ABSTRACT

Cigarette smoking is the most common cause of lung cancer. The greatest risk of lung cancer is among those who started smoking early in life and continued throughout their lives. However, the molecular mechanisms responsible for the susceptibility to lung cancer in the young smoker are not clear. Recently, several groups have reported that the hypermethylation of CpG islands is associated with exposure to tobacco smoke. We studied the association between the age at starting smoking and hypermethylation of p14, p16, and RASSF1A promoters in 204 primary non-small cell lung cancer patients. We also examined whether hypermethylation of the RASSF1A promoter is an independent prognostic factor. Methylation rates in the 204 samples were detected in 9% for p14, 27% for p16, and 32% for RASSF1A. There was no relationship between the hypermethylation of p14 and p16 and the age at starting smoking. However, hypermethylation of the RASSF1A promoter was found to be significantly associated with the age at starting smoking (P = 0.001). No relationship was found between the methylation status of the RASSF1A promoter and other smoking variables, such as pack-years, smoking status, and the duration of smoking. The age at starting smoking in patients with hypermethylation of RASSF1A was earlier than that of patients without hypermethylation of the RASSF1A promoter (19 ± 8 versus 25 ± 7; P = 0.001). Young smokers who started smoking before age 19 were 4.23 times [95% confidence interval (CI) = 1.03–9.67; P = 0.001] more likely to have hypermethylation of the RASSF1A promoter than smokers who started smoking after the age of 19. Furthermore, hypermethylation of the RASSF1A promoter was found to be associated with a poor prognosis in non-small cell lung cancer patients at stages 1 and 2 (P = 0.02 and 0.01, respectively; Log-rank test). The hazard of failure was 3.27 times higher for patients with hypermethylation of the RASSF1A promoter than for those without hypermethylation of the RASSF1A promoter (95% CI = 1.42–8.71; P = 0.01). Young smokers who started the habit before the age of 19 also had a poorer prognosis than those who started after the age of 19 (hazard ratio = 2.14, 95% CI = 1.22–9.11; P = 0.02). Our results suggest that starting cigarette smoking at an early age is associated with hypermethylation of the RASSF1A promoter and that hypermethylation of the RASSF1A promoter may be an independent prognostic factor in primary non-small cell lung cancer.

INTRODUCTION

Lung cancer is one of the most common malignancies in the world, and smoking has been definitively established as the major cause of lung cancer. The occurrence of lung cancer depends on the duration of smoking, and its risk is greatest among those who started smoking early in life and continued smoking throughout their lives. However, the molecular mechanism responsible for the increased risk of lung cancer in young smokers remains a mystery.

Recently, several groups have reported that DNA hypermethylation is associated with exposure to tobacco smoke (1–5). The prevalence of hypermethylation at the D17S5 locus was found to be significantly higher in smokers than in nonsmokers in both tumors and non-tumorous lung tissues (1). The de novo methylation of the p16 promoter was reported to occur at a high frequency in lung cancer induced by the inhalation of cigarette smoke in F344/N rats (2). Hypermethylation of the p16 promoter was also detected in 94% of rat adenocarcinomas induced by tobacco-specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (3). Kim et al. (4) reported that hypermethylation of the p16 gene is associated with the duration of smoking in primary NSCLC.3 Recently, aberrant promoter hypermethylation of the p16 gene was detected in the bronchial epithelium and sputum of current and former smokers (5).

The aberrant methylation of normally unmethylated CpG islands is an epigenetic change that induces the transcriptional inactivation of tumor suppressor genes. The promoter methylation of several tumor suppressor genes has been reported in various tumors, including lung cancer. Among the common targets for aberrant methylation in NSCLC are the promoter regions of the p14, p16, and RASSF1 genes. Hypermethylation at the 5′ flanking region of the p16 gene has been identified as a mechanism of p16 inactivation in NSCLC (3, 6). Recently, an aberrant methylation at the human p14 promoter has been reported in colorectal cancer cell lines (7) and many primary tumors, such as nasopharyngeal carcinoma (8), colorectal carcinoma (9), esophageal carcinoma, and lung cancer (10). Loss of heterozygosity at chromosome 3p21.3 is one of the most common and one of the earliest events that occur in the pathogenesis of lung cancer (11–13). RASSF1A (Ras association domain family 1A) gene is a candidate tumor suppressor gene at 3p21.3. Although the lack of expression of RASSF1A is common in lung cancer, mutations of RASSF1A are rare (14, 15). It has been reported that the RASSF1A gene is frequently inactivated in primary lung cancers by the de novo methylation of CpG islands in the promoter region (10, 14–17).

To investigate the association between the age at starting smoking and the hypermethylation of tumor suppressor genes known to be important to lung cancer carcinogenesis, we determined the methylation status of the p14, p16, and RASSF1A promoters using MSP in 204 primary NSCLC patients. In this study, we also further studied the relationship between the promoter methylation of these genes and patient’s overall survival.

MATERIALS AND METHODS

Tumor Specimens.

A total of 204 primary tumor samples was obtained from NSCLC patients who had been treated by surgical resection from January 1994 through September 2001 in the Department of Thoracic Surgery at Samsung Medical Center, Seoul, Korea. Written informed consent for the use of paraffin-embedded tissues was obtained from all patients before operation. Of the patients, 93 patients had stage I disease, 61 stage II disease, 39 stage III disease, and 11 patients stage IV disease.

DNA Extraction from Paraffin Tissue Blocks.

Formalin-fixed, paraffin wax-embedded tissues were cut into slides at 10-μm thickness. Before DNA extraction, the tissues were stained with H&E to locate the tumor areas. Areas

3 The abbreviations used are: NSCLC, non-small cell lung cancer; MSP, methylation-specific PCR; OR, odds ratio.
corresponding to tumor and the surrounding normal lung tissue were micro-dissected separately. Before DNA extraction, xylene was added to the micro-dissected tissues to remove the paraffin with vigorous vortexing. After centrifuging at full speed for 5 min, the supernatant was removed. After ethanol precipitation, the tissue pellet was resuspended in lysis buffer ATL (Qiagen), and DNA was isolated according to the manufacturer’s instructions (Qiagen).

Methylation Analysis. The methylation status of the p14, p16, and RASSF1A promoters was analyzed by MSP, as described previously by Herman et al. (18). Two sets of primers were designed, one specific for DNA methylated at the promoter region of each gene and the other specific for unmethylated DNA. The primers used for MSP have been described previously (9, 15, 18).

Statistical Analysis. The association between the methylation status of the p14, p16, and RASSF1A promoters and the clinicopathological characteristics was analyzed using Wilcoxon’s rank-sum test and Fisher’s exact test (or the Mantel-Haenszel χ² test). Multivariate logistic regression was conducted to estimate the relationships between hypermethylation of a gene and covariates. The effect of methylation on patient survival was estimated by the Kaplan-Meier method, and differences between two groups were compared using the Log-rank test. Cox proportional hazard regression analysis was performed to estimate the hazard ratio after controlling for potential confounding factors.

RESULTS

Hypermethylation of RASSF1A Is Associated with the Age at Starting Smoking. Methylation status of the CpG islands at the promoter regions of p14, p16, and RASSF1A genes was analyzed by MSP in paraffin-embedded NSCLC tissue (Fig. 1). Hypermethylation of the p14 and p16 promoters was detected in 18 of 204 (9%) and 55 of 204 (27%) primary NSCLCs, respectively (data not shown). The RASSF1A promoter was methylated in 65 of 204 (32%) primary tumors examined (Table 1). Hypermethylation of the p14 promoter was not associated with any smoking variables, including the age at starting smoking (20 ± 8 versus 19 ± 9; P = 0.32), pack-years smoked, duration of smoking, and smoking status (data not shown). Hypermethylation at p16 promoter was significantly associated with pack-years smoked, and the duration of smoking (data not shown), but not with the age at starting smoking (19 ± 8 versus 20 ± 6; P = 0.15). Hypermethylation of the RASSF1A promoter was strongly associated with the age at starting smoking (25 ± 7 versus 19 ± 8; P = 0.001). Young smokers who started the smoking before age 19 had a higher prevalence of hypermethylation of RASSF1A than those who started smoking after age 19 (46 versus 22%; data not shown). The age at starting smoking of adenocarcinoma patients was younger in patients with hypermethylation of the RASSF1A than in those without hypermethylation of RASSF1A (age 21 versus age 32, P = 0.003; Fig. 2). In cases of squamous cell carcinoma, the age of exposure to tobacco smoke was also significantly different (age 21 versus age 21, P = 0.003). The starting age of smoking in squamous cell carcinoma without hypermethylation of RASSF1A was older than that of those with hypermethylation of RASSF1A (age 21 versus age 16, P = 0.04).

Table 1. Univariate analysis of RASSF1A methylation in 204 primary NSCLC

<table>
<thead>
<tr>
<th>Methylation</th>
<th>Absent (n = 139)</th>
<th>Present (n = 65)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67 ± 10</td>
<td>65 ± 11</td>
<td>0.75</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>82</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>57</td>
<td>21</td>
<td>0.23</td>
</tr>
<tr>
<td>Pack-years&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57 ± 43</td>
<td>61 ± 37</td>
<td>0.51</td>
</tr>
<tr>
<td>Years smoked&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37 ± 17</td>
<td>40 ± 15</td>
<td>0.16</td>
</tr>
<tr>
<td>Starting age&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25 ± 7</td>
<td>19 ± 8</td>
<td>0.001</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>16</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>56</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>67</td>
<td>36</td>
<td>0.25</td>
</tr>
<tr>
<td>Histology&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoca</td>
<td>62</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Squamous</td>
<td>53</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>22</td>
<td>4</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* P; Wilcoxon’s rank-sum test for continuous variables and of Fisher’s exact (or χ²) test for categorical variables.
* Mean ± SD.
* Histology data are missing for three patients.

Fig. 1. Methylation analysis of p14, p16, and RASSF1A promoters in NSCLC. MSP for p14, p16, and RASSF1A was performed using unmethylation-specific (U) and methylation-specific (M) primer sets. Numbers, sample identification numbers. The DNA from the peripheral blood lymphocytes of healthy individuals was treated with SssI methyltransferase (New England Biolabs, Inc., Beverly, MA), subjected to bisulfite modification, and used as a positive control for methylated alleles. DNA from normal lymphocytes was used as a negative control for methylated alleles. Negative control samples without DNA were included for each PCR set.

Fig. 2. Mean age at starting smoking according to histology and RASSF1A methylation status. Bars, the mean age at starting smoking in each histology. The mean age at starting smoking for 204 patients was age 25 for those without hypermethylation of RASSF1A and age 19 for those with hypermethylation of RASSF1A, and this difference was statistically significant (P = 0.001). Among adenocarcinoma patients, the age of exposure to tobacco smoke was also significantly different (age 32 versus age 21, P = 0.003). The starting age of smoking in squamous cell carcinoma without hypermethylation of RASSF1A was older than that of those with hypermethylation of RASSF1A (age 21 versus age 16, P = 0.04).
in squamous cell carcinoma (40 versus 26%, respectively), and this difference was statistically significant ($P = 0.03$). Tumor stage was not associated with RASSF1A methylation status (data not shown).

**Multivariate Logistic Regression Analysis.** Logistic regression analysis was performed to control for the potential confounding effects of variables, such as age, sex, stage, and histology, and to calculate OR. The age at starting smoking was significantly associated with hypermethylation of the RASSF1A promoter (Table 2a). Young smokers who started smoking below age 19 had an increased risk of hypermethylation at the promoter region of RASSF1A gene compared with those who started smoking after age of 19 (OR = 4.23, 95% CI = 1.03–9.67; $P = 0.001$). Hypermethylation of RASSF1A occurred at 2.23 times higher prevalence in adenocarcinoma than in squamous cell carcinoma (95% CI = 1.34–11.23; $P = 0.03$).

**Hypermethylation of RASSF1A and Patient Survival.** Data were stratified by disease stage, because stage is an important risk factor in NSCLC. Kaplan-Meier survival estimates in stages I and II are shown in Fig. 3. Hypermethylation of the RASSF1A promoter was significantly associated with a poor prognosis in stages I and II ($P = 0.02$ and 0.01, respectively). Median survival among stage I cases was 40 and 56 months for patients with and without hypermethylation of the RASSF1A promoter, respectively (Fig. 3a). Among stage II cases, median survival was also significantly different: 19 and 34 months for patients with and without hypermethylation of the RASSF1A, respectively (Fig. 3b). Cox proportional hazard regression analysis was performed to determine whether hypermethylation of the RASSF1A promoter is an independent prognostic factor, after controlling for potential confounding factors (Table 2b). Patients with hypermethylation of the RASSF1A promoter had a significantly unfavorable prognosis (hazard ratio = 3.27, 95% CI = 1.42–8.71; $P = 0.01$) than those without hypermethylation of the RASSF1A promoter. Young smokers also showed poorer survival compared with those who started smoking late in life (hazard ratio = 2.14, 95% CI = 1.22–9.11; $P = 0.02$).

**DISCUSSION**

The reason that adolescent smokers are susceptible to the development of lung cancer could be linked to the immature development of lung. Alternatively, it might be because of the cumulative effect of cancer-causing chemicals on lung tissue. However, the molecular mechanism responsible for increasing the risk of lung cancer among young smokers remains elusive. In this study, we investigated the association between the age at starting smoking and the aberrant methylation of the CpG islands at promoter regions of the p14, p16, and RASSF1A genes that may play a role in the development of lung cancer. The prevalence of hypermethylation at the promoter region of three genes according to the present study is consistent with the findings of other groups (4, 9, 10, 14, 15). We found that the prevalence of hypermethylation of p14 and p16 promoters was similar for those who started smoking before 19 years of age and those who started smoking after 19 years of age. However, hypermethylation of RASSF1A promoter was found to occur more frequently in patients that started smoking early in life, and it is likely that the hypermethylation of a gene in those that started smoking while young occurs in a gene-specific manner. However, it is not clear what affects the increased susceptibility to de novo methylation of a specific gene in young smokers. The susceptibility to hypermethylation of a specific gene in young smokers could be attributed to nonrandom changes in the environment around a gene. Several factors, including the activity of DNA methyltransferase and local-specific factors, such as SP1 transcription factor, chromatin structure, proximity to a methylation center, and the preexisting methylation status of CpG islands (19), may affect the methylation status of a gene.

The increased activity of DNA methyltransferase is one of the likely factors for the increased susceptibility to the CpG island hypermethylation of a specific gene in young smokers. Tobacco smoke contains numerous carcinogens, including 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone, polyaromatic hydrocarbons, and metals, such as chromium, cadmium, and nickel. Smoking increases the activity of DNA methyltransferase and thereby induces DNA methylation (20). In addition, increased DNA methyltransferase drives de novo hypermethylation of susceptible loci (5 of 12 CpG islands) on a gene-specific basis (19).

Another possible factor is cis-acting elements that block the spread of methylation from a methylation center and thereby protect CpG...
islands from methylation. Multiple SP-1 sites are located at both the 5' and 3' boundaries to potentially protect the islands from the spread of methylation from a methylation center. Exposure to tobacco smoke in young age could cause selective changes in a limited set of key regulatory transcription factors, such as SP1 protein. The cis-acting element that normally maintains islands free of methylation could be easily disrupted by exposure to tobacco smoke in adolescence. In addition, binding proteins like those that may interact with the SP-1 sites at the island borders could also be disrupted by exposure to tobacco smoke. Accordingly, if these binding proteins were deficient in function or amount in adolescent smokers, the CpG islands of a specific gene might be rendered vulnerable to methylation.

The histone that helps maintain chromatin structure around the promoter region of a gene may also affect the genetic susceptibility to hypermethylation. A fifth histone, H1, is bound to the DNA on the inside of the solenoid structure of condensed chromatin. Histone H1 has a clear preference for methylated CpG, irrespective of the DNA sequences. One variant of histone H1, H1e, is known to exert a specific inhibitory effect on DNA methyltransferase activity in vitro by binding to CpG-rich DNA around promoter regions. Thus, if tobacco smoke affects the function of H1e, the chromatin structure around a CpG island may be changed, and this may play a role in gene-specific hypermethylation in young smokers. The developmental status of chromatin structure during adolescence can also affect the genetic susceptibility to methylation of a gene, because patterns of higher order chromatin organization are established during development. Interestingly, chromatin patterns in young and senescent human fibroblasts are different. A global shift in chromatin structure occurs in several regions of chromatin through development. Thus, a high prevalence of hypermethylation of RASSF1A promoter in smokers that started smoking early in life may be secondary to a lack of heterochromatinization at the promoter region of the RASSF1A gene during youth.

We also studied the prognostic significance of RASSF1A methylation and found that the prognosis of patients with hypermethylation of RASSF1A was poorer than those without. This observation is consistent with those of others (15, 17). We stratified data by the age at starting smoking to determine whether hypermethylation of the RASSF1A promoter is an independent prognostic factor irrespective of the age at starting smoking. Among those who started smoking at <19 years, those with hypermethylation of the RASSF1A promoter showed poorer survival than those without hypermethylation of the RASSF1A promoter. Hypermethylation of the RASSF1A promoter was also associated with poorer survival among patients who started smoking late in life. These results suggest that hypermethylation of the RASSF1A is an independent prognostic factor in NSCLC.

In conclusion, this study demonstrates that early initiation of smoking is associated with hypermethylation of the RASSF1A promoter in NSCLC and that hypermethylation of the RASSF1A promoter may affect a patient’s survival in NSCLC. Additional work is required to understand local-specific factors responsible for the increased susceptibility to hypermethylation of a specific gene in young smokers.

ACKNOWLEDGMENTS

We thank Jin-Hyuk Kim for manuscript reading. We also thank Sanghui Kim for assistance in data collection and management.

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Hypermethylation of \textit{RASSF1A} Promoter Is Associated with the Age at Starting Smoking and a Poor Prognosis in Primary Non-Small Cell Lung Cancer

Duk-Hwan Kim, Jin Seuk Kim, Yong-Ick Ji, et al.