Mutational and Epigenetic Evidence for Independent Pathways for Lung Adenocarcinomas Arising in Smokers and Never Smokers

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Abstract

Genetic and epigenetic alterations are considered to play important roles in lung cancer. Recent studies showed that EGFR and K-RAS mutations exhibited a mutually exclusive pattern in adenocarcinoma of the lung, suggesting the presence of two independent oncogenic pathways. However, it is unknown how epigenetic alterations were involved in lung carcinogenesis mediated by EGFR or K-RAS mutation. In this study, we examined the relationship between genetic and epigenetic alterations in 164 cases of lung adenocarcinoma. Somatic mutations were determined by direct sequencing of EGFR exons 18 to 21 and K-RAS codons 12 and 13. Methylation status of p16INK4a, RASSF1A, APC, RARβ, and CDH13, frequently methylated in lung cancer, was determined by methylation-specific PCR and the degree of methylation was defined as the methylation index. Multivariate analysis adjusted for age, sex, and smoking dose showed that the probability of having EGFR mutation was significantly lower among those with p16INK4a and CDH13 methylation than in those without [p16INK4a; odds ratio (OR), 0.07; 95% confidence interval (95% CI), 0.02-0.33; CDH13: OR, 0.34; 95% CI, 0.15-0.77] and the methylation index was significantly lower in EGFR-mutant cases than in wild type (OR, 0.70; 95% CI, 0.52-0.95). By contrast, K-RAS mutation was significantly higher in p16INK4a methylated cases than in unmethylated cases (OR, 4.93; 95% CI, 1.54-15.7) and the methylation index was higher in K-RAS mutant cases than in wild type with marginal significance (OR, 1.46; 95% CI, 0.95-2.25). Our results indicate that the differences in the evolvement of epigenetic alterations between the EGFR- and K-RAS-mediated tumorigenesis and suggest the specific interaction of genetic and epigenetic changes in tumorigenesis of lung cancer. (Cancer Res 2006; 66(3): 1371-5)

Introduction

Adenocarcinoma is the most frequent subtype of non–small-cell lung in both sexes and its genetic and epigenetic alterations have been investigated (1). Epidemiologic studies show that tobacco smoking is a well-known risk factor of lung cancer (2). However, adenocarcinoma is less associated with tobacco exposure than other types of lung cancers. Tobacco-associated carcinogens cause specific types of genetic alterations including G-to-T transversion of TP53, K-RAS mutation, and loss of heterozygosity at several loci in smoking-related adenocarcinoma of the lung (1, 3). Recently, a novel genetic alteration, activating mutations in the tyrosine kinase domain of the EGFR gene, was shown to be present in a subset of pulmonary adenocarcinomas showing significant association with tumor responsiveness to gefitinib (4, 5). Of interest, EGFR mutations are less frequent in adenocarcinomas associated with smoking, unlike K-RAS and TP53 mutations, and show a mutually exclusive pattern with K-RAS mutation, suggesting the presence of two independent pathways for the development of adenocarcinoma in respect to molecular features (6–8).

Aberrant methylation of various tumor suppressor genes has been established as one of the important mechanisms of human cancers (9). We previously reported that frequent methylation of p16INK4a, RASSF1A, APC, RARβ, and CDH13 occurred in lung cancer; furthermore, methylation of p16INK4a, RASSF1A, and APC was closely related to smoking status as was K-RAS mutation in adenocarcinoma of the lung (10, 11). However, the relationship between genetic and epigenetic alterations during lung cancer tumorigenesis is not clear. In colorectal carcinoma, Toyota et al. (12) proposed that tumors with simultaneous methylation of multiple CpG islands, termed CpG island methylator phenotype (CIMP), could be recognized as a novel pathway for tumor development. In their study, K-RAS mutations were related to colorectal carcinoma with CIMP. By contrast, TP53 mutations were related to colorectal carcinoma with non-CIMP, suggesting that activation of oncogenes by mutation and inactivation of tumor suppressor genes by aberrant methylation were related to the underlying mechanism of generating molecular diversity in cancer. The CIMP notion may help us not only to understand the mechanism of carcinogenesis but also to determine the therapeutic strategy; methyltransferase inhibitors may be an appropriate agent for tumors with CIMP (12). Furthermore, Nagasaka et al. (13) reported that there were differences in methylation between BRAF and K-RAS mutant colorectal carcinomas. These facts prompted us to study the interrelationship between genetic and epigenetic alterations in lung cancer pathogenesis.

In this study, we examined the relationship between somatic mutations of EGFR and K-RAS genes and DNA methylation of tumor suppressor genes to investigate the specific association between these alterations in adenocarcinoma of the lung.
Materials and Methods

Clinical samples and DNA extraction. Surgically resected specimens of 164 Japanese cases of adenocarcinoma of the lung were used for this study. Eighty-seven cases were male and 77 were female. Genomic DNAs were isolated from frozen tumor tissues by SDS/proteinase K digestion (Life Technologies, Inc., Rockville, MD), phenol-chloroform extraction, and ethanol precipitation. We previously examined the DNA methylation and EGFR mutation status for some cases in our previous reports (7, 11, 14). Newly analyzed cases included 42 cases for methylation and 40 cases for EGFR or K-RAS mutation analyses. According to our previous study (11), smoking status was divided into three groups: never smokers (<100 cigarettes/lifetime), light smokers (smokers with exposure of <30 pack years), and heavy smokers (smokers with exposure of ≥30 pack years). Our cases consisted of 78 cases of never smokers, 26 cases of light smokers, and 60 cases of heavy smokers. Institutional Review Board permission and informed consent were obtained for all cases.

Methylation-specific PCR assay. The methylation status of p16INK4a, RASSF1A, APC, RARβ1, and CDH13 genes was determined by methylation-specific PCR assay as previously described (15, 16). Briefly, 1 μg of genomic DNA was modified by sodium bisulfite, which converts all unmethylated cytosines to uracils while methylated cytosines remain unchanged. PCR amplification was done with sodium bisulfite–treated DNA as template as previously described, using specific primers for the methylated and unmethylated forms of each gene, and DNA from peripheral blood lymphocytes and buccal mucosa brushes, each from 10 healthy subjects, along with water.

Mutation analysis for EGFR and K-RAS genes. EGFR and K-RAS mutations were examined using PCR-based direct sequencing for four exons of the tyrosine kinase domain (exons 18-21) of EGFR gene and exon 2 of K-RAS gene as previously described (7, 14, 17). PCR products for each exon were incubated using ExoSAP-IT (Amersham Biosciences Corp., Piscataway, NJ) and sequenced directly using Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA) with ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Data analysis. Frequencies of mutation or methylation of two groups were compared using χ² test or Fisher exact test where appropriate. The methylation index, a reflection of the methylation status of all of the genes tested, is defined as the total fraction of genes methylated. The methylation indices of different groups were compared using the Mann-Whitney U test. Multivariate logistic regression model was used to further explore the effect of methylation on EGFR or K-RAS mutation with consideration of the effect of potential confounders. For all tests, P < 0.05 was regarded as statistically significant. Adjustment of multiple comparison was not considered because of exploratory faction of the study. All the analyses were done with STATA version 8 (College Station, TX).

Results

The presence of EGFR and K-RAS mutations and the methylation status of p16INK4a, RASSF1A, APC, RARβ1, and CDH13 genes were examined and correlated in 164 cases of adenocarcinomas of the lung. Examples of mutation and methylation analyses are shown in Fig. 1. Somatic mutations were detected in 74 (45%) cases for EGFR and in 16 cases (10%) for K-RAS genes (Table 1). The exon locations of the EGFR mutation were as follows: 1 mutation was present in exon 18, 33 mutations in exon 19, 7 mutations in exon 20, and 33 mutations in exon 21. K-RAS mutations were present in codon 12 in 15 cases and in codon 13 in one case. Aberrant methylation was found in 31 cases (18.9%) for p16INK4a, 53 cases (32.3%) for RASSF1A, 69 cases (42.1%) for APC, 60 cases (36.6%) for RARβ1, and 48 cases (29.3%) for CDH13 genes (Table 1). Univariate analysis for total cases showed an inverse relationship between EGFR mutation and p16INK4a and CDH13 methylation (P < 0.0001 for p16INK4a, P = 0.003 for CDH13), and the methylation index was lower in EGFR mutant cases (mean ± SE, 1.2 ± 0.1) than in wild-type cases (1.9 ± 0.1; P = 0.0007). We next evaluated the effect of risk factors other than methylation for EGFR mutation to show that sex and smoking degree were significantly associated with EGFR mutation (sex, P = 0.004; smoking, P < 0.001; Table 2). We then did a multivariate analysis adjusted for age, sex, and smoking degree and showed that the probability of having EGFR mutation among patients with adenocarcinoma was significantly lower in p16INK4a and CDH13 methylated cases than in those without methylation [p16INK4a: odds ratio (OR), 0.07; 95% confidence interval (95% CI), 0.02-0.33; P = 0.001; CDH13: OR, 0.34; 95% CI, 0.15-0.77; P = 0.009; Table 3]. Of note, EGFR mutation and p16INK4a methylation were mutually exclusive except for two cases that exhibited both p16INK4a methylation and EGFR mutation (Table 3). In addition, the methylation index was significantly lower in EGFR mutant cases (mean ± SE, 1.2 ± 0.1) than in those having the wild-type form (1.9 ± 0.1; OR, 0.70; 95% CI, 0.52-0.95; P = 0.023). Univariate analysis showed a significant relationship between K-RAS and p16INK4a gene (P = 0.0008) and the methylation index was higher in K-RAS mutant.
cases (mean + SE, 2.3 + 0.3) than in K-RAS wild type (1.5 + 0.1; \( P = 0.022 \)). As shown in Table 2, sex and smoking status also affected the frequency of K-RAS mutation (sex, \( P = 0.027 \); smoking, \( P = 0.011 \)); multivariate analysis was done, showing that the probability of having K-RAS mutation was significantly higher in \( p16^{INK4a} \) methylated cases than in unmethylated cases (OR, 4.93; 95% CI, 1.54-15.7; \( P = 0.007 \)). In addition, the methylation index was higher in K-RAS mutant cases than in wild-type cases; (c) the probability of having K-RAS mutations was significantly higher in \( p16^{INK4a} \) methylated cases than in those without methylation; and (d) the methylation index was marginally higher in K-RAS mutant cases than in wild-type cases. Whereas the precise mechanisms are unknown, these findings indicate that genetic and epigenetic alterations did not simply accumulate stochastically but interacted systematically for cancer development. In addition, these facts provide further evidence for our hypothesis that \( EGFR \) and K-RAS mutations target different subsets of lung adenocarcinomas based on smoking status and that the origins of adenocarcinomas arising in smokers and never-smokers travel down different pathways (8).

Epigenetic alteration of tumor suppressor genes, especially \( p16^{INK4a} \) methylation, might be one of the important mechanisms for K-RAS-related tumorigenesis but one that is rarely involved in the \( EGFR \)-related pathway. Indeed, transformation of primary cells

### Table 2. Impact of potential risk factors other than methylation for \( EGFR \) and K-RAS mutations

<table>
<thead>
<tr>
<th></th>
<th>( EGFR )</th>
<th>( K-RAS )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mu/W</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>30/37</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Female</td>
<td>44/33</td>
<td>2.53 (1.35-4.77)</td>
</tr>
<tr>
<td>Age 1-y increase</td>
<td>1.00 (0.97-1.03)</td>
<td>0.922</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>52/26</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Light</td>
<td>11/15</td>
<td>0.37 (0.15-0.91)</td>
</tr>
<tr>
<td>Heavy</td>
<td>11/49</td>
<td>0.11 (0.03-0.25)</td>
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NOTE: Mu, mutant type; W, wild-type.
by ras requires inactivation of senescence-related genes such as p16INK4a or TP53 (18), suggesting that K-RAS mutant cells would need inactivation of senescence-related genes by some mechanisms, such as methylation for multistep tumorigenesis of human cancers including lung carcinoma (19).

As mentioned, Toyota et al. (12) reported similar findings that K-RAS mutations were frequent in colorectal carcinomas with CIMP; by contrast, TP53 mutations were infrequent in colorectal carcinomas with CIMP, and inversely correlated with p16INK4a methylated colorectal carcinomas. In addition, p16INK4a methylation is one of the significant features of CIMP group (19). They hypothesized that TP53 mutant colorectal carcinomas may evolve along a distinct pathway characterized by chromosomal instability, including a significant degree of gene amplification and deletion (12). Thus, epigenetic alterations may be weakly involved in the TP53-related pathway. In our study, the relationship reported between TP53 mutation and p16INK4a methylation in colorectal carcinomas was similarly observed between EGFR mutation and p16INK4a methylation in lung cancers. As we did not examine the correlation of TP53 status with methylation status, further study is needed to investigate the interaction of TP53 and epigenetic alterations in lung cancer. Of note, TP53 mutation was not specifically associated with EGFR or K-RAS mutation or RASSF1A methylation in lung cancers (6, 20).

About the relationship between K-RAS mutation and p16INK4a methylation, our results were contradictory to those of a previous report. Divine et al. (21) showed that K-RAS mutation and p16INK4a methylation were independent events in lung adenocarcinoma cases from United States. For this discrepancy, two reasons may be considered. First, it is known that there are differences in the frequencies of K-RAS mutation in lung cancer among ethnic groups (7). This fact suggests that genetic or environmental differences may play a role in K-RAS mutations. Second, Divine et al. used a two-stage methylation-specific PCR assay, which increased the sensitivity to detect methylated alleles by >50-fold over the conventional methylation-specific PCR (22). Moreover, they enriched tumor cells by microdissection whereas we used frozen tumor tissues that contained nonmalignant cells. These factors may explain the discrepancies between their results and ours. Indeed, the rate of p16INK4a methylation in adenocarcinoma cases from United States or Australia was reported to be ~22% to 30% (10, 16, 23) by conventional methylation-specific PCR assay. In colorectal carcinomas, Nagasaka et al. (13) reported that the presence of BRAF mutations was closely related to microsatellite instability and showed high frequencies of promoter methylation in multiple genes, including p16INK4a, compared with those with K-RAS mutations. This result seems to be contradictory to that reported by Toyota et al. (12). However, because BRAF is downstream of RAS gene, these two reports suggested that p16INK4a methylation might be associated with RAS signaling for carcinogenesis. From another viewpoint, the difference derived from tissue specificity should be considered. Tobacco smoke is closely related to K-RAS mutation and p16INK4a methylation in lung adenocarcinoma (7, 16) but not in colorectal carcinomas. In addition, the rate of BRAF mutation was not frequent (1.9-3%) in lung cancers compared with colorectal carcinomas (13, 24-26). These facts suggested that the pathogeneses of colorectal and lung adenocarcinomas could be different, resulting in the difference of the relationship between K-RAS mutation and p16INK4a methylation in these two different kinds of cancers.

We showed that the relationship between EGFR mutation and p16INK4a methylation was mutually exclusive except for two cases that exhibited both EGFR mutation and p16INK4a methylation. The p16INK4a alteration is frequently observed in lung cancers due to aberrant methylation, point mutation, and homozygous deletion. Thus, further study should be done on these genetic alterations of p16INK4a gene in lung cancers to clarify the relationship between EGFR mutation and p16INK4a alteration.
In conclusion, our results showed important differences in the epigenetic alterations accompanying EGFR or K-RAS mutations. This might suggest that genetic and epigenetic changes specifically interact to promote tumorigenesis of lung adenocarcinoma. Although further clarification is required, our findings shed light on understanding the novel molecular pathogenesis by genetic and epigenetic interactions in adenocarcinoma of the lung.

References

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