RESEARCH ARTICLE

Correlation Between EGFR Mutations and Serum Tumor Markers in Lung Adenocarcinoma Patients

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Abstract

Background: Mutations affecting the epidermal growth factor receptor (EGFR) are good predictors of clinical efficacy of EGFR tyrosine kinase inhibitors (TKI) in patients with non-small cell lung cancer. Serum carcinoembryonic antigen (CEA) levels are also regarded as predictive for the efficacy of EGFR-TKI and EGFR gene mutations. This study analyzed the association between EGFR gene mutations and clinical features, including serum tumor marker levels in lung adenocarcinomas patients. Patients and Methods: A total of 70 lung adenocarcinoma patients with complete clinical data and pathological specimens were investigated. EGFR gene mutations at exons 19 and 21 were assessed. Serum tumor markers were detected by protein chip-chemiluminescence at the corresponding time, and correlations were analyzed. Results: Mutations of the EGFR gene were detected in 27 of the 70 patients and the serum CEA and CA242 concentrations were found to be significantly associated with the incidence of EGFR gene mutations ($P<0.05$). The AUCs for CEA and CA242 were 0.724 (95% CI: 0.598~0.850, $P<0.05$) and 0.769 (95% CI: 0.523~0.800, $P<0.05$) respectively. Conclusions: Serum CEA and CA242 levels are associated with mutations of the EGFR gene in patients with lung adenocarcinomas.

Keywords: Lung cancer - adenocarcinoma - epidermal growth factor receptor - tumor markers

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Introduction

Lung cancer is one of the most common tumors, the incidence and mortality rates of male were 35.5 and 31.2 per 100, 000 people respectively, which also common in female, and the incidence rate of Chinese women has risen to 21 per100, 000 people. The latest statistics show lung cancer accounts for 13% (1.6 million) of the total tumor cases and 18% (1.4 million) of the deaths in the world (Jemal et al., 2011). In China, the lung cancer incidence rate is 61.4 per 100,000 people (Parkin et al., 2005), of which about 80% are non-small cell lung cancer (NSCLC). Most patients have been in advanced stage when diagnosed. The median survival time is 6 to 12 months approximately, 1-year survival rate is about 20~50% (Schiller et al., 2002) and 5-year survival after diagnosed less than 15% (Jemal et al., 2010). Patients in early stage may be cured with surgery, but about 70% patients have local invasion or distant metastasis. The treatment choice is limited. The median survival time was only 8 ~10 month with first-line chemotherapy containing platinum (Green et al., 2004), the efficiency rate of the second-line chemotherapy was 16.3% and the third-line only 2.3%, median survival was only 4 months (Massarelli et al., 2003). Furthermore, the patients often could not tolerate the side effects of chemotherapy. In order to improve the effect of non-small cell lung cancer treatment and prolong patient survival time, new therapy way or drug is needed. With more research about cancer biology, the treatment about lung cancer strategies achieved great progress, for example epidermal growth factor receptor (EGFR).

ECFR is a proto-oncogene c-erb-1 (HER-1) receptors with tyrosine kinase activity, found over-expression and/or mutation in many tumors, transducting signal to control the proliferation and differentiation of tumor, also involving in angiogenesis, tumor invasion and local or distant metastasis. EGFR tyrosine kinase inhibitors have been found to have a upstanding disease control rate, with relatively minor adverse reactions. So this target therapy is another option in addition to conventional lung cancer treatment. Better effect are found in women, nonsmokers, Asian ethnicity and adenocarcinoma, which may be related to EGFR gene mutations (Pfister et al., 2004; Park et al., 2006; Jiang et al., 2009). Gene mutation can predict the effect of EGFR-TKI, but how to detect EGFR gene sometime may be a problem. Sometime Insufficient tumor tissue sample or difficult to obtain the primary tumor specimen led to the failure of detection of EGFR gene mutation (Costa et al., 2007). EGFR mutation detection in blood cells is now feasible, but the results could not be completely representative the feature of primary tumor because of tumor heterogeneity (Kim et al., 2008). And

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Table 1. Patient Characteristics

Characteristics		No. of patient	%
Gender	Women	38	54.3
	Man	32	45.7
Age,y	<65	43	61.4
	≥65	27	38.6
Stage(UICC)	I	6	8.6
	II	10	14.3
	III	26	37.1
	IV	28	40
ECOG PS	0-1	65	92.9
	2-3	5	7.1
Smoking history	Ever	24	34.3
	Never	46	65.7

we do not know will be there changes in EGFR mutation after chemotherapy (Han et al., 2011). So many questions and expensive testing cost reduced the value of EGFR genetic testing.

Serological tumor marker detection is relatively simple, noninvasive, economic, and reproducible. Currently there are several markers considered to predict the efficacy of EGFR-TKI, such as carcinoembryonic antigen (CEA) (Okamoto et al., 2005), polypeptide specific antigen (TPS) (Chen et al., 2010), amphiregulin and transforming growth factor-α (TGF-α) (Addison et al., 2010). and CEA was studied mostly and found to predict the efficacy of TKI (Okamoto et al., 2005). But there are few reports explaining the relationship between the EGFR gene mutation and the serum level of tumor markers only in lung adenocarcinoma patients. In this study, serum tumor markers and EGFR gene mutations were detected in lung adenocarcinoma patients at the corresponding time period, and the correlation were analyzed.

Materials and Methods

Patients

Seventy primary lung adenocarcinoma patients, who hospitalized at department of respiratory, oncology and thoracic surgery in Henan Provincial People's Hospital between January 1, 2009 and December 31, 2010, entered this retrospective study. These patients had complete clinical data and pathological specimens, in which, CT or ultrasound-guided needle biopsies in 32 cases (45.7%), bronchoscopy biopsies specimens in 13 cases (18.6%), surgical specimens in 16 cases (12.9%), pleural effusion sediment samples in 9 cases (12.9%). These patient were cytologically or histologically proven according to 1999 World Health Organization (WHO) lung and pleural histological type revised scheme (Tsuchiya et al., 2009) and staged According to the International Union Against Cancer (UICC) 1997 TNM staging criteria (Mountain et al., 1997).

For clinical TNM staging, all patients had undergone a computer tomography (CT) examination of thorax, enhanced CT scan if necessary, ultrasonic of the abdomen, a bone scintigram, and a brain enhanced CT or magnetic resonance imaging. The serum tumor markers levels were measured by chemiluminescence enzyme immunoassay detailed in Table 1. This study complied with the guidelines of the local ethics committee.

PCR method for analysis of EGFR mutations in lung cancer tissues

DNA extraction: DNA was isolated from the samples by xylene and ethanol precipitation in the biopsies embedded in paraffin according to the kit manufacturer's instructions (Amplly, Xiameng Biotechnology Co. Ltd, China).

PCR Amplification and sequencing: The two exons of EGFR (19 and 21) were amplified by PCR using the following forward and reverse primers (Invitrogen Biotechnology Co. Ltd, Shanghai, China)

EGFR exon 19: F: 5'-GCAATATCAGCCTTAGG TGCGGCGC-3', R: 5'-CATAGAAAGTGAACATTTAG GATGTG-3'; EGFR exon 21: F: 5'-CTAACGTTCGCCA GCCATAAGTCC-3', R: 5'-GCTGCGAGCTCACCCAG AATGTCTGG-3'.

A total of 25μl PCR reaction system included the following: 2μl DNA, 1μl 10xPCR buffer (Amplly Biotechnology Co., Ltd, XiaMeng, China), 1μl dNTP mixture (2.5 mmol/l), 1μl primer, 0.4μl Taq Polymerase (5 U/μl, Promega), the volume was made up with deionized water.

PCR reaction procedures were performed using 28 cycles of 30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C and extension for 5 min at 72°C. Sequencing reactions were performed on an ABI3700 genetic analyzer after PCR products were purified. Sequence variations were determined by Seqscape software (Applied Biosystems) with the EGFR reference sequence (NM_005228.3, National Center for Biotechnology Information, all were done in Guangzhou Yingwei Chuangjin Biotechnology Co. Ltd.

Serum tumor marker measurement

The collection and detection of serum samples were dealed by protein chip-chemiluminescence way with the instructions.

Serum specimen collection: 2ml venous blood was fasted and syringed into clean and dry tube without anticoagulant. After standing until natural precipitation (no less than 100μl) for about 1 hour, the specimens were centrifuged (2000 rpm × 5 min) and the supernatant were sucked out to measure. Hemolysis and jaundice specimens should not be detected. Fat blood samples were centrifuged (20000 rpm × 3 min) firstly, the lower serum was take to test after removing the upper oil.

Serum specimen measured: 100ul serum is added to the chip sub-grid, tumor markers were determined according to the Assay Kit purchased from Huzhou Shu Kang Biotechnology Co. Ltd (accuracy of each batches not more than 15% and not more than 15% between day imprecision).

Detection principle: Solid phase was coated on a substrate of tumor markers monoclonal antibody, which captured the serum tumor marker antigen. Tumor markers were detected quantitatively through the concentration of enzyme-labeled antibody. Normal reference value were provided by the Shu Kang Biotechnology Co. Ltd. Carbohydrate antigen 19-9 (CA199)<35.00 U/ml, CEA antigen (CEA)<5.00 ng/ml, carbohydrate antigen 242 (CA242)<20.00 U/ml, cancer antigen 125 (CA125)<35.00

Table 2. Comparison of Serum Tumor Markers Between the EGFR Mutation Group and the Wild

Group	Sample (n)	CA199	CEA	CA242	CA125	CA153
the wild	43	16.28±13.14	5.14±7.35	9.62±29.96	45.29±74.78	8.74±7.72
themutation	27	58.85±74.13	35.36±40.39	30.94±47.33	106.33±190.28	17.30±25.58
t or t'		2.95	3.849	2.092	1.592	1.691
P		0.006	0.001	0.043	0.122	0.102

Table 3. The Relationship Between the Clinical Characteristic and EGFR Mutation

Clinical Characteristics	sample (n)	EGFR mutation (n)	χ^2	*P*
Gender				
female	38	20	6.935	0.008
male	32	7		
Age				
<65	43	16	0.87	0.768
≥65	27	11		
Stage				
I,II	16	3	3.717	0.083
III,IV	54	24		
PS score				
0-1	65	25	0.005	0.946
12-2-3	5	2		
Smoker				
no	46	21	16.162	0
yes	24	2		
CA199				
normal	54	17	5.012	0.025
increase	16	10		
CEA				
normal	40	8	13.586	0
increase	30	19		
CA242				
normal	58	17	12.368	0.001
increase	12	10		
CA125				
normal	28	17	0.348	0.607
increase	20	10		
CA153				
normal	67	24	5.932	0.053
increase	3	3		

Table 4. Logistic Multivariate Regression Analysis Between Clinical Characteristic and EGFR Mutation

Factor	EGFR mutation		
	OR	*P*	95%CI
Gender	-0.751	0.794	0.088-6.430
Age	1.293	0.771	0.230-7.270
Stage	15.639	0.032	1.269-192.762
PS score	-0.209	0.289	0.012-3.784
Smoking history	-0.026	0.013	0.001-0.468
CA199	1.504	0.7	0.188-12.007
CEA	16.954	0.003	2.596-110.705
CA242	15.014	0.048	1.020-220.986

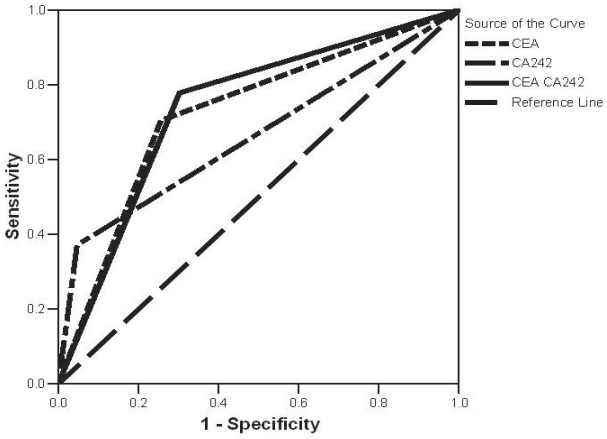

Figure 1. CEA and CA242 ROC Curves

U/ml, human growth hormone (HGH) <7.5 ng/ml, cancer antigen 15-3 (CA153)<35.00 U/ml.

Statistics

Using SPSS 12.0 statistical software for data processing. Measurement data were described as mean±standard deviation, t test or t' test was used for statistics in independent samples after testing homogeneity of variance. The methods of χ^2 test, Fisher exact test and multivariate Logistic regression were used to analysis the correlation between EGFR mutations and clinical factors. Receiver operating characteristic curve (ROC) was draw to assess the value of tumor marker in predicting EGFR mutation. All significance levels were used two-sided test, and $P<0.05$ was considered statistically significant.

Results

Characteristics of EGFR gene mutations

Mutations at EGFR gene were detected in 27 of the 70 patients. A deletion at exon 19 was observed in 15 patients (10 with E746-A750del, 3 with L747-T751>P and 3 with L747-P753>S). At exon 21, point mutation of L858R(2573T>G) was found in 10 patients and L861Q (2828T>A) in two.

Comparison of serum tumor markers between the EGFR mutation group and the wild

The serum levels of CA199, CEA and CA242 at the EGFR mutation group were all higher than these at the wild with significant difference ($P<0.05$), while there were no significant differences between the CA125 or CA153 (Table 2).

Relationship between clinical characteristics and EGFR gene mutation

A single factor χ^2 test showed that EGFR mutations was associated with the gender, smoking history and the serological level of CA199, CEA and CA242 ($P<0.05$), not with the age, PS score and the serological level of CA125 or CA153 (Table 3). The further multivariate logistic analysis showed that the patient of non-smoking, III+IV peroid, high serological CEA and CA242 level had higher rate at the EGFR mutation ($P<0.05$) (Table 4).

Draw the ROC curve and calculate the area under the curve

The areas under the curve of CEA and CA242 were 0.724 (95% CI: 0.598~0.850, $P<0.05$) and 0.769 (95% CI: 0.523~0.800, $P<0.05$) respectively. When the CEA cut-off point for 5.00 ng/ml, the sensitivity Se=70.4%, and the specificity Sp=74.4%; When CA242 cutoff point of 20.00U/ml, the sensitivity Se=37%, and the specificity Sp=95.3%. When CEA combined CA242, the area under the curve and was 0.738 (95% CI: 0.616 ~ 0.859, $P=0.001$), the sensitivity Se=77.8%, the specificity=69.8% (Figure 1).

Discussion

Paez et al. (2004) firstly pointed out that some gene mutations may be beneficial to the EGFR-TKI efficacy. The later studies confirmed the speculation and found that the mutations at the exons 18 to 21 of EGFR intracellular tyrosine kinase catalytic domain related to the EGFR-TKI efficacy. These gene mutations are more common in lung adenocarcinoma, female patients, non-smokers and peoples in East Asia (Pfister et al., 2004; Park et al., 2006; Jiang et al., 2009). The most common mutations in adenocarcinoma are the deletion mutation at exon 19, point mutation at exon 18, 21 and duplication or insertion at exon 20, and 88% of which at exons 19 and 21 (Mu et al., 2006). Now these mutations are used to predict the efficacy of EGFR-TKI (Kim et al., 2008; Mok et al., 2009).

In this study, the rate of EGFR mutations accounted for about 38.6% (27/70), 21.4% (15/70) at exon 19 and 17.1% (12/70) at 21 exons, which have been reported in literature (Mu et al., 2006). The mutation rate was similar to the result (37.6%, 50/133) reported by Hao et al. (2009), but lower than Dong et al. (2005) (47.5, 29/61). After the analysis of data in Taiwan, Dong estimated that the mutation of EGFR gene in lung adenocarcinoma might reach about 50% in China (Dong et al., 2003), which is much higher than our results. The differences may be due to the selected samples, which were fresh tissue (stored quickly in liquid nitrogen after surgical resection) in the Dong's report (Dong et al., 2006), while paraffin-embedded tissue in the Ying's (Yin et al., 2009) and ours. A number of studies have prompted that the EGFR gene mutation in women was significantly higher than those in men. In the present study, univariate χ^2 analysis suggested that EGFR mutations were different in different genders, but the latter logistic multivariate analysis showed no difference, similar to the result of Shojid et al. (2007) and Dong et al. (2009). Konsakad et al. (2004) analysised three factors including gender, smoke and adenocarcinoma and found that EGFR mutation rate was not related to the gender, but other two factors were influential factor independently. Many Asian women contributing to a high proportion of adenocarcinoma do not smoke, so the female factors may not be an independent factor. That Miller et al (Miller et al., 2004) found the efficacy of gefitinib had no relatioship with the gender can prove this viewpoint. Patients with EGFR mutation in non-smokers was 35%, significantly higher than the current smokers and the former (3% and 13%) (Yang et al., 2005). In this paper, both univariate χ^2 test and logistic multivariate analysis showed that the smoking history was an independent factor predicting the gene mutation at EGFR.

Most studies suggested that EGFR domain gene mutations involved the effect of EGFI-TKI. The EGFR-TKI domain mutations reported were about 486 types, summarized for 87 species, and new mutation is found endlessly (Kosaka et al., 2004; Mu et al., 2006). It is necessary to test all mutations already found in order to predict the effect of EGFR-TKI in clinical application? The EGFR gene mutation rarely is co-existing with K-ras gene mutation, but there is really existing (Bronte et al., 2010). So should the K-ras gene mutation be tested too? The results of B.R.21 clinical trial showed there was no correlation between erlotinib response and EGFR gene mutation, which suggest that EGFR mutation may be not an indicator to predict the effect of EGFR-TKI (Shepherd et al., 2005).

In advanced NSCLC patients, specimen for EGFR gene mutation usually is not adequate, and patients surely do not benefit from EGFR-TKI if the EGFR gene have no mutation after detection? Are there any more convenient and simple indicator to predict efficacy of EGFR-TKI? In this study, single-factor test suggested that EGFR gene mutation related with the gender, the smoking history and the serum levels of CA199, CEA and CA242, while multivariate analysis showed EGFR gene mutations only associated with the smoking history, the clinical stage and the serum levels of CEA and CA242. Japanese scholars found patients with elevated serum CEA levels had better effect to gefitinib. The patients of high serological CEA levels had higher EGFR mutation rate in recurrent lung adenocarcinoma patients after surgery, and the higher level of serological CEA, the higher mutation rate at EGFR gene (Okamoto et al., 2005). But the specimens for gene test were surgical specimens before disease recurrence, and may not representative all biological characteristic of recurrent tumor (Han et al., 2010). There were a few analogical reports about the relation between the serological markers and the curative effect of EGFR-TKI, but lack of EGFR mutation test. In our study, the serum CEA level in EGFR gene mutation group were significantly higher than the wild. Both univariate and multivariate analysis $\chi 2$ test showed that serum CEA level was correlated with EGFR mutation; the higher serum CEA level, the more EGFR gene mutation rate.

CEA, a representative tumor markers produced by tumor cells, is significantly higher in lung adenocarcinoma, women and the non-smokers patients (Tufman et al., 2010), which are the features of patient with high EGFR mutation rate. Are there any relationship between them? Carcinoembryonic antigen-related cell adhesion molecule (CEACAM), one member of CEA gene family, belongs to the immunoglobulin superfamily of adhesion molecule anchoring at the cell membrane. CEACAM1 is considered to be anti-oncogene, but expressing in lung cancer, especially in lung adenocarcinoma, can promote tumor angiogenesis (Ou et al., 2009). CEACAM5 and CEACAM6 are considered to be oncogene. The complex of CEA and its ligand can lead the error differentiation of tumor cells and avoid apoptosis. The excess expression of CEACAM6 can prevent tumor cell from anoikis, and

avoiding anoikis is regarded an important mechanism at tumor formation and metastasis. When CEACAM6 gene was silent by RNA interference method, pancreatic tumor cells were promoted to anoikis, and the transfer ability was suppressed after the pancreatic cancer cells of silent CEACAM6 gene were transfered to nude mice (Bedi et al., 1995). Like CEACAM6, CEACAM5 also can facilitate tumor cells avoiding anoikis. When both expression increasing, the normal cell polarity and organizational structure are disintegrated, the cell differentiation and maturation are impeded, and tumor may form (Duxbury et al., 2004). In tumor cells, the molecules, such as Akt and STAT3, downstreams of the EGFR pathway, phosphorylate a large number of proteins to regulate tumor cell survival and apoptosis (Cappuzzo et al., 2004; Sordella et al., 2004). If CEA is one of the increasing protein after the activation of EGFR pathway, and the serum levels of CEA may be is a message of the EGFR mutation. These require more research to determine.

In our study, the serum levels of CA242 and CA199 at EGFR gene mutation group were significantly higher than these in the wild-type group. Though single-factor test found that both were related with EGFR gene mutations, Multivariate analysis showed that only CA242 was related with EGFR mutation, and the higher serum level of CA242, the more mutation rate of EGFR. After calculated with AUC area, there were high level specificity, when CA242 took 20.00 U/ml for cut-off point. CA242 is a tumor associated antigen belonging to sialomucins component. CA242 has a analogous molecular structure with CA199, but pertaining to a different epitope family, and there are no relationship between the two antigen. These two glycopeptide antigens are studied more in digestive diseases, especially in pancreatic disease (Lamerz et al., 1999). Both serum levels significantly raised up in NSCLC, especially in adenocarcinoma. The serum levels in advanced disease were higher than the nonage, and the patient prognosis were poor when the level increased (Yang et al., 2007). There are few reports about CA242 and EGFR gene mutation, and need more further research.

In summary, this study suggests that EGFR gene mutation rate was low in lung adenocarcinoma patients with smoking, but high in elevated serum levels of CEA and CA242. So, in addition to the patient's gender, smoking history et al., the serum levels of CEA, CA242, especially CEA, can be used to forcast the EGFR gene mutation, which guide the clinical treatment in the lung adenocarcinoma patient.

Short summary, studies suggested that EGFR mutations involved the effect of EGFI-TKI. Specimen for EGFR mutation sometimes is not adequate for detection in advanced NSCLC patients. Patients with elevated serum CEA levels were found had better effect to gefitinib and patients with high serological CEA levels had higher EGFR mutation rate in recurrent lung adenocarcinoma patients, the higher level of serological CEA, the higher mutation rate at EGFR. There were a few analogical reports about the relation between the serological markers and the curative effect of EGFR-TKI but lack of EGFR mutation test. In our study, EGFR mutations were detected with PCR way, while serum tumor markers (CA199, CEA, CA242, CA125, HGH, CA153) were detected by protein chip-chemiluminescence way in 70 lung adenocarcinoma patients ,and the correlation were analyzed .EGFR gene mutation rate was low in lung adenocarcinoma patients with smoking, but high in elevated serum levels of CEA and CA242. So, in addition to the patient's gender, smoking history et al., the serum levels of CEA, CA242, especially CEA, can be used to forcast the EGFR gene mutation, which guide the clinical treatment in the lung adenocarcinoma patient.

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