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Hypermethylation of the large tumor suppressor genes in Japanese lung cancer

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Abstract. *Large tumor suppressor* (*LATS*) *1* and *2* are tumor suppressor genes implicated in the regulation of the cell cycle. The methylation statuses of the promoter regions of these genes were studied in Japanese lung cancers. The methylation statuses of the promoter regions of *LATS1* and *LATS2* were investigated by methylation-specific PCR. The findings were compared to clinicopathological features of lung cancer. Methylation-specific PCR showed that the *LATS1* promoter region was hypermethylated in 95 out of 119 (79.8%) lung cancers. The methylation status of *LATS1* was significantly associated with squamous histology (p=0.0267) and smoking status (never smoker vs. smoker; p=0.0399). *LATS1*-ummethylated patients harbored more EGFR mutations (p=0.0143). The *LATS2* promoter region was hypermethylated in 160 out of 203 (78.8%) lung cancers. However, the methylation status had no association with the clinicopathological characteristics of the lung cancers cases. Both the *LATS1* and *LATS2* methylation statuses did not correlate with survival of lung cancer patients. Thus, the EGFR methylation status of the *LATS* genes has limited value in Japanese lung cancers.

Introduction

Large tumor suppressor (*LATS*), which encodes a putative serine/threonine kinase, has been identified as a tumor suppressor gene in *Drosophila* (1,2). Deterioration of the *LATS* gene function results in promotion of cell proliferation and tumor formation in *Drosophila* (2). Two mammalian homologues of the *Drosophila LATS*, *LATS1* and *LATS2* have been identified. *LATS1*-deficient mice developed soft tissue sarcomas or ovarian stromal cell tumors, suggesting that *LATS1* is a tumor suppressor gene (3). Overexpression of *LATS1* causes G2-M arrest through the inhibition of CDC2 kinase activity *in vitro* (4). Furthermore, overexpression of *LATS1* significantly suppresses tumorigenicity *in vivo* by inducing apoptosis (4,5).

LATS2 overexpression results in cell cycle arrest in the G2/M phase via inhibition of Cdc2-cyclin B kinase activity leading eventually to apoptosis (6), inhibition of G1/S transition via down-regulation of Cdk2-cyclin E kinase activity (7), or apoptosis via down-regulation of Bcl-2 and Bcl-xL (8). *LATS2* binds to Mdm2 and inhibits its E3 ubiquitin ligase activity, resulting in the stabilization of p53 (9).

DNA methylation is an essential mechanism for the regulation of genes which contain a defined CpG island, and *LATS2* hypermethylation has been recently associated with an aggressive phenotype in breast cancers (10). Down-regulation of the *LATS2* gene is associated with poor prognosis in acute lymphoblastic leukemia (11). More recently, *LATS2* gene tumor-specific mutations and down-regulation have been reported in non-small cell carcinoma (12). These findings have led us to analyze the potential role of the promoter hypermethylation of the *LATS1* and *2* genes in non-small cell lung cancer (NSCLC) patients. In this study, the methylation statuses of the promoter regions of these genes were studied in Japanese lung cancers. The methylation statuses of the promoter regions of *LATS1* and *2* were investigated by methylation-specific PCR. The findings were compared to the clinicopathological features of the lung cancer cases.

Patients and methods

Patients. The study group included lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School. The lung tumors were classified according to the general rule for clinical and pathological recording of lung cancer in Japan (13). All tumor samples were immediately frozen and stored at -80˚C until assayed. Since Strazisar *et al* revealed that *LATS2* mutations were predominantly found in the squamous cell histotype of lung cancer while no mutations were found in adenocarcinoma (12), we mainly focused on squamous cell carcinoma for the *LATS2* sequencing study. The clinical and pathological characteristics of the 178 lung cancer patients for the *LATS2* sequencing analysis were as follows: 159 (89.3%) were male

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Table I. Clinicopathological data of 119 lung cancer patients.

Factors	*LATS1* gene status		P-value
	Methylated patients	Unmethylated patients	
Mean age (years)	64.5±8.9	66.3±13.3	0.2211
Stage			
I	38 (40.0%)	11 (45.8%)	0.6470
II-IV	57 (60.0%)	13 (54.2%)	
Lymph node metastasis			
N0	54 (56.8%)	16 (66.7%)	0.4880
N^+	41 (43.2%)	8 (33.3%)	
Smoking			
Never smoker	22 (23.2%)	11 (45.8%)	0.0399
Smoker	73 (76.8%)	13 (54.2%)	
EGFR mutation			
Wild-type	77 (81.1%)	13 (54.2%)	0.0143
Mutation	18 (18.9%)	11 (45.8%)	
Pathological subtypes			
SCC	36 (37.9%)	3 (12.5%)	0.0267
Non-SCC	59 (62.1%)	21 (87.5%)	
Age			
≤65	39 (41.1%)	9 (37.5%)	0.8190
>65	56 (58.9%)	15 (62.5%)	
Gender			
Male	75 (78.9%)	15 (62.5%)	0.1872
Female	20 (21.1%)	9 (37.5%)	

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

and 19 were female. One hundred and sixteen (65.2%) patients were diagnosed as squamous cell carcinomas, 42 were adenocarcinomas and 17 were adenosquamous cell carcinomas. One hundred and sixty-five (92.7%) were smokers and 13 were non-smokers. The clinicopathological characteristics of the lung cancer patients in the methylation analyses for *LATS1* and *2* are listed in Tables I and II, respectively. The samples from these patients were previously sequenced for EGFR (13-16).

PCR assays for LATS2 mutations. Total RNA was extracted from lung cancer tissues using the Isogen Kit (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. The RNA concentration was determined using a spectrophotometer and adjusted to a concentration of 200 ng/ml. Approximately 10 cases were excluded for each assay, since the tumor cells were too few to sufficiently extract tumor RNA. RNA (1 μg) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD, USA) with 0.5 μg oligo $(dT)_{12\text{-}16}$ (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The reaction mixture was incubated at 42˚C for 50 min and then at 72˚C for 15 min. We then used 1 μl of each DNA for PCR analysis. The PCR reactions were performed using the LA-Taq Kit (Takara Bio Inc., Shiga, Japan) in a 25-μl reaction volume. The primer sequences for the *LATS2* gene for exon 8 (including the S1073 region) were as follows: forward primer 5-CGACCCCGTAGATGAAGAAA-3 and reverse primer 5-AGCGATGCTGAGTCCTGTT-3 (454 bp, 3448-3901). The cycling conditions were as follows: initial denaturation at 94˚C for 5 min, followed by 40 cycles at 94˚C for 45 sec, 60˚C for 45 sec and 72˚C for 45 sec. The products were purified using the Qiagen PCR Purification Kit (Qiagen, Valencia, CA, USA). These samples were sequenced by ABI PRISM 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review.

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

The primer sequences for the *LATS1* gene for methylated (M) sequences were as follows: forward primer 5-GGAGTT CGTTTTGTC-3 and reverse primer 5-CGACGTAATAACG AACGCCTA-3. The primer sequences for the *LATS1* gene for unmethylated (U) sequences were as follows: forward primer 5-TAGGTTGGAGTGTGGTGGT-3 and reverse primer 5-CCC

Table II. Clinicopathological data of 203 lung cancer patients.

Factors	*LATS2* gene status		P-value
	Methylated patients	Unmethylated patients	
Mean age (years)	66.1±8.9	64.3±10.9	0.4076
Stage			
I	70 (43.8%)	22 (51.2%)	0.3946
II-IV	90 (56.2%)	21 (48.8%)	
Lymph node metastasis			
N0	95 (59.4%)	27 (62.8%)	0.7286
N^+	65 (40.6%)	16 (37.2%)	
Smoking			
Never smoker	35 (21.9%)	14 (32.6%)	0.1622
Smoker	125 (78.1%)	29 (67.4%)	
EGFR mutation			
Wild-type	126 (78.8%)	35 (81.4%)	0.8332
Mutation	34 (21.2%)	8 (18.6%)	
Pathological subtypes			
SCC	80 (50.0%)	18 (41.9%)	0.3920
Non-SCC	80 (50.0%)	25 (58.1%)	
Age			
≤65	68 (42.5%)	22 (51.2%)	0.3876
>65	92 (57.5%)	21 (48.8%)	
Gender			
Male	123 (76.9%)	31 (72.1%)	0.5490
Female	37 (23.1%)	12 (27.9%)	

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

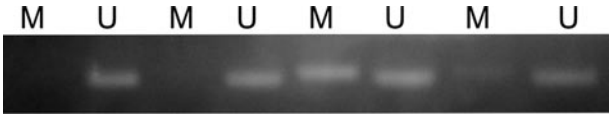

Figure 1. The results of the methylation-specific PCR analysis of *LATS2* in 4 lung cancers. M, methylated; U, unmethylated.

AACATAATAACAAACACCT-3. The primer sequences for the *LATS2* gene for methylated (M) sequences were as follows: forward primer 5-ATTTCGGTTTATTGTAATTTTC-3 and reverse primer 5-AACCAACATAATAAAACCCCG-3. The primer sequences for the *LATS2* gene for unmethylated (U) sequences were as follows: forward primer 5-TTTGTTTTTT GGGTTTAAGT-3 and reverse primer 5-CCAACATAATA AAACCCCA-3. The cycling conditions were as follows: initial denaturation at 94˚C for 5 min, followed by 40 cycles at 94˚C for 45 sec, 58˚C (*LATS1* and *LATS2*, M) or 53˚C (*LATS1*, U) or 50˚C (*LATS2*, 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's 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 the Spearman's and χ^2 tests. The overall survival of lung cancer patients was examined by the Kaplan-Meier method, and differences were examined by the log-rank test. Analysis was carried out using the StatView software package (Abacus Concepts Inc., Berkeley, CA, USA), and differences were considered significant at a p-value <0.05.

Results

LATS2 gene mutation status in Japanese lung cancer patients. We sequenced for the exon 8 of the *LATS2* gene in 178 NSCLC samples. No mutations were found in the 178 patients from the direct sequencing using cDNA samples.

LATS gene methylation statuses in Japanese lung cancer patients. Methylation-specific PCR showed that the *LATS1* promoter region was hypermethylated in 95 out of 119 (79.8%) lung cancers. The methylation status of *LATS1* was significantly associated with squamous histology (squamous cell carcinoma, 92.3% vs. non-squamous cell carcinoma, 73.8%; p=0.0267) and smoking status (never-smoker, 66.7% vs. smoker, 84.9%; p=0.0399). However, *LATS1* methylation status did not correlate with gender (p=0.1872), age (p=0.8190), lymph node

EGFR metastasis (p=0.4880) and pathological stages (I vs. II-IV; p=0.6470). *LATS1* ummethylated patients harbored more EGFR mutations (p=0.0143). *LATS1* methylation status did not correlate with patient survival (log-rank test; p=0.4109).

The *LATS2* promoter region was hypermethylated in 160 out of 203 (78.8%) lung cancers (Fig. 1). However, the methylation status revealed no associations with the clinicopathologic characteristics of the lung cancers. The *LATS2* methylation status did not correlate with patient survival (log-rank test; p=0.4598).

Discussion

We found that the *LATS* gene family was hypermethylated in Japanese lung cancers. We did not find correlations between the methylated statuses and gender, pathological stages and survival in Japanese NSCLC. Although the *LATS1* methylation status was correlated with smoking status, squamous histology and EGFR mutations, the methylation status of the *LATS* genes was of limited value in Japanese lung cancers. In addition, we did not find a *LATS2* mutation at exon 8, suggesting that an ethnic difference may exist.

The *LATS* tumor suppressor family has been shown to play an important role in the control of tumor development and the cell cycle (3-5,18,19). Mechanistic studies concerning *LATS1* revealed that it might control tumorigenesis by negatively regulating the cell cycle. Ectopic expression of *LATS1* in human cancer cell lines leads to the down-regulation of cyclin A and B at the protein level (5), and/or inactivation of CDC2 kinase, thereby blocking cells at G2/M and preventing tumor development in nude mice (4,5). Ectopic expression of *LATS1* in human tumor cell lines has also been shown to induce apoptosis by up-regulating the level of BAX protein (5) or up-regulating caspase-3 activity (4), indicating that *LATS1* may also control tumorigenesis by inducing apoptosis. Although it is unclear whether smoking induces the methylation of *LATS1*, the methylation also occurred more frequently in squamous cell carcinoma. Notably, a chromosomal alteration was frequently noted at chromosome 6q24 (20) where the *LATS1* gene is localized (18).

LATS2, also known as *KPM* (21), is the second mammalian member of the *LATS* tumor suppressor gene family (22). Human *LATS2* has been mapped onto human chromosome 13q11-12 (21), a hot spot (67%) for loss of heterozygosity in NSCLC (23). *LATS2* encodes a putative Ser/Thr protein kinase. The *LATS2* protein shares 85% sequence identify to human *LATS1* proteins in the kinase domain (21,22).

LATS2 has a role in the maintenance of mitotic fidelity and genomic stability, since *LATS*$^{-/-}$ mutant embryonic cells exhibit an increased frequency of cytokinetic defects, accumulation of micronuclei, supernumerary centrosomes and aneuploidy (24,25). *LATS2* also functions as an inducer of apoptosis through down-regulation of anti-apoptotic proteins of the Bcl-family (8). More recent findings implicate *LATS2* as the key mediator of the G1 tetraploidy checkpoint, while *LATS2* translocates into the nucleus by mitotic apparatus dysfunction and inactivates Mdm2 (9). Although down-regulation of the *LATS2* gene has been reported in several cancers (10,11) including lung cancer (12), in our analysis we did not find any correlation between *LATS2* methylation and clinicopathological features. We did not find a *LATS2* mutation at exon 8. An ethnic difference between the studies concerning mutant *LATS2* may exist.

In conclusion, the *LATS2* mutation in Japanese lung cancers appears to be extremely rare, and the methylation status of the *LATS* genes is of limited value in Japanese lung cancers.

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