

# Mutations of the *Epidermal Growth Factor Receptor* Gene in Lung Cancer: Biological and Clinical Implications

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## ABSTRACT

Recently it has been reported that mutations in the tyrosine kinase domain of the *epidermal growth factor receptor* (*EGFR*) gene occur in a subset of patients with lung cancer showing a dramatic response to *EGFR* tyrosine kinase inhibitors. To gain further insights in the role of *EGFR* in lung carcinogenesis, we sequenced exons 18–21 of the tyrosine kinase domain using total RNA extracted from unselected 277 patients with lung cancer who underwent surgical resection and correlated the results with clinical and pathologic features. *EGFR* mutations were present in 111 patients (40%). Fifty-two were in-frame deletions around codons 746–750 in exon 19, 54 were point mutations including 49 at codon 858 in exon 21 and 4 at codon 719 in exon 18, and 5 were duplications/insertions mainly in exon 20. They were significantly more frequent in female ( $P < 0.001$ ), adenocarcinomas ( $P = 0.0013$ ), and in never-smokers ( $P < 0.001$ ). Multivariate analysis suggested *EGFR* mutations were independently associated with adenocarcinoma histology ( $P = 0.0012$ ) and smoking status ( $P < 0.001$ ), but not with female gender ( $P = 0.9917$ ). In adenocarcinomas, *EGFR* mutations were more frequent in well to moderately differentiated tumors ( $P < 0.001$ ) but were independent of patient age, disease stages, or patient survival. *KRAS* and *TP53* mutations were present in 13 and 41%, respectively. *EGFR* mutations never occurred in tumors with *KRAS* mutations, whereas *EGFR* mutations were independent of *TP53* mutations. *EGFR* mutations define a distinct subset of pulmonary adenocarcinoma without *KRAS* mutations, which is not caused by tobacco carcinogens.

## INTRODUCTION

Non-small-cell lung cancer (NSCLC) frequently overexpresses receptors of the erbB family including the epidermal growth factor receptor (*EGFR*) encoded by *erbB-1* (*HER1*; ref. 1, 2). The *EGFR* is a 170 kilodaltons receptor tyrosine kinases (TK) that dimerizes and phosphorylates several tyrosine residues after binding of several specific ligands (1). These phosphorylated tyrosines serve as the binding sites for several signal transducers that initiate multiple signaling pathways resulting in cell proliferation, migration, and metastasis, evasion from apoptosis, or angiogenesis, all of which are associated with cancer phenotypes (1). Downstream pathways include ras-raf-MEK-ERK (raf-mitogen-activated protein kinase kinase-extracellular signal-regulated kinase), phosphatidylinositol-3 kinase-AKT and PAK-JNKK-JNK (p21-activated protein kinase-c-Jun NH<sub>2</sub> terminal kinase kinase-c-Jun NH<sub>2</sub> terminal kinase; ref. 1). Gefitinib is an orally administered small molecule that specifically inhibits *EGFR* tyrosine phosphorylation (3). Clinical trials revealed that there was a significant variability in response to gefitinib. Good clinical response has been observed most frequently in women, nonsmokers, patients with adenocarcinomas, and Japanese patients (4, 5). However, it has

not been possible to predict gefitinib sensitivity by levels of *EGFR* overexpression as determined by immunohistochemistry (6) or immunoblotting (7). The factor(s) that determine gefitinib sensitivity has long been an enigma. It has been reported recently that activating mutations of *EGFR* are present in a subset of pulmonary adenocarcinomas and that tumors with *EGFR* mutations are highly sensitive to gefitinib (8, 9). Furthermore, the incidence of *EGFR* mutations is higher in Japanese than in Caucasian patients (8). In this study, we searched for *EGFR* mutations in a large cohort of unselected Japanese NSCLC to correlate them with clinical and pathologic features including *KRAS* or *TP53* mutations.

## MATERIALS AND METHODS

**Patients.** Primary tumor samples were obtained from 277 unselected patients with lung cancer who underwent potentially curative pulmonary resection at the Department of Thoracic Surgery, Aichi Cancer Center Hospital from May, 2000 through November, 2000 and from January, 2001 through December, 2002, after obtaining appropriate approval from the institutional review and patients' written informed consent. These cases corresponded to 82% of all consecutive cases. Inclusion of the cases into this study was dependent on availability of frozen tumor material. About 20 cases were excluded because tumor cells were too few to sufficiently extract tumor RNA because of inflammation and/or necrosis. There were 159 males and 118 females with an age at diagnosis ranging from 26 to 89 (median 64) years. One hundred fifty-nine patients had stage I disease, 39 had stage II, 74 had stage III and 5 had stage IV diseases. There were 224 adenocarcinomas, 35 squamous cell carcinomas, 9 large cell carcinomas, 5 adenosquamous carcinomas, 3 small cell carcinomas, and 1 carcinoid. There were 115 never-smokers and 162 ever-smokers including current and former smokers. Smoking history was obtained by interviewing each patient at admission or first outpatient visit.

**Molecular Analysis of Lung Cancer Specimens.** Tumor samples were obtained at the time of surgery, rapidly frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Frozen tissue of the tumor specimens were grossly dissected to enrich as much tumor cells as possible by a surgical pathologist (Y. Y.). We isolated total RNA using the RNeasy kit (Qiagen, Valencia, CA).

The first four exons (exons 18–21) of the seven exons (exons 18–24) that code for TK domain of the *EGFR* gene that includes all of the mutations reported thus far (8, 9) were amplified with primers F1 (5'-AGCTTGTG-GAGCCTTACACC-3') and R1 (5'-TAAAATTGATTCCAATGCC-ATCC-3'), in a one-step reverse transcription-PCR setup with Qiagen OneStep reverse transcription-PCR kit (Qiagen, Valencia, CA). The cDNA sequence of *EGFR* gene was obtained from GenBank (accession number NM005228). Reverse transcription-PCR conditions were available after request. Reverse transcription-PCR products were diluted and cycle-sequenced with the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were electrophoresed on an ABI PRISM 3100 (Applied Biosystems). Both the forward and reverse sequences obtained were analyzed by BLAST and chromatograms by manual review.

***KRAS* and *TP53* Gene Analysis.** We had previously examined the same cohort for *KRAS* mutations and *TP53* mutations (10, 11). Briefly, *TP53* gene (exon 4 through 10) and *KRAS* gene (exons 1 and 2) were amplified and directly sequenced with ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

**Statistical Analysis.** For comparisons of proportions, the  $\chi^2$  test or Fisher's exact test were used. The Kaplan-Meier method was used to estimate the probability of survival as a function of time, and survival differences were

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**Note:** T. Kosaka and Y. Yatabe contributed equally to the present study.

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analyzed by the log-rank test. The two-sided significance level was set at  $P < 0.05$ . To identify which independent factors jointly had a significant influence on the incidence of EGFR mutations, the logistic regression modeling technique was used. We did all analyses using a StatView (version 5, SAS Institute Inc., Cary, NC) software on a Macintosh computer.

## RESULTS

**EGFR Mutations in Unselected Lung Cancer Specimens.** Of 277 unselected patients who underwent surgical resection of their tumors, we found that 111 patients (40%) had mutations in exons 18–21 of the *EGFR* gene. There were 52 deletion mutations, 54 point mutations, and 5 duplication/insertion mutations. In 14 tumors, corresponding cDNA from normal lung tissue far from the tumors was also sequenced, which confirmed that all these mutations were somatic. Details of the resulting changes in EGFR protein as a consequence of these mutations are illustrated in Fig. 1.

All of the 52 deletion mutations occurred around codons 746–750 in exon 19. About half (25 of 52) of deletion mutations were simple deletions of five amino acid residues ELREA from codon 746 to 750. However, 22 deletions were coupled with point mutations or insertions, yielding various changes in amino acid sequences as shown in Fig. 1. It is noted that, in all cases, such alterations were in-frame. Forty-six of the 54 point mutations were from a T to a G transversion at the second nucleotide of codon 858 in exon 21 resulting in substitution of leucine with arginine residue. Four of the point mutations occurred at codon 719 in exon 18. We noted that one tumor with a mutation at codon 719 and three tumors with mutations at codon 858 had another mutation occurring at codons 709, 768, 776, and 790, respectively. For rare mutations (all 5 insertions, E709H, T790M, S768I, R776C, V769L), we resequenced and confirmed that these mutations were actually present. In summary, 52 of the 111 (47%) *EGFR* mutations were deletions around codons 746–750 and 49 (44%) were L858R, altogether accounting for 91% of all of the *EGFR* mutations found. The four major classes of mutations (*i.e.*, deletions, L858R, mutations at codon 719, duplications/insertions) never occurred simultaneously. Furthermore, it is of note that only mutant sequences were present in chromatograms in 19 of 52 deletions, 13 of 46 in L858R, and 1 of 4 codon 719 mutations.

**Relationship between EGFR Mutations and Clinical-Pathologic Features.** *EGFR* mutations were significantly more frequent in females (59%) than males (26%;  $P < 0.001$ ), in never-smokers (66%) than ever-smokers (22%;  $P < 0.001$ ), and in patients with adenocarcinomas (49%) than in those with nonadenocarcinomas (2%;  $P < 0.001$ ). There was only one patient with an *EGFR* mutation of 53 nonadenocarcinoma patients. This patient was a 61-year-old male with adenosquamous carcinoma. Because female patients tended to be never-smokers and were likely to have adenocarcinoma, we did logistic regression analysis to determine which of these three variables independently contributed to the *EGFR* mutations. The result suggested that smoking status and adenocarcinoma histology independently affected *EGFR* mutations whereas female gender did not (smoking status, odds ratio 3.949,  $P < 0.001$ ; histologic type, odds ratio 27.486,  $P = 0.0013$ ; gender odds ratio 0.996,  $P = 0.9917$ ).

**Further Analysis of Patients with Adenocarcinoma.** *EGFR* mutations were found almost exclusively in adenocarcinomas with only one exception; hence, we did more detailed analysis limited to this subset of patients (Table 1). *EGFR* mutations were also significantly frequent in female, nonsmoking patients. When we divided ever-smokers into 3 categories depending on smoke exposure, there was a trend that the higher the exposure, the lower the incidence of *EGFR* mutations. *EGFR* mutations were significantly more frequent in well to moderately differentiated adenocarcinomas (58%) than in poorly

I. Deletions					52
719	740	750	760	860	
	*	*	*	*	
G . . .	KIPVAIKELREATSPKANKEILD . . .	FGLAKLLG			
A) Simple deletions					
G . . .	KIPVAIK-----TSPKANKEILD . . .	FGLAKLLG . . . .			30
G . . .	KIPVAIKE-----SPKANKEILD . . .	FGLAKLLG . . . .			25
G . . .	KIPVAIKE-----PKANKEILD . . .	FGLAKLLG			1
G . . .	KIPVAIKELREAT-----LD . . .	FGLAKLLG			3
G . . .	KIPVAIKELREAT-----LD . . .	FGLAKLLG			1
B) Deletion plus point mutation					
G . . .	KIPVAIKE-----PSPKANKEILD . . .	FGLAKLLG			2
G . . .	KIPVAIKE-----QKANKEILD . . .	FGLAKLLG			1
C) Deletion plus insertion					
G . . .	KIPVAIK-----RTPSPKANKEILD . . .	FGLAKLLG			20
G . . .	KIPVAIK-----VASSKANKEILD . . .	FGLAKLLG			1
G . . .	KIPVAIK-----APKANKEILD . . .	FGLAKLLG			1
G . . .	KIPVAIKE-----QSPKANKEILD . . .	FGLAKLLG			1
G . . .	KIPVAIKE-----QHPKANKEILD . . .	FGLAKLLG			3
G . . .	KIPVAIKE-----PTSPKANKEILD . . .	FGLAKLLG			1
G . . .	KIPVAIKE-----SKANKEILD . . .	FGLAKLLG			2
G . . .	KIPVAIKELREA-----SLD . . .	FGLAKLLG			10
G . . .	KIPVAIKELREA-----SLD . . .	FGLAKLLG			1
II. Point mutations					54
719	740	750	760	860	
	*	*	*	*	
G . . .	KIPVAIKELREATSPKANKEILD . . .	FGLAKLLG			
Codon 719					
S . . .	KIPVAIKELREATSPKANKEILD . . .	FGLAKLLG			4
C . . .	KIPVAIKELREATSPKANKEILD . . .	FGLAKLLG +E709H			2
A . . .	KIPVAIKELREATSPKANKEILD . . .	FGLAKLLG			1
Codon 858					
G . . .	KIPVAIKELREATSPKANKEILD . . .	FGRACKLLG			49
G . . .	KIPVAIKELREATSPKANKEILD . . .	FGRACKLLG +T790M			46
G . . .	KIPVAIKELREATSPKANKEILD . . .	FGRACKLLG +S768I			1
G . . .	KIPVAIKELREATSPKANKEILD . . .	FGRACKLLG +R776C			1
Codons 768 and 769					
S768I+V769L					1
III. Duplications / insertions					5
	740	750	760	770	
	*	*	*	*	
G . . .	KIPVAIKELREATSPKANKEILDEAYVMASVNDP				
	↑		↑	↑	
	KIPVAI		EAFQ	TLA	
				ASV	
				Y	
					1
					1
					1
					1
					1

Fig. 1. Analysis of 111 *EGFR* mutations in the TK domain of the *EGFR* gene found in unselected cases with lung cancer.

differentiated adenocarcinomas (30%;  $P < 0.001$ ). There were five bronchioloalveolar cell carcinomas (BAC) in our cohort, of which three harbored *EGFR* mutations (60%), according to the World Health Organization classification of lung cancers (which states that BAC is a true noninvasive cancer without stromal or pleural invasion; ref. 12). It seemed that *EGFR* mutations were associated neither with age of the patients nor with stage of diseases. There was no difference in incidence of *EGFR* mutations between both sexes in patients of age 50 (average age of menopause in Japan) or younger, although the number of patients of this age group was small (2 of 7 males, 2 of 7 females).

Our preliminary study indicated that patients with *EGFR* mutations survived for a longer period after gefitinib treatment than those without *EGFR* mutations.<sup>5</sup> However, *EGFR* mutations also might have prognostic impact on patients with pulmonary adenocarcinoma, even when the patients were not exposed to gefitinib because *EGFR*

<sup>5</sup> T. Mitsudomi, T. Kosaka, H. Endoh, Y. Horio, T. Hida, S. Mori, S. Hatoaka, M. Shinoda, T. Takahashi, Y. Yatabe, submitted for publication.

Table 1 Relationship between EGFR mutations and clinical and pathologic features in a subset of patients with adenocarcinoma

Variables	Category	EGFR		P
		Mutation (%)	Wild-type	
N		110	114	
Gender	Male	40	71	<0.001
	Female	70	43	
Age	≤64	51	60	0.3481
	> 64	59	54	
Smoking status	Never-smoker (pack years = 0)	76	36	<0.001
	Ever-smoker	34	78	
	Pack years <20	11	9	
	20–50	15	40	
	>20	8	29	
Differentiation	Well to moderately differentiated	89	65	<0.001
	Poorly differentiated	21	49	
Stage	IA and IB	69	70	0.8383
	IIA through IV	41	44	
Survival	3-year survival rate	86%	91%	0.9933
KRAS mutation	Mutated	0	26	<0.001
	Wild-type	97	73	
TP53 mutation	Mutated	37	42	0.4634
	Wild-type	59	54	

There were five BACs in our cohort, of which three harbored EGFR mutations.

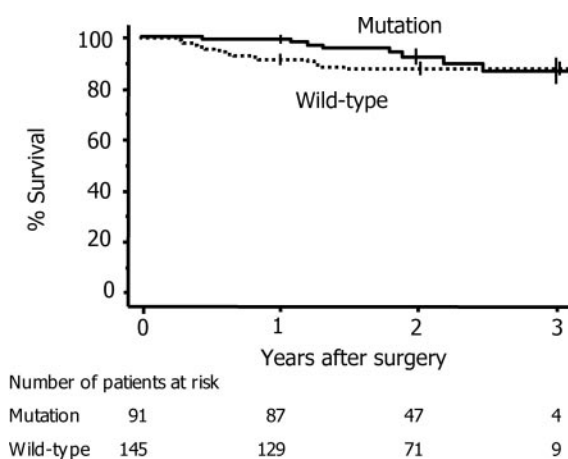


Fig. 2. Effect of EGFR mutations on survival of patients with adenocarcinoma calculated from the day of surgery. Patients later treated with gefitinib and those whose surgery was done for recurrent or second primary cancers were excluded.

mutations defined subsets of pulmonary adenocarcinoma with distinct features as described. Therefore, we did survival analysis in patients excluding those who were treated with gefitinib when they had recurrent diseases. The Kaplan-Meier curve (Fig. 2) indicated that EGFR mutations did not affect prognosis of the patients ( $P = 0.9933$ ), although the follow up period was relatively short (median follow up, 788 days)

**KRAS and TP53 Gene Mutational Analysis.** Of 224 patients with adenocarcinoma, KRAS and TP53 data were available for 196 and 192 patients, respectively. KRAS mutations were present in 26 of 196 patients (13%; 22 at codon 12, 1 at codon 13, and 3 at codon 61). TP53 mutations were present in 79 of 192 (41%). KRAS and TP53 mutations were significantly more frequent in ever-smokers, respectively [20% versus 6% for KRAS ( $P = 0.0054$ ) and 54% versus 30% for TP53 ( $P < 0.001$ )]. Interestingly, EGFR mutations were never found in tumors with KRAS mutations, showing a mutually exclusive relationship. By contrast, EGFR mutations and TP53 mutations seemed to occur independently. Figure 3 shows the relationship among the three mutations by a Venn diagram in 192 patients in whom information about the status of these three genes was available.

TP53 mutations seemed more widely distributed in tumors without EGFR mutations (Fig. 4). Of seven mutations either at codon 157, 248, or 273 in which strong and selective adduct formation of ben-

zo(a)pyrene diol epoxide, one of the major tobacco carcinogens, occurs (13), six were in tumors without EGFR mutations (Fig. 3). Furthermore, of 16 mutations caused by a G to a T transversions characteristic of mutations caused by aromatic polycyclic hydrocarbons (14), 15 were in tumors without EGFR mutations (Fig. 3).

## DISCUSSION

Adenocarcinoma is the most predominant histologic subtype, and its incidence is increasing in Japan. Registration of resected lung cancer in Niigata prefecture, Japan, revealed that the incidence of adenocarcinoma is 71% of 1211 patients operated on from 2001 to 2002 (15). In our institution, adenocarcinoma accounted for 54% of 975 patients who were operated on from 1965 through 1995, 69% of 522 from 1996 through 2000, and 76% of 407 from 2001 through 2003. Considerable evidence indicates that the EGFR pathway also plays an important role in both the pathogenesis and the progression of lung cancer (1).

We found that 40% of 277 unselected patients with lung cancer carried mutations in the TK domain of the EGFR gene. More than 90% of the mutations were either deletions around codons 746–750 in

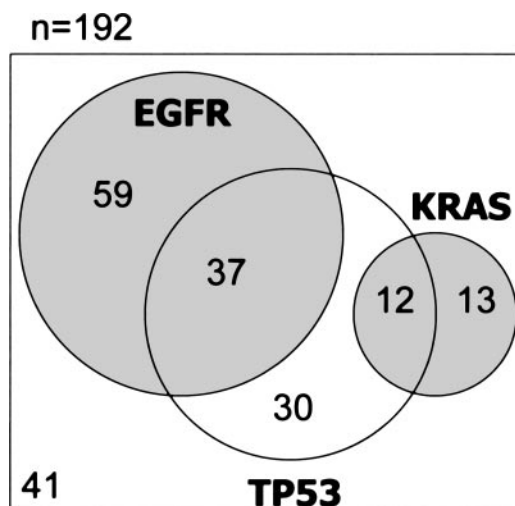
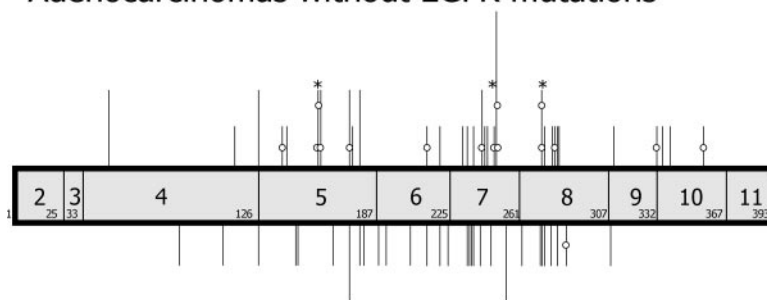


Fig. 3. The Venn diagram illustrating relationship among EGFR mutations, KRAS mutations and TP53 mutations in patients with adenocarcinoma ( $n = 192$ ). Diameters of each circle are roughly proportional to the number of mutations.

## Adenocarcinomas without EGFR mutations



## Adenocarcinomas with EGFR mutations

Fig. 4. Distribution of *TP53* gene mutations in adenocarcinomas without *EGFR* mutations ( $n = 42$ ) or with *EGFR* mutations ( $n = 32$ ). Numbers below show codons of exon boundaries. Asterisks show codons 157, 248, and 273, where strong and selective benzo(a)pyrene diol epoxide adduct formation is reported to occur (13). White circles indicate where *TP53* mutations were caused by a G to T transversion.

exon 19 or L858R in exon 21, which all flank the ATP-binding pocket that is important for TK activity (8, 9). We also noted that in about 30% of the cases with *EGFR* mutations, only bands derived from mutant allele were detected on chromatogram. This is somewhat puzzling considering the heterozygous nature of the *EGFR* mutations reported thus far (8, 9) and the presence of stromal cells in resected tumor specimens. This finding may suggest that loss of wild-type alleles or amplification of mutant alleles accompanied with mutations in these cases, as indicated by Minna *et al.* (16).

*EGFR* mutations were almost exclusively present in adenocarcinoma. Mutations were more prevalent in females and nonsmokers, confirming and extending the results of previous reports (8, 9). It is noteworthy that these characteristics and Japanese ethnicity are all predictors of gefitinib sensitivity at least by univariate analysis (4, 5). Multivariate analysis suggested that nonsmoking status and adenocarcinoma histology independently contributed to *EGFR* mutations but female gender did not. The fact that premenopausal women did not show higher incidence of *EGFR* mutations further suggested that apparent difference between female and male was caused by a difference in lifestyle including smoking habit rather than involvement of sexual environment.

Previously described genetic alterations in lung cancer are almost always more frequent in smokers than nonsmokers. For example, mutations of the *TP53* gene (17), *KRAS* genes (18), or deletion of the short arm of chromosome 3 (19) are known to be more frequent in smokers, as was the case in the present study for the first two. A plausible explanation for the reason why *EGFR* mutations are associated with nonsmoking status are not possible at this time, but it is natural to assume that *EGFR* mutations are caused by carcinogen(s) other than those contained in tobacco smoke. In Taiwan, human papilloma virus type, 16 of 18 infections (20) or cooking oil fume (21) have been investigated as a cause of lung cancer occurring in nonsmoking women. These observations might be relevant with preferential *EGFR* mutations in nonsmoking women. Nevertheless, *EGFR* mutations should provide a clue for pathogenesis of adenocarcinoma occurring in nonsmokers and should ultimately lead to discovery of effective prevention.

We were able to confirm higher incidence of *EGFR* mutations in Japanese patients. Lynch *et al.* found *EGFR* mutation in 2 of 25 unselected United States patients (9), and Paez *et al.* (8) did so in 1 of 61 United States patients and 15 of 58 Japanese patients. The reason for this marked difference between Japanese and United States patients is not very clear. However, difference in incidence of nonsmoking patients between Japanese and American female patients with lung cancer may partly account for this. In our cohort, 83% of female patients and 10% of male patients were never-smokers. This trend is common in Japan. For example, Toyooka *et al.* (22) and Minami *et al.* (23) reported that the proportion of never-smoking women in lung

cancer patients is 96% and 75%, respectively. This makes quite a contrast with the fact that only 15% of 706 United States female and 6% of 1,347 male patients with lung cancer are never-smokers (24).

We found that *EGFR* mutations and *KRAS* mutations known to play an important role in pathogenesis of adenocarcinoma of the lung (25) were strictly mutually exclusive, reminding us of a similar exclusionary relationship between retinoblastoma and p16 inactivation in lung cancer (26). This finding may be explained by the fact that the *KRAS*-mitogen-activated protein kinase pathway is one of the downstream signaling pathways of *EGFR* (1). Because it has been shown that L858R and delL747-P753ins S are activating mutations that result in markedly increased phosphorylation of *EGFR* when EGF was added (8, 9), tumors with *KRAS* mutations that already have activated further downstream effectors do not need to have *EGFR* mutations. The high incidence of *EGFR* mutations in lung adenocarcinomas may explain why *KRAS* mutations are lower in Japanese than in Caucasian patients. In the present study, *KRAS* mutations were found in 13% of adenocarcinomas, whereas they were present in 33% of Dutch cases (25). This may be also at least partially attributable to the difference in smoking status, because *KRAS* mutations were more frequent in smokers as reported previously (18). In contrast, the incidence of *TP53* mutations was not associated with *EGFR* mutations, although *TP53* mutations also occurred more frequently in smokers (17). However, *TP53* mutations in tumors without *EGFR* mutations showed characteristics of mutations caused by tobacco carcinogens in terms of sites or base substitution patterns (13, 14).

We also noted that well to moderately differentiated adenocarcinomas had a significantly higher incidence of *EGFR* mutations than poorly differentiated ones. This observation might be relevant to the fact that adenocarcinomas showing BAC feature show higher sensitivity to gefitinib (27). However, when we used the strict criteria as stated by the World Health Organization Classification of lung tumors (12), our cohort included only five BAC, of which three had *EGFR* mutations. Unfortunately, these strict criteria are not applied by many pathologists, leading to considerable confusion between BAC and adenocarcinoma with BAC features in the literature. Alternatively, we proposed terminal respiratory unit type adenocarcinoma that is characterized by morphological resemblance to type II pneumocytes, Clara cells, and/or bronchioles as well as expression of thyroid transcription factor-1 and surfactant proprotein B (refs. 28, 29). In the World Health Organization classification, most nonmucinous bronchioloalveolar, mixed bronchioloalveolar and acinar subtypes, and some papillary subtypes belong to the terminal respiratory unit type adenocarcinoma (28, 29). We found that most adenocarcinoma with *EGFR* mutations were categorized into terminal respiratory unit type adenocarcinoma.<sup>6</sup>

<sup>6</sup> Y. Yatabe, T. Kosaka, T. Takahashi, T. Mitsudomi, submitted for publication.

EGFR mutations were not associated with stage of disease, suggesting that EGFR mutations occurs relatively early in clinical course and are associated with pathogenesis of adenocarcinoma rather than progression.

In conclusion, we found a high incidence of EGFR mutations in Japanese patients with pulmonary adenocarcinoma, especially in those who never smoked. EGFR mutations were never present in tumors with KRAS mutations, indicating possibilities of genotype-oriented approach for pulmonary adenocarcinoma.

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