# Multiple Gene Methylation of Nonsmall Cell Lung Cancers Evaluated With 3-Dimensional Microarray

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**BACKGROUND.** Aberrant DNA methylation of the CpG islands for cancer-related genes is among the earliest and most frequent alterations in cancer and may be useful for diagnosing cancer or evaluating recurrent disease.

**METHODS.** In this study, a 3-dimensional (3-D), polyacrylamide gel-based DNA microarray coupled with linker-polymerase chain reaction (PCR) was developed to detect hypermethylation of CpG islands in multiple genes from a large group of different samples. The authors determined the frequency of aberrant promoter methylation of 15 genes in 28 resected primary nonsmall cell lung cancers (NSCLCs) and in 12 corresponding nonmalignant lung tissues.

**RESULTS.** Methylation frequencies in the tumor samples were detected in 18% of samples for the breast cancer 1 gene *BRCA1*, in 43% of samples for the tissue inhibitor of metalloproteinase 3 gene *TIMP-3*, in 38% of samples for the cyclin-dependent kinase inhibitor 4A gene *p*16INK4a, in 54% of samples for the cadherin 13 gene *CDH13*, in 50% of samples for the death-associated protein kinase gene *DAPK*, in 11% of samples for the E-cadherin gene *ECAD*, in 25% of samples for the insulin-like growth factor binding protein 7 gene *IGFBP7*, in 18% of samples for the Ras association domain family 1 gene *RASSF1*, in 68% of samples for the adenomatous polyposis coli gene *APC*, in 7% of samples for the cyclin-dependent kinase inhibitor gene *p15*, in 18% of samples for the *CD44* cell adhesion molecule gene, in 29% of samples for the human Mut-L homolog gene *h*MLH, in 32% of samples for the human telomerase reverse transcriptase gene *h*TERT, in 64% of samples for the calcitonin gene-related polypeptide $\alpha$ gene *CALCA*, and in 54% of samples for the estrogen receptor gene *ER*; however, methylation was not observed in the majority of corresponding nonmalignant tissues. Six samples in from 28 tumors had $>6$ genes methylated, and 1 sample had 13 genes methylated. Methylation of these genes was correlated with some clinicopathologic patient characteristics.

**CONCLUSIONS.** This study demonstrated that a 3-D microarray could be used to detect DNA hypermethylation and provided a high-throughput platform for DNA hypermethylation analysis. ***Cancer* 2008;112:1325–36.** © *2008 American Cancer Society.*

**KEYWORDS: polyacrylamide gel, DNA microarray, linker-polymerase chain reaction, DNA methylation.**

Lung cancer is one of the most prevalent cancers and is the leading cause of cancer deaths in the world. It is believed that >1.2 million individuals died from lung cancer in 2001 and that the majority were from low- and middle-income countries.[1] Although enormous progress has been made in understanding the molecular and cellular biology of lung cancer, a lot of work needs to be done to gain a complete understanding of the pathogenesis and early diagnosis of this disease.


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It is well known that silencing of tumor suppressors or other cancer-associated genes by methylation of CpG islands, which are located in the promoter and/or 5′ regions on many genes, is a common feature of human cancer.[2–6] Aberrant gene methylation also has been identified frequently in nonsmall cell lung cancer (NSCLC). This includes the cyclin-dependent kinase inhibitor 4A gene *p*16INK4a, the human Mut-L homolog gene *hMLH1*, the cadherin 13 gene *CDH13*, the death-associated protein kinase 1 gene *DAPK1*, *Ras*, the adenomatous polyposis coli gene *APC*,[7–12] and so on. It has been suggested that aberrant methylation status may become a useful marker to identify individuals who are at high risk of developing lung cancer. The detection of aberrant promoter hypermethylation in cancer is useful for diagnosing cancer and for detecting recurrence.

Several methods have been developed to evaluate the methylation status of cancer-related genes, such us Southern blot analysis,[13] bisulfite genomic DNA sequencing,[14] restriction enzyme-polymerase chain reaction (PCR) analysis,[15] methylation-specific PCR,[5] and methylation-sensitive single nucleotide primer extension,[16] and have offered useful and powerful tools for studying the phenomenon of DNA methylation. However, each method was restricted to the evaluation of DNA methylation on a gene-by-gene level. DNA microarrays based on fluorescence or isotope labeling[17–19] have achieved great successes in providing a high-throughput platform to evaluate the methylation status of different CpG sites within a specific gene or in a group of different genes within a single sample. Recently, to identify useful methylation markers related a particular disease, we developed a microarray technology that can screen the methylation status of a given gene from a group of different samples. PCR product microarrays of bisulfited genomic DNA[20] have been developed in which the bisulfited DNA from a single gene has to be amplified first from different clinical samples; then, the PCR products are spotted onto a microarray. Our group has made several efforts to improve the efficiency of the microarray platform. First, whole genomic DNA from several samples was immobilized onto array slides or nylon membranes,[19,21] and a bisulfite treatment process was performed directly on the microarray surface to detect the methylation status of multiple genes. This method demonstrated feasibility for species with a limited genome size; however, it remained difficult to detect human methylation with this method because of its low sensitivity for detecting particular genes and because of the small amount of immobilized genomic DNA on the planar surface of the microarray. Therefore, DNA methylation detection technology needs to be developed for the evaluation of multiple samples and genes.

Three-dimensional (3-D), functionalized, hydrophilic polyacrylamide gels with a porous structure can improve the amount of immobilized nucleic acids, which have been used as support substrates in microarray analyses.[22–24] Xiao et al.[25] improved this technique by using N,N,N′,N′-tetramethylethylenediamine (TEMED) to ensure the consistency of each samples' concentration and viscosity in the spotting. The objective of the current study was to determine the DNA methylation status of multiple genes in a group of NSCLC samples by using a 3-D polyacrylamide gel microarray coupled with linker-PCR. The frequencies of aberrant promoter methylation of 15 genes in 28 resected primary NSCLC samples and in 12 corresponding nonmalignant lung tissues were estimated successfully by using the 3-D gel-based DNA microarray spotted with universal PCR products.

## MATERIALS AND METHODS

### Experimental Guide

DNA segments of the 5′-untranslated region and the first exon were selected for the investigated target genes. Figure 1 shows that genomic DNA was restricted with *Mse*I from human lung tumor tissue, corresponding nonmalignant lung tissues, and whole blood cells from healthy individuals. The cleaved ends of DNA were ligated to unphosphorylated linkers and then digested with the methylation-sensitive endonuclease *Bst*UI. The digested products were amplified by linker-PCR as targets. The primer was labeled with acrylamide at its 5′ end. PCR products were purified and immobilized to glass slides through polyacrylamide gel and acrylamide. Consequently, a 3-D target DNA microarray was formed. Indocarbocyanine (Cy3)-labeled oligonucleotide probes synthesized to completely match with the target DNA fragment were hybridized with the 3-D microarray. If the interrogated CpG islands were hypermethylated, then the fragments could not be digested by *Bst*UI restriction endonuclease and could be amplified by linker-PCR. The hybridized signals of the linker-PCR products and matched oligonucleotides were green, or else black.

### Gene Selection

In total, 15 genes were selected for 3-D, microarray-based examination of methylation abnormalities. The gene profile was composed of the CD44 cell adhesion molecule, the insulin-like growth factor binding protein-7 gene *IGFBP7*, the *p15* cyclin-dependent kinase inhibitor gene, the Ras association domain fam-

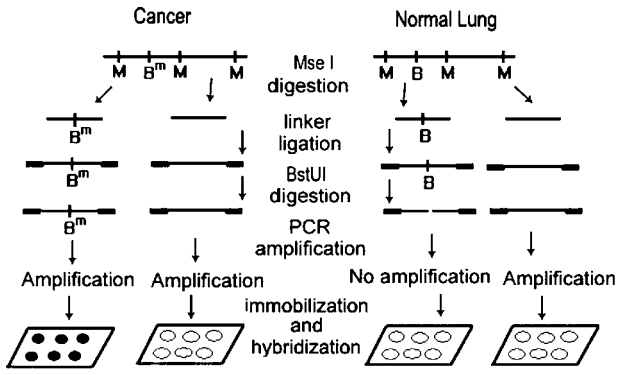



**FIGURE 1.** Schematic outline of the procedure for the preparation of the DNA targets. DNA samples were restricted with *Mse*I. The cleaved ends of DNA were ligated to unphosphorylated linkers and then digested with the methylation-sensitive endonuclease *Bst*UI. The digested products were amplified by polymerase chain reaction (PCR) analysis as targets. If the interrogated CpG islands were methylated, then they could be digested by *Bst*UI restriction endonuclease and could be amplified by linker-PCR. The hybridized signals of the linker-PCR products and the matched oligonucleotides were green. The *Mse*I-digested fragments without endonuclease *Bst*UI sites also could be amplified by linker-PCR. However, these PCR products could not be captured by the probes on the microarray and did not influence the results of this assay. M indicates MseI restriction enzyme recognition sites; B, unmethylated *Bst*UI endonuclease recognition sites; $B^m$, methylated *Bst*UI recognition sites.

ily 1 gene *RASSF1*, the breast cancer 1 gene *BRCA1*, the estrogen receptor gene *ER*, the calcitonin gene-related polypeptide α gene *CALCA*, the E-cadherin gene *ECAD*, *CDH13*, the human telomerase reverse transcriptase gene *h*TERT, *DAPK1*, tissue inhibitor of metalloproteinase 3 *TIMP-3*, *hMLH1*, *p*16INK4a, and *APC*. The panel included genes that have been reported in part as targets for epigenetic silencing in lung cancer.[9,26,27] *IGFBP7*, *h*TERT, and *hMLH1* reportedly are involved in DNA repair, the inactivation of which results in increased mutagenecity. *CD44*, *ER*, and *TIMP-3* often have been related to metastasis of cancer.[28,29] The *DAPK* gene reportedly is involved in apoptosis, whereas *APC* and *p*16INK4a affect the cell cycle and frequently are inactivated in human cancers.[30]

### Study Population and DNA Extraction

Primary tumor samples (n = 28) and samples of randomly selected, corresponding, nonmalignant lung tissues (n = 12) were obtained from patients with NSCLC who underwent curative resection at DeZhou Tumor Hospital (Shandong, China) and at the People's Hospital of CangZhou (Hebei, China) between June 2003 and March 2005. Tumors were snap-frozen in liquid nitrogen and stored at −70 °C until genomic DNA preparation. Whole blood cells from healthy individuals and lung tumor tissues were obtained from GuLou Hospital (Nanjing, China). Genomic DNA was extracted from whole blood cells and from lung tumor tissues by the standard method using proteinase K digestion and phenol/chloroform extraction.

### Preparation of DNA Targets

The preparation process for targets is illustrated in Figure 1. The DNA derived from the blood cells of healthy individuals was divided into 2 parts. One aliquot was treated with methylase SssI (SM) as a positive control under the conditions recommended by the supplier (New England Biolabs, Ipswich, Mass). The other aliquot was not treated with SM and was used as a negative control. The positive control generated in this way had 100% methylated cytosine in the test CpG sites, whereas the negative control had all unmethylated cytosine residues in the test CpG sites. Approximately 2 μg of DNA were restricted to complete with 5 U of *Mse*I (New England Biolabs) at 37 °C overnight in 50 μL reaction mixture. This enzyme restricts bulk DNA into small fragments. Because the recognition site (T*TAA) of *Mse*I rarely occurs in GC-rich regions, most CpG islands remain intact after the restriction. The digests were purified with QIAquick column (Qiagen, Gaithersburg, MD). The ligation[31] step began with the annealing of H-12 (5′-TAA TCC CTC GGA) to H-24 (5′-AGG CAA CTG TGC TAT CCG AGG GAT; 100 μM each) by mixing equal amounts of each oligonucleotide in a microcentrifuge tube and allowing the mixture to be cooled from 55 °C to room temperature over 1 hour. The use of universal linkers allows the subsequent amplification of all methylated fragments in ligated DNA samples. Then, the annealed linker primers (10 μM per sample) were added to the purified DNA to ligate the cleaved ends of the DNA fragments by incubating overnight with T4 DNA ligase (New England Biolabs) in 50 μL of reaction mixture at 16 °C. The mixture was held at 65 °C for 20 minutes to stop the reaction. The ligated products were purified with a QIAquick column (Qiagen).

The ligated DNA was digested with the methylation-sensitive endonucleoase *Bst*U (New England Biolabs). The digest was used for PCR without further purification. Genomic fragments that contained methylated sites were protected from the digestion and could be amplified by linker-PCR. Many of these linker-ligated *Mse*I fragments were expected to be present in the tumor sample because of aberrant methylation in the test site, whereas the same unmethylated fragments were digested and were not

present in the normal amplicon after PCR.[31] PCR reactions were performed in a 30-μL volume that contained 10 μM SH24 (acrylamide-5′-AGG CAA CTG TGC TAT CCG AGG GAT), 20 μM primer, 0.3 U *Taq* DNA polymerase, and 0.6 μL deoxyribonucleoside triphosphate (10 mM) in a buffer provided by the supplier. The tubes were incubated for 5 minutes at 72 °C to fill in 5′-protruding ends of the ligated DNA and were subjected to 25 cycles of amplification consisting of 1 minute at 95 °C and 3 minutes at 62 °C in a PTC-225 thermocycler (MJ Research, Watertown, Mass). The final extension was lengthened to 10 minutes.

### Printing and Postprocessing Microarray

Acrylamide-modified slides were prepared as described in previous reports.[23,25] Glass slides were cleaned by soaking in 10% aqueous nitric acid for 2 hours. Then, slides were rinsed with water and acetone and air dried. The cleaned slides were soaked in 10% 3 methacryloxypropyltrimethoxysilane (Sigma Chemical Company, St. Louis, MO) in acetone for 1 hour, washed in acetone, and air dried.

Purified, acrylamide-modified PCR products (at the desired concentration, from 0.8 μM to 10 μM) were prepared that contained 3% acrylamide monomer (acrylamide:bisacrylamide, 29:1), 30% glycerol, and 1% ammonium persulfate (APS). The completely mixed solutions were spotted on the modified glass slide using Smartarrayer-48 (Capital Biochip Corporation, China). After spotting, the glass slide was placed onto a humid sealed chamber in which a well containing TEMED was deposited in advance. The pressure in the sealed chamber was reduced to approximately 1000 pascals, and this pressure was maintained for 0.5 hours at room temperature. Under this pressure, TEMED was vaporized and diffused onto the slide surfaces to induce the copolymerization between acrylamide groups and acryl groups.

### Hybridization

After the attachment, to obtain single-stranded DNA (ssDNA) for hybridization analysis, double-stranded DNA immobilized on the slides was denatured at 95 °C for 5 minutes and cooled immediately or displaced in 0.1 M of NaOH for 10 minutes. During hybridization, the Cy3-labeled target solution was applied to the microarray and spread evenly by placing a coverslip on top, and the hybridization was performed in a humid glass chamber that was sealed with plastic film at room temperature for 2 hours.[32] The sequences of the 15 gene probes are listed in the Table 1.

**TABLE 1**
**Summary of Probe Sequences and Annealing Temperatures***

| Gene | Sequence: Cy3 5′ to 3′ | Temperature (°C) |
|---|---|---|
| *CD44* | CAGGCACCCCGCGACACTC | 49.5 |
| *p15* | GAGGCGCGCGATCCAGGTA | 59.8 |
| *IGFBP7* | CACGGGCGGGGCCCCCGTG | 49.5 |
| *hMLH1* | AGTGGCGCCTGACGTCGCGT | 54.2 |
| *BRCA1* | GACTGCGCGGCGTGAGCTCG | 52.5 |
| *ER* | CCAACGCGCAGGTCTAC | 39.1 |
| *ECAD* | ATAGACGCGGTGACCCTC | 55.1 |
| *CDH13* | CCACCTCCGCGGGGCTCG | 47.9 |
| *CALCA* | TAGTCCGCGATTTATAC | 39.8 |
| *DAPK1* | CAGGCGCGGCTCCCCGGTC | 53.4 |
| *p16* | ACGGCCGCGGCCCGGGGT | 58.3 |
| *h*TERT | CACCCTGGGAGCGCGAGCGG | 55.2 |
| *RASSF1* | GGAGAGCCGCGCAATGGA | 65 |
| *TIMP* | GCGCGCTGCCCTCCGAGT | 58.9 |
| *APC* | CGGAGAAGCGCGGTGTAG | 59.1 |

Cy3 indicates indocarbocyanine; *CD44*, cell adhesion molecule gene; *p15*, cyclin-dependent kinase inhibitor gene; *IGFBP7*, insulin-like growth factor binding protein 7 gene; *hMLH1*, human mut-L homolog 1 gene; *BRCA1*, breast cancer 1 gene; *ER*, estrogen receptor gene; *ECAD*, E-cadherin gene; *CDH13*, cadherin 13 gene; *CALCA*, calcitonin gene-related polypeptide α gene; *DAPK1*, death-associated protein kinase 1 gene; *p16*, cyclin-dependent kinase inhibitor gene; *h*TERT, human telomerase reverse transcriptase gene; *RASSF1*, Ras association domain family 1 gene; *TIMP*, tissue inhibitor of metalloproteinase 3 gene *TIMP3*; *APC*, adenomatous polyposis coli gene; C, cytosine; A, adenine; G, guanine; T, thymine.

* The Cy3 fluorescence molecule was modified to the 5′ terminal of every probe.

### Posthybridization and Scan

After hybridization, the slide was subjected to electrophoresis under 5–30 V/cm for 5–60 minutes in 1 × Tris-borate-ethylenediamine tetracetic acid buffer at room temperature. In this assay, 5 V/cm for 10 minutes was the right condition. After electrophoresis was completed, the slide was rinsed in water and dried under a stream of nitrogen. Images of the slides were captured by a scanner (ScanArray Lite; Packard BioScience Company, Meriden, Conn) and were analyzed with Genepix Pro 3.0 software. Because the mixture of the point samples was ropy, the spots were not uniform. The mean fluorescence intensities were used in this assay.

### Statistical Analysis

CpG island methylation status within the NSCLC series was correlated with clinicopathologic data that were acquired previously by using a commercially available statistical package (SPSS version 10.0; SPSS Inc, Chicago, Ill). Associations between risk factors (age, tumor grade, stage, pathology) and methylation of samples were determined by using the Fisher exact test. Differences were considered statistically significant if the $P$ value was $<.05$.

### CpG Island Methylation in Tissue DNA by Combined Bisulfite Restriction Analysis

A combined bisulfite restriction analysis (COBRA) assay was used to verify the feasibility of the polyacrylamide gel DNA array. The COBRA assay can determine the methylation status of CpGs within CpG islands. The proportion of methylated DNA versus unmethylated DNA was determined by measuring the relative intensities of cut and uncut PCR products. For that determination, COBRA was performed according to a previously published protocol.[33] In brief, bisulfite-sequence PCR was performed as described previously.[18] Approximately 100 ng of bisulfite-modified DNA were amplified with primer pairs (5′-AAA GAG GAG GGG TTG GTT GGT TAT TA-3′, 5′-TAC CTA ATT CCA ATT CCC CTA CAA ACT-3′). For this assay, the *p*16INK4a gene and 5 samples were interrogated by COBRA. PCR products were digested with the restriction enzyme *Bst*UI (New England Biolabs) and were separated on 2% agarose gels.

## RESULTS

### Sensitivity

Incomplete methylation-sensitive digestion may occur during sample preparation and may result in the false-positive detection of DNA methylation after PCR amplification. The feasibility of the 3-D microarray for assessing hypermethylation in multiple genes in numerous lung tumors was determined first. Three samples, 1 negative control (N) and 2 positive controls (P and P1), were analyzed by using a *p*16INK4a probe to assess the veracity of this assay (Fig. 2). In these 3 samples, N was from normal peripheral blood DNA treated with SM and *Bst*UI, P was from normal peripheral blood DNA treated with *Mse*I restriction enzyme, and P1 was from normal peripheral blood DNA treated sequentially with SM, *Mse*I, and *Bst*UI. The same amounts of the 3 samples were spotted on the glass slides to form the microarray and were hybridized with *p16* probes. Hybridization of each sample was repeated 6 times in a line marked P1, P, and N, respectively. The hybridization results are shown in Figure 2. The fluorescence intensities of samples P and P1 were approximately 20,000 and 18,000, respectively, whereas the intensity of sample N was only approximately 600. There was no difference in fluorescence intensity between P and P1, and the same was true of N, although the difference was nearly 100 times between the 2 positive samples and the negative sample. This assessment demonstrated that there were no false-positive results in the polyacrylamide gel array.

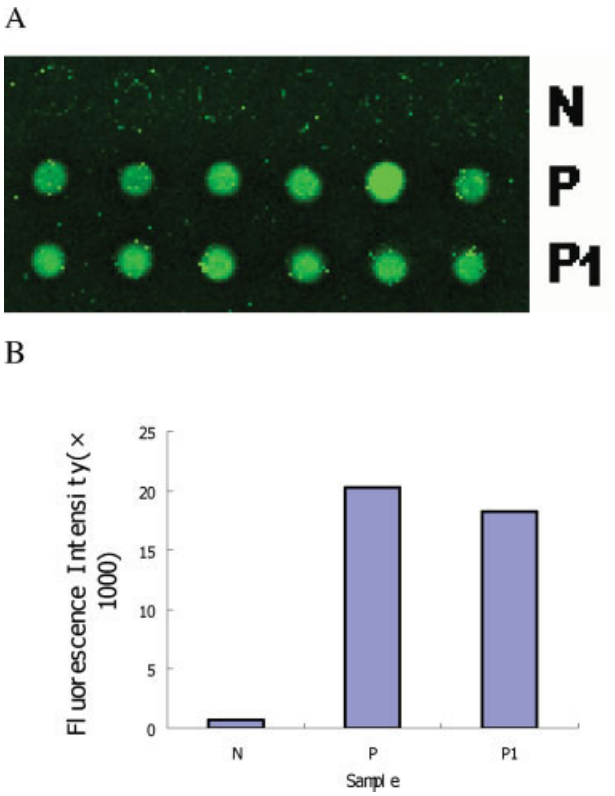



**FIGURE 2.** Three samples, 1 normal tissue sample (negative control) (N) and 2 positive control samples (P and P1) were detected by a *p*16INK4a probe to validate the sensitivity of the polyacrylamide gel array. (A) Hybridization results of the gel array. (B) The fluorescence intensities of the hybridization. Note that N was from a normal peripheral blood DNA sample that was treated with methylase SssI and *Bst*UI restriction endonuclease; P was from a normal peripheral blood DNA sample that was treated with *Mse*I restriction enzyme; and P1 was from a normal peripheral blood DNA sample that was treated sequentially with methylase SssI, *Mse*I restriction enzyme, and *Bst*UI restriction endonuclease.

For the same amount of each sample, a strongly methylated sample will contain less uncut MseI fragments than a less methylated sample. Hence, internal control to adjust these differences should be established. An indodicarbocyanine (Cy5)-labeled probe (Cy5-5′-GTTTCGCTCTGTTGCCCA) from one of the uncut MseI fragments was hybridized to the target DNA to measure cross-linking efficiency and DNA loading. The same amounts of positive and negative PCR products were dotted onto the glass; then, and Cy3 and Cy5 probes were mixed and hybridized to the DNA target. Figure 3 shows the hybridization results, in which we observed that the Cy5 intensity in the negative sample was only a little higher than that in the positive samples. Because there was only from 5% to 10% CpG dinucleotide of the whole genomic DNA, the majority of the geno-

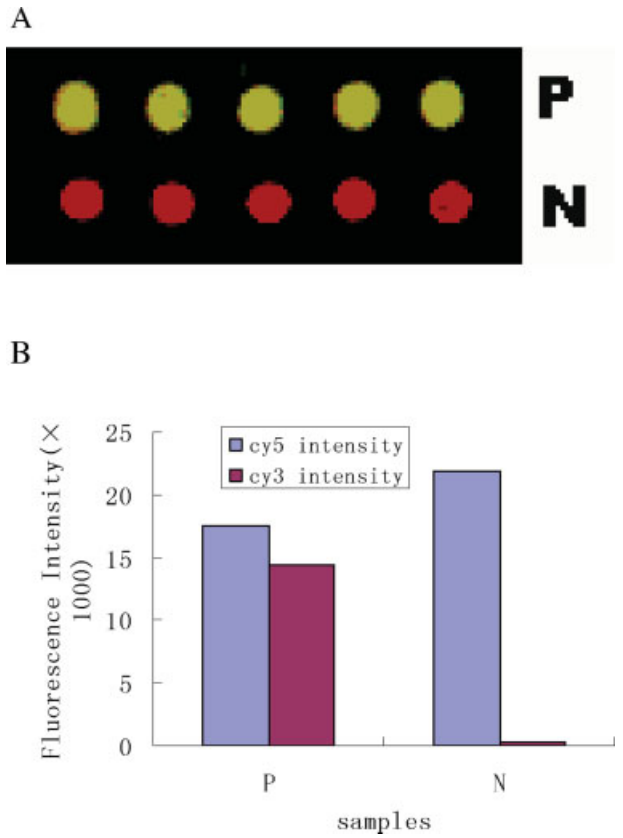



**FIGURE 3.** The cohybridization was conducted to measure cross-linking efficiency. The same amounts of positive and negative polymerase chain reaction products were dotted on the glass; then, indocarbocyanine (cy3) and indodicarbocyanine (cy5) probes were mixed and hybridized to the DNA target. P indicates positive control; N, negative control.

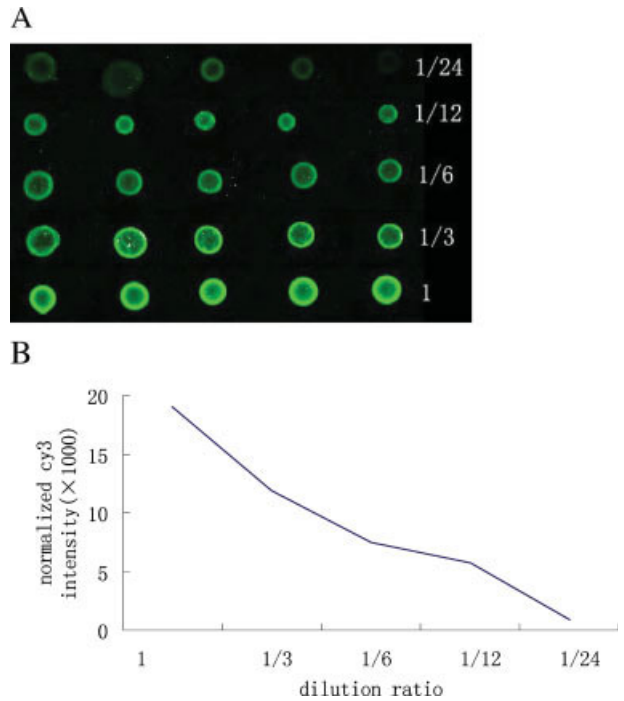



**FIGURE 4.** Dilution experimental results on linker-polymerase chain reaction (PCR) product polyacrylamide gel array fixed with mixtures of positive and negative PCR products hybridized with *p16* probe. (A) Hybridization image. (B) Normalized hybridization signals quantified with the average intensity of 5 spots for each mixture sample. Cy3 indicates indocarbocyanine.

mic DNA was uncut MseI fragments. Therefore, perhaps the difference raised by various degrees of methylation in the same amount DNA could be omitted in this assay.

To study the sensitivity of this method further and to accurately determine the presence, absence, and degree of methylation in multiple genes, a positive control was diluted with a negative control (unmethylated DNA [N1]) in proportions of 1:1, 1:3, 1:6, 1:12 and 1:24 to determine which ratios of methylated DNA in tumor tissues could be detected. The concentration of the positive control was 8 μg/μL, which was the same amount as positive and negative controls described above. In addition, as discussed above, the diluted PCR products were mixed with 3% acrylamide monomer (acrylamide:bisacrylamide, 29:1), 30% glycerol, and 1% APS and were hybridized to a *p16* probe. Here, normalized signal intensity was introduced to determine the methylation threshold of this assay. Because different DNA amounts produced different Cy3 intensities, it was difficult to estimate whether there was methylation in those samples that had a low degree of methylation if their concentrations were not same. Normalized signal intensity also could quantitatively calculate the degree of methylation in a sample. The normalized signal intensity was equal to the average signal intensity divided by its DNA concentration. Figure 4A shows the hybridization results of different concentration of positive control, and Figure 4B shows the normalized Cy3 intensity value. In Figure 4B, it can bee seen that, when the PCR product concentration ratio was 1:12, the normalized Cy3 intensity largely reduced when the PCR production concentration ratio decreased. However, when the concentration ratio was 1:24, there was only back fluorescence intensity. We considered the normalized Cy3 intensity of the concentration ratio of 1:12 as the methylation threshold for this assay. To perceptually determine the degree of methylation in those samples, the normalized Cy3-intensity segments between the methylation threshold and the positive control were divided into 3 levels with the methylation degree (percents) set from 0% to 30% (methylation zone I), from 30% to 60% (methylation zone II), and from 60% to 100%

(methylation zone III), from the lowest to the highest percentage of methylation. The degree of methylation for each sample was classified into 1 of these 3 zones.

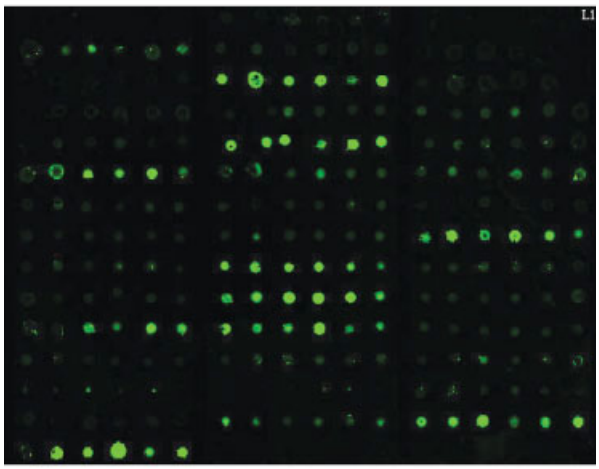
### Frequency of Methylation in Primary NSCLCs and Corresponding Nonmalignant Lung Tissues

Methylation profiles and prevalence within the different exposure groups were determined by polyacrylamide gel array (Fig. 5A). Figure 5 shows the results of hybridization of the gene *hMLH1*. In the array, 43 samples were interrogated, including 1 positive sample, 2 negative control samples, 28 resected NSCLCs, and 12 corresponding nonmalignant lung tissues. Every sample had 6 repeated dots. For 1 line, there were 3 samples spotted. An illustration+ of the dot positions is provided in Figure 5B.

In the corresponding nonmalignant lung tissue samples, hypermethylation of *BRCA1*, *IGFBP7*, *p15*, *CD44*, *TIMP3*, and *ER* was not detected; whereas low frequencies were observed in *CDH13* (3 samples), *RASSF1* (2 samples), *APC* (1 sample), *h*MLH (2 samples), *h*TERT (2 samples), *p*16INK4a (2 samples), *DAPK1* (2 samples), *CALCA* (3 samples), and *ECAD* (1 sample) (Table 2). The detailed results of CpG island hypermethylation were scored, and the results are summarized in Figure 6, which illustrates that the degrees of DNA methylation in all nonmalignant lung tissue samples were distributed in methylation zone I, except for N6, in which the *CDH13* gene was distributed in methylation zone II. In 12 samples, 3 nonmalignant lung tissues had methylation of the *CDH13* gene, which had the highest percents in the 15 genes. For the sample N3, DNA hypermethylation occurred in 5 of 15 genes, and that sample had the highest DNA hypermethylation of the 12 samples.

The overall hypermethylation frequencies detected in lung tumor were 18% for *BRCA1*, 54% for *CDH13*, 25% for *IGFBP7*, 18% for *RASSF1*, 68% for *APC*, 7% for *p15*, 18% for *CD44*, 29% for *h*MLH, 32% for *h*TERT, 38% for *p*16INK4a, 50% for *DAPK1*, 64% for *CALCA*, 11% for *ECAD*, 43% for *TIMP-3*, and 54% for *ER*. Three samples had methylation of only 1 gene, and those samples had the lowest degree of methylation of all the NSCLC samples. Four tumors samples (24%) had methylation of 50% of the genes, and the highest percent of gene methylation in a tumor sample was 60% (6 of 15 genes). In addition, we observed that at least 1 of the 15 genes was methylated in all of the tumor samples, and at least 1 tumor had 13 methylated genes. Overall, it appeared that there was no significant correlation with methylation status among the genes. However, in the tumor sam-

A



B

| | | |
|---|---|---|
| N | N | T1 |
| T2 | T3 | T4 |
| T5 | T6 | T7 |
| T8 | T9 | T10 |
| T11 | T12 | T13 |
| T14 | T15 | T16 |
| T17 | T18 | T19 |
| T20 | T21 | T22 |
| T23 | T24 | T25 |
| T26 | N3 | N2 |
| N24 | N7 | N6 |
| N9 | N14 | N20 |
| N10 | N21 | N12 |
| N1 | T27 | T28 |
| P | | |

**FIGURE 5.** Hybridization of the human Mut-L homolog gene (hMLH1). (A) There are 15 lines in this photograph of the polyacrylamide gel array. Because the 2 negative controls and the tumor sample T1 had no methylated CpG islands and had low background fluorescence intensities, the first line often was neglected. (B) Drawing of the dot positions from the array for each sample. Note that, because of edge effects (eg, the hybridization solution dried out or the coverslips moved), there often were fewer hybridized probes that there were dots at the edges of the glass slides. Consequently, the fluorescence intensities of the dots at the edges were lower. When the fluorescence intensities were calculated, the lines of dots on the left and right edges were cut off. T indicates tumor; N, corresponding normal tissue; P, positive control.

**TABLE 2**
**Frequency of CpG Island Methylation in Nonsmall Cell Lung Cancers and Corresponding Nonmalignant Lung Tissues From the Same Group of Patients**

| | Frequency of methylation, % | |
|---|---|---|
| Gene | Tumor, N = 28 | Nonmalignant tissue, N = 12 |
| *BRCA1* | 5 (18) | 0 (0) |
| *CDH13* | 15 (54) | 3 (25) |
| *IGFBP7* | 7 (25) | 0 (0) |
| *RASSF1* | 6 (21) | 2 (17) |
| *APC* | 19 (68) | 1 (8) |
| *p15* | 2 (7) | 0 (0) |
| *CD44* | 5 (18) | 0 (0) |
| *hMLH1* | 8 (29) | 2 (17) |
| *h*TERT | 9 (32) | 2 (17) |
| *p16* | 10 (38) | 2 (17) |
| *DAPK1* | 14 (50) | 2 (17) |
| *CALCA* | 18 (64) | 3 (25) |
| *ECAD* | 3 (11) | 1 (8) |
| *TIMP* | 12 (43) | 0 (0) |
| *ER* | 15 (54) | 0 (0) |

*BRCA1* indicates breast cancer 1 gene; *CDH13*, cadherin 13 gene; *IGFBP7*, insulin-like growth factor binding protein 7 gene; *RASSF1*, Ras association domain family 1 gene; *APC*, adenomatous polyposis coli gene; *p15*, cyclin-dependent kinase inhibitor gene; *CD44*, cell adhesion molecule gene; *h*MLH, human mut-L homolog gene; *h*TERT, human telomerase reverse transcriptase gene; *p16*, cyclin-dependent kinase inhibitor gene; *DAPK1*, death-associated protein kinase 1 gene; *CALCA*, calcitonin gene-related polypeptide α gene; *ECAD*, E-cadherin gene; *TIMP*, tissue inhibitor of metalloproteinase 3 gene *TIMP3*; *ER*, estrogen receptor gene.

ples T7, T9, T22, and T23 had very high degrees of methylation.

For most samples, the degree of methylation was distributed in methylation zone I, which had the lowest degree of methylation. However, overall, 82 of 420 CpG islands (19.5%) were in methylation zone I, which had the highest percent of the 3 methylation zones: The lowest percent was 4.7% (20 of 420 CpG islands) in methylation zone III, and methylation zone II had 10.9% (46 of 420 CpG islands) methylation. Simply put, with higher degrees of methylation, lower numbers of samples were distributed. However, most of the high levels of methylation often appeared in highly methylated genes or samples, such as the genes *CDH13*, *APC*, *CALCA*, and *p16* and in NSCLC tissue samples T9, T15, T22, and T23.

### Correlation Between Aberrant Methylation and Clinicopathologic Features in Patients With NSCLC

We analyzed the methylation changes in the tumors and the clinical data obtained from these patients (Table 3). Overall, we observed that there were few correlations between NSCLC and gene methylation. According to pathologic types, NSCLC included adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and adenosquamous cell carcinoma. For adenosquamous cell carcinoma, there were 8 genes with methylation frequency that was higher than in the other 3 types. To summarize our findings regarding other clinical parameters, the frequency of gene methylation was greater among men (10 of 15 genes) than among women; was greater for patients with well differentiated tumors (8 of 15 genes) than for patients with undifferentiated or poorly differentiated tumors; and, with regard to vascular permeation, patients with lymphatic permeation had a greater degree of gene methylation (11 of 15 genes).

The *APC* gene is a tumor suppressor gene that is associated with both familial and sporadic cancer and reportedly has high rates of allelic loss in lung cancer. In our assay, *APC* methylation also was observed more frequently in men (15 of 17 men; 88%) than in women (4 of 11 women; 36%). The difference in the frequency of *APC* methylation between men and women was significant ($P = .01$). Lymphatic permeation is a well established prognostic indicator in patients with resected NSCLC, and we observed that lymphatic permeation was involved with tumor in 83% of samples with *p*16INK4a methylation. There was a significant difference ($P = .013$) in the frequency of *p*16INK4a methylation between samples with and without lymphatic permeation. It has been reported that aberrant hypermethylation of the *p*16INK4a gene, which plays a key role in cell cycle regulation, is an early event in lung cancer and a potential biomarker for early diagnosis.[34] In the current study, 2 significant differences were observed between the frequency of *p*16INK4a methylation and patient characteristics. Other than lymphatic permeation, the frequency of *p*16INK4a methylation was statistically significant ($P = .037$) in adenosquamous cell carcinoma among the 4 pathology categories. However, the other gene that also had statistical significance in adenosquamous cell carcinoma was *ECAD* ($P = .023$). *ER*, which is member of the nuclear receptor superfamily, was associated with a higher risk for cancer. With our assay, methylation of *ER* was associated significantly with well differentiated tumors ($P = .024$).

### *p*16INK4a Methylation Analysis by COBRA

For DNA methylation analysis, we used 5 samples with different degrees of methylation and subjected them to COBRA. Those 5 samples included 3 tumor samples (T22, T23, and T24) and 2 normal tissue samples (N1and N14). PCR products were digested with *Bst*UI, which cut the fragment only if the CGCG target site had been retained through bisulfite-mediated deamination. Thus, enzymatic cleavage

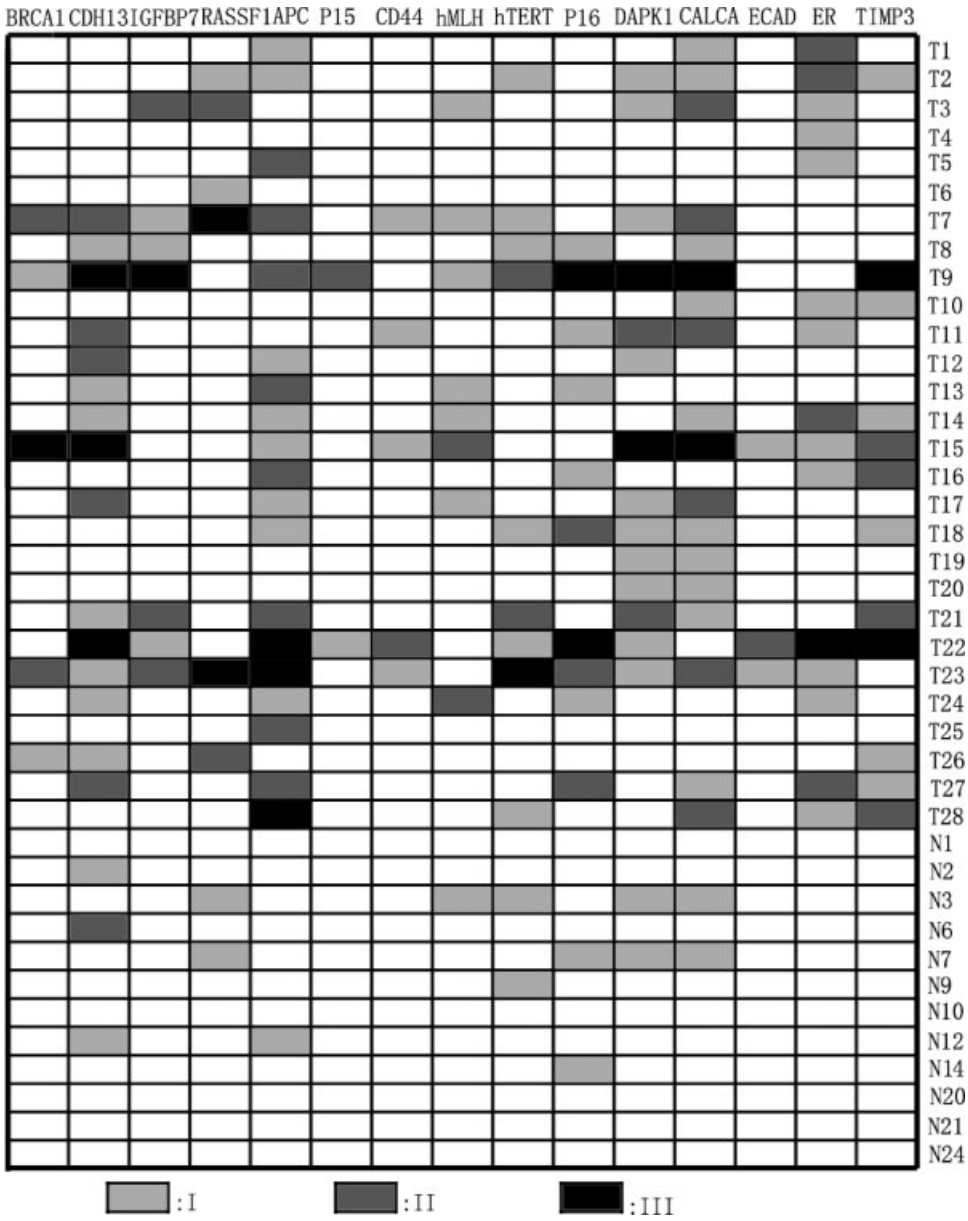



**FIGURE 6.** Summary of the methylation status of the 15 genes that were studied in resected primary nonsmall cell lung cancers and corresponding nonmalignant lung tissues. White boxes represent samples that were not methylated; boxes with different degrees of gray represent samples that were methylated at different levels or zones (I, II, and III). *BRCA1* indicates breast cancer 1 gene; *CDH13*, cadherin 13 gene; *IGFBP7*, insulin-like growth factor binding protein 7 gene; *RASSF1*, Ras association domain family 1 gene; *APC*, adenomatous polyposis coli gene; *p15*, cyclin-dependent kinase inhibitor gene; *CD44*, cell adhesion molecule gene; *h*MLH, human Mut-L homolog gene; *h*TERT, human telomerase reverse transcriptase gene; *p16*, cyclin-dependent kinase inhibitor gene; *DAPK1*, death-associated protein kinase 1 gene; *CALCA*, calcitonin gene-related polypeptide $\alpha$ gene; *ECAD*, E-cadherin gene; *ER*, estrogen receptor gene; *TIMP3*, tissue inhibitor of metalloproteinase 3 gene; T, tumor; N, corresponding normal tissue.

indicated methylation, and the lack of digestion indicated absence of methylation. In Figure 7, the upper band is the unmethylated band, and the lower bands are methylated bands. We observed partial digestion of theT22, T23, T24, and N14 amplicons, as expected and in agreement with the existence of the methylated *p16* allele in those samples. In the polyacrylamide gel oligo array assay, the methylation zones, in turn, were I, II, and III for COBRA: The unmethylated band was very weak in T22 and was the strongest in N14. The N23 and N24 amplicons largely were undigested and, thus, were considered predominantly unmethylated. In contrast, the PCR amplicon generated from N1 was completely undigested,

**TABLE 3**
**Correlation Between Clinical Data and DNA Methylation of the Different Genes**

| | Gene, % methylated (*P* value) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Characteristic** | **BRC** | **CD13** | **IGF** | **RAS** | ***APC*** | ***p15*** | ***CD44*** | **hML** | **hTER** | ***p16*** | **DAPK** | **CAL** | ***ECAD*** | ***ER*** | **TIMP** |
| Sex | | | | | | | | | | | | | | | |
| Men, n = 17 | 18 | 65 | 24 | 18 | 88 (.01) | 12 | 18 | 29 | 47 | 41 | 59 | 71 | 12 | 47 | 53 |
| Women, n = 11 | 18 | 36 | 27 | 18 | 36 | 0 | 18 | 27 | 18 | 27 | 36 | 64 | 9 | 73 | 18 |
| Age, y | | | | | | | | | | | | | | | |
| ≤60, n = 16 | 31 | 44 | 31 | 19 | 69 | 0 | 19 | 31 | 44 | 31 | 38 | 56 | 13 | 56 | 25 |
| >60, n = 12 | 0 | 67 | 17 | 17 | 67 | 17 | 17 | 25 | 25 | 42 | 67 | 83 | 8 | 58 | 58 |
| Differentiation | | | | | | | | | | | | | | | |
| Poor, n = 13 | 15 | 62 | 15 | 8 | 85 | 0 | 15 | 23 | 46 | 38 | 62 | 77 | 0 | 46 | 46 |
| Moderate, n = 9 | 11 | 44 | 22 | 22 | 44 | 11 | 11 | 44 | 22 | 11 | 44 | 78 | 11 | 44 | 33 |
| Well, n = 6 | 33 | 50 | 50 | 33 | 67 | 17 | 33 | 17 | 33 | 67 | 33 | 33 | 33 | 100 (.024) | 33 |
| Pathology | | | | | | | | | | | | | | | |
| Adeno, n = 15 | 13 | 60 | 20 | 7 | 73 | 7 | 13 | 40 | 33 | 33 | 47 | 73 | 7 | 53 | 40 |
| SCC, n = 7 | 14 | 43 | 14 | 29 | 57 | 0 | 14 | 14 | 43 | 14 | 71 | 86 | 0 | 29 | 43 |
| Large cell carcinoma, n = 3 | 33 | 33 | 33 | 33 | 33 | 0 | 0 | 33 | 0 | 33 | 0 | 33 | 0 | 100 | 0 |
| Adeno-SCC, n = 3 | 33 | 67 | 67 | 33 | 100 | 33 | 67 | 0 | 67 | 100 (.037) | 67 | 33 | 67 (.023) | 100 | 67 |
| Lymphatic permeation | | | | | | | | | | | | | | | |
| 1, n = 6 | 17 | 83 | 50 | 17 | 83 | 33 | 33 | 17 | 50 | 83 (.013) | 33 | 67 | 33 | 83 | 67 |
| 0, n = 22 | 18 | 45 | 18 | 18 | 64 | 0 | 14 | 32 | 32 | 23 | 55 | 64 | 5 | 50 | 32 |

BRC indicates breast cancer 1 gene *BRCA1*; CD13, cadherin 13 gene *CDH13*; IGF, insulin-like growth factor binding protein 7 gene *IGFBP7*; RAS, Ras association domain family 1 gene *RASSF1*; *APC*, adenomatous polyposis coli gene; *p15*, cyclin-dependent kinase inhibitor gene; *CD44*, cell adhesion molecule gene; hML, human mut-L homolog gene *h*MLH; hTER, human telomerase reverse transcriptase gene *h*TERT; *p16*, cyclin-dependent kinase inhibitor gene;.

DAPK, death-associated protein kinase 1 gene *DAPK1*; CAL, calcitonin gene-related polypeptide α gene *CALCA*; *ECAD*, E-cadherin gene; *ER*, estrogen receptor gene; TIMP, tissue inhibitor of metalloproteinase 3 gene *TIMP3*; Adeno, adenocarcinoma, SCC, squamous cell carcinoma.

which was consistent with no methylation in the polyacrylamide gel oligo array assay.

## DISCUSSION

A low capacity for immobilizing DNA is the common shortcoming that blocks the development of microarray-based techniques for DNA methylation analysis.[34–37] Hou et al.[38] tried to enrich the targets by using controlled pore glass beads to increase hybridization signals, but the signals were not high enough. Polyacrylamide gel arrays could provide high immobilizing capacity and a solution-like environment in which hybridization and other processes resemble a homogeneous liquid phase reaction rather than a heterogeneous, liquid-solid interface reaction. TEMED was introduced to ensure the consistency of sample concentrations, viscosity, and frequency of immobilizing after spotting. The ssDNA that is immobilized from direct PCR products could satisfy the analysis requirement. However, successful linker-PCR was necessary for detecting DNA methylation, and concentrated or precipitated PCR products using ethanol sometimes may help the assay produce a good result. In the current study, applying electrophoresis instead of the conventional washing in the posthybridization procedure effectively removed the nonbinding, labeled compounds and reinforced the capability for detecting the single nucleotide mismatch. Because of the large capacity of the immobilizing ssDNA, DNA methylation detection could be useful on a genomic scale.

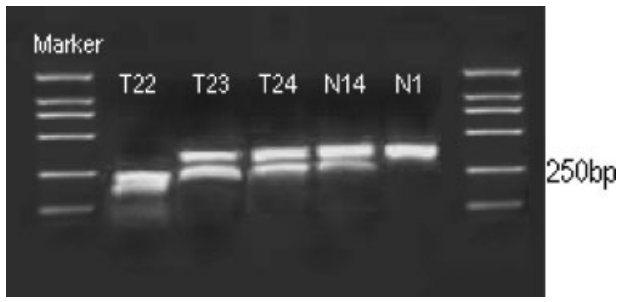



**FIGURE 7.** *p16* Methylation analysis of genomic DNA for 5 selected samples by combined bisulfite restriction analysis. Restriction patterns are shown that were obtained with the 330 base pair (bp) amplicon of the *p16* promoter. Partial digestion was observed with DNA of tumor samples T23 and T24 and normal tissue sample N14. Analysis of T22 revealed a very weak unmethylated band that resulted from cleavage and, thus, could be considered densely methylated. Although the polymerase chain reaction amplicon generated from sample N1 was completely undigested, it had no methylation.

It was reported previously that genes were inactivated in lung cancers by aberrant methylation. In the current report, the frequency of methylation of 15 genes was described along with the correlation of these methylation changes with clinicopathologic characteristics and molecular abnormalities. The methylation of *TIMP-3*, *p*16INK4a, *DAPK1*, *APC*, *CDH13*, *RASSF1*, *p15*, *ECAD* has been described previously in lung cancer cell lines and in a small number of primary lung tumors. Our results were similar to the previously reported data.[9,10,39] Moreover, DNA methylation has been detected in other interrogated genes. However, we observed CpG hypermethylation in the genes *CHD13*, *RASSF1*, *APC*, *h*MLH, *h*TERT, *p*16INK4a, *DAPK1*, *CALCA*, and *ECAD* in some of the corresponding, matched, nonmalignant lung tissues. When we compared the specific genes that were methylated in tumor samples and nonmalignant tissue samples from the same patient (Fig. 5) (Table 2), we observed that not all genes that were methylated in the nonmalignant tissues were methylated in the corresponding tumors from the same patients. We cannot exclude the possibility that this may have been caused in some samples by contamination from adjacent malignant cells. However, the possibility that methylated alleles in the nonmalignant lung samples represented premalignant changes may be the main explanation. Sabine et al.[9] also discussed this possibility in their report.

The association between methylation of these interrogated genes in NSCLC and clinicopathologic parameters in these patients was important for understanding the pathogenesis of the disease. To our knowledge, methylation of *RASSF1*, *CD44*, *ER*, *h*MLH, *h*TERT, *CALCA*, *BRCA1*, *IGFBP7*, and *p15* has not been reported previously in lung cancer. According to our results, methylation most of these genes them had no significant relation to clinicopathologic parameters of the patients. Methylation of *ER* was observed more frequently in women, whereas methylation of *APC*, *TIMP-3*, and *p*16INK4a was more common in men. The reason for this difference between the sexes most likely was attributable to the greater frequency of smoking among men than among women in China. The difference was significant compared with that reported by Sabine et al.,[9] with more frequent methylation of *TIMP-3* observed among women. Methylation of *p*16INK4a was more frequent in squamous cell carcinomas than in adenocarcinomas. The worse prognosis and higher rate of metastasis in patients with adenocarcinomas than in patients with squamous cell carcinomas may have been connected to the hypermethylation of *p*16INK4a. All of the results described above demonstrate that the methylation of certain genes may be associated with some clinicopathologic patient characteristics. These clinical correlations need to be confirmed in other independent studies. It was reported[40] that longer survival was associated with *ECAD* methylation in breast cancers. Because of the lack of the survival data in our studies, we could not calculate whether there were similar results in lung cancer. However, in the current study, we observed that *ECAD* methylation differed significantly in adenosquamous cell carcinoma.

In conclusion, polyacrylamide gel microarray coupled with linker-PCR was a simple, robust, and low-cost method for detecting CpG island methylation in a set of samples. It was a fast and highly sensitive measurement for detecting CpG island methylation. The results from our study of methylation in *CD44*, *IGFBP7*, *p15*, *RASSF1*, *BRCA1*, *ER*, *CALCA*, *ECAD*, *CDH13*, *h*TERT, *DAPK1*, *TIMP-3*, *h*MLH, *p*16INK4a, and *APC* stress the high frequency of methylation in primary resected NSCLCs and demonstrate that methylation may be the most common mechanism for inactivating cancer-related genes in NSCLC.

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