EGFR point mutation in non-small cell lung cancer is occasionally accompanied by a second mutation or amplification

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Activating mutations of *EGFR* are found frequently in a subgroup of patients with non-small cell lung cancer (NSCLC) and are highly correlated with the response to gefitinib and erlotinib. In the present study, we searched for mutations of *EGFR*, *HER2* and *KRAS* in 264 resected primary NSCLC from Japanese patients and determined whether there is a correlation between genetic alterations of these genes and clinicopathological factors, together with 85 tumors that we reported previously. *EGFR* mutations were found in 102 of the total 349 tumors, and seven tumors had two missense mutations. Reverse transcription–polymerase chain reaction of *EGFR* and subsequent subcloning analyses identified that the double mutations occurred in the same allele. Furthermore, in 202 NSCLC analyzed by Southern blotting, we identified 11 tumors with gene amplification of *EGFR,* with eight tumors containing a mutation in *EGFR*. Sequence analysis detected only weak or no signals of the wild-type allele in the eight tumors, strongly suggesting that the mutated allele was amplified selectively. These findings indicate that a dual genetic change of *EGFR* can occur in the same allele either with a possible second-hit mutation or with amplification, which may imply a more selective growth advantage in a cancer cell. Meanwhile, *HER2* mutations and amplifications were found in six of 349 tumors and three of 202 tumors, respectively, and *KRAS* mutations in 21 of 349 tumors. Mutations of the *EGFR* and *HER2* genes were more frequently found in female never or light-smoking patients with adenocarcinoma, and there were no tumors that had two or more mutations simultaneously among *EGFR*, *HER2* and *KRAS*. The current study further demonstrates that a double genetic event in *EGFR* can occasionally occur in lung cancer, thus providing new clues for understanding the involvement of epidermal growth factor receptor signaling cascades in the pathogenesis of NSCLC. (*Cancer Sci* 2006; 97: 753–759)

Non-small cell lung cancer is one of the leading causes of death from cancer in Japan and Western countries.[1,2] Although the prognosis for patients with advanced NSCLC has not improved in the last 20 years,[3] the molecular and biological characteristics of lung cancer have been studied intensively[4,5] and many molecules associated with the pathogenesis of lung cancer have been identified as promising new targets for lung cancer therapy.[6] Among these molecules, EGFR, one of the ERBB family of receptors, is thought to be a promising target because of its frequent overexpression (range 40–80%) in NSCLC[7] and its relationship with poor prognosis.[8] Small molecules of EGFR-TKI, such as gefitinib and erlotinib, have been developed, examined for the treatment of NSCLC, and shown to have antitumor activity.[9] Several clinical studies have revealed that Japanese female never-smoking patients with adenocarcinoma have a higher response rate to gefitinib.[10,11] Recently, activating mutations of the TK domain of *EGFR* were found in a unique subgroup of NSCLC and were highly correlated with the response to EGFR-TKI.[12–14] Subsequent analyses using a large series of NSCLC confirmed somatic mutations in the similar subgroup of patients as known clinical predictors of EGFR-TKI sensitivity.[15–18] Moreover, recent studies have shown that increased copy numbers of the *EGFR* gene are associated with a better response to EGFR-TKI.[17,19] HER2, another member of the ERBB family, can heterodimerize with EGFR and has also been shown to have somatic mutations in the TK domain in several cancers including NSCLC.[20,21] However, the involvement of *HER2* alterations in the pathogenesis of NSCLC is not clearly understood.

After earlier reporting 17 mutations of *EGFR* in 85 NSCLC,[22] we further extended our analysis with another 264 NSCLC. In a total of 349 tumors, we found 102 with *EGFR* mutation, six with *HER2* mutation, and 21 with *KRAS* mutation. We also identified seven tumors with double mutations of *EGFR* and eight tumors with both mutation and amplification of *EGFR*. We therefore investigated the double genetic event of EGFR in detail. Our results provide new insights into the involvement of alterations of the receptor TK family in NSCLC development.

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Abbreviations: EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; NSCLC, non-small cell lung cancer; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; SSCP, single-strand conformation polymorphism; TK, tyrosine kinase; TKI, tyrosine kinase inhibitor.

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Table 1. Clinicopathological characteristics of 349 primary non-small cell lung carcinoma patients

Parameters	*n*	%
Age (years)		
Mean	65 (range 29–85)	
Sex		
Male	226	65
Female	123	35
Histology		
Adenocarcinoma	242	69
Squamous cell carcinoma	89	26
Large cell carcinoma	7	2
Adenosquamous carcinoma	3	0.9
Pleomorphic carcinoma	1	0.2
Mucoepidermoid carcinoma	1	0.2
Unclassified non-small cell carcinoma	6	1.7
Smoking history		
Never or light smokers†	131	38
Heavy smokers	201	57
Undetermined	17	5
P-stage		
I or II	226	65
III or IV	97	28
Undetermined	26	7

†Pack-year < 20.

Materials and Methods

Samples

Primary lung tumor tissues were obtained from 349 randomly selected patients with NSCLC who underwent potentially curative pulmonary resection at Nagoya University Hospital, Nagoya First Red Cross Hospital, Nagoya Second Red Cross Hospital, Kasugai Municipal Hospital and Social Insurance Chukyo Hospital in Japan from August 1999 through to November 2003. In each case, tumor and corresponding normal lung tissue samples (taken as far as possible from the neoplastic area) were snap frozen and stored at −80°C. Ethical approval was obtained from each of the five hospitals and fully informed written consent was obtained from all patients prior to tissue collection.

The clinicopathological characteristics of the 349 primary NSCLC patients are summarized in Table 1. The study population consisted of 226 men and 123 women, with a mean age of 65 years (range 29–85 years). Histological type was determined according to the third World Health Organization/International Association for the Study of Lung Cancer classifications, with 242 adenocarcinomas, 89 squamous cell carcinomas, seven large cell carcinomas, three adenosquamous carcinomas, one pleomorphic carcinoma, one mucoepidermoid carcinoma, and six unclassified NSCLC. Smoking history was obtained by anamnesic data at admission or first outpatient visit. There were 107 never smokers, 24 light smokers (pack-year < 20) and 201 heavy smokers (pack-year ≥ 20), with 17 patients of undetermined smoking status. P-stage was determined according to the TNM staging system. One hundred and eighty patients were classified at stage I, 46 at stage II, 88 at stage III and nine at stage IV. The P-stage of 26 patients was not determined.

DNA extraction and direct sequencing

Genomic DNA was prepared from portions of frozen tumors or non-cancerous lung tissues by a standard technique.[23] Genomic DNA (20 ng) was amplified by PCR. The first four (exons 18–21) of seven exons (exons 18–24) encoding the TK domain of EGFR were examined. The primer sequences were 5′-GGCACTGCTTTCCAGCATGG-3′ and 5′-CAGCTTGCAAGGACTCTGGG-3′ for exon 18, 5′-CATGTGGCACCATCTCACAA-3′ and 5′-CCTGAGGTTCAGAGCCATGG-3′ for exon 19, 5′-ATGCGAAGCCACACTGACGT-3′ and 5′-GTATCTCCCTTCCCTGATTA-3′ for exon 20, and 5′-GGCATGAACATGACCCTGAA-3′ and 5′-ACAGCTAGTGGGAAGGCAGC-3′ for exon 21. Mutations in exon 20 of *HER2* and codons 12 and 13 of *KRAS* were examined by direct sequencing. The primer sequences were 5′-GCCATGGCTGTGGTTTGTGATGG-3′ and 5′-ATCCTAGCCCCTTGTGGACATAGG-3′ for exon 20 of *HER2*, and 5′-GGCCTGCTGAAAATGACTGA-3′ and 5′-GTCCTGCACCAGTAATATGC-3′ for codons 12 and 13 of *KRAS*.

The PCR products were sequenced using ABI PRISM 3100 (Applied Biosystems, Foster City, CA, USA) with a PCR primer and a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) as described previously.[24]

Mutation-specific PCR and SSCP analyses for L858R EGFR mutation

Mutation-specific PCR analysis for the L858R mutation was carried out using a forward primer with T substituted to G at the 3′ end (5′-AAGATCACAGATTTTGGGCG-3′) and a reverse primer (5′-ACAGCTAGTGGGAAGGCAGC-3′). Genomic DNA (20 ng) was amplified by touch-down PCR. The conditions used were 10 cycles at 95°C for 30 s, 67–57°C (decreasing 1°C/cycle) for 30 s, and 72°C for 30 s, followed by 20 cycles at 95°C for 30 s, 57°C for 30 s and 72°C for 30 s, and a 7-min extension at 72°C. The PCR products were then applied to a 3% agarose gel, and the band of expected size detected by ultraviolet light was defined as positive.

Single-strand conformation polymorphism analysis was carried out for exon 21 to cover the L858R mutation on mutation detection enhancement gels (FMC Bioproducts, Rockland, ME, USA) containing 10% glycerol with a primer set of 5′-CAGGGCATGAACTACTTGGA-3′ and 5′-TTGCCTCCTTCTGCATGGTA-3′, as described previously.[25]

Cloning of EGFR cDNA

Total RNA was extracted from tumor samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was generated using SuperScript II (Invitrogen) and random primers (Takara Bio, Tokyo, Japan) according to the manufacturer's instructions. RT-PCR was carried out using the primer set 5′-TAAGATCCCGTCCATCGCCA-3′ and 5′-TTGCCTCCTTCTGCATGGTA-3′, covering exons 18–21 of *EGFR*. Amplified products were subcloned into pGEM-T Easy Vectors (Invitrogen) using XL-1 and XL-1 *mutS* strains (Stratagene, La Jolla, CA, USA) as competent cells. More than 25 clones were picked up for each sample, plasmids were purified using QIAprep Spin Miniprep KIT (Qiagen, Valencia, CA, USA), and the inserts were sequenced bidirectionally.

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Southern blot analysis

A total of 7 μg of genomic DNA digested with *Eco*RI endonuclease (New England Biolabs, Beverly, MA, USA) was electrophoresed on a 0.7% agarose gel, transferred to Hybond N+ membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), hybridized with ^{32}P-labeled probes synthesized with random primers, and subjected to autoradiography after washing, as described previously.[24] The DNA probes of *EGFR*, *HER2* and β-*actin* were prepared by PCR with primers set of 5′-GGCACTGCTTTCCAGCATGG-3′ and 5′-CCTGAGGTCAGAGCCATGG-3′ for *EGFR*, 5′-GCCATGGCTGTGGTTTGTGATGG-3′ and 5′-ATCCTAGCCCCTTGTGGACATAGG-3′ for *HER2*, and 5′-CTGTGGCATCCACGAAACTA-3′ and 5′-AGGAAAGACACCCACCTTGA-3′ for β-*actin*.

Hybridized membranes were exposed to scientific imaging films (Eastman Kodak, Rochester, NY, USA) or BAS-IP (Fuji Photo Film, Tokyo, Japan), which were analyzed using a BAS 2500 image analyzer (Fuji Photo Film) to quantify the intensity of signals. The samples with *EGFR* copies more than 3.5-fold the normal lung DNA were defined as gene amplification positive.

Statistical analyses

The variables evaluated in the present study were investigated for association with mutation status of *EGFR*, *HER2* and *KRAS* using Fisher's exact test or the χ^2-test. To identify which independent variables had a significant influence on *EGFR* mutation, a logistic regression model was used. $P < 0.05$ was regarded as statistically significant. All analyses were carried out using StatView version 5 software (SAS Institute, Cary, NC, USA).

Results

EGFR mutations in Japanese NSCLC

We previously reported that 17 tumors had *EGFR* mutations identified by direct sequencing of exons 18, 19 and 21 among 85 resected Japanese NSCLC.[22] In the present study, we first carried out direct sequencing of exon 20 for the 85 tumors and detected another four tumors with mutations (Fig. 1). Taken together, we identified 21 tumors with *EGFR* mutations and further extended our analysis using another 264 tumors. We then identified 72 tumors with *EGFR* mutations in exons 18–21. In total, we found that 93 (27%) of 349 tumors had *EGFR* mutations by direct sequencing (Fig. 1).

As some recent reports suggest that SSCP or mutation-specific PCR analysis may be more sensitive than direct sequencing,[18] we used mutation-specific PCR to search for the mutation L858R in exon 21, which is one of the most frequent *EGFR* mutations, in 349 NSCLC. After confirming that all tumors with L858R detected by direct sequencing were positive for the mutation, we found another nine with the L858R mutation in 349 tumors. In total, we detected 102 (29%) *EGFR*-mutated tumors (95 tumors with a single mutation and seven with double mutations) among 349 NSCLC, with the overall number of mutations being 109 (Fig. 1; Table 2). The mutations consisted of three different types: in-frame deletions, single-nucleotide substitutions, and in-frame duplications or insertions. Forty-four tumors had an in-frame deletion at codons 746–750 in exon 19, with eight of these

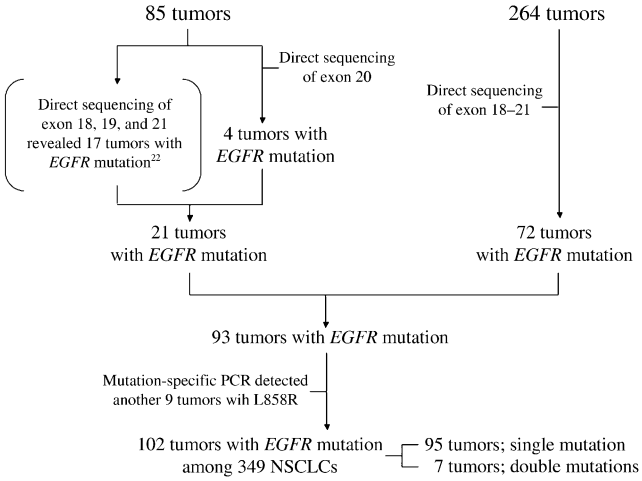

Fig. 1. A schematic representation of mutation analysis of the epidermal growth factor receptor (EGFR) gene. We previously reported 17 tumors with *EGFR* mutation detected by direct sequencing of exons 18, 19 and 21 among 85 non-small cell lung carcinomas (NSCLC; as indicated in parentheses), and further carried out direct sequencing of exon 20. Direct sequencing analysis of a further 264 NSCLC revealed 93 tumors with *EGFR* mutation among a total of 349 NSCLC. As the L858R mutation was then detected by mutation-specific polymerase chain reaction (PCR) in another nine tumors, we identified a total of 102 tumors with *EGFR* mutations (95 tumors with a single mutation and seven tumors with double mutations of *EGFR*).

deletions being accompanied by a point mutation or insertion. Fifty-three tumors had missense mutations in exons 18, 20 or 21, and the L858R mutation was the most common missense mutation. Five tumors had an in-frame duplication or insertion of one or three amino acids at codons 769–776 in exon 20. None of the mutations were present in the corresponding normal lung tissue, indicating that these mutations were somatic. Single-nucleotide polymorphisms were identified at 2361 (G/A) and 2457 (G/A) in exon 20 (data not shown).

Double mutations of *EGFR* on the same allele

To determine whether the double point mutations of *EGFR*, which were identified in the same sample, resulted from mutational events on the same allele or different alleles, we carried out cDNA cloning analysis. After extraction of total RNA from five samples available among the seven tumors that had double mutations, we carried out RT-PCR and TA-cloning using transformation into XL-1 bacteria, picked up at least five to 10 subclones for each sample, and sequenced the inserts bidirectionally. To avoid unsolicited mismatch repairs of PCR heteroduplex products during transformation, we also used the XL-1 *mutS* strain of bacteria. Collectively, over 30% clones were double-mutation positive, whereas most of the rest were wild type, possibly from RNA of contaminated normal cells or from the remaining wild-type allele (Table 3). Thus, the subcloning analyses revealed that all five tumors had mRNA containing both mutations, indicating that the mutations were localized in the same allele.

Gene amplification of EGFR

We carried out Southern blotting analysis of 202 tumors and found that 11 (5.4%) tumors had amplification of the *EGFR* gene. Among them, amplification was found in eight (8%) of

Table 2. One hundred and two tumors with epidermal growth factor receptor gene mutations among 349 non-small cell lung carcinomas

Mutation type	Exon	Nucleotide change	Amino acid change	$n^{\dagger}$
In-frame deletion	19	del(2236–2250)	E746-A750 del	18
	19	del(2235–2249)	E746-A750 del	15
	19	del(2240–2254)	L747-T751 del	3
	19	del(2240–2257)	L747-P753 del S ins	3
	19	del(2237–2251)	E746-T751 del A ins	2
	19	del(2237–2254)2255C > T	E746-S752 del V ins	1
	19	del(2239–2247)2248G > C	L747-A750 del P ins	1
	19	del(2240–2257)2261A > C	L747-K754 del ST ins	1
	Total			44
Single-nucleotide substitution	21	2573 T > G	L858R	43
	20	2303 G > T	S768I	2
	18 + 21	2125 G > A + 2573 T > G	E709K + L858R	1
	18 + 21	2126 A > G + 2573 T > G	E709G + L858R	1
	18 + 21	2126 A > C + 2573 T > G	E709A + L858R	1
	18 + 20	2155 G > A + 2303 G > T	G719S + S768I	1
	18 + 20	2155 G > T + 2303 G > T	G719C + S768I	1
	20 + 20	2303 G > T + 2305 G > T	S768I + V769L	1
	20 + 20	2303 G > T + 2320 G > A	S768I + V774M	1
	21	2582 T > G	L861Q	1
	Total			53
In-frame duplication and/or insertion	20	2304–2312 ins TGTGGCCAG	VAS 769–771 ins	1
	20	2308–2316 ins GACAACGTG	DNV 770–772 ins	1
	20	2311–2319 ins GCACCGTGG	APW 771–773 ins	1
	20	2314–2316 ins/dup AAC	N 772 ins	1
	20	2320–2328 ins/dup AACCCCCAC	NPH 774–776 ins	1
	Total			5
Overall tumors				102
Overall mutations				109

†Includes 17 cases reported previously:[22] one of G719S, six of del(2235–2249), three of del(2236–2250), one of del(2240–2254), one of del(2237–2254)2255C > T, and five of L858R. del, deletion; dup, duplication; ins, insertion.

Table 3. Analysis of five tumors with double mutations of the epidermal growth factor receptor gene

Sample	5′ mutation	3′ mutation	No. colonies		Same allele
			Total	5′ Mt/3′ Mt	
KD641	G719S	S768I	52	16 (31%)	Yes
KD826	G719C	S768I	40	38 (95%)	Yes
KD530	S768I	V769L	26	14 (54%)	Yes
KD514	E709A	L858R	28	14 (50%)	Yes
KD921	E709G	L858R	36	16 (44%)	Yes

Mt, mutation.

96 patients with *EGFR* mutations and three (3%) of 106 patients without *EGFR* mutations. The characteristics of tumors with amplification of *EGFR* are summarized in Table 4. Because sequence analysis demonstrated that the mutation signals of these tumors were dominant, with five being completely lost for wild-type signal, the amplification was suggested to have originated from the mutant allele (data not shown).

Alteration of *HER2* and *KRAS* mutations

To determine whether alterations of other molecules involved in the EGFR signaling cascades may have accounted for the pathogenesis of NSCLC, we analyzed mutation and amplification of *HER2* and mutation of *KRAS*. We found six (1.7%) mutations in exon 20 in the TK domain of *HER2* among 349 NSCLC (Table 5). Four tumors had YVMA 776–779 insertion mutations and two had G776V C (cystein) insertions. The corresponding normal lung tissues did not have the same mutations as the tumors, indicating that these alterations were somatic. We carried out Southern blotting analysis of 202 tumors including five tumors with *HER2* mutation and found amplification of *HER2* in three tumors without *HER2* mutation (data not shown). Meanwhile, *KRAS* mutations at codons 12 and 13 were present in 21 (6%) of the 349 NSCLC. There were no tumors that had two or more mutations simultaneously among the *EGFR*, *HER2* and *KRAS* mutations.

Relationship between clinicopathological features and genetic alterations

In univariate analysis of the correlation with clinicopathological features, *EGFR* mutations were significantly more frequent in women than in men (54 vs 15%; $P < 0.0001$), in patients with adenocarcinoma than in other histological subtypes (42 vs 1%; $P < 0.0001$), in never or light smokers (pack-year < 20) than in heavy smokers (pack-year ≥ 20) (54 vs 14%; $P < 0.0001$), and in patients with early stage (I or II) than in advanced stage (III or IV) (33 vs 21%; $P = 0.024$) (Table 6). *HER2* mutations were more frequent in female never or light smokers, and in early stage patients with adenocarcinoma, but did not reach

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Table 4. Clinicopathological features and mutation status of 11 non-small cell lung carcinoma with epidermal growth factor receptor (EGFR) gene amplification

Sample	Histology	Sex	Age (years)	Smoking (pack-years)	P-stage	*EGFR* amplification	*EGFR* mutation	*HER2* mutation	*KRAS* mutation
KD190	Ad	M	63	45	IIIA	+	–	–	–
KD215	Ad	M	71	8	IA	+	NPH 774–776 ins	–	–
KD280	Sq	M	73	50	IIIB	+	–	–	–
KD284	Ad	M	51	0	IIIA	+	E746-A750 del	–	–
KD377	Sq	F	77	57	IIIA	+	–	–	–
KD534	Ad	F	70	0	ND	+	L858R	–	–
KD564	Ad	F	75	0	IA	+	APW 771–773 ins	–	–
KD566	Ad	M	72	0	IB	+	E746-A750 del	–	–
KD587	Ad	F	75	0	IIIA	+	L747-P753 del S ins	–	–
KD591	Ad	M	50	0	IIIA	+	L858R	–	–
KD938	Ad	M	78	20	IB	+	L858R	–	–

Ad, adenocarcinoma; del, deletion; F, female; ins, insertion; M, male; ND, not determined; Sq, squamous cell carcinoma.

Table 5. Clinicopathological features of six tumors with *HER2* mutations among 349 non-small cell lung carcinomas

Sample	Histology	Sex	Age (years)	Smoking (pack-years)	P-stage	Nucleotide change	Amino acid change
KD318	Ad	F	68	0	IA	2325–2336 ins ATACGTGATGGC	YVMA 776–779 ins
KD583	Ad	F	36	2	IIIA	2337–2339 ins TGT	G776V C ins
KD617	Ad	M	60	4	IB	2325–2336 ins ATACGTGATGGC	YVMA 776–779 ins
KD1007	Ad	F	54	0	IA	2325–2336 ins ATACGTGATGGC	YVMA 776–779 ins
KD1024	Ad	F	71	0	IB	2337–2339 ins TGG	G776V C ins
KD1027	Ad	M	67	45	IIA	2325–2336 ins ATACGTGATGGC	YVMA 776–779 ins

Ad, adenocarcinoma; F, female; ins, insertion; M, male.

Table 6. Univariate analysis of the relationship between clinicopathological features and epidermal growth factor receptor (EGFR), *HER2* and *KRAS* gene mutations

Variable	*EGFR* mutation			*HER2* mutation			*KRAS* mutation		
	n	%	*P*-value*	*n*	%	*P*-value*	*n*	%	*P*-value*
Total (*n* = 349)	102	29		6	1.7		21	6	
Sex			<0.0001			NS (0.19)			NS (0.85)
Male (*n* = 226)	35	15		2	0.9		14	6	
Female (*n* = 123)	67	54		4	3.2		7	6	
Histology			<0.0001			NS (0.18)			0.0061
Adenocarcinoma (*n* = 242)	101	42		6	2.5		20	8	
Non-adenocarcinoma (*n* = 107)	1	1		0	0.0		1	1	
Smoking history†			<0.0001			0.037			NS (0.14)
Never or light smoker‡ (*n* = 131)	71	54		5	3.8		4	3	
Heavy smoker (*n* = 201)	29	14		1	0.5		15	7	
Undetermined (*n* = 17)	2	12		0	0.0		2	12	
P-stage†			0.024			NS (0.67)			NS (0.07)
I or II (*n* = 226)	75	33		5	2.2		17	8	
III or IV (*n* = 97)	20	21		1	1.0		2	2	
Undetermined (*n* = 26)	7	27		0	0.0		2	8	

†Patients whose smoking history (*n* = 17) and P-stage (*n* = 26) could not be determined were excluded from the analyses. ‡Pack-years < 20. *Fisher's exact test. NS, not significant.

statistical significance except in those with a lower smoking history. *KRAS* mutations were more frequent in heavy smoking and early stage patients with adenocarcinoma. In multivariate analysis, *EGFR* mutations were found to be independently associated with women, a lower smoking history and adenocarcinoma histology (Table 7).

Discussion

In the present report, we searched for mutations in the *EGFR*, *HER2* and *KRAS* genes in primary NSCLC resected from Japanese patients. Among the 349 NSCLC tumors, we found 109 *EGFR* mutations in 102 tumors, with seven tumors

Table 7. Logistic regression analysis of the association† between epidermal growth factor receptor (EGFR) gene mutation and clinicopathological features

Variable	Odds ratio	95% CI	*P*-value
Female	2.68	1.34–5.39	0.0056
Adenocarcinoma	18.4	4.32–78.4	<0.0001
Smoking history			
<20 pack-years	2.52	1.25–5.08	0.0098
Undetermined	3.5	0.58–21.3	0.17
P-stage			
I or II	1.81	0.92–3.56	0.088
Undetermined	1.07	0.33–3.46	0.91

†Subjects without information on smoking and P-stage were assigned dummy variables, and lack of information was adjusted in a model. CI, confidence interval.

harboring two missense mutations, six *HER2* mutations in six tumors and 21 *KRAS* mutations in 21 tumors. Notably, all the double missense mutations of *EGFR* were in the same allele, and both mutation and amplification of *EGFR* were detected in eight tumors, indicating that two genetic events that target *EGFR* occasionally occur in NSCLC.

Previous studies showed that in-frame deletional mutations in exon 19 and L858R mutation in exon 21 account for approximately 90% of *EGFR* mutations.[12–18] Although the direct sequencing method is accurate and can pick up various kinds of mutation within target regions, it is an expensive and complicated examination tool. Moreover, it may yield a false-negative result when the genomic DNA used is extracted directly from frozen tissues, which sometimes may be contaminated with a significant amount of genomic DNA from non-malignant cells. In this regard, Marchetti *et al*. reported that SSCP analysis was more sensitive than direct sequencing, especially for missense mutations of *EGFR*.[18] We used a mutation-specific PCR method for detecting the L858R mutation and still found another nine mutations in 349 tumors. We also used SSCP analysis for the nine tumors with L858R mutation detected only by mutation-specific PCR, as well as 94 tumors that included 20 *EGFR*-mutated and 74 wild-type tumors, and detected the L858R mutation in all but one of the nine tumors. Thus, there were several tumors with the L858R mutation that were detected only by mutation-specific PCR or SSCP analyses, indicating that these two methods are more sensitive than the direct sequencing method and may be equally useful for the first-step screening of L858R mutation.

We identified seven tumors harboring double missense mutations of *EGFR* that have been reported by other groups.[15,16,26] However, no report thus far has analyzed how the double mutations occur and whether they are involved in NSCLC development. We analyzed these tumors with the cloning method and found that all of the tumors had mRNA with two mutations. This indicates that *EGFR* was mutated at two different positions in the same allele, although we could not determine whether the two mutations occur sequentially or at one time. However, it may well be that a second-hit mutation of *EGFR* occurred in the same allele, which might give a greater growth advantage than a cancer cell with only a first-hit mutation of *EGFR*. To determine this, an experiment should be conducted to show whether transfectants made using an expression construct with an *EGFR* double mutation can induce more active biological properties than with a single mutation.

Gene amplification of *EGFR* has also been shown to be a good predictor of EGFR inhibitor response.[17,19,27] In the present study, the frequency of gene amplification was 5.4%, which seems to be consistent with previous reports using Southern blotting analysis,[28] although other studies using FISH analysis have indicated more frequent amplification.[17,29] Considering the contamination by genomic DNA from normal cells, FISH may be more sensitive than Southern blotting for detecting amplification, although reagents for detection and criteria of definition of amplification levels of *EGFR* vary among researchers, which seem to have caused confusion in the comparison of *EGFR* studies using FISH. Together with *EGFR* mutation status, we identified eight (8%) amplifications among 96 tumors with *EGFR* mutation and three (3%) among 106 without *EGFR* mutations. As sequence analysis demonstrated that the mutation signals of all of these eight tumors were dominant, of which five completely lost the wild-type signal, it suggested strongly that the mutated allele was amplified selectively.

Overexpression and amplification of *HER2* have been found frequently in several tumors such as breast cancer,[30,31] and mutations of *HER2* have also been reported in lung adenocarcinomas.[20,21] In the present study, we found six (1.7%) in-frame duplication or insertion mutations in 349 NSCLC and three (1.5%) amplifications of *HER2* in 202 tumors. KRAS is a downstream molecule of EGFR/HER2 in the signaling cascades, and we found 21 (6%) mutations of *KRAS* among 349 tumors. In agreement with a previous report,[21] there were no tumors with two or more simultaneous mutations among *EGFR*, *HER2* and *KRAS*, suggesting that the mutations are mutually exclusive.

In the present study, *EGFR* mutations were significantly more frequent in women and in lighter smokers with adenocarcinoma by multivariate analysis. Although *HER2* mutation and amplification were infrequent, at least one genetic alteration of *EGFR* or *HER2* was found in 64 (68%) out of 94 tumors categorized as female, adenocarcinoma, and light smoking history. These results indicated that constitutive activation of the EGFR signaling cascades is a key event in the pathogenesis of this subset of lung cancer. Furthermore, we demonstrated that a double genetic event of *EGFR*, containing a possible second-hit mutation or amplification, occasionally occurs on the same allele. Hence, we speculate that the second genetic event for *EGFR* might provide a greater growth advantage to one of the cancer cells with a single mutation. Taken together, our findings may provide new clues to understand the multistep activation mechanisms of *EGFR* that are involved in the pathogenesis of NSCLC and sensitivity and resistance to EGFR-TKI.

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