysis of Noncovalent Interactions. *ChemBioChem*, **22**, 408-415. https://doi.org/10.1002/cbic.202000380

[2] Campos-Carrillo, A., *et al.* (2020) Circulating Tumor DNA as an Early Cancer Detection Tool. *Pharmacology & Therapeutics*, **207**, Article ID: 107458. https://doi.org/10.1016/j.pharmthera.2019.107458

[3] Maity, A.K., *et al.* (2022) Novel Epigenetic Network Biomarkers for Early Detection of Esophageal Cancer. *Clinical Epigenetics*, **14**, Article No. 23. https://doi.org/10.1186/s13148-022-01243-5

[4] Villa, C. and Stoccoro, A. (2022) Epigenetic Peripheral Biomarkers for Early Diagnosis of Alzheimer's Disease. *Genes*, **13**, Article No. 1308. https://doi.org/10.3390/genes13081308

[5] Luo, X.-J., *et al.* (2021) Novel Genetic and Epigenetic Biomarkers of Prognostic and Predictive Significance in Stage II/III Colorectal Cancer. *Molecular Therapy*, **29**, 587-596. https://doi.org/10.1016/j.ymthe.2020.12.017

[6] Barault, L., *et al.* (2018) Discovery of Methylated Circulating DNA Biomarkers for Comprehensive Non-Invasive Monitoring of Treatment Response in Metastatic Colorectal Cancer. *Gut*, **67**, 1995-2005. https://doi.org/10.1136/gutjnl-2016-313372

[7] Jankowska, A.M., Millward, C.L. and Caldwell, C.W. (2015) The Potential of DNA Modifications as Biomarkers and Therapeutic Targets in Oncology. *Expert Review of Molecular Diagnostics*, **15**, 1325-1337. https://doi.org/10.1586/14737159.2015.1084229

[8] Schumacher, A., *et al.* (2006) Microarray-Based DNA Methylation Profiling: Technology and Applications. *Nucleic Acids Research*, **34**, 528-542.

https://doi.org/10.1093/nar/gkj461

[9] Li, Y. and Tollefsbol, T.O. (2011) DNA Methylation Detection: Bisulfite Genomic Sequencing Analysis. In: Tollefsbol, T., Ed., *Epigenetics Protocols. Methods in Molecular Biology*, Vol. 791, Humana Press, Totowa, 11-21. https://doi.org/10.1007/978-1-61779-316-5_2

[10] Nakato, R. and Sakata, T. (2021) Methods for ChIP-seq Analysis: A Practical Workflow and Advanced Applications. *Methods*, **187**, 44-53. https://doi.org/10.1016/j.ymeth.2020.03.005

[11] Moore, L.D., Le, T. and Fan, G. (2013) DNA Methylation and Its Basic Function. *Neuropsychopharmacology*, **38**, 23-38. https://doi.org/10.1038/npp.2012.112

[12] Razin, A. and Cedar, H. (1991) DNA Methylation and Gene Expression. *Microbiological Reviews*, **55**, 451-458. https://doi.org/10.1128/mr.55.3.451-458.1991

[13] Levenson, V.V. (2010) DNA Methylation as a Universal Biomarker. *Expert Review of Molecular Diagnostics*, **10**, 481-488. https://doi.org/10.1586/erm.10.17

[14] Locke, W.J., *et al.* (2019) DNA Methylation Cancer Biomarkers: Translation to the Clinic. *Frontiers in Genetics*, **10**, Article 1150. https://www.frontiersin.org/articles/10.3389/fgene.2019.01150 https://doi.org/10.3389/fgene.2019.01150

[15] Liang, T.-J., *et al.* (2017) APC Hypermethylation for Early Diagnosis of Colorectal Cancer: A Meta-Analysis and Literature Review. *Oncotarget*, **8**, 46468-46479. https://doi.org/10.18632/oncotarget.17576

[16] Li, B.-Q., Liu, P.-P. and Zhang, C.-H. (2017) Correlation between the Methylation of APC Gene Promoter and Colon Cancer. *Oncology Letters*, **14**, 2315-2319. https://doi.org/10.3892/ol.2017.6455

[17] Li, Q., Wei, W., Jiang, Y., Yang, H. and Liu, J. (2015) Promoter Methylation and Expression Changes of BRCA1 in Cancerous Tissues of Patients with Sporadic Breast Cancer. *Oncology Letters*, **9**, 1807-1813. https://doi.org/10.3892/ol.2015.2908

[18] Ibrahim, J., Peeters, M., Van Camp, G. and de Beeck, K.O. (2023) Methylation Biomarkers for Early Cancer Detection and Diagnosis: Current and Future Perspectives. *European Journal of Cancer*, **178**, 91-113. https://doi.org/10.1016/j.ejca.2022.10.015

[19] Kim, S.Y., *et al.* (2019) Aberrantly Hypermethylated Tumor Suppressor Genes Were Identified in Oral Squamous Cell Carcinoma (OSCC). *Clinical Epigenetics*, **11**, Article No. 116. https://doi.org/10.1186/s13148-019-0715-0

[20] Van Tongelen, A., Loriot, A. and De Smet, C. (2017) Oncogenic Roles of DNA Hypomethylation through the Activation of Cancer-Germline Genes. *Cancer Letters*, **396**, 130-137. https://doi.org/10.1016/j.canlet.2017.03.029

[21] Shireby, G., *et al.* (2022) DNA Methylation Signatures of Alzheimer's Disease Neuropathology in the Cortex Are Primarily Driven by Variation in Non-Neuronal Cell-Types. *Nature Communications*, **13**, Article No. 5620. https://doi.org/10.1038/s41467-022-33394-7

[22] Celarain, N. and Tomas-Roig, J. (2020) Aberrant DNA Methylation Profile Exacerbates Inflammation and Neurodegeneration in Multiple Sclerosis Patients. *Journal of Neuroinflammation*, **17**, Article No. 21. https://doi.org/10.1186/s12974-019-1667-1

[23] Fernández-Sanlés, A., *et al.* (2021) DNA Methylation Biomarkers of Myocardial Infarction and Cardiovascular Disease. *Clinical Epigenetics*, **13**, Article No. 86. https://doi.org/10.1186/s13148-021-01078-6

[24] Willmer, T., Johnson, R., Louw, J. and Pheiffer, C. (2018) Blood-Based DNA Methylation Biomarkers for Type 2 Diabetes: Potential for Clinical Applications. *Frontiers in Endocrinology*, **9**, Article 744. https://doi.org/10.3389/fendo.2018.00744 https://www.frontiersin.org/articles/10.3389/fendo.2018.00744

[25] Ghasemi, S. (2020) Cancer's Epigenetic Drugs: Where Are They in the Cancer Medicines? *The Pharmacogenomics Journal*, **20**, 367-379. https://doi.org/10.1038/s41397-019-0138-5

[26] Giri, A.K. and Aittokallio, T. (2019) DNMT Inhibitors Increase Methylation in the Cancer Genome. *Frontiers in Pharmacology*, **10**, Article 385. https://www.frontiersin.org/articles/10.3389/fphar.2019.00385 https://doi.org/10.3389/fphar.2019.00385

[27] Xu, W.S., Parmigiani, R.B. and Marks, P.A. (2007) Histone Deacetylase Inhibitors: Molecular Mechanisms of Action. *Oncogene*, **26**, 5541-5552. https://doi.org/10.1038/sj.onc.1210620

[28] Rugo, H.S., *et al.* (2020) The Promise for Histone Methyltransferase Inhibitors for Epigenetic Therapy in Clinical Oncology: A Narrative Review. *Advances in Therapy*, **37**, 3059-3082. https://doi.org/10.1007/s12325-020-01379-x

[29] Tomasetti, M., Gaetani, S., Monaco, F., Neuzil, J. and Santarelli, S. (2019) Epigenetic Regulation of miRNA Expression in Malignant Mesothelioma: MiRNAs as Biomarkers of Early Diagnosis and Therapy. *Frontiers in Oncology*, **9**, Article 1293. https://www.frontiersin.org/articles/10.3389/fonc.2019.01293 https://doi.org/10.3389/fonc.2019.01293

[30] Kamińska, K., *et al.* (2019) Prognostic and Predictive Epigenetic Biomarkers in Oncology. *Molecular Diagnosis & Therapy*, **23**, 83-95. https://doi.org/10.1007/s40291-018-0371-7

[31] Jin, Y., Allen, E.G. and Jin, P. (2022) Cell-Free DNA Methylation as a Potential Biomarker in Brain Disorders. *Epigenomics*, **14**, 369-374. https://doi.org/10.2217/epi-2021-0416

[32] Soler-Botija, C., Gálvez-Montón, C. and Bayés-Genís, A. (2019) Epigenetic Biomarkers in Cardiovascular Diseases. *Frontiers in Genetics*, **10**, Article 950. https://www.frontiersin.org/articles/10.3389/fgene.2019.00950 https://doi.org/10.3389/fgene.2019.00950

[33] Matthaios, D., *et al.* (2016) Methylation Status of the APC and RASSF1A Promoter in Cell‐Free Circulating DNA and Its Prognostic Role in Patients with Colorectal Cancer. *Oncology Letters*, **12**, 748-756. https://doi.org/10.3892/ol.2016.4649

[34] Zhu, X., *et al.* (2015) Hypermethylation of *BRCA*1 Gene: Implication for Prognostic Biomarker and Therapeutic Target in Sporadic Primary Triple-Negative Breast Cancer. *Breast Cancer Research and Treatment*, **150**, 479-486. https://doi.org/10.1007/s10549-015-3338-y

[35] Xie, B., *et al.* (2017) Elevation of Peripheral BDNF Promoter Methylation Predicts Conversion from Amnestic Mild Cognitive Impairment to Alzheimer's Disease: A 5-Year Longitudinal Study. *Journal of Alzheimer's Disease*, **56**, 391-401. https://doi.org/10.3233/JAD-160954

[36] Chang, N.-T., Delacruz, L. and Shiao, S.-Y.P.K. (2014) Meta-Analyses of Epigenetics Risk Factors for Heart Disease Prevention: NOS3 Human Gene Variations across Different Race-Ethnicity Groups. http://hdl.handle.net/10755/335205

[37] Sun, T., Chi, Q. and Wang, G. (2016) [Research Progress of NOS3 Participation in Regulatory Mechanisms of Cardiovascular Diseases]. *Journal of Biomedical Engineering*, **33**, 188-192. (In Chinese)

[38] Sandovici, I., *et al.* (2011) Maternal Diet and Aging Alter the Epigenetic Control of a Promoter-Enhancer Interaction at the *Hnf4a* Gene in Rat Pancreatic Islets. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 5449-5454. https://doi.org/10.1073/pnas.1019007108

[39] Mansouri, A., *et al.* (2019) MGMT Promoter Methylation Status Testing to Guide Therapy for Glioblastoma: Refining the Approach Based on Emerging Evidence and Current Challenges. *Neuro-Oncology*, **21**, 167-178. https://doi.org/10.1093/neuonc/noy132

[40] Jha, A.K., *et al.* (2012) Promoter Hypermethylation of *p*73 and *p*53 Genes in Cervical Cancer Patients among North Indian Population. *Molecular Biology Reports*, **39**, 9145-9157. https://doi.org/10.1007/s11033-012-1787-5

[41] Rowland, T.J., Bonham, A.J. and Cech, T.R. (2020) Allele-Specific Proximal Promoter Hypomethylation of the Telomerase Reverse Transcriptase Gene (TERT) Associates with TERT Expression in Multiple Cancers. *Molecular Oncology*, **14**, 2358-2374. https://doi.org/10.1002/1878-0261.12786

[42] Schneider, G., Bowser, M.J., Shin, D.-M., Barr. F.G. and Ratajczak, M.Z. (2014) The Paternally Imprinted DLK1-GTL2 Locus Is Differentially Methylated in Embryonal and Alveolar Rhabdomyosarcomas. *International Journal of Oncology*, **44**, 295-300. https://doi.org/10.3892/ijo.2013.2153

[43] Kingshott, G., *et al.* (2021) Alteration of Metabolic Conditions Impacts the Regulation of IGF-II/H19 Imprinting Status in Prostate Cancer. *Cancers*, **13**, Article No. 825. https://doi.org/10.3390/cancers13040825

[44] Chanda, K. and Mukhopadhyay, D. (2020) *LncRNA Xist*, X-Chromosome Instability and Alzheimer's Disease. *Current Alzheimer Research*, **17**, 499-507. https://doi.org/10.2174/1567205017666200807185624

[45] Benetatos, L., *et al.* (2010) CpG Methylation Analysis of the MEG3 and SNRPN Imprinted Genes in Acute Myeloid Leukemia and Myelodysplastic Syndromes. *Leukemia Research*, **34**, 148-153. https://doi.org/10.1016/j.leukres.2009.06.019

[46] Phokaew, C., Kowudtitham, S., Subbalekha, K., Shuangshoti, S. and Mutirangura, A. (2008) LINE-1 Methylation Patterns of Different Loci in Normal and Cancerous Cells. *Nucleic Acids Research*, **36**, 5704-5712. https://doi.org/10.1093/nar/gkn571

[47] Bollati, V., *et al.* (2011) DNA Methylation in Repetitive Elements and Alzheimer disease. *Brain, Behavior, and Immunity*, **25**, 1078-1083. https://doi.org/10.1016/j.bbi.2011.01.017

[48] Saeliw, T., *et al.* (2022) LINE-1 and *Alu* Methylation Signatures in Autism Spectrum disorder and Their Associations with the Expression of Autism-Related Genes. *Scientific Reports*, **12**, Article No. 13970. https://doi.org/10.1038/s41598-022-18232-6

[49] Yu, W., Zhang, L., Wei, Q. and Shao, A. (2020) O^6-Methylguanine-DNA Methyltransferase (MGMT): Challenges and New Opportunities in Glioma Chemotherapy. *Frontiers in Oncology*, **9**, Article 1547. https://doi.org/10.3389/fonc.2019.01547 https://www.frontiersin.org/articles/10.3389/fonc.2019.01547

[50] Cai, M.-Y., *et al.* (2011) H3K27me3 Protein Is a Promising Predictive Biomarker of Patients' Survival and Chemoradioresistance in Human Nasopharyngeal Carcinoma. *Molecular Medicine*, **17**, 1137-1145. https://doi.org/10.2119/molmed.2011.00054

[51] Sato, T., *et al.* (2013) PRC2 Overexpression and PRC2-Target Gene Repression Relating to Poorer Prognosis in Small Cell Lung Cancer. *Scientific Reports*, **3**, Article No. 1911. https://doi.org/10.1038/srep01911

[52] Wang, Y. and Shi, J. (2021) P53 and DNA Methylation in the Aging Process. *Jour-*

nal of Behavioral and Brain Science, **11**, 83-95.
https://doi.org/10.4236/jbbs.2021.114007

[53] Flavahan, W.A., Gaskell, E. and Bernstein, B.E. (2017) Epigenetic Plasticity and the Hallmarks of Cancer. *Science*, **357**, eaal2380.
https://doi.org/10.1126/science.aal2380

[54] Ehrlich, M. (2019) DNA Hypermethylation in Disease: Mechanisms and Clinical Relevance. *Epigenetics*, **14**, 1141-1163.
https://doi.org/10.1080/15592294.2019.1638701

[55] Peinado, M.A. (2011) Hypomethylation of DNA. In: Schwab, M., Ed., *Encyclopedia of Cancer*, Springer, Berlin, 1791-1792.
https://doi.org/10.1007/978-3-642-16483-5_2923

[56] Yokoyama, A.S., Rutledge, J.C. and Medici, V. (2017) DNA Methylation Alterations in Alzheimer's Disease. *Environmental Epigenetics*, **3**, Article ID: dvx008.
https://doi.org/10.1093/eep/dvx008

[57] Parris, T.Z., *et al.* (2014) Frequent *MYC* Coamplification and DNA Hypomethylation of Multiple Genes on 8q in 8p11-p12-Amplified Breast Carcinomas. *Oncogenesis*, **3**, e95. https://doi.org/10.1038/oncsis.2014.8

[58] Sharrard, R.M., Royds, J.A., Rogers, S. and Shorthouse, A.J. (1992) Patterns of Methylation of the *c-myc* Gene in Human Colorectal Cancer Progression. *British Journal of Cancer*, **65**, 667-672. https://doi.org/10.1038/bjc.1992.142

[59] Wei, X., Zhang, L. and Zeng, Y. (2020) DNA Methylation in Alzheimer's Disease: In Brain and Peripheral Blood. *Mechanisms of Ageing and Development*, **191**, Article ID: 111319. https://doi.org/10.1016/j.mad.2020.111319

[60] De Souza, R.A.G., *et al.* (2016) DNA Methylation Profiling in Human Huntington's Disease Brain. *Human Molecular Genetics*, **25**, 2013-2030.
https://doi.org/10.1093/hmg/ddw076

[61] Magwai, T., *et al.* (2021) DNA Methylation and Schizophrenia: Current Literature and Future Perspective. *Cells*, **10**, Article No. 2890.
https://doi.org/10.3390/cells10112890

[62] Lindsey, J.C., *et al.* (2007) Epigenetic Deregulation of Multiple *S*100 Gene Family Members by Differential Hypomethylation and Hypermethylation Events in Medulloblastoma. *British Journal of Cancer*, **97**, 267-274.
https://doi.org/10.1038/sj.bjc.6603852

[63] Zheleznyakova, G.Y., Cao, H. and Schiöth, H.B. (2016) *BDNF* DNA Methylation Changes as a Biomarker of Psychiatric Disorders: Literature Review and Open Access Database Analysis. *Behavioral and Brain Functions*, **12**, Article No. 17.
https://doi.org/10.1186/s12993-016-0101-4

[64] Kinde, B., Wu, D.Y., Greenberg, M.E. and Gabel, H.W. (2016) DNA Methylation in the Gene Body Influences MeCP2-Mediated Gene Repression. *Proceedings of the National Academy of Sciences of the United States of America*, **113**, 15114-15119.
https://doi.org/10.1073/pnas.1618737114

[65] Sproul, D. and Meehan, R.R. (2013) Genomic Insights into Cancer-Associated Aberrant CpG Island Hypermethylation. *Briefings in Functional Genomics*, **12**, 174-190.
https://doi.org/10.1093/bfgp/els063

[66] Mazzone, R., *et al.* (2019) The Emerging Role of Epigenetics in Human Autoimmune Disorders. *Clinical Epigenetics*, **11**, Article No. 34.
https://doi.org/10.1186/s13148-019-0632-2

[67] Zouali, M. (2021) DNA Methylation Signatures of Autoimmune Diseases in Human

B lymphocytes. *Clinical Immunology*, **222**, Article ID: 108622. https://doi.org/10.1016/j.clim.2020.108622

[68] Han, L., *et al.* (2016) DNA Methylation and Hypertension: Emerging Evidence and Challenges. *Briefings in Functional Genomics*, **15**, 460-469. https://doi.org/10.1093/bfgp/elw014

[69] Zhang, Y., *et al.* (2021) DNA Methylation in Atherosclerosis: A New Perspective. *Evidence-Based Complementary and Alternative Medicine*, **2021**, Article ID: 6623657. https://doi.org/10.1155/2021/6623657

[70] Yan, Y.-Y., *et al.* (2021) Cell-Free DNA: Hope and Potential Application in Cancer. *Frontiers in Cell and Developmental Biology*, **9**, Article 639233. https://www.frontiersin.org/articles/10.3389/fcell.2021.639233 https://doi.org/10.3389/fcell.2021.639233

[71] Grace, M.R., Hardisty, E., Dotters-Katz, S.K., Vora, N.L. and Kuller, J.A. (2016) Cell-Free DNA Screening: Complexities and Challenges of Clinical Implementation. *Obstetrical & Gynecological Survey*, **71**, 477-487. https://doi.org/10.1097/OGX.0000000000000342

[72] Liebs, S., Keilholz, U., Kehler, I., Schweiger, C. and Haybäck, J. (2019) Detection of Mutations in Circulating Cell-Free DNA in Relation to Disease Stage in Colorectal Cancer. *Cancer Medicine*, **8**, 3761-3769. https://doi.org/10.1002/cam4.2219

[73] Gao, Q., *et al.* (2022) Circulating Cell-Free DNA for Cancer Early Detection. *The Innovation*, **3**, Article ID: 100259. https://doi.org/10.1016/j.xinn.2022.100259

[74] Sánchez-Herrero, E., *et al.* (2022) Circulating Tumor DNA as a Cancer Biomarker: An Overview of Biological Features and Factors That May Impact on ctDNA Analysis. *Frontiers in Oncology*, **12**, Article 943253. https://www.frontiersin.org/articles/10.3389/fonc.2022.943253 https://doi.org/10.3389/fonc.2022.943253

[75] Oellerich, M., *et al.* (2017) Using Circulating Cell-Free DNA to Monitor Personalized Cancer Therapy. *Critical Reviews in Clinical Laboratory Sciences*, **54**, 205-218. https://doi.org/10.1080/10408363.2017.1299683

[76] Telekes, A. and Horváth, A. (2022) The Role of Cell-Free DNA in Cancer Treatment Decision Making. *Cancers*, **14**, Article No. 6115. https://doi.org/10.3390/cancers14246115

[77] Zeng, H., He, B., Yi, C. and Peng, J. (2018) Liquid Biopsies: DNA Methylation Analyses in Circulating Cell-Free DNA. *Journal of Genetics and Genomics*, **45**, 185-192. https://doi.org/10.1016/j.jgg.2018.02.007

[78] Cisneros-Villanueva, M., *et al.* (2022) Cell-Free DNA Analysis in Current Cancer Clinical Trials: A Review. *British Journal of Cancer*, **126**, 391-400. https://doi.org/10.1038/s41416-021-01696-0

[79] de la Cruz, F.F. and Corcoran, R.B. (2018) Methylation in Cell-Free DNA for Early Cancer Detection. *Annals of Oncology*, **29**, 1351-1353. https://doi.org/10.1093/annonc/mdy134

[80] Dumitrescu, R.G. (2012) Epigenetic Markers of Early Tumor Development. In: Dumitrescu, R. and Verma, M., Eds., *Cancer Epigenetics. Methods in Molecular Biology*, Vol. 863, Humana Pres, Totowa, 3-14. https://doi.org/10.1007/978-1-61779-612-8_1

[81] Tang, Q., Cheng, J., Cao, X., Surowy, H. and Burwinkel, B. (2016) Blood-Based DNA Methylation as Biomarker for Breast Cancer: A Systematic Review. *Clinical Epigenetics*, **8**, Article No. 115. https://doi.org/10.1186/s13148-016-0282-6

[82] Nassar, F.J., Msheik, Z.S., Nasr, R.R. and Temraz, S.N. (2021) Methylated Circulating Tumor DNA as a Biomarker for Colorectal Cancer Diagnosis, Prognosis, and Prediction. *Clinical Epigenetics*, **13**, Article No. 111. https://doi.org/10.1186/s13148-021-01095-5

[83] Wang, Y., *et al.* (2007) Identification of Epigenetic Aberrant Promoter Methylation of *RASSF1A* in Serum DNA and Its Clinicopathological Significance in Lung Cancer. *Lung Cancer*, **56**, 289-294. https://doi.org/10.1016/j.lungcan.2006.12.007

[84] Constâncio, V., Nunes, S.P., Henrique, R. and Jerónimo, C. (2020) DNA Methylation-Based Testing in Liquid Biopsies as Detection and Prognostic Biomarkers for the Four Major Cancer Types. *Cells*, **9**, Article No. 624. https://doi.org/10.3390/cells9030624

[85] Luo, H., Wei, W., Ye, Z., Zheng, J. and Xu, R.-H. (2021) Liquid Biopsy of Methylation Biomarkers in Cell-Free DNA. *Trends in Molecular Medicine*, **27**, 482-500. https://doi.org/10.1016/j.molmed.2020.12.011.

[86] Alexander, M., *et al.* (2017) Case-Control Study of Candidate Gene Methylation and Adenomatous Polyp Formation. *International Journal of Colorectal Disease*, **32**, 183-192. https://doi.org/10.1007/s00384-016-2688-1

[87] Si, J., *et al.* (2021) Epigenome-Wide Analysis of DNA Methylation and Coronary Heart Disease: A Nested Case-Control Study. *eLife*, **10**, e68671. https://doi.org/10.7554/eLife.68671

[88] Ku, J.-L., Jeon, Y.-K. and Park, J.-G. (2011) Methylation-Specific PCR. In: Tollefsbol, T., Ed., *Epigenetics Protocols. Methods in Molecular Biology*, Vol. 791, Humana Press, Totowa, 23-32. https://doi.org/10.1007/978-1-61779-316-5_3.

[89] Leitão, E., *et al.* (2018) Locus-Specific DNA Methylation Analysis by Targeted Deep Bisulfite Sequencing. In: Jeltsch, A. and Rots, M., Eds., *Epigenome Editing. Methods in Molecular Biology*, Vol. 1767, Humana Press, New York, 351-366. https://doi.org/10.1007/978-1-4939-7774-1_19

[90] Hofner, M., *et al.* (2020) Chapter Ten—Multiplex Analyses Using Methylation Sensitive Restriction Enzyme qPCR. In: Tollefsbol, T., Ed., *Epigenetics Methods*, Academic Press, Cambridge, 181-212. https://doi.org/10.1016/B978-0-12-819414-0.00010-0

[91] Bibikova, M., *et al.* (2011) High Density DNA Methylation Array with Single CpG Site Resolution. *Genomics*, **98**, 288-295. https://doi.org/10.1016/j.ygeno.2011.07.007

[92] Brooks, J.D., *et al.* (2010) DNA Methylation in Pre-Diagnostic Serum Samples of Breast Cancer Cases: Results of a Nested Case-Control Study. *Cancer Epidemiology*, **34**, 717-723. https://doi.org/10.1016/j.canep.2010.05.006

[93] Issa, J.-P.J. (2007) DNA Methylation as a Therapeutic Target in Cancer. *Clinical Cancer Research*, **13**, 1634-1637. https://doi.org/10.1158/1078-0432.CCR-06-2076

[94] Yang, X., *et al.* (2014) Gene Body Methylation Can Alter Gene Expression and Is a Therapeutic Target in Cancer. *Cancer Cell*, **26**, 577-590. https://doi.org/10.1016/j.ccr.2014.07.028

[95] Chang, L., *et al.* (2014) Elevation of Peripheral BDNF Promoter Methylation Links to the Risk of Alzheimer's Disease. *PLOS ONE*, **9**, e110773. https://doi.org/10.1371/journal.pone.0110773

[96] Gao, L., Zhang, Y., Sterling, K. and Song, W. (2022) Brain-Derived Neurotrophic Factor in Alzheimer's Disease and Its Pharmaceutical Potential. *Translational Neurodegeneration*, **11**, Article No. 4. https://doi.org/10.1186/s40035-022-00279-0

[97] Issa, J.-P.J. and Kantarjian, H.M. (2009) Targeting DNA Methylation. *Clinical Cancer Research*, **15**, 3938-3946. https://doi.org/10.1158/1078-0432.CCR-08-2783

[98] Da Costa, E.M., McInnes, G., Beaudry, A. and Raynal, N.J.M. (2017) DNA Methylation-Targeted Drugs. *The Cancer Journal*, **23**, 270-276. https://doi.org/10.1097/PPO.0000000000000278

[99] Cheng, Y., *et al.* (2019) Targeting Epigenetic Regulators for Cancer Therapy: Mechanisms and Advances in Clinical Trials. *Signal Transduction and Targeted Therapy*, **4**, Article No. 62. https://doi.org/10.1038/s41392-019-0095-0

[100] Flotho, C., *et al.* (2009) The DNA Methyltransferase Inhibitors Azacitidine, Decitabine and Zebularine Exert Differential Effects on Cancer Gene Expression in Acute Myeloid Leukemia Cells. *Leukemia*, **23**, 1019-1028. https://doi.org/10.1038/leu.2008.397

[101] Griffiths, E.A. and Gore, S.D. (2008) DNA Methyltransferase and Histone Deacetylase Inhibitors in the Treatment of Myelodysplastic Syndromes. *Seminars in Hematology*, **45**, 23-30. https://doi.org/10.1053/j.seminhematol.2007.11.007

[102] Sorrentino, V.G., *et al.* (2021) Hypomethylating Chemotherapeutic Agents as Therapy for Myelodysplastic Syndromes and Prevention of Acute Myeloid Leukemia. *Pharmaceuticals*, **14**, Article No. 641. https://doi.org/10.3390/ph14070641

[103] Tiffon, C., *et al.* (2011) The Histone Deacetylase Inhibitors Vorinostat and Romidepsin Downmodulate IL-10 Expression in Cutaneous T-Cell Lymphoma Cells. *British Journal of Pharmacology*, **162**, 1590-1602. https://doi.org/10.1111/j.1476-5381.2010.01188.x

[104] Moore, D. (2016) Panobinostat (Farydak): A Novel Option for the Treatment of Relapsed or Relapsed and Refractory Multiple Myeloma. *Pharmacy and Therapeutics*, **41**, 296-300.

[105] Hontecillas-Prieto, L., *et al.* (2020) Synergistic Enhancement of Cancer Therapy Using HDAC Inhibitors: Opportunity for Clinical Trials. *Frontiers in Genetics*, **11**, Article 578011. https://www.frontiersin.org/articles/10.3389/fgene.2020.578011 https://doi.org/10.3389/fgene.2020.578011

[106] Ramaiah, M.J., Tangutur, A.D. and Manyam, R.R. (2021) Epigenetic Modulation and Understanding of HDAC Inhibitors in Cancer Therapy. *Life Sciences*, **277**, Article ID: 119504. https://doi.org/10.1016/j.lfs.2021.119504

[107] Girard, N., *et al.* (2014) 3-Deazaneplanocin A (DZNep), an Inhibitor of the Histone Methyltransferase EZH2, Induces Apoptosis and Reduces Cell Migration in Chondrosarcoma Cells. *PLOS ONE*, **9**, e98176. https://doi.org/10.1371/journal.pone.0098176

[108] Cherblanc, F.L., Chapman, K.L., Brown, R. and Fuchter, M.J. (2013) Chaetocin Is a Nonspecific Inhibitor of Histone Lysine Methyltransferases. *Nature Chemical Biology*, **9**, 136-137. https://doi.org/10.1038/nchembio.1187

[109] Morgado-Pascual, J.L., Rayego-Mateos, S., Tejedor, L., Suarez-Alvarez, B. and Ruiz-Ortega, M. (2019) Bromodomain and Extraterminal Proteins as Novel Epigenetic Targets for Renal Diseases. *Frontiers in Pharmacology*, **10**, Article1315. https://www.frontiersin.org/articles/10.3389/fphar.2019.01315 https://doi.org/10.3389/fphar.2019.01315

[110] Flesher, J.L. and Fisher, D.E. (2021) G9a: An Emerging Epigenetic Target for Melanoma Therapy. *Epigenomes*, **5**, Article No. 23. https://doi.org/10.3390/epigenomes5040023

[111] Ishiguro, K., *et al.* (2021) Dual EZH2 and G9a Inhibition Suppresses Multiple Mye-

loma Cell Proliferation by Regulating the Interferon Signal and IRF4-MYC Axis. *Cell Death Discovery*, **7**, Article No. 7. https://doi.org/10.1038/s41420-020-00400-0

[112] Madakashira, B.P. and Sadler, K.C. (2017) DNA Methylation, Nuclear Organization, and Cancer. *Frontiers in Genetics*, **8**, Article 76. https://www.frontiersin.org/articles/10.3389/fgene.2017.00076 https://doi.org/10.3389/fgene.2017.00076

[113] Pfeifer, G.P. (2018) Defining Driver DNA Methylation Changes in Human Cancer. *International Journal of Molecular Sciences*, **19**, Article No. 1166. https://doi.org/10.3390/ijms19041166

[114] Lee, H.Y., *et al.* (2012) Potential Forensic Application of DNA Methylation Profiling to Body Fluid Identification. *International Journal of Legal Medicine*, **126**, 55-62. https://doi.org/10.1007/s00414-011-0569-2

[115] Forat, S., *et al.* (2016) Methylation Markers for the Identification of Body Fluids and Tissues from Forensic Trace Evidence. *PLOS ONE*, **11**, e0147973. https://doi.org/10.1371/journal.pone.0147973

[116] Yokoi, K., Yamashita, K. and Watanabe, M. (2017) Analysis of DNA Methylation Status in Bodily Fluids for Early Detection of Cancer. *International Journal of Molecular Sciences*, **18**, Article No. 735. https://doi.org/10.3390/ijms18040735

[117] Alzahrani, S.M., Al Doghaither, H.A. and Al-Ghafari, A.B. (2021) General Insight into Cancer: An Overview of Colorectal Cancer (Review). *Molecular and Clinical Oncology*, **15**, Article No. 271. https://doi.org/10.3892/mco.2021.2433

[118] Devall, M.A., *et al.* (2022) DNA Methylation Analysis of Normal Colon Organoids from Familial Adenomatous Polyposis Patients Reveals Novel Insight into Colon Cancer Development. *Clinical Epigenetics*, **14**, Article No. 104. https://doi.org/10.1186/s13148-022-01324-5

[119] Kong, C. and Fu, T. (2021) Value of Methylation Markers in Colorectal Cancer (Review). *Oncology Reports*, **46**, Article No. 177. https://doi.org/10.3892/or.2021.8128

[120] Cocco, E., *et al.* (2019) Colorectal Carcinomas Containing Hypermethylated MLH1 Promoter and Wild-Type BRAF/KRAS Are Enriched for Targetable Kinase Fusions. *Cancer Research*, **79**, 1047-1053. https://doi.org/10.1158/0008-5472.CAN-18-3126

[121] Wang, D., *et al.* (2021) Development of a Liquid Biopsy Based Purely Quantitative Digital Droplet PCR Assay for Detection of MLH1 Promoter Methylation in Colorectal Cancer Patients. *BMC Cancer*, **21**, Article No. 797. https://doi.org/10.1186/s12885-021-08497-x

[122] Müller, D. and Győrffy, B. (2022) DNA Methylation-Based Diagnostic, Prognostic, and Predictive Biomarkers in Colorectal Cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, **1877**, Article ID: 188722. https://doi.org/10.1016/j.bbcan.2022.188722

[123] Jurisic, V., *et al.* (2023) Analyses of $P16^{INK4a}$ Gene Promoter Methylation Relative to Molecular, Demographic and Clinical Parameters Characteristics in Non-Small Cell Lung Cancer Patients: A Pilot Study. *Molecular Biology Reports*, **50**, 971-979. https://doi.org/10.1007/s11033-022-07982-1

[124] Li, M., Wang, C., Yu, B., Zhang, X., Shi, F. and Liu, X. (2019) Diagnostic Value of RASSF1A Methylation for Breast Cancer: A Meta-Analysis. *Bioscience Reports*, **39**, Article ID: BSR20190923. https://doi.org/10.1042/BSR20190923

[125] Ramalho-Carvalho, J., Henrique, R. and Jerónimo, C. (2018) Methylation-Specific PCR. In: Tost, J., Ed., *DNA Methylation Protocols. Methods in Molecular Biology*, Vol. 1708, Humana Press, New York, 447-472.

https://doi.org/10.1007/978-1-4939-7481-8_23

[126] Liu, P., Lin, C.-W., Park, Y. and Tseng, G. (2021) MethylSeqDesign: A Framework for Methyl-Seq Genome-Wide Power Calculation and Study Design Issues. *Biostatistics*, **22**, 35-50. https://doi.org/10.1093/biostatistics/kxz016

[127] Hai, L., Li, L., Liu, Z., Tong, Z. and Sun, Y. (2022) Whole-Genome Circulating Tumor DNA Methylation Landscape Reveals Sensitive Biomarkers of Breast Cancer. *MedComm*, **3**, e134. https://doi.org/10.1002/mco2.134

[128] Gao, Y., *et al.* (2022) Whole-Genome Bisulfite Sequencing Analysis of Circulating Tumour DNA for the Detection and Molecular Classification of Cancer. *Clinical and Translational Medicine*, **12**, e1014. https://doi.org/10.1002/ctm2.1014

[129] Taiwo, O., *et al.* (2012) Methylome Analysis Using MeDIP-seq with Low DNA Concentrations. *Nature Protocols*, **7**, 617-636. https://doi.org/10.1038/nprot.2012.012

[130] Irizarry, R.A., *et al.* (2009) The Human Colon Cancer Methylome Shows Similar Hypo- and Hypermethylation at Conserved Tissue-Specific CpG Island Shores. *Nature Genetics*, **41**, 178-186. https://doi.org/10.1038/ng.298

[131] Irizarry, R.A., *et al.* (2008) Comprehensive High-Throughput Arrays for Relative Methylation (CHARM). *Genome Research*, **18**, 780-790. https://doi.org/10.1101/gr.7301508

[132] Tang, Q., Holland-Letz, T., *et al.* (2016) DNA Methylation Array Analysis Identifies Breast Cancer Associated RPTOR, MGRN1 and RAPSN Hypomethylation in Peripheral Blood DNA. *Oncotarget*, **7**, 64191-64202. https://doi.org/10.18632/oncotarget.11640

[133] Kitchen, M.O., *et al.* (2018) HumanMethylation450K Array-Identified Biomarkers Predict Tumour Recurrence/Progression at Initial Diagnosis of High-Risk Non-Muscle Invasive Bladder Cancer. *Biomarkers in Cancer*, **10**, Article ID: 1179299X17751920. https://doi.org/10.1177/1179299X17751920

[134] Yu, M., *et al.* (2012) Tet-Assisted Bisulfite Sequencing of 5-Hydroxymethylcytosine. *Nature Protocols*, **7**, 2159-2170. https://doi.org/10.1038/nprot.2012.137

[135] Orozco, L.D., *et al.* (2015) Epigenome-Wide Association of Liver Methylation Patterns and Complex Metabolic Traits in Mice. *Cell Metabolism*, **21**, 905-917. https://doi.org/10.1016/j.cmet.2015.04.025

[136] Miyata, K., Naito, M., Miyata, T., Mokuda, S. and Asahara, H. (2017) Bisulfite Sequencing for DNA Methylation Analysis of Primary Muscle Stem Cells. In: Ryall, J., Ed., *Skeletal Muscle Development. Methods in Molecular Biology*, Vol. 1668, Humana Press, New York, 3-13. https://doi.org/10.1007/978-1-4939-7283-8_1

[137] Hong, S.R. and Shin, K.-J. (2021) Bisulfite-Converted DNA Quantity Evaluation: A Multiplex Quantitative Real-Time PCR System for Evaluation of Bisulfite Conversion. *Frontiers in Genetics*, **12**, Article 618955. https://www.frontiersin.org/articles/10.3389/fgene.2021.618955 https://doi.org/10.3389/fgene.2021.618955

[138] Ziller, M.J., Hansen, K.D., Meissner, A. and Aryee, M.J. (2015) Coverage Recommendations for Methylation Analysis by Whole-Genome Bisulfite Sequencing. *Nature Methods*, **12**, 230-232. https://doi.org/10.1038/nmeth.3152

[139] Wojdacz, T.K., Hørning Møller, T., Thestrup, B.B., Kristensen, L.S. and Hansen, L.L. (2010) Limitations and Advantages of MS-HRM and Bisulfite Sequencing for Single Locus Methylation Studies. *Expert Review of Molecular Diagnostics*, **10**, 575-580. https://doi.org/10.1586/erm.10.46

[140] Kaneda, A., Takai, D., Kaminishi, M., Okochi, E. and Ushijima, T. (2003) Methylation-Sensitive Representational Difference Analysis and Its Application to Cancer Research. *Annals of the New York Academy of Sciences*, **983**, 131-141. https://doi.org/10.1111/j.1749-6632.2003.tb05968.x

[141] Khanna, A., Czyz, A. and Syed, F. (2013) EpiGnome™ Methyl-Seq Kit: A Novel Post-Bisulfite Conversion Library Prep Method for Methylation Analysis. *Nature Methods*, **10**, iii-iv. https://doi.org/10.1038/nmeth.f.369