Methods of DNA methylation analysis

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Purpose of review

To provide guidance for investigators who are new to the field of DNA methylation analysis.

Recent findings

Epigenetics is the study of mitotically heritable alterations in gene expression potential that are not mediated by changes in DNA sequence. Recently, it has become clear that nutrition can affect epigenetic mechanisms, causing long-term changes in gene expression. This review focuses on methods for studying the epigenetic mechanism DNA methylation. Recent advances include improvement in high-throughput methods to obtain quantitative data on locus-specific DNA methylation and development of various approaches to study DNA methylation on a genome-wide scale.

Summary

No single method of DNA methylation analysis will be appropriate for every application. By understanding the type of information provided by, and the inherent potential for bias and artifact associated with, each method, investigators can select the method most appropriate for their specific research needs.

Keywords

DNA methylation microarray, epigenetic, epigenomic

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Abbreviations

^{5m}C	5-methylcytosine
MCA	methylated CpG island amplification
MeDIP	methylated DNA immunoprecipitation
MSDK	methylation-specific digital karyotyping
MSP	methylation sensitive PCR
RLGS	restricted landmark genome screening

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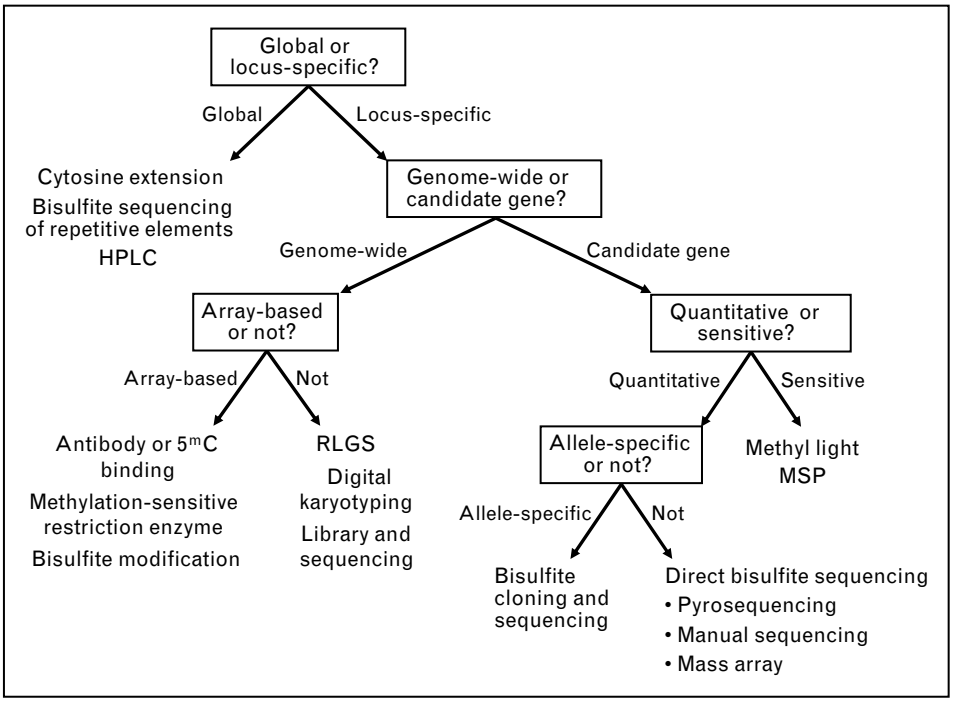
Introduction

Nutrients induce acute changes in gene expression by affecting transcriptional and posttranscriptional mechanisms, and the field of nutrient–gene interactions has been an active area of investigation for many years. More recently, it has become clear that nutrition can also affect epigenetic mechanisms, causing long-term changes in gene expression [1]. For example, there is increasing evidence that epigenetic mechanisms play a role in the developmental origins of health and disease [2], in which nutrition and other environmental factors affect developmental pathways to cause persistent changes in gene expression, metabolism and chronic disease susceptibility.

Epigenetics is the study of mitotically heritable alterations in gene expression potential that are not mediated by changes in DNA sequence [3]. Epigenetic regulation is critical for mammalian development and cellular differentiation, and epigenetic dysregulation causes human developmental diseases and cancer [4,5], and potentially a wide range of adult-onset chronic diseases [2]. In the mammalian nucleus, DNA is associated with histone proteins in a highly regulated and dynamic complex called chromatin. Several molecular mechanisms, including methylation of cytosines within CpG dinucleotides, various covalent modifications of histone proteins and cell-specific combinations of autoregulatory DNA-binding proteins, interact synergistically to regulate regional chromatin conformation (and transcriptional competence) in different cell types. From a clinical nutrition perspective, it is advantageous to focus on CpG methylation in initial studies of epigenetic regulation. CpG methylation is often highly correlated with locus-specific transcriptional activity [3]. As DNA methylation requires diet-derived methyl donors and cofactors, nutrition can affect this epigenetic modification [6]. Lastly, the covalent modification of cytosines within DNA is highly stable and very little DNA is required for its analysis. Hence, clinical samples of limited quantity and dubious quality can often be assessed for this epigenetic modification.

This article will not address the mechanisms by which CpG methylation affects transcriptional activity. Rather, our goal is to provide guidance for investigators who are new to the field of DNA methylation analysis. We will describe different approaches for analysis of DNA methylation, elaborate a 'decision tree' to aid in method selection and describe recent methodological advances in this field.

Figure 1 A decision tree to guide the selection of appropriate DNA methylation analysis methods



MSP, methylation sensitive PCR.

Methods for DNA methylation analysis

The appropriate approach for analysis of DNA methylation depends upon the goals of the study. Figure 1 shows a simplified decision tree to aid in the selection of appropriate methods for DNA methylation analysis.

A major advance in DNA methylation analysis was the development of a method for sodium bisulfite modification of DNA to convert unmethylated cytosines to uracil, leaving methylated cytosines unchanged [7]. This allows one to distinguish methylated from unmethylated DNA via PCR amplification and analysis of the PCR products. During PCR amplification, unmethylated cytosines amplify as thymine and methylated cytosines amplify as cytosine (Fig. 2a). Most methods for analyzing DNA methylation at specific loci are based on this approach.

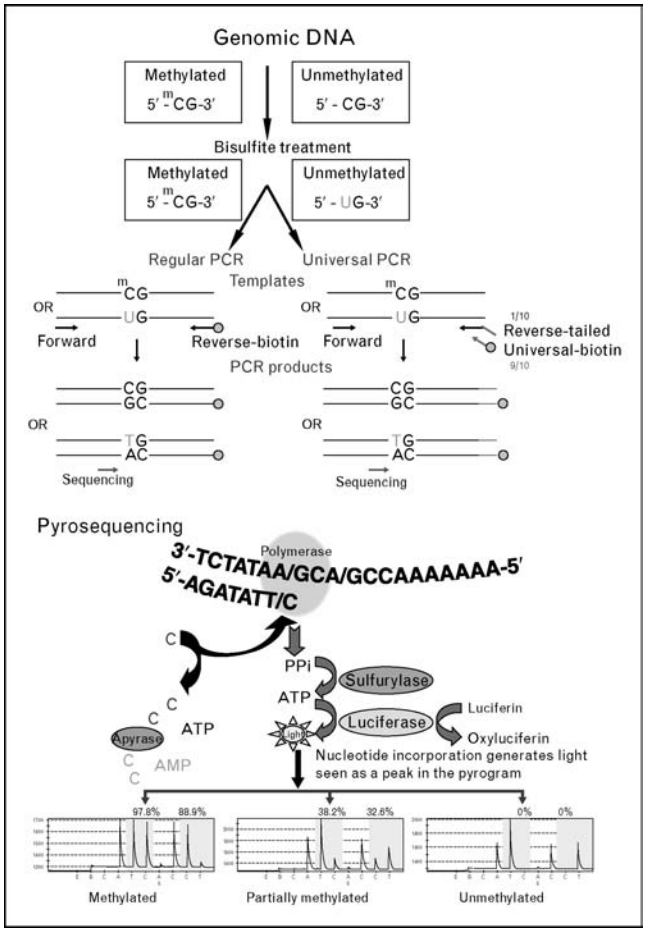
Global DNA methylation analysis

In mammals, 70–80% of all CpG dinucleotides are methylated and this methylation occurs predominantly in repetitive elements and regions in which CpG density is low. Conversely, CpG-rich regions called 'CpG islands', often found in gene promoters, are generally unmethylated. Interest in correlating the genomic 5-methylcytosine content with diet, lifestyle and clinical outcomes, such as in cancer patients after treatment with hypomethylating agents, is widespread [8]. High-performance liquid chromatography (HPLC) [9] is a classical method to quantify global DNA methylation and is highly quantitative and reproducible. This method requires large amounts of high-quality genomic DNA, however, and is not suitable for high-throughput analyses. To circumvent these problems, several bisulfite-based PCR methods have been developed to approximate global DNA methylation by assessing repetitive DNA elements such as *Alu* elements and long interspersed nucleotide elements (*LINE*) [10,11]. These methods require very little DNA and can be applied to paraffin-embedded tissue. As global methylation analyses provide no information on the genomic positions at which methylation is altered, however, it is difficult to link such changes to functional outcomes.

Gene-specific methylation analysis

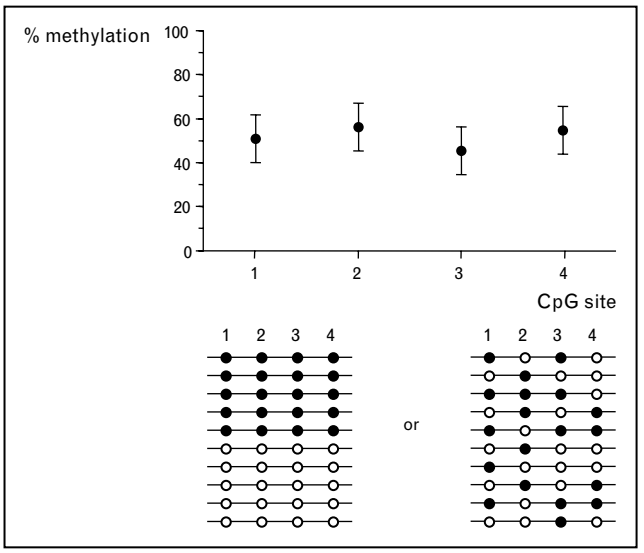
Gene-specific methylation analysis methods can be characterized as either 'candidate gene' or 'genome wide' approaches. We will discuss candidate gene approaches first; these can be further divided into 'sensitive' and 'quantitative' approaches. In the sensitive methods, methylated or unmethylated alleles are detected by designing primers overlapping multiple CpG dinucleotides. In quantitative methods, primers are designed to amplify both methylated and unmethylated alleles with equal efficiency, and the methylation level is analyzed by various approaches.

Figure 2 Outline of bisulfite-pyrosequencing analysis



(a) Bisulfite modification induces C to T polymorphisms at each unmethylated CpG site. Regular PCR or universal PCR is used to amplify genes of interest. (b) Percentage methylation at specific CpG sites can be detected and quantified by a pyrosequencer using a sequencing-by-synthesis method.

Figure 3 Divergent patterns of allele-specific CpG methylation



These can give identical results when CpG methylation is analyzed nonallele specifically. The top panel shows hypothetical results for percentage methylation at four CpG sites. The lower panel shows two very different possibilities for allele-specific data that would be consistent with these results.

Sensitive methods

Methylation sensitive PCR (MSP) [12] is a rapid and very sensitive technique to screen for methylation. Following bisulfite modification, PCR is performed using two sets of primers designed to amplify either methylated or unmethylated alleles. MSP has the advantages of being highly sensitive (able to detect one methylated allele in a population of more than 1000 unmethylated alleles) [12], and can be used on DNA samples of limited quantity and quality. MSP is not quantitative, however. Variations of MSP called MethylLight [13] or quantitative analysis of methylated alleles (QAMA) [14] were recently developed using real-time PCR for methylation detection. These assays were designed to detect completely methylated or unmethylated alleles, denying the reality of partial allelic methylation. The design of primers is essential for reliable results; ideally, the 'methylated' and 'ummethylated' primer sets should be designed for the same CpG sites and include multiple CpG sites at the 3′ ends.

Quantitative methods

Except for methylation-sensitive restriction analysis by Southern blotting, all quantitative methods for measuring DNA methylation at specific loci are based on modification of DNA by sodium bisulfite. Depending upon the type of question being investigated, it may or may not be important to obtain allele-specific data. For example, without allele-specific data, it is impossible to determine whether partial methylation at a specific locus is due to mosaic methylation of individual alleles or complete methylation of a subpopulation of alleles (Fig. 3).

Allele-specific bisulfite sequencing

Bisulfite sequencing is the gold standard for mapping allele-specific methylation across CpG sites within post-bisulfite PCR products [15]. In this method, a region of interest is amplified from bisulfite-modified DNA using PCR primers not overlapping CpG sites, to amplify both methylated and unmethylated alleles. PCR products are ligated to a cloning vector and transfected to competent cells. Antibiotic resistant colonies are grown on agar plates, selected individually and expanded by growing in LB medium. The plasmid DNA is isolated and sequenced. If a sufficient number of clones is sequenced, this method can be quantitative. As each clone represents a single allele, the data provide information on allele-specific methylation. Such information is particularly useful in the study of differentially methylated regions at genomically imprinted loci. This technique is labor-intensive, however, and costly for large sets of samples.

Quantitative but non allele-specific methods

An alternative approach employing direct radioactive sequencing of postbisulfite PCR products and quantitation by phosphor-imaging was recently validated [16•]. Instead of sampling a subset of alleles, as in the clone-and-sequence approach, direct sequencing averages across all alleles produced from the PCR step and is therefore more sensitive and quantitative. This method is cumbersome to perform on a large number of samples.

Bisulfite PCR followed by restriction analysis (COBRA) has been used as a quantitative technique for methylation detection [17]. After bisulfite modification and PCR amplification, the PCR product is digested with a restriction enzyme (whose recognition sequence is affected by bisulfite modification) and quantitated using gel electrophoresis and densitometry. This method provides data only for specific restriction enzyme cutting sites and is relatively time-consuming compared with MSP (due to the additional enzyme digestion step).

Bisulfite-pyrosequencing

Like other methods described above, bisulfite-pyrosequencing [18] relies on bisulfite conversion and PCR amplification (Fig. 2). To facilitate the conversion of PCR products to single-stranded DNA for later pyrosequencing, the PCR reaction is performed with either one primer biotinylated or using a tailed primer in combination with a biotin-labeled universal primer in the same reaction. (This avoids biotin-labeling each primer for each assay.) The sequencing primer is then annealed to single-stranded DNA and the samples are ready for pyrosequencing analysis. Pyrosequencing is a primer extension method for the analysis of short to medium-length DNA sequences. Incorporation of a nucleotide into the template strand leads to the release of pyrophosphate, which is quantified with a luciferase reaction. The signal produced is proportional to the amount of pyrophosphate released; therefore, the percentage of unconverted C and converted T nucleotides at each CpG site can be detected and quantified. This method has the advantages of introducing an internal control (DNA sequence including a control for unconverted cytosines) and allowing accurate quantitation of multiple CpG methylation sites in the same reaction. The only significant drawback is that only 25–30 base pairs can be sequenced in each reaction, limiting the number of CpG sites that can be assessed.

Bisulfite-PCR followed by MALDI-TOF MS

Another high-throughput method utilizes base-specific cleavage of nucleic acids and analyzes samples by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) [19]. In this method, regions of interest are PCR amplified from bisulfite-modified DNA using primers with a T7 RNA polymerase tag. The PCR products are in-vitro translated into a single-stranded RNA using T7 polymerase and subsequently cleaved by an endoribonuclease such as RNase A. Different cleavage patterns for the methylated and unmethylated CpG positions are then quantitated by mass spectrometry [19]. Compared with pyrosequencing, this method offers the possibility of detecting more CpG sites in a single amplicon (a maximum of ~800 bp). This sophisticated method, however, is technically challenging.

In all of these PCR-based methods, primer design is the key for successful amplification. Primers for quantitative bisulfite-PCR methods should ideally be free of CpGs. If this is not possible due to high CpG density, one CpG site can be included at the 5′ end of each primer. Such 'degenerate' primers should be synthesized as Y (C/T) in the forward strand and R (G/A) in the reverse stand. Postbisulfite PCR primers should incorporate sufficient cytosines in the original sequence to prevent amplification of unmodified DNA. The main concern for bisulfite-PCR is PCR bias, which occurs because methylated and unmethylated DNA molecules sometimes amplify with greatly differing efficiencies [20]. To test for PCR bias, a mixing experiment using known quantities of methylated and unmethylated genomic DNA is highly recommended for each assay [21]. The selection of the annealing temperature is possibly the most critical variable for minimization of PCR bias [21].

Methods for genome-wide analysis

Just as gene expression microarrays accelerated and revolutionized the study of transcriptional regulation, rapidly improving technologies are increasingly enabling researchers to assess locus-specific DNA methylation on a genome-wide scale.

Nonmicroarray-based genome-wide analysis

Several genome-wide DNA methylation analysis methods do not require microarrays. A classical method is Restriction Landmark Genome Scanning (RLGS) [22] – a two dimensional DNA gel electrophoresis technique. In combination with methylation-sensitive restriction enzymes (*NotI* or *AscI*), this technique provides methylation profiles of thousands of loci at once. It has limited genome coverage (up to 10% of CpG islands) and sensitivity (requires 30% methylation to be detectable), however. Nevertheless, this technique has been used to identify genes showing tissue-specific methylation in normal tissues [23] and genes aberrantly methylated in cancer [24].

Another nonarray-based method – methylation-specific digital karyotyping (MSDK) – was recently developed [25]. MSDK is conceptually similar to serial analysis of gene expression (SAGE) and relies on the cleavage of

genomic DNA with a methylation-sensitive enzyme (*AscI*). After *NlaIII* digestion, short sequence tags are sequenced and mapped to genomic locations. Although it requires a large number of sequencing reactions, MSDK requires no special device other than a DNA sequencer. Just like RLGS, however, it has limited genome coverage and requires a relatively large amount of DNA.

Another method to annotate the genomic methylation landscape is to construct unmethylated and methylated domains using limiting digestion with *McrBC* or other restriction endonucleases. DNA fragments are transfected into *Escherichia coli* and plasmid DNA from individual colonies analyzed by sequencing [26]. This method is relatively unbiased and high-resolution, but time-consuming. Recently, such massively parallel sequencing technology has been successfully applied in a genome-wide annotation of multiple histone modifications in a high-resolution and high-throughput manner [27••]. Consensus is growing that similar techniques will soon surpass array-based approaches as the method of choice for genome-wide analysis of DNA methylation.

Microarray-based genome-wide analysis

Three main classes of microarray-based methods have been developed to map ^{5m}C patterns in genomes: methods enriching for highly methylated regions using an antibody specific for ^{5m}C or methyl-binding proteins; methods based upon bisulfite modification; and methods utilizing methylation-sensitive restriction enzymes.

Methylated DNA immunoprecipitation (MeDIP) [28] immunoprecipitates DNA using an antimethylcytosine antibody, then hybridizes the immunoprecipitated DNA to microarrays. This technique is independent of the specific methylation-sensitive restriction sites within the target sequence, but requires large amounts of genomic DNA and antibody. Ligation-mediated PCR (LM-PCR) has been used to perform MeDIP with limited quantities of DNA [28]. LM-PCR is very inefficient, using blunt ends in the adaptor-ligation, however, and potentially causes bias towards GC-poor regions. Recently, methylated CpG island recovery assay (MIRA) has been used for genome-wide methylation analysis in cancers [29]. This method is based on purification of methylated DNA by methyl CpG binding protein columns. Methyl CpG binding proteins to coat the columns, however, are not yet commercially available. Both MeDIP and MIRA may lack sensitivity in genomic regions with a relatively low density of CpG sites.

Bisulfite-PCR and specially designed oligonucleotide arrays have been used to quantify the bisulfite-induced C to T changes at defined genomic positions. Although this method requires gene-specific PCR, it can interrogate multiple CpG sites within hundreds of genes at once [30]. This approach does not represent the entire genome, and primer design is challenging due to the T richness of DNA sequences after bisulfite conversion.

Various array-based strategies have been developed using combinations of methylation-sensitive and methylation-insensitive restriction enzyme digestion, followed by ligation-mediated PCR to enrich for methylated or unmethylated fragments. Differential methylation hybridization (DMH) [31] is one of the first described methods. Genomic DNA is digested with *MseI* (methylation-independent), ligated with linkers, then digested with *BstUI* or *HpaII* (both methylation-sensitive) to remove unmethylated fragments. The digested DNA is amplified by primers complementary to the linker sequence and the products are labeled and hybridized to arrays. This method is relatively simple and requires little DNA. Only CpGs within the restriction enzyme sites are analyzed, however, and incomplete digestion could lead to false positive results.

A strategy named methylated CpG island amplification [32] combined with microarray (MCA) addresses the problem of false positivity by using methylation-sensitive and insensitive isoschizomers (enzymes with the same recognition sequence). The principle is simple: DNA is first incubated with a methylation-sensitive restriction enzyme (*SmaI*) that digests unmethylated DNA, but leaves methylated sites intact. The same DNA is then digested with a methylation-insensitive *SmaI* isoschizomer (*XmaI*). The key is that whereas *SmaI* leaves blunt ends, *XmaI* cleavage produces 'sticky ends'. Following ligation of adapters that anneal to the *XmaI* cut sites, adapter-specific PCR results in amplification of methylated regions. This MCA product is then labeled and hybridized to microarrays (L. Shen *et al.* unpublished). This method has several distinct advantages – genome amplification is limited to the target molecules (methylated DNA) and each *SmaI* fragment is amplified only if the two *SmaI* sites are both unaffected by the methylation-sensitive enzyme and digested by the methylation-insensitive enzyme (i.e. adapter ligation is truly methylation-sensitive). This dramatically reduces false positives. Coverage is still limited, however, to CpGs within *SmaI* sites.

Conclusion

Continued rapid improvements in technology make the study of DNA methylation more accessible and exciting. Clearly, no one method of DNA methylation analysis will be appropriate for every application. By understanding the type of information provided by, and the inherent potential for bias and artifact associated with, each method, investigators can select the method most appropriate for their specific research needs. In the next few years, high-throughput methods of genome-wide

methylation analysis will be commercialized and become widely available. Methylation changes identified in microarray experiments will nonetheless continue to require validation by one or more of the locus-specific methods described here. Moreover, the relative subtlety of DNA methylation changes induced by nutritional exposures will motivate continued development of analytical methods that are both highly sensitive and quantitatively accurate.

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- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 639–640).

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