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Methylation-Specific PCR

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1. Introduction

1.1. Significance of DNA Methylation as an Epigenetic Phenomenon

Methylation of the DNA is an important epigenetic (i.e., not associated with alteration in the primary structure of the DNA) phenomenon, which plays important roles in regulation of gene expression, maintenance of genome integrity, and genomic imprinting. Although not only other nucleotides, but also proteins and lipids can be methylated, in the context of the present discussion, "methylation" designates only that of cytosine residues that are located 5' to guanines (CpG cytosines). A methyl residue is added to the 5 position of the pyrimidine ring of cytosine (5-methylcytosine) in the course of DNA replication, a process mediated by DNA(cytosine-5)-methyltransferases.

1.2. Other Methods for DNA Methylation Analysis

Investigation on DNA methylation had long been performed by Southern hybridization with methylation-sensitive endonucleases. Some endonuleases digest double-strand DNA at their cognitive sequences only when cytosines within are free of methylation, whereas others do so, regardless of the presence or absence of 5-methylcytosines. Genomic DNA is digested by a methylation-sensitive endonuclease and electrophoresed on agarose gel and probed with specific DNA probe. If the cytosines in the endonuclease recognition site is methylated, the DNA is left uncleaved at that site, thus appears band(s) of larger-than-expected size(s).

The main problems with Southern blotting with methylation-sensitive endonucleases is that only the cytosines in the context of available methylation-sensitive endonucleases [i.e., *Hpa*II (CCGG), *Hha*I (GCGC)] could be examined; also, relatively large amount of DNA (5–10 μg) is required for each analysis. The latter problem could be circumvented by the use of polymerase chain reaction (PCR). Since cytosine methylation should not be conserved after PCR (5-methylcytosines should be converted to cytosines), digestion by methylation-sensitive endonucleases should be per-

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formed *before* amplification by polymerase chain reaction (PCR). Primers should be positioned upstream and downstream of the recognition site of a methylation-sensitive endonuclease. DNA digested by a methylation-sensitive endonuclease is amplified with the primers: If the restriction site is methylated, the template DNA should be left uncleaved, thus the PCR product should be generated; and if the site is unmethylated, because the template is cut into fragments, there should be no product. Even if the template DNA is fully unmethylated at the restriction site, however, only slight incomplete digestion may lead to amplification because of high sensitivity of PCR. Thus, interpretation of the results should be made with caution for this method.

In 1992, Frommer et al. ***(1)*** described a method called bisulfite sequencing. This strategy of identifying the presence and absence of cytosine methylation has greatly contributed to the advancement of research on DNA methylation. Treatment of denatured DNA by sodium bisulfite deaminates cytosines at their 4 position, and converts cytosines to uracils. When the bisulfite-treated DNA is subjected to PCR, uracils are converted to thymines. 5-Methylcytosines are resistant to deamination by sodium bisulfite, and converted to cytosines after PCR. Thus, after PCR following bisulfite treatment, unmethylated cytocines are converted to thymines, while methylated cytosines are changed to cytosines (*see* **Fig. 1**). This means that cytosine methylation, which is an epigenetic modification of the DNA, can be translated into difference in primary structure (base composition) of the DNA. In bisulfite sequncing, the amplified products are cloned and sequenced. Because all the non-CpG cytosines are uniformly unmethylated, only the 5-methylcytosines in CpGs should remain cytosines on sequencing. Comparison with the original DNA sequence shows which cytosines are methylated.

1.3. Basic Concept of Methylation-Specific PCR

Methylation-specific PCR (MSP), which was first described by Herman et al. ***(2)*** in 1996, is an application of bisulfite sequencing method. For a sequence in a gene containing CpGs, the allele on which those CpGs are methylated and another on which those CpGs are unmethylated should give different sequences after bisulfite modification. When a primer set that are complementary to the sequence with methylated CpGs, but are not complementary to the originally same sequence with unmethylated CpGs, is used for PCR, only the sequence (allele) with methylated CpGs should be amplified. The same is true for the primer pair specific for sequence with unmethylated CpGs. The interpretation of the result is simple: If PCR product of the expected size is seen on agarose gel electrophoresis, the sample is considered to contain the methylated or unmethylated allele of the gene, depending on the primer pair used. Usually, primer pairs specific for methylated and unmethylated sequences, respectively, are used for the same gene, and the amplified products are run side-by-side on agarose gel for comparison.

1.4. Application of MSP

The greatest advantage of MSP over other methods of DNA methylation analysis is its simplicity. In contrast to Southern blotting with methylation-sensitive restriction enzymes, MSP requires much less amount of DNA, isotope use is usually unneces-

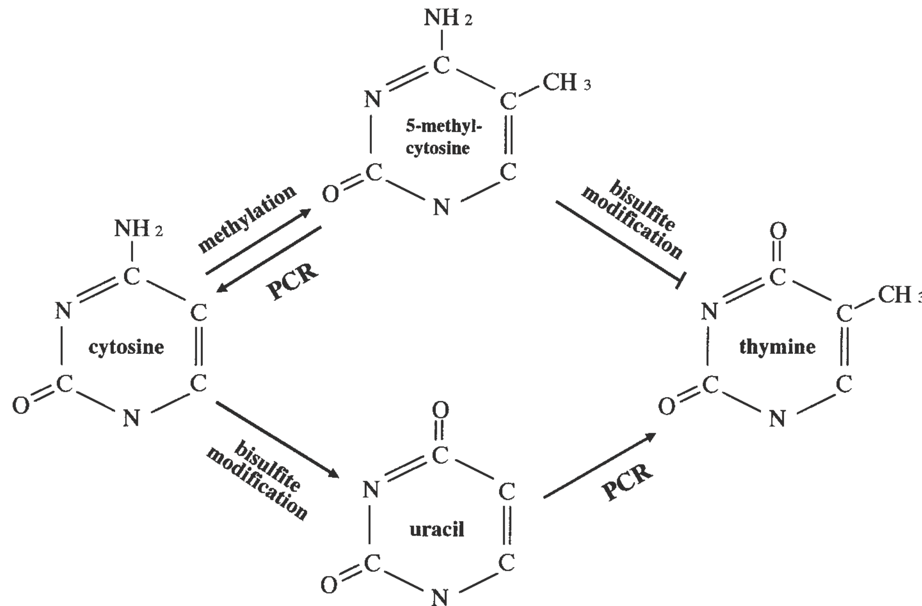

Fig. 1. Conversion of pyrimidines residues by chemical and biochemical reactions. Changes between pyrimidine residues by methylation, deamination, and PCR are being shown. Cytosines in DNA strands can be methylated to be 5-methylcytosines by DNA(cytosine-5)-methyltrasnferases in vivo, or by other methylases in vitro. Sodium bisulfite deaminates cytosines to uracils, whereas 5-methylcytosines are resistant to modification. PCR with dCTP as nucleotide source converts 5-methylcytosines to cytosines and uracils to thymines, respectively.

sary, and any CpGs, regardless of the sequence around, can be evaluated. Also, interpretation of the results is much more straightforward. MSP is superior to bisulfite sequencing in that it does not require cloning and sequencing, which usually take several days, and can be done in 1 or 2 d. All these characteristics fit well to researches that examine a large number of clinical samples at a time, and indeed it has been widely used for detection of aberrant hypermethylation and inactivation of tumor suppressor genes in cell lines and tumor samples ***(3)***. This method can also be used to evaluate methylation status of any DNA sequences, such as viral genes ***(4)***, and imprinted X-linked and autosomal genes ***(5*** and ***6)***.

2. Materials

2.1. Bisulfite Modification of the DNA

1. Hydroquinone (Sigma, St. Louis, MO).
2. Sodium bisulfite (Sigma).
3. Wizard DNA purification resin (Promega, Madison, WI), or similar product.
4. CpGenome DNA Modification Kit (Intergen, Purchase, NY).
5. 3 *M* Sodium hydroxide (NaOH).

6. 70 % Ethanol.
7. TE Buffer: 10 m*M* Tris-HCl, 0.1 m*M* ethylenediamine tetraacetic acid (EDTA), pH 7.5.
8. 5 *M* Ammonium acetate (NH_4OA_c).
9. 5 *M* Sodium chloride (NaCl).

2.2. PCR with Methylation-Specific Primers

1. 10X PCR reaction buffer: 100 m*M* Tris-HCl (pH 8.3), 500 m*M* KCl, 15 m*M* $MgCl_2$, (TaKaRa, Osaka, Japan).
2. dNTP mixture: 2.5 m*M* each (TaKaRa).
3. *Taq* DNA polymerase (TaKaRa).

3. Methods

The procedure of the MSP consists of two steps: bisulfite modification of the sample DNA, and PCR with methylation-specific primers. Chemical modification of double-strand DNA by sodium bisulfite consists of four steps:

1. Denaturation of the double-strand DNA by NaOH (*see* **Note 1**);
2. Sulfonation of the 6 position of cytosine by sodium bisulfite (cytosine sulfonate);
3. Hydrolytic deamination at 3 position by hydroquinone (uracil sulfonate);
4. Alkali desulfonation by NaOH (uracil).

These reactions can be carried out with the protocol described below. Alternatively, a kit for bisulfite modification (CpGenome DNA Modification Kit, Intergen) is commercially available. It is important to note that each single-strand DNA (the sense strand and the antisense strand), generated as the result of initial denaturation, is modified independently by sodium bisulfite in the subsequent steps. This means that the sense and antisense strands are no longer complementary to each other after modification. Bisulfite-modified DNA can be stored at –20°C and used for repeated analyses.

PCR with methylation-specific primers is not different from ordinary PCR, but needs some precautions (*see* **Note 2**). There may be three sets of methylation-specific primers for each gene, or a region of a gene: primers that amplify sequences on which CpGs are methylated (M-primers); primers that amplify sequences on which CpGs are unmethylated (U-primers); and primers that are designed for the region without CpGs and should amplify the gene regardless of methylation status (C-primers, C for common). M- and U-primers are the ones used for MSP, and C-primers are for bisulfite sequencing. There could be another set of primers, W-primers (W for wild-type), which amplify DNA not modified by sodium biulfite, or escaped modification for some technical defects. PCR with the forward M-primer and the reverse M-primer should detect, if present, the gene that are methylated at these CpGs. PCR with the forward M-primer and the reverse C-primer, or the other way around, should also work for the purpose. Because selecting regions fit for M- and U-primers is sometimes difficult, while the region without CpGs can usually be found, combination of either the forward M-primer or the forward U-primer, and the reverse C-primer may be a practical choice.

In fact, it is fairly easy to perform MSP on a gene for which workable methylation-specific primer sequences are already reported. If one wishes to examine a gene for which MSP has not been performed by others, the difficult part is selecting methylation-specific primers. The primers used in MSP should be designed so that they dis-

M-primer: 5'-*C*G AG*C*GTAGTATTT TT*C*GG*C*-3'
WT: 5'-ACCCAGAGGCCGCGAGCGCAGCACCTCCCGGCGCCAGT -3'
U-primer: 5'-GGTTG*T* GAG*T*G TAGTATTT TT*T*GG*T*-3'

Fig. 2. Example of primer selection for MSP. MSP primers for the human androgen receptor gene (HUMARA) is being shown ***(5)***, as an example of primer designing. WT: Unmodified sense strand sequence rich in CpG and non-CpG cytosines. M-primer: Primer specific for the methylated allele. All the cytosines except for those preceding guanines are changed to thymines, while cytosines 5' to guanines remain unchanged. The *T*s are thymines converted from cytosines. The *C*s are CpG cytosines that are methylated and are not to be converted to thymines. U-primer: Primer specific for the unmethylated allele. All the cytosines are changed to thymines. The *T*s are thymines converted from cytosines. The *T*s are CpG cytosines that are unmethylated and are to be converted to thymines.

criminate between methylated and unmethylated sequences *and*, at the same time, between bisulfite-modified and biulfite-unmodified sequences. Thus, the primers should be designed for the region rich in both CpGs and non-CpG cytosines. Both U- and M-primers must contain Ts that are non-CpG Cs in the unmodified (wild-type) sequence. U-primers must have Ts located 5' to Gs, at their 3' ends, and M-primers must contain Cs in the CpG context at their 3' ends. **Fig. 2** shows, as an example, the genomic sequence and the methylation-specific primers designed for the human androgen receptor gene ***(5)***. If the purpose of the investigation is to know whether the expression of a gene is regulated by methylation, the part of the gene to be examined is the promoter region of the gene, where clustering of CpGs (CpG islands) is found about half of the genes. Sometimes, however, the CpG island in the 5' region of a gene may extend to the 5' untranslated region (5'-UTR) or even to the coding region.

3.1. Bisulfite Modification of the DNA

1. Take 1 μg of DNA and add double-distilled water to 50 μL in a 1.5-mL tube.
2. Add 3.5 μL of 3 *M* NaOH (final concentration of 0.2 *M*), and incubate at 37°C or 10 min.
3. Freshly prepare 10 m*M* hydroquinone, and add 35μL of it to the tube.
4. Freshly prepare 3 *M* sodium bisulfite, adjust to pH 5.0 by adding 3 *M* NaOH, add 520 μL of it to the tube, mix well, overlay with mineral oil, and incubate at 50°C for 16 h or longer.
5. Put the tube on ice, add 5 μL of Wizard DNA purification resin (or similar DNA purification resin), mix, and incubate at room temperature for 10 min, spin at 5000*g* for 10 s at room temperature, discard the supernatant.
6. Put 1 mL of 70% ethanol, mix by Vortex, spin at 5000*g* for 10 s, and discard the supernatant. Do this two more times, and remove the supernatant completely.
7. Add 50 μL of TE, mix well, incubate at 50°C for 5 min, spin at 12,000*g* for 1 min, and transfer the supernatant to a fresh 1.5-mL tube.
8. Add 5.5 μL of 3 *M* NaOH, mix, and incubate at room temperature for 5 min.
9. Add 10 μL of 5 *M* NH_4OAc and mix (for neutralization).
10. Add 1 μL of 5 *M* NaCl and 200 μL of 100% ethanol, mix well, keep the tube at –20°C for 1 h. Spin at 12 000*g*, at 4°C for 5 min, discard the supernatant, rinse the pellet with 70% ethanol, dry the pellet, and dissolve in 20–50 μL of TE, and store at –20°C.

3.2. PCR with Methylation-Specific Primers

1. Make mixture of the following for the number of samples to be examined, and put the mixture into a 0.5-mL tube for PCR (*see* **Note 3**).
   a. 10X PCR reaction buffer (15 m*M* Mg^{2+}) 2 μL;
   b. 2.5 m*M* dNTP mixture 2 μL;
   c. forward primer (10 μ*M*) 1 μL;
   d. reverse primer (10 μ*M*) 1 μL;
   e. ddH_2O 10 μL.
   Overlay with mineral oil.
2. Add the bisulfite-modified DNA (2 μL), or a control sample (*see* **Note 4**) into the tube.
3. Put the tubes on a thermal cycler and start denaturation at 95°C for 5 min.
4. Make mixture of the following for the number of tubes.
   a. *Taq* DNA polymerase (5 U/μL) 0.2 μL;
   b. ddH2O 1.8 μL.
   Take a tube from the thermal cycler, add 2 μL of the *Taq* mixture to each tube through mineral oil, and put the tube back to the heat block. This should be done as quick as possible.
5. Go through 30–40 cycles of the following amplification (*see* **Note 3**).
   a. Denaturation 95°C for 30 s;
   b. Annealing 55°C for 30 s;
   c. Extension 72°C for 30 s.
6. Take 5–10 μL of the reaction solution and run on 2–3% agarose gel.

4. Notes

1. Complete denaturation of the DNA in the first step of bisulfite modification is important, because unmethylated cytosines in undenatured DNA cannot be converted to uracils, thus leads to misinterpretation that they are methylated. For bisufite sequencing of specific genes or plasmids, digestion of the template DNA by a endonuclease that cleaves outside the region to be amplified is recomended, in order to ensure complete denaturation ***(1)***. This pretreatment of DNA does not fit to examination for clinical samples, with which more than one genes may be examined for methylation. Another strategy for ensuring initial denaturation is to shear DNA through fine gage needles. Our experience with MSP for various genes *without* such pretreatment, however, did not show inconsistent results, and it seems that pretreatments may not be necessary. One can always check, if needed, the validity of bisulfite treatment of the given gene by PCR with W-primers or by sequencing MSP product.
2. By experience amplification efficiency with MSP is lower than with ordinary PCR, and one often needs to optimize PCR condition, in the direction of lowering stringency, to see clearer bands. The situation sometimes becomes tricky, because the M-primers and U-primers are designed to recognize sequences only partially different to each other. When one lowers the stringency of PCR and undergo a larger number of cycles to get clearer bands, they may notice that specificity of the reaction is lost: the same band can be seen with the control samples with the opposite specificity (e.g., M controls for U-primers). This may be the greatest pitfall for methylation analysis with MSP.
3. The factors that appear to influence PCR efficiency are reaction buffer [Mg^{2+} concentration, addition of demethyl sulfoxide (DMSO)], annealing temperature, and hot start regimens. We usually start with commercially available 10X PCR reaction buffer (10 m*M*

Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, final concentration), annealing temperature of 55°C, and hot start by adding *Taq* polymerase after incubating the reaction for 5 min at 95°C. When no product of the expected size is seen after agarose electrophoresis, elevation of $MgCl_2$ concentration, addition of DMSO (5% of the volume) should be tried. Elevating the annealing temperature may also help. Hot start appears to be critical for MSP, thus we usually do not even try nonhot start protocols. When ordinary hot start protocol of adding *Taq* after incubation at 95°C does not work, use of reagents designed for hot start, such as Platinum *Taq* (Gibco-BRL, Rockville, MD) may still help. Optimization of PCR conditions for MSP seems much more difficult than for ordinary PCR, and it may sometimes be wiser to redesign primer sequences before comparing all the possible conditions.

4. Not only for initial optimization of MSP but also for each experiment, as for any PCR, control templates are necessary. To obtain the perfect controls for MSP is sometimes difficult, since one needs both DNA that is methylated at the CpGs of the gene of interest, and DNA that is unmethylated in the same region. The ideal controls for U-primers are the PCR-amplified DNA segment, because PCR products are fully unmethylated. If one methylates the PCR product with methylases in vitro, that would be a good control for M-primers. There are several kinds of commercially available bacterial methylases with different sequence specificity. For example, Sss I methylase (New England Biolabs, Beverly, MA) methylates all the CpG cytosines. All these preparations are time consuming, but may be necessary for analysis of nonhuman (i.e., viral) genes. When DNA from some sources (e.g., cell lines) in which the gene in question is known to be either methylated or unmethylated is available, that could serve as controls. When one possesses DNA that is unmethylated for the gene, then in vitro treatment with methylases can provide controls for methylated gene.

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