Efficacy of epidermal growth factor receptor-tyrosine kinase inhibitors for Chinese patients with squamous cell carcinoma of lung harboring *EGFR* mutation

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ABSTRACT

Objective: Epidermal growth factor receptor (*EGFR*) mutation mostly occurred in lung adenocarcinoma, rarely in squamous cell carcinoma (SQCC). EGFR mutation rate in SQCC varied in previous reports, and the efficacy of *EGFR* tyrosine kinase inhibitors (TKIs) in SQCC harboring EGFR mutation has not yet been fully evaluated. The aim of this study was to investigate the efficacy *EGFR*-TKIs for Chinese patients with SQCC of lung harboring *EGFR* mutation.

Patients and methods: Two cohorts of patients were analyzed. The first cohort included 146 consecutive post-operation SQCC patients from January 2008 to October 2012. The second cohort included 63 patients with advanced SQCC receiving EGFR-TKIs treatment. *EGFR* mutation analysis was performed with Real-time PCR method. The pathologic diagnosis was validated with immunohistochemistry (IHC) for patients harboring activated *EGFR* mutation. And the efficacy of EGFR-TKIs in squamous cell carcinoma of lung (SQCC) was evaluated in patients with activated *EGFR* mutations.

Results: In the first cohort, 146 resected patients, EGFR mutations were detected in 3 patients, with the mutation rate of 2.0%. In cohort two, 63 patients treated with EGFR-TKIs, 15 patients possessed activated EGFR mutations. The response rate and disease control rate in these patients was 26.7% and 66.7% respectively. 5 patients had disease control over 6 months. The progression free survival (PFS) in *EGFR*-mutated patients was 3.9 months.

Conclusions: In Chinese SQCC patients, *EGFR* mutation rate was extremely low. EGFR-TKIs seemed to be less effective in EGFR-mutated SQCC patients, but some patients could still obtain benefit from EGFR-TKIs. To identify this part of patients, further study was warranted in the future.

KEYWORDS

Squamous cell carcinoma of lung (SQCC); immunohistochemistry (IHC); epidermal growth factor receptor mutation (EGFR mutation); epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs)

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Introduction

The presence of activating epidermal growth factor receptor (*EGFR*) mutation was associated with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) treatment response in patients with advanced non-small-cell lung cancer (NSCLC) (1). Recent phase III clinical studies of advanced NSCLC have demonstrated that *EGFR* mutations are the most effective predictor of clinical outcome in response to EGFR-TKIs in first-line treatment. The response rate ranged from 70% to 85% in *EGFR* mutant patients, with the progression-free survival (PFS) of 8 to 13 months (2-5). However, *EGFR* mutations occur almost exclusively in lung adenocarcinoma and the majority data on EGFR-TKIs sensitivity was derived from this histology. Due to the low mutation rate in squamous cell carcinoma of lung (SQCC) (6), *EGFR* mutation testing is not routinely recommended for SQCC in National Comprehensive Cancer Network (NCCN) guideline. Until now, no molecular targeted agents have been specifically developed for SQCC treatment.

EGFR mutation rate varied in previous reports, ranging

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from 1% to 15% (7-10). Several relative large series studies of surgically-resected SQCC found no *EGFR* mutations (11,12). But a meta-analysis from Asian reported that *EGFR* mutation rate was 10% in SQCC (13). As is known, the frequency of *EGFR* mutation is much higher in Asian population (14). Whether ethnic difference also existed in Chinese SQCC patients is not yet known. Another pool analysis from Japan demonstrated that the PFS of EGFR-TKIs in *EGFR* mutant SQCC was only 3 months, with the response rate of 30% (15). The research indicated that there was a subgroup of NSCLC patients harboring *EGFR* mutations with poor efficacy after EGFR-TKIs treatments, which was different from that of adenocarcinoma patients.

In the traditional diagnosis of NSCLC subtypes, no detailed pathologic analysis was provided. Poorly differentiated carcinoma might appear indistinguishable with morphologic diagnosis only (16). Great efforts had been made on distinguishing subtypes of NSCLC through immunohistochemical biomarkers these years (17-20). In 2011, a new multidisciplinary classification of lung adenocarcinoma has been developed, taking into account histologic, molecular, and radiologic features, as well as prognostic and predictive information for treatment selection (21). Several studies have already demonstrated that immunohistochemistry (IHC) of TTF-1 and P63 can effectively identify the tumor cell type in samples of NSCLC (17,18). Recently, another research have shown that combined with Napsin A, P63 and TTF-1 could be sufficient to reliably sub-classify poorly differentiated NSCLC (22). However, in pre-IHC era, the lack of precision in morphologic diagnosis of NSCLC subtypes might account for the variability of reported *EGFR* mutation rate in SQCC.

Whether *EGFR* mutations do arise in SQCC was still a controversial topic. And the efficacy of EGFR-TKI was still disputable in *EGFR* mutant SQCC. Here, we performed a retrospective study to estimate the *EGFR* mutation rate in IHC-verified SQCC and to evaluate the efficacy of EGFR-TKIs in Chinese patients with advanced SQCC harboring *EGFR* mutations.

Patients and materials

Study cohorts

Two cohorts of patients with SQCC were enrolled onto the research. All patients were treated at the Cancer Center of Sun Yat-Sen University (Guangzhou, China) from 1 January 2004 to 31 August 2012.

The first cohorts consisted of 146 consecutive patients with diagnosis of SQCC who had received radical resection from 1 January 2008 to 1 January 2012. A representative formalin-fixed, paraffin-embedded (FFPE) tumor block was collected for IHC reassessment and *EGFR* mutation analysis.

The second cohort included 67 patients with advanced SQCC who had received EGFR-TKIs (Gefitinib or Erlotinib) treatment in the course of disease from January 2004 to 31 August 2012. All the patients were diagnosed with advanced lung SQCC by bronchofiberscope biopsy or percutaneous lung biopsy. Only 63 patients had adequate specimens for further analysis. The FFPE tumor blocks of the 63 patients were also collected and subjected to IHC reassessment for pathological diagnosis, as described later. *EGFR* mutation analysis was also performed on this cohort of patients. The efficacy of EGFR-TKIs was all available.

The basic clinical data of the two cohorts of patients was collected, including age, gender, Eastern Cooperative Oncology Group performance status (ECOG PS), tumor stage, smoking status. In the second cohort, the response evaluation of patients with EGFR-TKIs was recorded. The prognosis of the patients in this cohort was retrospectively analyzed, including response rate and PFS.

The study was approved by the Institutional Review Board of Sun Yat-Sen University Cancer Center (Guangzhou, China). All the patients had provided written informed consent before samples were collected.

EGFR mutation analysis

The FFPE tumor blocks were cut into 5 μm consecutive sections for DNA extraction. The EGFR Scorpion ARMS Kit (DxS Ltd, Manchester, UK) was used to detect *EGFR* mutations by real-time PCR, which enabled to detect the low-level mutant DNA in the background of wild-type DNA based on the allele-specific and real-time PCR technologies. 2 ng DNA was added to each 25 μL assay reaction in 96-well plate. The plate was sealed and loaded into Stratagene MX3005P real-time PCR system (Agilent Technologies, Santa Clara, Canada). Obtained data was analyzed using MxPro v4.0 software (Agilent Technologies, Santa Clara, Canada).

IHC analyses

IHC staining was performed using mouse monoclonal anti-human antibodies for reconfirming the pathological diagnosis, including P63 (DAKO, 1:80), TTF-1 (DAKO, 1:600) and Napsin A (DAKO, 1:200). Sections with 5-μm-thick were cut from the FFPE and then routinely deparaffinized and rehydrated. For antigen retrieval, slides were heated in a microwave oven for 30 minutes in citrate buffer solution (pH=7.4) and cooled slowly at room temperature for 20 minutes. After blocking the activity of endogenous peroxidase with 3% hydrogen peroxide for 8 minutes, the sections were treated with primary antibodies and incubated for 12 hours. Subsequently, the slides were rinsed

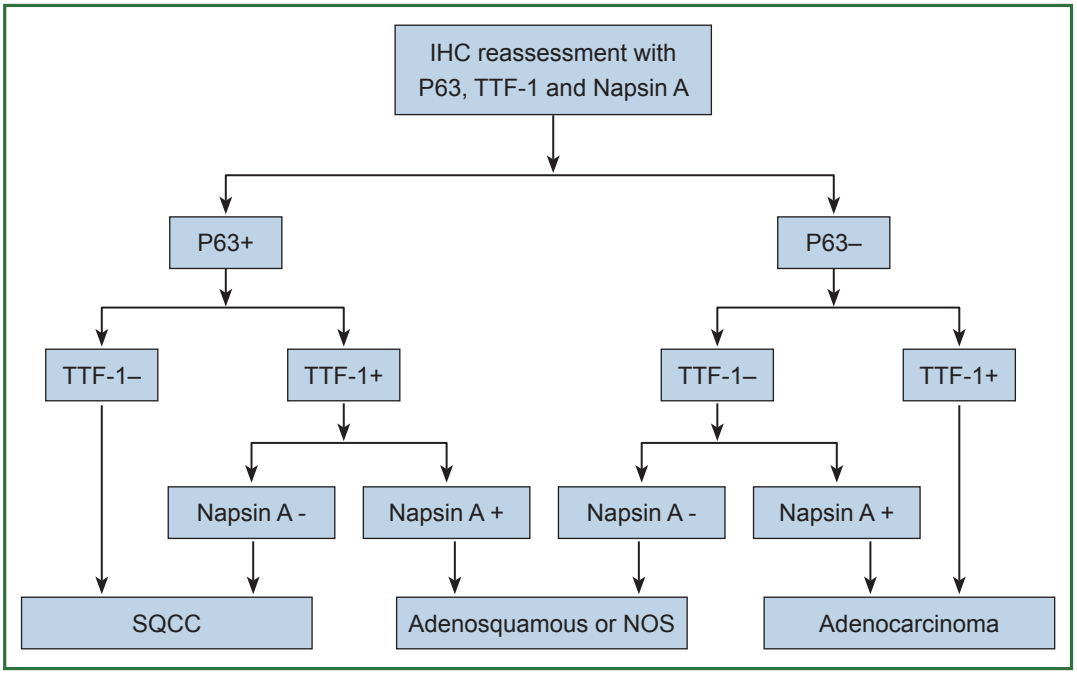

Figure 1. Immunohistochemistry reassessment chart for pathologic diagnosis with P63, TTF-1 and Napsin A.

in PBS three times and incubated in biotinylated secondary antibodies. After incubation, slides were washed again with PBS and then visualized using diaminobenzidine. Finally, Mayer's hematoxylin was used to counterstain the sections, which were then dehydrated and mounted.

P63-diffuse/TTF-1-negative profile supported SQCC. The cases with diffuse P63 and weak/focal co-expression of TTF-1 were further confirmed as SQCC by negative Napsin A. The profiles that supported adenocarcinoma were TTF-1-positive and P63-negative. TTF-1 and P63 double-negative profile was interpreted as indeterminate but favoring adenocarcinoma because negative P63 is highly unusual for SQCC, whereas TTF-1-negative adenocarcinoma art not uncommon. Double-negative carcinomas were further evaluated with Napsin A. Reactivity for P63 and TTF-1 in distinct cell population generally supported biphenotypic differentiation, such as adenosquamous carcinoma. Two pathologists who don't know the information of the patients were asked to independently assess the expression. The reassessment process was shown in (Figure 1).

Statistical analysis

The *EGFR* mutation rate in lung SQCC was analyzed in the first cohort. In the second cohort, PFS with EGFR-TKIs was calculated from the beginning receiving TKIs to the time of disease progression. Patients who had not progressed at the time of statistical analysis were censored at the time of last follow-up (31 August 2012). Univariate analysis by log-rank test was conducted on PFS to evaluate the effects of clinical factors relating to prognosis. Multivariate analysis was performed with Cox regression analysis. And chi-square test was used for response rate and disease control rate analysis. Survival analysis was depicted by Kaplan-Meier method. A P value <0.05 used to denote statistical significant, and all reported P values were two sided. All statistical analyses were performed with SPSS 16.0.

Results

Patient characteristics

In the first cohort, of the 146 patients with diagnosis of SQCC after radical resection, 129 patients (88.4%) were male and 17 female (11.6%), with the median age 59 years old (range: 30-81 years old). All the patients were stage I to IIIA.

In the second cohort, 63 patients with advanced lung SQCC were retrospectively analyzed, including 50 male (79.4%) and 13 female (20.6%). The median age was 54 years (range: 23-75 years). All the patients received Erlotinib (n=33) or Gefitinib (n=30) as second or third line treatment. The basic clinical characteristics of the two cohorts are listed in (Table 1).

IHC reassessment and EGFR mutation analysis

All the patients in the two cohorts had adequate specimens for IHC reassessment. The pathologic diagnosis of all the patients were verified to be SQCC with $P63^{+}$/$TTF\text{-}1^{+/-}$/Napsin A^{-} (Figure 2).

In cohort one, *EGFR* mutations were detected in only 3 patients (2.0%). All the 3 patients were L858R mutations. No

Table 1. The basic clinical characteristics of patients in two study cohorts.

Characteristics	Cohort one (n=146)		Cohort two (n=63)	
	Cases (n)	Percentage (%)	Cases (n)	Percentage (%)
Age [years]				
Median	59 [30-81]		54 [23-75]	
Gender				
Male	129	88.3	50	79.4
Female	17	11.7	13	20.6
Smoking				
Smoker	95	65.1	40	63.5
Nonsmoker	51	34.9	23	36.5
ECOG PS				
0-1	146	100	39	61.9
2	0	0	24	38.1
Staging				
I-II	119	81.5	0	0
IIIA	27	18.5	0	0
IIIB-IV	0	0	63	100
Prior chemotherapy				
0	–	–	0	0
1	–	–	33	52.4
2 or more	–	–	30	47.6
EGFR-TKI treatment				
Gefitinib	–	–	30	47.6
Erlotinib	–	–	33	52.4

ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor.

other mutations were detected. Hence, the *EGFR* mutation rate in Chinese lung SQCC was only 2.0%.

In cohort two, all 63 patients had additional samples for *EGFR* mutations analysis. *EGFR* mutations were detected in 15 patients (23.8%), including exon 19 deletions (n=13) and L858R mutation (n=2). The other 48 patients were *EGFR* wild-type.

Efficacy of EGFR-TKIs in verified advanced lung SQCC

In the 63 patients of verified SQCC, 5 patients achieved partial response (PR), among whom 1 patient received Gefitinib and 4 patients received Erlotinib. 25 achieved stable disease (SD), including 9 with Gefitinib and 21 with Erlotinib. The response rate and disease control rate were 7.9% and 47.6%, respectively.

At the end of data cut off, 4 patients (all SD) had not experienced progression at the last follow-up (31 Aug 2012). The median PFS of all patients was 2.5 months (95% CI: 1.3-3.7 months). Results of univariate analysis for PFS are shown in (Table 2). No factor was correlated significantly with PFS. Although the response rate of *EGFR*-mutant patients was better than that of *EGFR* wild-type patients (26.7% *vs.* 2.1%, P=0.002), the disease control rate between the two groups was not significantly different (66.7% *vs.* 41.7%, P=0.09) (Table 3). The PFS of *EGFR*-mutant patients were numerically longer than that of *EGFR* wild-type patients (3.9 *vs.* 1.9 months, respectively). No significant difference was observed between these two groups of patients (P=0.19) (Figure 3).

Discussion

Although the incidence of lung SQCC is decreasing as a consequence of changes in tobacco consumption habits (23,24), it is still the second most common type of NSCLC (25). Encouraging new target agents have provided great benefit to patients with adenocarcinoma, but, unfortunately, there was no effective targeted therapy for lung SQCC to date. EGFR-TKIs were now recommended as first-line treatment for NSCLC patients with sensitive *EGFR* mutation, which was mainly in lung adenocarcinoma. The frequency of *EGFR* mutation in lung SQCC varied in previous reports and in different ethics, ranging from 1% to 15% (7-10). In the China Edition of NCCN guideline, *EGFR* mutation detecting is recommended for SQCC basing on a meta-analysis. Hence, discrepancy existed in the guideline recommendation in different ethnicity.

Recently Natasha R. *et al.* retrospectively analyzed 95 biomarker-verified SQCC and reported that *EGFR* mutations do not occur

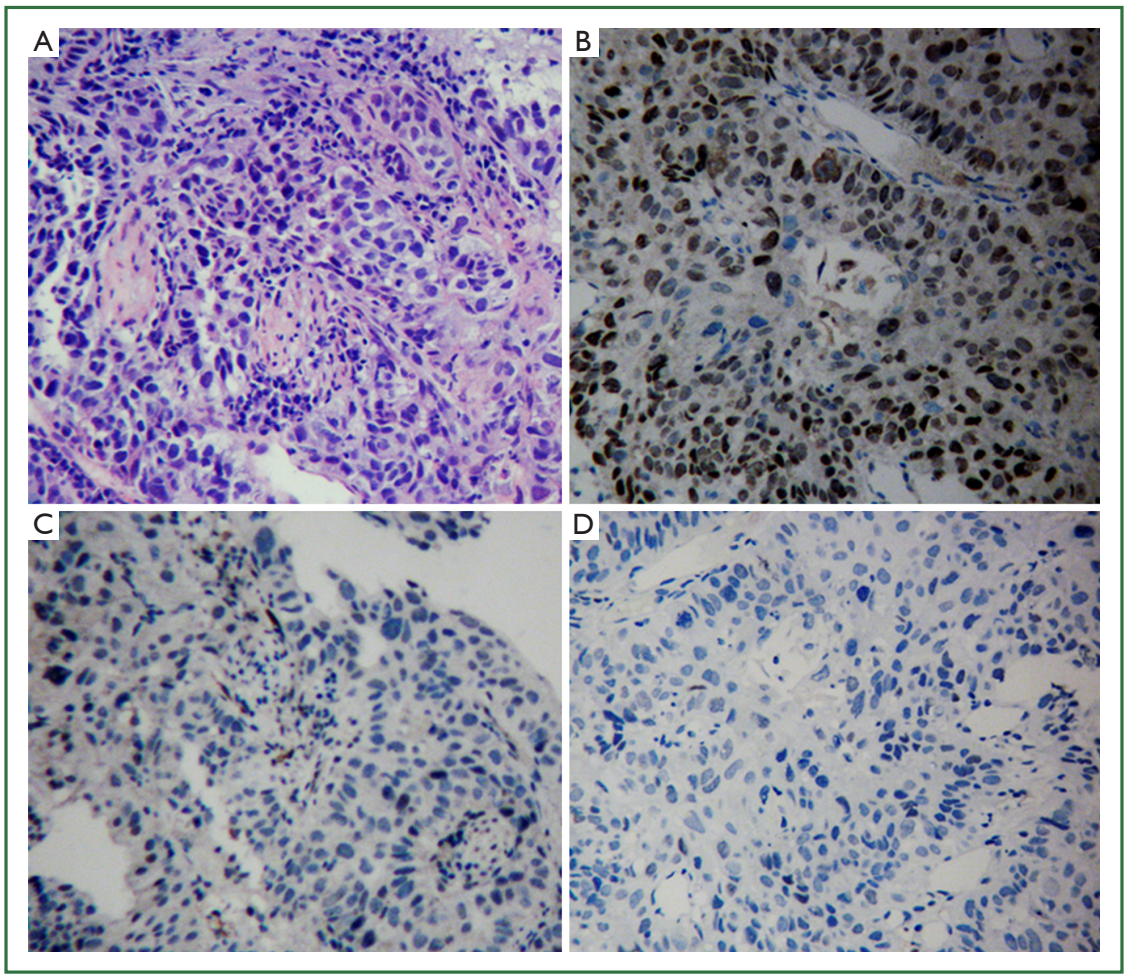

Figure 2. Immunohistochemical reassessment of pathological diagnosis in EGFR mutation patients to be squamous cell carcinoma. (A) Representative image of HE stain (20×); (B) Representative image of a P63 positive sample with deep brown-stained nuclei; (C) Representative image of negative Napsin A without stained; (D) Representative image of positive TTF-1 sample without stained.

in pure SQCC, and occasional detection of *EGFR* mutations in samples of SQCC was due to diagnosis of adenosquamous carcinoma or adenocarcinoma (26). However, another similar study was conducted by Miyamae *et al.* (9), demonstrated that *EGFR* mutations were detected in 3.4% among 87 validated lung SQCC specimens. To prove this controversial topic in Chinese population, we reassessed the pathologic diagnosis of the specimens and evaluated the *EGFR* mutation rate in Chinese squamous cell lung cancer patients in our study, which was the first time of *EGFR* mutation rate screening in Chinese squamous cell lung cancer with large cohort of patients. All the patients in cohort one had received operation and the large specimens were used for testing. All the patients were validated with IHC to be true SQCC. Only 3 patients possessed *EGFR* mutation. Hence, *EGFR* mutation did exist in true SQCC, with extremely low mutation rate of 2.0%, which was much lower than the previous reports of 10% (13). Recently, another research about comprehensive genomic analysis of lung SQCC reported 2 patients with *EGFR* mutation from 178 verified SQCC patients (27). The result was consistent with our study. It seemed that in SQCC patients, the frequency of *EGFR* mutation was similar in different ethnicity.

Although *EGFR* mutation was rare in lung SQCC, it was reported that the PFS of EGFR-TKIs in *EGFR* mutant SQCC was 3 months, with response rate and disease control rate of 30% and 70% respectively in a pool analysis (12). It seemed that *EGFR*-mutant SQCC could still obtain survival benefit from target therapy. However, the efficacy of EGFR-TKIs in *EGFR* mutant and *EGFR* wild-type SQCC patients has not yet been fully compared before. Here, we retrospectively analyzed 63 advanced SQCC patients who had received EGFR-TKIs treatment, including 15 *EGFR* mutant patients and 48 *EGFR* wild-type patients. The response rate in *EGFR* mutant patients was much higher (26.7% *vs.* 2.1%, P=0.002), but the disease control rate was not significantly different between the two groups (66.7% *vs.* 41.7%, P=0.09). The response rate and disease control rate in *EGFR* mutant patients were consistent with

Table 2. Univariate analysis of progression free survival in the second cohort patients.

Items	Cases (n)	Median PFS (months) (95% CI)	Univariate analysis P
Age (years)			0.24
≤54	32	1.8 (0.8-2.8)	
>54	31	3.3 (2.3-4.2)	
Gender			0.56
Male	50	2.3 (0.4-4.1)	
Female	13	2.6 (1.1-4.1)	
Smoking history			0.70
Smoker	23	2.5 (0.6-4.4)	
Never-smoker	40	2.0 (1.6-2.3)	
Previous chemotherapy			0.74
1 regimen	33	2.0 (0.6-3.3)	
≥2 regimens	30	3.0 (1.3-4.7)	
ECOG PS			0.32
0-1	39	2.6 (1.3-3.9)	
2	24	1.9 (0.2-3.7)	
EGFR mutation status			0.19
EGFR mutant	15	3.9 (1.5-6.3)	
EGFR wild-type	48	1.9 (0.7-3.2)	

ECOG PS, Eastern Cooperative Oncology Group performance status.

Table 3. The response rate and disease control rate in EGFR mutation and EGFR wild-type patients.

Group	Patients (n)	Response rate		Disease control rate	
		%	P value	%	P value
EGFR mutation	15	26.7	0.002	66.7	0.09
EGFR wild-type	48	2.1		41.7	

EGFR, epidermal growth factor receptor.

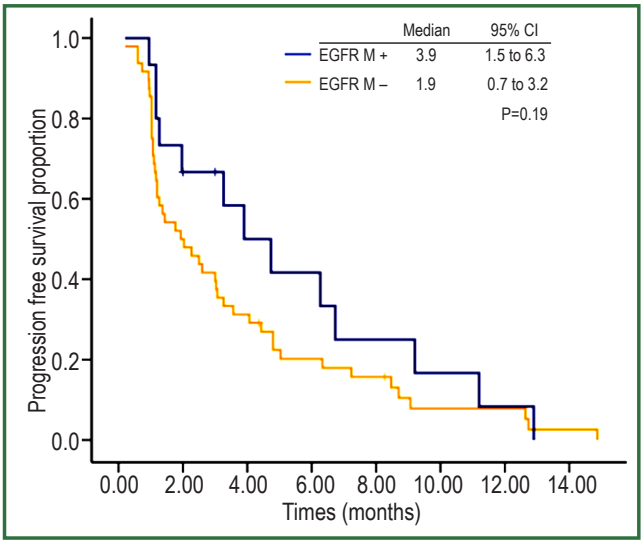

Figure 3. Kaplan-Meier plots showing progression free survival. The PFS between patients with EGFR mutation positive (EGFR M+) and EGFR mutation negative (EGFR M-) was not significantly different (3.9 *vs.* 1.9 months, P=0.19).

the report of pool analysis. The PFS was numerically longer in *EGFR* mutant patients (3.9 *vs.* 1.9 months), but no significant difference was observed (P=0.19), which might be caused by the relative small sample size. The results indicated that the efficacy of EGFR-TKIs in SQCC was limited, which was much poorer with historical comparison of *EGFR* mutant in Asian adenocarcinoma of 30% (14). There were few treatment options for patients with advanced lung SQCC. Platinum-based doublet chemotherapy was still the first choice. EGFR-TKIs could still be taken as a salvage treatment.

Interestingly, in the 48 wild-type *EGFR* patients, 1 achieved PR in response to erlotinib and another 19 patients achieve SD. The sensitivity of *EGFR* mutation tests was suspected to be one of the possible reasons for the response of EGFR-TKIs in patients without detectable *EGFR* mutations. In our study, sensitive method was used to detect *EGFR* mutation. Besides, it had been reported that *EGFR* mutations were evenly distributed in lung tumors. In the heterogeneity of *EGFR* mutation analysis by Yatabe *et al.* identical *EGFR* mutations were found throughout

individual tumors in a trans-sectional analysis and no discordant mutation patterns were detected among paired primary and metastatic site samples (28). The study indicated that *EGFR* mutation detection with different sizes or location merely affected the results. Another possible reason is that EGFR-TKIs might target additional pathways other than *EGFR* mutations, which still needed further study to validate.

The BR.21 (NCT00036647) and TRUST (NCT 00949910) study had proved the efficacy and safety of erlotinib in second-line or third-line treatment comparing with placebo (29,30). Recently, the Tailor study (NCT00637910) indicated that previously treated wild-type *EGFR* patients could not obtain PFS benefit from the second-line treatment of erlotinib when comparing with docetaxel (31). However, the subgroup analysis in SQCC patients showed similar PFS.

In the 15 patients harboring *EGFR* mutations, 5 patients failed to response to EGFR-TKIs treatment. According to pervious study, the presence of T790M mutation might account for the lower efficacy (15,32). Recently, a comprehensive genomic analysis of lung SQCC has demonstrated the complex genomic alternations on the core cellular pathway of SQCC. The PI3K/RTK/RAS signaling pathway possessed 69% of alternation, which might affect the efficacy of EGFR-TKIs (27). Besides, coexistence of PI3K mutation and *EGFR* mutation has been reported (33), which might also help to explain the poor efficacy of TKI in SQCC. Hence, it is suggested that combined analysis of KRAS, PI3KCA, MET and non-sensitizing *EGFR* mutation was necessary before treatment.

There are several major limitations of our study. First, this is a retrospective study. All the data were collected retrospectively. And the frequency of EGFR mutation rate of SQCC was from the early stage patients. Second, small sample size in cohort two might affect the statistically analysis. There were only 66 patients treated with *EGFR* TKIs analysis, and only 15 patients possessed *EGFR* mutation. Thirdly, due to the small specimens, we didn't have adequate samples for further molecular analysis.

In conclusion, lung SQCC validated with new WHO criteria did possess *EGFR* mutation, but the frequency of *EGFR* mutation in Chinese patients was only 2.0%, which was much lower than the previous reports. In *EGFR* mutant lung SQCC patients, the PFS was numerically longer than that of *EGFR* wild-type patients. The low mutation rate and limited efficacy don't justify routine test for all SQCC specimens. However, EGFR-TKIs could be taken as a salvage treatment for advance lung SQCC patients. Due to the small sample size of retrospective study, prospective studies with large cohort are warranted in the future.

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