Highly Sensitive Detection of *EGFR* T790M Mutation Using Colony Hybridization Predicts Favorable Prognosis of Patients with Lung Cancer Harboring Activating *EGFR* Mutation

Yoshihiko Fujita, PhD, Kenichi Suda, MD,† Hideharu Kimura, MD, PhD,* Kazuko Matsumoto, MD, PhD,* Tokuzo Arao, MD, PhD,* Tomoyuki Nagai, MD, PhD,* Nagahiro Saijo, MD, PhD,* Yasushi Yatabe, MD, PhD,‡ Tetsuya Mitsudomi, MD, PhD,† and Kazuto Nishio, MD, PhD**

Introduction: Approximately 50% of lung cancer patients with epidermal growth factor receptor (*EGFR*)-mutations (deletion in exon 19 or L858R) who develop acquired resistance to EGFR tyrosine kinase inhibitors (TKIs) reportedly carry a secondary *EGFR* T790M mutation. This mutation has been suggested to be present in tumor cells before EGFR-TKI treatment in a small population of individuals. Here, we use a highly sensitive colony hybridization technique in an attempt to evaluate the actual incidence of T790M in pretreatment tumor specimens.

Methods: DNA was extracted from surgically resected tumor tissues of 38 patients with the *EGFR* mutation and examined for the presence of T790M, using a standard polymerase chain reaction based method followed by a modified colony hybridization (CH) technique with an analytical sensitivity of approximately 0.01%. Associations between the T790M status and clinical characteristics including time to treatment failure (TTF) for EGFR-TKI were evaluated.

Results: The T790M mutation analysis of the specimens from the 38 patients detected 30 mutants (79%). The median TTF was 9 months for the patients with pretreatment T790M and 7 months for the patients without the T790M mutation ($p = 0.44$). When the patients with T790M were divided into strongly positive and modestly positive subgroups in terms of the frequency of positive signals observed using CH technique, the 7 patients with strong positivity had a TTF that was significantly longer than that of the 8 patients without T790M ($p = 0.0097$) and of the 23 patients with modest positivity ($p = 0.0019$).

Conclusions: Our highly sensitive CH method showed that a subgroup of non–small-cell lung cancer patients with the *EGFR* mutation harbored the rare T790M allele before EGFR-TKI treatment. A high proportion of T790M allele may define a clinical subset with a relatively favorable prognosis.

Key Words: Non–small-cell lung cancer, Epidermal growth factor receptor mutation, Colony hybridization.

(*J Thorac Oncol.* 2012;7: 1640–1644)

Non–small cell lung cancer (NSCLC) patients with an activating mutation within the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) respond dramatically to EGFR tyrosine kinase inhibitors (TKIs).[1,2] The common activating mutations include in-frame deletions in exon 19 (dels) and a point mutation in exon 21 (L858R), all of which lead to the sustained activity of EGFR tyrosine kinase. Unfortunately, almost all patients with these activating mutations eventually develop acquired resistance while receiving EGFR-TKI treatment.[3,4] A secondary mutation in exon 20 (T790M) is observed in approximately 50% of such cases.[5,6] The T790M mutation alters the relative affinity of the mutant receptor in favor of adenosine triphosphate (ATP), abrogating the effect of EGFR-TKIs.[7]

The mechanism by which the secondary mutation emerges remains unclear. One possibility is that a minor population of tumor cells with the T790M mutation exists among the pretreatment tumor cells. Indeed, the T790M mutation has been detected in a few pretreatment tumor samples.[8,9] However, the frequency of T790M mutations in these pretreatment samples was insufficient to explain why the T790M mutation occurs in approximately half the EGFR-TKI–resistant cases. We hypothesized that the actual population of tumor cells harboring the T790M mutation may be below the detection limit and may be assessable using a more sensitive assay system.

Here, we report the development of a highly sensitive colony hybridization (CH) method that detects low levels of the T790M mutation. We used this method to assess the T790M mutation in surgically resected tumor samples from NSCLC patients to resolve: (1) the frequency of tumor

*Department of Genome Biology, Kinki University School of Medicine, Osaka, Japan; †Department of Thoracic Surgery; and ‡Department of Pathology and Molecular Diagnostics, Aichi Cancer Center Hospital, Nagoya, Japan.

Disclosure: Tetsuya Mitsudomi, MD, PhD, has received lecture fees from AstraZeneca, Chugai, Boehringer-Ingelheim and Pfizer. The other authors declare no conflicts of interest.

Address for correspondence: Kazuto Nishio, MD, PhD, Department of Genome Biology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589–8511, Japan. E-mail: knishio@med.kindai.ac.jp

ISSN: 1556-0864/12/0711-1640

samples carrying the T790M mutation before EGFR-TKI treatment; and (2) its association with the clinical outcome.

PATIENTS AND METHODS

Patients and Sample Collection

Primary tumor samples were collected from NSCLC patients who underwent potentially curative pulmonary resection at the Department of Thoracic Surgery, Aichi Cancer Center Hospital, Japan, between March 1997 and January 2007. Informed consent was obtained from each patient before the operation. All the tumor samples were snap-frozen and stored at −80°C. For each specimen, the frozen sections were thawed and stained with hematoxylin and eosin to examine the proportion of tumor cells in the tissue. The genomic DNA contained at least 20% of the tumor content in our cohort. To extract the genomic DNA, the tissues were incubated with 1 × (polymerase chain reaction) PCR buffer containing 100 μg/mL proteinase K for 1 hour at 54°C. Next, the solution was incubated for 3 minutes at 95°C. As a preliminary experiment, we had previously assessed the presence of *EGFR* mutations in exons 18 to 21 using a direct sequencing analysis with previously described primers and protocols.[10] Patients with *EGFR* mutations were enrolled in the study. Patients who had received EGFR-TKI treatment before surgery were not eligible. The complete clinical data records including age, sex, pathological diagnosis, clinical stage, and dates of surgery and relapse were obtained from each patient's medical records and were reviewed by the physicians.

Scorpion Amplification Refractory Mutation System Assay

The Therascreen EGFR Mutation Kit (QIAGEN, Tokyo, Japan), which is designed to detect 29 types of *EGFR* mutations including T790M, was used for the Scorpion Amplification Refractory Mutation System (SARMS) assay, according to the manufacturer's instructions.

Colony Hybridization Assay

A CH assay was performed to detect the T790M *EGFR* mutation according to a previously described method, with some modifications.[11] Exon 20 of the *EGFR* gene was amplified for each DNA sample with a forward primer, 5′–CCATGAGTACGTATTTTGAAACTC–3′ (*EGFR*_Ex20_f) and a reverse primer, 5′–CATATCCCCATGGCAAACTCTTGC–3′ (*EGFR*_Ex20_r) using PrimeSTAR® HotStart DNA Polymerase (Takara Bio Inc., Ohtsu, Japan). The amplified 458 bp PCR fragment was cloned into a TOPO blunt cloning vector (Invitrogen, Carlsbad, CA). The resultant plasmid was used to transform *Escherichia coli* (TOP10) cells, which were then plated on Luria Broth-agar containing kanamycin. Blue/white selection using 40 mg/mL X-gal (Wako, Osaka, Japan) was performed to subtract the number of cells harboring an empty vector from the total number. The number of white colonies was used as a parameter to calculate the proportion of T790M colonies. After overnight incubation, the colonies (typically 1000–5000 colonies per plate) were transferred to a Hybond-N^+ membrane (GE Healthcare, Buckinghamshire, United Kingdom) and hybridized using oligonucleotide probes labeled with γ-^{32}P-ATP. The sequences of the oligonucleotide probes were 5′–CTCATCA*C*GCAGCTCATGCC–3′ for the wild-type product and 5′–CTCATCA*T*GCAGCTCATGCC–3′ for T790M (the altered nucleotide is underlined). The membrane was incubated for 16 hours at 37°C in hybridization solution containing 6 × saline-sodium citrate, 0.5% sodium dodecyl sulphate, 5 × Denhardt's solution, and 100 μg/mL of salmon sperm DNA, to which 8×10^5 count per minute/mL of γ-^{32}P-ATP-labeled probe had been added. After hybridization, the membranes were washed twice for 30 minutes at 55 °C in 6 × saline-sodium citrate and exposed to film. The black dots visualized on the film were judged as positive colonies. An assay was performed to evaluate positive cases until at least two independent positive colonies were obtained. Cases in which no signal was screened among 10,000 white colonies were judged as negative cases. To confirm the results, the positive colonies were selected to extract the plasmids and the inserted DNA was sequenced using the *EGFR*_Ex20_f primer.

To assess the sensitivity and specificity of both the wild-type and the T790M oligonucleotide probes, plasmids containing wild-type and T790M sequences were made as described above, using DNAs extracted from A549 and H1975 cell lines, respectively, as templates. Five control DNA solutions (#1–5) were prepared at different ratios of T790M to wild-type plasmid DNA as follows: #1, 0 %; #2, 100 %; #3, 1%; #4, 0.1%; #5, 0.01%. For each solution, the plasmid DNA was adjusted to 5 pg (approximately 1,000,000 copy equivalent)/μL.

Statistical Analyses

The Fisher's exact test was used to assess the relationship between the T790M mutation status and different characteristics, including sex and the primary mutation status. The Mann-Whitney test was used to assess the relationship between the T790M mutation status and age. Time to treatment failure (TTF) was defined as the interval between the initiation of EGFR-TKI treatment until the documentation of resistance to EGFR-TKI therapy, relapse, or death after having achieved a partial response or complete response. The survival curves were calculated according to the Kaplan–Meier method, and comparisons were performed using StatView software (version 5; SAS Institute, Cary, NC). p Value less than 0.05 was considered statistically significant.

RESULTS

Assessment of CH Assay Sensitivity

We assessed the sensitivity and specificity of both the wild-type and T790M oligonucleotide probes using control DNA solutions (#1–5). When the probe specific for the wild-type sequence was used, many dots were visualized for the control DNA solution #1 (wild-type alone), whereas no unambiguous dots were observed for control DNA solution #2 (T790M alone) (Fig. 1*A*). Meanwhile, the T790M probe produced no visible dots for control DNA solution #1 whereas many dots were vividly visualized for control DNA solution #2 (Fig. 1*B*). The results showed that these probes, even if their sequences differed from each other by only one base, specifically hybridized the complementary clones under the reaction conditions used. We

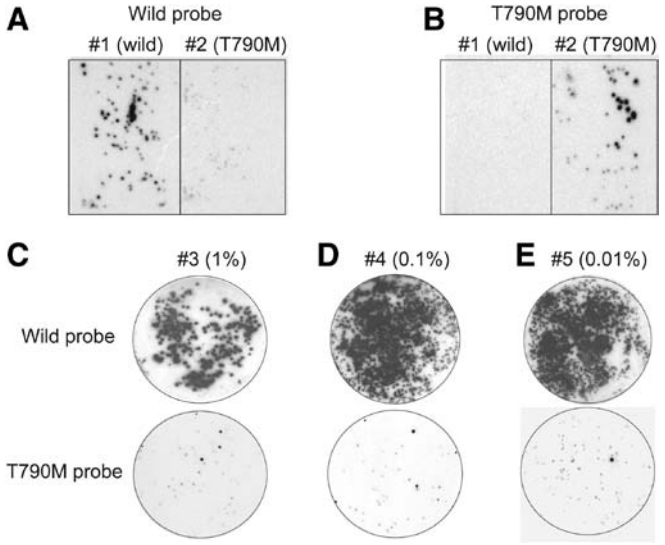

FIGURE 1. Representative allele-specific oligonucleotide colony hybridization for the quantitation of the T790M mutation. *A* and *B*, Hybridizing clones (black dots) are shown for control DNA solutions #1 (wild) and #2 (T790M) using (*A*) the wild-type probe and (*B*) the T790M probe. *C*, *D*, and *E*, Colonies for control DNA solutions #3 (1 % T790M), #4 (0.1% T790M), and #5 (0.01 % T790M) were hybridized with both wild type- and T790M- specific oligonucleotides.

additionally performed sensitivity assays using the serially diluted T790M DNA solutions (#3–5) as shown in Fig. 1*C*, *D*, and E. Many dots indicating the wild-type clones and a small number of dots indicating the T790M clones were observed in each case. The positive signal ratios for T790M in each DNA solution were as follows: for solution #3, three of 260 white colonies (1.15%); for solution #4, two of 1760 white colonies (0.11%); and for solution #5, two of 10,000 white colonies (0.02%). These ratios reflect the actual proportions of T790M clones included in each solution: #3, 1 %; #4, 0.1 %; and #5, 0.01 %. Direct sequencing was performed for DNAs isolated from several visualized colonies to confirm their positivity (data not shown).

Patient Characteristics

Thirty-eight patients with *EGFR* mutation were enrolled in this study (Table 1). The histological type in all the patients was adenocarcinoma. The primary mutation status was pre-assayed for each patient, using direct sequencing. Twenty-two patients (57.9 %) had a deletional mutation in exon 19, two patients (5.3 %) had a point mutation in exon 18 (n = 1, G719A; n = 1, G719C), one patient (2.6 %) had a 6 bp insertion in exon 19 and 13 (34.2 %) had a point mutation in exon 21 (n = 12, L858R; n = 1, L858R + E709G). All patients had prospectively received treatment with gefitinib.

Detection of *EGFR* T790M in Tumor Tissues

The *EGFR* mutations detected in the tumor samples are summarized in Table 1. The SARMS assay detected no T790M mutation in any of the patients. Using the CH assay, however, the T790M mutation was found in 30 of the 38 patients (78.9%). The positive colonies detected for patient no. 27 are shown as an example in Supplementary Figure 1 (Supplemental Digital Content 1, http://links.lww.com/JTO/A326). The mutation was confirmed by direct sequencing in at least two colonies per patient. Among the 30 positive cases, the positive clones were present at a low ratio to the total

TABLE 1. Detection of EGFR T790M Mutation in NSCLCs with Activating Mutation

					T790M detection			
No.	Age	Sex	Stage	Primary Mutation	SARMS	CH	T790M/Total Colonies (%)	
1	80	F		L858R	-	+	22/5,112	(0.43)
2	68	F		Del	-	+	6/5,531	(0.11)
3	59	F		L858R	-	+	2/4,987	(0.04)
4	68	M		L858R	-	+	2/4,313	(0.05)
5	69	F		Del	-	+	3/1,993	(0.15)
6	61	M		L858R	-	+	25/2,788	(0.90)
7	52	M		Del	-	+	33/5,269	(0.63)
8	69	F		Del	-	+	2/2,278	(0.09)
9	59	M		L858R	-	+	3/4,245	(0.07)
10	79	F		Del	-	+	2/4,832	(0.04)
11	71	F		L858R	-	+	12/2,294	(0.52)
12	51	F		L858R	-	+	31/1,702	(1.82)
13	73	F		Del	-	+	8/1,298	(0.62)
14	66	F		Del	-	+	12/2,359	(0.51)
15	52	F		Del	-	+	6/5,021	(0.12)
16	63	F		L858R	-	+	20/1,805	(1.11)
17	53	F		L858R	-	+	2/1,465	(0.14)
18	68	F		L858R	-	+	3/3,810	(0.08)
19	61	M		G719C	-	+	2/1,990	(0.10)
20	78	M		G719A	-	+	3/1,482	(0.20)
21	74	M		Del	-	+	2/2,277	(0.09)
22	67	M		Del	-	+	2/6,032	(0.03)
23	64	F		Del	-	+	6/3,998	(0.15)
24	57	F		L858R	-	+	3/5,756	(0.05)
25	50	F		L858R	-	+	3/5,215	(0.06)
26	71	M		Del	-	+	5/2,774	(0.18)
27	36	M		Del	-	+	2/9,443	(0.02)
28	61	F		Del	-	+	2/4,308	(0.05)
29	80	M		Del	-	+	4/5,490	(0.07)
30	62	F		Del	-	+	3/3,541	(0.08)
31	56	M		Del	-	-	0/10,823	(0)
32	50	F		L858R	-	-	0/10,456	(0)
33	45	M		Del	-	-	0/11,642	(0)
34	70	F		Del	-	-	0/12,309	(0)
35	64	F		Del	-	-	0/11,769	(0)
36	63	F		Ins	-	-	0/12,013	(0)
37	61	M		Del	-	-	0/12,468	(0)
38	54	M		Del	-	-	0/12,233	(0)

SARMS, Scorpion Amplification Refractory Mutation System; CH, colony hybridization method; Del, deletional mutations in exon 19; Ins, insertional mutation in exon 19.

TABLE 2. Patient Characteristics and T790M Mutation Status

		T790M Status		
		Positive	Negative	(*P* value)
Sex				0.69
Male	16	12	4	
Female	24	20	4	
Age				0.46
≥60	25	21	4	
>60	13	9	4	
Primary mutation				0.29
Del	22	6	16	
L858R	13	1	12	
Others	3	1	2	

colony number (0.02%–1.82%). No relationships between the T790M mutation status and other clinical characteristics including age, sex, and primary mutation status were detected (Table 2).

TTF Survival Analysis

In our cohort of 38 patients, the median TTF in patients with the T790M mutation (n = 30) was not statistically different from that in the patients without the mutation (n = 8) (p = 0.44), being 10 months and 8 months, respectively (Fig. 2*A*). When the patients were subdivided into three groups based on the detection rate for T790M colonies (nil: 0 %, n = 7; low: 0%–0.5 %, n = 23; and high: ≥ 0.5 %, n = 8), the median TTF for the patients with a high proportion was 41 months, which was significantly longer than that for the low-proportion group (7 months, p = 0.0019) or the nil-proportion group (7 months, p = 0.0097) (Fig. 2*B*). We also compared the TTF among the patients according to their mutation statuses (exon 19 del, n = 22; L858R, n = 13; and others, n = 3) and found no statistical differences (Supplementary Fig. 2, Supplemental Digital Content 2, http://links.lww.com/JTO/A327).

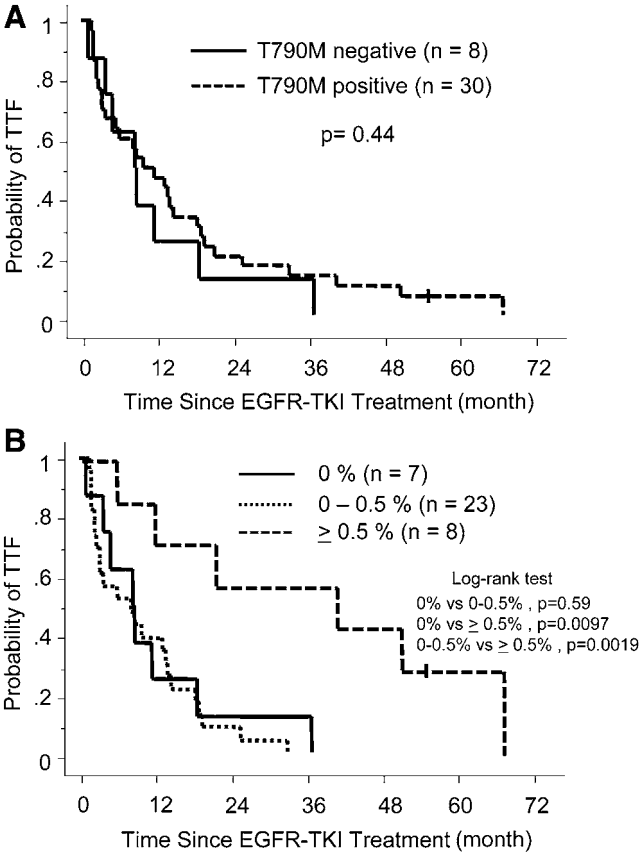

FIGURE 2. Kaplan–Meier curves for time to treatment failure (TTF) are shown for patients according to (*A*) their T790M status, and (*B*) the proportion of T790M colonies detected in surgically resected, pre-TKI treatment samples.

DISCUSSION

Our findings have shown that the T790M mutation is detectable in tumor tissues obtained from EGFR-TKI naive patients and that the CH is a useful method for detecting the T790M mutation. Through this method, we identified the T790M mutation in 30 of 38 untreated samples (79%), a much higher proportion of T790M mutant tumors than previously reported.[8,9] We initially used SARMS, a highly sensitive detection method, but the *EGFR* T790M mutation was not detected in any of the untreated samples examined in the present cohort. A possible reason for the higher proportion of T790M in this study may be the high sensitivity of our CH method. This method enables a very large number of colonies carrying different hybrid plasmids to be rapidly screened for a specified DNA sequence.[11,12] The sensitivity at which a single mutant genomic clone can be detected among normal clones has been estimated to be 1:1000 to 1:10,000.[13] Indeed, we detected positive signals when a mutant genomic clone was diluted with normal clone to 0.01%. Moreover, unlike other sensitive assays such as SARMS, the CH method produces no false-positive results, because colonies of interest can be determined to be either positive or negative by sequencing. Given that the T790M mutation occurs in *cis* with the activating mutation (dels or L858R) on the same allele,[6] the exact incidence rate of the T790M mutation may be corrected by the frequency of the activating mutations, which may vary among the patients.

This is the first report showing a positive correlation between the presence of T790M before EGFR-TKI treatment and patient outcome. To our surprise, the median TTF for seven patients with a high proportion (≥ 0.5%) was 41 months, which was significantly longer than that for 23 patients with a low proportion (0%–0.5 %, 7 months) and eight patients with a nil proportion (7 months) (Fig. 2*B*). This data suggested that the abundance, but not the presence, of T790M in untreated cancer cells impacted the benefits of EGFR-TKI treatment. This result was unexpected, because the outcome of the patients with an activating mutation who acquire a resistance mutation is likely to worsen.[8,14,15] The results of a study by Oxnard et al.[16] may support our findings. They showed that EGFR-TKI resistant patients with T790M identified in rebiopsy specimens had a relatively favorable prognosis compared with patients without the T790M mutation.[16] In a basic research study, the acquisition of T790M was associated with a slowdown in tumor growth,[6]

possibly explaining the longer TTF among the patients with a high ratio of T790M. Another explanation is that the patients who are devoid of T790M may acquire some other mechanism of resistance, leading to their poorer prognosis.[16]

NSCLC cells harboring the T790M mutation may be present before EGFR-TKI exposure as a small *drug-tolerant* subpopulation in cells with activating mutations.[17] The proportion of T790M mutant alleles may increase gradually during treatment, eventually becoming present at an easily detectable level in half of the patients at the time of clinical progression to EGFR-TKI resistance.

In conclusion, using a highly sensitive CH method, we showed that a subgroup of NSCLC patients with the *EGFR* mutation harbored the rare T790M allele before EGFR-TKI treatment. A high proportion of T790M alleles may define a clinical subset with a relatively favorable prognosis.

ACKNOWLEDGMENT

We thank Kusuo Tatsumi, Yukiko Yamamoto, and Norinaga Kakishita for support with the radioisotope work; Yoshihiro Mine for the sequencing work; and Tomoko Kitayama for technical support.

REFERENCES

1. Mitsudomi T, Morita S, Yatabe Y, et al.; West Japan Oncology Group. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 2010;11:121–128.
2. Maemondo M, Inoue A, Kobayashi K, et al.; North-East Japan Study Group. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010;362:2380–2388.
3. Rosell R, Moran T, Queralt C, et al.; Spanish Lung Cancer Group. Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009;361:958–967.
4. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;361:947–957.
5. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352:786–792.
6. Chmielecki J, Foo J, Oxnard GR, et al. Optimization of dosing for EGFR-mutant non-small cell lung cancer with evolutionary cancer modeling. *Sci Transl Med* 2011;3:90ra59.
7. Yun CH, Mengwasser KE, Toms AV, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc Natl Acad Sci USA* 2008;105:2070–2075.
8. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med* 2008;359: 366–377.
9. Rosell R, Molina MA, Costa C, et al. Pretreatment EGFR T790M mutation and BRCA1 mRNA expression in erlotinib-treated advanced non-small-cell lung cancer patients with EGFR mutations. *Clin Cancer Res* 2011;17:1160–1168.
10. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res* 2004;64:8919–8923.
11. De Meyer SF, Pareyn I, Baert J, Deckmyn H, Vanhoorelbeke K. False positive results in chimeraplasty for von Willebrand Disease. *Thromb Res* 2007;119:93–104.
12. Saitoh M, Niijima M, Takiguchi Y, et al. An early event of EGFR mutation in pleomorphic carcinoma of the lung. *Int J Clin Oncol* 2011;16: 770–773.
13. Mao L, Hruban RH, Boyle JO, Tockman M, Sidransky D. Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. *Cancer Res* 1994;54:1634–1637.
14. Inukai M, Toyooka S, Ito S, et al. Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. *Cancer Res* 2006;66:7854–7858.
15. Su KY, Chen HY, Li KC, et al. Pretreatment epidermal growth factor receptor (EGFR) T790M mutation predicts shorter EGFR tyrosine kinase inhibitor response duration in patients with non-small-cell lung cancer. *J Clin Oncol* 2012;30:433–440.
16. Oxnard GR, Arcila ME, Sima CS, et al. Acquired resistance to EGFR tyrosine kinase inhibitors in EGFR-mutant lung cancer: distinct natural history of patients with tumors harboring the T790M mutation. *Clin Cancer Res* 2011;17:1616–1622.
17. Sharma SV, Lee DY, Li B, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 2010;141:69–80.