RESEARCH ARTICLE

Serum Carcinoembryonic Antigen Levels before Initial Treatment are Associated with EGFR Mutations and EML4-ALK Fusion Gene in Lung Adenocarcinoma Patients

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Abstract

Background: Epidermal growth factor receptor (EGFR) mutations and echinoderm microtubule associated protein like 4-anaplastic lymphoma kinase (EML4-ALK) define specific molecular subsets of lung adenocarcinomas with distinct clinical features. Our purpose was to analyze clinical features and prognostic value of EGFR gene mutations and the EML4-ALK fusion gene in lung adenocarcinoma. Patients and Methods: EGFR gene mutations and the EML4-ALK fusion gene were detected in 92 lung adenocarcinoma patients in China. Tumor marker levels before first treatment were measured by electrochemiluminescence immunoassay. Results: EGFR mutations were found in 40.2% (37/92) of lung adenocarcinoma patients, being identified at high frequencies in never-smokers (48.3% vs. 26.5% in smokers; *P*=0.040) and in patients with abnormal serum carcinoembryonic antigen (CEA) levels before the initial treatment (58.3% vs. 28.6%, *P*=0.004). Multivariate analysis revealed that a higher serum CEA level before the initial treatment was independently associated with EGFR gene mutations (95%CI: 1.476~11.343, *P*=0.007). We also identified 8 patients who harbored the EML4-ALK fusion gene (8.7%, 8/92). In concordance with previous reports, younger age was a clinical feature for these (*P*=0.008). Seven of the positive cases were never smokers, and no coexistence with EGFR mutation was discovered. In addition, the frequency of the EML4-ALK fusion gene among patients with a serum CEA concentration below 5ng/ml seemed to be higher than patients with a concentration over 5ng/ml (*P*=0.021). No significant difference was observed for time to progression and overall survival between EML4-ALK-positive group and EML4-ALK-negative group or between patients with and without an EGFR mutation. Conclusions: The serum CEA level before the initial treatment may be helpful in screening population for EGFR mutations or EML4-ALK fusion gene presence in lung adenocarcinoma patients.

Keywords: Carcinoembryonic antigen - lung adenocarcinoma - EGFR mutations - EML4-ALK fusion gene

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Introduction

Lung cancer is the most common cause of cancer-related deaths, where it is associated with a 5-year worldwide survival rate of less than 15% (Jemal et al., 2011). Among new diagnoses of pulmonary carcinoma, Non-small-cell lung cancer (NSCLC) accounts for approximately 80% (Ozgul et al., 2013). Despite great efforts to improve the survival of patients with NSCLC, the outcomes are still considered unsatisfactory. In recent years, epidermal growth factor receptor (EGFR) gene mutations and EML4-ALK fusion gene have been discovered in a subset of NSCLC, especially among patients with pulmonary adenocarcinomas (Soda et al., 2007; Yatabe et al., 2007). The classification of lung cancer has been refined using molecular genetic data, because the difference of gene subtypes often means the corresponding treatment strategies.

EGFR mutations predominantly occur in patients with the features of female sex, non-smoking, adenocarcinoma histology and East Asian ethnicity (Yatabe et al., 2007; Zhang et al., 2013; Usuda et al., 2014). EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib, has been proved to be superior to cytotoxic chemotherapy in advanced lung cancer patients carrying the EGFR activating mutation, and EGFR mutation is a good predictor of clinical efficacy of TKIs (Maemondo et al., 2010; Fukuoka et al., 2011). An elevated serum carcinoembryonic antigen (CEA) level was generally considered to be a negative prognostic factor (Horinouchi et al., 2012). However, the abnormal serum CEA level at diagnosis was a good prognostic factor for TKIs treatment in lung cancer patients (Okamoto et al., 2005). Therefore, it was surprising, and correlation between EGFR mutations and serum tumor markers in lung adenocarcinoma patients may exist.

In 2007, the fusion oncogene of echinoderm

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microtubule-associated protein like 4 (EML4) and anaplastic lymphoma kinase (ALK) was first identified in patients with NSCLC (Soda et al., 2007). Unlike EGFR mutations, ALK gene rearrangement is infrequent in the entire NSCLC patient population (Li et al., 2013), and clinicopathologic characteristics and prognostic value of EML4-ALK fusion gene in lung cancer remain to be elucidated, especially in Chinese lung cancer patients. Furthermore, there is no report describing the relativity between EML4-ALK fusion gene and serum tumor marker.

In the current study, we retrospectively investigated the correlation between the presence of EGFR gene mutations or ALK gene rearrangement and clinical features, including the serum tumor marker levels, in lung adenocarcinoma patients. Prognostic value of EGFR gene mutations and ALK gene rearrangement was also evaluated.

Materials and Methods

Patients and samples

Specimens were obtained from 92 primary lung adenocarcinoma patients, who were under diagnosis and treatment at the Affiliated Tumor Hospital of Zhengzhou University during 2009~2010. Patients didn't receive chemotherapy, radiotherapy or targeted therapy before the specimens obtained. All biopsies had been proven lung adenocarcinoma by at least two pathologists and adequate tissues were ready for biomarker studies. We retrospectively reviewed clinical features of patients, including age, gender, smoking history, tumor diameter, differentiation, clinical stage and serum tumor marker Level before the initial treatment. The tumor stage for each patient was performed according to the AJCC Cancer Staging Manual, 7th edition. Time to progression (TTP) was calculated from the date of initial treatment to, either the date of relapse or radiologic progression, or the date of last contact. The overall survival (OS) was defined as the interval from the date of initial treatment to the date of death from any cause, or the last follow-up. This study was approved by the Ethical Committee of Zhengzhou University, and informed consent was obtained from all patients.

EGFR and EML4-ALK mutation analysis

Frozen tissues were suspended in 1 ml TRIzol reagent for total RNA extraction, according to the manufacturer's protocol. Total RNA was quantified using a spectrophotometer, and its quality was assessed by agarosegel electrophoresis. Mutational analysis of the EGFR gene was carried out by using reverse transcription-polymerase chain reaction (RT-PCR), and detailed process was the same as previous study (Nagai et al., 2005). The EGFR mutations were analyzed by using a Real-Time PCR Detection Kit to detect 3 specific in-frame deletion mutations in exon 19 (A, L747-A750del; B, E746-A750del; C, L747-S752del) and 2-point mutations in exon 21 (D, L858R; E, L861Q). EML4-ALK fusion gene was also screened with RT-PCR as described previously (Li et al., 2013). When the chimeric transcripts were detected, the products were confirmed to be EGFR mutations or EML4-ALK fusion by direct sequencing.

Table 1. Clinical Features of Genotype-specific Subsets of Patients with Lung Adenocarcinoma

Clinical features	EGFR mutation			EML4-ALK fusion gene		
	Positive	Negative	*P*	Positive	Negative	*P*
Age (years)			0.095			0.008
Median	59	54		49	57	
Range	37-76	40-76		40-62	37-76	
Gender			0.160			0.473
Male	16	32		3	45	
Female	21	23		5	39	
Smoking history			0.040			0.250
No	28	30		7	51	
Yes	9	25		1	33	
Clinical stage			0.358			0.260
Stage I+II	20	35		3	52	
Stage III+IV	17	20		5	32	
Tumor diameter			0.811			1.000
≤3.0 cm	13	18		3	28	
>3.0 cm	24	37		5	56	
Differentiation			0.349			0.108
Poor	10	20		5	25	
Moderate or well	27	35		3	59	
CEA			0.004			0.021
<5.0 ng/ml	16	40		8	48	
≥5.0 ng/ml	21	15		0	36	
CYFRA2-11			0.967			1.000
<3.3 ng/ml	18	27		4	41	
≥3.3 ng/ml	19	28		4	43	
NSE			0.580			1.000
<15.0 ng/ml	23	31		5	49	
≥15.0 ng/ml	14	24		3	35	

EGFR, epidermal growth factor receptor; EML4-ALK, echinoderm microtubule-associated protein like 4 and anaplastic lymphoma kinase; CEA, carcinoembryonic antigen; CYFRA 21-1, cytokeratin-19 fragments; NSE, neuron-specific enolase

Measurement of serum tumor marker Levels

Blood samples were collected within 2 weeks before the initial treatment, and the serum tumor marker level was measured by electrochemiluminescence immunoassay. According to the manufacturer's instructions, the normal range of serum CEA level was determined as <5.0 ng/ml, and the standard cutoff values for cytokeratin-19 fragments (CYFRA 21-1) and neuron-specific enolase (NSE) are 3.3 ng/ml and 15.0 ng/ml, respectively.

Statistical analysis

Statistical analyses were performed using SPSS, v.19.0 (IBM). Mann-Whitney test was used for comparing median age between mutation positive Group and negative Group. For categorical variables, Pearson $\chi 2$ test or Fisher's exact test was used to assess the association between gene mutation status and patients' clinical factors. Logistic regression models were used to ascertain independent predictive factors for EGFR mutations. Continuous data was described as mean ± standard deviation, and t test or t' test was used after homogeneity of variance. Kaplan-Meier method was used to estimate TTP and OS, and the difference between the two groups was compared

Table 2. Comparison of Serum Tumor Marker Level Between Mutation Positive Group and Negative Group

	CEA	CYFRA 2-11	NSE
EGFR			
Positive	23.47±35.01	4.32±3.29	17.81±10.54
Negative	8.53±16.91	6.19±16.68	17.36±11.71
P	0.020	0.506	0.852
95%CI	(2.49, 27.39)	(-7.39, 3.67)	(-4.31, 5.21)
EML4-ALK			
Positive	2.07±1.29	3.25±1.39	13.78±3.77
Negative	15.73±27.58	5.64±13.64	17.90±11.61
P	0.000	0.622	0.323
95%CI	(-19.71, -7.61)	(-12.03, 7.23)	(-12.36, 4.11)

95%CI, 95% confidence interval

Table 3. Multivariable Analysis of the Predictive Factors for EGFR Mutations

Factor		OR	95%CI	*P*
Age (years)	>65 year/≤65 year	2.446	(0.755,7.925)	0.136
Gender	Female/Male	1.293	(0.381,4.386)	0.680
Smoking history	No/Yes	1.838	(0.499,6.772)	0.360
Clinical stage	III+IV/I+II	1.307	(0.469,3.645)	0.609
Tumor diameter	≤3.0 cm/>3.0 cm	1.385	(0.489,3.924)	0.540
Differentiation	Moderate or well/Poor	1.842	(0.652,5.201)	0.249
CEA	≥5.0 ng/ml/<5.0 ng/ml	4.092	(1.476,11.343)	0.007

OR, Odds Ratio; 95%CI, 95% confidence interval

by using log-rank test. Multivariate regression was done with Cox's proportional hazard regression model. All significance levels were used two-sided test, and $P<0.05$ was considered statistically significant.

Results

Clinical characteristics of the EML4-ALK fusion gene

As shown in Table1, we identified 8 patients who harbored the EML4-ALK fusion gene (8.7%, 8/92), which was confirmed by DNA sequencing. The median age of EML4-ALK-positive group was much lower than EML4-ALK-negative patients (49 year vs. 57 year, $p=0.008$). Seven of the positive cases were never smokers (12.1%, 7/58), and one patient had light smoking history (10 cigarettes a day, 10 years). Moreover, all of EML4-ALK-positive patients were along with the normal CEA level ($p=0.021$). No associations were identified between ALK gene rearrangement and gender, clinical stage, tumor diameter, differentiation, serum CYFRA2-11 level, or serum NSE level of the patients.

EGFR mutations and clinical characteristics of patients

Of the 92 patients studied, we identified 37 cases of EGFR mutations at exons 19 and exons 21. Deletion mutations at exon 19 and point mutations of exon 21 were detected in 20 cases and 17 cases, respectively. No double mutations were detected in these EGFR exons, and no coexistence with EML4-ALK fusion gene was discovered. In concordance with previous reports, the EGFR mutation rate in never smokers was much higher than that in former or current smokers (48.3% vs. 26.5%; $P=0.040$). In addition, the abnormal serum CEA level before the initial treatment was associated with a higher frequency of EGFR mutations ($P=0.004$). There were no significant associations between EGFR mutation and age, clinical stage, tumor diameter, differentiation, serum CYFRA2-11 level, or serum NSE level (Table1).

Table 4. Factors Associated with TTP and OS

	TTP		OS	
Factor	Median (months)	*P*	Median (months)	*P*
Age		0.272		0.368
<65 year	38.8		51.1	
≥65 year	55.2		*	
Gender		0.711		0.555
Male	40.3		54.9	
Female	38.1		48.2	
Smoking history		0.580		0.319
No	38.1		52.3	
Yes	43.7		51.1	
Clinical stage		0.000		0.000
Stage I+II	48.5		*	
Stage III+IV	13		33.4	
Tumor diameter		0.813		0.660
≤3.0 cm	38.1		52.3	
>3.0 cm	40.1		48	
Differentiation		0.002		0.043
Poor	23.9		43.3	
Moderate or well	45		*	
CEA		0.179		0.391
<5.0 ng/ml	41.2		52.3	
≥5.0 ng/ml	32.5		51.1	
CYFRA2-11		0.024		0.171
<3.3 ng/ml	55.2		*	
≥3.3 ng/ml	33.8		48.2	
NSE		0.874		0.446
<15.0 ng/ml	39.1		47.7	
≥15.0 ng/ml	38.8		52.3	
EGFR mutations		0.350		0.348
Positive	32.5		48	
Negative	41.2		54.9	
EML4-ALK		0.378		0.746
Positive	26.5		52.3	
Negative	40.1		51.1	

*indicates that the median survival time can't be calculated because more than half of patients in this group is alive. TTP, time to progression; OS, overall survival

Comparison of serum tumor marker level between mutation positive group and negative group

The mean pre-treatment serum levels of CEA in patients with EGFR mutation were significantly higher than that observed in patients without EGFR mutation (23.47±35.01 vs. 8.53±16.91, $p=0.020$). On the contrary, the mean pre-treatment serum levels of CEA at the EML4-ALK-positive group were much lower than these at EML4-ALK-negative group (2.07±1.29 vs. 15.73±27.58, $p=0.000$). There were no significant differences between gene mutations status and the serum CYFRA 2-11 or NSE levels (Table2).

Multivariable analysis of the predictive factors for EGFR mutations

As given in Table3, the logistic regression analysis revealed that a higher serum CEA level before the initial treatment was the only independent factor associated with

Table 5. Multivariate Analysis of TTP and OS

Variables		TTP			OS		
		HR	95% CI	*P*	HR	95% CI	*P*
Age	≥65 year/<65 year	0.967	(0.455,2.057)	0.931	0.822	(0.328,2.059)	0.675
Gender	Female/Male	1.667	(0.825,3.371)	0.155	1.524	(0.647,3.586)	0.335
Smoking history	Yes/No	1.094	(0.490,2.444)	0.826	0.900	(0.330,2.460)	0.838
Clinical stage	III+IV/I+II	5.936	(3.187,11.056)	0.000	5.089	(2.470,10.482)	0.000
Tumor diameter	≤3.0 cm/>3.0 cm	2.233	(1.134,4.397)	0.020	2.201	(1.008,4.805)	0.048
Differentiation	Poor/Moderate or well	1.629	(0.907,2.926)	0.103	1.633	(0.812,3.283)	0.169
CEA	<5.0 ng/ml/≥5.0n g/ml	1.058	(0.567,1.976)	0.860	0.875	(0.404,1.897)	0.735
CYFRA2-11	≥3.3 ng/ml/<3.3 ng/ml	2.635	(1.418,4.898)	0.002	1.762	(0.867,3.581)	0.117
EGFR mutations	Negative/Positive	0.674	(0.357,1.273)	0.224	0.768	(0.351,1.679)	0.508
EML4-ALK	Negative/Positive	1.226	(0.418,3.598)	0.710	1.867	(0.516,6.758)	0.341

TTP, time to progression; OS, overall survival; HR, hazard ratio

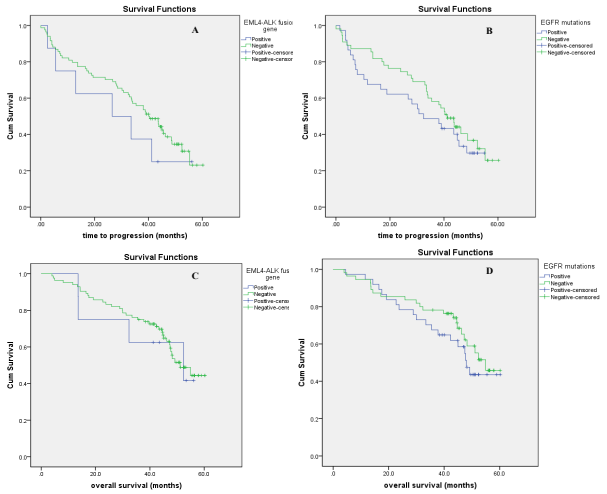

Figure 1. A: TTP for EML4-ALK-positive Patients versus EML4-ALK-negative Patients (26.5 months vs. 40.1 months, p=0.378); B: TTP for Patients with EGFR Mutations versus Patients without EGFR Mutations (32.5 months vs. 41.2 months, *p*=0.350); C: OS for EML4-ALK-positive versus EML4-ALK-negative Patients (52.3 months vs. 51.1 months, *p*=0.746); D: OS for Patients with EGFR Mutations versus Patients without EGFR Mutations (48.0 months vs. 54.9 months, *p*=0.348)

the presence of EGFR gene mutations with an odds ratio of 4.092 (95%CI: 1.476~11.343, *P*=0.007).

Survival analysis

At univariate analysis (Table 4), stage III+IV, poor differentiation and abnormal CYFRA2-11 level with shorter TTP was statistically significant ($p<0.05$). However, only stage III+IV and poor differentiation were significantly associated with shorter OS ($p<0.05$). The median TTP for EML4-ALK-positive group was 26.5 months, which was not significantly different compared to the EML4-ALK-negative group with 40.1 months ($p=0.378$, Figure 1A). Similarly, the median TTP for patients with EGFR mutation was 32.5 months, which was not significantly different compared to patients without EGFR mutation with 41.2 months ($p=0.350$, Figure 1B). The difference in median overall survival times between EML4-ALK-positive group and EML4-ALK-negative group or between patients with EGFR mutation and patients without EGFR mutation was not statistically significant ($p=0.746$ and $p=0.348$, respectively, Figure 1C and Figure 1D).

At multivariate analysis (Table 5), stage III+IV, tumor diameter and abnormal CYFRA2-11 level were independent prognostic factors for reduced TTP ($p<0.05$), and stage III+IV and tumor diameter were independent prognostic factors for OS ($p<0.05$). EML4-ALK fusion gene and EGFR mutations were not independent prognostic factors for TTP and OS ($p>0.05$).

Discussion

The utility of EGFR TKIs and ALK inhibitors provides a new therapeutic strategy for patients with NSCLC. In particular, EGFR TKIs have been widely used in clinical treatment. However, specific genotype often means that patients may be sensitive to some therapeutic method but resistant to others. So genotype screening is an important step for targeted therapy.

We identified 20 cases of EGFR mutations at exon 19 and exon 21 in 17 cases, which accounted for 40.2% (37/92) of lung adenocarcinoma patients. According to previous reports, EGFR mutations occur predominantly in exons 19 and 21, and mutations at exon 18 and exon 20 are rare (Choi et al., 2013; Gahr et al., 2013). The incidence rate of EGFR mutations in our study was similar to other results of Asian ethnicity, lung adenocarcinoma patients (Yatabe et al., 2007). In concordance with previous reports, EGFR mutations were identified at high frequencies in never-smokers ($p=0.040$). However, we couldn't find that gender was statistically associated with the presence of EGFR gene mutations ($p=0.160$). The difference with other studies may be due to male patients with higher incidence of squamous cell carcinoma (Cook et al., 2009). Interestingly, the abnormal serum CEA level before the initial treatment was significantly associated with the frequency of EGFR mutations by univariate and multivariate analysis ($p=0.004$ and $p=0.007$, respectively).

CEA was recognized as a tumor marker for NSCLC, especially for adenocarcinoma (Bergman et al., 1993). The serum CEA level fluctuated along with the progress and treatment of disease (Ishiguro et al., 2010). Consequently, serum tumor mark level before the first treatment was chosen for this study. An elevated serum CEA level generally indicated that patients had poor prognosis (Horinouchi et al., 2012). On the contrary, Okamoto's study demonstrated patients with a serum CEA concentration of over 5 ng/ml were more sensitive

to gefitinib treatment than those with a concentration of below 5 ng/ml (Okamoto et al., 2005). Qin's study also found that progress-free survival in high CEA group was significantly higher than in low CEA group among advanced NSCLC patients treated with gefitinib (Qin et al., 2013). It was perplexing, and they hypothesized that anti-apoptotic signal of the EGFR mutation may somehow elevate the expression level of CEA protein. Shoji' report explained part of the reasons, which described that the rate of EGFR gene mutations significantly increased as the serum CEA level increased in patients with recurrent lung adenocarcinoma (p=0.040) (Shoji et al., 2007). CEA is a member of the immunoglobulin superfamily and plays a role in cell adhesion and transform (Benchimol et al., 1989). When CEA is over-expressed on the cell surface, it is thought to play a role in tumourigenesis by inhibition of apoptosis and disruption of cell differentiation (Screaton et al., 1997; Ordonez et al., 2000). Target blockade of CEA with antibodies can inhibit the cell migration, invasion, and adhesion in several tumor cell lines (Blumenthal et al., 2005). EGFR is the cell surface receptor for members of EGF-family, and it can be activated by binding of its specific ligands, including epidermal growth factor and transforming growth factor α (TGFα) (Herbst, 2004). EGFR mutations can lead to EGFR overexpression or overactivity, which involved in tumourigenesis. The detailed mechanisms are unclear, and may include the following (Ravindranath et al., 2001; Scaltriti et al., 2006): 1) regulating cell proliferation and survival by mitogen-activated protein kinase (MAPK) pathway; 2) involving in cell growth, apoptosis resistance, invasion, and migration through phosphatidylinositol 3-kinase/Akt pathway; 3) promoting STAT3 persistent activation, which contributes to oncogenesis or tumor progression; 4) inducing tumor vessel formation by modulating the expression of VEGF and bFGF. If CEA is downstream signaling molecule of EGFR pathway, correlation between EGFR mutations and CEA expression may exist.

EML4-ALK fusion gene was found in 8 (8.7%, 8/92) cases of lung adenocarcinoma patients. The incidence rate of ALK gene rearrangement varied from 1.6% to 13.5%, and the wave may be attributed to the selected samples, ethnicity and measurement method (Immunohistochemistry, fluorescence in situ hybridization or RT-PCR) (Li et al., 2013; Wu et al., 2013). Clinical features in patients harboring the EML4-ALK gene included younger age, mutual exclusion with EGFR mutations and no or light history of smoking, which was consistent with previous reports (Shaw et al., 2009). We also found that the mean pre-treatment serum CEA level was significantly associated with EML4-ALK fusion gene (p=0.021). EGFR mutations and EML4-ALK translocation are called driver mutations, because they are both responsible for both the initiation and maintenance of lung cancer (Planchard, 2013). Although previous reports have indicated that ALK fusion can occur concurrently with EGFR mutations, the presence of double mutations is very rare, and EML4-ALK and EGFR mutation are considered to be mutually exclusive in general (Dearden et al., 2013; Miyanaga et al., 2013). Therefore, the opposite mechanism to EGFR mutations may exist, which can lead to the down-regulation of CEA expression.

In the clinical diagnosis and treatment process, acquiring adequate tissue for mutation analysis is often not feasible, particularly in patients with advanced disease. The efficiency of EGFR-TKIs is unclear in patients without mutational analysis (Aydiner et al., 2013). Combined with the previous reports (Okamoto et al., 2005), which proved that the serum CEA level was an independent predictive factor of clinical efficacy of EGFR-TKIs, patients with elevated serum CEA level before the initial treatment may be screened for EGFR-TKI treatment under the condition that we couldn't obtain specimens for gene detection. A recent phase III clinical trial had showed that crizotinib, the first clinically available tyrosine-kinase inhibitor (TKI) targeting ALK, was superior to standard chemotherapy in advanced non-small-cell lung cancer patients with EML4-ALK fusion gene (Shaw et al., 2013). The incidence rate of EML4-ALK fusion gene is low, and detection of the ALK gene rearrangement for all advanced lung cancer patients is impractical because of financial and technical problems. Besides younger age, no or light history of smoking, adenocarcinoma and no coexistence with EGFR mutations (Dearden et al., 2013; Li et al., 2013), the normal CEA levels before the initial treatment is also a clinical feature of patients with EML4-ALK fusion gene. These clinical characteristics can help us select high mutation population for ALK gene detection and improve the efficiency of molecular diagnosis.

Our study has some limitations. EGFR mutations at exon 18 and exon 20 weren't detected, although these mutations were rare according to previous reports. This may have led to bias. Also, the sample size of patients with EML4-ALK was small. Larger studies are required to confirm and extend these findings.

In summary, serum CEA level before the initial treatment is associated with EGFR mutations and EML4-ALK fusion gene in patients with lung adenocarcinoma. EML4-ALK fusion gene and EGFR mutations weren't prognostic factors of TTP and OS in entire lung adenocarcinoma. Larger studies are further needed to demonstrate our present findings. Furthermore, serum CEA level before the initial treatment should be measured before initial treatment, and this level is helpful in screening genotype-specific subsets.

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