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FGFR1 amplification and *FGFR* gene fusion in resected squamous cell carcinoma of the lung.

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Abstract

The objective of the present study was to investigate the clinicopathologic characteristics and prognostic role of *FGFR1* amplification and *FGFR* fusion in patients with surgically resected squamous cell carcinoma of the lung (SCCL). Here, fluorescent in situ hybridization (FISH) was performed to detect *FGFR1* amplification and reverse transcriptase polymerase chain reaction (RT-PCR) was used to screen 15 known *FGFR* fusion variants in 108 patients with surgically resected SCCL. All cases were also analyzed for *EGFR*, *KRAS*, *HER2* and *BRAF* mutations. Clinical characteristics including age, sex, smoking status, stage, relapse-free survival (RFS) and overall survival (OS) were collected. Of 108 tumors screened, 14 (13.0%) *FGFR1* amplification was found. There were 4 (3.7%) patients that harbored *FGFR* fusion including 2 BAG4-*FGFR1* and 2 *FGFR3*-TACC3 fusion. Compared to the *FGFR1* amplification negative group, patients with *FGFR1* amplification were more likely to be smokers (100.0%, 14 of 14 patients, p=0.036), significantly associated with larger tumor (>3 cm) (88.2%, 13 of 14 patients, p=0.032). Patients with *FGFR1* amplification had worse RFS (p=0.013) and OS (p=0.021) than those without *FGFR1* amplification. There was no correlation between *FGFR* fusion and clinicopathologic characteristics. No significant difference in RFS or OS was found between patients with *FGFR* fusion and those without *FGFR* fusion. In conclusion, *FGFR1* amplification and *FGFR* fusion occurred in 13.0% and 3.7% of patients with surgically resected SCCL, separately. *FGFR1* amplification was correlated with poor prognosis and identified a distinct subset of SCCL with a higher prevalence among smokers with relative larger tumor (>3 cm). *FGFR* is a therapeutic target and patients with *FGFR1* amplification or *FGFR* fusion may benefit from *FGFR* targeted therapy which needs further clinical investigation.

Keywords: *FGFR1* amplification, *FGFR* fusion, Squamous cell carcinoma of the lung.

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Introduction

Lung cancer is the leading cause of cancer mortality worldwide resulting in more than 1 million deaths annually [1-3]. Because the activating mutation in the epidermal growth factor receptor (*EGFR*) is associated with dramatic responses to *EGFR* tyrosine kinase inhibitors, and the oncogenic fusion genes such as *ALK*, *ROS1* or *RET* rearrangement are related to sensitivity of treatment with crizotinib or vandetanib, identification of oncogenic driver genes and molecularly targeted therapies based on tumor biology in NSCLC are becoming increasingly important [4-7]. However, most of advances in personalized treatment was about adenocarcinoma of lung and effective targeted therapies for squamous cell carcinoma of the lung (SCCL) [6,8], accounting for approximately 25% of non-small-cell lung cancers (NSCLCs), remained elusive. As a result, it will be necessary to clarify different molecular alteration on SCCL for further individual treatment.

The *FGFR* family, comprising *FGFR1*, *FGFR*2, *FGFR*3 and *FGFR*4, play crucial roles in cancer development and are targets for dysregulation by amplification, point mutations, or translocation [5,9,10]. Amplified *FGFR1* has been reported in 20% of SCCL and inhibition of the *FGFR1* pathway with *FGFR* inhibitors was demonstrated to lead to significant tumor shrinkage, suggesting that *FGFR* inhibitors might be an effective therapeutic option in SCCL with *FGFR1* amplification [11,12]. Recently, *FGFR* fusion was identified in diverse cancers including glioblastoma, bladder cancer, head and neck cancer and SCCL [13,14]. Moreover, *FGFR* fusion

has been shown to sensitize cancer cells to *FGFR* kinase inhibitors, suggesting that a new subset of cancers maybe treatable with *FGFR* targeted therapy [13].

To date, there are few studies that constitute a comprehensive picture of *FGFR1* amplification, *FGFR* fusion, their protein expression and correlation to SCCL [15]. In the present study, we examined *FGFR1* gene amplification, *FGFR* gene fusion, as well as the expression of *FGFR1*/*FGFR*3 in a consecutive collection of SCCL tumor samples. The mutational status of known oncogenic genes, including *EGFR*, *KRAS*, *HER2* and *BRAF* were also examined. This detailed understanding of *FGFR* alterations and other oncogene mutations in SCCL might enable a more precise delineation of candidate target populations, facilitating clinical trial design and validation of predictive biomarkers.

Materials and Methods

Patients and tissues

From October 2011 to September 2013, we consecutively procured primary tumor samples and corresponding formalin-fixed, paraffin-embedded tumor blocks from SCCL patients who underwent pulmonary resection at the Department of Cardiothoracic Surgery, Xinhua Hospital, Shanghai Jiaotong University school of medicine. Subjects eligible for this study had to meet the following: confirmed SCCL diagnosis by hematoxylin-eosin (H&E) and IHC staining, each sample containing sufficient for comprehensive mutational analyses and with no neoadjuvant treatment. This research was approved by the Institutional Review Board of the Xinhua Hospital, Shanghai Jiaotong University School of medicine. Written informed consent from each patient was obtained. Clinical and pathologic data were prospectively collected for analyses including age at diagnosis, sex, smoking history, histologic type, pathologic TNM stage, tumor size, tumor differentiation. Patients were observed in clinic or by telephone for disease recurrence and survival from the date of diagnosis.

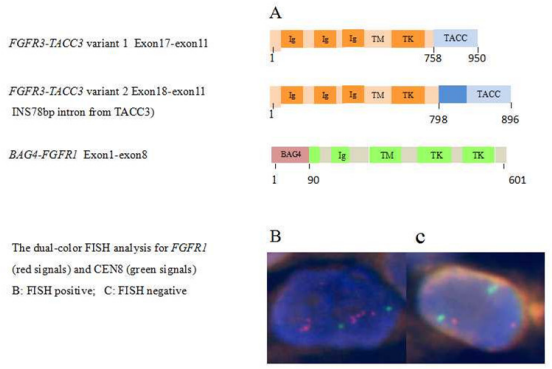

Figure 1. *FGFR fusion and FGFR1 amplification in SCCL. (A) FGFR fusion; (B) FGFR1 amplification positive; (C) FGFRQ fusion negative.*

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and FGFR fusions

Frozen tumor specimens were dissected and RNA/DNA was co-extracted following the standard instructions of RNA/DNA isolation Kit (TIANGEN BIOTECH, Beijing, China). Single-stranded RNA of each sample is reverse transcribed into complementary DNA (cDNA) by RevertAid First Strand cDNA Synthesis Kit (Fermentas, EU). For detection of *FGFR* fusions, primers were designed to cover all known fusion variants. Direct sequencing in both forwards and reverse directions was then performed in PCR amplification products. Details of RT-PCR are provided in the appendix.

Mutational analyses

EGFR (exons 18-21), *KRAS* (exons 2-3), *HER2* (exons 18-21), *BRAF* (exons 11-15) were amplified with KOD Plus Neo DNA polymerase (Toyobo, Osaka, Japan). All mutated cases were confirmed twice with independent PCR reactions.

Assessment of FGFR1 gene amplification

Fluorescent in situ hybridization (FISH) assay for *FGFR1* was performed by using *FGFR1* probe that hybridizes to the band 8p12-8p11.23 with Spectrum Orange (red) CEP8 with Spectrum Green (Abbott Molecular, Abbott Park, IL) following routine methods. FISH analysis was reinterpreted by two experienced evaluators blinded to the clinical data. At least 100 nuclei per patient were evaluated. Tissue samples with a *FGFR1*/CEP8 ratio of 1.0 were classified as normal and those with a *FGFR1*/CEP8 ratio between 1.0 and 2.0 were classified as having *FGFR1* gains. A *FGFR1*/CEN8 ratio of more than 4.0 was considered amplified. A minimum of 50 cells with both centromeric and *FGFR1* gene signals were scored to give conclusive data.

Immunohistochemistry analysis

To evaluate immunohistochemistry (IHC) analysis for screening and detecting SCCL with *FGFR* fusion genes, all of the included samples were subjected to IHC analysis with formalin-fixed paraffin-embedded slides sectioned. Details of IHC method is provided in the Appendix.

Results

FGFR1 amplification of SCCL by fluorescence in situ hybridization

A total of 108 cases met eligibility for this study. The characteristics for these patients are listed in Appendix Table A1. We analyzed the enrolled cases with gene copy number equal or above 4, a threshold that has been previously used to define *FGFR1* amplifications [12]. Among the 108 patients, *FGFR1* amplification was found in 13.0/% (14/108) of the cases (Figure 1). Details of the 14 cases with *FGFR1* amplification are listed in Table 1. The frequency of *FGFR1* amplification was significantly higher in current or ever

smokers than in never-smokers (100% *vs.* 0%; p=0.036). Patients with *FGFR1* amplification were also significantly associated with larger tumor (>3 cm) (92.9%, 13 of 14 patients, p=0.032). However, There was no significant association between increased *FGFR1* gene copy number (≥ 4) and other clinicopathological parameter (sex, p=0.593; age, p=0.577; stage, p=1.000; tumor differentiation, p=1.000) (Table 2).

Table 1. *Clinicopathologic Details of 18 SCCL Patients with FGFR1 amplification or FGFR3 Fusions.*

Patient No.	Age (years)	Sex	Smoking (pack-years)	Stage	Tumor Size (cm)	Lymph Node Status	Differentiation	*FGFR1* FISH	*FGFR*3 fusion
1	58	M	100	IIa	7.0	0	Moderate	amp	N
2	51	M	30	IIa	4.5	N1	Moderate	amp	N
3	57	M	100	IIIa	4.0	N2	Poor	amp	N
4	75	M	60	Ib	1.4	0	Poor	amp	N
5	64	M	80	IIb	9.0	0	Poor	amp	N
6	72	M	35	IIIa	5.2	N2	Poor	amp	N
7	64	M	120	IIa	5.5	0	Moderate	amp	N
8	66	M	40	Ib	4.0	0	Moderate	amp	N
9	64	M	40	IIa	4.0	N1	Poor	amp	N
10	45	M	38	IIIa	4.8	N2	Poor	amp	N
11	58	M	30	IIa	5.0	N1	Poor	amp	N
12	62	M	15	Ib	5.0	0	Moderate	amp	N
13	63	M	50	IIb	10.0	0	Poor	amp	N
14	61	M	160	Ib	3.5	0	Moderate	amp	N
15	49	M	20	IIIa	4.0	N2	Moderate	N	*FGFR*3-TACC3$^{\Delta}$
16	65	M	40	Ib	5.0	0	Poor	N	*FGFR*3-TACC3*
17	61	M	40	IIb	5.5	0	Moderate	N	BAG4--*FGFR1*
18	48	M	105	Ib	4	0	Poor	N	BAG4--*FGFR1*

SCCL: Squamous Cell Carcinoma Of Lung; *FGFR*3-TACC3*, *FGFR*3-TACC3 (F18:E11 INS78bp intron from TACC3); *FGFR*3-TACC3$^{\Delta}$, *FGFR*3-TACC3 (E17:E11); BAG4--*FGFR1*, BAG4--*FGFR1* (E1:E8)

Frequency and clinicopathologic characteristics of FGFR fusion in SCCL

All tumors were examined by RT-PCR and direct sequencing with primer sets covered 15 known *FGFR* fusion variants. Four *FGFR* fusions in 108 SCCL were identified. Of 108 squamous cell carcinomas, 4 (3.7%) harbored *FGFR* fusions which included two BAG4-*FGFR1* fusions and two *FGFR*3-TACC3 fusions. Details of the 4 *FGFR* fusion positive SCCLs are listed in Table 1. The histology of 4 tumors with *FGFR* fusions were confirmed by IHC bio-markers (p40 and TTF-1). We found that all of 4 *FGFR* fusion positive patients were current or ever smokers with lager tumor (>3 cm) while the frequency of was considerably higher than those in never smokers with tumors which were less than 3cm. This difference, however, failed to reach statistical significance (p=0.573 in smoking status and p=0.297 in tumor size). Similarly, there was no significant association between *FGFR* fusion and clinicopathological parameter (sex, p=1.000; age, p=1.000; stage, p=1.000; tumor differentiation, p=1.000) (Appendix Table A2).

Mutation analysis and IHC analysis

To explore the relationship of *FGFR1* amplification/*FGFR* fusion with other oncogene mutations in SCCL, testing for *EGFR*, *KRAS*, *HER2* and *BRAF* gene mutation was also arranged. Twenty-six patients were found to harbor *EGFR*, *HER2*, *KRAS* or *BRAF* mutation, including 4.6% (5/108) with an *EGFR* mutation, 1.9% (2/108) with a *KRAS* mutation and 0.9% (1/108) with an *HER2* mutation. *BRAF* mutation was not found in our study (Figure 2). All of the *FGFR1* amplification, *FGFR* fusion and oncogene mutation that we tested were excluded. Neither *FGFR1* amplification nor *FGFR* fusion was found to be correlated with the oncogene mutations above (Appendix Tables A3 and A4).

Furthermore, we detected the relationship between *FGFR1* amplification and *FGFR* fusion by IHC. Nevertheless, no significant correlation was found, either. (Appendix Table A5).

Table 2. *Clinicopathologic characteristics of FGFR1 amplification in SCCL.*

Characteristic	*FGFR1* Amp (+)	*FGFR1* Amp (-)	P
	No.of patients (%)	No.of patients (%)	
Age, years			
≤ 60	5 (35.7)	42 (44.7)	0.577
>60	9 (64.3)	52 (55.3)	
Sex			
Male	14 (100.0)	86 (91.5)	0.593
Female	0 (0.0)	8 (8.5)	
Smoking Status			
N	0 (0.0)	24 (25.5)	0.036
C/E	14 (100.0)	70 (74.5)	
Tumor size			
≤ 3 cm	1 (7.1)	36 (38.3)	0.032
>3 cm	13 (92.9)	58 (61.7)	
Stage			
I~II	11 (78.6)	71 (75.6)	1
III~IV	3 (21.4)	23 (24.5)	
Differentiation			
Well-moderate	6 (42.9)	40 (42.6)	1
Poor	8 (57.1)	54 (57.4)	

FGFR1 Amp: *FGFR1* Amplification; N: Never Smoker; C/E: Current/Ever Smoker

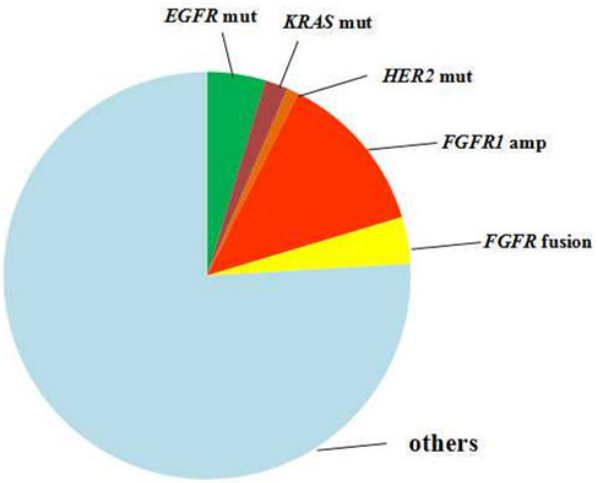

Figure 2. *FGFR1 amplification, FGFR fusion and other gene mutations in SCCL.*

Clinical outcome

The overall survival (OS) for all of the patients was 25.0 months for a median follow-up time of 25.2 months. As expected, median OS was longer in patients with stage I disease than in those with stage II to III disease (39.0 months *vs.* 19.5 months; p=0.011). A similar trend was also found in OS between SCCL patients without or with lymph node metastasis (26.2 months *vs.* 19.0 months; p=0.056). We did not observe significant differences in OS according to other clinicopathological parameters such as age, sex, smoking status or tumor tumor grade (p=0.261 for age, p=0.553 for sex, p=0.553 for sex, p=0.322 for smoking status and p=0.937 for tumor grade). We further detect the prognostic value of *FGFR1* gene amplification and *FGFR* fusion in the same panel of 108 cases. Survival for patients with *FGFR1* amplification was shorter than patients without amplification (25.0 months *vs.* 41.0 months; p=0.013) (Figure 3A). However, no significant difference in OS was found between patients with or without *FGFR* fusion (27.8 months *vs.* 25.0 months; p=0.749) (Figure 3C).

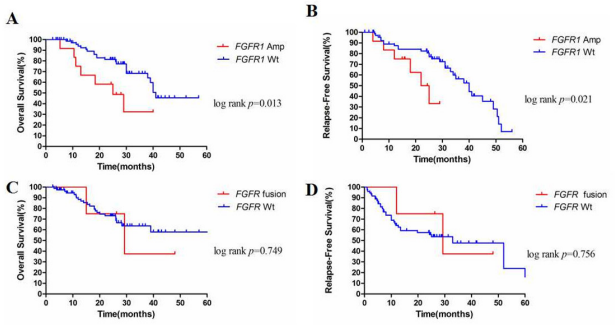

Figure 3. *Prognostic value of FGFR1 amplification and FGFR fusion in SCCL.*

The median recurrence-free survival (RFS) for the cohort was 15.8 months. Like OS, median RFS was longer in patients with stage I disease than in those with stage II to III disease (52.0 months *vs.* 12.7 months; p=0.029). A significant difference in RFS was also found between patients with or without lymph node metastasis (50.0 months for patients without lymph node metastasis *vs.* 11.7 months for patients with lymph node metastasis, p=0.040). Similar to OS, *FGFR1* amplified cases harbored worse RFS than those *FGFR1* wild-type patients (23.5 months *vs.* 40.0 months; p=0.021) (Figure 3B). Nevertheless, the difference in RFS between patients with or without *FGFR* fusion was not significant (29.3 months for patients with *FGFR* fusion *vs.* 33.0 months for patients without *FGFR* fusion, p=0.756) (Figure 3D).

Discussion

FGFR1 amplification and *FGFR* fusion seem to be of key importance in tumor progression and were illustrated to be new drivers for a range of cancers [16,17]. It was reported that *FGFR1* amplification was found in about 10% of breast cancer and located on chromosome 8p12 [18]. Other tumors, including bladder cancer, ovarian carcinoma and

rhabdomyosarcoma, were also tested and *FGFR1* amplification were found with lower frequency at the same region [19]. Recently, it is shown that gene rearrangement also contributes to the developments of solid tumors and the *FGFR* fusion gene was illustrated to play important roles in divers cancers [13]. The oncogenic potential of *FGFR1*-TACC1, *FGFR*2-BICC1 and *FGFR*3-TACC3 were confirmed by expression of the fusion kinases in NIH3T3 fibroblasts, Rat1A fibroblasts or astrocytes and conferred in vivo tumorigenesis of subcutaneous transplanted cells in immune-deficient mice [18,20].

In our study, we investigated whether *FGFR1* amplification and *FGFR* fusion were associated with the clinicopathologic parameters and their impact on survival in patients with operable SCCL. To the best of our knowledge, this may be the first report of multiple analysis on *FGFR1* amplification, *FGFR* fusion and oncogene mutations in a cohort of East Asians with SCCL, and we illustrated two important subtypes, showing negative prognostic impact of *FGFR1* amplification and demonstrating *FGFR* fusion as a unique subtype in resectable SCCL.

We demonstrated that patients with *FGFR1* amplification had identifiable clinicopathologic characteristics, including smokers status and relative larger tumor (>3 cm). In principle, our analysis showed similar results as shown by Weiss et al. They found that increased copy number status of *FGFR1* (≥ 4 signals) in lung SCC correlated significantly with the gender, smoking status and pathological subtypes but not with age, grade and lymph node status [12]. Interestingly, tumor size might be firstly recognized to be related with *FGFR1* amplification in our research. However, Lukas et al. reported no significant associations between *FGFR1* amplification and clinicopathological parameters [17]. The lack of standardized criteria for defining *FGFR1* amplification and the different number of cases may be the possible reason [12,17].

Another interesting founding in our research is that patients with *FGFR1* amplification had significantly shorter RFS and OS than those without *FGFR1* amplification. This result suggests that targeting the *FGFR1* pathway might improve survival in patients with *FGFR1* amplification who have resectable SCCL. Nowadays, several potent *FGFR* tyrosine kinase inhibitors including BGJ398 and AZD4547 have been in early clinical research [21,22]. However, there are more gene alterations that were found to be correlated with prognosis in adenocarcinoma than in SCCL. More comprehensive analysis will be needed to explore how to precisely predict the prognosis of patients with SCCL.

In the current study, we detected the *FGFR1*, *FGFR*2, *FGFR*3 gene fusions in SCCL patients and found the frequency of *FGFR* fusions was 3.7%, which is similar to previous studies [9]. *FGFR* fusions and *FGFR1* amplification were mutually exclusive with activating mutations in *EGFR*, *KRAS*, *BRAF* and *HER2*, showing the driver's role of those gene alteration in cancer development. Although a variety of fusion partners of *FGFR* were reported by recent studies [23,24], only one partner each for the *FGFR1* and *FGFR*3 fusions (BAG4-*FGFR1* and *FGFR*3-TACC3) were found. *FGFR*2 fusions were not identified in our study, indicating that *FGFR*3 or *FGFR1*, other than *FGFR*2 might be crucial for oncogenesis in SCCL. Unlike the previous study, we failed to find the correlation between *FGFR* gene fusion and clinicopathologic characteristics. This may be duo to different pathology subtypes of the cohort (the precious study showed the correlation in NSCLC, but we detected that in SCCL). The slightly lower number of cases that were analysed in our study may be another resean.

The methods for detection of gene fusions include RT-PCR, IHC, and FISH. Nevertheless, FISH assay was not available because the interval of two genes (*FGFR*3-TACC3 or BAG4-*FGFR1*) was too small to detect rearrangement. In current study, 14 known fusion variants of *FGFR* found in glioblastoma, cholangiocarcinoma, bladder cancer, thyroid cancer, oral cancer, breast cancer or head and neck squamous cell cancer by previous study were tested in 108 SCCLs [14]. Since RT-PCR could not be used to detect unknown fusion variants, it is possible that a few *FGFR* rearranged samples may have been missed. Therefore, more studies will be warranted to validate our findings in an independent cohort, especially in an Eastern population.

In conclusion, our data demonstrated that *FGFR1* amplification and *FGFR* fusion occurred in 13.0% and 3.7% of patients with lung squamous cell carcinoma, separately. *FGFR1* amplification was correlated with poor prognosis and identified a distinct subset of SCCL with a higher prevalence among smokers with relative larger tumor (>3 cm). Knowledge of these clinical characteristics will help clinicians select those patients most likely to harbor this genetic alteration and most likely to benefit from *FGFR* targeted therapy.

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