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How to Analyze DNA Methylation?

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Abstract
DNA methylation involves the addition of a methyl group to the 5 position of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring (cytosine and adenine are two of the four bases of DNA). DNA methylation also forms the basis of chromatin structure, which enables cells to form the myriad characteristics necessary for multicellular life from a single immutable sequence of DNA. DNA methylation plays multiple roles during development and serves to establish long-term gene silencing. Mapping of DNA methylation changes especially in CpG islands has become essential for the understanding of diverse biological processes. There is an important relationship between the degree of DNA methylation and gene activity in cancer. Hypermethylation of promoter CpG islands represents an alternative mechanism for inactivation of tumour suppressor genes, DNA repair genes, cell cycle regulators and transcription factors. By discovering sites of aberrant methylation in tumors, we can identify potential new biomarkers suitable for diagnostic and prognostic testing. A number of techniques for analysis of DNA methylation have been reported. The aim of this study was to review methodologies that can be used for monitoring of DNA methylation changes. We describe the advantages, disadvantages and potential use of these techniques.
Key words: DNA methylation, bisulfite conversion, MSP, MS-MLPA, restriction endonucleases

DNA Metilasyonunu Nasıl Analiz Edelim?

Özet
DNA metilasyonu, sitozin pirimidin halkasının 5' pozisyonuna veya adenin pürin halkasının 6 numaralı azotuna bir metil grubu eklenmesini tanımlar (sitozin ve adenin DNA'nın dört bazından ikisini oluşturmaktadır). DNA metilasyonu aynı zamanda hücrelerin, tek bir değişmez DNA dizisinden çok hücrelilerin yaşamı için gerekli olan çeşitli sayıdaki karakteristik özelliklerini oluşturmasına imkan tanıyan kromatin yapısının temelini de oluşturmaktadır. DNA metilasyonu, gelişim sırasında çok önemli rollerinin yanında uzun dönemli gen susturulmasında da görev almaktadır. CpG adacıkları başta olmak üzere DNA metilasyonundaki değişikliklerin haritalanması birçok biyolojik sürecin daha iyi anlaşılması için temel bir gereklilik halini almıştır. Kanserde DNA metilasyonunun derecesi ile gen aktivitesi arasında önemli bir ilişki vardır. Promoter bölgesindeki CpG adacıklarının aşırı şekilde metillenmesi tümör baskılayıcı genlerin, DNA tamir genlerinin, hücre döngüsü düzenleyicilerinin ve transkripsiyon faktörlerinin inaktivasyonu için alternatif bir mekanizma olarak karşımıza çıkmaktadır. Tümörlerde anormal metilasyon bölgelerinin keşfi ile birlikte prognostik ve diagnostik amaçlı yeni potansiyel biobelirteçler keşfedilebilir. DNA metilasyonunun analizi için birçok yöntem tanımlanmıştır. Bu çalışmanın amacı DNA metilasyonundaki değişiklikleri gözlemlemek için kullanılabilecek metodları gözden geçirmek, bu tekniklerin avantaj, dezavantaj ve potansiyel kullanımları tanımlamaktır.
Anahtar Kelimeler: DNA metilasyonu, bisülfit dönüşümü, MSP, MS-MLPA, restriksiyon endonukleazlar

The most widely studied epigenetic alteration is DNA methylation within the CpG context. DNA methylation refers to the covalent addition of a methyl group to position 5 of the cytosine ring. This process is mediated by DNA methyltransferases. Although most CpG sites in the human genome are methylated, CpG-dense regions known as CpG islands are usually unmethylated in normal tissue. This modification generally functions to repress gene expression and is important for the regulation of cellular differentiation and development (*1*). DNA methylation can also serve as a biomarker for many human diseases (*2*).

Monitoring of DNA methylation changes in human diseases has several advantages. Firstly, methylation analysis uses DNA, which is chemically more stable in comparison with RNA or proteins. Another advantage is easy evaluation, since the presence of DNA methylation indicates for example the presence of malignant cells. It is very expedient to analyze limited regions (CpG islands), and the cost is relatively low. Early studies of DNA methylation analyzed methylation mainly in tumor tissue, but an increasing number of studies are now using body fluids such as urine, peripheral blood and serum or plasma. The amount of DNA in serum or plasma is very low, which limits the number of available techniques. In contrast, the amount and quality of DNA extracted from peripheral blood leukocytes or whole blood are not usually a concern.

Methylation can be examined in specific selected loci, or a genome-wide approach can be used. Most loci-specific analyses are based on bisulfite conversion or methyl-sensitive digestion and PCR. HPLC (high-performance liquid chromatography) and ELISA (Enzyme-Linked ImmunoSorbent Assay) can be used for global DNA methylation analysis. The aim of this review is to provide a summary of the most frequently used techniques available for loci-specific DNA methylation analysis. We describe the applicability of selected methods and their limitations, advantages and disadvantages.

Bisulfite conversion based techniques

The most common techniques for DNA methylation analysis are based on bisulfite conversion. This technique involves treating DNA with bisulfite. In the bisulfite reaction all unmethylated cytosines are deaminated and sulfonated, converting them to uracils; methylated cytosines remain unchanged during this treatment. Thus, the sequence of the treated DNA will differ depending on whether the DNA was originally methylated or unmethylated. Also, the initially complementary DNA strands are no longer complementary after cytosine conversion. Thus, methods using bisulfite conversion are based upon these chemically-induced differences.

Bisulfite conversion based techniques usually rely on PCR amplification of the region of interest. DNA methylation can thus be analyzed in two different ways. Using methylation-specific primers, only methylated DNA is amplified. In contrast, using methylation nonspecific primers both methylated and unmethylated DNA is amplified. Methylation status is then determined using a second independent method such as for example MS-HRM or sequencing.

Bisulfite conversion is an easy and cost effective method. There are few disadvantages. The bisulfite reaction is relatively aggressive, which can become evident particularly during analysis of DNA isolated from formalin-fixed, paraffin- embedded (FFPE) tissue. For easy laboratory work there are several commercial kits for bisulfite modification, for example EZ DNA Methylation-Gold™ Kit (Zymo Research), CpGenome™ DNA Modification kit (Chemicon International), Methylamp DNA Modification Kit (Epigentek) and EpiTect Fast 96 Bisulfite Kit (Qiagen).

Methylation specific PCR (MSP)

MSP is now an established technology for monitoring of abnormal gene methylation in selected gene sequences (*3*). In gel based MSP assay the PCR primers are designed to specifically amplify selected regions of the gene of interest. If the sample DNA was originally unmethylated, an MSP reaction product will be detectable when using the primer set labeled as "U" (designed to be complementary to the unmethylated DNA sequence). No product will be generated using a primer set labeled as "M" (designed to be complementary to the derivative methylated DNA sequence. Conversely, an MSP product will be generated using the "M" primer set only if the sample was originally methylated, and the "U" primers will not allow amplification of such a template. In MSP as a control for the efficiency of chemical modification a "W" ("wild type") primer set is used. The wild type primer set anneals to any DNA (either unmethylated or methylated) that has not undergone chemical modification.

Standard rules as for classical PCR are used for primer design. Several MSP primer design programs exist, for example the free online version MethPrimer. The primers should be approximately 20-21 bp in length and should possess similar dissociation temperatures. In order to minimize primer dimer formation, primers should not be complementary, especially at the 3´ end. For analysis of promoter regions, the optimal area for primer selection is the most CG-rich region closest to the transcription site.

The three sets of primers used in MSP are derived from sequences closely related to each other, which introduces an opportunity for mis-priming. In order to avoid this and other PCR-related artifacts, the Hot Start version of PCR is recommended. The most frequently-used version of Hot Start PCR is an inhibition of polymerase using anti-Taq antibody. In this case Taq polymerase becomes activated when the complex of enzyme and antibody dissociates due to antibody denaturation.

MethyLight

The real time version of MSP is MethyLight. These assays can detect a few copies of methylated target in a background of several copies of unmethylated target (*4*). In the real time version, amplification with methylation-specific primers and probes is normalized to the total amount of input DNA to determine the fraction of DNA methylated for each region of interest. MethylLight is not only a highly specific, sensitive and reproducible method, but also requires a minimal amount of template DNA, making it compatible for analysis of poor quality DNA from paraffin embedded tissue or low concentration DNA isolated from plasma or serum. Integration of a MethylLight assay into a clinical lab would only require the presence of a real time PCR platform.

From MethylLight is derived also Quantitative MethyLight, which uses primers that amplify all target sequences, regardless of the methylation status. Competitive probes, one for the methylated target and one for the unmethylated target, are used to measure the relative level of methylated target.

HeavyMethyl

HeavyMethyl is another PCR-based technique, which uses an oligonucleotide blocker (*5*). When the DNA is methylated, the blocker oligonucleotides do not bind, leaving the primer-binding site accessible for the primers to bind and amplify the target. The amplifcation is detected with a methylation-specific oligonucleotide probe labeled with fluorescent dye (F) and quencher (Q). When the DNA is unmethylated, the blocker oligonucleotides bind, blocking access of the primers to their binding sites, and no PCR product is generated.

Methylation Sensitive High-Resolution Melting Analysis (MS-HRM)

Methylation detection using high-resolution melting analysis (HRM) is based on the melting properties of DNA in solution. HRM is carried out by gradually warming double- stranded DNA amplicon from approximately 50˚C to 95˚C. At some point in this process the melting temperature of the amplicon is reached and the double-stranded DNA dissociates. The principle of this method is that bisulfite-treated DNA templates with different contents of methyl cytosine can be distinguished by high-resolution analysis of differences in melting temperatures.

Most often HRM is done in real-time using fluorescent labeling. Melting analysis begins after the PCR process. The dyes that are used for HRM are known as intercalating dyes. They bind specifically to double stranded DNA and when they are bound they fluoresce brightly. In the absence of double stranded DNA they have nothing to bind to and they only fluoresce at a low level. At the beginning of the HRM analysis there is a high level of fluorescence in the sample because of the billions copies of the amplicon. But as the sample is heated up and the two strands of the DNA melt, the presence of double-stranded DNA decreases and thus fluorescence is reduced.

The principle of MS-HRM is the fact that after bisulfite treatment originally methylated DNA has more cytosine. Therefore, PCR products resulting from a template that was originally unmethylated will have a lower melting point than those derived from a methylated template. HRM also offers the possibility of determining the proportion of methylation in a given sample, by comparing it to a standard curve which is generated by mixing together different ratios of universally methylated and non-methylated control DNA.

HRM is sensitive and specific (*6*), and a relatively simple and cost-effective method. The advantage of this method is the analysis of all CpG loci in the amplicon.

Methylation specific sequencing

The development of sequencing technology has greatly expanded our understanding of how biological information is coded in the sequence of nucleotides of DNA. DNA sequencing ("reading" of DNA) is an overall term for the biochemical process of determining the precise order of nucleotides (A, C, G, T) within a DNA molecule. One of the biggest advantages of methylation specific sequencing of amplified products is the valuable control of bisulfite conversion.

Determining the order of nucleotides in the DNA is most commonly performed using Sanger's method. Sanger's method uses special properties of dideoxynucleotides which do not have a hydroxyl group at the 3' carbon of ribose. Dideoxynucleotides are able to be incorporated into the replicating DNA, but subsequently stop elongation of the chain. If we label each dideoxynucleotide with a different fluorescent dye, then various lengths and different color-labeled sequences will arise in the reaction mixture. The resulting sequence is then determined by capillary electrophoresis. Sanger's method is particularly applicable to the sequencing of short single-strand DNA sequences, and was used in the first sequencing project of the human genome (Human Genome Project, 2003).

Currently, pyrosequencing is often mentioned in connection with DNA sequencing. This is a very sensitive technology that can be applied to the quantification of sequence variations. Pyrosequencing can detect and quantify small changes in the degree of methylation. Pyrosequencing is based on the detection of light emitted during the synthesis of the complementary strand using exonuclease-deficient DNA polymerase. Pyrosequencing technology has already been used for the correlation of DNA methylation of tumor cells and gene expression, to measure cellular response to treatment agents that inhibit DNA methylation, and to assess changes in DNA methylation in tumor development and genetic imprinting (*7*).

In the last few years Next-Generation Sequencing (NGS) has become very important. At present we are aware of 5 commercially available NGS platforms. NGS platforms share identical technological features - massively parallel sequencing of clonally amplified products or different DNA molecules which are spatially separated in flow cells. One of the best known platforms is the 454 technology from Roche, which is based on pyrosequencing and emulsion PCR. Examples of other platforms are Solexa from the company Illumina and Solid from Applied Biosystems.

Methylation-sensitive endonuclease based techniques

Using methylation sensitive endonucleases is a classic approach in DNA methylation analysis. The endonucleases employed are frequently inhibited by methylation of their recognition site. This analysis includes treatment using a specific endonuclease followed by analysis of the treated DNA. The most commonly used restriction enzymes are the isoschizomers *HpaII* and *MspI*, which recognize the sequence CCGG. *HpaII* is blocked by methylation of either cytosine, whereas *MspI* is blocked only by methylation of the outer cytosine (*8*). Hence in mammalian genomes *HpaII* would be appropriate for detection of DNA methylation because of methylation especially in CpG sites, whilst in plant genomes use of *MspI* could be more successful.

The restriction endonucleases are only effective when DNA is not methylated. After digestion the cleaved DNA can be analyzed by electrophoresis and Southern blotting. Southern blotting was used as one of the first methods for analysis of DNA methylation. In the case of analysis using PCR, the PCR product will only be obtained when the DNA is methylated (not digested). Using a combination of restriction endonuclease with PCR appears a more sensitive method than Southern blot.

A recent study describes a "one-step methylation analysis" (*9*). This method is based on the combination of methylation sensitive endonucleases and quantitative real-time PCR in a single reaction. The first step is DNA cleavage, followed by inactivation of endonucleases and finally real-time PCR.

An important limitation of restriction enzyme-based techniques is that all analyses are limited to searching for methylation within the recognition site.

MS-MLPA (Methylation-Specific Multiplex Ligation-dependent Probe Amplification)

Methylation-specific MLPA (MS-MLPA) is a semi-quantitative method for methylation profiling. MS-MLPA is a variant of the MLPA technique in which copy number detection is combined with the use of a methylation-sensitive restriction enzyme *HhaI* (*10*). One of its major applications is methylation detection in imprinting

diseases such as Prader-Willi/Angelman and Beckwith-Wiedemann/RSS syndrome. MS-MLPA is also frequently used in tumor analysis.

MS-MLPA can determine the copy number and methylation status using PCR-based reaction. Each MS-MLPA reaction results in a combination of unique PCR amplification fragments which can be identified and quantified by capillary electrophoresis. In contrast to a standard multiplex PCR, MS-MLPA uses a single PCR primer pair for all its probes. The critical feature of MLPA and MS-MLPA is that only the probes that have hybridised to the sample DNA are amplified during the PCR. The MS-MLPA procedure can be divided into five steps (Fig.1). These steps in sequence are DNA denaturation and hybridisation of MLPA probes; ligation and digestion; PCR; separation of amplification products by capillary electrophoresis; and finally data analysis. MS-MLPA probes for methylation detection resemble other MLPA probes, except that their target sequence contains the restriction site of the methylation sensitive endonuclease *Hhal*. After hybridisation, the product is split into two tubes: one tube is processed as a standard MLPA reaction, providing information on copy number changes; the other is incubated with the *Hhal* endonuclease while hybridized probes are ligated. Hybrids of probes with unmethylated sample DNA are digested by the *Hhal* enzyme. Digested probes cannot be amplified exponentially during PCR and hence will not produce a signal during capillary electrophoresis. In contrast, if the sample DNA is methylated, the DNA probe hybrids are protected against *Hhal* digestion and the ligated probes will generate a peak (www.mlpa.com).

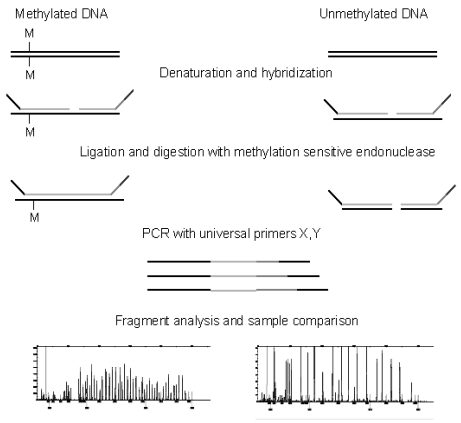

Figure I. MS-MLPA (Methylation Specific Multiplex Ligation-dependent Probe Amplification)

MLPA is a relative technique. No conclusion can be drawn from a single MLPA reaction. Comparison with reference DNA samples is essential. The optimal cut-off values for detecting a significant change in methylation of a sequence is probe dependent, and is also dependent on sample type and application. When interpreting MS-MLPA results we have to bear in mind that absence (or presence) of a particular analyzed CpG site does not necessarily mean that the whole CpG island is unmethylated (or methylated). It is also important to realize that a point mutation within the *Hhal* site of a MS-MLPA probe may result in a false positive methylation signal, as the restriction enzyme is not able to digest the probe-sample DNA hybrid.

Microarrays

Microarrays are used in the overall analysis of the DNA and for the detection of methylation in the promoter regions of selected genes. The principle of microarrays is immobilization of fragments of single-strand DNA on the positively charged nylon membrane and hybridization under appropriate conditions. Hybridization signals are usually detected using chemiluminescence.

Microarrays are commercially prepared and provided by different companies such as Illumina, NimbleGen, Agilent and Affymetrix. DNA probes, or the elements that make up the array, are a key factor in selecting the most appropriate platform to solve the issue. Some biochips, such as from Illumina, are tailored for DNA analysis after bisulfite modification, and some are suitable for analysis after DNA restriction or immunoprecipitation. The successful use of microarrays depends on application of effective bioinformatic strategies for data analysis, including data preprocessing, and hence the presence of an experienced bioinformatist is essential.

Immunoprecipitation methods

Immunoprecipitation methods allow isolation of the methylated DNA, thereby facilitating subsequent analysis. The principle of immunoprecipitation methods is the immobilization of denatured DNA on DEAE (diethylaminoethyl) membrane and subsequent incubation with a monoclonal antibody.

Immunoprecipitation of methylated DNA is routinely achieved using one of two methods: isolation of DNA methylated fragments using monoclonal antibodies specific for 5-methylcytosine (anti-5mC), or isolation of DNA fragments containing methylated CpG dinucleotides using methyl-binding domain protein (MBD protein) (*11*). Immunoprecipitated DNA can then be analyzed by the various methods mentioned above.

Conclusion

The importance of DNA methylation is obvious particularly in cancer: the evidence is the amount of published literature in recent years. A large number of methods that can be used for monitoring DNA methylation changes have been described in the literature. Any of these methods can be used in any situation, but it is always important to select the most appropriate method. When choosing the method, particular attention must be paid to the quantity and quality of isolated DNA, and laboratory options and equipment. Frequently the analyzed DNA is isolated from paraffin blocks. For analysis of such DNA the most appropriate method seems to be MS-MLPA, but the facilities of the laboratory must be kept in mind, since

besides the commonly used thermal cycler, a system of capillary electrophoresis for fragmentation analysis is also required. For analysis of DNA isolated from blood, the technique of bisulfite conversion followed by MSP could be used, or sequencing of the amplified products. It is important to remember that the correct choice of method is more likely to deliver reproducible results.

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