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 sequence of *EGFR* exons 18 to 21 and *K-RAS* codons 12 and 13. Methylation status of *$p16^{INK4a}$*, *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 *$p16^{INK4a}$* and *CDH13* methylation than in those without [*$p16^{INK4a}$*: 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 *$p16^{INK4a}$* 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 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 *$p16^{INK4a}$*, *RASSF1A, APC, RARβ*, and *CDH13* occurred in lung cancer; furthermore, methylation of *$p16^{INK4a}$*, *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.

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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 *p16^{INK4a}*, *RASSF1A, APC, RARβ*, 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 χ^2 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 *p16^{INK4a}, RASSF1A, APC, RARβ*, 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 *p16^{INK4a}*, 53 cases (32.3%) for *RASSF1A*, 69 cases (42.1%) for *APC*, 60 cases (36.6%) for *RARβ*, and 48 cases (29.3%) for *CDH13* genes (Table 1). Univariate analysis for total cases showed an inverse relationship between *EGFR* mutation and *p16^{INK4a}* and *CDH13* methylation ($P < 0.0001$ for *p16^{INK4a}*, $P = 0.003$ 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 *p16^{INK4a}* and *CDH13* methylated cases than in those without methylation [*p16^{INK4a}*: 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 *p16^{INK4a}* methylation were mutually exclusive except for two cases that exhibited both *p16^{INK4a}* 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 *p16^{INK4a}* gene ($P = 0.0008$) and the methylation index was higher in *K-RAS* mutant

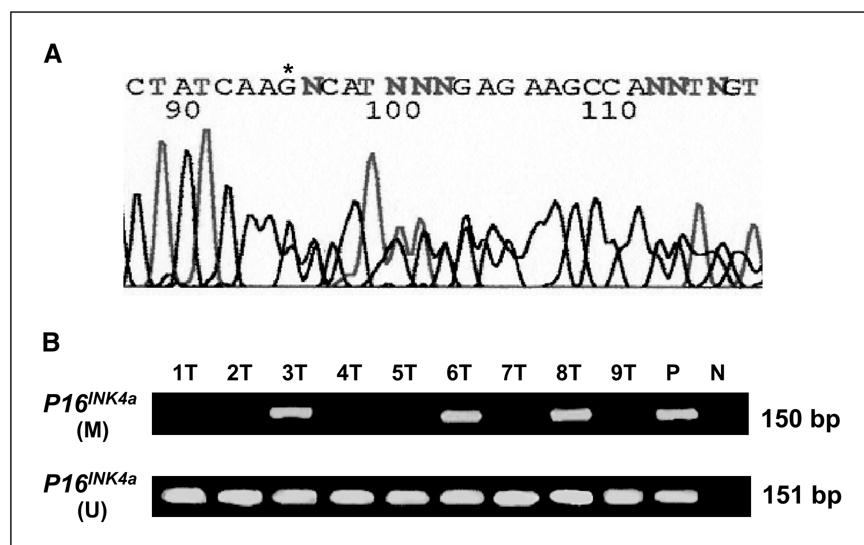

Figure 1. Examples of mutation and methylation analyses. *A,* the sequencing chromatogram showed in-frame deletion in exon 19 (delE746-A750). *, break point of in-frame deletion. *B, p16^{INK4a}* methylation was detected by methylation-specific PCR assay. In tumor samples, most of which consist of mixtures of tumor and nonmalignant cells, either the unmethylated band only or both methylated and unmethylated bands were present. *M,* methylated; *U,* unmethylated; *P,* positive control; *N,* negative control (water blank).

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Table 1. The rates of alterations of each gene in adenocarcinoma of the lung

Alteration	Total	Smoking status		
		Never (n = 78)	Light (n = 26)	Heavy (n = 60)
Somatic mutation				
EGFR	74 (45%)	52 (66.7%)	11 (42.3%)	11 (18.3%)
K-RAS	16 (10%)	3 (3.8%)	2 (7.7%)	11 (18.3%)
Aberrant methylation				
$p16^{INK4a}$	31 (18.9%)	7 (9.0%)	7 (26.9%)	17 (28.3%)
RASSF1A	53 (32.3%)	19 (24.4%)	8 (30.8%)	26 (43.3%)
APC	69 (42.1%)	25 (32.1%)	12 (46.2%)	32 (53.3%)
RARβ	60 (36.6%)	32 (41.0%)	10 (38.5%)	18 (30.0%)
CDH13	48 (29.3%)	18 (23.1%)	10 (38.5%)	20 (33.3%)
Methylation index	1.6	1.3	1.8	1.9

NOTE: The methylation index is defined as the total number of genes methylated in samples. The described value indicates the average of the methylation index in each group.

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 with marginal significance (OR, 1.46; 95% CI, 0.95-2.25; P = 0.086; Table 3). The majority of *EGFR* mutation were deletions in exon 19 and point mutations in exon 21. There was no significant relationship between mutational pattern of *EGFR* and methylation of any gene or the methylation index in this study. Somatic mutation or methylation of examined genes was not detected in DNA from samples of peripheral blood lymphocytes or buccal mucosa brushes from 10 healthy subjects.

Discussion

In this study, we investigated the interactions between genetic and epigenetic alterations in adenocarcinoma of the lung and did univariate and multivariate analyses. Our major findings are as follows: (*a*) the probability of having *EGFR* mutations was significantly lower in $p16^{INK4a}$ or *CDH13* methylated cases than in those without methylation; (*b*) the methylation index was lower in *EGFR* 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

	EGFR			*K-RAS*		
	Mu/W	OR (95% CI)	P	Mu/W	OR (95% CI)	P
Sex						
Male	30/57	1.00 (reference)		13/74	1.00 (reference)	
Female	44/33	2.53 (1.35-4.77)	0.004	3/74	0.23 (0.06-0.84)	0.027
Age						
1-y increase		1.00 (0.97-1.03)	0.922		1.04 (0.98-1.10)	0.229
Smoking						
Never	52/26	1.00 (reference)		3/75	1.00 (reference)	
Light	11/15	0.37 (0.15-0.91)	0.031	2/24	2.08 (0.33-13.2)	0.436
Heavy	11/49	0.11 (0.05-0.25)	<0.001	11/49	5.61 (1.49-21.1)	0.011

NOTE: Mu, mutant type; W, wild-type.

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Table 3. Adjusted ORs for *EGFR* and *K-RAS* mutations

	EGFR			*K-RAS*		
	Mu/W	OR (95% CI)	*P*	Mu/W	OR (95% CI)	*P*
Methylation status						
$p16^{INK4a}$						
U	72/61	1.00 (reference)	0.001	8/125	1.00 (reference)	0.007
M	2/29	0.07 (0.02-0.33)		8/23	4.93 (1.54-15.7)	
RASSF1A						
U	55/56	1.00 (reference)	0.488	9/102	1.00 (reference)	0.56
M	19/34	0.76 (0.36-1.63)		7/46	1.39 (0.46-4.19)	
APC						
U	45/50	1.00 (reference)	0.609	9/86	1.00 (reference)	0.8
M	29/40	1.21 (0.59-2.48)		7/62	0.87 (0.29-2.59)	
RARβ						
U	45/59	1.00 (reference)	0.941	9/95	1.00 (reference)	0.474
M	29/31	0.97 (0.47-2.01)		7/53	1.50 (0.50-4.53)	
CDH13						
U	61/55	1.00 (reference)	0.009	9/107	1.00 (reference)	0.302
M	13/35	0.34 (0.15-0.77)		7/41	1.78 (0.59-5.36)	
Methylation index, 1-unit increase		0.70 (0.52-0.95)	0.023		1.46 (0.95-2.25)	0.086

NOTE: M, methylated; U, unmethylated.

by ras requires inactivation of senescence-related genes such as $p16^{INK4a}$ 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 $p16^{INK4a}$ methylated colorectal carcinomas. In addition, $p16^{INK4a}$ 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 $p16^{INK4a}$ methylation in colorectal carcinomas was similarly observed between *EGFR* mutation and $p16^{INK4a}$ 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 $p16^{INK4a}$ methylation, our results were contradictory to those of a previous report. Divine et al. (21) showed that *K-RAS* mutation and $p16^{INK4a}$ 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 $p16^{INK4a}$ 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 $p16^{INK4a}$, 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 $p16^{INK4a}$ 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 $p16^{INK4a}$ 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 $p16^{INK4a}$ methylation in these two different kinds of cancers.

We showed that the relationship between *EGFR* mutation and $p16^{INK4a}$ methylation was mutually exclusive except for two cases that exhibited both *EGFR* mutation and $p16^{INK4a}$ methylation. The $p16^{INK4a}$ 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 $p16^{INK4a}$ gene in lung cancers to clarify the relationship between *EGFR* mutation and $p16^{INK4a}$ alteration.

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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.

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