REVIEW

DNA Methylation Tools and Strategies: Methods in a Review

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Abstract

DNA methylation is known as an important epigenetic change in plants and vertebrates genome. In this process, the methyl group transferred by DNA methyl transferase enzymes to cytosine at carbon residue 5 often in the CpG dinucleotide context. DNA methylation plays an important role in the natural development of the organism, genome stability maintenance and processes such as genomic imprinting and chromosome X inactivation in mammals. In addition, changes in DNA methylation pattern have seen in many diseases, including cancer. Analysis of DNA methylation has been useful for rapid disease diagnosis and progression. In recent decades, a revolution has taken place in the methods of DNA methylation analysis, and it is possible to study the pattern of gene methylation at a widespread, short and high resolution level. These methods can be divided into three general categories: (1) cut-based methods by methylation-sensitive enzymes; (2) sodium bisulfide based methods; (3) antibody based methods. Since the existence of different methods makes it difficult to select the appropriate approach, in this review, a number of common methods for examining the methylation pattern with the advantages and disadvantages will be discussed.

Keywords: Epigenetics- DNA methylation- restriction enzyme- sodium bisulfite

Introduction

DNA methylation known as the first epigenetic modifications reported in mammalian cells [1]. Epigenetics was first developed by Conrad Hal Waddington in the 1942s [2]. Epigenetics is an emerging area of research and is the study of stable and heritable alteration of gene expression without change of underlying DNA sequence [3-4]. Two most well characterized chromatin remodeling processes including: 1) DNA methylation (methyl group is covalently added to cytosine in DNA) 2) histone post-translational modification [5]. Epigenetic alteration triggered in response to environmental exposures to modulate the cellular function [6-7] which included adding a methyl group DNA methyltransferase covalently to 5'-carbon of cytosine mostly in CpG dinucleotide [8-9]. In mammalian genome, a large number of promoters co-localize with CpG islands (CGIs) [10]. Methylation of CGIs promoters lead to regulation of gene expression by regulation of protein-DNA interactions and finally presented by repression or high expression of underlying gene [10].

The methyl-CpG-binding proteins repress gene expression by histone deacetylase complex which remove acetyl groups from lysine residues at the N-terminal site of histones. It causes modification of histones, their function, and finally preventing access to transcription factors with DNA remodeling [11-12]. DNA methylation as mechanistically understood epigenetic modifications plays a critical role in mammalian development, mammalian X-chromosome inactivation, cellular memory, imprinting mechanism, pluripotency and differentiation control, cancer formation, cell cycle control, and apoptosis [13-14]. DNA methylation pattern assessment has emerged as an promising factor related to diabetes [15], cardiac disorder, neural disorders, and cancer [16-17]. Global or specific hypomethylation region of DNA demonstrated to be correlated with genomic instability [18]. It causes cancer formation by increasing mutation rate [19] specifically in tumor suppressor genes promoter region [20]. Therefore, investigation of DNA methylation pattern can be used for prediction, diagnosis and therapeutic

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outcome [21]. Human diseases related to genetics content and environmental factors interaction can be evaluated by DNA methylation studies. DNA methylation as an epigenetic biomarker provides more useful information than transcription-based biomarkers. Frequency of DNA methylation in early stages of cancer is higher than other situations [22-23].

In addition, methylated regions of DNA can be easily amplified and identified using PCR-based methods. Although epigenetic changes occur only in a small number of cells, it's suitable for early detection as a promising biomarker [24-25]. Different clinical samples for methylation studies can be used such as saliva, plasma, serum, urine and feces by non-invasive methods [26]. Although methylation approach is interesting, it suffers from some limitations. The main challenge faced by DNA methylation is variation among different cells derived from a same sample. Also, detection of 5-methylcytosine (5mC) from other types of DNA alterations, including hydroxymethyl cytosine, remain as challenge in many existing protocols [27-28]. Only some of the restriction enzymes are sensitive to methylated DNA; therefor, the restriction enzymes lose the ability to cut DNA at the specific site after DNA methylation. This is the choice method for methylation assessment. Another limitation of this method is DNA quantity to analysis which it should have high concentration [29-30]. Furthermore, CpG location could impact on the results in order to being underneath or far from enzyme recognition site [31-33]. However, early methylation analysis by PCR is a powerful method to overcome the DNA quantity challenge.

Other method has been developed based on bisulfite sequencing for methylation studies which need sulfonation at position six of pyrimidine rings and the fragment sequencing [34-35]. The molecular mechanism of this technique is based on exposure of sodium bisulfite and modification of cytosine into uracil. Sodium bisulfite does not affect methylated cytosine or 5-methylcytosine. Therefore, bisulfite can be analyzed by PCR, sequencing and microarray methods [36].

Differentiation between methylated DNA and non-methylated DNA using anti-methyl antibodies is another method. Immunoprecipitation method has been developed based on differentiation between methylated DNA and non-methylated DNA using anti-methyl antibodies [36].

Restriction landmark genomic scanning (RLGS)

Restriction landmark genomic scanning (RLGS) is a two-dimensional gel electrophoresis system [37]. Its known as one of the most reliable method to characterize CpG island methylation into nearly 2,000 restriction landmarks that has high probability of containing gene [38]. RLGS has been widely applied to characterize variation of methylation phenotypes and to identify candidate tumor suppressor genes, genome mapping, genomic amplifications, and degree of CpG island hypermethylation (39-40). During the initial step, DNA digested with a methylation-sensitive restriction enzyme such as NotI, AscI and MspI. It causes DNA to be cut only in methylated sites and then radionuclides added by DNA polymerase into the NotI half-site to visualize in next step by a two-dimensional electrophoresis process. The radioactive second-dimension gel is running with another restriction enzyme such as EcoRV at the first dimension and then the second dimension performed by Hinf1 restriction enzyme. The methylated and non-methylated fragment visualized by fragments comparison [41].

HpaII/MspI-PCR

The HpaII/MspI-PCR is commonly used for detection of 5mC in specific genes. This method is based on the sensitivity of restriction enzymes to methylation at their cleavage region. After digestion, restriction fragments amplified by PCR [42-43]. The sensitivity of this method is correlated with the number of cleavage sites which recognize by restriction enzymes. HpaII and SmaI are the most common methylation-sensitive enzymes in this method. MspI is isoschizomer for HpaII and XmaI and neoschizomer for SmaI. These isoschizomers are used in pairs for instance HpaII/MspI and recognize the same 5′-CCGG site. The main differences between them is sensitivity to methylation of cytosine [44]. MspI is not sensitive to methylation and remains undigested fragment which cannot be amplified in the PCR process. The other site, HpaII, is unable to digest target DNA if methylated CpG sequences overlap with the cleavage site [45].

Luminometric Methylation Assay (LUMA)

LUMA (Luminometric Methylation Assay) was first developed by Karimi et al. in 2006. This method is a high throughput method to the analysis of genomic DNA methylation based on the use of combined DNA cleavage by methylation-sensitive restriction enzymes and polymerase extension assay by pyrosequencing. The first step in this process is DNA digestion with a methylation-sensitive enzyme such as HpaII, and then all CCGG sites cleave with a non-methylation-sensitive enzyme such as MspI. EcoRI enzyme as an internal control could be used to resolve the problem of varying amounts of starting DNA [46-47]. After enzymatic digestion, the sticky ends of the cleaved fragment are amplified by labeled nucleotides. Signals from the labeled nucleotides reported by sequencing is analyzed to determine the methylation rate. Perhaps the most serious disadvantages of this method is due to its quantitative, rapid, internal control. Also, it is a very accurate method for determining the methylation percentage and can also be performed with low initial DNA content [48].

Methyl-sensitive cut counting

The basis of this approach is similar to the serial analysis of gene expression by next generation sequencing (NGS). It is a cost-effective method to detection of unmethylated CpG at single base-pair scale and can give a broad picture of hypomethylation of small fragments of the genome. This method limitation is being cytosine sensitive in the CCGG region which can easily be solved by employing several restriction enzymes with different cleavage sites. In the initial stage of the process DNA

is cleaved at non-methylated cytosine in CCGG region and then the sticky ends of these regions are filled using labeled nucleotides and identified as first adapter. At the second stage, DNA is cut using another restriction enzyme that cleave DNA about 20-30 bp near to the first adapter which known as second adapter. Following to this step a pair of primers based on the adaptor sequence undertaken to amplification and sequencing of cleaved DNA [49].

Methylated CpG Island Amplification and Microarray (MCAM)

MCA is a PCR-based methylation screening technique to identification of methylated CpG islands in a relatively large number of samples. In this method, CGIs selectively are targeted with oligonucleotide adaptors following to a digestion stage with a combination of methylation-sensitive enzyme such as SmaI and methylation-insensitive nucleases such as XmaI. DNA segment after that are amplified to generate million copies by PCR. Amplified fragments, showed the methylated fraction of the genome. Control and patient samples should be labeled with different fluorochromes and used in a comparative hybridization of reference and test samples on a microarray platform [50]. MCAM is a promising technique to study of cancer-related genes [51].

CpG methylation array profiling via bisulfite conversion

Bisulfite sequencing technology is recognized as the gold standard to determine methylated DNA. It was designed by Frommer et al. as a powerful technique with the ability to identify exact quantity or position of 5mC at a single base pair resolution [52]. With the advancement of technology and improvement of understanding of methylation processes, numerous methods have been developed for methylation analysis, but they are no longer widely used for screening because of their need for sequencing, high cost restriction, and low sensitivity (about 20%). Bisulfite sequencing technology converts C residues into U. Then uracil residues represent as thymine and amplified in the sense strand following PCR and sequencing. By contrast, 5mC distinguished from unmethylated cytosine residues because it do not respond to bisulfite exposure and displayed as cytosine residues on the amplified fragment [53].

Methylation-sensitive high-resolution melting (MS-HRM)

Methylation-sensitive high-resolution melting (MS-HRM) was first reported by Guldberg et al for genotyping studies [54]. This method is based on the difference of melting profiles between methylated and unmethylated PCR products when they subjected to thermal denaturation. Differences is due to PCR products GC content from unmethylated variant and in this way, they response to bisulfite modification in different patterns. Such approaches, although have failed to address detailed information about the methylation status of single cytosine within the fragment of interest, they can be analyzed by DNA sequencing [55]. This method is particularly useful to distinguish fully and partially methylated samples. In fact, MS-HRM is known as an pre-test to identify only methylated samples to next step of analysis process. MS-HRM is time and cost effective which has been attracting a lot of interests for both research and diagnostic settings [56].

Methylation-specific PCR (MSP)

Methylation-specific PCR (MSP) is a method that has facilitated analysis of methylation patterns of CpG islands located in the promoter. The main disadvantage of MSP is ability to assessing the methylation status in cell lines, and fresh or frozen tissues. In the initial stage of the process modification of cytosine's to uracil under sodium bisulfite conversion happened. Then, the bisulfite induced sequence differences determined and quantified accurately by PCR using two primer pairs for both unmethylated and methylated DNA. For methylated state, only the methylated state-specific primers are able to annealing to the target sequence and caused to amplification. The most important limitation of MSP is false- positive due to non-specific primers or annealing temperatures [57-58].

Whole genome bisulfite sequencing (WGBS)

Whole-genome bisulfite sequencing (WGBS) using next-generation sequencing technologies that is gold standard for analysis of DNA methylation status of whole genome [59]. This method is based on cytosine conversion into uracil with bisulfite exposure and after sequencing process, unmethylated cytosine represented as thymine. 5mC protected from conversion and record as cytosine (C). The most serious disadvantage of this method is analysis by sequencing process, experiment high cost, and needing large amounts of template DNA. WGBS can be carried out by Alu or LINE sequences. Another method is pyrosequencing, which is accurate method to determine of single-nucleotide polymorphism (SNP). The basis of this method is "sequencing by synthesis". Nucleotides incorporated by DNA polymerase in new strand and detected following to conversion of the pyrophosphate group. The ATP is used by luciferase to convert the luciferin and generated light which can be detected. Therefore, light is produced only when the soluble nucleotide acts as the complement of the target. DNA sequence can be determined with signal being produced. The main advantages of pyrosequencing is accuracy, flexibility, and parallel processing. Pyrosequencing is capable of analyzing 96 samples in just 20 minutes and does not require labeled primers, nucleotides and electrophoresis. This method has the ability to determine the frequency of alleles in samples, mutation analysis, methylation analysis, molecular haplotyping, correlation studies and single nucleotide polymorphism detection [60-61].

Combined Bisulfite Restriction Analysis (COBRA)

COBRA assay allows analyzing the sensitive quantification of DNA methylation status at a specific genomic locus. This method consists of major steps included: 1) bisulfite conversion of cytosine to uracil 2) amplification of converted DNA by locus-specific PCR 3)

digestion with restriction enzyme 4) restriction patterns assessment by Gel electrophoresis 5) quantification of restriction patterns. Methylated and unmethylated sequences have different response to bisulfite exposure and finally led to sequence differences and restriction patterns divergence. This method is quantitative, user friendly, needs low level of DNA concentration and could use for samples that are fixed with paraffin [62].

Methylation-sensitive single-nucleotide primer extension (Ms-SNuPE)

Methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) is quantitative assays with potential to rapid quantitation of methylation level at CpG sites. It used as a large-scale methylation screening assay. In this technique unmethylated cytosine at the first step converted to uracil with bisulfite while leaving methylated cytosine not altered. Then, Strand-specific PCR is carried out to provide a DNA template for quantitative methylation analysis using single-nucleotide primer extension. Ms-SNuPE performed with oligonucleotide (s) designed to hybridize immediately to upstream of the CpG islands. At least one of the PCR primers 3' end designed to anneal to Uracil that generate by bisulfite treatment. The visualization and quantitation of products should be performed by polyacrylamide gels electrophoresis. The advantage of this method is that it does not require a restriction enzyme and therefore CpG is not require to be in the enzyme recognition site [63-64].

MethyLight assays

MethyLight assays, is a probe-based, sodium-bisulfite-dependent, real-time PCR assay that allows rapid and sensitive quantification of the methylation pattern. This technique is compatible with small amount of template DNA and has sensitivity to very low frequencies of hypermethylated alleles. This advantage makes it suited for detection of methylated biomarkers in disease diagnosis [65-66]. Unlike MS-HRM, this method cannot detect heterogeneity because the primers and probes are designed for a specific methylation pattern. This assay has two steps including: methylation-specific priming and methylation-specific fluorescent probing. The methylation-specific probe can anneal to the methylated sequence. This assay can be performed via TaqMan probe which primers are methylated-specific and require a reference gene for quantitative PCR optimization [67-68].

HeavyMethyl (HM) assay

The HeavyMethyl (HM) assay is a real-time PCR method allows the qualitative and quantitative DNA methylation analysis of different samples including: fresh-frozen, formalin-fixed, and paraffin-embedded tissues and other samples such serum, plasma, and urine. The HM assay is depends on the use of a pair of primers flanking the sequence of interest and non-extendable oligonucleotide blockers are at overlapping primer-binding site. Therefore, they achieve methylation-specific amplification and detection [69].

Challenges of bisulfite-based methods

The deamination of all non-methylated cytosine not located in CpG is not completely accomplished in the target sequence. This may be due to the inappropriate timing and temperature for single stranded DNA amplification or the inadequate pH and concentration of the solutions or low DNA treatment time. Sulfite is recommended to solve this problem at least at 6-8 hours treatment time.

Affinity enrichment

Affinity enrichment is a technique that allows fast and easy analyze genome-wide DNA methylation patterns and methylation of mammalian promoters. This method is based on isolation of methylated DNA by antibody or with methyl-CpG binding domain (MBD) proteins. These methods are used in conjunction with microarrays or next-generation sequencing as high throughput [70]. Methylated DNA Immunoprecipitation (MeDIP) is depend on 5-methylcytidine antibody to enrichment of methylated cytosine and this method is more popular than MBD-based method [71]. Affinity enrichment detection has a variety of challenges. this methods suffers from sequence bias and false positive results [33]. The difference between using antibodies or proteins is that they are used for double-stranded and single-stranded DNA, respectively. Also, methylated-specific antibodies are used for CpG-rich regions while methyl-CpG binding domain is appropriate for detection of rare abundance CpG. Methylated-CpG Island Recovery Assay (MIRA) is another type of Affinity-based enrichment. It is based on use of MBD2b/MDB3L1 complex binding to double-stranded methylated DNA MIRA which is capable to detection of methylated DNA at low-density methylation of a single methylated CpG nucleotide [72]. New methods without the need for sulfate and enzymes offer numerous benefits such as speed, low cost and simplicity. One of these methods is High-performance liquid chromatography for DNA methylation analysis and the other is Methylated DNA recipitation combined Luciferase-fused Zinc finger Assay (MELZA).

High-performance liquid chromatography for DNA methylation analysis

An anion-exchange HPLC method for quantifying total DNA methylation was developed in 1980 by Kuo et al. which the basis of this method was mobility difference of cytosine and 5-methyl cytosine electrostatic properties. This method allows to characterize epigenetic changes during development and stress. It has potential to separate unmethylated cytosine from 5-methyl cytosine. This method may require large amounts of DNA which considered as its limitation [73-74].

Nanopore sequencing

Nanopore-based single-molecule device enables us to direct detection of methylation status of unamplified DNA sequence. At first, methylated DNA sequence recognized with methyl-CpG binding domain protein (MBD1). Then, DNA–MBD1 complex transported through two-

dimensional material nanopores (constriction of 19 nm diameter). The position of the methylation sites can be accurately determined by this method. Unmethylated DNA cannot be detected because of its smaller diameter [75].

Single-molecule real time (SMRT) sequencing

Single-molecule real time (SMRT) sequencing is using Polymerase enzyme kinetics [76]. In SMRT sequencing, fluorescently labeled nucleotides are incorporate into complementary nucleic acid strands. This method allow direct detection of 5-methylcytosine in the DNA template [77]. This method require methylated and unmethylated DNA template as control. Polymerase processivity is visualized by comparison of methylated template with control. Polymerase kinetics and processivity are affected by any modification of nucleotides and this change can be detected [77-78].

Methylated DNA precipitation combined Luciferase-fused Zinc finger Assay (MELZA)

This method is a combination of tow teqniques including: Methylated DNA Immunoprecipitation and Luciferase-fused Zinc finger Assay (MELZA). This method comprises the following 4 steps: 1) Capturing of methylated DNA using methyl CpG-binding domain 2) amplification of MBD-captured DNAs, which has a zinc-finger recognition site, by PCR via biotinylated primers 3) amplified sequences immobilization on magnetic beads biotin- streptavidininte reaction 3) quantification of immobilized fragment using luciferase-fused zinc finger protein [79-80].

In conclusion, today, a wide range of techniques has been set out for DNA methylation profiling. However, no specific method has been identified as the "gold standard" technique. Each method has its advantages and disadvantages. In recent years commercial kits and high throughput method have been attracting a lot of interest within the field of DNA methylation analysis. The first step in Methylation analysis is the identification of methylated sequence with microarrays or next-generation sequencing and then this sequences can be analysis by detail with other methods based on each interest. This review covered only some of the DNA methylation profiling methods to better understanding of each method. However, there are many challenges in choosing the best method.

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