The presence of activating epidermal growth factor receptor (EGFR) mutation was associated with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) treatment.
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 subclassify 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 bronchoscopy biopsy or percutaneous lung biopsy. Only 63 patients had adequate specimens for further analysis.

**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...
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 are 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).

**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-1−/Napsin A− (Figure 2).

In cohort one, EGFR mutations were detected in only 3 patients (2.0%). All 3 patients were L858R mutations. No
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 16 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
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

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Patients (n)</th>
<th>Response rate</th>
<th>Disease control rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>P value</td>
</tr>
<tr>
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<td>26.7</td>
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</tr>
<tr>
<td>EGFR wild-type</td>
<td>48</td>
<td>2.1</td>
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</table>

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).
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 respond 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 alteration, 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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References


