The Characterization of Gefitinib Sensitivity and Adverse Events in Patients with Non-small Cell Lung Cancer

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Abstract. *Background: Factors predicting gefitinib sensitivity and adverse events in non-small cell lung cancer (NSCLC) remain controversial. Patients and Methods: Correlations among clinicopathological characteristics, gefitinib sensitivity and adverse events were studied in 154 patients with NSCLC, whereas epidermal growth factor receptor (EGFR) mutations were analyzed in 44 patients. Results: Female, non-smoker, adenocarcinoma of stage I-II, and gefitinib effectiveness correlated with longer time to progression (TTP) and overall survival (OS), while the rate of interstitial lung disease in patients undergoing thoracic radiotherapy and stomatitis in females or those who never smoked were significantly higher. EGFR mutations were identified in 18 cases, and among 34 gefitinib-treated patients, 16 patients harboring mutations tended to do better, both in terms of TTP and OS. The results of the mutation analysis from surgical and non-surgical specimens were identical. Conclusion: Certain clinicopathological characteristics and EGFR mutations can be either predictive of gefitinib sensitivity or adverse events.*

The clinical stage of non-small cell lung cancer (NSCLC) is already advanced at diagnosis in more than 70% of the patients. Their prognoses are usually poor because this disease is commonly refractory to conventional chemotherapy. The onset and the proliferation of NSCLC often involve the epidermal growth factor receptor (EGFR), an ErbB family member, and a cascade of signaling pathways. EGFR is a component of signalling pathways involving tyrosine kinases (TK) regulating cell activation by forming monodimers or heterodimers with ErbB receptors after ligand binding. It is known that aberrations in these signaling pathways can lead to tumorigenesis.

Gefitinib (Iressa [ZD1839]; AstraZeneca Pharmaceuticals, Wilmington, DE, USA), reversibly inhibits TK by competing with ATP at an ATP binding site of the EGFR, and may, thus, exert anti-tumor effects. Fukuoka *et al.,* have reported that Japanese patients with NSCLC showed more favorable clinical responses to gefitinib compared to patients in other countries (27.5% *versus* 10.4%, respectively) (1). However, the molecular mechanisms underlying gefitinib sensitivity are not well-understood. Regarding adverse events, it has been reported that the occurrence of skin disease, digestive tract problems, liver dysfunction and body pain was significant, and, even more seriously, interstitial lung disease (ILD) was potentially fatal.

Recently, two groups have reported that somatic mutations in exon 18, 19 or 21, constituting a TK domain of the EGFR gene, are strongly correlated with sensitivity to gefitinib in patients with NSCLC (2, 3). Paez *et al.* showed that EGFR mutations in lung adenocarcinoma are more frequent in Japanese than in Caucasians (32% *versus* 3%), perhaps correlating with the superior response to gefitinib therapy in Japanese (3). Similar reports from several countries, especially in eastern Asia, confirmed racial differences in the frequency of EGFR mutations and in gefitinib sensitivity (4-14). Some reports noted better survival in patients with EGFR mutations, however, others found no significant differences in time to progression (TTP) and/or overall survival (OS) after gefitinib therapy in patients with or without EGFR mutations (4, 6, 8, 10). The reasons for these discrepancies are not known.

In the studies conducted to date, mutation analysis had been mostly confined to surgically-resected specimens. However, pleural effusion and biopsy specimens obtained by transbronchial biopsy (TBB) or needle biopsy have been used for pathological diagnosis, but there have been few

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attempts at mutational analyses using these small amounts of material (15). It was considered that if small specimens could suffice for mutation analysis, the number of patients eligible for such studies would be increased. Therefore, the aim of the present study was to identify predictive factors for gefitinib sensitivity and risk factors for adverse events, and additionally to test whether tumor cells derived from biopsies or cytology specimens are suitable for mutation analysis of the *EGFR* gene.

Patients and Methods

Study design and patient characteristics. One hundred and fifty-four consecutive Japanese patients with NSCLC treated with gefitinib in our Institutes, from September 2002 through March 2005, were entered into this study. The clinicopathological characteristics and adverse events associated with gefitinib therapy were evaluated retrospectively. TTP and OS were also analyzed. After informed consent had been obtained from all patients, EGFR mutations were analyzed in 34 out of the 154 patients, and in an additional 10 patients whose outcomes were not established (nine were not given gefitinib and one stopped due to severe nausea), because their specimens were applicable for the mutation analysis, such as polymerase chain reaction (PCR) amplification or direct sequencing. The specimens were obtained by surgery (n=22), TBB (n=13), lymph node biopsy (n=2), needle biopsy (n=2), or came from pleural effusion (n=1). In four patients, it was possible to compare results from two specimens obtained by different procedures (needle biopsy and autopsy in one case and TBB and surgery in three cases). The therapeutic effect of gefitinib was determined according to the Response Evaluation Criteria in Solid Tumors (RECIST) (30). Partial responses (PR) and complete responses (CR) were considered as defining therapy responders. TTP and OS were defined as the duration from initiation of gefitinib therapy to the confirmation of progressive disease (PD) and to the time of death, respectively. Clinical stages I to II and III to IV were categorized as early and advanced, respectively.

Mutational analysis. Histopathological reviews and preparation of genomic DNA were carried out using paraffin-embedded sections. Constituents in the specimens other than tumor cells were manually eliminated so that the latter always represented >50% of the entire specimen. Genomic DNA was extracted using Takara DEXPAT™ (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. Cells collected from pleural effusion were treated with QIAmp DNA Mini kit (Qiagen, Valencia, CA, USA). The entirety of exons 18, 19, 20 and 21 of the *EGFR* gene were amplified by nested PCR using primers from Sigma Genosys (Hokkaido, Japan) as described elsewhere (3). Each amplified fragment, which was confirmed as a single amplicon, was purified with a QIA quick PCR purification kit (Qiagen) and bidirectionally sequenced with a Big Dye Terminator Cycle Sequencing kit using the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The final sequence result was confirmed by independently repeated amplifications and DNA-sequencing analyses.

Statistical analysis. The Pearson's χ^2 test or Fisher's extract test was used for statistical analyses. A logistic regression model was used for multivariate analysis. The Mann-Whitney test was used for analyzing clinical responses. The mean durations of TTP and OS were calculated using the Kaplan-Meier method. Comparisons between two groups were made using log-rank tests. The two-sided significance level was set at $p<0.05$.

Table I. *Clinicopathological characteristics of the gefitinib-treated patients.*

Gefitinib-treated cases (n=154)	No.	
Age (mean±SD, years)		
	65.3±11.8 (all)	
	64.5±12.6 (male)	
	66.5±10.6 (female)	
Gender		
Male	92	
Female	62	
Histology		
ADC	121	
SCC	18	
LCC	7	
SCLC+ADC	2	
ASC	6	
Smoking history (Male/Female)		
Never smoked	61	(13/48)
Smoker	93	(79/14)
Stage		
I-II	19	
III-IV	135	
Prior chemotherapy		
0	50	
≥1	104	
Clinical response (evaluable patients=151)		
CR	6	
PR	56	
NC	37	
PD	52	

ADC: adenocarcinoma, SCC: squamous cell carcinoma, LCC: large cell carcinoma, SCLC: small cell carcinoma, ASC: adenosquamous cell carcinoma.

Results

Gefitinib sensitivity. The clinicopathological characteristics of the gefitinib-treated patients are given in Table I. The mean age was 65.3 years (30 to 92 years; male 64.5 years, female 66.5 years. Out of the 154 patients, 62 (40.3%) were female. One hundred and twenty-one diagnoses were of adenocarcinoma (78.6%), out of which 19 were early stage (12.3%). Sixty-two patients were responsive to gefitinib (40.3%); 61 patients had never smoked (39.6%); and 104 had a history of prior chemotherapy to gefitinib (67.5%). Among these variables, female gender (TTP: 3.8 *versus* 1.8 months, $p=0.031$; OS: 8.7 *versus* 4.7 months, $p=0.006$), diagnosis of adenocarcinoma (TTP: 3.0 *versus* 1.8 months, $p=0.037$; OS: 6.8 *versus* 2.5 months, $p=0.0008$), early stage (TTP: 11.4 *versus* 2.3 months, $p=0.005$; OS: 13.2 *versus* 5.0

Table II. *Adverse events due to gefitinib.*

Variable	Number of patients		
	Total (154)	Mutation (15)	Wild-type (18)
Lung injury	18	0	2
Liver dysfunction	14	2	3
Skin eruption	53	9	9
Diarrhea	11	1	2
Stomatitis	6	2	1
Nausea, appetite loss	12	1	0
Hematuria	3	0	0
Edema	3	0	1
Pancytopenia	2	0	0
Renal dysfunction	1	0	0
Hemorrhage	2	0	0
Anemia	1	1	0

Table III. *Clinicopathological characteristics of the mutation-analyzed cases.*

Mutation-analyzed cases (n=44)			
	Total	Mutation	
	44	18	
Age (mean±SD)	63.6±11.2	(All)	
	62.8±12.8	(Mutation)	
	64.3±10.1	(Wild-type)	
Gender			
Male	18	8	$p=0.691$
Female	26	10	
Smoking history			
Never smoked	29	13	$p=0.462$
Smoker	15	5	
Histology			
ADC	39	18	$p=0.060$
SCC	2	0	
LCC	1	0	
SCLC+ADC	1	0	
ASC	1	0	
Stage			
I-II	13	2	$p=0.026$
III-IV	31	16	
Prior chemotherapy			
No	11	3	$p=0.241$
Yes	33	15	
Sample analyzed			
Surgical material	22	7	
Non-surgical	18	10	
Both	4	1	
Clinical response (Evaluable = 34, Mutation = 16)			
CR	5	5	$p=0.003$
PR	18	10	
NC	7	1	
PD	4	0	

ADC: adenocarcinoma, SCC: squamous cell carcinoma, LCC: large cell carcinoma, SCLC: small cell carcinoma, ASC: adenosquamous cell carcinoma.

months, $p=0.004$), never smoked (TTP: 5.6 *versus* 1.8 months, $p=0.001$, OS: 9.2 *versus* 4.1 months, $p=0.0001$); and responsiveness to gefitinib (TTP: 9.4 *versus* 1.5 months, $p<0.0001$, OS: 9.4 *versus* 3.8 months, $p<0.0001$) were significantly correlated with longer duration of TTP and OS, while prior chemotherapy was not (TTP: 2.5 *versus* 3.5 months, $p=0.133$; OS.5.6 *versus* 5.9 months, $p=0.208$).

Adverse events due to gefitinib. Adverse effects of gefitinib are summarized in Table II. Occurrence of skin rash was the most common (34.4%). Nausea resulted in discontinuation of gefitinib in one case. The incidence of ILD was significantly higher in patients who had received prior thoracic radiotherapy (odds ratio 3.974, $p=0.016$), and six out of 18 patients who developed ILD died. Stomatitis was developed much more frequently in women and patients who had never smoked (female, odds ratio 7.982, $p=0.028$; never smoked, odds ratio 8.214, $p=0.026$).

EGFR mutations. An EGFR mutation was identified in 18 out of 44 cases analyzed (40.9%) as shown in Table III. Each mutation site is shown in Figure 1. Mutations were identified in nine out of 13 TBB specimens (69.2%), seven out of 22 surgically-resected specimens (30.4%), and one out of two lymph node biopsy specimens (50.0%). No mutations were found in two needle biopsy specimens. One case of a cytology specimen derived from malignant pleural effusion harbored the L858R mutation. No mutations were identified in three cases of either TBB or surgically-resected specimens, while in one case the same mutation (E746_A750 deletion) in exon 19 was found in both needle biopsy and autopsy specimens of the metastatic lymph node. There were six cases of in-frame deletion mutations and two cases of insertion mutations in exon 19, as well as ten substitution mutations (two cases in exon 18, one in exon 19, three in exon 20, three in exon 21, one in both exon 18 and 19) (Figure 1). Unexpectedly, one patient who had not received gefitinib therapy had the T790M substitution mutation in exon 20. This mutation have been previously reported to be newly acquired in gefitinib resistance (16).

The clinical stage of almost all 18 patients with mutations was advanced (94.4%, 17 cases; $p=0.018$). The presence of EGFR mutations was significantly associated with clinical response to gefitinib ($p=0.0008$). Fifteen tumors harboring

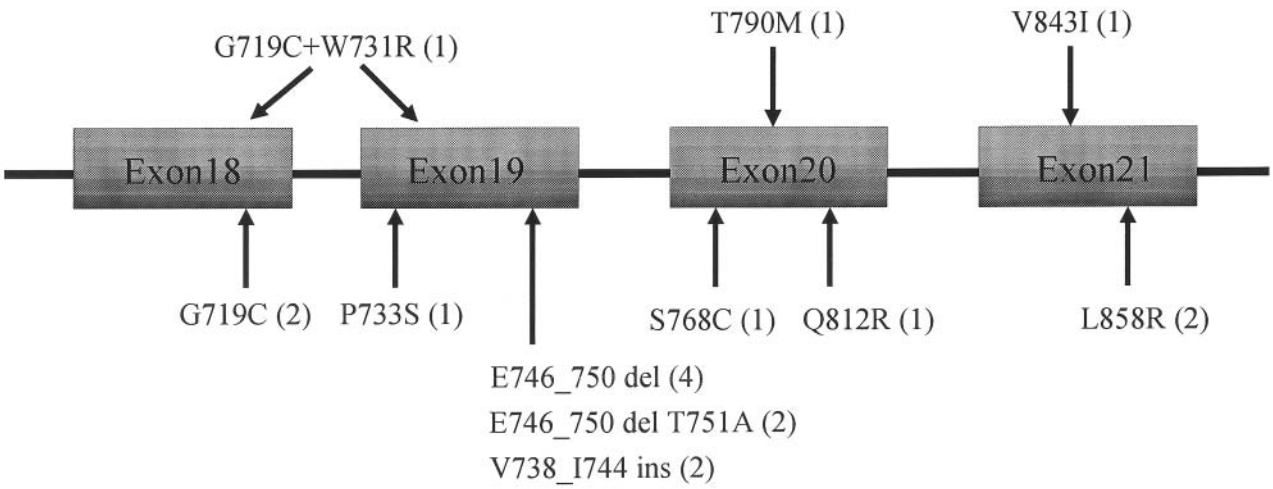

Figure 1. *Mutation sites of exons 18-21 in the EGFR gene. The number of cases identified with the respective mutation is shown in parenthesis.*

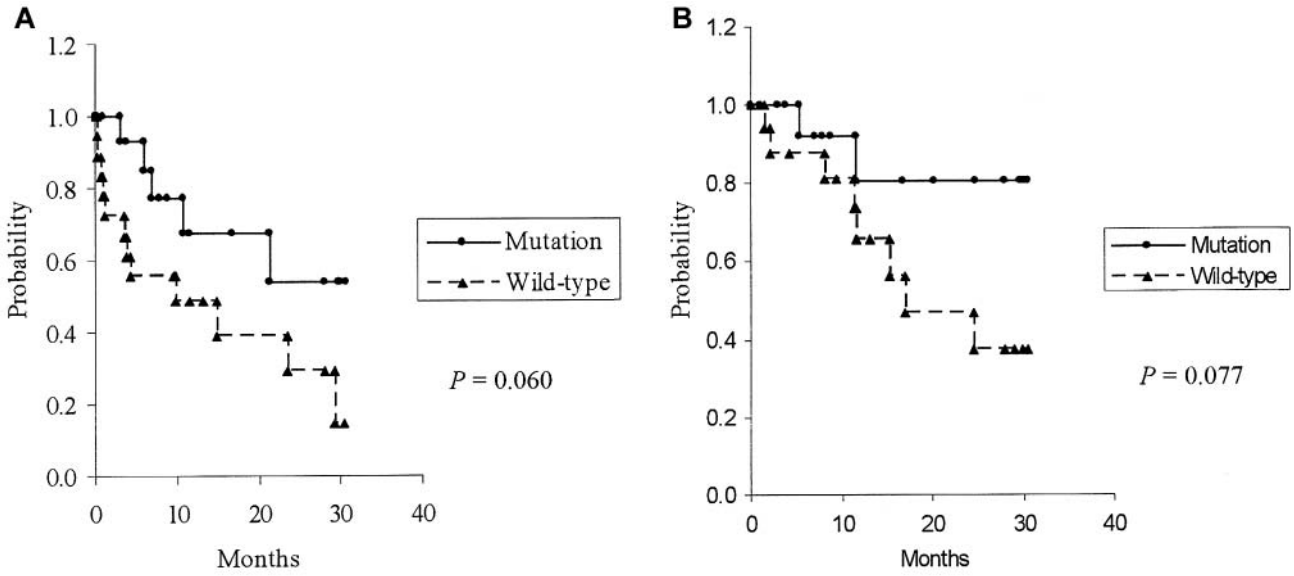

Figure 2. *Kaplan-Meier plots of (A) time to progression and (B) overall survival after gefitinib therapy, depending on EGFR mutation status.*

EGFR mutations were responsive to gefitinib and an additional one showed SD. The remaining two patients could not be included because one had not received gefitinib and the other was withdrawn due to severe nausea.

All mutations were found in adenocarcinoma ($p=0.06$). Eleven out of 15 (73.3%) patients had received chemotherapy prior to gefitinib. There were no significant differences in the mean age (mutation; 62.8 *versus* 64.3), gender (female; 10 out of 26 *versus* 16 out of 26; $p=0.691$), smoking history (never smoked; 13 out of 29 *versus* 16 out of 29; $p=0.462$), or prior chemotherapy (17 out of 35 *versus* 18 out of 35; $p=0.587$).

Univariate analysis was performed using the Kaplan-Meier method to evaluate TTP and OS for those gefitinib-treated patients (16 mutation and 18 wild-type cases) whose prognoses could be precisely estimated. The mutation group experienced prolonged TTP (mean, 13.70 *versus* 10.52 months; $p=0.060$) and OS (mean, 15.02 *versus* 13.87 months; $p=0.077$) (Figure 2).

EGFR mutations and adverse events due to gefitinib. Concerning possible correlations between EGFR mutations and adverse events associated with gefitinib therapy, it was found that lung injuries were developed in the two wild-type

cases, but not in the mutation group ($p=0.169$). The overall incidence difference of adverse events was not statistically significant between the two groups. Adverse events were seen in 11 out of 15 mutation cases (73.3%) and one patient was obliged to discontinue gefitinib due to gastrointestinal tract problems.

Evaluation of small amounts of samples. One malignant pleural effusion sample and 17 biopsy specimens, including TBB (n=13), lymph node biopsy (n=2), needle biopsy (n=2) and 22 surgical specimens, were used for mutation analyses (Table III). Mutations were detected in 7 surgical samples (30.4%), while out of the 18 non-surgical specimens 11 (61.1%) showed mutations. In order to validate these results, mutation analyses were also performed on three surgical specimens, for which the corresponding non-surgical specimens had not shown any mutations, and on an autopsy specimen whose lymph node biopsy had revealed a deletion mutation in exon 19. Identical results were obtained for both the surgical and non-surgical specimens.

Discussion

The aim of the present study was to identify predictive factors for gefitinib sensitivity and patient prognosis, as well as risk factors for adverse events associated with gefitinib therapy. Female gender and never having smoked have been recently identified as candidate risk factors for stomatitis. Stomatitis is a common adverse event of gefitinib therapy and often impairs quality of life (QOL); both female gender and non-smoking are also predictive factors for the effect of gefitinib (17, 18).

In the present study, EGFR mutations were identified in 18 out of 44 patients (40.9%). Although the mutation rate established here was similar to the previous reports from eastern Asia (17, 18), the overall mutation rate in this study was slightly higher (18.9% to 47.6%; mean 36.0%), whereas in clinically responsive cases it was relatively low (52.4% to 82.8%; mean 74.4%) compared to those previous reported (4, 5, 7-9, 11-14). Possible reasons for the lower mutation rate in clinically responsive cases may include: (i) other factors defining gefitinib sensitivity, (ii) the remaining tumor acquired an EGFR mutation after we had obtained specimens, and (iii) the small number of cases analyzed influenced the result. Other factors defining gefitinib sensitivity could include increased *EGFR* or *HER2* gene copy number and protein phosphorylation of Akt, PTEN, ERK1/2, or STAT5 (19-27). Increased copy number of the HER3 or 4 genes should be also evaluated because their products can form heterodimers with EGFR, whilst protein expression or phosphorylation in signaling pathways other than Akt, PTEN, ERK1/2 and STAT5 might be important. Small biopsy specimens might not have reflected the major characteristics of individual tumors, because the proportion of tumor cells harboring EGFR mutations within analyzed specimens may have been so low that insufficient cells with mutations were included. In addition, some reports have shown that the detection rate of EGFR mutation by new methods seemed to be superior to that by direct sequencing methods (28, 29). Although the direct sequencing method was the only one used in the present study, the differences found are unlikely due to specimen size, because the feasibility of using small specimens for EGFR mutational analysis was evaluated, and surgical materials and the corresponding non-surgical and small specimens revealed consistent results in mutation analyses. Furthermore, the detection efficiency of EGFR mutations in small specimens such as biopsy fragments or cells recovered from pleural effusion was comparable to that in surgical materials. However, it might be necessary to examine more cases because the feasibility was evaluated for 4 pairs and only one mutation case could be verified.

The group of patients with EGFR mutations experienced better TTP and OS. However, this difference was not statistically significant. This could also be explained by the same factors defining gefitinib sensitivity with those described as possible reasons for the lower mutation rate in clinically responsive cases. Another explanation may be that the sample of patients was enriched with gefitinib-responsive cases because of the retrospective nature of the study. Because clinical responses would yield significant differences in OS, as well as in TTP, and no mutations were found in any cases but one refractory to gefitinib, larger-scale mutation analysis eliminating "selectivity bias" could lead to statistical significance in TTP and OS. A third explanation could be that analytical or technical limitations, such as employing direct sequencing or using paraffin-embedded tissues may have affected the mutation detection rate; the development of new analytical methods aims to overcome such limitations (28, 29).

Conclusion

Certain clinicopathological characteristics, as well as EGFR mutations were identified which can be either predictive factors for gefitinib sensitivity or risk factors for adverse events associated with gefitinib therapy. EGFR mutations could be identified from biopsy or cytology specimens in patients with advanced NSCLC. These data might contribute to establishing optimum gefitinib therapy for NSCLC patients, especially at advanced stages.

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