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Advances in Genomic DNA Methylation Analysis

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Abstract: Genomic DNA methylation is one of three epigenomic mechanisms controlling DNA expression. It is involved in various aspects of development and other biological phenomena of eukaryotic organisms. Several experimental approaches have been worked out to analyze DNA methylation. The main methodologies may be broadly classified into two categories, Polymerase Chain Reaction (PCR) based and non-PCR based methylation methods. PCR techniques are more powerful and reliable in analysis of genomic DNA methylation profiles. Three main processes are required in the PCR based analysis, detection of methylated cytosine and this is be done by methylation-sensitive enzymes or sodium bisulphite treatment, then followed by PCR amplification and sequencing. The present methods have their drawbacks, advantages and potential applications. Currently DNA methylation profiles of genomes are analyzed by sophisticated integrated technologies. The aim of present study is to review the early and up to date methods which have been invented for analysis of genomic DNA methylation.

Key words: DNA methylation, 5-methylcytosine, PCR, sodium bisulphite, methylation sensitive enzymes

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INTRODUCTION

Nowadays it is generally accepted that methylome is referring to DNA methylation profile or map of the genome and there are increasing evidence of its biological importance in gene expression (Beck and Rakyan, 2008; Eckhardt *et al.*, 2006; Hocquette, 2005; Lister and Ecker, 2009). DNA methylation have been discovered in genomic DNA of most eukaryotic species and recent molecular studies have indicated it is an ancient property of these organisms and is characterized by conservative phylogenetic features (Feng *et al.*, 2010; Jeltsch, 2010; Zemach *et al.*, 2010). Studies on DNA methylation analysis have shown how the concert of this epigenomic phenomenon starts from fertilization of ovum and continues to play an important role in the development of the eukaryotic organisms (Bird, 2002). Analysis of DNA methylation has demonstrated its role in coordination with other epigenomic mechanisms in initiating various biological developmental processes including, X chromosome inactivation (Boumil and Lee, 2001; Mohandas *et al.*, 1981), cellular differentiation and organ formation (Ibrahim *et al.*, 2003; Shiota, 2004; Valledor *et al.*, 2007). Moreover, DNA methylation studies illustrated changes in DNA methylation patterns involved or associated with aging (Brunet and Rando, 2007; Ibrahim *et al.*, 2004; Richardson, 2003) and various health problems, for example cancer (Costello *et al.*, 2000; Gohar, 2010; Ibrahim *et al.*, 2009, 2010a, b; Liu *et al.*, 2006; Saleh *et al.*, 2010) and psychiatric disorders (Ibrahim, 2010b). Another important application of DNA methylation analysis is identification of heavy metal effects on this vital epigenomic mechanism and on the status of methylome, for example nickel (Ni^{2+}) was found to induce carcinogenesis through several processes including altering DNA methylation profile (e.g., DNA hypermethylation) and DNA methyltransferase (DNMT) inhibition (Gohar and Mohammadi, 2010). Considering the importance of DNA methylation and its impact on various biological phenomena, methods were devised to analyze DNA methylation, these methods include mainly Polymerase Chain Reaction (PCR) based and non PCR methods. The aim of this article is to review non-specific biochemical, immunological and enzymatic methods and the current powerful PCR analytical methods which have been invented and introduced by investigators for more specific analysis of genomic DNA methylation.

Early biochemical and immunological methods for detection DNA methylation: Several biochemical methods have been reported in the literatures for detection DNA methylation. The prerequisite of these methods is to hydrolyze the extracted DNA; this can be performed following specific chemical or enzymatic treatments (Table 1). It was found that hydrolysis of heat dried DNA could be carried out following treatment with 98% formic acid for 30 min at 175°C, drying in a desiccators in presence of potassium hydroxide and then the residue is dissolved in HCL before analyzing the nitrogen bases of nucleic acids (Vischer and Chargaff, 1948), it is also possible to perform hydrolysis in presence of 72% perchloric acid at 100°C for 1 h (Wyatt, 1951;

Table 1: Methods for chemical and enzymatic hydrolysis of DNA

Method	Hydrolyzing agent	References
Chemical hydrolysis	Hydrochloric acid	Vischer and Chargaff (1948)
	Perchloric acid	Wyatt (1951), Marshak and Vogel (1951)
Enzymatic hydrolysis	Pancreatic and venom nucleases	Ford *et al.* (1980), Dhananjaya and Souza (2010)
	Micrococcal nucleases	Michalon *et al.* (1993), Fuentes-Mascorro *et al.* (2000)

Table 2: Biochemical and immunological methods for detection DNA methylation

Method of analysis	References
Thin layer chromatography (TLC): Kieselgel, Cellulose, Dextran gel, DEAE	Coffey and Newburgh (1963), Jacobson (1964)
Paper chromatography	Gerlach *et al.* (1965); Marshak and Vogel (1951), Vischer and Chargaff (1948)
High-performance liquid chromatography	Christman (1982), Gehrke *et al.* (1984), Gomes and Chang (1983), Kuo *et al.* (1980), Patel and Gopinathan (1987)
Spectroscopic assays: Mass spectroscopy and Nuclear Magnetic Resonance spectroscopy (NMR)	Razin and Cedar (1977), Van de Ven and Hilbers (1988), Del Gaudio *et al* (1997)
Immunoassay	Sano *et al.* (1980), Achwal *et al.* (1984)

Marshak and Vogel, 1951). Another way to hydrolyze DNA is by enzymatic methods to get the mononucleotides. Researchers have used several DNA hydrolyzing enzymes from various sources, e.g., venom, pancreas, micrococcal nucleases (Dhananjaya and Souza, 2010; Ford *et al.*, 1980; Fuentes-Mascorro *et al.*, 2000; Michalon *et al.*, 1993; Telford and Stewart, 1989). The products of DNA hydrolysis could be analyzed by paper chromatography (Vischer and Chargaff, 1948) and by various matrices used in thin layer chromatography, high performance liquid chromatography (Eick *et al.*, 1983; Del Gaudio *et al.*, 1997; Gehrke *et al.*, 1984; Gomes and Chang, 1983; Kuo *et al.*, 1980) and spectroscopic assays (Razin and Cedar, 1977; Van de Ven and Hilbers, 1988). The investigators were able to make use of reverse-phase chromatographic system to separate deoxyribonucleoside monophosphates, identify the specificity of *in vitro* methylation of DNA and quantitation of the extent to which specific restriction endonuclease sites are methylated *in vivo* (Christman, 1982). Whereas Patel and Gopinathan (1987) were able by using high-performance liquid chromatographic method to separate five major bases (cytosine, thymine, guanine, adenine and uracil) and three minor methylated bases (5-methylcytosine, N^6-methyladenine and 7-methylguanine) and estimating 5-methylcytosine in DNAs of φX174 and pBR322. Another interesting approach was to use immunological assays for detection of DNA methylation, these methods were carried out by means of rabbit anti 5-methylcytosine antibodies (Achwal *et al.*, 1984; Sano *et al.*, 1980). These methods were found useful in detection of various components of DNA including 5-methyl cytosine. The aforementioned methods are summarized in Table 2.

It is worth noting the early important observation of possible use of sodium bisulphite for identification of 5-methyl cytosine (Hayatsu *et al.*, 1970; Shapiro *et al.*, 1970, 1973). This observation found very useful applications in more recent techniques which combined both sodium bisulphite reactions with 5-methylcytosine and PCR technologies.

Detection of DNA methylation by specific restriction enzymes: One of interesting tools used for investigating DNA methylation of genomic DNA is utilization of methylation sensitive and insensitive isoschizomeric restriction endonucleases. One of first work about possible discrimination by restriction enzymes between methylated and unmethylated cytosines was carried out by Bird and Southern (1978), they showed that *Hpa II, Ava I, Hha I* and *Hae II* were able to distinguish *Xenopus laevis* somatic (erythrocyte) rDNA from amplified rDNA. Amplified rDNA is cleaved at many sites by each enzyme whilst somatic rDNA is relatively resistant to digestion. They suggested the difference was attributed to the presence of 5-methylcytosine rendering many restriction sites in somatic rDNA resistant to nuclease attack and indicated that the major methylated sequence in eukaryotic DNA is CpG and recognition sites for all four enzymes contain this sequence. The importance of isoschizomeric restriction endonucleases was then established in DNA methylation analysis by Singer and coworkers. They reported that the restriction enzymes *Hpa II* and *Msp I* both recognize the sequence 5'-CCGG-3'. However, *Hpa II* cuts mouse liver DNA to fragments four times larger than does *Msp I*. The size of DNA cut by *Msp I* was close to that predicted from base composition and nearest neighbor analysis; they indicated that the most probable explanation of these results is that in mouse the site 5'-CCGG-3' is highly methylated. Thus, when genomic DNA is digested with *Hpa II*, it cleaves the sequence 5'-CCGG-3' only if the internal cytosine residue is unmethylated, whereas digestion with *Msp 1*, it cleaves the same sequence regardless of methylation (Singer *et al.*, 1979).

PCR-based methods of DNA methylation analysis: Several investigators observed the power of PCR technology for analysis of genomic DNA (Bej *et al.*, 1991), then the efforts of researchers interested in DNA methylation have been directed and succeeded in introducing novel PCR based methods for DNA

Table 3: PCR based methods for the analysis of DNA methylation

Method	References
Sodium bisulphite conversion of DNA and direct sequencing	Frommer *et al.* (1992), Shen *et al.* (2007), Yang *et al.* (2004)
Combined bisulphite restriction analysis (COBRA)	Kneip *et al.* (2009), Xiong and Laird (1997)
Methylation-specific PCR (MSP)	Clark *et al.* (1994), Herman *et al.* (1996), Jeong and Lee (2005)
Methylation-sensitive single-nucleotide primer extension (Ms-SNuPE)	Gonzalgo and Jones (1997, 2002)
Bisulphite sequencing PCR and pyrosequencing	Colella *et al.* (2003), Tost *et al.* (2003), Uhlmann *et al.* (2002), Reed *et al.* (2008, 2010)
Bisulphite Differential Denaturation PCR (BDD-PCR)	Rand *et al.* (2006)
Methylation-sensitive arbitrarily primed polymerase chain reaction (AP-PCR)	Liang *et al.* (2002)
LUminometric Methylation Assay (LUMA)	Karimi *et al.* (2006)
DNA Methylation Analysis by MethyLight Technology	Trinh *et al.* (2001)
Methylated CpG island amplification (MCA)	Toyota *et al.* (1999)

methylation analysis. DNA methylation analysis requires means for detection methylated cytosine and this can be done by methylation-sensitive enzymes and/or sodium bisulphite treatment, then followed by PCR amplification and sequencing.

Table 3 shows the current methods of PCR based techniques used for investigation of DNA methylation. In the following PCR based methods for DNA methylation analysis will be discussed.

Sodium bisulphite-PCR techniques: As early as 1970, it was noticed the importance of sodium bisulphite for identification of 5-methyl cytosine. This analytical method is based on the property of bisulphite to selectively deaminate cytosine residues. The bisulphite-mediated cytosine deamination was discovered independently in 1970 by two research groups, first group working at the University of Tokyo, Japan (Hayatsu *et al.*, 1970) and the other group in New York University (Shapiro *et al.*, 1970), who also reported that 5-methylcytosine was deaminated by bisulfite only very slowly. Other investigators demonstrated that in single-stranded DNA, sodium bisulphite preferentially deaminates cytosine residues to uracil, compared with a very slow rate of deamination of 5-methyl cytosine to thymine (Shapiro *et al.*, 1973; Frommer *et al.*, 1992). Researchers found it was possible to make use of this difference in bisulphite reactivity for genomic sequencing of 5 methyl cytosine residues (Frommer *et al.*, 1992). This procedure is called nowadays bisulphite genomic sequencing and involves the following steps: (1) total genomic DNA is either sheared mechanically or cleaved by restriction enzymes, (2) fully denatured by alkali, (3) treated with sodium bisulphite under conditions such that cytosine is converted stoichiometrically to uracil, but 5-methylcytosine remains unchanged, (4) any region of interest in the bisulphite-reacted DNA is subjected to PCR amplification to yield a fragment in which all uracil (formerly cytosine) and thymine residues have been amplified as thymine and only 5-methylcytosine residues have been amplified as cytosine, (5) the amplified DNA then cloned into a vector
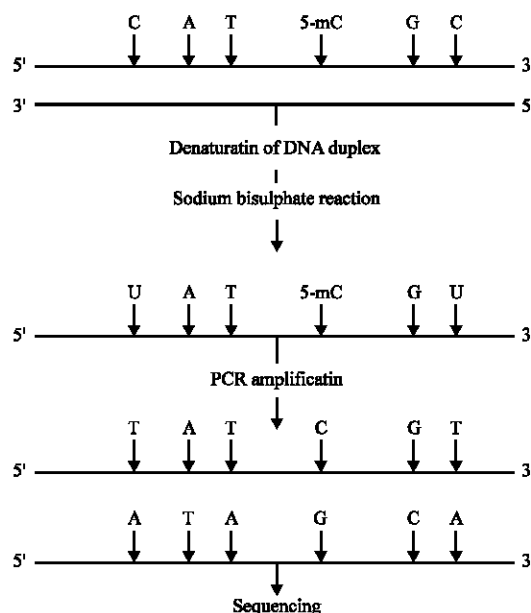

Fig. 1: Scheme illustrating sodium bisulphite-PCR technique for identification of 5- methylcytosine in DNA sequence. 5-mC, C, T, A, G, U indicate 5-methylcytosine, cytosine, thymine, adenine, guanine and uracil respectively, arrows show the position of nitrogen bases on the DNA strands or the direction of the reactions

and (6) followed by sequencing. The results of obtained DNA sequences will show that all cytosines in the original sequence are changed into thymines and comparison of the modified sequence with the unmodified one it becomes possible to analyze the methylated cytosines (Frommer *et al.*, 1992; Olek *et al.*, 1996). Figure 1 illustrates the essential steps required for DNA methylation analysis by sodium bisulphite techniques. Sodium bisulphite technique has been improved by various investigators (Paulin *et al.*, 1998); however the main basic features of the method remained the same. Thus, based on sodium bisulphite techniques several new methods were described.

Methylation-specific PCR (MSP): This procedure involves initial modification of DNA by sodium bisulphite, converting all unmethylated, but not methylated, cytosine to uracil and subsequent amplification with primers specific for methylated versus unmethylated DNA, in other words primers bind specifically to bisulfite-converted methylated or unmethylated DNA, leading to specific amplification. Advantages of this technique are its high relative sensitivity, ease of design and low complexity of the reaction. MSP method requires only small quantities of DNA, hence it is sensitive to 0.1% methylated alleles of a given CpG island locus and can be performed on DNA extracted from paraffin-embedded samples. Another important feature of MSP technique, it can eliminate the false positive results inherent to previous PCR-based approaches which relied on differential restriction enzyme cleavage to distinguish methylated from unmethylated DNA (Clark *et al.*, 1994; Herman *et al.*, 1996; Jeong and Lee, 2005).

Combined bisulfite restriction analysis (COBRA): Additional improvement has been introduced to MSP method by utilization of restriction enzymes to digest PCR products amplified from bisulphite-converted DNA, this is used to reveal methylation dependent sequence differences in PCR products of sodium bisulphite-treated DNA as described earlier, this method is called combined bisulphite restriction analysis (COBRA) method (Xiong and Laird, 1997). The investigators showed some features of this method which include ease of use and the possible application of this technique to determine quantitatively DNA methylation levels at specific loci in small amount of extracted genomic DNA. However, it is worth noting that this method is subjected to a single restriction step after PCR amplification, this might be considered a disadvantage, since all DNA is amplified, without a preferred amplification of methylated DNA. Kneip *et al.* (2009) used *Tsp*509I assay to overcome this limitation via digestion of unmethylated background during PCR cycling. They found this leads to highly sensitive and specific detection of methylated DNA as shown in comparative analysis with MSP on predefined DNA mixtures (Kneip *et al.*, 2009).

Methylation-sensitive single nucleotide primer extension (Ms-SNuPE): Another claimed improvement of sodium bisulphite method demonstrated that following genomic DNA treatment with sodium bisulphite to convert unmethylated cytosine to uracil while leaving 5-methylcytosine unchanged, amplification of the desired target sequence was then performed using PCR primers specific for bisulphite-converted DNA and then the resulting product was isolated and used as a template for methylation analysis at the CpG site(s) of interest (Gonzalgo and Jones, 1997, 2002). This method is named methylation-sensitive single nucleotide primer extension (Ms-SNuPE). The SNuPE assay utilizes internal primer(s) which anneal to a PCR-generated template and terminate immediately 5' of the single nucleotide to be assayed. The investigators demonstrated several advantages of Ms-SNuPE over existing methods because it is quantitative, does not use restriction enzymes and many CpG sites can be analyzed in each primer extension reaction by using a multiplex primer strategy.

Bisulphite differential denaturation PCR (BDD-PCR): As a result of an investigation about potential application of differential denaturation temperature in PCR of bisulphite-treated DNA, Bisulphite Differential Denaturation PCR (BDD-PCR) was invented for the selective amplification of unmethylated sequences following bisulphate treatment of DNA. The use of differential denaturation in PCR is particularly suited to amplification of undermethylated sequences following treatment with bisulphite, since bisulphite selectively converts cytosines to uracil while methylated cytosines remain unchanged. Thus, amplicons derived from unmethylated DNA retain fewer cytosines and their lower G+C content allows for their amplification at the lower melting temperatures, while limiting amplification of the corresponding methylated amplicons (Rand *et al.*, 2006). The authors indicated that BDD-PCR has the potential to be used to selectively amplify and detect aberrantly demethylated genes, such as oncogenes, in cancers and can be effectively utilized in improving the specificity of methylation specific PCR (MSP) by limiting amplification of DNA that is not fully converted, thus preventing misinterpretation of the methylation versus non-conversion.

Bisulphite sequencing PCR and pyrosequencing: Researchers working in DNA methylation analysis have realized that all technologies either allow precise quantification of methylation of several CpGs at once but are labor intensive, precluding the analysis of large numbers of samples, or concern only one CpG or a few CpGs, yielding little information on the methylation patterns of an entire region (Dupont *et al.*, 2004; Fakhrai-Rad *et al.*, 2002; Ronaghi, 2001). Thus recognizing the drawbacks of aforementioned methods, new approaches have been introduced to overcome these negative aspects. The new techniques used pyrosequencing, which is a sequencing-by-synthesis

technology that relies on the luminometric detection of pyrophosphate release on nucleotide incorporation through a cascade consisting of four enzymes. Initial reports demonstrated the feasibility and reliability of this technology for the precise quantification of methylation at each single CpG analyzed (Colella *et al.*, 2003; Tost *et al.*, 2003; Uhlmann *et al.*, 2002). A recent study compared between bisulfite sequencing PCR (BSP) and pyrosequencing for detection and to quantify DNA hypomethylation and hypermethylation (Reed *et al.*, 2010), the findings of the study indicated that although both methods can reliably detect increased, decreased and mixed methylation of DNA, but BSP appears to be more sensitive than pyrosequencing at detecting strong hypermethylation of DNA, on the other hand BSP and pyrosequencing equally detected hypomethylation and mixed methylation of DNA.

LUminometric methylation assay (LUMA): Karami *et al.* (2006) reported method for DNA methylation analysis which is based on combined DNA cleavage by methylation-sensitive restriction enzymes and polymerase extension assay by Pyrosequencing™. The investigators emphasized that the method is quantitative, highly reproducible and easy to scale up, since no primary modification of genomic DNA, such as bisulphite treatment is needed, the total assay time is only 6 h. In addition, the assay requires only 200-500 ng of genomic DNA and incorporates an internal control to eliminate the problem of varying amounts of starting DNA (Karimi *et al.*, 2006).

Methylation-sensitive arbitrarily primed polymerase chain reaction (AP-PCR): A simple and reproducible fingerprinting method was developed called methylation-sensitive arbitrarily primed polymerase chain reaction (AP-PCR) to screen for DNA methylation changes. This technique relies on digesting genomic DNA with methylation-sensitive and -insensitive restriction enzymes (e.g., *Hpa II* and *Msp I*) prior to AP-PCR amplification. The researchers investigated normal and tumor DNAs to identify differential methylation. After the PCR products were resolved on high-resolution polyacrylamide gels, regions of genomic DNA that showed hypo- and hypermethylation associated with tumors were detected. These fragments were then isolated, cloned and sequenced. Novel CpG islands were found to be frequently hypermethylated in bladder and colon tumors (Liang *et al.*, 2002).

DNA methylation analysis by methylight technology: MethyLight technology uses real time PCR analysis

for bisulphate based methylation analysis and was invented by Trinh *et al.* (2001). They reported that this method is a sensitive, fluorescence-based real-time PCR technique that is capable of quantitating DNA methylation at a particular locus by using DNA oligonucleotides that anneal differentially to bisulfite-converted DNA according to the methylation status in the original genomic DNA. Three oligonucleotides (forward and reverse primers and inter-positioned probe) are used in MethyLight, any one or more of which can be used for methylation discrimination, thus allows for a high degree of specificity, sensitivity and flexibility in methylation detection. Furthermore using real time PCR technology reduces the risk of contamination and handling errors associated with manual manipulation of PCR products. The researchers summarized the required steps for MethyLight technology as following: (1) determining the site of interest for methylation analysis, (2) designing methylation-specific primers and fluorogenic probes, (3) isolating the genomic DNA of interest, (4) converting the DNA with sodium bisulfite, (5) performing real time methylation analysis and (6) data processing. However the investigators indicated that MethyLight technology is not designed to offer high-resolution methylation information over a significant area of DNA sequence like bisulfite sequencing or to distinguish in detail different methylation patterns that may be present at the same location (Trinh *et al.*, 2001).

Methylated CpG island amplification (MCA): Another method which utilizes methylation sensitive restriction digestion for DNA methylation analysis was reported by Toyota and coworkers. They used restriction enzymes that have differential sensitivity to 5-methyl-cytosine, followed by adaptor ligation and PCR amplification, methylated CpG rich sequences can be preferentially amplified (Toyota *et al.*, 1999). This method was called Methylated CpG island Amplification (MCA), which is useful for both methylation analysis and cloning differentially methylated genes. The investigators reported that MCA coupled with modified representational difference analysis (RDA) originally reported by Lisitsyn *et al.* (1993), is a useful technique to study methylation and to isolate CpG islands differentially methylated in cancer and were able to isolate 33 clones differentially methylated in colorectal cancer, including several known genes. They showed that these clones are useful markers to identify novel genes silenced by hypermethylation in cancer and may also be useful markers for early detection and prediction of prognosis in colorectal cancer.

Other approaches of DNA methylation analysis: Sodium bisulphite conversion of genomic DNA has led to a large number of new methylation analysis techniques, including some of above mentioned techniques. In this section more examples of methods used for DNA methylation analysis are discussed. One of the studies reported a simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements (Yang *et al.*, 2004), the researchers used bisulphite treatment of DNA and simultaneous PCR of multiple DNA repetitive elements, such as Alu elements and Long Interspersed Nucleotide Elements (LINE). The PCR products were used for direct sequencing in order to quantitate DNA methylation. By restriction digestion or pyrosequencing, the assay was reproducible with a standard deviation of only 2% between assays.

For certain loci, primer design can be challenging for reaching acceptable specificity, to deal with this obstacle, HeavyMethyl technology was invented and it is based on amplification of bisulfite-treated DNA using methylation-unspecific primers in combination with methylation-specific blocking oligonucleotides (Cottrell *et al.*, 2004). Then researchers thought about using blocking conditions, because this might lead to increased assay flexibility and allows for the selective amplification of even such loci where specific MSP is hardly possible, thus Headloop PCR was developed by Rand and coworkers; the amplification of specific sequences after bisulfite treatment is suppressed through an extension at the 5' end of the primers (Rand *et al.*, 2005).

Investigators have aimed to analyze DNA methylation patterns on a genome-wide scale, to perform this task several techniques have been developed, but none of them has yet reached wide acceptance. Rauch and coworkers described a genome-wide DNA methylation detection method that depends neither on restriction endonucleases nor on specific antibodies. This method is based on the methylated-CpG island recovery assay (MIRA), which they previously applied for testing the methylation status of specific genes and it makes use of the high affinity of the MBD2/MBD3L1 complex for methylated DNA. The authors have used MIRA to detect cell type-dependent differences in DNA methylation on a microarray platform (Rauch *et al.*, 2006).

Molecular biologists interested in DNA methylation analysis noticed the possibility of utilization of single cell gel electrophoresis assay (comet assay). This assay was originally designed for detection and quantization of DNA damage at the individual cell level. Following gel electrophoresis under alkaline conditions, DNA is released from the nucleus forming a comet head and tail

and subsequent to fluorescent staining, the intensity of the stain is measured and related to DNA content, with DNA damage being quantified by visual grading or computer image analysis (Heaton *et al.*, 2002). This assay was modified to detect changes in the levels of DNA methylation in single cells. Several investigators used the difference in methylation sensitivity of the isoschizomeric restriction endonucleases *Hpa II* and *Msp I* to demonstrate the feasibility of the comet assay to measure the global DNA methylation level of individual cells (Wentzel *et al.*, 2010).

Recent paper reported new novel method for DNA methylation analysis, the technique is called methylation specific quantum dot fluorescence resonance energy transfer (MS-qFRET) is a nanotechnology assay that enables the detection of methylation and its changes in a sensitive quantifiable manner. It utilizes quantum dot-mediated fluorescence resonance energy transfer to achieve highly sensitive detection of DNA methylation. Template DNA is first treated with sodium bisulfite such that unmethylated cytosines are converted to uracil while methylated cytosines remain unconverted. Thereafter, the converted template is amplified using biotinylated methylation-specific primers. Quantum dots, functionalized with streptavidin, serve both as a scaffold to capture amplicons and as a donor for transferring energy to the Cy5 acceptor that is incorporated into the amplicons during PCR. Thus, the status of DNA methylation can be determined according to the level of Fluorescence Resonance Energy Transfer (FRET) (Bailey *et al.*, 2010).

Finally it is worth mentioned an investigation which showed another approach for genomic DNA methylation analysis. A method was reported which utilized the denaturing high-performance liquid chromatography (DHPLC) to analyze the overall degree of methylation of a genomic region through differential elution profiles based on temperature- dependent resolution of heteroduplexes from homoduplexes (Couvert *et al.*, 2003; Xiao and Oefner, 2001), but this method does not give any information about the methylation status of individual CpGs.

CONCLUSIONS

Genomic DNA methylation is considered one of three epigenomic mechanisms regulating various biological functions. The analysis of DNA methylation patterns has traditionally been challenging. A number of methods have been invented for DNA methylation analysis, however sodium bisulphite based PCR and methylation-sensitive restriction digestion methods remained the most practical

for such analysis in combination with DNA sequencing technologies.

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