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NARRATIVE REVIEWS

Epigenetic Technologies in Forensic Sciences: A New Frontier for Human Identification

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Abstract

Epigenetics, the study of heritable changes in gene function that occur without altering the DNA sequence, has emerged as a transformative field in modern biology. These molecular modifications, particularly DNA methylation and histone alteration scan, persist through cell divisions and play a critical role in regulating gene expression. In recent years, the surge in epigenetic research has opened new doors not only in medicine and developmental biology but also in forensic science. What makes epigenetics especially compelling in forensics is its ability to reveal information beyond genetic code. Forensic science has advanced to the point where it can now extract detailed clues about a person's age and appearance directly from a DNA sample. By examining specific epigenetic markers, chemical modifications that influence gene activity, scientists can infer characteristics, such as eye color, skin tone, and even approximate age. This cutting-edge approach provides a vital new tool in criminal investigations, especially in cases where traditional DNA profiling offers limited information, helping to build a more complete profile of a suspect or victim when other methods fall short. This review explores the major types of epigenetic modifications, their forensic applications, and the key markers used in identifying physical traits and biological age. It also discusses the technological requirements and current limitations of epigenetic analysis in forensic investigations as well as the most recent advancements in detecting and interpreting histone modifications.

1. Introduction

Epigenetic processes control the biochemical chain of events that starts with DNA nucleotide sequence and leads to the production of different RNA types, known as transcription. Messenger RNA (mRNA) generates the proteins (i.e., the process of translation) that serve as main cell signaling molecules. Differences in the functioning of this cascade among individuals were originally attributed to differences in the sequence of nucleotides. Certainly, changes in the sequence can impact the structure and synthesis of RNAs and the proteins they produce, leading to consequences for cellular processes and well-being. This reasoning is the basis for the investigation of genetic variants that can be inherited and are linked to illness conditions. Nevertheless, this viewpoint fails to acknowledge the intricate intricacy in the mechanisms through which a gene is transcribed into an RNA product. Gene transcription or expression is a continuously controlled process influenced by epigenetic signals that affect the functioning of the genome. The clear suggestion is that changes in the epigenetic program can lead to diversity in the transcription of DNA within a cell. The discovery that epigenetic signals are influenced by environmental factors resulted

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in a remarkable fusion of the biology and social sciences, demonstrating how much progress has been made in the study of human development and health, moving beyond the longstanding debate of nature versus nurture [1].

2. Epigenetics

Epigenetics is the investigation of inheritable characteristics, or a consistent alteration of cell function, that occurs without modifications to DNA sequence. Epigenetics often entails a modification that persists across cell division and impacts the control of gene expression. These impacts on cellular and physiological characteristics may arise from environmental influences or be a natural element of development. They have the potential to cause cancer [2]. The term "epigenetics" also refers to the process of changes: functionally important modifications to the genome that do not involve mutation of the nucleotide sequence. Some methods that cause these modifications include DNA methylation and histone modification as shown in Table 1. These mechanisms can modify how genes are expressed without changing the actual DNA sequence. In addition, RNA sequences that do not code for proteins are found to have an important function in controlling gene expression. Gene expression can be regulated by repressor proteins that bind to silencer sections of DNA [3].

A report from the World Health Organization (WHO) states that about 13 million fatalities occur each year because of environmental factors, and up to 24% of diseases are caused by preventable exposures. The roster of environmental hazards to human health encompasses a significant quantity of environmental contaminants. For example, the third National Report on Human Exposure to Environmental Chemicals by the US Center for Disease Control and Prevention (CDC) discovered 148 environmental chemicals that may be identified in the blood and urine of a sample of the US population. The list of contaminants assessed included metals, phytoestrogens, polycyclic aromatic hydrocarbons, dioxin-like compounds, polychlorinated biphenyls, phthalates, and other categories of pesticides [4].

3. Epigenetic strategy

3.1. DNA methylation

The addition of a methyl group to the C5 site of cytosine (5-methylcytosine) is the main epigenetic change that occurs in DNA. This alteration mainly happens at sites that contain CpG (a sequence of cytosine (C) and guanine (G) nucleotide, linked by a phosphate backbone) dinucleotides, frequently in regulatory sequences that inhibit gene expression [5]. CpG methylation is crucial for suppressing the activity of transposons and repetitive sequences as well as for imprinting and inactivating the X-chromosome [6]. The existence of a methylated cytosine might hinder transcription by blocking the attachment of transcription factors or may facilitate the attachment of other transcriptional inhibitors, such as histone-modifying proteins, including histone deacetylases (HDACs). Cytosine methylation at CpG dinucleotides is performed by a group of enzymes called DNA methyltransferases (DNMTs). This group includes the *de novo* methyltransferases DNMT3a and DNMT3b, as well as DNMT1, which identifies and methylate the daughter strand that is not already methylated during DNA replication [7]. While demethylation is recognized as a crucial process that takes place at specific phases of development, the mechanism of DNA demethylation is not as well understood as methylation [8].

3.2. Histone acetylation

Histone acetylation includes adding an acetyl group to lysine residues in the extending histone tails. It is commonly linked to transcriptional activation and is regulated by two opposing groups of enzymes: histone acetyl transferases (HATs), which add acetyl groups, and histone deacetylases (HDACs), which remove them [9]. HATs transfer an acetyl group to the ε-amino group of lysine utilizing acetyl CoA as a cofactor, which cancels out the positive charge on lysine, reduces the strength of the histone–DNA connection, and allows genes to be accessed [10]. HDACs

Table (1): Comparative summary of epigenetic strategies.

Epigenetic strategy	Mechanism	Enzymes involved	Biological functions
DNA methylation	Addition of a methyl group to the C5 site of cytosine in CpG dinucleotides	DNMT1 (maintenance), DNMT3a, and DNMT3b (de novo)	Suppression of transposons, X-chromosome inactivation, genomic imprinting, and gene silencing
Histone acetylation	Addition of an acetyl group to lysine residues on histone tails, which neutralizes their positive charge and relaxes chromatin structure to promote gene transcription	HATs (add), HDACs (remove)	Chromatin relaxation, gene activation, and transcriptional regulation
Histone phosphorylation	Addition of phosphate groups to serine, threonine, or tyrosine residues	Protein kinases (add), phosphatases (remove)	DNA damage response, transcriptional regulation, and cell division, apoptosis
Histone ubiquitination	Addition of ubiquitin (76 aa protein) to histones	E1 (activating), E2 (conjugating), and E3 (ligating enzymes)	Transcriptional regulation, genome stability, DNA repair, and proteasomal degradation

are enzymes that regulate gene expression by removing acetyl groups from lysine residues of both histone and non-histone proteins [11].

3.3. Histone phosphorylation

Histone phosphorylation was initially identified in 1967. All four main histones can be phosphorylated by various protein kinases and dephosphorylated by phosphatases. Phosphorylation occurs on serine, threonine, and tyrosine, and has been found to be linked to DNA damage response (H2A.X S139 and H4S1), regulation of transcription (H3S10, H3S28, H2BS32, H3T6, H3T11, H3Y41, H2BS36, H2BY37, H4S1, and H4S47), cell division (H3S10 and H3S28), reproductive cell division (H4S1 and H2BS10), and programmed cell death (H2BS10, H2AXS139, and H3T45) [12].

There are numerous indications of communication between histone phosphorylation and other post-translational modifications (PTMs). The crosstalk can occur in one of the two conditions. First, an existing histone phosphorylation can attract another enzyme that modifies histones, thereby facilitating the corresponding alteration. For instance, the addition of a phosphate group to histone H3 serine 10 (H3S10) increases the following acetylation of nearby H3K14 by the HAT Gcn5 acetyltransferase, which helps to facilitate transcription [12]. However, an existing histone phosphorylation can prevent another enzyme that modifies histones from binding to the nearby modification. For example, the addition of a phosphate group to H3S10 impacts the ability of HP1 to bind to methylated H3K9 [13].

3.4. Histone ubiquitination

Knowledge of histone ubiquitination dates back to more than 30 years. However, its roles are still not as well understood as other histone modifications. Ubiquitin is a 76-amino acid (aa) regulatory protein that is widely present in all eukaryotes. Several physiological activities are regulated by the ubiquitination post-translational modification of target proteins, such as protein degradation, cell-cycle control, stress response, DNA repair, immunological response, signal transmission, transcriptional regulation, endocytosis, and vesicle trafficking. The ubiquitin-proteasome system (UPS) is a highly organized system for breaking down proteins. It consists of several protein components (ubiquitin-activating E1 enzymes, ubiquitin-conjugating E2 enzymes, ubiquitin-protein E3 ligases, and the 26S proteasome) that work together to ensure the timely and efficient breakdown of target proteins. The addition of ubiquitin molecules to histones is important for controlling several processes in the nucleus, such as maintaining stability of the genome and regulating transcription [14].

Forensic epigenetics has traditionally concentrated on DNA methylation; however, histone modifications offer supplementary insights for forensic casework. These modifications are reversible and context-dependent, reflecting both cellular identity and environmental exposures. Forensic Applications under Exploration Body Fluid and Tissue Identification where Histone marks exhibit variability across different cell types. Variations in H3K4me3 or H3K27me3 may differentiate epithelial cells from the blood or semen [15]. Research indicates that cell-type-specific histone modifications could serve as biomarkers when DNA methylation profiles are inadequate and provide a more stable signal in highly degraded samples because of the physical protection of nucleosome core [16]. Age estimation, which is a pattern of histone modification, alter with aging, such as H3K9ac, H3K14ac, and H4K8ac often decrease over time as deacetylase enzymes may remain active, or acetylation is lost passively [17]. These signatures could complement DNA methylation clocks in forensic age prediction. Environmental and lifestyle exposures where histone states are responsive to stress, diet, smoking, and toxins, potentially offer insights into lifestyle factors pertinent to victim's identification. At post-mortem interval (PMI) estimation, histone modifications degrade in a predictable manner post-mortem. Monitoring changes in acetylation and methylation could enhance the precision of PMI estimation, compared to morphological methods. H3K4me3 and H3K27me3 exhibit greater stability and demonstrate slower and more predictable decay patterns. This stability renders them potentially excellent biomarkers for estimating PMI. Notably, the ratio of H3K36me3 to H3K27me3 is proposed as a particularly stable marker for estimation of PMI over extended periods [18].

Histone modifications represent a valuable source of forensic information, potentially providing insights into tissue identity, PMI, age, and disease state that may complement or even surpass the capabilities of DNA methylation. Nevertheless, this field remains in its early stages of development. Currently, DNA methylation is a more practical and established tool for routine forensic casework. The future likely resides in multi-omic approaches, wherein DNA methylation, histone modification, and potentially RNA expression data are integrated to construct a highly powerful and precise biological profile of a sample for forensic investigation [19].

4. Forensic Requirements of Epigenetics

Forensic DNA analysis has many criteria that are influenced by the limited quality and quantity of DNA usually found in crime scene evidence. This affects the selection of markers to be studied and the technology that can be employed. These criteria also pertain to forensic epigenetics. Furthermore, there are extra technological difficulties because of the numerical results of epigenetic analysis, as opposed to forensic genetic analysis, which is mostly based on quality. The finite quantity of human biological material found at crime scenes limits the number of individual DNA testing that can be conducted. As a result, it is necessary to have multiplex genotyping methods in forensic analysis that can analyze many epigenetic markers at the same time. This is because individual markers usually do not provide sufficient information that is helpful

for forensic purposes. However, the current technologies for analyzing many epigenetic markers at the same time, such as DNA methylation microarrays and whole-genome bisulfite sequencing, are not appropriate for forensic trace analysis because of the significant quantity of high-quality DNA they need. Meanwhile, the existing methods for analyzing epigenetics, such as bisulfite pyrosequencing, methylation quantitative polymerase chain reaction (PCR), and EPITYPER®, have limitations in their ability to handle DNA that is of low quality or quantity. These methods can only analyze a small number of markers (<20), which is often not enough to fully investigate a forensic question of interest [20].

Quantities of DNA collected from crime scene traces are often small, usually in the picogram–nanogram range. Thus, it is necessary to have advanced technology in forensics that can accurately detect variations in DNA, such as levels of DNA methylation. Techniques such as methylation Snapshot, despite their restricted ability to handle numerous samples at once, can presently detect DNA input as low as a few nanograms per PCR [21].

However, the most modern epigenetic techniques necessitate bisulfite conversion before marker analysis; the effectiveness of converting unmethylated cytosines into uracil greatly relies on DNA input. Usually, bisulfite conversion kits need at least 50–200-ng DNA to work well. Decreased DNA input results in higher technical variance, which in turn leads to a wider range of errors in the subsequent DNA methylation study. Advanced technologies capable of analyzing several DNA methylation markers from low-quality/quantity DNA are not currently available. Crime scene evidence can include many types of cells. Although cell/tissue-type composition is often not limiting in genetic analysis, it can provide challenges in epigenetic analysis. Forensic epigenetic assays must function equally well in all forensically relevant cell or tissue types, or if that is not possible, then it must be customized for specific tissue types, which require determining the tissue type before doing epigenetic analysis. Certain DNA methylation sites might exhibit significant variations among various tissues, which should be considered when applying pre-existing predictive marker sets and prediction models to a sample that may come from a different tissue source [22,23].

5. DNA Methylation in Forensics

DNA methylation has various effects on an individual's lifestyle, health, physical appearance, etc. It provides information about socioeconomic status, diet, physical activity, alcohol consumption, drug use, and smoking habits. DNA methylation levels have proven useful in determining the age and predicting visible traits (such as hair, skin, and eye color) to help narrow down the list of potential suspects in a crime. Forensic epigeneticists have developed innovative methods for profiling and analyzing DNA methylation. However, there are challenges due to the quantitative nature of epigenetic analysis [24,25].

6. Epigenetic Markers for Identifying Traits in Forensic Investigations

The existing epigenetic biomarkers are found to coincide with biomarkers of other traits. An instance is the estimation of age using methylation-specific biomarkers, which provide overlapping information about the sample source in relation to health- or other lifestyle-related information. Additional epigenetic markers that are more precise are required to accurately predict observable traits, as the current markers are insufficient to clearly determine the origin of the sample [26].

7. Approaches and Strategies for DNA Methylation Analysis

The currently accessible methods for examining DNA methylation of many CpG sites in a precise and efficient manner require high-quality DNA and a significant number of epigenetic markers. The below are mentioned the most modern technique for forensic methylation analysis:

- DNA methylation microarrays
- DNA methylation bisulfite genomic sequencing
- Bisulfite pyrosequencing
- Methylation quantitative PCR
- Methylation mass spectrometry
- Bar-coded bisulfite amplicon sequencing

7.1. DNA methylation microarrays

The DNA methylation microarray is a screening technology that uses methylation-sensitive restriction enzymes to analyze methylated fragments and examine them on a CpG island microarray. The concept of differential methylation hybridization, the initial array-based technique, genomic DNA is initially fragmented using a methylation-insensitive restriction enzyme, such as *Mse* I. The *Mse* I restriction enzyme is not expected to affect any CpG islands because its recognition site is the TTAA sequence. Following digestion using *Mse I*, the adapters are attached to the end of each DNA fragment and processed using methylation-sensitive enzymes, specifically *Bst UI* and *Hpa II*. The resulting methylated fragments stay intact and are amplified by PCR with primers that are unique to the ligated adapters. Following the process of differential labelling, the amplicons are then co-hybridized to a CpG island array [27]. Age may be determined by analyzing six CpG candidates (cg00481951, cg19671120, cg14361627, cg08928145, cg12757011, and cg07547549) in saliva using the Illumina 450K microarray platform. To determine blood, saliva, semen, vaginal fluid, and menstrual blood [28], DNA methylation microarrays provide cost-effective and reliable investigation of numerous biologically significant genomic areas. In addition to the need for significant quantities of high-molecular weight DNA, another drawback of the approach is that the methylation-sensitive restriction

enzymes do not examine every cytosine. This approach is somewhat inaccurate when it comes to the upper limit of the fragments that undergo hybridization. There are a few drawbacks to this strategy, the restricted number of informative restriction sites can be crucial in forensic sample analysis when the phenotypic results are influenced by a methylation alteration at a specific CpG that is not located within the restriction site of a methylation-sensitive restriction enzyme. In addition, owing to the nature of the test, DNA methylation microarrays are unable to accurately differentiate between low and high levels of CpG methylation.

7.2. DNA methylation bisulfite genomic sequencing

Methods for profiling genome-wide DNA methylation are categorized into four groups: (1) techniques that use restriction enzymes sensitive to DNA methylation (such as MRE-seq), (2) methods that utilize methyl cytosine-specific antibodies (such as methylated DNA immunoprecipitation using MeDIP-seq), (3) approaches that employ methyl-CpG-binding domains to enrich methylated DNA at specific sites of interest, and (4) those based on sodium bisulfite treatment. Nevertheless, the initial three techniques enable the identification of methylation across measured regions of varying sizes, spanning from 100 bp to 1,000 bp. Techniques that utilize sodium bisulfite treatment, which changes unmethylated cytosines to thymine (via uracil) while leaving methylated cytosines unaffected, can assess DNA methylation at the level of individual nucleotides. In the rest of this section, we concentrated on sequencing techniques that are based on bisulfite conversion [29,30]. This chemical conversion enables precise, base-by-base mapping of DNA methylation patterns. BGS is customized to analyze specific regions, such as targeted PCR amplicons, or scaled up to assess larger sections of the genome.

Whole Genome Bisulfite Sequencing (WGBS) builds upon the principles of BGS by applying this treatment across the entire genome. Instead of focusing on particular regions, all DNA is subjected to bisulfite conversion and then sequenced using high-throughput methods [31]. WGBS combines the process of converting DNA molecules with bisulfite with high-throughput sequencing. To carry out WGBS, the genomic DNA is initially fragmented in a random manner to the desired size (200 bp). The broken DNA is transformed into a sequencing library by attaching adaptors that contain 5-methyl-2'-deoxycytidine (5mC). The sequencing library is then processed using bisulfite. This therapy efficiently changes unmethylated cytosines to uracil. Following the PCR amplification of the library treated with bisulfite, it is subjected to high-throughput sequencing [32,33]. Following the PCR, uracil is depicted as thymine. An accurate remembrance of cytosine methylation necessitates not only enough sequencing depth but also heavily relies on the quality of bisulfite conversion and library amplification. The advantage of this broad method is that it usually covers more than 90% of the CpGs in the human genome in a fair representation. It enables the detection of non-CG methylation as well as the identification of partially methylated domains (PMDs), areas at distant regulatory elements with low methylation (LMRs), and DNA methylation valleys (DMVs) in embryonic stem cells. Although WGBS has its benefits, it is still the most expensive method and the standard library preparation requires a relatively large amount of DNA (100 ng–5 ug). Because of this, it is not typically used for a large number of samples. In order to accurately detect differences in methylation between samples, a high sequencing depth is necessary, which results in a significant increase in sequencing cost [34].

7.3. Bisulfite pyrosequencing

Pyrosequencing is a method that utilizes a sequencing-by-synthesis device that is intended to measure single-nucleotide polymorphism (SNP). Creating artificial C/T SNP using bisulfite modification allows for the real-time assessment of DNA methylation at both local and global levels. Changes in DNA methylation are linked to aging as well as age-related illnesses, such as cancer and cardiovascular, neurodegenerative, and autoimmune diseases. Given its widespread occurrence in various clinical diseases, measuring the amount of DNA CpG methylation, both overall and at specific genes, can provide insights into the control of genes related to pathological illnesses. The capacity to identify and measure the methylation pattern of DNA has the potential to function as an early detection indicator and possible therapeutic target for various diseases. Here, we offer a comprehensive technical procedure for pyrosequencing together with important details about assay design and system intricacies that serve as a solid basis for newcomers in the field [35].

Pyrosequencing, a sequencing technology that provides real-time results, is widely regarded as a reliable method for accurately measuring the quantity of alleles at the level of individual bases. Quantitative bisulfite pyrosequencing measures the degree of DNA methylation by examining fake "C/T" SNPs at CpG sites in a particular pyrosequencing test. The bisulfite pyrosequencing methylation assay design is particular to the DNA strand, and the primer design should not include any CpG sites and should be devoid of frequent mutations. In addition, pyrosequencing experiments need to be checked for any bias in amplification during bisulfite PCR in order to achieve accurate and consistent sequencing quantification. Pyrosequencing analysis provides a consistent determination of average methylation at several CpG sites inside the pyrosequencing test directly from a PCR result, quickly and correctly for multiple samples simultaneously [36].

7.4. Methylation quantitative PCR

The key characteristic of the quantitative multiplex methylation-specific PCR (QM-MSP) technology, which allows for the accurate measurement of DNA methylation, is a two-step PCR process used to simultaneously analyze a

group of up to 12 genes in small amounts of DNA obtained from clinical samples. During the initial stage, a maximum of 12 genes are examined using a pair of primers specific to each gene. These primers, one forward and one reverse, amplify both methylated and unmethylated copies of the same gene at the same time and in multiple copies, all inside a single PCR reaction. This amplification step, which does not rely on methylation, generates amplicons with a maximum of 109 copies per microliter after 36 cycles of PCR. In the next stage, amplicons from the initial reaction (STEP 1) are measured using real-time PCR (RT-PCR) and a standard curve. This is done by employing two different fluorophores (6FAM and VIC) to identify methylated or unmethylated DNA for each gene. These measurements are taken in the same well. One modified copy is detected in 100,000 reference gene copies. Methylation is observed on a continuous scale. To determine whether a sample is positive for the gene panel, the maximum degree of normal DNA methylation is identified using receiver operating characteristic (ROC) analysis. This analysis maximizes specificity and sensitivity of the assay to differentiate between normal/benign DNA and malignant DNA. QM-MSP is used on clinical samples of fresh or fixed ductal cells, ductal fluid, nipple fluid, fine needle aspirates, core biopsies, and tumor tissue sections [37].

Yu *et al.* demonstrated that a quantitative assay for specific mRNAs (QASM assay) is a PCR-based method that employs fluorescence-based RT-PCR to precisely measure the level of methylation at individual CpG sites [38]. This assay has exceptional analytical specificity, sensitivity, and reproducibility. Moreover, it is a streamlined assay that does not necessitate an input control reaction (such as ALU-C4) or fully methylated DNA reference. It is important to mention that the measurement of methylation is highly accurate, compared to bisulfite pyrosequencing, which is commonly recognized as the most trustworthy method for assessing methylation levels.

The QASM technique was developed to identify the methylation status of an individual CpG dinucleotide. It has the potential to provide information about methylation at a single-base level as long as the surrounding sequence allows for the designing of primers and paired probes that specifically target the CpG dinucleotide of interest while avoiding other CpG dinucleotides. This specificity is achieved by reducing the complexity of DNA through bisulfite treatment. The acquisition of this specific methylation data is accomplished by bisulfite genome sequencing or bisulfite pyrosequencing, methods that were previously deemed impractical and expensive for validating low-throughput samples in large populations. The QASM assay demonstrated excellent analytical sensitivity, specificity, and repeatability for measuring individual CpG sites. Hence, the QASM test is employed to ascertain the methylation status of individual CpGs, not only in CpG-poor regions but also in CpG-rich regions or repetitive sequences, provided that the probes are precisely positioned over the target CpG and undergo selective amplification [38].

7.5. Methylation by mass spectrometry

Mass spectrometry (MS) is now an essential tool in molecular and cellular biology because of its exceptional specificity, sensitivity, and efficiency as well as its capability to provide precise molecular weight and structural data [39]. This method has demonstrated significant promise and widespread use in the identification, characterization, and quantification of proteome and metabolome [40]. Although MS-based DNA/RNA analysis has been used to determine PCR amplicons and identify SNP, its application for detecting DNA/RNA oligomers has not been investigated extensively. A probable explanation is inclination for the DNA/RNA phosphodiester backbone to break into smaller fragments because of ionization processes, which are influenced by size of the molecules. This trend decreases the ionization efficiency of larger DNA/RNA oligomers, resulting in reduced sensitivity and mass resolution when attempting to directly detect these oligonucleotides [41].

In recent years, there has been a growing use of MS detection for analyzing nucleobases in damaged oligonucleotides. This method is used to study DNA/RNA damages, adducts, and epigenetic alterations. The utilization of MS tests for nucleobases, as opposed to DNA/RNA oligomers, has opened up new opportunities for achieving the level of sensitivity necessary for routine DNA/RNA detection. A typical workflow for this approach, involving hydrolysis of DNA to its constituent nucleosides for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, is shown in Figure 1. However, these methods are generally prioritized for the comprehensive examination of entire genome (global methylation), rather than specific

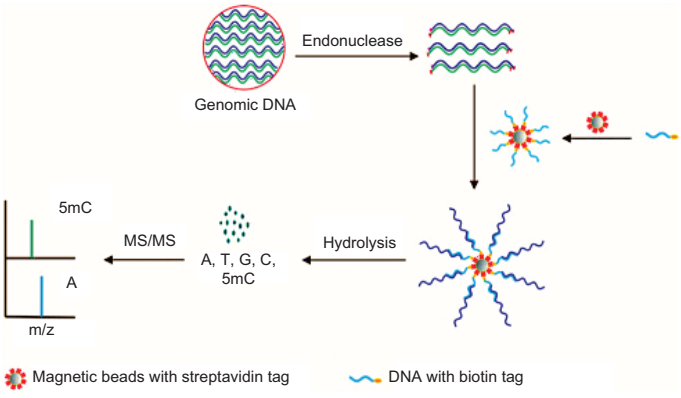

Figure (1): Schematic workflow for quantifying global DNA methylation using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Genomic DNA is fragmented by endonucleases and then hydrolyzed into individual nucleosides. This process releases standard deoxyribonucleosides (deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine) and the epigenetic marker 5-methyl-2'-deoxycytidine (5mC). The mixture is then separated by LC and analyzed via MS/MS. The specific and quantitative nature of MS/MS allows for the precise calculation of the global methylation level based on the ratio of 5mC to total cytosine.

Table (2): Summary of approaches and strategies for DNA methylation analysis.

Method	Principle/mechanism	Applications	Advantages	Limitations
DNA methylation microarrays	Uses methylation-sensitive restriction enzymes; fragments hybridized on CpG arrays (e.g., Illumina 450K)	Epigenetic age prediction, and tissue source ID (saliva, semen, and blood)	Cost-effective for large-scale analysis; targets many CpG sites	Limited to enzyme recognition sites; can't quantify low/high methylation accurately
Bisulfite genomic sequencing (WGBS)	Sodium bisulfite converts unmethylated cytosine to uracil; sequenced to detect methylation at base level	Genome-wide methylation profiling; stem cell studies, PMDs, LMRs, and DMVs	Highest resolution; single-base accuracy; >90% CpG coverage	Expensive; requires large DNA amounts and deep sequencing
Bisulfite pyrosequencing	Bisulfite-treated DNA analyzed by sequencing-by-synthesis; detects artificial C/T SNPs	Site-specific methylation quantification; aging and disease biomarker detection	Accurate, reproducible, real-time, and multiple-sample compatible	Primer design is complex; risk of PCR bias; moderate throughput
Quantitative methylation-specific PCR (QM-MSP, QASM)	Two-step PCR + real-time fluorescence detection; targets methylated/ unmethylated sequences	Gene panel methylation (cancer, biopsy samples); CpG-specific methylation analysis	High sensitivity/ specificity; works on small clinical samples; scalable	Requires careful design; less suited for genome-wide analysis
Methylation mass spectrometry	Detects modified nucleobases with high-resolution MS; avoids direct DNA/RNA oligomer analysis	Detection of DNA/RNA damage, adducts, and epigenetic marks	Very specific and sensitive; useful for nucleobase profiling	Not suitable for full sequences; complex instrumentation; ionization limits
Bar-coded bisulfite amplicon sequencing (BBA-Seq)	Uses nested PCR + illumina barcoded adapters on bisulfite-treated DNA	Epigenetic aging, sample tracking, and passage estimation	High coverage (~3,900×); allows correlation studies; flexible data analysis	Complex data analysis; requires bioinformatics tools (TrimGalore, Bismark, R/Python)

DNA/RNA sequences [42,43]. Direct mass spectrometric analysis of oligonucleotides is ineffective for epigenetic mapping, as it cannot accurately detect the location of histone PTMs or DNA methylation in polymers, as it cannot distinguish between isomeric peptides and oligonucleotides differing only at modification site [44].

7.6. Bar-coded bisulfite amplicon sequencing

Converted DNA treated with bisulfite was utilized for a nested PCR. The second PCR incorporated barcoded Illumina adapters, enabling the differentiation of donors and passages. The amplicons were combined and subjected to sequencing using the Illumina MiSeq platform with v2 nano reagents in 250 paired-end mode. The bisulfite-converted sequencing data were examined using the software tools TrimGalore, Bismark53, and bowtie254. The average sequencing coverage of amplicons was around 3,900 reads per amplicon. Additional pattern analysis and visualization were conducted using customized Perl (Practical Extraction and Report Language) and R scripts as well as the R tool ggplot2 for generating area plots. The Pearson correlation between adjacent CpGs was calculated using the scipy program in Python. The resulting correlation values were then shown as a heatmap using the seaborn utility. Simply said, the BBA-Seq amplicons were analyzed to determine their most probable passage number (ranging from 0 to 50) by examining the sequence of methylation and unmethylated CpG sites. The probabilities of passing numbers were determined using linear regression models for each CpG site, obtained from the training dataset (Table 2). Finally, the mean passage number for each sample was computed using all sequencing reads [45–47].

8. Conclusion

The convergence of epigenetics and forensic science marks a transformative moment in modern investigative biology. As we delve deeper into the dynamic layers of gene regulation, we uncover a powerful toolkit for understanding not just who someone is but also what they have become through environmental influence, age, lifestyle, and more. This review traces the intricate pathways of DNA methylation, histone modifications, and their profound implication in forensic context from predicting a suspect's age or physical traits to identifying tissue origin in trace biological evidence.

Despite the remarkable potential, the road ahead is paved with both promises and challenges. The sensitivity of epigenetic methods to DNA quality and quantity presents a technological bottleneck, especially when working with degraded forensic samples. Current analytical tools, while robust in controlled laboratory settings, must evolve to meet the stringent demands of real-world forensic applications. Nonetheless, with the accelerating pace of innovation spanning microarrays, bisulfite sequencing, mass spectrometry, and high throughput barcoding, there is optimism that forensic epigenetics will soon shift from experimental possibility to standardized practice.

In essence, epigenetics does not merely add a new layer to forensic science, it also redefines it. By embracing the nuances of gene expression as forensic identifiers, we open the door to a future where justice is not just served by fingerprints and short tandem repeat (STR) profiles but also by the very signatures life writes onto our genome.

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