Usefulness of Nanofluidic Digital PCR Arrays to Quantify T790M Mutation in *EGFR*-mutant Lung Adenocarcinoma

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Abstract. *Aim: The present pilot study assessed the usefulness of nanofluidic digital polymerase chain reaction (PCR) arrays in epidermal growth factor receptor (EGFR)-mutant lung adenocarcinoma after tyrosine kinase inhibitor (TKI) resistance. Patients and Methods: We enrolled 12 patients with primary lung adenocarcinoma with sensitive EGFR mutation-confirmed T790M status by re-biopsy after TKI resistance. Nanofluidic digital PCR arrays were used to quantify T790M in genomic DNA from the pre-treatment primary site and in serum cell-free DNA (cfDNA). Results: On digital PCR, quantified T790M at the pre-treatment primary site was higher in re-biopsy-positive T790M patients (n=4) than in re-biopsy-negative patients (n=8) (0.78%±0.36% vs. 0.07%±0.09%, p<0.01). T790M at the pre-treatment primary site correlated with progression-free survival (PFS) after gefitinib therapy (r=0.67, p=0.016). Conclusion: Use of digital PCR to quantify T790M at the primary site of EGFR-mutant lung adenocarcinoma predicted T790M emergence in re-biopsies after TKI resistance and PFS after gefitinib therapy.*

Abbreviations: NSCLC, Non-small-cell lung cancer; *EGFR*, epidermal growth factor receptor; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; PFS, progression-free survival; OS, overall survival; PCR, polymerase chain reaction; RR, response rate; DCR, disease control rate; cell-free DNA, cfDNA.

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Sensitive mutations in epidermal growth factor receptor (*EGFR*), such as 19 deletion and L858R in adenocarcinoma, are promising targets for EGFR tyrosine kinase inhibitors (EGFR-TKIs) (1-2). First-generation EGFR-TKIs, such as gefitinib and erlotinib, are recommended for treating *EGFR*-mutated adenocarcinoma (3-4). Recent randomized phase III studies (5-8) showed that progression-free survival (PFS) was significantly longer in patients with non-small-cell lung cancer (NSCLC) and sensitive mutations who received EGFR-TKIs as first-line therapy than in those who received platinum-based chemotherapy. Therefore, evaluation of sensitive *EGFR* mutations in patients with adenocarcinoma is essential in selecting appropriate therapies.

Unfortunately, most patients develop recurrence within 10-16 months after initial EGFR-TKI treatment (9). Approximately 60% of patients with acquired resistance to EGFR-TKIs have the second gatekeeper T790M mutation (T790M) (9-11). The T790M substitution alters proper binding of the drug to the ATP pocket of *EGFR* and/or restores the affinity for ATP *versus* the drug to that of wild-type *EGFR* (12, 13). Other reported mechanisms responsible for acquired resistance are *MET* amplification in 5% to 10% of cases (9, 14) and small-cell cancer transformation in less than 5% of cases (9, 15). The primary mechanism by which tumor cells acquire T790M remains controversial. One hypothesis is that a small number of tumor cells with T790M are present among pretreatment tumor cells, and tumor cells with T790M might be enriched during proliferation after TKI treatment (12). Researchers produced a model for studying acquired resistance in which T790M was developed *in vitro* by prolonged exposure of *EGFR*-mutated NSCLC cell lines to EGFR-TKIs (16, 17). In addition, the role of pre-existing T790M in these cell lines was investigated (18). The salient issue is whether the gatekeeping T790M mutation occurs *de novo* or whether pre-existing T790M is selected during EGFR-TKI exposure.

Table I. *Patients' characteristics (n=12).*

	Re-biopsy-positive T790M group (n=4)	Re-biopsy-negative T790M group (n=8)	*p*-Value
Age	68.8±13.0	59.3±16.8	0.24
Gender Male/Female	1/3	3/5	0.66
PS 0/1	2/2	7/1	0.41
Smoking history			
Current/former/never	0/0/4	0/1/7	0.71
EGFR mutation			
19Del/L858R	3/1	5/3	0.66
Gefitinib			
1st/2nd/3rd line	1/3/0	2/3/3	0.32
RR (%)	50	76	0.67
DCR (%)	100	100	-
Site of re-biopsy			
Lung/Lym/other*	3/1/0	5/2/1	0.75

PS, Performance status; *EGFR*, epidermal growth factor receptor; 19Del, exon 19 deletion; L858R, exon 21 L858R; RR, response rate; DCR, disease control rate; Lym, lymph node; *cerebrospinal fluid.

Table II. *Digital PCR array quantification of T790M (n=12).*

Case	EGFR mutation at primary site (standard method)	*Quantification of T790M (digital PCR)		T790M at recurrence site (standard method)
		Primary site	Serum cfDNA	
1	19del	1.21	0.02	+
2	L858R	0.83	0.00	+
3	19del	0.70	0.05	+
4	19del	0.33	0.00	+
5	19del	0.24	0.00	–
6	19del	0.21	0.02	–
7	L858R	0.20	0.03	–
8	L858R	0.07	0.03	–
9	19del	0.07	0.00	–
10	19del	0.04	0.00	–
11	19del	0.00	0.00	–
12	L858R	0.00	0.00	–

cfDNA, Cell-free DNA; *EGFR*, epidermal growth factor receptor; PCR, polymerase chain reaction; *Quantification of T790M, ratio of the number of positive T790M molecules to the number of positive exon 2 molecules (control).

There have been a number of advances in the clinical detection and assessment of T790M, including detection of a small T790M fraction among a large *EGFR* wild-type background. However, the sensitivity of traditional methods is still insufficient. Digital polymerase chain reaction (PCR) arrays are based on limiting-dilution analysis and can detect single molecules, thus enabling extremely sensitive detection and quantification. Wang *et al.* (19) reported that nanofluidic digital PCR can quantify *EGFR* copy number and can be optimized to a limit of 0.02% in genomic DNA prepared from formalin fixed, paraffin-embedded samples of early-stage resectable NSCLC tumors.

We evaluated the usefulness of nanofluidic digital PCR arrays in assessing *EGFR*-mutant lung adenocarcinoma at a pretreatment primary site and in serum cell-free DNA (cfDNA) after TKI resistance.

Patients and Methods

Digital PCR arrays. Nanofluidic digital PCR arrays (BioMark HD System, Fluidigm Japan K.K., Tokyo, Japan) were used to quantify T790M in genomic DNA from the pre-treatment primary site and in serum cfDNA after TKI resistance. The test kit uses a PCR-based assay in which specific areas of DNA containing mutations are targeted by Amplification Refractory Mutation System (ARMS®; QIAGEN KK, Tokyo, Japan) primers. Scorpions® technology (QIAGEN KK) is then used to amplify and detect those specific areas of DNA. Techniques based on ARMS primers are less time-consuming and more sensitive than screening technologies, such as DNA sequencing. Numbers of mutant molecules were estimated by counting the number of positive chambers in the digital PCR chip and were corrected using the Poisson equation. We assessed the ratio of the number of positive T790M molecules to the number of positive exon 2 molecules (control).

Clinical samples. We enrolled 12 patients with *EGFR*-mutant lung adenocarcinoma who had developed tumor recurrence after previous curative surgery. All patients had been treated with EGFR-TKIs during the period from January 2008 to January 2013 and later developed acquired resistance. After confirmation of EGFR-TKI resistance, re-biopsied specimens from the recurrent site were obtained from all 12 patients who were then classified as clinically T790M-positive or -negative, using standard clinical methods, such as the direct sequence method and the peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp method (20). Nanofluidic digital PCR arrays were used for highly sensitive T790M detection and quantification in 12 sample pairs of formalin-fixed, paraffin-embedded slides of tissue from the surgically resected primary site before EGFR-TKI treatment and cfDNA from peripheral blood after emergence of EGFR-TKI resistance. Using the QIAamp FFPE Tissue Kit (QIAGEN KK), we extracted DNA from formalin fixed, paraffin-embedded slides. The DNA was stored at 4˚C until use. Whole-blood samples were centrifuged at 250 × *g* for 10 min at room temperatureand the supernatants were stored at −80˚C until DNA extraction. cfDNA was extracted from the pellet of the supernatant with a QIAamp Circulating Nucleic Acid Kit (QIAGEN KK), according to the manufacturer's protocol, and was stored at 4˚C until use.

Clinical outcomes. We retrospectively analyzed the clinical characteristics, response rate (RR) and disease control rate (DCR) for EGFR-TKIs among the enrolled 12 patients. The PFS of patients treated with EGFR-TKIs was defined as the period from the date EGFR-TKI therapy was started to the date when the earliest sign of disease progression was confirmed clinically by findings from

Table III. *T790M positivity in re-biopsies after TKI resistance by digital PCR array (n=12).*

	T790M with digital PCR at primary site >0.285%	T790M with digital PCR at primary site ≤0.285%
Re-biopsy-positive T790M group	4	0
Re-biopsy-negative T790M group	0	8

PCR, Polymerase chain reaction; TKI, tyrosine kinase inhibitor. Sensitivity: 100%; specificity: 100%.

computed tomography or magnetic resonance imaging according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Overall survival (OS) was defined as the period from the date of EGFR-TKI initiation to the date of death from any cause.

Statistical analysis. Statistical analyses were conducted using the SPSS software for Windows, version 12.0 (SPSS Inc., Tokyo, Japan). Differences in clinical characteristics, RR, DCR and adverse events between patient groups were compared using the Fisher's exact test. To predict T790M positivity in re-biopsies, we determined the cut-off level of T790M at the pretreatment primary site. Survival curves were drawn by the Kaplan-Meier method and statistical analysis was performed using the log-rank test. Using Spearman's test, we analyzed correlations of T790M at the primary site with both PFS and OS after EGFR-TKI therapy.

This single-center study was conducted at Toho University (Tokyo, Japan) and was approved by the university Research Ethics Committee (authorization number: 25037).

Results

Characteristics of patients with and without T790M on re-biopsy. Patients were divided into 2 groups according to clinical T790M status after TKI resistance, as confirmed by standard techniques for re-biopsied specimens. The re-biopsy-positive T790M group comprised 4 patients and the re-biopsy-negative T790M group comprised 8 patients. There were no significant differences between these groups in clinical characteristics, including age, sex, performance status, clinical stage, smoking history, *EGFR* mutation status, RR and DCR of gefitinib therapy (Table I). Re-biopsy specimens were obtained from lung or lymph nodes, with the exception of 1 patient in the re-biopsy-negative group from whom cerebrospinal fluid was collected.

Outcomes in patients with and without T790M. We retrospectively analyzed PFS on gefitinib and OS among both groups. The re-biopsy-positive T790M group had a tendency toward better PFS as compared with the re-biopsy-negative T790M group (median, 688 *vs*. 319 days, respectively; p=0.16). Median OS was 1,874 days in the re-biopsy-positive T790M group and 1,310 days in the re-biopsy-negative T790M group (p=0.11).

Digital PCR array quantification of T790M at the pretreatment primary site. T790M was analyzed by digital PCR in 12 samples of formalin-fixed, paraffin-embedded slides of surgically-resected tissue from primary sites obtained at initial curative surgery. Digital PCR arrays were able to detect and quantify T790M in 10 of the 12 samples (83.3%) from pretreatment primary sites (Table II). On digital PCR, the quantified levels of T790M at the pretreatment primary site were significantly higher in the re-biopsy-positive T790M group (n=4) than in the re-biopsy-negative T790M group (n=8) (0.78%±0.36% *vs*. 0.07%±0.09%, respectively; p=0.0025; Figure 1).

Prediction of T790M emergence after EGFR-TKI resistance. To identify emergence of T790M mutation in re-biopsied specimens, we calculated the cut-off level for digital PCR-quantified highly sensitive T790M at the pretreatment primary site. The receiver operating characteristic curve indicated that the optimal cut-off level for pretreatment T790M in the prediction of clinical T790M emergence after EGFR-TKI resistance was 0.285%. The sensitivity and specificity were both 100% (Table III).

Correlation of digital PCR-quantified T790M level at the primary site with PFS and OS after gefitinib therapy. Using the Spearman's test, we analyzed the correlations of digital PCR-quantified level of T790M at the primary site with PFS and OS after gefitinib therapy. T790M level at the pretreatment primary site was significantly positively correlated with PFS after gefitinib therapy (r=0.67, p=0.016; Figure 2) but not with OS after gefitinib therapy (r=0.49, p=0.11).

Digital PCR array quantification of T790M at cfDNA. Digital PCR was used to analyze T790M in peripheral blood (cfDNA) from the same 12 patients after emergence of EGFR-TKI resistance. T790M was detected in 5 of the 12 cfDNA samples (41.7%) and was quantified as 0.00%-0.05% (Table II). The quantified levels of T790M in serum cfDNA after TKI resistance did not significantly differ between the re-biopsy-positive T790M group and re-biopsy-negative T790M group (0.018%±0.023% *vs*. 0.01%±0.014%, respectively; p=0.11).

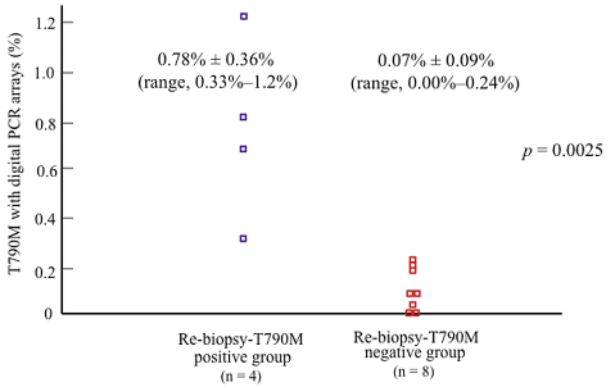

Figure 1. *Quantification of T790M at the pretreatment primary site by digital PCR array. T790M levels at the pretreatment primary site were significantly higher among T790M-positive patients (n=4) than among T790M-negative patients (n=8) (0.78%±0.36% vs. 0.07%±0.09%; p=0.0025). PCR, polymerase chain reaction.*

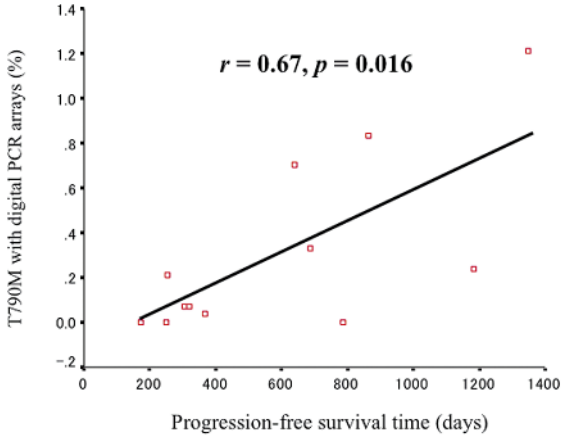

Figure 2. *Correlation between T790M level at the primary site, quantified by digital PCR array and PFS after gefitinib therapy. T790M level was significantly positively correlated with PFS after gefitinib therapy (r=0.67, p=0.016). PCR, polymerase chain reaction; PFS, progression-free survival.*

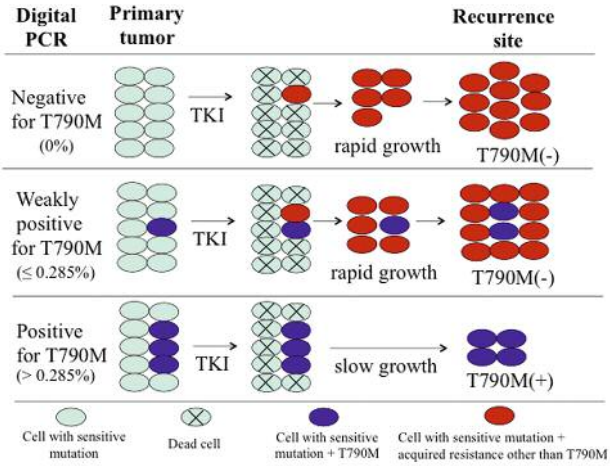

Figure 3. *A hypothesis regarding acquired tyrosine kinase inhibitor (TKI) resistance using digital polymerase chain reaction (PCR). In pretreatment T790M-negative patients (confirmed by digital PCR), tumor cells with acquired resistance but without T790M mutation grow rapidly after the death of sensitive tumor cells. In patients with a weakly positive result for T790M, both tumor cells with acquired resistance but without T790M mutation and tumor cells with T790M survive; however, the former grow rapidly and become dominant. In T790M-positive patients, tumor cells with T790M grow slowly resulting in indolent tumor progression and longer progression-free survival (PFS) on gefitinib.*

Discussion

The current standard testing method is able to detect the T790M mutation in fewer than 3% of primary tumors before TKI treatment (21). Recently, the results of highly sensitive methods have been reported. The T790M mutation was detected in 31.5% of primary tumors by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (21), in 65% of tumors by laser microdissection and peptide-nucleic acid-clamping PCR (22) and in 79% of tumors by colony hybridization (23). In our study, nanofluidic digital PCR arrays detected T790M in 83.3% of pre-treatment primary sites, which was a higher rate than in previous reports. This suggests that an extremely small number of cancer cells with *de novo* T790M -undetectable by standard testing methods- is present in the vast majority of pretreatment *EGFR*-mutant tumors.

Without re-biopsy, it is difficult to identify the primary mechanism of TKI resistance in a patient. Using highly sensitive nanofluidic digital PCR arrays, we found that the quantified level of T790M at the pre-treatment primary site was significantly higher in re-biopsy-positive T790M patients than in T790M-negative patients (p=0.0025). Although the present findings are retrospective, a quantified T790M level greater than 0.285% at the pretreatment primary site on digital PCR was accurate in predicting clinical T790M emergence after TKI resistance. These results suggest, because of selection pressure, a minute population of *de novo* T790M cells at the pre-treatment primary site gradually increases after EGFR-TKI therapy. The higher prevalence of T790M at the pretreatment primary site on digital PCR likely increases the risk of TKI resistance associated with acquired T790M.

Oxnard *et al.* (24) reported that outcomes were better in TKI-resistant patients with T790M identified in re-biopsy specimens than in TKI-resistant patients without T790M.

In addition, pre-clinical data showed that growth was slower in *EGFR*-mutant cell lines that acquired T790M than in parental cell lines (25). Our results confirm these earlier findings. Using a colony hybridization method, Fujita *et al*. (23) reported that time-to-TKI treatment failure was significantly longer for patients with a high proportion of T790M colonies detected in pre-treatment samples than those with a low proportion of or no T790M colonies. In our study, the digital PCR-quantified level of T790M at the pretreatment primary site was significantly positively correlated with PFS after gefitinib therapy (r=0.67, p=0.016).

One hypothesis regarding acquired TKI resistance is shown in Figure 3. First, in digital PCR-confirmed T790M-negative patients, tumor cells with acquired resistance but without T790M mutation grow rapidly after the death of sensitive tumor cells. Second, in patients with a weakly positive result for T790M on digital PCR, tumor cells with acquired resistance other than T790M grow rapidly and become dominant, while tumor cells with acquired resistance other than T790M, such as *MET* amplification, and tumor cells with T790M, survive after the death of sensitive tumor cells. Third, in patients with a positive result for T790M on digital PCR, tumor cells with T790M grow slowly resulting in indolent tumor progression and long PFS on gefitinib. Further studies are required in order to test this hypothesis (Figure 3).

A novel non-invasive method using droplet digital PCR to genotype plasma cfDNA has been reported (26). However, in 3 out of 9 patients (33.3%), plasma cfDNA genotyping could not detect T790M at the time of disease progression, probably because indolent progression was limited to the thoracic cavity in those 3 patients. In our study, nanofluidic digital PCR arrays could not detect T790M in 2 of 4 serum cfDNA samples (50%) from patients with re-biopsy-positive T790M. In addition, tumor progression in these cfDNA false-negative patients was limited to pulmonary metastases. Moreover, the quantified levels of T790M in serum cfDNA after TKI resistance did not significantly differ between re-biopsy-positive and -negative T790M patients. Indolent T790M-positive tumor cells, in addition to growing slowly, might spread only within the thoracic cavity, which would impede detection using cfDNA in peripheral blood. Future research should aim to develop a non-invasive digital PCR technique for repeated detection and quantification of the T790M mutation.

The major limitation of the present study is that it is a retrospective single-Center study with a small sample size. A multicenter study is needed in order to confirm the usefulness of digital PCR. Third-generation TKIs, such as AZD9291 (27) and CO-1686 (28), are expected to overcome T790M-associated EGFR TKI resistance. In the near future, new treatment strategies using digital PCR should be established for patients with *EGFR*-mutant NSCLC and the T790M mutation. In conclusion, use of digital PCR to quantify T790M at the primary site of *EGFR*-mutant lung adenocarcinoma was useful in predicting T790M positivity in re-biopsies after TKI resistance and progression-free survival after gefitinib therapy.

Conflicts of Interest

No conflicts of interest to declare.

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