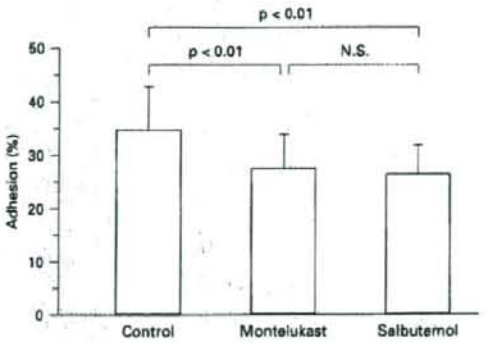

Fig. 2. Effects of montelukast (1 μM) and salbutamol (1 μM) on IL-5 -induced (100 pM) eosinophil adhesion to rh-ICAM-1. Data are expressed as means ± SEM from six experiments. N.S. = Not significant.

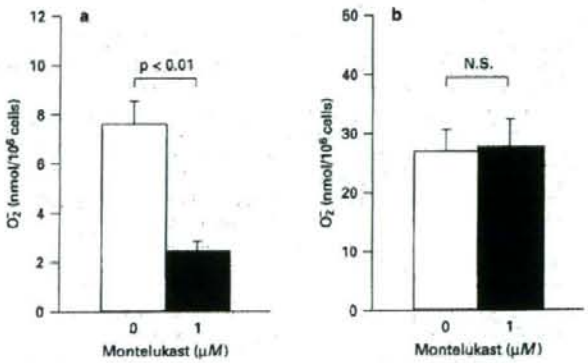

Fig. 3. Effects of montelukast (1 μM) on **a** LTD_4- (100 nM) or **b** PMA-induced (1 ng/ml) eosinophil O_2^- generation. Data are expressed as means ± SEM from six experiments. N.S. = Not significant.

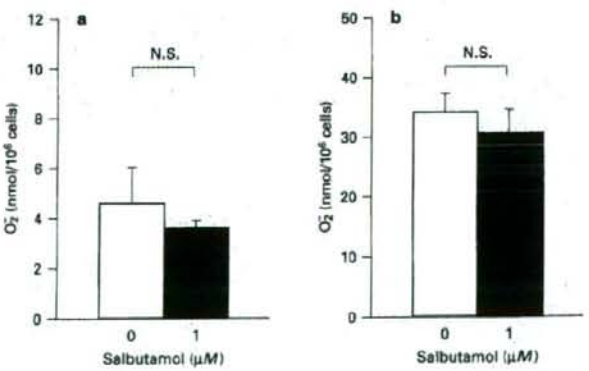

Fig. 4. Effects of salbutamol (1 μM) on **a** LTD_4- (100 nM) or **b** PMA-induced (1 ng/ml) eosinophil O_2^- generation. Data are expressed as means ± SEM from six experiments. N.S. = Not significant.

Discussion

β_2-Agonists have been shown to modulate several functions of eosinophils in response to a variety of inflammatory mediators [20]. In this study, we observed that IL-5-induced adhesion of eosinophils to rh-ICAM-1 was partially inhibited by salbutamol, a representative inhaled β_2-agonist. This result is consistent with our previous observation that IL-5-induced eosinophil adhesiveness was attenuated by tulobuterol, a β_2-agonist [21]. The fact that montelukast, an LTRA, partially attenuated IL-5-induced adhesion of eosinophils to ICAM-1 is not what we expected. Although the exact mechanism remains unknown, both IL-5 and ICAM-1 have the ability to amplify eosinophil generation of cysLTs [27–29]. The combination of these two factors may induce the generation of cysLTs in vitro, which in turn augments the adhesion of eosinophils [11]. Nonetheless, these observations suggest that both β_2-agonists and LTRAs may downmodulate the interaction of eosinophils with ICAM-1 in the presence of IL-5.

This study confirms our previous observations that LTD_4 directly induced activation of the respiratory burst and adhesion to ICAM-1 of eosinophils. These effects of LTD_4 were significantly inhibited by montelukast, indicating that LTD_4 acts mainly via cysLT1 receptors expressed on eosinophils. In contrast to montelukast, a high concentration of salbutamol failed to modify LTD_4-induced activation or adhesion of eosinophils, suggesting that LTD_4-induced activation and adhesion of eosinophils are insensitive to β_2-agonists. These results suggest that LTRAs may provide preferential effects on the regulation of airway inflammation in asthma, especially under the condition that cysLTs are highly involved. Neither montelukast nor salbutamol modified PMA-induced O_2^- generation from eosinophils, suggesting that eosinophil activation via protein kinase C is insensitive to these pharmacological agents.

There is evidence that a cysLT contributes to the accumulation of eosinophils in the airways of asthmatics. There is also increasing evidence that the addition of an LTRA, but not an LABA, to ICS additionally reduces the number of eosinophils in sputum, blood and exhaled nitric oxide in asthma [22, 23]. Our results that an LTRA, but not a β_2-agonist, modified eosinophil activation induced by a cysLT may provide new insights into the mechanisms by which antiasthma therapy regulates eosinophilic inflammation in asthma. When activated, a variety of inflammatory cells involved in asthmatic inflammation are capable of generating cysLTs at sites of allergic inflammation, and therefore, eosinophils are likely to be exposed to LTD_4 [30, 31]. Meanwhile, LTD_4 induces the respiratory burst of eosinophils and enhances the interaction between eosinophils and ICAM-1, which is constitutively expressed on airway epithelium [11, 12]. An oxygen metabolite, hydrogen peroxide, also augments both the expression of ICAM-1 on endothelial cells [32] and eosinophil adhesion to ICAM-1 [24]. Interaction with ICAM-1 enhances the effector functions of eosinophils, including the generation of cysLTs [33]. Finally, the chemotactic response [12, 14], enhanced survival [15] and interaction between ICAM-1 and eosinophils would be augmented by newly generated cysLTs. Thus, an LTRA can modify the adhesion and other effector functions of eosinophils induced by cysLTs, and thereby may effectively contribute to the downregulation of eosinophilic inflammation in asthma. Our observations also raise the possibility that eosinophilic inflammation caused by cysLTs is insensitive to β_2-agonists. Despite its effects on eosinophil activation induced by a variety of mediators including IL-5, it is unlikely that β_2-agonists, even at high concentrations, are capable of regulating the cysLT-dependent mechanisms of eosinophilic inflammation in asthma. These differential pharmacological properties would explain the eventual manifestations of the effects of LTRAs and β_2-agonists, especially in combination with ICS, on the regulation of airway inflammation in asthma.

A combination of ICS and an LABA preferentially improves symptoms and pulmonary function as compared with ICS plus an LTRA; however, underlying eosinophilic inflammation is a fundamental feature of asthma and may result in airway remodeling [1–3]. Recent evidence suggests that the addition of an LTRA, but not an LABA, to ICS further reduces airway inflammation of asthma [22, 23]. Our observations partially explain such differential effects of LTRAs and LABAs on inflammation in asthma and raised the possibility that LTRAs may provide preferential effects in terms of prevention of airway remodeling.

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Gefitinib for non-small-cell lung cancer patients with epidermal growth factor receptor gene mutations screened by peptide nucleic acid-locked nucleic acid PCR clamp

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This study was prospectively designed to evaluate a phase II study of gefitinib for non-small-cell lung cancer (NSCLC) patients with epidermal growth factor receptor (EGFR) mutations. Clinical samples were tested for EGFR mutations by peptide nucleic acid-locked nucleic acid PCR clamp, and patients having EGFR mutations were given gefitinib 250 mg daily as the second treatment after chemotherapy. Poor PS patients omitted chemotherapy. Of 107 consecutive patients enrolled, samples from 100 patients were informative, and EGFR mutations were observed in 38 patients. Gefitinib was given to 27 patients with EGFR mutations, and the response rate was 78% (one complete response and 20 partial responses; 95% confidence interval: 58–93%). Median time to progression and median survival time (MST) from gefitinib treatment were 9.4 and 15.4 months, respectively. Grade 3 hepatic toxicity and skin toxicity were observed in one patient each. There were significant differences between EGFR mutations and wild-type patients in response rates (78 *vs* 14%, $P=0.0017$), and MST (15.4 *vs* 11.1 months, $P=0.0135$). A Cox proportional hazards model indicated that negative EGFR mutation was a secondary prognostic factor (hazards ratio: 2.259, $P=0.036$). This research showed the need for screening for EGFR mutations in NSCLC patients.

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Clinical Studies

Gefitinib is an orally active epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that competes with ATP for the ATP-binding site in the cytoplasmic tail of EGFR (Brehmer *et al*, 2005). Gefitinib was studied in two trials: the Iressa® Dose Evaluation in Advanced Lung Cancer (IDEAL)-1 and IDEAL-2 trials (Fukuoka *et al*, 2003; Kris *et al*, 2003). Patients enrolled in the IDEAL-1 and IDEAL-2 trials were required to have failed only one prior platinum-containing regimen, and a platinum plus docetaxel, respectively. In the IDEAL trials, the response rates ranged from 9 to 19%. Grades 3 and 4 toxicities were relatively uncommon. Based on the IDEAL trials, gefitinib received registration approval by the US Food and Drug Administration (FDA) for the second- and third-line treatment of non-small-cell lung cancer (NSCLC) (Siegel-Lakhai *et al*, 2005). The Iressa® Survival Evaluation in Lung Cancer (ISEL) trial investigated gefitinib in second- and third-line NSCLC patients to investigate the survival benefit of gefitinib monotherapy compared with placebo. A total of 1692 patients who were refractory to or could not tolerate chemotherapy were enrolled. The results showed significantly greater tumour shrinkage in the gefitinib arm, but the overall survival durations were similar in both arms: 5.6 months in treated patients *vs* 5.1 months in patients received placebo. This failure of gefitinib to show a survival advantage over placebo resulted in controversy about the registration (Thatcher *et al*, 2005; Twombly, 2005).

In 2004, it was shown that mutations in the *EGFR* gene are significantly associated with response to two tyrosine kinase inhibitors, gefitinib (Lynch *et al*, 2004; Paez *et al*, 2004). The majority of EGFR tyrosine kinase domain mutations occur in two 'hot spots', exons 19 and 21. In exon 19, deletions eliminate four highly conserved amino acids (LREA). In exon 21, a missense point mutation substitutes an amino acid at position 858 (L858R). Among various mutations found in the EGFR tyrosine kinase domain, only the following have so far been positively associated with a response to gefitinib or erlotinib from retrospective analyses: G719C (exon 18), some of the common exon 19 deletions (LREA), L861Q (exon 21) and L858R (exon 21) (Pao and Miller, 2005). All such mutations result in conformational changes that lead to increased sensitivity to tyrosine kinase inhibitors.

Several retrospective studies have shown that higher rates of these mutations were found in females, in never-smokers, in Asians and in patients with adenocarcinomas (Mitsudomi *et al*, 2005; Tokumo *et al*, 2005). And a better response to gefitinib has been reported in patients harbouring EGFR mutations (Taron *et al*, 2005). These results indicate that screening of patients for EGFR tyrosine kinase domain mutations before treatment with gefitinib or other EGFR inhibitors may predict the clinical benefit of the

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treatment. However, approaches frequently required biopsy or surgical specimens, as well as skilful techniques (Lynch *et al*, 2004; Paez *et al*, 2004; Mitsudomi *et al*, 2005; Pao and Miller, 2005; Tokumo *et al*, 2005; Twombly, 2005). We developed a method, peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp, capable of detecting EGFR mutations in the presence of 100-fold background levels of wild-type EGFR from normal cells (Nagai *et al*, 2005). Because of its high sensitivity and specificity, PNA-LNA PCR clamp was considered suitable to detect EGFR mutations both in histological samples such as surgical specimens, and in cytological samples such as sputum and pleural effusions.

This phase II study was prospectively designed to evaluate the effect of gefitinib in NSCLC patients with *EGFR* gene mutations screened by PNA-LNA PCR clamp.

PATIENTS AND METHODS

The two-step protocol of this phase II study, that is (i) testing for EGFR mutations by PNA-LNA PCR clamp, and (ii) administering gefitinib to NSCLC patients with EGFR mutations, were approved by the Institutional Review Board (IRB) of Saitama Medical University Hospital. This study was performed in accordance with the Declaration of Helsinki (1964, amended in 2000) of the World Medical Association.

Primary entry criteria and testing for EGFR mutations

Consecutive NSCLC patients who were admitted in our single institution and gave written informed consent for testing for *EGFR* gene mutations by PNA-LNA PCR clamp, which was designed to detect 11 different EGFR mutations. Detection rate (sensitivity) by PNA-LNA PCR clamp is 89% and its accuracy (specificity) is 100%. In PNA-LNA PCR clamp, existence of other types of EGFR mutation is realised by seeing escape of the inhibition of amplification by the clamp primer, and, in this case, a direct sequencing method is employed to seek other types of EGFR mutation. Finally, overall sensitivity and specificity of this system is 97 and 100%, respectively, using clinical samples (submitting). The cytology specimens were divided into pathology samples (the main sample) and PNA-LNA PCR clamp samples (a small aliquot). When the pathologist confirmed a pathology sample to contain cancer cells (i.e. rated as classes IV or V), the cells in the PNA-LNA PCR clamp samples, which had been collected and stored in the AL buffer (a buffer containing protein denaturant: Qiagen, Hilden Germany), were then subjected to the analysis. While, the paraffin-embedded tissue specimens were serially thin-sliced: one slice was used to confirm the presence of cancer cells under microscopy, whereas the others were investigated by the PNA-LNA PCR reaction.

The PNA-LNA PCR clamp method has been described in detail (Nagai *et al*, 2005). Briefly, primers used were

F18: 5′-GGTAGCTGTTCAGTTAAAGAACACC-3′ and
B18: 5′-CCTTTGGTCTGTGAATTGGTC-3′ for exon 18,
F19: 5′-CTGGATGAAATGATCCACACG-3′ and
B19: 5′-TGGGTAGATGCCAGTAATTGC-3′ for exon 19, and
F21: 5′-CTGGATGGAGAAAAGTTAATGGTC-3′ and
B21: 5′-CAGCAAGTACCGTTCCCAAAG-3′ for exon 21.

PCR primers were designed manually or by using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) so that the T_m values were between 55 and 60°C. Fluorogenic probes containing LNA were manually designed and confirmed by the LNA T_m prediction tool (http://lna-tm.com/) to have T_m values between 54 and 56°C. Peptide nucleic acid clamp primers, 14- to 18-mer in length, were designed according to the guidelines (Ugozzoli *et al*, 2004). LNA-containing oligos were synthesised by IDT (Coralville, IA, USA), and PNA oligos were synthesised by Greiner Japan, Tokyo, Japan. For PNA-LNA PCR clamp, PCR primers (200 nM each), fluorogenic probes (100 nM each) and a PNA clamp primer (5 μM) were added to the Basic Mixture containing 25 mM TAPS pH 9.3, 50 mM KCl, 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 200 μM each of dNTPs and 1.25 U of Takara Ex Taq HS (Takara Bio, Shiga, Japan). For PCR reactions, PCR and the real-time amplification monitoring for the PNA-LNA PCR clamp were performed using Smart Cycler II (Cepheid Sunnyvale, CA, USA). PCR cycling was a 30-s hold at 95°C followed by 45 cycles of 95°C for 3 s and 62°C (exons 18 and 19) or 56°C (exon 21) for 30 s.

Secondary entry criteria and treatment schedule

After testing for EGFR mutations by PNA-LNA PCR clamp, patients who satisfied the following inclusion criteria were enrolled: (a) EGFR mutations, (b) inoperable stage III–IV and recurrence after operation, (c) measurable region(s), (d) adequate bone marrow (white blood cell count $\geqslant 4000\,mm^{-3}$; platelet count $\geqslant 100\,000\,mm^{-3}$; hemoglobin $\geqslant 9.5\,g\,dl^{-1}$), total bilirubin $\leqslant 1.5\,mg\,dl^{-1}$, transaminases less than twice the upper limit of normal, and serum creatinine level $\leqslant 1.5\,mg\,dl^{-1}$, (e) age 20 years, (f) no medical problems severe enough to prevent compliance with the study requirements, and (g) secondary informed consent to be treated by gefitinib.

Gefitinib (250 mg p.o. daily) was given as the second treatment after disease was on progression by cytotoxic chemotherapy for PS 0–2 patients with EGFR mutations. In the case of poorer PS owing to advanced disease, the first line chemotherapy was omitted and gefitinib was administered as the first therapy. The other patients not enrolled into the phase II study were clinically treated by appropriate therapies according to our institutional manual, and their data on EGFR mutation status and survival time were collected and analysed.

Evaluation

Patients were evaluated by physical examination, chest X-ray, bone scintiscan, computed tomography (CT) of the head, chest and abdomen, and fiberoptic bronchoscopy, and clinical stages were then determined according to the tumour-node-metastasis system. Chest X-rays were assessed at least every 2 weeks after the initial evaluation, and a chest CT was planned to evaluate tumour response and tumour progression. Tumour response was classified in accordance with Response Evaluation Criteria in Solid Tumours.

Before the first course, each patient was subjected to a complete blood cell count (CBC), serum chemistry for renal and hepatic functions, electrolyte analysis and urinalysis. CBC, serum chemistry, electrolyte analysis and urinalysis were assessed at least once a week after the initial evaluation. The NCI Common Toxicity Criteria (version 3) was used to grade organ system damage.

Statistical analysis

The primary end point of this study was the response to gefitinib for patients with EGFR mutations. Sample size was determined to be 25 patients with *EGFR* gene mutations. We chose a 75% response rate as a desirable target level and a 50% response rate as uninteresting. Our design had a power in excess of 90% and less than 10% type I error. A total number of patients to be tested by PNA-LNA PCR clamp was decided to be more than 100 patients because about 30% of Japanese NSCLC patients were reported to have EGFR mutations in previous articles (Mitsudomi *et al*, 2005; Tokumo *et al*, 2005).

Secondary end points were survival, side effects and clinical usefulness of PNA-LNA PCR clamp. Differences in response to gefitinib and survival after gefitinib therapy between patients with wild *EGFR* genes and those with mutant *EGFR* genes were assessed to indicate a clinical usefulness of screening by PNA-LNA PCR

clamp. Furthermore, differences in overall survival from the initial treatment between the groups, and whether EGFR mutations were a prognostic factor were also investigated. Survival curves were drawn using the Kaplan–Meier method, and Logrank was calculated for evaluating survival differences between the groups. A Cox proportional hazards model (multiple variate) using EGFR mutations, sex, stage and PS was also employed using the data from all the patients enrolled by the primary entry criteria. All the analyses were calculated by SPSS® 11.0J.

RESULTS

From September 2004 to October 2005, samples from 107 Japanese NSCLC patients were tested by PNA-LNA PCR clamp but two patients refused consent for checking for EGFR mutations (Figure 1). One hundred patients (93%) of the 107 patients provided adequate samples for evaluation of EGFR mutation status, and samples from seven patients did not provide enough DNA. PNA-LNA PCR clamp detected EGFR mutations in 38 patients (38%; 95% confidence interval (CI): 28–48%) who were 15 male and 23 female patients (Table 1). Their median age was 62 years old, and, of the 38 patients, 33 patients had adenocarcinoma. Exon 19 deletions, L858R and L861Q were found in 25 (66%), 12 (32%) and 1 (2%) patients, respectively (Figure 1). On the other hand, 62 patients (51 men/11 women; median age: 66 years; adenocarcinoma: 43 patients) were judged to have wild-type EGFR. There were significant differences between EGFR mutation-positive and EGFR mutation-negative groups with regard to sex (male *vs* female: $P=0.00001$), histology (adenocarcinoma *vs* non-adenocarcinoma: $P=0.02$) and smoking (>20 pack-years *vs* <20 pack-years: $P=0.003$) (Table 1).

Phase II study

Of the 38 patients with EGFR mutations, gefitinib was given to 27 patients. The other 11 patients were not treated by gefitinib because they did not meet the secondary entry criteria.

Four patients and 23 patients were given gefitinib as the first-line and the second-line treatment, respectively. All of the 27 patients were assessed for response. One patient showed a complete response (CR) and 20 patients showed partial responses (PRs). The overall response rate was 78% (95% CI: 62–94%) (Table 2). The response rate in the 23 patients treated by gefitinib after chemotherapy was 74% (95% CI: 56–92%). When patients were stratified by EGFR mutation types, response rates were 75% (15 out of 20 patients) for exon 19 deletions, and 86% (six out of seven patients) for L858R. There were no significant differences in the response rates between the mutation types (χ^2 test: $P=0.557$).

For the 27 patients, median time to progression (TTP) from the gefitinib treatment was 9.4 months. And median survival time (MST) from the gefitinib treatment was 15.4 months (Figure 2).

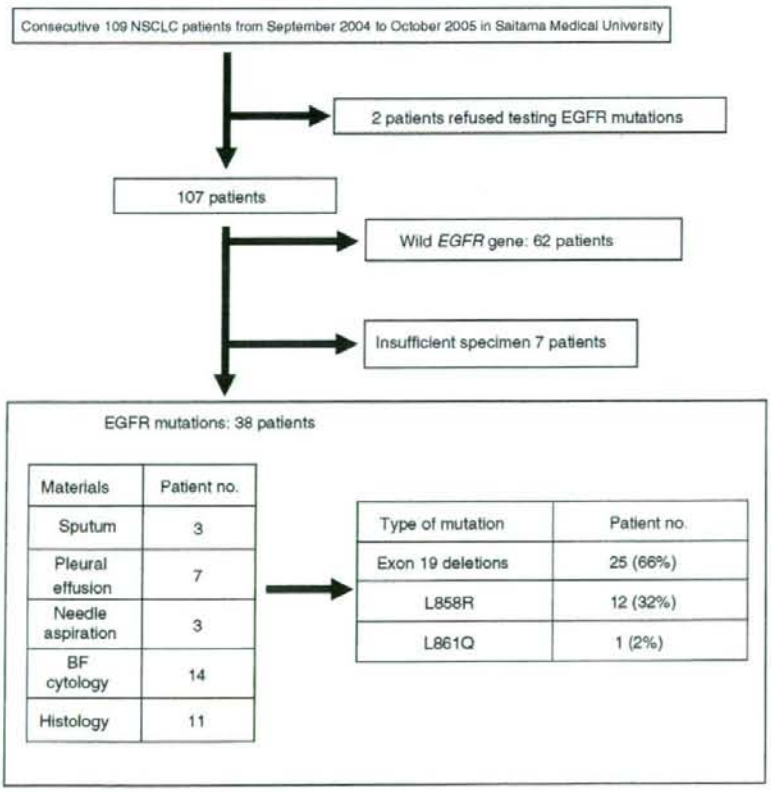

Figure 1 Patients entered and source of specimen and type of EGFR mutation.

Table 1 Patients' characteristics

	Mutation ($n=38$)	Wild-type ($n=62$)	P-value
Male/female	15/23 pts	51/11 pts	0.00001
Median age (years) (s.d., range)	62 (10.0, 44–79)	66 (12.0, 32–81)	0.09
Stage			
I	1 pt (2.6%)	3 pts (4.8%)	0.175
II	1 pt (2.6%)	2 pts (3.2%)	
III	9 pts (23.7%)	23 pts (37.1%)	
IV	23 pts (60.5%)	22 pts (35.5%)	
Relapse	4 pts (10.5%)	12 pts (19.4%)	
Histology			
Adenocarcinoma	33 pts (86.8%)	43 pts (69.4%)	0.02
Squamous cell carcinoma	2 pts (5.3%)	12 pts (19.4%)	
Adenosquamous	1 pt (2.6%)	1 pt (1.6%)	
Large cell carcinoma	0 pt (0.0%)	1 pt (1.6%)	
Undifferentiated	2 pts (5.3%)	5 pts (8.1%)	
Smoking			
>20 pack-years	13 pts (34.2%)	40 pts (64.5)	0.003
ECOG PS			
0–2	34 pts (89.5%)	55 pts (88.7%)	0.906
3–4	4 pts (10.5%)	7 pts (11.3%)	
Treatments[a]			
Operation	10 pts (26.3%)	23 pts (37.1%)	0.948
Chemotherapy	30 pts (78.9%)	43 pts (69.4%)	
Irradiation	2 pts (5.3%)	6 pts (9.7%)	

ECOG PS = Eastern co-operative oncology group performance status; Pts = patients.
[a]All the treatments which were given to patients for the intervening periods of the diseases.

There were also no significant differences in survival time after the gefitinib treatment between the patients with exon 19 deletions and L858R (Kaplan–Meier, logrank: $P<0.455$). The 21 patients with CR/PR had a longer TTP and overall survival (14.4+ and 19.1+ months, respectively) than patients with stable disease/progression (3.1 and 5.6 months, respectively).

All 27 eligible patients were assessable for toxicity (Table 3). Grade 3 drug-related hepatic toxicity was observed in one patient (4%), and Grade 3 skin toxicity occurred in one patient (4%). Other gastrointestinal toxicities were mild and acceptable. No lung toxicities were observed.

Clinical usefulness of PNA-LNA PCR clamp

To investigate the clinical usefulness of PNA-LNA PCR clamp screening, patients with EGFR mutations detected by the test were compared to those with wild EGFR. The response rates were

Table 2 Efficacy of gefitinib in patients with EGFR mutation

	CR	PR	s.d.	PD	Response
Prior chemotherapy (+)	1	16	5	1	17 pts/23 pts (74%) (95% CI: 56–92%)
Prior chemotherapy (−)	0	4	0	0	4 pts/4 pts (100%)
Exon 19 deletions	1	14	4	1	15 pts/20 pts (75%)
L858R	0	6	1	0	6 pts/7 pts (86%)
Total	1	20	5	1	21 pts/27 pts (78%) (95% CI: 62–94%)

CI = confidence interval; CR = complete response; EGFR = epidermal growth factor receptor; PD = progressive disease; PR = partial response; Pts = patients; s.d. = standard deviation.

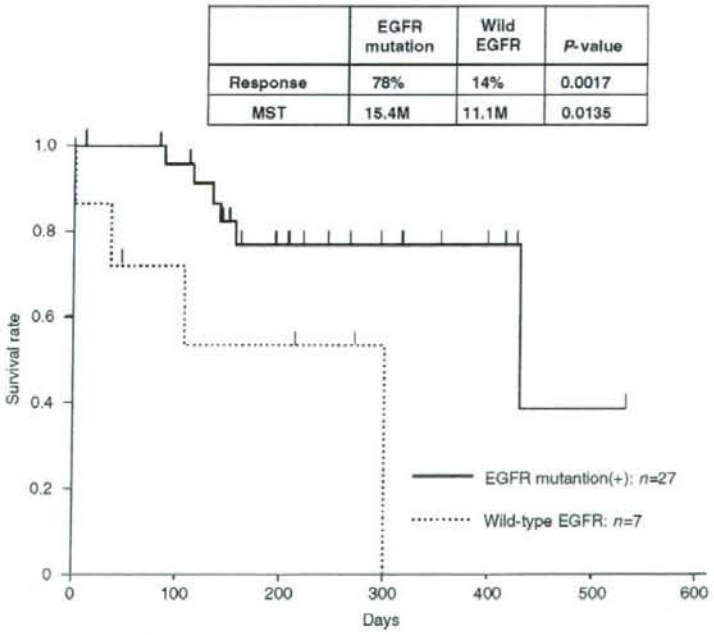

Figure 2 Survival time curves *after gefitinib treatment* in patients with and without EGFR mutation are shown.

significantly different between patients with EGFR mutations (78%) and patients with wild EGFR (14%) (χ^2 test, $P=0.0017$, Figure 2). Median survival time after the gefitinib treatment was significantly different between patients with EGFR mutations (15.4 months) and those with wild-type EGFR (11.1 months) (Kaplan–Meier, logrank: $P=0.0135$, Figure 2).

Furthermore, to clarify whether EGFR mutation status tested by PNA-LNA PCR clamp could be a prognostic factor for NSCLC patients, the relationship between EGFR mutation status and overall survival were evaluated using 99 patients except for one patient who was lost in follow-up. Figure 3 shows the comparison of overall survival after the initial treatments between NSCLC patients with EGFR mutations and those with wild-type EGFR by the Kaplan–Meier method. Overall survival after the initial treatment was significantly different between the groups (EGFR mutations: 19.1 months and wild-type EGFR: 10.7 months, logrank: $P<0.0108$). The Cox proportional hazards model (multiple variate) was also applied using EGFR mutations, sex, stage and PS. The latter three factors are well known as prognostic factors in NSCLC patients (Brundage *et al*, 2002). The Cox proportional hazards model indicated that detecting EGFR mutations was a secondary prognostic factor (Table 4).

Table 3 Side effects of gefitinib in patients with EGFR mutation

	No. of patients with CTC grade ($n=27$)		
	2	3	4
Haematologic toxicity			
Neutropenia	1	0	0
Thrombocytopenia	0	0	0
Anaemia	0	0	0
Other toxicities			
Diarrhoea	5	0	0
Nausea and vomiting	2	0	0
Acne/acneform	9	1	—
Abnormal liver function (AST, ALT)	1	1	0
Abnormal renal function	0	0	0
Acute lung injury	0	0	0

ALT = alanine aminotransferase; AST = aspartate aminotransferase; CTC = common toxicity criteria; EGFR = epidermal growth factor receptor.

DISCUSSION

With PNA-LNA PCR clamp, we were able to determine EGFR mutation status in a majority of the NSCLC patients using clinical samples such as sputum and BF cytology. To determine EGFR mutations, direct sequencing or PCR-single strand conformational polymorphism methods are frequently employed (Lynch *et al*, 2004; Paez *et al*, 2004; Mitsudomi *et al*, 2005; Pao and Miller, 2005; Tokumo *et al*, 2005; Twombly, 2005). However, these methods are time-consuming and require specimens that consist mostly of cancer cells. Another approach that analysis of an increased *EGFR* gene copy number, based on fluorescence *in situ* hybridisation analysis, could be used as a predictive marker for sensitivity to gefitinib (Bell *et al*, 2005; Hirsch and Witta, 2005; Takano *et al*, 2005). However, this method also needs specimens consisting mostly of cancer cells, significant operation time and skilful technicians who have intertechnician variability. Thus, these methods can be employed only at some academic medical centres. The preferred and practical method is one that can sensitively, specifically and quickly detect EGFR mutations from specimens used for the diagnosis of lung cancers without removing contaminating normal cells. Peptide nucleic acid-locked nucleic acid PCR clamp can rapidly (within 2 hours) detect EGFR mutations from all specimens used to diagnose lung cancers, that is, sputum, pleural effusion and bronchial washing which contain many normal cells. This method is able to sensitively and

Table 4 Cox proportional hazards analysis

	Hazards ratio	*P*-value
Mutation	2.259	0.036
Performance status	1.542	0.002
Male/female	1.053	0.887
Stage	1.029	0.319

A Cox proportional hazards model (multiple variate) using EGFR mutations, sex, stage and PS was employed using the data from all the patients ($n=99$*) enrolled by the primary entry criteria. *Data missing; one patient.

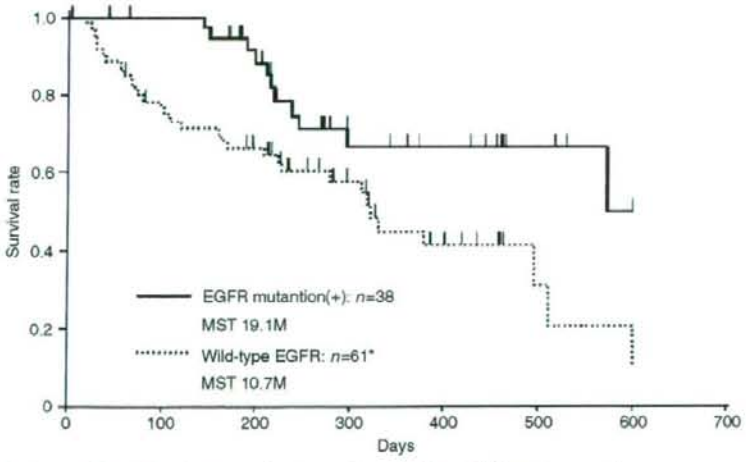

Figure 3 Overall survival curves *after the initial treatments* in patients with and without EGFR mutation are shown.

specifically detect all 11 types of EGFR mutations (Nagai *et al*, 2005) in the presence of 100-fold wild-type EGFR background levels. These 11 mutations account for more than 95% of EGFR mutations found (Lynch *et al*, 2004; Twombly, 2005).

PNA-LNA PCR clamp prospectively detected EGFR mutations in 38% (95% CI: 28–48%) of the consecutive patients with NSCLC. Patients who were EGFR mutation-positive were mostly women (61%) and had adenocarcinomas (87%), and significantly lower smoking index (34%). These results were consistent with the results of previous retrospective reports (Mitsudomi *et al*, 2005; Tokumo *et al*, 2005). Some clinical studies are trying to select patients to gefitinib treatment by clinicopathologic features of adenocarcinoma and non-smoker without testing EGFR mutations. Our data indicate such an approach is not feasible. For example, when selecting patients with adenocarcinoma and smoking >20 pack-years, 15 of the 38 patients with EGFR mutations (39%) would be missed, whereas 13 of the 62 patients without EGFR mutations (21%) would be mistakenly included.

Furthermore, the presence of EGFR mutations detected by the PNA-LNA PCR clamp was found to be a prognostic factor in Japanese patients with NSCLC in this prospective screening. A Cox proportional hazards model indicated that detecting EGFR mutations was a significant prognostic factor and was superior to sex or stage, indicating that incorporating the PNA-LNA PCR clamp into clinical studies and clinical practice is critical.

This phase II study clearly showed the favourable response to gefinitib in NSCLC patients with EGFR mutations. The response rate was 78% and the lower limit of the 95% confidential interval of response was 62%. In contrast to previous retrospective analyses (Riely *et al*, 2006; Hirsch *et al*, 2006), patients with exon 19 deletions were equally responsive compared to those with L858R in this study. This might be due to our small sample size, so these data need to be confirmed in a larger trial. In EGFR mutation-positive patients treated by gefitinib, TTP (9.4 months) of after the gefitinib treatment and MST (19.1 months) after the initial treatment were longer than in patients treated with the regimens using platinum doublet. Detection of EGFR mutations clearly differentiates gefinitib-sensitive patients from gefinitib-insensitive patients with regard to response rate and survival times.

Four patients with EGFR mutations received gefitinib as the first line treatment because they could not be given chemotherapy owing to poor PS. Two patients had meningitis carcinomatosa. One had multiple brain metastases. And one had repeated aspiration pneumonia owing to recurrent nerve palsy. All of these patients showed PR and obtained better PS. Their survival times were 190, 183+, 278+ and 296+ days, respectively, and all returned home. This experience taught us the usefulness of testing for EGFR mutations in patients with poor PS owing to advanced disease. Thus, even in Europe and the US where frequencies of EGFR mutations are low, incorporating testing for EGFR mutations in clinical practice may provide a huge benefit to some patients.

In conclusion, our study prospectively demonstrated the clinical benefit of gefitinib given to NSCLC patients with good PS as the second-line treatment harbouring EGFR mutations, and, also, gefitinib given to NSCLC patients with poor PS as the first-line treatment showed a favourable response. To attain this benefit, screening clinical samples at the time of diagnosis is imperative, and PNA-LNA PCR clamp is a good method to achieve this aim.

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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 for 154 patients with NSCLC, whereas EGFR mutations were analyzed for 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. Conclusions: Certain clinicopathological characteristics and EGFR mutations can be either predictive of gefitinib sensitivity or adverse events. Also, small-sized specimens may be applicable for the mutational analysis.*

The clinical stage is already advanced at diagnosis in more than 70% of non-small cell lung cancer (NSCLC) patients and 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 signalling 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 signalling 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.* reported that Japanese patients with NSCLC showed more favorable clinical responses to gefitinib compared to patients in other countries (27.5% *versus* 10.4%) (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 were significant, and, even more seriously, interstitial lung disease (ILD) was potentially fatal.

Recently, two groups 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, confirm racial differences in the frequency of EGFR mutations and in gefitnib 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 so far, mutation analysis has 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 attempts at

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Key Words: Gefitinib, epidermal growth factor receptor gene mutation, interstitial lung disease, stomatitis, EGFR, NSCLC.

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mutational analyses using these small amounts of material (15). If small specimens can suffice for mutation analysis, the number of patients eligible for such studies could 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 patients' characteristics. In our institutes, from September 2002 through March 2005, 154 consecutive Japanese patients with NSCLC treated with gefitinib 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, EGFR mutations were analyzed in 34 of the 154 patients, and in an additional 10 patients whose outcomes were not established (nine not given gefitinib and one where it was discontinued due to severe nausea) because their specimens were applicable for the mutation analysis such as Polymerase Chain Reaction (PCR) amplification or direct sequencing. The patients whose analysis did not work were eliminated from the analysis. 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). Partial responses (PR) and complete responses (CR) were together taken 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 other than tumor cells in the specimens 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 QIAamp 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)		
Age (mean±SD, years)		
65.3±11.8 (all)		
64.5±12.6 (Male)		
66.5±10.6 (Female)		
Gender	No.	
Male	92	
Female	62	
Histology	No.	
ADC	121	
SCC	18	
LCC	7	
SCLC+ADC	2	
ASC	6	
Smoking history	No.	Male/Female
Never smoked	61	13/48
Smoker	93	79/14
Stage		No.
I-II	19	
III-IV	135	
Prior chemotherapy	No.	
0	50	
≥1	104	
Clinical response (evaluable patients=151)		
CR	6	
PR	56	
NC	37	
PD	52	

Abbreviation (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, respectively). Of the 154 patients, 62 (40.3%) were female. One hundred and twenty-one diagnoses were of adenocarcinoma (78.6%), of which 19 were early stage (12.3%), and 62 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 months, $p=0.004$); never smoked (TTP: 5.6 *versus*

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

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 of 18 patients who developed ILD died. Stomatitis developed much more frequent 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 of 44 cases analyzed (40.9%) as shown in Table III. Each mutation site is shown in Figure 1. Mutations were identified in nine of thirteen TBB specimens (69.2%), seven of 22 surgically-resected specimens (30.4%), and one 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 nonetheless had the T790M substitution mutation in exon 20. This mutation was previously reported to be newly acquired in gefitinib resistance (16).

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.

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 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 in the other it was withdrawn due to severe nausea.

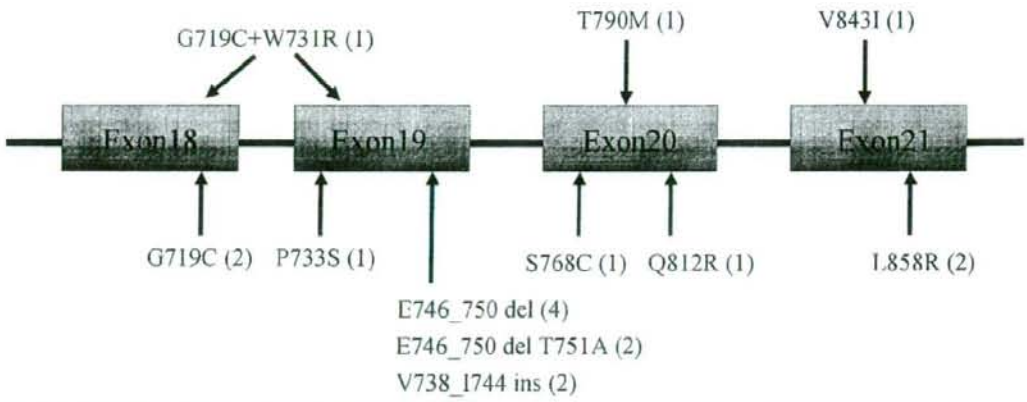

Figure 1. *Mutation sites of exons 18 to 21 in the EGFR gene. The number of cases identified with the respective mutation is shown in parenthesis.*

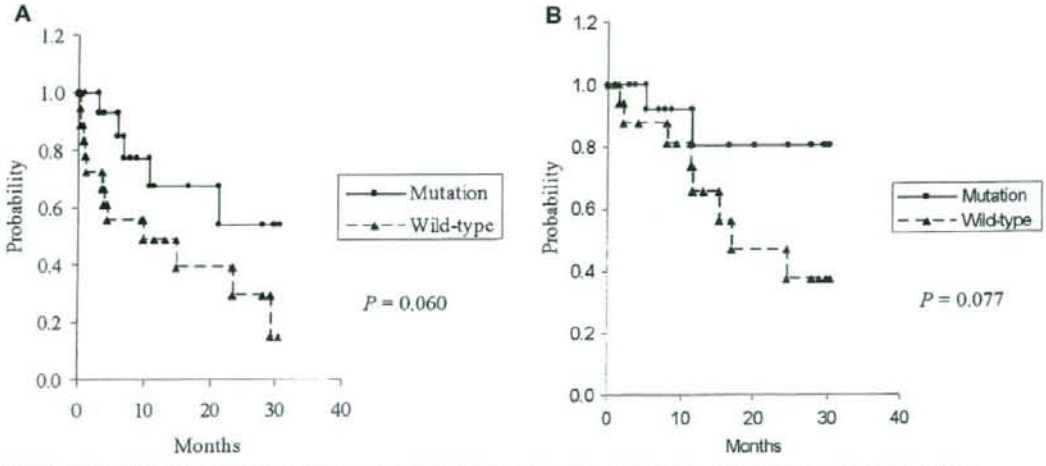

Figure 2. *Kaplan-Meier plots of (A) time to progression and (B) overall survival after gefitinib therapy, depending on EGFR mutation status.*

The mutations were all found in adenocarcinoma (p=0.06). Eleven 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 of 26 *versus* 16 of 26; p=0.691), smoking history (never smoked; 13 of 29 *versus* 16 of 29; p=0.462), or prior chemotherapy (17 of 35 *versus* 18 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 developed in two wild-type cases, but not in the mutation group (p=0.169). The overall incidence of adverse events was not statistically significantly different between the two groups. Adverse events were seen in 11 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 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 are newly identified as candidate risk factors for stomatitis. Stomatitis is a common adverse event of gefitinib therapy and often impairs quality of life (QOL), and 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 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 gefinitib 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 signalling pathways other than Akt, PTEN, ERK1/2 and STAT5 might be important. Small biopsy specimens might not have faithfully reflected the major characteristics of individual tumors, in that 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 showed that the detection rate of EGFR mutation by new methods seemed to be superior to that by direct sequencing methods (28, 29). Whilst the direct sequencing method was the only one used in the present study, the differences we found are unlikely due to specimen size as 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 as those described in 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 may 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

We have identified certain clinicopathological characteristics as well as EGFR mutations 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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Involvement of the platelet-activating factor receptor in host defense against *Streptococcus pneumoniae* during postinfluenza pneumonia

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van der Sluijs, Koenraad F., Leontine J. R. van Elden, Monique Nijhuis, Rob Schuurman, Sandrine Florquin, Takao Shimizu, Satoshi Ishii, Henk M. Jansen, René Lutter, and Tom van der Poll. Involvement of the platelet-activating factor receptor in host defense against *Streptococcus pneumoniae* during postinfluenza pneumonia. *Am J Physiol Lung Cell Mol Physiol* 290: L194–L199, 2006. First published August 12, 2005; doi:10.1152/ajplung.00050.2005.—Although influenza infection alone may lead to pneumonia, secondary bacterial infections are a much more common cause of pneumonia. *Streptococcus pneumoniae* is the most frequently isolated causative pathogen during postinfluenza pneumonia. Considering that *S. pneumoniae* utilizes the platelet-activating factor receptor (PAFR) to invade the respiratory epithelium and that the PAFR is upregulated during viral infection, we here used PAFR gene-deficient ($PAFR^{-/-}$) mice to determine the role of this receptor during postinfluenza pneumococcal pneumonia. Viral clearance was similar in wild-type and $PAFR^{-/-}$ mice, and influenza virus was completely removed from the lungs at the time mice were inoculated with *S. pneumoniae* (*day 14* after influenza infection). $PAFR^{-/-}$ mice displayed a significantly reduced bacterial outgrowth in their lungs, a diminished dissemination of the infection, and a prolonged survival. Pulmonary levels of IL-10 and KC were significantly lower in $PAFR^{-/-}$ mice, whereas IL-6 and TNF-α were only trendwise lower. These data indicate that the pneumococcus uses the PAFR leading to severe pneumonia in a host previously exposed to influenza A.

virus; bacteria; pneumonia; inflammation

ALTHOUGH INFLUENZA A INFECTION alone may lead to pneumonia, secondary bacterial infections during and shortly after recovery from influenza are much more common causes of pneumonia (12, 28). Bacteria such as *Staphylococcus aureus* and *Haemophilus influenzae* are known to cause postinfluenza pneumonia, but *Streptococcus pneumoniae* is the most prominent pathogen causing secondary bacterial pneumonia in recent decades (28). Primary infection with this pathogen is usually less severe than secondary infection (16). Influenza is known to increase adherence of and subsequent colonization with bacterial respiratory pathogens. Bacteria may adhere to the basal membrane after disruption of the airway epithelial layer by the cytopathic effect of the virus (17) but may also bind to specific receptors in the airway epithelium induced by influenza virus (6, 11). Because the platelet-activating factor receptor (PAFR) has been described to be upregulated during viral infections (10) and since the PAFR is able to bind phosphorylcholine, a cell wall component of *S. pneumoniae* (3, 5, 12), it has been suggested that the PAFR may play a critical role during secondary bacterial pneumonia (11).

The PAFR, a G protein-coupled receptor, is mainly expressed on macrophages, monocytes, neutrophils, and epithelial cells (8, 9, 22, 24). Activation of epithelial cells leads to upregulation of the PAFR at the cell surface, which facilitates colonization and invasion of *S. pneumoniae* (3, 9). A recent study by McCullers and Rehg (11) investigated the potential role of the PAFR in pneumococcal pneumonia following influenza A infection. These authors showed that PAFR blockade during secondary pneumococcal pneumonia does not prevent lethal synergism between influenza virus and *S. pneumoniae* (11). Moreover, administration of the PAFR antagonist CV-6209 resulted in enhanced bacterial outgrowth, even in mice with primary pneumococcal pneumonia (11). These findings contrast with earlier studies reporting that administration of PAFR antagonists reduced pneumococcal outgrowth in rabbits (2, 3). In line, our laboratory recently demonstrated that PAFR gene deficient ($PAFR^{-/-}$) mice display a diminished bacterial outgrowth and a reduced lethality after intranasal infection with *S. pneumoniae* (20). To obtain further insight in the role of the PAFR during secondary bacterial pneumonia, we inoculated $PAFR^{-/-}$ mice and wild-type mice with *S. pneumoniae* on *day 14* after influenza virus infection and studied host defense against primary influenza virus infection and secondary *S. pneumoniae* infection.

MATERIALS AND METHODS

Mice. $PAFR^{-/-}$ mice were generated as described (7) and backcrossed seven times to a C57BL/6 background. Wild-type C57BL/6 mice were obtained from Harlan Sprague-Dawley. Pathogen-free 8-wk-old female C57BL/6 mice and $PAFR^{-/-}$ mice were maintained at biosafety *level 2* during the experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Experimental infection protocol. Influenza A/PR/8/34 (VR-95; ATCC, Rockville, MD) was grown on LLC-MK2 cells (RIVM,

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