ORIGINAL RESEARCH

EGFR with TKI-sensitive mutations in exon 19 is highly expressed and frequently detected in Chinese patients with lung squamous carcinoma

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Abstract: Recently, tyrosine kinase inhibitors (TKIs) have been recommended as a first-line treatment for advanced non-small cell lung cancer (NSCLC), significantly improving the treatment outcomes of lung adenocarcinoma patients with the EGFR mutation. However, the application of TKIs for lung squamous cell carcinoma (SCC), the second largest pathological subtype of NSCLC, remains controversial because available data for the EGFR mutation profile and frequency in SCC patients are limited. In this study, 89 bronchoscopic–biopsy specimens from Chinese SCC male patients were assayed for EGFR exon 19 mutation, using improved polymerase chain reaction-denature gel gradient electrophoresis. EGFR exon 19 mutations were detected in 77 of 89 (86.5%) patients, and included six kinds of point mutations (11.6%) and two deletions (Del_747-751 [64.9%] and Del_746-751 [23.3%]). We found that the proportion of mutated EGFR varied from 0.98% to 100% in positive specimens and increased with the development of the disease. The difference of proportion between Stage IV patients and Stage II patients or Stage III patients was significant ($P<0.001$). These results provided valuable clues to explain the reason why patients harboring the same mutation responded distinctly to TKI treatment. Del_747-751 and Del_746-751 were the dominant mutations in the assayed SCC patients (76.4%), and both belong to the EGFR–TKI-sensitive mutation. Recently research demonstrated that Del_746-751 patients have better response to EGFR-TKI than Del_L747-751 patients. However, our study indicated that majority of SCC patients (55.5%) carried Del_L747-751. We suggest that the unique clinic features of SCC should be further studied to reveal the mechanism of poorer treatment outcome of EGFR–TKI therapy, and that a better treatment plan and more specific, potent targeted drugs for lung SCC need to be developed.

Keywords: lung squamous carcinoma, EGFR exon 19, mutation profile, PCR-DGGE, tyrosine kinase inhibitors

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Introduction

Lung cancer is a malignancy associated with high mortality, and the situation is especially serious in China compared to the world. In 2015, 733,300 new lung cancer cases were reported and 610,200 lung cancer-related deaths occurred in China.[1] The World Health Organization (WHO) predicts that annual number of deaths from lung cancer in China is estimated to reach 1 million by 2025.[2] Approximately 70%–80% of lung cancer is non-small cell lung cancer (NSCLC), and squamous cell carcinoma (SCC) is the second largest subgroup of NSCLC.[3–5] Tyrosine kinase inhibitors (TKIs) have been recommended as a first-line treatment option for advanced adenocarcinoma patients, and have significantly improved the treatment outcomes for patients.[6] EGFR–TKIs combined with chemotherapy were approved as a second-line treatment option in USA

and Europe for advanced squamous carcinoma patients.[6] Currently, SCC patients still have limited treatment options in addition to chemotherapy,[7] thus resulting in a poorer treatment outcome as compared to adenocarcinoma patients.[7,8]

The EGFR signaling pathway is associated with several key cellular metabolic processes.[9–12] An aberrant EGFR pathway contributes crucially to tumor initiation and progress.[12] EGFR mutations can be identified in 80% of NSCLC patients,[13] especially in the Asian race, never smokers, women, and patients with adenocarcinoma,[10] therefore adenocarcinoma patients with EGFR mutation can benefit from TKI therapy.[12] The mutation frequency of EGFR in SCC was reported to be only ~1%–15%,[14] and EGFR mutation analysis and TKI treatment are not routinely recommended for SCC patients.[14,15] For example, the European Society for Medical Oncology (ESMO) recommended the EGFR mutation test for nonsquamous carcinoma patients.[16] However, these recommendations were based on limited data on the EGFR mutation profile and frequency of SCC, especially in Chinese and other Asian populations.[7,15] When we searched the PubMed database using keywords "EGFR" and "lung squamous carcinoma," few results were returned.

Recently, several studies demonstrated that the SCC patients could benefit from TKI therapy,[17,18] although the treatment outcome, the progression-free survival (PFS), and overall survival (OS) were not as good as those of the adenocarcinoma patients.[18] Furthermore, the discrepancy among different studies and/or patients was significant. For example, Xu et al[19] reported 26 Chinese SCC EGFR mutation-positive patients who were treated with TKI, and the PFS was 3.98 months (95% CI, 3.22–4.63 months). In another study,[20] 53 Japanese SCC patients were treated with TKI, and the median PFS of EGFR-positive patients (n=20) was 1.4 months (95% CI, 0.7–5.8 months), while the PFS of EGFR-negative patients (n=33) was 1.8 months (95% CI, 1.0–2.4 months). Though no statistical significance in median PSF was detected between EGFR-positive and -negative groups (P=0.1734), the range of PFS of EGFR-positive (8.3-fold) patients is much wider than that of EGFR-negative patients (2.4-fold),[20] which suggested that greater interindividual difference existed in EGFR-positive patients.

The EGFR mutational profile was obtained by direct sequencing,[21] high-throughput sequencing,[22,23] and some polymerase chain reaction (PCR)-based methods,[20] which will ultimately determine whether or not the TKI[12] treatment can be applied to specific patients. However, the abovementioned methods have some ramifications in terms of sensitivity, cost, and time[24] and, as a result, no method is highly recommended for regular clinical diagnosis of lung cancer.[25–28] Most studies detected the EGFR mutation using genomic DNA extracted from paraffin specimens.[24] EGFR was overexpressed in advanced-stage NSCLC patients at both protein and mRNA levels,[29] but the ratio of mutated EGFR to wild type expressed in SCC still needs to be determined.

This study aimed to investigate the mutation status of EGFR exon 19 by PCR-denature gel gradient electrophoresis (DGGE) to provide information for understanding the mechanism of poorer treatment outcome of EGFR–TKI therapy for SCC patients by providing a clear view of the mutation profile and frequency in Chinese SCC patients.

Materials and methods

In this study, we assayed EGFR mutations in Chinese patients with lung squamous carcinoma using PCR-DGGE and direct sequencing of PCR products.

Sample collection

Specimens were collected from patients who underwent bronchoscopic biopsy for lung cancer in Shanghai Pulmonary Hospital from 2013 to 2015, with the approval of the Ethics committee of Tongji University. All patients involved in this study provided written informed consent for the use of their tissue samples in this study. The specimens that were confirmed to be SCC by pathological examination were used for the following experiments. Detailed clinical information is shown in Table 1. All specimens were immediately preserved in the RNA store reagent (Tiangen Biotech, Beijing, China) at 4°C until total RNA extraction.

Chemical and reagents

The chemicals used in this study were purchased from Sigma-Aldrich (St Louis, MO, USA).

Table 1 Clinicopathological features of patients with lung squamous cell carcinoma

Variables (n)	Number of patients (%)
Median age (min–max)	64 (41–81)
Cigarette smoking	
Smoker	45 (50.5)
Nonsmoker	35 (39.3)
No data available	9 (10.1)
TNM stage	
I	4 (4.4)
II	37 (41.5)
III	22 (24.7)
IV	26 (29.2)
Total	89

Abbreviation: TNM, tumor node metastasis.

Total RNA extraction and reverse transcription

Total RNA Extraction Kit (Tiangen Biotech) was used to extract the total RNA. Approximately 1,000 ng of total RNA was reverse transcribed in 20 µL volume using FastQuant cDNA first chain synthesis Kit (Tiangen Biotech) and the manufacturer's instructions were followed throughout the operation. cDNA were stored at −20°C for future experiments.

PCR and DGGE

PCR and DGGE were performed according to the methods reported by Shahi et al,[24] with two modifications, ie, 20 cycles for first-round PCR, and use of cDNA as the template for PCR instead of gDNA. Universe High-Fidelity Hot Start DNA Polymerase (Biotool, Houston, TX, USA) was used as the PCR enzyme. DGGE analysis was performed on the Universal Mutation detection system (D-Code™; Bio-Rad, Hercules, CA, USA). A 30%–60% denaturation gradient gel was used, and samples were allowed to run for 16 hours at 120 V at 58°C. The gels were stained with GelRed (GelRed™; Biotium, Fremont, CA, USA) and images were documented using Gel Documentary XR+ System (Gel Doc™ XR; Bio-Rad) and quantified using Gel-pro Analyzer 4.0 (Media Cybernetics, Rockville, MD, USA). The samples were analyzed in triplicate and always with positive and negative controls (Figure 1).
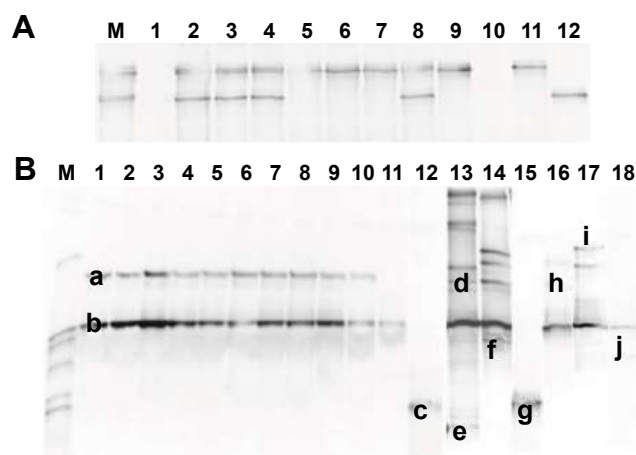

Figure 1 DGGE gel images of mutation analysis of EGFR exon 19.
Notes: (**A**) Result of DGGE system verification. M: EGFR exon 19 mutation detection marker. 1: No template; 2–4: specimen 203 that harbored deletion 1 mutation (positive sample [PS]); 5–7: specimen 231, no mutation in exon 19 (negative sample [NS]); 8: specimen 203 that harbored deletion 1 mutation (positive sample [PS]); 9: specimen 231, no mutation in exon 19 (negative sample [NS]); 10: no template; 11: negative control (using wild type exon 19 fragment cloned in plasmid as PCR template); 12: positive control of Del-1 (using exon 19 harboring Del-1 mutation cloned in plasmid as PCR template). (**B**) Detection of EGFR exon 19 mutation in squamous cell carcinoma specimens by DGGE. M: marker; 1: 203; 2: 23; 3: 89; 4: 177; 5: 25; 6: 244; 7: 38; 8: 71; 9: 5; 10: 202; 11: 468; 12: 10; 13: 466; 14: 476; 15: 473; 16: 481; 17: 482; 18: 483. a: deletion 1; b: wild type; c: deletion 2; d: M2; e: M4; f: M5; g: M1; h: M2; i: M3; j: M6.
Abbreviations: DGGE, denature gel gradient electrophoresis; PCR, polymerase chain reaction.

Sequence analysis

After DGGE, each candidate band was cut from the gel, crushed, and soaked in 30 µL Tris–EDTA buffer overnight at 4°C, individually, and then the buffer was used as a template for cloning PCR. PCR primers were same as PCR-DGGE without the GC clamp. PCR products were cloned using Lethal Based Fast Cloning Kit (Tiangen Biotech) and sequenced by Sunny Biotechnology Co. (Shanghai, China). Sequencing results were analyzed by BLAST.

Statistical analysis

Statistical analysis was performed with SPSS 17.0 or GraphPad Prism 5.0. Two groups of data were analyzed using unpaired t-test to determine the significance. For three or more groups of data, one-way analysis of variance was adopted. Results were presented as mean ± SD. $P<0.05$ was considered statistically significant.

Results

Characteristics of patients involved in this study

Since no sufficient specimens from female SCC patients have accumulated due to the low incidence of SCC in the Chinese female population, only the specimens from male patients were analyzed in this study. The detailed characteristics such as patients' age, smoking history, and tumor node metastasis stage are shown in Table 1.

Mutation profile of EGFR exon 19

In 89 SCC specimens assayed, 41 patients (46%) were in early stage (I–II) and 48 were in advanced stage (III–IV) (54%). Two kinds of deletions (c.2240-2254 and c.2237-2251) and six point mutations were identified in EGFR exon 19 regions. After being verified by the database of catalogue of somatic mutations in cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic) (Table 2), five point mutations were identified for the first time in all kinds of cancer (Table 2). In early-stage patients (I–II), seven different kinds of mutations were identified, whereas only four kinds of mutations were found in advanced-stage patients (III–IV) (Tables 2 and 3). Fifty of 89 patients (56.2%) harbored the c.2240-2254 deletion (Table 2) and 18 patients (20.2%) harbored the c.2237-2251 deletion (Table 2), which accounted for 76.4% of the total number of SCC patients studied; and both deletions are EGFR–TKI-sensitive.[30] Nine out of 89 patients (11.6%) were found to have point mutations; three of these patients were identified to have either a double mutation or a combination of point mutations. Twelve patients (15.5%) carried no mutation in exon 19 (Table 3).

Table 2 EGFR exon 19 mutation profile of SCC patients

Mutation type	Nomenclature	Nucleotide changes	Amino acid changes	Number of patients (%)	References
Deletion	Del-1	c.2240-2254_del	Leu747-Thr751_del	50 (64.9)	24 COSP4242*
	Del-2	c.2237-2251_del	Glu746-Thr751_del	18 (23.3)	18
Point mutation	M1	c.2225T>C, c.2230A>G, 2237A>G, 2238A>G*	val742Ala, Ile744Val, Glu746Gly, Glu746Gly*	1 (1)	This study
	M2	c.2274A>G*	Glu758Glu*	4 (4.4)	This study
	M3	c.2210A>G,* c.2274A>G	Lys737Arg, Glu758Glu	1 (1)	This study
	M4	c.2212G>T* c.2225T>C, c.2230A>G, c.2237A>G, c.2238A>G c.2274A>G	Val737Phe,* val742Ala, Ile744Val, Glu746Gly, Glu746Gly* Glu758Glu	1 (1)	This study
	M5	c.2238A>G,* c.2246A>T* c.2274A>G	Glu746Gly,* Glu749Val, Glu758Glu	1 (1)	This study
	M6	c.2234A>C, c.2280C>T	Lys747Thr, Leu760leu	1 (1)	SCV000062071.2*

Note: *Accession number of catalogue of somatic mutations in cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic).
Abbreviation: SCC, squamous cell carcinoma.

In Stage I, 50% of patients harbored deletion 1 (n=4). In Stage II patients, 48.6% of patients had deletion 1 (n=18), and 24.3% had deletion 2 (n=9). For Stage III patients, 68.1% of patients (n=15) carried deletion 1 and 4.5% of patients (n=1) had deletion 2. For Stage IV patients, 57.6% (n=15) of patients harbored deletion 1 and 30.7% carried deletion 2 (n=8) (Table 3).

Point mutations in EGFR exon 19 were identified in 25% of Stage I patients (n=4), 13.5% of Stage II patients (n=37), and 9.1% of Stage III patients (n=22). Two kinds of point mutations were identified in three patients, and no point mutation was detected in any of the Stage IV patients assayed (n=26) (Table 3; Figure 1B).

Among the 89 patients involved in this study, there were 45 smokers, 35 non-smokers, and the remaining 9 patients did not have smoking information. For smoking patients, their

Table 3 Distribution of eight kinds of mutation in different SCC stages

SCC stage	Number of patients	Del-1	Del-2	M1	M2	M3	M4	M5	M6
I	4	2	0	0	1	0	0	0	0
II	37	18	9	1	1	1	1	0	1
III	22	15	1	0	2	0	0	1	0
IV	26	15	8	0	0	0	0	0	0
Total	89	50	18	1	4	1	1	1	1

Abbreviation: SCC, squamous cell carcinoma.

average smoking history was 34.4±10.7 years and the average daily consumption was 30.7±13.8 cigarettes/day (Table 4). Compared with smoking patients (42 patients [93.3%]), nonsmoking patients had a much lower incidence of the exon 19 mutation (27 patients [77.1%]). Furthermore, 7 (15.5%) smoking patients and only 2 (5.7%) nonsmoking patients were identified as harboring point mutations. But no significant difference was observed in mutation frequency and mutation type between smoking and nonsmoking patients (Table 4).

Quantification of DGGE bands

Each patient's wild type and mutated EGFR band in DGGE gel was quantified, and the proportion of mutated EGFR was 12.67%±10.60% in Stage I specimens, 19.00%±17.89% in Stage II specimens, 16.63%±17.13% in Stage III specimens, and 37.49%±21.1% in Stage IV specimens. The significant difference between Stage IV and Stage II (P=0.001) or Stage III (P=0.001) was identified (Figure 2).

Discussion

In this study, we detected two kinds of deletion and six point mutations in EGFR exon 19 from 89 Chinese male SCC patients. Out of the six point mutations identified, five mutations (M1–M5) are reported, for the first time, to be associated with cancer. In this study, 85% SCC

Table 4 Comparison of EGFR exon 19 mutational profile between smoking and nonsmoking patients

Smoke history in years	Number of patients	Number of patients without mutation	Del-1	Del-2	M1	M2	M3	M4	M5	M6
<25	7	0	6 (85.7%)	1 (14.2%)	0	0	0	0	0	0
~30	19	0	9 (47.3%)	7 (36.8%)	0	1 (5.2%)	1 (5.2%)	0	0	1 (5.2%)
~40	11	3 (27.2%)	4 (36.3%)	1 (9%)	0	2 (18.1%)	0	0	1 (9%)	0
>50	8	1 (12.5%)	6 (75%)	0	0	1 (12.5%)	0	0	0	0
Nonsmoker	35	8 (22.8%)	19 (54.2%)	6 (17.1%)	1 (2.8%)	0	0	1 (2.8%)	0	0
No data available	9	0	6 (66.6%)	3 (33.3%)	0	0	0	0	0	0

patients were harboring the EGFR mutations; this figure is much higher than that reported previously in other studies (15%–45%).[15,31,32] The significant difference may be due to the different methods adopted. In most previous studies, genomic DNA extracted from paraffin-embedded tumor specimens was used to detect mutations.[21] The lower concentration of genomic DNA analyzed leads to poor sensitivity and inaccuracy of mutation detection.[33]

In this study, we used cDNA as the PCR template which was extracted and reverse transcribed from fresh specimens (0.050–0.100 g). The higher concentration of template improved the sensitivity of our system. Additionally, PCR-DGGE is more sensitive (~1%[24]) than direct sequencing (20%[15]) and more cost-effective than the high-throughput sequencing method.[4] Shahi et al[24] used the same PCR-DGGE method and detected 10 different kinds of EGFR exon 19 mutations in 55 out of 210 (16.2%) Caucasian NSCLC patients. Bircan et al[32] reported that 45.5% of Turkish male SCC patients (n=11) carried the EGFR exon 19 mutation. These results demonstrate that the EGFR exon 19 mutation rate is significantly different among the ethnic groups.
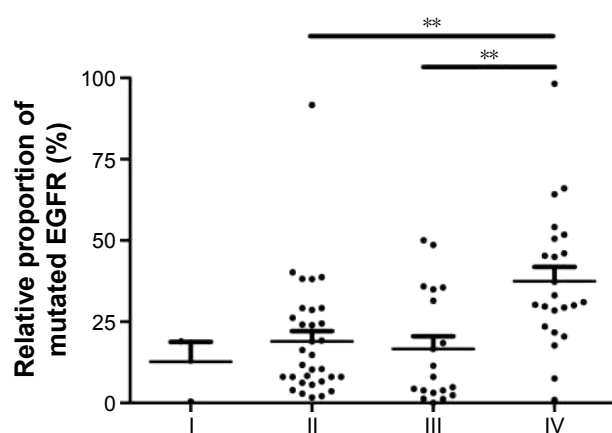
Figure 2 Comparison of the proportion of mutated EGFR between different SCC stage patients.
Note: **$P < 0.01$.
Abbreviation: SCC, squamous cell carcinoma.

The EGFR mutation detection and profile are crucial for the determination of the lung cancer treatment strategies. The EGFR mutational profile of NSCLC, especially SCC, is not well studied in the Chinese population.[34]

In this study, among the EGFR mutation-positive patients, 88.3% of patients (68 of 77) carried the EGFR–TKI-sensitive mutation – deletion 1 (64.9%) and deletion 2 (23.3%). It is an interesting finding because adenocarcinoma patients harboring this mutation can significantly benefit from the EGFR–TKI treatment,[30,35] and several studies have demonstrated that the SCC patients could benefit from TKI treatment.[17,18]

Kaneda et al[36] reported that the Del-E746 patients have a better response to EGFR–TKI compared to Del-L747 patients. In 89 Chinese SCC patients assayed in this study, the frequency of the 746-751-deletion and 747-751-deletion was 20.2% and 56.2%, respectively. In 11 Turkish SCC patients, three deletions occurred in the 731-744 regions (27.3%) and two occurred in the 750-760 regions (18.2%).[37] These results suggest that more specific EGFR–TKI should be developed for Chinese SCC patients.

In 37 Stage II patients, five kinds of point mutations were detected in 13.5% patients, one kind of point mutation was detected in 22 Stage III patients (13.6%), but no point mutation was identified in 26 Stage IV patients. In other words, in patients harboring the EGFR exon 19 mutation, the frequency of deletion increased from 72.9% (Stage II), 72.7% (Stage III) to 88.4% (Stage IV). The deletion mutation became increasingly dominant with the development of the disease, which agreed with the finding in Caucasian (100%)[24] and Turkish SCC patients (100%).[32]

In this study, one point mutation c.2274A>G was identified in 5 out of 9 patients harboring the point mutation. Though this is a synonymous mutation (Glu (GAA)758Glu (GAG)), GAG (4.39) appears more frequently in high-expressing genes than GAA (2.95).[38] Synonymous mutation can play an important role in cancer,[39] ie, KRas,[40,41] by affecting the gene expression,[39,42] especially in oncogenes. The

role of new identified point mutation c.2274A>G warrants further study.

Another interesting finding in this study was that the mutated EGFR mRNA in total EGFR mRNA varied from 0.98% to 100%, which possibly explained the discrepancy of EGFR–TKI treatment outcome among EGFR-positive SCC patients.[14] In the present study, the proportions of mutated EGFR in total were significantly higher in Stage IV (37.49%) than in Stage II (19.00%) (P=0.001) and Stage III (16.63%) (P=0.001) (Figure 2). Selvaggi et al[29] reported that EGFR protein was highly expressed in Stage III (50%) compared to Stage I (20%) and Stage II (25%) in NSCLC patients. Our results further suggest that mutated EGFR protein may be the main contributor to the higher expression of EGFR in advanced-stage SCC or NSCLC, and this issue deserves further attention.

Based on the above-mentioned findings, the EGFR–TKI treatment for Chinese SCC patients and the best time point of the treatment should be reconsidered and studied.

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Disclosure

The authors report no conflicts of interest in this work.

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