

# Relationship Between T790M Allele Frequency and Therapeutic Effects Before and After EGFR-TKI Administration Using Droplet Digital PCR in Non-small-cell Lung Cancer With *EGFR* Mutation

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## Abstract

**Background/Aim:** This study aimed to investigate the effectiveness of sequential treatment with afatinib and osimertinib by clarifying the correlation between therapeutic effects and *EGFR* T790M mutant allele frequency.

**Patients and Methods:** From August 2013 to July 2019, tumor samples from before and after epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) administration were collected from patients from eight institutions. We measured T790M mutant allele frequency using droplet digital polymerase chain reaction using biopsy specimens from patients mainly treated with afatinib and analyzed the T790M to EGFR-activating mutation ratio (T/A ratio) in pre- and post-biopsy tissue.

**Results:** Among 36 patients (afatinib group: n=24, first-generation EGFR-TKI group: n=12) with preserved pre- and post-biopsy tissue, the median T/A ratios before (pre-T/A ratio) and after EGFR-TKI administration (post-T/A ratio)

*continued*



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in the afatinib group were 0.005 and 0.014, and those in the first-generation EGFR-TKI group were 0.026 and 0.352, respectively. The results of a Mann–Whitney *U*-test revealed that the difference between the pre-T/A and post-T/A ratios was not higher in the afatinib group than in the first-generation EGFR-TKI ( $p=0.0372$ ). No significant difference in progression-free or overall survival was found between the two groups.

**Conclusion:** Compared with first-generation EGFR-TKI treatment, treatment with afatinib did not affect changes in the T/A ratio.

**Keywords:** Afatinib, EGFR-TKI, osimertinib, digital PCR, *EGFR* mutation.

## Introduction

Compared with platinum-based chemotherapy, epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have been reported to significantly improve progression-free survival (PFS) in patients with advanced non-small-cell lung cancer (NSCLC) harboring EGFR-activating mutation (1-4). Afatinib, a second-generation EGFR-TKI, is known for its selective inhibitory effects on both *EGFR* mutations and the ErbB receptor family (5, 6). Furthermore, afatinib has demonstrated a significant prolongation of PFS and time-to-treatment failure compared with gefitinib in patients with advanced NSCLC with *EGFR* mutations (7, 8). In cases of acquired resistance to EGFR-TKI, *EGFR* T790M mutation accounts for approximately half of all cases (9-11). Osimertinib, a third-generation EGFR-TKI, inhibits both EGFR-TKI sensitizing mutations and *EGFR* T790M resistance mutations (12). The FLAURA trial reported longer PFS and overall survival (OS) with osimertinib than with gefitinib or erlotinib in a first-treatment setting (13, 14).

Numerous other uncommon *EGFR* mutations have also been reported, and compound mutations in which two or more mutations coexist within the tyrosine kinase domain are frequently identified (15-19). Afatinib, which has demonstrated efficacy in patients with NSCLC who harbor major uncommon *EGFR* and compound mutations as well as in patients with common *EGFR* mutations, is often used (20).

Whether the T790M mutation should be considered a *de novo* mutation that exists prior to EGFR-TKI treatment (clonal selection hypothesis) or a second hit that appears

during EGFR-TKI treatment (second hit hypothesis) remains a topic of debate. However, with the development of more highly sensitive mutation detection methods (21, 22), a small number of T790M mutations are believed to be present before EGFR-TKI treatment, even if they are below the detection limit of conventional methods. If the clonal selection hypothesis is true, it is thought that the T790M mutation is selectively increased by EGFR-TKIs, after which, osimertinib is administered to achieve a high therapeutic effect. It is hypothesized that the afatinib to osimertinib sequence therapy would be very effective. However, few studies have been conducted on sequential treatment with afatinib and osimertinib in patients with *EGFR* mutation-positive advanced NSCLC. In the GIO-TAG trial, the median duration of sequential treatment with afatinib and osimertinib for patients with *EGFR* mutation who acquired T790M mutation during treatment with afatinib was 27.6 months, and this treatment may be a particularly attractive option in Asian patients with *EGFR* mutation exon19 deletion (23). Therefore, whether sequential therapy with afatinib and osimertinib prolongs OS compared with first-line treatment with osimertinib remains a clinical question.

We previously reported that patients with EGFR-mutated NSCLC with high T790M allele frequency before EGFR-TKI administration (pre-T790M) had significantly shorter PFS than did those with low pre-T790M allele frequency (24). We also found that pre-T790M abundance may not necessarily confer post-T790M mutation, but the number of specimens after EGFR-TKI administration was small, and the prognosis in paired specimens was not evaluated. In addition, most of

the patients in that study received first-generation EGFR-TKIs as the first-line treatment.

In the present study, to investigate the correlation between therapeutic effects and *EGFR* T790M mutant allele frequency, we assessed pre- and post-T790M allele frequencies using droplet digital polymerase chain reaction (ddPCR) in biopsy specimens obtained from patients mainly treated with afatinib.

## Patients and Methods

**Study design and patient population.** Patients aged 20 years or older with pathologically diagnosed stage IIIB to IV NSCLC with sensitizing *EGFR* mutation were eligible for enrollment. In addition, both pre- and post-EGFR-TKI treatment tissue samples had to be available, and all prior treatment before re-biopsy had to involve EGFR-TKI treatment only, excluding prior chemotherapy. From August 2013 to July 2019, patients were accrued from eight institutions. We collected the following clinical data for analyses: sex, age, disease stage, smoking status, performance status (PS), and T790M mutation status. We defined PFS as the time from treatment initiation to disease progression or death from any cause. In this study, the switching of EGFR-TKIs due to adverse events was considered as continuation of EGFR-TKI treatment. In such cases, PFS was defined as the time from first-line EGFR-TKI treatment initiation to disease progression or death during subsequent EGFR-TKI treatment. In addition, we defined OS as the time from treatment initiation to death from any cause. This study was approved by the central institutional review boards and ethics committees of all participating institutions, and written informed consent was obtained from all patients before the study began.

**Sample collection.** Four 10-mm-thick sections were obtained from each formalin-fixed paraffin-embedded (FFPE) specimen for the ddPCR. Genomic DNA was extracted from the FFPE specimens using the QIAmp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

**Probes and primers for ddPCR and detection of *EGFR* mutations.** Primers and probes were procured from MBL-IDT K.K. (Nagoya, Japan). Fluorescent probes targeting wild-type and mutant sequences were conjugated to tetrachlorofluorescein (TET; lex 522 nm/ $\lambda$ em 539 nm) or 6-carboxyfluorescein (FAM; lex 494 nm/ $\lambda$ em 522 nm) fluorophores with ZEN/IABkFQ double quencher, respectively. The primers and probes listed in the reference (25) were used.

A multiplex assay was developed to identify three common *EGFR* mutations and each corresponding wild-type sequence (25). This study followed that established method.

**Data analysis for ddPCR and quantification of T790M.** RainDrop Analyst software (Bio-Rad Laboratories, Hercules, CA, USA) was used to analyze the droplet event data according to the manufacturer's instructions. Briefly, sample data were loaded with a drop-size gating template (Bio-Rad Laboratories). Data from the positive control sample were used to create the compensation matrix in RainDrop Analyst. The compensation matrix was applied to data from each sample to eliminate crosstalk fluorescence signals from the TET and FAM fluorophores. The sizes and locations of wild-type and mutant-specific gates were established by manual selection of the area containing wild-type or mutant-specific clusters in the positive control.

For each unknown sample, ddPCR-positive droplet events were counted within each gate. The number of events within each gate was converted to the number of events per assay based on the total number of intact drops.

In this study, we excluded samples that had fewer than 10 events/assay with EGFR-activating mutation. The T790M to EGFR-activating mutation ratio (T/A ratio) was calculated using the following method. The fractional abundance of EGFR-activating mutation and T790M were measured based on the droplet count by ddPCR, and the T/A ratio was defined as the ratio of T790M to EGFR-activating mutation.

**Statistical analysis.** The relationship between T790M allele frequency and the patients' clinicopathological

characteristics was analyzed using the  $\chi^2$  or Fisher's exact test, depending on the numbers of patients. Differences in continuous data were compared using the Mann-Whitney *U*-test, and PFS and OS were estimated using the Kaplan-Meier method. To compare the survival curves among the patient subgroups, the log-rank test was used. All statistical analyses were performed using EZR software (26) (Jichi Medical University, Shimotsuke, Japan), which is a modified version of R commander designed to add the statistical functions frequently used in biostatistics. All *p*-values <0.05 were considered statistically significant.

## Results

The patients' characteristics are shown in Table I. The median age of the patients was 68 years, 69.4% were women, 58.3% had no history of smoking, 52.8% had exon19 deletion, and 47.2% had exon21 L858R mutation. Twelve patients had received first-generation EGFR-TKIs and 24 had received afatinib. The detection rate of the T790M mutation in clinical practice was 66.7% in the first-generation EGFR-TKI group and 45.8% in the afatinib group. The actual number of patients treated with osimertinib was six (50.0%) in the first-generation EGFR-TKI group and nine (37.5%) in the afatinib group. The T790M detection rates among 36 patients with preserved pre- and post-biopsy tissue before and after EGFR-TKI treatment were 80.6% and 75.0%, respectively. The median pre- and post-T/A ratios in the first-generation EGFR-TKI group were 0.026 and 0.352, and those in the afatinib group were 0.005 and 0.014, respectively (Figure 1A). The results of the Mann-Whitney *U*-test revealed that the difference in the T/A ratio was significantly higher in the first-generation EGFR-TKI group than in the afatinib group (*p*=0.0372, Figure 2A). No significant difference in PFS or OS was found between the first-generation EGFR-TKI group [PFS: 309.5 days (95% confidence interval (CI)=150-1000); OS: 1,566 days (95%CI=472-NA)] and the afatinib group [PFS: 459.5 days (95%CI=269-832);

Table I. *The study participants' baseline characteristics.*

| Characteristics             | 1 <sup>st</sup> generation EGFR-TKIs* | Afatinib**   |
|-----------------------------|---------------------------------------|--------------|
| Number of patients          | 12                                    | 24           |
| Median age in years (range) | 69.5 (55-85)                          | 67.0 (38-81) |
| Sex                         |                                       |              |
| Male                        | 5 (41.7%)                             | 6 (25.0%)    |
| Female                      | 7 (58.3%)                             | 18 (75.0%)   |
| Smoking                     |                                       |              |
| Never                       | 3 (25.0%)                             | 18 (75.0%)   |
| Smoker                      | 2 (16.7%)                             | 5 (20.8%)    |
| Unknown                     | 7 (58.3%)                             | 1 (4.2%)     |
| Stage                       |                                       |              |
| III                         | 0 (0%)                                | 2 (8.3%)     |
| IV                          | 11 (91.7%)                            | 16 (66.7%)   |
| Postoperative recurrence    | 1 (8.3%)                              | 6 (25.0%)    |
| Histology                   |                                       |              |
| Adenocarcinoma              | 12 (100%)                             | 23 (95.8%)   |
| NSCLC                       | 0 (0%)                                | 1 (4.2%)     |
| ECOG PS                     |                                       |              |
| 0-1                         | 8 (66.7%)                             | 23 (95.8%)   |
| ≥2                          | 3 (25.0%)                             | 1 (4.2%)     |
| Unknown                     | 1 (8.3%)                              | 0 (0%)       |
| EGFR mutation status        |                                       |              |
| Exon19 deletion             | 7 (58.3%)                             | 12 (50.0%)   |
| Exon21 L858R                | 5 (41.7%)                             | 12 (50.0%)   |
| T790M mutation in practice  | 8 (66.7%)                             | 11 (45.8%)   |
| Cases of osimertinib use    | 6 (50.0%)                             | 9 (37.5%)    |

ECOG: Eastern Cooperative Oncology Group; PS: performance status; EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitor; NSCLC: non-small-cell lung cancer. In this study, the switching of EGFR-TKIs due to adverse events was considered as continuation of EGFR-TKI treatment. \*Gefitinib, erlotinib, and gefitinib/erlotinib were included. \*\*Afatinib + bevacizumab and gefitinib/afatinib were included.

OS: 1,883 days (95%CI=804-NA); *p*=0.743 and *p*=0.832, respectively] (Figure 3A).

The results of the analysis of the T/A ratio by *EGFR* mutation type (exon19 deletion vs. exon21 L858R) showed that in the exon19 deletion population, the median pre- and post-T/A ratios were 0.005 and 0.009 in the afatinib group and 0.022 and 0.313 in the first-generation EGFR-TKI group, respectively (Figure 1B). The difference between the pre- and post-T/A ratios in the exon19 deletion population was significantly higher in the first-generation EGFR-TKI than in the afatinib group (*p*=0.0465, Figure 2B). No significant difference in PFS or OS was observed in the exon19 deletion population

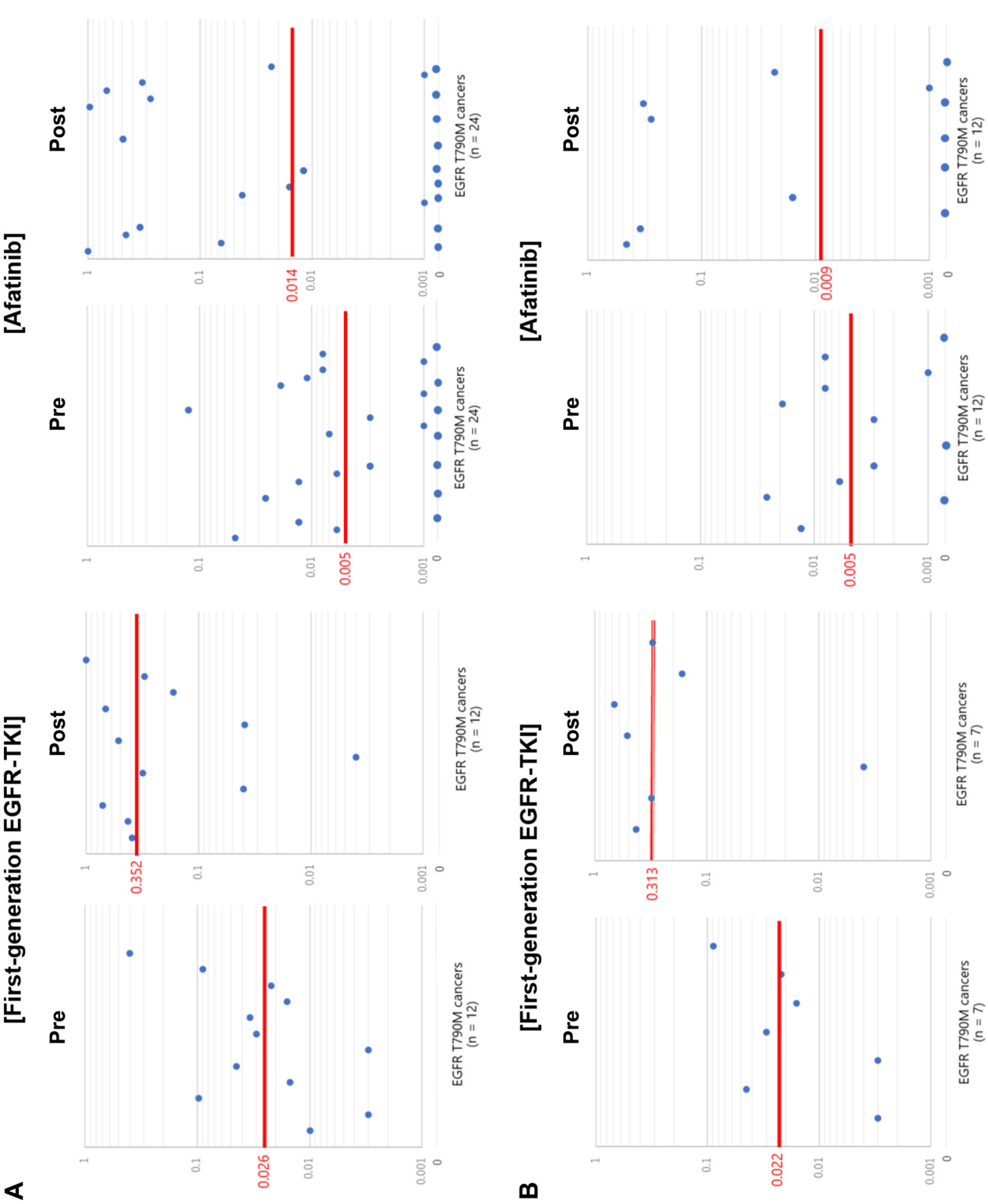


Figure 1. Continued

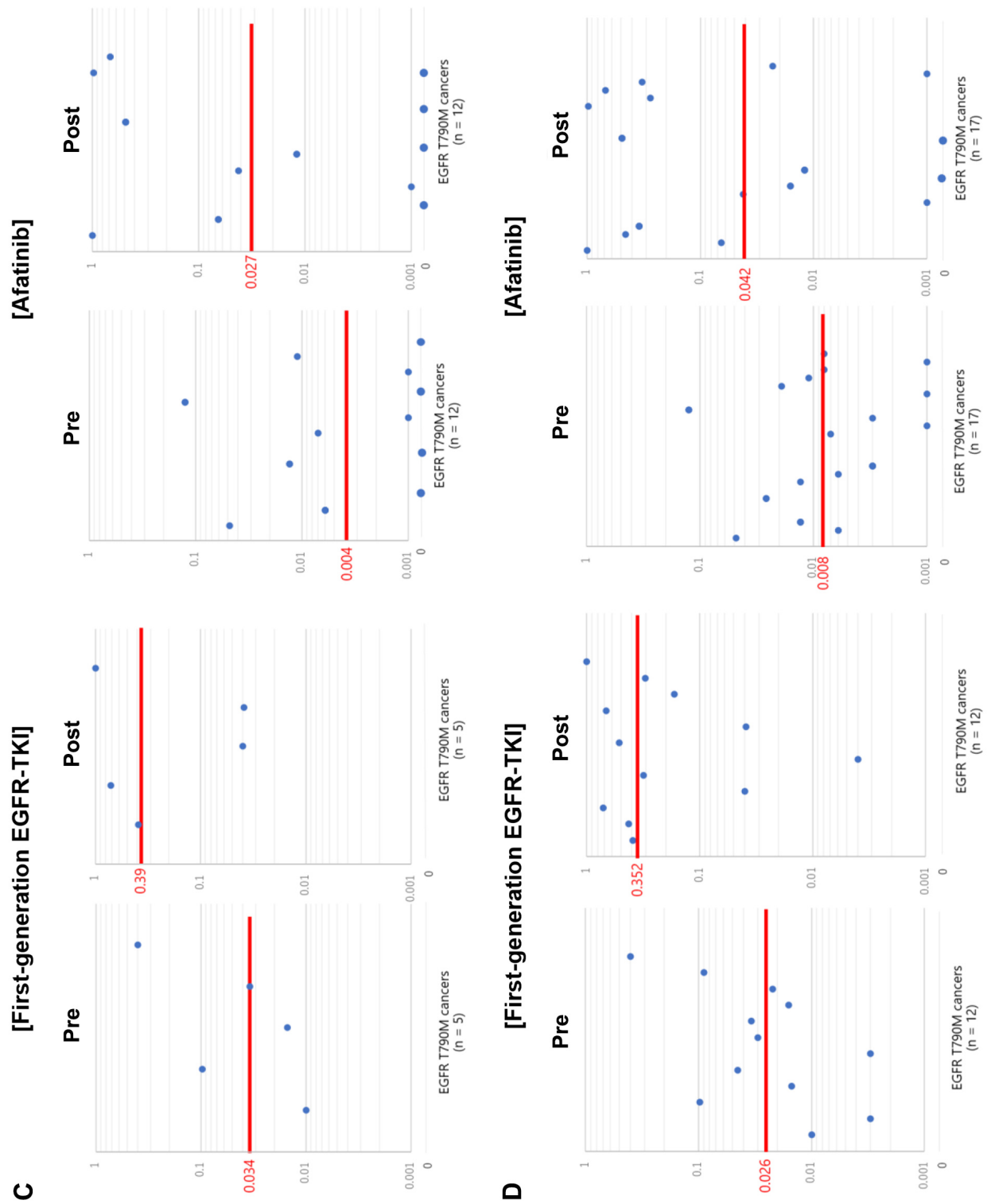


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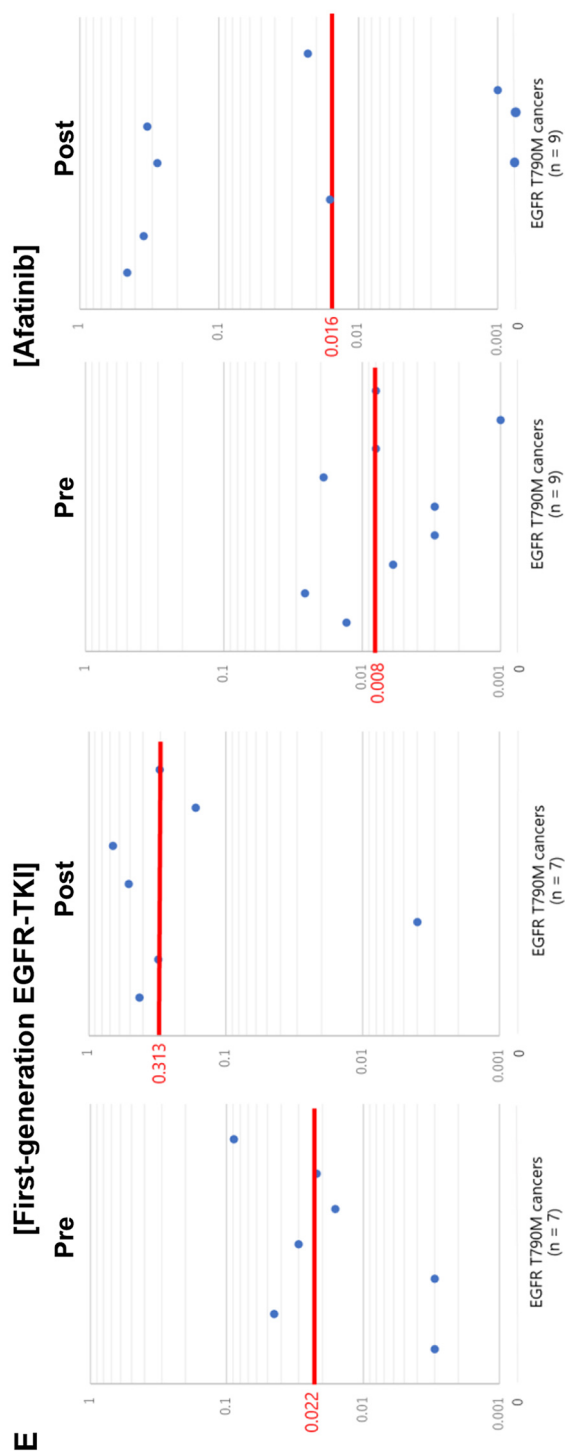


Figure 1. Pre- and post-T790M to EGFR-activating mutation ratio (T/A) ratios in the first-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) and afatinib groups. (A) Total population, (B) exon21 L858R population, (C) exon19 deletion population, (D) EGFR T790M negative cases, (E) total population except pre-T790M negative cases.

between the first-generation EGFR-TKI group [PFS: 278 days (95%CI=150-588); OS: 1,617 days (95%CI=167-NA)] and the afatinib group [PFS: 470 days (95%CI=216-916); OS: 1,956 days (95%CI=398-NA);  $p=0.739$  and  $p=0.652$ , respectively] (Figure 3B).

The median pre- and post-T/A ratios in the exon21 L858R population of the afatinib group were 0.004 and 0.027, and those of the first-generation EGFR-TKI group were 0.034 and 0.390, respectively (Figure 1C). No significant difference in the pre- or post-T/A ratio in the exon21 L858R population was found between the two groups ( $p=0.429$ ; Figure 2C). No significant difference in PFS or OS was observed in the exon21 L858R population between the first-generation EGFR-TKI group [PFS: 444 days (95%CI=139-NA); OS: 1,566 days (95%CI=472-NA)] and the afatinib group [PFS: 452.5 days (95%CI=212-1011); OS: 1,065 days (95%CI=481-NA);  $p=0.956$  and  $p=0.976$ , respectively] (Figure 3C).

In this study, T790M-negative cases were observed only in the afatinib group. As pre-T790M-negative cases did not fit the rationale of this study, we compared the T/A ratios between the two groups in the total and exon19 deletion populations, except for pre-T790M-negative cases. The median pre- and post-T/A ratios in the first-generation EGFR-TKI group were 0.026 and 0.352, and those in the afatinib group were 0.008 and 0.042, respectively (Figure 1D). The results of the Mann-Whitney *U*-test showed no significant difference in the pre- and post-T/A ratios between the two groups ( $p=0.223$ ; Figure 2D). In addition, no significant difference in PFS or OS was found between the first-generation EGFR-TKI [PFS: 309.5 days (95%CI=150-1,000); OS: 1,566 days (95%CI=472-NA)] and the afatinib group [PFS: 506.0 days (95%CI=252-916); OS: 1,883 days (95%CI=673-NA);  $p=0.569$  and  $p=0.748$ , respectively] (Figure 3D). The median pre- and post-T/A ratios in the exon19 deletion population of the afatinib group were 0.008 and 0.016, and those in the first-generation EGFR-TKI group were 0.022 and 0.313, respectively (Figure 1E). The difference between the pre- and post-T/A ratios in the first-generation EGFR-TKI group tended to be higher than that

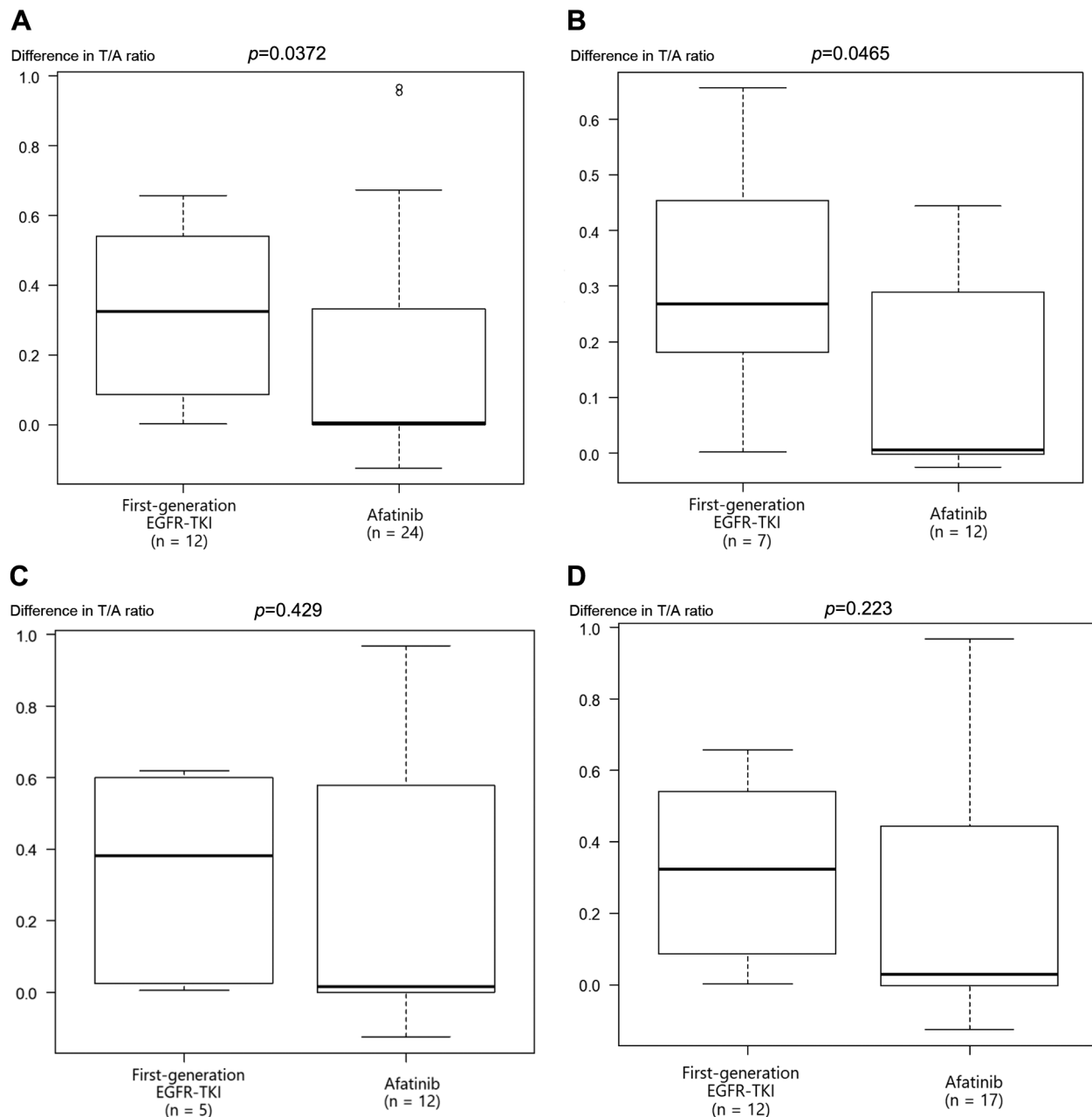


Figure 2. Continued

the afatinib group, but no significant difference was found ( $p=0.072$ , Figure 2E). No significant difference in PFS and OS was found between the exon19 deletion population of the first-generation EGFR-TKI group [PFS: 278 days (95%CI=150-588); OS: 1,617 days (95%CI=167-NA)] and the afatinib group [PFS: 358 days (95%CI=41-916); OS:

1,118 days (95%CI=348-NA);  $p=0.923$  and  $p=0.952$ , respectively] (Figure 3E).

In short, excluding pre-T790M-negative cases, no significant change in the T/A ratio was found between the two groups in the overall or exon19 deletion population.

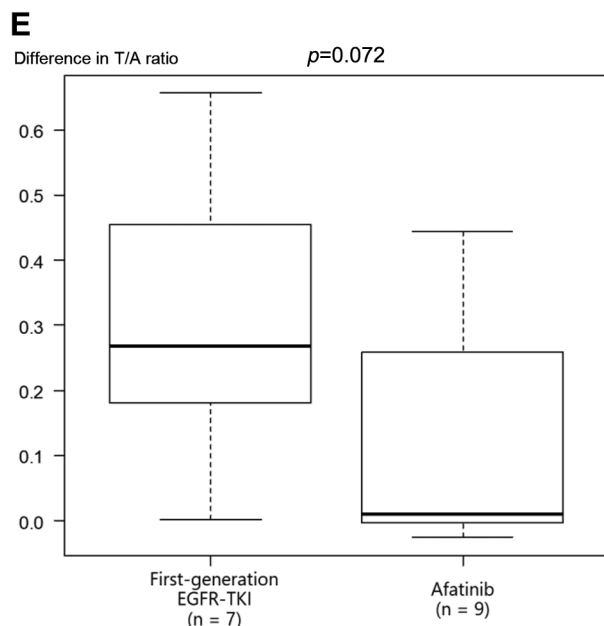


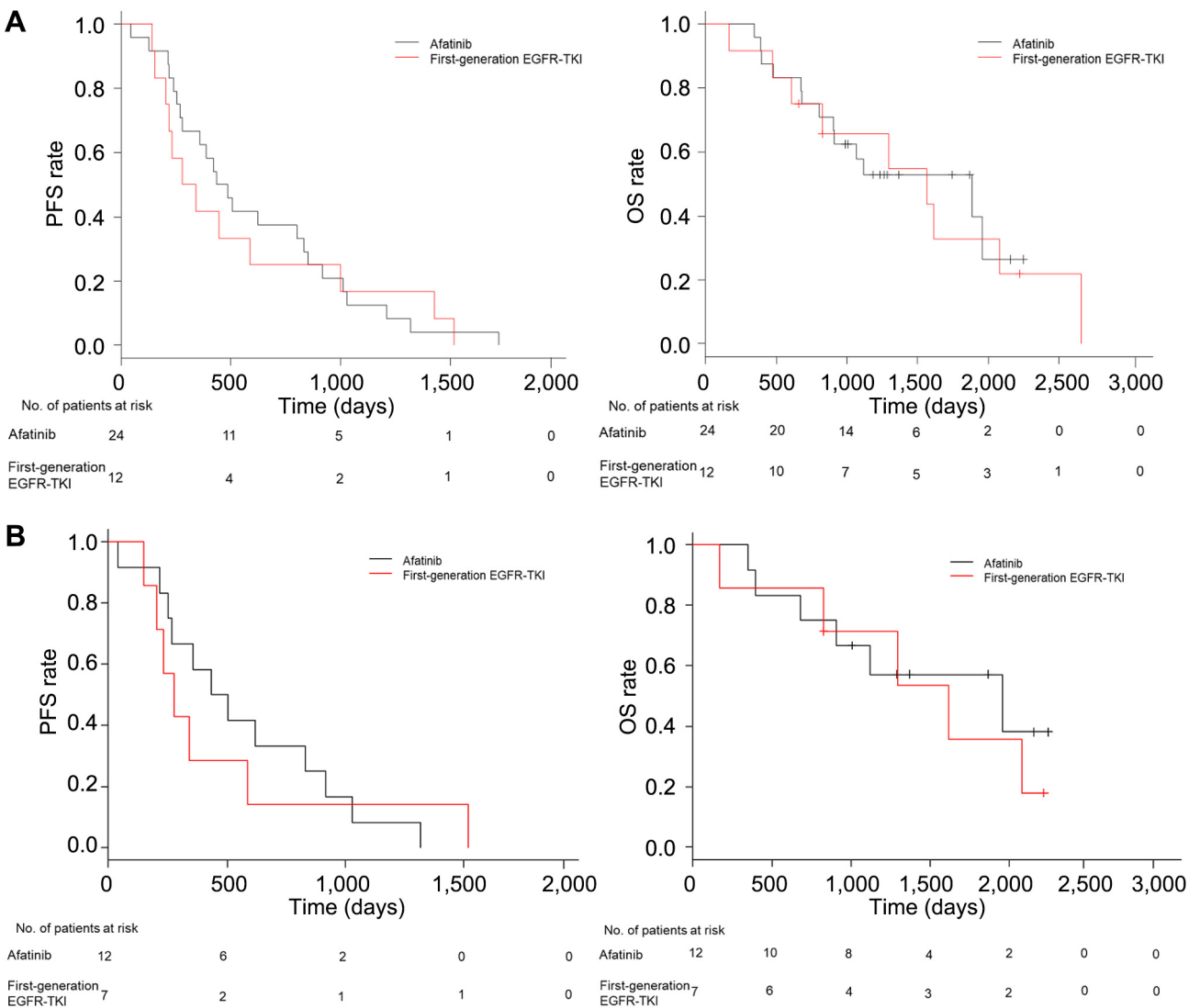
Figure 2. Box plot of a comparison of T790M ratios using the Mann-Whitney U test in the first-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) and afatinib groups. (A) Total population, (B) exon19 deletion population, (C) exon21 L858R population, (D) total population except pre-T790M negative cases, (E) exon19 deletion except pre-T790M negative cases.

## Discussion

T790M mutation is the major acquired resistance mutation to first-generation EGFR-TKI or afatinib with an incidence of up to 60% in patients with EGFR mutation-positive NSCLC, after which the only effective EGFR-TKI available is osimertinib. Real-world data suggests that sequential therapy with afatinib followed by osimertinib may enhance the efficacy of osimertinib in patients with the T790M mutation, potentially due to the effects of prior afatinib treatment (23, 27). Several studies reported that both high ratio of T790M mutation and high mutation allele frequency of T790M were associated with better osimertinib treatment outcomes in TKI-relapsed patients with EGFR mutation positive NSCLC (28, 29). The present study did not show differences in allele frequency of T790M mutation, PFS, and OS between afatinib and first-generation EGFR-TKI in EGFR-mutant NSCLC, using paired

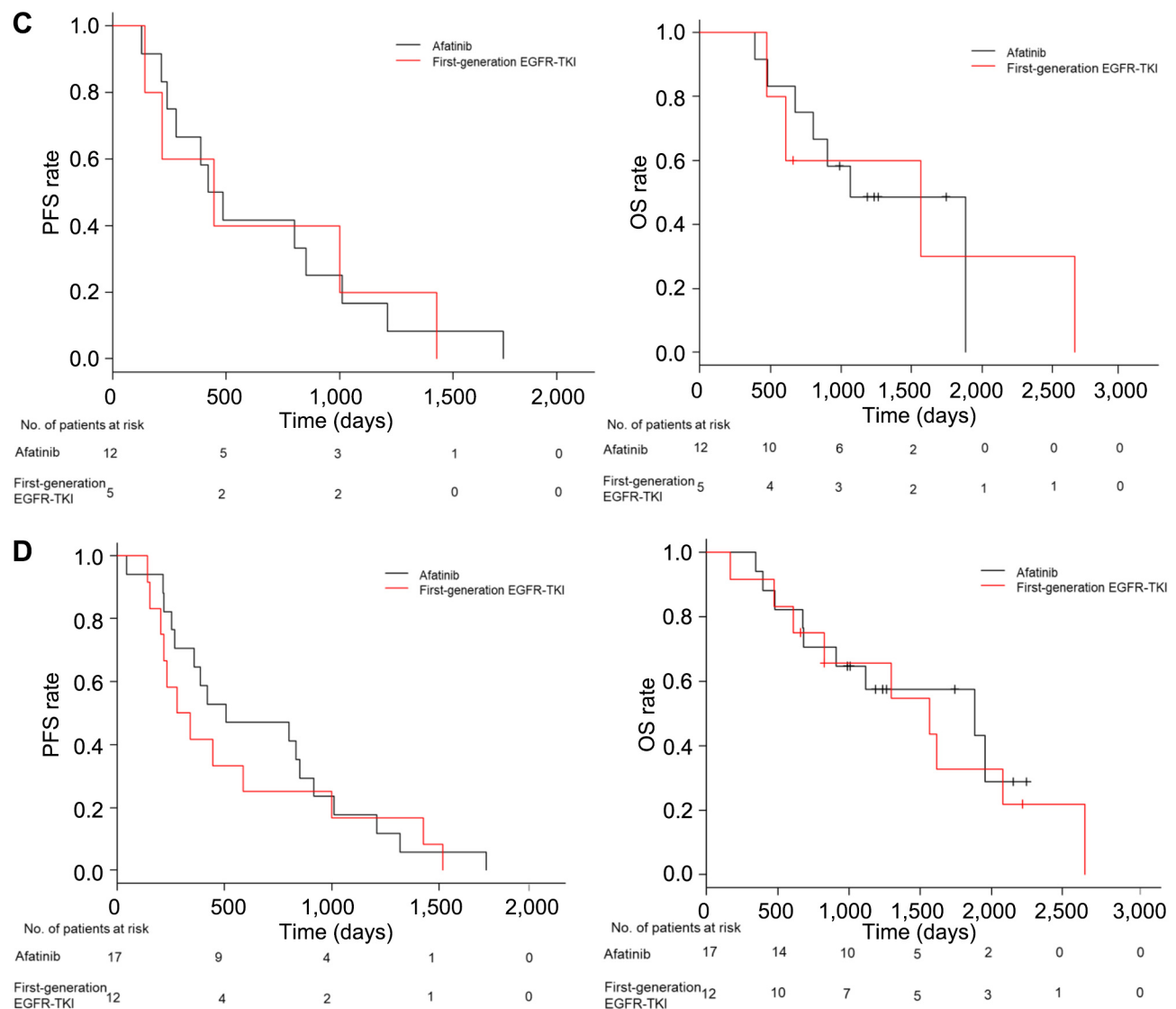
samples before and after treatment. In this study, we directly compared the pre- and post-treatment T/A ratios between afatinib and first-generation EGFR-TKI in EGFR-mutated NSCLC, and the mean pre- and post-T/A ratios of afatinib and first-generation EGFR-TKI were consistent with previous studies (30-32).

In our study, the high detection rate of the T790M mutation was observed in both pre and post samples, consistent with a previous report (22). In the first-generation EGFR-TKI group, the T790M mutation was detected in all samples before and after EGFR-TKI, whereas it was not detected in some samples in the afatinib group. Specifically, the T790M mutation was not observed in seven cases before treatment (29.1%) and in nine cases after treatment (37.5%), with four of these cases (16.6%) showing no detection of the mutation in both pre- and post-treatment samples. The post-T/A ratio in both groups increased compared to the pre-T/A ratio. Both the pre- and post-T/A ratios were higher in the first-generation EGFR-TKI group compared to the afatinib group. Additionally, the changes in the T/A ratios before and after treatment were also significantly greater in the first-generation EGFR-TKI group than in the afatinib group. These significant changes were observed in the exon19 deletion population as well as in the overall population, whereas those in the exon21 L858R population did not significantly differ between groups. These differences in change by treatment may be due to several cases of undetectable T790M mutation in the afatinib group. Additionally, there were no cases in the first-generation EGFR-TKI group in which the T/A ratio was lower in the post- than in the pre-biopsy specimens, but there were several cases in the afatinib group in which the T/A ratio was lower in the post- than in the pre-biopsy specimens. Afatinib is known to exhibit inhibitory activity against the T790M mutation (33), therefore it is possible that the T790M mutation may have been eliminated in these patients who received afatinib treatment. Our result did not reveal that tumor cells harboring T790M mutation undergo selection and enrichment during afatinib treatment compared to first-generation EGFR-TKI,



although the T/A ratio increased during TKI treatment. The reason for the nonsignificant increase in the T/A ratio after resistance to afatinib treatment was likely responsible for the development of acquired resistance apart from T790M mutation. It is well known that EGFR mutation positive tumors often contain cooccurring alterations in genes, such as TP53, PI3KCA, RB1, or ERBB2. Several studies have shown that the T790M mutation has features of spatial and temporal heterogeneity in the tumors in individuals with EGFR-mutant NSCLC who have

acquired resistance to EGFR-TKIs (34). Another possible reason could be tumor heterogeneity, as most specimens are biopsy specimens. Furthermore, EGFR-mutant NSCLC with positive PD-L1 expression exhibited a lower rate of T790M mutation detection after first- or second-generation EGFR-TKIs (35-38). Although we were unable to examine PD-L1 expression due to insufficient sample volume, patients with PD-L1-positive EGFR-mutant NSCLC may not benefit with regard to long-term survival with sequential afatinib and osimertinib (39). This study was

Figure 3. *Continued*

unable to prove the hypothesis that afatinib selectively enriches T790M-positive cancer cells more effectively than first-generation EGFR-TKIs.

Our study demonstrated a non-significant trend toward longer PFS in the afatinib group compared to the first-generation EGFR-TKI group, both in the overall population and in the exon19 deletion population. There was no significant difference in OS between the two groups. In our population, the incidence of T790M mutation in clinical

practice was 66.7% in the first-generation EGFR-TKI group and 45.8% in the afatinib group. Additionally, among those with acquired resistance to EGFR-TKIs, the rate of osimertinib was also higher in the first-generation EGFR-TKI group compared to the afatinib group (50.0% vs. 37.5%). The differences in both the incidence of T790M mutation and the use of osimertinib had a significant impact on OS in our study. A clinical trial has been conducted to evaluate the efficacy of sequential therapy

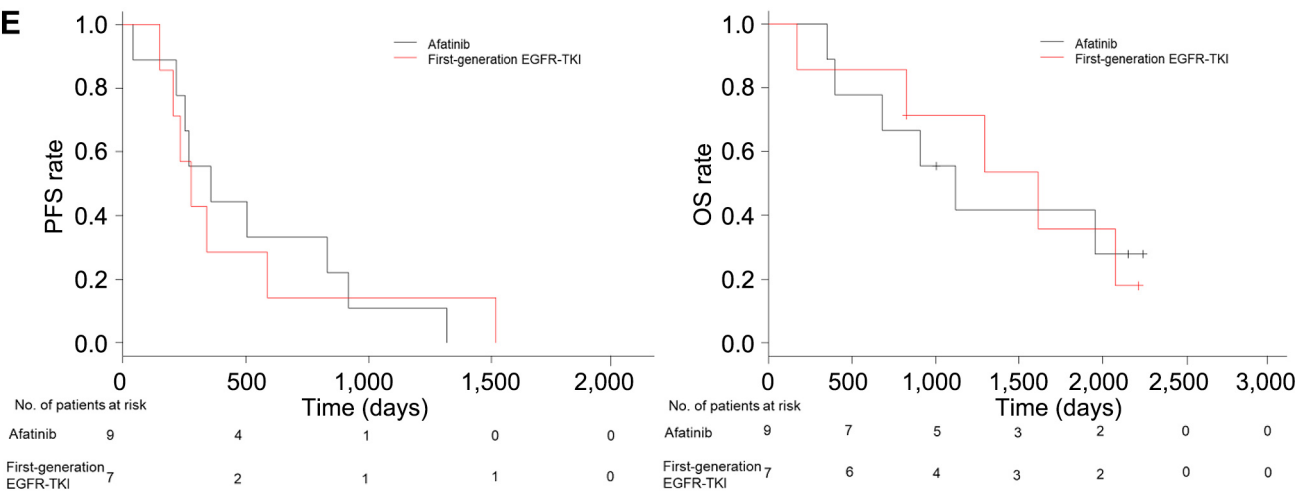


Figure 3. Kaplan–Meier curves for progression-free survival (PFS) and overall survival (OS) in the first-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) and afatinib groups. (A) Total population, (B) exon19 deletion population, (C) exon21 L858R population, (D) total population except pre-T790M negative cases, (E) exon19 deletion except pre-T790M negative cases.

with afatinib and osimertinib. A randomized phase II study comparing switching after 8 months of afatinib treatment to treatment with osimertinib without progressive disease and osimertinib monotherapy has been conducted and it was recently reported that the primary endpoint, the 2-year progression-free survival did not improve. Considering PD-L1 expression and resistance mechanisms other than the EGFR mutation in EGFR mutation-positive NSCLC, treatment strategies are likely to be osimertinib-based combination therapies (40, 41).

**Study limitations.** First, this is a retrospective study with a small sample size. Second, the patients were selected based on the availability of sufficient tissue samples. Third, we were unable to examine genetic mutations other than the T790M mutation, or PD-L1 expression. Therefore, it is not clear whether patients treated with afatinib in our study are representative of positive EGFR-mutant NSCLC.

Our study found no significant difference in PFS, OS, or T790M enrichment between afatinib and first-generation EGFR-TKIs in EGFR-mutated NSCLC. While afatinib did not selectively increase the T790M mutation, the findings highlight the complexity of resistance mechanisms and the need for personalized treatment strategies. Future

research should explore molecular profiling, co-occurring mutations, and sequential therapy approaches to optimize outcomes in EGFR-TKI-treated patients.

### Conflicts of Interest

HK, Honoraria: Boehringer Ingelheim, AstraZeneca, Chugai Pharmaceutical. YK, Honoraria: AstraZeneca, Chugai Pharmaceutical, Boehringer Ingelheim. Research funding: Boehringer Ingelheim, Chugai Pharmaceutical. KS, Honoraria: MSD, Eli Lilly Japan, AstraZeneca, Kyowa Kirin, Nippon Kayaku, Chugai Pharmaceutical, Taiho Pharmaceutical, Ono Pharmaceutical. MT, Honoraria: AstraZeneca, Chugai Pharmaceutical, Boehringer Ingelheim, Ono Pharmaceutical, Pfizer, Eli Lilly. Research funding: Chugai Pharmaceutical, MSD, Ono Pharmaceutical, Eisai, Daiichi-Sankyo, Yansen. NI, Honoraria: AstraZeneca, Boehringer Ingelheim. HS, Honoraria: AstraZeneca, Chugai Pharmaceutical, Boehringer Ingelheim, MSD. MK, Honoraria: MSD, Eli Lilly Japan, AstraZeneca, Chugai Pharmaceutical, Boehringer Ingelheim Japan, Shionogi Pharmaceutical, Ono Pharmaceutical. YS, Honoraria: MSD, Eli Lilly, AstraZeneca, Kyowa Kirin, Nippon Kayaku, Chugai Pharmaceutical, Taiho Pharmaceutical, Ono Pharmaceutical, Boehringer Ingelheim,

Pfizer, Novartis, Takeda, Bristol Myers Squibb, TK, Honoraria: Chugai Pharmaceutical, MSD. All other Authors declare no conflicts of interest.

## Authors' Contributions

Koichi Ogawa (KO) and Hiroyasu Kaneda (HK) contributed to the concept and design of this study. Motohiro Tamiya (MT), Nobuhisa Ishikawa (NT), Kenichi Minami (KM), Hidekazu Suzuki (HS), Yosuke Eguchi (YE), Masaki Kanazu (MK), and Yuki Sato (YS) collected the samples and associated data. Yasuhiro Koh (YK) conducted the experiments. KO led the data analysis with statistical advice from Kenji Sawa (KS). KO wrote the first draft of the paper. KO, HK, KS, Yoshiya Matsumoto, MT, NT, KM, HS, YE, MK, YS, YK, and Tomoya Kawaguchi (TK) contributed to and approved the final manuscript.

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