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RESEARCH ARTICLE

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Clinical outcomes of EGFR-TKI in advanced lung squamous cell carcinoma and EGFR-TKI remodel tumor immune microenvironment

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ABSTRACT

Background: Clinical data is scarce in epidermal growth factor receptor (EGFR)-mutated lung squamous cell carcinoma (LUSC), and the resistance mechanisms to EGFR-tyrosine kinase inhibitor (TKI) is rarely studied. This study aimed to assess the efficacy of EGFR-TKI treatment in EGFR-mutated LUSC patients .

Methods: Data of a cohort of 99 LUSC patients who were treated with EGFR-TKI and were followed up to October 31, 2023.

Results: The objective response rate (ORR) of EGFR-mutated LUSC patients was higher than that of EGFR wild-type patients (44.4% vs 4.4%, $p<0.001$). The progression-free survival (PFS) of EGFR-mutated LUSC patients receiving EGFR-TKI treatment was significantly longer than that of EGFR wild-type patients (6.4 months vs. 1.3 months; $p<0.001$). Resistance mechanisms to EGFR-TKI in EGFR-mutated LUSC patients included secondary T790M mutations, 19 deletion-insertion mutations, MET amplification, histological transformation, and loss of EGFR mutations. The tumor immune microenvironment (TIME) of EGFR-mutated LUSC showed a downregulation of CD4 ($p=0.047$) and CD8 ($p=0.14$), and an upregulation of PD-L1 ($p=0.0021$) after EGFR-TKI treatment failure.

Conclusions: EGFR-mutated LUSC patients receiving EGFR-TKIs treatment had higher ORR and longer PFS than EGFR wild-type LUSC patients.

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Introduction

Lung cancer stands as the foremost cause of cancer-related fatalities worldwide, with squamous cell carcinoma being a prevalent histological subtype, constituting approximately 25%–30% of all non-small cell lung cancer (NSCLC) cases [1,2]. Within NSCLC, the epidermal growth factor receptor (EGFR) mutation is a common driver mutation, with an occurrence rate of about 4.4%–5% in lung squamous cell carcinoma (LUSC) [3–5]. Molecular targeted therapy has emerged as a pivotal treatment option for advanced NSCLC patients, with EGFR tyrosine kinase inhibitors (TKI) serving as the primary treatment for advanced NSCLC patients harboring EGFR mutations [6]. Nevertheless, the progression-free survival (PFS) of EGFR-positive LUSC patients undergoing EGFR-TKI therapy is notably shorter than that of their lung adenocarcinoma counterparts, and there is considerable variability in reported EGFR-TKI efficacy in different studies [7–9]. Lewis et al. [10] followed up eight EGFR-mutant LUSC patients, and their disease control rate (DCR) was 87.5% and PFS was 4.9 months after receiving EGFR-TKIs as first- or second-line therapy. In another meta-analysis [11], a total of 115 EGFR-mutated LUSC patients received EGFR-TKIs were included and the pooled analysis of them showed that their objective response rate (ORR), DCR, PFS and overall survival (OS) 39.1%, 71.3%, 5.6 and 15.0 months, respectively. The precise efficacy of EGFR-TKI treatment in EGFR-positive LUSC necessitates further elucidation through additional clinical investigations.

EGFR-TKI resistance, culminating in disease progression, poses an inevitable hurdle for EGFR-positive lung

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cancer patients subjected to TKI therapy. In lung adenocarcinoma, secondary T790M mutation represents the most common mechanism observed with 1st and 2nd generation EGFR-TKIs. [12]. Nonetheless, research into the drug resistance mechanisms in LUSC remains sparse. Currently, platinum-based chemotherapy remains the preferred option for NSCLC patients confronting EGFR-TKI resistance in the absence of standard targeted therapies. Regrettably, the PFS for patients undergoing chemotherapy merely extends to 4 to 5 months [13], underscoring the pressing need for alternative therapies to enhance patients' quality of life and prolong their survival. In recent years, immune checkpoint inhibitors (ICI) have emerged as a pivotal component of lung cancer treatment. While first-line ICIs have limited benefits for EGFR-mutated NSCLC patients [14], clinical investigations are underway to assess ICI efficacy in EGFR-mutated NSCLC patients with TKI resistance [14,15]. Nevertheless, no specific research has been conducted regarding EGFR-mutated LUSC patients. This study retrospectively gathers clinical data from LUSC patients treated with EGFR-TKI to probe into the efficacy of EGFR-TKI treatment, potential resistance mechanisms in LUSC patients with EGFR-TKI resistance, and the effectiveness of ICI therapy in EGFR-positive LUSC patients. Our aim is to provide valuable insights for the therapeutic decisions of patients facing EGFR-TKI resistance.

Materials and methods

Study cohort

Between January 2015 and January 2022, we identified a total of 6505 patients diagnosed with LUSC at Zhejiang Cancer Hospital in Hangzhou, China. Among them, 99 patients with advanced LUSC received EGFR-TKI treatment. We categorized these patients based on their EGFR mutation status, with 54 having EGFR-positive and 45 having EGFR-negative LUSC, all of whom received EGFR-TKI therapy. We gathered clinical records, treatment history, and survival data by reviewing medical records, and our follow-up extended until October 31, 2023. Additionally, we divided tumor samples into two groups: pre-TKI samples, taken from EGFR-positive LUSC patients before EGFR-TKI treatment, and post-TKI samples, obtained from EGFR-positive LUSC patients who developed resistance to EGFR-TKI therapy. We included a total of 22 pre-TKI and 11 post-TKI formalin-fixed paraffin-embedded (FFPE) samples from 30 EGFR-positive patients, with 6 paired pre-TKI and post-TKI samples from 3 patients at Zhejiang Cancer Hospital. The study design is illustrated in Figure 1.

Molecular analysis

All patients in this study had confirmed LUSC based on pathological examination. We retrospectively collected the EGFR gene mutation status from medical history data and their gene mutation status using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) and next-generation sequencing (NGS) before initiating EGFR-TKI treatment. Furthermore, we retrospectively collected NGS data from 9 EGFR-positive LUSC patients who developed resistance to EGFR-TKI therapy. NGS was employed to detect gene abnormalities in accordance with the guidelines of the College of American Pathologists (CAP) [16,17].

Multiplex immunofluorescence staining

For multiplex fluorescence immunohistochemistry (mIHC) analysis, a five-color fluorescent kit based on tyramide signal amplification (TSA) was employed according to the manufacturer's protocol (abs50029, Absin Bioscience, China) [18]. In brief, all slides were subjected to deparaffinization in xylene, rehydration, and tap water washing before undergoing epitope retrieval/microwave treatment in Tris–EDTA buffer (pH 9; HKI0004, HaoKebio, Hangzhou, China). Endogenous peroxidase was then blocked using hydrogen peroxide (HKI0046, HaoKebio, Hangzhou, China), and protein blocking was performed with fetal bovine serum (HKW2084, HaoKebio, Hangzhou, China). Each antigen was labeled in one round, including primary antibody incubation, secondary antibody incubation, and TSA signal amplification, followed by labeling with the next antibody. Primary antibodies CD4 (1:600; Abcam; Catalog number: ab133616; Clone numbers: EPR6855), CD8 (1:200; Abcam; Catalog number: ab237709; Clone numbers: CAL66), Foxp3 (1:400; Abcam; Catalog number: ab215206; Clone numbers: EPR22102-37), PD-L1 (1:250; Abcam; Catalog number: ab205921; Clone numbers: 28–8) were incubated for 2 h at room temperature. Subsequently, secondary antibody incubation was performed for 10 min, followed by TSA signal amplification with the five-color fluorescent kit (abs50029, Absin Bioscience, China), which included fluorophores DAPI, Opal 520 (CD8), Opal 570 (CD4), Opal 620 (PD-L1), Opal 700 (Foxp3), Opal Polymer HRP Rb, and TSA coumarin system. After the final TSA cycle, DAPI was counterstained at a 1:200 dilution for 10 min. The slides were scanned using the Pannoramic SCAN 150 (3D Histech, HUN). Visiopharm (Visiopharm, DK) in conjunction with hematoxylin and eosin (HE) staining was utilized to identify cancerous tissue in the scanned

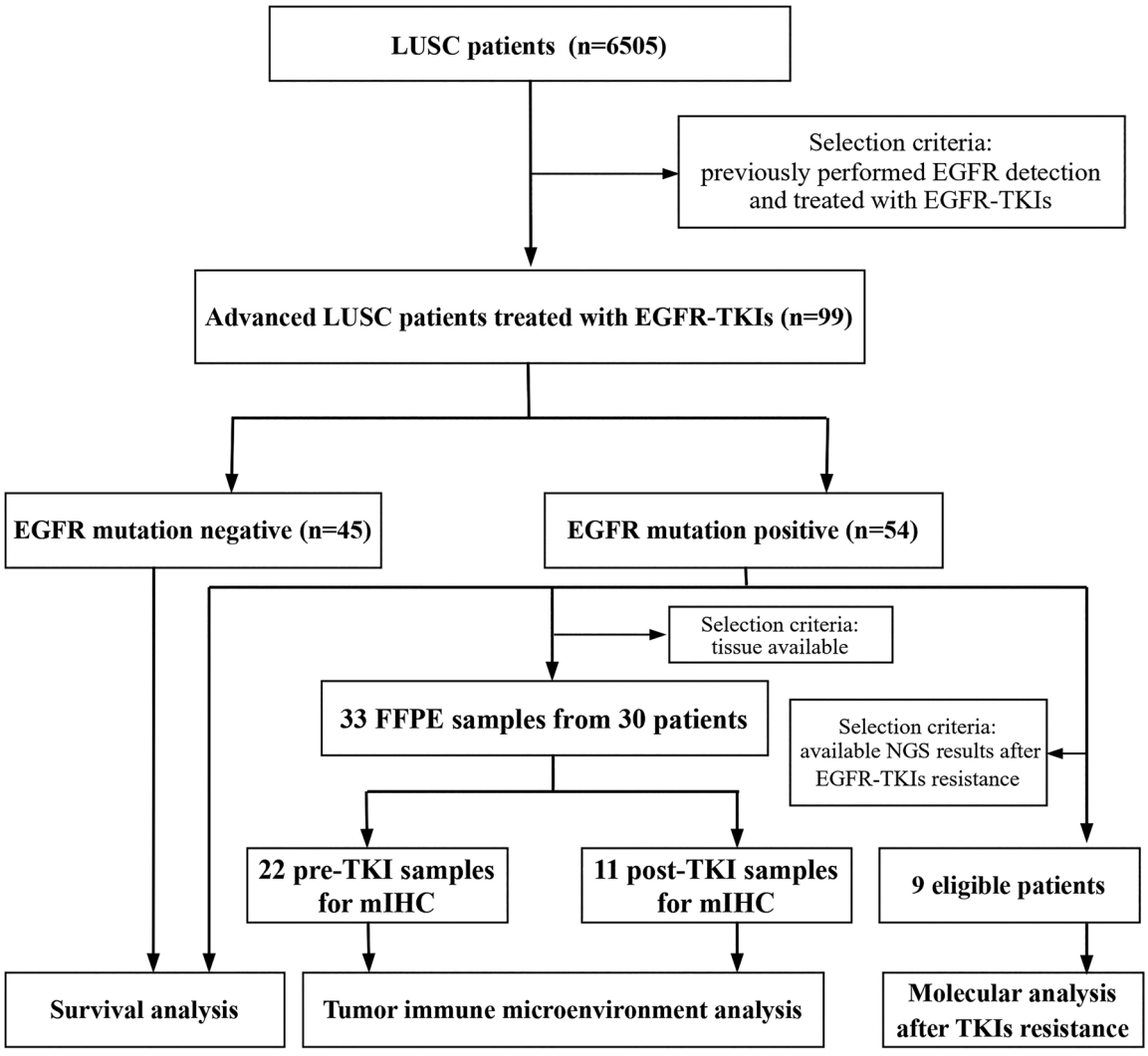

Figure 1. Sample collection and study design of study.

images and calculate the proportion of positive cells for each marker.

Statistical analysis

The correlation between EGFR mutation status and clinical features was assessed using the Pearson Chi-square test. Survival curves were estimated using the Kaplan-Meier method and compared through the log-rank test. Prognostic factors were analyzed in univariate and multivariate analyses using the Cox proportional hazards model, with hazard ratios (HR) and 95% confidence intervals (CIs) calculated. In the case of mIHC analysis, a normal distribution test was conducted on the expression data for each marker. For normally distributed data (CD4, CD8, PD-L1), t-tests were applied, while for non-normally distributed data (Foxp3), the Mann-Whitney U test was employed. The comparison of the proportion of each tumor microenvironment immune type (TMIT) based on categorical variables was analyzed using the Chi-square test. A significance level of $p < 0.05$ was considered statistically significant. Statistical analysis was carried out using SPSS (version 25.0) and R (version 4.2.1).

Treatment and follow-up

Response evaluation was conducted in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST guideline 1.1) [19], which defined complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD), and objective response rate (ORR). Progression-free survival (PFS) for patients who received EGFR-TKI therapy was calculated from the date of TKI therapy initiation to the date of disease progression or death. Immunotherapy progression-free survival (ICI-PFS) for patients who received ICI therapy was calculated from the date of ICI therapy initiation to the date of disease progression or death. Overall survival (OS) was defined as the time from the

diagnosis date of advanced LUSC to the date of death or the last follow-up visit (October 31, 2023). By the last follow-up, 98 patients had reached the endpoint of EGFR-TKI treatment, and 6 patients were still alive.

Results

Patient characteristics

In our study, we included 99 patients with LUSC who underwent EGFR-TKI treatment. Their median age was 64 years (range 39–90 years), and there was a predominance of males (75.7%) over females (24.3%). Among these patients, 54 were EGFR positive, and their mutation profiles were as follows: 26 (48.1%) had the 19del mutation, 19 (35.2%) had the L858R mutation, 3 (5.6%) had the primary T790M mutation, 2 (3.7%) had the G719X mutation, 1 (1.9%) had the G719X + L861Q mutation, 1 (1.9%) had the A702S mutation, 1 (1.9%) had the E709A mutation, and 1 (1.9%) had the F723L mutation. Table 1 presents the clinical characteristics of the LUSC patients. The results showed that the EGFR positive group had a higher proportion of female patients, never-smokers, and patients with brain metastases compared to the EGFR negative group (40.7% vs. 4.4%, $p < 0.001$; 53.7% vs. 11.1%, $p < 0.001$; 24.1% vs. 2.2%, $p = 0.002$, respectively). Among the 54 EGFR positive patients, 29 (53.7%) received EGFR-TKI as their first-line therapy, while 25 (46.3%) received it as second- or subsequent-line therapy. Of these, 37 (68.5%) received first-generation TKI (8 gefitinib and 29 icotinib), 11 (20.4%) received afatinib, and 6 (11.1%) received osimertinib. Among the 45 EGFR-negative patients, 44 (97.8%) attempted EGFR-TKI therapy as second- or subsequent-line treatment upon disease progression following chemoradiotherapy; 3 (6.7%) received icotinib, and 42 (93.3%) received afatinib.

Clinical outcomes and correlation factors of TKI efficacy

The optimal efficacy of EGFR-TKI in the EGFR-positive group showed that 1/54 (1.9%) patient achieved CR, 23/54 (42.6%) patients achieved PR, 21/54 (38.9%) patients achieved SD, and 9/54 (16.7%) patients achieved PD. The evaluation of TKI efficacy in the EGFR-negative group showed that no patient achieved CR, 2/45 (4.5%) patients achieved PR, 14/45 (31.1%) patients achieved SD, and 29/45 (64.4%) patients achieved PD. The ORR was significantly higher in the EGFR-positive group than in the EGFR-negative group (44.4% vs 4.4%, $p < 0.001$), and the PFS was also longer in the EGFR-positive group than in the EGFR-negative group (6.4 months vs 1.3 months; $p < 0.001$; Figure 2A), but their OS was similar (18.3 months vs 17.3 months; $p = 0.56$; Figure 2B). The PFS for LUSC patients with the 19del mutation, L858R mutation, and other mutation sites were 6.7, 9.2, and 3.6 months, respectively ($p = 0.097$; Figure 2C).

Among all 99 LUSC patients received EGFR-TKI treatment, patients with liver metastases had a shorter PFS compared to those without liver metastases (1.1 months vs. 3.8 months, $p = 0.021$, Figure 2D). Univariate and multivariate analyses confirmed that LUSC patients with the 19del mutation, L858R mutation, and those without liver metastases exhibited better clinical outcomes with EGFR-TKI therapy (Table 2). Subgroup analysis, considering clinical characteristics, generally favored EGFR positive patients, except for those with poor ECOG PS (Figure 2E).

Molecular analysis

We retrospectively collected NGS data from 9 EGFR-positive LUSC patients following the failure of EGFR-TKI treatment. As depicted in Figure 3, among them, 2 patients (2/9) had secondary T790M mutation, 2 patients (2/9) had mesenchymal to epithelial transition factor (MET) amplification, one patient (1/9) had secondary T790M mutation and EGFR deletion-insertion mutation, one patient (1/9) experienced histological transformation from squamous cell carcinoma to adenocarcinoma and EGFR deletion-insertion mutation, one patient (1/9) experienced EGFR mutation loss, and 2 patients (2/9) had no explicit resistance mechanisms. Additionally, these patients had a high incidence of tumor protein 53 (TP53) mutations (6/9, 66.6%). In this study, we only obtained the EGFR gene mutation status at baseline from medical history data, while the other genetic mutations status was unknown, so that we did not further analyze the possible secondary mutations, which still need to be further explored in future research.

Differences of TIME between pre- and post-TKI LUSC

To elucidate the remodeling effect of EGFR-TKI on tumor immune microenvironment (TIME) and its potential impact on ICI efficacy, we analyzed changes in the expression of common immune markers before and after TKI therapy failure. In our study, we had access to 22 pre-TKI samples and 11 post-TKI samples, which were subjected to mIHC. Figure 4A provides an example of mIHC results from two paired samples obtained from one patient before and after TKI

Table 1. Clinical characteristics.

Characteristic	All cases ($n=99$)	EGFR mutation		P value
		positive ($n=54$)	negative ($n=45$)	
Age, *n* (%)				0.397
<65	53	31 (57.4)	22 (48.9)	
≥65	46	23 (42.6)	23 (51.1)	
ECOG, *n* (%)				0.343
0–1	85	48 (88.9)	37 (82.2)	
2–3	14	6 (11.1)	8 (17.8)	
Gender, *n* (%)				0.000
Female	24	22 (40.7)	2 (4.4)	
Male	75	32 (59.3)	43 (95.6)	
Smoking history, *n* (%)				0.000
Yes	65	25 (46.3)	40 (88.9)	
No	34	29 (53.7)	5 (11.1)	
TNM stage, *n* (%)				0.091
IIIB-IIIC	25	10 (18.5)	15 (33.3)	
IV	74	44 (81.5)	30 (66.7)	
Brain metastasis, *n* (%)				0.002
Yes	14	13 (24.1)	1 (2.2)	
No	85	41 (75.9)	44 (97.8)	
Bone metastasis, *n* (%)				0.076
Yes	38	25 (46.3)	13 (28.9)	
No	61	29 (54.7)	32 (71.1)	
Liver metastasis, *n* (%)				0.219
Yes	15	6 (11.1)	9 (20.0)	
No	84	48 (88.9)	36 (80.0)	
Local therapy, *n* (%)				0.503
Yes	58	30 (55.6)	28 (62.2)	
No	41	24 (44.4)	17 (37.8)	
Treatment line of TKIs, *n* (%)				0.000
First line	30	29 (53.7)	1 (2.2)	
Second line and after	69	25 (46.3)	44 (97.8)	
Type of TKIs, *n* (%)				0.000
Gefitinib/Icotinib	40	37 (68.5)	3 (6.7)	
Afatinib	53	11 (20.4)	42 (93.3)	
Osimertinib	6	6 (11.1)	0	
Best response to TKIs, *n* (%)				0.000
CR/PR	26	24 (44.4)	2 (4.4)	
SD/PD	73	30 (55.6)	43 (95.6)	

treatment. We examined four markers (CD4, CD8, PD-L1, and Foxp3) and observed alterations in their expression within the TIME following EGFR-TKI therapy. In comparison to pre-TKI samples, post-TKI samples exhibited a significant decrease in CD4 expression ($p=0.047$), a decrease in CD8 expression with no statistical significance ($p=0.14$), a significant increase in PD-L1 expression ($p=0.0021$), and a slight increase in Foxp3 expression without statistical significance ($p=0.31$, Figure 4B). As reported in previous studies [20,21], we categorized LUSC TIME into four Tumor Microenvironment Immune Types (TMIT) based on the median expression values of CD8 and PD-L1. Type I tumors (CD8-high/PD-L1-high) may benefit from anti-PD-1/PD-L1 therapy, while type II tumors (CD8-low/PD-L1-low) are less likely to benefit from anti-PD-1/PD-L1 therapy. Our findings revealed that post-TKI LUSC had a higher proportion of TMIT I and a lower proportion of TMIT II compared to pre-TKI LUSC ($p=0.000$, Figure 4C). Overall, although post-TKI LUSC exhibited reduced infiltration of CD4/CD8+ T cells compared to pre-TKI LUSC, PD-L1 expression was significantly upregulated. Subsequently, we conducted a detailed analysis of the treatment history of 8 EGFR-positive LUSC patients who received ICI therapy. Among them, 3 patients received ICI as first-line therapy, and the best therapeutic effect showed that 1 patient (1/3) achieved PR, and 2 patients (2/3) achieved SD. 5 patients received ICI as subsequent treatment after TKI resistance, and the best therapeutic effect showed that all 5 patients (5/5) achieved SD. The ICI-PFS of patients received ICI as first-line therapy is 4.9–10.8 months, while the ICI-PFS of patients received as subsequent treatment after TKI resistance is 6.0–36.5 months (Figure 5).

Discussion

In this study, we observed that a higher percentage of female (40.7% vs. 4.4%, $p<0.001$) and non-smoking patients (53.7% vs. 11.1%, $p<0.001$) exhibited EGFR positivity among LUSC patients compared to those who were EGFR negative. EGFR-positive patients had a higher risk of developing brain metastases than EGFR-negative patients (24.1% vs. 2.2%, $p=0.002$), consistent with earlier research [22,23]. The ORR for

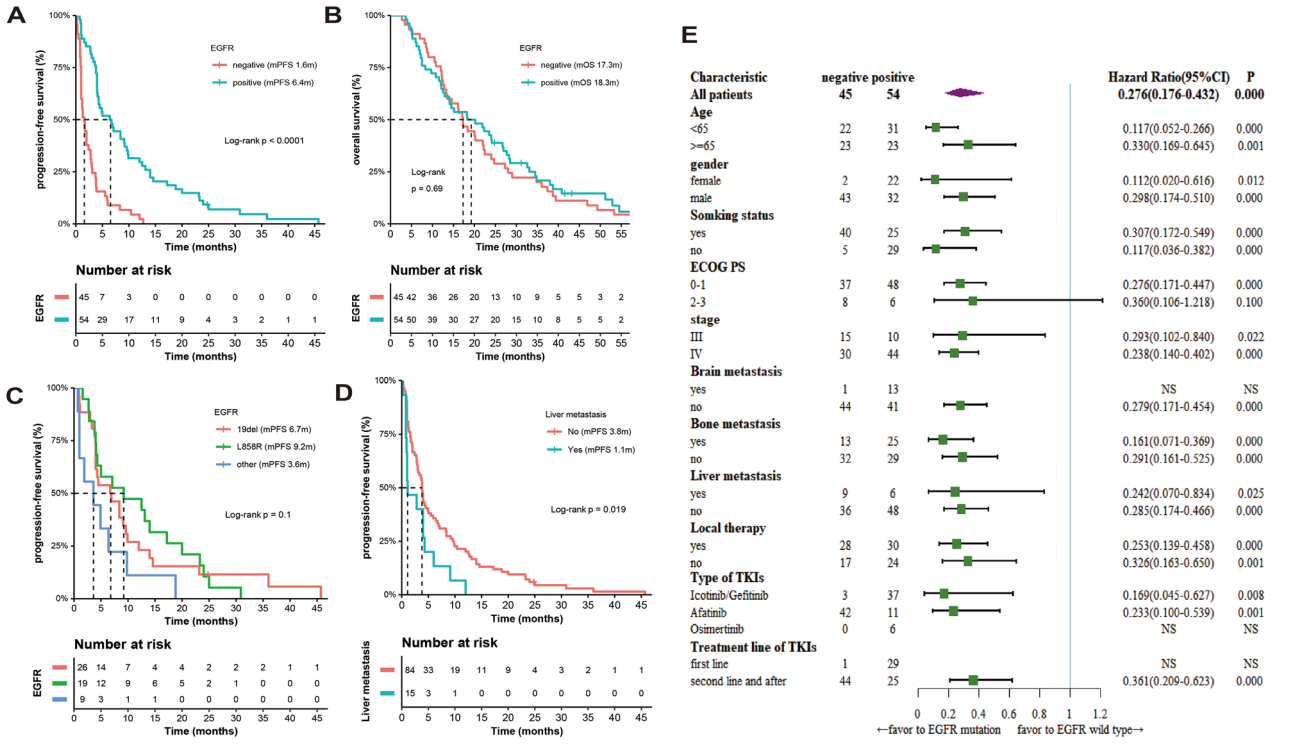

Figure 2. Kaplan-Meier Curves of progression-free survival (PFS, A) and overall survival (OS, B) stratified by EGFR mutation status. Kaplan-meier curves of PFS stratified by EGFR mutation types (C) and liver metastasis (D). (E) Forest plot of subgroup analysis by baseline characteristics for TKI-PFS in patients with EGFR-mutated positive or negative.

EGFR-TKI treatment in the EGFR-positive group was significantly higher than in the EGFR-negative group (44.4% vs 4.4%, $p < 0.001$). Interestingly, two EGFR-negative patients achieved PR after receiving EGFR-TKIs, which may be due to EGFR downstream pathway mutations or bypass pathway activation. The PFS in EGFR-positive LUSC patients was significantly longer than that in EGFR-negative LUSC patients (6.4 vs. 1.3 months, $p < 0.001$). However, a limitation that should be noted is that the EGFR-negative group received EGFR-TKIs in later lines on average than the EGFR-positive group which may be associated with lower ORR and survival. In addition, the OS was similar between the two groups (18.3 vs. 17.3 months, $p = 0.56$). This similarity in OS may be due to the fact that some EGFR-positive patients were not eligible for post-line chemotherapy after TKI failure, and the small sample size in our study. Previous studies have reported a PFS of 10–14 months and an ORR of 50%–80% in EGFR-positive lung adenocarcinoma [24–26], which is notably better than what was observed in EGFR-positive LUSC patients in this study. These findings indicate that EGFR-positive LUSC patients can benefit from EGFR-TKI therapy, although its efficacy in LUSC is lower compared to lung adenocarcinoma.

In the EGFR-positive group, the majority of EGFR activating mutations consisted of exon 19 deletion mutations (48.1%) and exon 21 L858R mutations (35.2%). Only about 10% of patients received a 3rd generation EGFR-TKI and the PFS for EGFR-TKI treatment in patients with 19del and L858R mutations in LUSC was longer than in cases with other EGFR mutations (9.2 vs. 6.7 vs. 3.6 months, $p = 0.097$). Previous studies have shown that TKI in EGFR 19del mutations NSCLC patients are more effective than those with other EGFR mutations [27,28]. In this study, EGFR L858R mutations patients had the best TKI efficacy, which suggests that unlike EGFR-mutated lung adenocarcinoma, in EGFR-mutated LUSC patients, the L858R mutations have better TKI efficacy compared to other EGFR mutations.

In all LUSC patients received EGFR-TKI therapy, patients with liver metastases had a shorter TKI-PFS compared to those without liver metastases (1.1 vs. 3.8 months, $p = 0.021$). Previous research has suggested that this could be attributed to higher levels of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) expression in the tumor microenvironment of liver metastases when compared to other types of metastases [29–31]. HGF enhances ErbB3/PI3K/AKT signaling by activating c-MET [32], and VEGF stimulates tumor angiogenesis and facilitates the proliferation of EGFR mutant tumor cells [33–35]. In a phase III randomized, double-blind clinical trial

Table 2. Uni- and multivariate analyses of clinical parameters on PFS to EGFR-TKI.

	Univariate analysis		Multivariate analysis	
Characteristic	Hazard Ratio(95%CI)	*P*	Hazard Ratio(95%CI)	*P*
Gender (female/male)	1.655(1.032–2.653)	0.037*	0.783(0.360–1.701)	0.536
Age (<65/≥65)	0.882(0.591–1.318)	0.541		
ECOG PS(0–1/2–3)	1.643(0.921–2.901)	0.093		
Somking status (yes/no)	0.635(0.415–0.972)	0.037*	1.011(0.512–1.997)	0.975
Stage (III/IV)	1.073(0.677–1.702)	0.765		
Brain metastasis (yes/no)	0.732(0.413–1.298)	0.351		
Bone metastasis (yes/no)	0.974(0.645–1.473)	0.902		
Liver metastasis (yes/no)	0.521(0.296–0.915)	0.023*	0.469(0.261–0.840)	0.011*
Local therapy (yes/no)	0.971(0.646–1.467)	0.887		
Treatment line of TKIs (first line/ second line and after)	2.299(1.453–3.636)	0.000*	1.588(0.827–3.051)	0.165
EGFR mutation types				
Without	1		1	
EGFR 19del	0.254(0.147–0.437)	0.000*	0.213(0.089–0.510)	0.001*
EGFR L858R	0.231(0.128–0.417)	0.000*	0.230(0.090–0.588)	0.002*
Other EGFR mutation	0.518(0.250–1.072)	0.076	0.758(0.324–1.770)	0.521
Type of TKI				
Icotinib/Gefitinib	1		1	
Afatinib	2.064(1.340–3.178)	0.001*	0.709(0.329–1.527)	0.379
Osimertinib	0.672(0.262–1.723)	0.408	0.666(0.204–2.174)	0.501

involving 449 advanced NSCLC patients with EGFR mutations, which included 224 patients treated with erlotinib plus an anti-VEGF receptor antibody and 225 patients treated with erlotinib plus a placebo, the results indicated that in the group of patients with liver metastases, those treated with erlotinib and anti-VEGF receptor antibodies had a better PFS compared to those treated with erlotinib plus a placebo [36]. Therefore, EGFR-TKI therapy is more effective in LUSC patients without liver metastasis than that in LUSC patients with liver metastasis.

Many EGFR-positive LUSC patients chose not to undergo re-biopsy, leading to a loss of genetic testing data in some cases. Ultimately, we could only obtain NGS data from 9 EGFR-positive patients after they experienced EGFR-TKI treatment failure. Because we lack data on genetic mutations other than the EGFR gene at baseline, we only considered the mechanisms currently known to be associated with EGFR-TKI resistance, the findings indicated that 3 cases (33.3%, 3/9) had acquired T790M mutations, 2 cases (22.2%, 2/9) had EGFR deletion-insertion mutations, 2 cases (22.2%, 2/9) exhibited mesenchymal to epithelial transition factor (MET) amplification, 1 case (11.1%, 1/9) underwent histological transformation from squamous cell carcinoma to adenocarcinoma, and 1 case (11.1%, 1/9) experienced EGFR mutation loss. Acquired T790M mutations remained the most common resistance mechanism in EGFR-positive LUSC patients treated with 1st and 2nd generation EGFR-TKIs, although the frequency of these mutations was lower than that observed in lung adenocarcinoma [37]. Similarly, Chang et al. [8] reported NGS results for 8 EGFR-mutated female LUSC patients after developing resistance to TKIs. Among them, 2 patients had acquired T790M mutations, 1 patient had MET amplification, and the others did not exhibit any explicit secondary mutations that could be used for further treatment guidance. As is well known, histological transformation is one of the resistance mechanisms of NSCLC targeted therapy and there have been many reported cases of adenocarcinoma transforming into other tissue types such as small cell carcinoma and squamous cell carcinoma [38]. In this study, one patient experienced a histological transformation from squamous cell carcinoma to adenocarcinoma, indicating that the transformation from squamous cell carcinoma to adenocarcinoma also can lead to TKI resistance in LUSC patients. Interestingly, one patient experienced disease progression after receiving gefitinib for 14.6 months, and a subsequent second blood biopsy revealed the loss of EGFR mutations. This suggests that the loss of EGFR mutations may be one of the mechanisms contributing to TKI treatment failure. Additionally, although TP53 mutations status at baseline was unknown, 6 out of 9 patients (66.6%, 6/9) were found to have TP53 mutations at the time of resistance. Actually, previous studies have demonstrated that TP53 mutations are associated with a faster development of resistance mechanisms in EGFR-positive NSCLC and may cooperate with other genomic events to facilitate the acquisition of resistance mutations to EGFR-TKIs [39]. This explains the complex nature of resistance mechanisms observed in LUSC patients in our study. The high likelihood of secondary resistance mutations and the complexity of resistance mechanisms underscore the importance of re-biopsy after TKI treatment failure for clinical physicians in diagnosing and formulating effective treatment plans.

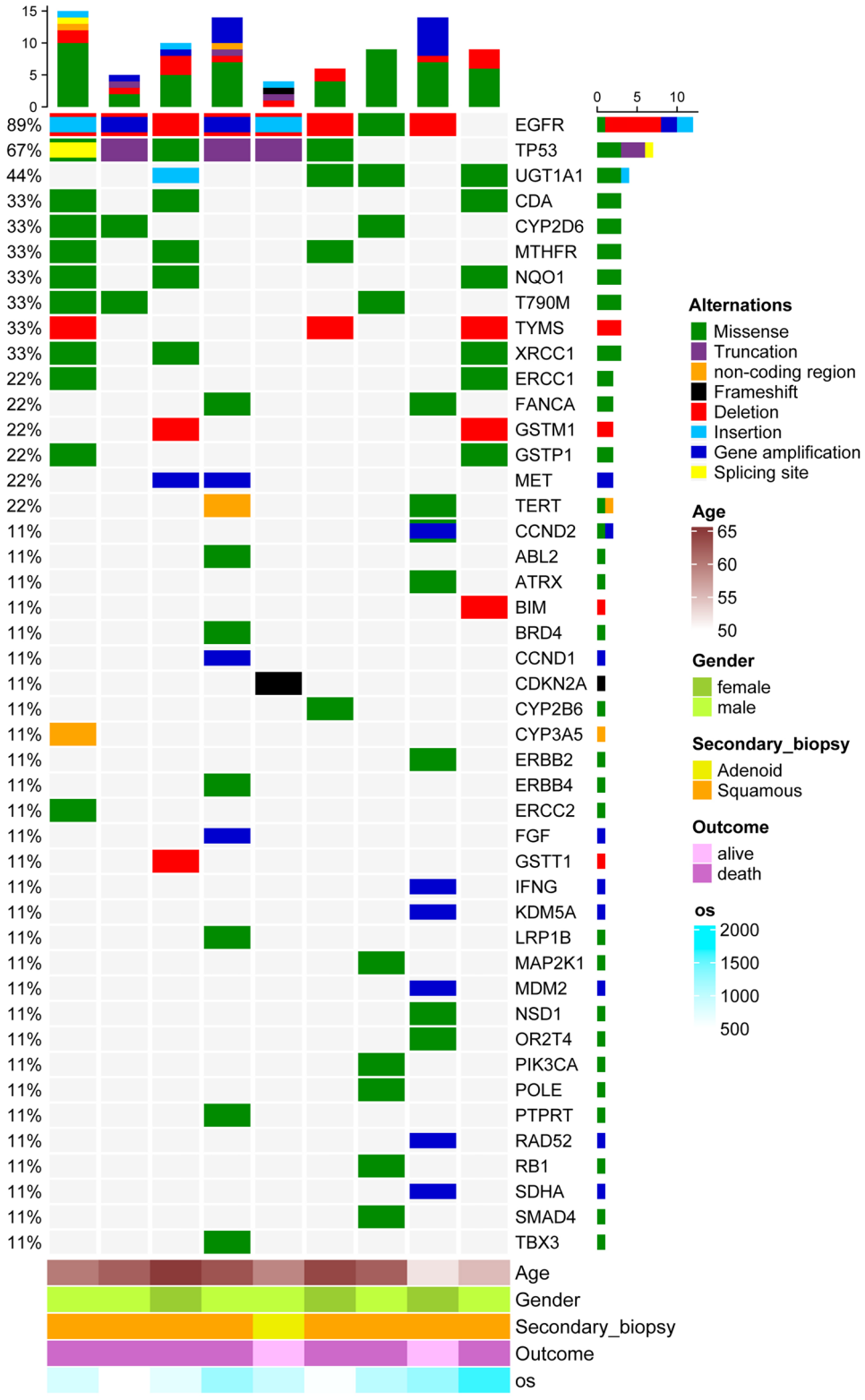

Figure 3. co-Mutation plot of various types of mutations in 9 EGFR-positive LUSC patients after TKI failure; each column represents one patient; the mutation rates of each gene were marked on the left in percentage and grouped according to their protein functions; patient characteristics were shown at the bottom with different colors.

Since 2015, anti-PD1/PD-L1 immunotherapy has become a crucial treatment for lung cancer, either as a standalone therapy or in combination with chemotherapy. For instance, a clinical trial involved 272 previously treated LUSC patients, randomly assigning them to receive either ICI (135 patients) or

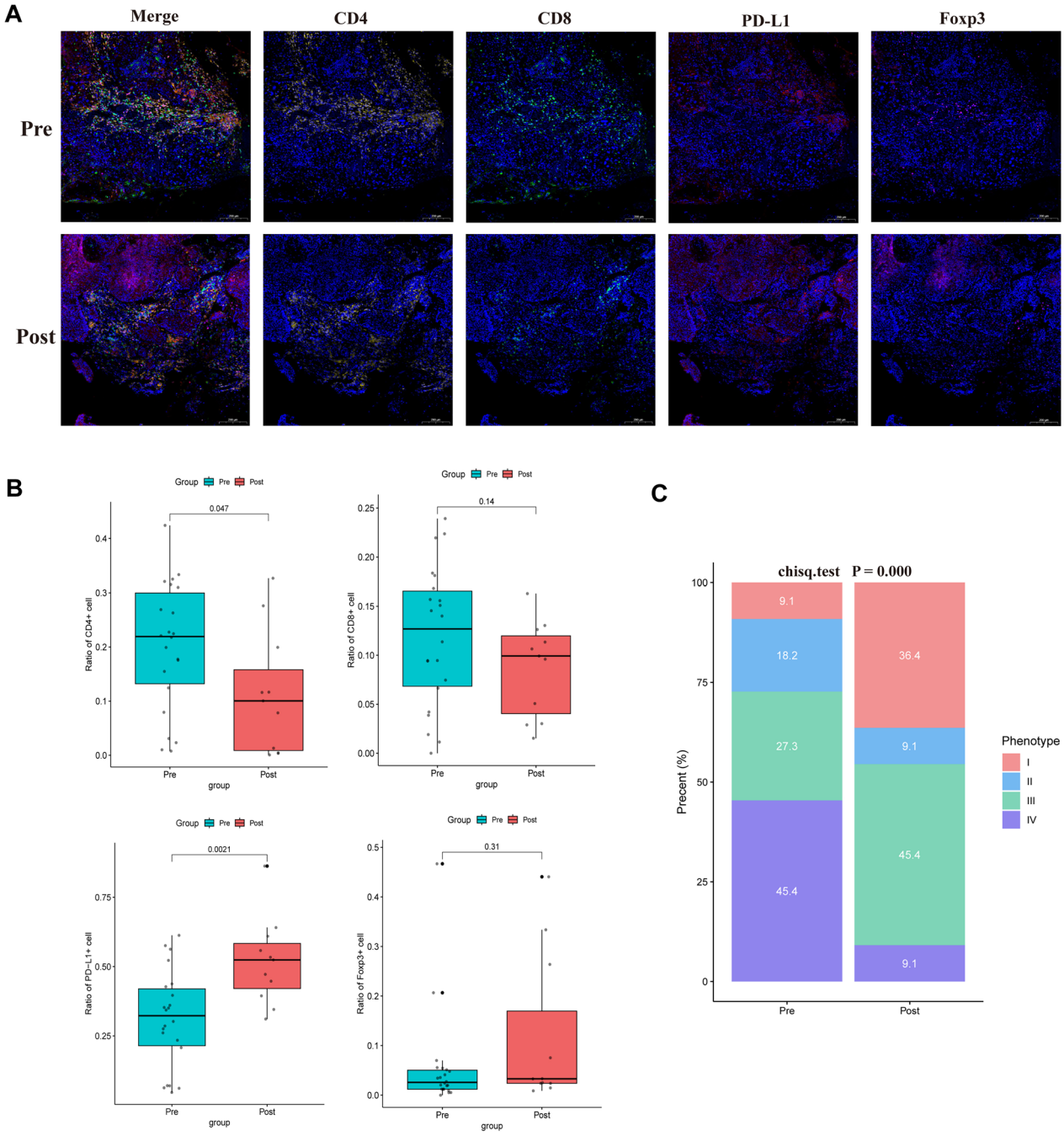

Figure 4. (A) Example of immunofluorescent staining of tumor tissue samples from LUSC patients. pre-TKI samples, taken from patients before EGFR-TKI treatment, and post-TKI samples, taken from patients who developed resistance to EGFR-TKI. (DAPI antibody is expressed in blue, CD4 antibody is expressed in yellow, CD8 antibody is expressed in green, PD-L1 antibody is expressed in red, and foxp3 antibody is expressed in purple). (B) Comparison of the infiltration levels of CD4+, CD8+, PD-L1+, and foxp3+ cells between pre-TKI and post-TKI tumors in EGFR-positive LUSC cohort. (C) The proportion of TMIT between pre-TKI and post-TKI tumors is compared.

docetaxel (137 patients). The results of this trial demonstrated that the efficacy of ICI was superior to chemotherapy in second-line treatment, with a longer overall survival (OS) observed in the ICI group compared to the chemotherapy group (9.2 vs. 6.0 months, $p < 0.001$) [40]. However, it was initially believed that anti-PD1/PD-L1 immunotherapy had limited effectiveness in EGFR-positive NSCLC [41]. To the best of our knowledge, this is the first study to report on the TIME of EGFR-positive LUSC patients after TKI therapy failure. In this study, we collected 22 pre-TKI samples and 11 post-TKI samples and assessed the TIME using mIHC. The results revealed that the expression of CD4+ ($p = 0.047$) and CD8+

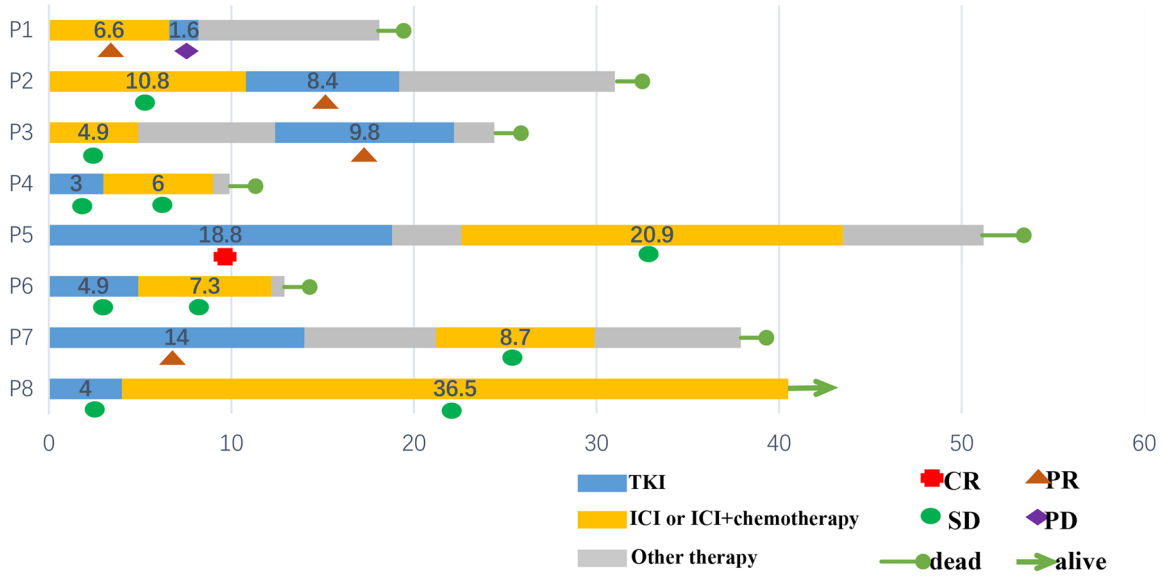

Figure 5. Treatment history and survival data (OS and PFS) of 8 EGFR-positive LUSC patients who received immunotherapy.

($p = 0.14$) was reduced, while the expression of PD-L1 significantly increased ($p = 0.0021$) in post-TKI samples compared to pre-TKI samples. According to prior research, reduced lymphocyte infiltration is associated with poorer immune response, while increased PD-L1 expression suggests improved efficacy of immunotherapy [42]. In an effort to predict the effectiveness of ICI therapy following TKI failure, we established four TMITs based on previous studies [20]. These include TMIT I tumor (CD8-high/PD-L1-high), TMIT II tumor (CD8-low/PD-L1-low), TMIT III tumor (CD8-low/PD-L1-high), and TMIT IV tumor (CD8-high/PD-L1-low), determined by the levels of CD8 and PD-L1 expression. The findings indicated that the proportion of TMIT I in the post-TKI group was significantly higher than that in the pre-TKI group (36.4% vs 9.1%, $p < 0.001$), while the proportion of TMIT II was lower (9.1% vs 18.2%, $p < 0.001$). This suggests that LUSC patients who have experienced TKI failure may derive benefit from ICI therapy.

Subsequently, we conducted a retrospective analysis of the history of ICI therapy in EGFR-positive LUSC patients. However, our study only included 8 patients who received ICI treatment, with 3 patients undergoing ICI as first-line therapy and 5 patients receiving ICI as subsequent treatment after TKI resistance. Previous studies have shown high rates of pneumonitis when ICI is given prior to EGFR-TKIs [43], but the 3 patients who received ICI as first-line treatment did not develop pneumonia while receiving EGFR-TKIs in this study. Due to the limited number of cases, we could only provide a descriptive analysis. The findings indicated that the ICI-PFS was greater than 6 months for those receiving ICI as subsequent treatment after TKI resistance, and two patients even achieved remarkable ICI-PFS durations of 20.9 and 36.5 months. Hence, ICI therapy emerges as a potential strategy for EGFR-positive LUSC patients after TKI failure. Moreover, in a previous meta-analysis conducted by Qian et al. [44] the authors examined 7 RCTs that compared ICI therapy alone or in combination with other therapies to other treatment modalities in EGFR-positive NSCLC patients after TKI failure. The results indicated that ICI monotherapy did not improve OS or PFS in NSCLC patients. However, patients who received ICI in combination with other therapies had better PFS compared to those treated with conventional chemotherapy. Although these RCTs primarily focused on patients with adenocarcinoma, we believe that ICI-based combination therapy is also a promising option for LUSC patients. However, further clinical data is needed to validate this hypothesis.

Conclusions

In summary, our study revealed that EGFR-positive LUSC patients have better ORR and PFS with TKI therapy compared to EGFR-negative LUSC patients. The resistance mechanisms to EGFR-TKI of EGFR-positive LUSC are complex and diverse, with secondary T790M mutations being the most common. And the histological transformation from squamous cell carcinoma to adenocarcinoma is also one of its resistance mechanisms. Furthermore, our findings suggest that ICI therapy is a promising strategy for EGFR-positive LUSC patients after experiencing TKI failure. This study enhances our comprehension of LUSC and establishes a foundation for further exploration of this rare patient group.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Review Board of Zhejiang Cancer Hospital (Permits No.IRB-2023-565) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study is a retrospective study and most of patients have died. The exempt written informed consent for this study was approved by the Ethics Committee of Zhejiang Cancer Hospital. All patient data were de-identified to maintain confidentiality and comply with privacy regulations.

Authors contributions

HL conceived the experiments and helped to coordinate support and funding. ZC, JG and JC searched the literatures, analyzed data and wrote manuscript. LY, SH and LC supported writing manuscript. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The relevant data supporting the conclusions of this article will be available by contacting the corresponding authors upon reasonable request.

References

[1] Cheng TY, Cramb SM, Baade PD, et al. The international epidemiology of lung cancer: latest trends, disparities, and tumor characteristics. J Thorac Oncol. 2016;11(10):1653–1671. doi:10.1016/j.jtho.2016.05.021.

[2] Youlden DR, Cramb SM, Baade PD. The international epidemiology of lung cancer: geographical distribution and secular trends. J Thorac Oncol. 2008;3(8):819–831. doi:10.1097/JTO.0b013e31818020eb.

[3] Wang Z, Shen Z, Li Z, et al. Activation of the BMP-BMPR pathway conferred resistance to EGFR-TKIs in lung squamous cell carcinoma patients with EGFR mutations. Proc Natl Acad Sci U S A. 2015;112(32):9990–9995. doi:10.1073/pnas.1510837112.

[4] Heist RS, Sequist LV, Engelman JA. Genetic changes in squamous cell lung cancer: a review. J Thorac Oncol. 2012;7(5):924–933. doi:10.1097/JTO.0b013e31824cc334.

[5] Perez-Moreno P, Brambilla E, Thomas R, et al. Squamous cell carcinoma of the lung: molecular subtypes and therapeutic opportunities. Clin Cancer Res. 2012;18(9):2443–2451. doi:10.1158/1078-0432.Ccr-11-2370.

[6] Wu YL, Zhou C, Liam CK, et al. First-line erlotinib versus gemcitabine/cisplatin in patients with advanced EGFR mutation-positive non-small-cell lung cancer: analyses from the phase III, randomized, open-label, ENSURE study. Ann Oncol. 2015;26(9):1883–1889. doi:10.1093/annonc/mdv270.

[7] Xu J, Chu T, Jin B, et al. Epidermal growth factor receptor tyrosine kinase inhibitors in advanced squamous cell lung cancer. Clin Lung Cancer. 2016;17(4):309–314. doi:10.1016/j.cllc.2015.11.009.

[8] Chang Q, Qiang H, Qian J, et al. Epidermal growth factor receptor mutation status and response to tyrosine kinase inhibitors in advanced chinese female lung squamous cell carcinoma: a retrospective study. Front Oncol. 2021;11:652560. doi:10.3389/fonc.2021.652560.

[9] Shukuya T, Takahashi T, Kaira R, et al. Efficacy of gefitinib for non-adenocarcinoma non-small-cell lung cancer patients harboring epidermal growth factor receptor mutations: a pooled analysis of published reports. Cancer Sci. 2011;102(5):1032–1037. doi:10.1111/j.1349-7006.2011.01887.x.

[10] Lewis WE, Hong L, Mott FE, et al. Efficacy of targeted inhibitors in metastatic lung squamous cell carcinoma with EGFR or ALK alterations. JTO Clin Res Rep. 2021;2(11):100237. doi:10.1016/j.jtocrr.2021.100237.

[11] Liu Y, Zhang Y, Zhang L, et al. Efficacy of epidermal growth factor receptor-tyrosine kinase inhibitors for lung squamous carcinomas harboring EGFR mutation: a multicenter study and pooled analysis of published reports. Oncotarget. 2017;8(30):49680–49688. doi:10.18632/oncotarget.17915.

[12] Tripathi SK, Pandey K, Rengasamy KRR, et al. Recent updates on the resistance mechanisms to epidermal growth factor receptor tyrosine kinase inhibitors and resistance reversion strategies in lung cancer. Med Res Rev. 2020;40(6):2132–2176. doi:10.1002/med.21700.

[13] Smit EF, van Meerbeeck JP, Lianes P, et al. Three-arm randomized study of two cisplatin-based regimens and paclitaxel plus gemcitabine in advanced non-small-cell lung cancer: a phase III trial of the European Organization for Research and Treatment of Cancer Lung Cancer Group–EORTC 08975. J Clin Oncol. 2003;21(21):3909–3917. doi:10.1200/jco.2003.03.195.

[14] Reck M, Mok TSK, Nishio M, et al. Atezolizumab plus bevacizumab and chemotherapy in non-small-cell lung cancer (IMpower150): key subgroup analyses of patients with EGFR mutations or baseline liver metastases in a randomised, open-label phase 3 trial. Lancet Respir Med. 2019;7(5):387–401. doi:10.1016/s2213-2600(19)30084-0.

[15] Garassino MC, Cho BC, Kim JH, et al. Durvalumab as third-line or later treatment for advanced non-small-cell lung cancer (ATLANTIC): an open-label, single-arm,

phase 2 study. Lancet Oncol. 2018;19(4):521–536. doi:10.1016/s1470-2045(18)30144-x.
[16] Yohe S, Thyagarajan B. Review of clinical next-generation sequencing. Arch Pathol Lab Med. 2017;141(11):1544–1557. doi:10.5858/arpa.2016-0501-RA.
[17] McCombie WR, McPherson JD, Mardis ER. Next-generation sequencing technologies. Cold Spring Harb Perspect Med. 2019;9(11):a036798. doi:10.1101/cshperspect.a036798.
[18] Zhang W, Hubbard A, Jones T, et al. Fully automated 5-plex fluorescent immunohistochemistry with tyramide signal amplification and same species antibodies. Lab Invest. 2017;97(7):873–885. doi:10.1038/labinvest.2017.37.
[19] Schwartz LH, Liti√®re S, de Vries E, et al. RECIST 1.1-Update and clarification: from the RECIST committee. Eur J Cancer. 2016;62:132–137. doi:10.1016/j.ejca.2016.03.081.
[20] Ock CY, Keam B, Kim S, et al. Pan-cancer immunogenomic perspective on the tumor microenvironment based on PD-L1 and CD8 T-cell infiltration. Clin Cancer Res. 2016;22(9):2261–2270. doi:10.1158/1078-0432.Ccr-15-2834.
[21] Teng MW, Ngiow SF, Ribas A, et al. Classifying cancers based on T-cell infiltration and PD-L1. Cancer Res. 2015;75(11):2139–2145. doi:10.1158/0008-5472.Can-15-0255.
[22] Shin DY, Na II, Kim CH, et al. EGFR mutation and brain metastasis in pulmonary adenocarcinomas. J Thorac Oncol. 2014;9(2):195–199. doi:10.1097/jto.0000000000000069.
[23] Li L, Luo S, Lin H, et al. Correlation between EGFR mutation status and the incidence of brain metastases in patients with non-small cell lung cancer. J Thorac Dis. 2017;9(8):2510–2520. doi:10.21037/jtd.2017.07.57.
[24] Wu YL, Zhou C, Hu CP, et al. Afatinib versus cisplatin plus gemcitabine for first-line treatment of Asian patients with advanced non-small-cell lung cancer harbouring EGFR mutations (LUX-Lung 6): an open-label, randomised phase 3 trial. Lancet Oncol. 2014;15(2):213–222. doi:10.1016/S1470-2045(13)70604-1.
[25] Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med. 2009;361(10):947–957. doi:10.1056/NEJMoa0810699.
[26] Zhou C, Wu YL, Chen G, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. Lancet Oncol. 2011;12(8):735–742. doi:10.1016/s1470-2045(11)70184-x.
[27] Pretelli G, Spagnolo CC, Ciappina G, et al. Overview on therapeutic options in uncommon EGFR mutant non-small cell lung cancer (NSCLC): new lights for an unmet medical need. Int J Mol Sci. 2023;24(10):8878. doi:10.3390/ijms24108878.
[28] Zheng Q, Huang Y, Zhao H, et al. EGFR mutation genotypes affect efficacy and resistance mechanisms of osimertinib in T790M-positive NSCLC patients. Transl Lung Cancer Res. 2020;9(3):471–483. doi:10.21037/tlcr.2020.03.35.
[29] Chen DS, Hurwitz H. Combinations of bevacizumab with cancer immunotherapy. Cancer J. 2018;24(4):193–204. doi:10.1097/ppo.0000000000000327.
[30] Maemura M, Iino Y, Yokoe T, et al. Serum concentration of hepatocyte growth factor in patients with metastatic breast cancer. Cancer Lett. 1998;126(2):215–220. doi:10.1016/s0304-3835(98)00014-7.
[31] Shiota G, Okano J, Kawasaki H, et al. Serum hepatocyte growth factor levels in liver diseases: clinical implications. Hepatology. 1995;21(1):106–112. doi:10.1002/hep.1840210119.
[32] Turke AB, Zejnullahu K, Wu YL, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell. 2010;17(1):77–88. doi:10.1016/j.ccr.2009.11.022.
[33] Goel HL, Mercurio AM. VEGF targets the tumour cell. Nat Rev Cancer. 2013;13(12):871–882. doi:10.1038/nrc3627.
[34] Le X, Nilsson M, Goldman J, et al. Dual EGFR-VEGF pathway inhibition: a promising strategy for patients with EGFR-mutant NSCLC. J Thorac Oncol. 2021;16(2):205–215. doi:10.1016/j.jtho.2020.10.006.
[35] Fukumura D, Kloepper J, Amoozgar Z, et al. Enhancing cancer immunotherapy using antiangiogenics: opportunities and challenges. Nat Rev Clin Oncol. 2018;15(5):325–340. doi:10.1038/nrclinonc.2018.29.
[36] Nakagawa K, Garon EB, Seto T, et al. Ramucirumab plus erlotinib in patients with untreated, EGFR-mutated, advanced non-small-cell lung cancer (RELAY): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol. 2019;20(12):1655–1669. doi:10.1016/s1470-2045(19)30634-5.
[37] Mok TS, Wu Y-L, Ahn M-J, et al. Osimertinib or platinum–pemetrexed in EGFR T790M–positive lung cancer. New Eng J Med. 2017;376(7):629–640. doi:10.1056/NEJMoa1612674.
[38] Park S, Shim JH, Lee B, et al. Paired genomic analysis of squamous cell carcinoma transformed from EGFR-mutated lung adenocarcinoma. Lung Cancer. 2019;134:7–15. doi:10.1016/j.lungcan.2019.05.024.
[39] Vokes NI, Chambers E, Nguyen T, et al. Concurrent TP53 mutations facilitate resistance evolution in EGFR-mutant lung adenocarcinoma. J Thorac Oncol. 2022;17(6):779–792. doi:10.1016/j.jtho.2022.02.011.
[40] Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non–small-cell lung cancer. N Engl J Med. 2015;373(2):123–135. doi:10.1056/NEJMoa1504627.
[41] Attili I, Passaro A, Corvaja C, et al. Immune checkpoint inhibitors in EGFR-mutant non-small cell lung cancer: A systematic review. Cancer Treat Rev. 2023;119:102602. doi:10.1016/j.ctrv.2023.102602.
[42] Gettinger SN, Choi J, Mani N, et al. A dormant TIL phenotype defines non-small cell lung carcinomas sensitive to immune checkpoint blockers. Nat Commun. 2018;9(1):3196. doi:10.1038/s41467-018-05032-8.
[43] Wiest N, Majeed U, Seegobin K, et al. Role of immune checkpoint inhibitor therapy in advanced EGFR-mutant non-small cell lung cancer. Front Oncol. 2021;11:751209. doi:10.3389/fonc.2021.751209.
[44] Qian X, Guo X, Li T, et al. Efficacy of immune checkpoint inhibitors in EGFR-Mutant NSCLC patients with EGFR-TKI resistance: A systematic review and meta-analysis. Front Pharmacol. 2022;13:926890. doi:10.3389/fphar.2022.926890.