DNA-Directed Methylation Analysis Methodology

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Abstract

Epigenetics alterations, especially DNA methylation, play a critical role in control of gene expressions. Abnormal patterns of methylation are observed in early-stages of many cancers. Therefore, methylation analysis is useful in primary detection of tumors. Advances knowledge about the functional role of aberrant epigenetic modifications as potential biomarkers for cancer, have attracted considerable interest to pursue such investigations. Currently, many methodologies are available to distinguish methylation patterns, however, none is considered as the ‘gold-standard’ technique. This paper is an overview on some convenient methylation analysis methods.

Introduction

DNA methylation, an important epigenetics alteration, exists in all living creatures and plays a very important role in human development, control of genome expression and diseases (1). DNA methylation adds a methyl (CH₃) group at the fifth carbon position of a cytosine within a cytosine-guanine (CpG) dinucleotide (2). Aberrant changes in DNA methylation status are ubiquitous in the many of human cancer and the detection of these changes can be informative for cancer diagnosis(3). DNA methylation is important for different processes in eukaryotic cells, such as X-chromosome inactivation, genomic imprinting and suppression of repetitive sequences, also it has found that changes in methylation profile have a substantial impact on carcinogenesis processes (4). Research illustrated that global hypermethylation as well as gene-specific hypermethylation lead to cancer in human, especially in the case of tumor suppressor genes. Genome-wide or global hypomethylation lead to chromosomal instability, consequently the rate of mutation increases. Furthermore, promoter hypermethylation, especially in tumor suppressor genes, prevents transcription factors from binding to the promoters and consequently suppresses gene transcription (Figure 1) (5). Therefore, analysis of CpG methylation is very helpful in diagnosis and progression of cancers as well as classification of disease. Moreover, DNA methylation as a very stable biomarker can be easily collected and detected in different type of samples such as saliva, plasma, serum, urine and stool in negligibly invasive methods(6). DNA methylation biomarkers as epigenetics changes offer several important benefits over expression-based biomarkers. For instance, the frequency of DNA methylation changes in the early stage of cancers is significantly...
more than conventional genetics changes. Furthermore, they are easily amplified and detected using PCR-based methods even if such changes are present only in a small number of cells (7). However, there are different limitations for DNA methylation analysis, especially for individual methylated CpG sites. Moreover, heterogeneity of methylation profiles in various cells within one sample is yet another limitation of DNA methylation analysis techniques (8). Furthermore, distinguishing 5-mC and other types of DNA modifications in mammalian DNA including 5-hydroxy methyl cytosine (5-hmC) is a challenge in many available protocols (9). Assays for DNA methylation analysis must be able to detect this small modification in a sequence-specific manner, a task that is hampered by the fact that DNA methylation is not replicated and amplified during the process of the polymerase chain-reaction (PCR), therefore, assays for DNA methylation analysis must be able to detect and fix this small chemical modification in a sequence-specific manner (10).

Nowadays, there are several approaches for the detection and validation of DNA methylation analysis (Figure 2). However, no single method has emerged as the ‘gold-standard’ technique. Each method has its advantages and disadvantages. By understanding the type of information provided, an investigator can select the method most suitable for his/her specific research needs. This paper firstly considers some convenient methylation analysis methods based on bisulfite, affinity enrichment and restriction enzymes and then considers quantitative and more recent methods.

**Methylation-specific PCR (MSP)**

Bisulfite converts unmethylated cytosines into thymines, which means that it turns an epigenetics difference into a genetic difference (11). These conversions can be recognized by several methods combined with PCR, sequencing or microarray. One of the most popular methods based on using bisulfite is methylation-specific PCR (MSP) which utilizes two sets of distinct methylation-specific primer for the sequence of interest. This is a very sensitive and rapid method for the analysis of CpG methylation, however, is not a quantitative approach yet (12).

**Bisulfite sequencing**

Another Bisulfite-based method is bisulfite sanger sequencing. In this approach, firstly, cytosine converts to uracil by bisulfite, then PCR is performed using two sets of primers including methylation-specific and non-specific primers in which all uracils are replaced by thymine. Consequently, it produces methylation-specific single-nucleotide differences which then can be recognized by sanger sequencing after alignment against the reference sequence (13).

**Affinity enrichment**

Affinity-based methods usually use antibodies (MeDIP) or methyl-specific proteins such as methyl-binding domain of human methyl-CpG-binding-domain protein 2 (MBD2) (MIRA) for enrichment of the methylated DNA (14,15). These techniques are typically combined with array or next-generation sequencing or real-time PCR (16). The downstream methods which use such an approach result in enrichment of the sample with methylated DNA. Affinity-based methods allow for rapid genome-wide analysis of DNA methylation, but they are not sensitive for individual methylated CpG sites. Moreover, they produce false-positive responses due to cross-reactivity of antibodies.

**Restriction enzyme-based approaches**

These approaches use some restriction enzymes which are sensitive to methylation, for example HpaII is inhibited using the methylation of DNA, or conversely McrBC has tendency to methylate the DNA (17). Restriction enzyme-based methods are highly sensitive however, such methods subject to limitation since the coverage of
genomic DNA is very low and each restriction enzyme covers only 2-8% of the genome. Moreover, it requires long intact and large amount of DNA with high-quality (18).

**Quantitative methods**

Combined Bisulfite-Restriction Analysis (COBRA) is a simple quantitative method to analyze the methylation patterns. In this method, after bisulfite modification and PCR amplification, the PCR product is digested with a restriction enzyme and quantitated using gel electrophoresis and densitometry. However, this is only for specific restriction enzyme cutting sites, relatively time-consuming and Gel-based analysis (19). Real-time MSP protocols use the benefits of real-time PCR by fluorescent-labeled MSP primers and TaqMan probes. Briefly, bisulfite-converted DNA is amplified using a 5′ fluorescent reporter dye and a 3′ quencher dye (20). There are several quantitative approaches based on real-time such as methyl light (21), heavy methyl (22) and QAMA (23) which are suitable for the detection of specific patterns of methylation in primary tumor tissues, and more importantly, for the recognition of very low levels of methylated circulating DNA. FRET-based techniques are other tools have shown great potential for diagnostic and screening of methylation pattern in cancer.

A new FRET-based technique has been improved using the quantum dots. This approach is highly sensitive whereby only a very low amount of DNA is required (24). Recently, bisulfite pyrosequencing has been improved for highly quantitative analysis of methylated DNA. After bisulfite treatment and PCR, the degree of every methylation at each CpG position in a sequence is determined from the ratio of T and C (25). Analysis of methylation profiles in DNA by pyrosequencing combines a rapid and simple reaction protocol with high-throughput, reproducible and accurate protocol of methylation at several CpG sites.

**Microarray-based methods**

High-throughput studies of DNA methylation profile, especially for human epigenome project, are important in methylation analysis methodologies. There are three different array-based technologies including (i) bead arrays (e.g. from Illumina), (ii) short oligonucleotide arrays (25-mer oligonucleotides, from Affymetrix) and (iii) long oligonucleotide arrays (60-mer oligonucleotides, from NimbleGen and Agilent). Moreover, there exist two popular Bead chips technologies including GoldenGate™ (26) and Infinium® (27). GoldenGate™ bead chips can analysis methylation state of 1536 specific CpG sites in 371 genes (one to nine CpG sites per gene) measured in a single reaction by 200 ng of bisulfite-treated genomic DNA (26). Infinium® bead chips are new high-density approaches which can assay over 480K CpG sites. This enables up to 96 samples to be run in parallel (high sample throughput) (27).

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**Figure 2.** The general outline of some conventional and new methods for the study of DNA methylation. MSP: Methylation-specific PCR, COBRA: Combined Bisulfite-Restriction Analysis, QAMA: Quantitative Analysis of Methylated Alleles, SMRT: Single-Molecule Real-Time Sequencing.
Newer methods

Conventional methods usually use bisulfite or restriction enzymes with the potential of generating false positive results. In addition, such methods are time-consuming and costly. New methods are bisulfite-free/enzyme-free which would conquer these limitations and possess several advantages, such as speed, low cost and convenience. The nanopore-based assay is a novel method which selectively detects methylated DNA/methyl-binding protein complex through a 19 nm nanopore with significantly deeper and prolonged nanopore ionic current blockade, while unmethylated DNA is undetectable due to smaller diameter. It is a simple, direct and single-molecule electrical detection approach to early cancer detection (28). SMRT sequencing is yet another novel technique which can detect not only N6-methylcytosine, but also 5-methyladenine, and 5-hydroxy methyl cytosine in DNA methylation, without bisulfite conversion. In SMRT sequencing, DNA polymerases enzyme incorporate fluorescently labeled nucleotides into complementary DNA strands. The arrival times and durations of the fluorescence pulses yield information about polymerase kinetics and allow direct detection of modified nucleotides in the DNA template (29). MELZA utilize a luminescent assay using an engineered proteins including luciferase fused to zinc finger proteins. It is a new approach developed for DNA methylation analysis without bisulfite treatment. In this method DNA is fragmented by restriction of enzymes. Then methylated DNA fragments are captured using the methyl-binding proteins and finally amplified by PCR. Consequently, the amplicons are quantitatively recognized through the luciferase activity (30).

Conclusion

There is no single technique to completely achieve all required standards for the analysis of DNA methylation patterns. The choice of the suitable approach depends on several parameters including quantity, nature and the quality of samples. Furthermore, the availability of equipments and the expertise of researchers are among key issues to consider. The best selection of a method for the analysis of methylation patterns may be a combination of several approaches to enable generating accurate, sensitive and reproducible results.

References

20. Rand K, Qu W, Ho T, Clark SJ, Molly P. Conversion-specific detection of DNA methylation using real-time polymerase chain reaction (ConLight-MSP) to avoid false positives. Methods, 2002;27: 114e120.


