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Comparison of T790M Acquisition Between Patients Treated with Afatinib and Gefitinib as First-Line Therapy: Retrospective Propensity Score Matching Analysis

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Abstract

Afatinib, a second-generation, irreversible pan-HER inhibitor, shows better suppression of T790M-positive lung cancer cells than gefitinib in preclinical studies. However, whether the effect of afatinib on T790M acquisition differs from that of gefitinib when used clinically as first-line therapy remains unclear. To reaffirm the preclinical efficacy of afatinib on T790M-positive lung cancer cells, H1975 cells and established PC-9 cells resistant to gefitinib and erlotinib by T790M were used. In total, 398 patients with second biopsy at progression with stage IIIB/IV non–small cell lung cancer with EGFR mutation, treated with afatinib or gefitinib as first-line therapy, were retrospectively reviewed. Propensity score matching was used to balance covariates. Afatinib inhibited the growth of lung cancer cells with low T790M allele frequencies, which are resistant to gefitinib, but not those with high T790M allele frequencies. Afatinib and gefitinib showed similar efficacy in terms of progression-free survival (PFS) (11.5 vs 13.4 months, $P = .08$) and overall survival (OS) (29.3 vs 28.5 months, $P = .76$). T790M patients had better PFS and OS than those without T790M. There was no significant difference in the cumulative T790M acquisition ratio over time between afatinib and gefitinib (48.8% vs 59.3%, $P = .317$). The median time to acquire T790M was 12.9 months for afatinib and 15.7 months for gefitinib ($P = .342$). Although afatinib inhibited the growth of lung cancer cells with low T790M allele frequencies in preclinical studies, this could not be translated into clinical efficacy in terms of lowering the rate or delaying the time of T790M acquisition.

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Introduction

Afatinib is a second-generation potent EGFR-TKI that covalently binds to all homo- and heterodimers formed by the members of the HER family, such as EGFR, HER2, ErbB3, and ErbB4 [1]. In both cell-free kinase and cell proliferation assays, afatinib shows much better inhibitory activity on EGFR harboring L858R/T790M mutations compared to first-generation EGFR-TKIs [1–4]. Research shows that the activity of afatinib on the T790M mutation was lower than that on single sensitizing mutations such as L858R or E19del, but it remained within a range that indicated similar effectiveness [5]. In addition, afatinib reduced tumor volume by approximately half in transgenic mice with L858R/T790M mutation-driven cancer [2].

Although afatinib was expected to overcome acquired resistance to first-generation EGFR-TKI, it failed to show an overall survival (OS) benefit over placebo in patients with advanced EGFR-mutant lung cancer following previous EGFR-TKI therapy [6]. This discordance with preclinical data might be explained by amplification of T790M.

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The irreversible EGFR-TKIs were initially effective *in vitro* and *in vivo* against EGFR mutant tumors containing T790M, while resistance arose when T790M was amplified over time [7]. The efficacy of afatinib on T790M seems to be dependent on the T790M allele frequency, not just positivity of T790M. This implies that the clinical activity of afatinib could also vary according to the T790M allele frequency. The improvement of progression-free survival (PFS), tumor response rate, and disease-related symptoms in the LUX-Lung 1 clinical trial [6] despite no OS benefit could be understood in a similar context.

Therefore, we assumed that afatinib may reduce the rate or delay the time of T790M acquisition during first-line EGFR-TKI therapy because the T790M amplification that decreases afatinib efficacy probably occurs in the later phase. Accordingly, retrospective studies revealed that the T790M acquisition by afatinib (20.0%) was lower than that by gefitinib (52.8%) or erlotinib (44.6%) [8]. However, this is controversial due to contradictory reports showing that the frequency of T790M at the time of progression was not different according to the type of EGFR-TKIs [9,10].

In this study, we reaffirmed the preclinical activity of afatinib using EGFR-mutant lung cancer cells with different T790M allele frequencies and examined whether the cumulative ratio of T790M over time or the median time to acquire T790M differed between patients treated with afatinib and gefitinib as first-line therapy.

Material and Methods

Cell Culture and Reagents

The H1975 cell line was obtained from the American Type Culture Collection (Rockville, MD). The PC-9 cells were kindly provided by Dr. Kazuto Nishio (National Cancer Center Hospital, Tokyo, Japan). PC-9/GR (gefitinib-resistant cell line) and PC/ER (erlotinib-resistant cell line) have been established in previous studies [11,12]. Cells were cultured in RPMI1640 medium containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C in an atmosphere of 5% CO_2. Tests for mycoplasma contamination were negative. Afatinib was purchased from Selleck Chemicals (Houston, TX).

Cell Viability Assay

Cells (5×10^3) were seeded in 96-well sterile plastic plates, incubated overnight, and then treated with the drugs. After 72 hours, 15 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well, and the plates were incubated for 4 hours. Crystalline formazan was solubilized by adding 100 μl of 10% (w/v) sodium dodecyl sulfate and incubating for 24 hours, after which absorbance at 595 nm was spectrophotometrically recorded using a microplate reader. The results were representative of at least three independent experiments, with the error bars signifying standard deviation (SD). The IC_{50} values were calculated using the GraphPad Prism software (La Jolla, CA). To validate the long-term effects of afatinib, cells were treated with afatinib for 72 hours, and the medium was replaced with drug-free medium. After incubation for 5 days, attached cells were stained with a 0.2% trypan blue solution containing 50% methanol.

T790M Mutation Analysis

Peptide nucleic acid (PNA)–mediated PCR clamping assay (PNAClamp EGFR Mutation Detection kit, PANAGENE Inc., Dadjeon, Korea) was used to detect T790M mutation. The detection of T790M mutation was performed as previously described [11].

Study Population

This retrospective study was approved by the Institutional Review Board at the University of Ulsan, Asan Medical Center (2018-0541). The flow diagram of patient enrollment is illustrated in Figure 1*A*. From January 2008 to December 2017, 418 patients with locally advanced or metastatic adenocarcinomas of the lung underwent second biopsy after progression during first-line therapy with either gefitinib or afatinib at Asan Medical Center, Seoul, Korea, and Koshin University, Busan, Korea. A total of 398 patients whose second biopsy results were available were included in this study. Clinical information, such as age, gender, lung cancer staging according to AJCC 7th edition, first biopsy EGFR mutation type and second biopsy T790M status, OS, and PFS during TKI therapy, was retrospectively reviewed using electronic medical records.

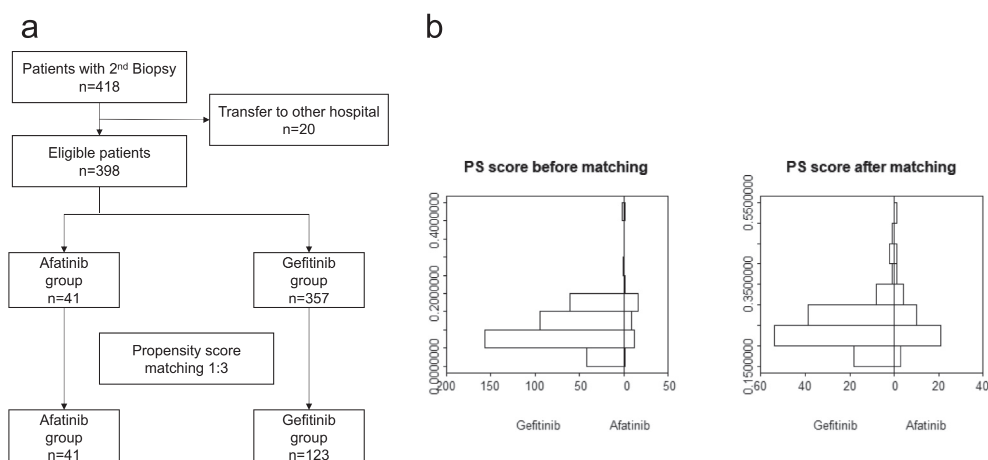

Figure 1. The process of patient enrollment in the study. (a) A total of 418 patients with second biopsies were identified, and 20 were transferred to another hospital. There were 398 eligible patients, where 41 were treated with afatinib and 357 received gefitinib. Propensity score matching resulted in 164 patients. (b) The propensity score before matching has an uneven distribution between afatinib and gefitinib; however, after matching, the distribution ratio between each score value becomes 1:3 between afatinib and gefitinib.

Table 1. Clinical Characteristics of 398 Patients with Adenocarcinoma of the Lung, with Second Biopsies After TKI Medication in the Presence of EGFR Mutation and Its Propensity Score–Matched Results

	Before Propensity Score Matching (*n* = 418)			After Propensity Score Matching (*n* = 164)		
Characteristics	Afatinib	Gefitinib	*P* Value	Afatinib	Gefitinib	*P* Value
N	41	357		41	123	
Age mean ± SD	59.2 ± 12.3	59.8 ± 10.8	.737	59.2 ± 12.3	60.9 ± 11.5	.417
Gender			.047			.928
Male	21 (51.2%)	122 (34.2%)		21 (51.2%)	66 (53.7%)	
Female	20 (48.8%)	235 (65.8%)		20 (48.8%)	58 (46.3%)	
Stage			.080			.992
IIIB	7 (17.1%)	93 (25.1%)		7 (17.1%)	21 (17.1%)	
IVA	7 (17.1%)	95 (26.6%)		7 (17.1%)	20 (16.3%)	
IVB	27 (65.9%)	169 (47.3%)		27 (65.9%)	82 (66.7%)	
1st biopsy			.324			.506
EGFR mutations						
E19del	27 (65.9%)	212 (59.4%)		27 (65.9%)	88 (71.5%)	
L858R	11 (26.8%)	131 (36.7%)		11 (26.8%)	31 (25.2%)	
Others	3 (7.3%)	14 (3.9%)		3 (7.3%)	4 (3.3%)	
T790M (+)	20 (48.8%)	146 (40.9%)	.422	20 (48.8%)	73 (59.3%)	.317

Propensity Score Matching

Of the 398 patients, 41 were on afatinib and 357 on gefitinib. Due to the discrepancy in patient baseline characteristics (Table 1), we used propensity score (PS) matching to identify similar baseline characteristics treated with afatinib or gefitinib. The matching was based on age, gender, AJCC 7th edition stage for lung cancer, and EGFR mutation type, with a ratio of 1:3 for afatinib versus gefitinib. The PS matching yielded 164 patients with no statistical difference in age, gender, stage, and first biopsy EGFR status.

Statistical Analysis

Statistical analysis was performed using R 3.5.1. To minimize the differences of baseline characteristics, PS matching was applied with optimal matching, implemented with the *MatchIt* [13] and *optmatch* [14] packages in R. Comparisons between two groups were performed using the *t* test for continuous data, and Pearson's chi-square test for categorical data. Cumulative distribution of T790M mutation over time was presented, and the *P* value was calculated using Pearson's chi-square test for the ratio, while the time to onset was calculated using Mann-Whitney-Wilcoxon test. The Kaplan-Meier method was used to plot OS and PFS according to the medication and the T790M status. The log-rank test was used to compare survival curves to calculate the *P* value. Subgroup analysis of OS and PFS and T790M status was performed using a univariate Cox proportional-hazard model. Univariate Cox analysis was performed to calculate hazard ratios (HRs) and 95% confidence interval (CI) along *P* values, where a two-tailed *P* value of <.05 was set as statistically significant. *P* values lower than .2 in univariate Cox analysis were included in the multivariate Cox analysis, where *P* values of <.05 were set as significant. The above was implemented with the *survival* [15] package in R.

Results

The Effect of Afatinib on the Growth of EGFR-Mutant Lung Cancer Cells with Different T790M Allele Frequencies

Gefitinib- and erlotinib-resistant cells were established as in previous studies [16,17]. In a similar study [17], PC-9/GR (H) and PC-9/ER (H) cells showed increased T790M allele frequencies, and they also were more resistant to afatinib compared to PC-9/GR (L) and PC-9/ER (L) (Figure 2, *A-C*). These results suggest that the

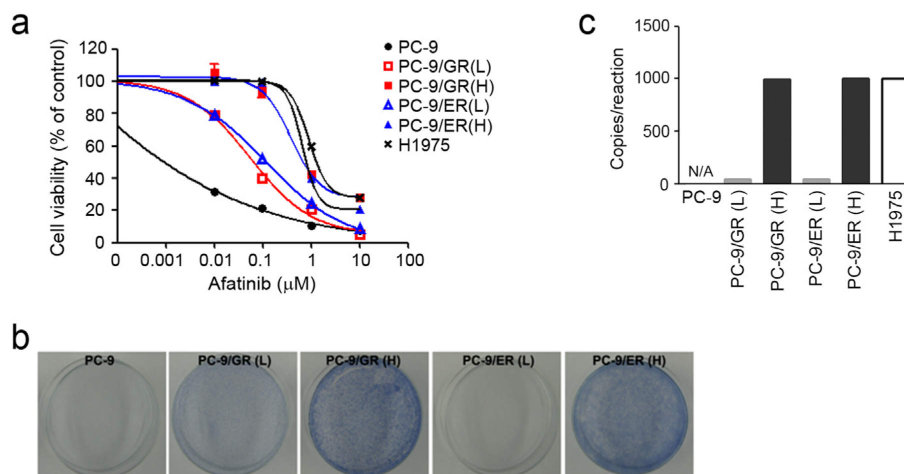

Figure 2. The efficacy of afatinib according to T790M mutation allele frequencies. (a) Cells were treated with afatinib, and sensitivity to afatinib was determined by MTT assay. (b) Cells were treated with 0.1 μM afatinib for 72 hours, and the medium was replaced with drug-free medium. After incubation for 5 days, attached cells were stained with trypan blue solution. (c) T790M mutation from each cell was detected by using PNAClamp EGFR Mutation Detection kit. The rate of T790M mutation was displayed as copies/reaction.

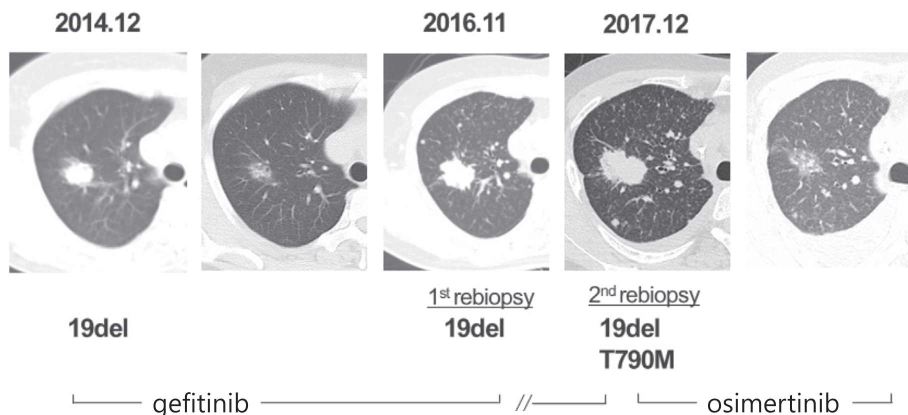

Figure 3. A case showing the different results of T790M by the same test over time with continued EGFR-TKI beyond progression. Increased T790M allele frequency would result in the positivity of T790M on second rebiopsy.

activity of afatinib on T790M-containing cells differs according to T790M allele frequency.

Elapsed Time Effect of Pharmacological Pressure on the Positivity of T790M

A male patient receiving gefitinib for 2 years developed resistance, and the first rebiopsy with PNA clamping assay showed T790M-negative results, although enough tumor cells were retrieved for analysis (Figure 3). His symptoms were very mild because only primary lung mass grew without any new lesions. Hence, we continued the administration of gefitinib for another 1 year and took the second rebiopsy when his respiratory symptoms, such as cough and dyspnea, were aggravated with further growth of lung mass. Interestingly, T790M turned out to be positive on the same test, and his disease responded well to subsequent osimertinib. Increased T790M allele frequency over time might affect the test result in this case.

Baseline Characteristics of Patients After Propensity Matching

We retrospectively reviewed the medical records of 398 patients: those who had at least more than two biopsies, the second biopsy performed after progression of the disease, while on TKI treatment with an initial EGFR mutation. The baseline characteristics in the prematched and pos-matched cohorts are presented in Table 1. Before PS matching, female/male ratio, disease stage, and type of EGFR mutation were not equal between afatinib and gefitinib groups. After PS matching, the female proportion in the gefitinib group changed from 65.8% to 46.3% (P = .928). The stages of that group were also adjusted (stage IIIB from 25.7% to 17.1%, stage IVA from 26.3% to 16.3%, and stage IVB from 47.3% to 66.7%). The types of EGFR mutation in the gefitinib group were also matched (P = .506) with E19del from 59.4% to 71.5%, and L858R from 37.7% to 25.2%. Distribution of patient characteristics before and after PS matching is illustrated in Figure 1*B* and Supplemental Figure 1, where Raw Treated (afatinib) and Raw Control (gefitinib) have different density distribution histograms, while Matched Treated (afatinib) and Matched Control (gefitinib) have similar density distribution histograms.

The Comparison of T790M Acquisition in Afatinib and Gefitinib Groups

The cumulative T790M acquisition ratios for afatinib and gefitinib were 48.8% and 59.3%, respectively (P = .317). The median time to acquire T790M was 12.9 months for afatinib, while it was 15.7 months

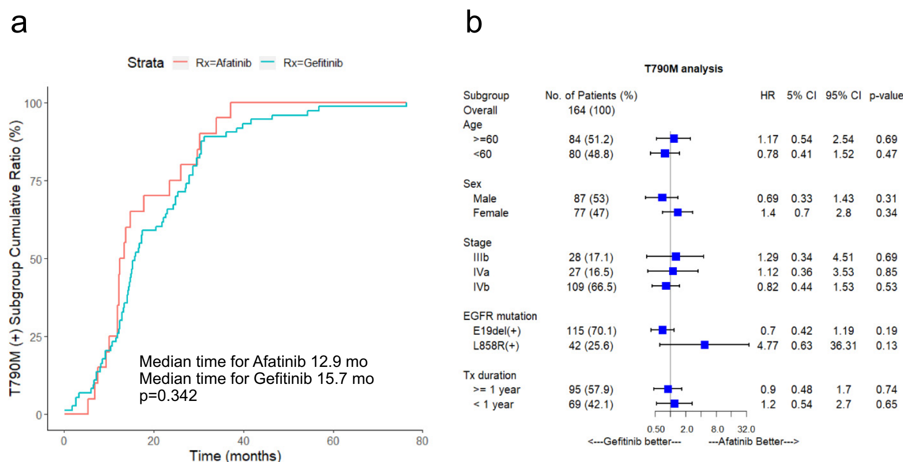

Figure 4. T790M mutation results by afatinib and gefitinib. (a) Cumulative ratio of T790M mutation over time for afatinib and gefitinib is plotted, where each ratio plateaus at 48.8% and 59.3% for afatinib and gefitinib, respectively. (b) The forest plot for T790M mutation subgroup analysis using univariate Cox analysis. There is no statistically significant factor causing increased rate or delayed time of T790M mutation.

for gefitinib (P = .342) (Figure 4*A*). The forest plot using subgroup analysis shows there was no statistically significant factor involved in determining T790M mutation on either medication. However, there was a slight trend of lower rates of T790M mutation in afatinib, with an HR of 4.77 [95% CI 0.63-36.31, P = .13] in the L858R mutation group for gefitinib (Figure 4*B*).

Survival According to Drugs and the Status of T790M

The median OS was 29.3 months in the afatinib group and 28.5 months in the gefitinib group, respectively, with an HR of 0.77 [95% CI 0.55-1.55, P = .76] (Figure 5*A*). The median PFS was 11.5 months for the afatinib group and 13.4 months for the gefitinib group, respectively, with an HR of 0.73 [95% CI 0.51-1.04, P = .08] (Figure 5*B*). Regardless of used drugs, the median OS and PFS were better in T790M-positive patients: OS—21.2 months [95% CI 15.9-29.3 months] versus 36.1 months [95% CI 29.7-60.8 months], HR of 0.48 [95% CI 0.32-0.71, P = .002] (Figure 5*C*); PFS—8.1 months [95% CI 6.6-13.0 months] versus 13.7 months [95% CI 12.6-15.7 months], HR of 0.68 [95% CI 0.50-0.93, P = .015] (Figure 5*D*).

Discussions

Our case demonstrates that pharmacological pressure for selection and amplification over time might raise the initial test-negative level of T790M to the level detectable by the same test. The positive response to osimertinib verified the authenticity of T790M-mediated resistance in this patient. We can clearly see this phenomenon in cell line studies (Figure 2). Engelman et al. established gefitinib-resistant cells (H3255/GR) by prolonged exposure to gefitinib in gefitinib-sensitive mother cells (H3255) [18]. Initial direct sequencing of H3255/GR could not detect T790M, while a highly sensitive HPLC-based technique confirmed the presence of T790M. In our cell line models, we did not detect T790M in PC-9/GR or ER (L) by direct sequencing, which has low sensitivity (Figure 2*C*). However, T790M was detected using the more sensitive pyrosequencing method or by direct sequencing of PC-9/GR or ER (H), which provides greater T790M amplification. Semiquantitative pyrosequencing revealed that PC-9/GR and ER (L) had around 14% T790M, while more than 50% T790M was found in PC-9/GR or ER (H) [17]. Hence, we concluded that the positivity of T790M is decided by both the test sensitivity and T790M allele frequency. Taken together, these findings raise the clinically difficult question of whether later retesting is required for initial T790M test-negative patients while keeping EGFR-TKI, despite disease progression, like in our case. This question is currently difficult to answer, but it seems worth trying, at least in asymptomatic or mildly symptomatic patients presenting slow disease progression because changing to cytotoxic chemotherapy is the only option in these patients and second-line chemotherapy is usually not so effective. In addition, the slowly growing tumor is more likely to harbor T790M.

Some preclinical experiments [1–4], including ours, indicated that afatinib could be more effective than gefitinib in the clinical setting. However, there were no significant differences between afatinib and gefitinib in terms of PFS and OS in our study. PFS of around 11.5 months and OS of around 29 months by EGFR-TKI in our study were similar to those reported in other studies [19–24]. As expected, both survival indices were better for patients with T790M-mediated resistance, reflecting the slow-growing nature of T790M-positive lung cancer (Figure 5, *C* and *D*). In multivariate Cox analysis (Supplemental

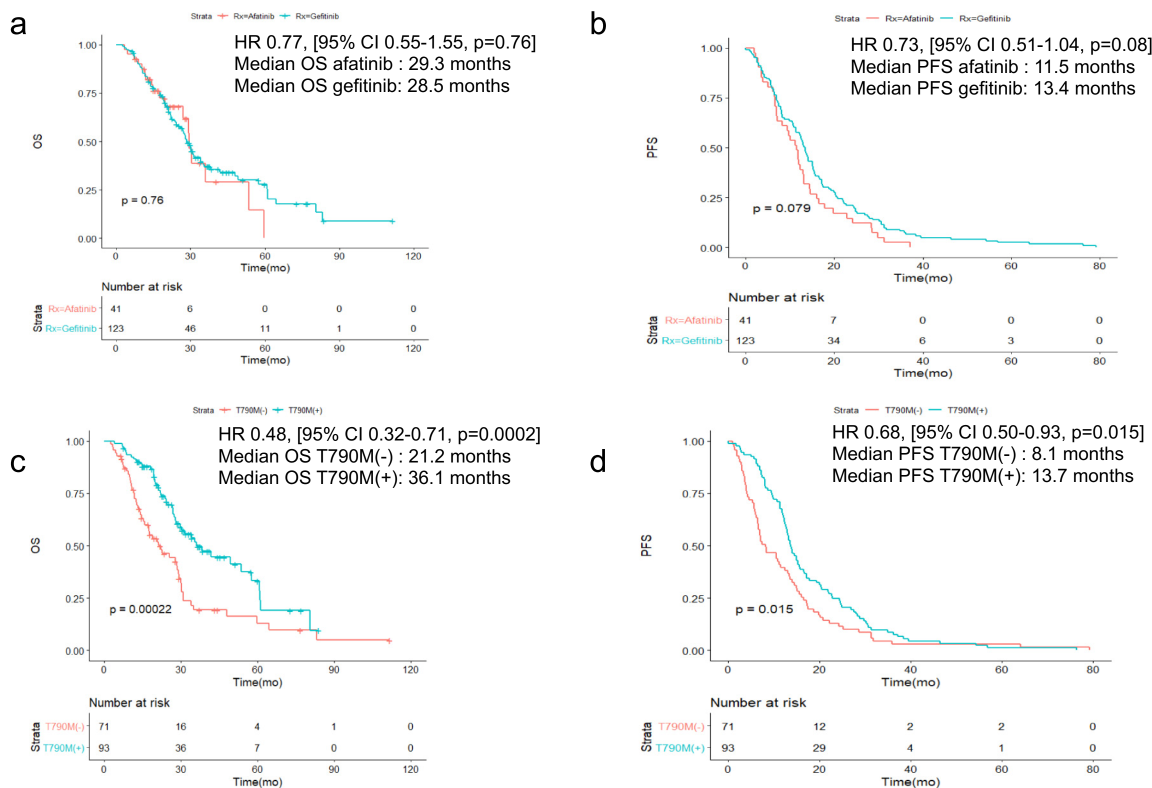

Figure 5. Kaplan-Meier survival plots for OS and PFS. (a) OS is compared between afatinib and gefitinib treatment. (b) PFS is compared between afatinib and gefitinib treatment. (c) OS is compared between those with or without T790M mutation. (d) PFS is compared between those with or without T790M mutation.

Figures 2 and 3), the poor prognostic factors, which had statistically significant effects on OS, were male sex [HR = 1.65, 95% CI 1.07-2.56, P = .03] and stage IVb [HR = 3.02, 95% CI 1.47-6.24, P < .01], whereas the types of EGFR mutation did not affect survival (Figure 5 and Supplemental Figure 3), which was also observed in another recent study using propensity score matching [25].

The results are important in a therapeutic setting because there is a possibility that afatinib is effective in T790M-positive lung cancer detected by sensitive techniques, mostly used at present if amplification is not accompanied. Ercan et al. depicted this situation in their study [7]. Actually, our study was initiated by the question of whether it can be translated into clinics. Disappointingly, afatinib did not show any statistically significant difference compared to gefitinib in terms of cumulative ratio of T790M and median time to T790M acquisition, although the afatinib group did present a slightly longer time and slightly higher rate for T790M acquisition (Figure 5*B*), with an HR of 4.77 [95% CI 0.63-36.31, P = .13]. Further, we could not find any significant factors determining T790M acquisition while on afatinib or gefitinib.

One limitation of our study is the small number of patients in the afatinib group. Afatinib has more side effects than gefitinib [23,26–29], which affect the clinician's choice of EGFR-TKIs. There is a tendency to favor gefitinib in female patients who are more concerned about skin problems [29] and less drug tolerability in real-world practice, which seems to be the reason why more female patients are included in the gefitinib group. EGFR mutations are more common in female patients, and therefore, the number of patients treated with afatinib came to be relatively small. From Table 1, it was evident that direct comparison despite uneven baseline characteristics between two groups might cause biased analysis, so the process of PS matching was needed. However, due to the small size of the afatinib group, even with a 1:3 ratio assigned to gefitinib, approximately 66% of the gefitinib data was discarded. This led to significantly less statistical power in our analysis. To overcome this issue, more patients need to be enrolled in the afatinib group in the following investigations.

In conclusion, afatinib has the inhibitory effect on the growth of the lung cancer cells with low T790M frequency. However, it could not be translated into the clinical efficacy to lower the rate or delay the time of T790M acquisition.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.04.004.

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