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RESEARCH

Biological Characteristics and Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors Efficacy of EGFR Mutation and its Subtypes in Lung Adenocarcinoma

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Abstract Mutation of epidermal growth factor receptor (EGFR) gene has been reported to be present in lung adenocarcinoma (LAC). In this study, we extensively investigated the impact of patients' biological characteristics on EGFR mutation and the impact of EGFR mutation subtypes on targeted therapy of advanced LAC. We examined EGFR exons18to21status in169 LAC patients by direct sequencing to study the impact of patients' biological characteristics on the EGFR mutational spectrum. And then, 59 patients with advanced LAC harboring EGFR exon 19 deletions(del 19) or exon 21 point mutation(L858R) mutations received first-line treatment of gefitinib or erlotinib, the efficacy of treatment, and the progression-free survival (PFS) of these patients were recorded. The frequency of the EGFR mutation and its subtypes and the variables associated with the EGFR mutation after removing the confound factors were investigated by the logistic analysis using all samples ($n=169$). The EGFR mutation was significantly associated with well-differentiated tumor and excessive household cooking fumes($P<0.05$). The deletions in exon 19 were more frequently associated with well-differentiated tumor ($P<0.05$). The overall frequency of the EGFR mutation was 49 %. Then the impact of EGFR mutation subtypes on targeted therapy were investigated by the retrospective analysis on 59 advanced LAC patients with del 19 or L858R mutations and treated first-line with erlotinib or gefitinib. The deletions in exon 19 got longer PFS ($P<0.05$). But there were no differences in PFS between erlotinib therapy and gefitinib therapy. EGFR mutations were more frequently in high tumor differentiation and excessive household cooking fumes LAC. The del 19 mutation rate is relatively high with a high differentiation degree in advanced lung adenocarcinoma. The deletions in exon 19 may benefit more from first-line targeted therapy of advanced LAC compared with exon 21 point mutation L858R. There was no significant difference between the efficacy of gefitinib and erlotinib treatments associated with EGFR mutation and its subtypes.

Keywords Lung adenocarcinoma(LAC) · Epidermal growth factor receptor (EGFR) · Erlotinib · Gefitinib · Exon 19 deletions(del 19) · Exon 21 point mutation(L858R)

Abbreviations

LAC	Lung adenocarcinoma
EGFR	Epidermal growth factor receptor
del 19	Exon 19 deletions
L858R	Exon 21 point mutation
PFS	Progression-free survival

Introduction

Lung cancer is a common malignancy and is the primary cause of all cancer-related deaths, world-wide. The major forms of lung cancer include adenocarcinoma and squamous carcinoma, and the incidence of lung adenocarcinoma has increased rapidly in recent years [1]. EGFR mutations represent the most common mutations of non-small cell lung carcinoma (NSCLC) [2, 3], especially in lung adenocarcinoma [4]. Numerous studies have reported that EGFR mutations are more prevalent in the Asian population, and in women and non-smokers [5, 6], however, this gender difference remains unclear and further studies are required. Therefore, it is critical to study the relevant factors associated with EGFR mutations.

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Small molecule tyrosine kinase inhibitors (TKIs), such as erlotinib (Tarceva, Roche Pharmaceuticals) and gefitinib (Iressa, AstraZeneca) represent a breakthrough in the treatment of advanced NSCLC, and are approved as first-line treatments of patients with advanced NSCLC with EGFR dominant mutations [7–9]. Previous clinical trials revealed that treatment of advanced NSCLC patients harboring EGFR mutations with gefitinib, significantly prolonged the survival of non-smoking and Asian patients compared with supportive treatment [10]. However, in an independent study with a large patient cohort, it was found that erlotinib could benefit all feature subsets of patients [11]. Although both drugs have similar mechanisms of action, the benefit of each drug for specific patient subsets still needs to be determined. In this study, we investigate the efficacy of gefitinib and erlotinib treatment in advanced NSCLC patients with EGFR mutations, in order to facilitate the choice of drug for treatment in clinical practice.

Material and Methods

Tissue Specimens and Patient Cases

A hundred and sixty-nine lung adenocarcinoma specimens were collected from patients at the Department of Respiratory Medicine,Xiangya Hospital of Central South University, China between September 2009 and April 2011. Tumor specimens, including paraffin blocks or frozen tissues of primary tumors and metastases tissue from bronchoscopic, lymph node and percutaneous needle biopsies.

Patients were histopathologically diagnosed as primary lung adenocarcinoma, newly diagnosed and untreated, no history of other tumor. The patients' clinical data, including demographic information, degree of tumor differentiation, smoking status, passive smoking status, performance status, exposure to household cooking fumes, biopsy site (primary tumor/metastasis) and imaging studies, were recorded (Table 1).

Of the 169 patients with advanced lung adenocarcinoma, 60 (one patient left the study due to severe gastrointestinal reaction) harbored mutations in EGFR (del 19 or L858R) as detected by direct sequencing, and accepted first-line treatment with gefitinib or erlotinib. The timing and order of different EGFR TK inhibitors depended on the physicians' discretion. Gefitinib was taken 250 mg daily orally while erlotinib was taken 150 mg daily. Baseline assessments were usually performed 2 weeks before treatment. Chest computed tomography scan (including liver and adrenal glands) was done every 2–3 months as routine clinical practice and as needed to confirm response and disease progression.

Table 1 General statistical information of patients

Feature	The number of cases(N=169)	
Age (years)	No.	%
M±SD	53.21±12.338	
Distribution	21–80	
Sex		
Male	88	52.1
Female	81	47.9
Smoking		
No	112	66.3
Yes	57	33.7
Passive Smoking		
No	92	54.4
Yes	77	45.6
Household cooking fumes		
No	89	60.3
Yes	58	39.7
Tumor differentiation		
Low	35	23.6
Media	80	54.1
High	33	22.3
Biopsy site		
Primary tumor	141	83.4
Metastases	28	16.6

The cut-off date for data collection was September 31, 2012. The primary endpoint was Progression Free Survival (PFS). PFS was measured from the first day of gefitinib or erlotinib treatment until the first objective or clinical sign of disease progression, or death in the absence of documented progression. Informed consent was obtained from all patients in accordance with the guidelines of Central South University, and the study protocols were approved by the Ethics Committee of Central South University in July 2009.

DNA Extraction

Paraffin-embedded tissues (10 μm thick) were prepared and dried following deparaffinization with xylene and washing with anhydrous ethanol. Fresh tissue (20 mg) was removed and cut into pieces. DNA was extracted using TIANamp FFPE DNA Kit (Tiagen Biotech, Beijing) in accordance with the manufacturer's instructions.

Amplification and Sequencing of Polymerase Chain Reaction (PCR)

The primer sequence of the EGFR18-21 exon was designed in accordance with previous publications [5, 12–14]. All primer

sequences were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Primers for PCR amplification in nested reactions for exons 18, 19, 20and 21 of EGFR were as follows: exon 18 (first PCR, sense 5′-CAAATGAGCTGGCAAGTGCCGTGTC-3′ and antisense 5′-GAGTTTCCCAAACACTCAGTGAAAC-3′; nested PCR, sense 5′-CAAGTGCCGTGTCCTGGCACCC AAGC-3′ and antisense 5′-CCAAACACTCAGTGAAAC AAAGAG-3′); exon 19 (first PCR, sense 5′-GCAATATC AGCCTTAGGTGCGGCTC-3′ and antisense 5′-CATAGA AAGTGAACATTTAGGATGTG-3′; nested PCR, sense 5′-GTGCATCGCTGGTAACATCC-3′ and antisense 5′-TGTG GAGATGAGCAGGGTCT-3′); Exon 20(first PCR, sense 5′-CCATGAGTACGTATTTTGAAACTC-3′ and antisense 5′-CATATCCCCATGGCAAACTCTTGC-3′; nested PCR, sence 5′-GAAACTCAAGATCGCATTCATGC-3′ and antisense 5′-GCAAACTCTTGCTATCCCAGGAG-3′) exon 21 (first PCR, sense 5′-CTAACGTTCGCCAGCCATAAGT CC-3′ and antisense 5′-GCTGCGAGCTCACCCAGAATGT CTGG-3′; nested PCR, sense 5′-GCTCAGAGCCTGGCAT GAA-3′ and antisense 5′-CATCCTCCCCTGCATGTGT-3′). All PCR assays were carried out in a 25-μL volume that contained 2 μl of genomic DNA, 1 μl of primer 1 F,1 μl of primer 1R and 2×Taq PCR MasterMix (Tiangen Biotech (Beijing) Co.,LTD.). DNA was amplified for 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a 5-min extension at 72 °C. For PCR amplification, whole genomic DNA was extracted from paraffin specimens and nested PCR was performed for two amplification cycles. For fresh specimens, whole genomic DNA was extracted and general PCR amplification was performed. PCR amplification products were confirmed by gel electrophoresis. DNA bidirectional sequencing was performed by Shanghai Shenggong Biological Engineering Technology & Services Co., Ltd. Software ChromasPro1.5 was used to interpret gene sequencing. The Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/blast.cgi) was used to compare the DNA sequence to the standard, wild-type DNA sequence in order to identify mutated sites. Variations observed at the corresponding locations of both forward and reverse nucleotide sequences were noted as putative gene mutations.

Statistical Analysis

Software SPSS 17.0 was used for statistical analysis. All tests were two-sided, and the significance level was $P=0.05$. EGFR mutations and the biological characteristics of selected cases were analyzed with logistic regression. Efficacy of gefitinib and erlotinib treatment was assessed by Kaplan-Meier survival analysis.

Results

Correlation of EGFR Mutations with Clinical and Pathological Features of Advanced Lung Adenocarcinoma Patients

Analysis of tissue specimens from 169 patients with advanced lung adenocarcinoma, identified EGFR mutations in 83 cases (mutation rate, 49.1 %). Of these, three patients harbored exon 18 mutations (3.6 %), 42 patients contained exon 19-deletion mutations (50.6 %), 34 patients harbored exon 21 mutations (41 %) and four patients contained both exon 19-deletion and exon 21 mutations (4.8 %). The T790M mutation was not detected.

Univariate analysis revealed that the presence of EGFR mutations was associated with tumor biopsy site (primary tumor/metastasis), smoking, passive smoking, household cooking fumes and tumor differentiation degree ($P<0.05$), and was not related to age or sex (Table 2). Logistic regression analysis demonstrated that EGFR mutation and tumor differentiation of lung adenocarcinomas were positively correlated (odds ratio [OR]=1.346) (1.016, 1.785) ($P<0.05$). EGFR mutation was also positively correlated with the presence of household cooking fumes (OR=2.866) (1.163, 7.064) ($P<0.05$) (Table 3). Univariate analysis of differences between clinical and pathological features of advanced lung adenocarcinoma and del 19/L858R, revealed no significant differences in del 19/L858R and gender, age, biopsy site (primary tumor/metastasis), smoking, passive smoking, household cooking fumes ($P>0.05$) but del 19 were more frequently associated with well-differentiated tumor ($P<0.05$) (Table 4).

Correlation Between the Presence of EGFR Mutation (del 19/L858R) and the Efficacy of Treatment with EGFR-TKIs (Erlotinib/Gefitinib)

Of the 60 patients included in this analysis, one patient terminated treatment owing to severe gastrointestinal side effects, and was not included in the analysis. The remaining 59 patients were treated with EGFR-TKIs (gefitinib, 27 patients; erlotinib, 32 patients), with no significant differences in age or sex distribution between these two treatment groups ($P>0.05$.) All patients were assessed for treatment efficacy up to the last follow-up date (August 1, 2012; Table 5).

Assessment of efficacy at 7 months revealed disease control rates (DCR) of 75.7 % for del 19 ($P<0.05$) and 50 % for L858R ($P<0.05$). PFS in the del 19 group was significantly longer than that in L858R group ($P<0.05$; Fig.1a). A difference in PFS between gefitinib and erlotinib treatment groups was also observed. For advanced lung adenocarcinoma patients harboring EGFR mutations (del 19 or L858R), the PFS of patients treated with erlotinib was extended compared with that of the gefitinib treatment group; however, this difference

Table 2 Univariate analysis of the correlation of clinical and pathological features of advanced lung adenocarcinoma to EGFR mutation status

	Negative	Positive	Frequency (%)	χ^2 value	P-value
Sex					
Male	48	40	45.5	0.983	0.321
Female	38	43	53.1		
Biopsy site					
Primary tumor	67	74	52.5	3.867	**0.049**
Metastasis	19	9	32.1		
Smoking					
No	49	63	56.25	6.769	**0.009**
Yes	37	20	40		
Passive Smoking					
No	38	54	58.7	7.420	**0.006**
Yes	48	29	37.7		
Cooking oil fumes ($n=147$)					
No	57	32	36.0	16.947	**<0.001**
Yes	17	41	70.7		
Differentiation	Mann–Whitney U test			$Z=-2.864$	**0.004**
Poorly differentiated	26	9	25.7		
Moderately differentiated	40	40	50		
Well-differentiated	12	21	63.6		
Age (year)					
<60	55	60	52.2	1.35	0.254
≥60	31	23	42.6		

The entries in bold show that the presence of EGFR mutations was associated with tumor biopsy site (primary tumor/metastasis), smoking, passive smoking, household cooking fumes and tumor differentiation degree ($P<0.05$)

was not statistically significant ($P>0.05$) (Fig. 1b). For EGFR del 19 lung adenocarcinoma patients, there was no significant difference in PFS between patients treated with erlotinib or gefitinib ($P>0.05$) (Fig. 1c). Similarly, for EGFR L858R lung adenocarcinoma patients, there was no significant difference in PFS between patients treated with erlotinib or gefitinib ($P>0.05$) (Fig. 1d).

Discussion

Although there are limited studies describing the relationship between household cooking fumes and EGFR mutations, in accordance with our results, several groups have shown that household cooking fumes and the incidence of lung adenocarcinoma are related [15–18]. Furthermore, we demonstrate that household cooking fumes are correlated with EGFR mutations ($B=1.517$, OR=4.561, 95 % CI: 1.810 to 11.492; $P=0.001$). This may reflect the different traditional life between China and the West, since women represent a high-risk population for adenocarcinoma and are the main group exposed to cooking fumes. However, in this study, we observed no difference in exposure to household cooking fumes between men and women (32 % vs 47.2 %).

Table 3 Logistic regression analysis of the correlation of EGFR mutation status to patient biological characteristics

	B	Odds ratio (95 % confidence interval)	P-value
Biopsy	−0.507	0.602 (0.213, 1.702)	0.339
Differentiation	0.626	1.870 (1.045, 3.346)	**0.035**
Smoke	−0.260	0.771 (0.303, 1.958)	0.584
Passive Smoking	−0.536	0.585 (0.253, 1.351)	0.209
COF ($n=147$)	1.517	4.561 (1.810, 11.492)	**0.001**

The entries in bold show that EGFR mutation was positively correlated with the differentiation of lung adenocarcinomas and excessive household cooking fumes

The effect of household cooking fumes on EGFR mutation may be related to the presence of aldehydes (tt-DDE, trans, trans-2,4-decadienal). Indeed, previous reports in Taiwan revealed that the total concentration of aldehydes (tt-DDE, trans, trans-2,4-decadienal), polycyclic aromatic compounds and acrylic thiamine in kitchen fumes was very high (86 %) [19]. In support of this, exposure of animals to tt-DDE led to respiratory epithelial pre-cancerous lesions. At the molecular level, tt-DDE exposure leads to phosphorylation of Rb protein and abnormal expression that may increase the risk of tumorigenesis [20] and potentially lead to EGFR abnormalities. However, the specific mechanism of action remains unclear and requires further study because of the complex composition of household cooking fumes.

Among the 169 cases of lung adenocarcinoma, we observed that EGFR mutations were positively correlated to the degree of lung adenocarcinoma differentiation. Similar conclusions were obtained by other groups in China [21,

Table 4 Relationship between EGFR mutation sites (del 19 and L858R) and the clinical and pathological features of lung adenocarcinoma ($n=76$)

	Exon 19 deletions	Exon 21 point mutations	χ^2 value	P-value
Sex				
Male	20	18	0.213	0.645
Female	22	16		
Biopsy site	Fisher's exact test			
Primary tumor	36	31		0.723
Metastasis	6	3		
Smoking				
No	30	28	1.241	0.265
Yes	12	6		
Passive Smoking				
No	28	19	0.926	0.336
Yes	14	15		
Cooking oil fumes ($n=67$)				
No	13	17	2.363	0.124
Yes	23	14		
Differentiation ($n=66$)	Mann–Whitney U test		$Z=-2.537$	**0.011**
Poorly differentiated	4	4		
Moderately differentiated	14	23		
Well-differentiated	17	4		
Age (year)				
<60	24	16	0.111	0.738
≥60		116		

The entries in bold show that the deletions in exon 19 were more frequently associated with well-differentiated tumor ($P<0.05$)

22], Japan [23] and Hong Kong [24], based on the study of NSCLC. In contrast, there have been no reports from Western scholars, and further studies with larger patient cohorts are necessary to determine whether these results are related to race.

Within the group of advanced lung adenocarcinoma patients receiving first-line erlotinib or gefitinib treatment, EGFR del 19 mutations were observed in 37 cases and L858R mutations in 22 patients. Patients harboring del 19 mutations exhibited higher DCR in the seventh, ninth and 11th assessment. Survival analysis demonstrated that PFS of the del 19 group (14.2 months) was significantly different to that of the L858R group (9.6 months) ($P<0.05$). Previous clinical studies also demonstrated higher treatment efficacy in patients harboring the EGFR del 19 deletion mutation and longer PFS (OR: 3.08; 95 % CI, 1.63–5.81; $P=0.001$) compared with patients with L858R point mutations in NSCLC [25]. The EGFR del 19 mutation occurs at lysine residue 745 (K745), which is a key ATP binding site. Deletion of several downstream amino acids would change the configuration of the EGFR catalytic site (αC-helix) and modify the ability of the receptor tyrosine kinase to bind ATP, which may improve the sensitivity of tumor cells to small molecule inhibitor drugs. The majority of mutations in exon 21 were substitution mutations (the main mutation being L858R), occurring in the highly conservative DFG motif close to the kinase activation loop [26]. However its relationship to key ATP binding sites is not as close to that of the αC-helix, and may explain why patients with EGFR del 19 deletion mutations are more sensitive to treatment with erlotinib or gefitinib compared with patients with the L858R point mutation. In the present study, we observed no difference in the efficacy between first-line erlotinib and gefitinib treatment in advanced lung adenocarcinoma patients with EGFR advantage mutations (del 19 deletion mutation or L858R point mutation). A study by Kim et al. (2010) compared the efficacy of erlotinib and gefitinib treatments in 467 patients with NSCLC who did not respond to chemotherapy. The majority of patients did not have EGFR detection, and no significant differences in reaction rates, median PFS and overall survival were observed between the two treatment groups [27]. In 2012, the same group performed

Table 5 The relationship between EGFR mutation sites (del 19 and L858R) and therapeutic efficacy of tyrosine kinase inhibitors in patients with advanced lung adenocarcinoma ($n=59$)

Time (month)	del 19 (37)		L858R (22)		P-value
	PD	DCR	PD	DCR	
1	0	100	0	100	
3	2	94.6	2	90.9	0.624
5	2	89.2	4	72.7	0.152
7	5	75.7	5	50.0	**0.044**
9	1	73.0	2	40.9	**0.015**
11	2	67.6	1	36.4	**0.020**

The entries in bold show that patients harboring del 19 mutations exhibited higher DCR in the seventh, ninth and 11th assessment

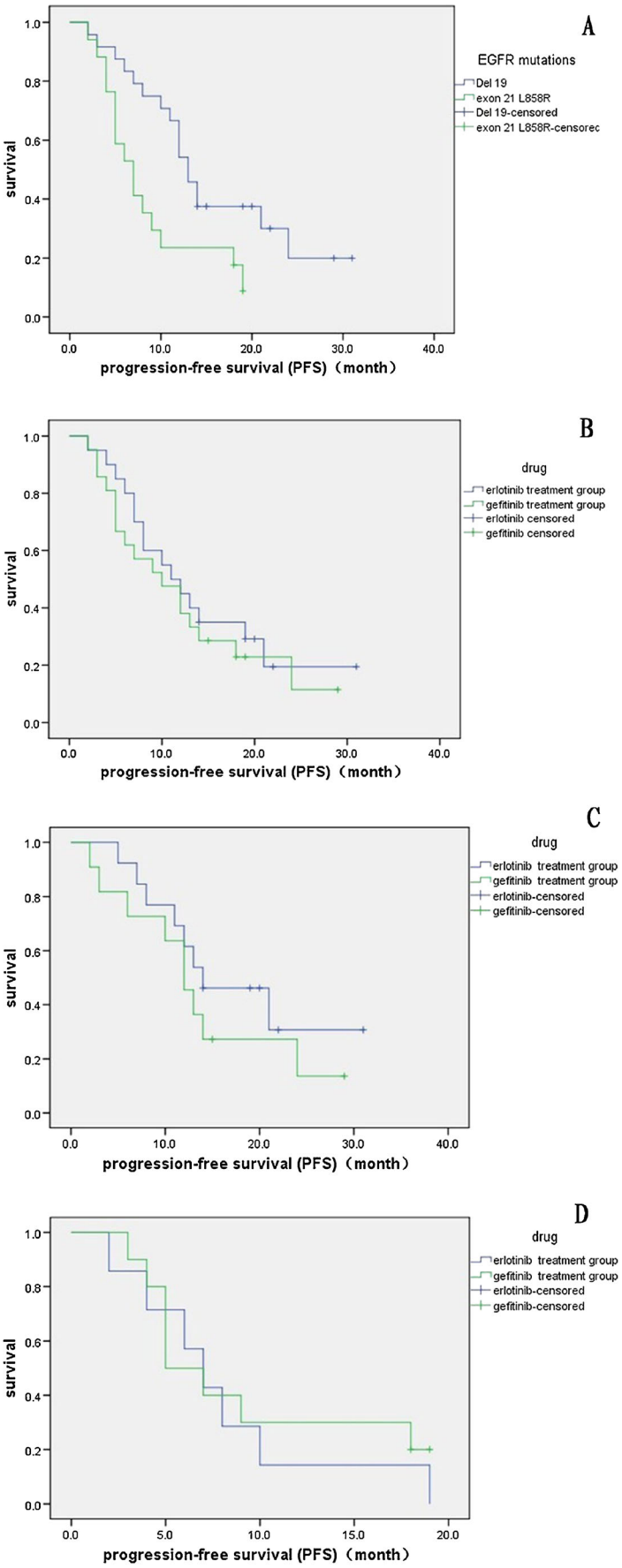

Fig. 1 **a** Comparison of the difference in progression-free survival (PFS) periods of advanced lung adenocarcinoma patients (del 19 and L858R groups) following first-line treatment with EGFR-tyrosine kinase inhibitors (TKIs). The PFS of del 19 group was longer than that of the L858R group (both median PFS periods were 14.2 months vs 9.6 months) ($P<0.05$). **b** Comparison of the difference in PFS periods of advanced lung adenocarcinoma patients with EGFR advantage mutations in the erlotinib treatment and gefitinib treatment groups. The difference between these two PFS was not statistically significant ($P>0.05$). **c** Comparison of the difference in PFS periods of advanced lung adenocarcinoma patients with EGFR del 19 mutations in the erlotinib treatment and gefitinib treatment groups. The difference between these two PFS was not statistically significant ($P>0.05$). **d** Comparison of the difference in PFS periods of advanced lung adenocarcinoma patients with EGFR L858R point mutations in the erlotinib treatment and gefitinib treatment groups. The difference between these two PFS was not statistically significant ($P>0.05$)

a random comparison and observation study on the efficacy of erlotinib and gefitinib treatment in patients with EGFR mutations or advantage mutations. No significant differences were observed in the reaction rates, median PFS period and overall survival period between the two treatment groups, regardless of EGFR mutation status [28]. This may be due to the very similar therapeutic mechanism of action of erlotinib and gefitinib. The selectivity of these two drugs according to EGFR mutation sites had no guiding significance.

In summary, in advanced lung adenocarcinoma patients, exposure to household cooking fumes and high degree of tumor differentiation may be positive predictors of EGFR mutation. While non-smoking and no passive smoking exposure also had certain reference value, gender and age should not be used as predictors of EGFR mutation status in lung adenocarcinoma patients. Within the population with EGFR advantage mutations, a higher efficacy of first-line erlotinib or gefitinib treatment in advanced lung adenocarcinoma patients with EGFR del 19 deletions compared with patients with L858R point mutations may be predicted. However, since there is no difference between first-line treatment with erlotinib or gefitinib in advanced lung adenocarcinoma patients with EGFR advantage mutations, from a clinical standpoint, it is not necessary to choose specific small molecule TKIs according to EGFR mutation sites.

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