Clinical Cancer Research

Ultra-Sensitive Detection of the Pretreatment EGFR T790M Mutation in Non–Small Cell Lung Cancer Patients with an EGFR-Activating Mutation Using Droplet Digital PCR

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Abstract

Purpose: The resistance to the EGFR tyrosine kinase inhibitors (TKI) is a major concern in non–small cell lung cancer (NSCLC) treatment. T790M mutation in EGFR accounts for nearly 50% of the acquired resistance to EGFR-TKIs. Earlier studies suggested that T790M mutation was also detected in TKI-naïve NSCLCs in a small cohort. Here, we use an ultra-sensitive droplet digital PCR (ddPCR) technique to address the incidence and clinical significance of pretreatment T790M in a larger cohort.

Experimental Design: ddPCR was established as follows: wild-type or T790M mutation-containing DNA fragments were cloned into plasmids. Candidate threshold was identified using wild-type plasmid, normal human genomic DNA, and human A549 cell line DNA, which expresses wild type. Surgically resected tumor tissues from 373 NSCLC patients with EGFR-activating mutations were then examined for the presence of T790M using ddPCR.

Results: Our data revealed a linear performance for this ddPCR method ($R^2 = 0.998$) with an analytical sensitivity of approximately 0.001%. The overall incidence of the pretreatment T790M mutation was 79.9% (298/373), and the frequency ranged from 0.009% to 26.9%. The T790M mutation was detected more frequently in patients with a larger tumor size ($P = 0.019$) and those with common EGFR-activating mutations ($P = 0.022$), as compared with the others.

Conclusions: The ultra-sensitive ddPCR assay revealed that pretreatment T790M was found in the majority of NSCLC patients with EGFR-activating mutations. ddPCR should be utilized for detailed assessment of the impact of the low frequency pretreatment T790M mutation on treatment with EGFR-TKIs. *Clin Cancer Res; 21(15); 3552–60. ©2015 AACR.*

Introduction

The EGFR tyrosine kinase inhibitors (TKIs) have been demonstrated to be effective in treating non–small cell lung cancer (NSCLC) patients with EGFR-activating mutations (1, 2). The superiority of EGFR-TKIs in progression-free survival over platinum-based chemotherapies for EGFR-mutated lung cancers has been reported in several phase III trials as first-line

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

The prevalence of a *de novo* EGFR T790M mutation in patients with EGFR-mutant non–small cell lung cancer (NSCLC) remains unclear. This "pretreatment T790M" mutation was underestimated due to the limitation in detection sensitivity of conventional DNA sequencing despite its potential importance. Here, we demonstrate the prevalence of the pretreatment T790M mutation in 373 surgically resected tumor samples from patients with NSCLC harboring EGFR-activating mutations using a droplet digital PCR system. Approximately 80% of patients with NSCLC harboring EGFR-activating mutations had the pretreatment T790M mutation primarily at an ultra-low allele frequency between 0.001% and 0.1%. The EGFR T790M mutation was detected more frequently in NSCLCs with larger tumor sizes and those with common EGFR-activating mutations. These data suggest that the pretreatment T790M mutation widely exists in EGFR-mutant NSCLC, and high-resolution mutational profiling using an ultra-sensitive method can open the possibility of developing strategies for personalized cancer therapies in patients with NSCLC.

treatments (3–7). However, a majority of the responders eventually develop acquired resistance to EGFR-TKIs (8–10). Both basic and clinical approaches have been applied to elucidate and overcome this acquired resistance. These studies have uncovered several mechanisms of acquired resistance as candidates: EGFR secondary mutations (primarily the T790M gatekeeper mutation), mesenchymal-to-epithelial transition factor receptor (MET) amplification, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutation, small cell lung cancer transformation, PTEN loss, high-level hepatocyte growth factor expression, fatty acid synthase /NF-κB pathway activation, epithelial–mesenchymal transition, and loss or splice variants of BIM. Among those, a secondary missense T790M mutation is observed in nearly half of all cases that are resistant to EGFR-TKIs (10).

There is some controversy surrounding the T790M mutation as to whether it is acquired after EGFR-TKI treatment or it is a *de novo* mutation that preexists before EGFR-TKI treatment. This "pretreatment T790M" mutation was identified in tumors as a minor clone before exposure to EGFR-TKIs and was found concurrently with other activating EGFR mutations (11). Using conventional DNA sequencing such as the Sanger method, the pretreatment T790M mutation was found in 1% to 8% of patients concurrently with an EGFR-activating mutation (12–15). Utilizing more sensitive detection methods such as the Scorpion Amplification Refractory Mutation System, the reported incidence of pretreatment T790M was higher: 2% to 79% in EGFR-activating mutation-positive samples (11, 15–20). The evidence is so far limited for the clinical relevance of pretreatment T790M identified at extremely low frequencies. Costa and colleagues reported that progression-free survival (PFS) to erlotinib was shorter for patients with T790M mutation than for those without T790M mutation (20). The results of meta-analysis have also suggested that the pretreatment T790M mutation has the potential to be a predictive marker for the effect of EGFR-TKIs in EGFR-activating mutation-positive patients on their PFS (21). The pretreatment T790M mutation may be underestimated, despite its potential importance, due to the limitation in detection sensitivity of conventional DNA sequencing. Thus, the prevalence of the pretreatment T790M mutation needs to be addressed in a larger patient cohort by a clinically adaptable state-of-the-art technique with higher sensitivity.

Digital PCR (dPCR) is a highly sensitive gene mutation detection method that is based on the compartmentalization and amplification of single DNA molecules (22, 23). Quantification of compartments with endpoint fluorescence after the PCR process reveals the number of copies of target DNA. Droplet digital PCR (ddPCR) is one such dPCR technology based on compartmentalization of DNA into droplets (24). In ddPCR, individual DNA fragments are compartmentalized into more than a million droplets, which are then amplified in parallel. By using this system, the detection of one *KRAS* mutant among 200,000 *KRAS* wild-types has been demonstrated for genomic DNA from tumor cell lines (25), and this technologic advantage in highly sensitive mutation detection has been adopted in clinical research as well (26–30).

Here, we report the advantage of a picodroplet-based dPCR method that detects a mutant allele of interest present at an ultra-low level, and this method was used to assess the T790M mutation in 373 surgically resected EGFR-mutant tumor samples from NSCLC patients enrolled in a Japan molecular epidemiology lung cancer (JME) study (31). By using this ultra-sensitive quantitative assay, we aimed to accurately detect the pretreatment T790M mutation and its clinicopathologic correlation in a large cohort of patients with NSCLC.

Materials and Methods

Study design and patients

The JME study (UMIN000008177) is a prospective, multicenter molecular epidemiology study to address associations between driver mutations and smoking and other environmental factors. Eligible subjects are newly diagnosed patients with stage I to IIIB NSCLC who have undergone surgery. Full details of the study design have been published previously (31). Planned objectives of this JME substudy were evaluations of the T790M mutation status in pretreatment tumor samples using ultra-sensitive ddPCR and comparison with that determined by conventional methods.

This study was approved by the Institutional Review Board of the National Hospital Organization of Japan. All the patients provided written informed consent. From July 2012 to December 2013, 958 patients were recruited from 43 institutions and 901 samples were successfully analyzed. Somatic mutations were examined by multiplex-targeted deep sequencing (described in Supplementary Materials and Methods) and mutations in *EGFR* were validated by sensitive PCR methods by an independent clinical laboratory (SRL, Tokyo, Japan). Three hundred and seventy-three samples harboring an EGFR-activating mutation based on the Cycleave PCR method (32) were analyzed by ddPCR.

Sample collection

Details are described in Supplementary Materials and Methods.

Droplet digital PCR

Figure 1 describes the use of ddPCR (RainDance Technologies, Lexington, MA) to quantify the amount of mutant and wild-type alleles in formalin-fixed, paraffin-embedded (FFPE) samples. In ddPCR, the input DNA sample extracted from FFPE samples is compartmentalized into more than one million droplets with RainDrop Source and the reaction is carried out individually within each droplet. Droplets are PCR amplified and fluorescently labeled, followed by counting endpoint fluorescence in an automated droplet flow cytometer (with RainDrop Sense). All droplet event data from each sample were converted to a two-dimensional histogram displaying 6-carboxyfluorescein (FAM) intensity on the *x*-axis and tetrachlorofluorescein (TET) intensity on the y-axis after spectral compensation. Droplet events within the wild-type gate and the mutant gate were counted as the concentration of allelic prevalence. Details of this procedure are provided in Supplementary Materials and Methods. The sequences of primers and probes for detection of the T790M mutation are given in Supplementary Table S1.

Deep sequencing

Details are described in Supplementary Materials and Methods.

Data analysis

Details are described in Supplementary Materials and Methods. Supplementary Figure S1 illustrates the decision-making flowchart for determining the presence or absence of the T790M mutation. Assays were considered "positive" if the measured event was ≥10 events/assay. Assays were considered "negative" if the event within a gated region was <10 events/assay. An assay was deemed inconclusive if the amount of amplifiable DNA (describe below) was less than 50 ng, at which point an additional assay was performed using 8 μL of DNA (double the volume of the initial assay).

Determination of the amount of amplifiable DNA

The number of PCR-positive droplets (total amount of the wild-type and mutant clusters) reveals the quantity of amplifiable target DNA fragments. The theoretical fraction of positive droplets was estimated as previously described (27, 28), and a Poisson distribution of genomic DNA was expected in the droplets.

Statistical analysis

To analyze the relationship between the pretreatment T790M mutation and the patients' characteristics, the χ^2 test was used, depending on the numbers of the patients. *P* values < 0.050 were considered to be statistically significant. All statistical analyses were performed using Prism software (GraphPad Software, Inc.).

Results

ddPCR for detecting the EGFR T790M mutation

We developed assays for detecting the EGFR T790M mutation; the assay was designed to test the specificity of both the wild-type and T790M oligonucleotides using a TET-labeled wild-type sequence probe and a FAM-labeled mutant sequence (2369C>T) probe, respectively. An *EGFR* wild-type sample produces only TET-labeled droplets, whereas an EGFR T790M mutant produces both FAM- and TET-labeled droplets (Fig. 1).

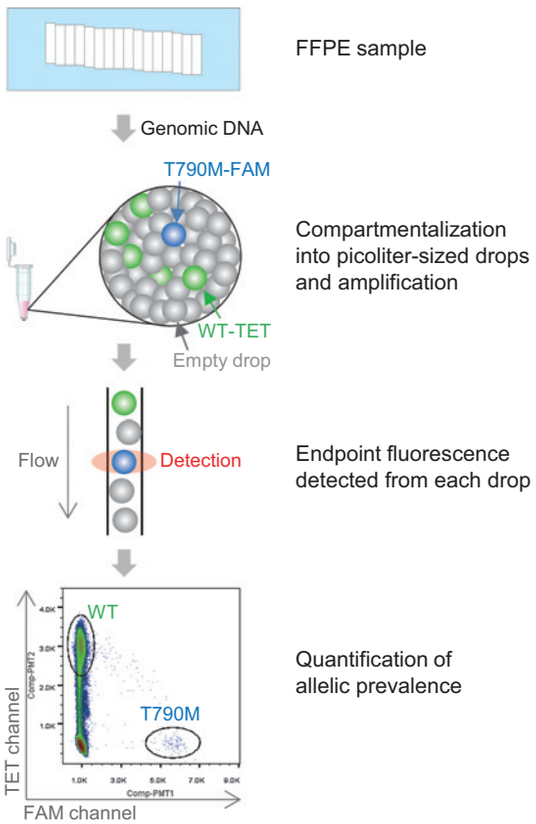

Figure 1.
Detection of mutant alleles using ddPCR. Genomic DNA from a FFPE sample is compartmentalized into millions of droplets containing 0 or 1 copy of target DNA (T790M mutant or wild type). After PCR cycling, droplets containing the endpoint fluorescence from each probe targeting mutant or wild-type DNA are detected using a flow cytometer. The detection of these signals allows quantification of allelic prevalence. Assays were considered "positive" if the measured event was ≥10 events/assay. FFPE, formalin-fixed paraffin-embedded; WT, wild-type; FAM, 6-carboxyfluorescein; TET, tetrachlorofluorescein.

Assessment of ddPCR assay sensitivity

The performance of our ddPCR assay was assessed using a plasmid containing the mutant sequence that was serially diluted with a plasmid containing the wild-type sequence. Results of the two-dimensional histogram from the ddPCR assay are summarized in Supplementary Fig. S2A. The expected copy number of mutant alleles (i.e., 0, 20, 200, or 2,000) spiked into the 200,000 wild-type alleles plotted against the actual copy number of mutant alleles observed in the samples is shown in Supplementary Fig. S2B. Regression analysis of the copy number of observed mutant DNA versus the copy number of expected mutant DNA produced a slope of 1.259, and a determination coefficient (R^2) of 0.998. The observed concentration of DNA matches the expected concentration over the range of 1% to 0.01% (Supplementary Fig. S2C). An ultra-rare mutation as low as 0.001% was successfully detected by the ddPCR assay (Supplementary Fig. S3).

The limit of blank (LOB) is the primary characteristic of an assay that determines the lower limit of detection, and the LOB was defined by the frequency of positive droplets

measured in wild-type samples as well as normal human genomic DNA. The number of false-positive droplet events using 200,000 copies of wild-type plasmid DNA controls, 400 ng of normal human genomic DNA (obtained from Clontech), and 400 ng of EGFR wild-type A549 cell line genomic DNA were measured for eight negative control experiments. Supplementary Table S2 shows the raw data for LOB analysis. The LOB was determined by evaluating the 95% one-tailed upper limit of the model distribution, as performed by investigators previously (28). The number of false events of mutant droplets detected per analysis is: seven from wild-type plasmid DNA, nine from human normal genomic DNA, and seven from A549 genomic DNA. Representative two-dimensional histograms from control samples are shown in Supplementary Fig. S4. We also performed ddPCR assay on formalin-fixed cell block using EGFR wild-type A549 cells as well as adjacent normal tissue samples as a control for formalin-fixed samples (Supplementary Fig. S5 and Supplementary Table S3). The LOB was comparable with the control experiment using unfixed samples, suggesting that formalin fixation and embedding in paraffin are unlikely to cause artificial mutation calls on our ddPCR assay.

Patient characteristics

Characteristics of all the patients who participated in the JME study were described elsewhere (31). The characteristics in this study are shown in Table 1. Patients had a median age of 69 years (range 37–87 years) and 276 patients (74.0%) were female. Never smokers were 280 (75.1%) and adenocarcinoma was the dominant histologic type (96.8%). Stages I, II, III, and IV were present in 290 (77.7%), 43 (11.5%), 32 (8.6%), and 8 (2.1%) patients, respectively.

Table 1. Patient characteristics

Patients, *n*	***n* = 373 (%)**
Age	
Median	69
Range	37–87
Gender	
Male	97 (26.0)
Female	276 (74.0)
Smoking status	
Never smoker	280 (75.1)
Smoker	93 (24.9)
Histologic type	
Adenocarcinoma	361 (96.8)
Squamous cell carcinoma	12 (3.2)
Stage	
I	290 (77.7)
II	43 (11.5)
III	32 (8.6)
IV	8 (2.1)
Tumor status	
T1	238 (63.8)
T2, T3, T4	135 (36.2)
N0	314 (84.2)
N1, N2	59 (15.8)
M0	365 (97.9)
M1	8 (2.1)
EGFR mutation status	
Common[a]	354 (94.9)
Uncommon[b]	19 (5.1)

[a]EGFR L858R and exon19 deletion.
[b]G719A, S, or C; L861Q; and G719A/L861Q double mutation.

The *EGFR* mutation status was assayed for each patient using the Cycleave PCR method by an independent medical laboratory and deep sequencing (the MiSeq sequencer, Illumina) in-house. Three hundred and fifty-four patients had the *EGFR* common mutation (199 patients had the L858R mutation and 155 patients had an exon 19 deletion). The ratio of exon 19 deletion and L858R mutation is different from previous reports (33), possibly owing to the fact that patients with non-smoking history and those with smoking history were accrued in a 1:1 fashion in JME study instead of all comers. Nineteen patients had the *EGFR* uncommon mutation [14 patients had G719 mutation (G719A = 8; G719S = 5; G719C = 1), 4 patients had the L861Q mutation, and 1 patient had the G719S/L861Q double mutation]. Moreover, 5 patients had EGFR T790M mutation. Exon 20 insertion was not assessed in this study.

ddPCR analysis of the pretreatment EGFR T790M mutation in DNA from lung cancer patients harboring EGFR-activating mutations

We first studied the EGFR T790M assay in 5 patients with lung cancer with the pretreatment EGFR T790M mutation in addition to the sensitizing EGFR mutation in their tumors detected by the MiSeq sequencer as a positive control for our assay. More than one thousand copies of the T790M-mutant allele were detected (Supplementary Table S4 and Supplementary Fig. S6), and the ratios of wild-type to mutant clones were identical between the MiSeq sequencer and ddPCR (Supplementary Table S4). These experiments were repeated over two nonconsecutive days by different operators. The regression analysis revealed a slope of 1.035 and a correlation coefficient (R^2) of 0.999 (Supplementary Fig. S7), demonstrating that our ddPCR assay provides a reliable and quantifiable measure of EGFR T790M-mutant alleles within an FFPE sample.

To demonstrate the possibility of detecting and quantifying T790M mutation alleles from pretreatment tumor samples of patients with lung cancer, we performed a ddPCR analysis of 373 tumor DNA samples. Using 10 events/assay as our threshold for a positive result, 298 of 373 (79.9%) cases could be correctly identified as positive for the EGFR T790M mutation. Representative positive and negative plots of patient data are shown in Fig. 2A. The percentage of T790M-mutant allele frequency in the 298 positive cases was shown in Fig. 2B, and the ratios of mutant alleles are summarized in Table 2. Among the 298 positive cases, six samples (2.0%) presented at a high ratio (>1%) that could be detected with the MiSeq sequencer. However, one of the six was not called by MiSeq or the Cycleave PCR method that was performed by SRL. The mutant ratio of this sample was 1.4%, which is the threshold of sensitivity of both methods. Sixteen samples (5.4%) presented at a moderate ratio (>0.1%) that could be detected with the Scorpion Amplification Refractory Mutation System. A total of 282 samples (94.6%) presented at a low ratio (<0.1%) that could not be detected with any of the commercially available methods used in this study. One sample (0.3%) presented at an ultra-low ratio (<0.01%). Sixteen samples were reanalyzed by ddPCR by another operator on a different day, and then checked for the reproducibility of the frequency of the T790M-mutant allele. The regression analysis demonstrated a slope of 1.021 and a correlation coefficient (R^2) of 0.998 (Supplementary Fig. S8), suggesting that our ratio data are a reproducible, independent range of ratios.

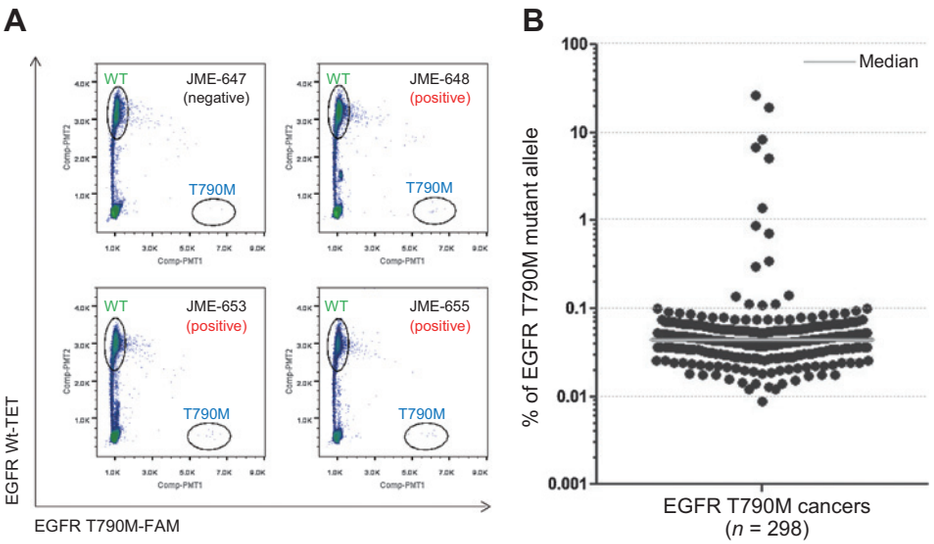

Figure 2.
Detection of EGFR T790M mutation alleles in NSCLC patient samples harboring EGFR activation mutations. A, representative positive and negative plots detected for patients are shown as an example. Four representative two-dimensional histograms of ddPCR assay from primary assay using 4 μL of genomic DNA. Results of primary mutation call are indicated under the sample ID. B, frequency of alleles is shown for 298 T790M mutant-positive NSCLC patients.

Correlations with clinicopathologic factors

Clinicopathologic correlations with the pretreatment T790M mutation were investigated. As shown in Table 3, no statistical difference was found between the pretreatment T790M mutation and such clinical parameters as age, gender, smoking status, histologic type, or tumor stage. Statistical differences were found in the T790M mutation-positive/negative ratio [79.9% (n = 298)/20.1% (n = 75)] versus both tumor status and EGFR-activating mutation status. Pretreatment T790M mutation-positive patients had significantly larger tumors than T790M-negative patients [T2-4: 88.9% (120/135), $P = 0.019$]. Although the T790M mutation was detected in 81.1% (287/354) of patients with EGFR common mutations, the T790M mutation was found to significantly decrease in patients with uncommon EGFR mutations [57.9% (11/19), $P = 0.022$]. We also investigated the relationship between concurrent gene mutation and the T790M mutation (Table 3). Among the 69 patients who had coincident *EGFR* and *p53* mutations, 61 patients (88.4%) had the T790M mutation ($P = 0.096$). Moreover, all 11 patients with *PIK3CA* and *EGFR* mutations had the T790M mutation (P = 0.097). These results, while not statistically significant, suggest that tumors with the pretreatment T790M mutation may manifest greater genomic complexity.

Assessment of ddPCR data

Amplifiable DNA concentrations in the FFPE samples varied from 13.2 ng/assay to 3837.8 ng/assay (data not shown). Figure 3A shows that the ddPCR assessment of amplifiable DNA concentration was independent of the number of mutant copies per assay, suggesting that the number of mutant copies is not due to the counting of false-positive droplets. However, Fig. 3B reveals that the distributions of amplifiable DNA concentrations were not similar for samples with mutations identified in the tumor DNA (light gray bar) or for samples with wild-type identified in the tumor DNA (dark gray bar). The combined distribution (black bar) shows that more than 50% of the samples had less than 100 ng of amplifiable DNA per assay. The frequency of wild-type samples was significantly higher in lower-amplifiable DNA than in overall (Supplementary Table S5). Most of the samples called wild-type were distributed in lower-amplifiable DNA, suggesting that samples called wild-type could contain false-negatives due to insufficient amplifiable DNA.

Table 2. Allele frequency of the EGFR T790M mutation in pretreatment NSCLC tumors harboring EGFR-activating mutations

% of MT allele	Samples, *n* (%)
≥10%	2 (0.5)
≥1%	4 (1.1)
≥0.1%	10 (2.7)
≥0.01%	281 (75.3)
≥0.001%	1 (0.3)
Negative	75 (20.1)

Discussion

We report the ultra-sensitive and quantitative analysis of the pretreatment EGFR T790M mutation in NSCLC samples via a picodroplet dPCR assay. Our results suggest the presence of a minor pretreatment EGFR T790M mutation in approximately 80% of NSCLC samples. Most of the pretreatment EGFR T790M mutations were below 0.1% in their frequency, which cannot be detected with commonly used methods in the clinical setting.

A number of efforts have been made to detect the pretreatment T790M mutation in recent studies (11–20). Using a ddPCR method, we identified the T790M mutation in 79.9% (n = 373) of samples, which was a far higher frequency than in previous reports that used direct sequencing (1.0%–7.5%, n = 34–783; refs. 11–15), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (1.9%–31.2%, n = 48–579; refs. 15,19), TaqMan assay (34.9%, n = 129; ref. 16), and an allele-specific selective androgen-receptor modulators assay (38.5%, n = 26; ref. 18). A higher frequency of the T790M mutation was observed using more sensitive methods (17,20). Costa and colleagues identified the T790M mutation in 65.3% in 95 patients with EGFR-mutant NSCLC using peptide-nucleic acid-clamping PCR with an analytical sensitivity of approximately 0.02% (20). Fujita and colleagues also identified the T790M mutation in 78.9% (n = 38) using colony hybridization

Table 3. Patient characteristics and T790M mutation status

Characteristics	Patients ($n = 373$; %)	Positive ($n = 298$, 79.9%; %)	P (χ^2)	Negative ($n = 75$, 20.1%; %)	P (χ^2)
Age					
<65	120 (32.2)	93 (31.2)	0.790	27 (36.0)	0.519
≥65	253 (67.8)	205 (68.8)		48 (64.0)	
Gender					
Male	97 (26.0)	77 (25.8)	0.961	20 (26.7)	0.905
Female	276 (74.0)	221 (74.2)		55 (73.3)	
Smoking status					
Never smoker	280 (75.1)	228 (76.5)	0.665	52 (69.3)	0.301
Smoker	93 (24.9)	70 (23.5)		23 (30.7)	
Histologic type					
Adenocarcinoma	361 (96.8)	288 (96.6)	0.920	73 (97.3)	0.803
Squamous cell carcinoma	12 (3.2)	10 (3.4)		2 (2.7)	
Stage					
I	290 (77.7)	227 (76.2)	0.695	63 (84.0)	0.330
II	43 (11.5)	37 (12.4)		6 (8.0)	
III	32 (8.6)	27 (9.1)		5 (6.7)	
IV	8 (2.1)	7 (2.3)		1 (1.3)	
Tumor status					
T1	238 (63.8)	178 (59.7)	0.280	60 (80.0)	0.007
T2, T3, T4	135 (36.2)	120 (40.3)		15 (20.0)	
N0	314 (84.2)	246 (61.8)	0.572	68 (90.7)	0.148
N1, N2	59 (15.8)	52 (17.4)		7 (9.3)	
M0	365 (97.9)	291 (97.7)	0.859	74 (98.7)	0.648
M1	8 (2.1)	7 (2.3)		1 (1.3)	
EGFR mutation status					
Common[a]	354 (94.9)	287 (96.3)	0.382	67 (89.3)	0.064
Uncommon[b]	19 (5.1)	11 (3.7)		8 (10.7)	
p53 mutation status					
Wild-type	304 (81.5)	237 (79.5)	0.521	67 (89.3)	0.101
Mutant	69 (18.5)	61 (20.5)		8 (10.7)	
PIK3CA mutation status					
Wild-type	362 (97.1)	287 (96.3)	0.592	75 (100)	0.132
Mutant	11 (2.9)	11 (3.7)		0 (0)	

[a]EGFR L858R and exon 19 deletion.
[b]G719A, S, or C; L861Q; and G719/L861Q double mutation.

with an analytical sensitivity of approximately 0.01% (17). Results of these two studies were comparable with ours; our results support their hypotheses in a larger cohort with a different and state-of-the-art detection method and these results may open up possibilities for the use of third-generation EGFR-TKIs as a first-line therapy in EGFR-mutant NSCLC.

The clinical significance of the minor pretreatment T790M mutation identified through a more sensitive assay has been discussed. In patients who have undergone surgery, the presence of the pretreatment T790M mutation has been associated with a favorable prognosis (17). This result is consistent with several studies that demonstrated that patients with the T790M mutation had a longer survival time among the patients who acquired EGFR-TKI resistance (34), and the cultured tumor cells harboring both the EGFR-activating and T790M mutations manifested slower growth (35), suggesting the prognostic value of T790M mutation. In contrast, five other studies have demonstrated a shorter PFS in advanced NSCLC patients harboring both the EGFR-activating and T790M mutations who were treated with EGFR-TKI (12, 15, 16, 18, 20), suggesting the predictive value of T790M mutation. However, the clinical significance of the pretreatment T790M mutation in patients with early-stage NSCLC has not previously been addressed on a large scale using an ultrasensitive method. JME study, the main study from which samples were obtained for this study, is a prospective, multicenter molecular epidemiology study and 10-year follow-up of enrolled patients is preplanned. Thus, detailed clinical information from this study cohort will be obtained during the follow-up period and analyses of the correlation between the pretreatment T790M mutation and clinical outcomes such as relapse, response to drugs, and survival will be performed.

The current study using an ultra-sensitive assay in a large cohort uncovered at least two genetic associations between clinicopathological features and the pretreatment T790M mutation. First, the T790M mutation was more frequently detected in patients with larger tumors (88.9%, $P = 0.019$), suggesting that larger tumors tend to include a greater number of rare T790M subclones. Second, the T790M mutation was detected significantly less frequently in patients with uncommon EGFR mutations (57.9%, $P = 0.022$), suggesting that the pretreatment T790M mutation may be associated with particular genetic backgrounds, the underlying genomic and biologic mechanisms of which should be further clarified. We also found that there is a tendency for the T790M mutation to correlate with mutations in *TP53* and *PIK3CA*; 88.4% ($P = 0.096$) or 100% ($P = 0.097$) of patients with *p53* or *PIK3CA* mutations had the T790M mutation, suggesting that *p53* and *PIK3CA* mutations are somehow associated with the pretreatment T790M mutation. Further investigation using follow-up clinical data is needed to understand the clinical significance of these concurrent mutations.

Some third-generation EGFR-TKIs have undergone evaluation in several clinical trials. These new drugs target the EGFR T790M

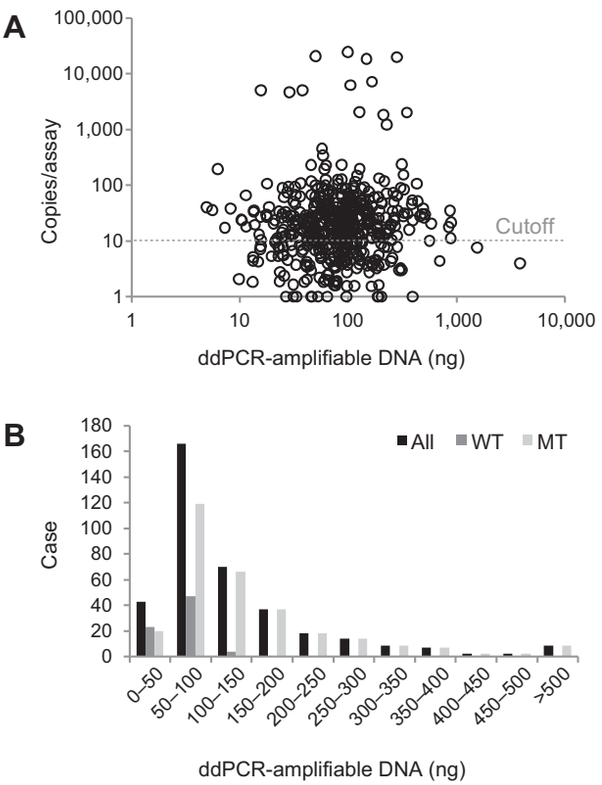

Figure 3.
Distribution of amplifiable DNA concentrations in the 373 analyzed samples. A, correlation between the quantity of amplifiable DNA and the number of mutant alleles per assay as determined by ddPCR. B, distribution of amplifiable DNA concentrations in the 373 analyzed tumor samples (black bar), T790M mutations (dark gray bar), and wild-type samples (light gray bar).

mutation as well as EGFR-activating mutations while sparing wild-type EGFR (10). A phase I study of CO-1686, a third-generation EGFR-TKI, is ongoing (NCT01526928). Preliminary results demonstrated promising antitumor activity with 58% of tumor shrinkage in patients with the T790M mutation (36). A preliminary phase I trial has performed for AZD9291, an oral, irreversible, and selective third-generation inhibitor of both EGFR-activating and T790M mutations (NCT01802632). This trial demonstrated partial tumor shrinkage in 91 of 177 (51.4%) patients receiving the drug; 132 of these patients had tumors that carried the T790M mutation, and 89 of those (67.4%) responded to treatment, with many other patients achieving disease stability (37, 38). Third-generation EGFR-TKIs as a first-line treatment may have the potential to eradicate tumors harboring EGFR-activating and pretreatment T790M mutations, and this should be evaluated in future studies. An ultra-sensitive and quantitative assay such as ddPCR assay would be advantageous in precisely diagnosing patients harboring low frequency pretreatment T790M mutation who seem to benefit from third-generation EGFR-TKIs.

Technical challenges remain with this type of highly sensitive method when dealing with very small quantities of amplifiable DNA. In the case of tissue-derived DNA, sensitivity depends, at least in part, on the amount of DNA being analyzed. For example, using 10 events/assay as the threshold, the estimated sensitivities of 50 ng and 100 ng of amplifiable DNA are 0.032% and 0.016%, respectively. Quality of DNA is also a bottleneck for high-throughput genotyping. In particular, the quality of DNA extracted from FFPE, a major source of tumor DNA samples in clinical settings, varies substantially, thus leading to unreliable sequencing data (39). ddPCR is also affected by the quality of DNA (28), suggesting that our actual detection rates described here might be higher than 80%. On the other hand, there was a recent report discussing artificial mutation calls resulting from formalin-fixed samples when using sensitive molecular assays (40). Although analytical cut-off of our ddPCR assay has been validated on the FFPE fixed tumor cell line as well as adjacent normal tissue samples, it needs to be determined according to the true clinical significance. In fact, T790M mutation with ultra-low frequency (11.42 mutant copies/130,383.89 wild-type copies: 0.009%) was detected in one patient and clinical significance of this ultra-rare frequent mutation remains unknown. This will be addressed in the near future when 10-year follow-up data are obtained.

To our knowledge, this is the largest study thus far using an ultra-sensitive method for detecting the pretreatment T790M mutation that has demonstrated associations between the incidence of the pretreatment T790M mutation and clinicopathological as well as genetic features in early-stage NSCLC. The data shown in this study suggest the potential of ddPCR as a high-resolution diagnostic tool for selection of patients with the aim of enabling more personalized therapies, and further validation based upon these initial results is needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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