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FORENSIC ASPECTS OF DNA METHYLATION: A REVIEW

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ABSTRACT

Epigenetic mechanisms include DNA methylation, histone modification and chromatin remodeling and play an important role in the regulation of gene expression. Among these, DNA methylation is widely studied and emerging field providing leads to solve criminal cases in the field of forensic genetics. DNA methylation in eukaryotes involves the addition of a CH_3 (methyl) group to the cytosine base of DNA in the presence of enzyme DNA methyltransferase and S-adenosylmethionine as methyl group donor. These epigenetic changes occur without interfering with the sequence of base pairs in the DNA. Methylation occurs at the cytosine base which is immediately followed by guanine base which is called CpG dinucleotide sites. Several factors can affect methylation of DNA, including ageing, lifestyle, sex, disease, environmental factors, etc. Differential DNA methylation patterns can be used by forensic scientists to predict the age, sex, body fluid/tissue type, to differentiate between monozygotic twins, and to identify species from biological evidences recovered from crime scenes. The present review article focuses on how DNA methylation occurs, various factors affecting DNA methylation and its applications in the field of forensic science.

Keywords: Epigenetics, DNA Methylation, Gene, CpG sites, tDMRs, Forensic Science.

INTRODUCTION

Epigenetics is a broad term that includes various modifications to the genome, which are reversible and play a significant role in the regulation of gene expression without changing the nucleotide sequence of DNA (Kader and Ghai, 2015). According to Waddington, epigenetics can be defined as "a set of interactions between genes and the surrounding environment, which determines the phenotype of physical traits in an organism" (Sweatt *et al*., 2013; Tatarinova

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and Kerton, 2012). Many other biologists and cancer researchers, have defined epigenetics as "meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself" (Sweatt *et al.,* 2013). Various modifications to the genome which falls under epigenetics are: DNA methylation, histone modification and chromatin remodeling (Tammen *et al.,* 2013). The present article is focused on DNA methylation markers and their significance in the field of forensic science since these epigenetic markers play an imperative role in the study of gene expression. These markers are heritable, can last for generations and also expressed in the phenotype of an individual (Richards, 2006).

DNA methylation involves the addition of a methyl group to the 5´position of cytosine within DNA sequence by the enzyme DNA methyltransferase and the methyl group is donated by S-Adenosylmethionine (SAM) for this reaction. After methylation, cytosine is converted into 5-methylcytosine (5-mC) (Auclair and Weber, 2012; Jeltsch, 2002; Kader and Ghai, 2015; Rawat and Kushwaha, 2016; Tammen *et al.,* 2013; Vidaki and Kayser, 2018). Approximately 3% of DNA is methylated in the human genome. The addition of methyl group to only one strand of the double-stranded DNA is called hemi-methylation and addition to both the strands is called full methylation. In the human genome, methylation commonly occurs in CpG dinucleotides but it is also reported outside of these CpG dinucleotides (Kader and Ghai, 2015; Tammen et al., 2013). These CpG sites can be defined as regions of DNA where cytosine base is immediately followed by a guanine base (Li and Zhang, 2012). Approximately 30 million CpG dinucleotides are reported in the human genome which may be methylated or unmethylated and nearly 60-90% of CpG sites are methylated. Unmethylated sites are referred as CpG islands because they are grouped in regions and usually present around the regulatory region of many genes (Kader and Ghai, 2015; Tammen et al., 2013; Vidaki *et al.,* 2013). It is observed that DNA methylation of CpG island located at the promoter region of gene can cause silencing of gene expression. Some studies also proposed that DNA methylation has no correlation with the regulation of gene expression (Kader and Ghai, 2015).

It is a natural phenomenon or it can be induced by various factors like ageing, smoking habits, lifestyle and environmental exposure etc. (Vidaki *et al.,* 2013). Studies related to DNA methylation can be used for the diagnoses and treatment of complex diseases like cancer, diabetes, and neurological disorders. It is the most commonly used phenomenon among other epigenetics in leading forensic investigations, which gives rise to forensic epigenetics. Forensic epigenetics includes the prediction of age, sex, body fluid identification, differentiating monozygotic twins, and also determination of tissue by DNA methylation profiling (Rawat and Kushwaha, 2016; Vidaki and Kayser, 2017). The features of DNA methylation which are useful for forensic investigations are differentially methylated regions (DMR) and methylation variable position (MVP) (Lee *et al.,* 2016).

FACTORS AFFECTING DNA METHYLATION

DNA methylation is substantially affected by a variety of endogenous and exogenous factors for example diets/nutrition, ageing, environmental exposures, and lifestyle.

Age

Several studies have been carried out in different regions, that has proven the association of hypo and hyper DNA methylation with ageing. Generally, it is observed that there is decrease in DNA methylation in human genome but certain promoter genes show hypermethylation (Kader and Ghai, 2015; Tammen et al., 2013; Vidaki and Kayser, 2018). Studies performed by Bjornsson *et al.* (2008) found that there are changes in DNA methylation within same individual with respect to time. Boks et al. (2009) performed age related studies on DNA methylation level of 280 high quality probes and they observed that 58 probes were significantly related to age. Christensen et al. (2009) performed studies on 119 solid tissues and found that there is high correlation of CpG island with age and methylation. In this study, authors observed that loci present in CpG islands get methylated with age and loci outside of that lost methylation with age.

Nutrition and Diet

Dietary factors play a vital role in the field of epigenetics. It has been observed that several bioactive food components in diet of an individual can affect gene expression by altering DNA methylation (Tammen *et al*., 2013). A number of dietary bioactive components have been identified that has potential effect on epigenetics (Hardy and Tollefsbol, 2011). During the process of DNA methylation methyl group is donated by SAM and availability of SAM is determined by one-carbon metabolism (Hardy and Tollefsbol, 2011; Tammen *et al.,* 2013; Kader and Ghai, 2015). This pathway required vitamin B_6, $B_{12,}$ betamine, folate and choline along with some amino acids which are methionine, cysteine, glycine and serine. If any of these components of pathway is missing, there is alteration of DNA methylation (Tammen et al., 2013). There are several other bioactive components which alter epigenetic mechanisms in different ways like epigallocatechin-3-gallate (EGCG), a polyphenol found in green tea, curcumin found in curry and resveratrol found in grapes, they all can reduce global DNA methylation in cancer cell line by competitive inhibition of DNA Methyltransferase (Hardy and Tollefsbol, 2011; Tammen *et al.,* 2013). Studies performed by Davis *et al.* (2000) on Caco-2 cells (in vitro) and rat liver and colon (in vivo) proposed the effect of dietary selenium and arsenic on DNA methylation. Zhang and Chen (2011) in their research article mentioned that genistein which is soy-derived bioactive isoflavones, activates genes which suppress tumor causing genes by modulating chromatin configuration and DNA methylation which finally affects cancer cell survival.

Life style

It has been observed that lifestyle of an individual can alter epigenetics mechanisms. For example, there are few evidences that tobacco smoking may be related to DNA methylation (Lim and Song, 2012). Tobacco smoke is carcinogenic and is also associated with disease like cardiovascular disease and other chronic respiratory problems. Tobacco smoke cause hypomethylation in both utero and adult exposure. Genes that are targeted by tobacco smoke include cancer, cell cycle and metabolism related genes (Martin and Fry, 2018). So many individuals are habitual of consuming alcohol regularly, this can also alter epigenetic mechanism. Alcohol ingestion can inhibit folate-mediated methionine synthesis, due to which there is deficiency of methionine which is an active component of one-carbon pathway for synthesis of SAM (Mason and Choi, 2005).

Environmental Factors

A plethora of work has been carried out which described the effect of environmental factor on epigenetics. Exposure to environmental toxins and carcinogenic agents may lead to alteration in DNA methylation. There are few examples such as: Aflatoxin B_1(AFB_1) which is a mycotoxin and contaminate food such as corn, peanuts, and grain. Wu *et al*. (2013) performed experiment to examine the relation between AFB_1 exposure and global DNA methylation. They concluded that exposure of AFB_1 has epigenetic effect on hepatocellular carcinoma development and also on alteration in DNA methylation (Martin and Fry, 2018). Air pollutants include a wide variety of toxic substances like, sulfur oxides, nitrogen oxides, ozone, carbon monoxide and harmful chemicals which can alter epigenetic mechanism. Bind *et al.* (2012) performed experiment on 704 elderly men to demonstrate the effect of air pollutants on blood markers (fibrinogen, C-reactive protein, intercellular molecule-1 and vascular cell adhesion molecule). They observed an increase in gene expression which is due to alteration in DNA methylation level (Martin and Fry, 2018).

Forensic DNA Methylation assays

In forensic investigations, DNA is used as an indispensable tool for the absolute identification of the perpetrator. There are various methods of DNA Typing, evolving from RFLP technique to PCR – based techniques (Butler, 2005). In forensic science where the quality and quantity are limited, non- destructive methods are preferred. DNA methylation is a recent method which is used in forensic science for the dissemination of justice. DNA methylation profiling is an efficient, convenient, non-destructive, have high sensitivity, high specificity and can be used to analyze multiple tissue in a single assay (Kader and Ghai, 2015). Forensic biologist has to answer so many challenging questions by analyzing biological evidences to help investigation officer in solving crime. Few examples of such questions are: - discrimination between monozygotic twins, distinguish between undetectable body fluids and tissue samples, prediction of

age from biological samples, determination of sex from biological samples, etc. (Rana, 2018). Forensic biologists use methylation profile of DNA for answering questions which are discussed above (Kader and Ghai, 2015).

FORENSIC APPLICATIONS OF DNA METHYLATION

Detection and Identification of Body Fluids

Biological fluids such as semen, saliva, urine, sweat, vaginal secretions and blood recovered from the crime scene plays a vital role in forensic proceedings (Kader and Ghai, 2015). They link victim and perpetrator to the crime scene. Various DNA typing assays such as STR profiling are used to generate DNA profiles from the biological evidences recovered from the crime scene and compared it with the DNA profiles of the victim and the suspect (Goodwin et al., 2011). One such approach is DNA methylation profiling which can identify body fluids. The time period from which the biological fluid was deposited can also be estimated. One such attempt was made by Frumkin *et al.* (2011) in which successful differentiation of blood, semen, skin samples and saliva was made. In a study conducted by Choi *et al.* (2014) biological fluids can be identified by varying degree of methylation that are specific in nature. The authors described a new method of body fluid identification which includes analysis of tDMRs (Tissue specific differentially methylated regions) and identifying body fluid specific bacteria. Sugimoto et al. (2009) studied that maturation arrest (MA) patients has higher VASA TDMR methylation among patients in which VASA gene expression has undergone gene silencing. Staples (2020) described the use of five tissue specific methylated markers to differentiate saliva from other biological fluids by using chemometrics. Park et al. (2014) described that DNA methylation can be used to differentiate biological fluids and it was also termed as Next Generation Serology (NGS). Lee *et al.* (2012) described the use of DACT1, USP49, HOXA4, PFN3, and PRMT2 loci by bisulfate sequencing to differentiate vaginal fluid, menstrual blood, seminal fluid, blood and saliva. Similarly, Antunes *et al.* (2016) observed that vaginal fluid can be differentiated from other biological fluids by the use of bisulphite-modified pyrosequencing from PFN3A marker.

Sex Identification

Sex typing is also very important aspect of forensic investigation. The evidence recovered from the scene of crime is analyzed to identify sex. It is routinely done by STR assays by amplifying AMELX and AMELY markers in DNA (Butler, 2005). However, sex can also be predicted from varying DNA methylation patterns. It started from Naito and colleagues in 1993 by studying varying degrees of methylation at DXZ4 locus of the X-chromosome. Authors observed that inactive X chromosome shows hypomethylation and active X chromosome shows hypermethylation at DXZ4 locus. This is also applicable for the detection of sex reverse patients (Vidaki *et al.,* 2013). In a study conducted by Boks et al.

(2009), Illumina Golden Gate bead array was used for estimating sex from DNA methylation patterns. The authors observed that 56 autosomal probes were hypermethylated in females by Mann - Whitney U test on Illumina Golden Gate bead array. Lee *et al.* (2012) differentiated male-female by HOXA4 tDMR from saliva samples.

Age Prediction

Aging is a natural phenomenon occurs in all living organisms. This phenomenon can be characterized by progressive change in molecular, cellular and physiological function in organisms (Daunay *et al*., 2019; Rana, 2018; Vidaki and Kayser, 2018). Age prediction from biological evidences is very useful in forensics for identification of human remains in case of mass disaster and missing person. It is also useful to solve criminal as well as civil cases by limiting the range of search of suspect (Correia Dias *et al*., 2020; Lee *et al*., 2016). DNA methylation has been proven highly correlated epigenetic marker in human genome with respect to human aging process (Freire-Aradas *et al.,* 2020; Lee *et al.,* 2016). Other DNA biomarkers for age prediction are: telomere length, single joint T-cell receptor excision circle (sjTREC) rearrangements and mitochondrial mutations (Daunay *et al.,* 2019). Dias et al. (2020) performed experiment on 29 fresh bone samples (4 from females and 25 from males) and 31 tooth samples (23 from living and 8 from deceased) by using bisulphite Sanger sequencing method on methylation level of 43 CpG sites located at ELOVL2, FHL2, PDE4C, MIR29B2C and EDARADD genes. They observed that there is positive correlation of all CpG sites located at ELOVL2, FHL2, PDE4C genes with age and negative correlation of all CpG sites located at MIR29B2C and EDARADD genes with age. They also collect 31 fresh bone samples (26 males and 5 females) and 24 tooth samples (16 from living and 8 from deceased) and perform multiplex SNaPshot assay on methylation data obtained from 5 CpG sites located at ELOVL2, FHL2, MIR29B2C, TRIM59 and KLF14 genes. They observed positive correlation of CpG sites at ELOVL2, FHL2, TRIM59 and KLF14 genes with age and negative correlation of CpG sites located at MIR29B2C gene with age. Aradas *et al.* (2020) performed experiment on 84 blood samples and use four different technologies: EpiTYPER, MiSeq, Pyrosequencing and SNaPshot for detection of DNA methylation level for CpG sites located at three genes ELOVL2, FHL2 and MIR29B2C which are related to age. They observed that DNA methylation patterns and subsequent predictions of age from EpiTYPER, MiSeq and Pyrosequencing systems are comparable while SNaPshot system gives higher predictive error.

Authenticating DNA Sample

Forensic investigations which deal with DNA analysis, first of all, biological evidences are collected from the crime scene and then DNA is extracted by the DNA investigators to generate a DNA profile from it. It is generally believed that evidences containing DNA recovered from the scene of crime will be biological in origin. However, according

to Frumkin *et al*., 2010, in- vitro DNA can be easily contrived by any person and it can be mixed with other biological evidences at the scene of crime which can result into false positive identification and can result into conviction of innocent. Artificial biological samples can easily be synthesized such as blood, tissue, biological fluids, etc. (Kader and Ghai, 2015). Differentiating in-vitro and artificially synthesized DNA is a difficult task. In-vitro synthesized DNA by techniques such as Whole genome amplification (WGA) can be introduced at the scene of crime by anyone with malicious intent (Frumkin *et al.,* 2010; Melchior *et al.,* 2008). These DNA samples whether in-vitro or naturally occurring, cannot be differentiated by using commercial DNA typing assays. However, artificial and natural DNA can be differentiated on the basis of the DNA Methylation (Kader and Ghai, 2015). To determine the authenticity of the DNA, it can be subjected to DNA methylation assays by various ways such as sodium bisulphite treatment and then subjected to PCR amplification. DNA samples can be authenticated by identifying various tDMRs which are known to be hyper and hypomethylated. In a study conducted by Frumkin *et al.,* 2010, it was not possible to differentiate between in- vitro synthesized and natural DNA by normal DNA typing assays. The authors developed an authentication assay to differentiate naturally occurring and artificial DNA by examining DNA methylation patterns and concluded that artificially synthesized DNA will eternally be unmethylated whereas, naturally occurring DNA will have varying methylated patterns (Frumkin *et al.,* 2010).

Tissue Specific Differentially Methylated Regions (tDMRs)

The source of tissue determination plays an important role in forensic science. The tissue recovered from the crime scene is analyzed forensically to extract useful information. If the source of the tissue is known then it is easy to proceed further as different tissues will be treated differently to obtain useful information from it. Therefore, it becomes very important to determine the source of the tissue. Eckhardt et al. (2006) described that DNA methylation patterns are tissue specific, thus they serve as an excellent marker for the identification of body tissue. The authors generated methylation profiles by bisulphite DNA sequencing of chromosome 6, 20 and 22 from 12 different tissues. In humans, different tissues will have different patterns of methylation on the same chromosome (Choufani *et al.,* 2011**).** Frumkin et al. (2011) identified tissue with low DNA methylation gave low signal and that with high methylation gave strong signals in electropherogram. The determination of the source of the tissue evidence recognized at the crime scene is crucial in reconstruction of the events (An et al., 2012). Kader and Ghai (2015) reported that there are certain regions in the chromosome which are differentially methylated called tDMRs. These tDMRs shows different patterns of methylation on the basis of the type of tissue or cell.

Human Migration and Ethnic Communities

There are a large number of ethnic communities of human beings. They occur in various biographical distributions. There are various cases related to human

migration in forensic science. The migration pattern, ethnic community and geographical distributions can be established by DNA methylation assays (Race and Genetics Working Group, 2005). Different human races have different DNA methylation patterns. These differences are from the birth of an individual (Rana, 2018). Elliot et al. (2014) described that AHRR locus of the autosomal chromosome for European population is hypomethylated and for south Asians is hypermethylated. These differences are mainly due to smoking trends. Whereas, Adkins et al. (2011) observed that there are 13.7% differences in the autosomal CpG island patterns of the African -Americans and Caucasian – Americans.

Forensic Psychology

Psychology deals with the scientific study of human behavior. Forensic psychology involves applications of laws and principles of psychology for the dissemination of justice. According to a study conducted by Day and Sweatt (2010), repetitive criminal behavior or chronic personality disorders can be due to epigenetic factors specially DNA methylation. According to authors, recent studies show that DNA methylation may take part in formation and storage of memory. Poor upbringing of children and anti-social behavior can affect DNA methylation pattern of the brain which will finally be reflected in the behavior of a person through gene expression (Khulan *et al.,* 2014; Szyf, 2011; Wu *et al.,* 2014). Psychological factors such as depression, stress, social behaviors and etc. plays a significant role in change in DNA methylation patterns (Kader *et al.,* 2018). A study conducted by Kader *et al*. (2018) described the DNA methylation patterns of four genes, *NR3C1, SLC6A4, BDNF* and *OXTR* in regulating psychological behavior.

Species Identification

Species identification is an important aspect in forensic science investigation. One cannot differentiate the origin of biological evidences recovered from the crime scene by mere observation. It may have human origin and non-human origin. There are various methods for species identification such as immunological assays. DNA methylation can also be used for species identification. Razin and Friedman (1981) described that DNA of some prokaryotes at cytosine base are hypomethylated. On the other hand, some prokaryotes DNA is methylated at the adenine base only. In case of eukaryotes, methylation is only observed at the cytosine base. A study conducted by Herraez *et al.* (2013) compared humans and primate samples with respect to their CpG DNA methylation patterns. Their study showed significantly different methylation patterns of primates and humans. A recent study conducted by Wilkinson *et al.* (2021) compared DNA methylation profiles of 712 known age bats to observe the phenomenon of difference in life span of different species. Provateris *et al.* (2018), described the DNA methylation patterns in insects. According to the authors, DNA methylation is negligible or lost in insects belonging to collembola, dipteral and strepsitera.

Distinguish Between Monozygotic Twins

It's a great challenge for forensic biologist to distinguish between monozygotic twins as theoretically they possess identical genomic DNA sequence (Kader and Ghai, 2015; Rawat and Kushwaha, 2016; Vidaki and Kayser, 2018; Li *et al.,* 2013; Lee *et al.,* 2016). So, to differentiate them with standard DNA profiling method (STRs, VNTRs, SNPs profiling) is not possible (Rana, 2018; Vidaki and Kayser, 2018). Many researchers proposed the use of DNA methylation method to distinguish between monozygotic twins (Kader and Ghai, 2015). Li et al. (2013) performed studies on 22 pairs of monozygotic twins in which 9 pairs are of males and 13 pairs are of females, with methylation Bead Chip including 27,578 CpG sites from which 92 CpG sites are selected which shows significant difference in methylation status of monozygotic twins. Fraga *et al.* (2005) performed studies on lymphocytes of 80 Caucasian twins in which 30 are males and rest are females. X chromosome inactivation was analyzed in female monozygotic twins and they observed that 19% of all female twins shows a skewed pattern of methylation which varied between siblings. And there was methyl-cytosine difference in 35% of the twin pairs. They also use amplification on inter-methylated sites (AIMS) which shows 0.5-35% difference in bands between the twin pairs (Kader and Ghai, 2015).

Table-1: Studies based on the DNA methylation

Samples	Objective/Aim	Findings	Reference
43 different samples from cells and tissues	DNA methylation profiling of human chromosomes 6, 20 and 22	Established reference DNA methylation profiles from human chromosome 6, 20 and 22 and established that DNA methylation patterns are tissue specific and serves as an excellent marker for tissue identification	Eckhardt *et al.* (2006)
Blood of 23 monozygotic twins, 23 dizygotic twins and 96 healthy individuals	The relationship of DNA methylation with age, gender and genotype in twin and healthy controls	Observed that 56 autosomal probes were hypermethylated in females by Mann - Whitney U test on Illumina Golden Gate bead array.	Boks *et al.* (2009)
131 patients with azoospermia or severe oligozoospermia	Tissue-specific differentially methylated regions of the human VASA gene are potentially associated with maturation arrest phenotype in the testis	Maturation arrest (MA) patients has higher VASA TDMR methylation among patients in which VASA gene expression has undergone gene silencing.	Sugimoto *et al.* (2009)
Blood, dry saliva stains, smoked cigarette butts, hair, skin	Authentication of forensic DNA samples	Concluded that in-vitro DNA cannot be distinguished from natural DNA by simple DNA typing assays such as STR typing and developed an authentication assay and concluded that artificially	Frumkin *et al.* (2010)

		synthesized DNA will eternally be unmethylated whereas, naturally occurring DNA will have varying methylated patterns	
Blood, Semen, Skin, Saliva	DNA methylation-based forensic tissue identification	Successful in differentiation of blood, semen, skin and saliva samples.	Frumkin *et al.* (2011)
Blood, saliva, urine, vaginal secretion, semen and skin epidermis	DNA methylation–based forensic tissue identification	Described DNA methylation assay to determine tissue source from single as well as mixed samples.	Frumkin *et al.* (2011)
201 Newborns, 107 African- American, 94 Caucasian	Racial differences in gene-specific DNA methylation levels are present at birth	Observed 13.7 % differences in the CpG island of the autosomal chromosome between Caucasian-Americans and African -Americans. 2% of the autosomal CpG islands show different pattern in male and female	Adkins *et al.* (2011)
Venous blood, saliva, semen, menstrual blood and vaginal fluid	Potential forensic application of DNA methylation profiling to body fluid identification	Described the use of DACT1, USP49, HOXA4, PFN3, and PRMT2 loci by bisulfate sequencing to differentiate vaginal fluid, menstrual blood, seminal fluid, blood and saliva.Differentiated male-female by HOXA4 tDMR from saliva samples.	Lee *et al.* (2012)
192 samples	Differences in smoking associated DNA methylation patterns in South Asians and Europeans.	Performed DNA Methylation assay on 192 samples from a town in England and observed varying methylation patterns in 29 samples with respect to CpG sites. Identified link between smoking habits and ethnic group at AHRR locus.	Elliott *et al.* (2014)
Blood, saliva, semen and vaginal secretions	Identification of body fluid- specific DNA methylation marker for use in forensic science	Eight DNA methylation markers that are cg26107890 and cg20691722 for saliva, cg23521140 and cg17610929 for semen and cg01774894 and cg14991487 for vaginal secretions were evaluated in 80 body fluid samples by pyrosequencing method. All markers showed high specificity for specific body fluid.	Park *et al.* (2014)
Blood, saliva and semen from 20 males and menstrual blood and vaginal fluid from 14 females	Body fluid identification by integrated analysis of DNA methylation and body fluid-specific microbial DNA	Developed multiple PCR systems for identification of semen, saliva, vaginal fluid. Unmethylation at USP49, DACT1 and PFN3 tDMRs help to distinguish	Choi *et al.* (2014)

		semen. Saliva can be identified by saliva specific bacteria, *Veillonellaatypica* and *Streptococcoussalivarius*. Vaginal fluid differentiates by hypomethylation at PFM3 tDMR and presence of *Lactobacillus crispatus* and *Lactobacillus gasseri*.	
	A novel approach identifies new differentially methylated regions (DMRs) associated with imprinted genes	Established new strategy to locate DMRs associated genes. Their study identified known DMRs and new DMRs for NAP1L5 and ZNF597 genes.	Choufani *et al.* (2015)
Blood, semen, buccal swab and vaginal swab	Forensic discrimination of vaginal epithelia by DNA methylation analysis through pyrosequencing	Observed that vaginal epithelia can be differentiated from other biological fluids by the use of bisulphite-modified pyrosequencing from PFN3A marker.	Antunes *et al.* (2016)
84 blood samples	A comparison of forensic age prediction models using data from four DNA methylation technologies.	use four different technologies: EpiTYPER, MiSeq, Pyrosequencing and SNaPshot for detection of DNA methylation level for CpG sites located at three genes ELOVL2, FHL2 and MIR29B2C which are related to age. They observed that DNA methylation patterns and subsequent predictions of age from EpiTYPER, MiSeq and Pyrosequencing systems are comparable while SNaPshot system gives higher predictive error.	Aradas *et al.* (2020)
29 fresh bone samples and 31 tooth samples 31 fresh bone samples and 24 tooth samples	DNA methylation age estimation from human bone and teeth	Use bisulphite Sanger sequencing method on methylation level of 43 CpG sites located at ELOVL2, FHL2, PDE4C, MIR29B2C and EDARADD genes. They observed that there is positive correlation of all CpG sites located at ELOVL2, FHL2, PDE4C genes with age and negative correlation of all CpG sites located at MIR29B2C and EDARADD genes with age. Performed multiplex SNaPshot assay on methylation data obtained from 5 CpG sites located at ELOVL2, FHL2, MIR29B2C, TRIM59 and	Correia Dias *et al*. (2020)

		KLF14 genes. They observed positive correlation of CpG sites at ELOVL2, FHL2, TRIM59 and KLF14 genes with age and negative correlation of CpG sites located at MIR29B2C gene with age.	
Blood, semen, vaginal epithelial cells and saliva	Identification of saliva using DNA methylation analysis for forensic use	Identify hypermethylation of five loci cg-9652652, cg-11536474, cg-3867465, cg-10781408 and cg-10122865 with several other adjacent CpG sites in saliva which can be used to distinguish saliva from other body fluids.	Staples (2020)

CONCLUSIONS

DNA methylation assays, among other epigenetics mechanisms has been proven as an informative technique in the field of forensic science. DNA methylation is affected by certain factors such as ageing, lifestyle, and various environmental factors. Some of the factors cause hypomethylation, some cause hypermethylation and some of them cause both hypo and hypermethylation. As pattern of DNA methylation is tissue specific and unique in every individual, therefore DNA methylation assays can be helpful in distinguishing fluids/tissue, monozygotic twins, in age prediction, sex identification, species identification, human behavior studies, etc. Forensic examiner should take some cautions while selecting CpG sites for DNA methylation studies and also at the time of interpretation of profile of DNA methylation. DNA methylation profile in combination with conventional methods of DNA profiling like STR profiling may prove a great asset in solving forensic cases. In future, more extensive studies are needed on epigenetics which can locate new reliable and stable markers for forensic applications.

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