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LINE-1 Hypomethylation Is Associated With Malignant Traits and Cell Proliferation in Lung Adenocarcinoma

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Abstract. *Background/Aim: Long interspersed nuclear element-1 (LINE-1) methylation status is a marker for global DNA methylation. However, the relationship between LINE-1 methylation and the biology of lung adenocarcinoma remains unclear. Here, we aimed to examine the role of LINE-1 in lung cancer. Materials and Methods: LINE-1 methylation levels were quantified by bisulfite pyrosequencing of resected tumor specimens from 162 patients with lung adenocarcinoma. The relationships of LINE-1 methylation with clinicopathological factors, gene mutations, and Ki-67 immunoreactivity were investigated. Results: LINE-1 hypomethylation was associated with tumor invasion and advanced stage. TP53 mutations were more frequently detected in the LINE-1 hypomethylation group than in the hypermethylation group. LINE-1 hypomethylation was associated with poor recurrence-free survival, high maximum standardized uptake value in positron-emission tomography, and high Ki-67 expression in tumors. Conclusion: LINE-1 hypomethylation was associated with high-grade malignancy and poor prognosis in lung adenocarcinoma, but was not related to driver mutations.*

Despite recent developments in molecular target therapy in cancer treatment, lung cancer is still the leading cause of cancer-related mortality worldwide. The overall 5-year survival rate is reported to be 2-30% (1). Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases, and adenocarcinoma is the most common cell type in NSCLC (2). Recent studies of lung adenocarcinoma biology have demonstrated high rates of tumor-suppressor mutations, such as *TP53* mutations (3). Moreover, driver oncogene mutations, such as epidermal growth factor receptor (*EGFR*) mutations and echinoderm microtubule-associated protein-like 4/anaplastic lymphoma kinase (*EML4-ALK*) rearrangements, have been discovered and could be effective therapeutic targets (4, 5).

With the development of next-generation sequencing, gene profiling of lung adenocarcinoma has greatly advanced (6). Accordingly, it has become essential to elucidate the cellular and molecular basis of cancer in order to utilize new molecular targets and biomarkers for treatments (7). Recently, epigenetic alterations, such as aberrant DNA methylation within the CpG dinucleotide, have been widely investigated as relevant genetic traits of cancer (8). Among such alterations, hypermethylation in the promoter regions of tumor-suppressor genes causes gene silencing and thus, has a major role in carcinogenesis (9). Promoter hypermethylation may have application as a marker or predictor in several cancers (10, 11). In contrast, global hypomethylation in the CpG dinucleotides of the cancer genome is also commonly observed in human cancers (11). Because of its associations with global loss of imprinting and increased chromosomal instability, global hypomethylation is thought to play a critical role in carcinogenesis.

Long interspersed nuclear element 1 (*LINE-1*) is a family of non-long terminal repeat retrotransposons interspersed throughout genomic DNA; these sequences comprise 17% of the human genome (12). *LINE-1* is composed of a 5'-untranslated region (UTR), two open-reading frames, and a 3'-UTR, and *LINE-1* elements make up much of the CpG methylation in the 5'-UTR regions in normal somatic cells. Thus, *LINE-1* methylation levels are thought to represent the global DNA methylation status (13). In recent studies, *LINE-1* hypomethylation has been shown to associate with the clinicogenetic features of early stage NSCLC (14) and other carcinomas (15-17).

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However, the relationships between *LINE-1* methylation levels and mutation status or cancer malignant traits in NSCLC are unclear. Therefore, in the current study, we investigated possible correlations of *LINE-1* methylation levels with cancer-related gene mutations and tumor malignant behaviors in lung adenocarcinoma.

Materials and Methods

Patients and samples. Tumor tissues and corresponding normal tissues were obtained from 181 consecutive patients who underwent resection for NSCLC without pre-operative therapy between April 2009 and December 2013 at the Department of Surgery and Science, Kyushu University Hospital (Fukuoka, Japan). This study included 97 men and 84 women, with a mean age of 68.0 years (range=37-85 years) at surgical resection. Tumor samples and corresponding non-malignant lung tissue samples (most distant from the tumor) were gained immediately after resection, frozen in liquid nitrogen. They were stored at −80˚C.

The tumor cell type was diagnosed based on the World Health Organization (WHO) histological classification of lung tumors, fourth edition (18). Pathological staging was determined according to the 7th edition of the TNM staging system (19). A routine check-up, involving a physical examination, chest X-rays, blood cell count measurements, serum chemistry, and serum tumor markers that included carcinoembryonic antigen (CEA) and cytokeratin fragment 19 (CYFRA), was performed four times per year for the first 3 years, and they were also checked twice a year thereafter. Computed tomography was performed twice a year for the first 2 years and once a year thereafter. The magnetic resonance imaging (MRI) for brain and bone scintigraphy or fluorodeoxyglucose positron-emission tomography (PET) were done annually. This study was approved by the Kyushu University Institutional Review Board for Clinical Research (approval no. 2020-113).

Bisulfite treatment and LINE-1 methylation analysis. *LINE-1* methylation levels were evaluated by pyrosequencing. Total DNA was extracted from the tissue samples using ISOGEN (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's recommendations. Fifty nanogram of the genomic DNA was used for the modification with sodium bisulfite with an EpiTect Bisulfite kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. DNA methylation levels of *LINE-1* were measured by a bisulfite pyrosequencing analysis (PyroMark Q24; Qiagen). The detailed protocol was described previously (20). Briefly, the nucleotide dispensation order was: GCT CGT GTA GTC AGT CG. This assay quantified the methylation levels of three CpG sites in positions 331-318 of *LINE-1*. A percentage of C nucleotide relative to the sum of C and T nucleotides at each CpG site was calculated. The relative amounts of C in the three adjacent CpG sites was averaged and was taken as the overall *LINE-1* methylation level; assays were performed in triplicate.

Mutation analysis. To detect *TP53* mutations, exons 5-9 of the *TP53* gene were amplified by polymerase chain reaction (PCR) using *TP53* primers from Nippon Gene, and mutations in *TP53* were detected in 125 patients with adenocarcinoma by PCR direct sequencing, as previously described (21). To detect *EGFR* mutations, the peptide nucleic acid-locked nucleic acid (PNA-LNA; Mitsubishi Chemical Medience, Tokyo, Japan) PCR clamp method was performed, using genomic DNA extracted from formalin-fixed paraffin-embedded sections of 156 adenocarcinoma surgical specimens (22).

To analyze *ALK* rearrangements, immunohistochemistry for *ALK* was performed for 156 patients with adenocarcinoma using an *ALK* detection kit (Nichirei Bioscience, Tokyo, Japan). The method was based on the intercalated antibody-enhanced polymer method with the 5A4 clone as the primary anti-*ALK* antibody. Immunohistochemistry results were scored as 0 (no specific staining within a tumor), 1+ (faint staining intensity in >10% of tumor cells without background staining), 2+ (moderate staining intensity), or 3+ (strong staining intensity) (23). The scoring was confirmed by two pathologists at Kyushu University Hospital.

Tissue preparation and Ki-67 immunohistochemistry. Primary lung carcinomas were fixed immediately in 10% (v/v) formalin after resection. After embedding in paraffin, serial 3-μm-thick sections were prepared from each sample and reserved for hematoxylin and eosin staining and immunohistochemical staining. Immunohistochemical staining for Ki-67 was performed as follows: Endogenous peroxidase was terminated at room temperature using 3% hydrogen peroxide in methanol for 30 min. The slides were blocked with normal goat serum before the slides were incubated with mouse monoclonal antibodies against Ki-67 (Dako, CA, USA) at a dilution of 1:100 at 4˚C overnight. The sections were then treated with goat anti-mouse immunoglobulin for 60 min at room temperature. Ki-67 immunostaining was performed by the streptavidin-biotin-peroxidase complex method using diaminobenzidine as a chromogen. The counterstaining with hematoxylin was performed afterwards.

To calculate the Ki-67 index, we observed five high-power fields in which more than 200 cancer cells could be counted, and the rate of stained cell nuclei among all nuclei in the field was determined. Ki-67 expression was categorized as 'positive' if more than 10% of cancer cell nuclei were stained and as 'negative' when 10% or fewer of the nuclei were stained (24).

Statistics. We calculated the value of T/N by dividing *LINE-1* methylation levels in tumor tissue (%) by *LINE-1* methylation levels in normal lung tissue (%) for each case. *LINE-1* methylation levels in lung adenocarcinomas were categorized into two groups by splitting the value of T/N at the median: >0.967 (hypermethylation) and ≤0.967 (hypomethylation).

Intergroup differences in patient characteristics, such as age, sex, smoking history, tumor invasion, and gene mutation status, were assessed using Student's *t*-tests and χ^2 tests. Mann–Whitney *U*-tests were used to compare pathological stages and tumor differentiation. Survival curves were estimated using the Kaplan–Meier method and assessed by log-rank tests. Recurrence-free survival (RFS) was defined as the time interval from the operation to the detection of recurrence or to death from any cause, whichever occurred first. A univariate survival analysis was done using the Cox proportional hazards model. In multivariate survival analysis, age, sex, and procedure were analyzed as variables in a stepwise manner. Statistical differences were considered to be significant if the *p*-value was below 0.05. All statistical data were analyzed using JMP statistical software version 9.0.2 (SAS Institute Inc.).

Results

LINE-1 methylation levels in NSCLC tissues and corresponding non-neoplastic lung tissues. We first examined

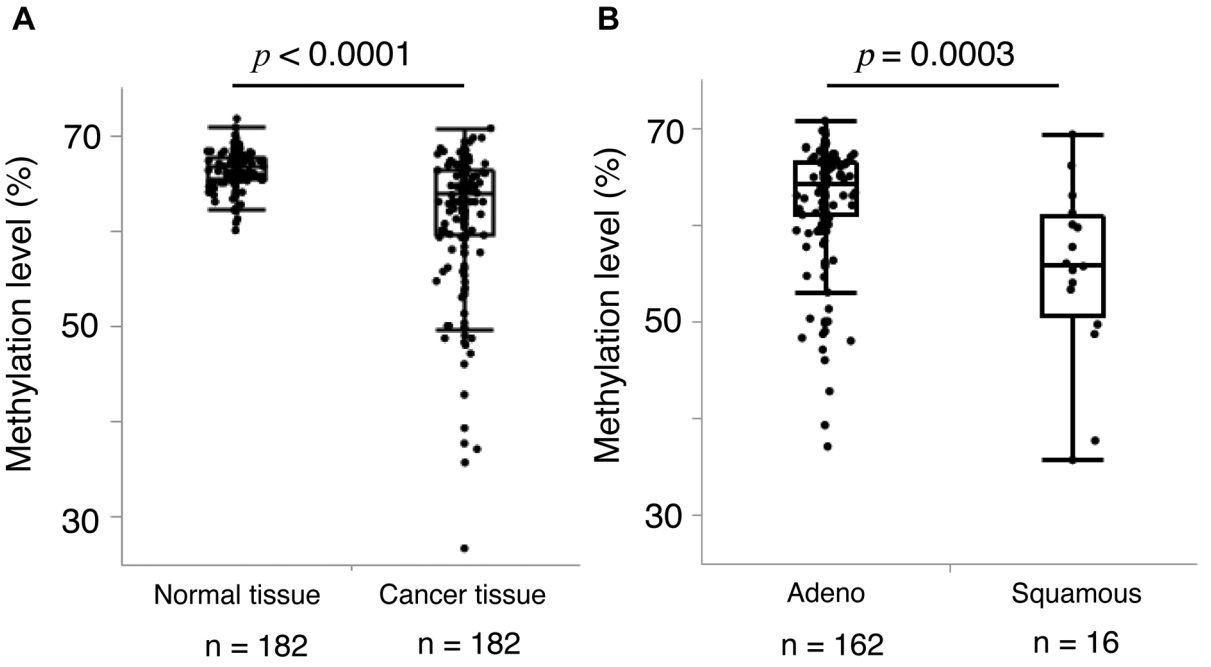

Figure 1. *Long interspersed nuclear element 1 (LINE-1) methylation levels in non-small-cell lung cancer tissues and corresponding non-neoplastic lung tissues were significantly different (A). LINE-1 methylation levels were significantly higher in squamous cell carcinoma than in adenocarcinoma (B).*

LINE-1 methylation levels in NSCLC tissues and corresponding non-neoplastic lung tissues using bisulfite pyrosequencing. *LINE-1* methylation levels were significantly higher in carcinoma tissues (64.0%±7.1%) than in corresponding normal lung tissues (66.6%±1.8%; $p<0.0001$; Figure 1A). Moreover, *LINE-1* methylation levels were significantly lower in squamous cell carcinoma (55.8%±10.7%) than in adenocarcinoma (64.6%±6.0%; $p=0.0003$; Figure 1B).

Relationships between LINE-1 methylation levels and clinicopathological factors. In univariate analysis, no significant correlations were found between tumor *LINE-1* methylation levels and clinicopathological factors, such as age, sex, and smoking history (Table I). We found significantly more patients with malignant features, such as high histological grade ($p=0.026$), pleural invasion ($p=0.0001$), lymphatic invasion ($p=0.0078$), vascular invasion ($p=0.0010$), and high pathological TNM stage ($p=0.0048$) in the *LINE-1* hypomethylation group than in the hypermethylation group (Table I).

Relationships between LINE-1 hypomethylation and tumor-related mutations in lung adenocarcinoma. We found *TP53* mutations in 18 of 125 (14.4%) lung adenocarcinomas. The *LINE-1* hypomethylation group had significantly more *TP53* mutation-positive tumors (14/63) than the hypermethylation group (4/62, $p=0.012$; Table II). There were no significant differences in the number of tumors harboring *EGFR* mutations (available samples: n=153, $p=0.68$) or *ALK* fusions (available samples: n=156, $p=0.21$) between the groups.

Influence of LINE-1 methylation level on survival. Survival analysis was performed in 162 patients with lung adenocarcinoma who underwent curative resections. Median follow-up time was 594 days (range=11–1594 days). *LINE-1* hypomethylation in lung adenocarcinomas was associated with poor prognosis in terms of RFS ($p=0.0007$; Figure 2A). In a subgroup analysis of stage IA cases, RFS was also significantly worse for patients with *LINE-1* hypomethylation ($p=0.042$; Figure 2B). Subgroup analysis showed that *LINE-1* methylation had no prognostic value in patients with stage IB ($p=0.22$; Figure 2C) and stage IIA-IIIA ($p=0.62$; Figure 2D) lung adenocarcinoma.

Relationship between LINE-1 methylation levels and maximum standardized uptake value (SUV-max). Next, we examined the relationship between *LINE-1* methylation levels and the SUV-max in PET images. We were able to assess data for SUV-max of tumors from the clinical charts of 155 patients. The SUV-max level was significantly higher in hypomethylation cases than in hypermethylation cases ($p<0.0001$; Figure 3A). Moreover, *LINE-1* methylation levels in tumor sites were correlated with Ki-67 expression ($p<0.0001$, $R^2=0.173$; Figure 3B, left). The SUV-max level

Table I. *Clinicopathological characteristics according to LINE-1 methylation level in patients with lung adenocarcinoma (n=162).*

Characteristic			*LINE-1* methylation level		*p*-Value
		Number (n=162)	High (n=81)	Low (n=81)	
Age (years)		69 (37-85)	70 (37-84)	68 (48-85)	**0.81**
Gender	Male	82 (50.6%)	38 (46.3%)	44 (53.7%)	0.35
	Female	80 (49.4%)	43 (53.8%)	37 (46.2%)	
Smoking history	Never	79 (48.8%)	44 (55.7%)	35 (44.3%)	0.16
	Current or former	83 (51.2%)	37 (44.6%)	46 (55.4%)	
Histological grade	G1	84 (51.8%)	51 (60.7%)	33 (39.3%)	0.026
	G2	57 (35.2%)	22 (38.6%)	35 (61.4%)	
	G3	21 (13.0%)	8 (38.1%)	13 (61.9%)	
	G4	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Pleural invasion	Negative	128 (79.0%)	75 (58.6%)	53 (41.4%)	0.0001
	Positive	34 (21.0%)	6 (17.6%)	28 (82.4%)	
Lymphatic invasion	Negative	140 (86.4%)	76 (54.3%)	64 (45.7%)	0.0078
	Positive	22 (13.6%)	5 (22.7%)	17 (77.3%)	
Vascular invasion	Negative	110 (67.9%)	65 (59.1%)	45 (40.9%)	0.0010
	Positive	52 (32.1%)	16 (30.8%)	36 (69.2%)	
Pathological stage	IA	96 (59.2%)	56 (58.3%)	40 (41.7%)	0.031
	IB	27 (16.7%)	11 (40.7%)	16 (59.3%)	
	IIA-IV	39 (24.1%)	14 (35.9%)	25 (64.1%)	

Data are presented as n (%) or median (range). *LINE-1*: Long interspersed nuclear element-1.

Table II. *Relationship between LINE-1 methylation level and gene mutations in lung adenocarcinoma.*

Characteristic			*LINE-1* methylation level		*p*-Value
		Number (n=162)	High (n=81)	Low (n=81)	
TP53 mutation (n=125)	Negative	107 (85.6%)	58 (54.2%)	49 (45.8%)	0.012
	Positive	18 (14.4%)	4 (22.2%)	14 (77.8%)	
EGFR mutation (n=153)	Negative	76 (49.7%)	36 (47.4%)	40 (52.6%)	0.68
	Positive	77 (50.3%)	39 (50.6%)	38 (49.4%)	
EML4-ALK fusion (n=155)	Negative	146 (93.6%)	73 (50.0%)	73 (50.0%)	0.21
	Positive	10 (6.4%)	3 (30.0%)	7 (70.0%)	

Data are presented as n (%) or median (range). *LINE-1*: Long interspersed nuclear element-1; *EGFR*: epidermal growth factor receptor; *EML4-ALK*: echinoderm microtubule-associated protein-like 4/anaplastic lymphoma kinase.

was also correlated with Ki-67 expression ($p<0.0001$, $R^2=0.382$; Figure 3B, right).

Discussion

Global hypomethylation, or genome-wide hypomethylation, has been shown to be related to carcinogenesis and cancer development (11). In addition to being connected with abnormal expression of cancer-related genes, global hypomethylation is also thought to be related to genomic instability in cancers (25). Recent studies have reported that *LINE-1*, as a marker of global methylation, is hypomethylated in several cancers compared with adjacent normal tissues (14). In NSCLC, *LINE-1* methylation levels are generally decreased in cancer tissues compared with normal tissues (14, 25-27). In accordance with these previous studies, our current findings showed that *LINE-1* was significantly hypomethylated in lung cancer tissues compared with that in adjacent normal lung tissues.

In this study, we found that *LINE-1* methylation levels were significantly lower in squamous cell carcinoma than in adenocarcinoma. Only two other studies have reported histological differences in *LINE-1* methylation levels in

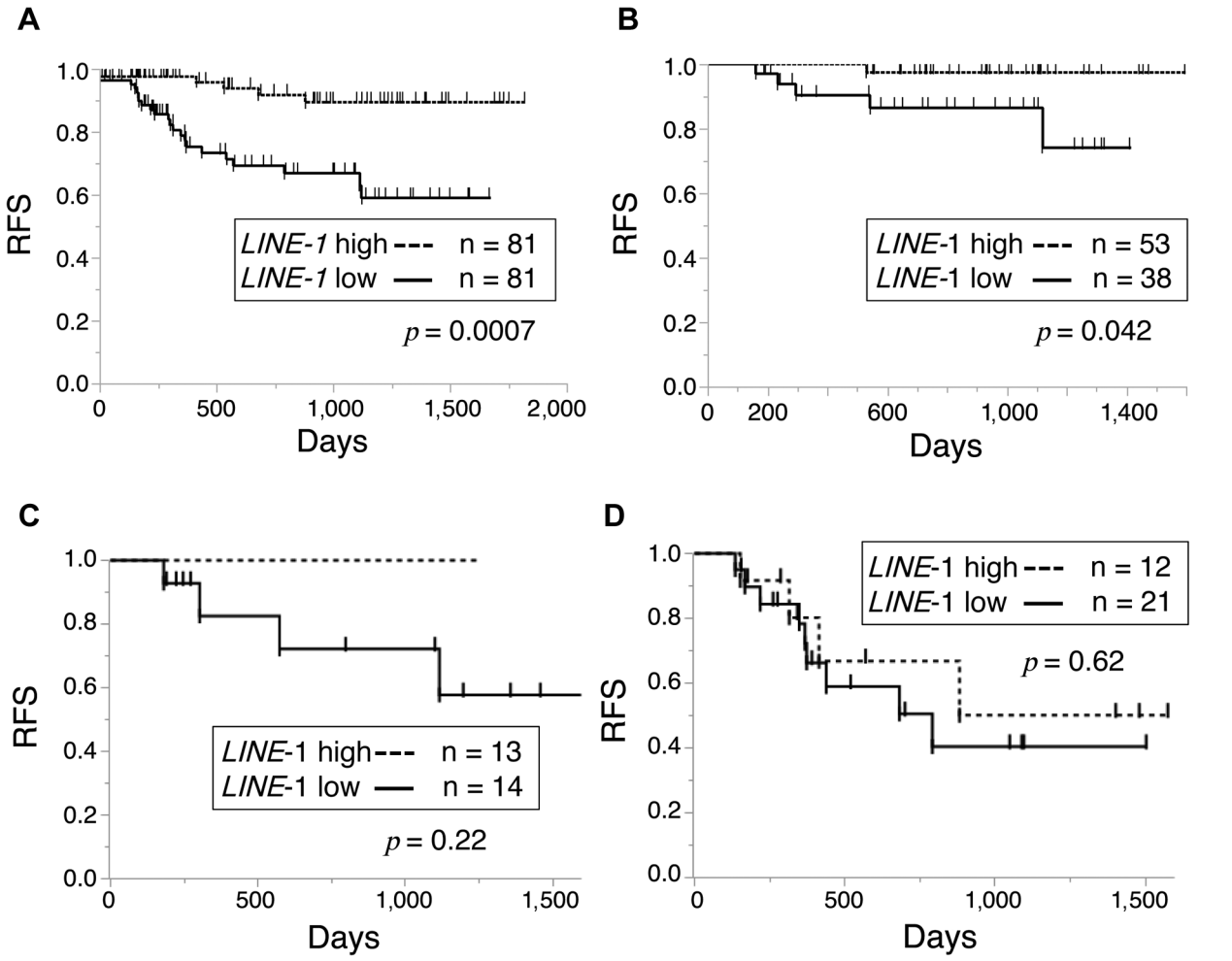

Figure 2. *Kaplan–Meier curves of recurrence-free survival (RFS) according to LINE-1 methylation levels in lung adenocarcinoma. (A) Kaplan–Meier curves of RFS for patients with all pathological stages. In a subgroup analysis, Kaplan–Meier curves of RFS in stage IA cases (B) showed significant differences. Kaplan–Meier curves of RFS in stage IB (C) and stage IIA-IIIA (D) are shown.*

NSCLC. For example, in their analysis of 246 patients with NSCLC, Saito *et al*. reported that the median value of *LINE-1* methylation was 87.9% in adenocarcinomas and 64.2% in squamous cell carcinomas (14). In another study, the mean *LINE-1* methylation level was also significantly lower in squamous cell carcinoma than in adenocarcinoma (37.8% *versus* 63.4%, respectively; $p<0.001$) (27). The two studies also showed that *LINE-1* hypomethylation was concomitant with smoking habit, suggesting a possible relationship between *LINE-1* hypomethylation and tobacco smoking. In fact, tobacco smoke has been shown to be a strong modifier of DNA methylation (28). However, in our analysis of patients with adenocarcinoma, we did not find any connection between *LINE-1* hypomethylation and tobacco smoking. In a study by Imperatori *et al*., the cut-off level for dichotomizing the methylation status was determined by the *LINE-1* methylation percentage in tumor samples (58% was provided by a model-based cluster algorithm). In contrast, in this study, we used the median of the ratio of the methylation level in cancer cells to that in normal cells. This difference may account for the different results observed in our study and the previous study with regard to the relationship between *LINE-1* methylation and tobacco smoking.

In our study, *LINE-1* hypomethylation was found to be associated with tumor malignant features and poor RFS after surgery in patients with lung adenocarcinoma. The prognostic difference was particularly significant in Stage I patients. Saito *et al*. previously reported that *LINE-1* hypomethylation was an independent marker for poor prognosis in surgical patients with stage IA NSCLC (14). Additionally, the study by Imperatori demonstrated similar results in stage I NSCLC (27). However, both studies did not report the prognostic significance of *LINE-*

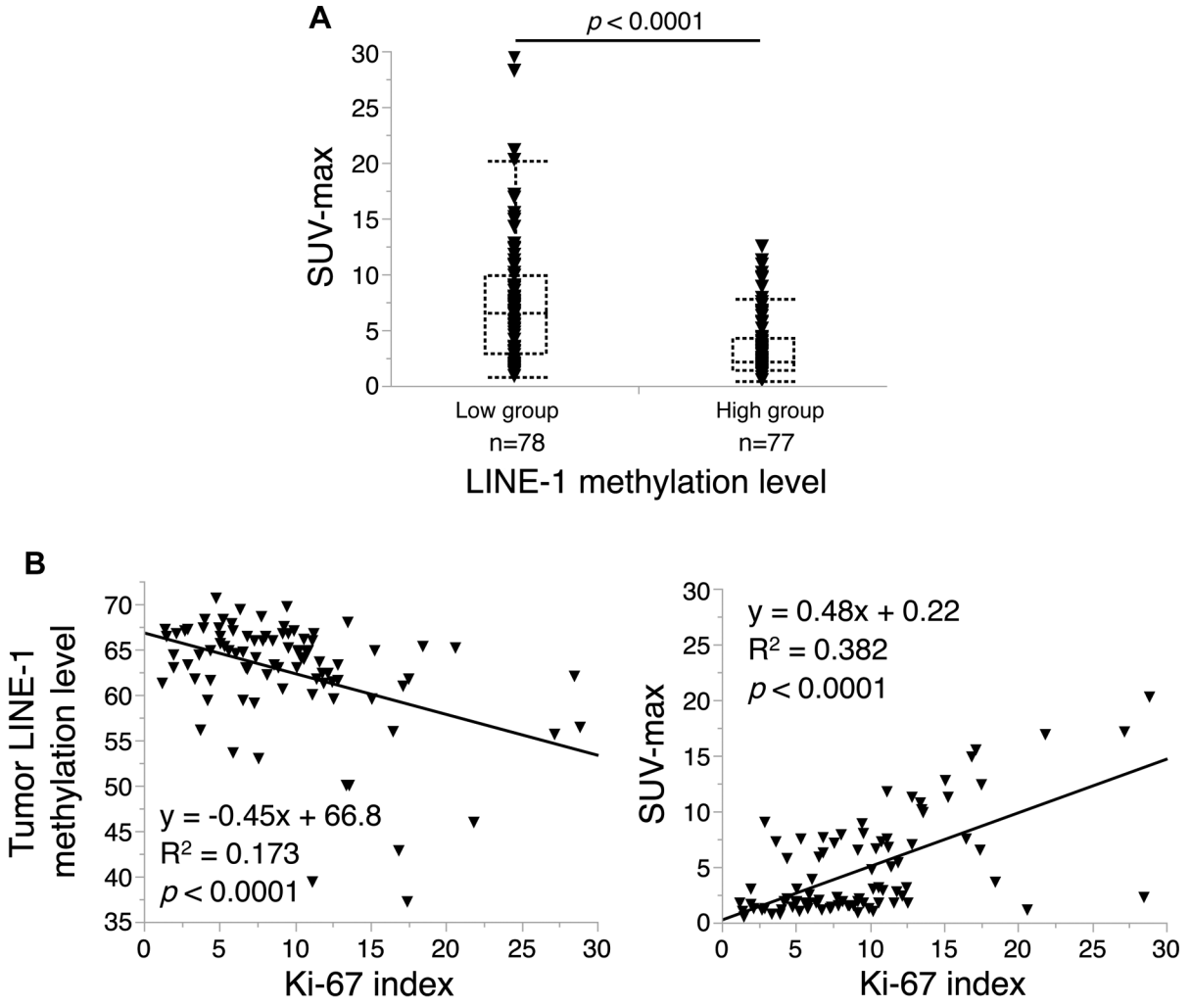

Figure 3. *Relationship between LINE-1 methylation levels and SUV-max values in FDG-PET imaging (A); p-values are shown. Association of Ki-67 index with LINE-1 methylation levels (left) and SUV-max (right) in lung adenocarcinoma. The coefficient of determination (R2) and p-value are shown.*

1 methylation in lung adenocarcinoma only. In a study of 211 patients with lung adenocarcinoma using pyrosequencing, Ikeda *et al*. found that hypomethylation of *LINE-1* was associated with advanced cancer stage and vascular invasion of the tumor (29). They also demonstrated that the lowest quartile of the *LINE-1* methylation level in tumors was associated with poor disease-free survival. In their subgroup analyses, this association was only found in patients with stage II and stage III disease, but not in patients with stage I disease. Conversely, in the current study, we found a significant relationship between *LINE-1* hypomethylation and poor survival in stage I patients. In another study, Rhee *et al*. reported the opposite relationship between *LINE-1* methylation and prognosis in patients with stage I adenocarcinoma (30). They observed this tendency in two different analyses using different definitions of hypomethylation, *i.e.,* according to the percentage of all *LINE-1* methylation assays and according to the ratio of tumor to normal tissue methylation level. However, from our literature search, their study is the only study to have demonstrated a connection between *LINE-1* hypomethylation and good survival in patients with lung cancer.

In this study, we demonstrated that the frequency of *TP53* mutations was significantly correlated with *LINE-1* hypomethylation in lung adenocarcinomas. Our data supported the findings of a previous report by Imperatori *et al*. They showed a correlation between p53 immunoreactivity and *LINE-1* hypomethylation in NSCLC, although the detection method for *TP53* mutations was different from the method used in our study. Several other studies showed that hypomethylation of *LINE-1* is associated with *TP53* mutations in solid tumors other than lung cancer, such as esophageal squamous cell carcinoma and hepatocellular carcinoma (19, 31). However, the cause of

the relationship between *LINE-1* hypomethylation and *TP53* mutation has not yet been elucidated. Genomic instability caused by loss of p53 function may contribute to global DNA hypomethylation in cancers.

We found no association between *LINE-1* hypomethylation and *EGFR* mutations or *ALK* rearrangements. Similarly, the study by Imperatori *et al*. also showed no correlation between *LINE-1* hypomethylation and driver mutations in lung adenocarcinoma. These findings suggest that global methylation may have little association with the development of lung adenocarcinoma harboring driver mutations.

In the current study, the SUV-max value of FDG-PET imaging was significantly higher in hypomethylation cases than in hypermethylation cases, and the SUV-max value was correlated with Ki-67 expression in tumors. Moreover, *LINE-1* methylation level was inversely correlated with Ki-67 expression. To the best of our knowledge, this study is the first to show an interactive association between *LINE-1* hypomethylation and the SUV-max with regard to clinical outcomes in patients with lung adenocarcinoma. Our group previously reported that the SUV-max was associated with the expression of Ki-67, which reflects cancer aggressiveness (32). Thus, our current findings suggested that low global methylation levels in cancers may induce tumor proliferation and therefore, may contribute to the malignant traits in lung adenocarcinoma.

In conclusion, we showed that *LINE-1* hypomethylation was significantly associated with malignant features in lung adenocarcinoma. Patients with *LINE-1* hypomethylation had poor RFS, especially those with stage I disease. Thus, our findings suggested that *LINE-1* methylation in tumors may predict prognosis in patients with lung adenocarcinoma.

Conflicts of Interest

All Authors declare no conflicts of interest in association with this study.

Authors' Contributions

H.K., T.O., H.K., and Y.M. participated in the study conception and design. H.K., S.S., and M.K. participated in the data acquisition; H.K., Y.M. and H.K. participated in the data analysis; S.O. and Y.O. participated in the pathological examination and revision; H.K., T.O., T.T. and M.M participated in the drafting of manuscript; All Authors approved the final draft.

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