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Clinical Utility of Liquid Biopsy for EGFR Driver, T790M Mutation and EGFR Amplification in Plasma in Patients with Aacquired Resistance to Afatinib

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Abstract

Background: Cell-free DNA (cfDNA) genotyping in plasma using the cobas EGFR Mutation Test v2 (cobas) is the first liquid biopsy as a companion diagnosis to identify the EGFR T790M mutation (T790M) after the failure of treatment of EGFR-tyrosine kinase inhibitor (TKI) (1st generation, gefitinib [G] and erlotinib [E] and afatinib [A]). This study investigated the clinical utility of a liquid biopsy for patients who acquired resistance to afatinib.

Methods: We prospectively collected plasma from 51 patients who had acquired resistance to afatinib between April 2015 and November 2016 to evaluate the frequency of T790M using the cobas and digital droplet PCR (UMIN000025112). Additionally, we retrospectively reviewed 38 patients who tested by cobas in plasma after G/E failure to compare for T790M detection between A and with G/E.

Results: The detection rate of EGFR sensitive mutation (EGFR sens.) and T790M in plasma in patients treated with A (A group) as an initial EGFR-TKI was lower than with G/E followed by A (G/E→A), although the differences were not significant (EGFR sens.: 41% (A) vs. 67% (G/E→A), *P*=0.1867; and T790M: 8% (A) vs. 17% (G/E→A), *P*=0.5798). In first-line setting, the detection rate for EGFR-sens. and T790M in plasma by cobas was lower in A group than in G/E group, although there was no significant difference (EGFR sens.: 34% (A) vs. 52% (G/E), *P*=0.2072; and T790M: 10% (A) vs. 27% (G/E), *P*=0.1161).

Conclusion: The detection of EGFR sens. and T790M in plasma by cobas might be insufficient in patients treated with afatinib than with G/E.

Background

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have been a standard first-line therapy for non-small cell lung cancer (NSCLC) patients with EGFR-sensitive mutations (1–3). First-generation EGFR-TKIs, gefitinib and erlotinib, reversibly bind to and inhibit EGFR signaling. In contrast, second-generation ErbB family blockers, such as afatinib and dacomitinib, irreversibly block the signaling from all relevant homo-dimers and hetero-dimers of the ErbB family of receptors (EGFR/ErbB1, HER2/ErbB2, ErbB3, and ErbB4). Second-generation EGFR-TKIs have been reported to show a significantly longer PFS than first-generation EGFR-TKIs in clinical trials (4, 5).

The emergence of the EGFR T790M point mutation is the most common mechanism of acquired resistance to the EGFR-TKIs gefitinib, erlotinib and afatinib (6, 7). Osimertinib, a third-generation and irreversible mutant-selective EGFR-TKI, has been approved for advanced NSCLC patients harboring *EGFR* mutations, including the T790M mutation, based on the results of the AURA3 trial (8). On the other hand, EGFR wild-type amplification has also been reported as a mechanism or resistance to EGFR-TKIs, including osimertinib (9, 10).

Cell-free DNA (cfDNA) genotyping in plasma using the cobas EGFR Mutation Test v2 (cobas test) is the first liquid biopsy to be approved as a companion diagnostic system test to identify patients with the

EGFR T790M (T790M) mutation. cfDNA genotyping in plasma is a more easily accessible method of detecting T790M mutation than tissue-based biopsies. However, the AURA3 trial observed that only 51.2% of T790M-positive patients, as evaluated using tumor tissues, were also T790M-positive when assessed using cfDNA in plasma (11), implying that the sensitivity of the cfDNA assay was insufficient to identify all T790M mutant-positive patients. Nevertheless, few reports have investigated the clinical utility of a liquid biopsy for detecting T790M mutation in patients with acquired resistance to afatinib, since most patients enrolled in the AURA3 trial were treated with G/E.

Therefore, we planned to investigate the clinical utility of a liquid biopsy for detecting T790M mutation in EGFR-mutated NSCLC patients with acquired resistance to afatinib. In addition, we exploratory evaluated the difference in T790M detection in plasma from subjects treated with first-generation EGFR-TKIs, and an EGFR wild-type amplification status in patients with acquired resistance to afatinib.

Methods

Patients

We studied two patient populations: a study arm consisting of prospective observational patients, and a control arm consisting of retrospective patients. For the study arm, we prospectively collected plasma samples from 51 patients who had been treated with afatinib and had experienced progression during afatinib treatment between April 2015 and November 2016 at 13 institutions (UMIN000025112) **(Figure 1A)**. The inclusion criteria were as follows: 1) a diagnosis of NSCLC, 2) a diagnosis of EGFR mutation, 3) the presence of progressive disease (PD) as assessed using the RECIST criteria, and 4) treatment with afatinib as the last EGFR-TKI to be administered prior to PD. Patients who had been treated with G/E as the last EGFR-TKIs before RECIST-PD were excluded **(Figure 1A)**. The presence of T790M mutation and/or EGFR driver mutation was evaluated in these patients using the cobas test and digital droplet PCR (ddPCR).

In addition, to evaluate the difference in the detection of EGFR-driver and T790M mutation in plasma samples from patients treated with first-generation EGFR-TKIs (G/E) and those treated with afatinib, we retrospectively collected data on 33 patients who had been treated with G/E as their initial EGFR-TKIs and whose plasma samples had been analyzed using the cobas test to evaluate the presence of T790M mutation; these patients were regarded as a control arm. Moreover, if sufficient cfDNA samples were available after cobas testing and ddPCR for the T790M mutation, EGFR copy number variation (CNV) was evaluated in an exploratory manner using ddPCR.

In all the patients, the patient characteristics, efficacy of EGFR-TKIs, and the T790M status in plasma, objective response rate (ORR), and progression-free survival (PFS) after treatment with EGFR-TKIs were reviewed. The protocol for this study was approved by the institutional review board of each institution, and the study was registered as a clinical trial (Clinical trial information: UMIN000025112).

EGFR mutation assay in plasma

To assess the presence of EGFR mutations in plasma, approximately 20 mL of whole blood was collected using K2-EDTA collection tubes; within 4 hours after blood collection, the samples were then centrifuged to separate the plasma from the peripheral blood cells at 1500 ×*g* for 10 min at 4℃, and the plasma supernatant was transferred to conical tubes and stored at -80℃ until transport. The plasma samples were transported at -80°C to one of two *commercial* laboratories (SRL Inc., Tokyo, Japan, and G&G Science Inc., Fukushima, Japan). The mutant allele frequency and EGFR copy number were measured using the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA). Isolated cfDNA was amplified using ddPCR Supermix for Probes (Bio-Rad) using EGFR T790M/L858R (PrimePCR ddPCR Mutation Assay; Bio-Rad), EGFR del19 assay (TaqMan Mutation Detection Assay; Thermo Fisher Scientific) for del19 EGFR mutations, and EGFR (RPP30 reference gene) for gene CNV (PrimePCR ddPCR Copy Number Assay; Bio-Rad), according to the manufacturers' protocols. The digital PCR data were analyzed using Quanta Soft analytical software (version 1.7.4, Bio-Rad).

Statistical analysis

All the statistical analyses were performed using the JMP version 11 statistical software package (SAS Institute, Cary, NC, USA). Differences in the baseline characteristics between the groups and comparison of the detection rate in EGFR driver mutation and T790M between the groups were compared using the Fisher exact tests for categorical data. The PFS was calculated from the date of therapy initiation to disease progression. The survival probabilities were estimated using the Kaplan–Meier method, where differences in the variables were calculated using the log-rank test.

In this study, 5% was considered significant with an alpha error. The cut-off date was August 30, 2018. The target number of cases was set at 100 at the time of planning. Although there are few reports about the detection rate of T790M by cfDNA in plasma with afatinib resistant cases, the positive rate of T790M by first generation EGFR-TKIs is reported to be about 50%. On the other hand, in afatinib-resistant cases, it was reported that the T790M mutation was found in 48%, although it was analyzed in a small number of cases. Therefore, in this study, the threshold of T790M detection rate was set to 35% and the expected value was set to 50%. When the number of cases is calculated with α = 0.05 and the detection rate = 0.80, the required number of cases is 68. The success rate of EGFR gene mutation due to Cobas version 2 in plasma samples was about 70-80%, and the target enrollment was set at 100 considering some incompatibility. However, due to difficulties in registering cases, the final number of cases was 51. In this study, 5% was considered significant as anderror.

Results

Patient characteristics

Between April 2015 and November 2016, a total of 51 patients were enrolled in this study. The characteristics of the patients in the study arm are summarized in Table 1. The median age of the patients was 68 (range, 36–81) years. Twenty-eight (55%) were male. All the patients had adenocarcinoma (Ad). Forty-five (88%) patients had an ECOG PS score of 0 or 1, while 6 (12%) patients had an ECOG PS or 2 or 3. Thirty-two (63%) had a 19del mutation, 13 (25%) had L858R, and 6 (12%) had minor EGFR mutations. Thirty-nine (76%) patients received treatment with afatinib as an initial EGFR-TKI therapy (A group), and 12 (26%) patients received afatinib as a second or subsequent EGFR-TKI therapy after having received gefitinib and/or erlotinib (G/E→A group). Among the 39 patients who were treated with afatinib in an initial EGFR-TKI setting (A group), 16 (41%) patients were positive for a driver mutation and 3 (8%) patients were positive for T790M in plasma using the cobas test at the time of progression. In the G/E→A group, 8 patients (67%) were positive for a driver mutation and 2 (17%) patients were positive for T790M in plasma (Fig. 2A). The detection rates for EGFR driver and T790M mutations in plasma were lower in the A group than in the G/E→A group, although the differences were not significant.

Table 1
Patient characteristics (N = 51).

Characteristics	N = 51	%
Age, median (range)	68 (36–81)	
Sex (Male/Female)	28/23	55/45
Histology (Adenocarcinoma/others)	51/0	100/0
Smoking (Yes/No)	25/26	49/51
EGFR mutation (19del/L858R/others)	32/13/6	63/25/12
Stage at the diagnosis (III/IV/postoperative recurence)	1/45/5	2/88/10
Performance status 0–1/2	45/6	88/12
Treatment line with afatinib Initial/ Second or subsequent	39/12	76/26

Comparison of cobas test and ddPCR for the detection of T790M mutation

Nineteen (37%) of the 51 patients had T790M mutation copies in plasma when assessed using ddPCR, and the T790M copy number ranged from 80 to 375000 (Fig. 2B). Among these patients, 5 patients who had more than 400 copies/mL also tested positive for the T790M mutation using the cobas test. In one

patient who continued to receive afatinib treatment after RECIST-PD, blood samples were collected serially (Fig. 2C). The result of the first cfDNA analysis for T790M mutation using the cobas test was negative. However, the copy numbers for EGFR driver mutation and T790M increased as the site of metastasis progressed, and a second cobas test using cfDNA resulted in a positive result for T790M mutation.

Regarding the concordance between the results for plasma and for tissue in patients who tested negative for T790M mutation using the cobas test, 8 of the 14 patients who were T790M negative according to the cobas test and T790M copy positive underwent a rebiopsy to determine the T790M mutation status, and 3 of these patients were found to have the T790M mutation in their tissue samples (Fig. 2D). In contrast, 20 of the 32 patients who were T790M negative according to the cobas test and T790M copy negative underwent a rebiopsy, and 5 patients were found to have the T790M mutation in their tissue samples. The presence of a T790M mutation copy as detected using ddPCR did not influence the T790M mutation status in tissue samples from patients who tested negative for T790M using the cobas test (ddPCR T790M copy number positive vs. negative :37.5% vs. 40%, $P = 0.6508$).

Detection rate of EGFR driver mutation and T790M between Gefitinib/Erlotinib and Afatinib groups

Next, we evaluated whether the types of EGFR-TKIs (G/E or A) affected the detection of EGFR driver and T790M mutations in plasma. Among the 51 patients who were enrolled in the prospective observational study, 29 patients who were treated with afatinib in a first-line setting and who had major EGFR mutations (Ex19 del and L858R) were selected (A arm). In addition, we retrospectively collected data from 33 patients who had been treated with only first-generation EGFR-TKIs (G/E) as a first-line setting and whose plasma samples had been assessed using the cobas test (G/E arm). The patient characteristics are shown in Table 2. Males, smokers, and EGFR 19del were significantly higher in the A arm than in the G/E arm (males: 68.9% vs. 27.2%, $P < 0.01$; smoking: 44.8% vs. 24.2%, $P = 0.01$; EGFR 19del: 79.3% vs. 51.5%, $P = 0.01$).

Table 2
Patient Characteristics (N = 62).

	Afa (N = 29).	G/E (N = 33).	P value
Age, median (range)	66 (36–80)	68 (41–80)	
Sex (Male/Female)	20/9	9/24	0.0010
Histology (Adenocarcinoma/others)	29/0	33/0	1.0
Smoking (Yes/No)	13/16	8/25	0.0126
Mutation (19del/ L858R)	23/6	17/16	0.0225
Stage (III, IV/postoperative recurrence)	25/4	23/10	0.1208
Treatment with Osimertinib (Yes/No)	9/20	15/18	0.2448

Regarding the efficacies of EGFR-TKIs, no significant difference in PFS was observed between the A arm and the G/E arm (16.4 vs. 13.5 months, $P = 0.5580$) (Fig. 3A). Regarding the presence of EGFR-driver and T790M mutations in plasma, the detection rates for EGFR-driver mutation and T790M mutation in plasma using the cobas test in patients treated with afatinib were lower than in those treated with G/E, although the differences were not significant (EGFR driver mutation: 34% [A arm] vs. 52% [G/E arm], $P = 0.2072$; and T790M: 10% [A arm] vs. 27% [G/E arm], $P = 0.1161$) (Fig. 3B). In addition, among patients who tested positive for the EGFR driver mutation in plasma, the detection rate for T790M mutation was not significant between the G/E and A arms (52.9%[G/E arm] vs. 20% [A arm], $P = 0.1241$).

After initial EGFR-TKIs failure, 9 (31%) patients in the A group and 15 (45%) patients in the G/E group received osimertinib treatment. No significant difference in the PFS after osimertinib treatment was seen between the patients in the A and G/E groups (9.0 months [G/E] vs. 9.0 months [A], $P = 0.7696$, Fig. 3C).

Assessment of EGFR wild-type CNV in plasma

In this study, we also analyzed the EGFR wild-type (wt) copy number gain in cfDNA in an exploratory manner using digital PCR. Twenty-three patients had a sufficient cfDNA volume for the assessment of EGFR-wt copy number. Two of the 23 patients had the T790M mutation in plasma as detected using the cobas test. An EGFR-wt copy number gain and loss were detected in one patient each (EGFR CNV: 1.28 and 23.9) (Fig. 4A **and B)**. Interestingly, the patient who had a high EGFR CNV had been treated with osimertinib after afatinib but had not responded to the osimertinib treatment.

Discussion

In this study, we examined the detection rates for EGFR-driver and T790M mutations in plasma using the cobas test in patients with EGFR-mutated NSCLC who had acquired resistance to afatinib in a real-world setting. The detection of T790M mutation using the cobas test was in complete agreement with the

positive results (more than 400 copies/mL) for T790M mutation that were obtained using ddPCR. In addition, this study also showed that the detection of EGFR driver and T790M mutations in plasma was lower in patients treated with first-line afatinib than in those with first-line G/E (34.5% vs. 51.5%), although the difference was not significant.

Some reports have shown that in 40%-60% of patients with acquired resistance to afatinib, the emergence of T790M mutation was detected using tissue analyses, similar to the results for patients with acquired resistance to G/E (12, 13). However, Lee et al. recently reported that the occurrence of T790 M mutation in patients treated with afatinib is significantly lower than that in patients treated with gefitinib and erlotinib (14). In addition, Yoon et al. reported that although no significant difference was observed, the cumulative acquisition of the T790M mutation in patients treated with afatinib was lower than for patients treated with gefitinib (48.8% vs. 59.3%, $P = .317$), since afatinib had a greater inhibitory activity, compared with G/E, against *EGFR*-mutant cell lines harboring common mutations (including T790M) in vitro (15). These results might be consistent with our data. Regarding the T790M status in plasma in patients treated with afatinib, Hochmair et al. reported that the T790M mutation was detected using a liquid biopsy in 47 out of 67 patients who had been treated with afatinib (16). However, they did not use the cobas test, which is a companion diagnostic system test for T790M mutation, but rather the droplet digital PCR system, which is a more sensitive assay than the cobas test. In fact, in this study, among the 46 patients who tested negative for the T790M mutation in their plasma samples using the cobas test, T790M mutation copies were detected using droplet digital PCR in 14 patients. Therefore, the frequency of T790M mutation depends on the types of assays used for T790M mutation.

The detection rates for EGFR driver and T790M mutations using the cobas test in our study were lower than those reported in the pooled AURA extension and AURA 2 data (EGFR driver mutations: 76–85% vs. 40%-51%, T790M mutations: 61% vs. 5.7%-27.2%, respectively) (17). One possible reason is the difference in terms of patient populations. The AURA studies included only patients with measurable disease to enroll the clinical trials. However, our study only included patients who acquired resistance to an initial TKI treatment, either afatinib or G/E. In addition, the positivity of EGFR driver and T790M mutations in plasma is related to the disease burden, and disease burden can affect positivity. Our data might be closer to the real-world setting for EGFR-mutated NSCLC. Additionally, Sacher et al. reported that when no sensitizing mutation is detected in patients with known EGFR-mutant lung cancer and acquired resistance, plasma genotyping for T790M can be uninformative (18). To analyze the T790M mutation status in plasma more thoroughly, we evaluated the frequency of T790M mutation in EGFR driver mutation-positive NSCLC patients in plasma, but the detection of T790M mutation in EGFR driver mutation-positive patients was similar between the G/E and A arms.

Additionally, this study also showed that among 23 patients who had a cfDNA volume that was sufficient for assessment, one patient had EGFR wild-type amplification. EGFR wild-type amplification has also been reported as a mechanism of resistance to EGFR-TKIs, including osimertinib (9, 10). Indeed, the patient with EGFR wild-type amplification exhibited primary resistance to osimertinib.

The present study had several limitations. First, the sample size was relatively small. In addition, some of the patient characteristics, such as sex, smoking history and EGFR mutation type, differed between the A and G/E arms. However, previous reports have shown that these characteristics did not affect the occurrence of T790M mutation after EGFR-TKIs. Second, the timing for re-biopsy using either tissue or liquid samples was not regulated. Furthermore, tissue analyses for EGFR T790M and EGFR amplification were not performed in all the patients at the same time as the plasma test.

Conclusions

The cobas test in plasma after in patients treated with afatinib could detect T790M, but it is necessary to accumulate more cases in a real-world setting. Further examination of the clinical utility of liquid biopsy, including the cobas test, for T790M mutation and EGFR amplification is needed for the introduction of liquid biopsies into clinical practice.

Abbreviations

EGFR: Epidermal growth factor receptor

TKIs: tyrosine kinase inhibitors

NSCLC: non-small cell lung cancer

PFS: progression free survival

cfDNA: cell free DNA

RECIST: response evaluation criteria in solid tumours

ddPCR: Droplet Digital polymerase chain reaction

CNV: copy number variation

ECOG PS: Eastern Cooperative Oncology Group Performance Status

PD: preogressive disease

Declarations

Ethics approval and consent to participate.

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. The full protocol was approved by the institutional review board in each institution. All participants in the study arm provided written informed consent before enrollment.

Consent for publication.

Not applicable.

Availability of data and material.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests.

Dr. Yoshida has obtained research grants from Nippon Boehringer Ingelheim. Dr. Hida has obtained research grants from Novartis Pharma, Chugai Pharmaceutical, Taiho Pharmaceutical, AstraZeneca, Nippon Boehringer Ingelheim, Pfizer, Janssen Pharmaceutical, and Astellas, and has received personal fees from Novartis Pharma, Chugai Pharmaceutical, Taiho Pharmaceutical, AstraZeneca, Nippon Boehringer Ingelheim, and Pfizer. Dr Ito has obtained personal fees from Nippon Boehringer Ingelheim, AstraZeneca, Pfizer, Eli Lilly, Chugai Pharmaceutical, MSD, Ono Pharmaceutical, Taiho Pharmaceutical. All the other authors have no conflicts of interest. Dr. Ogutri has obtained research grant from Nippon Boehringer Ingelheim and speaker's bureau from Chugai Pharmaceutical, Taiho Pharmaceutical, Boehringer Ingelheim, AstraZeneca, Eli Lilly. Dr Kimura has obtained speaker's bureau from Chugai Pharmaceutical, Taiho Pharmaceutical, Boehringer Ingelheim, AstraZeneca, Novartis Pharma, Meiji Seika Pharmaceutical, MSD, Kyorin Pharmaceutical, Ono Pharmaceutical. Dr Kubo has obtained honoraria from Boehringer Ingelheim.

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Authors' contributions.

YO, TY, KI and TH are conducting the study as described in the protocol and drafted this manuscript. YO, TY, KI and TH participated in the trial design and planning of the statistical analyses. YO and TY are conducting the translational research associated with this study. YO, TY, KA, TO, NI, SM, KI, TK, EK, TM, AK, TK, TA, TT and TH are contributing the recruitment of participants and data collection and critically reviewed the manuscript. TY and TH are co-principal investigator of the study. All authors approved the final version of the manuscript.

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Figures

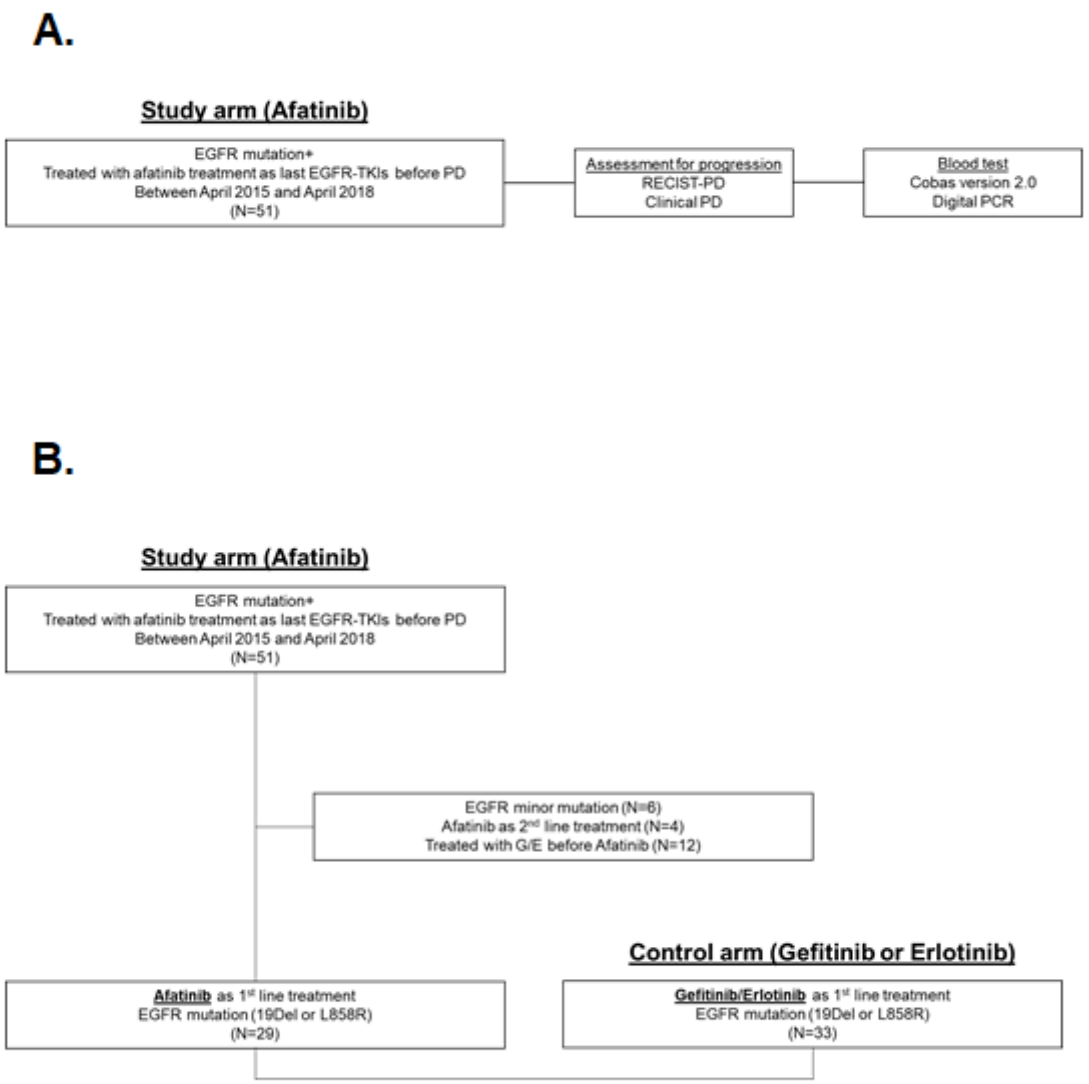

Figure 1

Patient enrollment in the study. (A) A total of 51 patients who had been treated with afatinib and had experienced progression during afatinib treatment were eligible for the prospective observational study. (B) Plasma samples from a total of 29 patients who had been initially treated with afatinib and 33 patients who had been initially treated with G/E were analyzed using the cobas test to evaluate the presence of T790M mutation.

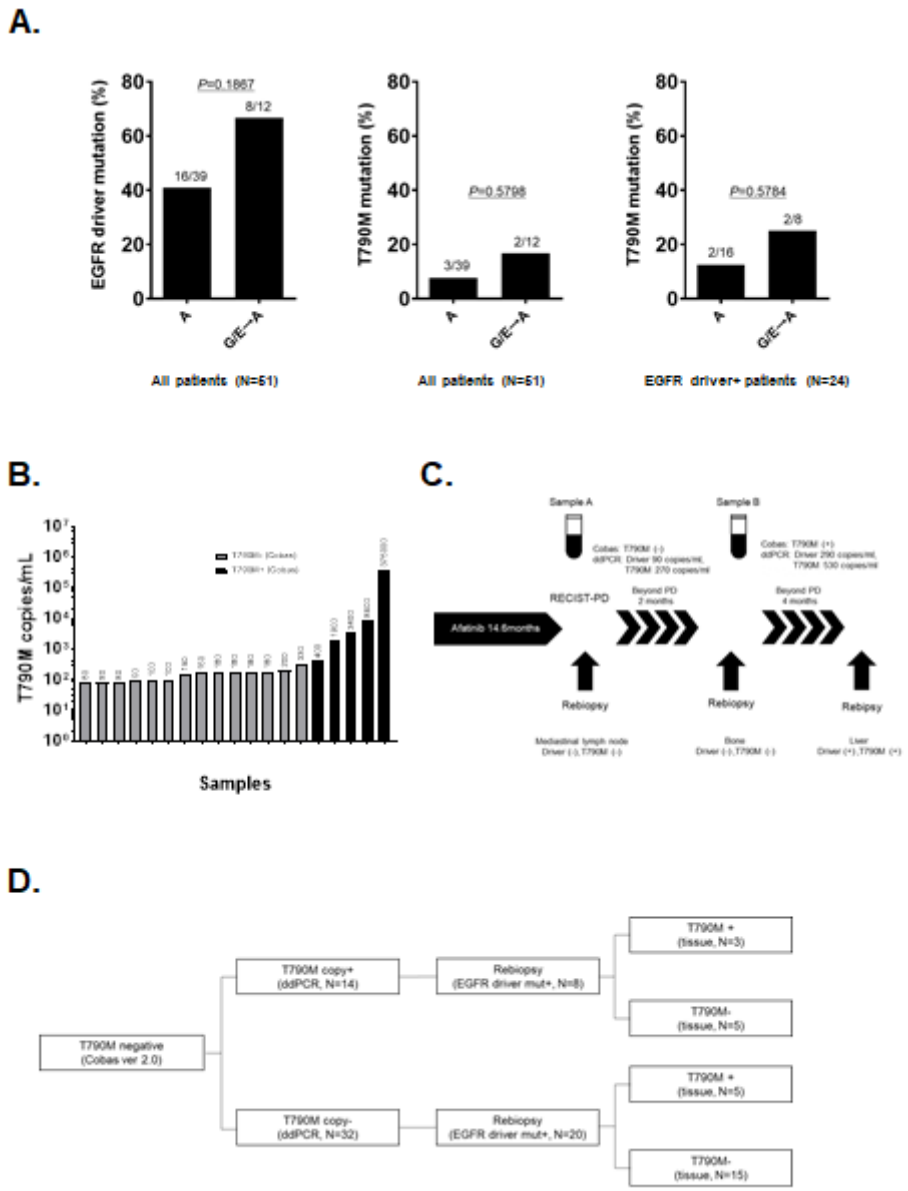

Figure 2

(A) Frequency of EGFR driver and T790M mutations in plasma for all the patients and of T790M mutation in plasma for patients who had tested positive for EGFR driver mutation in plasma according to first-line treatment with afatinib and first-line treatment with G/E. (B) T790M mutation copy numbers in plasma as assessed using ddPCR. (C) Serial analysis for T790M in plasma samples obtained from one patient who continued to receive afatinib treatment after RECIST-PD. (D) T790M mutation status as assessed using ddPCR and tissue samples in patients with negative T790M results using the cobas test.

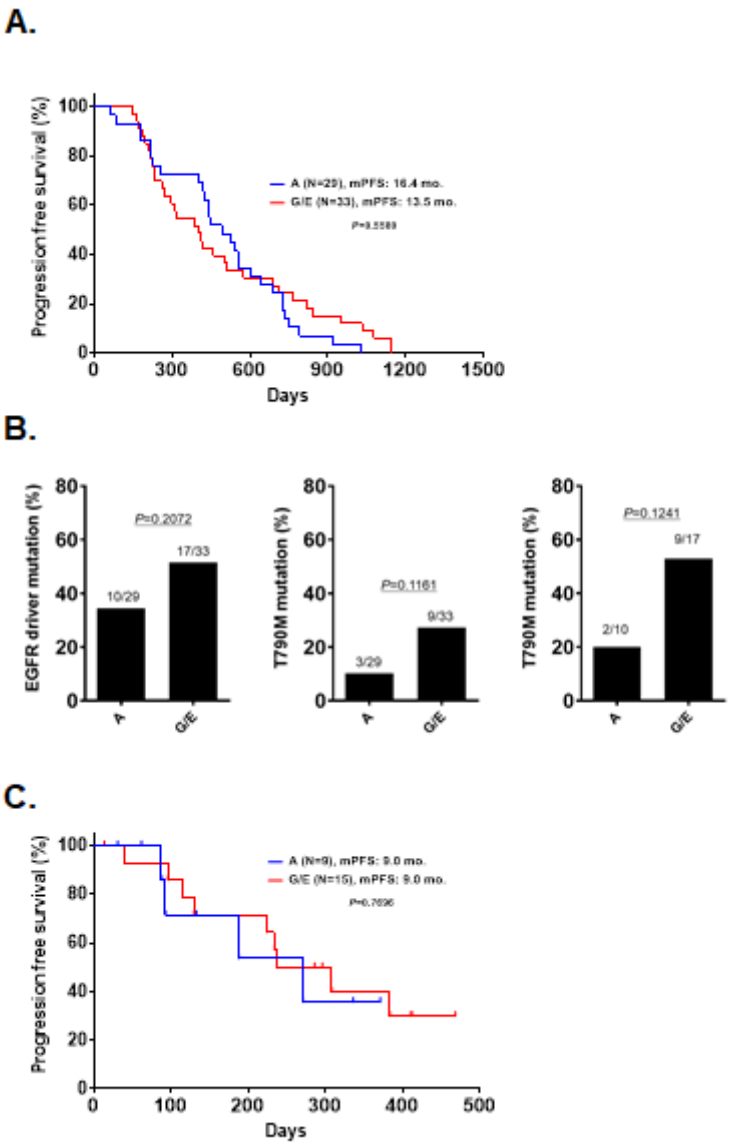

Figure 3

(A) PFS of patients treated with G/E or afatinib in a first-line setting. (B) Frequencies of EGFR driver and T790M mutations in plasma in all patients and of T790M mutations in plasma in patients who tested positive for EGFR driver mutation in plasma according to first-line afatinib and first-line G/E treatment. (C) PFS of patients treated with osimertinib after G/E and afatinib failure.

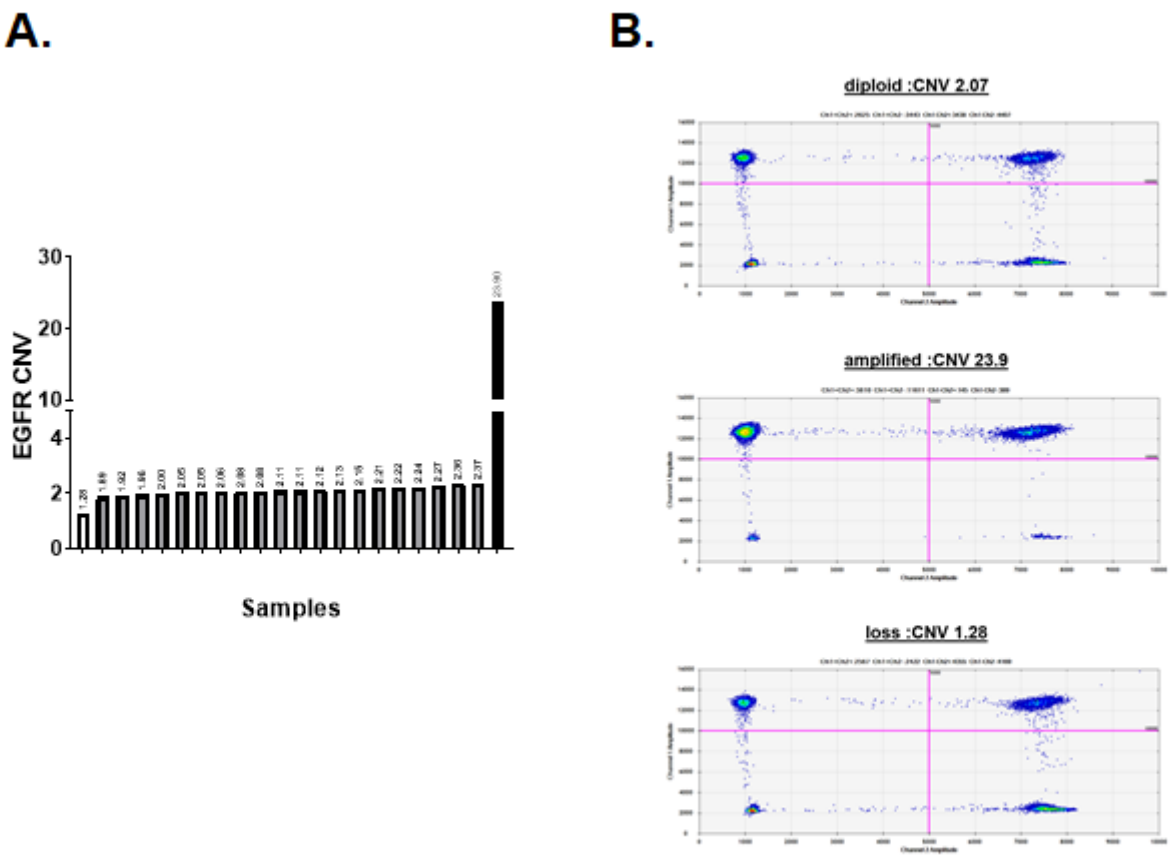

Figure 4

(A) PFS of patients treated with G/E or afatinib in a first-line setting. (B) Frequencies of EGFR driver and T790M mutations in plasma in all patients and of T790M mutations in plasma in patients who tested positive for EGFR driver mutation in plasma according to first-line afatinib and first-line G/E treatment. (C) PFS of patients treated with osimertinib after G/E and afatinib failure.