An Alternative Method for Screening EGFR Mutation Using RFLP in Non-small Cell Lung Cancer Patients

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Introduction: Epidermal growth factor receptor (EGFR) mutations are strong determinants of tumor response to EGFR tyrosine kinase inhibitors in non-small cell lung cancers (NSCLCs). Currently available methods of EGFR mutation detection rely on direct sequencing. Here, we describe the use of an alternative way to screen EGFR mutations.

Methods: A total of 109 frozen tumor specimens from NSCLC patients were obtained. For mutational analysis of EGFR exons 18, 19, and 21, reverse transcription-polymerase chain reaction was performed on the cDNA using original primers designed for restriction fragment length polymorphism (RFLP).

Results: EGFR mutations were detected in 37 patients (34%) by both RFLP and direct sequencing except one case in which it was detected only by RFLP. EGFR mutations were more frequently observed to be significant by multivariate analysis in patients with adenocarcinoma (OR = 5.56), no-smoking history (OR = 4.34), and 65-year-old or younger (OR = 2.64), but not in women (OR = 1.14). Among 37 patients, 18 were treated with gefitinib and 9 responded to the treatment. One patient without any mutation responded.

Conclusion: RFLP is a useful method for screening EGFR mutations and can also be applied to predicting the sensitivity of NSCLC patients to EGFR-tyrosine kinase inhibitors.

Key Words: EGFR mutation, Non-small cell lung cancer, RFLP.

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Lung cancer is the most common cause of death in both men and women worldwide, with non-small cell lung cancer (NSCLC) accounting for approximately 80% of these cases.[1] Recently, two drugs, gefitinib (Iressa) and elrotinib (Tarceva), which target the epidermal growth factor receptor (EGFR) tyrosine kinase (TK), were approved in different countries to treat NSCLC.[2,3]

In 2004, three separate studies reported that mutations in the EGFR gene in lung carcinomas made the disease more responsive to treatment with TK inhibitors.[4–6] Since then, a multitude of data has emerged from different groups around the world.

Most EGFR somatic mutations were exclusively detected in adenocarcinomas, including bronchiolo-alveolar carcinomas. The mutations were detected in exons 18, 19, and 21, which encode the intracellular kinase domain. The mutations detected in exon 18 had substitution of the amino acid G719 in the P-loop, whereas those detected in exon 21 had substitution of an amino acid in the activation domain (L858 and L861). The mutations in exon 19 were in-frame deletions that may alter the structure of αC helices. All of the EGFR mutations affect amino acids near the ATP-binding pocket that is targeted by gefitinib. Functional assays revealed that the hotspot mutants of EGFR had a higher EGF-independent activation than did the wild-type EGFR.[4–7]

EGFR mutations are predominantly found in Asians, women, adenocarcinomas, and never-smokers, which explains the association between the clinical predictors and gefitinib sensitivity.[4–6,8–10]

Direct gene sequencing is a standard method for detecting gene mutations. However, it is not suitable for clinical pretherapeutic screening of patients because it is time-consuming, costly, and sometimes unreliable. Thus, an easy and reliable method for detecting EGFR mutations that can be used clinically is needed.

The aim of this study was to establish an easy and reliable method with which to screen EGFR mutations. We studied a large series of consecutive NSCLC patients for EGFR mutations in exons 18, 19, and 21 using a comparative approach between 2 techniques: direct sequencing of polymerase chain reaction (PCR) products and restriction fragment length polymorphism (RFLP) analysis.

PATIENTS AND METHODS

Cell Lines and Plasmids Containing Wild-Type and Mutant EGFR Genes

Three NSCLC cell lines, SK-MES-1, H1650, and H1975, were purchased from American Type Culture Col-

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lection (Manassas, VA). SK-MES-1 has wild-type EGFR. H1650 contains an E746-A750 deletion mutation in exon 19. H1975 contains an L858R point mutation in exon 21 and a secondary T790M point mutation which is related to the resistance to gefitinib and erlotinib in exon 20.[11,12] pL420 plasmids containing wild-type, G719C and L858R mutant EGFR genes (generous gifts from Dr. Matthew Myerson, Dana-Farber Cancer Institute, Boston) were used to validate the RFLP assay for corresponding point mutations in exon 18 and exon 21, respectively.

Extraction of Nucleic Acids and Restriction Fragment Length Polymorphism for EGFR Mutants

Genomic DNA was isolated from tumors and lung cancer cell lines using the DNeasy Mini Kit (Qiagen, Munich, Germany), according to the manufacturer's protocol. Total RNA was also isolated from the same samples using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized using an Omniscript Reverse Transcription kit (Qiagen).

We have designed original primers against cDNA for RFLP to detect mutations (Figure 1). The following primers containing appended M13 forward or reverse primer tails for direct sequencing were used for PCR amplification: exon 18 (forward, 5′-TGTAAAACGACGGCCAGTCCCTGGGGATC-GGCCTCTTCATGCGA-3′; reverse, 5′-CAGGAAACAGCT-ATGACCTATACACCGTGCCGAACGCACCGGGG-3′), exon 19 (forward, 5′-TGTAAAACGACGGCCAGTGATCA-AAGTGCTGGGCTCC-3′; reverse, 5′-CAGGAAACAGCT-ATGACCACGGTGGAGGTGAGGCAGAT-3′), exon 21 (forward, 5′-TGTAAAACGACGGCCAGTAACACCGCA-GCATGTCAAGAT-3′; reverse, 5′-CAGGAAACAGCTA-TGACCATTCCAATGCCATCCACTTGAT-3′), exon 20 (forward, 5′-TGTAAAACGACGGCCAGTCCTCGATGA-AGCCTACGTGATG-3′; reverse, 5′-CAGGAAACAGCTA-TGACCGGCAGCCGAAGGGTATGAGCTG-3′). The PCR reaction was performed on 1 μL of template cDNA, as prepared above, to which were added 10× buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$), 0.2 mM of both dNTP and 0.25 U Ampli*Taq* Gold, and 0.2 μM forward and reverse primers in a 50 μL reaction volume. The "hot start" PCR cycling parameters were: one cycle of 95°C for 15 minutes, 40 cycles of 95°C for 20 seconds, 60°C for 30 seconds, and 72°C for 1 minute, followed by one cycle of 72°C for 3 minutes.

On the other hand, for the additional experiment to compare the sensitivity of the assay between cDNA and genomic DNA, we also performed RFLP against genomic DNA for exon 19 and exon 21. We chose external and nested primers designed by Paez JG[5] for PCR on genomic DNA. We use the following primers in external PCR: Exon 19, (forward, 5′-AAATAATCAGTGTGATTCGTGGAG-3′; reverse, 5′-GAGGCCAGTGCTGTCTCTAAGG-3′), Exon 21, (forward, 5′-GCAGCGGGTTACATCTTCTTTC-3′; reverse, 5′-CAGCTCTGGCTCACACTACCAG-3′). And we used in nested PCR: Exon 19, (forward, 5′-GTGCATCGCT-GGTAACATCC-3′; reverse, 5′-TGTGGAGATGAGCAG-GGTCT-3′), Exon 21, (forward, 5′-GCTCAGAGCCTGG-CATGAA-3′; reverse, 5′-CATCCTCCCCTGCATGTGT-3′). External-round PCR reaction was performed on 0.1 μg of genomic DNA with the same protocol as described above. For nested-round PCR reaction, 3 μL of the external-round PCR product was amplified in a second 50 μL reaction mixture using nested primers assembled as external-round PCR reaction, described above.

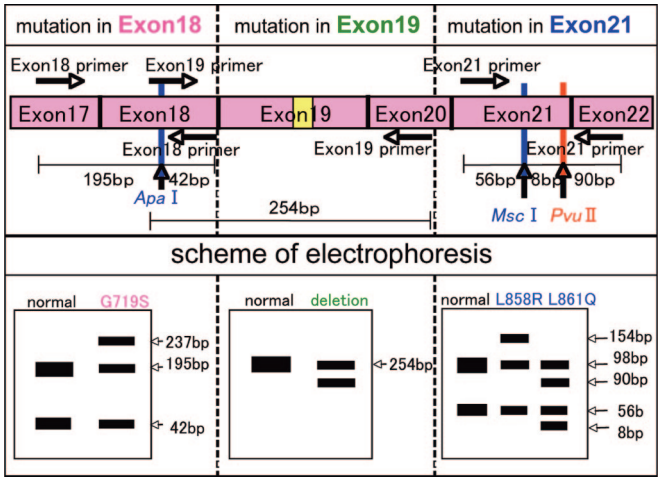

FIGURE 1. Scheme of digestion of PCR products and gel electrophoresis. PCR products were digested with corresponding enzymes (without digestion for exon 19), then were run on 2% agarose gel and the existence of mutations was assessed. Both *Apa*I and *Msc*I digest wild type EGFR allele, while *Pvu*II digests mutant EGFR allele.

Mutation Assay for G719X in Exon 18

The restriction enzyme *Apa*I digests the GGGCCC sequence in the amplicon of the wild-type allele. In contrast, the mutant allele was not digested because of the base substitution of G to X at the second base of GGGCCC. The PCR products after digestion were run on 2% agarose gel and the existence of the mutation was assessed (Figure 1).

Mutation Assay for Deletion in Exon 19

Because the range of exon 19 deletions containing commonly deleted codons 746 to 751 was reported to be from 9 to 18 bp, differences in the sizes of the PCR products enabled us to distinguish mutant from wild-type. The PCR products were run on 2% agarose gel and the existence of exon 19 mutations was assessed (Figure 1).

Mutation Assay for L858R and L861Q in Exon 21

The restriction enzyme *Msc*I was used to digest the TGGCCA sequence in the amplicon of the wild-type allele. In contrast, mutant type (L858R) was not digested because of the base substitution of T to G at the first base of TGGCCA. On the other hand, 2582T>G mutation creates a new *Pvu*II restriction site, CAGCTG, that can be used for a PCR-RFLP assay to distinguish L861Q mutant allele from wild-type. The PCR products were digested simultaneously with the restriction enzymes *Msc*I and *Pvu*II and run on 2% agarose gel, and the existence of these mutations was assessed (Figure 1).

Mutation Assay for T790M in Exon 20

The restriction enzyme *Nla*III was used to digest the CATG sequence in the amplicon of the mutant type (T790M) allele because of the base substitution of C to T at the third base of CACG. In contrast, wild-type allele was not digested. The PCR products after digestion were run on 2% agarose gel and the existence of the mutation was assessed (Figure 1).

EGFR Gene Sequencing

EGFR gene mutations in the cDNA samples were examined using PCR-based direct sequencing for exons 18, 19, and 21 to confirm the results of RFLP analysis. Sequencing was performed using the Applied Biosystems PRISM dye terminator cycle sequencing method with an ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer Corp., Foster City, CA) at the Central Research Center of Keio University Hospital.

Patients and Clinical Samples

Tumor samples from 109 patients diagnosed as having primary NSCLC by histopathological examination were obtained from Keio University (82 samples) and Kawasaki Municipal Hospital (27 samples). Ninety-one frozen tumor specimens were obtained either by surgery ($n = 48$), computed tomography-guided needle lung biopsy ($n = 32$) or ultrasonography-guided needle lung biopsy ($n = 5$), or TBLB ($n = 6$). Fourteen samples from pleural effusion, three samples from pericardial effusion, and one sputum sample were also obtained. Malignant effusion collected by pleurocentesis or cardiocentesis was centrifuged and the cell pellet was collected after removal of the supernatant. All samples were stored at −80°C until the DNA and RNA extraction procedures described above were performed.

All patient samples were collected or tested with informed consent, as approved by our respective institutional review boards. Clinical parameters for the patients were obtained from their medical records.

Clinical information was available for all 109 patients and Table 1 summarizes the demographic and clinical data of the study cohorts.

TABLE 1. Clinicopathological Features of All Patients

Variables	Subset	No.	(%)
No. patients		109	
Age (yr)	Mean	64.9	
	Range	30–85	
Sex	Male	59	(54.1)
	Female	50	(45.9)
Stage	I	18	(16.5)
	II	7	(6.4)
	III	33	(30.3)
	IV	51	(46.8)
Smoking history	Never smoker	37	(34.0)
	Ever-smoker	72	(66.0)
Tumor type	Adenocarcinoma	79	(72.6)
	AWBF	1	(0.9)
	BAC	5	(4.6)
	Squamous cell carcinoma	13	(11.9)
	Large cell carcinoma	1	(0.9)
	LCNEC	1	(0.9)
	Adenosquamous carcinoma	1	(0.9)
	Pleomorphic	2	(1.8)
	Others	5	(5.5)
Tumor samples	Resected tumor	48	(44.0)
	CT-guided lung biopsy	32	(29.4)
	US-guided lung biopsy	5	(4.6)
	TBLB	6	(5.5)
	Pleural fluid	14	(12.8)
	Pericardial fluid	3	(2.8)
	Sputum	1	(0.9)
EGFR mutations		37	(33.9)
	Exon 18		1/37, 2.7%
	Exon 19	22	22/37, 59.5%
	Exon 21	14	14/37, 37.8%

AWBF, adenocarcinoma with bronchiolo-alveolar carcinoma features; BAC, bronchiolo-alveolar carcinoma; EGFR, epidermal growth factor receptor; LCNEC, large cell neuroendocrine carcinoma; TBLB, transbronchial lung biopsy.

Statistical Analyses

The association between EGFR mutational status and tumor response to gefitinib was assessed using the χ^2 test. Multivariate analysis using logistic regression models was performed to assess the associations among histologic subtypes, gender, smoking history, age, and mutational status. All analyses were performed using Stat View (version 5, SAS Institute Inc., Cary, NC) software on a Macintosh computer.

RESULTS

Patterns of PCR-RFLP for EGFR Mutations on Gel-Electrophoresis

Figure 1 presents the predicted gel-electrophoresis patterns for PCR-RFLP samples. We first confirmed the patterns of electrophoresis for EGFR mutations using vectors containing an EGFR exon 18 or 21 point mutation as well as wild-type or cell lines containing an exon 19 deletion mutation and wild-type genes. G719S and L858R mutant vectors were clearly distinguished from wild-type by PCR-RFLP (Figure 2*A* and Figure 2*C*, respectively). On the other hand, a shorter band from the deleted allele in exon 19 and a longer band from the wild-type allele were observed by reverse transcription-polymerase chain reaction from H1650 (Figure 2*B*).

Sensitivity of PCR-RFLP Analysis of EGFR Mutations in Exon 19 and Exon 21

We evaluated the sensitivity of our RFLP assay in exon 19 or in exon 21 by combining SK-MES-1 cells with H1975 or H1650, respectively, in different ratios (Figure 3*A*). In exon 19, a shorter band from the deleted allele was detected up to the level of 1×10^2-fold dilution. In exon 21, the mutant allele at the 154 bp band was also detected up to the level of 1×10^2-fold dilution. The band of 154 bp indicates digested mutant alleles, and the band of 98 bp indicates wild-type alleles.

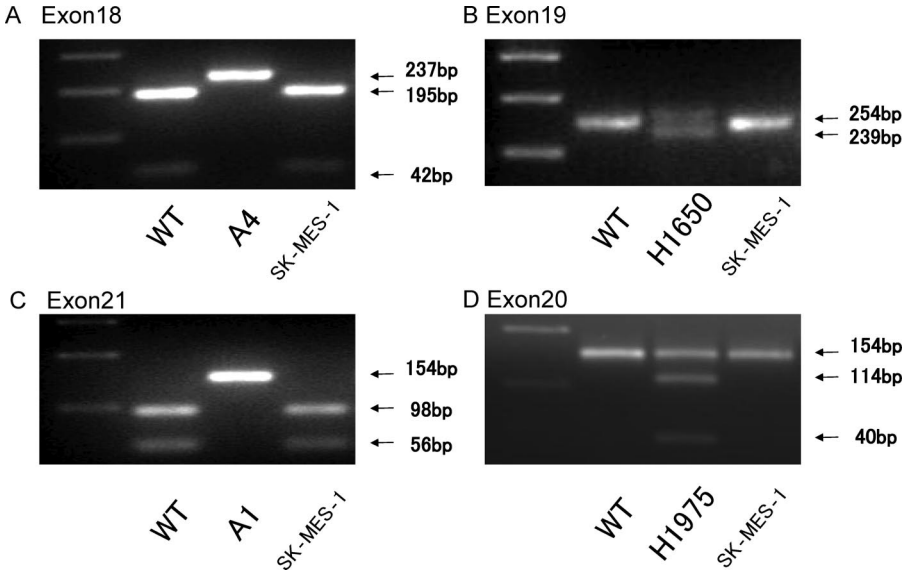

FIGURE 2. Demographic data of EGFR mutations in exon 18, 19, 21, and 20 by PCR-RFLP. *A*, The PCR products of exon18 treated with *Apa*I. *B*, The PCR products of exon19. *C*, The PCR products of the exon21 treated with *Msc*I and *Pvu*II, simultaneously. *D*, The PCR products of exon20 treated with *Nla*III. WT, EGFR (wild type) vector; A4, EGFR (G719S) vector; H1650, lung cancer cell line with EGFR mutation (del E746-A750); A1, EGFR (L858R) vector; H1975, lung cancer cell line with EGFR mutation (L858R and T790M); SK-MES-1, lung cancer cell line without EGFR mutation.

We also compared the sensitivity of cDNA and genomic DNA samples in pleural effusion from NSCLC patients for RFLP assay.

As malignant pleural effusion usually contains many hematopoietic cells such as macrophages and lymphocytes in addition to tumor cells, as a consequence, dilution of

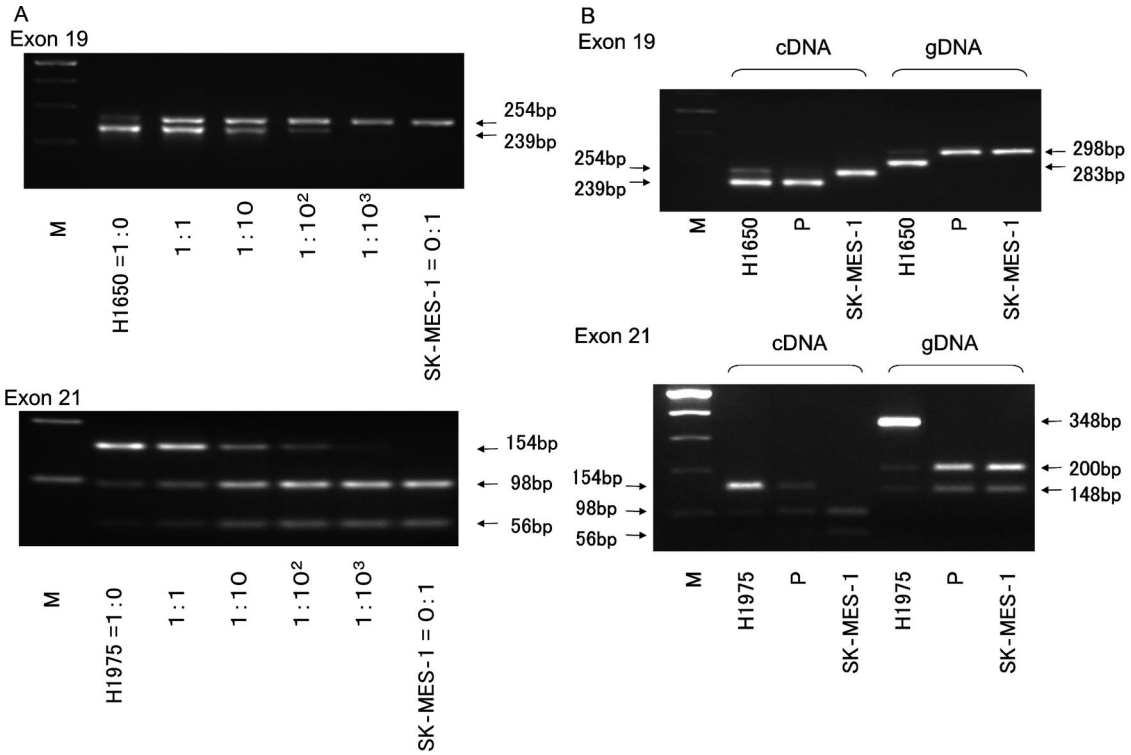

FIGURE 3. Sensitivity of PCR-RFLP analysis of EGFR mutations. *A*, In exon 19, H1650 was mixed with SK-MES-1 from 1- to 10^3-fold. A shorter band from the deleted allele was detected up to the level of 1×10^2-fold dilution. In exon 21, H1975 was mixed with SK-MES-1 from 1- to 10^3-fold. The mutant allele at the 154 bp band was detected up to the level of 1×10^2-fold dilution. SK-MES-1, lung cancer cell line without EGFR mutation; H1650, lung cancer cell line with EGFR mutation (del E746-A750); H1975, lung cancer cell line with EGFR mutation (L858R). *B*, PCR-RFLP was performed using either cDNA or genomic DNA (gDNA). The mutant allele in exon 19 was only detected by using cDNA in the case of malignant pleural effusion. In exon 21, the mutant allele (a 154 bp digested fragment) can be distinguished readily by using cDNA in the case of malignant pleural effusion of NSCLC. H1650; lung cancer cell line with EGFR mutation (del E746-A750), H1975; lung cancer cell line with EGFR mutation (L858R), SK-MES-1; lung cancer cell line without EGFR mutation, P; the case of malignant pleural effusion of NSCLC, M; marker.

genomic DNA derived from tumor cells occurs. To minimize the dilution caused by contaminated hematopoietic or other nontumor cells, we have chosen cDNA instead of genomic DNA for PCR/RFLP, taking into consideration that hematopoietic cells usually do not express the EGFR gene. Indeed, we could detect the mutation bands only when cDNA was used but not when genomic DNA was used from NSCLC patients with malignant pleural effusion (Figure 3*B*).

Results of PCR-RFLP Analysis of EGFR Mutations in Exons 18, 19, 21, and 20 in Clinical Samples

Only 1 patient (patient 56) showed 2 fragments (237 bp and 195 bp, corresponding to mutant and wild-type alleles, respectively) by RFLP using *Apa*I for exon 18 (Figure 4*A*). We found a novel point mutation in codon 719 (2146G>C [G719D]) confirmed by direct sequencing.

In exon 19, we found 22 deletion mutations. Patients who had a deletion mutation showed two fragments corresponding to wild-type and deletion mutant alleles (Figure 4*B*).

In exon 21 using *Msc*I and *Pvu*II, we found 14 point mutations in codon 858 by RFLP. Patients who had a 2573T>G point mutation showed 3 fragments (154 bp and 98 bp, corresponding to L858R mutant and wild-type alleles, respectively) (Figure 4*C*). No L861Q mutation was observed in our specimens.

In exon 20 using *Nla*III, we found 2 point mutations in codon 790 (2369C>T [T790M]) by RFLP. Patients who had a 2369C>T point mutation showed 2 fragments (154 bp, 114 bp, and 40 bp, corresponding to T790M mutant and wild-type alleles, respectively) (Figure 4*D*).

Finally, we found 37 EGFR mutations (34%) in exons 18, 19, and 21 in the present study.

EGFR Mutations and Clinicopathologic Features

(Table 2) The mutation status was significantly correlated with pathologic subtype (adenocarcinoma including bronchiolo-alveolar carcinomas versus nonadenocarcinoma, odds ratio = 5.56, $p = 0.035$), smoking status (never-smokers versus ever-smokers; odds ratio = 4.34, $p = 0.007$) and age (65-year-old or younger versus older than 65 years; odds ratio = 2.64, $p = 0.037$) but not with gender (female versus male; odds ratio = 1.14, $p = 0.813$) by logistic multivariate analysis.

Indeed the female never-smoker patients with adenocarcinoma had a high mutation rate (18/27, 66.6%), whereas the young never-smokers with adenocarcinoma had a higher mutation rate (13/15, 86.7%). Moreover, the young female never-smoker patients with adenocarcinoma had the highest mutation rate (10/11, 90.9%), whereas the elderly male ever-smokers with nonadenocarcinoma had the lowest mutation rate (1/11, 9.1%).

EGFR mutations were not influenced by disease extent (TNM stage) (data not shown).

EGFR Mutations and Clinical Outcome in Patients Treated with Gefitinib

(Table 3) Among 109 patients, 36 were treated with gefitinib (250 mg/d) and evaluated for their response. Eight of 36 patients were treated with gefitinib as an initial treatment. The results of the evaluation showed that 10 patients responded to the treatment. Nine of 10 responsive patients had mutations in exon 19 or exon 21, whereas one had no mutations. The response rate to gefitinib in patients with EGFR deletion mutations in exon 19 and L858R point mutation in exon 21 was 58.3% (7 of 12) and 33.3% (2 of 6, 1 of 7 patients was not evaluable due to discontinuation of treatment because of side effect), respectively. The patient

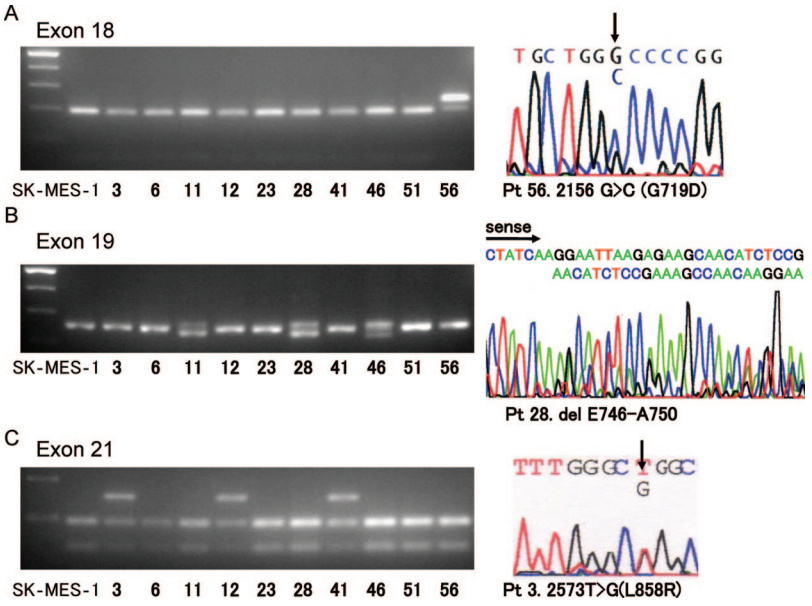

FIGURE 4. Result of PCR-RFLP analysis of EGFR mutations in exon 18, 19, and 21 in clinical samples. *A*, In exon 18, only patient 56 showed 2 fragments (237 bp and 195 bp, corresponding to mutant and wild type alleles, respectively). Direct sequencing analysis of PCR products is shown. Point mutation 2156G>C (G719D) (arrow) is detected in patient 56. *B*, In exon 19, patients 11, 28, and 46 showed 2 fragments corresponding to wild type and deletion mutant alleles. In-frame deletion was detected in patients 28. *C*, In exon 21, patients 3,12, and 41 showed 2 fragments (154 bp and 98 bp, corresponding to L858R mutant and wild type alleles, respectively). No L861Q mutation was observed in our specimens. Point mutation 2573T>G (L858R) was detected in patients 3, 12, and 41.

TABLE 2. Correlation of EGFR Mutations with Clinicopathologic Features

	EGFR Mutation		Odds Ratio	*p*
	+	−		
(A) Gender and EGFR mutation status				0.813
Female	22	28	1.14	
Male	15	44		
(B) Histology and EGFR mutation status				0.035
Adenocarcinoma (with BAC)	35	50	5.56	
Nonadenocarcinoma	2	22		
(C) Smoking habit and EGFR mutation status				0.007
Never smoker	21	16	4.34	
Ever-smoker	16	56		
(D) Age and EGFR mutation status				0.037
≤65	25	30	2.64	
>65	12	42		

EGFR, epidermal growth factor receptor; BAC, bronchiolo-alveolar carcinoma.

TABLE 3. Response to Gefitinib and EGFR Mutation Status

	EGFR Mutation		
Response	+	−	
CR	1	0	
PR	8	1	
SD	5	1	
PD	4	16	
CR/PR	9	1	
SD/PD	9	17	$p = 0.003$
CR/PR/SD	14	2	
PD	4	16	$p < 0.00001$

CR, complete response; EGFR, epidermal growth factor receptor; PD, progressive disease; PR, partial response; SD, stable disease.

with G719D mutation in exon 18 was not treated with gefitinib. On the other hand, 2 patients had T790M mutation and both also had activating mutations (data not shown). One patient who had L858R mutation was not treated with gefitinib. Another patient who had deletion mutation in exon 19 was resistant to gefitinib.

EGFR mutations were more frequently observed in samples from the patients who showed complete or partial responses (9 of 10 cases, 90.0%) than in samples from patients with stable disease or progressive disease (9 of 26, 34.6%; $p = 0.003$). Alternatively, the response rate to gefitinib in patients with EGFR mutations was 50% (9 of 18).

Comparison of Detection of Mutations by RFLP and Direct Sequencing

EGFR mutations were detected in 37 patients. They were detected by both RFLP and direct sequencing methods in 36 cases, whereas it was detected only by RFLP but not by direct sequencing in one case.

DISCUSSION

In the present study, we have described a reliable PCR-RFLP assay for the detection of mutations occurring in the EGFR TK domain. We have also analyzed a large series of NSCLCs for mutations in the TK domain of the EGFR gene by RFLP to assess the actual incidence of this genetic abnormality and its distribution according to histologic type, sex, smoking history, age, and TNM system parameters. In addition, we have analyzed the relationship between EGFR mutations and clinical outcome in patients treated with gefitinib.

In our analysis of 109 cases, most of the EGFR mutations were present in adenocarcinomas. Approximately 41% of adenocarcinomas showed the EGFR mutation, whereas the corresponding figure was 8% for nonadenocarcinomas. In univariate analysis, EGFR mutations were also significantly more frequent in women, younger patients, and never-smokers (data not shown). However, when the histotype, sex, smoking history, and age were tested by multivariate analysis against the presence of mutations in EGFR as a dependent variable, histotype, history of never smoking, and younger age (≤65) remained significant, while female sex did not.

EGFR mutations were more frequently observed in the samples from younger patients (45.5%) than older patients (22.2%). Tomizawa et al. also reported that EGFR mutation was significantly more frequent in younger patients (38%) than in older patients (12%, $p < 0.0001$).[13] For lung cancer, young adults are generally defined as being 40 or 45 years and under, whereas elderly patients are defined as 65 or 70 years and older. When we used 45 years as a cutoff value for age, there was no significant difference between the younger and the older patients regarding EGFR mutations, either by univariate or multivariate analysis. On the other hand, when 65-year-old was used as the cutoff value, the younger, nonelderly patients had a significantly higher prevalence of the EGFR mutation. Indeed, we found that the nonelderly (≤65 years) female never-smoking patients with adenocarcinoma had the highest mutation rate (90.9%), however, this mutation rate was almost identical to the value found in the nonelderly never-smokers with adenocarcinoma independent of sex (86.7%). Moreover, we found only one mutation in the elderly ever-smoker patients with nonadenocarcinoma (1/16, 6.3%) or in males with the above 3 indexes (9.1%). Together with the findings of multivariate analysis, the results suggest that being female may not be a significant independent factor for predicting EGFR mutations, as has been reported elsewhere. This may be partially explained by the fact that female was the predominant sex in young and adenocarcinoma patients.[14]

EGFR mutations were more frequently observed in samples from patients who showed a complete or partial response than in samples from patients with a stable or progressive disease, supporting the findings of many previous reports (Table 3). The exon 19 deletion mutations are re-

ported to be more predictive of gefitinib response or demographics compared with the exon 21 mutation.[9,15,16] The response rate in our analysis was also higher in patients with the exon 19 deletion mutations (58.3%) compared with those with L858R (33.3%).

In tumors from patients not treated with either gefitinib or erlotinib, the 2369C>T mutation (T790M) seems to be extremely rare. We have identified only 2 cases of this mutation in 109 tumors (1.8%).

Large-scale screening requires a rapid and sensitive technique. At the present time, EGFR mutation detection is most commonly performed by direct DNA sequencing of the EGFR kinase domain. However, direct sequencing has several disadvantages with regard to clinical use. The most notable of these is a low detection rate when DNA from clinical samples is used, presumably because of the presence of high rates of contaminated normal and fibrous tissues in tumor samples. Detection of mutations by this method requires at least 30% of the mutated DNA in a sample.[17]

Some investigators have attempted to improve the sensitivity of detection of EGFR mutations in samples containing a mixture of tumor and normal cells. Wookey et al. reported that the ARMS method (Scorpion Amplified Refractory Mutation System technology) was superior to the direct sequencing method and introduced the WAVE method for detecting EGFR mutations.[18] Moreover, EGFR mutations were detectable using the ARMS method in serum DNA from patients with NSCLC.[19] One attempt involved the detection of EGFR mutations using a LightCycler PCR assay.[20] SSCP assay is more sensitive than direct sequencing and is a more rapid method.[21] Recently, 2 rapid and sensitive methods have been demonstrated: the peptide nucleic acid-locked nucleic acid PCR clamp method[22] and mutant-enriched PCR assay.[23] In these studies, EGFR mutations were detected in the presence of 1000-fold and 2000-fold wild-type EGFR genes, respectively.

Indeed, some of the above methods seem to be more sensitive than our method, however, our method does not require special equipment such as real-time PCR machines using multiple dyes like in the Scorpion ARMS and peptide nucleic acid-locked nucleic acid clamp PCR methods. Thus, compared with the other techniques, ours is relatively simple, cost-effective, fast, and reliable as we can see the mutated bands directly. The sensitivity for detection of mutations by our method is also sufficiently high as it is able to detect mutations in samples containing as few as 1% mutated cancer cells (Figure 3*A*). Although a variety of different mutations are seen spanning the entire EGFR TK domain, 94% reside in exons 18 (5%), 19 (48%), and 21 (41%).[24] Using the PCR-RFLP analysis, more than 90% of the mutations in the EGFR gene can be immediately recognized.

Specimens of lung tumor usually contain substantial proportions of normal cells, such as fibrous tissue and peripheral blood cells. Also, normal cells, such as inflammatory cells or mesothelial cells, are also contained in the pleural effusion of lung cancer patients, in addition to tumor cells. Contaminated wild-type DNA interferes with accurate analysis. Most epithelial cells express EGFR, whereas cells of hematopoietic origin are usually EGFR-negative.[25] Using cDNA instead of genomic DNA for the PCR-RFLP, we can minimize the influence of the contaminated hematopoietic or other nontumor cells, which have no EGFR expression. We compared the sensitivity of cDNA and genomic DNA samples in pleural effusion from NSCLC patients when using the RFLP assay. We were able to detect the mutation bands only when a cDNA sample was used (Figure 3*B*). Thus, PCR-RFLP using cDNA is more sensitive than using DNA, particularly when analyzing contaminated samples.

In conclusion, mutations in the EGFR TK domain define a new molecular type of lung carcinoma that is likely to respond to EGFR TK inhibitors. Good clinical independent predictive factors are suggested to be a nonsmoking history, younger age (≤65), and histotype of adenocarcinoma, but not female sex. The PCR-RFLP assay described here is a rapid and reliable method for the screening of EGFR kinase domain mutations in lung cancer patients with various types of samples, as we can minimize the influence of contaminating cells having no EGFR expression using cDNA. The sensitivity, cost, and simplicity of the procedure are satisfactory for genetic testing of lung cancer patients at the clinical laboratory level. RFLP will be one of the useful assays for predicting the sensitivity of NSCLC patients to EGFR-TKIs.

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REFERENCES

1. Jemal A, Murray T, Ward E, et al. American Cancer Society Cancer statistics, 2005. *Cancer J Clin* 2005;55:10–30.
2. Tanovic A, Alfaro V. Gefitinib. Current status in the treatment of non-small cell lung cancer. *Drugs Today (Barc)* 2004;40:809–827.
3. Ross JS, Schenkein DP, Pietrusko R, et al. Targeted therapies for cancer 2004. *Am J Clin Pathol* 2004;122:598–609.
4. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–2139.
5. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–1500.
6. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and elrotinib. *Proc Natl Acad Sci USA* 2004;101:13306–13311.
7. Sordella R, Bell DW, Haber DA, et al. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* 2004;305:1163–1167.
8. Han SW, Kim TY, Hwang PG, et al. Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small cell lung cancer patients treated with gefitinib. *J Clin Oncol* 2005;23:2493–2501.
9. Mitsudomi T, Kosaka T, Endoh H, et al. Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small cell lung cancer with postoperative recurrence. *J Clin Oncol* 2005;23:2513–2520.
10. Takano T, Ohe Y, Sakamoto H, et al. Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small cell lung cancer. *J Clin Oncol* 2005;23:6829–6837.

11. Kobayshi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small cell lung cancer to gefitinib. *N Engl J Med* 2005; 352:786–792.
12. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005;2:e73.
13. Tomizawa Y, Iijima H, Sunaga N, et al. Clinicopathologic significance of the mutations of the epidermal growth factor receptor gene in patients with non-small cell lung cancer. *Clin Cancer Res* 2005;11:6816–6822.
14. Kuo CW, Chen YM, Chao JY, et al. Non-small cell lung cancer in very young and very old patients. *Chest* 2000;117:354–357.
15. Cappuzzo F, Varella-Garcia M, Shigematsu H, et al. Increased HER2 gene copy number is associated with response to gefitinib therapy in epidermal growth factor receptor-positive non-small cell lung cancer patients. *J Clin Oncol* 2005;23:5007–5018.
16. Jackman DM, Yeap BY, Sequist LV, et al. Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients treated with gefitinib or erlotinib. *Clin Cancer Res* 2006;12:3908–3914.
17. Bosari S, Marchetti A, Buttitta F, et al. Detection of p53 mutations by single-strand conformation polymorphisms (SSCP) gel electrophoresis: a comparative study of radioactive and nonradioactive silver-stained SSCP analysis. *Diagn Mol Pathol* 1995;4:249–255.
18. Wookey A, Ellison G, Donald E. Comparison of methods for the detection of mutations in the epithelial growth factor receptor gene. *In 96th Ann Meeting Am Assoc Cancer Res* 2005;46:1250, abstract 5287.
19. Kimura H, Kasahara K, Kawaishi M, et al. Detection of epidermal growth factor receptor mutations in serum as a predictor of the response to gefitinib in patients with non-small cell lung cancer. *Clin Cancer Res* 2006;12:3915–3921.
20. Sasaki H, Endo K, Konishi A, et al. EGFR mutation status in Japanese lung cancer patients: genotyping analysis using LightCycler. *Clin Cancer Res* 2005;11:2924–2929.
21. Marchetti A, Martella C, Felicioni L, et al. EGFR mutations in non-small cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol* 2005;23:857–865.
22. Nagai Y, Miyazawa H, Huqun, et al. Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. *Cancer Res* 2005;65:7276–7282.
23. Asano H, Toyooka S, Tokumo M, et al. Detection of EGFR gene mutation in lung cancer by mutant-enriched polymerase chain reaction assay. *Clin Cancer Res* 2006;12:43–48.
24. Chan SK, Gullick WJ, Hill ME. Mutations of the epidermal growth factor receptor in non-small cell lung cancer—search and destroy. *Eur J Cancer* 2006;42:17–23.
25. Real FX, Rettig WJ, Chesa PG, et al. Expression of epidermal growth factor receptor in human cultured cells and tissues: relationship to cell lineage and stage of differentiation. *Cancer Res* 1986;46:4726–4731.

