

EGFR mutations in patients with lung adenocarcinoma in southwest China: are G719S/A and L861Q more likely detected in tumors derived from smokers?

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Background: The clinical characteristics of epidermal growth factor receptor (EGFR) hotspot mutations, such as deletions in exon 19, substitution of L858R in exon 21, and mutations in exon 20, have been widely reported in nonsmall cell lung cancer. However, the clinical features of other low frequency EGFR mutations in these four exons (especially the relationship with smoking history), eg, substitutions of G719S/A/C in exon 18 and L861Q in exon 21, remain unclear. This study investigated the relationship between G719S/A/C and L861Q mutations (in exon 18 and 21) and smoking history.

Methods: Specimens from 194 patients with lung adenocarcinoma were analyzed for EGFR mutations in exons 18–21 by high-resolution melting curve analysis and amplification refractory mutation technology to establish the relationship between G719S/A/C and L861Q mutations and smoking history.

Results: Ninety-six of 194 tumors (49.5%) were confirmed to be EGFR mutation-positive. Among these mutations, 71 of 104 (68.3%) were from never smokers, six of 17 (35.3%) were from former smokers, and 19 of 73 (26.0%) were from current smokers ($P < 0.001$). The mutation rate in heavy smokers (5/23, 21.7%) was significantly lower than in light smokers (20/67, 29.9%) and never smokers (71/104, 68.3%, $P < 0.001$). Seven low frequency EGFR mutations (four substitutions of G719S, and G719 A, respectively, and three of L861Q in exon 21) were identified. Five of these mutations were derived from smokers (one former light smoker, one current heavy smoker, and three current light smokers). Four of these patients had been treated with tyrosine kinase inhibitors and all had a partial response, with median overall survival (14.5 months) and median progression-free survival (6.8 months), being longer than in patients with similarly staged lung adenocarcinoma without EGFR mutation or treatment with tyrosine kinase inhibitors (6.8 and 3.1 months, respectively, according to data from an as yet unpublished study at our institution).

Conclusion: This study provides further evidence that smoking status, include years of smoking and number of cigarettes smoked per day, plays an important role in EGFR mutation in patients with lung adenocarcinoma. Five of seven specimens with G719S/A or L861Q mutations coming from smokers indicates that there may be a relationship between G719S/A or L861Q mutation and smoking history. However, regardless of the influence of smoking, the effectiveness of tyrosine kinase inhibitors was satisfactory in four patients harboring G719S/A and L861Q EGFR mutation.

Keywords: EGFR, lung adenocarcinoma, G719S/A, L861Q, smoking history

Introduction

Epidermal growth factor receptor (EGFR) is a target of the tyrosine kinase inhibitors (TKIs). Patients with nonsmall cell lung cancer (NSCLC) harboring EGFR mutations are particularly responsive to the small molecule TKIs, ie, gefitinib and erlotinib.^{1–3} In recent years, TKIs have become a first-line therapy for NSCLC in the National Comprehensive Cancer Network clinical practice guidelines.⁴ This has led to EGFR mutation analysis becoming a routine molecular test in many hospitals. Most TKIs bind to domains in EGFR encoded by exons 18–21,⁵ and the currently reported EGFR mutations include substitutions of G719 in exon 18, in-frame deletions in exon 19, in-frame duplications or insertions in exon 20, and substitutions of L858R or L861Q in exon 21.^{6,7} As reported in other studies, approximately 85%–90% of EGFR mutations are deletions in exon 19 and point mutations of L858R in exon 21,⁸ and these are the best characterized mutations in patients with NSCLC who respond favorably to treatment with gefitinib or erlotinib.⁷

A number of studies have found that cancer patients who smoke while receiving treatment for their malignancies have poorer outcomes than their nonsmoking counterparts.⁹ In a prospective study, Browman et al¹⁰ showed that patients with stage III/IV head and neck cancer who continue to smoke during radiotherapy have lower rates of response and decreased survival compared with nonsmoking patients. The importance of smoking history is also clear from our understanding of the clinical significance of EGFR gene mutations. Although somatic EGFR mutations are more common in patients with NSCLC who have never smoked cigarettes, patients with a limited smoking history are more likely to harbor EGFR mutations than those with a heavy smoking history.¹¹ For example, D'Angelo et al¹² tested 2142 lung adenocarcinoma specimens for the presence of EGFR exon 19 deletions and substitution of L858R. They identified mutations in 15% of tumors from former smokers (181/1218), 6% from current smokers (20/344), and 52% from subjects who had never smoked (302/580; $P=0.001$ for smokers versus nonsmokers). Therefore, smoking history has been recognized as having a significant influence on EGFR mutations.

The EGFR mutations described above, including deletions in exon 19, substitutions of L858R in exon 21, T790M and insertions in exon 20, are common, and have been widely reported. However, other low frequency mutations, including substitutions in G719, S768, and L861, are also part of the spectrum of EGFR mutations.^{13,14} These low frequency mutations have been analyzed in many institutions, but only for a small number of cases, and their clinical significance,

particularly their correlation with smoking history, remains unknown.

In this study, we analyzed 194 lung adenocarcinoma specimens for EGFR mutations using high-resolution melting curve analysis and amplification refractory mutation system (ARMS) technology. Specimens with mutations of G719S/A, L861Q, and insertions in exon 20 of EGFR were analyzed and confirmed by direct sequencing. The clinical features of these mutations and their relationship with smoking history are described in this paper.

Materials and methods

Patients and tissues

The primary tumors for this study were obtained from 194 consecutive patients with lung adenocarcinoma treated surgically at Daping Hospital, Third Military Medical University, Chongqing, People's Republic of China, between July 2009 and December 2012 (Table 1). The patient population consisted of 112 men (57.7%) and 82 women (42.3%) of mean age 58.2 (range 26–81) years. The patients were categorized on the basis of their smoking history¹⁵ as 104 never smokers (53.6%), 17 former smokers (8.8%), and 73 current smokers (37.6%). We further categorized them according to number of cigarettes smoked per day,¹⁶ ie, 104 never smokers (0 per day), 23 heavy smokers (>20 per day), and 67 light smokers (≤ 20 per day).

DNA isolation

All samples were obtained from paraffin-embedded sections of tumor specimens, all of which were confirmed to contain

Table 1 Clinical characteristics of patients with lung adenocarcinoma and genetic screening

Characteristic	Patients (n = 194)	EGFR mutations	χ^2	P
Age, years				
Mean (%)	58.2 years	96 (49.5)		
Gender				
Males, n (%)	112 (57.7)	36 (32.1)		
Females, n (%)	82 (42.3)	60 (73.2)	31.878	<0.001
Smoking status, n (%)				
Never smokers*	104 (53.6)	71 (68.3)		
Former smokers	17 (8.8)	6 (35.3)		
Current smokers	73 (37.6)	19 (26.0)	32.605	<0.001
Cigarettes/day, n (%)				
Never smokers [#]	104 (53.6)	71 (68.3)		
Light smokers	67 (34.5)	20 (29.9)		
Heavy smokers	23 (11.9)	5 (21.7)	32.718	<0.001

Notes: *Never smokers (<100 lifetime cigarettes); former smokers (quit \geq one year ago), and current smokers (quit <one year ago);¹⁵ [#]never smokers (0 cigarettes/day); light smokers (≤ 20 cigarettes/day); and heavy smokers (>20 cigarettes/day).¹⁶

Abbreviation: EGFR, epidermal growth factor receptor.

tumor cells by microscopy. Tumor targets were manually dissected from 8 μm unstained histological sections. DNA was isolated from each target using a QIAamp[®] DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity of DNA isolated was assessed using ultraviolet spectrophotometry. The DNA extracted was stored at -20°C until use.

High-resolution melting analysis

Polymerase chain reaction (PCR) amplification was performed using a LightCycler[®] 480 (Roche, Basel, Switzerland). An EGFR gene mutation detection kit (Suzhou MicroDiag Biomedicine, Jiangsu, People's Republic of China) was used to identify mutations in exons 18–21, and each reaction contained 60 ng of sample DNA (using the same amount as the negative control), and 10 μL of 2 \times reaction buffer (containing forward and reverse primers and a high-resolution melting master). PCR cycling conditions were 95°C for five minutes, 50 cycles of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 25 seconds. After amplification, a post-melting curve program was initiated by heating to 95°C for one minute, cooling to 40°C for one minute, increasing the temperature to 65°C for one second, and then from 65°C to 95°C while continuously measuring fluorescence at 15 acquisitions per degree of temperature. At the end of the high-resolution melting program, fluorescent melting curves were analyzed using the LightCycler 480 gene scanning software (Roche, Mannheim, Germany). The mutant samples were identified by comparison with the wild-type sequence. All 194 samples were analyzed using the high-resolution melting method.

ARMS technology

An ADx-ARMS kit (Amoy Diagnostics, Xiamen, People's Republic of China) detected a total of 29 EGFR mutation hotspots in exons 18–21. The assay was performed using the LightCycler 480 according to the manufacturer's protocol. PCR cycling conditions were 95°C for five minutes, 15 cycles of 95°C for 25 seconds, 64°C for 20 seconds, 72°C for 20 seconds (for mutation enrichment), 31 cycles of 95°C for 25 seconds, 60°C for 35 seconds, and 72°C for 20 seconds. All of the 194 samples were analyzed using ADx-ATMS, and the results were then compared with those from high-resolution melting analysis.

Sequencing analysis

Primers for EGFR exons 18–21 (Table 2) were synthesized by Invitrogen (Shanghai, People's Republic of China), and PCR was performed using Hotstart Taq polymerase (Roche,

Germany). The total PCR volume of 25 μL contained 1 \times Hotstart Taq buffer, 1.5 mM MgCl_2 , 200 μM dNTP, 300 nM of each primer, 1 unit Hotstart Taq polymerase, 30 ng genomic DNA, and double-distilled H_2O . The thermal cycling conditions were 95°C for five minutes, 34 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for one minute, and a six-minute final extension at 72°C . The PCR products were then sent to Invitrogen for direct sequencing. G719S/A/C and L861Q mutations and insertions in exon 20 were further confirmed by direct sequencing.

Evaluation and statistical analysis

Responses of four patients harboring G719S/A and L861Q mutations to TKIs were evaluated according to the Response Evaluation Criteria in Solid Tumors. Overall survival was assessed from the date of starting TKI therapy until death or the last follow-up. Progression-free survival was assessed from the date of starting TKI therapy until the date of disease progression or death.

We analyzed differences in EGFR mutation rates according to clinical characteristics, in particular smoking history, using the Pearson's χ^2 test. A P value < 0.05 was considered to be statistically significant. All analyses were performed using Statistical Package for Social Sciences software version 18.0 (SPSS Inc, Chicago, IL, USA).

Results

Substitutions of L858R in exon 21 more common than in-frame deletions in exon 19

Exons 18–21 were analyzed with high-resolution melting analysis and ARMS technology using genomic DNA extracted from formalin-fixed, paraffin-embedded tissue specimens from the 194 patients with lung adenocarcinoma (Table 1). High-resolution melting and ARMS analysis showed that 96 tumors (49.5%) were EGFR mutation-positive, which is consistent with other studies. Substitution of L858R in exon 21 was most common, accounting for 53.1% (51/96) of all mutations identified, followed by in-frame deletions in exon 19 in 38.5% (37/96). Two insertions in exon 20 and seven low frequency mutations, ie, substitutions of G719S/A in exon 18 or L861Q in exon 21, comprised the remainder (Table 3 and Figure 1).

EGFR mutation rate influenced by smoking

In order to reveal the influence of smoking history on the EGFR mutation rate in patients with lung adenocarcinoma,

Table 2 Primers corresponding to exons 18, 19, 20, and 21 of epidermal growth factor receptor used for sequencing analysis (GenBank database accession number, NC_000007.13)

Primers	Sense (5'–3')	Antisense (5'–3')
Exon 18	GAGGGCTGAGGTGACCCTTGT	CTGTGCCAGGGACCTTACCTTATACA
Exon 19	ATCCCAGAAGGTGAGAAAGATAAAATTC	CAGCAAAGCAGAAACTCACATCG
Exon 20	GAAGCCCACTGACGTGCCT	CCCTTCCCTGATTACCTTTGCGA
Exon 21	GCAGCCAGGAACGTA CTGGTG	TCCTTACTTTGCCTCCTTCTGCA

we considered these two variables together, and found that 71 of 96 (74.0%) EGFR mutation-positive lung adenocarcinomas came from never smokers (71 of 104, 68.3%, Table 1). Six of 17 (35.3%) former smokers and 19 of 73 (26.0%) current smokers also had EGFR mutations. EGFR mutations were more frequently detected in lung adenocarcinomas from never smokers than those from smokers (68.3% versus 27.9%; hazard ratio 0.404; 95% confidence interval 0.277–0.530; $\chi^2 = 31.645$, $P < 0.001$). The difference in EGFR mutation rates was not statistically significant between former smokers and current smokers (35.3% versus 26.0%; $\chi^2 = 1.523$; $P = 0.467$), which might be attributable to the small number of former smokers included in the study.

We further categorized our 194 patients into never smokers ($n = 104$), heavy smokers ($n = 23$), and light smokers ($n = 67$), and investigated the influence of number of cigarettes smoked per day on EGFR mutation rate. The EGFR mutation rate in heavy, light, and never smokers were 5/23 (21.7%), 20/67 (29.9%), and 71/104 (68.3%), respectively, with significant differences (hazard ratio 0.397; 95% confidence interval 0.271–0.515; $\chi^2 = 32.718$, $P < 0.001$).

Table 3 Characteristics of patients with different epidermal growth factor receptor mutations

	19 Del	L858R	Exon 20	Uncommon mutations
Total patients (n = 96)	37	51 [#]	2 [*]	7 [#]
%	38.5	53.1	2.1	7.3
Gender				
Males	14	17	1	5
Females	23	34	1	2
Smoking status				
Never smokers	28	40	1	2
Former smokers	2	3	0	1
Current smokers	7	8	1	4
Cigarettes per day				
Never smokers [#]	28	40	1	2
Light smokers	8	7	1	4
Heavy smokers	1	4	0	1

Notes: *Insertions in exon 20; [#]a patient harbored both G719S and L858R mutations; uncommon mutations are substitutions of G719 in exon 18 and L861 in exon 21.

Abbreviations: 19 Del, exon 19 mutation of EGFR; L858R, Substitutions of L858 in exon 21 of EGFR.

Five of seven G719S/A or L861Q mutations were from smokers

Seven of the 194 patients with lung adenocarcinoma in this study had low frequency EGFR mutations, including four substitutions of G719 in exon 18 and three of L861 in exon 21 (Table 4). Direct sequencing confirmed that these mutations were G719S, G719A, G719 A, and G719S ($n = 1$ each), and L861Q ($n = 3$), respectively. Five of these mutations (5.6%) were derived from smokers (one former light smoker, one current heavy smoker, and three current light smokers) and two (1.9%) from never smokers, but the difference was not statistically significant because of the small number of patients. Interestingly, among these mutations, four substitutions of G719S and G719 A in exon 18 were all derived from smokers with a smoking history at least 20 pack-years.

Partial response in TKI-treated patients with G719S/A and L861Q EGFR mutations

The response of four patients with G719S/A or L861Q mutations treated with TKIs was also evaluated in this study (Table 4). Treatment of the other three patients was still in progress at the time of completion of the study. The four treated patients all showed a partial response to TKIs, with prolonged overall survival (14.5 months versus 6.8 months) and progression-free survival (6.8 months versus 3.1 months) compared with patients with similarly staged lung adenocarcinoma without EGFR mutation and treatment with TKI (data from an as yet unpublished study at our institution). The fact that all four patients had a more than 20-year smoking history indicates that patients with lung adenocarcinoma and G719S/A or L861Q mutations of EGFR can respond well to TKIs, regardless of their smoking history.

Discussion

Multiple prospective studies have demonstrated that mutations in the tyrosine kinase domain of EGFR are the best predictors of response to TKIs and the progression-free survival benefit.^{2,17} Mutations in EGFR have been most frequently identified in patients with lung adenocarcinoma,¹⁸

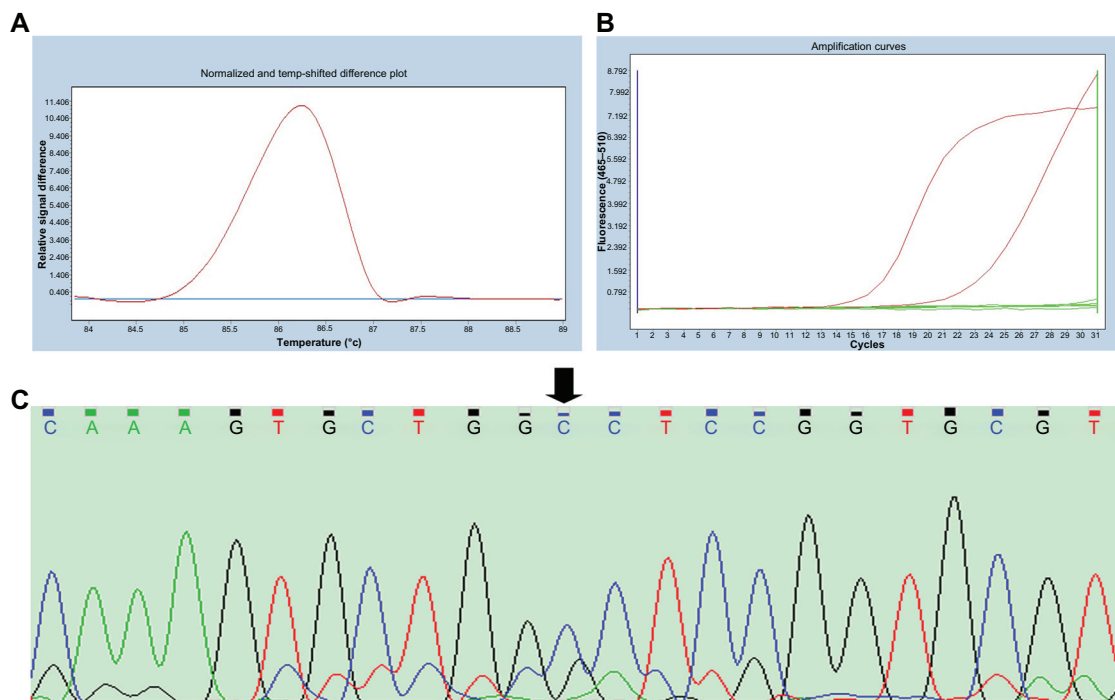


Figure 1 Representative results for HRM, ARMS, and sequencing. **(A)** Hotspot mutation in exon 18 detected using HRM. **(B)** Substitution of G719 mutation detected using ARMS. **(C)** Substitution of G719 mutation detected using direct sequencing. Black arrow indicates substitution site.

Abbreviations: HRM, high-resolution melting; ARMS, amplification refractory mutation system.

particularly in those with no smoking history.¹⁹ Pao et al²⁰ found that EGFR mutations were commonly detected in patients with NSCLC who had never smoked as opposed to former or current smokers (7/15 versus 4/81; $P = 0.0001$), and that tumors likely to be mutation-positive could be identified by specific clinical characteristics. Pham et al¹⁵ identified EGFR mutations in 34 of 67 (51%) never smokers, 29 of 151 (19%) former smokers, and two of 47 (4%) current smokers. Significantly fewer EGFR mutations are found in people who have smoked for more than 15 pack-years or who have stopped smoking less than 25 years ago.

Our studies found that 71 of 96 (74.0%) EGFR mutation-positive lung adenocarcinomas were derived from patients

who had never smoked, whereas only six of 17 (35.3%) and 19 of 73 (26.0%), respectively, were from former smokers and current smokers harboring EGFR mutations. Moreover, EGFR mutations were found in only five of 23 (21.7%) heavy smokers, which is a significantly lower mutation rate than in light smokers (20/67, 29.9%) and never smokers (71/104, 68.3%). These results further confirm that smoking history has a significant influence on the probability of EGFR mutation.

Two classes of EGFR mutations, ie, deletions in exon 19 and substitutions of L858R in exon 21, are the most frequent, representing 85%–90% of all EGFR mutations reported,¹⁵ and are also the mutations which show the best response to gefitinib or erlotinib. In this study, these two mutations were

Table 4 Characteristics of seven patients harboring low frequency EGFR mutations

Number	Age (years)	Gender	Mutation type	Smoke	Smoking history (years)	Disease stage	Effectiveness of TKIs	
							OS (months)	PFS (months)
p075	61	Male	G719S	CLS	20	III	19	8
p087	66	Male	G719A	CHS	40	IIIb	16	6
p089	58	Male	L861Q	CLS	21	IV	13	6
p161	63	Male	G719A	FLS	20*	II	10	7
P405 ^a	79	Female	L861Q	NS	–	IIIb	–	–
P426 ^a	57	Female	L861Q	NS	–	IV	–	–
P448 ^a	62	Male	G719S/L858R	CLS	42	IV	–	–

Notes: G719S/A, substitutions of G719 in exon 18 of EGFR; L861Q, substitutions of L861 in exon 21 of EGFR; *TKI treatment in progress; *quit smoking more than 20 years earlier.

Abbreviations: EGFR, epidermal growth factor receptor; CLS, current light smokers; CHS, current heavy smokers; FLS, former light smokers; NS, never smokers; OS, overall survival; PFS, progression-free survival; TKIs, tyrosine kinase inhibitors.

representing 38.5% (37/96) and 53.1% (51/96), respectively, accounting for 91.6% of all EGFR mutations found. These two types of mutation, as well as T790M and insertions in exon 20, have been extensively reported and their clinical significance is widely known.

However, other mutations in the EGFR tyrosine kinase domain, including amino acid substitutions at G719, S768, and L861, have been reported to be uncommon. Wu et al¹⁴ identified 78 (12.4%) patients with uncommon mutations among 1261 patients with NSCLC, and also confirmed that the G719S/A/C and L861Q mutations were sensitive to EGFR TKIs. The response rate to TKIs was 57.1% and the median progression-free survival was 6.0 months in their patients with G719S/A/C or L861Q mutations. In our study, EGFR mutations consisted of two “hotspots” (88/96, 91.6%), two insertions in exon 20, and (7/96, 7.3%) low frequency mutations, substitutions at G719S/A in exon 18 and L861Q in exon 21. Of interest is that five of these mutations were identified in smokers (one former light smoker, one current heavy smoker, and three current light smokers), in particular substitutions of G719S and G719A in exon 18. Whether this finding is coincidental or whether there is a relationship between G719S and G719A EGFR mutations and smoking history in patients with lung adenocarcinoma was unclear in our study because of the small number of patients with G719S/A mutations included, and further research is needed to clarify this issue.

Although the frequency of EGFR mutation is significantly higher in patients who have never smoked, mutations in former and current smokers account for a considerable proportion of all mutations, being 13% for former smokers and 5% for current smokers, according to a report by Pietanza et al.²¹ The EGFR mutation rates of 35.3% and 26.0% seen in our study for former smokers and current smokers, respectively, are still significant.

In this study, we evaluated the effectiveness of TKIs in the treatment of four patients with lung adenocarcinoma and harboring G719S/A or L861Q EGFR mutations. These patients all partially responded to TKIs, and their median overall survival (14.5 versus 6.8 months) and progression-free survival (6.8 versus 3.1 months), respectively, were markedly longer than in patients with similarly staged lung adenocarcinoma but without EGFR mutation or treatment with TKI. Further, their smoking history (all had smoked for more than 20 years) indicated that patients with lung adenocarcinoma and G719S/A or L861Q EGFR mutations can respond well to TKIs, regardless of smoking history. Our results add to the data indicating that identification of EGFR

mutations should be considered in all patients with lung adenocarcinoma, regardless of their smoking history. Meanwhile, the relationship between low frequency mutations of EGFR, especially G719S/A/C in exon 18, and smoking history might be important and influence the clinical treatment and prognosis of patients with lung adenocarcinoma and a lengthy smoking history.

The techniques currently used to identify EGFR mutations in patients with lung cancer include PCR single-strand conformation polymorphism,²² high-resolution melting analysis,²³ ARMS technology,²⁴ mutant-enriched PCR, peptide nucleic acid-locked nucleic acid PCR clamping,²⁵ and direct sequencing. These methods come with distinct advantages and disadvantages. In this study, high-resolution melting and ARMS were used to detect EGFR mutations in patients with lung adenocarcinoma, and the results might offer guidance when planning treatment with EGFR TKIs. Our results show that both high-resolution melting analysis and ARMS technology have similar sensitivity for detection of EGFR mutations (96 of 194 tumors were EGFR mutation-positive on both techniques in our study). These two methods for detection of EGFR mutation are convenient and reliable, and should reduce the rate of false results which can result from other complicated and less sensitive methods, such as traditional sequencing. Therefore, these two methods can be used widely and safely for detection of EGFR mutations in patients with lung adenocarcinoma.

Disclosure

The authors report no conflicts of interest in this work.

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