Methylation of the *DLEC1* gene correlates with poor prognosis in Japanese lung cancer patients

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Received September 7, 2009; Accepted December 2, 2009

DOI: 10.3892/ol_00000050

Abstract. The incidence of chromosome 3p gene alterations is one of the most frequent and earliest documented events in lung cancer. This study aimed to investigate promoter methylation in the *deleted in lung and esophageal cancer 1* (*DLEC1*) gene, as well as the *p16* and *CDH1* genes in Japanese lung cancer cases. The methylation status of the promoter regions of *DLEC1*, *p16* and *CDH1* was investigated using methylation-specific PCR. The findings were compared to the clinicopathological features of lung cancer. Methylation-specific PCR showed that the *DLEC1* promoter region was methylated in 65 out of 116 (56%) lung cancers. Patients with *DLEC1*-methylated cancer were associated with a significantly worse prognosis than those with unmethylated cancer (p=0.0368; hazard ratio=1.83). The *p16* methylation status correlated with squamous histology (p=0.03) and smoking status (never smoker vs. smoker; p=0.0122). Patients with *p16* ummethylated cancer harbored more *EGFR* mutations (p=0.0071). The *CDH1* promoter region was hypermethylated in 65 out of 118 (55.1%) lung cancer cases. However, the *CDH1* methylation status was not associated with the clinicopathological characteristics of the lung cancer types. *p16* and *CDH1* methylation status did not correlate with survival in the lung cancer patients. Thus, in our Japanese cohort, the methylation status of the *DLEC1* gene was a marker of poor prognosis independent of stage.

Introduction

Alleic loss of chromosome 3p is one of the most frequent and earliest documented events in lung cancer with a wide range of 3p mutations (12-26), suggesting the presence of multiple tumor-suppressor genes on 3p (1-3). The chromosome locus 3p22.3 is a 'hot spot' for chromosomal aberrations and loss of heterozygosity in cancers, including lung cancer (4,5). Further analysis led to the identification of the *DLC1* gene (6), which was later renamed *deleted in lung and esophageal cancer 1 (DLEC1)* on 3p22.3. Loss of *DLEC1* expression has been observed in lung, esophageal, renal, ovarian and nasopharyngeal carcinoma cell lines and primary tumors. Moreover, functional analyses strongly suggest that *DLEC1* is a tumor-suppressor gene (6-8). Promoter hypermethylation has been shown to be responsible for the silencing of *DLEC1* in ovarian cancer and nasopharyngeal carcinoma (7,8). Furthermore, there have been methylation analyses reported for hepatocellular carcinoma (4), gastric (9) and lung cancers (5).

This study investigated whether the promoter hypermethylation of *DLEC1* plays a role in lung cancer in Japanese patients, and whether it has any prognostic significance. The methylation status of the promoter regions of *DLEC1* was investigated using methylation-specific PCR. The findings were compared to the clinicopathological features of lung cancer.

Patients and methods

Patients. The study group included lung cancer patients who had undergone surgery at the Nagoya City University Hospital. Written informed consent was obtained. Approval was granted by the Institutional Ethics Committee of the Nagoya City University Graduate School of Medical Sciences. The lung tumors were classified according to the general criteria for the clinical and pathological record of lung cancer in Japan (10). Tumor samples were immediately frozen and stored at -80˚C until assayed. The clinical and pathological characteristics of the 116 lung cancer patients for *DLEC1* sequencing analysis follow. Eighty-nine patients (76.7%) were male, 27 were female. Thirty-eight patients (32.8%) had squamous cell carcinomas; 65, adenocarcinomas and 10 had adenosquamous cell carcinomas. Eighty-five (73.8%) were smokers and 31 were non-smokers. The clinical and pathological characteristics of the lung cancer patients for the methylation analyses are documented in Tables I, II and III, respectively. The samples from these patients were previously sequenced for *EGFR* (11-14).

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Key words: methylation, *DLEC1* gene, lung cancer

Table I. Clinicopathological data of 116 lung cancer patients.

	DLEC1 gene status		
Factors	Methylated cases	Unmethylated cases	p-value
Mean age (years)	64.5±8.9	66.3±13.3	0.2211
Stage			0.7028
I	24 (36.9%)	21 (41.2%)	
II-IV	41 (63.1%)	30 (58.8%)	
Lymph node metastasis			0.5734
N0	34 (52.3%)	30 (58.8%)	
N^+	31 (47.7%)	21 (41.2%)	
Smoking			0.2910
Never smoker	20 (30.8%)	11 (21.6%)	
Smoker	45 (69.2%)	40 (79.4%)	
EGFR status			0.1657
Wild-type	49 (75.4%)	44 (86.3%)	
Mutation	16 (24.6%)	7 (13.7%)	
Pathological subtypes			0.4269
SCC	19 (29.2%)	19 (37.3%)	
Non-SCC	46 (70.8%)	32 (62.7%)	
Age			0.3478
≤65	32 (49.2%)	20 (39.2%)	
>65	33 (50.8%)	31 (60.8%)	
Gender			0.9999
Male	50 (76.9%)	39 (76.5%)	
Female	15 (23.1%)	12 (23.5%)	

N^+, lymph node metastasis-positive; SCC, squamous cell carcinoma.

Methylation-specific polymerase chain reaction analysis. DNA was prepared from tissue samples using standard methods. Bisulfite modification of genomic DNA was performed using the MethylCode Bisulfite Conversion Kit (Invitrogen, CA, USA). Briefly, 500 ng of genomic DNA was denatured by incubation with CT Conversion Reagent at 98˚C for 10 min and 68˚C for 2.5 h and at 4˚C for several min. Modified DNA was purified by the Spin Column and then eluted with Elution Buffer.

The primer sequences for the *DLEC1* gene for methylated (M) sequences were: forward, 5-GTTTCGTAGTTCGGTT TCGT C-3 and reverse, 5-CGAAATATCTTAAATACGCA ACG-3 (107 bp). The primer sequences for the *DLEC1* gene for unmethylated (U) sequences were: forward, 5-TAGTTTT GTAGTTTGGTTTTGTT-3 and reverse, 5-ACAAAATATCT TAAATACACACAACA-3. The primer sequences for the *CDH1* gene for methylated (M) sequences were: forward, 5-GGTGAATTTTTAGTTAATTAGCGGTAC-3 and reverse, 5-CATAACTAACCGAAAACGCCG-3. The primer sequences for the *CDH1* gene for unmethylated (U) sequences were: forward,5-GGTAGGTGAATTTTTAGTTAATTAGTGGTA-3 and reverse, 5-ACCCATAACTAACCAAAAACACCA-3. The primer sequences for the *p16* gene for methylated (M) sequences were: forward, 5-TTATTAGAGGGTGGGGTG GATTGT-3 and reverse, 5-G ACCCCGAACCGCGACCG TAA-3. The primer sequences for the *p16* gene for unmethylated (U) sequences were: forward, 5-TTATTAGAGGGT GGGGTGGATTGT-3 and reverse, 5-CAACCCCAAACC ACAACCATA-3. The cycling conditions were: initial denaturation at 94˚C for 5 min, followed by 40 cycles at 94˚C for 45 sec, 65˚C (*p16*, M), 60˚C (*p16*, U), 58˚C (*DLEC1*, M), 57˚C (*CDH1*) or 55˚C (*DLEC1*, U) for 45 sec, and 72˚C for 45 sec.

Statistical analysis. Statistical analyses were carried out using the Mann-Whitney U test for unpaired samples and the Wilcoxon signed-rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's and the χ^2-test. Survival of the lung cancer patients was examined by the Kaplan-Meier method, and differences were examined by the log-rank test. Analyses were carried out using the Stat-View software package (Abacus Concepts Inc., Berkeley, CA, USA), and differences were considered significant at $p<0.05$.

Results

DLEC1 gene methylation status in Japanese lung cancer patients. Methylation-specific PCR showed that the *DLEC1* promoter region was methylated in 65 out of 116 (79.8%) lung

Table II. Clinicopathological data of 221 lung cancer patients.

Factors	*p16* gene status		
	Methylated cases	Unmethylated cases	p-value
Mean age (years)	66.1±8.9	64.3±10.9	0.4076
Stage			0.2726
I	39 (43.3%)	67 (51.5%)	
II-IV	51 (56.7%)	63 (48.5%)	
Lymph node metastasis			0.1207
N0	51 (56.0%)	87 (66.9%)	
N^+	40 (44.0%)	43 (33.1%)	
Smoking			0.0122
Never smoker	15 (16.5%)	41 (31.5%)	
Smoker	76 (83.5%)	89 (68.5%)	
EGFR status			0.0071
Wild-type	80 (87.9%)	94 (72.3%)	
Mutation	11 (12.1%)	36 (27.7%)	
Pathological subtype			0.030
SCC	49 (53.8%)	54 (38.6%)	
Non-SCC	42 (46.2%)	86 (61.4%)	
Age			0.2198
≤65	37 (40.7%)	64 (49.2%)	
>65	54 (59.3%)	66 (50.8%)	
Gender			0.6141
Male	74 (81.3%)	101 (77.7%)	
Female	17 (18.7%)	29 (22.3%)	

N^+, lymph node metastasis positive; SCC, squamous cell carcinoma.

cancer types. The methylation status of *DLEC1* was not associated with squamous histology (squamous cell carcinoma 29.2% vs. non-squamous cell carcinoma 37.3%; p=0.4269) and smoking status (never smoker 30.8% vs. smoker 21.6%; p=0.291). In addition, the *DLEC1* methylation status did not correlate with gender (p=0.9999), age (p=0.3478), lymph node metastasis (p=0.5734) and pathological stages (I vs. II-IV; p=0.7028). *DLEC1* methylation independently existed with *EGFR* mutations (p=0.1657).

The *p16* promoter region was methylated in 91 out of 221 (78.8%) lung cancer types (Table II). The methylation status was correlated with squamous histology (p=0.03), smoking status (never smoker vs. smoker; p=0.0122) and *EGFR* wild-type (p=0.0071). However, the *p16* methylation status did not correlate with survival (p=0.6215).

The CDH promoter region was methylated in 65 out of 118 (78.8%) lung cancer types (Table III). A higher frequency of methylation cases of the *CDH1* promoter region was associated with younger patients (p=0.0491). However, the methylation status did not correlate with survival (p=0.8011).

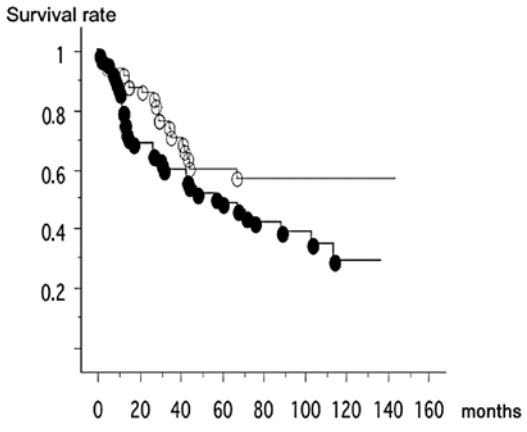

Figure 1. Of the 65 *DLEC1* methylated cases, 37 patients succumbed to the disease (mean survival, 62.4 months). Of the 51 unmethylated cases, 18 patients succumbed to the disease (mean survival, 51.1 months). Thus, patients with *DLEC1* methylated cancer were significantly associated with poor survival (log-rank test, p=0.0407). Black ovals, unmethylated cases; white ovals, methylated cases.

DLEC1 gene methylation status and survival in lung cancer patients. Of the 65 *DLEC1* methylated cases, 37 patients succumbed to the disease, while of the 51 unmethylated cases, 18 patientts succumbed to the disease. Thus, patients with *DLEC1* methylated cancer were significantly associated with poor survival (log-rank test, p=0.0407) (Fig. 1). In the

Table III. Clinicopathological data of 118 lung cancer patients.

	CDH1 gene status		
Factors	Methylated cases	Unmethylated cases	p-value
Mean age (years)	66.6±8.9	62.7±11.2	0.0491
Stage			0.5669
I	22 (33.8%)	21 (39.6%)	
II-IV	43 (66.2%)	32 (60.4%)	
Lymph node metastasis			0.5780
N0	34 (52.3%)	31 (58.5%)	
N^+	31 (47.7%)	22 (41.5%)	
Smoking			0.4073
Never smoker	15 (23.1%)	16 (30.2%)	
Smoker	50 (76.9%)	37 (69.8%)	
EGFR status			0.2594
Wild-type	54 (83.1%)	39 (73.6%)	
Mutation	11 (16.9%)	14 (26.4%)	
Pathological subtypes			0.0514
SCC	37 (56.9%)	40 (75.5%)	
Non-SCC	28 (43.1%)	13 (24.5%)	
Age			0.0275
≤65	24 (36.9%)	29 (54.7%)	
>65	41 (63.1%)	24 (43.3%)	
Gender			0.5096
Male	52 (80.0%)	39 (73.6%)	
Female	13 (20.0%)	14 (26.4%)	

N^+, lymph node metastasis positive; SCC, squamous cell carcinoma.

multivariate analysis, pathological stage [stage I vs. stage III-IV, p=0.0038, relative risk, 2.495 (1.342-4.636)] and *DLEC1* methylation status were independent prognostic factors [p=0.0348, relative risk, 1.865 (1.046-3.362)].

Discussion

We demonstrated that the *DLEC1* gene was hypermethylated in Japanese lung cancer patients. We did not find any correlations between methylation status and gender, pathological stage and smoking status in Japanese NSCLC patients. However, the *DLEC1* methylation status was correlated with survival in Japanese lung cancer patients.

DLEC1 is a candidate tumor-suppressor gene found in multiple cancer types (3-5,7,15,16). *DLEC1* suppresses tumor growth or reduces the invasiveness of cancer cells. In ovarian cancer cell lines, the number of colonies formed in *DLEC1* transfectants was significantly lower than that in mock transfectants (8), which showed that *DLEC1* suppressed the growth of ovarian cancer cells and/or inactivation of CDC2 kinase, thereby blocking cells at the G2/M phase and preventing tumor development in nude mice (4,5). The demethylating agent 5-aza caused the loss of mRNA expression in lung cancer cell lines (16). *DLEC1* methylation is cancer-specific, as it was only rarely detected in matched normal lung tissue (5). As there is no antibody available for *DLEC1*, we were unable to determine whether methylated tumors show a loss or reduced *DLEC1* protein expression. However, a loss of *DLEC1* RNA expression was previously shown in 8 out of 30 primary lung cancer types, although this loss was not due to gene mutations (6). No correlation between *DELC1* methylation and clinical parameters of gastric cancer was found (17), similar to our investigation.

It was demonstrated that *p16* methylation occurs more frequently in squamous cell carcinoma (16,18). These previous results are consistent with our present findings. Several studies have demonstrated a significant association between DNA methylation and tobacco smoking (20,21). Methylation of the p16INK4A gene was induced in rats treated with tobacco-specific NKK[4-N-methyl-N-nitrosamino-1-3-prydil 1-1-butanone (NNK), polyaromatic hydrocarbon] (20). Lung tumors that were induced in F344/N rats after exposure to cigarette smoke by inhalation displayed *de novo* methylation of p16INK4a (21).

The correlation between *CDH1* gene methylation and survival is controversial. Although Nakata *et al* demonstrated a marginal correlation between *CDH1* methylation and survival (p=0.0473) (16), Kim *et al* demonstrated that *CDH1*

methylation itself did not correlate with either survival or clinicopathological factors (22).

Preclinically, the majority of ovarian cancer cell lines siginificantly up-regulated DLEC1 transcripts after histone deacetylase (HDAC) inhibitor treatment (8). Moreover, exposure to the HDAC inhibitor PXD101 (belinostat) had varying effects on hepatocellular carcinoma cell lines (23). Thus, several HDAC inhibitors were found to exhibit antiproliferative activity and induce apoptosis in human cancer cells (24). Moreover, the restoration of tumor-suppressor genes, such as *DLEC1*, by HDAC inhibitors may contribute to antitumor effects.

Acknowledgements

The authors would like to thank Mrs. Tomomi Shibata for the excellent technical assistance. This work was supported by the Grand-in-Aid for Research, Nagoya City University (2006) and Grants-in-Aid for Scientific Research, Japan Society for the Promotion of Science (JSPS) (nos. 21591820 and 21390394).

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