Presence of *Epidermal Growth Factor Receptor* Gene T790M Mutation as a Minor Clone in Non–Small Cell Lung Cancer

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Abstract

The threonine-to-methionine substitution at amino acid position 790 (T790M) of the *epidermal growth factor receptor* (*EGFR*) gene has been reported in progressing lesions after gefitinib treatment in non–small cell lung cancer (NSCLC) that causes sensitive tumors to become resistant to gefitinib. Alternatively, the *EGFR* T790M mutation might be present in small fractions of tumor cells before drug treatment, and the tumor cells harboring the T790M mutation might be enriched during the proliferation after drug treatment. We developed a mutant-enriched PCR assay to detect small fractions of cells with T790M mutation and used this technique to detect mutations in 280 NSCLCs, including gefitinib-treated 95 cases. Although the direct sequencing detected only 1 T790M mutant case, the mutant-enriched PCR (confirmed to enrich one mutant out of 1×10^3 wild-type alleles) detected 9 additional cases among 280 cases. As linkage to clinicopathologic factors, the T790M mutation showed no bias for sex, smoking status, or histology but was significantly more frequent in advanced tumors (9 of 111 cases) than in early-stage tumors (1 of 169 cases; $P = 0.0013$). Among gefitinib-treated cases, gefitinib-sensitive mutations were found in 30 cases. The T790M mutation was present in 3 of 7 no-responders with the gefitinib-sensitive mutation and was not present in 19 responders ($P = 0.014$). Our results indicate that the T790M mutation is sometimes present in a minor population of tumor cells during the development of NSCLC and suggest that the detection of small fractions of T790M mutant alleles may be useful for predicting gefitinib resistance of NSCLCs with sensitive *EGFR* mutations. (Cancer Res 2006; 66(16): 7854-8)

Introduction

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is highly expressed in epithelial tumors, including lung cancers (1), and its intracellular signaling cascade leads to a multitude of effects, including cell proliferation, cell differentiation, angiogenesis, metastasis, and antiapoptosis (2). *EGFR* mutations, predominantly in exons 19 and 21, have been found in non–small cell lung cancer (NSCLC) and have been reported to be related to the responsiveness of tumors to gefitinib (3–5), although some controversial findings have been reported (6, 7). In addition, recent studies have reported the appearance of a threonine-to-methionine substitution at amino acid position 790 (T790M) in *EGFR* in tumors that acquire resistance to gefitinib after gefitinib treatment (8, 9). Previous reports on T790M showed that this amino acid substitution was due to a C-to-T base pair change at the second letter of codon790 of *EGFR* (8–11). The introduction of vectors carrying T790M to cells also caused gefitinib resistance, indicating that T790M causes sensitive tumors to become resistant to EGFR tyrosine kinase inhibitors (EGFR-TKI; refs. 8, 9). In these reports, the T790M mutation was not found in tumors before treatment but in progressing lesions. Our results also suggested that *de novo* T790M mutation was rarely detected in NSCLC by direct sequencing (2 of 397 cases) that can only detect the mutation being present in a relativity major clone of tumors. However, the T790M mutation might exist in a small fraction of tumor cells before drug treatment, and the tumor cells harboring this mutation might be enriched over time during treatment with gefitinib or erlotinib (9).

To detect a small fraction of mutant alleles among a large amount of wild-type alleles in clinical samples, a sensitive assay, rather than direct DNA sequencing, is essential. Mutant-enriched PCR is a highly specific and sensitive technique that can detect one copy of mutant allele among as many as 10^3 to 10^4 copies of the wild-type alleles (12). The use of this assay has been proven using various kinds of clinical specimens, including biopsy and pleural fluid specimens (13), and we recently applied this assay to detect the *EGFR* exon 19 deletions and the L858R mutation. Thus, the establishment of a mutant-enriched PCR assay to detect a small fraction of T790M mutant alleles would be useful for detecting the presence of the T790M mutation in tumors as a minor clone. Furthermore, the effect on gefitinib responsiveness caused by a minor fraction with the T790M mutation might be of clinical importance.

In this study, we examined the presence of the *EGFR* T790M mutation in NSCLC using highly sensitive assay and determined the presence of any correlations between the mutation status and clinicopathologic factors, including the gefitinib effect on treated cases.

Materials and Methods

Construction of plasmids containing *EGFR* exon 20 alleles. pCR2.1-TOPO plasmids containing *EGFR* exon 20 wild-type or exon 20 T790M mutant-type, derived from human primary lung cancer and nonmalignant lung tissue, were used to validate the sensitivity of mutant-enriched PCR assay for the T790M *EGFR* mutation. The sequences of each plasmid were confirmed by sequencing.

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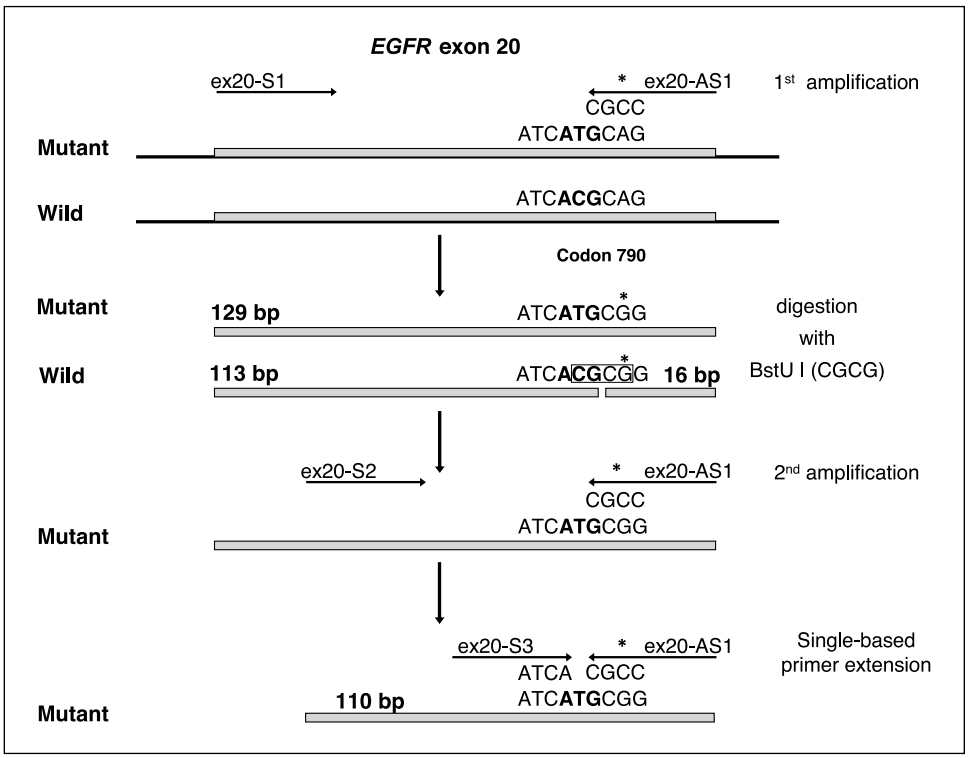

Figure 1. The principle of the mutant-enriched PCR assay for the *EGFR* T790M mutation. First-round PCR was done using ex20-S1 and ex20-AS1 primers. To enrich PCR amplicons containing T790M mutation, the first PCR product was digested with *Bst*UI resulting in digestion of only wild-type. The second PCR amplification was done with ex20-S2 and ex20-AS1 primers, and the amplified product was analyzed by a single-base primer extension reaction. *Asterisks,* mismatched nucleotide (T to C) in the primer.

Mutant-enriched PCR analysis for T790M of *EGFR* mutation in exon 20. A mutant-enriched PCR assay is a two-step PCR with intermittent restriction digestion to eliminate wild-type genes selectively, thus enriching the mutated genes (12). The sequences of the primers for PCR are as follows: ex20-S1, 5′-ACTGACGTGCCTCTCCCTCC-3′ (forward); ex20-S2, 5′-CCTCCAGGAAGCCTACGTGA-3′ (forward); and ex20-AS1, 5′-CGAAGGG-CATGAGCCGC-3′ (reverse). The ex20-AS1 primer harbors one mismatched site (T to C) to introduce a new CGCG sequence for PCR amplification of wild-type alleles. The restriction enzyme *Bst*UI was used to digest the CGCG sequence in the amplicon of the wild-type. In contrast, T790M mutant alleles were not digested because of the base substitution of C-to-T nucleotide at first base of CGCG, resulting in the enrichment of mutant alleles. In this assay, the first round of amplification was done for 30 cycles (20 seconds at 94°C, 30 seconds at 60°C, 20 seconds at 72°C) using 10 to 50 ng of sample DNA, 150 μmol/L deoxynucleotide triphosphate, 2 pmol of each primer, and 0.25 unit of HotStarTaq DNA polymerase (Qiagen, Inc., Valencia, CA) using ex20-S1 and ex20-AS1 primers. After digestion using *Bst*UI, the second-round PCR was done using ex20-S2 and ex20-AS1 primers (Fig. 1). The nucleotide of second letter of *EGFR* codon 790 was examined by single-base primer extension reaction and by direct sequencing to confirm results independently.

Single-base primer extension reaction. A single-base primer extension reaction was done using an ABI Prism SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA). The reaction mix contained 3 μL of second-round PCR product (as the template), 5 μL of SNaPshot Ready Reaction Premix, and the following primers: ex20-S3 (5′-TCCACCGTGCAGCT-CATCA-3′) or ex20-AS1 (Fig. 1). The products were separated and detected by capillary electrophoresis on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). The data were analyzed using GeneMapper Software version 3.7 (Applied Biosystems).

Clinical samples, DNA extraction, and *EGFR* and *KRAS* sequencing. We examined 280 specimens from clinical samples of NSCLC patients. The patient characteristics are shown in Tables 1 and 2. Two-hundred and eighty specimens consisted of 233 cases obtained by surgical resection, 26 cases of lung biopsies, 1 of lymph node biopsy, and 20 of pleural fluid from patients. Ninety-five cases were treated with gefitinib, and their response to drug was recorded.[5] The permission of the Institutional Review Board and informed consent from each patient were obtained. Clinicopathologic staging was determined according to International Union Against Cancer tumor-node-metastasis classification of malignant tumors (14). Tumor response was assessed as complete response, partial response, no change, or progressive disease according to the WHO criteria (15). DNAs of clinical samples were obtained from frozen specimens, paraffin-embedded specimens, and pleural fluid as mentioned in our previous report (13). As nonmalignant samples, 53 peripheral lung tissue specimen corresponding tumors, including 3 cases with the T790M mutation, were obtained. The concentration of DNA was quantified using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). *EGFR* mutations in exons 18 to 21 and *KRAS* mutations in exon 2 were examined using direct sequencing as described previously (16). DNA sequencing was done using Applied Biosystems Prism dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA) with ABI Prism 3100 Genetic Analyzer (Applied Biosystems) in Central Research Laboratory, Okayama University Medical School (Okayama, Japan).

Statistical analyses. The differences of significance among categorized groups were compared using Fisher's exact test. The statistical test was two sided, and the probability value <0.05 was defined as being statistically significant.

Results

Sensitivity of a mutant-enriched PCR for *EGFR* T790M mutation. To evaluate the sensitivity of the mutant-enriched PCR assay, we used serially diluted plasmid DNAs containing *EGFR* mutant and wild-type alleles. The mutant-enriched PCR assay for the *EGFR* T790M mutation was able to detect one copy of mutant allele in the presence of 1×10^3 copies of wild-type alleles (data not shown).

[5] S. Ichihara et al., submitted for publication.

Table 1. Patient characteristics

Variables	Subset	*n* (%)
Age (range, 28-91)	<68	145 (52)
	>69	135 (48)
Sex	Male	191 (68)
	Female	89 (32)
Smoking history	Never	95 (34)
	Ever	185 (66)
Disease stage	I	137 (49)
	II	32 (11)
	III	51 (18)
	IV	52 (19)
	Recurrent disease	8 (3)
Histology	Adenocarcinoma	205 (73)
	Squamous cell carcinoma	59 (21)
	Others	16 (6)
Gefitinib treatment	On	95 (34)
	Off	185 (66)

Direct sequencing and mutant-enriched PCR assay for T790M mutation. *EGFR* mutations were found at exon 18 in 7 cases (1 of V715A, 4 of G719A, 1 of G719S, and 1 of G721D), at exon 19 in 45 cases (44 cases of in-frame deletion and 1 D761Y mutation), at exon 20 in 3 cases (1 T790M mutation and 2 insertions), and at exon 21 in 43 cases (L858R mutation) out of a total 280 cases of NSCLCs that were examined by direct sequencing. These mutations included three cases that harbored double mutation: T790M plus L858R, D761Y plus L858R, and exon 20 insertion plus L858R. *KRAS* mutations were found in 19 cases. This mutational spectrum was consistent with previous reports, suggesting the absence of a selection bias in the present cohort.

We examined the presence of the *EGFR* T790M mutation in the same 280 specimens using the mutant-enriched PCR assay. The representative examples of the mutant-enriched PCR assay followed by single-base primer extension reaction and direct sequencing are shown in Fig. 2. The T790M mutation was detected in only 1 (0.36%) of 280 cases by direct sequencing as we previously reported (10). On the other hand, the mutant-enriched PCR assay detected 9 (3.2%) additional cases with T790M mutations among the 280 cases (Table 2). No mutations were found in 53 corresponding nonmalignant peripheral lung tissues, including nonmalignant lung tissues from the patients with three cases of T790M-positive tumors. We confirmed our results by doing the mutant-enriched PCR assay in triplicate. All the mutant cases showed a C-to-T change despite the possibility of another nucleotide change if a random PCR error had occurred. These findings strongly suggest that the mutant-enriched PCR assay was highly specific.

The frequency and state of *EGFR* T790M mutation and clinicopathologic factors. The clinicopathologic factors of T790M mutant cases are shown in Table 2. T790M mutations were found in four cases that also harbored an exon 19 deletion or an L858R mutation, in two cases with *KRAS* mutations, and in four cases without any *EGFR* or *KRAS* mutation at examined exons. In addition, we examined the presence of *EGFR* exon 19 deletions and L858R mutation as a minor clone in six *EGFR* wild-type cases by the mutant-enriched PCR for these alterations (13) and found no mutation. As sex or smoking status, there were no significant differences for the rate of T790M mutation in sex [8 (4.2%) of 191 men and 2 (2.2%) of 89 women] or in smoking status [8 (4.3%) of 185 ever smoker and 2 (2.1%) of 95 never smoker]. The T790M mutation was present in 6 (2.9%) of 205 adenocarcinomas, 2 (3.4%) of 59 squamous cell carcinomas, 1 (11.1%) of 9 adenosquamous carcinomas, 1 (16.7%) of 6 large cell carcinomas, and none of 1 spindle carcinoma, indicating no predominant histology for the T790M mutation. About disease stage, no T790M-positive case was present in 137 cases of stage I, 1 (3.1%) in 32 stage II, 5 (9.8%) in 51 stage III, 4 (7.7%) in 52 stage IV, and none in 8 recurrent disease cases. The T790M mutation was preferentially present in advanced cases [stages III and IV and recurrent disease; 9 (8.1%) of 111 cases] compared with early cases [stages I and II; 1 (0.59%) of 169 cases; $P = 0.0013$]. By contrast, there was no difference in single

Table 2. The characteristics of 10 patients with the T790M mutation

Patient	Sex	Age	Pathology	Smoking	Direct sequencing		Stage	Response	Progression-free survival	Overall survival
					EGFR	*KRAS*				
1	M	70	AD	S	Wild-type	Wild-type	IIIB	PD	11	297*
2	M	56	AD	S	Wild-type	G12V	IV	NC	122	178*
3	M	35	SQ	S	Wild-type	G12D	IV	PD	21	91*
4	M	80	AD	S	L858R	Wild-type	IIA	NE	10	109*
5	F	77	ADSQ	NS	T790M+L858R	Wild-type	IIIA	NC	48	261*
6	M	68	LC	S	Wild-type	Wild-type	IV	NC	60	107*
7	M	53	AD	S	del745(3)-750(2)	Wild-type	IV	NC	32	77
8	M	48	SQ	S	Wild-type	Wild-type	IIIA	NC	99	406*
9	F	56	AD	NS	L858R	Wild-type	IIIA	NC	353	353
10 †	M	71	AD	S	Wild-type	Wild-type	IIIA	NA	NA	NA

Abbreviations: AD, adenocarcinoma; SQ, squamous cell carcinoma; ADSQ, adenosquamous carcinoma; LC, large cell carcinoma; S, smoker; NS, never smoker; PD, progressive disease; NC, no change; NE, not evaluable; NA, not available.

*Death.

†Patient 10 was not treated with gefitinib.

exon 19 deletion and L858R mutation between advanced cases [28 (25.2%) of 111 cases] and early cases [58 (34.3%) of 169 cases].

Ninety-five cases were treated with gefitinib, and tumor responsiveness was found in 23 cases. Gefitinib-sensitive *EGFR* mutation was present in 19 cases and no *EGFR* mutations in 4 cases. Among 10 cases harboring the T790M mutation, 9 cases were treated with gefitinib. Two patients showed progressive disease, 6 no change, and 1 not evaluable, indicating that there was no-responder even in cases harboring a minor population of the T790M mutation (Table 2). Gefitinib-sensitive mutations were found in 30 cases consisting of 7 no-responders, 19 responders, and 4 not evaluable cases. The T790M mutation was found in 3 of 7 no-responders and was not found in 19 responders with gefitinib-sensitive mutations, indicating that the T790M mutation in cases with gefitinib-sensitive mutation was significantly frequent in gefitinib-resistant cases (P = 0.014). Of note, two of these three T790M mutant cases were not detected by direct sequencing, showing that the cells harboring the T790M mutation represented a minor population.

Discussion

We found the presence of the *EGFR* T790M mutation in a minor tumor population in 3.6% of the NSCLCs examined in this study. In contrast, the majority of the exon 19 deletions and the L858R mutation were found in major populations of the tumor cells as reported in our previous study (13). These *EGFR* mutations were identified by direct sequencing and mutant-enriched PCR assays at almost equal frequency (13). Although *EGFR* exon 19 deletions and L858R mutations cause the constitutive activation of *EGFR*, the introduction of the *EGFR* T790M mutation into cells does not substantially alter the EGFR tyrosine kinase activity or the status of EGFR signal transduction cascade molecules as shown using standard cell-based assays *in vitro* (8, 9, 17). Bell et al. (17) reported that the presence of a germ-line *EGFR* T790M mutation in the patients with lung cancer is associated with a relatively late onset of familial NSCLC, suggesting that the T790M mutation may possess the potential attenuated function for oncogenesis. These findings suggest that the *EGFR* T790M mutation does not play a major causative or proliferative role in tumorigenesis and that the selective proliferation of cells harboring a T790M mutation does not normally occur. Thus, the T790M mutation tends to be present as a minor clone if the T790M mutation occurs late during carcinogenesis, when tumor cells exhibit more genomic instability than at earlier stages. Indeed, the rate of T790M mutation is significantly higher in advanced tumors than in early-stage tumors, but no difference was present in drug-sensitizing mutations between disease stages. These facts also suggest that the T790M mutation was just "bystander" mutations being not involved in tumor initiation during the development of NSCLC initiated by some other mechanisms, such as an *EGFR*-activating mutation or a *KRAS* mutation, which was also seen in six of our cases. Other mechanisms may have been responsible for carcinogenesis in our other four cases with wild-type of *EGFR* and *KRAS*.

In gefitinib-treated cases, gefitinib-sensitive *EGFR* mutant cases harboring T790M minor clone showed no response to gefitinib. Similarly, a minor proportion of cells harboring an imatinib-resistant BCR-ABL mutation was reported in the patient with chronic myelogenous leukemia; a major proportion of mutant cells was later found when resistance became apparent (18). Taking together our results, we hypothesized that gefitinib treatment may cause the selection of T790M mutant cells and that even a small fraction of T790M-positive tumor cells at the beginning of treatment could lead to clinical gefitinib resistance as a result of the selective proliferation of T790M mutant cells (Fig. 3). Recent studies indicate that several drugs can overcome the resistance caused by the T790M mutation, suggesting that the development of second-generation EGFR inhibitors is imminent (19). Thus, the detection of a minor T790M-positive clone may be useful not only for predicting gefitinib resistance but also for effectively preventing the emergence of resistance through the appropriate usage of next-generation EGFR inhibitors.

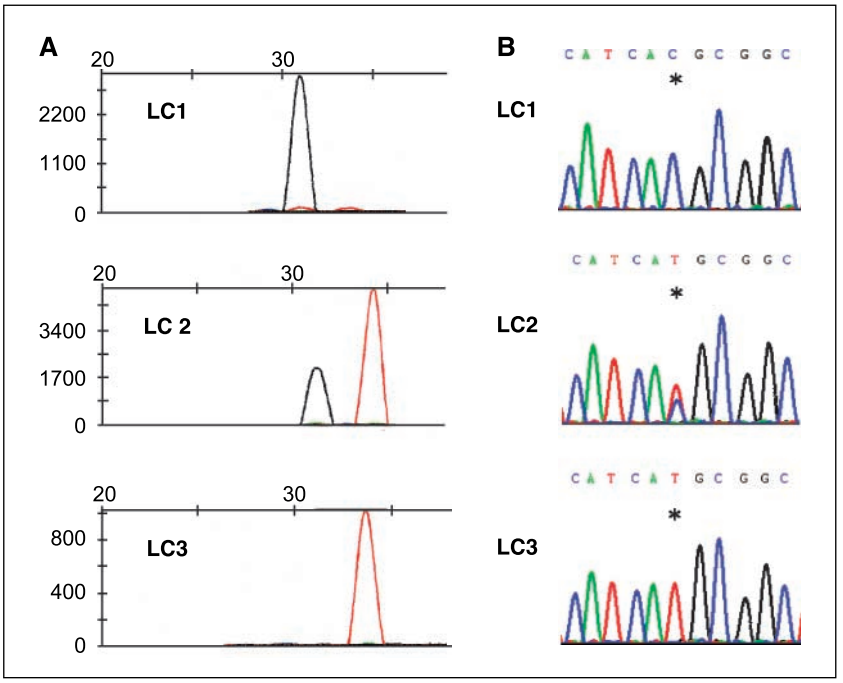

Figure 2. Representative examples of the mutant-enriched PCR assay. *A,* chromatogram of second letter in codon 790 determined by a single-base primer extension assay after enrichment of mutant alleles. *Black* and *red peaks,* C (wild-type nucleotide) and T (mutant-type nucleotide), respectively. Only wild-type sequence (C) was detected in LC1. Both wild-type (C) and mutant (T) sequences were detected in LC2. Only mutant sequence (T) was detected in LC3. *B,* corresponding sequence chromatograms for the product of the mutant-enriched PCR assay.

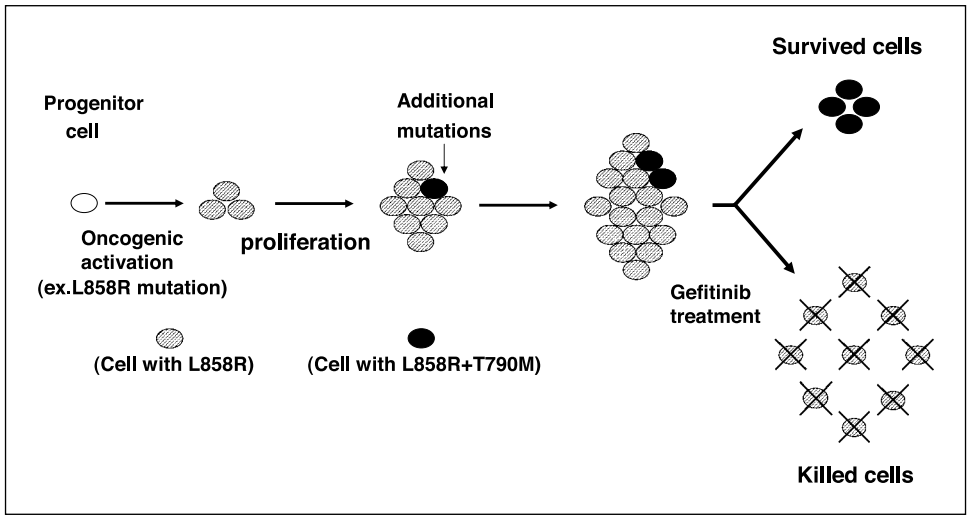

Figure 3. Schema showing a hypothesis for the T790M mutation-related resistance to EGFR-TKI (gefitinib or erlotinib).

We reported that the exon 20 insertion mutations showed no bias for sex or smoking status (20), which is different from exon 19 deletions and the L858R mutation that are frequently present in women and never smokers. It is interesting that the T790M mutation is also an alteration in exon 20 and shows a similar association in sex and smoking status to exon 20 insertions. Of note, although 3.6% of the NSCLCs harbored the T790M mutation in our series, as shown using the mutant-enriched PCR assay described above, a more sensitive assay might reveal an even higher percentage. Thus, the actual frequency at which the T790M mutation appears as a minor clone in NSCLC is unknown.

In conclusion, we developed a mutant-enriched PCR assay to detect the *EGFR* T790M mutation and found that the T790M mutation is sometimes present as a minor population in tumor cells and can cause the emergence of resistance after gefitinib administration. The detection of a small fraction of T790M-positive tumor cells may be useful for predicting the clinical course of the gefitinib-treated NSCLC patients and to establish EGFR-targeted therapy using new EGFR inhibitors.

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