Lung adenocarcinoma resistance to therapy with EGFR-tyrosine kinase inhibitors is related to increased expression of cancer stem cell markers *SOX2*, *OCT4* and *NANOG*

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Abstract. The present study aimed to explore the relationship between the efficacy of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) and the expression of the cancer stem cell (CSC)-related markers. Specimens of 72 cases of lung adenocarcinoma with significantly different therapeutic effects of the first-generation EGFR-TKI treatment were collected. The patients were divided into a sensitive group [progression-free survival (PFS) longer than 26 months] and a resistant group (PFS less than 5 months) according to the efficacy of first-line EGFR-TKI treatment. The expression of CSC-related markers (*OCT4*, *SOX2*, *NANOG*) in tumor tissues of the two groups was detected by immunohistochemical (IHC) staining, immunofluorescence and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). IHC staining was quantified using H-scores. The unpaired nonparametric t-test was used to detect the differences in the results of IHC and RT-qPCR analyses between the groups. The Chi-square test was used to detect the differences in the clinical characteristics between the two groups. The t-test revealed that the IHC H-scores of *SOX2* (P=0.003), *OCT4* (P=0.036) and *NANOG* (P=0.032) were significantly higher in the resistant group than in the sensitive group. The results of RT-qPCR revealed that the relative levels of *SOX2* (P=0.018), *OCT4* (P=0.035) and *NANOG* (P=0.044) were significantly higher in the resistant group than in the sensitive group. The number of male patients, patients who smoked, patients with stage IV lung adenocarcinoma disease, and patients with poor differentiation was higher in the resistant group than in the sensitive group, with statistically significant differences. The poor efficacy of first-generation EGFR-TKIs for lung adenocarcinoma appears to be related to the increased expression of CSC markers.

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Abbreviations: EGFR, epidermal growth factor receptor; TKIs, tyrosine kinase inhibitors; IHC, immunohistochemistry; PFS, progression-free survival; RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction; NSCLC, non-small cell lung cancer; CSCs, cancer stem cells; TNM, tumor size, lymph node and distant metastasis; *SOX2*, sex-determining region Y-box 2; *OCT4*, octamer-binding transcription factor 4; *NANOG*, *NANOG* homeobox; ARMS, amplification refractory mutation system

Key words: cancer stem cells, epidermal growth factor receptor mutation, tyrosine-kinase inhibitors

Introduction

Lung cancer is one of the most malignant tumors and the leading cause of cancer-related deaths worldwide (1). Despite the development of anticancer therapies, such as chemotherapy, targeted therapies, and radiotherapy, the 5-year survival rates are still not optimistic. Primary and acquired resistance has been considered as a key factor in reducing the efficacy of current cytotoxic therapies in the treatment of non-small cell lung cancer (NSCLC). Studies have demonstrated the presence of a subset of cells called cancer stem cells (CSCs), which have stem-like properties, as observed in lung cancer (2).

Although epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are effective for lung adenocarcinoma patients with EGFR mutations, the patients invariably develop resistance to these drugs after a period of treatment (3). Recently, various mechanisms of resistance to EGFR-TKIs have been revealed, such as secondary mutations (T790M) (4), aberrated activation of the bypass pathways (c-Met, HGF, AXL) (5,6), and abnormal downstream pathways (K-RAS mutations, loss of PTEN, mutations of BRAF and PIK3CA mutation) (3,7). However, another possible mechanism is associated with targeting drug resistance, which involves CSCs. Several *in vitro* and *in vivo* experiments have revealed that CSCs are the source of drug resistance and therapeutic failure for lung cancer (2,8). The correlation between CSCs and

the efficacy of targeted drugs is still not clear. Stem-related transcription factors, such as *OCT4*, *SOX2* and *NANOG*, are considered as the critical regulators of self-renewal and pluripotency of stem cells, playing an important role in tumor proliferation and differentiation (9-11). Thus, the expression of CSC-related markers can reflect the number of CSCs in cancer tissues (12,13).

Hence, in the present study the analysis of the expression of cancer stem cell-related markers were investigated and two groups of patients with significant differences in clinical outcomes after treatment with first-generation EGFR-TKIs were retrospectively analyzed, aiming to explore the relationship between the efficacy of targeted drug therapy and CSCs and provide potential targets for the development of new therapeutic strategies.

Materials and methods

Patients and tissue samples. The pathological tissue samples of patients with lung cancer were collected from the tissue bank of the Shanghai Chest Hospital. The pathological tissues of the patients analyzed in this study were obtained before treatment with EGFR-TKI drugs. The present study was approved by the Ethics Committee of Shanghai Chest Hospital. All patients provided their informed consent.

In this retrospective study, patients with significantly different therapeutic effects after the treatment with first-generation EGFR-TKIs from January 2014 to December 2016 at Shanghai Chest Hospital were identified. The patients were divided into a sensitive group [progression-free survival (PFS) >26 months] and a resistant group (PFS <5 months) according to the efficacy of first-generation EGFR-TKI treatment. At present, the average PFS of first-generation targeted drugs is 10-13 months (14-16). Therefore, the cutoff value of sensitivity and resistance for targeted drugs should be set as two times the average upper line and one half the lower limit of the average. Other eligibility criteria included the following: EGFR-sensitive mutation (either exon 19 deletion, L858R in exon 21, or other sensitive mutations) and patients receiving first-generation EGFR-TKIs (including gefitinib, icotinib and erlotinib) as the first-line therapy. PFS was calculated from the date of initiation of EGFR-TKI therapy until the date of progression or last follow-up. The exclusion criteria were as follows: Non-first-line EGFR-TKI therapy, incomplete medical history information, loss to follow-up, history of other malignancies, and EGFR exon 20 insertion. In the present study, lung cancer staging was conducted on the baseline status of all patients enrolled according to the AJCC 8th edition TNM staging system. Tumor EGFR mutation status was determined by the amplification refractory mutation system (ARMS). The clinical characteristics, including age, sex, smoking status [a 'smoker' is defined as a patient who has smoked >100 cigarettes during his lifetime (17,18)], EGFR mutation status, TNM stage, tumor differentiation status and type of EGFR-TKIs, are summarized in Table I.

Immunohistochemical staining. The formalin-fixed, paraffin-embedded tissues were sectioned at a thickness of 4 μm using a slicer and baked in an oven at 65˚C for >2 h. The paraffin sections were deparaffinized using xylene and washed with gradient alcohol, and then distilled water. The sections were placed in the antigen repair solution, heated in a microwave oven, and cooled to room temperature for antigen repair. The endogenous peroxidase was removed with 3% hydrogen peroxide. Then, the goat serum was used for blocking to eliminate unspecific staining. The slides were incubated overnight at 4˚C with various primary antibodies. To visualize antigens, peroxidase-labeled antibodies (code K5007; REAL EnVision Rabbit/Mouse; Dako) were applied at room temperature for 60 min. The slices were rinsed in phosphate-buffered saline (PBS), stained with DAB (liquid DAB + substrate, DAKO), and re-stained with hematoxylin. Two independent observers examined the stained slides in a blind fashion.

Immunohistochemical (IHC) staining was evaluated using the histological score (H score) (19). The intensity of IHC staining was classified as strongly positive, moderately positive and weakly positive according to the color depth of the positive cells, and the corresponding values were 1, 2, and 3. The H score was calculated as follows: H Score = Staining intensities (SI) x percentage of positive cells= [1 x (% cells 1+) + 2 x (% cells 2+) + 3 x (% cells 3+)]. This score ranged from 0 to 300 (300 indicated strong staining in 100% of the tumor cells).

The antibodies used were as follows: Human anti-*SOX2* and anti-*OCT4* were purchased from Abclonal (cat. nos. A0561 and A7920, respectively). Human anti-*NANOG* was obtained from Abcam (product code ab109250). Human anti-*SOX2*, anti-*OCT4*, and anti-*NANOG* were used at a 1:100 dilution in PBS for IHC staining.

Immunofluorescence staining. The paraffin sections were stained with indirect immunofluorescence staining after dewaxing and antigen repair. They were dyed with indirect immunofluorescence dyeing of indirect method after deparaffinization and antigen repair. The histological sections were washed with PBS at pH 7.3. Then, polyclonal rabbit antibodies against *SOX2* (dilution of 1:100), *OCT4* (dilution of 1:100), and *NANOG* (dilution of 1:100) were added to each section. The sections were maintained overnight at 4˚C overnight. After washing three times with PBS, the sections were stained with donkey antibodies against rabbit immunoglobulin G (Jackson/Alexa Fluor® 488; cat. no 711-545-152; dilution of 1:100). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). As a control, the primary antibodies were replaced with PBS.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the paraffin-embedded tumor tissues using an FFPE RNA Kit (product code R6954-01; Omega Bio-tek, Inc.). RNA was reverse-transcribed using Hifair II 1st Strand cDNA Synthesis SuperMix for qPCR (product no. 11123ES60; Yeasen) according to the manufacturer's protocols. Approximately 1 μg of cDNA sample was used for each PCR analysis. RT-qPCR was performed with UNICON® qPCR SYBR-Green Master Mix (product no. 11199ES08; Yeasen) on an AB (Applied Biosystems; Thermo Fisher Scientific, Inc.) ViiA 7 instrument. The *GAPDH* housekeeping gene was used as a control. The thermocycling conditions of the RT-qPCR consisted of an initial denaturation step at 95˚C for 30 sec, followed by

Table I. Epidemiological, clinicopathological and mutation status data in sensitive and resistant patients treated with first-generation EGFR-TKIs.

Characteristics	No. of cases (%)	Sensitive group, n (%)	Resistant group, n (%)	P-value
Age (years)				
≤60	42	15 (48.4)	27 (65.9)	0.137
>60	30	16 (51.6)	14 (34.1)	
Sex				
Male	28	8 (25.8)	20 (48.8)	0.048
Female	44	23 (74.2)	21 (51.2)	
Smoking status				
Smoker[a]	23	4 (12.9)	19 (46.3)	0.003
Never-smoker	49	27 (87.1)	22 (53.7)	
EGFR mutation status				
19del	35	15 (48.4)	20 (48.8)	0.288
21L858R	34	16 (51.6)	18 (43.9)	
Others	3	0 (0.0)	3 (7.3)	
TNM stage				
IIB	1	1 (3.2)	0 (0.0)	0.003
IIIA/IIIB	25	17 (54.8)	8 (19.5)	
IV	46	13 (41.9)	33 (80.5)	
Differentiation				
Well	14	10 (32.3)	4 (9.8)	0.009
Moderate	26	13 (41.9)	13 (31.7)	
Poor	32	8 (25.8)	24 (58.5)	
Type of EGFR-TKIs				
Gefitinib	31	17 (54.8)	15 (36.6)	0.679
Icotinib	22	8 (25.8)	15 (36.6)	
Erlotinib	17	6 (19.4)	11 (26.8)	

[a]Smoker, is defined as a patient who has smoked >100 cigarettes during his lifetime. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

40 cycles at 95˚C for 10 sec and 60˚C for 34 sec. The dissolution curve stage is set by default by the instrument (1 cycle). The expression of tested genes relative to *GAPDH* was determined using the method of ΔΔCq (20). The expression levels of CSC-related markers in each sample were calculated. The primer sequences of each gene were as follows: PrimerBank ID: 325651854c1, *SOX2* forward, 5'-GCCGAGTGGAAACTTTTGTCG-3' and reverse, 5'-GGCAGCGTGTACTTATCCTTCT-3'; PrimerBank ID: 4505967a1, *OCT4* forward, 5'-CTTGAATCCCGAATGGAAAGGG-3' and reverse, 5'-GTGTATATCCCAGGGTGATCCTC-3'; PrimerBank ID: 153945815c1, *NANOG* forward, 5'-TTTGTGGGCCTGAAGAAAACT-3' and reverse, 5'-AGGGCTGTCCTGAATAAGCAG-3'; PrimerBank ID: 378404907c1, *GAPDH* forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Statistical analysis. The unpaired nonparametric t-test was used to detect the differences in the results of IHC and RT-qPCR analyses between the two groups. The results of t-tests were reported as the mean ± standard deviation. The chi-square test was used to detect the differences in the distribution of clinicopathological factors between the two groups. The correlation of the expression of *OCT4*, SOX2, and *NANOG* with the clinicopathological features was determined using Pearson's correlation coefficient test. $P<0.05$ was considered to indicate a statistically significant difference. The statistical data were obtained using an SPSS software package, version 24.0 (IBM Corp.).

Results

Patients and clinical characteristics. A total of 72 patients who met the inclusion criteria were included in the analyses. Of the 72 lung adenocarcinoma patients with EGFR mutation who received first-generation EGFR-TKI treatment, 31 were sensitive (sensitive group) and 41 were resistant to first-generation EGFR-TKIs (resistant group). The median age of the entire cohort was 58 (31-76) years. The demographics of the whole cohort are presented in Table I. The expression of *SOX2*, *OCT4* and *NANOG* in 72 lung adenocarcinoma samples was analyzed by IHC staining, immunofluorescence staining and RT-qPCR.

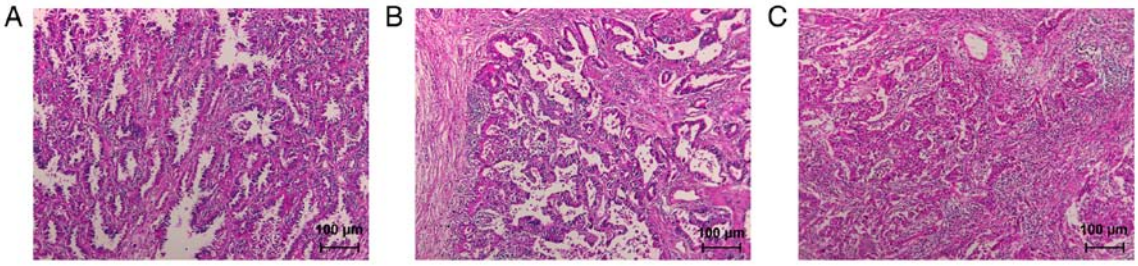

Figure 1. According to the integrity of the gland structure, cell heterogeneity, and mucosal secretion, lung adenocarcinoma was classified as follows: (A) well differentiated, (B) moderately differentiated, and (C) poorly differentiated. Original magnification, x100.

According to the integrity of glandular structures, cellular heterogeneity, and mucus secretion status, lung adenocarcinomas were classified as well, moderately, and poorly differentiated. In well-differentiated tumors, the malignant glands were composed of tall columnar or mucous epithelium, with large, round tumor nuclei and prominent nucleoli; the glandular structures were easily demonstrared under routine microscopy (Fig. 1A). In moderately differentiated tumors, glandular development was not as good as that in well-differentiated tumors (Fig. 1B). In poorly differentiated tumors, the adenocarcinoma was composed of a neoplastic cell population with very poor glandular development. The gland structure was unclear under routine microscopy, and the tumor cells had obvious abnormalities (Fig. 1C).

Correlations between clinical features and efficacy of EGFR-TKIs and the expression of SOX2, OCT4 and NANOG. The association between clinicopathological features of the 72 patients with lung adenocarcinoma and the sensitivity of first-generation EGFR-TKIs are presented in Table I. The chi-square test revealed that men (P=0.048), smokers (P=0.003), patients with stage IV (P=0.003), and patients with poor differentiation (P=0.009) were more likely to be in the resistant group, with statistically significant differences. No obvious correlation was revealed between the sensitivity of first-generation EGFR-TKIs and age, EGFR mutation status, and type of EGFR-TKIs of patients (Table I).

The correlation of the expression of *SOX2*, *OCT4* and N*ANOG* with clinicopathological features of patients with lung adenocarcinoma treated with first-generation EGFR-TKIs was evaluated. As revealed in Table II, the expression of *SOX2* was significantly correlated with PFS (R=-0.256, P=0.030), TNM stage (R=0.257, P=0.029), and differentiation (R=0.347, P=0.003). The expression of *OCT4* was positively associated with PFS (R=-0.242, P=0.041), TNM stage (R=-0.359, P=0.002), and differentiation (R=0.269, P=0.022). The expression of *NANOG* was significantly asscoiated to PFS (R=-0.289, P=0.014) and differentiation (R=0.258, P=0.029).

The association between clinicopathological features of the 72 patients with lung adenocarcinoma and the expression of *SOX2*, *OCT4* and N*ANOG* analyzed using the chi-square test is presented in Table III. An association was observed between the efficacy of EGFR-TKI therapy and the expression of *OCT4* and *NANOG* (P=0.039 and P=0.004, respectively), TNM stage and the expression of *SOX2* and *OCT4* (P=0.034 and P=0.007, respectively), differentiation and the expression of *SOX2* and *NANOG* (P<0.001 and P=0.008, respectively).

IHC and immunofluorescence staining. The expression levels of *SOX2*, *OCT4* and N*ANOG* were examined in the tissue samples from the two groups of patients with lung adenocarcinoma having significantly different prognosis to elucidate whether the expression of lung CSC-related markers was associated with the efficacy of EGFR-TKIs in lung adenocarcinomas. The representative confocal images of *SOX2*, *OCT4* and N*ANOG* with immunofluorescence staining in lung adenocarcinoma specimens are presented in Fig. 2. IHC staining revealed the expression of *SOX2* in 58 lung adenocarcinoma cases; 55 cases exhibited cells positive for *OCT4*, and 50 cases exhibited the expression of *NANOG*. The staining intensities of *SOX2*, *OCT4* and N*ANOG* are presented in Fig. 3. The color reactions were observed in the nucleus and cytoplasm of the tumor cells. The staining intensity was divided into four grades based on the staining depth of positive cells: Unstained (negative), weakly positive (+), moderately positive (++), and strongly positive (+++). The tissue sections of each sample were stained by IHC staining, and the H-score was calculated. The results of the t-test revealed that the expression levels of *SOX2* (Fig. 4A, P=0.003), *OCT4* (Fig. 4B, P=0.036), and *NANOG* (Fig. 4C, P=0.032) were significantly higher in the resistant group than in the sensitive group.

PCR detection for SOX2, OCT4 and NANOG. The relative expression levels of *SOX2*, *OCT4* and N*ANOG* were detected using RT-qPCR. RT-qPCR could not be performed in five patients due to the small tissue sample. The *GAPDH* housekeeping gene was used as a control. The expression level of each tested gene relative to the expression of *GAPDH* was calculated using the ΔΔCq method. Significant differences in the expression level of each tested gene in the two groups were identified using the unpaired nonparametric t-test. The results revealed that the relative levels of *SOX2* (Fig. 5A, P=0.018), *OCT4* (Fig. 5B, P=0.035) and *NANOG* (Fig. 5C, P=0.044) were significantly higher in the resistant group than in the sensitive group. The RT-qPCR results were consistent with the IHC analysis results.

Discussion

Lung cancer is one of the most common malignant tumors, accounting for nearly 30% of all cancer-related deaths (21). The clinical use of first-generation EGFR-TKIs has revealed significant efficacy for advanced lung adenocarcinoma patients with EGFR-sensitive mutation and an increased survival rate. However, two groups of patients had significantly different

Table II. Correlation of *OCT4*, *SOX2* and *NANOG* expression with clinicopathological features determined by Pearson's correlation test.

Parameters	PFS	Age	Sex	Smoking status	EGFR mutation status	TNM stage	Differentiation	Type of EGFR-TKIs
SOX2 expression								
R	-0.256[a]	0.064	-0.104	0.036	0.047	0.257[a]	0.347[a]	0.004
P-value	0.030	0.595	0.385	0.767	0.692	0.029	0.003	0.976
OCT4 expression								
R	-0.242[a]	-0.077	-0.175	0.170	0.139	0.359[a]	0.269[a]	0.183
P-value	0.041	0.520	0.141	0.152	0.244	0.002	0.022	0.125
NANOG expression								
R	-0.289[a]	0.057	0.027	-0.128	0.012	-0.074	0.258[a]	0.054
P-value	0.014	0.634	0.819	0.286	0.923	0.539	0.029	0.655

[a]P<0.05 was considered significant. PFS, progression-free survival; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; *SOX2*, sex determining region Y-box 2; *OCT4*, octamer-binding transcription factor 4; *NANOG*, Nanog homeobox.

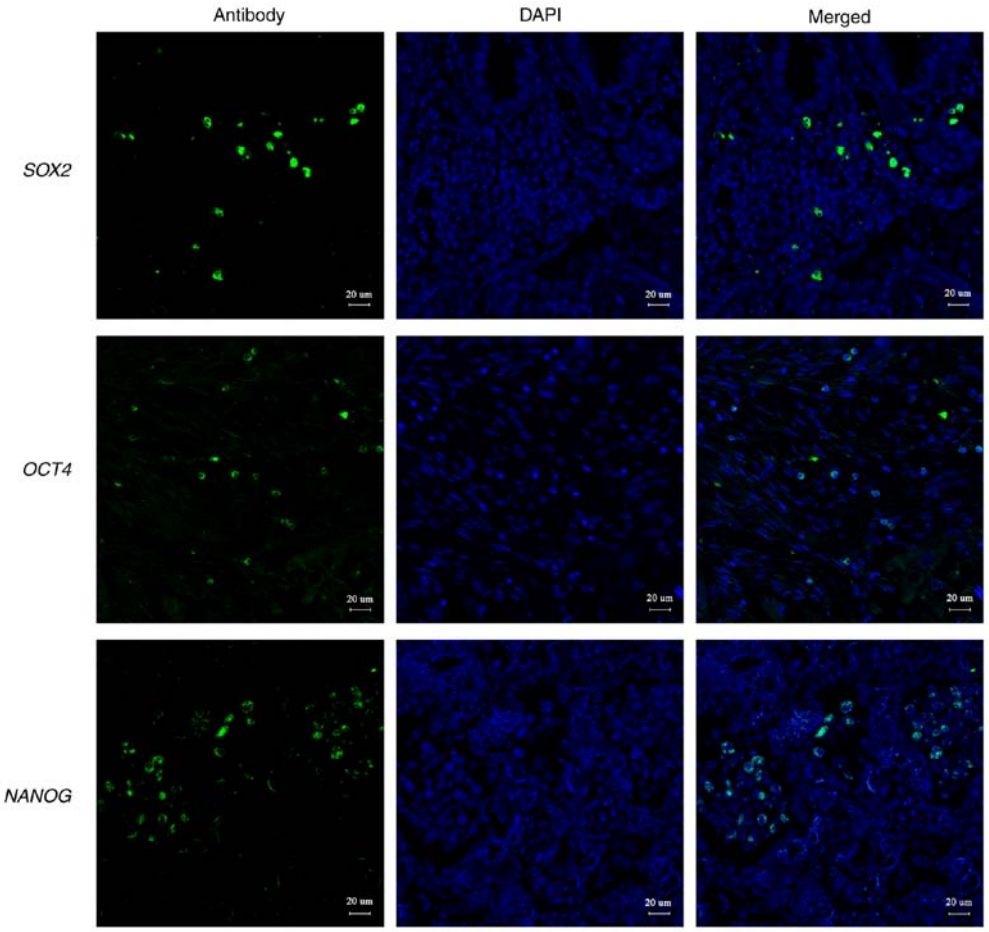

Figure 2. Representative confocal images of *SOX2*, *OCT4* and N*ANOG* with immunofluorescence staining in lung adenocarcinoma specimens. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). In the monochromatic-colored immunofluorescence images, all cancer cells expressing the corresponding tumor stem cell markers (*SOX2*, *OCT4* and N*ANOG*) reflected green fluorescence. Original magnification, x400. *SOX2*, sex-determining region Y-box 2; *OCT4*, octamer-binding transcription factor 4; *NANOG*, *NANOG* homeobox.

therapeutic effects after using the targeted drugs. These included patients with rapid progression or a long period of disease stabilization without progression. At present, the resistance to EGFR-TKI therapy, either intrinsic or acquired, is a major barrier to a complete cure. The median PFS for patients treated with EGFR-TKIs is only ~10-13 months (14-16).

Table III. Association between SOX2, OCT4 and NANOG expression[a] and clinicopathological factors in 72 patients with lung adenocarcinoma analyzed by chi-square test.

	SOX2			*OCT4*			*NANOG*		
Characteristics	Positive	Negative	P-value	Positive	Negative	P-value	Positive	Negative	P-value
Efficacy									
Resistant (PFS <5 months)	36	5	0.074	35	6	0.039[c]	34	7	0.004[d]
Sensitive (PFS >26 months)	22	9		20	11		16	15	
Age (years)									
≤60	34	8	0.920	33	9	0.606	26	16	0.100
>60	24	6		22	8		24	6	
Sex									
Male	24	4	0.378	24	4	0.137	19	9	0.816
Female	34	10		31	13		31	13	
Smoking status									
Smoker[b]	19	4	0.763	20	3	0.234	14	9	0.289
Never-smoker	39	10		35	14		36	13	
EGFR mutation status									
19del	27	8	0.572	25	10	0.455	24	11	0.978
21L858R	29	5		27	7		24	10	
Others	2	1		3	0		2	1	
TNM stage									
IIB	1	0	0.034[c]	0	1	0.007[d]	0	1	0.137
IIIA/IIIB	16	9		15	10		20	5	
IV	41	5		40	6		30	16	
Differentiation									
Well	6	8	P<0.001[d]	8	7	0.059	5	9	0.008[c]
Moderate	24	2		21	5		21	5	
Poor	28	4		26	5		24	8	
Type of EGFR-TKIs									
Gefitinib	25	7	0.637	21	10	0.236	22	10	0.742
Icotinib	20	3		18	5		15	8	
Erlotinib	13	4		16	2		13	4	

[a]The expression of SOX2, OCT4 and NANOG were evaluated by H score of immunohistochemistry (positive, H Score >0; negative, H score =0). [b]Smoker, is defined as a patient who has smoked >100 cigarettes during his lifetime. PFS, progression-free survival; EGFR, epidermal growth factor receptor; [c]P<0.05; [d]P<0.005. TKI, tyrosine kinase inhibitor; *SOX2*, sex determining region Y-box 2; *OCT4*, octamer-binding transcription factor 4; *NANOG*, Nanog homeobox.

In the present study, IHC and RT-qPCR analyses were used to detect and analyze the expression of CSC-related markers in patients with lung adenocarcinoma having significantly different effects after treatment with EGFR-TKIs. The results revealed that the level of CSC markers was higher in the EGFR-TKI resistant group than in the EGFR-TKI sensitive group, and the difference was statistically significant. In addition, the expression of *SOX2* and *OCT4* was significantly correlated with PFS, TNM stage, and differentiation, whereas the expression of *NANOG* was significantly associated to PFS and differentiation.

CSCs play an important role in tumor development, infiltration, metastasis and recurrence (2,22). *SOX2*, *OCT4* and N*ANOG* are the core transcriptional regulators of CSCs and constitute the core transcriptional network to maintain pluripotency and self-renewal ability of CSCs (9,23,24). These transcription factors can reprogram somatic cells into pluripotent stem cells and contribute to drug resistance and poor prognosis (25,26). Several previous studies indicated that *OCT4*, *SOX2* and *NANOG* were involved in the occurrence, drug resistance, and development of tumors (11,23,24,27). Shien *et al* investigated the molecular and cellular profiles of cells with acquired resistant to EGFR-TKI in EGFR-mutant lung cancers. In their study, gefitinib-resistant sublines were established by exposing EGFR mutant cell lines to gefitinib using stepwise escalation and high-concentration exposure methods. They found that the sublines established by the high-concentration exposure methods had CSC-like properties (28). The targeted drugs could kill some of the rapidly dividing cancer cells with sensitive EGFR mutations. However,

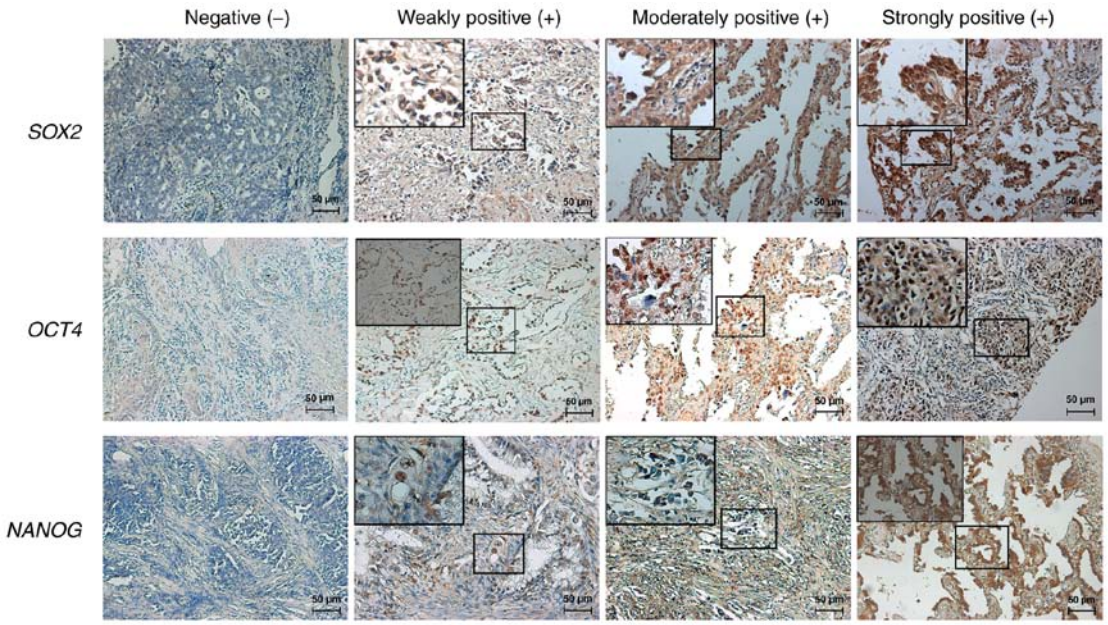

Figure 3. Representative images of *SOX2*, *OCT4* and N*ANOG* with IHC staining in lung adenocarcinoma specimens. The staining intensity of *SOX2*, *OCT4* and N*ANOG* is presented in the figure; '-' indicated negative; '+' indicated weakly positive; '++' indicated moderately positive, and '+++' indicated strongly positive. Original magnification, x200. *SOX2*, sex-determining region Y-box 2; *OCT4*, octamer-binding transcription factor 4; *NANOG*, *NANOG* homeobox; IHC, immunohistochemical.

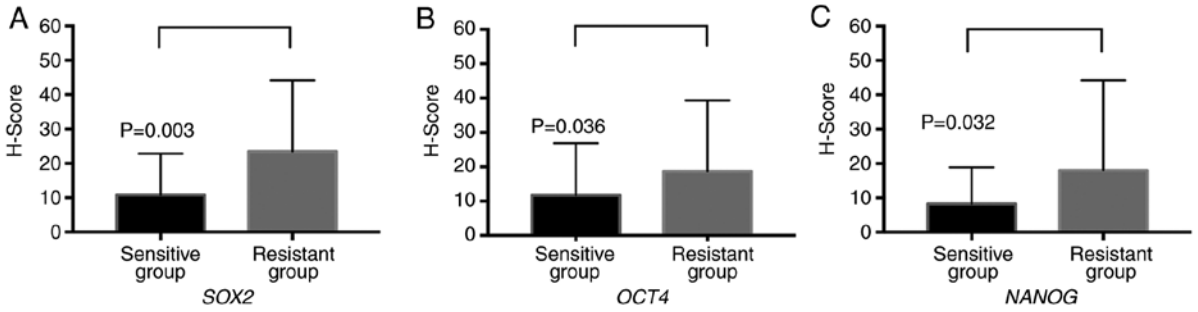

Figure 4. Immunohistochemistry is used to semi-quantitatively evaluate the expression of *SOX2* (A), *OCT4* (B) and N*ANOG* (C) in each sample. The expression of *SOX2*, *OCT4 a*nd N*ANOG* was evaluated using the t-test after calculating the H score. P-values are indicated. *SOX2*, sex-determining region Y-box 2; *OCT4*, octamer-binding transcription factor 4; *NANOG*, *NANOG* homeobox.

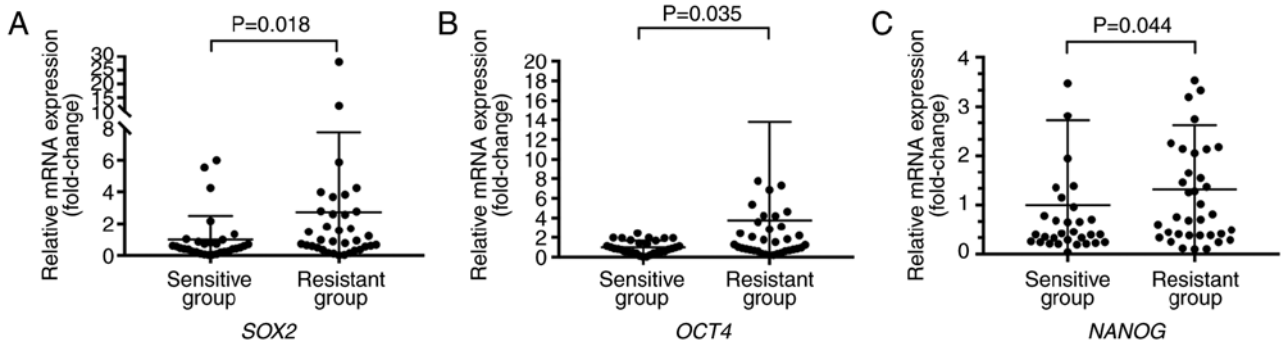

Figure 5. Expression of *SOX2* (A), *OCT4* (B) and *NANOG* (C) is assessed by RT-qPCR. Symbols represent individual samples, with the mean and SD indicated. The significance was evaluated using the unpaired nonparametric t-test, with P-values indicated. *SOX2*, sex-determining region Y-box 2; *OCT4*, octamer-binding transcription factor 4; *NANOG*, *NANOG* homeobox.

CSCs not sensitive to various treatments could be further replicated, self-renewed, and evolved into more drug-resistant lung cancer cells after a period of EGFR-TKI therapy.

In the present study, it was revealed that the number of male patients, patients who smoked, patients with stage IV, and patients with poor differentiation was significantly higher

in the resistant group than in the sensitive group. These results were consistent with the findings of other previous studies (29-34). Since a majority of the smokers were men in the present study, it is possible that smoking may make the interaction between sex and the efficacy of EGFR-TKIs confusing. In addition, some studies have demonstrated that the expression of CSC markers was related to clinical and pathological parameters in patients with lung cancer disease (35,36). For instance, Li *et al* revealed that the high expression level of CSC markers was significantly associated with poorer differentiation, higher TNM stage and worse prognosis of lung cancer (35).

The present study also had some limitations and biases that should be acknowledged. First, the sample size of this study was relatively small. The follow-up studies should have a larger sample size for further verification. Second, this study suffered from selection bias due to its retrospective nature. In addition, the mechanisms discussed in this study required further experiments.

Therefore, the present study concluded that the therapeutic effect of first-generation EGFR-TKIs was highly correlated with the levels of CSC-related markers. The markers of CSCs were highly expressed in EGFR-TKI resistant patients.

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Availability of data and materials

The data used and/or analyzed in this study can be obtained from the corresponding author on reasonable request.

Authors' contributions

XZha and FH designed and supervised the study, and edited the manuscript. BH and CL designed the experiments and analyzed the data. FH, LZ and XY performed all experimental work. HZ, XZhe and YS supported administration and drafted the work or revised it critically for important intellectual content. All authors read, approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shanghai Chest Hospital. All patients provided their informed consent. Investigations involving human patients in this study were performed according to the principles of the Declaration of Helsinki and the ethical standards of the national research committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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