Picoliter-Droplet Digital PCR-based Analysis of Cell-free Plasma DNA to Assess *EGFR* Mutations in Lung Adenocarcinoma That Confer Resistance to Tyrosine Kinase Inhibitors

Running Title: *EGFR* T790M mutations assessed by picoliter-ddPCR

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Abstract

Purpose: To evaluate the utility of analyzing cell-free plasma DNA (cfDNA) by picoliter-droplet digital PCR (picoliter-ddPCR) to detect *EGFR* mutations that confer resistance to tyrosine kinase inhibitors (TKIs) used for treatment of lung adenocarcinoma (LADC).

Experimental Design: Thirty-five LADC patients who received EGFR-TKI therapy, including ten who received tumor re-biopsy after development of resistance, were subjected to picoliter-ddPCR-cfDNA analysis to determine the fractions of cfDNAs with TKI-sensitive (L858R and inflame exon 19 deletions) and -resistant (i.e., T790M) mutations, as well as their concordance with mutation status in re-biopsied tumor tissues.

Results: cfDNAs from 15 (94%) of 16 patients who acquired resistance were positive for TKI-sensitive mutations. Seven (44%) were also positive for the T790M mutation, with fractions of T790M (+) cfDNAs ranging from 7.4% to 97%. T790M positivity in cfDNA was consistent in eight of ten patients for whom re-biopsied tumor tissues were analyzed, whereas the remaining cases were negative in cfDNA and positive in re-biopsied tumors. Prior to EGFR-TKI therapy, cfDNAs from 9 (38%) and 0 of 24 patients were positive for TKI-sensitive and T790M mutations, respectively. Next-generation sequencing of cfDNA from one patient, who exhibited innate resistance to TKI despite a high fraction of TKI-sensitive mutations and the absence of the T790M mutation in his cfDNA, revealed the presence of the L747P mutation, a known driver of TKI resistance.

Conclusions: Picoliter-ddPCR examination of cfDNA, supported by next-generation sequencing analysis, enables non-invasive assessment of EGFR mutations that confer resistance to TKIs.

Implications for Practice

Non-invasive monitoring of the predominance of tumors harboring the secondary T790M mutation in the activating mutation in *EGFR* gene is necessary for precise and effective treatment of lung adenocarcinoma (LADC). Because cells harboring the T790M mutation are resistant to EGFR-TKIs, the predominance of tumor cells harboring the T790M mutations influences the choice of whether to use conventional or next-generation TKIs. Digital PCR-based examination of cfDNA is a promising method; however, its feasibility, including consistency with examination of re-biopsied tumor tissue, has not been fully proven. We present a picoliter-ddPCR technology as a candidate method for testing cfDNA and assessing the predominance of T790M-mutant tumors.

Introduction

EGFR (epidermal growth factor receptor) is a driver gene of non-small cell lung cancer (NSCLC), particularly lung adenocarcinoma (LADC). Activating somatic mutations in this gene define a subset of cases that respond to specific EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib and erlotinib [1,2]. The most frequent mutations in EGFR occur in the exons encoding the kinase domain of EGFR, including various types of in-frame deletions in exon 19 (19del) and a point mutation in exon 21 leading to the substitution of leucine for arginine at position 858 (L858R). Tumors harboring these TKI-sensitive mutations nearly always acquire resistance to TKIs within 2 years [3,4]. The most common mechanism of resistance, accounting for 60% of cases, is the occurrence of the secondary mutation T790M (replacing a gatekeeper amino acid) in the *EGFR* allele harboring the TKI-sensitive mutation [5]. To overcome resistance to conventional EGFR-TKIs, a new generation of drugs (including AZD9291, CO-1686, and HM61713) that suppress the kinase activity of EGFR proteins harboring secondary T790M substitutions is currently being developed [6-9]. Phase I clinical trials have demonstrated that progressed NSCLC patients who are diagnosed with T790M-positive tumors by genetic testing of re-biopsied tumor tissues respond to these new drugs [10]. However, because the new drugs bind their targets irreversibly, they are associated with severe side effects that are not observed during conventional EGFR-TKI therapy. In addition, other mutations in EGFR also confer resistance [11]. Therefore, to achieve precise and effective treatment of *EGFR* mutation-positive NSCLC patients, it is necessary to monitor the predominance of *EGFR* mutations that confer TKI resistance during therapy; the

choice between conventional and next-generation EGFR-TKIs must be made based on the identities of the *EGFR* mutations conferring TKI resistance [6,7].

Circulating plasma cell-free DNA (cfDNA), which is released into plasma from tumor tissues or circulating tumor cells (CTCs), represents a non-invasive liquid biopsy that could provide genetic information about CTCs and residual tumor cells [12-14]. cfDNA is particularly attractive for use in the lung cancer clinic due to the occasional difficulty of obtaining tumor tissues with high cellularity [15,16]. Indeed, *EGFR* mutations present in tumor cells can be detected in the cfDNA of NSCLC patients using digital PCR [17-20] and next-generation sequencing (NGS) [21,22]. In particular, TKI-sensitive and T790M mutations in the cfDNA of NSCLC patients have been successfully detected using a digital PCR-based method called BEAMing (beads, emulsion, amplification, and magnetics) [15,21,23,24]. Thus, cfDNA represents a promising source of material for non-invasive monitoring of tumor burden. However, several issues need to be resolved before these methods can be applied in the lung cancer clinic, including the concordance of T790M mutation status between cfDNA and re-biopsied lung cancer tissues, as well as their compatibility with *EGFR* mutation tests currently performed for biopsied tumor tissue. Regarding the latter point, routine *EGFR* mutation tests, such as the Scorpion/ARMS assay, provide information about the presence/absence and location of driver mutations, but not the exact sequence of the mutants, in each tumor [15,21]. Therefore, cfDNA examination during EGFR-TKI therapy should utilize this information.

In this study, we established a picoliter-droplet digital PCR (ddPCR) system to quantify TKI-sensitive and -resistant *EGFR* mutations in the cfDNA of patients who were treated with the conventional EGFR-TKIs. Our picoliter-ddPCR system has several advantages. First, the major types of *EGFR* exon 19 in-frame deletions can be detected in a single assay using a common probe, which enables us to detect > 95% of known mutations [25] without prior information about the exact mutant DNA sequence, which cannot be provided by the routine tests. Second, picoliter-ddPCR is performed in millions of droplets, including hundreds to thousands of droplets containing a single molecule of template DNA, preventing inaccuracy due to the inclusion of two or more *EGFR* DNA molecules in a droplet; therefore, this assay yields accurate estimates of the fraction of mutant DNA. Third, the assay is simple and rapid, consisting only of the PCR and detection procedures (**Supplementary Fig. 1**), making it feasible for routine use in the lung cancer clinic.

We examined cfDNA samples from 35 LADC patients who received EGFR-TKI therapy: 16 provided cfDNA after developing resistance to EGFR-TKIs, including five who also provided cfDNA before TKI-therapy, and 19 provided cfDNA before EGFR-TKI therapy. We examined the samples using picoliter-ddPCR to determine the fraction of cfDNAs with EGFR-T790M mutation to assess the predominance of T790M-positive tumor cells and the concordance of T790M mutation status between cfDNA and re-biopsied tumor tissues.

Materials and methods

Picoliter-droplet digital PCR (picoliter-ddPCR)

The assay for detecting representative exon 19 in-frame deletions employed two TaqMan probes (**Fig. 1A, Supplementary Table 1**). The FAM-labeled wild-type probe was designed to hybridize to a region in *EGFR* exon 19, where in-frame deletions occur; therefore, this probe does not hybridize with deletion alleles. The VIC-labeled reference probe was designed to hybridize to a region in *EGFR* exon 19, where in-frame deletions do not occur; therefore, this probe hybridizes with both wild-type and deletion alleles. This assay was designed to detect 34 representative exon 19 deletions covering >95% of deletion mutations in LADC [25]: qpK745_E749, K745_T751>K, qpE746_A750, E746_R748>E, E746_A750>IP, E746_T751>IP, E746_T751>I, E746_T751>V, E746_T751>A, E746_T751>VA, E746_S752>I, E746_751T>E, E746_S752>A, E746_S752>V, E746_P753>VS, E746_A755>E, qpE746_T751, qpE746_S752, E746_S752>D, qpE746_S753, qpL747_E749, qpL747_A750, L747_A750>P, qpL747_T751, L747_T751>Q, L747_T751>P, L747_T751>S, qpL747_S752, L747_S752>Q, qpL747_P753, L747_P753>V, L747_P753>S, L747_P753>Q, and S752-I 759del. The assays to detect the L858R and T790M mutations also employed two TaqMan probes, one specific for the mutant allele and the other for the wild-type allele. For the L858R mutation, the mutant-specific probe detected the 2753T>G mutation, the predominant form of the L858R mutation. For the T790M mutation, a probe that detected the 2369C>T mutation was used [26].

Digital PCR was performed using the RainDrop Digital PCR System (RainDance Technologies, Billerica, MA, USA), in which PCR takes place in millions of droplets with

volumes of ~5 pL [27-29] (**Supplementary Fig. 1**). PCR solutions (40 µL) were prepared by mixing 20 µL of QuantStudio® 3D Master Mix (Life Technologies, Grand Island, NY, USA), 4 µL of 10× Droplet Stabilizer (RainDance Technologies), 2 µL of TaqMan SNP Genotyping Assay, and DNA. The mixture was subjected to emulsification, followed by PCR: 95°C × 10 min (1 cycle); 45 cycles of 95°C × 15 s and 60°C × 1 min; 98°C × 10 min; and a 10°C hold. The endpoint fluorescence signal (i.e., the fluorescence intensities of VIC [red] and FAM [green]) of each individual droplet was measured and visualized as clusters in a 2-dimensional histogram. Spectral compensation was applied to each sample to eliminate contamination of fluorescence signals between the VIC and FAM fluorophores. Compensation factors, as well as the respective thresholds to define droplets positive for exon 19 deletions and the L858R and T790M mutations, were set based on data from positive-control cell lines.

Analysis of genomic DNA from lung cancer cell lines

Genomic DNA extracted from cell lines was used to assess the accuracy and reproducibility of picoliter-ddPCR. The II-18 lung cancer and ACC-MESO-1 mesothelioma cell lines were obtained from the RIKEN BioResource Center (Tsukuba, Japan). The NCI-H1975 lung cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The PC−9 lung cancer cell line was obtained from the late Dr. Yoshihiro Hayata of Tokyo Medical University, who established this cell line. In the II-18, H1975, and PC−9 cell lines, the alterations of cancer-related genes were consistent with those in previous reports and in the COSMIC database, confirming cell line authenticity [30-32].

We confirmed a lack of *EGFR* mutations in ACC-MESO-1 by real-time PCR. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). After DNA was sheared to a size range of 2ï 4 kb using a CovarisÊ S2 System (Covaris, Massachusetts, USA), DNA fragments were purified using the MinElute PCR Purification kit (Qiagen). Quantitative and qualitative analyses of the purified products were performed on a 2100 Bioanalyzer (Agilent Technologies, Boeblingen, Germany). Genomic DNA from those cell lines, as well as H1975 DNA serially diluted from 50% to 1.56% with ACC-MESO-1 DNA, was subjected to picoliter-ddPCR in triplicate. The amounts of DNA used in picoliter-ddPCR are described in the legend of **Supplementary Fig. 2**. The linear correlation coefficient values (R^2) were calculated using the IBM SPSS Statistics software (version 20.0: IBM, New York, NY, USA). The picoliter-ddPCR experiments were designed and performed following the essential requirements in the MIQE guidelines for ddPCR [33].

Feasibility assessment of picoliter-ddPCR

The feasibility of the picoliter-ddPCR assay described above was examined using DNA from three *EGFR*-mutant lung cancer cell lines: PC−9, harboring an exon 19 deletion; II-18, harboring the L858R mutation; and NCI-H1975, harboring the T790M and L858R mutations [34,35]. Detection of *EGFR*-mutated DNA was reproducible, and background mutations were not detected (**Supplementary Fig. 2A**). The quantitative nature of the assay was validated using serially diluted DNA (**Supplementary Fig. 2B**, $R^2 = 0.96$), and the limit of detection was defined as more than 0.75% mutant alleles. The calculated fraction of

T790M alleles in all TKI-sensitive alleles was reproducible when as little as 2 ng of DNA was used in picoliter-ddPCR (**Supplementary Fig. 2C**); under these conditions, hundreds of droplets positive for *EGFR* wild-type alleles were detected. Thus, we concluded that this method was suitable for examining the proportion of alleles with TKI-sensitive mutations that also harbored the T790M mutation.

Analysis of cfDNA from NSCLC patients

Peripheral blood samples were collected after obtaining written informed consent from 35 LADC patients receiving EGFR-TKI therapy. From 16 of the 35 patients, blood samples were collected after the confirmation of resistance. Five of these 16 patients also provided blood samples before the administration of EGFR-TKI, therefore, a total of 21 blood samples were collected from the 16 patients who acquired resistance (i.e., 16+5=21). From the remaining 19 patients, blood samples were collected only before the administration of EGFR-TKI. Thus, a total of 40 (i.e., 21+19) peripheral blood samples were collected from 35 LADC patients. In addition, peripheral blood samples were also collected from two patients with LADC harboring the *EML4-ALK* fusion; these samples were used as *EGFR* mutation-free controls. From each patient, blood samples were collected in two 5 mL EDTA-containing Vacutainers and spun to separate plasma within 30 min of collection. Plasma samples were kept frozen at -80°C until DNA extraction. Plasma samples obtained from Patients 1, 8, 10, and 11 before the initiation of EGFR-TKI therapy were also analyzed in this study. cfDNA was extracted from 2 ml of plasma using the QIAamp Circulating

Nucleic Acid kit (Qiagen, USA) and quantitated using a NanoDrop spectrophotometer (Thermo Fisher Scientific/Life Technologies) and a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). cfDNA was used in picoliter-ddPCR without being sheared. Reaction mixtures containing 4 ng of cfDNA, as determined using a Qubit fluorometer (and corresponding to the amount of cfDNA obtained from 1 ml of plasma in the majority of cases), were subjected to picoliter-ddPCR in triplicate (Patients 1ï 3, 13, and 17) or in single reactions. This study was approved by the institutional review board of the National Cancer Center. The study was registered in the UMIN Clinical Trial Registry (UMIN 000017581).

Threshold setting for judgment of positivity

To set the threshold for calling *EGFR* mutations in patient cfDNA, cfDNA from two patients with LADC harboring the *EML4-ALK* fusion were subjected to picoliter-ddPCR. These samples were considered negative for *EGFR* mutations because the *ALK* fusion is mutually exclusive with *EGFR* mutations in lung cancer [36]. Only a few droplets among millions were positive for *EGFR* mutations (mean = 2.5, standard error [SE] = 1.4) (**Supplementary Fig. 3**). It remained possible that a few *EGFR* mutations were present, even in *ALK* fusion-positive LADC patients; however, to avoid false-positive results, the threshold for a positive call was tentatively set to ten droplets, based on the following equation: mean + 5 × SE = 9.5. Using this threshold, the rate of false-positive droplet detection was predicted to be less than 0.0002%.

EGFR mutation analysis in biopsied tumor tissue

Formalin-fixed, paraffin-embedded (FFPE) tumor tissues from patients were submitted for high-resolution melting analysis (HRMA) or Scorpion-ARMS-based diagnosis of *EGFR* mutations during the course of standard clinical practice between December 2003 and June 2015. EGFR-TKI-resistant tumor tissues were obtained by re-biopsy of pericardium (Patient 1), primary disease (Patients 2 and 9), liver metastasis (Patients 3, 5, and 7), pleural effusion (Patients 8 and 10), and lymph node (Patients 4 and 6), and were subjected to Scorpion-ARMS-based clinical examination for T790M mutations.

Genome-capture deep sequencing using a next-generation sequencer

Nucleotide sequences of *EGFR* were examined by targeted genome-capture and massively parallel sequencing using a MiSeq sequencer and a 90-gene targeted panel, the NCC oncopanel (Cat No. 931196, Agilent). One microgram of cfDNA was subjected to enrichment using the probes. The mean depth of sequencing was 1027.

Results

Picoliter-ddPCR to detect *EGFR* mutations in cfDNA

Picoliter-ddPCR assays to detect exon 19 deletions and L858R and T790M mutations were designed as shown in **Fig. 1A**. In this analysis, each cfDNA sample was subjected to two picoliter-ddPCR assays, one for the T790M mutation and the other for either

a TKI-sensitive exon 19 deletion or a L858R mutation, chosen based on a routine clinical test of tumor samples before EGFR-TKI therapy. The assay for exon 19 deletions was designed to detect loss of the wild-type signal and could, therefore, detect representative in-frame deletion mutations in exon 19. The L858R and T790M assays were designed to detect wild-type and mutant alleles with each probe. Based on data from these two picoliter-ddPCR assays, the proportion of T790M cfDNA among all tumor cfDNA, as represented by TKI-sensitive mutations, was deduced as shown in **Fig. 1B**.

Study cohort consisting of 35 LADC patients who received EGFR-TKI therapy

We prepared a cohort of 35 patients with advanced LADCs (**Table 1**). Their tumor tissues obtained before EGFR-TKI therapy were diagnosed positive for TKI-sensitive *EGFR* mutations; all patients received molecular-targeted therapy with EGFR-TKIs (**Table 1**). In 16 cases (Patients 1ï16: TKI-resistant cohort), cfDNA was obtained after the confirmation of resistance to EGFR-TKIs (**Fig. 2**). From five cases (Patients 1, 8, 10, 11, and 12), cfDNA was also available from before EGFR-TKI therapy. In the other 19 cases (Patients 17ï35: pre-TKI cohort), cfDNA was obtained only before initiation of EGFR-TKI therapy (**Supplementary Fig. 4**).

cfDNA analysis for TKI-sensitive and T790M mutations

We first examined cfDNA obtained after acquisition of resistance in 16 cases of the TKI-resistant cohort. cfDNA from 15 (94%) and seven (44%) patients were positive for TKI-sensitive and T790M mutations, respectively (**Fig. 2 and 3, detailed data in Table 1 and Supplementary Table 2**). In 10 of these 16 cases, re-biopsied tumor tissues were subjected to a T790M test after they became resistant to EGFR-TKI (Patients 1ï 10 in **Fig. 2**), and seven (Patients 1ï 7) are positive and the remaining three (Patients 8-10) were negative for T790M mutation, respectively. The positivity and negativity of T790M mutation between cfDNA and re-biopsied tumors was concordant in eight patients (concordance rate is 0.8), while, in the remaining two cases, only re-biopsied tumors, but not the cfDNAs, showed the positivity. If we judge the test results of re-biopsied tumors as being correct, the present cfDNA assay was estimated to have a sensitivity of 71% (5 detected in cfDNAs / 7 detected in re-biopsied tumors), without giving false positives (**Supplementary Fig. 5**).

Among the 16 patients in the TKI-resistant cohort, cfDNA obtained before EGFR-TKI therapy was available from five (Patient 1, 8, 10, 11, and 12; **Fig. 2**). Three of them were positive for a TKI-sensitive mutation, whereas all were negative for the T790M mutation. Consistent with this, cfDNAs obtained from 19 patients before EGFR-TKI therapy (case 17ï 35) were all negative for T790M mutations, whereas six cases (32%) were positive for TKI-sensitive mutations (**Supplementary Fig. 4**). In total, 9 (36%) of the 24 cfDNA samples obtained before EGFR-TKI therapy were positive for TKI-sensitive mutations, whereas all were negative for the T790M mutation (**Fig. 3**), further supporting the idea that the T790M mutation is enriched after acquiring resistance to the EGFR-TKI therapy.

306

307 **Deduction of tumor predominance by plasma cfDNA profiling**

308 The predominance of TKI-resistant tumors was deduced from profiles of *EGFR*
309 mutations in cfDNA in each case. Seven cases (Patients 1ï 5, 14, and 15 in **Fig. 2**) acquired
310 EGFR-TKI resistance, associated with the occurrence of the T790M mutation in cfDNAs. In
311 these cases, the proportion of T790M mutant tumor alleles ranged from 7.4% to 97% (**Table**
312 **1**). Thus, the proportion of tumor cells harboring T790M mutations differed among the cases
313 following acquisition of resistance.

314 cfDNAs obtained both before and after acquisition of EGFR-TKI resistance were
315 analyzed in five of the cases (Patients 1, 8, 10, 11, and 12). The fraction of cfDNAs with
316 TKI-sensitive mutations was similar or greater than that before therapy in all of these cases
317 (shown by circle size in **Fig. 4A, B,** and **Fig. 4C**). Therefore, tumor progression during
318 EGFR-TKI therapy often (but not always) results in an increase in the proportion of cfDNA
319 from tumor cells, whereas the T790M mutation appears after acquisition of resistance in a
320 subset of cases (shown by black area in **Fig. 4A, B,** and **D**).

321 Patient 12 had the highest fraction (69.3%) of TKI-sensitive mutant DNAs among
322 the TKI-resistant cohort, but did not have the T790M mutation (**Table 1**). Notably, this case
323 had a high fraction (60.6%) of TKI-sensitive mutant DNAs before EGFR-TKI therapy and
324 maintained a high fraction of TKI-sensitive mutant DNAs throughout the therapy (**Fig. 4C**).
325 This patient did not respond to gefitinib and was the only case in this cohort with progressed
326 disease (**Table 1**). To determine the mechanism underlying this innate EGFR-TKI resistance,

the patientôs cfDNA was further analyzed by deep-target sequencing. NGS results revealed the L747P (c.2239_2240TT>CC) mutation at a high allele frequency (69%), but no in-frame deletion mutation in EGFR exon 19 (**Fig. 4C**). L747P is a known driver mutation in *EGFR* that is mis-diagnosed as an exon 19 deletion mutation by routine *EGFR* mutation tests [11].

Discussion

In this study, we evaluated the ability of picoliter-ddPCRï based analysis of plasma cfDNA to detect *EGFR* mutations conferring resistance to EGFR-TKIs. T790M mutations were detected in 7 (44%) of 16 cfDNA samples of patients with confirmed resistance to EGFR-TKI therapy, but in none of 24 cfDNA samples obtained before EGFR-TKI therapy. Thus, our assay results also demonstrated that the T790M mutation is enriched in cfDNA after acquiring resistance to the EGFR-TKI therapy. In ten patients whose tumor tissues were re-biopsied after confirmation of resistance, the concordance rate of T790M mutation status between cfDNA and re-biopsied tumor tissue was 0.8, and the sensitivity was 71%. This result was similar to that of a recent study using a conventional ddPCR method [37]. In our study, two case (Patients 6 and 7) exhibited negativity in cfDNA but positivity in tumors, while the reason for the discordance is unclear. cfDNA have been thought to reflect tumor predominance throughout the body [14,16,38]. However, intra- and/or inter-tumor heterogeneity might have caused low levels of T790M-mutated cfDNA in these cases; a single re-biopsied tissue examined does not necessary represent the status of tumors in the body (i.e., due to the nature of cfDNA as a biomarker). On the other hand, the negativity in those two

cfDNA samples might also be due to a low sensitivity of the present picoliter-ddPCR assay using the stringent threshold value set for this study (i.e., the ability of the assay). Thus, this study supports the utility of picoliter-ddPCR for cfDNA-based monitoring of T790M mutation in tumors during EGFR-TKI therapy, however, the sensitivity of the assay would be able to be improved by further adjusting the amounts of cfDNA subjected to ddPCR as well as threshold values.

The predominance of T790M mutation-positive tumor cells among all tumor cells informs the choice of whether to use next-generation TKIs that suppress the activity of EGFR-T790M mutants. Notably, the fractions of T790M-mutant cfDNA among all tumor cfDNA, which led us to deduce predominance of T790M-positive tumor cells, differed among the seven cases that were positive for this mutation (**Table 1**). Hence, not only positivity but also the fractions of T790M cfDNAs might help considering the therapeutic strategy. In case 14, most tumor cells were deduced to have acquired the T790M mutation (**Fig. 4D**); therefore, this patient might benefit from next-generation EGFR-TKIs. On the other hand, in some patients, such as Patients 3 and 15, T790M-mutant cfDNA constituted only a minor fraction of tumor cfDNA; therefore, T790M mutation might have occurred in only subset of tumor cells. Indeed, co-occurrence of more than one resistance mechanism (e.g., *HER2* and *MET* amplification) within a single tumor has been observed [39]. Therefore, these patients might benefit from next-generation EGFR-TKIs; however, detailed monitoring of tumor shrinkage and the proportion of T790M alleles would be necessary to assess therapeutic effects. Strategies for treating patients with EGFR-TKI resistance using next-generation EGFR-TKIs

or other drugs are being actively discussed and tested in clinical trials [40]. Co-monitoring of cfDNA with TKI-sensitive and T790M mutations will help to establish criteria for drug selection.

By combining NGS analysis of cfDNA, we deduced the molecular mechanism of one patient who did not respond to gefitinib, indicating that their tumor cells were innately resistant to this drug despite the absence of the T790M mutation. NGS analysis revealed the presence of a 2 bp indel (c.2239_2240delinsCC) in exon 19, causing an amino acid substitution at codon 747 from leucine to proline, L747P, but the absence of T790M and any other known TKI-sensitive EGFR mutations in cfDNA. Our picoliter-ddPCR analysis of cfDNA and the Scorpion-ARMs test of tumor tissues prior to gefitinib therapy mis-diagnosed this case as being positive for exon 19 deletion mutation due to mis-priming of the oligonucleotides used for PCR [11]. The EGFR-L747P mutation is a rare driver mutation conferring innate resistance of tumor cells to conventional EGFR-TKIs [41]. These findings suggest the utility of NGS to detect diverse mutations by complementing the ddPCR assay focused on TKI-sensitive and T790M *EGFR* mutations. Resistance to EGFR-TKIs have been revealed to be caused not only by *EGFR*-T790M mutations, but also by other alterations in *EGFR* and those in other genes, such as *MET* and *HER2* amplifications [39]. Thus, establishment of a comprehensive cfDNA analysis method that enable detection not only of TKI-sensitive and T790M *EGFR* mutations but also other genetic alterations conferring resistance is necessary for non-invasive diagnosis for EGFR-TKI resistance in lung cancer

clinic. Usage of picoliter-ddPCR focusing on hot-spot mutations, complemented by NGS analysis, will be a way to perform such an analysis.

TKI-sensitive mutations were detected in most (15/16, 94% in **Fig. 3**) of cfDNA samples obtained after confirming resistance. Notably, cfDNA obtained from patients who developed new extrapleural tumors upon disease progression following EGFR-TKI therapy exhibited high (>10%) fractions of cfDNA with TKI-sensitive mutations ($P = 0.00070$ by Fisherôs exact test; **Supplementary Table 3**). This finding confirms the utility of cfDNA to deduce the tumor burden in progressed cases, as suggested by previous studies [17,22]. On the other hand, only a subset of cfDNA samples obtained before EGFR-TKI therapy were positive for TKI-sensitive mutation (9/24; 38 % in **Fig. 3**), despite the extrapleural growth of the tumors (**Supplementary Table 4**). The reason for this difference is unknown, but one possible explanation is that epithelial-to-mesenchymal transition (EMT) of tumor cells, which often occurs contemporaneously with acquisition of resistance to EGFR-TKIs, might increase the amount of plasma cfDNA by promoting the dissemination of tumor cells into plasma [42,43].

This study has a few limitations. First, the sample size was small, particularly for cfDNA samples with corresponding re-biopsied tumor tissues. Hence, the utility, feasibility and robustness of this picoliter-ddPCR assay should be further examined in a clinical trial assessing subsequent response to treatments by conventional and the third-generation TKIs by prospectively including a large set of samples. To this end, we have initiated a large-scale prospective study to validate the concordance of T790M predominance between cfDNA and

410 re-biopsied tumor tissues. Second, this study focused on determining the predominance of the
411 T790M mutation in cfDNA from advanced patients in order to monitor the burden of
412 T790M-positive tumor cells during therapy. Due to the stringent criteria used here to judge
413 positivity, the sensitivity of the assay was lower than other assays that focus on diagnosis of
414 early-stage tumors [24,44]. Increasing the amount of cfDNA used in the assay and/or setting
415 more appropriate thresholds according to the cfDNA amounts should make our assay more
416 sensitive.

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Figure legends

Figure 1. Picoliter-ddPCR for *EGFR*-cfDNA

A. Assay design: Left, an assay to detect exon 19 deletions (19DEL). The assay was designed to detect the loss of wild-type sequence and could therefore detect multiple in-frame deletions in exon 19. Wild-type DNA generated signals from both the wild-type probe (FAM: green) and reference probe (VIC: red), whereas deletion alleles generated a signal only from the 19DEL reference probe. Right, assays to detect the L858R and T790M mutations. Mutant DNA generated signals from the mutation-specific (FAM: green) probe, whereas wild-type DNA generated a signal from the wild-type probe (VIC; red).

B. Assessment of the predominance of T790M-positive tumor cells among all tumor cells. cfDNA was subjected to T790M and 19DEL or L858R mutation assays. The fraction of T790M-cfDNAs among 19DEL/L858R-mutant (i.e., tumor-derived) cfDNAs reflects the predominance of T790M-positive (i.e., TKI-resistant) tumor cells among all tumor cells. The result from case 1 after acquisition of resistance (one of the triplicated assays in Supplementary Table 2) is shown as a representative. FAM and VIC intensities are shown in arbitrary units.

Figure 2. Therapeutic process of LADC patients in the TKI-resistant cohort

The content and duration of therapy, as well as the timing of *EGFR* mutation tests, are illustrated for 16 patients whose cfDNA was obtained after acquisition of resistance to EGFR-TKI therapy. From Patients 1, 8, 10, 11, and 12, cfDNA samples were also obtained

572 before EGFR-TKI therapy. All patients were diagnosed as having tumors harboring
573 TKI-sensitive mutations, based on analysis of biopsied tissue samples. In Patients 1ï 10,
574 re-biopsied tumor tissues obtained after acquisition of resistance to EGFR-TKIs were also
575 tested for T790M mutations. Positivity for TKI-sensitive (TKI-mut) and T790M mutations in
576 cfDNA and re-biopsied tumors is shown on the right.
577
578
579 **Figure 3. Positivity for *EGFR* mutations in cfDNA**
580 Percent of cfDNA samples positive for TKI-sensitive and T790M mutations are
581 shown. Sixteen samples were obtained after the acquisition of resistance to EGFR-TKI
582 therapy (Patients 1ï 16), and 24 samples were obtained before EGFR-TKI therapy (Patients 1,
583 8, 10, 11, 12 and 17ï 35).
584
585
586 **Figure 4. *EGFR*-cfDNA profile of representative patients who acquired resistance to**
587 **EGFR-TKI**
588 **A.** Circle graph showing fractions of tumor cell-derived cfDNA, as well as fractions of
589 T790M-mutant cfDNA.
590 **B.** Profile of four cases who provided cfDNA both before EGFR-TKI therapy and after
591 acquisition of EGFR-TKI resistance. (-) indicates no positivity for the T790M mutation.
592 **C.** Results of NGS analysis of cfDNA of case 12 after expressing resistance to EGFR-TKI

593 therapy, shown in the Integrated Genomics Viewer. L747P mutant alleles were detected in

594 69% (2922/4232) of sequencing reads, whereas no T790M reads were observed (0/4274).

595 **D.** Profile of three representative cases who provided cfDNA only after resistance acquisition

596 to EGFR-TKI therapy. ñ?ò indicates no results.

597

Supplementary Figure 1. Workflow of picoliter-ddPCR

More than 4 million picoliter-sized droplets are created from 40 μL of PCR solution (A), followed by thermal cycling (B). PCR produces a small fraction of fluorescence-positive droplets (<1/1000), and endpoint fluorescence is measured in each droplet (C). Fluorescence of droplets was visualized as clusters in a two-dimensional histogram to enable counting of fluorescence-positive droplets (D).

Supplementary Figure 2. Quantitative performance of picoliter-ddPCR

A. Detection of exon 19 deletions, L858R, and T790M in genomic DNA by picoliter-ddPCR. DNA (40 ng) from the PC－9, II-18, and NCI-H1975 cell lines was subjected to picoliter-ddPCR in multiplicate, as indicated in parentheses. Data are expressed as means ± SE.

B. Quantitative detection of T790M alleles among wild-type alleles. DNA from NCI-H1975 cells harboring the T790M mutation was serially diluted with DNA from ACC-MESO-1 mesothelioma cells, which harbor the wild-type *EGFR* gene; 40 ng of DNA was used in the T790M assay (in triplicate).

C. Reproducible estimation of the proportion of TKI-sensitive alleles containing the T790M mutation using the picoliter-ddPCR assay for detecting the T790M and L858R mutations. Various concentrations of NCI-H1975 DNA, which has the same copy number of the T790M and L858R alleles, were subjected to the T790M and L858R assays. The T790M/L858R fraction was calculated as in **Fig. 1B**. Data are expressed as the means ± SE.

Supplementary Figure 3. Analysis of cfDNA obtained from two LADC patients without the *EGFR* mutation.

cfDNA from two patients with LADC harboring the *EML4-ALK* fusion was subjected to picoliter-ddPCR for the exon 19 deletion and the L858R and T790M mutations. DNA (4 ng, as quantitated on a Qubit fluorometer) was subjected to picoliter-ddPCR in multiplicate (as indicated in parentheses). Data are expressed as the means ± SE.

Supplementary Figure 4. Therapeutic process of LADC patients in the pre-TK cohort

The content and duration of therapy, as well as the timing of *EGFR* mutation tests, are illustrated for 19 patients whose cfDNA was obtained before EGFR-TKI therapy. All patients were diagnosed as having tumors with TKI-sensitive mutations, based on analysis of biopsied tumor tissue samples.

Supplementary Figure 5. Cross-tabulation of T790M analysis of cfDNAs and re-biopsied tumor tissues.

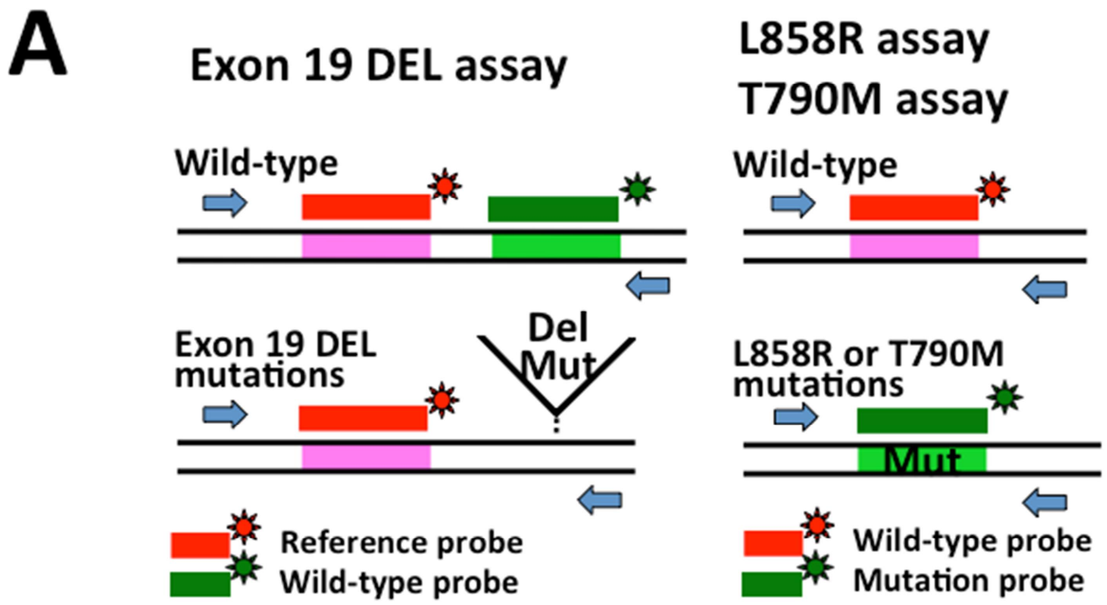
Detection of T790M mutation using cfDNA; sensitivity, specificity, positive predictive value (PPV), and negative predictive value were shown. T790M mutation statuses in the corresponding re-biopsied tumor tissues are used for standard test.

Table 1. Study subjects for picoliter-ddPCR analysis of cfDNA

Case	Age	Sex	Smoking (pack years)	EGFR-TKI sensitive mutation in tumor	Stage	EGFR-TKI (best response)	Duration of therapy (months)	cfDNA before resistance acquisition		cfDNA after resistance acquisition			T790M mutation in re-biopsied tumor tissue
								TKI-sensitive mut (%)	T790M mut (%)	Sensitive mut (%)	T790M mut (%)	Deduced T790M (+) tumor %	
Case with resistance to EGFR-TKI therapy (TKI-resistant cohort)													
1	62	Female	Never	19DEL	IV	Erlotinib (PR)	6.6	5.8	Negative	30.7*	15.0*	49	Positive
2	74	Female	Never	19DEL	IV	Erlotinib (PR)	12.4	-	-	24.7*	9.5*	39	Positive
3	67	Female	Never	L858R	IV	Gefitinib (PR)	12	-	-	4.0*	0.86*	22	Positive
4	64	Male	Never	19DEL	IV	Gefitinib (PR)	8.2	-	-	37.0	21.5	58	Positive
5	71	Female	Never	L858R	IV	Gefitinib (NE) & Erlotinib (NE)	18.0 & 22.0	-	-	17.5	8.5	49	Positive
6	57	Male	Never	19DEL	IV	Gefitinib (NE)	35.5	-	-	Negative	Negative	-	Positive
7	65	Female	2	19DEL	IV	Erlotinib (SD) & Afatinib (SD)	25.6 & 4.8	-	-	3.4	Negative	-	Positive
8	65	Male	Never	19DEL	IV	Erlotinib (PR)	10	Negative	Negative	3.0	Negative	-	Negative
9	64	Male	Never	L858R	Post-operative relapse	Gefitinib (SD)	22	-	-	10.3	Negative	-	Negative
10	58	Male	38	L858R	IV	Erlotinib	-	Negative	Negative	9.3	Negative	-	Negative
11	47	Female	20	19DEL	IV	Gefitinib (SD)	7.1	3.2	Negative	3.0	Negative	-	-
12	69	Female	Never	19DEL	IV	Gefitinib (PD)	1.6	60.6	Negative	69.3	Negative	-	-
13	68	Male	Never	19DEL	IV	Erlotinib (SD)	8.6	-	-	1.1*	Negative	-	-
14	65	Female	Never	L858R	IV	Gefitinib (PR) & Erlotinib (SD)	6.4 & 5.3	-	-	5.8	5.6	97	-
15	53	Female	Never	19DEL	IV	Gefitinib (PR)	12.3	-	-	16.0	1.2	7.4	-
16	51	Female	Never	19DEL	IV	Erlotinib (SD) & Gefitinib (SD)	31.5 & 3.2	-	-	10.8	Negative	-	-
Case before EGFR-TKI therapy (pre-TKI cohort)													
17	72	Female	Never	L858R	Post-operative relapse	Gefitinib	-	Negative	Negative	-	-	-	-
18	56	Female	Never	L858R	IV	Erlotinib	-	Negative	Negative	-	-	-	-
19	52	Male	50	L858R	Post-operative relapse	Gefitinib	-	Negative	Negative	-	-	-	-
20	67	Male	30	L858R	Post-operative relapse	Erlotinib	-	Negative	Negative	-	-	-	-
21	58	Female	26	19DEL +T790M	Post-operative relapse	Gefitinib	-	Negative	Negative	-	-	-	-
22	58	Female	Never	19DEL	IV	Gefitinib	-	3.2	Negative	-	-	-	-
23	51	Male	29	19DEL	Post-operative relapse	Erlotinib	-	21.4	Negative	-	-	-	-
24	53	Female	Never	19DEL	Post-operative relapse	Afatinib	-	Negative	Negative	-	-	-	-
25	64	Male	Never	19DEL	Post-operative relapse	Gefitinib	-	Negative	Negative	-	-	-	-
26	64	Male	55	19DEL	Post-operative relapse	Gefitinib	-	14.6	Negative	-	-	-	-
27	60	Female	Never	19DEL	Post-operative relapse	Gefitinib	-	Negative	Negative	-	-	-	-
28	63	Female	Never	L858R	Post-operative relapse	Gefitinib	-	Negative	Negative	-	-	-	-
29	50	Female	Never	19DEL	Post-operative relapse	Gefitinib	-	Negative	Negative	-	-	-	-
30	72	Female	Never	L858R	Post-operative relapse	Gefitinib	-	Negative	Negative	-	-	-	-
31	63	Male	20	19DEL	Post-operative relapse	Gefitinib	-	Negative	Negative	-	-	-	-
32	58	Male	38	L858R	IV	Erlotinib	-	17.8	Negative				
33	52	Female	18	19DEL	Relapse after chemoradiotherapy	Afatinib	-	Negative	Negative	-	-	-	-
34	55	Female	Never	19DEL	IV	Gefitinib	-	68.1	Negative				
35	54	Male	Never	19DEL	IV	Gefitinib	-	0.8	Negative	-	-	-	-

-: not examined, *: average of three replicates, PR: partial response, SD: stable disease, NE: not evaluated.

Figure 1


A
Exon 19 DEL assay
L858R assay
T790M assay
Wild-type
Wild-type
Exon 19 DEL mutations
Del Mut
L858R or T790M mutations
Mut
Reference probe
Wild-type probe
Wild-type probe
Mutation probe

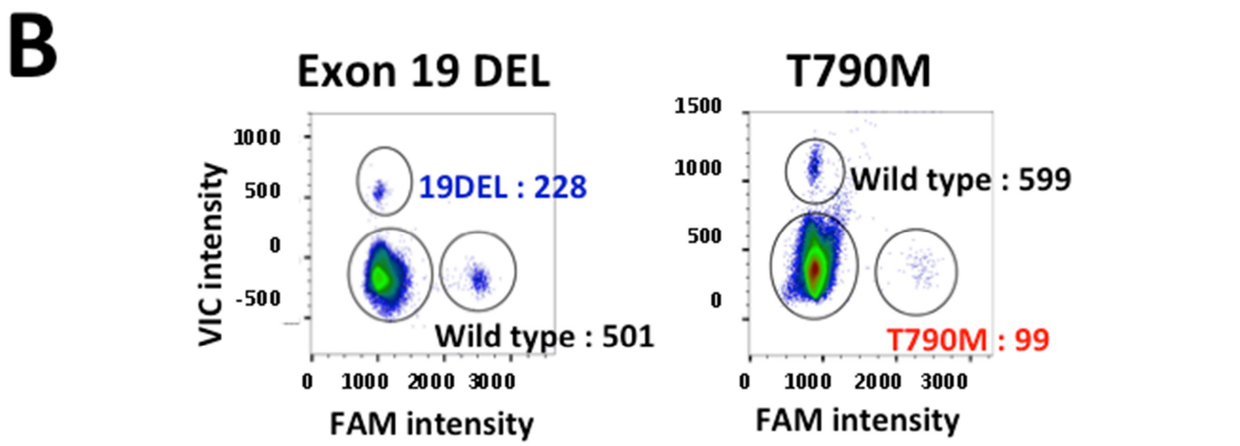
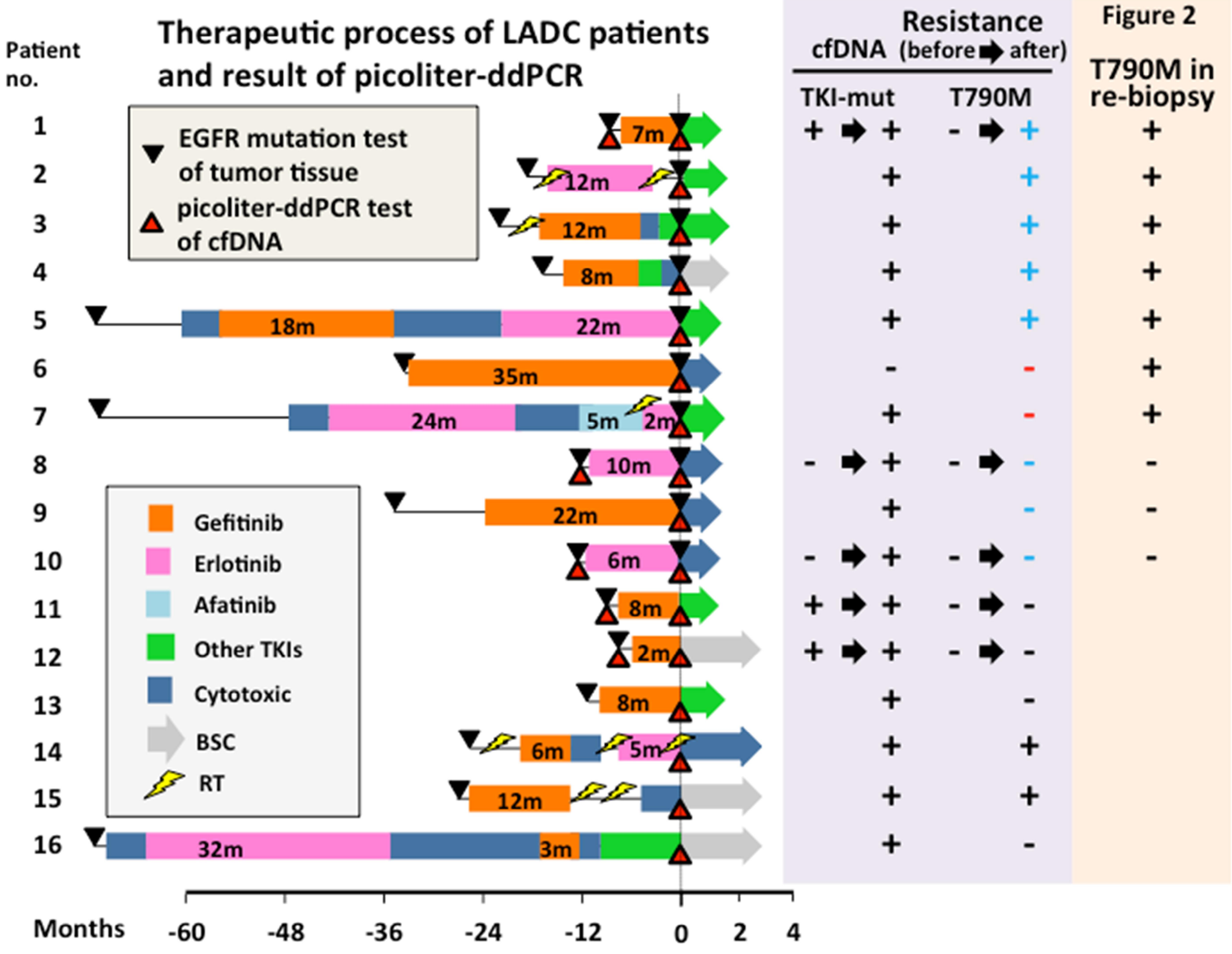

B
Exon 19 DEL
T790M
VIC intensity
19DEL : 228
Wild type : 501
Wild type : 599
T790M : 99
FAM intensity
FAM intensity
Tumor cfDNA in all cfDNA
31.3% (228/(501+228))
T790M+ tumor cfDNA in all cfDNA
14.2% (99/(599+99))
T790M+ tumor cfDNA in tumor cfDNA
45.4% (14.2/31.3)

Figure 2

Therapeutic process of LADC patients and result of picoliter-ddPCR



Patient no.	Resistance cfDNA (before ➡ after) TKI-mut			T790M			T790M in re-biopsy
1	+	➡	+	-	➡	+	+
2			+			+	+
3			+			+	+
4			+			+	+
5			+			+	+
6			-			-	+
7			+			-	+
8	-	➡	+	-	➡	-	-
9			+			-	-
10	-	➡	+	-	➡	-	-
11	+	➡	+	-	➡	-	
12	+	➡	+	-	➡	-	
13			+			-	
14			+			+	
15			+			+	
16			+			-	

Figure 3

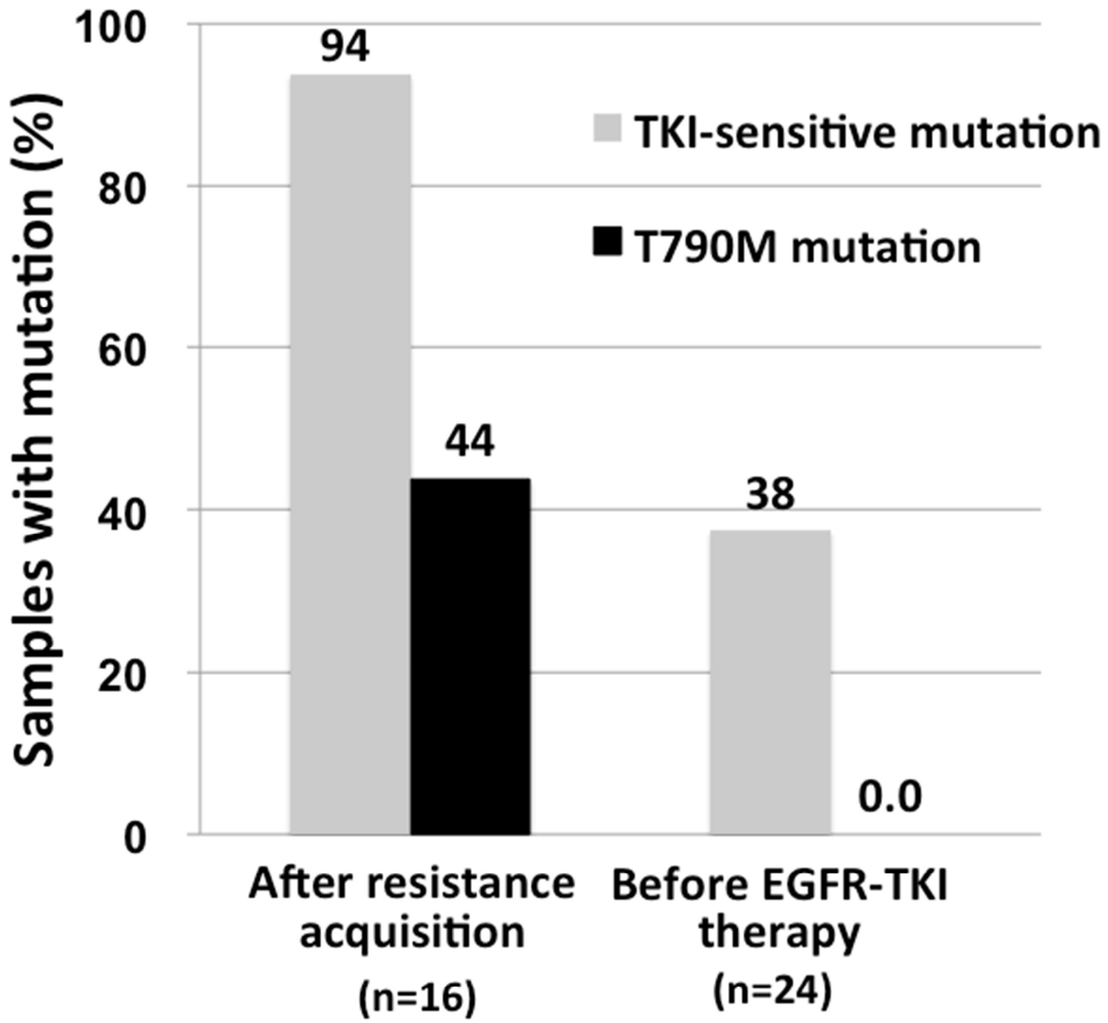
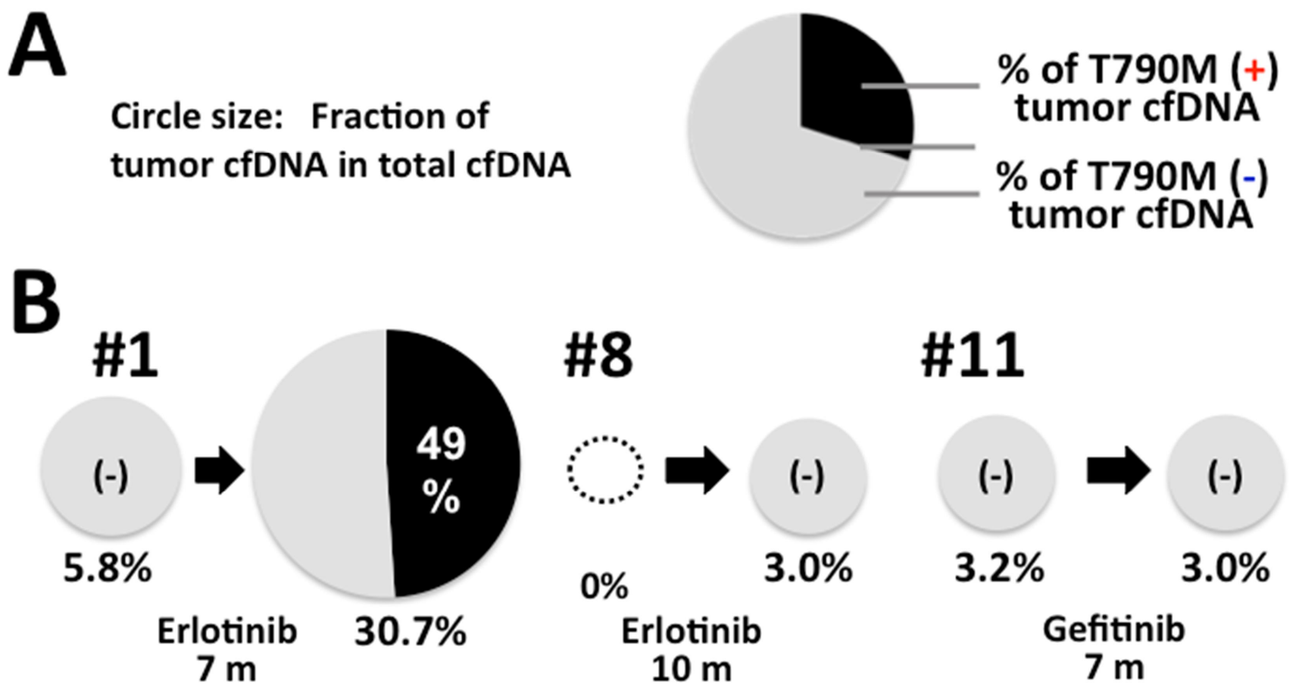

100
80
60
40
20
0
Samples with mutation (%)
94
44
38
0.0
TKI-sensitive mutation
T790M mutation
After resistance acquisition
(n=16)
Before EGFR-TKI therapy
(n=24)

Figure 4


A
Circle size: Fraction of tumor cfDNA in total cfDNA
% of T790M (+) tumor cfDNA
% of T790M (-) tumor cfDNA
B
#1
(-)
5.8%
49 %
30.7%
Erlotinib 7 m
#8
0%
Erlotinib 10 m
(-)
3.0%
#11
(-)
3.2%
Gefitinib 7 m
(-)
3.0%
C
#12
% fraction of tumor cfDNA in total cfDNA
100
80
60
40
20
0
0
30
60
Days
Gefitinib discontinuation
19del assay
T790M assay
L747P
UUA>CCA
69%
(2922/4232)
T790M negative (0/4274)
D
#3
?
Erlotinib 12 m
22%
4.0%
#7
?
Gefitinib 12 m
7.4%
16.0%
#13
?
Gefitinib 6 m
Erlotinib 5 m
97%
5.8%