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

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Abstract Cases of non–small-cell lung cancer (NSCLC) carrying the somatic mutation of epidermal growth factor receptor (EGFR) have been shown to be hyperresponsive to the EGFR tyrosine kinase inhibitor gefitinib (IRESSA). If EGFR mutations can be observed in serum DNA, this could serve as a noninvasive source of information on the genotype of the original tumor cells that could influence treatment and the ability to predict patient response to gefitinib. Serum genomic DNA was obtained from Japanese patients with NSCLC before first-line gefitinib monotherapy. Scorpion Amplified Refractory Mutation System technology was used to detect EGFR mutations. Wild-type EGFR was detected in all of the 27 serum samples. EGFR mutations were detected in 13 of 27 (48.1%) patients and two major EGFR mutations were identified (E746A750del and L858R). The EGFR mutations were seen significantly more frequently in patients with a partial response than in patients with stable disease or progressive disease ($P = 0.046$, Fisher's exact test). The median progression-free survival was significantly longer in patients with EGFR mutations than in patients without EGFR mutations (200 versus 46 days; $P = 0.005$, log-rank test). The median survival was 611 days in patients with EGFR mutations and 232 days in patients without EGFR mutations ($P > 0.05$). In pairs of tumor and serum samples obtained from 11 patients, the EGFR mutation status in the tumors was consistent with those in the serum of 8 of 11 (72.7%) of the paired samples. Thus, EGFR mutations were detectable using Scorpion Amplified Refractory Mutation System technology in serum DNA from patients with NSCLC. These results suggest that patients with EGFR mutations seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients without EGFR mutations.

Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future (1). Targeting the epidermal growth factor receptor (EGFR) is an appealing strategy for the treatment of non–small-cell lung cancer (NSCLC) as EGFR has been found to be expressed, sometimes strongly, in NSCLC tumors (2). Mutations of EGFR tyrosine kinase have been reported in NSCLC patients with dramatic responses to gefitinib (IRESSA), an EGFR tyrosine kinase inhibitor (3, 4). Studies have reported that EGFR mutations are strong determinants of tumor response to EGFR tyrosine kinase inhibitors (5–7). Approximately 30 mutations in exons 18 to 21 of EGFR were detected in a lung tumor specimen (3–8). The two most common NSCLC-associated EGFR mutations are the 15-bp nucleotide in-frame deletion in exon 19 (E746_A750del) and the point mutation replacing leucine with arginine at codon 858 in exon 21 (L858R; refs. 5, 8). These two mutations account for ~90% of all EGFR mutations and could explain the dramatic responders to gefitinib. Most EGFR mutations have been identified retrospectively from operative resected tumor samples. However, it is sometimes difficult to obtain tumor samples from patients with inoperable NSCLC in prospective studies; thus, it is necessary to establish a method to detect mutant EGFR, especially the two major mutations, from other more readily accessible patient samples.

Recently, PCR technology for the amplification of small amounts of DNA has made it possible to identify the same alterations, which are typically observed in DNA from resected or biopsied tumor cells, using serum samples from patients with various types of tumor, including NSCLC (9, 10). The detection of EGFR mutations in serum DNA may provide a noninvasive and repeatable source of genotypic information

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that could influence treatment and prognosis, especially in patients with NSCLC treated with gefitinib. However, it is well known that interfusion of normal cells with tumor cells prevents the detection of mutations in the tumor cells. Therefore, it is necessary to enhance the sensitivity of the detection of EGFR mutations from tumor-derived DNA mixed with normal cells.

Scorpion primers are used in a fluorescence-based method for the specific detection of PCR products (11). A Scorpion is a specific probe sequence that is held in a hairpin loop configuration by complementary stem sequences at the 5′ and 3′ ends of the probe. Scorpion can be used in combination with the Amplified Refractory Mutation System (ARMS) to enable the detection of single-base mutations (11, 12). ARMS technology is used for allele discrimination and additional mismatches are introduced near the 3′ terminus of the primers to enhance specificity. For the detection of known mutations, the Scorpion-ARMS method is highly sensitive and fast (13). Our hypothesis was that the ARMS and Scorpion methods could enhance the sensitivity of the detection of EGFR mutations from the wild type.

The aims of this study were to develop a highly sensitive assay for the detection of EGFR mutations in serum DNA, to compare the mutation status in serum to tumors from a subset of their patients, and to clarify the relationship between the EGFR mutation status in serum DNA and clinical manifestations, and in particular the responsiveness to gefitinib.

Materials and Methods

Patients and clinical trials. This study was carried out as a correlative study in a multicenter clinical phase II trial of gefitinib monotherapy at the Department of Respiratory Medicine, Kanzawa University Hospital; the Department of Internal Medicine, Kouseiren Takaoka Hospital; the Department of Internal Medicine, Shinminato Municipal Hospital; the Department of Internal Medicine, Fukuiken Saiseikai Hospital; the Department of Respiratory Medicine, Toyama City Hospital; the Department of Respiratory Medicine, Ishikawa Prefectoral Hospital; and the Department of Respiratory Medicine, Kanazawa Municipal Hospital. According to Simon's minimax design, our study, with a sample size of 25, had an 80% power to support the hypothesis that the true objective response rate was >30% and a 5% significance to deny the hypothesis that the true objective response rate was <10%. Assuming an inevaluability rate of <20%, we projected an accrual of 30 patients. The study was conducted with the approval of the appropriate ethical review boards based on the recommendations of the Declaration of Helsinki for biomedical research involving human subjects. Japanese patients with stage IIIB or IV histologically or cytologically proven chemotherapy-naïve NSCLC were enrolled in this trial. Gefitinib was orally given to all patients at a fixed dosage of 250 mg/d. Efficacy was assessed using the Response Evaluation Criteria in Solid Tumors guidelines (14). The analysis of the samples in this study was done blinded to the clinical outcome.

Blood sample collection and DNA extraction. Blood samples from the 27 patients with NSCLC were collected before the initiation of gefitinib administration. Separated serum was stocked at −80°C until use. Serum DNA was extracted and purified using a Qiamp Blood Kit (Qiagen, Hilden, Germany) with the following protocol modifications. One column was used repeatedly until the whole sample had been processed. The resulting DNA was eluted in 50 μL of sterile bidistilled buffer. The concentration and purity of the extracted DNA were determined by spectrophotometry. The extracted DNA was stocked at −20°C until use.

Scorpion ARMS primers for the detection of E746_A750del and L858R. We used an EGFR Scorpion Kit (DxS Ltd., Manchester, United Kingdom), which combined two technologies (i.e., ARMS and Scorpion) to detect mutations in real-time PCR reactions. Four kinds of scorpion primers for the detection of E746_A750del, L858R, and wild type in both exon 19 and exon 21 were designed and synthesized by DxS. The sequences of the scorpion primer for E746_A750del and L858R were based on the GenBank-archived human sequence for EGFR (accession no. AY588246). All reactions were done in 25-μL volumes using 1 μL of template DNA, 7.5 μL of reaction buffer mix, 0.6 μL of primer mix, and 0.1 μL of Taq polymerase. All reagents are included in this kit. Real-time PCR was carried out using SmartCycler II (Cepheid, Sunnyvale, CA) under the following conditions: initial denaturation at 95°C for 10 minutes, 50 cycles of 95°C for 30 seconds, and 62°C for 60 seconds with fluorescence reading (set to FAM that allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. Data analysis was done with Cepheid SmartCycler software (Ver. 1.2b). The cycle threshold (Ct) was defined as the cycle at the highest peak of the second derivative curve, which represented the point of maximum curvature of the growth curve. Both Ct and maximum fluorescence (Fl) were used for interpretation of the results. Positive results were defined as follows: Ct ≤45 and Fl ≥50. These analyses were done in duplicate for each sample and reviewed by two investigators blinded to any clinical information. To confirm the sensitivities for the detection of E746_A750del and L858R, we used the standard DNA that was included in the EGFR Scorpion Kit. Standard DNA with E746_A750del and L858R at a volume of 1, 10, 100, 1,000, or 10,000 pg and the mixture of standard DNA with wild type at 10,000 pg and standard DNA with E746_A750del and L858R at a volume of 1, 10, 100, 1,000 or 10,000 pg were used. For quantification, a standard curve was generated by plotting the cycle number of Ct against the log of the DNA volume of the known standards. The linear correlation coefficient (R^2) values and the formula of the slopes were calculated. DNA (10,000 pg) for the positive control was extracted from a Japanese human adenocarcinoma PC-9 cell line known to contain E746_A750del, a Japanese human adenocarcinoma 11_18 cell line known to contain L858R, and a human epidermoid carcinoma A431 cell line known to contain wild-type exon 19.

Tissue sample collection and DNA extraction. Tumor specimens were obtained on protocols approved by the Institutional Review Board. Twenty paraffin blocks of tumor material, obtained from 15 patients at the time of diagnoses (and before treatment), were collected retrospectively. Eleven tumor samples were collected from the primary cancer via transbronchial lung biopsy, one was resected intraoperatively, and nine were from metastatic sites (four from bone, three lymph nodes, one brain, and one colon). All specimens underwent histologic examination to confirm the diagnosis of NSCLC. DNA extraction from tumor samples was done using a DEXPAT kit (TaKaRa Biomedicals, Shiga, Japan).

PCR amplification and direct sequencing. Amplification and direct sequencing were done in duplicate for each sample obtained from serum and tissue specimens. PCR was done in 25-μL volumes using 15 μL of template DNA, 0.75 units of Ampli Taq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 2.5 μL of PCR buffer, 0.8 mmol/L deoxynucleotide triphosphate, 0.5 μmol/L of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. The sequences of primer sets and schedules of amplifications were followed as previously described (12). The amplification was done using a thermal cycler (Perkin-Elmer, Foster City, CA). Sequencing was done using an ABI prism 310 (Applied Biosystems, Foster City, CA). The sequences were compared with the GenBank-archived human sequence for EGFR (accession no. AY588246).

Statistical analysis. Fisher's exact test was used to assess the relationship between the presence of EGFR mutations in patients with NSCLC and different characteristics, including gender, tumor histology, and response to gefitinib. Regarding analyses of response to gefitinib, patients were categorized into the two groups: (*a*) partial response and (*b*) stable disease or progressive disease (Response Evaluation Criteria

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in Solid Tumors criteria). We compared Kaplan-Meier curves for overall survival and progression-free survival using the standard log-rank test. Overall survival was defined as the time from the initiation of gefitinib administration to death from any cause; patients known to be still alive at the time of the analysis were censored at the time of their last follow-up. Progression-free survival was defined as the time from the initiation of gefitinib administration to first appearance of progressive disease or death from any cause; patients known to be alive and without progressive disease at the time of analysis were censored at the time of their last follow-up. $P = 0.05$ was considered statistically significant. The statistical analyses were done using the StatView software package version 5.0.

Results

Patients and extracted DNA from serum. Twenty-eight patients were enrolled between October 23, 2002 and August 3, 2003 (Table 1). All patients were evaluated for response and followed for progression-free survival and overall survival. Blood samples (2 mL) were collected from 27 of these patients before the initiation of gefitinib administration. These 27 patients represented a subset of that phase II study. Serum DNA was extracted in all 27 samples at a median concentration of 70.0 ng/mL (range, 0-1,720.0 ng/mL).

Sensitivity of the EGFR Scorpion. Preliminary experiments were done to evaluate the sensitivity of the EGFR Scorpion kit (Fig. 1A-C). All curves using E746_A750del and L858R standard DNA (volumes of 1-10,000 pg) increased up to 45 cycles (Fig. 1A). When wild-type standard DNA and distilled water were used as negative controls, the curves did not increase and continued flat at 50 cycles (Fig. 1A and C). When diluted E746_A750del and L858R standard DNA were mixed with wild-type standard DNA at ratios from 10^0 to 10^{-5}, all curves that indicated the presence of E746_A750del and L858R increased up to 45 cycles (Fig. 1B and D). Standard curves in the range of measured volumes in this study were linear with r^2 values from 0.987 to 0.998. Both slopes of curves were almost parallel (Fig. 1E). The Ct of diluted mutant standard DNA mixed with wild-type DNA was close to that of mutant standard DNA alone. Although the peak fluorescence levels of diluted E746_A750del standard DNA mixed with wild-type DNA were lower than without wild-DNA standard, the presence of E746_A750del was clearly detected at ratios less than 10^{-4}. The peak fluorescence levels of diluted L858R standard DNA mixed with wild-type DNA were equivalent to those without wild-DNA standard. Curves of DNA with the mutations at an amount of up to 1 pg were unaffected by interfusion of DNA of wild-type EGFR. There were no significant differences between either the minimum detectable volume of the mutations or the minimum detectable ratio of wild type to the mutations.

In the cell-based experiments using genomic DNA of human cancer cell lines, the signal using DNA derived from the PC-9 cells was detected whereas the signal using DNA from the A431 cells was, as expected, not detected (Fig. 1D and E).

EGFR mutation status of serum DNA detected by EGFR scorpion. The E746_A750del or L858R status of serum DNA derived from 27 patients with NSCLC was examined. Wild-type exons 19 and 21 were detected from all serum samples. E746_A750del was detected in samples of 12 patients. L858R was detected in 1 patient (Table 2). In total, EGFR mutations were detected in 13 of 27 (48.1%) patients. The histologic subtypes of the original tumors are summarized in Table 3A in the 27 patients who were assessed for EGFR mutation in serum. Eleven of 23 (47.8%) cases of adenocarcinoma, one of two cases of squamous-cell carcinoma, and one of two cases of large-cell carcinoma were positive for EGFR mutations. An EGFR mutation was more frequently detected in the samples from female patients than those from males [7 of 10 (70%) versus 6 of 17 (35%); Table 3B].

EGFR mutation status in serum (EGFR Scorpion) and response to gefitinib. EGFR mutations were more frequently observed in the samples from the patients who showed a partial response (7 of 9 cases, 77.8%) than in samples from patients with stable disease or progressive disease (6 of 18 cases, 33.3%; $P = 0.046$, Fisher's exact test; Table 3C).

EGFR mutation status in serum (EGFR Scorpion) and effect on survival. Median progression-free survival and overall survival of all the patients treated with gefitinib were 98 and 306 days, respectively. Patients with EGFR mutations in serum showed a significantly longer median progression-free survival compared with the patients without EGFR mutations (200 versus 46 days, $P = 0.005$; Fig. 2A). The patients with EGFR mutations showed a longer median overall survival compared with the patients without EGFR mutations, although there was no statistical significance (611 versus 232 days, $P = 0.078$; Fig. 2B). These results suggest that patients who were serum EGFR mutation positive seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients who were EGFR mutation negative.

EGFR mutation in serum analyzed by direct sequencing and in comparison with EGFR Scorpion. The deletional mutation (E746_A750del) was detected by direct sequencing in serum DNA extracted from 10 of 27 patients (37.0%). No point mutation in exons 18, 19, and 21 was detected in the PCR

Table 1. Patient characteristics

	(*n*)
No. patients	27
Age (y)	
Median	64
Range	44-87
Sex	
Male	17
Female	10
Performance status	
0	19
1	6
2	2
Stage	
IIIB	3
IV	24
Histology	
Adenocarcinoma	23
Squamous-cell carcinoma	2
Large-cell carcinoma	2
Response	
Partial response	9
Stable disease	8
Progressive disease	10

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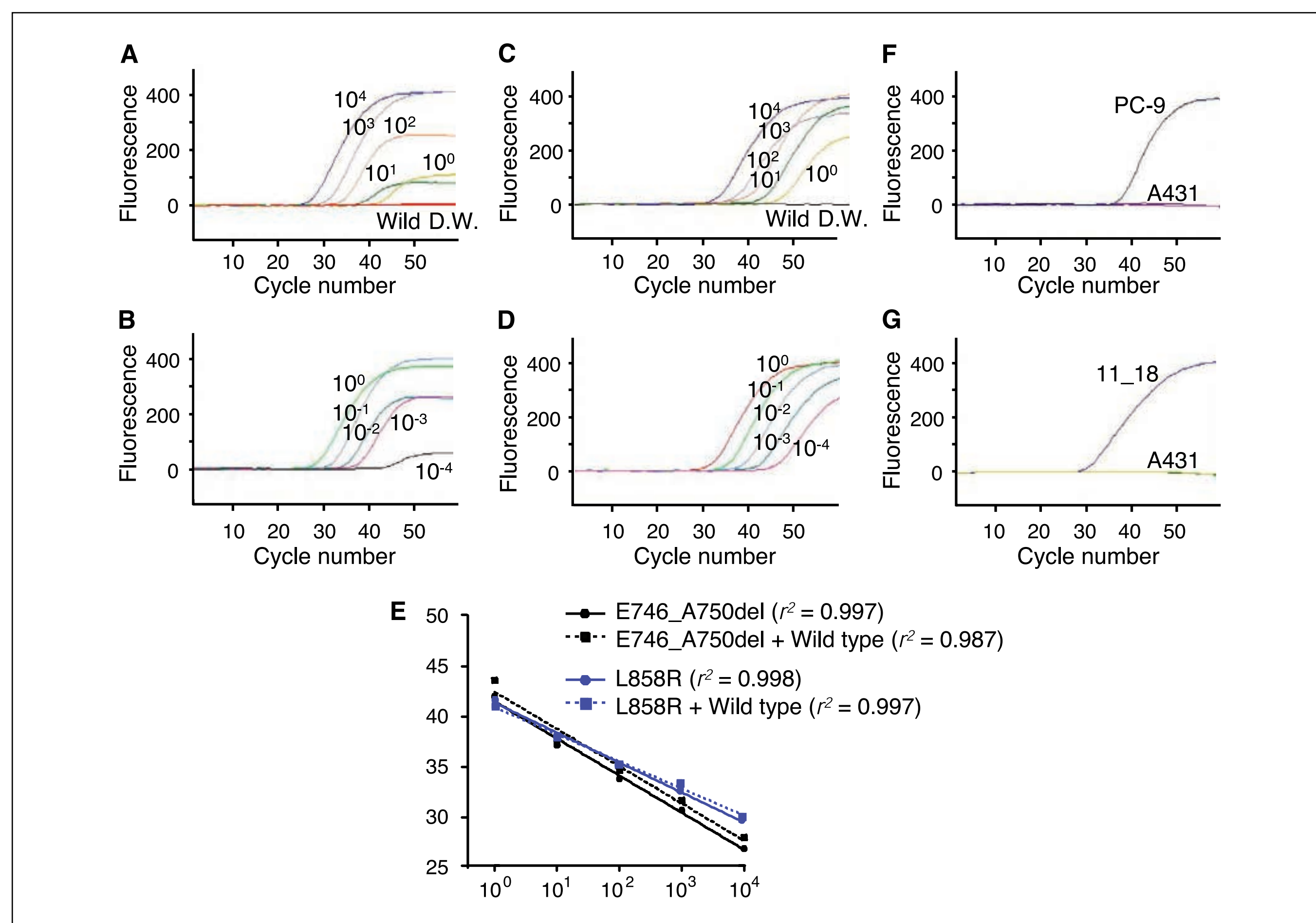

Fig. 1. Sensitivity of detection for mutations of E746_A750del and L858R using the EGFR Scorpion kit (*A* and *B,* E746_A750del; *C* and *D,* L858R). Standard DNA with E746_A750del (*A*) and L858R (*C*) were used at various volumes of 10,000 pg (10^4), 1,000 pg (10^3), 100 pg (10^2), 10 pg (10^1), and 1 pg (10^0). Standard DNA with wild-type (*Wild*) and distilled water (*D.W.*) were used as negative controls in the same experiment. Standard DNA with E746_A750del (*B*) and L858R (*D*) at concentrations from 1 to 10,000 pg were mixed with 10,000 pg of standard DNA with wild-type at a ratio of 1:1 (10^0), 1:10 (10^{-1}), 1:100 (10^{-2}), 1:1,000 (10^{-3}), and 1:10,000 (10^{-4}). *E,* standard curves were derived by plotting the Ct of each curve (shown in *A-D*) against the log of the standard DNA volume (*black lines,* E746_A750del; *blue lines,* L858R). *F,* PC-9 with E746_A750del and A431 with wild-type. *G,* 11_18 with L858R and A431.

products from serum samples. The serum EGFR status detected by direct sequence was not correlated statistically with histologic type, gender, response to gefitinib (Table 3), or survival (progression-free survival, P = 0.277; overall survival, P = 0.859). EGFR mutation status, as assessed by direct sequence, was consistent with those assessed by EGFR Scorpion in 15 of 27 (55.6%) of the paired samples. In four cases, EGFR mutation status (E746_A750del) was positive by direct sequence and negative by EGFR Scorpion. Eight cases were negative by direct sequence and positive by EGFR Scorpion. Thus, the sensitivity of EGFR Scorpion seems to be higher than that of direct sequencing due to the use of the specific primers for EGFR mutations in this kit.

EGFR mutations in tumors in comparison with those in serum. Twenty tumor samples were obtained from 15 patients retrospectively. Sequencing of EGFR exons 19 and 21 was done in samples from 12 of these under the same PCR conditions (Table 4; the other three samples were not evaluated because of low amplification of PCR products). EGFR mutations were detected in four cases (25.0%); three were the 15-bp deletion (E746_A750del) in exon 19 and one was the L858R point mutation in exon 21. Tumor histology of patients with EGFR mutations was adenocarcinoma in three and large-cell carcinoma in one. The responses to gefitinib in these four patients were two partial response, one stable disease, and one progressive disease.

Pairs of tumor samples and serum samples were obtained retrospectively from 11 patients (Table 4). The EGFR mutation status in the tumors was consistent with those in the serum of 8 of 11 (72.7%) of the paired samples. The E746_A750del mutation was positive in the tumor and negative in the serum in two patients, and the E746_A750del mutation was negative in the tumor and positive in the serum in one patient.

Discussion

Our findings have shown that EGFR mutations were detectable in serum samples obtained from patients with NSCLC and that the EGFR Scorpion kit consisting of ARMS and Scorpion technology is a useful method for detection of EGFR mutations. The EGFR mutation status in serum detected by the EGFR Scorpion was correlated statistically with responsiveness to, and the progression-free survival of, gefitinib treatment. Our finding supports the hypothesis that the EGFR

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mutation status from serum DNA is useful to predict the responsiveness to gefitinib.

The mutation rate observed in our study seems to be relatively high (48%) although we have detected only two major mutations. EGFR mutations have been detected at a higher frequency in lung tumors from female patients, those with adenocarcinoma histology, nonsmokers, and patients of Asian origin (6, 8). However, previous reports show that the mutation rate of EGFR in operative samples of Japanese patients was from 26% to 59% (4, 6, 15, 16). The EGFR mutation rate in our study is equivalent to that observed in these reports. It can be speculated that the high sensitivity and specificity of the EGFR Scorpion allowed us to detect the EGFR mutations even in serum. Another possible reason is the high number of patients with adenocarcinoma in our study (23 of 27, 85.2%). Previous studies have shown that very few patients with nonadenocarcinoma, including squamous cell carcinomas and large-cell carcinomas, have EGFR mutations (3–8). Our results were in line with the previous studies and showed that no patients with squamous cell carcinoma or large-cell carcinoma had the mutations.

Table 2. Patients' characteristics and EGFR mutant status detected from serum DNA using the EGFR ARMS-Scorpion method

Response	Gender	Histology	Exon 19		Exon 21	
			Wild	E746_A750del	Wild	L858R
PR	M	Ad	+	–	+	+
PR	F	Ad	+	+	+	–
PR	M	Ad	+	–	+	–
PR	F	Ad	+	+	+	–
PR	M	Ad	+	+	+	–
PR	F	Ad	+	–	+	–
PR	M	Ad	+	+	+	–
PR	F	Ad	+	+	+	–
PR	F	Ad	+	+	+	–
SD	M	Large	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Ad	+	–	+	–
SD	F	Ad	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Ad	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Scc	+	+	+	–
PD	F	Scc	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Large	+	+	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	+	+	–
PD	M	Ad	+	–	+	–

Abbreviations: SD, stable disease; PD, progressive disease; PR, partial response; M, male; F, female; Ad, adenocarcinoma; Large, large-cell carcinoma; Scc, squamous-cell carcinoma; +, curve detected by SmartCycler; –, curve not detected by SmartCycler.

Table 3. Frequency of EGFR mutations in serum DNA from patients with NSCLC according to histology (A), gender (B), and response to gefitinib (C)

	EGFR Scorpion kit			Direct sequence		
	+	–		+	–	
(A) Histology and EGFR mutant states						
Ad	11	12		8	15	
Non-Ad	2	2	$P > 0.999$	2	2	$P > 0.999$
(B) Gender and EGFR mutant states						
Female	7	3		5	5	
Male	6	11	$P = 0.120$	5	12	$P = 0.415$
(C) Response to gefitinib and EGFR mutant states						
PR	7	2		4	5	
SD/PD	6	12	$P = 0.046$	6	12	$P = 0.683$

NOTE: A total of 27 samples were obtained from 28 patients before treatment.

We identified 12 deletion mutations and a single point mutation (L858R). Previous reports have shown that the frequency of detection of E746_A750del is almost equivalent to that of L858R (15, 16). It seems that the rate of detection of L858R in our study was very low compared with the rate of E746_A750del. The sensitivity for detection of L858R using the Scorpion ARMS method is very high and equivalent to that of E746_A750del. We thus consider that it is unlikely that the low-frequency L858R mutation could be due to assay-related false-negative findings. On the other hand, it also seems unlikely that either sampling method or the patients' eligibility criteria are biased toward the high rate of E746_A750del. Therefore, we have not been able to clarify the moot point. Further analyses in much larger groups of patients will be necessary to clarify the frequency of the major two mutations in serum DNA. Unfortunately, parallel tumor tissue investigations were done only on a small subset of the participating patients. Furthermore, findings in the serum were divergent from those obtained from the primary tissue in 3 of 11 patients from whom the paired samples were obtained. Therefore, this study is at best hypothesis-forming and will require follow-up analysis in much larger groups of patients.

Some investigators reported that mutations in the EGFR tyrosine kinase domain enhanced responsiveness to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib, and seemed to be associated with the prolonged survival of the patients who received these drugs (7, 8). In a placebo controlled study showing a survival advantage for NSCLC patients who received erlotinib, Tsao et al. (17) showed that the presence of an EGFR mutation might increase responsiveness to erlotinib, but was not indicative of a survival benefit, and concluded that EGFR mutation analysis was not necessary to identify patients in whom treatment with EGFR inhibitors was appropriate. Our results are not in line with their conclusions. In their study, the rate of mutation analysis was low and 107 of 731 patients

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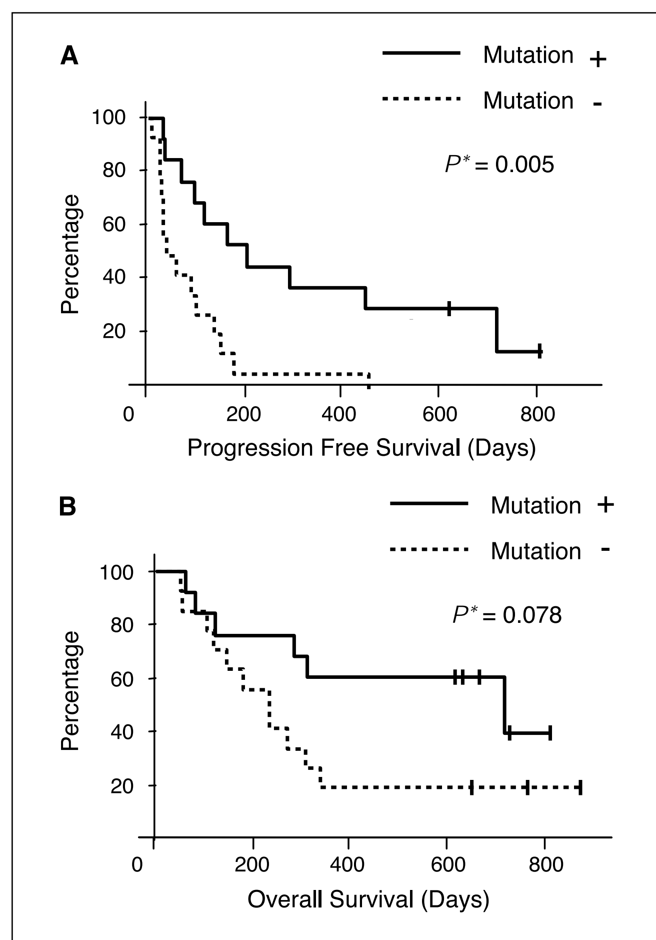

Fig. 2. Progression-free survival (*A*) and overall survival (*B*) with respect to the EGFR mutation status of NSCLC. *, log-rank test.

enrolled in their study were successfully analyzed for EGFR mutation. Sensitivity for detecting EGFR mutation in their study might be unstable as interfusion of normal cells in tumor cells decreases the sensitivity for detecting tumor-derived mutations using direct sequencing. They propose that additional processes (such as microdissection) to enrich tumor cell DNA might increase the rate of detection of new mutations; however, it seems that their results are insufficiently robust to reach this conclusion. Therefore, we propose the use of EGFR mutation analysis from serum DNA, which is easily collected and repeatable, to show that EGFR mutation status using the EGFR Scorpion kit correlates with the responsiveness to gefitinib.

EGFR mutation in NSCLC is reported to be somatic (3, 4). It is well known that the concentration of free circulating DNA in serum is higher in patients with tumors than in healthy volunteers (18) and it seems that the detected mutational EGFR in serum was tumor derived. This is the first report analyzing EGFR mutations from serum DNA and evaluating EGFR mutation status and clinical outcome (response and survival) with gefitinib. No other studies have analyzed EGFR mutations from samples other than actual tumor samples. The mutation in two patients was positive in the tumor and negative in the serum, and the mutation in one patient was negative in the tumor and positive in the serum. We have tried to explain the discrepancy why tumor and serum were not better correlated as follows. In cases of positive in the tumor and negative in the serum, the volumes of mutant DNA extracted from the serum were under the detectable limit using the Scorpion ARMS method, or a very small amount of DNA derived from an actual tumor was circulating in the bloodstream. A previous study showed that 73% of patients with at least one molecular event, such as a hypermethylation of the tumor suppressor gene *p16*, in their tumor DNA had the same alteration in plasma DNA (10). In a case of negative in the tumor and positive in the serum, wild-type DNA interfered with

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Table 4. EGFR mutation status in tumor samples and serum samples. Pairs of both tumor samples and serum samples were obtained from 11 patients

Gender	Histology	Response	EGFR mutation status				
			Tumor sample	EGFR Scorpion kit (serum sample)			
				Exon 19		Exon 21	
				Wild	Mutation	Wild	Mutation
M	Large	SD	Wild	+	–	+	–
F	Scc	PD	Wild	+	–	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	PR	L858R	+	–	+	+
F	Ad	SD	Wild*	+	+	+	–
M	Large	PD	E746-A750del	+	+	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	SD	E746-A750del*	+	–	+	–
F	Ad	PR	E746-A750del*	+	–	+	–
M	Ad	PD	Wild	+	–	+	–

*Patients who have different states of EGFR mutation from tumor-derived DNA and serum-derived DNA.

the detection of mutant DNA in the tumor samples using the direct sequencing method. The rate of the mutations in serum DNA detected by the Scorpion ARMS was compared with that in tumor tissues detected by the direct sequencing method as a current standard method. DNA from tumor samples consisted of a mixture of the mutant DNA and wild-type DNA because the EGFR mutation status was always heterogeneous, and the complete removal of normal cells, such as normal epithelial cells and inflammatory cells, from tumor specimens is very difficult. Parallel tumor tissue investigations were done on only a small subset of these patients, which is a recognized limitation in the present study. A larger study is necessary to evaluate the consistency of the mutation status from tumor and serum. On the other hand, it is sometimes difficult to obtain tumor samples from patients with inoperable NSCLC in prospective studies. We showed that patients who were EGFR mutation positive in the serum DNA using the Scorpion ARMS method seem to have better outcomes with gefitinib treatment in terms of progression-free survival, overall survival, and response, despite the nonconformity between the mutation states of tumor and serum DNA in some of the patients. We anticipate that the detection of EGFR mutations in serum DNA using the Scorpion ARMS will be equivalently useful as a feasible approach for predicting tumor response to gefitinib.

Two groups have reported alternative methods for detection of EGFR mutations. One group used the LightCycler PCR assay (19) and the other postulated that the SSCP assay was more sensitive than direct sequencing and was a rapid method (20). Further studies are needed to validate these assays for detection of EGFR mutations and to clarify the most sensitive assay. Although the direct sequence method is common in reported studies, the EGFR mutation status in serum DNA by direct sequencing did not correlate with the responsiveness to and survival benefit of gefitinib in our study. These results indicate that the EGFR Scorpion kit is superior to the direct sequencing method for detection of an EGFR mutation in serum as a predictive marker.

One limitation of the EGFR Scorpion kit is that it is only able to detect mutations targeted by the designed Scorpion primers. EGFR mutations are not solely at these two sites but clustered around the ATP-binding site in exons 18, 19, and 21 (3–8). Moreover, the secondary mutation, a substitution of methionine for threonine at position 790 (T790M), leads to gefitinib resistance in NSCLC patients who have EGFR mutations and are responsive to treatment with gefitinib (21–23). Mutations in *K-ras*, a known downstream signaling molecule in the EGFR signaling pathway, are more frequent in patients who develop disease progression with treatment with either gefitinib or erlotinib (24). These mutation states may also be critical factors for the treatment of gefitinib. To clarify the usefulness of serum DNA as a source of genotypic information, the Scorpion primers need to be designed for detection of these mutations, and further studies using these primers are required.

In conclusion, the two major mutations of EGFR, E746_A750del and L858R, were detected in serum DNA with the EGFR Scorpion kit from patients with NSCLC. These results suggest that patients who were EGFR mutation positive seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients who were EGFR mutation negative. In the near future, a controlled clinical trial is necessary to confirm these conclusions.

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