Long Interspersed Nucleotide Element 1 Hypomethylation Is Associated With Poor Prognosis of Lung Adenocarcinoma

Koei Ikeda, MD, PhD, Kenji Shiraishi, MD, PhD, Ayami Eguchi, MT, Hidekatsu Shibata, MD, PhD, Kentaro Yoshimoto, MD, PhD, Takeshi Mori, MD, PhD, Yoshifumi Baba, MD, PhD, Hideo Baba, MD, PhD, and Makoto Suzuki, MD, PhD

Departments of Thoracic Surgery, and Gastroenterological Surgery, Graduate School of Medical Science, Kumamoto University, Kumamoto, Japan

Background. Genome-wide DNA hypomethylation is known to play important roles in genomic instability and carcinogenesis. Methylation in long interspersed nucleotide element 1 (LINE-1) is a good indicator of the global DNA methylation level within a cell. The aim of this study was to evaluate prognostic significance of LINE-1 hypomethylation in lung adenocarcinoma.

Methods. A consecutive series of 211 lung adenocarcinoma patients who underwent curative resections without any preoperative chemotherapy or radiotherapy at Kumamoto University Hospital between April 2010 and December 2012 were included. The LINE-1 methylation levels were quantified in tumor and noncancerous tissue by Pyrosequencing assay.

Results. Higher histologic grade and positive findings for vascular invasion were significantly associated with lower methylation levels. The disease-free survival in the hypomethylation group was significantly shorter than that of the non-hypomethylation group. The prognostic difference was more obvious in advanced cases (stage II, III) than in stage I cases.

Conclusions. The LINE-1 methylation level is associated with histologic grade and vascular invasion of lung adenocarcinoma. Additionally, LINE-1 hypomethylation is a useful biomarker to predict early recurrence of lung adenocarcinoma.


Adenocarcinoma (AD) is the most frequent subtype of non-small cell lung cancer (NSCLC) [1]. Although major improvements have been made in survival with NSCLC, the impact on long-term survival has remained modest [2]. A better understanding of the molecular pathogenesis of lung AD is now needed to identify biomarkers that would enable early detection and development of novel therapeutic targets.

In the last decade, aberrant gene function and transcriptional silencing by CpG island hypermethylation have been recognized as key components in the initiation and progression of cancer [3]. The DNA methylation leads to the silencing of genes that are critical in several pathways involved in human cancer [4, 5]. On the other hand, global DNA hypomethylation is reported to play an important role in genomic instability, leading to cancer development [6, 7]. Long interspersed nuclear element 1 (LINE-1) is a family of non-long terminal repeat retrotransposons comprising almost 21% of the human genome. Because of the high frequency in the genome, LINE-1 methylation is a useful marker of global methylation status [8, 9]; LINE-1 is heavily methylated in normal human tissues. In contrast, the hypomethylation is reported in many types of human neoplasms, and its prognostic significance for several types of neoplasm has been shown [10–15].

In this study, LINE-1 Methylation was quantified in 211 samples of curatively resected lung ADs by utilizing a bisulfit polymerase chain reaction (PCR)-pyrosequencing assay. The correlation with clinicopathologic factors and prognostic significance of LINE-1 hypomethylation was examined.

Material and Methods

Patients and Tissue Samples

Frozen samples of tumor and corresponding noncarcinoma lung tissue were obtained from a consecutive series of 211 lung AD patients who underwent curative resections without any preoperative chemotherapy or radiotherapy at Kumamoto University Hospital between April 2010 and December 2012. The patients consisted of 112 males and 99 females, ages from 28 to 87 years (68.6 ± 9.8 years). Information on smoking history (using the pack-year) was available for all cases. Disease stage was determined in accordance with the 7th edition of the TNM Classification for Lung and Pleural Tumors [16]. Pathologic...
Hypomethylation of Lung Adenocarcinoma Tissues

Methylation levels of resected lung cancer tissue and matched normal lung cancer tissue were quantified by using pyrosequencing. The mean methylation level of lung adenocarcinoma tissues was 70.3% ± 7.7%, which was significantly lower than that of matched normal lung tissues (normal lung: 73.5% ± 2.7%, p < 0.001, by the paired t test; Fig 1). The association between clinical characteristics and methylation levels of lung adenocarcinoma tissues is shown in Table 1. Higher histologic grade (p = 0.001) and positive findings for vascular invasion (p = 0.03) were significantly associated with lower methylation levels. Age, gender, smoking status, and epidermal growth factor receptor mutation were not associated with methylation levels.

Prognostic Significance of LINE-1 Methylation Level

Among the 211 cases of lung adenocarcinomas, 15 patients had tumor recurrences. The median follow-up period of patients was 15.2 months. For survival analysis, they were divided into 4 groups by their LINE-1 methylation levels: ie, the first quartile cases (Q1), 74.36% to 80.65%; the second quartile cases (Q2), 72.06% to 74.30%; the third quartile cases (Q3), 69.10% to 72.02%; and the fourth quartile cases (Q4), 18.86% to 69.09%. The disease-free survival of the 4 groups is shown in Figure 2A. The 2-year survival rate of Q1, Q2, Q3, and Q4

Statistical Methods

Data are expressed as the mean ± SD for continuous data and numbers and percentages for categoric data. All statistical analyses were performed using SPSS for Windows (version 15; Texas Instruments, Warrentville, IL). Differences between continuous variables were evaluated using 2-tailed Student t tests, and category data were compared using χ2 tests. Odds ratio (OR), 95% confidence intervals (CI), and corresponding p values were analyzed. A p value less than 0.05 was considered statistically significant. For the survival analysis, the Kaplan-Meier method was used to assess the survival time distribution, and the log-rank test was used. A multivariate, stage-stratified Cox proportional hazard model was constructed to compute a hazard ratio according to LINE-1 methylation status, containing sex (male versus female), age at surgery (≥70 vs <70), tobacco use (yes versus no), histologic grade (G1 vs G2–4) and pathologic stage (stage I vs stage II and III). An interaction was assessed by including the cross product of the LINE-1 variable and another variable of interest in a multivariate Cox model, and thereafter the Wald test was performed.
was 97.4%, 88.9%, 88.9%, and 81.2%, respectively. In a log-rank test, Q4 cases had significantly higher recurrence rates compared with Q1 cases (Fig 2A: \( p = 0.03 \)). The other combination of cases showed no significant difference. Thus, we defined Q4 cases as the hypomethylation group and the other cases (Q1 to Q3) as the non-hypomethylation group. The disease-free survival in the hypomethylation group was significantly shorter than that of the non-hypomethylation group (\( p = 0.03 \): Fig 2B).

In a subgroup analysis of stage I cases, there was no significant difference between the 2 groups (\( p = 0.48 \): Fig 2C). In contrast, the hypomethylation group of advanced stage (stage II, III) showed significantly shorter disease-free survival than the non-hypomethylation group (\( p = 0.05 \): Fig 2D). Additionally, adjuvant chemotherapy was not showing significant relationship with the prognosis of this group.

### Multivariate Analysis of Disease-Free Survival

A Cox regression analysis with disease-free survival was performed. We included the variables of age (over 70 years), gender (male), smoking history (positive), histologic grade (grade 2 or 3), pathologic stage (stage II or III), vascular invasion (positive), adjuvant chemotherapy (positive), and LINE-1 methylation status (hypomethylation group) in this analysis. The pathologic stage (OR = 23.70, \( p = 0.001 \)) and LINE-1 methylation status (OR = 8.40, \( p = 0.004 \)) were revealed to be independent prognostic factors (Table 2).

### Comment

In previous studies, LINE-1 hypomethylation was reported to be related to worse prognosis of NSCLC, and the methylation rate was significantly lower in squamous cell carcinoma than in AD [18, 19]. Squamous cell carcinoma and AD are the 2 major histologic subtypes of NSCLC, and their pathogenesis and clinical behavior are different. Therefore, we analyzed the prognostic significance of LINE-1 methylation level in lung AD cases and first demonstrated its association with early relapse. We used a quantitative pyrosequencing assay for LINE-1 methylation; that is a robust, accurate and reproducible method to quantitate methylations [20]. There was a significant difference between lung AD tissues and normal lung, and the methylation levels of LINE-1 in lung AD samples were significantly associated with their histologic grade and vascular invasion. The hypomethylation of LINE-1 seems to be associated with more aggressive features of lung AD and the hypomethylation group of lung AD cases had significantly shorter disease-free intervals after curative resection.

Although global hypomethylation is reported to be a worse prognostic factor in many types of neoplasms [10–15], the mechanisms that contribute to poor prognosis are not well known. Several reports describe the correlation between global hypomethylation and genomic instability [21, 22], which are known to indicate poor prognosis of NSCLC [23–25]. Transcriptional dysregulation might be another possible mechanism, and activation of proto-oncogenes might affect the aggressiveness of the tumor. Furthermore, in addition to its role as a surrogate marker for global DNA methylation, LINE-1 methylation status by itself likely has biological effects as retrotransposons [26, 27]. Increased expression of LINE-1 after hypomethylation may be associated with chromosomal breaks through an increase in nuclease activity, result in chromosomal instability, and lead to a variety of genomic alterations that could contribute to aggressive phenotype of cancers.

In our study, LINE-1 hypomethylation was demonstrated to be related to early recurrence of lung adenocarcinoma, especially in advanced cases that often received adjuvant chemotherapies. Because studies have shown that genomic instability is a negative predictive marker of response to cytotoxic agents [28–32], this might be the reason that LINE-1 hypomethylation cases showed poor prognosis. On the other hand, preclinical studies suggest that colon cancer cells with genomic instability and tumor xenografts are more sensitive to irinotecan compared with microsatellite stable cells [33–36]. Together this suggests that it might be necessary to treat lung AD differentially depending on the methylation status.

We recognize that there are some limitations to our study. For example, because of the short follow-up period, we could not demonstrate a correlation between LINE-1 methylation status and overall survival. Additionally, tumor recurrence was detected in only 3 stage I patients, and the association between their LINE-1 methylation status and tumor recurrence was unclear. A
larger prospective study would provide some insight into this possible relationship.

In conclusion, we demonstrated the significant prognostic effect of LINE-1 hypomethylation in lung AD cases. The LINE-1 hypomethylation was significantly correlated with their histologic grade and vascular invasion. The LINE-1 hypomethylation group showed significantly shorter disease-free survival than in the non-hypomethylation group.

Table 2. Multivariate Analysis of Disease-Free Survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>3.22 (0.42–24.84)</td>
<td>0.26</td>
</tr>
<tr>
<td>Older age</td>
<td>0.72 (0.20–2.64)</td>
<td>0.62</td>
</tr>
<tr>
<td>Smoking</td>
<td>2.38 (0.40–14.02)</td>
<td>0.34</td>
</tr>
<tr>
<td>Histologic grade</td>
<td>1.67 (0.39–7.08)</td>
<td>0.49</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>0.51 (0.09–2.78)</td>
<td>0.43</td>
</tr>
<tr>
<td>Stage</td>
<td>23.70 (4.15–135.42)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
<td>1.59 (0.36–6.33)</td>
<td>0.57</td>
</tr>
<tr>
<td>LINE-1 hypomethylation</td>
<td>8.40 (1.99–35.54)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

CI = confidence interval; LINE-1 = long interspersed nucleotide element 1.

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan (23592069) (24592096).

References

2. Sakai T, Tsushima T, Kimura D, Hatanaka R, Yamada Y, Fukuda I. A clinical study of the prognostic factors for postoperative early recurrence in patients who underwent...

INVITED COMMENTARY

Cure rates after resection of non-small cell lung cancer (NSCLC) remain unacceptably low. We are in desperate need of markers that can provide insight into tumor biology, predict a more aggressive disease course, and guide the use of adjuvant therapies. This work from Ikeda and colleagues [1] proposes that tumor hypomethylation of long interspersed nuclear elements 1 (LINE-1) could serve as that type of marker in resected adenocarcinoma of the lung. LINE-1 is widely recognized to be hypomethylated in tumor tissues compared with normal...