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ORIGINAL PAPER

EGFR Mutations are More Frequent in Well-Differentiated than in Poor-Differentiated Lung Adenocarcinomas

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Abstract Somatic mutations in epidermal growth factor receptor (EGFR) tyrosine kinase domain, particularly deletions in exon 19 and point mutation in exon 21, are associated with clinical outcome in patients with lung adenocarcinoma, suggesting that EGFR mutation would have an important role in clinical decision making. DNA was extracted from the excised specimens of 60 lung adenocarcinoma patients with phenol-chloroform and ethanol precipitation. Exon 19 and 21 were amplified by PCR, and direct sequenced from both sense and antisense directions. EGFR somatic mutations were present in 13 of 60 patients (21.67%), including seven cases of in-frame deletion in exon 19 around codon 746 and six cases of amino acid substitution in exon 21. Exon 21 mutation is more frequent in adenocarcinomas with bronchi-alveolar component than exon 19 deletions. Mutations were more prevalent in well-differentiated adenocarcinomas (9/27, 33.33%) than in moderate to poor-differentiated adenocarcinomas (4/33, 12.12%) ($P<0.05$). Adenocarcinomas with bronchi-alveolar components had higher mutation frequency (8/22,36. 36%) than those without bronchi-alveolar components (5/38, 13.16%) ($P<0.05$). In this study, female patients had more mutation rate than male patients. This trend was also observed in the patients with pathologic stage I–II compared with stage III–IV, but neither of them was statistically significant. Patients with cisplatin-based adjuvant chemotherapy had no significantly prolonged survival compared with single radical resection. But patients with EGFR mutation had relative longer survival. In conclusion, our study suggest that EGFR mutations may be a valuable prognostic factor for disease free survival of surgically treated lung adenocarcinoma patients independently from adjuvant chemotherapy.

Keywords Chemotherapy · Epidermal growth factor receptor · Lung adenocarcinoma · Polymerase chain reaction · Sequence analysis

Abbreviations

EGFR	Epidermal growth factor receptor
NSCLC	Non-small cell lung carcinoma
PCR	Polymerase chain reaction
MARK	Mitogen activated protein kinase
PI3K	Phosphatidylinositol 3 kinase

Introduction

Lung cancer is the leading cause of cancer-related death because of its high incidence, malignant behavior and lack of major advancements in treatment strategy. Non-small cell lung carcinoma (NSCLC) makes up 80% of all primary pulmonary malignant tumors and the frequency of adenocarcinoma has recently become higher and higher. There is a requirement for the development of more effective systemic treatment with fewer side-effects. Protein kinase activation by somatic mutation is a common mechanism of tumor genesis. Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase identified as being highly expressed in cancer cells including NSCLC by immuno-

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histochemical staining. The targeting of the ATP binding pocket of the EGFR tyrosine kinase, by the small molecule drugs such as gefitinib (Iressa), represents a promising new therapeutic strategy for chemo-refractory NSCLC patients in Japan and the United States. However, it is apparent that only a subset of patients responds to such treatment. A multitude of data from different groups described that EGFR tyrosine kinase domain gene mutation occurred in highly selected subpopulations of NSCLC patients: adenocarcinoma histology, never-smoke status, East Asian ethnicity and female gender [1–5]. Although a variety of different mutations are seen spanning the entire EGFR tyrosine kinase domain, 89% reside in exon 19 and exon 21. To determine whether EGFR tyrosine kinase domain mutations are early events in the pathogenesis of lung adenocarcinoma, we tested and analyzed the mutation of EGFR exon 19 and 21 for lung adenocarcinoma patients to find the relation between the mutation rate and clinic pathological features.

Materials and Methods

Tumor Collection and DNA Extraction

Surgically resected specimens of 60 primary lung adenocarcinoma patients and corresponding adjacent nonmalignant lung tissues were obtained from Tianjin Chest Hospital from 2000 to 2006. Complete clinical data were obtained from medical records. The patients consisted of 22 males and 38 females, none of whom had documented treatment with gefitinib before the operation, with an age at diagnosis ranging from 33 to 80 (median 63) years. There were 34 never-smokers and 26 ever-smokers including current and former smokers. Adenocarcinomas were histologically classified according to the presence or absence of bronchioalveolar components. Clinicopathologic staging was determined according to International Union Against Cancer tumor-node-metastasis classification of lung malignant tumors. Representative sections from tissue used for DNA extraction were stained with H&E to determine tumor histological subtype and ensure that specimens contained at least 50% tumor cells. Genomic DNA was isolated by digestion with proteinase K in 56°C followed by phenol-chloroform (1:1) extraction and ethanol precipitation.

PCR and Sequencing Analysis

EGFR mutations were detected using PCR-based direct sequencing of the 19 and 21 exon. PCR amplification was carried out with an initial 5-min denaturation at 95°C, followed by 35 cycles of 30 s at 95°C (denaturation), 30 s at 55–62°C (annealing), and 20 s at 72°C (extension), and then by 5 min at 72°C and a final cooling to 4°C. The primers and condition of PCR amplification are shown in Table 1. For one PCR reaction, 100 ng of genomic DNA were mixed with 0.5 μl of each primer at a concentration of 25 μmol/L, 1 unit of Taq DNA polymerase (Takara), 0.5 μL of 4 mol/L deoxynuleotide triphosphate, 2.5 μL of 10×PCR buffer including Mg^{2+} and 18.5 μL sterilized distilled water. The PCR products were confirmed by 2% agarose gel electrophoresis and then sent to Beijing Huada Genomics Institute for being purified and sequenced directly using ABI PRISM 3700XL Genetic Analyzer. Sequences with deletion or mutation were verified with both forward and reverse sequencing analyses in tumor and nonmalignant lung tissues.

Statistical Analyses

The sequencing results were observed by ABI Sequence Scanner software and compared with the original sequence of EGFR tyrosine kinase domain in Genbank to mark the position of nucleotide change. Differences in the characteristics of lung adenocarcinoma patients with or without EGFR mutations were compared by Chi-square test or Fisher's exact test. Probability values <0.05 were defined as being statistically significant. All statistical tests were two-sided.

Results

Mutations in Exon 19 and 21 of EGFR Tyrosine Kinase Domain

The distribution of nucleotide and protein sequence alterations, and the patient characteristics associated with these

Table 1 Primer sequences for EGFR exon 19 and 21, the corresponding annealing temperature (TA) and the size of expected PCR products

Exon no.	Primers	Primer sequence	TA (°C)	Product size (bp)
EGFR 21	Forward	5′GTTCGCCAGCCATAAGTCCT 3′	57	396
	Reverse	5′CAGAATGTCTGGAGAGCATC 3′		
EGFR 19	Forward 1st	5′ATCAGTGTGATTCGTGGAGC 3′	55	488
	Reverse 1st	5′GGCCAGTGCTGTCTCTAAGG 3′		
	Forward nested	5′GGCAGCATGTGGCACCATCT 3′	62	268
	Reverse nested	5′AGGATGTGGAGATGAGCAGG 3′		

abnormalities, are summarized in Table 2. We examined 43 cases of lung adenocarcinoma and found mutations in 13 cases (21.67%). All of the six deletion mutations occurred around codons 746–750 in exon 19. About half (four of seven) of deletion mutations were simple deletions of five amino acid residues ELREA from codon 746 to 750 (Fig. 1a), one case (case 13) was simple deletion from 747 to 751 (LREAT, Fig. 1b). Two deletions of six amino acid residues LREATS from codon 747 to 752 were coupled with substitution at 753 from proline to serine (P753S, Fig. 1c) or a point mutation from A to C at the third nucleotide of codon 758 resulting in substitution of glutaic acid with aspartic acid residue (E758D, Fig. 1d). Another six tumors had a point mutation with amino acid substitutions (2573T→G) within exon 21: leucine to arginine substitution at codon 858 (L858R, Fig. 2). It is noted that, in all cases, such alterations were in-frame. The mutations of EGFR exon 19 and 21 never occurred simultaneously. All sequence alterations in this study were heterozygous in tumor DNA. In each case with EGFR mutation, nonmalignant lung tissue from the same patient showed wild-type sequence, confirming that the mutations are somatic in origin. NSCLC-associated EGFR mutations are most frequently heterozygous. However, some authors reported mutations involving exon 19 that appeared to be homozygous [1, 6], and all of cases in our study showed heterozygous missense mutations in EGFR. The matched normal lung tissue showed no mutations, and thus EGFR mutations must be somatic in origin. The heterozygous nature of somatic mutations implies that they exert a dominant oncogenic effect in tumor cells. However, neither the previous studies nor ours used a laser capture technique to extract tumor DNA specifically; normal lung DNA might have been included.

Association Between Patient Characteristics and EGFR Mutation Status

Table 3 summarized the patient characteristics for all patients as well as the frequency of EGFR gene mutations in each category. EGFR mutation rate was higher in female patients with 28.95%, compared with 9.09% in male patients without statistical difference. The smoking status, ever or never smokers, did not modify the frequency of EGFR mutations. Similar to the previous reports [7, 8], patients who were adenocarcinoma with bronchi-alveolar component were more likely to have the EGFR mutation, with eight of 12 (36.36%) have mutations, compared with 5 of 38(13.16%) in those without bronchi-alveolar component. More than 80% of patients with L858R mutation in exon 21 were adenocarcinomas with bronchi-alveolar component, compared with 43% of patients with deletion in exon 19. Frequencies of mutations in well-differentiated adenocarcinomas and moderate to poor-differentiated adenocarcinomas were also statistically different ($P<0.05$) with well-differentiated adenocarcinomas more likely to have mutations. When stages were considered, nine patients (27.27%) of pathologic stage I–II had EGFR mutations, compared with 4 (14.81%) of stage III–IV. It demonstrated that lung adenocarcinoma with low stage had higher mutation rate, but we found no statistical significance.

Adjuvant Chemotherapy and Overall Survival

Within 42 days after surgery, 41 patients with stage IB, II and IIIA lung adenocarcinoma were assigned to receive Navelbine (25–30 mg/m^2 on day 1 and day 8) and cixplatin (80–100 mg/m2 on day 1) every 4 weeks for three cycles. But only 78% (32) of these patients received the three

Table 2 Mutational analysis of EGFR exon 19 and 21 in patients of lung adenocarcinoma

Patient no.	Gender	Age	Histological type	Smoking status	Mutation	Effect of mutation
13	Female	63	ADC+BAC	Never	Deletion 2240→2254	DelL747-T751
19	Female	60	ADC+BAC	Smoker	Deletion 2236→2250	DelE746-A750
23	Female	72	ADC+BAC	Never	Deletion 2239→2256 + substitution C→A 2274	DelL747-S752+E758D
35	Male	50	ADC	Smoker	Deletion 2235→2249	DelE746-A750
36	Female	69	ADC	Smoker	Deletion 2236→2250	DelE746-A750
41	Female	64	ADC	Never	Deletion 2240→2257	DelL747-P753(insSer)
82	Female	43	ADC	Smoker	Deletion 2235→2249	DelE746-A750
43	Female	58	ADC+BAC	Never	Substitution G→T 2573	L858R
49	Male	52	ADC+BAC	Smoker	Substitution G→T 2573	L858R
64	Female	67	ADC+BAC	Never	Substitution G→T 2573	L858R
65	Female	62	ADC+BAC	Never	Substitution G→T 2573	L858R
71	Female	55	ADC	Never	Substitution G→T 2573	L858R
77	Female	67	ADC+BAC	Never	Substitution G→T 2573	L858R

ADC adenocarcinoma, *ADC+BAC* adenocarcinoma with bronchio-alveolar component

Fig. 1 a–d The nucleotide sequence of heterozygous in-frame deletions in exon 19 by direct sequencing (*double peaks*). The *vertical arrow* ▶ indicates the site of mutation. Sequence alignment of selected regions were shown as following:

Exon 19 wild type (part):

TATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCC

Fig 1a:TATCAAG----------ACATCTCCGAAAGCCAACAAGGAAATCC

Fig 1b:TATCAAGGAAT----------CTCCGAAAGCCAACAAGGAAATCC

Fig 1c:TATCAAGGAAT----------CGAAAGCCAACAAGGAAATCC

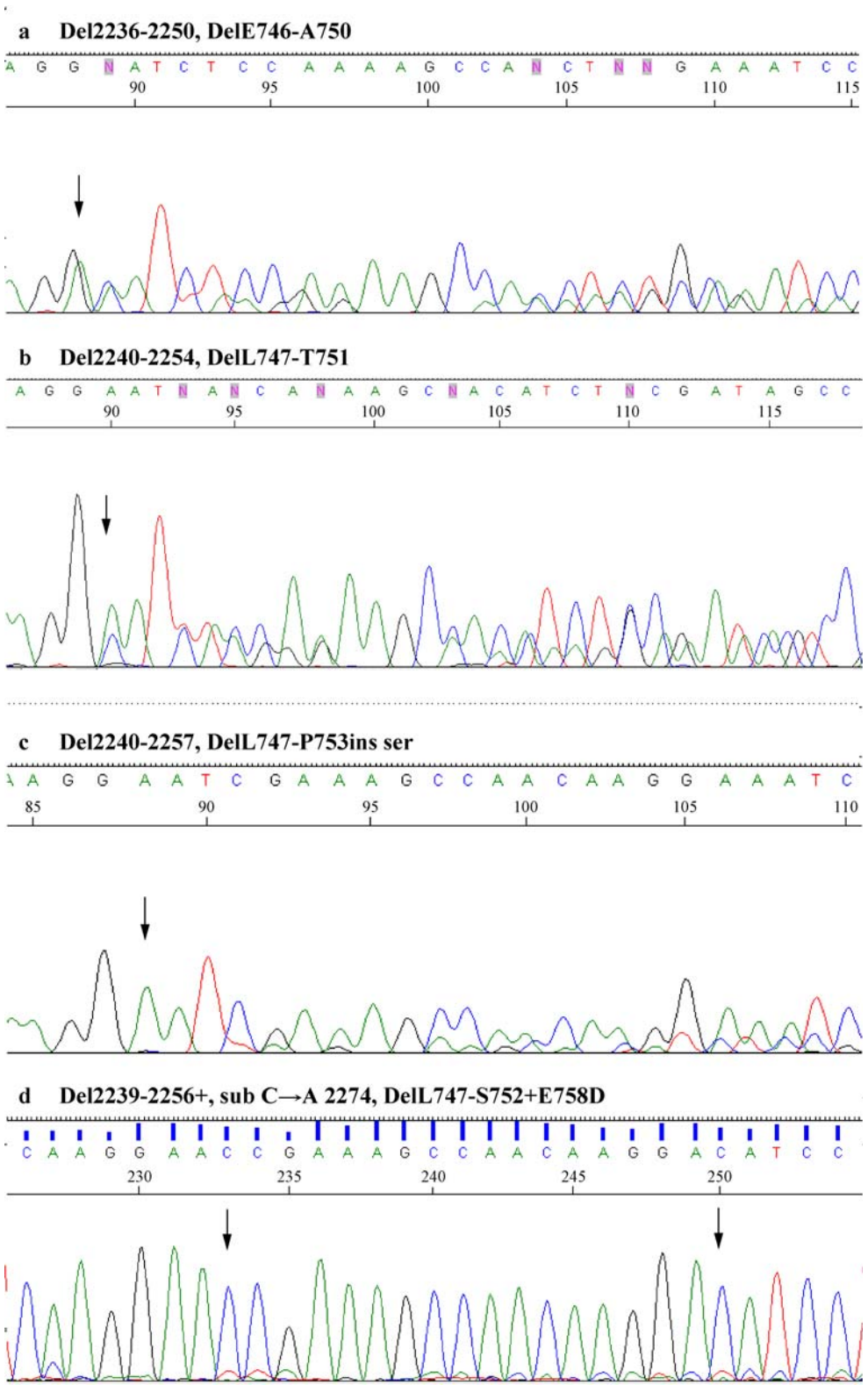
Fig 1d:TATCAAGGAA----------CCGAAAGCCAACAAGGACATCC

planned cycles of NP treatment. Nine patients (22%) stopped NP treatment because of personal choice or intolerable toxicity, such as neutropenia, thrombocytopenia and anemia. Therefore, most of these patients expended a long time to fully recover. The median duration of follow up for all 60 patients analyzed was 36.4 months (interquartile range=16.7–81.4 months). By June 2007, the number of neoplastic-related deaths was 23 (38%, 14 in the patients with adjuvant chemotherapy and nine in those who underwent surgery alone). It is noted that although only four patients (31%) with mutation received the complete adjuvant chemotherapy, the whole 13 patients with EGFR mutation were still alive without evidence of disease by the end of follow-up.

Discussion

The erb-B (HER) is one of several known receptor tyrosine kinase systems involved in cellular signaling and comprises four different receptors of which EGFR (erbB1) was the first discovered. The erb-B family of receptors transduce effects of approximately eleven different ligands, such as EGF, TGFα and some cellular factors. The structure of EGFR consists of an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic component containing a tyrosine kinase entity. Tyrosine kinases are central regulators of signaling pathways that control differentiation, transcription, cell cycle progression, apoptosis, motility and invasion. Extracellular ligand-binding induces conformational changes resulting in the formation of receptor dimmers and oligomers. Tyrosine kinase phosphorylation then ensues and heralds second messenger signal transduction via mitogen activated protein kinase (MARK), phosphatidylinositol 3 kinase (PI3K) and other pathways, leading to a multitude of effects including cell proliferation, cell differentiation, angiogenesis, metastasis, and antiapoptosis. EGFR protein overexpression, sometimes as a consequence of gene amplification, has been demonstrated in several epithelial tumor types and sometimes predicts a more aggressive phenotype and worse prognosis [9]. Mutations in several genes have been identified in key regions of the tyrosine kinase domain in several cancers [10]. EGFR mutations were identified in the tyrosine domain, with the majority clustering with exons 19 and 21. Mutations were either in-frame deletions or anino acid substitutions clustered around ATP binding pocket. Missense mutations changing leucine 858 arginine (L858R) and multiple deletions clustered in the region spanning codons 746 to 750, were found [11]. Interestingly, the deleted amino acid sequence (ELREA) flanks the ATP binding cleft of the tyrosine kinase—the very same target area of gefitinib. The L858R mutation lies close to a highly conserved region: the DFG motif. It's noted that the sequence alterations cluster around the active site of the kinase and that the substitution mutations lie in the activation loop and glycine-rich P-loop, and structural elements were known to be important for autophosphorylation in many protein kinases. Moreover, these mutations of EGFR doubled or tripled the EGF signal and prolonged the activation compared with the wide-type receptor. Consistent activating signals acted on the autoregulation of protein kinases and increased the sensitivity of tumor cell to gefitinib. These EGFR mutants selectively activate the Akt and signal transducers and activators of transcription signaling pathways, which promote cell survive. NSCLC cells expressing EGFR mutants underwent extensive apoptosis after knockdown of the mutant EGFR. Therefore, it is possible that inhibiting the mutant EGFR causes tumor cell apoptosis and a rapid therapeutic effect [12]. But there was different conclusion from other retrospective analysis which revealed that patients with EGFR tyrosine kinase domain mutations displayed better postoperative prognosis that patients with wild-type EGFR [13].

Because adenocarcinoma is the dominant histology for EGFR mutation and this type of tumor is found more commonly than in the past, our study was limited to lung



adenocarcinoma. In this report, EGFR gene mutation was detected in 21.67% of adenocarcinoma cases, relatively higher than that from Caucasian and similar to previous data from other researchers in our country [14–16]. Substitution mutation L858R in exon 21 was detected in six tumors, and others were detected multiple deletion mutations clustered in the region spanning codons 746 to 759 within the kinase domain. Most of these affected codons have already been reported to be sites for EGFR mutations, but we also identified a novel mutation (DelL747-

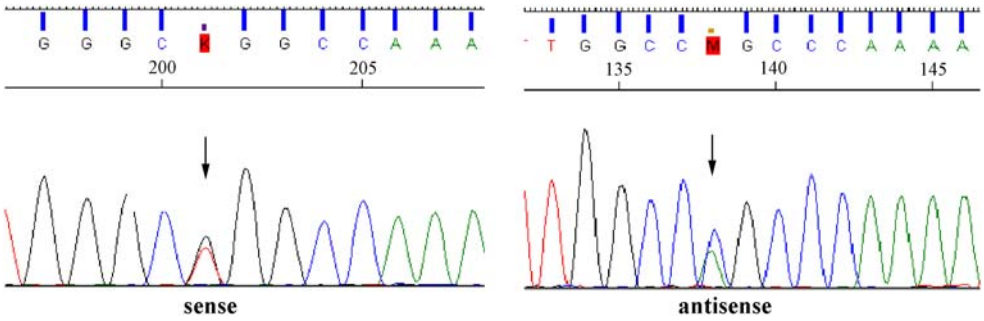


Fig. 2 Point mutation of exon 21 (L858R) (sense and antisense). The nucleotide sequence of heterozygous point mutation in exon 21 by direct sequencing (*double peaks*). The *vertical arrow* indicates the site of mutation (L858R, CTG→CGG)

S752+E758D) that has not been reported previously. The WHO classification defines bronchioloalveolar carcinoma as adenocarcinoma showing bronchioloalveolar extension without invasion. We found that much more invasive adenocarcinomas also demonstrated bronchioloalveolar components in some domain. A hypothesis of multistep carcinogenesis has been proposed where lung adenocarcinoma develops from atypical adenomatous hyperplasia to invasive adenocarcinoma through bronchioloalveolar carcinoma [17]. Previous reports showed that the presence of bronchioloalveolar features was associated with sensitivity to tyrosine kinase inhibitor [18–20]. The clinicalpathological characteristics of bronchioloalveolar carcinoma include its good prognosis and the prevalence of females and patients who have never smoked before, which were coincided with characteristics of the populations harbored EGFR mutations. In our study, mutations were more prevalent in well-differentiated adenocarcinoma, especially with bronchioloalveolar component than in moderate to poor-differentiated adenocarcinoma, confirming the hypothesis that oncogenic activation of EGFR may also contribute to the early step of lung adenocarcinoma development.

Table 3 Association between patient characteristics and EGFR mutation status

	Mutation	Wild type	Mutation rate (%)	χ^2	*P*-value
Sex					
Female	11	27	28.95	3.24	>0.05
Male	2	20	9.09		
Smoking status					
Never-smoker	9	25	26.47	1.07	>0.05
Ever-smoker	4	22	15.38		
Histology					
ADC+BAC	8	14	36.36	4.42	<0.05
ADC	5	33	13.16		
Differentiation					
Well	9	18	33.33	3.93	<0.05
Moderately to poorly	4	29	12.12		
TNM stage					
I–II	9	24	27.27	1.38	>0.05
III–IV	4	23	14.81		

*ADC*adenocarcinoma, *ADC+BAC*adenocarcinoma with bronchio-alveolar component

There was a tendency towards higher mutation ratio of female and never-smoker, but we were unable to show any statistical differences in EGFR mutation between various gender and smoking history. We also found that the low TNM stage had higher frequency of mutations than stage III and above with no statistical significance. But patients with mutation in this study demonstrated prolonged survival, compared with mutation-negative patients. A trend was observed that mutant tumors had slower progression and gave metastasis later. We need to increase the number of samples to draw reliable statistical conclusions.

To this today, radical surgery is the primary treatment for patients with stage I, II or IIIA non-small cell lung carcinoma. However, long-term survival of these patients after surgery alone is largely unsatisfactory, and the role of cisplatin-based adjuvant chemotherapy in patient survival has not yet been established [21]. In the majority of the patients who have undergo single radical resection, deaths is cancer-related and follows relapses at distant sites. Toxicity is the main cause of inadequate dose delivery in adjuvant chemotherapy, with an average of 50% of patients receiving the full course of treatment [22]. This study confirms and expands on findings of previous studies that the efficacy of adjuvant treatments on the overall survival of lung adenocarcinoma patients after completed resection remains unproven. But it is obvious that some patients had to stop their adjuvant chemotherapy because of intolerable toxicity or prolong their recovering time. So during the last decade, several new chemotherapeutic agents, including EGFR tyrosine kinase inhibitors, because available and have been extensively investigated in refractory and recurrent lung carcinoma.

Gefitinib is an small-molecule orally active EGFR tyrosine kinase inhibitor, which compete with and prevent the binding of adenosine triphosphate (ATP) at the ATP-binding region within the EGFR tyrosine kinase thereby inhibiting tyrosine residue phosphorylation and signaling. This drug, a kind of more effective treatment with fewer side-effects, has been licensed in China from 2005, and used for chemotherapy-refractory cases or recurrent cases after curative intent surgical resection. But a number of clinical

trials have recovered significant variability in the response to gefitinib, with higher responses seen in women, in non-smokers, in East Asians and in patients with adenocarcinoma [12]. After 2004, more and more researchers proved that EGFR mutations were associated with an improved response to treatment with EGFR tyrosine kinase inhibitor agents. But there was no significant difference in response to conventional chemotherapy by mutation status. Our data demonstrated that Chinese lung adenocarcinoma patients had higher EGFR mutation frequency and would obtain more beneficence from tyrosine kinase inhibitors, such as gefitinib.

In conclusion, we demonstrated that EGFR mutation was correlated with the higher differentiation and bronchioloalveolar feature of lung adenocarcinoma. This trend was observed in the low TNM stage, later metastasis and prolonged survival without statistically significant. This implied that EGFR mutation screening was of paramount importance to chemotherapic refractory patients, recurrent or high TNM stage patients. We believe that molecular profiling will likely be an efficient and effective method to provide individual predictive and prognostic genetic information for patient and oncologist.

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