DNA microarray: a high throughput approach for methylation detection

L. Gao a, L. Cheng a, J.N. Zhou b, B.L. Zhu a, Z.H. Lu a,b

a Key Laboratory for Molecular and Biomolecular Electronics of Ministry of Education, Southeast University, Nanjing 210096, China
b Jiangsu Cancer Hospital, Nanjing 210009, China

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Abstract

We described a DNA microarray-based method combined with bisulphite treatment of DNA and regular PCR to examine hyper-methylation in promoter 1A of APC gene. A set of oligonucleotide probes were designed and immobilized on the aldehyde-coated glass slides for detecting the methylation pattern of 15 selected CpG sites in the region. The methylation status of 30 colorectal tumor samples have been examined by both of methylation-specific PCR (MS-PCR) and the present microarray method. The methylation pattern of the 15 CpG sites for the samples have been obtained with the microarray. A total of 19 samples out of 30 were methylated by microarray, in which five samples cannot be detected by MS-PCR due to the methylated CpG patterns not accordant to the MS-PCR primers. The detecting ratio for methylation of APC gene of colorectal tumor samples increased from 46.7% with MS-PCR to 63.3% with the microarray, which successfully demonstrated that DNA microarray-based method not only can obtained the methylation patterns for the related genes, but also decrease the false-negative results of methylation status by the conventional MS-PCR for the investigated genes.

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1. Introduction

DNA methylation is an epigenetic modification that can play an important role in the control of gene expression in mammalian cells [1]. The enzyme involved in this process is DNA methyltransferase (DNMT), which catalyzes the transfer of a methyl group from S-adenosyl methionine to cytosine residues to form 5-methylcytosine within the context of the CpG dinucleotide. The distribution of CpG is non-random with the majority of the genome being CpG-poor. Certain regions of the genome, which are often clustered at the 5' end of genes, are rich in CpG dinucleotide and have been termed CpG islands. In mammalian cells, approximately 70% of the CpG residues in the mammalian genome which correspond to 3-5% of the cytosine residues in the whole genomic DNA is present as 5-methylcytosine [2]. In tumor cells, normal methylation patterns are frequently disrupted with global hypo-methylation accompanying region-specific hyper-methylation. Usually hyper-methylation can increase mutation rate and heritably silence genes whose promoters are associated with CpG islands. When these hyper-methylation events occur within the promoter of a tumor-suppressor gene, which controls cellular proliferation, they will silence the gene and provide the cell with a growth advantage in a manner akin to deletions or mutations. That is why neoplasia procedure is greatly induced by hyper-methylation of tumor-suppressor genes [3].

At present, a variety of methods have been used to evaluate the methylation status of genes: Southern blot, bisulphite genomic DNA sequencing, restriction enzyme-PCR, methylation-specific PCR (MS-PCR), methylation-sensitive single nucleotide primer extension (MS-SNuPE), denaturing high-performance liquid chromatography (DHPLC) and electrochemistry [3-5]. MS-PCR is a technique that was first described by Herman et al. on the basis of bisulphite genomic sequencing described by Frommer et al. [6]. It takes advantage of the sequence difference existing between methylated and unmethylated DNA after bisulphite treatment. The basic principle is: unmethylated cytosines are deaminated to uracil while the methylated ones remain cytosines after the treatment. Sequence differences will be obtained by two pairs of

primers specific for unmethylated or methylated sequences. MS-PCR is a rapid and qualitative method for determining the presence of methylation in a given gene. But on the other hand, the disadvantage of MS-PCR is that the methylation status can only be told when some CpG sites within the primer sequences are methylated concurrently, especially at the 3′ end. It is not capable of pattern recognition and identification of CpG dinucleotides outside the methylation-specific primers.

Recently, considerable advances have been made in hybridization-based microarray technology for genome-wide analysis of gene mutations and single nucleotide polymorphisms [7,8]. The technology that uses thousands of short oligonucleotides arrayed on glass slides for detection of all possible nucleotide changes in target DNA can also be applied in methylation detection. The methylation status of the DNA sequences can be obtained by the C/T discrimination since all unmethylated cytosines are transformed into thymine while those methylated cytosines remain the same after the bisulphite modification and PCR amplification of the treated DNA. According to previous SNP microarray studies the single base difference can be easily told by proper probes under proper hybridization procedure.

In this study, we fabricated a DNA microarray composed of a group of oligonucleotide probes that included all types of methylation patterns of 15 adjacent CpG sites in the promoter 1A of APC gene. A set of four oligonucleotide probes with 15 bases for every CpG site were designed to fabricate a DNA microarray to determine the methylation patterns (Fig. 1). Thirty samples of colorectal tumor were used to examine the aberrant methylation. The targets were derived from PCR products of bisulphite-modified DNA. MS-PCR method was also performed with the same 30 samples as a comparison.

2. Materials and methods

2.1. Samples preparation

Thirty sporadic colorectal tumors and corresponding normal control samples were supplied by Jiangsu Cancer Hospital (Nanjing, China). Genomic DNA was isolated by standard phenol/chloroform extraction methods from fresh tissues. Two micrograms of genomic DNA of each sample was bisulphite-treated following the protocol presented by Frommer et al. [6]. Shortly, genomic DNA was denatured in 0.3 M NaOH at 37 °C for 10 min. The bisulphite reaction was carried out in 3 M sodium bisulphite and 0.5 mM hydroquinone (Sigma Chemical Co., USA) at 50 °C for 20 h. DNA was recovered by a desalting column (DNA Clean-Up System, Promega Inc., USA) and desulphonated in 0.3 M NaOH at room temperature for 15 min, neutralized by ammonium acetate, alcohol precipitated, dried and then resolved in 20 μl of deionized water. The modified DNA was used immediately or stored at −20 °C for further analyses. For the preparation of positive control DNA targets, a normal DNA sample was treated with SssI methyltransferase (New England Biolabs) that methylates cytosine residues within all CpG dinucleotides in vitro prior to modification by bisulphite. DNA from the bisulphite nucleotide sequencing experiment was used as templates in the following experiments.

2.2. Methylation-specific PCR (MS-PCR)

Two pairs of primers were employed in MS-PCR procedure [9]. Primer sequences for the unmethylated reaction were UNMF: 5′-GTG TTT TA T TGT GGA GTG TGG GTT-3′ (forward) and UNMR: 5′-CCA A TC AAC AAA CTC CCA ACA A-3′ (reverse), which amplify a 108-bp product, and for the methylated reaction, MF: 5′-TA T TGC GGA GTG CGG GTC-3′ (forward) and MR: 5′-TCG ACG AAC TCC CGA CGA-3′ (reverse), which amplify a 98-bp product. The 5′ position of the forward unmethylated and methylated primers corresponds to bp 696 and 702 of GenBank sequence no. U02509, respectively. Both reverse primers originate from bp 782 of this sequence. The PCR reactions were performed in a total volume of 25 μl containing 1× Taq PCR buffer (75 mM Tris–HCl pH 9.0, 20 mM ammonium sulfate, 0.1 ml/l Tween), 1.5 mM MgCl2, 400 nM each primer, 200 μM dNTPs, 1 U Taq polymerase (Sigma), and 1 μl bisulphite-treated DNA. PCR amplification was performed as follows: 95 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 60 °C for 40 s, and 72 °C for 30 s, and ended with an extension of 72 °C for 5 min and quick chill to 4 °C on a PTC-225 thermocycler (MJ Research). Products amplified with both types of primers were examined on 1.5% agarose gel (Fig. 2).

Fig. 1. Probe strategy for isolated CpG dinucleotide (A) and adjacent CpG dinucleotides (B).
Fig. 2. Methylation-specific PCR of APC promoter 1A in colorectal tumor tissues. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated genes; the presence of product in those lanes marked M indicates the presence of methylated genes. All of the normal tissues analyzed are unmethylated at the APC promoter 1A. Those samples presented both a clear methylated lane and a faint unmethylated lane can be interpreted as a mixture of tumor cells and normal cells in investigated tissues. S: DNA marker (DL 2000, TaKaRa).

2.3. Microarray procedure

We selected 15 CpG dinucleotides closed to the first exon region in the microarray experiment including nine isolated CpG dinucleotides (#1, #2, #3, #4, #5, #6, #7, #14 and #15) and six in couples (#8–9, #10–11 and #12–13) (Fig. 3). The total 48 probes include all types of methylation patterns of the 15 CpG sites. Oligonucleotide probes were synthesized and purified by Shenyao Inc. (Shanghai, China). The oligonucleotides were suspended in 3× SSC to a final concentration of 40 pmol/μl and then printed in quadruplicate as microdots (100 μm in diameter) onto the aldehyde-coated glass slides using a PixSys5500 microarrayer (Cartesian Technolgy Inc.). After overnight immobilization the slides were washed thoroughly to remove unbound oligonucleotides with 0.1% SDS and also treated with a NaBH4 solution for 30 min before hybridization procedure.

A 411 bp segment spanning the promoter 1A and exon 1A of the APC gene was amplified by asymmetric PCR as the hybridization target. Primer sequences for target amplification were PF: 5′-TGT GTT GTA AAA A TT A TA GTA AT-3′ (forward) and PR: 5′-ACA CCT CCA TTC TA T CTC C-3′ (reverse). The 5′ position of the forward and reverse primers corresponds to bp 475 and 866 of GenBank sequence no. U02509, respectively. And the reverse primer was labeled with fluorescent molecule TAMRA in the 5′ position. The asymmetric PCR reactions were performed in a total volume of 25 μl containing 1× Taq PCR buffer (75 mM Tris–HCl pH 9.0, 20 mM ammonium sulfate, 0.1 μl/l Tween), 1.5 mM MgCl2, 100 nM forward primer, 500 nM reverse primer, 200 μM dNTPs, 1 U Taq polymerase (Sigma), and 1 μl template DNA. Amplification conditions consisted of an initial 5 min denaturation at 94°C followed by 40 cycles of 30 s denaturation at 94°C, 30 s annealing at 48°C and 40 s extension at 72°C. Amplified products were examined on 1.5% agarose gel (Fig. 4). The amplified product, therefore, may contain a pool of DNA fragments with altered nucleotide sequences due to differential methylation status without any discrimination between methylated and unmethylated sequences. Six microlitres of fluorescent single strand PCR product was mixed with 3 μl hybridization buffer (3 M TEACl). The microarray hybridization was conducted in a moist hybridization chamber under a cover slip at 45°C for 2 h. The slide was rinsed and washed at room temperature with 2× SSC–0.1% SDS for a total of 10 min, followed by washing with 0.1× SSC–0.1% SDS at room temperature for 5 min, and dried by nitrogen air blow. The fluorescent images of the hybridized slides were obtained using a ScanArray Lite Microarray Analysis Systems (A Packard BioScience Company, USA). Signal data of both methylated and unmethylated probes were given by software Quantray. The net signal was determined by subtraction of this local background from the mean average intensity for each spot. Average signal intensity of each probe was derived from four replicate spots in a single array area. Statistical analyses were conducted using Excel.

3. Results

A representation of the MS-PCR analysis was shown in Fig. 2. 43.3% of the colorectal tumor samples (14/30) were found to be methylated by the approach. The primer sequences designed for the promoter 1A of APC gene spanned seven CpGs (#1, #2, #8, #9, #10 and #11). However, there are 29 CpG dinucleotides in the entire sequence of promoter

Fig. 3. Nucleotide sequences of a downstream region of the promoter 1A and exon 1A (GenBank accession no. U02509). The 15 CpG sites tested by DNA microarray are underlined and marked with numbers.
Fig. 4. Four hundred and eleven base pairs PCR product for hybridization. S: DNA marker (DL 2,000, TaKaRa).

It is possible that the negative samples might also be methylated in some other patterns of CpG sites that are missed by MS-PCR.

The above 16 ‘unmethylated’ samples from the MS-PCR results were re-examined by DNA microarray-based method. The hybridization result revealed that 5 out of the 16 samples were methylated in some CpG sites, which were missed by MS-PCR. After the re-examination by DNA microarray, a total of 19 samples out of 30 were methylated in the promoter 1A of APC gene. And the detecting ratio was increased by 16.6–63.3% compared with MS-PCR result.

In order to set up a standard control, we have prepared the positive sample by treating a normal DNA with SssI methyltransferase that methylates cytosine residues within all CpG dinucleotides. Both negative and positive control samples were hybridized to the DNA microarray. The scanned fluorescent signals of sample 11T, negative control and positive control are showed in Fig. 5. It shows obviously that the sample 11T was methylated at CpG sites #8–9, but not at CpG sites #10–11. The result gave a good example that the negative result from MS-PCR could be revealed positive by microarray.

According to Gitan’s research the methylation level of the target sequence had a linear relationship with the intensity ratio of methylated probe (M) and unmethylated probe (U) [10]. The average intensity of hybridization signals of every probe was derived from four replicate spots in an array area. Standardization curve then can be drawn according to the fluorescence intensity of methylated and unmethylated probes from control samples. The methylation levels in percentage for the test CpG sites can be approximately determined from the calibration curve. An example of the methylation level analysis for CpG #8–9 in sample 11T is shown in Fig. 6.

4. Discussion

Adenomatous polyposis coli (APC) gene is a tumor-suppressor gene that was isolated and cloned in 1991 [11,12]. APC gene locates in 5q21–22 and encodes a 2843 growth-regulatory protein called APC protein, which normally suppresses hyper-proliferation of epithelium [13]. As the tumorigenesis mechanism of colorectal cancer describes, APC gene is the only gatekeeper in colorectal epithelium proliferation control whose inactivation results in hyper-proliferation and then leads to the formation of small adenomas followed by K-ras and p53 gene mutation [14,15]. DNA hyper-methylation in the promoter region of APC gene increases mutation rate in exons and silence the gene so that it loses control of cellular proliferation. This excessive epigenetic modification in promoter region of APC gene acts as one of the steps in the Knudson ‘two-hit’ model of tumor formation. Further more, according to the Knudson ‘two-hit’ model of tumor formation, the inactivation pattern of APC gene could be biallel hyper-methylated or one allel hyper-methylated along with mutation or loss of heterozygosity (LOH) in the other [16–18].

It is obvious from above that the development of efficient approaches for methylation detection of APC gene is very important to early genetic diagnosis for colorectal tumors.

We can draw a conclusion from above description that the detecting efficiency of MS-PCR is mostly hampered by the inflexibility of fixed primers with limited CpG sites inside. Although it can be improved by application of nested PCR [19], abundant CpG dinucleotides in large range still cannot be screened site by site. DNA microarray technique showed great advantages in high-throughput analysis of DNA methylation. It will contribute significant information to our under-
standing of CpG island methylation in cancer. We hope that this microarray technique will assist researchers to study the relationship between methylation patterns and clinical phenotype in different kinds of tumors, thereby assist physicians in early diagnosis, therapy and prognosis of tumor diseases.

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References