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A Quantitative DNA Methylation Assay Using Mismatch Hybridization and Chemiluminescence[1]

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Objective To develop a quantitative method for methylation analysis of the p16 gene based on mismatch hybridization and chemiluminescence. **Methods** Genomic DNA was modified by sodium bisulfite to convert all unmethylated but not methylated cytosines to uracil, and subsequently a pair of primer having no CpG sites was designed for amplification target DNA containing methylated or unmethylated CpG sites. The PCR product spanning CpG sites were hybridized with two oligonucleotide probes which perfectly matched the methylated and unmethylated CpG sequences respectively, and the hybrids were detected by chemiluminescent method. The percentage of methylated target sequences could be estimated by calculating the ratio of signals obtained with two probes. **Results** The percentage of methylation of artificial mixtures DNA showed a linear relation. There was a negative correlation between the methyaltion index with p16 transcriptional mRNA of p16 gene in tumor cell lines. **Conclusion** Compared with existing methods, this assay is nonisotopic, rapid, simple, and can be widely applied to the study of DNA methylation.

Key words: Methylation; Chemiluminescence; Mismatch hybridization; p16 gene

INTRODUCTION

DNA methylation has profound effects on the mammalian genome. These effects include transcriptional repression, X chromosome inactivation, imprinting and suppression of parasitic DNA sequences[1]. Normal methylation patterns are frequently disrupted in tumor cells with global hypomethylation accompanying CpG islands hypermethylation. DNA methyllation in the promoter regions is a powerful mechanism for the suppression of gene activity. The epigenetic event could be considered as one of the 'hits' of Knudson model of tumor formation[2].

The detection of aberrant DNA methylation is helpful for the understanding of the fundamental mechanisms of oncogenesis and may provide the useful information for the early diognosis of cancers. Established methods for methylation analysis include methylation-sensitive restriction enzyme treatment followed by Southern blot and PCR[3], bisulfite sequencing[4]. However, these techniques require large amounts of DNA, and the analysis is limited by the availability of methylation-sensitive restriction enzymes recognizing particular CpG sites. Methylation specific PCR (MSP) [5] is sensitive, but this method is not quantitative and the PCR conditions must be strictly controlled to avoid false-positive results. Real-time PCR[6] is a quantitative technique, but specialized equipment and reagents are very costly. COBRA[7] and Ms-SNuPE[8] require the use of gel electrophoresis and radioisotopes, and are labor-intensive.

Here we introduce a novel method for quantitative detection of p16 promoter hypermethylation based on mismatch hybridization and chemiluminescence. Genomic DNA was modified by sodium bisulfite to convert all unmethylated but not methylated cytosines to uracil, and subsequently a pair of biotinylated primer having no CpG sites was designed for amplification target DNA containing methylated or unmethylated CpG sites. Two oligonucleotide probes, which perfectly matched the methylated and unmethylated CpG sequences respectively, were synthesized. The oligonucleotide probes were labelled

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with digoxigenin (Dig) and hybridized with PCR product spanning CpG sites. The hybrids were captured by a tube coated with streptavidin and detected by chemiluminescent method. The percentage of methylated target sequences could be estimated by calculating the ratio of signals obtained with two probes.

MATERIALS AND METHODS

Cell Lines and Demethylation Treatment

The tumor-derived cell lines T-24 and BIU-87 (human bladder cancer cell line), SPC-A1 (lung adenocarcinoma cell line) and Hep-G2 (liver cancer cell line) were obtained from the Typical Culture Center of Wuhan University. Cells were maintained at 37℃ in a atmosphere of 5% CO_2 in RPMI1640 (Gibco PRL, USA) with 10% fetal bovine serum. Cell lines were treated with 1 μmol/L 5-aza-2′-deoxycytidine (Sigma Chemical Co, USA) for 24 h, and cultured for 10 days. DNA and RNA were isolated and purified with TRIzol reagent kit (Gibco PRL, USA) according to the manufacturer's instructions.

Bisulfite Modification

Bisulfite modification of genomic DNA was performed as reported by Herman *et al*.[5]. Briefly, 1 μg of DNA was denatured with NaOH (final concentration 0.2 mol/L), and 10 mmol/L hydroquinone (Sigma, USA) and 3 mol/L sodium-bisulfite were added and incubated at 50℃ for 16 h. Afterward, modified DNA was purified using Wizard DNA purification resin (Promega, USA) followed by ethanol precipitation, and resuspended in distilled water. Treatment of genomic DNA with sodium-bisulfite converted the unmethylated cytosines (but not methylated cytosines) to uracil.

Polymerase Chain Reaction

The modified DNA was used as a template for PCR. A 273bp fragment of p16 gene was amplified. This fragment included a portion of the p16 gene CpG-rich promoter region. The primers recognized the bisulfite-modified template, but could not discriminate between methylated and unmethylated alleles. The sense and antisense primer sequences were as follows: p16A, 5′-Biotin-GAA GAA AGA GGA GGG GTT GG-3′ and p16B, 5′- CTA CAA ACC CTC TAC CCA CC-3′. All primers were obtained from Takara (Takara Biotechnology Co, Dalian). The PCR amplification conditions were as following: a 50 μL reaction mixture containing 50 ng template DNA, 300 ng of each primer, 0.25 mmol/L of each dNTP, 1×PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH9.0, 1.5 mmol/L $MgCl_2$, 0.1% TritonX-100). Reactions were hot-started at 95℃ for 10 min before 1.0 U Taq polymerase (Promega, USA) was added. Amplification was carried out in a T-Gradient thermoblock (Biometra, USA) for 30 cycles (30 s at 95℃, 30 s at 60℃, and 30 s at 72℃), followed by a final 6 min extension at 72℃. DNA from peripheral blood lymphocytes of healthy individuals and water blanks were used as negative controls for methylated genes. DNA from T-24 cells was used as a positive control for methylated alleles.

Design of Probe, Hybridization, and Detection

Two oligonucleotide probes were designed and labelled with digoxigenin. There were three sequence differences (corresponding to three CpG sites) between the two probes. The two probes (P_m and P_u) could perfectly match the methylated and unmethylated PCR products, respectively. P_m and P_u sequences were as follows: 5′-Dig-TCG ACC TCC GAC CGT AAC –3′(P_m) and 5′-Dig-TCA ACC TCC AAC CAT AAC –3′(P_u). After amplification, two aliquots of 5 μL PCR products were incubated with 1.5 pmol of each of two oligonucleotide probes in 30 μL of hybridization, respectively. The mixture was heated for 10 min at 95℃, then cooled to 48℃, and 20 μL was added to luminescent tube coated with streptavidin. Hybridization reactions were performed at 48℃ for 40 min with a total volume of 20 μL hybridization buffer.After hybridization, the luminescent tube was washed with 200 μL of washing buffer (phosphate-buffered saline and 0.1% Tween) at 48℃ twice and at room temperature three times. The hybrids were detected by a chemiluminescent method. Firstly, 200 μL of anti-Dig-peroxidase conjugates (Roche) was added, and this mixture was incubated for another 40 min at room temperature and washed with PBS three times. Finally, 200 μL chemiluminescent substrate (4×10^{-5} mmol/L Luminol, 3×10^{-5} mmol/L p-iodopheno, 5×10^{-4} mmol/L H_2O_2, and 0.1 mmol/L Tris-HCl pH8.0) was added and incubated for 2 min. The 6 sec relative luminescence unit (RLU) was recorded by a Lumat LB 9507 recorder (EG&G Berthold). The methylation index (%) in a sample was calculated using the equation: methylation index = $R_m/(R_m+R_U)\times100\%$. R_m: the RLU of methylated PCR products; R_u: the RLU of unmethylated PCR products.

Reverse Transcription-Polymerase Chain Reaction

Total RNA (about 2.5 μg) was isolated from cells and reverse transcribed using Oligo $(dT)_{15}$, M-MLV

reverse transcriptase (Promega, USA). Amplification of cDNA was performed using primers specific for p16, β-actin gene which was used as a control. Primer sequences for p16 and β-actin were: 5′-AGC CTT CGG CTG ACT GGC TGG-3′(p16 sense); 5′-GGC CCA TCA TCA TGA CCT GG-3′(p16 antisense), 5′-CAC CCC CAC TGA AAA AGA TGA –3′(β-actin sense), 5′-CAT CTT CAA ACC TCC ATG ACG –3′(β-actin antisense). PCR reactions were performed as reported by Gonzalez-Zulueta *et al*.[9]. Ten μL of PCR products were loaded onto a 2% agarose gel and visualized under UV illumination (Vilber Louramt, France).

RESULTS

Kinetics of Luminol-H_2O_2-HRP Chemiluminescent System

Five μL methylated PCR products was incubated with 1.5 pmol oligonucleotide probes P_m. Hybridization and detection were carried out as described under Materials and Methods. RLU was detected every 30 sec after the substrate was added. Kinetic curves of chemiluminescence were generated (Fig. 1). After 2 min, the RLU reached a peak value.

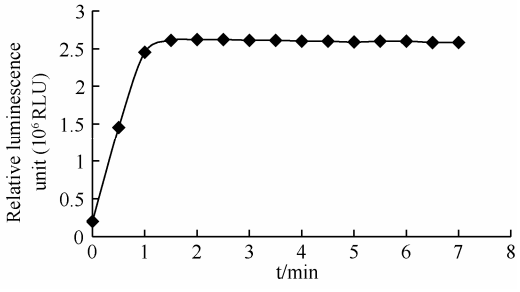

FIG. 1. Kinetic curves of chemiluminescence system.

Linear Range and Detection Limit

Under the experimental conditions as described above, serial dilutions of methylated PCR products were analyzed by hybridization with oligonucleotide probes P_m, and RLU was detected. Chemiluminescent signals were directly proportional to the concentration of PCR products. The linear range was from 1.25×10^{-3} to 1.0 pmol with a detection limit of 2.5×10^{-4} pmol ($2\times$SNR, signal-to-noise ratio). The correlation coefficient was 0.996. Two aliquots of 5 μL methylated and unmethylated PCR products were assayed 10 times with the method described. The CV was 5.2% and 6.4%, respectively (Fig. 2).

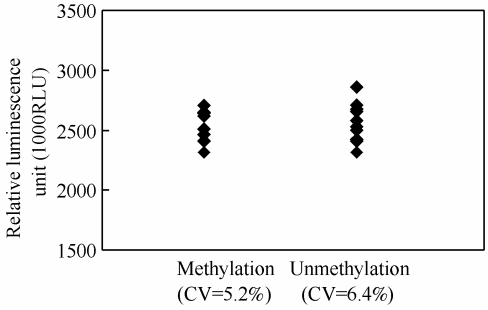

FIG. 2. Precision of chemiluminescence system.

Determination of Optimal Hybridization Temperature

For oligonucleotide probes, mismatch of a single base would greatly reduce the thermal stability of hybrids[10]. In this study, there were three sequence differences (corresponding to three CpG sites) between the two oligonucleotide probes, so the appropriate hybridization temperature would allow the discrimination of methylated from unmethylated PCR products. The two probes were hybridized with their match and mismatch sequences at different hybridization temperatures (46℃, 48℃, 50℃, and 52℃), then the chemiluminescence was measured as described above. The optimal hybridization temperature of 48℃ was chosen. Under the experim- ental conditions, the RLU values of probes hybridized with the mismatch sequences were very low (about background value), and had no effects on assay.

Measurement of Methylation Index in Artificial Mixtures of Methylated and Unmethylated p16 Sequences

To validate the measurement of the methylation index, the equivalent concentrations of methylated DNA (from T-24 cells) and unmethylated DNA (from healthy individuals) were mixed at different ratios (1:0, 3:1, 1:1, 1:3, 0:1). The DNA mixtures were treated with sodium bisulfite, amplified by PCR and analyzed by chemiluminescence to detect the proportions of the methylated alleles. Fig. 3 shows a plot of the observed values against the theoretical values. The values for the methylated alleles confirmed a good linearity of the quantitative analysis.

Methylation and Expression of the p16 Gene in Tumor Cell Lines

Herman *et al*.[11] reported a highly significant correlation between methylation of p16 gene and transcriptional silencing of the gene. In this study, the method was used to assay the methylation index of p16 gene in tumor cell lines before and after demethylation treatment with 5-aza-2′-deoxycytidine, and the

expression level of p16 gene was detected by RT-PCR at the same time.As shown in Fig. 4, the methyaltion index was negatively correlated with p16 transcriptional status. mRNA of p16 gene was not detected in BIU-87 human bladder cancer cell lines.

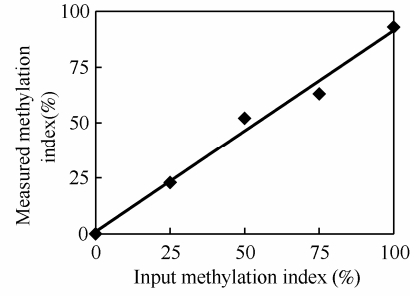

FIG. 3. Quantitative analysis of the proportion of methylated alleles in mixtures of methyl lated and unmethylated p16 sequences.

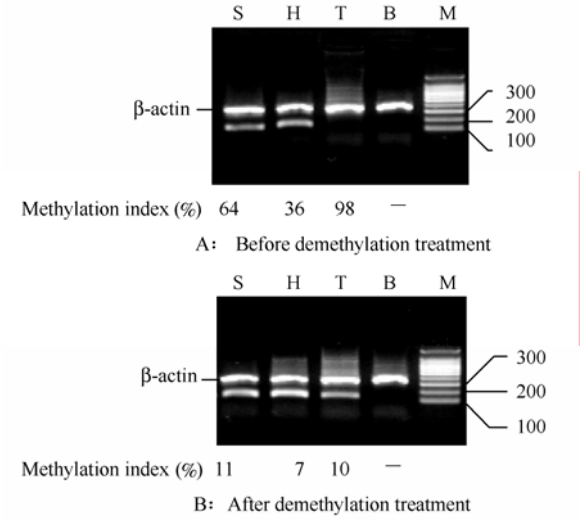

FIG. 4. Detection of methylation index and expre ssion level of p16 ene in tumor cell lines before and after demethylation treatment. S: SPC-A1, H: Hep-G2, T: T24, B: BIU-87, M: 100bp ladder.

DISCUSSION

DNA methylation alterations have been widely recognized as a contributing factor in human carcinogenesis. A significant number of tumor suppressor genes are transcriptionally silenced by promoter hypermethylation. To gain a deeper understanding of the DNA methylation patterns, quantitative data of methylation are necessary. In this study, we developed a novel method for quantitative detection of p16 promoter hypermethylation based on mismatch hybridization and chemiluminescence. Compared with existing methods, this assay is nonisotopic, rapid, and simple.

After methylated and unmethylated DNAs were modified by sodium bisulfite,the sequence differences could be found existed at the CpG sites. A pair of biotinylated primers having no CpG sites were designed for amplification target DNA contained methylated or unmethylated CpG sites. Because it was a competitive PCR, there was an equal amplify-cation efficiency for methylated and unmethylated DNA templates. The molar ratio of methylated products versus unmethylated products was invariable in PCR, and equal to the molar ratio of methylated versus unmethylated templates in original samples. To avoid an unnecessary amplification of the unconverted DNA, PCR primers were designed to be complementary to the sequences containing cytosine residues (but without CpG site) in the corresponding region of the original DNA.

There were three sequence differences (corresponding to three CpG sites) between the two probes (P_m and P_u). P_m and P_u could perfectly match the methylated and unmethylated PCR products, respectively. Sequencing results of methylated PCR products proved that all the three CpG sites were methylated (results not shown). An appropriate hybridization temperature was the key to enhance the specificity of hybridization reaction. Under the experimental conditions described, the RLU values of probes hybridized with the uncomplementary sequences was very low (about background value), and had no effects on assay. Additionally, total conversion of cytosines to uracils was also critical to the success of analyses. Maximum conversion rates of cytosines occurred at 50℃(16-18 h) [12], so the conditions must be strictly controlled.

Recent studies have shown that DNA methylation of the promoter region of certain cancer-associated genes is a potential early detection biomarker[13]. Because the concentration of free DNA in serum of cancer patients released by tumor cells is much higher than that in the blood of healthy individuals, examination of serum for circulating tumor DNA with abnormal methylation patterns offers a possible method for early detection of several cancers and serves as a point for early intervention and prevention strategies. There has been a considerable interest in the use of methylation analysis in the clinical detection of tumors, such as analysis of aberrant p16 methylation in sputum[13] or bronchoalvevlar lavage fluid for lung cancer diagnosis and methylation analysis in plasma/serum of lung[14] and liver[15] cancer patients. This method provides a quantitative tool for this type of analysis. In addition, the method can be applied to cancer screening for populations at risk.

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