

Analysis of *Epidermal Growth Factor Receptor* Gene Mutation in Patients with Non – Small Cell Lung Cancer and Acquired Resistance to Gefitinib

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Abstract Purpose: Non – small cell lung cancers carrying activating mutations in the gene for the epidermal growth factor receptor (EGFR) are highly sensitive to EGFR-specific tyrosine kinase inhibitors. However, most patients who initially respond subsequently experience disease progression while still on treatment. Part of this “acquired resistance” is attributable to a secondary mutation resulting in threonine to methionine at codon 790 (T790M) of EGFR.

Experimental Design: We sequenced exons 18 to 21 of the *EGFR* gene to look for secondary mutations in tumors with acquired resistance to gefitinib in 14 patients with adenocarcinomas. Subcloning or cycleave PCR was used in addition to normal sequencing to increase the sensitivity of the assay. We also looked for T790M in pretreatment samples from 52 patients who were treated with gefitinib. We also looked for secondary *KRAS* gene mutations because tumors with *KRAS* mutations are generally resistant to tyrosine kinase inhibitors.

Results: Seven of 14 tumors had a secondary T790M mutation. There were no other novel secondary mutations. We detected no T790M mutations in pretreatment specimens from available five tumors among these seven tumors. Patients with T790M tended to be women, never smokers, and carrying deletion mutations, but the T790M was not associated with the duration of gefitinib administration. None of the tumors had an acquired mutation in the *KRAS* gene.

Conclusions: A secondary T790M mutation of *EGFR* accounted for half the tumors with acquired resistance to gefitinib in Japanese patients. Other drug-resistant secondary mutations are uncommon in the *EGFR* gene.

Activating mutations in the gene for the epidermal growth factor receptor (EGFR) are present in a subset of pulmonary adenocarcinomas. Tumors with *EGFR* mutations are highly sensitive to gefitinib and erlotinib, small-molecule EGFR-specific tyrosine kinase inhibitors (1–3). These mutations occur in the tyrosine kinase domain of the *EGFR* gene. Deletion mutations in exon 19 and the substitution of leucine with arginine at codon 858 (L858R) account for ~90% of all these mutations (4). *EGFR* mutations are more prevalent in women,

never smokers, patients of Asian ethnicity, and those with adenocarcinoma histology (4). These features are the same as those of patients whose tumors have elevated sensitivity to EGFR-specific tyrosine kinase inhibitors. The response rates of lung cancers with an *EGFR* mutation are as high as 80% (5). Responses are often dramatic, and several reports have shown that patients with *EGFR* mutations survive significantly longer after gefitinib treatment than patients without mutations (6). However, it is also common for patients to show disease progression after presenting with an initial marked response to EGFR-specific tyrosine kinase inhibitors. The mean duration of the initial response is about 3 to 7 months (7, 8).

Recently, it has been reported by two groups that a secondary threonine-to-methionine mutation at codon 790 (T790M) of the *EGFR* gene is related to the acquired resistance to gefitinib and erlotinib (9, 10). Crystal structure modeling has shown that residue T790 is located in the ATP-binding pocket of the catalytic region of EGFR, and it seems to be critical for the binding of erlotinib and gefitinib (9). Substitution of the threonine at codon 790 with a bulkier residue, such as methionine, would result in steric hindrance to the binding of these two drugs. A secondary T790M mutation has been identified in one tumor (9) and in three of six tumors (10) with acquired resistance to gefitinib.

Imatinib is a tyrosine kinase inhibitor specific for BCR-ABL, KIT, and platelet-derived growth factor A, which is used to treat

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chronic myelogenous leukemia (CML) and gastrointestinal stromal tumor. Analogous secondary mutations in the kinase domains of these genes are considered to constitute one of the mechanisms of acquired drug resistance (11–14). The structural similarity between ABL and EGFR tyrosine kinases is fairly high, and the most common mutation related to acquired resistance is a threonine-to-isoleucine mutation at codon 315 (T315I), corresponding to T790M in the EGFR gene (15). In CML, 20 to 30 other mutations of the ABL gene have been identified as responsible for acquired resistance to imatinib (12, 16–19), so secondary EGFR gene mutations other than T790M are possible (Fig. 1).

Secondary mutations of the ABL gene have also been detected in pretreatment samples from some CML patients, although the fraction of mutant cells was very low (16, 20). The existence of a similar mechanism is expected for non-small cell lung cancer. Furthermore, we and others have reported that the T790M mutation of the EGFR gene exists as a major mutation independently of gefitinib treatment, although instances are very rare (21, 22).

It has also been reported that KRAS mutations are associated with a lack of sensitivity to gefitinib and erlotinib (23, 24). Therefore, it is possible that acquired KRAS mutations are also associated with acquired resistance.

In this study, we looked for the T790M mutation and other secondary mutations of the EGFR gene in tumors from patients who showed disease progression after presenting with an initial response to EGFR-specific tyrosine kinase inhibitor treatment and in tumors before gefitinib treatment. We also looked for KRAS mutations in the same tumors.

Materials and Methods

Patients. Patients with non-small cell lung cancer who initially responded but subsequently experienced disease progression while on gefitinib treatment were defined as having “acquired resistance.” A detailed definition of the effectiveness of gefitinib treatment was described in our previous study (25). Briefly, gefitinib treatment is judged to be effective when tumors show a decrease of at least a 30% in tumor diameter in imaging studies or when elevated carcinoembryonic antigen levels decrease to a level less than half the baseline level.

Fourteen tumor samples and 10 corresponding pretreatment tumor samples from eligible patients were obtained according to this definition at the time of diagnosis or treatment. The selection of patients depended only on whether a second tumor sample collected at the time of progression could be obtained. Appropriate approval from the institutional review board and the patients’ written informed consent were obtained. Patient characteristics and details of the samples are shown in Table 1. All patients had adenocarcinomas, and the median duration of gefitinib treatment was 367 days (range, 69–921 days). We also analyzed the samples of 52 patients who had been treated with gefitinib for recurrent disease after they had undergone pulmonary resection. This cohort was part of our previous study, and their clinical details are described elsewhere (25).

Subcloning mutational analysis of the EGFR gene. Genomic DNA and total RNA (if possible) were extracted from each sample (Table 1). Exons 18 to 21 of the EGFR tyrosine kinase domain were amplified using PCR or reverse transcription-PCR (RT-PCR) methods. PCR for genomic DNA was done using AmpliTaq Gold (Applied Biosystems, Foster City, CA) and the following primers: exon 18, 5'-GAGGTGACCC-TTGCTCTGTGT-3' (forward) and 5'-CCCAACACTCAGTGAACAAA-3' (reverse); exon 19, 5'-TGCCAGTTAACGTCCTCTCT-3' (forward) and 5'-ATGTTGAGATGAGCAGGGTCTA-3' (reverse); exon 20, 5'-TGAACCTC-AAGATCGCATTTCAT-3' (forward) and 5'-CATGGCAAACCTCTGCTATCC-3'

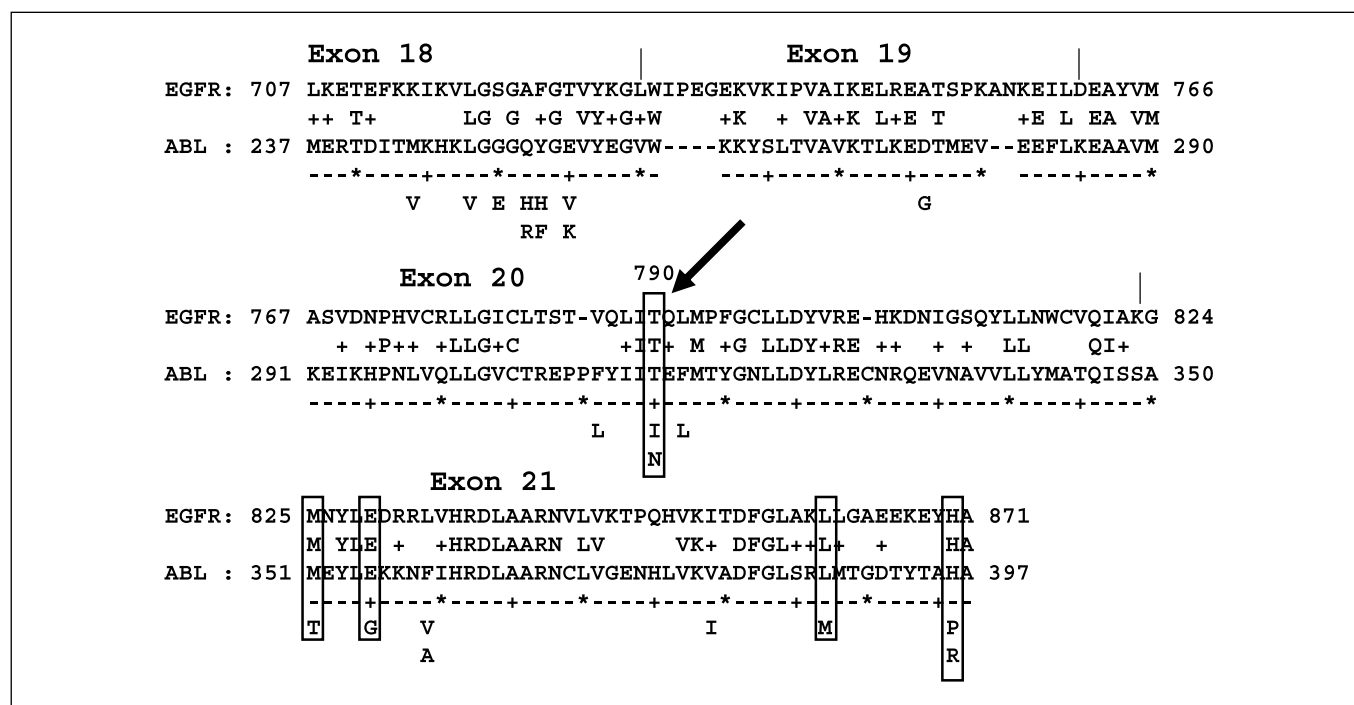


Fig. 1. Structural similarity between EGFR tyrosine kinase and ABL. This amino acid alignment was obtained using basic local alignment search tool, and both sequences were obtained from Genbank (accession nos.: EGFR, NM 005228; ABL, NM 005157). Top line, EGFR; bottom line, ABL. Vertical lines, boundaries between exons. Numbers at each end, codon numbers. Capital letters under the alignment, amino acid changes in ABL that have been reported as acquired imatinib resistance mutations. Square frames, qualifying codons as common codons in EGFR and ABL and as acquired resistance mutant codons in ABL. Arrow, location of codon 790 of EGFR and codon 315 of ABL.

Table 1. Patient characteristics and results of sequencing analysis

Patient no.	Sex	Smoking status	Prior treatment	Gefitinib response	Gefitinib treatment days	Analyzed specimen (state)	Nucleic acid	Activating mutation	T790M mutation	T790M (pre-gefitinib samples)
1	F	NS	S	E	642	LN (Fr)	RNA	Δ2	+	—
2	M	FS	S	E	368	PE (Al)	RNA	Δ3	—	—
3	M	NS	S	E	116	PE (Al)	RNA	Δ1	—	—
4	F	FS	CT	E	599	PE (CL)	RNA	Δ1	—	NA
5	F	NS	CRT	E	921	LU (Al)	RNA	Δ1	+	NA
6	F	NS	None	E	181	PE (Al)	RNA	Δ1	+	—
7	F	FS	CT	E	346	BO (Al)	RNA	Δ1	+	—
8	F	NS	S→CRT	E	623	LN (Al)	RNA	L858R	—	NA
9	M	FS	S	E	915	BR (Fr)	DNA	L858R*	—	—
10	M	FS	S→CRT	NE	69	PE (Al)	DNA	L858R	—	—
11	F	FS	None	E	560	LU (Fr)	RNA	L858R*	+	NA
12	F	NS	CT	E	239	PE (Al)	RNA	Δ1	+	—
13	F	NS	S	E	367	PE (Al)	RNA	L858R	—	—
14	F	NS	CRT	E	235	LN (Al)	RNA	Δ1	+	—

NOTE: Patients 1, 4, and 13 received gefitinib therapy twice. Pretreatment samples from patients 4, 5, 8, and 11 were not available. Patient 10 was defined as not evaluable according to our definition. However, this patient showed a 46% decrease in carcinoembryonic antigen and a marked reduction in pleural effusion on initial treatment before subsequent progression. Therefore, we regarded this case as eligible for this study.

Abbreviations: Al, alcohol fixed; BO, bone metastasis; BR, brain metastasis; CL, cell line; CRT, chemoradiotherapy; CT, chemotherapy; del, deletion; E, effective; F, female; Fr, frozen; FS, former smoker; ins, insertion; LN, lymph node; LU, lung tumor; M, male; NA, not available; NE, not evaluable; NS, never smoker; PE, pleural effusion; RT, radiotherapy; S, surgery; Δ1, del E746-A750; Δ2, del L747-P753 insS; Δ3, del L747-A750 insP.

*Patients 9 and 11 had another point mutation (L833V in patient 9 and R776H in patient 11).

(reverse); and exon 21, 5'-GAGCTTCTTCCCATGATGATCT-3' (forward) and 5'-GAAAATGCTGGCTGACCTAAAG-3' (reverse). The PCR conditions were as follows: 1 cycle of 95°C for 11 minutes, 45 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds followed by 1 cycle of 72°C for 4 minutes.

RT-PCR for RNA was done with primers 5'-AGCTTGTGGAGCCTCT-TACACC-3' (forward 1) and 5'-TAAATTTGATTCCAATGCCATCC-3' (reverse 1) in a one-step RT-PCR setup using Qiagen OneStep RT-PCR kits (Qiagen, Valencia, CA) as described previously (26). RT-PCR conditions were as follows: 1 cycle of 50°C for 30 minutes and 95°C for 15 minutes, 40 cycles of 94°C for 50 seconds, 62°C for 50 seconds, and 72°C for 1 minute followed by 1 cycle of 72°C for 10 minutes.

The PCR products were subcloned using TOPO TA Cloning kits (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each clone was then directly amplified with the same primers using AmpliTaq Gold and cycle sequenced using BigDye Terminator v3.1/1.1 cycle sequencing kits (Applied Biosystems). Subcloning PCR conditions were as follows: 1 cycle of 95°C for 11 minutes, 45 cycles of 95°C for 50 seconds, 62°C for 50 seconds, and 72°C for 70 seconds followed by 1 cycle of 72°C for 4 minutes.

The sequencing reaction products were electrophoresed using an ABI PRISM 3100 system (Applied Biosystems). Both forward and reverse sequences were analyzed with basic local alignment search tool, and the chromatograms were analyzed by manual review.

Cycleave real-time PCR assay. Details of the cycleave real-time PCR assay have been described previously (27). Briefly, genomic DNA was extracted, and exon 20 of the *EGFR* gene was amplified by real-time quantitative PCR assay on a SmartCycler (TaKaRa, Gifu, Japan) using Cycleave PCR Core kits (TaKaRa) with a T790M-specific cycling probe and a wild-type cycling probe. As few as ~5% of tumor cell molecules could be detected in this assay.

Mutational analysis of the *KRAS* gene. A RT-PCR direct sequence assay was done for RNA, and a cycleave real-time PCR assay was done for DNA. *KRAS* primers for PCR were 5'-GGCCTGCTGAAAATGACTGA-3' (forward 1) and 5'-TCTTGCTAAGTCTGAGCCTGTT-3' (reverse 3).

Codon 12 cycling probes and a wild-type cycling probe were used in cycleave real-time PCR assays. Direct sequencing was used to identify codon 12, 13, and 61 mutations.

Results

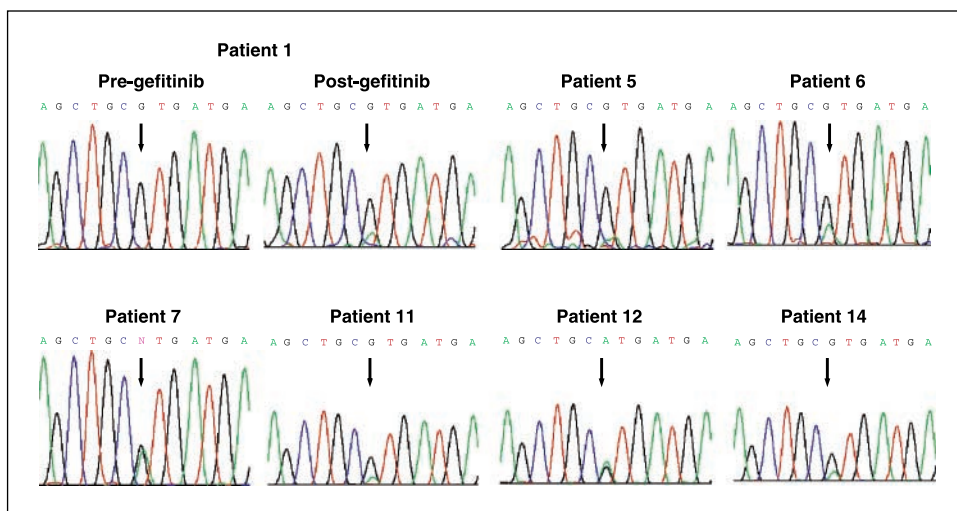
Detection of secondary mutations in the *EGFR* gene or the *KRAS* gene. For the analysis of secondary mutations, we first amplified exons 18 to 21 of the *EGFR* gene, which include the region homologous to the region of the *ABL* gene that contains all the secondary mutations thus far reported to be responsible for imatinib resistance in CML. All 14 tumors with acquired resistance had activating mutations of the *EGFR* gene, either deletion mutations, including codons 746 to 750 (nine patients), or L858R (five patients). Seven tumors had a secondary T790M mutation (Table 1; Fig. 2).

When we sequenced corresponding tumor samples that had been obtained before gefitinib treatment, the same activating mutations were always present, whereas T790M was not detected in any of the available pretreatment samples (samples for patients 4, 5, 8, and 11 were not available).

Mutant bands for T790M in the sample from patient 7 were as strong as the wild-type bands, and the mutant bands were stronger than the wild-type bands in patient 12 (Fig. 2). However, in most cases, the T790M mutant bands were weaker than the wild-type bands.

Two tumors had another point mutation as well as L858R (L833V in patient 9 and R776H in patient 11). L833 corresponds to F359 of *ABL*, where a secondary mutation to valine or alanine has been reported in CML (Fig. 1; ref. 12). However, the pretreatment sample of patient 9 revealed that L833V existed before treatment in the same ratio as the L858R band. The ratios of L833V and L858R bands were unchanged

Fig. 2. Sequencing chromatograms for *EGFR* exon 20. Secondary T790M mutations were observed in seven patients. Antisense strands of each chromatogram. Arrows, small peaks of the C→T substitution at nucleotide 2,369 (G→A on the antisense strand), which results in the T790M mutation. This substitution was observed only in posttreatment samples. T790M mutant bands were clearly detected on sequencing chromatograms, except in that of patient 5; in this patient, it was unclear because of artifacts.



before and after gefitinib treatment. Although the T790M mutant band was weaker than the L858R mutant band in patient 11, the intensity of the R776H mutant band was the same as that of the L858R mutant band and both mutations were heterozygous. We considered these point mutations to be primary mutations and not associated with “acquired” resistance.

To increase the sensitivity for the detection of T790M and other possible secondary mutations in the tyrosine kinase domain, each PCR product was subcloned and multiple subclones were amplified and sequenced directly. All the T790M mutations found by sequencing the noncloned PCR products were confirmed by this subcloning method, but no new T790M mutations were detected even when >50 clones were analyzed in samples from patients 2 and 3 (Table 2). Furthermore, we detected no secondary mutations in exons 18 to 21 other than T790M.

The T790M mutations were either present in clones with activating (or sensitizing) mutations or in other clones without activating mutations (Table 2). In three tumors (of patients 1, 5, and 14), T790M was present only in clones with activating mutations, whereas in the remaining four tumors (patients 6,

7, 11, and 12), T790M was present in both clones with and without activating mutations. No tumor carried the T790M mutation only in the wild-type clones. However, four of five T790M mutations were in clones without activating mutations in the tumor of patient 6.

We also looked for mutations in codon 12 (and codons 13 and 61 in RNA samples) in the *KRAS* gene. However, none of the samples from the tumors studied had *KRAS* mutations.

Relationship between T790M mutation and clinical and genetic features. T790M mutations were more frequent in women (women, 7 of 10; men, 0 of 4), who had never smoked (never smoker, 5 of 8; previous smoker, 2 of 6), and with deletion mutations (deletion, 6 of 9; L858R, 1 of 5). There was no difference in the incidence of T790M in the presence or absence of prior chemotherapy (with, 4 of 8; without, 3 of 6; Table 1).

We also compared the duration of gefitinib treatment, which is considered to correlate roughly with the time to progression, with the presence or absence of T790M. However, the median treatment times were almost identical (tumors with T790M, 346 days; tumors without T790M, 368 days; Fig. 3).

Analysis of corresponding tumor tissues before gefitinib treatment in patient 1. To determine whether rare T790M

Table 2. Analysis of acquired mutation using the subcloning method

Patient no.	Activating mutation	Total clones	Activating mutant clones		Wild-type clones	
			With T790M	Without T790M	With T790M	Without T790M
1	Δ2	21	8	10	0	3
2	Δ3	54	0	52	0	2
3	Δ1	51	0	50	0	1
4	Δ1	21	0	13	0	8
5	Δ1	51	3	39	0	9
6	Δ1	47	1	17	4	25
7	Δ1	20	4	5	1	10
8	L858R	18	0	14	0	4
9	L858R	20	0	14	0	6
10	L858R	20	0	5	0	15
11	L858R	21	5	10	1	5
12	Δ1	23	11	9	1	2
13	L858R	21	0	8	0	13
14	Δ1	19	7	8	0	4

mutant clones existed before gefitinib treatment, we analyzed the corresponding tumor tissues of patient 1, whose tissue after gefitinib treatment had a secondary T790M mutation. Tumor tissue was obtained at the time of operation. PCR products from the tumor before gefitinib treatment were subcloned, and 103 subclones were amplified and sequenced directly. However, at this sensitivity, we detected no clone carrying the T790M mutation. Among 103 clones, 92 (89%) had activating deletion mutations, suggesting that the mutant allele was amplified before gefitinib treatment. The incidence of clones with deletional mutations was similar (18 of 21, 85%) in a cervical lymph node taken after gefitinib resistance had developed.

To further explore of possible association of T790M with metastatic spread, we looked for the T790M mutation in hilar and mediastinal lymph nodes with metastases dissected at the time of surgery. Genomic DNA was extracted from lymph nodes from four stations (aortopulmonary, ascending aorta, main bronchus, and intrapulmonary) and analyzed using cycleave real-time PCR. However, we detected no T790M mutations.

Analysis of tumors for T790M before gefitinib treatment in 52 patients who were treated with gefitinib. The possible presence of T790M at a low frequency in tumors before gefitinib treatment might affect the tumor response or the time to progression after gefitinib treatment. In a previous study, we sequenced exons 18 to 23 of the *EGFR* genes of 52 patients who had been treated with gefitinib for recurrent disease after they had undergone pulmonary resection. None of them had the T790M mutation. Here, we used a cycleave real-time PCR assay, which is more accurate analysis than normal sequence, to investigate whether rare T790M mutant cells were present. However, we detected no T790M mutations in these 52 tumors.

Discussion

We studied 14 tumors with acquired resistance to gefitinib for secondary mutations occurring in the *EGFR* tyrosine kinase domain. Seven of the 14 tumors had a secondary T790M mutation, an incidence consistent with those of previous studies (9, 10). Whereas clones with activating mutations (deletion or L858R) might well have been eliminated by selection pressure during gefitinib treatment, those clones were always present in tumors that developed acquired resistance. In most cases, clones with the T790M mutation were not predominant.

The T790M mutations occur more frequently in women who had never smoked and who had a deletion-type mutation. Time to progression did not differ between tumors that acquired secondary T790M mutations and those that did not. However, these tendencies require careful interpretation because of the number of samples was small.

In a previous report, Kobayashi et al. (9) showed that the T790M mutation was observed with either wild-type or deletion mutation sequences, whereas Pao et al. (10) showed that both the T790M and L858R mutations were in the same allele. Our data showed that three samples had the T790M mutation only in the clones with activating mutation and four samples had the T790M mutation in the clones with and without activating mutation, whereas the most of T790M mutation was in the clones with activating mutation, except for

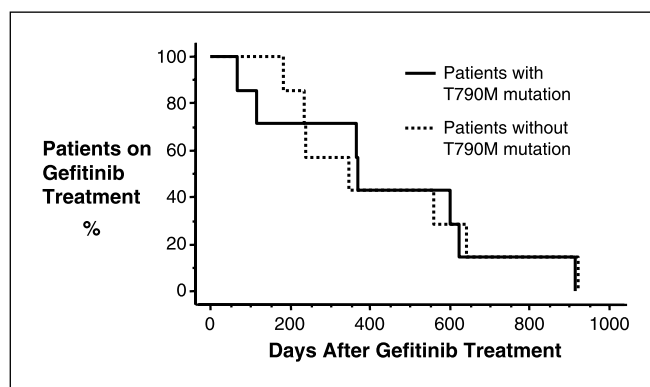


Fig. 3. Effect of the T790M mutation on the length of gefitinib treatment. The length of gefitinib treatment was considered to be roughly related to time to progression. Median treatment times were almost identical in both the presence and absence of the T790M mutation.

the samples of patient 6. It is possible that this could result from a PCR error or DNA repair error at the subcloning step. Bell et al. (28) have reported that artifactual PCR-generated allelic separation occurred with probability of $\sim 30\%$ in their analysis. However, it is also possible that the T790M mutation occurs in both alleles or that tumor heterogeneity exists.

In CML, 20 to 30 mutations in the *ABL* gene are responsible for acquired resistance to imatinib. Many types of mutations have been detected, and there are four distinguishable clusters (P-loop, T315, M351, and A-loop; ref. 29). Furthermore, secondary mutations in the *ABL* kinase domain are found in 50% to 90% of patients (29), many more than in patients with non-small cell lung cancer. We detected no novel mutations in the *EGFR* gene other than T790M. Two tumors had another point mutations together with L858R, L833V, or R776H. We considered these point mutations to be primary mutations and not associated with acquired resistance. However, these conclusions were based only on sequencing and subcloning methods, and we have no evidence of the functional effects of these mutations. There may be differences in the mechanisms of acquired resistance between non-small cell lung cancer and CML.

We previously reported that, in a series of 397 unselected patients with non-small cell lung cancer who had undergone surgery, 2 female patients with no history of smoking had L858R plus T790M mutations (21). Because these patients were not treated with gefitinib, T790M might well have conferred a growth advantage. These tumors were aggressive and later developed recurrent disease. One was treated with gefitinib but was refractory to treatment. A similar case was reported by another group (22). Inspired by this observation and because the secondary mutations related to imatinib resistance in CML were detected at low frequencies (0.01-0.9%) in pretreatment samples (16, 20), we attempted to detect minor clones with the T790M mutation in samples before gefitinib treatment. However, we could not detect the T790M mutation by assays that can detect mutant cells if there is about 1% to 5% at least. It remains unclear whether a more sensitive method would have detected rare clones with the T790M mutation in our samples.

Why tumors with T790M mutant cells acquire resistance to gefitinib despite the fact that mutant band for the T790M

mutation was almost always weaker than wild-type band remains unclear. It is possible that cells with the T790M mutation preexist at a very low frequency and gradually increase during gefitinib treatment by clonal selection as in cases of CML (16). It is also possible that amplification of the activating mutant allele occurs in resistant tumors and parts of them have the T790M mutation. Another possibility is that multiple coexisting mechanisms, including the T790M mutation, cause acquired resistance cooperatively or independently. A recent study suggested that increased internalization of ligand-bound EGFR is one of the mechanisms underlying acquired gefitinib resistance (30). It is also likely that *EGFR* gene amplification (31) by alteration of downstream molecules, such as AKT (32), might play a role in the acquisition of resistance to gefitinib.

Mutations in *KRAS* are associated with a lack of sensitivity to gefitinib and erlotinib (23). We looked for *KRAS* mutations because of the possibility that acquired *KRAS* mutations are associated with acquired resistance. There were no *KRAS* mutations in any tumor. The same finding has been reported in a previous study (10), suggesting that *KRAS* mutations are not associated with acquired resistance.

In conclusion, half of tumors with acquired resistance to gefitinib had secondary T790M mutations. No novel mutations in the *EGFR* gene were present in contrast to CML.

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