regulated by means of redox-dependent mechanisms. The transactivation of EGFR was observed to occur quickly, after <30 minutes of exposure to TNF-α in PC-9 cells (Fig. 4A and B). No additional induction of ligands, EGF and transforming growth factor-α, were detected by ELISA in the culturing medium of the cells even after 6 hours of 100 ng/mL TNF-α exposure (data not shown). From these observations, we think that this activation could occur independently of ligands but not through TNF-α-mediated ligands synthesis or proteolytic releasing of preexisting ligands from the disrupted cells. Although TNF-α induced the same levels of EGFR autophosphorylation in all three cell lines, this EGFR activation is minimally transmitted to the downstream Akt/NF-κB pathway in the resistant PC-9/ZD2001 cells (Fig. 4A). Moreover, an inhibitory effect of gefitinib on TNF-α-induced Akt/NF-κB activation was not observed although wortmannin, a phosphatidylinositol 3-kinase inhibitor, completely inhibited this signaling in PC-9/ZD2001 cells (Fig. 4B). These results suggest that the weak effect of EGFR on Akt/NF-κB signaling could occur between EGFR and phosphatidylinositol 3-kinase in PC-9/ZD2001 cells.

Several articles reported that the sensitivity to gefitinib is regulated by active mutant EGFR (30, 31), by the expression level of phosphatase and tensin homologue/MMAC/TEP (32), and by levels of Akt phosphorylation (13, 33, 34). Because the gefitinib-hypersensitive PC-9 cells originally had 15-bp deletion mutation in exon 19 of EGFR, they were thought to have a gefitinib-sensitive active mutant EGFR (35); however, because we found no alteration of the EGFR mRNA sequence in PC-9/ZD2001 cells (data not shown), we conclude that this gefitinib-resistant cell line was a good model for acquired gefitinib resistance. In our previous study, EGFR signaling mediated by transforming growth factor-α, an EGFR ligand, could not activate the mitogen-activated protein signaling pathway but could partially activate the Akt signaling cascade in PC-9/ZD2001. In PC-9/ZD2001R cells, the association between EGFR and mitogen-activated protein kinase signaling was completely reconstituted. On the basis of this result, we conclude that the decrease of EGFR signaling to the mitogen-activated protein kinase signaling pathway might contribute to acquired gefitinib resistance.[5] In this study, TNF-α significantly induced EGFR autophosphorylation but subsequent activation of the Akt signaling cascade was little observed in PC-9/ZD2001 (Fig. 4A and B). This decreased EGFR signaling on Akt could be partially caused by the decrease in EGFR expression but we have

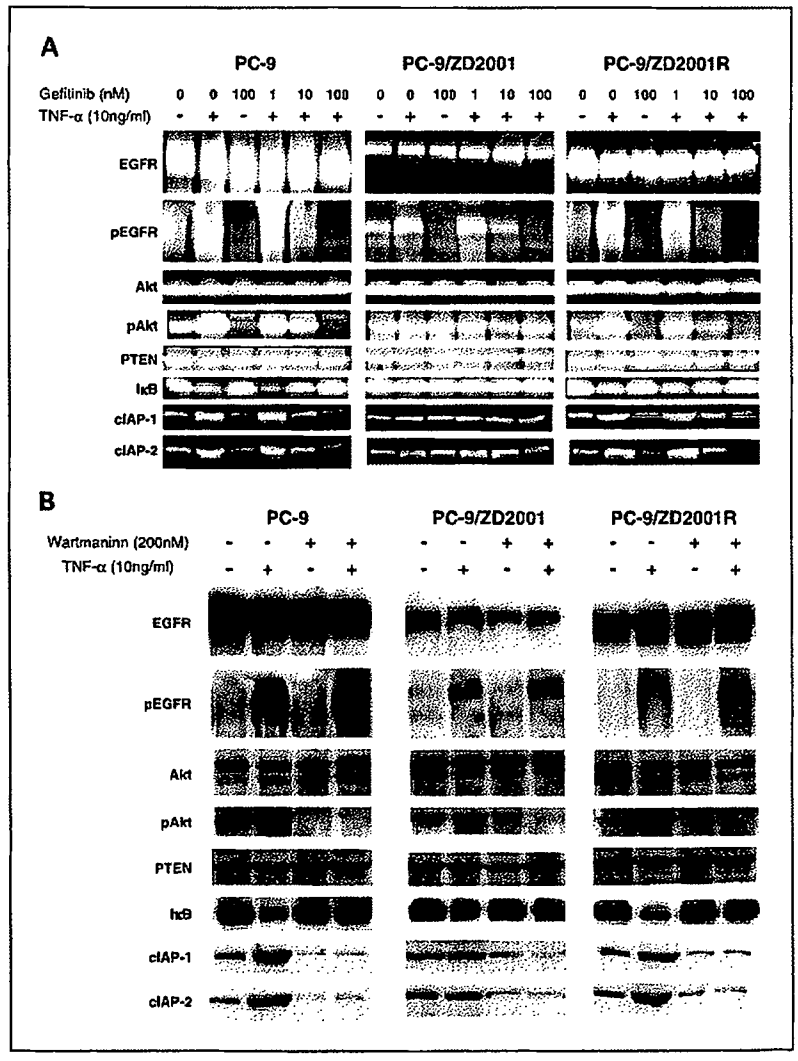

Fig. 4. Inhibitory effect of gefitinib on TNF-α-induced phosphorylation of Akt1 and degradation of IκB. Cells were treated with TNF-α with or without gefitinib (*A*) or wortmannin (*B*) simultaneously for 30 minutes at 37°C. Cell lysates were prepared and equivalent amounts of protein from each cell lysate were resolved with 10% SDS-PAGE, transferred to nitrocellulose membranes, and subjected to Western blotting with specific antibodies (as described in Materials and Methods). The EGFR and Akt1 membranes were stripped and reblotted with antibodies against phospho-EGFR (Tyr1045) and phospho-Akt, respectively. Expression of β-actin was used as internal control. Although treatment with TNF-α significantly phosphorylated EGFR in all three cell lines, downstream Akt/NF-κB activation was observed in PC-9 and PC-9/ZD2001R but weakly in PC-9/ZD2001. Gefitinib inhibited cross-talk signaling in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells (*A*). A phosphatidylinositol 3-kinase inhibitor, wortmannin, completely inhibited this signaling in all three cell lines (*B*).

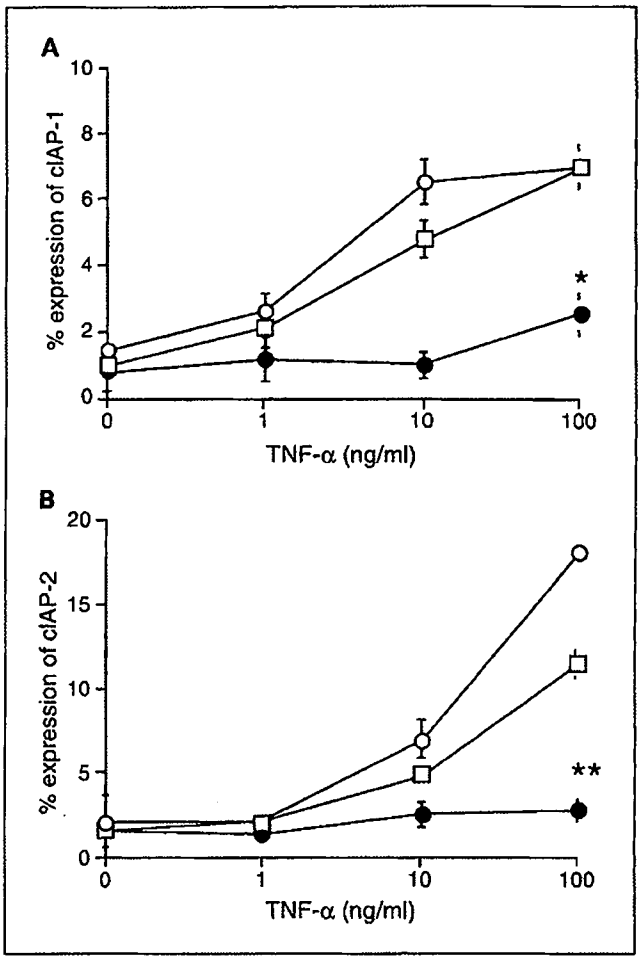

Fig. 5. TNF-α induced c-IAP1 and c-IAP2 mRNA expression in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells. The cells were exposed to the indicated concentrations of TNF-α for 12 hours; after which mRNA was isolated with the guanidium isothiocyanate method. Induction of c-IAP1 (*A*) and c-IAP2 (*B*) mRNA was measured with a fluorescence-based real-time RT-PCR method using specific primer sets (as described in Materials and Methods). The expression levels of c-IAP1 and c-IAP2 mRNA were significantly and dose-dependently increased by exposure to TNF-α in PC-9 and PC-9/ZD2001R cells but this enhancement was rarely observed in PC-9/ZD2001 cells. Results expressed as the percentage of each cell line compared with the internal control, expression of β-actin mRNA. ○, PC-9; ●, PC-9/ZD2001; □, PC-9/ZD2001R. Points, mean of three different experiments; bars, SD. *, $P < 0.001$.

no data to explain the discrepancy between transforming growth factor-α-mediated and TNF-α-mediated EGFR signaling in this cell line. Nevertheless, TNF-α-mediated cross-talk signaling to EGFR, although ligand independent, seems to cause downstream activation in a different way from that caused through ligand-mediated direct EGFR activation. Akt/NF-κB signaling is also known to be downstream of other receptors, such as other Her family receptors (36), platelet-derived growth factor receptor (37), and IFN receptor (38). We previously confirmed the expression of other Her family receptors, Her2 and Her3, in PC-9 cells. Possibly, signaling of these receptors may be able to modulate the TNF-α-mediated cross-talk signaling and Akt/NF-κB signaling. Various aspects of TNF-α-induced cross-talk signaling to EGFR, such as EGFR heterodimer formation with other Her family receptors and downstream signaling specificity, require further investigation.

Human IAPs, c-IAP1 and c-IAP2, have been reported to block the apoptotic events caused by caspase-8 activation by directly combining with caspase-3 and caspase-7 and restraining them (10, 27). IAPs play a key role in the resistance to apoptotic effect of TNF-α superfamily of proteins (39) and various anticancer drugs (40, 41); for this reason, IAPs are considered promising targets in anticancer therapy (42, 43). To evaluate TNF-α-mediated antiapoptotic signaling, we measured IAP induction in these cell lines by means of Western blotting analysis and real-time RT-PCR. As might be expected, IAPs and their mRNAs were markedly induced by TNF-α in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells (Fig. 5A and B). TNF-α-induced activation of caspase-3, but rarely of caspase-8, was significantly lower in PC-9 and PC-9/ZD2001R as compared with PC-9/ZD2001 (Fig. 3A and B). These results suggest that TNF-α precisely activates apoptotic signaling through caspase-8 in all three cell lines and that induction of IAPs blocks downstream signaling by inhibiting caspase-3 in PC-9 and PC-9/ZD2001R. In these cell lines, the induction of IAPs likely plays a key role in determining the sensitivity to TNF-α-mediated apoptosis among the antiapoptotic proteins that are induced by NF-κB-mediated transcription.

Several clinical studies of TNF-α as an anticancer treatment have been done, mainly in the 1970s; however, treatment with TNF-α was greatly limited by its side effects, particularly its toxicity to previously healthy organs (44–49). Recently, several new anticancer therapies using TNF-α have been developed, such as RGD-V29 (F4614) and TNF-erade (Biologic), in an attempt to reduce adverse effects (8, 9, 50, 51). We have shown that a NSCLC cell line with acquired resistance to gefitinib acquired collateral sensitivity to TNF-α. These data strongly suggest that treatment with TNF-α might be effective against tumors that have acquired resistance to gefitinib after long-term administration of this drug. Further analysis is required before clinical application.

In summary, the cross-talk signaling from TNFR to EGFR and subsequent IAP induction play important roles in the resistance to TNF-α-induced apoptosis in PC-9 cells. Because this signaling cascade is decreased in the gefitinib-resistant PC-9/ZD2001 cells, TNF-α did not activate the Akt/NF-κB cascade. This decrease of EGFR signaling to Akt/NF-κB pathway, which is related to gefitinib-acquired resistance, may contribute to the acquisition of hypersensitivity to TNF-α in this cell line.

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Epidermal growth factor receptor gene mutation in non-small cell lung cancer using highly sensitive and fast TaqMan PCR assay

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Summary Epidermal growth factor receptor (*EGFR*) gene mutations have been found in a subset of non-small cell lung cancer (NSCLC) with good clinical response to gefitinib therapy. A quick and sensitive method with large throughput is required to utilize the information to determine whether the molecular targeted therapy should be applied for the particular NSCLC patients. Using probes for the 13 different mutations including 11 that have already been reported, we have genotyped the *EGFR* mutation status in 94 NSCLC patients using the TaqMan PCR assay. We have also genotyped the *EGFR* mutations status in additional 182 NSCLC patients, as well as 63 gastric, 95 esophagus and 70 colon carcinoma patients. In 94 NSCLC samples, the result of the TaqMan PCR assay perfectly matched with that of the sequencing excluding one patient. In one sample in which no *EGFR* mutation was detected by direct sequencing, the TaqMan PCR assay detected a mutation. This patient was a gefitinib responder. In a serial dilution study, the assay could detect a mutant sample diluted in 1/10 with a wild-type sample. Of 182 NSCLC samples, 46 mutations were detected. *EGFR* mutation was significantly correlated with gender,

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smoking status, pathological subtypes, and differentiation of lung cancers. There was no mutation detected by the TaqMan PCR assay in gastric, esophagus and colon carcinomas. TaqMan PCR assay is a rapid and sensitive method of detection of *EGFR* mutations with high throughput, and may be useful to determine whether gefitinib should be offered for the treatment of NSCLC patients. The TaqMan PCR assay can offer us a complementary and confirmative test.

1. Introduction

Lung cancer is the deadliest cancer in many developed countries. Gefitinib (Iressa®, Astra Zeneca, London, UK), an inhibitor of the epidermal growth factor receptor (*EGFR*) tyrosine kinase, has been approved in Japan and the United States for the treatment of non-small cell lung cancer (NSCLC). Recently, erlotinib (Tarceva®, Roche, Basel, Switzerland), another inhibitor of *EGFR* tyrosine kinase, has been approved in the United States and Switzerland for the treatment of NSCLC. Gefitinib caused significant tumor shrinkage in 27.5% of Japanese NSCLC patients but in only 10.4% of Caucasian population [1–4]. Unfortunately, the addition of gefitinib to the traditional chemotherapy did not add any benefit to the patient survival [3], although overexpression of EGFR protein was seen in relatively high frequencies [5]. We and others have shown that the somatic mutation in tyrosine kinase (TK) domain of *EGFR* is associated with sensitivity of NSCLC to gefitinib [6–8]. Gefitinib targets the ATP-binding cleft with the TK domain and the reported mutations are either deletion or single amino acid substitutions in exon 18, 19, or 21 clustered around the ATP-binding pocket of the TK domain.

In vitro, *EGFR* mutations have been reported to confer enhanced tyrosine kinase activity in response to epidermal growth factor (EGF) and increased sensitivity to inhibition by gefitinib [6,7,9,10]. Thus, it is highly likely that *EGFR* mutation is a critical determinant of the patient's response to gefitinib. To determine the *EGFR* gene status may bring important information whether gefitinib is a therapeutic option for the NSCLC patient. If we can avoid unnecessary prescription of gefitinib in patients who are in fact non-responders, we will avoid fatal side effects of the drug and significantly reduce the health care cost. Fluorescent dye-based genotyping technology using the 5′ nuclease assay (TaqMan PCR assay) was developed as a large-scale and highly sensitive method in SNP scoring [11–16]. For SNP genotyping, one pair of TaqMan probes and one pair of PCR primers are used. Two TaqMan probes differ at the polymorphic site, with one probe complementary to the wild-type allele and the other to the variant allele. Recently, this method is being applied for genotyping of insertion/deletion polymorphism as a simple and cost-effective method [17].

We applied this genotyping technique with TaqMan probe to detect *EGFR* somatic mutations. Probes were designed according to the 13 different *EGFR* mutations including 11 that have already been reported. We show in this paper that this method is sensitive enough to detect the mutation in samples contaminated with 9-fold excess of wild-type samples. It is also fast and could be applied in large-scale screening.

2. Materials and methods

2.1. Patients and genomic DNA

NSCLC tissues were obtained by surgical excision between 1997 and 1999 from 67 patients at Nagoya City University Hospital in Japan. NSCLC tissues were also obtained from 27 patients at National Hospital Organization, Kinki-Chuo Chest Medical Center who were subsequently treated with gefitinib. Of 27 gefitinib treated samples, six transbronchial biopsy samples were obtained. These 94 samples were sequenced and also analyzed using TaqMan PCR assay. We have also analyzed additional 182 recent NSCLC cases, as well as 63 gastric, 95 esophagus and 70 colon carcinomas, operated between 2000 and 2003 at Nagoya City University Hospital using TaqMan PCR assay. The research was approved by the Institutional Review Board of each hospital. All the patients consented to the use of their tissues for the present analysis. The tissues were placed in liquid nitrogen immediately after resection or fixed by formalin and paraffin embedded. Genomic DNA was extracted using Wizard SV Genomic DNA Purification System (Promega) according to the manufacturer's instructions.

2.2. Genomic DNA PCR and DNA sequencing

All of 27 samples from National Hospital Organization, Kinki-Chuo Chest Medical Center were amplified by PCR reaction for genotyping analysis.

The primers for amplification of exon18, exon19, exon20, and exon21 were designed as previously described [7]. The reaction mixtures were contained with 1 μl of template DNA, 2 μl of 10× LA PCR Buffer II, 2 μl of 25 mM $MgCl_2$, 3.2 μl of 2.5 mM dNTP mixture, 0.4 μl of each primer (250 μM), 0.5 μl of TaKaRa LA Taq (5 U/μl), 10 μl of ddH_2O in a volume of 20 μl. Thermal cycling conditions were as follows: initial denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s; 64 °C for 30 s; and 72 °C for 1 min. The final extension was for 5 min at 72 °C. The PCR products were sequenced by ABI PRISM 3100 Genetic Analyzer® and analyzed by ABI PRISM SeqScape Software Version 2.1.1®.

2.3. Genotyping by the TaqMan PCR assay

The primers and TaqMan® MGB probes were designed with Primer Express 2.0 software (Applied Biosystems). The sequences of the allele-specific probes and primers used in the TaqMan PCR assay are shown in Table 1. TaqMan PCR and genotyping analysis were performed on Applied Biosystems 7500 Real Time PCR System (Applied Biosystems) in the manufacture's instructions. The reaction mixtures were amplified in 1 μl of genomic DNA (10 ng/μl) or 1 μl of 100-fold diluted PCR products, 5 μl of 2× TaqMan® Universal Master Mix (Applied Biosystems), 0.5 μl of 20× primer/probe mix (each final concentration of primer and probe is 9 μM and 2 μM), 3.5 μl of ddH_2O in a volume of 10 μl. PCR cycling conditions were as follows: one cycle at 95 °C for 10 min; and 40 cycle at 95 °C for 15 s and 58 °C for 1 min. The results were analyzed on Applied Biosystems 7500 Real Time PCR System using allelic discrimination assay program.

2.4. *EGFR* DNA amplification

The *EGFR* gene amplification was analyzed for 27 gefitinib treated patients by quantitative real-time PCR, performed on a PRISM 7500 sequence detector (Applied Biosystems) by using a QuantiTect SYBR Green kit (Qiagen Inc., Valencia, CA). We have quantified each tumor DNA by comparing the target locus to the reference *Line-1*, a repetitive element for which copy numbers per haploid genome are similar among all of the human normal and neoplastic cells. Quantification is based on standard curves from a serial dilution of human normal genomic DNA. The relative *EGFR* copy number level was also normalized to normal human genomic DNA as calibrator. Copy number change of *EGFR* gene relative to the *Line-1* and the calibrator were determined by using the formula $(T_{EGFR}/T_{Line\text{-}1})/(C_{EGFR}/C_{Line\text{-}1})$, where T_{EGFR} and $T_{Line\text{-}1}$ are quantity from tumor DNA by using *EGFR* and *Line-1*, and C_{EGFR} and $C_{Line\text{-}1}$ are quantity from calibrator by using *EGFR* and *Line-1*. PCRs for each primer set were performed in at least triplicate, and means were reported. Conditions for quantitative PCR reaction were as follows: one cycle of 50 °C for 2 min; one cycle of 95 °C for 15 min; 40 cycles of 95 °C for 15 s; 56 °C for 30 s; and 72 °C for 34 s. At the end of the PCR reaction, samples were subjected to a melting analysis to confirm specificity of the amplicon. Primers for *EGFR* gene were designed by using Primers 3[12] to span a 100–150 bp non-repetitive region at exon 28 and were synthesized by Invitrogen (Carlsbad, CA). Primer sequences for *EGFR* gene used in this study are as follows: forward, CCACCAAATTAGCCTGGACA; and reverse, CGCGACCCTTAGGTATTCTG. *EGFR* amplification (increased *EGFR* copy number) was defined as more than five copies.

2.5. Statistical analysis

For comparisons of proportions, the Fisher's Exact test was used. The two-sided significance level was at $P < 0.05$. We did all analyses using a Stat View (version 5, SAS Institute Inc., Cary, NC) software.

3. Results

3.1. Genotyping by genomic DNA sequencing

We have already published the *EGFR* genomic DNA sequencing data of 67 NSCLC samples [7,18]. Seventeen cases had a mutated allele and there were six different mutations. Other groups have also reported additional somatic mutations in the same region of the *EGFR* gene [6,8]. We first sequenced some additional samples to find previously unknown mutations. Twenty-seven NSCLC tumor samples from National Hospital Organization, Kinki-Chuo Chest Medical Center were subjected to conventional genomic DNA sequencing in exon 18, 19, 20, and 21. Nine of 27 cases (33.3%) had a mutation. Of these nine cases, two were novel mutations. One patient carried a 24 nucleotide in-frame deletion (2239–2262), removing amino-acid 747 through 754, and three nucleotides insertion at 2270, adding one asparagine (delL747–K754&insK757NK) as shown in Fig. 1A and B. This region overlaps with the other deletion mutations reported previously [6,7]. Another tumor had two mutations: one amino acid substitution in the exon 21: leucine–proline at codon 838 (L838P) (Fig. 1C); and a deletion

Table 1 Sequence of the mutation specific TaqMan probes and PCR primers

Mutation no.	Probe name	Nucleotide	Amino acid	Primer sequence (forward)	TaqMan probe	Primer sequence (reverse)
	WT1				VIC–ATTAAGAGAAGCAACATCT	
1	Del 1a	2235–2249del	E746–A750del		FAM–CGCTATCAAAACATCT	
2	Del 1b	2236–2250del			FAM–CTATCAAGACATCTCC	
3	Del 2	2254–2277del	S752–I759del		FAM–AGAAGCAACACTCGAT	
	WT2			CCCAGAAGGTGAGAAAGTTAAAATTC	VIC–CGAAAGCCAACAAG	CCCACACAGCAAAGCAGAAA
4	Del 3	2239–2247del, 2248G > C	L747–E749del, A750P		FAM–CAAGGAACCAACATC	
5	Del 4	2240–2257del	L747–S752del, P753S		FAM–AAGGAATCGAAAGCC	
6	Del 5	2238–2255del, 2237A > T	L747–S752del, E746V		FAM–CAAGGTTCCGAAAGC	
7	Del 6	2240–2251del	L747–A750del, T747S		FAM–TCAAGGAATCATCTCC	
	WT3				VIC–AAGTGCTGGGCTCC	
8	G719C	2155G > T	G719C	TGAGGATCTTGAAGGAAACTGAATTC	FAM–AAAGTGCTGTGCTCC	TGCCAGGGACCTTACCTTATACA
9	G719S	2155G > A	G719S		FAM–AAAGTGCTGTGCTCC	
	WT4				VIC–TTGGGCTGGCCAAA	
10	L858R	2573T > G	L858R	CCGCAGCATGTCAAGATCAC	FAM–TTGGGCGGGCCAA	TCCTTCTGCATGGTATTCTTTCTCT
	WT5				VIC–CCAAACTGCTGGGTG	
11	L861Q	2582T > A	L861Q		FAM–CCAAACAGCTGGGTG	
	WT6				VIC–ATTAAGAGAAGCAACATCT	
12	Novel Del	2239–2262del	L747–K754del	CCCAGAAGGTGAGAAAGTTAAAATTC	FAM–CTATCAAGGAAGCCAACAA–MGB	CCCACACAGCAAAGCAGAAA
12	Novel Ins	2265–2267 InsCAA	N756Ins		FAM–CCAACAACAAGGAAAT–MGB	
	WT7			GGAGGACCGTCGCTTGGT	VIC–CGCGACCTGGCAG–MGB	CCCAAAATCTGTGATCTTGACATG
13	L838P	2513T > C	L838P		FAM–CGCGACCCGGCAG–MGB	

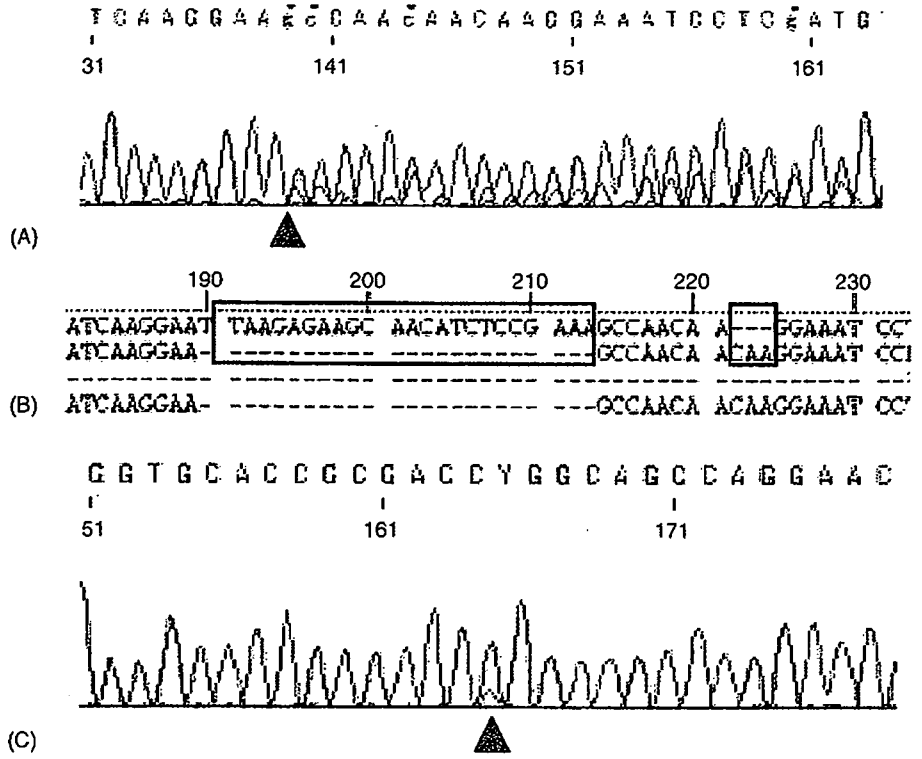

Fig. 1 (A) Data from direct sequencing showing a novel 24 nucleotide in-frame deletion (2239–2262), removing amino-acid 747–754 (arrow head: deletion start). (B) Nucleotide sequence of the novel insertion–deletion mutant as aligned with the wild-type sequence. Three nucleotides (CAA) are inserted at the position 2270 in the exon 19, adding one asparagine (delL747–K754&insK757NK) (box: insertion–deletion sequence, first line: wild-type sequence; second line: mutation type sequence). (C) DNA sequencing of case #6. The other mutation found in case #6. Amino acid substitution: leucine to proline at codon 838 (L838P) due to T–C substitution at position 2513 in exon 21 (arrow head: mutation point).

mutation (Del 5). The substituted amino acid is conserved among all the erbB family members. We designed TaqMan probes for these two novel mutations as described below. We have not determined whether these nine genomic abnormalities found in this cohort are somatic or genomic.

3.2. Analysis using TaqMan PCR assay

Next, we subjected these 94 NSCLC samples to TaqMan PCR assay. To detect all of these mutations specifically, we designed 13 sets of specific TaqMan probes. They targeted 11 previously reported mutations and the two novel mutations that were identified in this paper (Table 1). Each probe has been shown to anneal only to the correct sequence. Using this analysis, 27 cases were detected to have a mutation. In all of the cases except case #1, the results of TaqMan PCR were in complete agreement with the genomic DNA sequencing data (Table 2). Typical results of the TaqMan PCR assay and their corresponding DNA sequence data are shown in Fig. 2. In Table 2, cases #1 to #9 were responders for gefitinib (partial response), and cases #10 to #27 were non-responders.

Case #1 was determined to have a mutation carrying amino acid substitution in exon 21 (L858R) by the TaqMan PCR assay (Fig. 3A). The genomic DNA sequencing could not detect the mutation (Fig. 3B). As the PCR assay has suggested that the sample had a significant contamination with wild-type DNA, we did a serial dilution experiment to determine the relative content of the mutated alleles in this tumor. The standard curve was configured using a titration by 10% steps with the control synthetic oligos harboring the mutant sequence (Fig. 3C), and the approximation curve was calculated (Fig. 3D). In reference to this data, the data for the case #1 suggested that this tumor contained 11% mutated allele and 89% normal allele. The first sequence data was rechecked and a very small peak was found to be present which was compatible with a substitution of G for T at nucleotide 2573 (L858R) (Fig. 3B). The heterogeneous tumor cells or contamination with wild-type DNA from the normal tissue was suspected. The genomic DNA of case #1 was newly

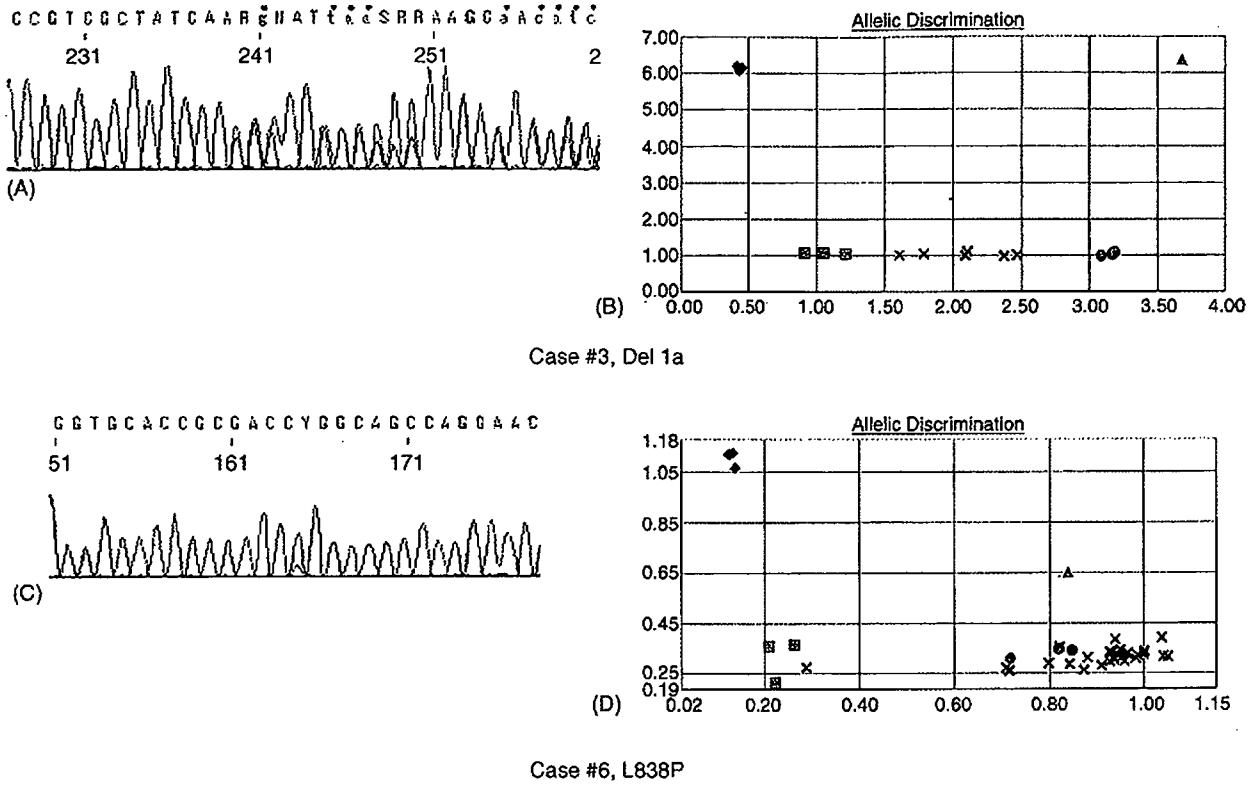

Fig. 2 In the TaqMan PCR assay, triangles indicate the samples with the somatic mutations. Diamonds indicate the mutation controls (artificial template oligo). Circles indicate the wild-type controls (artificial template oligo). Crosses indicate samples that turned out to be wild type. Squares indicate controls without DNA template. Both abscissa and ordinate are fluorescent intensity of each dye. Samples with mutation appear deviated from the abscissa. Heterozygous samples appear deviated from both abscissa and ordinate and are plotted roughly at 45° if the sample contained mutation and wild-type alleles at 1:1 ration. (A) Genotyping by DNA sequencing (case #3, Del 1a). (B) Genotyping by TaqMan PCR assay (case #3, Del 1a). (C) Genotyping by DNA sequencing (case #6, L838P). (D) Genotyping by TaqMan PCR assay (case #6, L838P).

prepared and sequenced again. The sequence data now showed a clear peak of the mutated allele (Fig. 3E). A repeat TaqMan PCR assay of the newly prepared DNA now revealed a proportion of 35% tumor DNA and 65% wild-type DNA (Fig. 3C). In nine gefitinib responders, six patients (66.7%) had *EGFR* mutation from TaqMan PCR assay. Of 27 gefitinib treated samples, six trans-bronchial biopsy samples were also evaluated by TaqMan PCR assay. In these six samples, the result from biopsy samples analysis perfectly matched with that of the surgical removed samples.

3.3. Analysis of *EGFR* DNA copy number

The *EGFR* gene amplification of 27 samples from patients who were treated with gefitinib at National Hospital Organization, Kinki-Chuo Chest Medical Center, was analyzed by quantitative real-time PCR. Four of 27 cases were found to have *EGFR* DNA amplifications (*EGFR* copy number >5) (Table 2). In these four cases, two had *EGFR* mutation. The two cases showed a clinical response to gefitinib. In the 10 cases with *EGFR* mutation in this cohort, only two had *EGFR* amplification. The *EGFR* amplification did not correlate with *EGFR* mutation status ($p=0.6125$). The *EGFR* amplification did not correlate with any of the clinicopathological factors. There was not any statistically significantly correlation between *EGFR* amplification and overall survival (data not shown).

3.4. *EGFR* mutation status in the additional 182 NCSLC samples

In addition to the 67 cases we have already reported and 27 cases we have sequenced in this paper, we genotyped additional 182 recently operated NSCLCs for *EGFR* mutations using TaqMan PCR assay. Forty-six mutations were detected (25.3%):

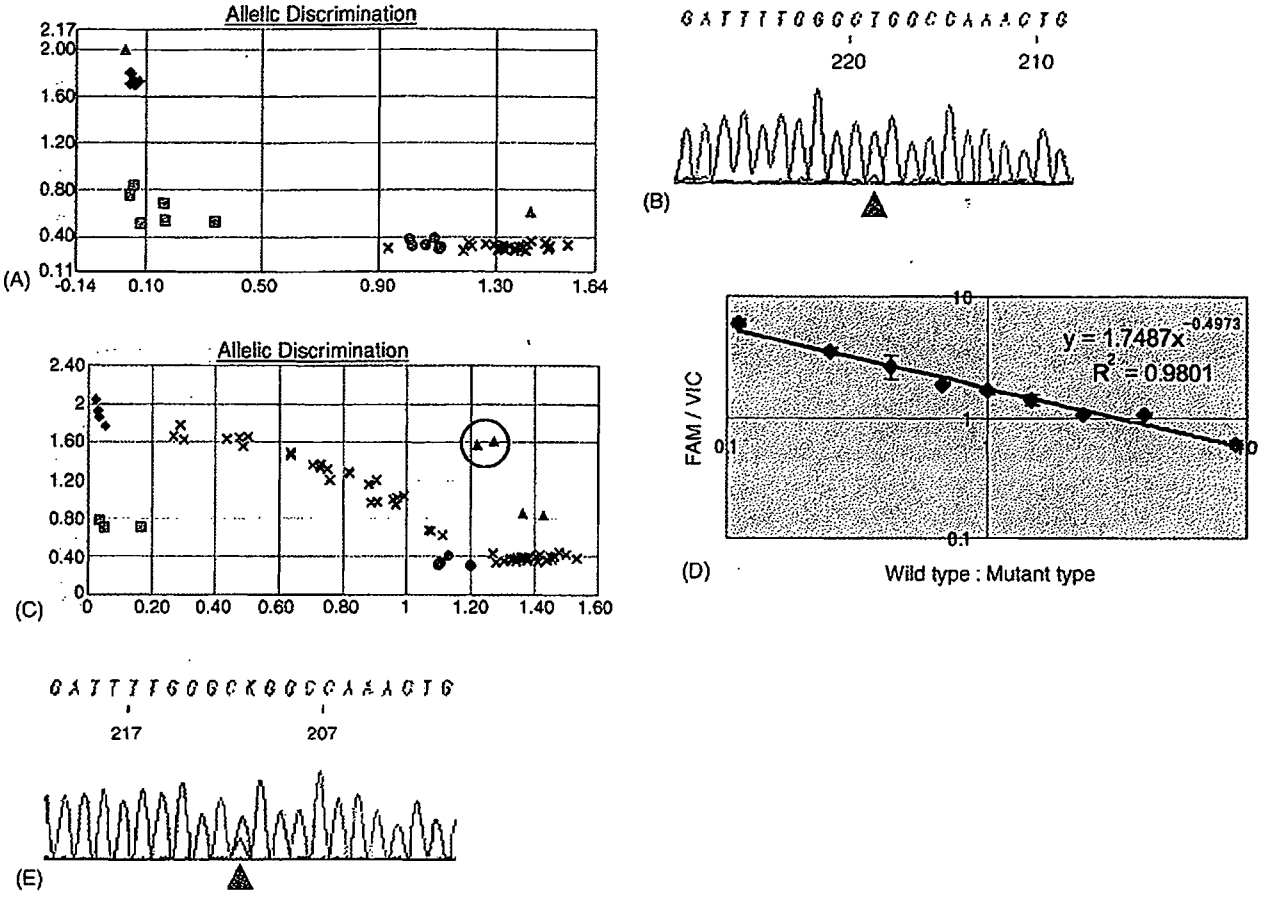

Fig. 3 (A) TaqMan analysis of case #1. Case #1 appeared to have mutation carrying amino acid substitution in exon 21 (L858R) by TaqMan PCR assay. The triangle deviating about 10% from the abscissa is the sample. (B) DNA sequencing of case #1. The first screening data by genomic DNA sequencing of case #1 shows a barely identifiable mutation peak representing a G for T at nucleotide 2573 (arrow head: mutation point). (C) TaqMan PCR assay of titrated samples. The standard curve was configured using a titration by 10% steps of the control synthetic oligos harboring mutated sequence by TaqMan PCR assay. The sample of case #1 is indicated as closed triangles which deviate about 10% from the abscissa. This showed that this tumor contained the mutated allele at the proportion of 11%. Closed triangles in the circle indicate the genomic DNA of case #1 that was newly prepared that was calculated as 35% of total DNA fraction. (D) The approximation curve calculated from the data in Fig. 3C. The *x*-axis indicates the wild type/mutant type ratio, and *y*-axis indicates FAM/VIC which is the degree of deviation from the *x*-axis relative to the 100% mutation control ($y = 1.7487x^{-0.4973}$, $R^2 = 0.9801$). (E) DNA sequencing of the case #1 that has been newly prepared. The sequence showed a clear peak of mutated allele that was substituted G—T at position 2573 (arrow head: mutation point).

20 patients had the L858R mutation; 22 had the deletion mutation in exon 19; two had the G719C mutation; and two patients had the L861Q mutation. Relationship between the *EGFR* mutation and clinical–pathologic factors in additional 182 NSCLC patients is shown in Table 3. Among those with *EGFR* mutation, 17/127 (13.4%) were male and 29/55 (52.7%) were female; 27/46 (58.7%) were never-smokers and 12/113 (10.6%) were ever-smokers; 45/97 (46.4%) were adenocarcinoma and 1/85 (1.2%) was non-adenocarcinoma; 35/80 (43.8%) were well differentiated and 11/85 (12.9%) were moderately or poorly differentiated. The mutation status were significantly correlated with gender (women versus men, $p < 0.0001$), smoking status (never-smokers versus ever-smokers, $p < 0.0001$), pathological subtypes (adenocarcinoma versus non-adenocarcinoma, $p < 0.0001$), and differentiation (well versus moderately or poorly, $p < 0.0001$). In 46 patients with *EGFR* mutations, 43 genomic DNA from matched normal lung tissues were available and were showed to be wild type by TaqMan PCR assay, suggesting that these mutations were somatic. There was no mutation detected by the TaqMan PCR assay in gastric, esophagus and colon carcinomas.

Table 2 Mutation status of the *EGFR* gene and *EGFR* copy number in 27 Gefitinib treated samples

Sample no.	Sequence	TaqMan	Copy number
1	WT	L858R	2.41
2	Del/Ins	Del/Ins	2.78
3	Del 1a	Del 1a	1.6
4	Del 1b	Del 1b	2.19
5	WT	WT	2.1
6	Del 5/L838P	Del 5/L838P	5.97
7	WT	WT	2.03
8	WT	WT	2.08
9	Del 3	Del 3	1.52
10	WT	WT	1.83
11	WT	WT	3.94
12	G719S	G719S	2.79
13	WT	WT	1.14
14	WT	WT	1.67
15	WT	WT	3.13
16	Del 1b	Del 1b	2.31
17	WT	WT	1.35
18	WT	WT	1.15
19	WT	WT	1.42
20	WT	WT	2.34
21	Del 1b	Del 1b	1.28
22	WT	WT	2.26
23	WT	WT	6.79
24	L858R	L858R	9.74
25	WT	WT	5.08
26	WT	WT	1.47
27	WT	WT	1.16

Case #1 to #9: responders; case #10 to #27: non-responders. *EGFR* amplification (increased *EGFR* copy number) was defined as more than five copies.

4. Discussion

Gefitinib was developed as an inhibitor of *EGFR* tyrosine kinase that is often over-expressed in many cancers. It showed a promising effect on a few cancers in phase I trial [1]. Subsequently, however, in phase II randomized trials in which the drug was used in combination with other traditional chemotherapy, the effect was marginal in patients with NSCLC [7]. Last year, our group and others have reported identification of genetic mutations in the *EGFR* kinase domain [6,7]. The mutation was seen in a subset of NSCLC with a good response to gefitinib. These reports triggered further studies on the *EGFR* mutation and the tumor's response to gefitinib and erlotinib [6–8]. All the groups identified recurrent mutations in the same region around the ATP binding pocket in *EGFR* tyrosine kinase domain. In vitro studies have reported that the kinase activity of *EGFR* or the sensitivity to gefitinib showed a strong association with *EGFR* gene mutation [7,9]. In our analysis, 6/9 (66.7%) gefitinib-responders had *EGFR* mutations. Thus, some of gefitinib-responders might have other mechanism besides *EGFR* mutations.

In this paper, we were unable to show any differences in *EGFR* amplification between tumors carrying the wild-type *EGFR* sequence and tumors carrying the mutant *EGFR* sequence, which is not surprising as it has been convincingly shown that *EGFR* mutation and not expression levels is responsible for the clinical response to *EGFR* tyrosine kinase inhibitors [6,7,19]. Hirsch et al. [20] reported that *EGFR* gene copy number correlated with EGFR protein expression, but not with prognosis in a cohort of patients not treated with gefitinib. Cappuzzo et al. [21] reported that high *EGFR* gene copy number was associated with better survival. However, there was not any statistically significantly correlation between *EGFR* amplification and overall survival in our analysis. Further study will be needed to delineate the relationships among *EGFR* mutation, *EGFR* gene copy number, *EGFR* mRNA expression, and gefitinib sensitivity.

Over the past three decades, the incidence of lung adenocarcinoma has increased worldwide. Most individuals with lung adenocarcinoma, especially women, are nonsmokers, a population that is sensitive to gefitinib. Reported risk factors for the development of lung adenocarcinoma include cigarette smoking, exposure to cooking fumes, air pollution, second-hand smoke, asbestos, and radon; nutritional status; genetic susceptibility; immunologic dysfunction; tuberculosis infection; asthma; and human papilloma virus [22]. In our analysis of recently operated 182 cases, most of the *EGFR* mutations were present in adenocarcinomas except one case. Mutations were more prevalent in females than in males and in nonsmokers than in smokers, confirming and extending the results of previous reports [6,8,9,23]. More recently, it has been reported that all of adenocarcinomas carrying *EGFR* mutations were well to moderately differentiated [24]. These data were comparable with those obtained in our analysis.

In this report, we used the TaqMan PCR assay based on allele specific probe. This method combines the amplification and detection step, and does not require any post-PCR processing. This makes the TaqMan PCR assay easy-to-use and allows high throughput operation. Furthermore, this method was highly sensitive to detect *EGFR* mutations. One gefitinib responded case with a base-substitution mutation could be detected by the TaqMan PCR assay, although it was undetectable at the first conventional genomic sequencing. When the mutated allele consisted only about 10% of total

Table 3 Relationship between the *EGFR* mutation and clinical–pathologic factors

Factors	Patients with *EGFR* mutation	Patients without *EGFR* mutation	*p*-Value
Gender			
Male	17 (13.4%)	110	<0.0001
Female	29 (52.7%)	26	
Age			
≦64	26 (31.7%)	56	0.0868
>64	20 (20%)	80	
Smoking status			
Never-smokers	27 (58.7%)	19	<0.0001
Ever-smokers	12 (10.6%)	101	
Lymph node metastasis			
N0	34 (27.9%)	88	0.3625
N+	12 (20.7%)	46	
Differentiation			
Well	35 (43.8%)	45	<0.0001
Moderately/or poorly	11 (12.9%)	74	
Pathological subtypes			
Adeno	45 (46.4%)	52	<0.0001
Non-adeno	1 (1.2%)	84	
Stage			
I	33 (31.1%)	73	0.0555
II–IV	13 (17.6%)	61	

N+: lymph node metastasis positive; Adeno: adenocarcinoma.

genomic DNA content, it was not detected by the sequencing. It is alleged that the detection limit in genomic sequencing is about 25% content in general. Because, in the clinical settings, it is not always possible to obtain samples carrying containing homogeneous tumor cells, this highly sensitive method is preferable to the conventional sequencing. Previous reports might underestimate the *EGFR* mutations. The present method can be most effectively used with 5–10 ng of DNA but can analyze as few as 1 ng of tumor DNA. To investigate the correlation between *EGFR* mutations and the response to drugs, large-scale statistical analysis is needed. In these clinical research areas, we believe that this TaqMan PCR assay with high throughput is one of the powerful tools.

In trade off to its accuracy, this method is effective only to the mutations that are already known. The probes cannot be designed to the sequences of unknown mutations. Thus, we must take into account that there is always at a false negative risk in TaqMan PCR assay. However, with the high pace of research [23–25], most of the mutations in *EGFR* gene will be identified in a few years. The good news is the presence of predominant mutations (L858R and exon 19 deletions) which comprise 75–90% of all the *EGFR* mutations. The TaqMan PCR assay presented in this paper can offer us a complementary and confirmative test with sequencing. In our 94 sequenced samples, 24/27 (88.9%) were these predominant mutations. With highly significant correlation between the clinical response to gefitinib (and erlotinib) and *EGFR* mutation, many future clinical trials may first need *EGFR* mutation data using high throughput assays like the one described in this paper.

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ORIGINAL REPORT

Epidermal Growth Factor Receptor Gene Mutations and Increased Copy Numbers Predict Gefitinib Sensitivity in Patients With Recurrent Non–Small-Cell Lung Cancer

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ABSTRACT

Purpose
To evaluate epidermal growth factor receptor (*EGFR*) mutations and copy number as predictors of clinical outcome in patients with non–small-cell lung cancer (NSCLC) receiving gefitinib.

Patients and Methods
Sixty-six patients with NSCLC who experienced relapse after surgery and received gefitinib were included. Direct sequencing of exons 18 to 24 of *EGFR* and exons 18 to 24 of *ERBB2* was performed using DNA extracted from surgical specimens. Pyrosequencing and quantitative real-time polymerase chain reaction were performed to analyze the allelic pattern and copy number of *EGFR*.

Results
Thirty-nine patients (59%) had *EGFR* mutations; 20 patients had deletional mutations in exon 19, 17 patients had missense mutations (L858R) in exon 21, and two patients had missense mutations (G719S or G719C) in exon 18. No mutations were identified in *ERBB2*. Response rate (82% [32 of 39 patients] *v* 11% [three of 27 patients]; $P < .0001$), time to progression (TTP; median, 12.6 *v* 1.7 months; $P < .0001$), and overall survival (median, 20.4 *v* 6.9 months; $P = .0001$) were significantly better in patients with *EGFR* mutations than in patients with wild-type *EGFR*. Increased *EGFR* copy numbers (≥ 3/cell) were observed in 29 patients (44%) and were significantly associated with a higher response rate (72% [21 of 29 patients] *v* 38% [14 of 37 patients]; $P = .005$) and a longer TTP (median, 9.4 *v* 2.6 months; $P = .038$). High *EGFR* copy numbers (≥ 6/cell) were caused by selective amplification of mutant alleles.

Conclusion
EGFR mutations and increased copy numbers were significantly associated with better clinical outcome in gefitinib-treated NSCLC patients.

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INTRODUCTION

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase of the *ErbB* family that has been implicated in cell proliferation and survival and is frequently overexpressed in many solid tumors, including non–small-cell lung cancer (NSCLC). Gefitinib (Iressa; AstraZeneca, Osaka, Japan) is an orally active, selective EGFR tyrosine kinase inhibitor that binds to the adenosine triphosphate–binding pocket of the EGFR kinase domain and blocks downstream signaling pathways. Two phase II studies, IRESSA Dose Evaluation in Advanced Lung Cancer 1 and 2 (IDEAL 1 and 2), have demonstrated that gefitinib monotherapy exerts an antitumor activity in patients with advanced NSCLC who had previously received platinum-based chemotherapy.[1,2] Gefitinib was approved in Japan for the treatment of inoperable or recurrent NSCLC in July 2002.

The IDEAL trials and retrospective studies have revealed that women, never smokers, patients with adenocarcinoma, and Japanese patients have higher response rates to gefitinib.[1-4] Among patients with adenocarcinoma, histologic subtypes have been studied; one study showed that responses were more frequent in patients with bronchioloalveolar carcinoma (BAC) features (38% *v* 14%; $P < .001$),[3] whereas another study showed that the response rate was higher in patients with a papillary-dominant subtype (76% *v* 21%; $P = .002$).[5]

Although no predictive molecular markers had been identified at the time of approval, somatic mutations in the kinase domain of *EGFR* have been subsequently linked to gefitinib sensitivity. According to three initial reports, 20 of 24 gefitinib-responsive tumors contained *EGFR* mutations, whereas 19 nonresponsive tumors did not contain any mutations.[6-8] The mutations were detected in exons 18 to 21 of *EGFR*, close to the region coding the adenosine triphosphate–binding pocket of the kinase domain, and most of them were observed in two hotspots: in-frame deletions including amino acids at codons 747 to 749 in exon 19 and an amino acid substitution at codon 858 (L858R) in exon 21. Analyses of surgically resected NSCLC tumors revealed that such mutations were more frequent among women, never smokers, patients with adenocarcinoma, and Japanese or East Asian patients,[7-13] consistent with the known clinical predictors of gefitinib sensitivity.

To evaluate the exact predictive value, we studied consecutive patients with recurrent NSCLC who received gefitinib therapy. To insure high-quality genetic analyses of the archived tissues, we used methanol-fixed, paraffin-embedded surgical specimens, which are known to preserve DNA better than formalin-fixed tissues,[14] and performed laser capture microdissection (LCM).

Recently, some other biomarkers of NSCLC have been studied. The *EGFR* and chromosome 7 copy numbers in NSCLC were assessed using fluorescence in situ hybridization (FISH), and more than 3.0 *EGFR* copies per cell (balanced polysomy or gene amplification) were detected in 39 (22%) of 183 patients.[15] A correlation between an increased *EGFR* copy number and gefitinib sensitivity was also proposed in another study.[16] In yet other studies, mutations in the kinase domain of *ERBB2 (HER2)*, a gene coding another receptor tyrosine kinase of the ErbB family, were detected in 16 (3.6%) of 445 patients with lung adenocarcinoma.[17,18] In the current study, we also analyzed the *EGFR* copy number and the presence of *ERBB2* mutations to assess their impact on clinical outcome.

The expression of EGFR and related proteins has been more widely studied using immunohistochemistry. Some studies suggested that high expression of phosphorylated Akt[19,20] or low expression of phosphorylated mitogen-activated protein kinase[20,21] was associated with better outcome in gefitinib-treated patients, but in general, methods, criteria, and results were inconsistent among studies. We thought that protein expression should be analyzed in another exploratory study, and in the current study, we focused on the genetic analyses.

PATIENTS AND METHODS

Patients

After searching the pharmaceutical records of the National Cancer Center Hospital, 279 patients with NSCLC who had begun receiving gefitinib monotherapy (250 mg/d) between July 2002 and May 2004 were identified. Seventy-three of these patients had undergone surgical resection of primary NSCLC at the hospital and subsequently relapsed. Recurrences were not necessarily confirmed pathologically but were diagnosed clinically. Seven patients were ineligible for inclusion in this study because methanol-fixed tissues were not available (n = 5) or their informed consent to the genetic analysis was not obtained (n = 2); consequently, 66 patients were included.

Genetic Analyses of EGFR and ERBB2

On a protocol approved by the institutional review board of the National Cancer Center, we performed mutational analyses of exons 18 to 24 of *EGFR* and exons 18 to 24 of *ERBB2* and analyzed the *EGFR* copy number. Methanol-fixed, paraffin-embedded surgical specimens of primary NSCLC were collected retrospectively, and DNA was extracted from bulk tumor tissue, laser capture microdissected tumor tissue, and normal lung tissue from each patient. LCM was performed using a PixCell II LCM system (Arcturus Engineering Inc, Mountain View, CA) according to a previously described method.[22] If appropriate, tumor cells were captured separately from two areas with different histologic subtypes, such as an area with a BAC subtype and another area with stromal invasion. Nested polymerase chain reaction (PCR) was performed to amplify exons 18 through 24 of *EGFR* using previously described primers,[6] and standard PCR was used to amplify exons 18 through 24 of *ERBB2*. Direct sequencing of the PCR products was performed using ABI PRISM 3700 and 3100 DNA Sequencers (Applied Biosystems, Foster City, CA). All sequencing reactions were performed in both forward and reverse directions, and single nucleotide substitutions, insertions, and deletions were detected using an application program named NAMIHEI.[23] Pyrosequencing was performed to verify the sequencing data of the hotspots of *EGFR* and to assess the proportion of mutant alleles in the laser-captured tumor cells using a Pyrosequencing PSQ 96MA (Pyrosequencing, Uppsala, Sweden).[24] On the basis of the proportion of mutant alleles, *EGFR* mutations were divided into two patterns: balanced heterozygous (BH) pattern (< 60%) and mutant-allele-dominant (MD) pattern (≥ 60%). The cutoff level of 60% was decided because if more than 60%, the superiority of the mutant over the wild-type sequences was obvious on the direct sequencing chromatograms. Quantitative, real-time, TaqMan duplex PCR was performed to analyze the *EGFR* copy number using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The *EGFR* primers were 5′-GGAGGACCGTCGCTTGGT-3′ and 5′-AACACCGCAGCATGTCAAGA-3′; the probe (5′-CACCGCGACCTGGCAGCCA-3′) was labeled with the reporter dye 6-carboxyfluorescein (FAM). RNaseP was coamplified in the same reaction mixture as the endogenous reference gene using TaqMan RNaseP Control Reagents (6-carboxyrhodamine [VIC] dye; Applied

Biosystems). The average *EGFR* copy number per cell was calculated from the differences in the threshold amplification cycles between *EGFR* and RNaseP. Peripheral-blood samples obtained from healthy volunteers were analyzed as normal controls. Decreased, normal, moderately increased, and highly increased *EGFR* copy numbers were defined as less than 1.5, 1.5 to 3.0, 3.0 to 6.0, and ≥ 6.0 copies per cell, respectively.

Pathologic Evaluation

We reviewed the histologic features of the 66 patients using hematoxylin and eosin–stained slides of tumor samples. Two board-certified pathologists (K.T. and Y.M.) who were unaware of the patients' outcome and mutational status examined all the specimens independently; in case of discrepancy, final diagnoses were established by consensus. Adenocarcinoma was categorized in two ways. The first categorization was based on the WHO's classification of lung tumors,[25] which includes four major subtypes of adenocarcinoma: papillary, acinar, BAC, and solid; the dominant subtype in the total tumor mass of each case was documented. The second categorization was based on a report from the Memorial Sloan-Kettering Cancer Center,[26] in which adenocarcinomas were classified into adenocarcinoma without BAC features (Ad), adenocarcinoma with BAC features (AwBF), BAC with focal invasion (BwFI), and pure BAC (PBAC). If two or more tumors were present in one patient, the diagnosis of the most invasive tumor in each case was documented.

Radiologic Evaluation

In patients who had measurable lesions, imaging studies were performed at baseline, approximately 4 weeks after the initiation of gefitinib treatment, and periodically thereafter throughout the treatment. One board-certified radiologist (U.T.) who was unaware of the patients' mutational status reviewed the baseline, first follow-up, and confirmatory imaging studies and classified the tumor responses into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) using standard bidimensional measurements.[27] Responders were defined as patients with CR or PR. In this study, SD was subdivided into minor response (MR) and no response. MR was defined as a ≥ 25% decrease in the sum of the products of the perpendicular diameters of all measurable lesions at any point during gefitinib treatment. Time to progression (TTP) was defined as the time from the start of gefitinib administration to confirmed disease progression or death.

Statistical Analysis

The associations among mutational status, *EGFR* copy number, patient characteristics, and tumor response to gefitinib were assessed using a χ^2 test. The differences in TTP and overall survival (OS) according to the patient subgroups were compared using Kaplan-Meier curves and log-rank tests. Multivariate analyses using logistic regression models and Cox proportional hazard models were performed to assess the association between the biomarkers and clinical outcome while adjusting for the baseline patient characteristics. All analyses were performed using the SPSS statistical package (SPSS version 11.0 for Windows; SPSS Inc, Chicago, IL).

RESULTS

Patient Characteristics

The patient characteristics are listed in Table 1. All of the patients were Japanese. The proportions of women (39%), never smokers (47%), and patients with adenocarcinoma (94%) in this study were higher than those in a database of more than 1,000 patients with advanced or recurrent NSCLC treated at our hospital during the four most recent years (27%, 27%, and 73%, respectively). Twenty-two patients (33%) had been included in our phase II trial for first-line gefitinib therapy for patients with recurrent NSCLC, and the others had been treated with gefitinib in clinical practice settings. The operations for primary NSCLC were performed between February 1994 and August 2003, and the median time from the operations to the start of gefitinib was 2.3 years (range, 0.6 to 9.1 years).

Table 1. Patient Characteristics

	Patients (n = 66)	
	No.	%
Age, years		
Median	65	
Range	32-80	
Sex		
Female	26	39
Male	40	61
Smoking history*		
Never smokers	31	47
Former smokers	12	18
Current smokers	23	35
Histologic diagnosis		
Adenocarcinoma	62	94
Papillary/acinar/BAC/solid†	30/18/9/5	45/27/14/8
Ad/AwBF/BwFI/PBAC	15/45/2/0	23/68/3/0
Squamous cell carcinoma	3	5
Pleomorphic carcinoma	1	2
Performance status		
0/1	22/28	33/42
2/3	12/4	18/6
Prior chemotherapy regimens		
0	37	56
1	14	21
≥ 2	15	23

Abbreviations: BAC, bronchioloalveolar carcinoma; Ad, adenocarcinoma without BAC features; AwBF, adenocarcinoma with BAC features; BwFI, BAC with focal invasion; PBAC, pure BAC.
*Never smokers were defined as subjects who have never had a smoking habit, and former smokers were defined as subjects who had stopped smoking at least 1 year before diagnosis.
†Dominant subtype.

Clinical Outcome

Sixty-four patients had measurable lesions at the start of gefitinib administration. CR and PR were observed in two and 32 patients, respectively. MR was observed in three of nine patients with SD. Twenty-one patients had PD, including six patients who died before the first follow-up imaging studies. Two patients had only unmeasurable bone lesions at baseline; one patient showed rapid symptom improvement and continued to receive gefitinib therapy without progression for 13.8+ months, whereas the other

patient developed new lesions and died on day 71. These patients were included in the analysis as a responder and a nonresponder, respectively. The overall response rate was 53%. Forty-one patients died, and the median follow-up time for the 25 survivors was 14.6 months (range, 10.3 to 32.3 months). Eleven patients were still receiving gefitinib without progression at the time of the analysis. The median TTP and the median survival time (MST) for all patients were 5.2 and 16.3 months, respectively.

EGFR *and* ERBB2 *Mutations*

Forty-three mutations in the *EGFR* tyrosine kinase domain were detected in 39 (59%) of the 66 patients. All the mutations detected in this study are shown in Table 2. Twenty patients had deletional mutations in exon 19, and 17 patients had missense mutations (L858R) in exon 21. In exons 18 and 20, five types of missense mutations were detected. Two of them (G719S and G719C) occurred at a codon considered to be a third hotspot.[6,7,9-12] The others (L703V, E709K, and S768I) were detected in patients who also had mutations at the hotspots. Because these mutations were not detected in the normal lung tissues from the same patients, they were considered to be somatic mutations. No somatic mutations were detected in exons 22 to 24. Silent single nucleotide polymorphisms were identified at nucleotides 2361 (G/A; Q787Q), 2370 (G/A; T790T), and 2457 (G/A; V819V) in exon 20, and at nucleotide 2709 (C/T; T903T) in exon 23, but the association between these polymorphisms and the somatic mutations was not observed. In this study, no mutations and no polymorphisms were detected in exons 18 to 24 of *ERBB2*.

All 43 mutations were detected in LCM samples, but 11 (26%) of these mutations were not detected in the bulk tumor samples. In 13 patients, LCM was performed at separate areas with different histologic subtypes, but no heterogeneity was identified; the same mutations were detected in nine patients, and no mutations were detected in four patients. Mutational analyses of synchronous double lung cancers were performed in two patients; one patient had a tumor with wild-type *EGFR* and a more invasive tumor with L858R + S768I, and the other patient had a tumor with a 9-bp deletion (del L747-E749) and a more invasive tumor with a 15-bp deletion (del E746-T751insA) + L703V.

Among the 39 patients with *EGFR* mutations, the proportion of mutant alleles ranged from 29% to 94%. Nineteen patients showed a BH pattern and 20 patients showed an MD pattern.

EGFR *Copy Number*

The *EGFR* copy number in the laser-captured tumor cells ranged from 1.27 to 31.2 per cell, and increased *EGFR* copy numbers (≥ 3.0 per cell) were observed in 29 patients (44%). The relation between the copy number and the proportion of mutant alleles is shown in Figure 1. Increased copy numbers were observed more frequently in patients with *EGFR* mutations than in patients with wild-type *EGFR* (56% [22 of 39 patients] *v* 26% [seven of 27 patients]; $P = .014$). High copy numbers (≥ 6.0 per cell) were observed only in patients with an MD pattern of mutations. The copy number and the proportion of mutant alleles among patients with *EGFR* mutations was positively correlated (Spearman correlation coefficient = 0.643; $P < .001$), implying that the mutant alleles were selectively amplified in patients with an MD pattern. One patient with an MD pattern had a tumor with only approximately one copy per cell, indicating a hemizygous mutation with a loss of wild-type allele. No alterations in the gene copy number were observed in normal lung tissues.

Table 2. *EGFR* Mutations Detected in the Present Study

Exons	Amino Acids	Nucleotides	No. of Patients
19	del E746-A750	del 2235-2249	12
	del E746-A750	del 2236-2250	5
	del E746-T751insA	del 2237-2251	1
	del L747-E749	del 2239-2247	1
	del E746-S752insV	del 2237-2255 + ins T	1
21	L858R	T → G at 2573	17
18	G719S	G → A at 2155	1
	G719C	G → T at 2155	1
	L703V	C → G at 2107	1*
	E709K	G → A at 2125	1†
20	S768I	G → T at 2303	2‡

Abbreviations: *EGFR*, epidermal growth factor receptor; del, deletion; ins, insertion.
*A patient with del E746-T751insA.
†A patient with L858R.
‡A patient with L858R and a patient with G719C.

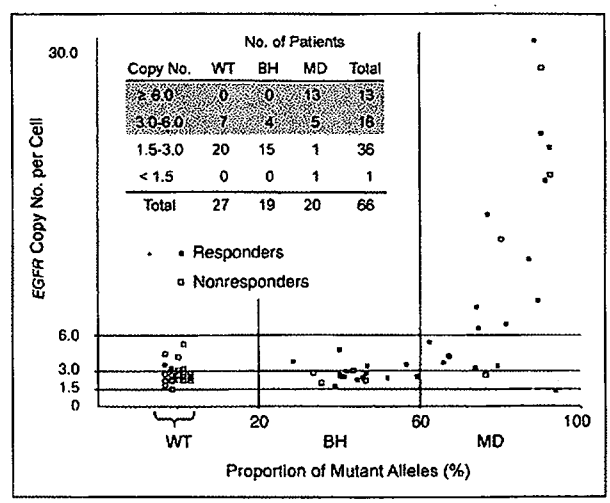

Fig 1. Relation between the epidermal growth factor receptor (*EGFR*) copy number and the proportion of mutant alleles. WT, patients with wild-type *EGFR*; BH, patients with a balanced heterozygous pattern of *EGFR* mutations; MD, patients with a mutant-allele-dominant pattern of *EGFR* mutations.

EGFR *Mutations,* EGFR *Copy Number, and Clinical Outcome*

The tumor responses to gefitinib according to the mutational status of *EGFR* are shown in Table 3. The response rates of patients with mutant and wild-type *EGFR* were 82% and 11%, respectively ($P < 10^{-7}$). Seven patients with *EGFR* mutations were nonresponders; three patients had PD at 0.3 (early death), 2.3, and 2.3 months, and four patients had SD. Three of the four patients with SD had MR (TTP, 2.5, 5.2, and 6.9 months), and the other patient continued to receive gefitinib therapy without progression for 24.2 months, whereas all SD tumors with wild-type *EGFR* progressed within 5 months without MR. Meanwhile, three patients with wild-type *EGFR* exhibited PR, and two of these patients were still receiving gefitinib therapy without progression at 10.9+ and 21.1+ months. The Kaplan-Meier plots of TTP and OS according to the presence of the *EGFR* mutations are shown in Figures 2 and 3, respectively. Patients with *EGFR* mutations had a significantly longer TTP and OS compared with those with wild-type *EGFR*.

Univariate analyses were performed to assess the correlations among patient characteristics, *EGFR* mutations, *EGFR* copy number, and clinical outcome (Tables 4 and 5). The response rates were significantly higher in women, never/former smokers, and patients with BAC features and were marginally higher in patients with a papillary-dominant subtype. The response rates among these subgroups were approximately consistent with the rates of *EGFR* mutations. An increased *EGFR* copy number was also significantly associated with a higher response rate and a longer TTP.

The results of multivariate analyses among 62 patients with adenocarcinoma are shown in Table 6. The presence of *EGFR* mutations was strongly associated with a higher response rate, a longer TTP, and a longer OS. An increased *EGFR* copy number was also a significant or marginally significant predictor of a higher response rate and a longer TTP. These results did not change substantially if any combinations of variables were included in the models.

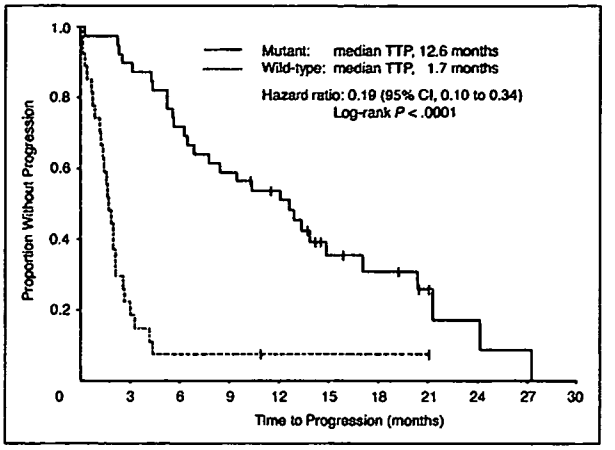

Fig 2. Kaplan-Meier plot of time to progression (TTP) according to epidermal growth factor receptor (*EGFR*) mutation status.

Among patients with wild-type *EGFR*, TTP was significantly longer in patients with increased *EGFR* copy numbers (median, 3.0 *v* 1.4 months; log-rank $P = .021$), and both of the two long-term responders had tumors with moderately increased *EGFR* copy numbers (3.20 and 3.45/cell). Among patients with *EGFR* mutations, TTP and OS were not significantly different according to the types of mutations, the presence of additional mutations, the proportion of mutant alleles, or the *EGFR* copy number (data not shown).

DISCUSSION

This study strongly implies that the mutational status of *EGFR* is a major determinant of gefitinib sensitivity in patients with NSCLC. The response rate was 82%, the median TTP was 12.6 months, and the MST was 20.4 months in gefitinib-treated patients with *EGFR*-mutant NSCLC. *EGFR* mutations might be a good prognostic factor independent of treatment, but these remarkable results suggest a

Table 3. *EGFR* Mutations and Tumor Response to Gefitinib

	Responders		Nonresponders			Responders/Total Patients	Response Rates (%)
	CR	PR	MR	SD	PD		
Mutant	2	30*	3	1	3†	32/39	82
DEL	0	18*	2	0	0	18/20	90
L858R	2	11	1	1	2†	13/17	76
G719	0	1	0	0	1	1/2	50
Wild-type	0	3	0	5	19	3/27	11
Total	2	33	3	6	22	35/66	53

Abbreviations: *EGFR*, epidermal growth factor receptor; CR, complete response; PR, partial response; MR, minor response; SD, stable disease without MR; PD, progressive disease; DEL, deletional mutations in exon 19; G719, G719S, or G719C.
*Including a clinical responder without measurable lesions.
†Including a patient who had no measurable lesions at baseline.

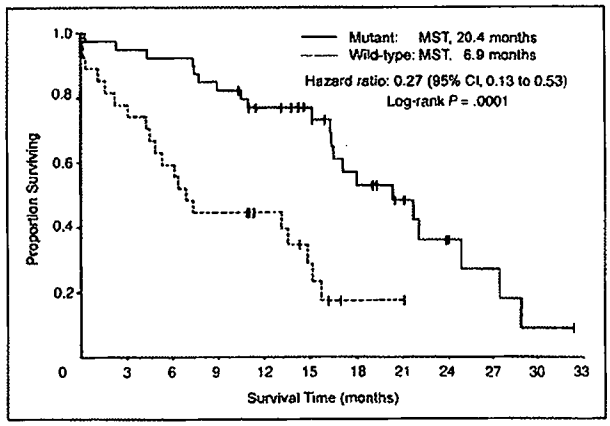

Fig 3. Kaplan-Meier plot of overall survival according to epidermal growth factor receptor (*EGFR*) mutation status. MST, median survival time.

survival benefit from gefitinib therapy in patients with *EGFR* mutations. Four of seven nonresponders with *EGFR* mutations also seemed to experience some clinical benefits because they had MR or a long SD (≥ 6 months). Among nine patients with SD, MR, or a long SD was observed only in patients with *EGFR* mutations. Although the sample size was too small to draw a firm conclusion, this finding suggests that *EGFR* mutations are also associated with clinical benefits in SD.

Table 4. *EGFR* Mutations Among Patient Subgroups

	EGFR Mutations		
	No. of Patients	%	*P*
Total	39/66	59	
Sex			.18
Female	18/26	69	
Male	21/40	53	
Smoking history			.003†
Never smokers	21/31	68	
Former smokers	10/12	83	
Current smokers	8/23	35	
Histologic diagnosis			—
Adenocarcinoma	38/62	61	
Squamous cell carcinoma	0/3	0	
Pleomorphic carcinoma	1/1	100	
Dominant subtype*			.059‡
Papillary	22/30	73	
Acinar	10/18	56	
BAC	5/9	56	
Solid	1/5	20	
BAC features*			.002
Yes	34/47	72	
No	4/15	27	

Abbreviations: *EGFR*, epidermal growth factor receptor; BAC, bronchioloalveolar carcinoma.
*Only patients with adenocarcinoma (n = 62).
†Comparison between never/former smokers and current smokers.
‡Comparison between patients with papillary-dominant adenocarcinoma and patients with other adenocarcinoma.

The *EGFR* mutations detected in this study were concentrated in three hotspots, deletions around codons 747 to 749, L858R, and G719S (or G719C), similar to the results of previous reports.[6-13] Some genetic variations existed among these mutations. Together with one of the hotspot mutations, additional missense mutations in exons 18 or 20 were detected in four patients. Among the 39 patients with *EGFR* mutations, an MD pattern was observed in 20 patients. Because the *EGFR* copy number in their tumor cells increased as the proportion of mutant alleles increased, this pattern was assumed to be caused not by homozygous mutations but by the selective amplification of the mutant alleles. Because one patient had a hemizygous mutation without amplification, the loss of wild-type alleles was also thought to be responsible for the pattern. The moderately increased copy number in patients with a BH pattern or wild-type *EGFR* can be explained by *EGFR* amplification and/or polysomy of chromosome 7.

Among the patients with *EGFR* mutations, three patients had PD and eight of the other 36 patients had tumor regrowth within 6 months. This suggests the presence of other factors associated with intrinsic or acquired resistance to gefitinib. Although any genetic alterations of *EGFR*-mutant tumors at the time of primary surgery were not significantly associated with clinical outcome, that might be because further alterations occurred after the primary surgery or after gefitinib administration. Recently, a secondary mutation (C → T at nucleotide 2369; T790M) in exon 20 was detected in patients with *EGFR*-mutant NSCLC who had tumor regrowth during gefitinib therapy after exhibiting an initial response to the agent; this mutation was thought to be associated with acquired resistance.[28,29] To elucidate the determinants and the mechanism of resistance to gefitinib, genetic analyses of tumor samples obtained after gefitinib treatment are needed.

In this study, three (11%) of the 27 patients with wild-type *EGFR* responded to gefitinib. Various explanations for this result are possible: (1) the mutational analyses of the responders were false-negative, (2) the *EGFR* mutations occurred in their tumors after the primary surgery, (3) the recurrent tumors originated from a source other than the analyzed tumor cells, or (4) other determinants of gefitinib sensitivity were present.

The results of multivariate analyses suggest that the *EGFR* copy number is another independent predictor of gefitinib sensitivity. It is noteworthy that an increased *EGFR* copy number was observed in two of the three responders with wild-type *EGFR*, and was significantly associated with a longer TTP among patients with wild-type *EGFR*. Because patients with *EGFR* mutations had favorable clinical outcome regardless of *EGFR* copy numbers, the impact of increased copy numbers on *EGFR*-mutant NSCLC was unclear. In the overall population, an increased *EGFR* copy number was significantly associated with a higher response

Table 5. Clinical Outcome Among Patient Subgroups (univariate analyses)

	Response Rate			Time to Progression		Overall Survival	
	No.	%	*P*	Median (months)	Log-Rank *P*	Median (months)	Log-Rank *P*
Total	66	53		5.2		16.3	
Sex			.033		.35		.30
Female	26	69		6.2		16.5	
Male	40	43		3.3		15.1	
Smoking history			.007		.026		.37
Never/former smokers	43	65		6.9		16.4	
Current smokers	23	30		2.6		15.1	
Dominant subtype*			.070		.28		.65
Papillary	30	67		7.7		16.4	
Others	32	44		4.2		15.7	
BAC features*			.012		.12		.19
Yes	47	64		6.5		16.5	
No	15	27		2.1		15.7	
Performance status			.77		.012		< .0001
0-1	50	52		5.2		17.1	
2-3	16	56		3.1		6.1	
EGFR mutations			< .0001		< .0001		.0001
Yes	39	82		12.6		20.4	
No	27	11		1.7		6.9	
EGFR copy number			.005		.038		.33
≥ 3.0	29	72		9.4		16.4	
< 3.0	37	38		2.6		15.7	

Abbreviation: BAC, bronchioloalveolar carcinoma; *EGFR*, epidermal growth factor receptor.
*Only patients with adenocarcinoma (n = 62).

rate and a longer TTP, but not with a longer OS, which might be because an increased copy number had an unfavorable impact on prognosis, as suggested by another study.[15] In chronic myeloid leukemia, as well as *BCR-ABL* mutations that were structurally corresponding to T790M in *EGFR*, an increased *BCR-ABL* gene copy number was reported as a determinant of resistance to imatinib, a BCR-ABL tyrosine kinase inhibitor.[30] Therefore, we should consider the possibility that an increased *EGFR* copy number is associated with not only sensitivity but also resistance to gefitinib.

Among adenocarcinomas, the presence of BAC features was significantly associated with gefitinib sensitivity and *EGFR* mutations, but the BAC component was relatively small in most of the responders. The dominant subtype associated with a higher response rate was not BAC but papillary; both of the two patients with BwFI had PD, and all three patients with pure papillary adenocarcinoma without BAC features had PR. The association between pathologic features and gefitinib sensitivity or *EGFR* mutations is also the subject of further investigation.

Table 6. Univariate and Multivariate Analyses of the Association Between Biomarkers and Clinical Outcome in Patients With Lung Adenocarcinoma (n = 62)

	Odds Ratios for Response		Hazard Ratios for TTP		Hazard Ratios for OS	
	Univariate	Multivariate*	Univariate	Multivariate*	Univariate	Multivariate*
EGFR mutations, yes *v* no	31.0	27.9	0.21	0.13	0.30	0.16
95% CI	7.2 to 134	3.7 to 209	0.11 to 0.38	0.06 to 0.29	0.15 to 0.62	0.06 to 0.39
P	< .001	.001	< .001	< .001	.001	< .001
EGFR copy number, ≥ 3.0 *v* < 3.0	4.0	4.6	0.57	0.42	0.80	0.59
95% CI	1.4 to 12	0.84 to 25	0.32 to 1.0	0.21 to 0.84	0.42 to 1.5	0.26 to 1.4
P	.011	.079	.050	.014	.49	.22

Abbreviations: TTP, time to progression; OS, overall survival; *EGFR*, epidermal growth factor receptor.
*In the multivariate analyses, age (continuous variable), sex (women *v* men), smoking history (never/former smokers *v* current smokers), dominant subtype (papillary *v* others), bronchioloalveolar carcinoma features (yes *v* no), performance status (0 to 1 *v* 2 to 3), prior chemotherapy (yes *v* no), *EGFR* mutations (yes *v* no), and *EGFR* copy number (≥ 3.0 *v* < 3.0) were included as factors.