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Review Article

DNA Methylation as a Biomarker for Body Fluid Identification

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Abstract

Current techniques for the forensic identification of body fluids are either enzyme or protein based. Because of their labile nature, these substrates are easily prone to environmental degradation, thereby limiting their use as a potential source of forensic evidence. Epigenetic changes, such as DNA methylation and histone acetylation, can be used for body fluid identification. Epigenetic markers as DACT1 and USP49 are currently being used for semen identification. Markers as cg26107890 and cg14991487 are used to differentiate saliva and vaginal secretions from other body fluids. However, such markers show overlapping methylation patterns. This review article aimed to highlight the feasibility of using DNA methylation of certain genetic markers in body fluid identification and its implications in forensic investigations.

The reviewed articles have employed molecular genetics techniques such as bisulfite sequencing PCR (BSP), methylation specific PCR (MSP), and Multiplex SNaPshot Microarray. Bioinformatics software such as Matrix Laboratory (MATLAB) and BiQ Analyzer has been used. Biological fluids have different methylation patterns which allows using DNA methylation to identify body fluids gives accurate results consuming minute amounts of precocious biological evidence material. Recent studies have incorporated next-generation sequencing (NGS) to discover more reliable markers that can differentiate between different body fluids. Nonetheless, new DNA methylation markers are yet to be discovered to accurately differentiate between saliva and vaginal secretions with high confidence.

Epigenetic changes are dynamic, and it is important to find stable DNA sequences that can be used as reliable biomarkers for the identification of different types of body fluids encountered at a crime scene.

Keywords: Forensic Sciences, DNA Methylation, Body Fluid Identification, Pyrosequencing, Epigenetics

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تميز بين سوائل الجسم المختلفة. ومع ذلك، لم يتم حتى الآن اكتشاف علامات مثيلة حمض نووي جديدة للتمييز بدقة وبثقة عالية بين اللعاب والإفرازات المهبلية. ويوجد حركة مطردة في دراسة التغيرات الفوق الجينية حيث أنه من المهم العثور على تسلسل مستقر في الحمض النووي يمكن استخدامه كمُعلِّمة حيوية موثوقة لتحديد الأنواع المختلفة من سوائل الجسم التي قد نواجها في مسرح الجريمة.

الكلمات المفتاحية: علوم الأدلة الجنائية، مثيلة الحمض النووي، تحديد هوية سوائل الجسم، تحديد التسلسل البايرو، علم الفوق الجيني.

1. Introduction

Identification of biological evidence found at a crime scene is crucial for forensic investigations. For example, identification of semen at a crime scene informs the investigators about the occurrence of a sexual assault, and may help to identify the perpetrator. Most identification techniques that are presently available are enzyme and protein based which can be subjected to degradation and tend to be non-specific and destructive with limited potentials for further storage of evidences [1]. For example, Phadebas® test is commonly used for the detection of saliva in forensic specimens. This test, which uses enzyme activity of the amylase present in saliva [2] is highly presumptive as amylase can be found in urine, serum, and some plants and bacteria [3]. Semen is identified by the presence of prostatic acid phosphatase (AP) and prostate-specific antigen (PSA). The seminal AP, in the presence of alpha-naphthyl acid phosphate and brentamine fast blue, will produce a dark purple color in less than a minute, while detection of PSA is done by immunochromatographic assays using anti-PSA antibody/antigen complexes [4]. Such protein based tests have limitations as they can be subjected to degradation or may exhibit inhibitory effects [3].

ميثلة الحمض النووي كمُعَلِّمة حيوية لتحديد هوية سوائل الجسم

المستخلص

تعتمد التقنيات المتاحة حالياً للتحديد الجنائي لهوية سوائل الجسم على مبادئ تحليلية مبنية على ركائز الأنزيم أو البروتين، وبسبب الطبيعة الغير مستقرة لهذه الركائز فإنها تكون عرضة للتحلل بفعل تأثرها السهل بالعوامل البيئية ما يحد من استخدامها كمصدر محتمل للأدلة الجنائية. ويمكن استخدام التغيرات الفوق الجينية مثل مثيلة الحمض النووي وأستلة بروتين الهيستون لتحديد هوية سوائل الجسم حيث تستخدم حالياً مُعَلِّمات فوق الجينية مثل: DACT1 و USP49 لتحديد السائل المنوي، كما تستخدم معلمات مثل cg26107890 و cg14991487 لتمييز اللعاب والإفرازات المهبلية عن سوائل الجسم الأخرى. ومع ذلك، تُظهِر هذه المعلمات تداخل مع معلمات مثيلة حمض نووي أخرى.

هدفت هذه المراجعة العلمية إلى تسليط الضوء على جدوى استخدام مثيلة الحمض النووي لمعلمات وراثية معينة في تحديد سوائل الجسم وأثر إدراجها في التحقيقات الجنائية. وقد استخدمت المقالات التي تم استعراضها تقنيات الوراثة الجزيئية مثل تسلسل ثنائي السلفيت(bisulfite sequencing PCR BSP)، ومثيلة تفاعل البلمرة المتسلسل المحددة (methylation specific PCR; MSP) والمصفوفة الدقيقة للنيوكليوتيدات الأحادية متعددة التكثير Multiplex SNaP-shot Microarray. وقد استخدمت برامج المعلوماتية الحيوية مثل برنامج مختبر ماتريكس (Matrix Laboratory MATLAB)، وبرنامج التحليل بي كيو BiQ Analyzer. إن السوائل الحيوية لديها أنماط مثيلة مختلفة ما يسمح بتحديد سوائل الجسم ويمكّن الحصول عل نتائج دقيقة من خلال استخدام كميات ضئيلة من عينات الأدلة الحيوية المجموعة مسبقاً. وقد أدرَجت الدراسات التي أجريت مؤخراً تقنية تحيد التسلسل النيوكليوتيدي الجيل القادم next-generation sequencing (NGS)، بغرض معرفة علامات أكثر موثوقية يمكن أن

2. Epigenetic changes

All human body cells develop from embryonic stem cells. Even though all nucleated cells share the same DNA sequence, they produce different proteins and perform different functions. This difference in gene expression is due to the epigenetic changes. These changes are functionally relevant, such as DNA methylation and histone acetylation [5]. DNA methylation involves attachment of a methyl (-CH_3) group to the carbon 5` of the cytosine ring at the CpG site (Figure-1). Epigenetic changes also occur due to environmental exposures, like diet and smoking. In mammals, epigenetic changes are involved in imprinting, reprogramming, gene silencing (Figure-2), X-chromosome inactivation and carcinogenesis [6].

Several studies have been concerned with the distribution of the methylated CpG sites within the gene regions and its possible interaction with the transcriptional machinery of that gene, particularly those methylated islands that are located around the promoter region of the gene (Figure-3) [8].

Furthermore, a comparison study of CG islands methylation patterns across different parts of individual genes revealed that the promoter areas (TSS1500, TSS200, and 5'-UTR) and the first exon were almost exclusively unmethylated; however, variable CG methylation levels were found in the gene body and 3'-UTR (Figure-4) [10]. Such epigenetic changes can be used as biomarkers for body fluid identification to overcome the limitations that arise from routinely used standard identification tests.

Moreover, Lokk et al., (2014) have shown that there are tissue-specific, differentially methylated regions (tDMRs) within the same gene that vary greatly between tissues with different functions (Table-1). Similarly, an earlier study in which the CpG island methylation data of sperm and other

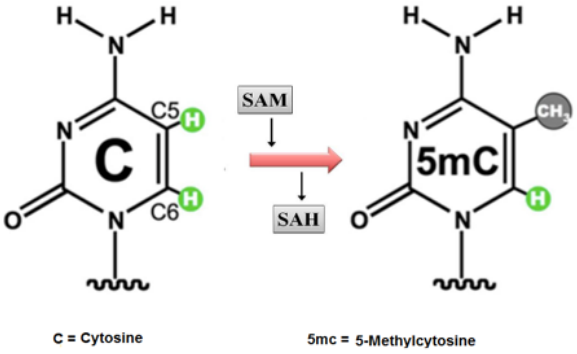

Figure 1- *Schematic DNA methylation. DNA methyltransferases use S-adenosyl-l-methionine (SAM) as the source of methyl groups, producing S-adenosyl homocysteine (SAH). DNA methyltransferases catalyze the addition of methyl groups to the 5'-position of the pyrimidine ring of cytosine [7].*

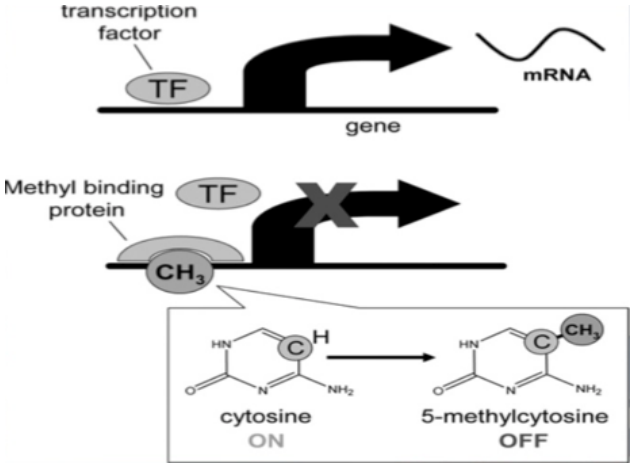

Figure 2- *Illustration of the role of DNA methylation in gene silencing.*

11 somatic tissues from Human Epigenome Project were analyzed, has shown that the CpG island methylation profiles are highly correlated between somatic tissues, while the methylation profile in sperm is quite distinct [11].

DNA methylation controls gene expression which in turn controls various cellular functions [5]. DNA methylation is a new approach in gene expression studies. Naito et al. (1993) were the first to use DNA methylation in forensic genetics [12]. They devised a method for female sex typing using epigenetics. This method relied on detection of repetitive sequence at DXZ4 region, which is highly methylated on the active X chromosome and hypomethylated on the

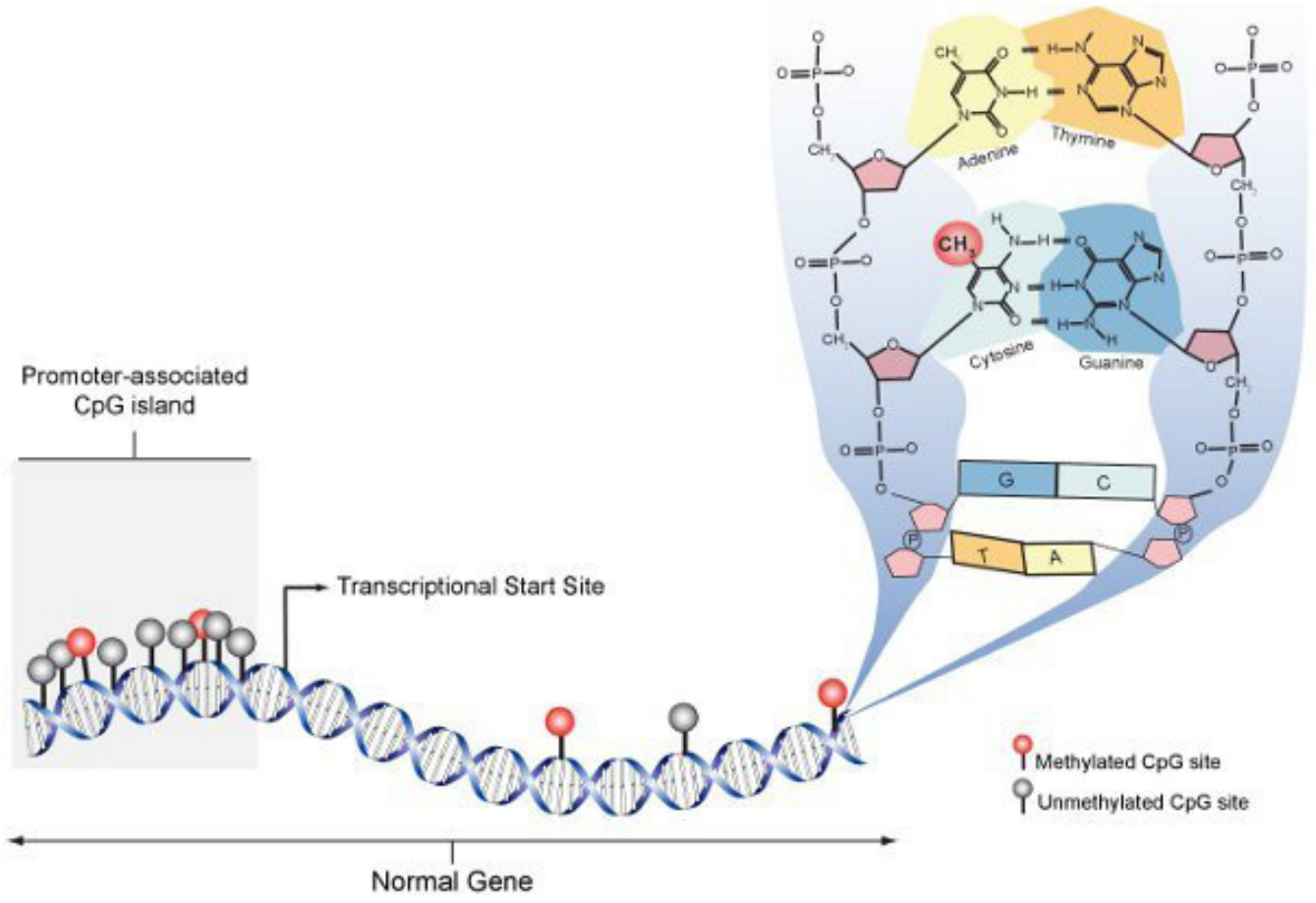

Figure 3- *Schematic diagram showing CpG methylation. In the normal cell, promoter-associated CpG islands are predominantly unmethylated (grey) whereas CpG sites within gene bodies are sparse and generally methylated (red). The panel on the right expands the molecular structure of DNA at an individual CpG site and shows methylation with a CH3 molecule at carbon 5 of cytosine [9].*

inactive X chromosome. This suggested method was very sensitive as it required only 50 pg of DNA for successful testing. In the case of sex-reversed individuals, this technique was proposed as a complementary technique [12].

A more recent study, which involved analysis of DNA methylation patterns as a distinguishing tool between neural and non-neural tissues, has suggested that tissue-specific methylation patterns are a critical aspect of the regulatory mechanisms of tissue-specific gene expression during different phases of development [13].

In addition to the role of DNA methylation in long term silencing of genes and silencing of repetitive elements (e.g., transposons) [14], as well as X chromosome inactivation, it was also shown that DNA methylation plays an important role in the establishment and maintenance of imprinted genes, suppression of viral genes, and other deleterious elements that have been incorporated into the genome of the host over time as well as carcinogenesis [15, 16].

It was suggested that gain of CpG methylation may represent a feature of cancer cells since methylated cytosines are highly unstable bases and thus will predispose to gene mutation. Methylated cytosines are often deaminated and converted to thymine, which in turn can lead to inactivation of tumor suppressor genes (TSGs) (e.g. CGA, which encodes an arginine residue, is changed to TGA, which specifies a top codon resulting in a prematurely truncated

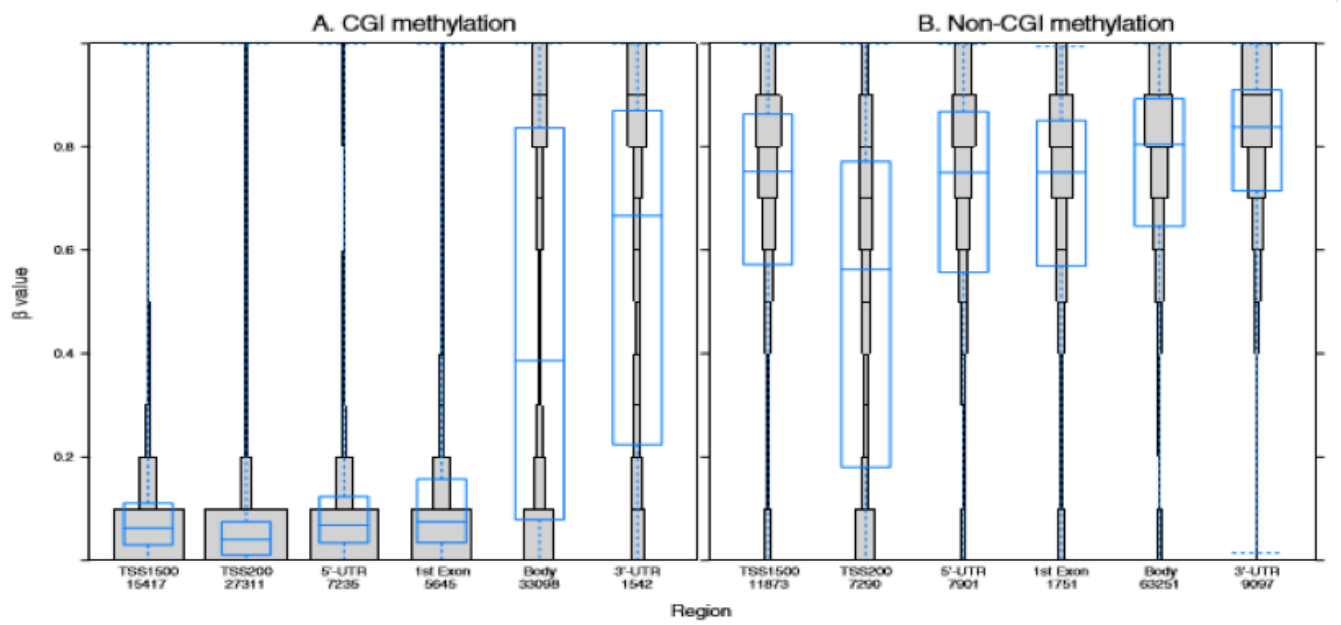

Figure 4- *DNA methylation distribution in CGI and non-CGI regions. Distribution of DNA methylation in specific gene regions. Each gene region is further divided into bins that correspond to beta values with 0.1 intervals. The area of each bin corresponds to total number of CpGs. The overall distribution and the mean of beta value of the CpGs in each gene region are shown as a box plot. (A) DNA methylation is low in promoter areas, but high in non-CGI regions of all gene areas (B). (A-B) The numbers on the x-axis correspond to total number of CpGs in each gene region; also the x-axis shows different genomic regions, and the y-axis shows the beta values [10].*

Table 1- *Tissue Specific Differentially Methylated Regions (tDMRs) - Data Summary [10].*

Tissues	Hypermethylated blocks	Hypermethylated blocks with gene annotation	Hypomethylated blocks	Hypomethylated blocks with gene annotation
Adipose (subcutaneous, abdominal)	84	65	376	301
Artery (coronary, splenic)	380	280	283	219
Bone, joint cartilage	129	73	168	104
Bone marrow (red, yellow)	175	150	1,300	1,028
Gastric mucosa	74	54	26	22
Lymph node	56	42	5	3
Tonsils	4,983	3,893	1,072	924
Bladder	379	274	752	566
Gall bladder	65	47	93	66
Aorta	628	453	1120	888
Medulla Oblongata	651	495	349	278
Ischiatic nerve	203	156	1,090	861

protein on translation (Figure-5) [17].

Moreover, in a recent research involving a genome-wide DNA methylation profiling of the right coronary arteries from patients with coronary heart disease, a high percentage of differentially methylated genes was detected within different vascular tissues. Additionally, this study identified hypomethylation of four CpG-sites located within the MIR10B gene sequence in the right coronary artery in the area of advanced atherosclerotic plaques in comparison with the other vascular tissues [18].

3. Potential application of DNA methylation in forensic science

DNA methylation is involved in complex biological processes like cell differentiation and aging [6]. Cell differentiation is regulated by gene expression which leads to the development of different types of cells. The gene expression is in turn regulated by DNA methylation, which is influenced by environment and age [19, 20]. In forensic investigations, knowing the origin of biological fluid is of great importance as it can reveal the type of crime that occurred and help reconstruct it. As each biological fluid is produced at different locations in the body and for different functions, they should have differential methylation patterns. This difference in methylation process can be used as a tool to identify the nature of the biological fluid retrieved at a crime scene. Most of the body-fluid presumptive identification tests are enzyme and/or protein based and consume a good quantity of available evidence material [1]. However, using DNA methylation to identify body-fluids will not only give accurate results but also conserve the trace amount of biological evidence material that may be utilized in other forensic investigations such as DNA typing and sequencing [1].

Another alternative to the enzyme based test is the use of mRNA from crime scene samples to identify the sample type; however, mRNA is more prone to degradation due to the abundant presence of ribonucleases [21]. In contrast, DNA is more stable, which renders it more useful for biological sample identification using DNA methylation pattern. Moreover, protein based assays are qualitative and not quantitative due to which statistical confidence level cannot be calculated.

DNA methylation, despite being criticized for showing differential methylation at several loci, in spite of that, recent studies have included new markers that showed either on or off signal in the target body fluid and thus gave an added quantitative value [1, 22].

DNA methylation-based identification is similar to the DNA profiling and requires the same equipment with no additional training. However, standard body-fluid identification tests use different protocols and equipment and, therefore, require skilled expertise [1]. In addition to body-fluid identification, DNA methylation levels can also be used to estimate the age, gender and ethnicity of the individual. Such information is of great value to either convict or exonerate a suspect [23-25].

4. Techniques for DNA methylation analysis

The most common method of analyzing DNA methylation is by chemically modifying cytosine residues using sodium bisulfate (Figure-6) [3, 9, 26]. The non-methylated cytosine(s) are converted to uracil whereas methylated cytosine(s) remain unchanged. The speed of bisulfite conversion can be fastened by using a highly-concentrated bisulfite solution at an elevated temperature [27]. After

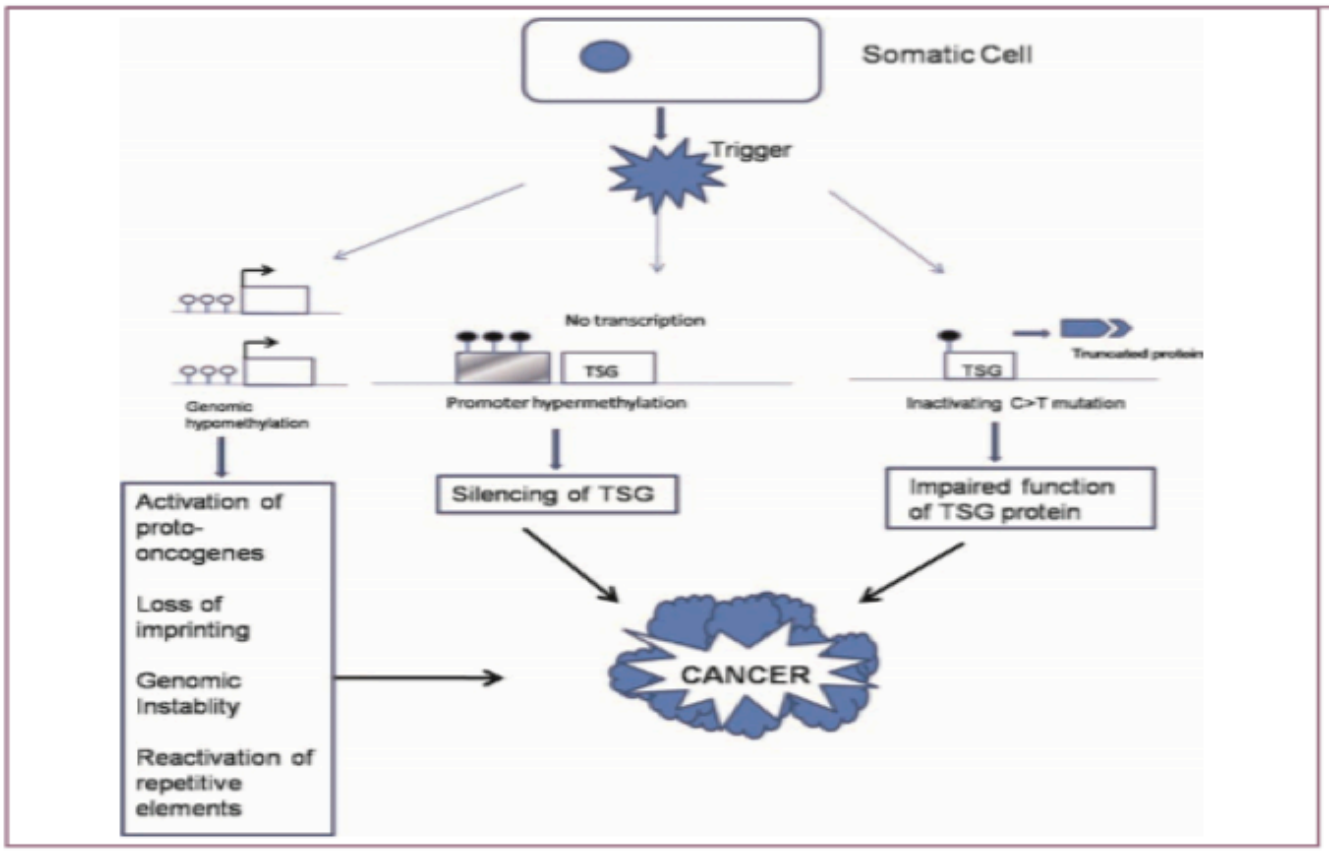

Figure 5- *DNA methylation and cancer. Examples of three mechanisms leading to tumorigenesis. (TSG= tumor suppressor gene) [17].*

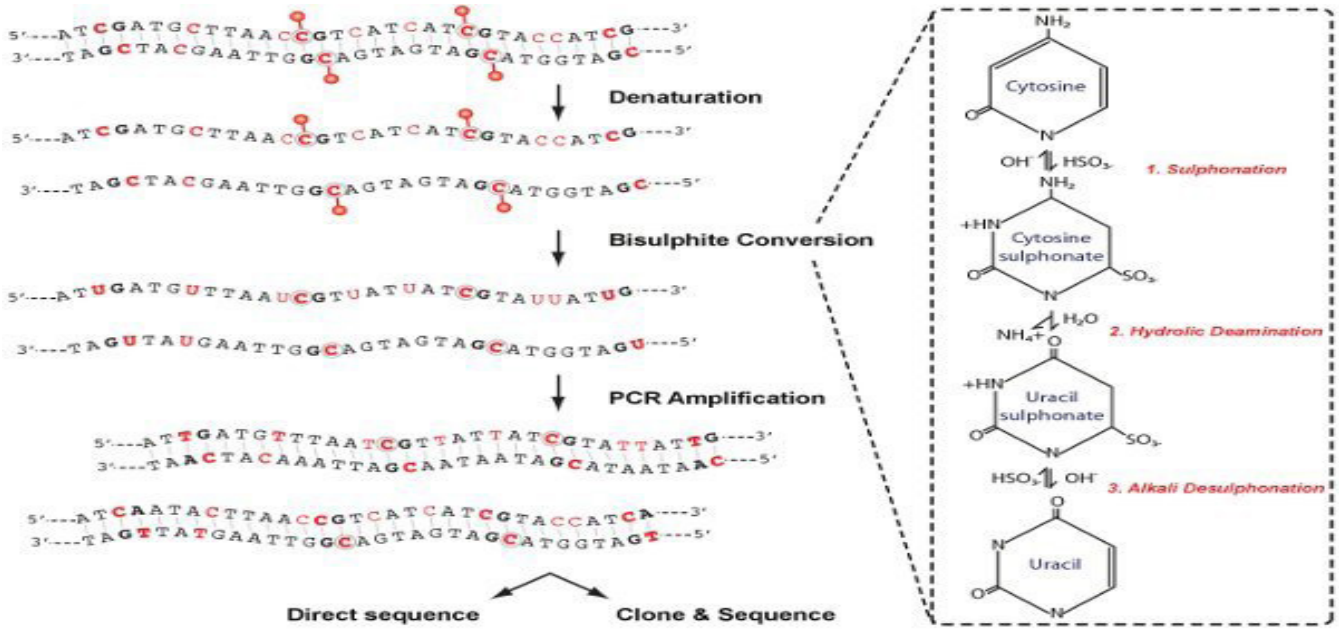

Figure 6- *Schematic representation of DNA methylation analysis technique. Analysis of DNA methylation includes four main stages; denaturation, bisulphite conversion, PCR amplification and analysis. In the right panel, modifications to the cytosine molecule that occur during bisulphite conversion are depicted [9].*

chemical treatment, the results can be analyzed using pyrosequencing. Madi et al. (2012) used bisulfite treatment to modify the DNA samples and then amplified the region of interest using PCR. The reverse primers were biotin labeled in order to produce biotinylated PCR products which can be used for pyrosequencing [3].

Methylation SNaPshot, the second most popular approach in the forensic field, involves the use of the methylation-sensitive single nucleotide primer extension (ms-SNuPE) technique. This method has the advantage of simultaneous analysis of multiple markers by designing multiplex methylation SNaPshot and thus, methylation levels at multiple CpG sites can be conveniently visualized in the electropherogram [28].

Park et al., (2014) have used sodium bisulfite treatment to modify the DNA samples. Modified DNA samples were then amplified using the InfiniumTM Methylation Assay kit (Illumina). Amplified DNA was then hybridized to Human Methylation 450 kTM bead array (Illumina) and scanned with the Illumina iSCAN system to identify body-fluid specific DNA methylation markers. Selected marker candidates were then validated using pyrosequencer [29].

During the last five years, several assays have been developed that combine different markers in what is called multiplex SNapshot microarray that is capable of screening for different DNA methylation markers together in one process which thus enables investigators to identify various types of body fluids [29-31].

Another approach for the analysis of methylation patterns is by using restriction enzymes. Some restriction enzymes will only cut the specific sequence if it is not methylated. The restriction enzyme approach relies on the presence of an apt recognition site at the CpG of interest. The methylation-sensitive restriction enzyme will only cleave this site when the CpG is not methylated [22]. Wasserstrom et al. (2013) used forward and reverse primers in order to amplify CpG Island that contained Hha1 recognition sequence and showed significant difference in methylation between semen and non-semen samples [32]. The drawback of using such a method is that some methylated sequences might not have restriction enzyme sites and the methylated sequences that are not present in restriction sites will not be detected [5].

Ultimately, bisulfite genomic sequencing is regarded as a gold-standard technology for the detection of DNA methylation because it provides a qualitative, quantitative and efficient approach to identify 5-methylcytosine at single base-pair resolution. Being a fundamental method of DNA methylation analysis, bisulfite genomic sequencing has been widely used in various research and clinical settings [33, 34].

To sum up, no single method of DNA methylation analysis will be appropriate for every application. Investigators can select the method most appropriate for their specific research needs based on understanding the type of information provided by, and the inherent potential for bias and artifact associated with each method [35].

5. Available biomarkers

Epigenetic changes are dynamic and they change with age and environment. It is important to identify candidate markers that can be used as biomarkers in forensic investigations. Candidate markers can be identified either by screening known regions of DNA with high methylation rate, like CpG islands and promoter regions, or by performing whole genome analysis [36].

There are lots of data available showing many promising candidate markers that can be successfully used to identify body fluids. Lee et al. (2012) have recommended DACT1 and USP49 as good candidate markers for semen identification [37]. Whereas, Madi et al. (2012) showed that ZC3H12D and FGF7 gave differential methylation patterns for semen and can be used for semen identification [3]. Park et al. (2014) were able to differentiate semen from non-semen samples using cg23521140 and cg17610929 as biomarkers [29]. Additionally, Frumkin et al. (2011) used L91762 and L68346 as semen identification markers [1]. Furthermore, Wasserstrom et al. (2013) devised a semen identification kit (Nucleix DSI-semen kit) using markers on chromosome 4 (25287119–25287254), chromosome 11 (72085678–72085798, 57171095–57171236, 1493401–1493538), and chromosome 19 (47395505–47395651) collectively [32].

Park et al. (2014) screened out markers (cg26107890 and cg20691722 for saliva and cg01774894 and cg14991487 for vaginal secretions) that could differentiate saliva and vaginal secretions from other biological fluids [29]. However, these markers showed overlapping methylation patterns between saliva and vaginal secretions, and thus more studies are needed to find reliable markers that can distinguish saliva and vaginal secretions from each other. In the same study, the researchers were able to differentiate blood from other biological fluids successfully using cg06379435 and cg08792630 as blood specific markers. Additionally, Madi et al. (2012) successfully differentiated blood from other biological fluids using C20orf117 as an identification marker, which is specific to white blood cells [3].

Forat et al. (2016) have selected 9 loci and referred to them as the most promising differentiating markers. These markers are cg26285698 and cg 03363565 for blood, cg09696411 for menstrual blood, cg21597595 and cg15227982 for saliva, cg14991487 and cg03874199 for vaginal secretions, and cg22407458 and cg05656364 for semen (Figure-7) [31].

6. Potential uses of DNA methylation as a forensic tool

The use of DNA methylation for body tissue analysis in forensic field is relatively new; it was first used by Frumkin et al. (2011). They analyzed many biological tissues using genetic loci that give different levels of DNA methylation. They used 1 ng of DNA with Hha1 methylation sensitive restriction enzyme to analyze DNA methylation levels. Using the Hha1 enzyme, they screened out 38 genomic loci that gave differential amplification patterns. Loci that had higher methylation were amplified more effectively than the loci which had lower methylation [1]. Wasserstrom et al. (2012) using the same aaproach as Frumkin et al., developed a kit for the identification of semen using DNA methylation pattern. This kit is commercially available as Nucleix DNA source identifier DSI-SemenTM kit and can replace the microscopic examination of samples used to differentiate semen from non-semen samples [32]. This kit employs 5 genomic loci that showed significant variation in DNA methylation between semen and non-semen samples. Included in this kit are three additional loci (Chr19: 5572820–5572916, Chr22: 16491586–16491695, Chr2: 52821303–52821462) that are used as controls for Hha1 digestion and PCR amplification. Around 135 tissue samples were used for the validation of this kit [32]. LaRue et al. (2012) conducted validation study on the DSI- SemenTM kit, which demonstrated the robustness and reliability of

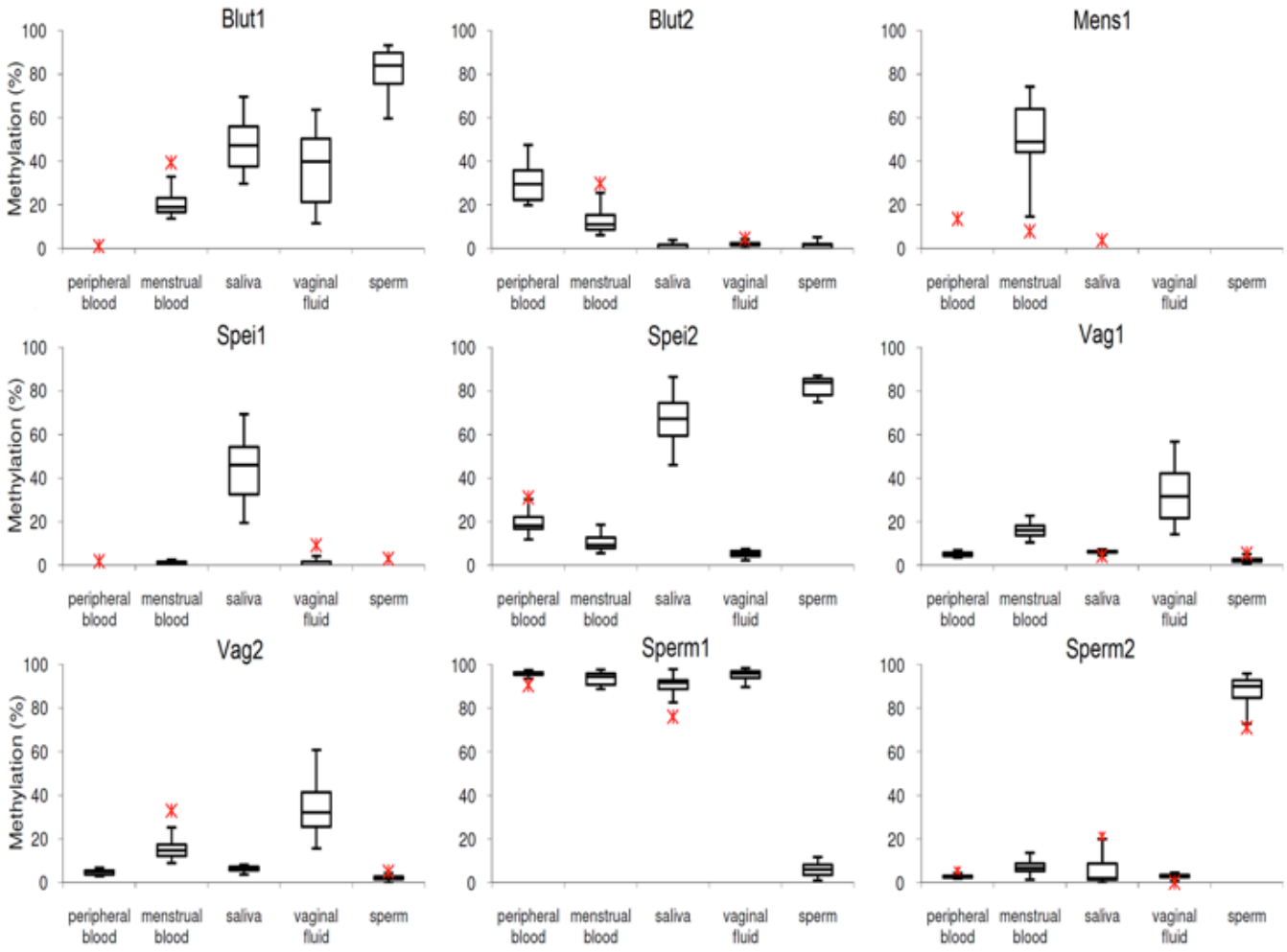

Figure 7- *Boxplot diagrams showing the discriminant power of the 9 human body fluid markers. They present the methylation rates for 20–22 samples per body fluid. Methylation values identified as outliers are marked with an asterisk. Only the lowest and highest outliers are shown [31].*

the kit and showed positive results for semen with as little as 31 pg of template DNA [38].

Park et al. (2014) conducted genome wide profiling for various body fluids and were able to identify novel DNA methylation markers for blood, saliva, semen and vaginal secretions. The authors were able to perform pyrosequencing with as little as 10 ng of pre-modified DNA [29].

Lee et al. (2012) studied 5 tissue-specific differentially methylated regions (tDMRs) to differentiate body fluids. Out of these 5 tDMRs, two regions were for the gene DACT1 and USP49, which were reported as semen specific markers in previous studies [37]. The tDMRs from DACT1 showed 93% unmethylation for semen samples but hypermethylation in other body fluids (blood, saliva, menstrual blood, vaginal fluid). On the other hand, tDMRs from USP49 showed hypomethylation in 97% of clones from semen and hypermethylation in other body fluid samples [37]. The other 3 tDMRs were selected as candidates for blood specific markers. These 3 tDMRs (HOXA4, PFN3, and PRMT2) showed varying methylation profiles for the body fluid samples. The tDMRs of PFN3 showed 80% methylation of CpG loci for all body fluids and 65% methylation of CpG loci for vaginal fluid. The tDMRs of PRMT2 showed hypomethylation for semen samples and

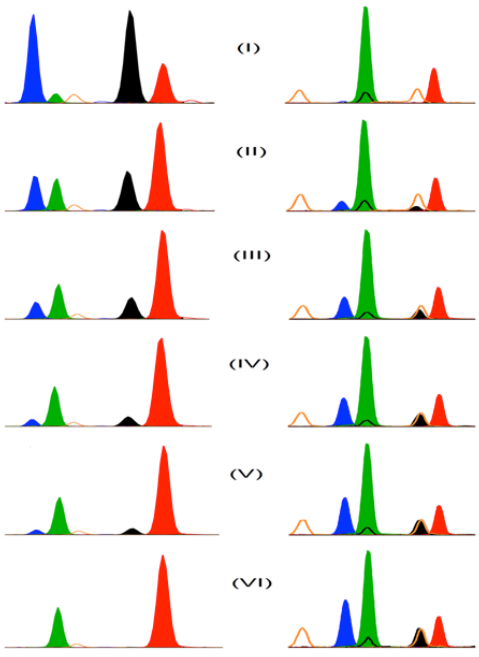

Figure 8- *Results of mixture experiments for both peripheral blood markers. The specific reaction of Blut1 (left) and Blut2 (right) with blood DNA in a mixture with DNA of the remaining 4 fluids is shown. Both markers are diplex assays. 100% mixture + 0% blood (I), 80% mixture + 20% blood (II), 60% mixture + 40% blood (III), 40% mixture + 60% blood (IV), 20% mixture + 80% blood (V), 0% mixture + 100% blood (VI). Blut1 is umethylated in pure peripheral blood (green and red signal). Blut 2 is methylated in blood (blue and black signal) [31].*

hypermethylation in more than half of samples from menstrual blood and vaginal fluid. This study suggested the use of tDMRs from DACT1, USP49, PRMT2, and PNF3 to differentiate semen from vaginal fluid. The tDMRs from DACt1 and USP49 can be used for semen identification [37].

Madi et al. (2012) studied 4 markers (C20orf117, BCAS4, FGF7 and ZC3H12D) to differentiate body fluids (blood, semen, saliva/buccal cells). Gene C20orf117 showed hypermethylation for blood, mainly white blood cells, and successfully differentiated it from other samples. Marker ZC3H12D showed hypomethylation for semen samples and successfully differentiated it from other body fluids. Marker BCAS4, which showed hypermethylation with semen samples in previous studies [39], also gave hypermethylation with saliva samples. The study used saliva samples that included epithelium lining of the inside of the cheek. The methylation profile generated for saliva samples were inclusive with epithelial cells of the buccal cavity. The marker FGF7 gave higher methylation values for semen samples than other body fluids. The authors suggested that FGF7 marker can be used to successfully differentiate semen samples from other body fluids [3].

In 2014, Wu et al. investigated the correlation of DNA methylation levels in blood and saliva in young girls and found that methylation levels in saliva DNA were generally much lower than those in WBC DNA. They concluded that different DNA sources should be investigated separately for DNA methylation studies [40].

More recently, Forat et al. (2016) were able to differentiate between blood and semen using two reciprocal markers for each body fluid. One of each pair of markers is specifically methylated in its corresponding body fluid while it is unmethylated in all other body fluids, whereas the other marker is specifically unmethylated in its target body fluid and shows methylation in all other body fluids. This approach allowed better analysis of complex mixtures with high sensitivity (Figure-8) [31].

Moreover, choi et al. (2014) have developed a successful multiplex protocol that enabled identification of semen as well as detection of certain bacterial DNA that further allowed identification of saliva and vaginal fluid [41].

7. Conclusion

Epigenetics is a new field with only a few decades of research, and the use of epigenetics in forensic science is a recent approach. Epigenetic changes of an individual DNA differ with age and environmental changes, a fact which

makes it important to select stable DNA sequences that can be used as biomarkers for forensic purposes. More comprehensive studies on epigenetics are required which can find more stable epigenetic markers for forensic application. Such studies will also reveal the function of DNA methylation in cell differentiation and aging and help better understand these complex biological processes, which will enlighten an array of epigenetic applications in forensic fields. Previous studies were unable to find unique markers that can differentiate saliva from vaginal secretions. Therefore, further studies are still needed that might incorporate next-generation sequencing to find out more reliable markers that can differentiate between saliva and vaginal secretion samples with greater accuracy and precision.

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