Promoter hypermethylation of tumor suppressor and tumor-related genes in non-small cell lung cancers

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Aberrant methylation of promoter CpG islands is known to be a major inactivation mechanism of tumor suppressor and tumor-related genes. To determine the clinicopathological significance of gene promoter methylation in non-small cell lung cancer (NSCLC), we examined the promoter methylation status of the *APC, DAP-kinase, E-cadherin, GSTP1, hMLH1, p16, RASSF1A* and *RUNX3* genes in 75 NSCLCs and corresponding non-neoplastic lung tissues by methylation-specific PCR (MSP). The frequencies of methylation in NSCLCs and corresponding non-neoplastic lung tissues were: 37% (28 of 75) and 48% (36 of 75) for *APC*, 28% (21 of 75) and 13% (10 of 75) for *DAP-kinase*, 29% (22 of 75) and 15% (11 of 75) for *E-cadherin*, 1% (1 of 75) and 0% (0 of 75) for *GSTP1*, 7% (5 of 75) and 0% (0 of 75) for *hMLH1*, 31% (23 of 75) and 0% (0 of 75) for *p16*, 43% (32 of 75) and 4% (3 of 75) for *RASSF1A*, and 20% (15 of 75) and 3% (2 of 75) for *RUNX3*, respectively. Methylation of *p16* was more frequent in squamous cell carcinomas than in adenocarcinomas ($P<0.05$), and was associated with tobacco smoking ($P<0.05$). On the contrary, methylation of *APC* and *RUNX3* was more frequent in adenocarcinomas than in squamous cell carcinomas ($P<0.05$). Thus, a different set of genes is thought to undergo promoter methylation, which leads to the development of different histologies. In addition, methylation of *p16, RASSF1A* and *RUNX3* was mostly cancer-specific ($P<0.05$), and may be utilized as a molecular diagnostic marker of NSCLCs. (Cancer Sci 2003; 94: 589–592)

Lung cancer is the leading cause of cancer deaths in the world, with over one million cases diagnosed every year.[1)] Lung cancers are divided into two major histological types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the latter consisting of adenocarcinoma (AdC), squamous cell carcinoma (SCC) and large cell carcinoma. There is a growing body of biological and epidemiological data suggesting that AdC and SCC are distinct etiological entities.[2,3)] There are no squamous cells in the normal bronchus, and SCCs are believed to arise from metaplastic cells present in the bronchi of smokers. By contrast, most AdCs may express features of Clara cells or type II pneumocytes. It is well known that the female/male occurrence ratio of AdC is significantly higher than that of SCC. SCC is more common in the central region, but AdC is more common in the peripheral region. Genetically, mutations in *p53* and *p16* are more common in SCC than AdC,[4,5)] although *K-ras* mutations are more frequent in AdC.[6)] Allelic imbalance is more frequent in SCC than AdC.[7,8)] Aberrant methylation of normally unmethylated CpG-rich areas (or islands) in or near the promoter region has been associated with transcriptional inactivation of tumor suppressor and tumor-related genes in human cancers, including NSCLC.[9–12)] Therefore, it is possible that methylation of different genes may contribute to the development of different histologies. In fact, we have recently determined that *p16* methylation is more frequent in SCC than AdC.[5)] In the present study, we extended our analysis to a number of tumor suppressor and tumor-related genes (*APC*, *DAP-kinase*, *E-cadherin*, *GSTP1*, *hMLH1*, *p16*, *RASSF1A* and *RUNX3*), which have been shown to be silenced by promoter hypermethylation in certain human malignancies, in a large number of NSCLCs. We compared the results of methylation analyses with the clinicopathological characteristics. In addition, the cancer specificity of methylation was analyzed for each gene to evaluate the possible usefulness of methylation detection as a molecular diagnostic tool.

Materials and Methods

Tissue samples and DNA extraction. A total of 75 pairs of NSCLCs and corresponding non-neoplastic lung tissues were obtained surgically. The NSCLC patients ranged in age from 39 to 86 years (average, 67.3 years). Written informed consent was obtained from all participants or their families. Tissue samples were immediately frozen and stored at −80°C until analysis. Genomic DNA was extracted using SepaGene (Sanko Junyaku Co., Tokyo).

Methylation-specific PCR (MSP). The promoter methylation status of the examined genes was determined by MSP, as described previously.[13)] DNA samples were treated with bisulfite to convert all unmethylated cytosines to uracils, while leaving methylated cytosines unaffected. Briefly, 2 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were then purified using a Wizard DNA purification resin (Promega, Madison, WI), treated with NaOH, precipitated with ethanol, and resuspended in a volume of 30 μl. Modified DNA was amplified in a total volume of 20 μl using GeneAmp PCR Gold Buffer (PE Applied Biosystems, Foster City, CA) containing 1.0 m*M* $MgCl_2$, 20 μM of each primer, 0.2 m*M* dNTPs, and 1 unit of *Taq* polymerase (Ampli*Taq* Gold DNA Polymerase, PE Applied Biosystems). After activation of the *Taq* polymerase at 95°C for 10 min, PCR was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles, each cycle consisting of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s, followed by a final 7 min extension at 72°C. A positive control and negative control (diluted water without DNA) were included for each amplification. The PCR products were then loaded onto a non-denaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination. MSP primer sequences are listed in Table 1.[14–16)] All DNA samples were checked for bisulfite modification using an unmethylated *E-cadherin* primer set.

Statistical analysis. Statistical comparisons were performed using either Fisher's exact test or a χ^2 test, as appropriate. A P value of less than 0.05 was considered statistically significant.

Results

Correlation between methylation and clinicopathological characteristics. We analyzed the correlation between gene methylation status and the clinicopathological characteristics of NSCLCs (Fig.

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Table 1. Summary of primer sequences

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing temperature (°C)	Product size (bp)
APC	TGTTTTGCGGATTTTTTT	GCAATAAAACACAAACCCCG	55	158
DAP-kinase	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCG	55	98
E-cadherin (M)	GGTGAATTTTTAGTTAATTAGCGGTAC	CATAACTAACCGAAAACGCCG	55	204
GSTP1	TTCGGGGTGTAGCGGTCGTC	GCCCCAATACTAAATCACGACG	55	91
hMLH1	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	55	115
p16	GGGTCGGAGGGGGTTTTTTC	CAACCGCCGAACGCACTCGA	55	97
RASSF1A	GTGTTAACGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA	55	93
RUNX3	ATAATAGCGGTCGTTAGGGCGTCG	GCTTCTACTTTCCCGCTTCTCGCG	55	117
E-cadherin (U)	GGTAGGTGAATTTTAGTTAATTAGTGGTA	ACCCATAACTAACCAAAAACACCA	55	211

M, methylated-sequence specific primers; U, unmethylated-sequence specific primers.

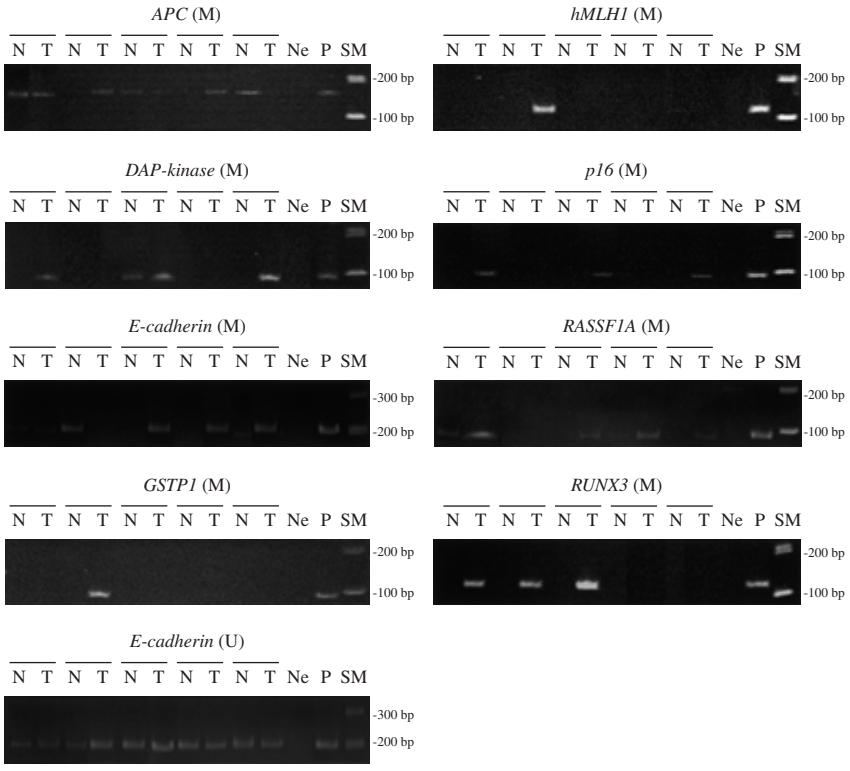

Fig. 1. Methylation analysis of eight genes in NSCLCs and the corresponding non-neoplastic lung tissues by MSP. The unmethylated form of *E-cadherin* was run as a control for DNA integrity. M, methylated-specific sequence; U, unmethylated-specific sequence; N, non-neoplastic lung tissue; T, tumor; Ne, negative control (water blanks); P, positive control; SM, size marker.

1 and Table 2). Methylation of *p16* was more frequent in SCCs than in AdCs ($P<0.05$), while *APC* and *RUNX3* methylation was more frequent in AdCs than in SCCs ($P<0.05$). Methylation of *p16* was detected more frequently in smokers than in non-smokers ($P<0.05$). The frequency of methylation became higher for the *p16*, *DAP-kinase* and *RASSF1A* genes as the Brinkman Index increased (the Brinkman Index is defined as the number of cigarettes smoked/day×years smoked), although the correlation between Brinkman Index and frequency of methylation was not statistically significant (Fig. 2).

Cancer specificity of promoter methylation. We compared the frequency of methylation between NSCLCs and corresponding non-neoplastic lung tissues for the *APC, DAP-kinase, E-cadherin, GSTP1, hMLH1, p16, RASSF1A* and *RUNX3* genes (Fig. 1 and Table 3). Methylation was significantly more frequent in NSCLCs than in the corresponding non-neoplastic lung tissues ($P<0.05$) for *DAP-kinase, E-cadherin, p16, RASSF1A* and *RUNX3*. Methylation of *p16, GSTP1* and *hMLH1* was not seen in any of the non-neoplastic lung tissues.

Discussion

We studied the promoter methylation status of the *APC, DAP-kinase, E-cadherin, GSTP1, hMLH1, p16, RASSF1A* and *RUNX3* genes in NSCLCs, and compared the results with the clinicopathological parameters. We found that *p16* methylation was more frequent in SCCs than AdCs ($P<0.05$), and was associated with tobacco smoking ($P<0.05$). *APC* and *RUNX3* methylation was more frequent in AdCs than SCCs ($P<0.05$). Kim *et al.*[17] have reported a significant association between *p16* methylation and SCCs. Toyooka *et al.*[18] have reported that methylation of *APC*, *RARβ* and *CDH13* is a feature of AdCs, and that the rates of methylation of the *p16* and *APC* genes are higher in ever-smokers compared to never-smokers. These findings indicate that differential methylation of specific genes may lead to the development of different histologies. Although it is unclear whether smoking induces DNA methylation, recent reports have indicated an association between DNA methylation and tobacco carcinogens in animal models.[19–21] Methylation of *p16, DAP-kinase* and *RASSF1A* is associated with tobacco smoking in head and neck squamous cell carcinoma.[22]

Table 2. Correlation between gene methylation and clinicopathological characteristics in NSCLCs

	Gene							
	APC	*DAP-kinase*	*E-cadherin*	*GSTP1*	*hMLH1*	*p16*	*RASSF1A*	*RUNX3*
	% methylation							
Age (years)								
<65 (*n*=23)	35	35	17	5	10	22	44	24
≥65 (*n*=52)	39	27	35	0	6	35	47	19
Sex								
Female (*n*=21)	43	29	33	0	4	19	30	12
Male (*n*=54)	35	30	28	2	8	35	48	23
Histology[1)]								
AdC (*n*=43)	47	23	33	2	5	16	42	28
SCC (*n*=29)	24 [2)]	35	24	0	10	48 [2)]	41	7 [2)]
Others (*n*=3)	33	67	33	0	0	67	67	33
TNM stage								
I (*n*=50)	38	24	24	0	4	30	46	22
II (*n*=6)	33	67	33	0	17	50	50	33
III (*n*=19)	37	32	42	5	11	26	32	11
Smoking history								
No (*n*=20)	50	25	35	0	10	10	35	25
Yes (*n*=55)	33	31	27	2	6	38 [2)]	46	18

1) AdC, adenocarcinoma; SCC, squamous cell carcinoma; Others, two large cell carcinomas and one adenosquamous cell carcinoma.
2) $P<0.05$ by χ^2 test.

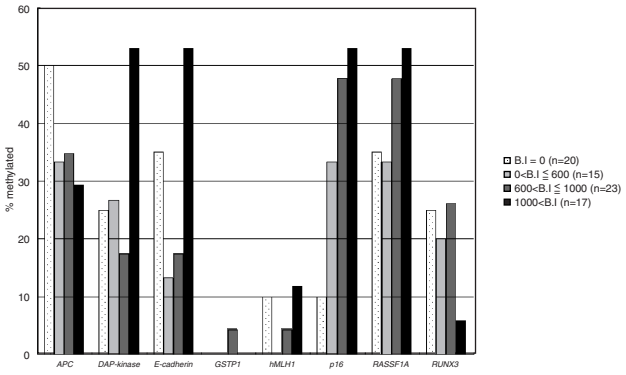

Fig. 2. Correlation between frequency of promoter methylation and Brinkman Index (B.I). Frequency of methylation became higher for the *p16*, *DAP-kinase* and *RASSF1A* genes as the B.I increased.

Table 3. Frequencies of gene methylation in NSCLCs and the corresponding non-neoplastic lung tissues

Gene	Frequency of methylation	
	Non-neoplastic lung tissue (%) (*n*=75)	Tumor (%) (*n*=75)
APC	48	37
DAP-kinase	13	28[1)]
E-cadherin	15	29[1)]
GSTP1	0	1
hMLH1	0	7
p16	0	31[1)]
RASSF1A	5	43[1)]
RUNX3	3	20[1)]

1) $P<0.05$ by χ^2 test.

In our present study, the methylation frequencies of *p16*, *DAP-kinase* and *RASSF1A* rose with increasing Brinkman Index, though histological differences were not statistically significant. Other environmental and genetic factors also influence an individual's susceptibility to gene methylation.[23, 24)]

Next, we compared the frequency of promoter methylation between NSCLCs and corresponding non-neoplastic lung tissues to evaluate the cancer specificity of gene methylation. Promoter methylation is not always cancer-specific, but is also present in non-neoplastic aging cells or tissues. For example, estrogen receptor (ER) methylation has been shown to increase during aging in normal human colonic mucosa.[25)] Methylation of *APC* and *E-cadherin* is frequently present in non-neoplastic gastric epithelia of elderly individuals,[14, 15)] while methylation of *RUNX3* and *TSLC1* is mostly cancer-specific.[26, 27)] Such differences in methylation patterns may correspond to type A (aging-specific) and type C (cancer-specific) methylation previously described for gastric and colorectal cancers.[28, 29)] However, the differences might depend on the position of the examined CpG sites.[30)] If critical CpG sites for the silencing of each gene are more precisely analyzed, age-related methylation may be cancer-specific. In the present study, methylation of the *DAP-kinase*, *E-cadherin*, *p16, RASSF1A* and *RUNX3* genes was found to be more frequent in NSCLCs than non-neoplastic lung tissues ($P<0.05$). Among them, the methylation of *p16*, *RASSF1A* and *RUNX3* in non-neoplastic lung tissues was absent or very rare, and so the methylation was regarded as a cancer-specific phenomenon. Therefore, in terms of clinical application, the detection of *p16*, *RASSF1A* and *RUNX3* methylation may be useful as a diagnostic tool in a variety of materials, including biopsy samples, sputum, peripheral blood and bone marrow.

In conclusion, the methylation of specific genes may lead to the development of different histologies in NSCLCs. We propose that the cancer-specific methylation of genes, such as *p16*, *RASSF1A* and *RUNX3*, can be utilized as a molecular diagnostic marker for NSCLCs.

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