were obtained in the studies using AA861, a 5-LO inhibitor that suppresses the production of $LTB_4$, and WEB2070, another PAF receptor antagonist (data not shown). These results indicate that the augmentation of eosinophil TBM due to IL-8–stimulated neutrophils is partly mediated by $LTB_4$, PAF, and TNF-α. The effects of these inhibitors on TBM of neutrophils alone or eosinophils alone were also examined: IL-8 (10 nM)–induced TBM of neutrophils was not modified by inhibitors for MMP9, LTB4, PAF, or TNF-α ($n = 6$, $P$ = n.s., data not shown). Similarly, eotaxin (3 nM)-induced TBM of eosinophils was not modified by inhibitors for MMP9, LTB4, or PAF ($n = 6$, $P$ = n.s., data not shown). Anti–TNF-α mAb slightly but significantly reduced eotaxin-induced TBM of eosinophils (migrated eosinophils: 48.1 ± 3.5 by isotype mouse IgG1 versus 39.3 ± 2.4 by eotaxin alone, $n = 6$, $P = 0.02$), suggesting that TNF-α may act as an autocrine activator and partly contribute to the TBM of eosinophils in this system. Finally, pretreatment of only neutrophils, but not eosinophils, with the various inhibitors did not modify the subsequent TBM of eosinophils in the presence of IL-8–activated neutrophils ($n = 3$, $P$ = n.s., data not shown).

### The Conditioned Medium from IL-8-Stimulated Neutrophils Induces the TBM of Eosinophils

To further examine whether the neutrophils transmigrated to IL-8 produce chemoattractants for eosinophils, neutrophils were added to the upper compartment of a chamber with a Matrigel-coated transwell insert and either the control medium or IL-8 (10 nM) was added to the bottom compartment. After a 2-h incubation in 5% $CO_2$ at 37°C, the bottom compartments were centrifuged at 4°C for 20 min at 700 × $g$. The supernatants were gently recovered and then examined for their ability to induce the TBM of eosinophils. The conditioned medium from a combination of IL-8 and neutrophils significantly induced the TBM of eosinophils as compared with the control medium or the condition medium from neutrophils in the absence of IL-8 (migrated eosinophils: 7.0 ± 0.6%, $P < 0.05$ versus the other two conditions, $n = 5$).

### Anti-CXCR2 Antibody Does Not Modify the Augmentation of Eosinophil TBM by IL-8–Stimulated Neutrophils

The augmented TBM of eosinophils observed with IL-8–stimulated neutrophils may be a consequence of modification of the CXCR2, an IL-8 ligand, which is expressed on activated eosinophils (24). Although addition of the anti-CXCR2 antibody partly attenuated the TBM of neutrophils in response to IL-8 (% inhibition: 53.3 ± 8.6%, $n = 5$, $P < 0.01$ versus isotype control), the augmented TBM of eosinophils in the presence of IL-8 and neutrophils was not significantly modified by this antibody ($n = 5$, $P$ = n.s., data not shown). An anti-CXCR1 antibody provided similar results ($n = 2$, data not shown).

## DISCUSSION

We showed that neutrophils stimulated with chemoattractant such as IL-8 augment the TBM of eosinophils. The reaction kinetics of the augmented TBM of eosinophils traced that of neutrophils. The augmented TBM of eosinophils by a combination of neutrophils and IL-8 was inhibited by an MMP-9 inhibitor, an $LTB_4$ receptor antagonist, PAF-antagonists, or an anti–TNF-α mAb. $LTB_4$, a chemotactic factor for both neutrophils and eosinophils (18, 25), by itself induced the TBM of eosinophils, and this TBM is augmented by the presence of neutrophils. There results provide a mechanism for our previous observation that a positive correlation between the concentrations of neutrophils and eosinophils in sputum from subjects with severe asthma (10), and suggest that neutrophils can regulate the accumulation of eosinophils through these mechanisms in the airways of asthma.

The mechanism by which neutrophils enhance the TBM of eosinophils is important. We showed that the inhibition of MMP-9 effectively suppresses the augmented TBM of eosinophils. There is evidence that IL-8 stimulates the release of MMP-9 from neutrophils (26). Moreover, the cellular source of MMP-9 may include eosinophils (27). The MMP-9 inhibitor, which is a selective inhibitor of MMP-9 (IC50 = 5 nM) (28), did not modify the TBM of neutrophils to IL-8 or eosinophils to eotaxin, suggesting that MMP-9 is not required for TBM induced by these ordinary and potent chemoattractants in these experimental conditions. Nonetheless, our results suggest that digestion of membrane by MMP-9 is involved in the mechanisms of augmented TBM of eosinophils induced by IL-8–stimulated neutrophils. The process of TBM of eosinophils would also require the presence of an activator that acts as either chemotactic or chemokinetic for eosinophils. Zuurbier and colleagues (29) reported that eosinophil migration across monolayers of lung epithelial cells in response to complement fragment 5a (C5a), but not to RANTES, PAF, or IL-8, was increased in the presence of neutrophils. They explained that neutrophils, but not eosinophils, rapidly inactivated C5a and decreased the activity of C5a that had diffused into the upper compartment, and thereby maintain a proper C5a chemotactic gradient in their trans-epithelial migration model. In contrast, our study suggested that neutrophils enhanced the TBM of eosinophils, at least in part, via the generation of activators for eosinophils: the inhibition of $LTB_4$, PAF, or TNF-α actions partially suppressed TBM of eosinophils. In addition to $LTB_4$, PAF is a chemotactic factor for both neutrophils and eosinophils (18, 24). The pharmacologic inhibitors may reduce migration of neutrophils and subsequently reduce migration of eosinophils, instead of acting to disrupt the effects of neutrophil-derived mediators on eosinophils. From this point of view, we observed that IL-8–induced TBM of neutrophils in this system was not modified by inhibitors for LTB4 or PAF. Furthermore, pretreatment of neutrophils with the various inhibitors did not modify the subsequent TBM of eosinophils in the presence of IL-8–activated neutrophils. TNF-α is not a chemotactic agent for neutrophils or eosinophils itself, but has been shown to be an activator for functions of both neutrophils and eosinophils such as adhesion or respiratory burst (25, 30). Our results that TNF-α is involved in the augmented TBM of eosinophils suggest that the activation of effector function(s) of either neutrophils or eosinophils may be sufficient to augment the TBM of eosinophils. We observed that anti–TNF-α mAb slightly but significantly reduced eotaxin-induced TBM of eosinophils, but not IL-8–induced TBM of neutrophils, suggesting some role of this cytokine as an autocrine activator for eosinophil migration in this system.

Increased concentrations of $LTB_4$ (31, 32), PAF (33), and TNF-α (34) have been reported in the airways of asthma. Our results suggest that neutrophils are activated via a combination of IL-8 and basement membrane and generate these mediators. In this context, we found that the conditioned medium from a combination of IL-8 and neutrophils, but not neutrophils alone or medium alone, induced the TBM of eosinophils, indicating that the neutrophils transmigrated to IL-8 produce chemoattractant(s) that result in subsequent migration of eosinophils. Taken together, the augmented TBM of eosinophils is likely a complex of the effects of both MMP-9, on the basement membrane, and eosinophil activators and chemoattractants such as $LTB_4$, PAF, and TNF-α.

Our assay simulates the initial phase of inflammation involving neutrophils and eosinophils, in which cells are stimulated and

migrate out of blood vessels to accumulate at the inflammation site. In the later phase of inflammation, the expression of surface receptors of cells may be modified to become responsive to molecules that they were initially unresponsive. A good example is the expression of CXCR2, an IL-8 ligand, in activated eosinophils (24). The augmented TBM of eosinophils in the presence of IL-8 and neutrophils was not significantly modified by anti-CXCR2 antibody, ensuring in this assay that eosinophils are activated not directly by IL-8 but via the activation of neutrophils. However, this antibody partly attenuates the TBM of neutrophils to IL-8. Similar results were observed with an anti-CXCR1 antibody. These results suggest that migration of neutrophils is not a sole contributing factor for the augmented eosinophil migration. Not only MMP-9 or chemoattractants released from transmigrated neutrophils, but also the eosinophil activators released from neutrophils activated by IL-8 and integrin-mediated signaling before transmigration, may be important in the subsequent migration of eosinophils. We speculated that a complex consisting of this priming process, and release of MMP-9 and chemoattractants from migrated neutrophils, results in the eventual manifestation of the enhanced transmigration of eosinophils.

Neutrophils may actively participate in the development of airway disease of asthma. Our results suggest that neutrophils migrated to IL-8 may lead eosinophils to accumulate in the airways of asthma and possibly aggravate this disease. Therefore, therapies that suppress functions or accumulation of neutrophils may be effective for severe asthma. Further study will be warranted to elucidate the detailed roles of neutrophils in the pathophysiology of the disease.

***Conflict of Interest Statement*:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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# Differential Effects of Salbutamol and Montelukast on Eosinophil Adhesion and Superoxide Anion Generation

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**Key Words**

$\beta_2$-Agonist · Cell adhesion · Eosinophil · Leukotriene antagonist · Superoxide anion

**Abstract**

***Background:*** $\beta_2$-Agonists, a representative class of bronchodilators used for asthma, have been shown to modulate some functions of eosinophils, including cell adhesion. Similarly, a leukotriene receptor antagonist (LTRA) may be beneficial in controlling inflammation in asthma, as cysteinyl leukotrienes (cysLTs) can cause accumulation or activation of eosinophils. Recent evidence suggests that the addition of an LTRA, but not a long-acting $\beta_2$-agonist, to inhaled corticosteroid additionally reduces the number of eosinophils in sputum and blood from patients with asthma. The present study examined whether a $\beta_2$-agonist and an LTRA differentially modify eosinophil adhesion and activation induced by cysLTs and other activators. ***Methods:*** Eosinophils were isolated from blood of healthy donors and then incubated in the presence or absence of salbutamol (albuterol) or montelukast. Eosinophils were then exposed to leukotriene $D_4$ ($LTD_4$) or another activator, and the generation of superoxide anion ($O_2^-$) was evaluated by cytochrome C reduction assay. Eosinophil adhesion was examined by an eosinophil peroxidase assay. ***Results:*** Montelukast, but not salbutamol (both at 1 $\mu M$), inhibited $LTD_4$-induced (100 n*M*) eosinophil adhesion to recombinant human intercellular adhesion molecule 1. Both drugs similarly and partially inhibited the 100 p*M* interleukin-5-induced adhesive response of eosinophils to recombinant human intercellular adhesion molecule 1. Montelukast, but not salbutamol, blocked $LTD_4$-induced eosinophil $O_2^-$ generation of eosinophils. Finally, neither salbutamol nor montelukast modified phorbol myristate acetate (1 ng/ml)-induced $O_2^-$ generation from eosinophils. ***Conclusion:*** These results confirm that $LTD_4$ directly induces activation of eosinophils via the cysLT1 receptor. Furthermore, the results suggest that a $\beta_2$-agonist has no effect on eosinophil adhesion and activation induced by cysLTs. These results explain the differential effects of an LTRA and a $\beta_2$-agonist in the treatment of eosinophilic inflammation in asthma.

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## Introduction

Eosinophils preferentially accumulate at sites of allergic inflammation and are believed to play a role in the pathophysiology of asthma. Recent studies have estab-

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lished that eosinophils are essentially involved in airway remodeling [1-3]. Moreover, it is theoretically conceivable that eosinophils are the major cellular source of cysteinyl leukotrienes (cysLTs) in asthma [4, 5].

Over the past decade, increasing evidence has suggested that cysLTs contribute to the accumulation of eosinophils in the airway tissues of asthmatics [6–9]. For example, leukotriene $E_4$ ($LTE_4$) inhalation increases the accumulation of eosinophils and, to a lesser extent, neutrophils in asthmatic individuals [6]. In this context, cysLTs exert various effects on human eosinophils in vitro [10–16]. We have reported that $LTD_4$ upregulates the expression of $\beta_2$-integrins on human eosinophils in vitro and augments eosinophil adhesion to tissue culture plates or to recombinant human intercellular cell adhesion molecule 1 (rh-ICAM-1) [11]. Furthermore, we observed that $LTD_4$ directly induces transendothelial migration, respiratory burst and degranulation, mainly via the cysLT1 receptor and $\beta_2$-integrin [12]. Clinical studies have examined whether leukotriene receptor antagonists (LTRAs) modulate eosinophil accumulation in the asthmatic airway. For example, pranlukast, a cysLT1 antagonist, reduces the number of eosinophils in the bronchial mucosa [17] and in sputum [18]. Montelukast, another LTRA, yielded similar results [19].

$\beta_2$-Agonists, a representative class of antiasthma drugs, can downmodulate some functions of eosinophils [20]. For example, we have reported that tulobuterol, a $\beta_2$-agonist, attenuates interleukin (IL)-5-induced adhesion of blood eosinophils [21]. Therefore, like an LTRA, a $\beta_2$-agonist may act to regulate eosinophilic inflammation in asthma. However, recent evidence suggests that the combination of inhaled corticosteroid (ICS) plus an LTRA exerts marked antieosinophilic or anti-inflammatory effects in asthma, as compared with an ICS/long-acting $\beta_2$-agonist (LABA) combination [22, 23], suggesting that eosinophilic inflammation induced by cysLTs in asthma may be insensitive to $\beta_2$-agonists. The present study was undertaken to examine whether $\beta_2$-agonists and LTRAs differentially modify eosinophil adhesion or activation induced by cysLTs and other activators.

## Materials and Methods

*Reagents*

Percoll was obtained from Pharmacia (Uppsala, Sweden). Anti-CD16 antibody-coated magnetic beads were purchased from Miltenyl Biotec (Auburn, Calif., USA). RPMI-1640 medium, PBS, newborn calf serum and fetal calf serum (FCS) were obtained from Life Technologies (Grand Island, N.Y., USA). $LTD_4$ was obtained from Cayman Chemical (Ann Arbor, Mich., USA), montelukast was provided by Merck & Co., Inc. (White Station, N.J., USA), and rh-IL-5 and rh-ICAM-1 were purchased from R&D Systems (Minneapolis, Minn., USA). Other reagents were purchased from Sigma (St. Louis, Mo., USA) unless stated otherwise.

*Eosinophil Separation*

Eosinophils were isolated from the peripheral blood of healthy volunteers aged 20–29 years, with an equal sex distribution. Eosinophils were isolated by negative immunomagnetic bead selection as described previously [24, 25]. Briefly, heparinized blood diluted with Hank's balanced salt solution (HBSS) without $Ca^{2+}$ was separated by centrifugation for 20 min at 700 *g* over 1.090 g/ml Percoll. Plasma, mononuclear cells and the Percoll were removed, and pelleted red blood cells were lysed by hypotonic shock. The resulting granulocytes were washed in HBSS supplemented with 2% newborn calf serum at 4°C and then incubated with anti-CD16 antibody-coated magnetic beads for 40 min at the same temperature. The cells were filtered through steel wool in a magnetic field (Miltenyl Biotec) to remove neutrophils bound to the magnetic beads. CD16-negative eosinophils (>98% purity and >99% viability) were collected, washed and resuspended in RPMI supplemented with 5% FCS (RPMI/FCS).

*Eosinophil Adhesion Assay*

Eosinophil adhesion to rh-ICAM-coated plates was assessed as residual eosinophil peroxidase (EPO) activity of adherent eosinophils, as previously described [11, 21, 26]. Briefly, rh-ICAM-1 was dissolved in 0.05 *M* $NaHCO_3$ coating buffer (15 m*M* $NaHCO_3$, 35 m*M* $Na_2CO_3$, pH 9.2), added to 96-well tissue culture plates (Corning Incorporated, Corning, N.Y., USA) and incubated at 4°C overnight [11]. Residual fluid was decanted and 200 µl/well HBSS/0.1% gelatin (HBSS/gel) was added to reduce nonspecific adhesion. After a 2-hour incubation at ambient temperature, the wells were decanted and ready for the addition of eosinophils [11]. Eosinophils (100 µl of $1 \times 10^5$ cells/ml in HBSS/FCS) were preincubated with either montelukast (1 µ*M*), salbutamol hemisulfate (1 µ*M*) or buffer alone (control) at 37°C for 20 min and then placed onto rh-ICAM-1-coated plates in the presence or absence of an activator and incubated for 20 min at 37°C.

After five washes with 37°C HBSS, 100 µl HBSS/FCS was added to the reaction wells. In the experiments to determine kinetics, the reaction was serially stopped by partial aspiration and washing of the corresponding wells every 5 min for the first 10 min and every 10 min for the next 50 min. As standards, 100 µl of serially diluted cell suspensions ($1 \times 10^3$, $3 \times 10^3$, $1 \times 10^4$, $3 \times 10^4$ and $1 \times 10^5$ cells/ml) were added to empty wells. EPO substrate (1 m*M* *o*-phenylenediamine, 1 m*M* $H_2O_2$ and 0.1% Triton X-100 in Tris buffer, pH 8.0) was then added to all wells. After 30 min incubation at room temperature, 50 µl of 4 *M* $H_2SO_4$ was added to stop the reaction, and the absorbance at 490 nm was measured. Each experiment was performed in quadruplicate using eosinophils from a single donor, and the mean value was used to determine the percentage of eosinophil adhesion, which was calculated from the log dose-response curve. Eosinophil viability after incubation exceeded 98% by trypan blue dye exclusion.

*Superoxide Anion Generation*

Eosinophil superoxide anion ($O_2^-$) generation was measured as the superoxide dismutase (SOD)-inhibitable reduction in ferricy-

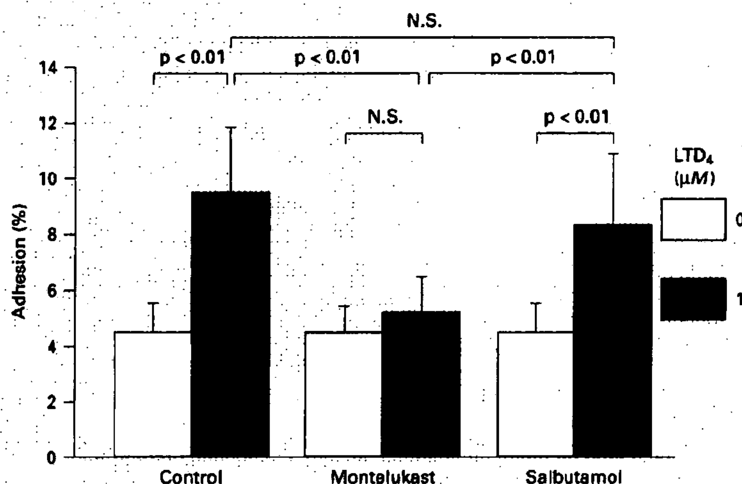


**Fig. 1.** Effects of montelukast (1 μ*M*) and salbutamol (1 μ*M*) on $LTD_4$-induced (100 n*M*) eosinophil adhesion to rh-ICAM-1. Data are expressed as means ± SEM from six experiments. N.S. = Not significant.

tochrome C in 96-well culture plates (Corning Inc.), as described previously [12, 26]. We initially added SOD (0.2 mg/ml in HBSS/gel; 20 μl) to SOD control wells and then HBSS/gel to all wells to bring the final volume to 100 μl. Eosinophil density was adjusted to $1.25 \times 10^6$ cells/ml of HBSS/gel mixed 4:1 with cytochrome C (12 mg/ml HBSS/gel), and then eosinophils, which were preincubated with either montelukast (1 μ*M*), salbutamol hemisulfate (1 μ*M*) or buffer alone (control) at 37°C for 20 min, were added to all wells in a volume of 100 μl. To initiate the reaction, the cells were incubated with $LTD_4$ (100 n*M*) or phorbol myristate acetate (PMA; 1 ng/ml). Immediately after adding the activator, the absorbance of the cell suspensions in the wells was measured at 550 nm in an Immuno-Mini (NJ-2300; Japan Intermed Co., Tokyo, Japan), followed by repeated readings over the next 240 min. Between readings, the plates were placed in a 5% $CO_2$ incubator at 37°C. Each reaction was performed in duplicate against an identical control reaction containing 20 μg/ml SOD. The results were adjusted to represent a 1-ml reaction volume, and $O_2^-$ generation was calculated at an extinction coefficient of $21.1 \times 10^3$ $M/l^{-1}$ $cm^{-1}$ as nanomoles of cytochrome C reduced per $1.0 \times 10^6$ cells per minute minus the SOD control. The maximum value during the incubation time was examined for the effects of drugs on eosinophil $O_2^-$ generation. Cell viability determined by trypan blue exclusion at the completion of each experiment remained at 95% after 240 min of incubation.

*Statistics*

Data are presented as means ± SEM. ANOVA with repeated measures was used to compare more than two variables. When the initial p value was below 0.05, Scheffe's post-hoc test was used to determine the significance of differences between groups. Student's t test was used to perform paired comparisons. Statistical significance was established at the $p < 0.05$ level.

## Results

As previously reported, 100 n*M* $LTD_4$ augmented the adhesive response of eosinophils to rh-ICAM-1 (fig. 1). Montelukast, at a clinically relevant concentration (1 μ*M*), significantly inhibited $LTD_4$-induced eosinophil adhesion to rh-ICAM-1 ($p < 0.01$, n = 6; fig. 1). In contrast, salbutamol (1 μ*M*) did not modify this adhesion of eosinophils ($p > 0.1$, n = 6; fig. 1). Neither montelukast nor salbutamol modified the spontaneous adhesion of eosinophils to rh-ICAM-1 ($p > 0.1$, respectively, n = 6; fig. 1).

In contrast to the differential effects observed with $LTD_4$-induced adhesion, both montelukast and salbutamol (1 μ*M*) similarly and partially inhibited the 100 p*M* IL-5-induced adhesive response of eosinophils to rh-ICAM-1 ($p < 0.01$, respectively, n = 6; fig. 2). There was no statistically significant difference between the effects of montelukast and salbutamol ($p > 0.1$).

As previously reported, 100 n*M* $LTD_4$ directly induced the generation of $O_2^-$ from eosinophils. This activation was significantly inhibited by montelukast (1 μ*M*) ($p < 0.01$, n = 6; fig. 3a). On the other hand, montelukast did not modify PMA-induced (1 ng/ml) generation of $O_2^-$ from eosinophils ($p > 0.1$, n = 6; fig. 3b). Salbutamol (1 μ*M*) did not modify $LTD_4$- or PMA-induced $O_2^-$ generation from eosinophils ($p > 0.1$, respectively, n = 6; fig. 4a, b). The baseline spontaneous generation of $O_2^-$ from eosinophils was not modified by montelukast or salbutamol (data not shown).

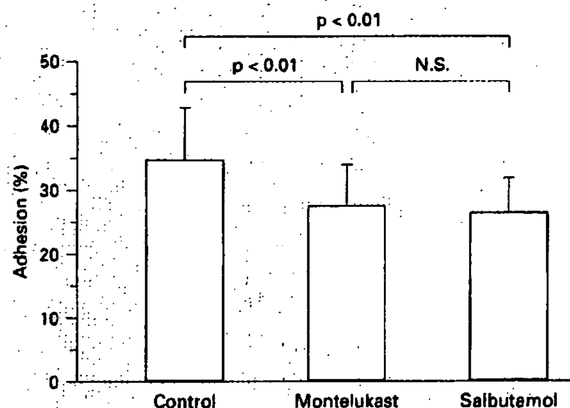


**Fig. 2.** Effects of montelukast (1 μ*M*) and salbutamol (1 μ*M*) on IL-5 -induced (100 p*M*) eosinophil adhesion to rh-ICAM-1. Data are expressed as means ± SEM from six experiments. N.S. = Not significant.

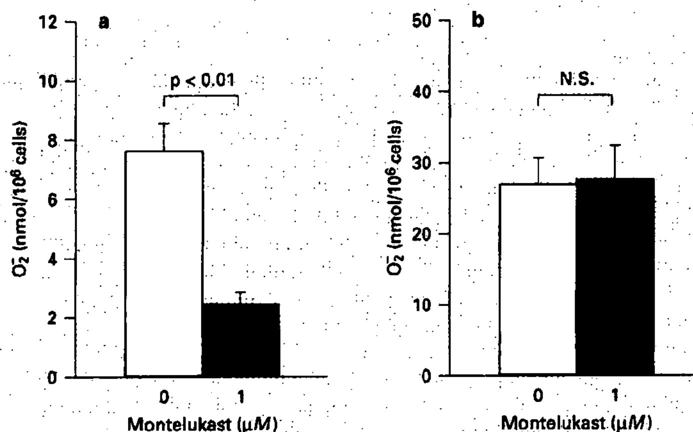


**Fig. 3.** Effects of montelukast (1 μ*M*) on **a** $LTD_4$- (100 n*M*) or **b** PMA-induced (1 ng/ml) eosinophil $O_2^-$ generation. Data are expressed as means ± SEM from six experiments. N.S. = Not significant.

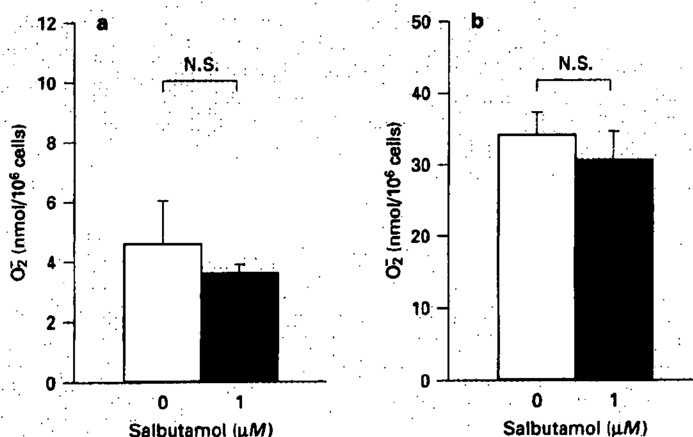


**Fig. 4.** Effects of salbutamol (1 μ*M*) on **a** $LTD_4$- (100 n*M*) or **b** PMA-induced (1 ng/ml) eosinophil $O_2^-$ generation. Data are expressed as means ± SEM from six experiments. N.S. = Not significant.

## Discussion

$\beta_2$-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 $\beta_2$-agonist. This result is consistent with our previous observation that IL-5-induced eosinophil adhesiveness was attenuated by tulobuterol, a $\beta_2$-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 $\beta_2$-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 $\beta_2$-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 $\beta_2$-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 $\beta_2$-agonists. Despite its effects on eosinophil activation induced by a variety of mediators including IL-5, it is unlikely that $\beta_2$-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 $\beta_2$-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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**Keywords:** lung neoplasms; EGFR; gefitinib; tyrosine kinase inhibitor; clinical trial; PNA-LNA PCR clamp

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 $\mu$M) 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 $\mu$M 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



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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 ($\chi^2$ 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).

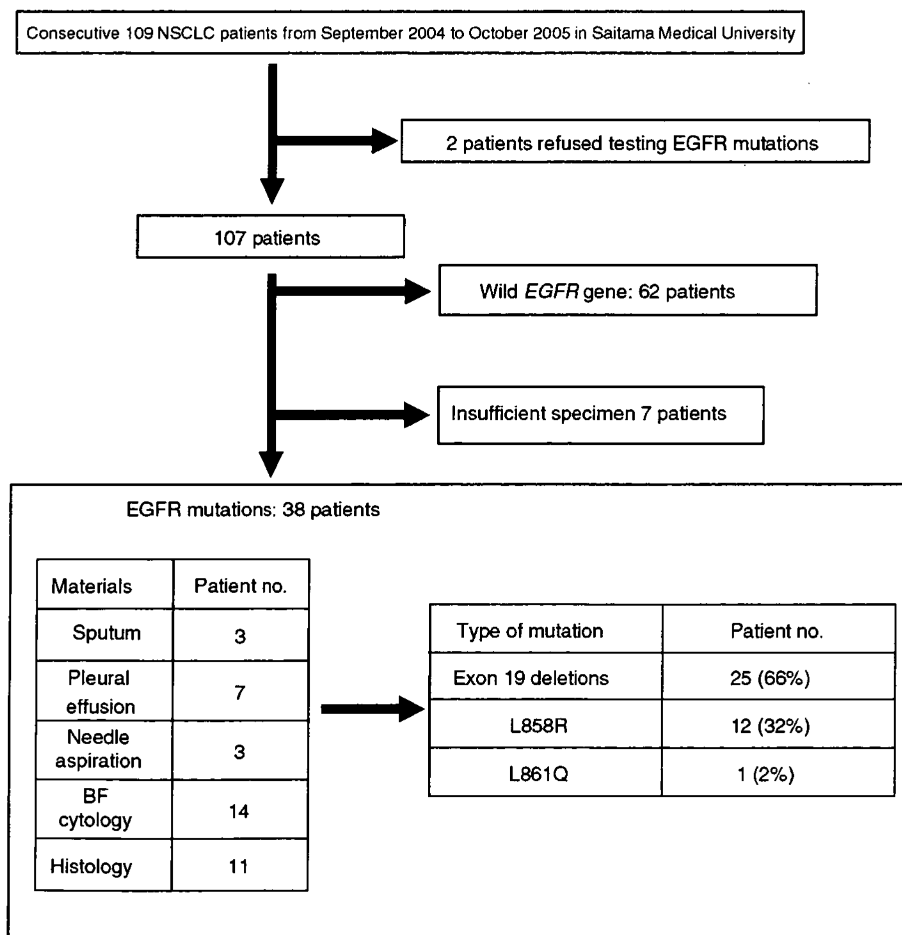


**Figure 1** Patients entered and source of specimen and type of EGFR mutation.

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**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.

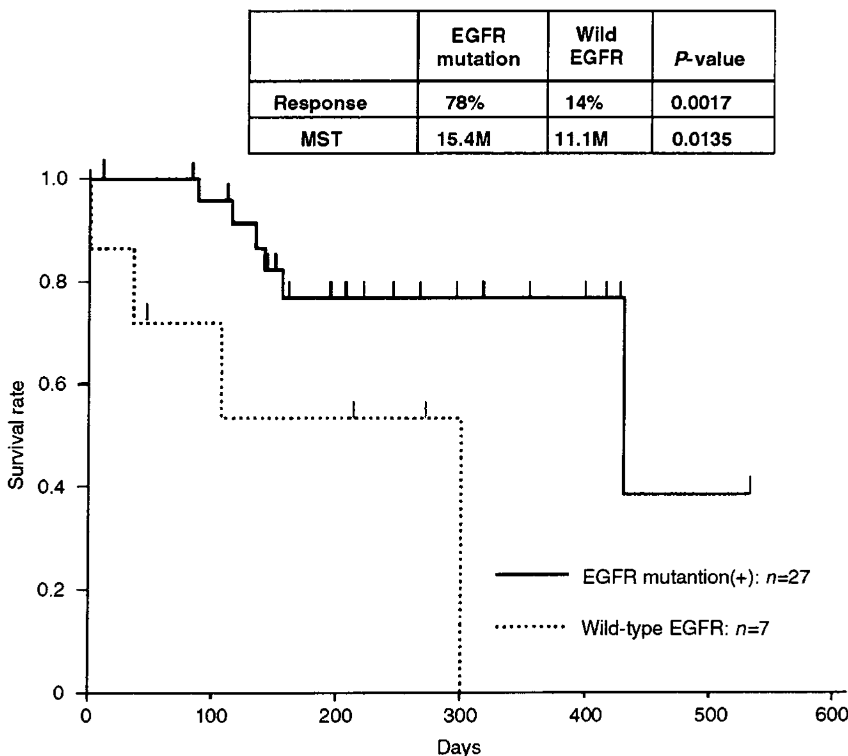


**Figure 2** Survival time curves *after gefitinib treatment* in patients with and without EGFR mutation are shown.



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significantly different between patients with EGFR mutations (78%) and patients with wild EGFR (14%) ($\chi^2$ 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.

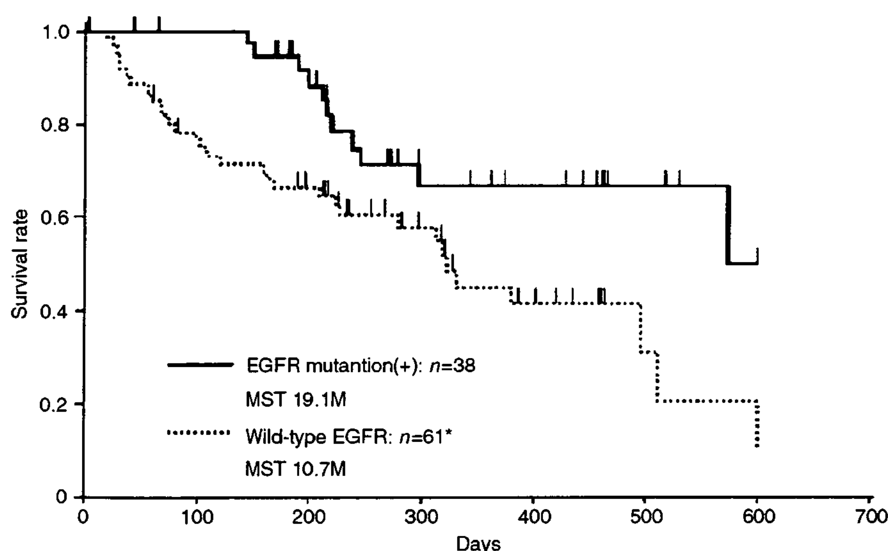


**Figure 3** Overall survival curves *after the initial treatments* in patients with and without EGFR mutation are shown.

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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.

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 $\chi^2$ 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).

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.

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.

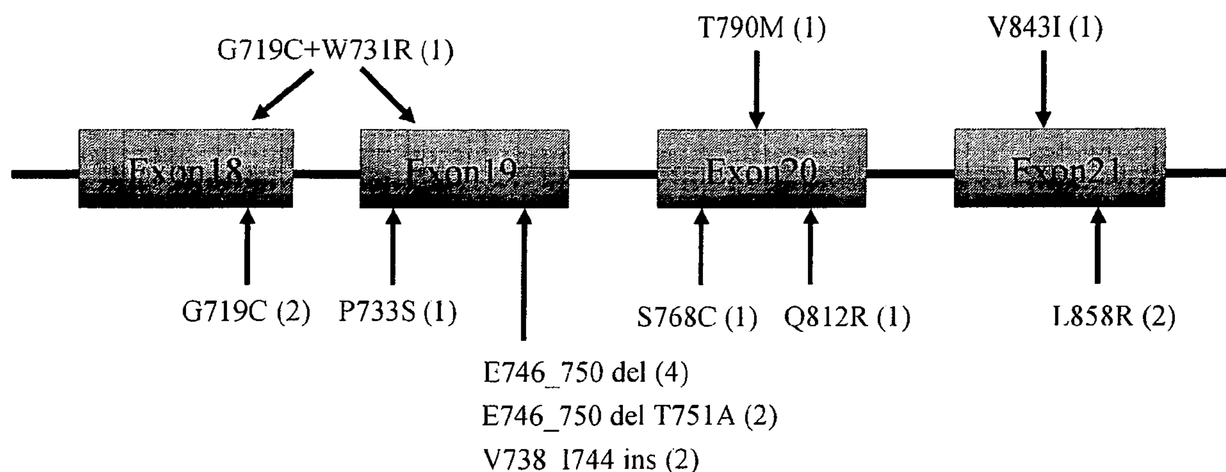


Figure 1. *Mutation sites of exons 18 to 21 in the EGFR gene. The number of cases identified with the respective mutation is shown in parenthesis.*

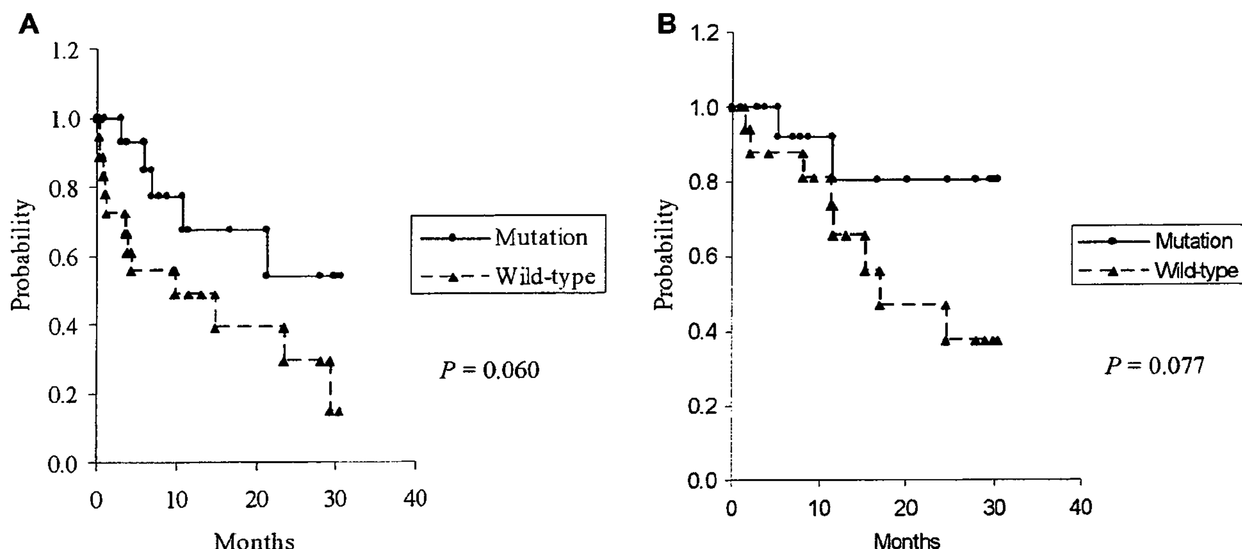


Figure 2. *Kaplan-Meier plots of (A) time to progression and (B) overall survival after gefitinib therapy, depending on EGFR mutation status.*

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.