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• GASTRIC CANCER •

Microarray-based method for detecting methylation changes of p16^{Ink4a} gene 5′-CpG islands in gastric carcinomas

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Abstract

AIM: Aberrant DNA methylation of CpG site is among the earliest and most frequent alterations in cancer. Several studies suggest that aberrant methylation of the CpG sites of the tumor suppressor gene is closely associated with carcinogenesis. However, large-scale analysis of candidate genes has so far been hampered by the lack of high-throughput approach for analyzing DNA methylation. The aim of this study was to describe a microarray-based method for detecting changes of DNA methylation in cancer.

METHODS: This method used bisulfite-modified DNA as a template for PCR amplification, resulting in conversion of unmethylated cytosine, but not methylated cytosine, into thymine within CpG islands of interest. Therefore, the amplified product might contain a pool of DNA fragments with altered nucleotide sequences due to differential methylation status. Nine sets of oligonucleotide probes were designed to fabricate a DNA microarray to detect the methylation changes of p16 gene CpG islands in gastric carcinomas. The results were further validated by methylation-specific PCR (MSP).

RESULTS: The experimental results showed that the microarray assay could successfully detect methylation changes of p16 gene in 18 gastric tumor samples. Moreover, it could also potentially increase the frequency of detecting p16 methylation from tumor samples than MSP.

CONCLUSION: Microarray assay could be applied as a useful tool for mapping methylation changes in multiple CpG loci and for generating epigenetic profiles in cancer.

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INTRODUCTION

The epigenetic event has been observed in GC-rich regions, called CpG islands, frequently located in the promoter and the first exon regions of genes. CpG island hypermethylation is closely associated with transcriptional inactivation of tumor suppressor genes, which is a common feature in human carcinomas[1]. Hypermethylated CpG islands therefore play a causal role in promoting tumor development and are useful molecular markers for cancer diagnosis and prognosis. p16, an inhibitor of the cyclin D-dependent protein kinases, is a classic tumor suppressor gene, and its inactivation is closely associated with carcinogenesis. Hypermethylation of the CpG islands of the p16 gene has been proposed as an alternative mechanism for the loss of p16 expression. p16 hypermethylation could be detected in each stage, which is consistent with the finding that aberrant methylation of p16 is a very early event in carcinogenesis[2]. Detection of promoter hypermethylation of cancer-related genes may be useful for cancer diagnosis or the detection of recurrence[3,4].

At present, several molecular biology methods are routinely used to determine the methylation status of a CpG island, such as Southern blot[5], bisulfite genomic DNA sequencing[6], restriction enzyme-PCR[7], methylation-specific PCR (MSP)[8], methylation-sensitive single nucleotide primer extension (MS-SNuPE)[9], electrochemistry[10], *etc*. Among these, bisulfite nucleotide sequencing is a standard technique for detailed mapping of methylated cytosine residues within a gene promoter. This meticulous method, developed by Frommer *et al*.[6], relies on the ability of sodium bisulfite to deaminate cytosine residues into uracil in genomic DNA, whereas the methylated cytosine residues are resistant to this modification. The target DNA is then amplified by PCR with specific primers to yield fragments in which all uracil residues are converted to thymine, whereas methylated cytosine residues are amplified as cytosine. The PCR products are sequenced and the methylation status of individual CpG sites is then analyzed by comparing it with the unmodified sequences of a given promoter. Using this conventional method, many investigators have addressed the importance of promoter CpG hypermethylation in the regulation of specific gene transcription in cancer[11-14]. The method, which requires cloning and sequencing of individual inserts, can be labor intensive and is restricted to the evaluation of DNA methylation on a gene-by-gene basis. Such an approach has given researchers a limited picture of complex epigenetic alterations in cancer. Clearly, it is of great importance to establish novel, reliable and high-throughput methods for the methylation detection of earlier cancer diagnosis.

For this purpose, considerable advances have been made in hybridization-based microarray technology for genome-wide analysis of gene mutations and single nucleotide polymorphisms[15-17]. In this new approach, oligonucleotides are arrayed on solid supports known as probes, and the labeled complex DNA mixtures to be interrogated are known as targets[18]. Recently, we developed an oligonucleotide microarray to analyze methylation patterns of several adjacent CpG sites[19]. The DNA microarray can successfully map the methylation pattern of p16^{Ink4a} gene. However, it can not be used to quantify the methylation level of promoter region of gene. Gitan *et al*.[20] have developed a methylation specific oligonucleotide (MSO) microarray to analyze methylation of human estrogen receptor (ER) α gene. The targets were derived from PCR products of bisulfite-modified DNA, whereas the probes used a series of

arrayed oligonucleotides that can discriminate between converted and unconverted nucleotides, that is, unmethylated and methylated cytosines, at CpG sites. The MSO microarray is a novel and powerful tool for determining the methylation level in multiple CpG island loci and for generating epigenetic profiles in cancer.

The aim of this study was to use oligonucleotides microarray method to analyze methylation changes of p16 gene CpG islands in gastric carcinomas. A 336 bp segment was selected in the 5' untranslated region and the first exon of the p16 gene, as the investigated target, which contain 32 CpG sites. Nine sets of oligonucleotide probes were designed to test 23 CpG sites within the island. Here, we described the oligonucleotide microarray procedure and its application for analyzing the methylation changes of p16 gene CpG islands in gastric tumor and corresponding normal tissues.

MATERIALS AND METHODS

Tissue samples

Eighteen gastric tumor and corresponding normal tissues were obtained from Gulou Hospital (Nanjing, China). Genomic DNA was isolated by standard methods using proteinase K digestion and phenol/chloroform extraction.

Bisulfite genomic sequencing

Bisulfite processing of DNA was performed as described by Frommer *et al*.[6] and the modifications introduced by Clark *et al*.[21]. Briefly, 1 μg of genomic DNA was digested by *Eco*R I and denatured in 0.35 mol/L NaOH at 37 ℃ for 20 min. Bisulfite reaction was carried out in 3.2 mol/L sodium bisulfite and 0.5 mmol/L hydroquinone (Sigma Chemical Co., USA) at 55 ℃ for 16-24 h. DNA was recovered by a desalting column (DNA Clean-Up System, Promega Inc., USA) and desulphonated in 0.2 mol/L NaOH at 37 ℃ for 15 min, neutralized by ammonium acetate, alcohol precipitated, dried and then dissolved in 30 μL of deionized water. After bisulfite processing, all unmethylated cytosine residues converted to uracil, whereas the methylated ones remained unchanged. For bisulfite genomic sequencing the sense strand of a 336 bp fragment of the p16 gene 5'-CpG island corresponding to nucleotides -128 to +208 relatively to the transcription start site[22] was amplified with primers which did not contain cytosine in a CpG context and consequently annealed to the methylated status of the island. The sequence of forward primer is 5'-AAA GAG GAG GGG TTG GTT GGT TAT TA-3' and that of backward primer is 5'-TAC CTA ATT CCA ATT CCC CTA CAA ACT-3'. (Forward primer position 5-30 and reverse primer position 310-336 in GenBank accession number U12818). PCR-reaction was performed in a buffer containing 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1 g/L Triton X-100, 50 g/L DMSO, 1.75 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP and 1 μL bisulphite treated DNA. Amplification conditions were as follows: at 95 ℃ for 5 min; followed by 35 cycles, each at 95 ℃ for 1 min, at 62 ℃ for 1 min, and extension at 72 ℃ for 30 s, and ended with an extension at 72 ℃ for 7 min and quickly chilled to 4 ℃ on a PTC-225 thermocycler (MJ Research). PCR products were gel purified and cloned into the pMD18-T vector according to the manufacturer's instructions (TAKARA). Part of the same PCR products was fluorescently labeled later for MSO microarray analysis. Plasmid DNA from 30 positive recombinant clones was isolated, and inserts were sequenced on an automated sequence analyzer (ABI377A, Applied Biosystem Inc., USA).

Oligonucleotide microarray

Nine sets of paired oligonucleotides used in this study were designed to include two or three CpG sites of the p16 CpG island to be interrogated (Table 1). These oligonucleotides were specific to the bisulfite-modified sequence of portion of the p16 CpG island. Each was synthesized with amino-linked C6 [$NH_2(CH_2)_6$] linker attached to its 5' end. These oligonucleotides were suspended in sodium carbonate buffer (0.1 mol/L, pH 9.0) to a final concentration of 80 μmol/L. Approximately, 1 nL (0.05-0.1 pmole) of each oligonucleotide was printed on the aldehyde-coated glass slides (DAKO) using a PixSys5500 microarrayer (Cartesian Technology Inc). After printed, the glass slides were incubated in a humid chamber at room temperature overnight, and then at 37 ℃ for 2 h. The slides were washed thoroughly in 1 g/L SDS to remove unbound oligonucleotides. After further treatment with a $NaBH_4$ solution for 15 min, the slides were ready for hybridization. For target labeling, PCR products of bisulfite-treated DNA were labeled with Cy3-dCTP (Amersham Pharmacia) by terminal transferase (TAKARA). The unincorporated dCTP was removed by passing the labeled target through a micro-Biospin column (Bio-Rad). The labeled products were resuspended in hybridization solution (1:3 dilution v/v). Then the mixture was denatured at 95 ℃ for 5 min, cooled to room temperature, and applied to the DNA microarray slides. Microarray hybridization was conducted in a moist hybridization chamber under a cover slip at 42 ℃ for 2 h. After hybridization, the slide was rinsed and washed at room temperature with 2×SSC-1 g/L SDS and 0.1×SSC-1 g/L SDS for a total of 15 min, respectively, and then dried by centrifugation at 600 rm for 5 min.

Table 1 Nucleotide sequences of methylated and unmethylated probes analyzed in oligonucleotide microarray

p16 CpG sites		Oligonucleotide sequences	Tm (℃)
#4-6	M:	5'-NH_2-$(T)_{10}$-CAACCGCCGAACGCAC-3'	56
	U:	5'-NH_2-$(T)_{10}$-CAACCACCAAACACAC-3'	50
#8-10	M:	5'-NH_2-$(T)_{10}$-CAACCGCCGAACGCAC-3'	61
	U:	5'-NH_2-$(T)_{10}$-CCACCACCCACTACCTA-3'	53
#11-13	M:	5'-NH_2-$(T)_{10}$-CCGCCGCCGACTCCAT-3'	61
	U:	5'-NH_2-$(T)_{10}$-CCACCACCAACTCCAT-3'	53
#15-17	M:	5'-NH_2-$(T)_{10}$-AACCGCGACCGTAACCAA-3'	58
	U:	5'-NH_2-$(T)_{10}$-AACCACAACCATAACCAA-3'	50
#18,19	M:	5'-NH_2-$(T)_{10}$-TCTACCCGACCCCGAACC-3'	60
	U:	5'-NH_2-$(T)_{10}$-TCTACCCAACCCCAAACC-3'	55
#20,21	M:	5'-NH_2-$(T)_{10}$-AACAACGCCCGCACCTC-3'	57
	U:	5'-NH_2-$(T)_{10}$-AACAACACCCACACCTC-3'	50
#22,23	M:	5'-NH_2-$(T)_{10}$-ACAACGCCCCCGCCTC-3'	59
	U:	5'-NH_2-$(T)_{10}$-ACAACACCCCCACCTC-3'	52
#24,25	M:	5'-NH_2-$(T)_{10}$-AACTATTCGATACGTTAAAC-3'	48
	U:	5'-NH_2-$(T)_{10}$-AACTATTCAATACATTAAAC-3'	39
#26-28	M:	5'-NH_2-$(T)_{10}$-ATCGACCTCCGACCGTAAC-3'	55
	U:	5'-NH_2-$(T)_{10}$-ATCAACCTCCAACCATAAC-3'	49

Image scanning and data processing

DNA microarray slide was scanned with ScanArray Lite microarray analysis systems (A Packard BioScience Company, USA) after the above treatment. Images acquired by the scanner were analyzed with software Genepix Pro 3.0. Each spot was defined by the positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined and a local background using mean pixel intensity was computed for each spot. The net signal was determined by subtraction of this local background from the mean average intensity for each spot. The data generated by the software were exported in a spreadsheet format and processed using Microsoft Excel. Statistical analyses were conducted using Origin 5.0 software.

Methylation-specific PCR (MSP)

The 5'-CpG island regions of the p16 gene were amplified with primers for methylated and unmethylated DNA, respectively. Primer pairs described in Table 2[8] were synthesized and purified by Shengyou Inc (Shanghai, China). PCR amplification was performed in a buffer containing 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1 g/L Triton X-100, 50 g/L DMSO, 1.75 mmol/L

$MgCl_2$, 0.2 mmol/L of each dNTP and 1 μL bisulfite treated DNA in a final volume of 30 μL. The amplification was carried out for 35 cycles (30 s at 95 ℃, 30 s at the annealing temperature listed in Table 2, and 30 s at 72 ℃), followed by a final 4-min extension at 72 ℃ and quickly chilled to 4 ℃ on a PTC-225 thermocycler (MJ Research). Products amplified with both types of primers were examined on 10 g/L agarose gel.

Table 2 MSP primers used for amplification of p16 gene CpG island. MS and US represent methylated and unmethylated sense primers, respectively. MA1 and MA2, UA1 and UA2 represent methylated and unmethylated antisense primers, respectively

Primer sets	Sequences (5'→3')	Size bp	Annealing temp (℃)
MS	TTATTAGAGGGTGGGGCGGATCGC	234	65
MA1	CCACCTAAATCGACCTCCGACCG		
US	TTATTAGAGGGTGGGGTGGATTGT	234	60
UA1	CCACCTAAATCAACCTCCAACCA		
MS	TTATTAGAGGGTGGGGCGGATCGC	150	65
MA2	GACCCCGAACCGCGACCGTAA		
US	TTATTAGAGGGTGGGGTGGATTGT	151	60
UA2	CAACCCCAAACCACAACCATAA		

RESULTS

Figure 1 outlines the MSO strategy for DNA methylation analysis. Test DNA samples were bisulfite-modified, PCR amplified products contained pools of DNA fragments with altered nucleotide sequences due to their differential methylation status. As shown, the unmethylated allele of a given DNA sequence was expected to have the unmethylated cytosine of the tested CpG sites converted to thymine, whereas these CpG sequences remained unchanged in the methylated allele. Target DNA was then hybridized to arrayed oligonucleotides probes specifically designed to discriminate between converted and unconverted nucleotides at these CpG sites.

A 336 bp segment was selected in the 5' untranslated region and the first exon of the p16 gene, as the investigated target, which contains 32 CpG sites (Figure 2). Nine sets of oligonucleotide probes were designed to test 23 CpG sites within the island, each set contained a pair of methylated and unmethylated oligonucleotides for interrogating 2 or 3 CpG sites in close proximity (Table 1). First, control DNA targets were used to test the accuracy and reproducibility of probes designed for microarray hybridization. We selected fully methylated and unmethylated ones as positive and negative controls from 36 positive recombinant clones. The positive control generated in this way remained 100% cytosine in the tested CpG sites, whereas the negative control had all cytosine residues converted into thymine in the tested CpG sites. Next, a series of microarray hybridization were performed with mixtures of Cy3 labeled positive and negative DNA targets at different proportions representing 0%, 25%, 50%, 75%, and 100% of DNA methylation

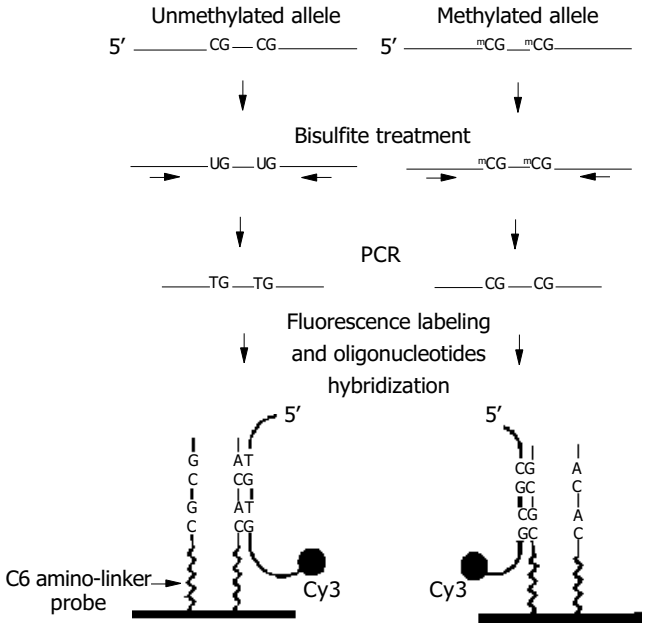

Figure 1 Schematic outline for analysis of DNA methylation based on oligonucleotide microarray[20]. Genomic DNA was bisulfite treated and amplified by PCR for a specific CpG island region of interest. The amplified product was labeled with Cy3 fluorescence dye and hybridized to oligonucleotide probes attached to a glass surface. At left an oligonucleotide probe was designed to form a perfect match with a target DNA containing the unmethylated allele. At right a probe was designed to form a perfect match with the methylated DNA target.

to test the linearity of the protocol. An example of the microarray analysis for CpG#26-28 is shown in Figure 3A. The average intensity of hybridization signals from the four replicate spots for the methylated (M) and unmethylated (U) alleles was then derived and used to calculate the intensity ratio of M/(M+U). In this case, a linear relationship (R_2 = 0.9882) was established, showing that the increase in DNA methylation was proportional to the increase in intensity ratios in the control samples. The result suggested that this set of oligonucleotide probes was optimal for the detection of methylation changes at CpG#26-28. This approach was used to test other oligonucleotide probes and to generate a set of standards for the calibration of DNA methylation changes in the test samples (Figure 3B). We noticed that the regression line for CpG#24, 25 was much higher on the *Y*-axis than the rest of the CpG sites. Moreover, its slope was much lower than the others. This higher nonspecific hybridization was likely due to the lower melting temperature of the unmethylated probe. An oligonucleotide sequence such as this would result in the compression of the usable scale and make the assessment of methylation status a little more challenging.

Microarray assay was used to analyze the methylation status of 18 gastric tumor and corresponding normal tissues. Figure 4

GGCTGGCTGGTCACCAGAGGGTGGGGCGGACCGCGTG^{4}**CG**CT^{5}**CG**G^{6}**CG**
GCTGCGGAGAGGGGGAGAGCAGGCAG^{8}**CG**GG^{9}**CG**G^{10}**CG**GGGAGCAGCA
TGGAGC^{11}**CG**G^{12}**CG**G^{13}**CG**GGGAGCAGCATGGAGCCTTCGGCTGACTGGC
TGGCCA^{15}**CG**GC^{16}**CG**^{17}**CG**GCC^{18}**CG**GGGT^{19}**CG**GGTAGAGGAGGTG^{20}**CG**G
G^{21}**CG**CTGCTGGAGG^{22}**CG**GGGG^{23}**CG**CTGCCCAA^{24}**CG**CAC^{25}**CG**AATAGTT
A^{26}**CG**GT^{27}**CG**GAGGC^{28}**CG**ATCCAGGTGGGTAGAGGGTCTGCAGCGGGAG
CAGGGGATGGCGGGCGACTCTGGAGGACGAAGTTTGCAGGGGAATTGG
AATCAGGTAGCGC

Figure 2 Nucleotide sequences of the 5' untranslated region and the first exon of the p16 gene (Genbank accession no.U12818.1 GI: 533724). The 23 CpG sites tested by oligonucleotide microarray are underlined and shown in bold.

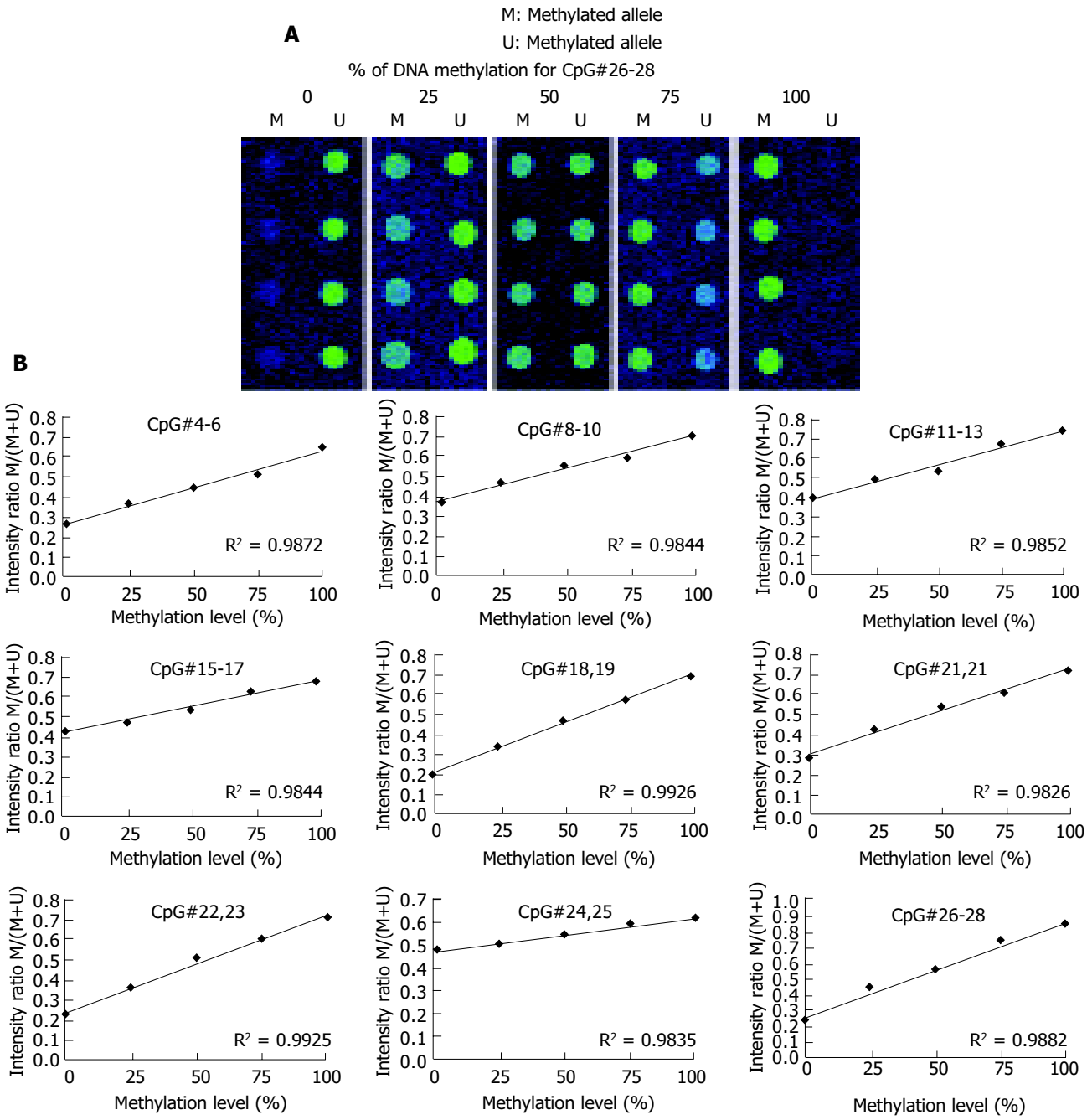

Figure 3 Standardization curve for microarray assays. A: Mixtures of fully methylated and unmethylated control DNA (from 36 positive recombinant clones) prepared and amplified by PCR using bisulfite primers for the p16 gene CpG island. B: A calibration curve for measuring methylation changes at the p16 gene CpG sites.

shows the representative examples of microarray results. By use of the standard curves derived from the aforementioned calibration controls, no methylation was detected in the normal tissues. Extensive methylation of the p16 CpG island was observed in 7 of 18 gastric tumor tissues (T1, T2, T3, T8, T9, T11, and T18), a modest degree of methylation was found in T10 and T15, whereas little or no methylation was seen in others (Figure 5).

To further validate the microarray findings in gastric tumors, methylaton-specific PCR (MSP) was conducted in 18 tumor and corresponding normal tissues. The primers MA1 and MA2 for MSP included CpG#16-19 and CpG#26-28 sites, respectively. Therefore, CpG#15-17 and CpG#26-28 sites were used for this conformation. A representation of the MSP analysis is shown in Figure 6. By use of this approach, two normal tissues were completely unmethylated (N4 and N8). MSP results of the 18 gastric tumor tissues completely matched with microarray results. Interestingly, the MSP results indicated that the methylation was not detected in T1 when the amplification was performed with primers MS and MA2, whereas the methylation could be found in T1 when the amplification was performed with primers MS and MA1 (Figure 6). A most possible reason was that some CpG sites were not methylated in the region of primer MA2. Figure 5 indicates that CpG#15-17 sites had no methylation in T1, whereas CpG#26-28 sites had 47% methylation in T1, which were consistent with the MSP results.

The above results indicated that microarray assay could potentially increase the frequency of detecting p16 methylation from tumor samples than MSP. MSP was a simple, sensitive, and specific method for determining the methylation status of virtually any CpG-rich region. However, methylation could not be detected when some CpG sites were not methylated in region of MSP primers. The issue could be easily overcome using microarray assay. Furthermore, microarray assay could estimate which CpG sites (or CpG-rich region) were easily methylated in certain tumors.

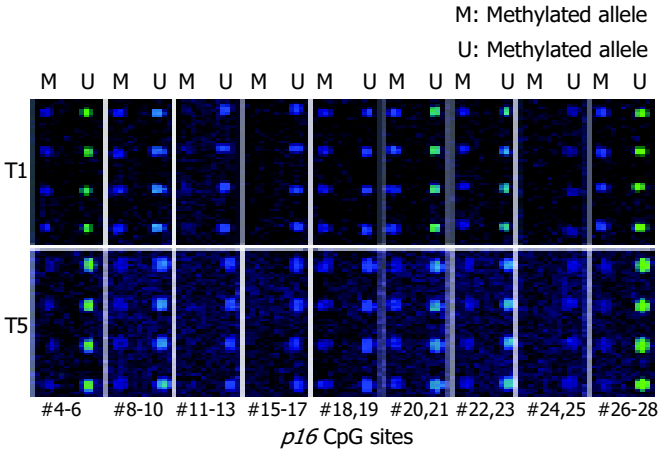

Figure 4 Methylation analysis of 23 CpG sites in p16 gene CpG island using oligonucleotide microarray.

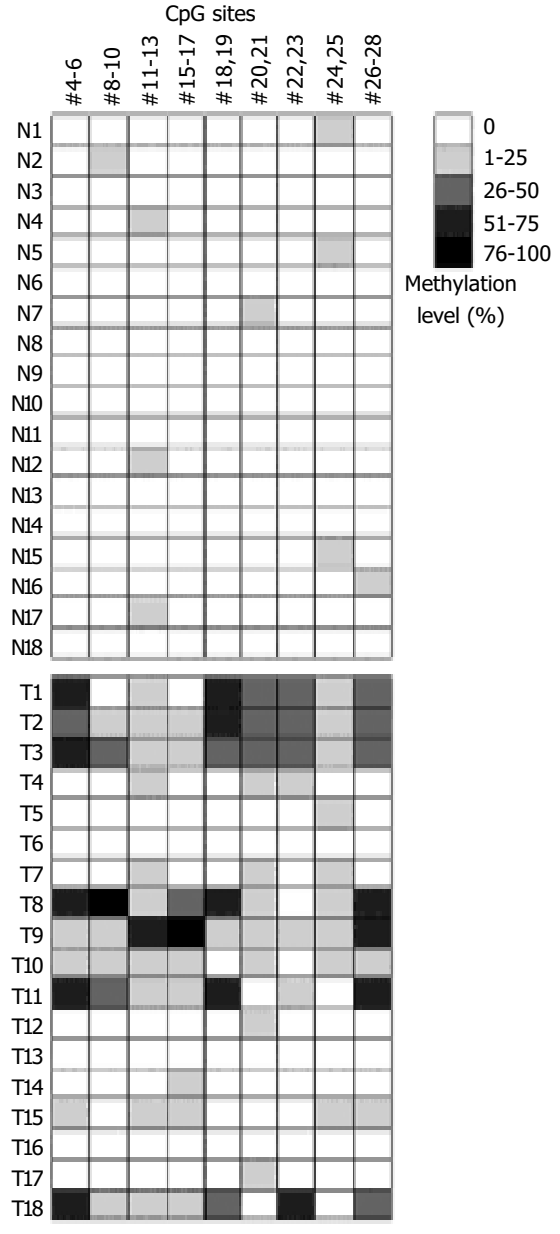

Figure 5 Methylation analysis of p16 gene CpG islands by oligonucleotide microarray. Summaries of the microarray results are shown for 18 gastric tumors and corresponding normal tissues. Gray scale shown at right represents the methylation levels in percentage determined from the calibration curve for the test CpG sites.

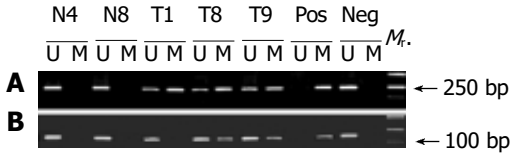

Figure 6 MSP analysis of the p16 gene CpG island in gastric tumor (T) and corresponding normal (N) tissues. M and U indicate amplification using methylated and unmethylated sequence-specific primers, respectively. DNA extracted from tumor and the corresponding normal tissues were amplified with primers MS and MA1 (A) and MS and MA2 (B), respectively. (Pos) Positive control; (Neg) negative control; (Mr.) DNA marker.

DISCUSSION

In this study, we have applied a microarray method to a comprehensive analysis of DNA methylation. The results indicated that the microarray was successfully used to map methylated CpG sites within the p16 gene CpG islands in clinical samples. The derived methylation information for gastric tumor and corresponding normal tissues was assessed quantitatively and independently validated by methylation-specific PCR.

This microarray-based analysis of DNA methylation is expected to provide new tools for research in this field. At present, most methylation assays have been limited to analyzing CpG islands of a few known genes and are restricted in throughput for a genome-wide analysis. Until recently, Gitan *et al*.[20] have developed a novel technique called MSO microarray that combines bisulfite DNA assay and oligonucleotides microarray for analysis of DNA methylation. The MSO microarray potentially allows rapid screening of multiple CpG sites in many gene promoters. CpG island hypermethylation has been reported to be linked to the silencing of more than 100 cancer-related genes. A DNA microarray can be generated to contain hundreds of oligonucleotides designed to discriminate between methylated and unmethylated sequences in these gene promoters. Bisulfite-treated genomic DNA from each of these loci can be amplified from investigated samples in a 96-well format to generate multiple targets for oligonucleotides hybridization.

As with other oligonucleotide microarrays, cross-hybridization between imperfect-match probes and targets could be observed. In addition, some probes might inherently diminish hybridization signals, probably due to decreased duplex stability of targets and probes[18]. Through careful data analysis, Gitan and his colleagues considered that cross-reactivity might also increase when oligonucleotide probes were designed to query methylation differences in one single CpG site. The issue is easily overcome by designing probes to include two or more CpG sites. This design consideration may limit the MSO assay's ability to detect methylation changes in single CpG sites. Adorjan *et al*.[23] have developed a microarray-based assay that can analyze methylation changes of single CpG sites. Several hundred CpG sites were screened in 76 samples from four different human tumor types and corresponding healthy controls. The results demonstrated that the microarray could be applied as a powerful tool to the assessment of selected CpG dinucleotides and quantification of methylation at each site. As shown in this study, the use of a simple control system could test the accuracy and reproducibility of the probes designed for microarray hybridization. This control system can also be used to calibrate the levels of methylation changes detected in the investigated samples by microarray assay.

In summary, microarray assay can be readily used to high-throughput analysis of DNA methylation. It will contribute significant information to our understanding of CpG island methylation in cancer.

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