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Epigenetic inactivation of the *RUNX3* gene in lung cancer

KOJI SATO[1], YOSHIO TOMIZAWA[1,3], HIRONOBU IIJIMA[1,3], RYUSEI SAITO[3], TAMOTSU ISHIZUKA[1], TAKASHI NAKAJIMA[2] and MASATOMO MORI[1]

[1]Department of Medicine and Molecular Science, [2]Department of Tumor Pathology, Gunma University Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511; [3]National Nishigunma Hospital, 2854 Kanai, Shibukawa, Gunma 377-8511, Japan

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Abstract. The silencing of tumor suppressor genes (TSGs) by aberrant hypermethylation occurs frequently in human cancer. Recently the *RUNX3* gene was identified as a TSG inactivated by hypermethylation. We examined *RUNX3* expression by reverse transcription-PCR and the methylation status of this gene by methylation specific-PCR in 43 lung cancer cell lines and 120 primary non-small cell lung cancer (NSCLC) tumor samples. *RUNX3* expression was absent in 10 (50%) of 20 small cell lung cancer (SCLC) cell lines, 8 (50%) of 16 adenocarcinoma (AdC) cell lines, and 1 (33.3%) of 3 squamous cell carcinoma (SqC) cell lines. The frequency of *RUNX3* methylation was significantly higher in AdC (7/16, 43.8%) than SCLC cell lines (1/20, 5%; p=0.032). *RUNX3* expression was restored by treatment with 5-aza-2'-deoxycytidine and/or trichostatin-A in AdC cell lines. These results indicated that *RUNX3* expression was regulated by aberrant hypermethylation in AdC cell lines. *RUNX3* methylation was detected in 30 (25%) of 120 primary NSCLC tumors. *RUNX3* methylation was significantly more frequent in non-smokers (16/43, 37.2%) than smokers (12/71, 16.9%; p=0.014), and in patients with AdC (26/72, 36.1%) than in patients with SqC (3/45, 6.7%; p<0.001). These results indicated that silencing of the *RUNX3* gene plays an important role in the pathogenesis of lung cancer, and aberrant methylation is an important mechanism of inactivation of the *RUNX3* gene in lung AdC.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the world (1). Human lung cancer is classified into two major histological subtypes, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The two types differ in clinical, therapeutic, and biological features, as well as genetic alterations such as *RB* and *p16* (2,3).

The runt-domain-related (RUNX) proteins are transcription factors which target the TGF-ß signaling pathway. RUNX proteins have been shown to interact with downstream SMAD protein in mediating the growth-suppressive effects of TGF-ß (4). In mammals, there are three families of runt-related genes, *RUNX1 (PEBP2αB/CBFA2/AML1), RUNX2 (PEBP2αA/CBFA1/AML3)*, and *RUNX3 (PEBP2αC/CBFA3/AML2)*. All three play important roles in both normal developmental processes and carcinogenesis (5,6). The *RUNX3* gene is a putative tumor suppressor gene located at 1p36, a region that frequently exhibits loss of heterozygosity events in several cancer types including lung cancer (7). Loss of *RUNX3* expression leads to the abnormal proliferation of gastric epithelial cells, a lack of responsiveness to apoptosis, and a growth-inhibitory effect induced by TGF-ß (8). Though structural mutations and deletions of *RUNX3* are rare, a reduction of *RUNX3* expression frequently occur in cancers including gastric and colorectal cancers (8,9). However, the expression of *RUNX3* in lung cancer has not been fully examined.

Aberrant hypermethylation of CpG islands is an important mechanism that results in a loss of gene expression (10). A reduction in the expression of the *RUNX3* gene caused by aberrant methylation has been frequently found (8,9). Aberrant methylation was also found in lung cancers (11,12). However, no study has analyzed the correlation between the expression and the methylation status of the *RUNX3* gene in lung cancer, especially subtypes of lung cancer such as SCLC, AdC and SqC.

In the present study, we examined *RUNX3* expression and methylation status in 43 lung cancer cell lines (20 SCLC and 23 NSCLC cell lines), and analyzed the methylation status of *RUNX3* in 120 primary NSCLC tumor samples. Furthermore, we analyzed the association between *RUNX3* methylation and clinicopathological characteristics in patients with NSCLC.

Materials and methods

Cell lines and cell culture. A total of 43 lung cancer cell lines were studied including 20 SCLCs (NCI-H187, -H209, -H378, -H524, -H526, -H740, -H865, -H1045, -H1092, -H1184,

Correspondence to: Dr Y. Tomizawa, Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan
E-mail: ytomizaw@showa.gunma-u.ac.jp

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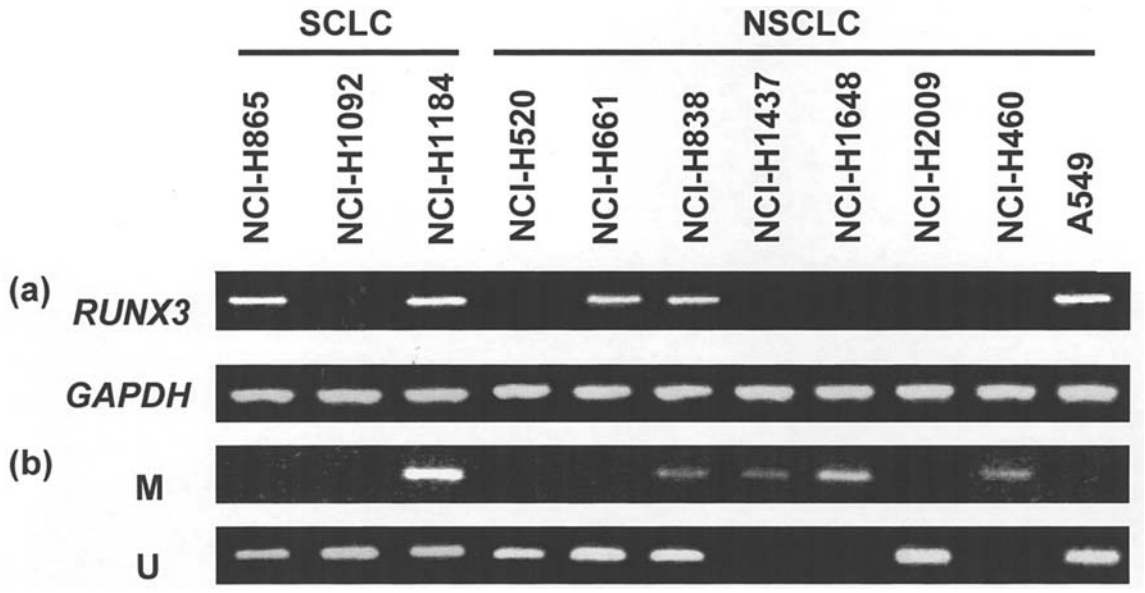

Figure 1. (a) Representative results of the RT-PCR-based analysis of the *RUNX3* gene in lung cancer cell lines. RT-PCR yielded the expected 870-bp DNA products. *GAPDH* was used as an internal control. (b) MSP-based analysis of *RUNX3* methylation in lung cancer cell lines. Lines labeled M and U denote products with methylated and unmethylated sequences, respectively. Two cell lines (A549 and NCI-H460) were used as PCR control described previously (22).

Table I. Incidence of loss of *RUNX3* mRNA expression in lung cancer cell lines.

Cell line	No.	Number of *RUNX3* mRNA-negative samples (%)
Small cell lung cancer	20	10 (50.0)
Non-small cell lung cancer	23	10 (43.8)
Adenocarcinoma	16	8 (50.0)
Squamous cell carcinoma	3	1 (33.3)
Large cell carcinoma	4	1 (25.0)
Total	43	20 (46.5)

-H1238, -H1339, -H1607, -H1618, -H1963, -H2107, -H2141, -H2171, -H2227, and HCC33) and 23 NSCLCs (NCI-H322, -H460, -H520, -H661, -H838, -H1264, -H1299, -H1395, -H1437, -H1648, -H1792, -H1819, -H2009, -H2087, -H2106, -H2122, HCC15, HCC44, HCC78, HCC95, HCC193, HCC515, and A549) established previously (13). The cells were grown in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37˚C.

Tissue specimens. Primary lung tumors were obtained from 120 patients with NSCLC. All patients underwent surgery at the Gunma University Hospital or National Nishigunma Hospital, Gunma, between August 1991 and March 2001. The tumors were histologically classified as AdCs (n=72), SqCs (n=45), large cell carcinomas (LCCs, n=2), Adenosquamous carcinoma (n=1) according to the Histological Typing of Lung Tumors of the World Health Organization (14). The tumors were frozen and stored at -80˚C until the extraction of DNA.

Extraction of DNA and RNA. Genomic DNA was prepared as previously described (15) or else with a QIAamp DNA mini kit (Qiagen, Tokyo, Japan) or DNeasy kit (Qiagen). RNA was prepared using an RNeasy kit (Qiagen). In all cases, the manufacturer's instructions were followed.

Reverse transcription (RT)-PCR. *RUNX3* mRNA expression was measured by RT-PCR. The RNA (3 μg) was reverse transcribed in a volume of 20 μl using SuperScript II (Invitrogen) according to the manufacturer's instructions. The cDNA (1 μl) was used for the analysis of *RUNX3* with specific primer sequences. The *RUNX3* primers were 5'-GAGTTTCACCCTG ACCATCACTGTG-3' (sense) and 5'-GCCCATCACTGGT CTTGAAGGTTGT-3' (antisense), which yielded 870-base pair (bp) PCR products (8). The gene for Glyceraldehyde 3' phosphate dehydrogenase (*GAPDH*), a house keeping gene, was used to check the integrity of the RNA in all samples (16). The PCR amplification consisted of denaturation at 95˚C for 15 min, 35 cycles of denaturation at 95˚C for 20 sec, annealing at 55˚C for 60 sec, and extension at 72˚C for 90 sec, and a final extension at 72˚C for 10 min. The PCR products were separated in 2% agarose gels with ethidium bromide and visualized under UV illumination.

Methylation-specific PCR (MSP). The methylation status of *RUNX3* was analyzed by MSP using bisulfite-modified genomic DNA. The modification of DNA with bisulfite was performed as described previously (17). Briefly, 1 μg of genomic DNA was denatured with 0.2 M NaOH. Then, 10 mM hydroquinone (Sigma) and 3 M sodium-bisulfite (Sigma) were added and the solution was incubated at 55˚C for 16 h. Treatment of genomic DNA with sodium bisulfite converts

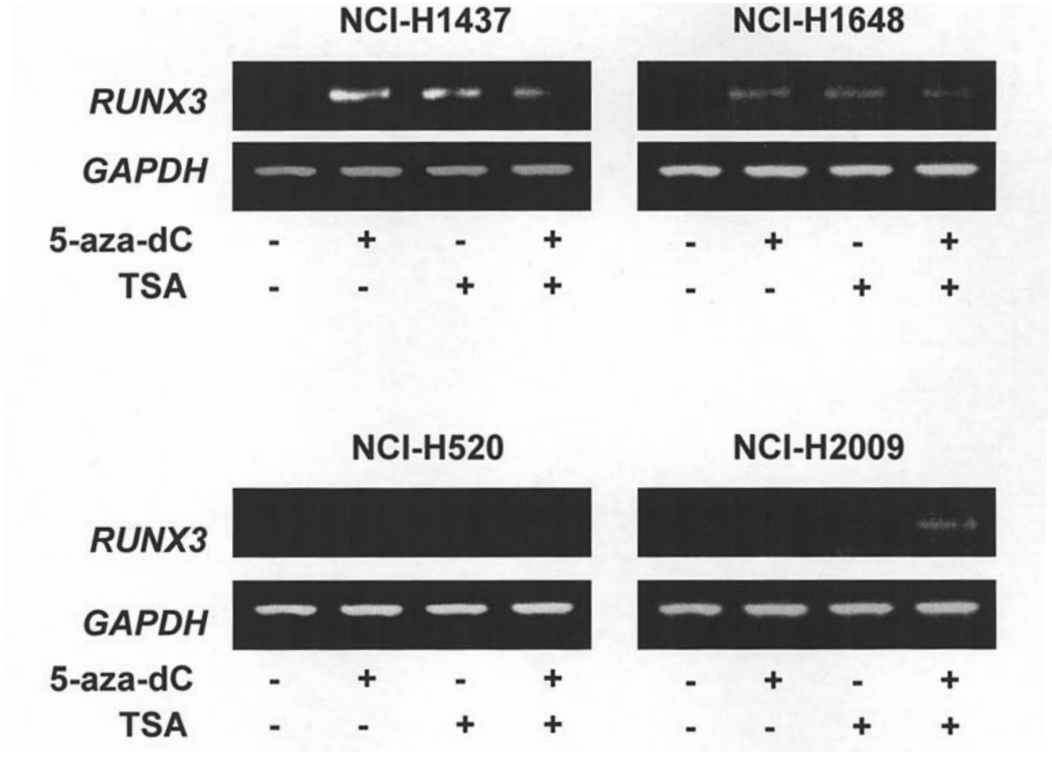

Figure 2. Restoration of *RUNX3* expression by 5-aza-dC and TSA in NCI-H520, -H1437, -H1648, and -H2009. *RUNX3* expression was restored by 5-aza-dC and TSA in NCI-H1437 and -H1648 but not in NCI-H520 and -H2009. *RUNX3* expression was restored by the combined treatment with 5-aza-dC and TSA in NCI-H1437, -H1648, and -H2009 but not in NCI-H520.

unmethylated (but not methylated) cytosines to uracil, which is then converted to thymidine during subsequent PCR to give sequence differences between methylated and unmethylated DNA. The modified DNA was used as a template for MSP with primers specific for either the modified methylated or the modified unmethylated *RUNX3* promoter sequences. Two distinct promoters, P1 and P2, regulate *RUNX3* transcription. The major *RUNX3* mRNA is transcribed from P2. The genomic region surrounding the P2 promoter contributes to a large CpG island (18). P2 possesses the hall mark characteristics of a GC-rich promoter. Thus, in theory it is rational that transcription from P2 should be regulated by DNA methylation. We examined the region using two sets of primers specific for MSP, described previously (8). The primer sequences for the methylated *RUNX3* gene were 5'-TTACGAGGGGCGGTCG TACGCGGG-3' (sense) and 5'-AAAACGACCGACGCGA ACGCCTCC-3' (antisense), and for the unmethylated allele were 5'-TTATGAGGGGTGGTTGTATGTGGG-3' (sense) and 5'-AAAACAACCAACACAAACACCTCC-3' (antisense). Each primer set generated a 220-bp product. PCR was performed for a total of 35 cycles at an annealing temperature of 63˚C for the methylated primers and 57˚C for the unmethylated primers using HotStar Taq DNA Polymerase (Qiagen). The PCR products were separated in 2% agarose gels with ethidium bromide and visualized under UV illumination.

5-aza-2'-deoxycytidine (5-aza-dC) and trichostatin-A (TSA) treatment. Cells were incubated with medium containing 10 μM 5-aza-dC (Sigma), or 0.5 μM TSA (Wako, Tokyo) for 48 h. In the synergistic study, the cells were cultured with 10 μM 5-aza-dC for 48 h and then with 0.5 μM TSA for another 24 h. We isolated total RNA with an RNeasy mini kit (Qiagen), and performed RT-PCR.

Statistical analysis. Fisher's exact test was used to examine the association of two categorical variables. A p-value of <0.05 was considered to be statistically significant. Statistical analysis was performed using StatView J-4.5 for Macintosh.

Results

RUNX3 expression and methylation status in lung cancer cell lines. We first analyzed the expression of the *RUNX3* gene in 43 lung cancer cell lines using the RT-PCR method. Representative results are shown in Fig. 1a. *RUNX3* expression was absent in 10 (50%) of 20 SCLC cell lines and 10 (43.5%) of 23 NSCLC cell lines (Table I). Among the NSCLC cell lines, *RUNX3* expression was absent in 8 (50%) of 16 AdC cell lines, 1 (33.3%) of 3 SqC cell lines, and 1 (25%) of 4 LCC cell lines. Loss of *RUNX3* expression was common to all the histological subtypes of lung cancer cell lines. We next analyzed the methylation status of the *RUNX3* gene in these 43 cell lines. Representative examples are illustrated in Fig. 1b. Methylation of *RUNX3* was detected in 1 (5%) of 20 SCLC cell lines and 8 (40%) of 23 NSCLC cell lines (Table II). Among the NSCLC cell lines, *RUNX3* methylation was observed in 7 (43.8%) of 16 AdC cell lines, none (0%) of 3 SqC cell lines, and 1 (25.0%) of 4 LCC cell lines. The frequency of *RUNX3* methylation was significantly higher in the AdC than SCLC cell lines ($p=0.032$).

Table II. Presence of methylated and unmethylated *RUNX3* alleles in lung cancer cell lines.

RUNX3 promoter genotype						
M[a] alleles	U[a] alleles	SCLC	NSCLC	AdC	SqC	LCC
+	+	1	1	1	0	0
+	-	0	7	6	0	1
-	+	19	15	9	3	3
-	-	0	0	0	0	0
Total		20	23	16	3	4

[a]M, methylated; U, unmethylated; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; AdC, adenocarcinoma; SqC, squamous cell carcinoma; LCC, large cell carcinoma.

Table III. Correlation between expression and methylation of *RUNX3* in lung cancer cell lines.

		RUNX3		*RUNX3* promoter genotype	
	Total	expression	Total	M[a] alleles	U[a] alleles
Small cell lung cancer	20	+	10	1[b]	10
		-	10	0	10
Non-small cell lung cancer	23	+	13	1[b]	13
		-	10	7	3
Adenocarcinoma	16	+	8	1[b]	8
		-	8	6	2
Squamous cell carcinoma	3	+	2	0	2
		-	1	0	1
Large cell carcinoma	4	+	3	0	3
		-	1	1	0

[a]M, methylated; U, unmethylated. [b]Both M allele and UM allele were exhibited.

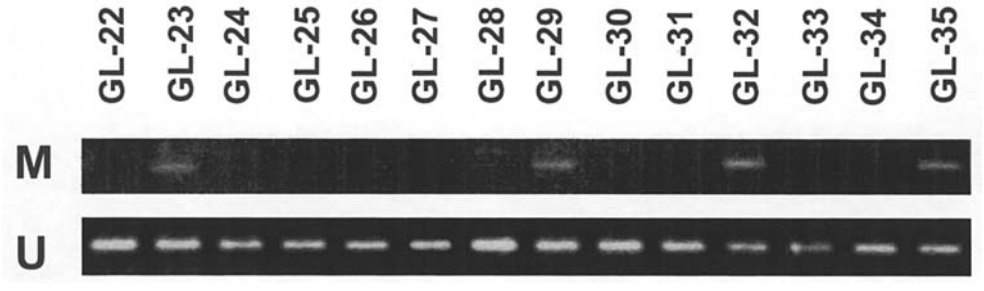

Figure 3. Representative results of the MSP-based analysis of *RUNX3* methylation in primary NSCLC tumors. Lines labeled M and U denote products with methylated and unmethylated sequences, respectively.

Both methylated and unmethylated alleles were found in one (NCI-H1184) of the SCLC cell lines and one (NCI-H838) of the AdC cell lines (Fig. 1b and Table II). Then we analyzed the correlation between the expression and the methylation status of the *RUNX3* gene. Unmethylated alleles were detected in all *RUNX3*-expressing SCLC and NSCLC cell lines including NCI-H1184 and -H838. Methylated alleles were detected in none (0%) of 10 SCLC, 7 (70%) of 10 NSCLC, 6 (75%) of 8 AdC, none (0%) of 1 SqC, and 1 (100%) of 1 LCC cell lines which were negative for the RUNX3 expression (Table III).

Restoration of RUNX3 expression by 5-aza-dC and TSA. To assess whether the loss of *RUNX3* expression in lung cancer was a result of aberrant methylation and/or histone

Correlation of *RUNX3* methylation with clinicopathological characteristics of patients with non-small cell lung cancer.

Characteristics	Subset	No.	*RUNX3*		p[b]
			M[a]	U[a]	
Gender	Male	77	15	62	0.051
	Female	43	15	28	
Age	≤69	72	16	56	NS
	>69	48	14	34	
Smoking history	Smoker	71	12	59	0.014
	Non-smoker	43	16	27	
	Unknown	6			
Stage	I	71	19	52	NS
	II	15	6	9	
	III	24	4	20	
	IV	8	1	7	
	Unknown	2			
Pathology	Adenocarcinoma	72	26	46	<0.001
	Squamous cell carcinoma	45	3	42	
	Large cell carcinoma	2	1	1	
	Adenosquamous cell carcinoma	1	0	1	
Differentiation	Poorly	21	3	18	0.018
	Moderately	60	12	48	
	Well	36	14	22	
	Unknown	3			
Vascular invasion	+	44	6	38	0.014
	-	68	23	45	
	Unknown	8			
Lymphatic permeation	+	59	13	46	NS
	-	54	16	38	
	Unknown	7			
Pleural involvement	+	57	14	43	NS
	-	58	16	42	
	Unknown	5			

[a]M, methylated; U, unmethylated. [b]Fisher's exact test.

deacetylation, we examined the effect of the demethylating agent 5-aza-dC and/or histone deacetylase inhibitor TSA on 4 cell lines without *RUNX3* expression. The results are shown in Fig. 2. The expression was restored by the treatment with 5-aza-dC and/ or TSA in two cell lines (NCI-H1437 and -H1648) in which aberrant methylation was detected by MSP. On the other hand, the expression was not restored by the treatment with 5-aza-dC or TSA in two cell lines (NCI-H520 and -H2009) in which no aberrant methylation was detected by MSP. Although the expression was not restored by treatment with the combination of 5-aza-dC and TSA in NCI-H520, it was restored by this treatment in NCI-H2009.

Correlation between RUNX3 methylation and clinicopathological features in primary NSCLCs. One hundred and twenty primary NSCLC tumor samples were examined for *RUNX3* methylation by MSP. Representative examples are illustrated in Fig. 3. *RUNX3* methylation was detected in 30 (25%) of the 120 tumors. All tumor samples had unmethylated alleles. We then analyzed the relationship between the methylation of *RUNX3* and the clinicopathological characteristics of these patients. The results are summarized in Table IV. *RUNX3* methylation was more predominantly detected in females (15/43, 34.9%) than males (15/77, 19.5%; p=0.051), in non-smokers (16/43, 37.2%) than smokers (12/71, 16.9%; p=0.014), in patients with AdC (26/72, 36.1%) than in

patients with SqC (3/45, 6.7%; p<0.001), in well-differentiated tumors (14/36, 38.9%) than in moderate to poorly differentiated tumors (15/81, 18.5%; p=0.018), and in tumors without vascular invasion (23/68, 33.8%) than in those with vascular invasion (6/44, 13.6%; p=0.014). There was no significant correlation of *RUNX3* methylation with age, stage, lymphatic permeation, or pleural involvement.

Discussion

We first examined the expression and methylation status of *RUNX3* in 43 lung cancer cell lines by RT-PCR and MSP. The expression was frequently absent in lung cancer cell lines, and the frequency of loss of expression was similar among the histological subtypes. On the other hand, though the aberrant methylation of *RUNX3* was frequently detected in AdC cell lines, it was rare in SCLC and SqC cell lines. Furthermore, the expression was restored by treatment with 5-aza-dC or 5-aza-dC plus TSA in the AdC cell lines NCI-H1437, -H1648, and -H2009, though the expression was not restored by this treatment in the SqC cell line NCI-H520. These results indicated that the expression was regulated by aberrant hypermethylation in AdC cell lines, while it was not regulated by hypermethylation in the SCLC or SqC cell lines. It has been reported that the *RUNX3* gene was inactivated by aberrant methylation in gastric, colorectal, bile duct, and pancreatic cancers (8,9,19,20) the predominant form of which is AdC. These previous results and the present study suggested that loss of *RUNX3* expression due to aberrant methylation is specific to the subtype of AdC in several human cancer types. One AdC cell line (NCI-H838) expressed *RUNX3* though it had a methylated allele (Fig. 1 and Table III). However, since unmethylated alleles were also present in this cell line, *RUNX3* expression was detected.

We next analyzed the methylation status of the *RUNX3* gene in 120 primary NSCLC tumors, and found the frequency of *RUNX3* methylation to be 25%. The frequency was slightly higher compared to previous findings (21,22). The discrepancy in the results might be due to the difference in the population. AdC was the predominant histological subtype in the present study. We then analyzed the relationship between methylation of the *RUNX3* gene and the clinicopathological characteristics of these patients. One interesting aspect of the present study is that *RUNX3* methylation was frequently observed in non-smokers. Genetic and epigenetic changes in lung cancer have been almost always more frequent in smokers than non-smokers, such as mutations of *p53* and *KRAS*, and aberrant hypermethylation of *p16* and *APC* (2,3,23,24). Although it is unclear why *RUNX3* methylation was predominantly detected in patients with no history of smoking, it is reasonable to assume that the methylation is caused by carcinogens other than those contained in tobacco smoke. Cheng *et al* investigated infections with the human papilloma virus as a cause of lung cancer in female non-smokers (25). Sakura *et al* reported the aberrant hypermethylation of multiple genes in gastric cancer associated with infections with the Epstein-Barr virus (26). Although the Epstein-Barr virus is rarely affected with lung carcinogenesis (27), *RUNX3* methylation might be induced by infections with some viruses. Recently, it was reported that mutations in the tyrosine kinase domain of the *EGFR* gene occurred in patients with no history of smoking (28,29). This is similar to the results of *RUNX3* methylation status in the present study. However, the mechanism of these molecular abnormalities and the association of *RUNX3* methylation with *EGFR* mutation is not known. Further analyses will be needed to clarify the carcinogenesis and progression of lung cancer in non-smokers. We also found that *RUNX3* methylation was preferentially observed in AdC rather than SqC in primary NSCLCs as the results of an analysis of lung cancer cell lines. Molecular differences between AdC and SqC have been described in previous studies, including allelic loss at 3p, mutations of *p53* and *RASSF1A* methylation (12,30,31). Yanagawa *et al* reported that methylation of *RUNX3* and *APC* occurred more frequently in AdC than SqC (21). The result of the present study was consistent with this previous study. Therefore, these results including the analysis of the cell lines suggested that methylational inactivation of the *RUNX3* gene plays an important role in the pathogenesis of lung AdC.

In conclusion, loss of *RUNX3* expression was frequently detected in all histological subtypes of lung cancer cell lines. The silencing mechanism was the aberrant hypermethylation in AdC cell lines, but not in SCLC cell lines. Methylation of *RUNX3* also frequently occurred in primary AdC, especially among patients who were non-smokers. These results indicated that silencing of the *RUNX3* gene plays an important role in the pathogenesis of lung cancer, and aberrant methylation is an important mechanism of inactivation of the *RUNX3* gene in lung AdC.

References

1. Parkin DM, Bray FI and Devesa SS: Cancer burden in the year 2000. The global picture. Eur J Cancer 37 (Suppl 8): 4-66, 2001.
2. Kelley MJ, Nakagawa K, Steinberg SM, Mulshine JL, Kamb A and Johnson BE: Differential inactivation of CDKN2 and Rb protein in non-small cell and small cell lung cancer cell lines. J Natl Cancer Inst 87: 756-761, 1995.
3. Shimizu E, Zhao M, Shinohara A, *et al*: Differential expressions of cyclin A and the retinoblastoma gene product in histological subtypes of lung cancer cell lines. J Cancer Res Clin Oncol 123: 533-538, 1997.
4. Ito Y and Miyazono K: RUNX transcription factors as key targets of TGF-beta superfamily signaling. Curr Opin Genet Dev 13: 43-47, 2003.
5. Ito Y: Oncogenic potential of the RUNX gene family: 'overview'. Oncogene 23: 4198-4208, 2004.
6. Lund AH and van Lohuizen M: RUNX: a trilogy of cancer genes. Cancer Cell 1: 213-215, 2002.
7. Nomoto S, Haruki N, Tatematsu Y, *et al*: Frequent allelic imbalance suggests involvement of a tumor suppressor gene at 1p36 in the pathogenesis of human lung cancers. Genes Chromosomes Cancer 28: 342-346, 2000.
8. Li QL, Ito K, Sakakura C, *et al*: Causal relationship between loss of RUNX3 expression and gastric cancer. Cell 109: 113-124, 2002.
9. Ku JL, Kang SB, Shin YK, *et al*: Promoter hypermethylation downregulates RUNX3 gene expression in colorectal cancer cell lines. Oncogene 23: 6736-6742, 2004.
10. Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K and Herman JG: Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 10: 687-692, 2001.
11. Zochbauer-Muller S, Fong KM, Virmani AK, Geradts J, Gazdar AF and Minna JD: Aberrant promoter methylation of multiple genes in non-small cell lung cancer. Cancer Res 61: 249-255, 2001.
12. Tomizawa Y, Iijima H, Nomoto T, *et al*: Clinicopathological significance of aberrant methylation of RARß at 3.24, RASSF1A at 3p21.3, and FHIT at 3p14.2 in patients with non-small cell lung cancer. Lung Cancer 46: 305-312, 2004.

RM, Johnson BE, Ihde DC, *et al*: NCI-Navy Medical Oncology Branch cell line data base. J Cell Biochem (Suppl) 24: 32-91, 1996.
14. WHO: Histological Typing of Lung Tumors. Vol. 1, 2nd edition, Geneva, 1981.
15. Yokota J, Wada M, Shimosato Y, Terada M and Sugimura T: Loss of heterozygosity on chromosome 3, 13, and 17 in small-cell carcinoma and chromosome 3 in adenocarcinoma of the lung. Proc Natl Acad Sci USA 84: 9252-9256, 1987.
16. Chen XQ, Bonnefoi H, Pelte MF, *et al*: Telomerase RNA as a detection maker in the serum of breast cancer patients. Clin Cancer Res 6: 3823-3826, 2000.
17. Sunaga N, Miyajima K, Suzuki M, *et al*: Different roles for Caveolin-1 in the development of non-small cell lung cancer versus small cell lung cancer. Cancer Res 64: 4277-4285, 2004.
18. Bangsow C, Rubins N, Glusman G, *et al*: The RUNX3 gene-sequence, structure and regulated expression. Gene 279: 221-232, 2001.
19. Goel A, Arnold CN, Tassone P, *et al*: Epigenetic inactivation of RUNX3 in microsatellite unstable sporadic colon cancers. Int J Cancer 112: 754-759, 2004.
20. Wada M, Yazumi S, Takaishi S, *et al*: Frequent loss of RUNX3 gene expression in human bile duct and pancreatic cancer cell lines. Oncogene 23: 2401-2407, 2004.
21. Yanagawa N, Tamura G, Oizumi H, Takahashi N, Shimazaki Y and Motoyama T: Promoter hypermethylation of tumor suppressor and tumor-related genes in non-small cell lung cancers. Cancer Sci 94: 589-592, 2003.
22. Li QL, Kim HR, Kim WJ, *et al*: Transcriptional silencing of the RUNX3 gene by CpG hypermethylation is associated with lung cancer. Biochem Biophys Res Commun 314: 223-228, 2004.
23. Suzuki H, Takahashi T, Koroshi T, *et al*: p53 mutation in non-small cell lung cancer in Japan. Cancer Res 52: 734-736, 1992.
24. Ahrendt SA, Decker PA, Alawi EA, *et al*: Cigarette smoking is strongly associated with mutation of the K-ras gene in patients with primary adenocarcinoma of the lung. Cancer 92: 1525-1530, 2001.
25. Cheng YW, Chiou HL, Sheu GT, *et al*: The association of human papilloma virus 16/18 infection with lung cancer among nonsmoking Taiwanese women. Cancer Res 61: 2799-2803, 2001.
26. Sakuma K: High-density methylation of p14(ARF) and p16(INK4A) in epstein-Bar virus-associated gastric carcinoma. Int J Cancer 112: 273-278, 2004.
27. Chen FF, Yan JJ, Lai WW, Jin YT and Su IJ: Epstein-Barr virus-associated non-small cell lung carcinoma. Cancer 82: 2334-2342, 1998.
28. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T and Mitsudomi T: Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 64: 8919-8923, 2004.
29. Marchetti A, Martella C, Felicioni L, *et al*: EGFR mutations in non-small cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 1: 857-865, 2005.
30. Fujita T, Kiyama M, Tomizawa Y, Kohno T and Yokota J: Comprehensive analyses of p53 gene mutation characteristics in lung carcinoma with special reference to histological subtypes. Int J Oncol 15: 927-934, 1999.
31. Sekido Y, Fong KM and Minna JD: Progress in understanding the molecular pathogenesis of human lung cancer. Biochim Biophys Acta 1378: F21-F59, 1998.