ORIGINAL STUDY

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Concomitant hypermethylation of multiple genes in non-small cell lung cancer (NSCLC)

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Abstract: Primary lung cancer remains the leading cause of cancer death worldwide. Promoter hypermethylation is a major inactivation mechanism of tumor-related genes, and increasingly appears to play an important role in carcinogenesis. In the present study, we used quantitative methylation-specific PCR (Q-MSP) assays to analyze promoter hypermethylation of nine genes in a large cohort of well-characterized non-small cell lung cancer (NSCLC) and explore their associations with the clinicopathological features of tumor. We found that there were significant differences in methylation levels for six of nine gene promoters between cancerous and noncancerous lung tissues. More importantly, with 100% diagnostic specificity, high sensitivity, ranging from 44.9% to 84.1%, was found for each of the nine genes. Interestingly, promoter hypermethylation of most genes was closely associated with histologic type, which was more frequent in squamous cell carcinomas (SCC) than in adenocarcinomas (ADC). In addition, highly frequent concomitant methylation of multiple genes was found in NSCLC, particularly in SCC. Our data showed that multiple genes were aberrantly methylated in lung tumorigenesis, and that they were closely associated with certain clinicopathological features of NSCLC, particularly of the histologic type, suggesting that these hypermethylated genes could be potential biomarkers in early detection of NSCLC in high-risk individuals, as well as in evaluating the prognosis of NSCLC patients. (*Folia Histochemica et Cytobiologica 2011, Vol. 49, No. 1, 132–141*)

Key words: DNA methylation, multiple genes, quantitative methylation-specific PCR (Q-MSP), non-small cell lung cancer (NSCLC)

Introduction

Worldwide, lung cancer kills more than one million people each year, and as the leading cause of death in men, and the second leading cause in women, it is a major health problem [1, 2]. Lung cancer is clinically classified into two major histological categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The former is the most aggressive subtype, accounting for 10–15% of all cases. NCLSC accounts for approximately 85–90% of lung cancers [2]. Despite surgery, approximately 30–40% of patients with NSCLC who have discrete lesions and histologically negative lymph nodes die of recurrent disease [3]. One reason for this problem is that this disease has proven difficult to diagnose early and treat successfully. In fact, many imaging and cytology-based strategies have been employed to achieve early detection of lung cancer and identified high-risk individuals. Although some of these strategies are indeed sensitive , none has been demonstrated to reduce lung cancer mortality. Fortunately, specific molecular markers would provide an alternative approach to complement imaging techniques for the early diagnosis of lung cancer. Among them, DNA methylation has emerged as a highly promising biomarker, and it is being actively studied in multiple cancers.

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Hypermethylation of gene promoter is an important mechanism of gene inactivation and acts as an alternative to mutational events, such as gene mutations and intragenic deletions, in oncogenesis [4, 5]. Hypermethylation of gene promoter is the earliest and commonest molecular event in human cancers, including NSCLC [6, 7], indicating that loss of gene transcription mediated by methylation events is recognized now as a crucial component in the initiation and progression of lung cancer. Taken together, hypermethylation of gene promoter may be an important biomarker for the early detection of lung cancer.

In the present study, we determined the DNA methylation profiles of nine genes using quantitative methylation-specific PCR (Q-MSP) in a cohort of clinically well-characterized NSCLC samples. The selected genes play important roles in T- and B-cell regulation in certain malignancies (CALCA), cell adhesion and/or invasion and/or metastasis (CDH1), apoptosis (DAPK1), development (EVX2 and IRX2), cell proliferation and/or differentiation (PAX6, RARβ, and TIMP3), and cell cycle control (RASSF1A).

The aim of this study was to identify a panel of genes that differentiate cancerous tissues from noncancerous tissues as excellent biomarkers for early detection and prognostic evaluation of lung cancer, and explore their associations with known or suspected cancer risk factors.

Material and methods

Clinical samples

With institutional approval, a series of 96 paraffin-embedded primary NSCLC samples and 15 noncancerous lung samples were randomly selected at the First Affiliated Hospital of China Medical University, People's Republic of China. None of these patients had received chemotherapy or radiotherapy before the surgery. Informed consent was obtained from each patient before the surgery. The histopathological diagnosis and subtype classification of NSCLC samples were examined and agreed upon by an experienced pathologist at the Department of Pathology of the Hospital. As shown in Table 1, the mean age of all NSCLC cases was 58.9 years. Those aged > 60 years accounted for 42%. There were more males than females (69% vs. 31%). Sixty-nine percent of patients had a smoking history. Ninety percent of NSCLC cases had surgical stage I and II disease and 84% had tumors < 5 cm. By histology, 69% were squamous cell carcinomas (SCC), the remaining 31% being adenocarcinomas (ADC). Cases with lymph node metastasis, pleural indentation and invasion or adhesion were 26/96 (27%), 21/96 (22%), and 31/96 (32%), respectively.

Table 1. Baseline patient and tumor characteristics of NSCLC cases

Characteristics	No. of patients (%)
Gender	
Male	66 (68.8)
Female	30 (31.2)
Age (mean years ± SD)	58.9 ± 9.2
≤ 60	56 (58.3)
> 60	40 (41.7)
Smoking history (pack-years)	
0	30 (31.3)
1–39	35 (36.5)
≥ 40	31 (32.2)
Tumor size (mean cm ± SD)	3.9 ± 1.7
1–3	37 (38.5)
3–5	44 (45.8)
> 5	15 (15.7)
Histologic type	
Adenocarcinoma	30 (31.3)
Bronchoalveolar cell (BAC)	6 (6.3)
Adenocarcinoma (non-BAC)	24 (25)
Squamous	66 (68.7)
Histologic stage	
I	54 (56.3)
II	32 (33.3)
III	10 (10.4)
Lymph node metastasis	
No	70 (72.9)
Yes	26 (27.1)
Pleural indentation	
No	75 (78.1)
Yes	21 (21.9)
Invasion or adhesion	
No	65 (67.7)
Yes	31 (32.3)

DNA isolation

Paraffin-embedded NSCLC samples were treated and DNA was isolated as previously described [8]. Briefly, after an eight-hour treatment at room temperature with xylene to remove paraffin, samples were incubated with 1% sodium dodecyl sulfate (SDS) and 0.5 mg/ml proteinase K at 48°C for 48 hours to digest tissues. A few mid-interval spiking aliquots of concentrated proteinase K facilitated digestion. DNA was subsequently isolated by standard phenol-chloroform extraction and ethanol precipitation.

Sodium bisulfite treatment

Genomic DNA from the primary tumors and non-cancerous lung samples was subjected to treatment of sodium bisulfite, as described previously [9]. Briefly, a final volume of 20 μl of H$_2$O containing ~2 μg genomic DNA, 10 μg salmon sperm DNA, and 0.3M NaOH was incubated at 50°C for 20 minutes to denature the DNA. The mixture was then incubated for 2–3 hours at 70°C in 500 μl of a freshly prepared solution containing 3M sodium bisulfite (Sigma, St. Louis, MO, USA), and 10 mM hydroquinone (Sigma, St. Louis, MO, USA). DNA was subsequently recovered by a Wizard DNA Clean-Up System (Promega Corp., Madison, WI, USA) following the instructions of the manufacturer, followed by ethanol precipitation, and re-suspension in 30 μl of deionized H$_2$O. After bisulfite treatment, all unmethylated cytosine residues converted to uracil, whereas the methylated cytosine residues remained unchanged. Bisulfite-treated DNA samples were stored at –80°C until use.

Quantitative methylation-specific PCR (Q-MSP) assay

The methylation status was determined by the fluorescence-based quantitative PCR assay as described previously [9]. Briefly, the Q-MSP assay was carried out in triplicate for each sample in a final reaction mixture of 20 μl containing 3 μl bisulfite-treated DNA, 600 nM each primer, 200 nM Taq-Man probe, 5.5 mM MgCl2, 1 U platinum Taq polymerase, 200 μM each of deoxyguanosine triphosphate, and 2% Rox reference. After an initial denaturation step at 95°C for two minutes, 40 cycles of 15 seconds at 95°C and one minute at 60°C for annealing and extension were run using a CFX96 Real-Time PCR Detection System (Bio-Rad, CA, USA). Normal leukocyte DNA was methylated *in vitro* with Sss I methylase (New England Biolabs, Beverly, MA, USA) to generate completely methylated DNA as a positive control. Each plate contained triplicate samples, multiple water blanks, as well as negative unmethylated controls and serial dilutions of positive methylated control to construct the standard curve. The internal reference gene β-actin was used to normalize the amount of input DNA. The primers and TaqMan probes used in the present study were designed using Methyl Primer Express Software v1.0 (Applied Biosystems) and presented in Table 2. The relative methylation level of each sample was calculated using the method described previously [9].

Table 2. Q-MSP primer and TaqMan probe sequences used in the present study

Genes	Forward primer sequence (5' → 3')	Probe sequence (5' → 3')	Reverse primer sequence (5' → 3')
CALCA	GTTTTGGAAGTATGAGGGTGACG	6FAM-ATTCCGCCAATACACAACAACCAATAAACG-TAMRA	TTCCCGCCGCTATAAATCG
CDH1	AATTTTAGGTTAGAGGGTTATCGCGT	6FAM-CGCCCACCCGACCTCGCAT-TAMRA	TCCCCAAAACGAAACTAACGAC
DAPK1	GGATAGTCGGATCGAGTTAACGTC	6FAM-TTCGGTAATTCGTAGCGGTAGGGTTTGG-TAMRA	CCCTCCCAAACGCCGA
EVX2	TCGTTGGCGGGTGGGTATAG	6FAM-CTTCACTCCAAACGCGTCTCCATCTCCCG-TAMRA	ACGCCGATAACAACCATTTTAACG
IRX2	GCGGGTCGTTTAGGTTAGTATTCG	6FAM-CCCTCCATCCACGCCCGACCGAAA-TAMRA	CGCCGAACAACGAACAAAACG
PAX6	ATATAGGACGGCGGGTTTAGGTTG	6FAM-CCCAAAATCCGACCGACTCCAACCCCTA-TAMRA	TTCCGACCGAACGAAAACCTAC
RARβ	GGGATTAGAATTTTTATGCGAGTTGT	6FAM-TGTCGAGAACGCGAGCGATTCG-TAMRA	TACCCCGACGATACCCAAAC
RASSF1A	GCGTTGAAGTCGGGGTTC	6FAM-ACAAACGCGAACCGAACGAAACCA-TAMRA	CCCGTACTTCGCTAACTTTAAACG
TIMP3	GCGTCGGAGGTTAAGGTTGTT	6FAM-AACTCGCTCGCCCGCCGAA-TAMRA	CTCTCCAAAATTACCGTACGCG
β-Actin	TGGTGATGGAGGAGGTTTAGTAAGT	6FAM-ACCACCACCCAACACACAATAACAAACACA-TAMRA	AACCAATAAAACCTACTCCTCCCTTAA

Data analysis

A positive gene methylation was defined when the ratio was above a certain cut-off value. The relative methylation level varied substantially among the nine genes and the cut-off points were thus studied for each gene individually. To determine cut-off value of each gene, we construct receiver operating characteristic (ROC) curves using MedCalc software (MedCalc, Belgium). The area under the ROC curve is a measure of the ability of a continuous marker to accurately classify cancerous and noncancerous tissue. Such a curve is a plot of sensitivity vs. 100 minus specificity values associated with all dichotomous markers that can be formed by varying the cut-off values used to define a marker 'positive gene methylation'. Logistic regression models were used to examine the association between gene methylation and the clinicopathological features of cases using the MedCalc software (MedCalc, Belgium). First, univariate models were examined, then multivariate models were developed that adjusted for the most important covariates, including age, pack-years, histologic type, and histologic stage.

Associations were expressed as odds ratios and their corresponding 95% confidence intervals (CI). Analyses were conducted to assess the association with NSCLC for each gene separately, then for concomitant hypermethylation of multiple genes. $p < 0.05$ was considered to be statistically significant. All statistical analyses were performed using the SPSS statistical package v11.5 (Chicago, IL, USA).

Results

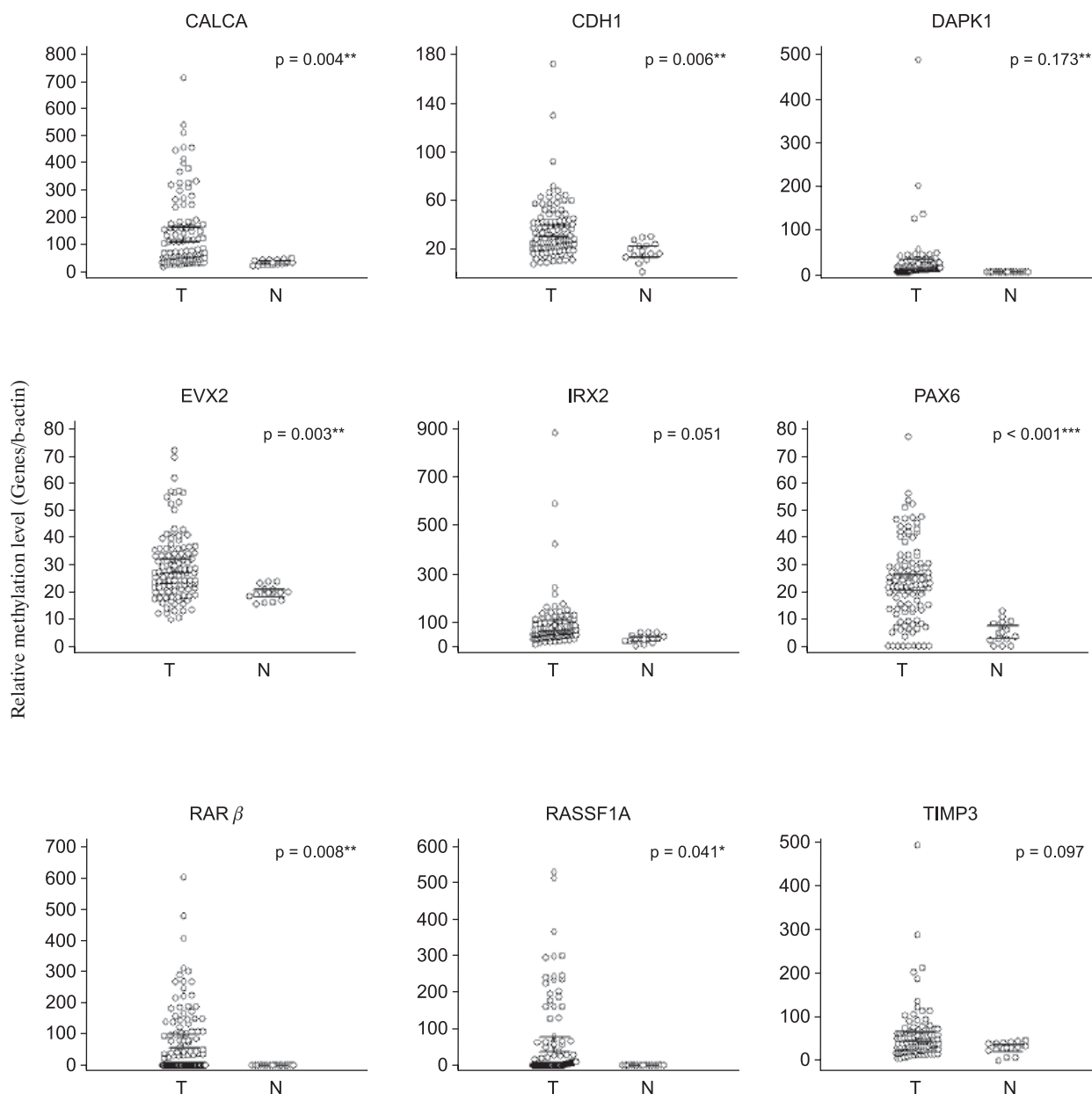### Frequent promoter hypermethylation in NSCLC

The methylation profiles of nine genes were assessed in their promoters using Q-MSP in a cohort of NSCLC samples, including CALCA, CDH1, DAPK1, EVX2, IRX2, PAX6, RARβ, RASSF1A, and TIMP3. As shown in Figure 1, the overall methylation level of each of the nine genes was higher in cancerous tissues than in noncancerous lung tissues. Among them, significant differences were found in CALCA ($p < 0.01$), CDH1 ($p < 0.01$), EVX2 ($p < 0.01$), PAX6 ($p < 0.01$), RARβ ($p < 0.01$), and RASSF1A ($p < 0.05$). The cut-off values for each gene were calculated using ROC curves to differentiate NSCLC from noncancerous lung tissues, respectively, and determine diagnostic sensitivity and specificity. As shown in Figure 2, with more weight toward specificity than sensitivity, 100% diagnostic specificity was obtained for all of these genes. The sensitivity of each individual gene

was 69.2% for CALCA, 45.8% for CDH1, 84.1% for DAPK1, 63.6% for EVX2, 52.3% for IRX2, 75.7% for PAX6, 54.2% for RARβ, 72.9% for RASSF1A, and 44.9% for TIMP3.

Association of promoter hypermethylation with clinicopathologic features of NSCLC

Among all clinicopathologic features, methylation levels varied substantially by histologic type (Table 3). Methylation level of most genes was higher in SCC than in ADC, including CALCA, CDH1, DAPK1, EVX2, IRX2, RARβ, and TIMP3, particularly in DAPK1 ($p < 0.01$) and TIMP3 ($p < 0.01$) (Table 3). Conversely, methylation level of RASSF1A was much higher in ADC than in SCC (Table 3), although no significant difference was observed ($p = 0.08$). There was no significant association between gene methylation and age (Table 3). Among all hypermethylated genes, methylation level of CALCA was only found to be significantly associated with smoking history ($p < 0.05$) (Table 3). Methylation level of all of these genes was not significantly associated with invasion and adhesion (Table 3). Table 3 also shows that there was a significantly lower methylation level of RASSF1A in the lymph node metastasis group than in the nonmetastasis group ($p < 0.05$). Moreover, methylation level of CALCA was found to be significantly associated with histologic stage ($p < 0.05$) (Table 3). However, no association was found between promoter hypermethylation and other clinocopathologic features, including gender, quantity of cigarette smoking (pack-years), tumor size, or pleural indentation (data not shown).

In univariate analyses, hypermethylation of most genes was more frequent in SCC compared to ADC, particularly in CDH1 (OR = 2.63, 95% CI = 1.05––6.60), DAPK1 (OR = 6.64, 95% CI = 1.85–23.8), IRX2 (OR = 2.89, 95% CI = 1.17–7.13), and TIMP3 (OR = 2.51, 95% CI = 1.01–6.21) (Table 4). Conversely, hypermethylation of RASSF1A was significantly frequent in ADC (OR = 0.31, 95% CI = 0.09––0.99) in the present study (Table 4). In addition, hypermethylation of CALCA (OR = 2.13, 95% CI = = 1.01–4.47) and TIMP3 (OR = 1.88, 95% CI = 1.01––3.49) was significantly associated with histologic stage (Table 4). Of note, hypermethylation of RARβ was negatively associated with smoking history (OR = = 0.50, 95% CI = 0.20–1.23), quantity of cigarette smoking (OR = 0.60, 95% CI = 0.36–1.01) and pleural indentation (OR = 0.41, 95% CI = 0.15–1.11) in NSCLC, although no statistical significance was noted (Table 4). Additionally, hypermethylation of RASSF1A (OR = 0.47, 95% CI = 0.24–0.90) was significantly negatively associated with histologic stage

Figure 1. Distribution patterns of promoter hypermethylation of the nine genes in NSCLC. Quantitative methylation-specific PCR (Q-MSP) assay was performed as described in Material and methods. The relative methylation level (on Y axis) is represented by ratios of candidate gene/β-actin × 1,000, except for EVX2 and PAX6/β-actin × 100. Horizontal lines indicate a 95% normal confidence interval for the sample mean. T — cancerous tissues; N — noncancerous lung tissues. *p < 0.05; **p < 0.01; ***p < 0.001

(Table 4). Similarly, hypermethylation of RASSF1A was also negatively associated with smoking history (OR = 0.58, 95% CI = 0.20–1.62), lymph node metastasis (OR = 0.47, 95% CI = 0.18–1.25), and invasion or adhesion (OR = 0.44, 95% CI = 0.17–1.11), respectively, although these associations did not reach statistical difference (Table 4). In order to assess the independent associations between gene methylation and age, pack-years, histologic type, and histologic stage, we conducted multiple multivariable logistic regressions (Table 5).

In multivariable analyses adjusting for the potential false discovery rate associated with multiple comparisons (nine different models), hypermethylation of CDH1 (OR = 3.14, 95% CI = 1.14–8.65), DAPK1 (OR = 8.07, 95% CI = 1.75–37.2), and IRX2 (OR = 3.54, 95% CI = 1.29–9.73) remained associated with histologic type, which was significantly more likely in SCC than in ADC (Table 5). However, after adjustment, there were no significant differences between hypermethylation of RASSF1A and TIMP3 and histologic type (Table 5). Interestingly,
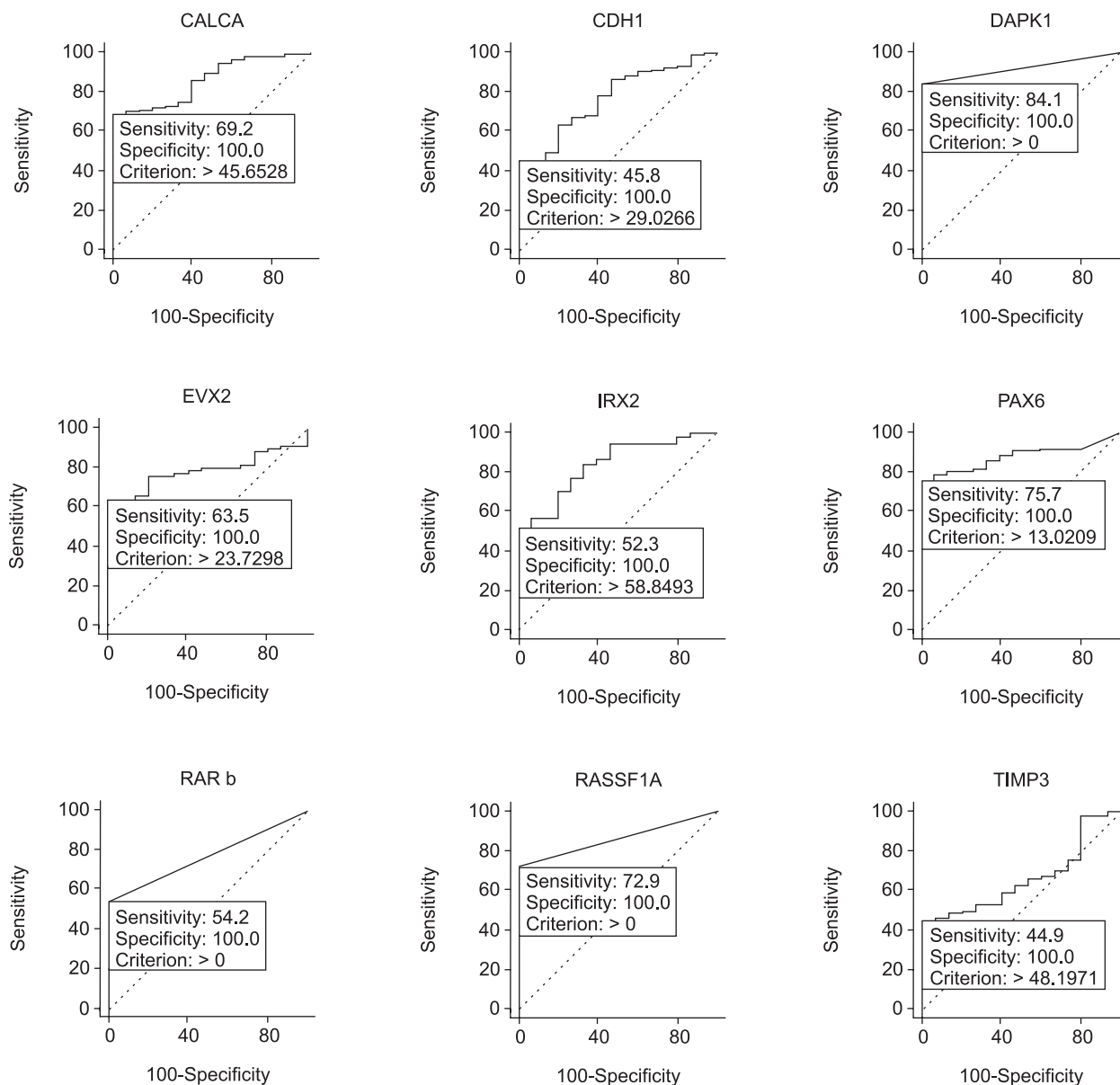
Figure 2. Receiver operating characteristic (ROC) curves for the nine genes in NSCLC. Complete DNA methylation data from all cancerous and noncancerous lung tissues were used for the analyses. The ROC curves plot sensitivity vs. 100%-specificity. The cut-off values for each of nine genes were determined based on ROC curves individually

hypermethylation of RARβ remained negatively associated with quantity of cigarette smoking after multivariable adjustment, and a statistical difference was noted (OR = 0.58, 95% CI = 0.34–0.99) (Table 5). Moreover, multivariable analyses indicated that no significant differences were found between hypermethylation of CALCA and TIMP3 and histologic stage (Table 5). However, hypermethylation of RASSF1A (OR = 0.55, 95% CI = 0.27–1.10) remained negatively associated with histologic type (Table 5), although this association did not reach statistical difference.

Concomitant hypermethylation of multiple genes and their associations with clinocopathologic features in NSCLC

Field cancerization effects in NSCLC will probably dictate the development of a gene panel for early detection of NSCLC that focuses on assessing the prevalence for promoter hypermethylation of multiple genes in individual specimens. In the present study, we identified a panel of the nine hypermethylated genes with 100% diagnostic specificity in NSCLC, and further analyzed methylation status of these genes in indivi-

Table 3. Correlation between methylation level of gene promoter and clinicopathological features of NSCLC [Median (P25, P75)]

Gene	Age (n = 96)				Smoking history (n = 96)				Histologic type (n = 96)			
	> 60 (n = 40)	≤ 60 (n = 56)	Z	P	Y (n = 66)	N (n = 30)	Z	P	SSC (n = 66)	ADC (n = 30)	Z	P
CALCA	77.5 (41.2, 183.7)	66.0 (39.4, 155.0)	0.845	0.472	107.2 (41.5, 197.8)	54.7 (35.7, 94.5)	1.486	0.024*	76.2 (46.4, 192.7)	53.1 (35.5, 158.3)	1.349	0.053
CDH1	25.0 (19.3, 38.6)	27.7 (20.0, 42.3)	0.725	0.670	27.1 (19.9, 41.4)	25.7 (18.5, 45.1)	0.716	0.685	31.9 (21.1, 42.9)	21.2 (14.4, 35.5)	1.349	0.053
DAPK1	7.7 (5.7, 15.8)	7.1 (3.1, 13.8)	1.001	0.269	7.3 (4.7, 13.8)	8.2 (2.5, 23.9)	0.812	0.525	8.8 (6.0, 17.2)	4.9 (0.00, 7.7)	1.872	0.002*
EVX2	28.4 (22.5, 36.4)	25.7 (21.0, 35.3)	1.035	0.234	28.2 (21.7, 36.1)	25.4 (19.1, 35.7)	0.771	0.593	29.2 (22.4, 36.8)	23.9 (19.8, 29.5)	1.294	0.07
IRX2	52.7 (41.6, 89.8)	62.6 (40.4, 102.2)	0.742	0.641	61.1 (41.1, 89.0)	59.0 (36.7, 99.1)	0.688	0.731	62.9 (43.9, 101.9)	49.1 (28.8, 73.7)	1.211	0.106
PAX6	25.8 (19.4, 42.0)	21.0 (11.8, 28.6)	1.208	0.108	23.8 (13.4, 31.7)	21.8 (14.7, 27.8)	0.743	0.639	22.5 (13.4, 32.0)	22.4 (15.7, 29.5)	0.592	0.875
RARβ	27.3 (0.00, 105.0)	37.5 (0.00, 136.4)	0.742	0.641	12.8 (0.00, 149.0)	43.6 (0.00, 108.2)	0.950	0.328	31.2 (0.00, 138.2)	34.5 (0.00, 124.2)	0.509	0.958
RASSF1A	2.31 (0.00, 59.1)	3.38 (0.03, 52.3)	0.604	0.859	1.8 (0.00, 52.9)	7.5 (0.5, 54.0)	1.032	0.237	1.49 (0.00, 33.2)	9.01 (1.2, 138.6)	1.266	0.08
TIMP3	43.6 (24.1, 70.1)	39.0 (22.8, 68.0)	0.587	0.882	40.1 (24.7, 70.6)	43.4 (20.2, 64.7)	0.482	0.974	49.1 (27.0, 72.3)	28.1 (15.7, 59.7)	1.638	0.009*

Gene	Lymph node metastasis (n = 96)				Invasion or adhesion (n = 96)				Histologic stage (n = 96)			
	Y (n = 26)	N (n = 70)	Z	P	Y (n = 31)	N (n = 65)	Z	P	≥ III (n = 10)	< III (n = 86)	Z	P
CALCA	95.2 (53.5, 263.8)	65.9 (38.8, 158.3)	1.077	0.197	109.6 (56.1, 292.9)	63.7 (36.3, 158.3)	1.005	0.265	168.5 (105.3, 258.5)	65.1 (38.0, 158.3)	1.420	0.035*
CDH1	27.9 (19.9, 40.9)	26.1 (18.9, 42.8)	0.679	0.745	26.1 (19.9, 40.4)	26.4 (19.7, 42.3)	0.600	0.864	35.3 (22.3, 41.3)	26.0 (19.8, 41.8)	0.738	0.648
DAPK1	8.6 (6.2, 20.0)	7.4 (3.5, 14.0)	0.967	0.308	7.7 (4.6, 12.3)	7.5 (3.8, 15.9)	0.496	0.967	8.6 (6.5, 14.1)	7.5 (4.0, 15.4)	0.988	0.283
EVX2	30.3 (21.6, 36.2)	26.3 (21.5, 34.6)	0.919	0.367	26.9 (22.5, 35.7)	28.3 (21.0, 36.2)	0.784	0.570	29.0 (25.3, 36.1)	27.0 (21.2, 35.9)	0.954	0.323
IRX2	62.4 (40.4, 83.7)	59.0 (41.0, 101.4)	0.632	0.820	57.3 (39.7, 100.8)	61.3 (41.1, 89.4)	0.566	0.906	62.1 (44.0, 101.7)	59.0 (40.7, 87.5)	0.501	0.963
PAX6	23.0 (12.5, 35.2)	22.5 (13.9, 29.7)	0.699	0.714	23.4 (14.0, 31.6)	22.1 (12.1, 30.3)	0.607	0.855	24.4 (17.9, 33.8)	22.3 (12.7, 30.4)	0.800	0.543
RARβ	33.6 (0.00, 185.5)	32.4 (0.00, 108.2)	0.842	0.477	38.7 (0.00, 184.5)	32.2 (0.00, 108.4)	1.055	0.216	14.6 (0.00, 185.6)	34.5 (0.00, 124.2)	0.515	0.953
RASSF1A	0.9 (0.00, 11.0)	6.7 (0.4, 63.9)	1.459	0.028*	1.2 (0.00, 25.8)	3.5 (0.3, 60.7)	0.807	0.533	0.00 (0.00, 21.5)	33.4 (0.2, 58.0)	1.100	0.178
TIMP3	52.1 (26.2, 73.3)	39.4 (21.2, 64.0)	0.895	0.400	34.3 (23.0, 71.9)	44.5 (24.5, 69.5)	0.714	0.688	43.5 (24.9, 69.1)	40.4 (22.9, 70.0)	0.661	0.774

*Significant at p < 0.05

Table 4. Promoter hypermethylation of individual genes in NSCLC — univariate associations with clinicopathological features (OR[†] and 95% CI)

Genes	Male vs. female	Age (per 10 years)	Smoking history	Pack-years[1]	SSC vs. ADC
CALCA	0.73 (0.28–1.90)	0.94 (0.58–1.52)	1.43 (0.57–3.57)	0.88 (0.51–1.51)	2.21 (0.89–5.47)
CDH1	2.13 (0.86–5.22)	0.83 (0.53–1.30)	1.41 (0.59–3.39)	0.91 (0.55–1.50)	2.63 (1.05–6.60)*
DAPK1	1.45 (0.43–4.87)	1.16 (0.60–2.24)	2.11 (0.64–6.92)	1.83 (0.84–3.98)	6.64 (1.85–23.8)*
EVX2	0.94 (0.38–2.33)	1.12 (0.70–1.80)	1.16 (0.47–2.85)	0.82 (0.48–1.41)	2.16 (0.89–5.29)
IRX2	1.29 (0.54–3.06)	0.76 (0.48–1.19)	1.06 (0.45–2.52)	0.91 (0.55–1.50)	2.89 (1.17–7.13)*
PAX6	1.04 (0.37–2.88)	1.19 (0.70–2.03)	0.78 (0.27–2.25)	0.93 (0.51–1.69)	1.04 (0.37–2.88)
RARβ	0.75 (0.31–1.81)	1.13 (0.72–1.77)	0.50 (0.20–1.23)	0.60 (0.36–1.01)	0.92 (0.39–2.19)
RASSF1A	0.75 (0.28–2.05)	0.98 (0.59–1.62)	0.58 (0.20–1.62)	0.73 (0.41–1.30)	0.31 (0.09–0.99)*
TIMP3	1.09 (0.46–2.60)	1.09 (0.70–1.71)	1.09 (0.46–2.60)	1.27 (0.76–2.11)	2.51 (1.01–6.21)*
Genes	**Tumor size[2]**	**Lymph node metastasis**	**Pleural indentation**	**Invasion or adhesion**	**Histologic stage[3]**
CALCA	1.01 (0.55–1.88)	1.74 (0.62–4.91)	1.18 (0.41–3.41)	2.44 (0.88–6.79)	2.13 (1.01–4.47)*
CDH1	1.20 (0.67–2.13)	1.02 (0.41–2.51)	1.79 (0.67–4.76)	0.96 (0.41–2.27)	1.11 (0.62–2.02)
DAPK1	1.81 (0.72–4.54)	5.17 (0.64–42.0)	1.63 (0.33–8.02)	1.70 (0.43–6.67)	1.24 (0.50–3.08)
EVX2	0.79 (0.43–1.43)	1.25 (0.48–3.28)	0.81 (0.30–2.21)	1.43 (0.57–3.61)	1.51 (0.78–2.93)
IRX2	1.57 (0.88–2.83)	1.17 (0.47–2.88)	1.37 (0.52–3.63)	0.86 (0.36–2.01)	1.14 (0.63–2.07)
PAX6	0.78 (0.40–1.54)	0.74 (0.26–2.09)	2.04 (0.54–7.69)	1.84 (0.61–5.56)	1.76 (0.79–3.93)
RARβ	0.78 (0.44–1.39)	1.43 (0.57–3.58)	0.41 (0.15–1.11)	1.44 (0.60–3.45)	0.94 (0.52–1.70)
RASSF1A	1.12 (0.59–2.14)	0.47 (0.18–1.25)	1.76 (0.53–5.84)	0.44 (0.17–1.11)	0.47 (0.24–0.90)*
TIMP3	1.67 (0.92–3.01)	2.05 (0.82–5.10)	1.16 (0.44–3.05)	0.69 (0.29–1.66)	1.88 (1.01–3.49)*

[†]OR — odds ratio with 95% confidence interval; *Significant at $p < 0.05$; [1]Pack-years (0; > 0 and < 40; ≥ 40); [2]Tumor size (>1 and ≤ 3; > 3 and ≤ 5; > 5); [3]Histologic stage (I, II, III)

Table 5. Promoter hypermethylation of individual genes in NSCLC — multivariable models assessing age, pack-years, histologic type, and histologic stage (OR[†] and 95% CI)

Genes	Age[1]	Pack-years[2]	SSC vs. ADC	Histologic stage[3]
CALCA	0.97 (0.59–1.59)	0.76 (0.42–1.35)	1.87 (0.70–5.01)	1.94 (0.87–4.31)
CDH1	0.81 (0.51–1.29)	0.81 (0.47–1.39)	3.14 (1.14–8.65)*	0.87 (0.45–1.65)
DAPK1	1.10 (0.55–2.20)	1.53 (0.67–3.48)	8.07 (1.75–37.2)*	0.60 (0.20–1.80)
EVX2	1.16 (0.71–1.88)	0.71 (0.40–1.25)	2.14 (0.80–5.72)	1.35 (0.66–2.77)
IRX2	0.74 (0.46–1.18)	0.80 (0.47–1.38)	3.54 (1.29–9.73)*	0.86 (0.44–1.65)
PAX6	1.24 (0.72–2.13)	0.88 (0.47–1.65)	0.80 (0.26–2.42)	1.93 (0.82–4.50)
RARβ	1.18 (0.74–1.88)	0.58 (0.34–0.99)*	1.11 (0.42–2.90)	0.99 (0.52–1.90)
RASSF1A	0.96 (0.57–1.65)	0.81 (0.44–1.50)	0.44 (0.13–1.54)	0.55 (0.27–1.10)
TIMP3	1.10 (0.69–1.77)	1.13 (0.66–1.93)	1.89 (0.70–5.05)	1.63 (0.85–3.13)

[†]OR — odds ratio with 95% confidence interval, adjusted for multiple comparisons; [1]Age (per 10 years); [2]Pack-years (0; > 0 and < 40; ≥ 40); [3]Histologic stage (I; II; III); *Significant at $p < 0.05$

duals. The results showed that the prevalence of concomitant methylation of five or more genes, six or more genes, seven or more genes, and eight or more genes in NSCLC were 65/96 (67.7%), 48/96 (50%), 37/96 (38.5%), and 23/96 (24%), respectively. In addition, there was a significantly increased frequency for concomitant hypermethylation of multiple genes in SCC compared to ADC (data not shown).

Discussion

Epigenetic gene silencing is a molecular mechanism of silencing a gene by methylating its promoter region. Epigenetic silencing is involved in initiation and progression of human cancer [10], including NSCLC [11, 12]. The aim of this study is to identify a panel of hypermethylated genes with high sensitivity and specificity for early detection of NSCLC and explore their associations with clinocopathologic features of cases using a Q-MSP approach. Hypermethylation of most of these genes, as a mechanism for their silencing, has been frequently reported in NSCLC [13–17]. Moreover, this study also involved three new methylation markers, EVX2, IRX2, and PAX6, which were highly specific for tumor-associated methylation, and little or no methylation was found in non-malignant lung tissue [18]. Our data showed that there were significant differences in the methylation level between cancerous and noncancerous lung tissues from six of nine genes, including CALCA, CDH1, EVX2, PAX6, RARβ, and RASSF1A (Figure 1). It is worth noting that 100% diagnostic specificity, and excellent sensitivity, ranging from 44.9% to 84.1%, was obtained for each of the nine genes (Figure 2), suggesting that these methylation markers play a role in tumor development and make them excellent candidates for early diagnosis and prognostic evaluation of NSCLC.

Our study found that promoter hypermethylation of most of the genes was strongly associated with histologic type. Among them, a number of genes were more frequently methylated in SCC than in ADC (Tables 3 and 4). Unlike what was observed in the present study, previous studies have indicated that a number of genes were more frequently methylated in ADC, not in SSC [17, 19, 20], which is new to the literature. Of note, similar to a previous study [21], the present study showed that hypermethylation of RASSF1A was negatively associated with smoking history, lymph node metastasis, invasion or adhesion. Importantly, RASSF1A was more frequently methylated in the early tumor stage (Table 4), suggesting that RASSF1A hypermethylation may be an early molecular event in the development of a lung tumor.

Our study did not find a significant association between hypermethylation of most of the genes and smoking history in NSCLC, except for CALCA gene. This accords with most studies [22–25], although tobacco smoking plays a central role in the development of lung cancer. However, several studies, as well as the present study, have reported aberrant methylation of some tumor-related genes was associated with tobacco smoking in lung cancer [19, 23, 26], such as p16 and CALCA genes. It is possible that smoking-associated lung cancer is a complex disease which involves many unique genetic and epigenetic alterations. Thus, a better understanding of the molecular mechanisms underlying this disease would undoubtedly improve the clinical outcomes of patients with NSCLC.

A very recent study showed that a number of important tumor-related genes are more frequently methylated in females compared to males in NSCLC, suggesting that certain methylation events are associated with gender [17]. However, these differences have not been consistently found in most of the previous studies [20, 27, 28]. Similarly, hypermethylation of all of these genes was also not significantly associated with gender in the present study. It is possible that the differences in the promoter hypermethylation associated with gender may be related to geographical or cultural differences, and, more importantly, differences in environmental carcinogen exposure [29–31]. The discrepant results might also have been caused by genetic differences between the study populations [32]. Moreover, it is possible that these differences are attributable to chance as a result of the relatively small cohort of study subjects. Further study with more subjects will thus be needed.

Concomitant methylation of multiple genes is common, particularly in cancerous tissues, which is strongly associated with lung cancer risk [33, 34]. In the present study, we identified a panel of the nine hypermethylated genes with excellent specificity and sensitivity, which were only methylated in cancerous tissues but not in noncancerous tissues. Among these nine genes, concomitant methylation of five or more genes was detected in 67.7% (65/96) of NSCLCs; methylation of eight or more genes was also detected in 24% (23/96) of NSCLCs, suggesting that methylation of these genes could be used as an indicator of a widespread methylation phenotype in NSCLC. Today, gene methylation in biological fluids is emerging as a biomarker that could be important for the early detection of lung cancer and monitoring prevention and intervention. Thus, our findings reinforce the use of this panel of hypermethylated genes as a biomarker for early detection and prognostic evaluation of lung cancer.

To sum up, we identified a panel of hypermethylated genes that differentiate cancerous tissues from noncancerous tissues, and demonstrated that promoter hypermethylation was strongly associated with histologic type. Importantly, highly frequent concomitant methylation of multiple genes was found in NSCLC, particularly in SCC, strongly suggesting that a methylated gene panel from sputum or plasma may have potential in noninvasively detecting NSCLC, as well as in evaluating the prognosis in post-operative NSCLC patients.

Acknowledgements

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