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Relationship between epidermal growth factor receptor gene mutation and copy number in Chinese patients with non-small cell lung cancer

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Abstract

Epidermal growth factor receptor (*EGFR*) gene mutation and copy number are useful predictive markers that guide the selection of non-small cell lung cancer (NSCLC) patients for *EGFR*-targeting therapy. This study aimed to investigate the correlation between *EGFR* gene mutation and copy number and clinicopathologic characteristics of Chinese patients with NSCLC. NSCLC specimens collected from 205 patients between November 2009 and January 2011 were selected to detect *EGFR* gene mutations with real-time polymerase chain reaction (RT-PCR) and to detect *EGFR* gene copy number with fluorescence *in situ* hybridization (FISH). *EGFR* mutations primarily occurred in females, non-smokers, and patients with adenocarinomas (all $P < 0.001$). Tissues from 128 (62%) patients were FISH-positive for *EGFR*, including 37 (18%) with gene amplification and 91 (44%) with high polysomy. *EGFR* gene mutation was correlated with FISH-positive status ($R = 0.340$, $P < 0.001$). Multivariate analysis showed that not smoking (OR = 5.910, 95% CI = 2.363–14.779, $P < 0.001$) and having adenocarcinoma (OR = 0.122, 95% CI = 0.026–0.581, $P = 0.008$) were favorable factors for *EGFR* gene mutation. These results show a high frequency of *EGFR* FISH positivity in NSCLC tissues from Chinese patients and a significant relevance between *EGFR* gene mutations and FISH-positive status. Among the FISH-positive samples, *EGFR* gene mutation occurred more frequently in samples with gene amplification compared to those with high polysomy, suggesting that *EGFR* mutation and gene amplification should be used as clinical decision parameters to predict response to *EGFR*-targeting therapy.

Key words Epidermal growth factor receptor, gene mutation, gene copy number, non-small cell lung cancer, correlation

Lung cancer is the leading cancer in men, comprising 17% of total new cancer cases and 23% of total cancer deaths[1]. The epidermal growth factor receptor (EGFR) family of proteins is well known to play an important role in lung carcinogenesis, tumor cell survival and proliferation[2]. Somatic gene mutations clustered within the tyrosine kinase domain of *EGFR* are common in non-small cell lung cancer (NSCLC) and occur most frequently in women, East Asians, non-smokers, and patients with adenocarcinomas[3-6]. Studies on agents targeting mutated *EGFR* sparked an interest in the predictive and prognostic significance of gene status[7]. The average response rate to therapy with anti-EGFR tyrosine kinase inhibitors (TKIs) was 75% for NSCLC patients with *EGFR* mutations [8]; however, a high response rate to anti-EGFR TKIs has also been observed in patients with increased *EGFR* gene copy number[9,10]. Molecular analysis using fluorescence *in situ*

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hybridization (FISH) indicated that increased *EGFR* gene copy number with balanced polysomy occurs in approximately 10% to 40% of patients with NSCLC [11]. Some studies have shown that *EGFR* mutations are highly predictive of drug response, and others indicated that *EGFR* gene copy number may be equally or more predictive of improved survival than *EGFR* mutation status [12]. Further studies suggested that *EGFR* gene mutation and amplification can occur simultaneously, and both were proposed as potential biomarkers of anti-EGFR TKI responsiveness[13,14].

Although the most useful biomarker for selecting candidates for anti- EGFR TKI therapy still remains controversial [15,16], concurrent analyses of gene mutation and copy number have revealed the relevance and association of these factors with clinical outcome. This study aimed *to* analyze the correlation between *EGFR* gene mutations and gene copy number in Chinese patients with NSCLC and to further clarify the relationship between clinicopathologic features and gene mutations and copy number.

Materials and Methods

Tumor specimens

Tumor specimens were obtained from 205 consecutive patients who underwent surgery for pathologically proven NSCLC between November 2009 and January 2011 at Sun Yat-sen University Cancer Center. Detailed demographic and clinical information of these patients was available at the Sun Yat-sen University Cancer Center Surveillance System. No patient underwent either neoadjuvant chemoradiotherapy or target therapy. Tumors were staged according to the International Association of the Study for Lung Cancer (IASLC) TNM staging system [17]. The tumor blocks were fixed in 10% buffered formaldehyde and embedded in paraffin. The blocks were cut in 4-μm consecutive sections and stained with hematoxylin and eosin (HE). Slides rich in viable tumor cells were submitted for FISH analysis.

Real-time PCR analysis of *EGFR* mutations

We used an EGFR kit (GP Medical Technologies Ltd, Beijing, China) to detect a deletion in exon 19 (delE746-A750) and mutation in exon 21 (L858R) with real-time polymerase chain reaction (RT-PCR). RT-PCR was performed as follows: initial activation of DNA polymerase at 50℃ for 2 min, denaturation at 95℃ for 10 min, 40 cycles of amplification at 95℃ for 15 s and at 62℃ for 60 s. The cycle threshold (Ct) was used for results interpretation and was defined as the cycle at the highest peak of the second derivative curve, which represented the maximum point of the growth curve [18]. Positive results were defined as Ct $\leqslant$ 34 on the growth curve. The samples with positive results (34 < Ct $\leqslant$ 38) were subjected to repeated RT-PCR for result validation.

FISH analysis of *EGFR* gene copy number

FISH assays were performed using the EGFR FITC Red/CEP 7 Rhodamine Green probe (GP Medical Technologies Ltd, Beijing, China) according to the manufacturer's instructions. Tumor tissue sections were deparaffinized in 2× xylene washes at room temperature for 10 min, and dehydrated orderly in 100%, 85%, and 70% ethanol for 2 min each. After incubation in 30% saline sodium citrate (SSC) at 50℃ for 20 to 30 min, sections were digested with proteinase K at 37℃ and rinsed in 2× SSC for 30 min. The EGFR/CEP 7 probe set was applied to the selected area on each section. The sections were incubated at 90℃ for 30 min for co-denaturation of chromosomal and probe DNAs, hybridized at 42℃ for 16 h, and washed in SSC at room temperature thereafter. Chromatin was counterstained with DAPI (0.15 mg/mL in Vectashield Mounting Medium). The sections were observed under an epifluorescence microscope using single-band filters, and images of each section were merged by the Smart Capture software (Vysis, Downers Grove, IL, USA).

FISH analyses were defined according to the previously published criteria by Cappuzzo *et al*.[10] Six FISH strata were classified according to the frequency of tumor cells with specific number of copies of the *EGFR* gene and chromosome 7 centromere: 1) disomy ($\leqslant$ 2 copies of *EGFR* per cell in > 90% of cells); 2) low trisomy ($\leqslant$ 3 copies in 10%–40% of cells); 3) high trisomy ($\leqslant$ 3 copies in > 40% of cells); 4) low polysomy ($\geqslant$ 4 copies in 10%–40% of cells); 5) high polysomy ($\geqslant$ 4 copies in > 40% of cells); 6) gene amplification (tight *EGFR* gene clusters and a ratio of *EGFR* gene to chromosome of $\geqslant$ 2, or $\geqslant$ 10 copies in $\geqslant$ 10% of cells). Gene amplification and high polysomy were defined as FISH-positive and all else were FISH-negative.

Statistical analyses

Chi-square (χ^2) test or Fisher's exact test was performed to determine the associations between the presence of *EGFR* mutations or FISH phenotypes and patients' characteristics. Multivariate analyses (logistic regression models) were used to determine the influence factors for *EGFR* mutations. A P value < 0.05 was considered significant.

Results

Patient characteristics

Patients' clinical characteristics are presented in Table 1. Of the 205 patients, 138 were men and 67 were women, with a median age of 59 years (range, 29–80 years); 119 were smokers, and 86 were non-smokers. According to the WHO classification[19], 147 patients had adenocarcinoma (including bronchioloalveolar carcinoma), 52 had squamous cell carcinoma, 3 had adenosquamous carcinoma, 2 had sarcomatoid carcinoma, and 1 had large-cell carcinoma (lymphoepithelioma-like carcinoma); 85 presented with stage I disease (23 with IA, 62 with IB), 30 with stage II disease (5 with IIA, 25 with IIB), 70 with stage III disease (51 with IIIA, 19 with IIIB), and 20 with stage IV disease.

EGFR gene mutations

In the 205 specimens of NSCLC, we detected *EGFR* mutations in 66 (32%) samples, including exon 19 deletion mutations in 34 (17%) and exon 21 point mutations in 32 (15%) samples (Figure 1). Of the 66 patients with mutations, 40 were women and 26 were men; 17 were smokers and 49 were non-smokers; 63 had adenocarcinomas and 3 had non-adenocarcinomas. There were significantly higher mutation rates in women, non-smokers, and patients with adenocarcinomas (all $P < 0.001$); no relationships were observed between *EGFR* mutation rates and age ($P = 0.412$) and histological differentiation ($P = 0.063$) (Table 1).

EGFR gene copy number

FISH analysis for *EGFR* gene copy number was performed on 205 specimens. FISH was positive in 128 (62%) of these 205 specimens, including 91 (44%) cases with high polysomy (Figure 2A) and 37 (18%) cases with amplification (Figure 2B). We also detected the disomy (Figure 2C) in 36 cases, low polysomy (Figure 2D) in 35 cases, low trisomy (Figure 2E) in 4

Table 1. Relationships between clinicopathologic characteristics and *EGFR* gene mutation and copy number in patients with non-small cell lung cancer (NSCLC)

Variable	Total	*EGFR* gene mutation			FISH-positive phenotype		
		Cases	%	*P*	Cases	%	*P*
Gender							0.112
Male	138	26	19	<0.001	81	59	
Female	67	40	60		47	70	
Age (years)							0.624
⩾ 59	111	33	35	0.412	71	64	
< 59	94	33	30		57	61	
Smoking status							0.209
Ever	119	17	14	<0.001	70	59	
Never	86	49	57		58	67	
Histological type				<0.001			0.095
ADC	147	63	43		97	66	
Non-ADC	58	3	5		31	54	
Differentiation				0.063			0.080
Grade 1	17	5	29		9	53	
Grade 2	116	46	40		80	69	
Grade 3	63	13	21		36	57	
Unknown	9	2	22		3	33	
TNM stage				0.038			0.127
IA	23	11	48		15	65	
IB	62	13	21		31	50	
IIA	5	0	0		2	40	
IIB	25	6	24		16	64	
IIIA	51	20	39		38	75	
IIIB	19	6	32		11	58	
IV	20	10	50		15	75	

EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; ADC, adenocarcinoma.

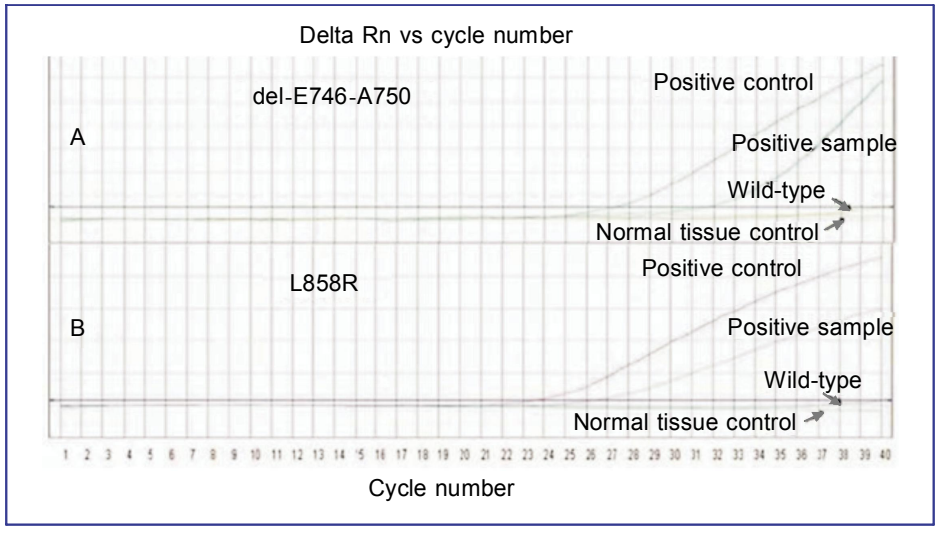

Figure 1. **Representative real-time polymerase chain reaction (RT-PCR) curves for mutations in *EGFR* exons 19 and 21.** The positive control curve shows the mutation. Two ascending curves denoted "positive sample" represent exon 19 del-E746-A750 mutation (A) and exon 21 L858R mutation (B). Smooth curves represent wild-type *EGFR*.

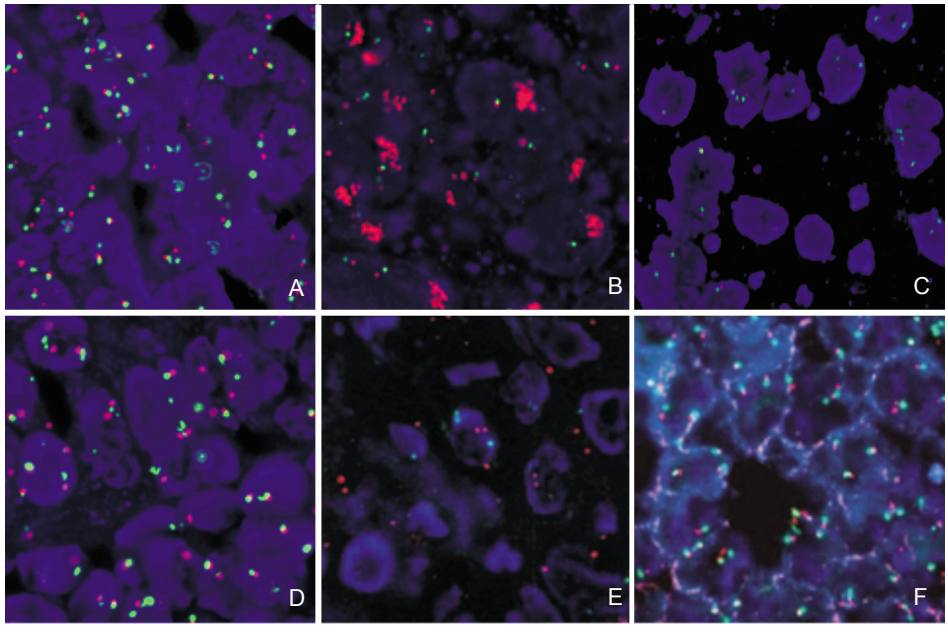

Figure 2. ***EGFR* gene copy number analyzed with fluorescence *in situ* hybridization (FISH).** Chromosome 7 centromere was labeled with FITC (green) and *EGFR* gene was labeled with rhodamine (red). A, *EGFR* high polysomy; B, *EGFR* amplification; C, *EGFR* disomy; D, *EGFR* low polysomy; E, *EGFR* low trisomy; F, *EGFR* high trisomy.

cases, and high trisomy (Figure 2F) in 2 cases. No relationship was found between *EGFR* gene copy number and patients' characteristics (Table 1). In the 2 cases of sarcomatoid carcinoma, one had amplification and the other had polysomy. In the 3 cases of adenosquamous carcinoma, 2 were detected with amplification and 1 with disomy.

Relationship between *EGFR* gene mutations and gene copy number

Comparison between gene mutations and copy number showed that 57 (86%) of the 66 samples with *EGFR* mutation were also FISH-positive (24 with amplification and 33 with high polysomy), suggesting a relationship between *EGFR* mutation and the FISH-positive phenotype ($P < 0.001$) (Table 2). In the NSCLC patients with *EGFR* mutation, stratified analysis showed that the FISH-positive phenotype occurred more frequently than the FISH-negative phenotype (Table 3). In these two groups, we found that the FISH-positive phenotype primarily occurred in cases with *EGFR* mutation and adenocarcinoma ($P < 0.001$) or tumor differentiation of grade >1 ($P < 0.001$) (Table 3).

Table 2. Relationship between *EGFR* mutation and the FISH-positive phenotype in patients with NSCLC

EGFR mutation	Total	FISH phenotype [cases (%)]		*R*	*P*
		Positive	Negative		
Presence	66	57 (86)	9 (14)	0.34	<0.001
Absence	139	71 (51)	68 (49)		

Abbreviations as in Table 1.

Table 3. Analysis of factors that influence *EGFR* FISH phenotype in patients with NSCLC

Variable	*EGFR* mutation	FISH phenotype [cases (%)]		*P*
		Negative	Positive	
Gender				
Male	–	54 (48)	58 (52)	0.001
	+	3 (11)	23 (89)	
Female	–	14 (52)	13 (48)	0.001
	+	6 (15)	34 (85)	
Age (years)				
⩾ 59	–	34 (44)	44 (56)	0.011
	+	6 (18)	27 (82)	
< 59	–	34 (56)	27 (44)	<0.001
	+	3 (9)	30 (91)	
Smoking status				
Ever	–	46 (45)	56 (55)	0.036
	+	3 (18)	14 (82)	
Never	–	22 (60)	15 (40)	<0.001
	+	6 (12)	43 (88)	
Histological type				
ADC	–	41 (49)	43 (51)	<0.001
	+	9 (14)	54 (86)	
Non-ADC	–	27 (49)	28 (51)	0.241
	+	0 (0)	3 (100)	
Differentiation				
Grade 1	–	7 (58)	5 (42)	0.294
	+	1 (20)	4 (80)	
Grade >1	–	61 (48)	66 (52)	<0.001
	+	8 (13)	53 (87)	
Stage				
Early (IA+IB)	–	35 (57)	26 (43)	0.001
	+	4 (17)	20 (83)	
Medium (IIA+IIB+IIIA)	–	22 (40)	33 (60)	0.010
	+	3 (11)	23 (89)	
Advanced (IIIB+IV)	–	11 (48)	12 (52)	0.037
	+	2 (12)	14 (88)	

Abbreviations as in Table 1.

Further analysis of the FISH-positive patterns revealed that amplification was more highly associated with *EGFR* mutation than high polysomy. In contrast, FISH-negativity was more associated with wild-type *EGFR* (P = 0.003) (Table 4). In patients with *EGFR* gene mutations, stratified analyses for influencing factors on FISH-positive phenotype showed that the frequency of amplification was related to *EGFR* mutation in patients

who were female (P = 0.004) or younger than 59 (P = 0.001), who had never smoked (P = 0.023), or who had adenocarcinoma (P = 0.019), grade > 1 (P = 0.017), or early stage disease (P = 0.029) (Table 5).

Multivariate analyses showed that never smoking (OR = 5.910, 95% CI = 2.36-14.78, P < 0.001) and adenocarcinoma (OR = 0.122, 95% CI = 0.026–0.581, P = 0.008) were related to *EGFR* mutations.

Table 4. Relationship between *EGFR* mutations and FISH phenotype in patients with NSCLC

FISH phenotype	Total	*EGFR* mutation [cases (%)]		*R*	*P*
		Presence	Absence		
High polysomy	91	33 (36)	58 (64)	0.375	<0.001
Amplification	37	24 (65)	13 (35)		
FISH negativity	77	9 (12)	68 (88)		

Abbreviations as in Table 1.

Table 5. Stratified analysis of factors influencing FISH-positive phenotype in patients with NSCLC

Variable	*EGFR* mutation	FISH-positive phenotype [cases (%)]		*P*
		High polysomy	Amplification	
Gender				
Male	–	45 (77)	13 (59)	0.127
	+	14 (23)	9 (41)	
Female	–	13 (41)	0 (0)	0.004
	+	19 (59)	15 (100)	
Age (years)				
⩾ 59	–	34 (64)	10 (56)	0.516
	+	19 (36)	8 (44)	
< 59	–	24 (63)	3 (16)	0.001
	+	14 (37)	16 (84)	
Smoking status				
Ever	–	44 (86)	12 (63)	0.045
	+	7 (14)	7 (37)	
Never	–	14 (35)	1 (6)	0.023
	+	26 (65)	17 (94)	
Histological type				
ADC	–	35 (52)	8 (27)	0.019
	+	32 (48)	22 (73)	
Non-ADC	–	23 (96)	5 (71)	0.120
	+	1 (4)	2 (29)	
Differentiation				
Grade 1	–	5 (83)	0 (0)	0.048
	+	1 (17)	3 (100)	
Grade >1	–	53 (62)	13 (38)	0.017
	+	32 (38)	21 (62)	
Stage				
Early (IA+IB)	–	24 (65)	2 (22)	0.029
	+	13 (35)	7 (78)	
Medium (IIA+IIB+IIIA)	–	25 (64)	8 (47)	0.233
	+	14 (36)	9 (53)	
Advanced (IIIB+IV)	–	9 (60)	3 (27)	0.098
	+	6 (40)	8 (73)	

Abbreviations as in Table 1.

Discussion

Anti-EGFR TKIs are well known and are used for monotherapy in NSCLC. *EGFR* mutations were reported as predictive factors associated with anti-EGFR TKI sensitivity and response to EGFR-targeting therapies [6,9,20-22]. Many studies reported an association between *EGFR* mutations and some clinical features in NSCLC. The rates of *EGFR* mutation in those studies were 44%−55% in patients with adenocarcinoma, 51%−68% in non-smokers, 42%−62% in females, and 30%−50% in Asian ethnicity, but in contrast, the mutation rates were 10% in smokers, 14% in males, and 8% in adenocarcinoma patients from Western countries[20,23-25]. In our current study of 205 specimens from Chinese patients with NSCLC, exon 19 deletion mutation and exon 21 point mutation were detected in 66 (32.2%) specimens by RT-PCR and occurred more frequently in adenocarcimoma (43%), females (60%), and non-smokers (57%), reflecting findings similar to those in the literature.

Although *EGFR* mutation was suggested to be the best predictor for TKI sensitivity, it should not be the only patient selection criterion for EGFR-targeting therapy; indeed, in studies with anti-EGFR TKIs, the survival benefit conferred by using *EGFR* mutation as a predictor is not only confined to individuals with tumor shrinkage, but also includes those with tumor stability or even disease progression [16]. Large randomized studies showed that *EGFR* gene copy number, as determined by FISH analysis, is probably an alternative for NSCLC patient selection and may show a survival benefit by predicting sensitivity to TKIs. In previous studies, high gene copy number or gene amplification tested by FISH was identified in 22%−45% of NSCLC patients [10,26,27]. Either *EGFR* FISH or mutation analyses could identify NSCLC with better outcome and response to TKIs[9,10,26,28]. In our study, we identified 128 (62%) FISH-positive cases from 205 specimens, including 37 cases with amplification and 91 cases with high polysomy, which was a higher frequency than that seen in other studies. However, as seen in other studies, *EGFR* gene copy number was not correlated with age, gender, smoking status, stage, histological type, and differentiation[28].

Different trials have demonstrated a significant association between the *EGFR* FISH-positive phenotype and *EGFR* mutations [10,26,28,29]. Our data have also demonstrated a positive correlation between the FISH-positive phenotype and gene mutations (R = 0.34, P < 0.001). Further analysis showed that histological type and grade could influence the FISH patterns when EGFR mutation status is taken into consideration.

Some authors consider high polysomy as significant gene alteration in carcinogenesis, though the biological mechanism was not clear. Moreover, *EGFR* amplification was closely associated with *EGFR* mutation[9,22,30,31]. Our study also demonstrated the relationship between *EGFR* mutation and amplification, though high polysomy tended to relate with wild-type *EGFR*. Stratified analyses demonstrated that amplification was associated with *EGFR* mutations in patients who are female or young, who have never smoked, or who have adenocarcinoma, grade >1 (non-well differentiation), or early stage disease. Some articles inferred that *EGFR* mutant alleles were amplified selectively, and *EGFR* amplification happened during invasive growth of lung adenocarcinoma with *EGFR* mutations. Moreover, *EGFR* copy number gain was a late event compared to gene mutation in NSCLC pathogenesis, implying that *EGFR* mutation resulted in a high *EGFR* copy number [26,32-34]. However, other authors thought that *EGFR* mutation and *EGFR* copy number gain are two independent events in NSCLC pathogenesis [35]. In our study, except for a number of observations already published in previous studies, we also found that early stage is associated with amplification in patients with *EGFR* mutations, which suggests that amplification may occur earlier in carcinogenesis than *EGFR* mutation.

Specific characteristics such as female gender, non-smoking status, and adenocarcinoma histology are well known to relate with *EGFR* mutation in East Asians with NSCLC [6,20]. However, multivariate analyses in our study only showed that non-smoking status and adenocarcinoma histology were related to *EGFR* gene mutation in Chinese patients with NSCLC. Thus, gender may not be a candidate selective factor for *EGFR*- targeting therapy in Chinese patients, but we need more evidence to prove that in further studies.

Conclusions

EGFR mutations are more common in Chinese NSCLC patients with adenocarcinoma histology and non-smoking status. Chinese NSCLCs are frequently *EGFR* FISH-positive. Among the FISH-positive patterns, gene amplification is related with *EGFR* gene mutation. Clinicopathologic characteristics including female gender, young age, non-smoking status, adenocarcinoma histology, high grade, and early stage are related to Chinese NSCLC patients with *EGFR* mutations and *EGFR* gene amplification simultaneously. Therefore, *EGFR* mutation and gene amplification should be used as clinical decision parameters to predict response to anti-*EGFR* TKI therapy.

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