Forensic Epigenetic Analysis: The Path Ahead

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Significance of the Study

- Epigenetic modifications of the genome are induced by several factors resulting in epigenomic variations, also referred to as epigenetic fingerprinting. Epigenomic variations have an impact on human developmental processes; alterations in DNA methylation patterns lead to several human genetic disorders, including various forms of cancer, and cause visible phenotypic changes. Multiple cell/tissue-specific, lifestyle-related epigenetic markers have been identified which have significant roles in forensic studies. This review describes the importance of epigenetic modifications in forensic medicine and highlights the requirements and the limiting factors of forensic epigenetic techniques for DNA methylation profiling analysis.

Keywords

Forensic medicine · DNA methylation · Epigenetic fingerprinting

Abstract

Unlike DNA fingerprinting, which scores for differences in the genome that are phenotype neutral, epigenetic variations are gaining importance in forensic investigations. Methylation of DNA has a broad range of effects on the life-style, health status, and physical appearance of individuals. DNA methylation profiling of forensic samples is useful in determination of the cell or tissue type of the DNA source and also for estimation of age. The quality and quantity of the biosample available from the crime scene limits the possible number of DNA methylation tests and the selection of the technology that can be used. Several techniques have been used for DNA methylation analysis for epigenetic in-

vestigations of forensic biological samples. However, novel techniques are needed for multiplex analysis of epigenetic markers as the techniques that are currently available require a large amount of high-quality DNA and are also limited in their multiplexing capacities that are often insufficient to fully resolve a forensic query of interest.

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Introduction

Ever since the discovery of DNA fingerprinting by Jeffreys et al. [1] using human myoglobin gene-based probes, applications have embraced all living forms besides humans, such as plants of economic importance, extinct animals, human pathogens, etc. [2–6]. Lately, the interest in forensic epigenetics has increased significantly given the emerging value of epigenetic variations in resolution of

Fig. 1. Several environmental factors affect the methylation status of the genome, resulting in individual epigenomic variations referred to as the epigenetic fingerprint. Epigenetic fingerprints can have a wide range of phenotypic associations such as age, individual lifestyle, health status, physical appearance, alcohol intake, etc., which help to resolve forensically relevant questions.

questions of forensic relevance [7]. Epigenetic modifications are among the most important heritable alterations in the DNA. The epigenetic process does not change the DNA sequence; it involves DNA base modifications such as cytosine 5-CpG-3 methylation and posttranslational histone modifications such as histone H3 methylation or acetylation [8]. It is involved in the regulation of gene expression as a response to brief or prolonged exposure to various environmental agents [9]. The methylated CpG islands near gene promoters make it compact and inaccessible to transcription factors leading to inactivation of the gene [10]. Epigenetics has a wide role in human development and it is a key regulator of genomic imprinting and X-chromosome inactivation. Misprogramming of epigenetic regulation and aberrant DNA methylation of key regulatory genes affect several human diseases. DNA methylation aberrations have been implicated in various forms of cancer and in numerous developmental disorders such as fragile X syndrome [11], Prader-Willi syndrome, Angelman syndrome [12], and Beckwith-Wiedemann syndrome [13].

Epigenetics is also relevant in the forensic field; the DNA methylation levels adjusted across the genome, as a response to various brief or prolonged environmental stimuli, result in individual epigenomic variations referred to as epigenetic fingerprint [14]. Compared to other epigenetic modifications such as chromatin structure changes or histone modifications, DNA methylation is preferred in forensics for both in vitro stability and high sensitivity in terms of the amounts of DNA required. In forensic investigations, differential DNA methylation profiles have been analyzed for tissue/cell type identification [15], sex determination [16], and estimation of age [17] and also to differentiate between monozygotic twins [18]. In this review, we discuss the importance of DNA methylation in forensic studies, as well as the requirements and the limiting factors of forensic epigenetic techniques, including the most important advantages and disadvantages, for DNA methylation profiling analysis.

Table 1. Advantages and disadvantages of various techniques for DNA methylation profiling analysis

DNA methylation technique	Advantages	Disadvantages
DNA methylation microarrays	Cost effective Easy to perform	Qualitative/semiquantitative No single site resolution Large amounts of sample DNA Sequence fragmentation may exclude some CpG sites
DNA methylation bisulfite genomic sequencing	Informative and precise Requires very small amounts of DNA FFPE-DNA is possible	Requires cloning of PCR products Large numbers of clones are required (≥ 15) Labor intensive Low-throughput analysis
Pyrosequencing	Highly quantitative Single-site resolution Fast run times	Short amplicons (≤ 500 bp) Dedicated and expensive equipment Problems with high-density CpG
Methylation quantitative PCR	Sensitive Quantitative and qualitative Equipment is easily accessible Can be multiplexed	Low precision No single-site resolution
Methylation mass spectrometry	Quantitative Single-site resolution, FFPE-DNA is possible Short amplicons (≤ 500 bp) Multiplexing capacity (limited)	Dedicated equipment High costs Sequence fragmentation may exclude some CpG sites
Methylation-sensitive single-nucleotide primer extension	High throughput Rapid quantitation of cytosine Methylation	Use of radioactive isotope labeling
Bar-coded bisulfite amplicon sequencing	Cost effective Less labor intensive High level of multiplexing is possible High-throughput capabilities	Possible PCR bias Bias in the DNA fragmentation and linker ligation

DNA Methylation in Forensics

Methylation of DNA has a broad range of effects on the individual lifestyle, health status, physical appearance, etc. [19]. It can also reveal information related to one's socioeconomic status, diet, physical activity, alcohol consumption [20], drug intake, and smoking status [21]. For the purpose of identifying the source of a biological trace left at a crime scene, DNA methylation levels have been found to be useful for estimation of age [17] and prediction of visible characteristics (such as hair, skin, and eye color) [22, 23] to narrow down the pool of suspects related to a crime (Fig. 1). Innovative methods have been developed by forensic epigeneticists for profiling and analysis of DNA methylation (Table 1). However, there are challenges given the quantitative outcome of the epigenetic analysis.

Requirements for Forensic Epigenetic Analysis

The limiting factor in the progress of forensic genetics and forensic epigenetics is the amount and quality of DNA recovered from a crime scene. The availability of multiple epigenetic markers and convenient technology for multiplex analysis of a large number of markers is also critical for forensic epigenetic advancement.

Epigenetic Biomarkers for Forensic Trait/Tissue Identification

For forensic trace analysis, there is a need for forensic trait-specific multiple DNA methylation markers. Forensic traits refer to anything which could strongly affect the methylation pattern of genomic DNA, e.g., lifestyle, aging, diet, physical activity, alcohol, drug intake, smoking, etc. [17–21]. The available epigenetic biomarkers have been reported to be overlapping with the biomarkers of other characteristics. One example is estimation of age

using methylation-specific biomarkers, which reveals intersecting information about the sample source related to health- or other lifestyle-related information [24]. More specific epigenetic biomarkers for the accurate prediction of visible characteristics are needed as the biomarkers presently available are not sufficient to explicitly envision the source of the sample.

Quality and Quantity of the Forensic Trace

Different methods for DNA methylation analysis demand large amounts of high-quality DNA, whereas the forensic biological samples available at crime scenes are extremely limited. Currently available technologies, such as DNA methylation microarrays and whole-genome bisulfite sequencing, require a large amount of high-quality DNA [25]. More advanced technologies for epigenetic marker analysis that are able to deal with a small amount of DNA or low-quality DNA, such as pyrosequencing and EpiTYPER[®], are limited in their potential of fewer than 20 markers by multiplex analysis [26]. The amount and quality of DNA recovered from crime scene traces is one of the important keys to resolving a forensic question of interest. Several of the techniques for DNA methylation analysis discussed below require >1 µg of high-quality DNA from cultured cells or fresh tissue samples for reliable results. The available DNA sample may be degraded during the bisulfite treatment for unmethylated cytosine conversion to uracil and it may further reduce the number of intact DNA molecules accessible for PCR analysis. Accordingly, the low quality and limited quantity of starting DNA molecules may result in stochastic amplification, which does not accurately reflect the distribution of methylation in the original DNA sample. For accurate DNA methylation analysis of forensic DNA samples, there is a need for more advanced methods for multiplex genotyping for the immediate simultaneous analysis of multiple epigenetic markers as analysis of every single epigenetic marker is time consuming and requires more DNA.

Sample Source Cell/Tissue Type

Prior to the analysis of any forensic traits mentioned earlier, the identification of cell or tissue type using genomics or epigenomics provides useful information for crime scene investigations as crime scene traces can consist of different types of cells. Information on the cell or tissue type would be beneficial before the selection of epigenetic markers for forensic trait analysis [27]. Several multiplex test assays have been developed for the identification and validation of tissue-specific epigenetic marker analysis. Several examples of DNA methylation mark-

ers analysis for cell and tissue type identification include validation of a set of 9 DNA methylation markers for the identification of blood, saliva, semen, vaginal fluid, and menstrual blood [28]. Similarly, using the Illumina 450K database, Lin et al. [29] identified a set of 8 tissue-specific epigenetic markers. In addition to that, Vidaki et al. [30] identified 2 novel semen-specific CpG markers (cg04382920 and cg11768416) for epigenetic forensics of samples derived from sexual assaults.

Forensic epigenetic methods have to perform equally well in all forensically relevant cell or tissue types as some DNA methylation sites are differentially methylated between different tissues, which is a point for consideration when applying previously established predictive marker sets with different tissue origin [20, 21].

Techniques for Forensic Epigenetics Analysis

The currently available technologies for analysis of DNA methylation of multiple CpG in a quantitative single-nucleotide resolution and high-throughput manner demand high-grade quality DNA and a large number of epigenetic markers. These are described below.

DNA Methylation Microarrays

DNA methylation microarray is a high-throughput screening method that utilizes methylation-sensitive restriction enzymes to profile methylated fragments and interrogate them to a CpG island microarray. The principle of differential methylation hybridization, the first array-based method, was described by Huang et al. [31] for genome-wide screening of hypermethylated CpG islands in tumor cells. Genomic DNA is first sheared with any methylation-insensitive restriction enzyme, such as *MseI*. The restriction enzyme *MseI* is unlikely to interfere with any CpG islands as the restriction recognition site is TTAA. After digestion with *MseI*, the adapters are ligated to the end of each DNA fragment and treated with methylation-sensitive enzymes, i.e., *BstUI* and *HpaII*. The resulting methylated fragments remain intact and are amplified by PCR with primers specific to the ligated adapters. After differential labelling, the amplicons are cohybridized to a CpG island array [32]. Hong et al. [33], used a small sample size ($n = 54$) and identified 6 age-associated CpG candidates (cg00481951, cg19671120, cg14361627, cg08928145, cg12757011, and cg07547549) in saliva, based on the Illumina 450K microarray platform. For the identification of blood, saliva, semen, vaginal fluid, and menstrual blood, Forat et al. [28] identified 150 candidate tissue-specific markers based on Illumina Human Methylation 450K Beadchip microarray data. DNA methylation microarrays

offer inexpensive and consistent analysis of many biologically relevant genomic regions. Besides the requirement of large amounts of high-molecular weight DNA, the other limitation to the technology is that the methylation-sensitive restriction enzymes do not interrogate every cytosine. This method is rather imprecise in terms of the higher limit of the fragments that are subjected to hybridization. The limited availability of informative restriction sites may be critical in forensic sample analysis when the phenotypic outcomes are determined by a methylation change at an isolated CpG that is not within the restriction site of a methylation-sensitive restriction enzyme. Additionally, because of the qualitative nature of the assay, DNA methylation microarrays cannot reliably distinguish low levels of CpG methylation from high levels of CpG methylation.

DNA Methylation Bisulfite Genomic Sequencing

DNA methylation bisulfite genomic sequencing is regarded as the gold standard for efficient detection of 5-methylcytosine at a single base pair resolution. In this method, treatment with sodium bisulfite converts unmethylated cytosine residues into uracil residues in single-stranded DNA, and the methylated cytosines are immune to this conversion. The converted cytosine is recognized as thymine in subsequent PCR amplification using specific methylation primers allowing methylated cytosines to be distinguished from unmethylated cytosines. The amplified PCR products are cloned before analysis of the methylation status through sequencing. All of the cytosine residues in the sequence represent previously methylated cytosines in the genome [34]. To produce the methylation maps of a single PCR amplicon, the traditional method of bisulfite sequencing relied on Sanger sequencing but advancements in next-generation sequencing techniques have enabled sequencing of the entire bisulfite-treated genome and it is much faster and cheaper. All of the upcoming technologies for DNA methylation detection discussed further in this article are extensions of bisulfite-based methods.

Bisulfite Pyrosequencing

Bisulfite pyrosequencing, another simple and easily available technique for forensic laboratories, also involves bisulfite conversion of the DNA strand using specific primers for amplification of the target gene region, and real-time DNA sequencing based on sequencing-by-synthesis technology. During nucleotide incorporation by DNA polymerase into a growing DNA strand, the released inorganic pyrophosphate (PP_i) reacts with APS in the presence of ATP sulfurylase, giving rise to ATP. Further,

in the presence of ATP and the substrate luciferin, the enzyme luciferase produces oxyluciferin which produces visible light that can be detected by a built-in CCD camera. Subsequently, the sequence and allelic contribution is depicted as a quantitative Pyrogram. Any unused ATP and unincorporated nucleotides are degraded by the enzyme apyrase prior to the release of the next nucleotide. This method allows detailed and high-resolution analysis of DNA methylation at specific CpG sites. Due to its single-CpG resolution, its highly quantitative nature, and its sensitivity, this technique is by far the most popular for forensic epigenetic analysis [35]. It has been applied successfully for analysis of small genomic regions (50–100 bp) that can contain multiple adjacent CpG sites. Initial developmental validation studies have shown that singleplex bisulfite pyrosequencing assays are very sensitive (down to 50–100 pg of DNA input, depending on the locus) and applicable to old stains and simulated case samples [36]. Pyrosequencing uses a high-throughput platform that can interrogate many CpG sites within an amplicon in real time. The incomplete conversion of bisulfite results in potential false-positive artifacts which may significantly impact the biological interpretation of assay outcomes. The advantages of the pyrosequencing method are the long read sizes and the fast run times. However, the disadvantages include its high cost and poor sensitivity.

Methylation Quantitative PCR

Methylation quantitative PCR, a real-time PCR assay, is a bisulfite treatment-based method for detection and quantitation of DNA methylation in the genomic DNA. These sequence differences are detected by fluorescence-based quantitative PCR using sequence-specific primers and a sequence-specific intervening probe which binds the amplicon. Methylation-quantitative PCR is a highly sensitive and quantitative assay to study the methylation level of entire PCR fragments that may contain several CpG [37]. The real-time qPCR method is one of the commonly used methods in forensics for quantitative and qualitative assessment of human-specific genomic DNA. Forensic epigenetic qPCR assays come in different variants, like SYBRTM Green-based qPCR, which is an epigenetic qPCR assay that targets 1 or a few CpG sites in the primer binding region and is carried out as single-plex reactions [37]. On the other hand, another forensic epigenetic qPCR assay, i.e., the TaqManTM probe-based assay, is more sensitive and, by using different fluorescent dyes, it can be multiplexed [38].

The use of droplet digital PCR (ddPCR) to detect DNA methylation has recently emerged as an advancement

over qPCR for DNA methylation detection. The ddPCR method is based on sample partitioning into 20,000 nanolitre-sized water-oil emulsion droplets, by randomly distributing a single sample, such that each droplet acts as a separate PCR reaction. Each droplet is then separately assessed and scored as positive or negative for fluorescence. ddPCR uses absolute quantification instead of the relative quantification used by qPCR. The Poisson limit theorem allows estimation of the starting number of targets per droplet distribution [39]. Van Wesenbeeck et al. [39] analysed 2 separate CpG sites in a set of colon adenoma FFPE samples with both the qPCR and the ddPCR methods and showed that qPCR and ddPCR assess methylation status equally well with a high DNA input. However, methylation detection is more accurate using ddPCR in low-input samples. In this procedure, methylation-independent primers will allow the detection of methylation which is independent of bisulfite conversion efficiency. Additionally, due to the accessibility of the ddPCR technology and its accuracy in high- as well as low-DNA input samples, this method could be used for studies involving degraded or stored forensic DNA samples.

Methylation Mass Spectrometry

The Agena Bioscience MassARRAY EpiTYPER® is a mass spectrometry-based method to assess DNA methylation of multiple CpG in genomic regions of 200–600 bp in a quantitative, single-nucleotide resolution and high-throughput manner. For region-specific bisulfite sequencing, this method uses matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for detection and quantitation of DNA methylation [40]. Bisulfite treatment of genomic DNA produces methylation-dependent sequence variations of cytosine to thymine (C/T) in the PCR amplified products. Subsequently, in the cleavage products generated from the reverse strand, these C/T variations appear as G/A variations by base-specific cleavage. The outcome of these G/A variations results in a mass difference of 16 Da per CpG site, which is detected by the MassARRAY system. Furthermore, the relative amount of methylation can be calculated by comparing the signal intensity between the mass signals of the methylated and nonmethylated templates in the mass spectrum. MassARRAY technology has gained popularity among forensic epigenetic protocols. Using EpiTYPER® MassARRAY technology, Freire-Aradas et al. [41] analyzed DNA methylation levels in a total of 177 CpG in 22 candidate genomic DNA in 725 European individuals and built a novel age prediction model. The significant advantage of this method is that the PCR

primers are not restricted to the methylation state of the genomic DNA; they bind independently to both methylated and nonmethylated DNA templates although sequence fragmentation may exclude some CpG sites.

Methylation-Sensitive Single-Nucleotide Primer Extension

The methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) technique is similar to other methylation quantitative assays that use treatment of genomic DNA with sodium bisulfite to discriminate unmethylated cytosines from methylated ones to generate a DNA template for quantitative methylation analysis for rapid quantitation of methylation at individual CpG sites [42]. The primers in the Ms-SNuPE method are designed to hybridize sequences upstream and terminate immediately 5- to the CpG site being interrogated. Once the primer has annealed to its appropriate target DNA sequence, a single-nucleotide extension reaction is performed in the presence of a DNA polymerase and an appropriate radiolabelled dNTP. The reaction products are electrophoresed on polyacrylamide gels for visualization and then quantitated by phosphorimager analysis for high-throughput methylation interrogation for a wide range of applications. The Applied Biosystems SNaPshot technology is an adapted version of the Ms-SNuPE technique which uses nonradioactive labelling for quantitation of methylation at individual CpG sites.

Ms-SNuPE can be used for high-throughput methylation analysis and rapid quantitation of cytosine methylation. This technique requires small amounts of DNA, and in a single primer extension reaction the methylation status of several CpG sites can be determined simultaneously using multiple oligonucleotides [43]. Limitations of Ms-SNuPE include the use of radioactive isotopes with analysis platforms for methylation quantitation and the number of CpG sites that can be measured (~2–4) per reaction.

Bar-Coded Bisulfite Amplicon Sequencing

A more advanced bisulfite-based technique, i.e., bar-coded bisulfite amplicon sequencing (BBA-seq) is the simplified version of the targeted bisulfite next-generation sequencing which might be suitable for forensic epigenetic applications including forensic epigenetic marker analysis. Like any other bisulfite-based assay, the first step in this method is sodium bisulfite treatment of genomic DNA, which converts unmethylated cytosines to uracils, while methylated cytosines are protected. Furthermore, uracils are amplified and sequenced as thymine residues in subsequent PCR amplification reactions. The BBA-seq proce-

dures are completed in 2 PCR cycles. The first PCR cycle is dedicated to target enrichment to amplify regions of interest from bisulfite-converted genomic DNA. The primers used for target enrichment are designed to have overhangs with partial linker sequences that are subsequently used to amplify barcoded libraries in the second round of the PCR cycle. Target enrichment PCR products of the first PCR cycle for each sample are pooled prior to a second round of the PCR cycle to concomitantly add the same multiplexing indices to all amplicons of interest. After sample barcoding, all PCR reactions of the second-round PCR cycle are purified and pooled for sequencing on the Illumina MiSeq [44]. Bernstein et al. [45] reported that the BBA-seq method performed DNA methylation quantitation with high precision and accuracy compared to a traditional bisulfite next-generation sequencing approach. DNA methylation was measured using both methods and nearly identical results were obtained at the H19 locus in mouse genomic DNA [45]. This sequencing approach is more cost effective and less labor intensive and provides insights into the variation of DNA methylation of neighbouring CpG sites on the same DNA strands. Additionally, a high level of multiplexing is possible with this method for analysis of the CpG in multiple cell preparations when compared to the traditional methods. Because of its multiplexing abilities and high-throughput capabilities this method will be valuable in forensic epigenetic studies for investigating methylation changes in selected epigenetic markers where base resolution and high quantitative accuracy are required. However, limitations to the BBA-Seq method may arise from either the original bisulfite-specific PCR bias or bias in the DNA fragmentation and linker ligation. Future improvements of the protocol to overcome these limitations will be beneficial as studies on epigenetics progress.

Conclusion

Epigenetic patterns are dynamic and get altered as a response to the environment and/or biological processes. Application of epigenetic analysis for forensic investigations has attracted the attention of forensic researchers in the past few decades. Epigenetic applications are new and currently limited in their ability to multiplexing of epigenetic markers. Multiplexing will yield information about multiple epigenetic markers/target loci for multiple DNA samples as analysis of a single epigenetic marker typically does not give enough forensically relevant information and analysis of every single epigenetic marker is time consuming and requires larger amounts of DNA.

Analysis of DNA methylation is by no means a trivial task, and no single technique fulfils all of the criteria for generating unambiguous data on DNA methylation. Some of these methods are technically challenging, and the choice of the DNA methylation procedure will often depend on the available equipment and expertise. Identification of epigenetic signatures of lifestyle, multiple tissue-specific, age-related, disease-associated epigenetic biomarkers, and more advanced methods for extraction of a high quality and quantity of DNA recovered from crime scene traces are needed for comprehensive analyses of forensic epigenetics to resolve a crime and identify unknown perpetrators of a crime.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interests to declare.

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