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Expression of EGFR and c-Met in NSCLC: Real-time quantitative PCR as a alternative to immunohistochemistry for prognosis

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ABSTRACT

This research is to investigate the effect of EGFR and c-Met in the tumorigenesis, proliferation and prognosis of non-small cell lung cancer (NSCLC). We present 61 cases of NSCLC here, and detected the expression level of EGFR and c-Met by immunohistochemistry (IHC) and quantitative real-time PCR (qRT-PCR). The relativity of these two groups and the relationship with clinicopathologic features and prognosis were analyze. Significant correlations was only found in high level of EGFR and c-Met expression and tumor differentiation in IHC. And there were significant correlations between the two genes expression and smoke status and histology type in qRT-PCR. Significant positive correlations were found between the expression status of EGFR and that of c-Met in protein and gene levels (r= 0.303, P = 0.018 and r = 0.352, P=0.005, respectively). Multivariate analysis revealed that expression status of EGFR and c-Met detected by qRT-PCR not IHC and lymph node invasion were independent prognostic factor.

KEYWORDS

Non-small cell lung cancer (NSCLC);
EGFR;
c-Met;
Immunohistochemistry;
Quantitative real-time PCR.

INTRODUCTION

Although the diagnosis and treatment for lung cancer has improved in recent years, but the situation of prognosis still poor. The survival rate of the patients with non-small cell lung cancer (NSCLC) was less than 15% after excised completely[1]. To guide clinicians in selecting treatment options for NSCLC patients, reliable markers predictive of poor clinical outcome are desirable.

Epidermal growth factor receptor (EGFR), a transmembrane receptor protein, is one of the tyrosine kinase receptor families. Hepatocyte growth factor receptor (c-Met), a kind of receptor with protein tyrosine kinases activity, belongs to tyrosine kinase SRC families. Both EGFR and c-Met are highly expressed in lung tumors and play important roles in the NSCLC progression[2, 3]. Recently, several papers suggest an in-

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teraction between HGF/MET and EGFR signaling pathways[4-7]. The current method of choice for EGFR and c-Met testing is immunohistochemistry (IHC), which assesses expression directly at the protein level. However, the results of IHC are not quantitative and there are no standardized scoring system and no uniformly accepted threshold for positivity. Thus, we investigated whether quantitative real-time PCR (qRT-PCR), an inherently quantitative method insensitive to interobserver variability and easily amenable to standardization, represents a useful alternative approach for scoring of the EGFR and c-Met status in NSCLC compared to IHC. The expression of EGFR and c-Met in patients with NSCLC was examined and correlated with clinical outcome data. We show that qRT-PCR was a alternative to IHC for predicting the prognosis of NSCLC.

MATERIALS AND METHODS

Study population

Collect integrated clinical data of 61 cases with NSCLC, without any treatment, between1998 and 2005 in the First Affiliate Hospital of Xi'an Jiaotong University and. The follow-up was end in the May 2008. Histological classification and differentiation grade were conducted according to 1999 WHO histological classification standards of lung cancer; staging was carried out according to newly revised TNM staging criteria of the International Union against Cancer in 1997.

Immunohistochemical staining

Tissue specimens were fixed in neutral buffered formalin (10% v/v formalin in water; pH 7.4) and embedded in paraffin wax. Serial sections of 4-μm thickness were cut and mounted on charged glass slides. The monoclonal antibody against EGFR (1:100; Cell Signaling Technology) and c-Met (1:200; Santa Cruz Biotechnology) were used respectively. The Streptavidin-Peroxidase technique (Golden Bridge International: SP-9000) was used as described[8]. An irrelevant rabbit antiserum served as a negative control. Sections were counterstained with Mayer's hematoxylin. Both of the percentage of positive cells and the strength of the staining were considerate in the follow methods. 5 magnification visions were selected random under the optical microscope, the calculation of results as followed: the percentage of positive cells in 0-5% was counted 0; the percentage of positive cells in 5-25% was counted 1; 26-50% was counted 2; 51-75% was counted 3; ¨R76% was counted 4. On the respect of staining strength, the score for tumor cells without stain is 0; straw yellow for 1; brown for 2; tan for 3. The staining index score was the sum of the items above. The score in 0-2 mean negative, while the score in >3 mean positive.

Genomic DNA extraction

For nucleic acid extraction from paraffin-embedded tissues, 5 mm sections were immersed in xylene for 30 min to remove paraffin, and washed in absolute and then in70% ethanol. All samples were subjected to digestion with 0.5% SDS and 0.5 mg/ml proteinase K at 37 °C overnight, extracted with phenol and precipitated with ethanol in the presence of sodium acetate. DNA concentration was quantitated by A 260 absorbance with a Bioanalyzer and NanoDrop™ spectrometers.

Qrt-PCR

The PCR cycling began with template denature at 95°C for 30s, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec, 72°C for 20 sec, and 78°C for 20 sec. In addition, final PCR products were resolved in agarose electrophoresis and a single band of expected size indicated the specificity of the reaction. After the verification, the efficiency of amplification was consistency. The expression levels of candidate genes were standardized using the Line-1 as an internal reference. The primers were listed in TABLE 1. Taken the average of the three parallel groups Ct value, and then calculated the deviation Ct value (¡÷Ct) between the Ct of c-Met and Line-1 and that between the Ct of EGFR and Line-1, separately. Relative DNA copy numbers

TABLE.1 : Primer sequence for Real-time Fluorescence Quantitative PCR

Primer	Primer sequence	Length of products
EGFR (F)	5'-GGGCAAAGAAGAAACGGAG-3'	89bp
EGFR (R)	5'-GTCCATCAGTGGGGAGTAAG-3'	
c-Met (F)	5'-TCATTGGTTCCAATCACAGCTCA-3'u	80bp
c-Met (R)	5'-GCCACCGAGACAGAGGCTAATC-3'	
Line-1 (F)	5'-CCGCTCAACTACATGGAAACTG-3'	135bp
Line-1 (R)	5'-GCGTCCCAGAGATTCTGGTATG-3'	

((RCNs) was calculated as the follow formula: DNA = $2^{-i\div Ct}\times 100$.

Statistical method

All of the data were analyzed by SPSS 17.0. The association between staining index and other categorical factors potentially predictive of prognosis was analyzed using the Fisher's exact test. The Spearman's rank correlation coefficient was used for analyzing the association of EGFR expression levels with c-Met expression status. The result of qRT-PCR was record as mean ±standard deviation and t test was used to compare the average RCNs of two groups. In order to analyze the relationship between RCNs and survival time for NSCLC patients, RCNs were dichotomized into low and high groups using the median expression value within cohort as a cutoff. Survival curve and median survival were estimated by the Kaplan-Meier method. Their differences were verified by log-rank test. Multivariate analysis was done using the Cox proportional hazard regression analysis. $P < 0.05$ means significant differences.

3. RESULTS

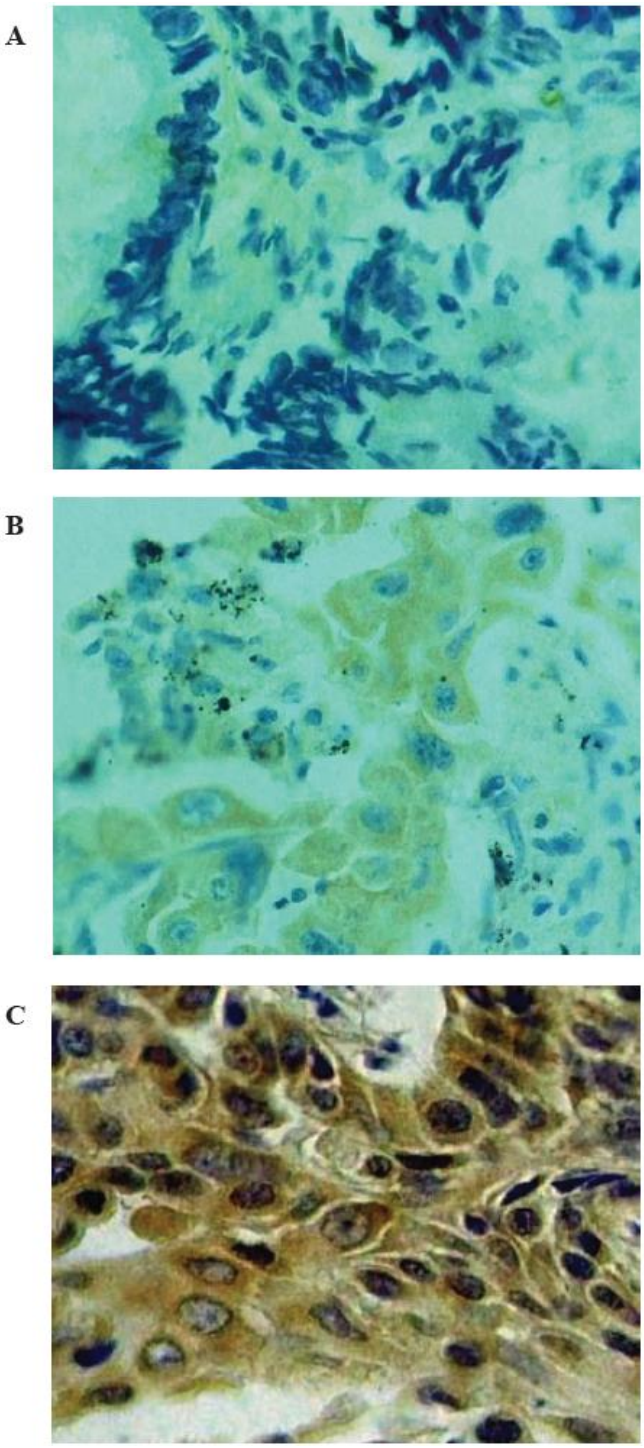
The expression of EGFR and c-Met in NSCLC and their relationships to clinicopathologic variables

Both EGFR and c-Met immunoreactivity were found primarily in the cytosol. Figure 1 shows representative expression patterns of EGFR and c-Met in NSCLC. There were significant correlations between the high level of EGFR and c-Met expression and the tumor differentiation. However, the high level rates were not significantly correlated with gender, age, smoking status, histology, T-stage, N-stage, and TNM stage (TABLE 2).

The relationship between the rcns and patients' clinical and pathological characters

There were significant different RCNs of EGFR and c-Met expression in group of histology and smoking stats. But in the respect of sex, age, differentiation, primary tumor, lymph node, TNM, there is no significant difference can be obtained ($p > 0.05$) (TABLE 3).

Figure 1. Immunohistochemical expression of EGFR and c-Met in NSCLC



A, negative staining in NSCLC; B, typical immunohistological features with high levels of c-Met expression in NSCLC. The c-Met staining shown cytoplasmic localization; C, typical immunohistological features with high levels of EGFR in NSCLC. The EGFR staining was present in the cytoplasm of tumor cells; Magnifications, ×200.

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TABLE 2 : Clinicopathologic variables and the expression status of EGFR and c-Met

Variables	Total	EGFRpositive (%)	P	c-Met positive(%)	P
Gender			0.215		0.552
Male	47	34（72.3）		26（55.3）	
Female	14	13（92.9）		9（64.3）	
Age			0.689		0.740
<60	29	23（79.3）		16（55.2）	
?60	32	24（75.0）		19（59.4）	
Smokingstatus			0.062		0.348
Yes	37	32（86.5）		23（62.2）	
No	24	15（62.5）		12（50.0）	
Histology			0.294		0.445
AC	26	22（84.6）		14（53.8）	
SCC	33	23（69.7）		19（57.6）	
Differentiation			0.027		0.017
High	26	16（61.5）		10（38.5）	
Poor	31	27（87.1）		21（67.7）	
T-stage			0.569		0.062
T_{1-2}	47	37（78.7）		30（63.8）	
T_{3-4}	14	10（71.4）		5（35.7）	
N-stage			0.795		0.580
N_0	33	25（75.8）		20（60.6）	
N_{1-2}	28	22（78.6）		15（53.6）	
TNM			0.567		0.199
? -?	45	36（80.0）		28（62.2）	
? -?	16	11（68.8）		7（43.8）	

AC, adenocarcinoma; SCC, squamous cell carcinoma.

Correlation test

There was statistically significant association of EGFR expression status with c-Met expression levels both in protein and DNA levels(r= 0.476, P<0.001 and r = 0.352, P=0.005, respectively).

Survival analysis

Kaplan-Meier analysis was used to calculate the impact of clinicopathologic features and protein expression on survival. High DNA copy numbers of EGFR and c-Met, and N stage were associated with decreased survival (P<0.05), whereas High expression of EGFR and c-Met detected by IHC were not significant. Cox regression analysis revealed a statistically significant correlation among N stage and High DNA copy numbers of c-Met (P <0.05, TABLE 4).

DISCUSSION

After the analysis of genomics and proteomics of lung cancer cell lines and gastric cancer cell lines, Guo et al. verified a complex signal transduction pathway related to susceptibility of targeted drug. And the center role of EGFR and c-Met in the pathway, besides more than 100 targets tyrosine kinase involved, has been revealed[9]. The relationship between the protein expression and DNA copy numbers of these two genes and their clinicopathologic significance was discussed in this paper.

Consistent with the result of Meert et al.[10], our immunohistochemistry assay showed the protein expression rate of EGFR and c-Met in poor differentiation NSCLC is higher than that of well differentiation. We

TABLE 3 : The relationship between the relative DNA copy numbers and pathological characters

Variables	Total	RCNs of EGFR	P	RCNs of c-Met	P
Gender			0.069		0.088
Male	47	0.14±0.17		0.12±0.07	
Female	14	0.25±0.23		0.28±0.32	
Age			0.213		0.583
<60	29	0.20±0.23		0.15±0.14	
?60	32	0.14±0.14		0.17±0.21	
Smoking status			0.013		0.032
Yes	37	0.22±0.22		0.20±0.21	
No	24	0.10±0.08		0.10±0.09	
Histology			0.026		0.015
AC	26	0.24±0.26		0.23±0.25	
SCC	33	0.12±0.08		0.11±0.06	
Differentiation			0.539		0.791
High	26	0.15±0.15		0.15±0.10	
Poor	31	0.18±0.23		0.16±0.22	
T-stage			0.575		0.872
T_{1-2}	47	0.16±0.19		0.16±0.19	
T_{3-4}	14	0.19±0.19		0.15±0.12	
N-stage			0.653		0.961
N_0	33	0.16±0.22		0.16±0.15	
N_{1-2}	28	0.18±0.15		0.16±0.21	
TNM			0.738		0.529
? -?	45	0.16±0.19		0.15±0.13	
? -?	16	0.18±0.18		0.19±0.27	

RCNs, Relative DNA copy numbers, presented as mean ±standard; AC, adenocarcinoma; SCC, squamous cell carcinoma.

further found that the DNA copy numbers of EGFR increased significantly in adenocarcinoma, which is in accord with Suzuki's research on the basis of 181 cases of NSCLC[11], but in the contrast to Dacia et al. detection of 199 cases of NSCLC[12]. The reason of this discrepancy may be the difference between the detection method and reagents. But it is more likely that EFGR is impacted by some abnormal signaling pathways or interacted with other factors (such as c-Met) in the process of transcription and translation in tumor cells. The further research will help to reveal the exact mechanism.

Our results showed that there was a significant positive correlation between the EGFR and c-Met in protein and DNA level, revealing a collaborative effect between EGFR and c-Met in the process of NSCLC. A similar result appeared in Nakamura's research which reported that there is highly correlation between Akt expression and phosphorylated EGFR and c-Met[13]. Moreover, Puri et al. reported that there is a synergy effect between ECG and HGF in the process of tumor cells proliferation, activation and the regulation of downstream signal-transmitting passageways[14]. It can be predicted that an mutual complementation between EGFR and c-Met in NSCLC.

There are also lots of reports about the relationship between the expression of EGFR and c-Met and clinical outcome, but the results were conflicting. For EGFR, Some investigators observed over-expression of EGFR associated with a favorable clinical outcome[15], while some shown EGFR over-expression in NSCLC to be associated with a shorter survival[16]. There were simi-

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TABLE 4 : Estimation the risk of death by Cox proportional hazards regression model

Variables	Univariate analysis		Multivariate analysis	
	P	Hazard ratio（95% CI）	P	Hazard ratio（95% CI）
Gender (female vs. male)	NS	1.170(0.497-2.757)	NS	2.233(0.629-7.927)
Age (?60 vs. <60)	NS	1.031(0.490-2.169)	NS	0.789 (0.306-2.032)
Smoking status (no vs. yes)	NS	0.643(0.282-1.462)	NS	1.057 (0.363-3.082)
Histology (SCC vs. AC)	NS	0.855(0.448-1.632)	NS	1.041 (0.466-2.323)
Differentiation (poor vs. s well)	NS	0.667(0.366-1.217)	NS	0.840 (0.409-1.725)
T-stage (T_{3-4} vs. T_{1-2})	NS	1.381(0.607-3.141)	NS	2.191 (0.484-9.916)
N-stage (N_{1-2} vs. N_0)	0.018	2.513(1.168-5.403)	0.005	4.159 (1.543-11.209)
TNM stage (? -? vs.? -?)	NS	1.116(0.488-2.556)	NS	0.466 (0.092-2.367)
EGFR gene copies (high vs. low)	0.046	2.249(1.015-4.982)	NS	2.075 (0.694-6.207)
c-Met gene copies (high vs. low)	0.027	2.450(1.105-5.431)	0.034	3.132 (1.087-9.024)
EGFR IHC (positive vs. negative)	NS	1.951(0.708-5.375)	NS	0.950 (0.187-4.821)
c-Met IHC (positive vs negative)	NS	1.236(0.579-2.638)	NS	1.708 (0.602-4.849)

AC, adenocarcinoma; SCC, squamous cell carcinoma; NS, not significant; vs., versus.

lar phenomena for c-Met[17-20]. It was reported no correlation between c-Met and prognosis in colorectal cancer[17], but it can predict poor prognosis in liver cancer, breast and ovarian cancers[18-20]. And Kanteti et al. reported highly DNA copy numbers of c-Met associated with a better prognosis[21]. We surmised that the reason for it was the method used for detecting the expression of biomarkers, so we compared two different assessment technique: IHC vs. qRT-PCR. qRT-PCR is simple, cost-effective, and rapidly produces quantitative, numerical, and reproducible results[22]. The most important advantage of qRT-PCR is the fact that interpretation of the results is straightforward, easily amenable to standardization, insensitive to interobserver variability, and does not require experienced pathologists, which was contrast to IHC. According to our results, expression of EGFR and c-Met detected by IHC failed to predict patients' prognosis in univariate analysis, whereas N-stage, expression status of EGFR and c-Met detected by qRT-PCR shown to be an independent prognostic indicator in patients with NSCLC. We showed that qRT-PCR was a alternative to IHC for predicting the prognosis of NSCLC, but the clinical significance needs more samples and further verification.

In summary, the current study suggested that the high expression of EGFR and c-Met in both protein and DNA levels were associated with important clinicopathologic parameters in NSCLC. There was a significant positive correlations between the expression status of EGFR and that of c-Met. Further, DNA copy numbers of EGFR and c-Met detected by qRT-PCR were the important prognostic indicator in cases of NSCLC. Therefore, EGFR/c-Met signal pathway may be attributed to the malignant transformation of NSCLC, and attention should be paid to a possible target for therapy.

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