Original Article

Clinical significance of EGFR and EGFRvIII expression in human esophageal carcinoma

Xiaoyi Duan[1], Suna Zhou[2], Mingxin Zhang[3], Peng Wang[4], Jia Zhang[5], Jiansheng Wang[6]

ABSTRACT

Objectives: Epidermal growth factor receptor (EGFR) and its mutated variant EGFRvIII are involved in the occurrence and development of malignancies. Our objective was to find a correlation between EGFR and EGFRvIII expression in esophageal carcinoma and clinical outcomes.

Methodology: Immunohistochemistry and Western blot analysis were applied to detect expression of EGFR and EGFR vIII in specimens of esophageal carcinoma patients. Patient-matched normal tissues served as the control.

Results: EGFR and EGFRvIII were detected in cell membrane and cytoplasm. A significantly higher expression of EGFR and EGFRvIII was observed in tumors as compared to normal tissues. Moreover, the expression of EGFR and EGFRvIII in esophageal carcinoma was significantly associated with the tumor location and degree of tumor invasion, tumor-node- metastasis (TNM) staging, pathological grade, and lymph node metastasis. However, there were no significant associations with age, invasiveness, tumor size, or growth pattern.

Conclusion: The over expression of EGFR or EGFRvIII is related with the malignant degree, and EGFR or EGFRvIII may be a novel promising indicator for early diagnosis of esophageal carcinoma.

KEY WORDS: Esophageal carcinoma, EGFR; EGFRvIII, Immunohistochemistry, Western blot analysis.

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INTRODUCTION

Esophageal carcinoma is the eighth most common type of malignancy in the world[1] and the fifth leading cause of cancer death in China. It has a poor prognosis due to the lack of early screening strategies and the advanced stage of the disease at the time of final diagnosis. It is therefore necessary to improve early diagnosis in order to advance the treatment of esophageal carcinoma.

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of tyrosine kinase receptors with growth promoting effects.[2] Epidermal growth factor receptor variant III (EGFRvIII), the most common mutated from of EGFR, plays an

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important role in development of malignant tumors. It is reported to be expressed in many types of cancer.[3] More importantly, EGFRvIII expression is specific to tumors and can not be detected in normal tissues. Therefore, EGFRvIII is an ideal detection parameter for malignant tumors; both EGFR and EGFRvIII are targets for currently available molecular targeting drugs. Consequently, assessment of the expression of these markers could potentially improve diagnosis and aid in targeted therapy decisions.[4,5] Until now, the expression of EGFRvIII in human esophageal carcinoma tissues has never been investigated.

It is reasonable to expect that evidence of EGFR or EGFRvIII expression would provide the best means of detecting malignancy and improving the benefits of therapy. Thus, in this study, we determined the expression of EGFR and EGFRvIII in esophageal carcinomas and analyzed the relationship of these markers and clinic-pathological characteristics.

METHODOLOGY

Patients and samples studied: This study examined esophageal carcinomas and corresponding adjacent normal tissues taken from 33 patients (22 males and 11 females) who had undergone surgical resection in the Department of Surgical Oncology of the First Affiliated Hospital of Xi'an Jiao tong University from June to November in 2006. But one point should be added: because of loss of samples due to inefficient protein extraction, we finally tested 25 pairs of specimens in the course of western blot. The mean age of the patients was 58 (range, 37–73). None of the patients had received radiation or chemotherapy preoperatively. This study was approved by the institutional ethics committee and received the informed consent of all patients.

Immunohistochemical detection of EGFRvIII, EGFR: Briefly, liquid nitrogen snap-frozen, formalin-fixed and paraffin-embedded tissues were used for immunohistochemical staining. Five micron sections were prepared from the blocks and fixed on the slides. Then sections were deparaffinized in xylene and rehydrated in a graded alcohol series. Endogenous peroxidase was blocked by soaking in 3% H_2O_2 for 30 minutes. After the slides have been washed, they were microwaved to induce antigen retrieval and then treated with a protein-blocking reagent for 20 minutes. Sections were incubated with rabbit EGFR polyclonal antibody (Santa Cruz, USA) or EGFRvIII polyclonal antibody (Bioss, Beijing, China) overnight at $4C^0$. Detection of immunostaining was performed using a SP and DAB kit (Zsbio, Beijing, CHN). Negative controls were prepared with a normal serum as the primary antibody, and known positive controls were included in each run.

Western blot analysis: 100 mg of tissue was minced and lysed by the addition of 1 mL of 1% phenylmethylsulfonyl fluoride (PMSF) at $4C^0$. Next, the samples were centrifuged at 12,000× g for 30 minutes at $4C^0$ to collect supernatants. After that, the concentration of extracted protein was measured using the Coomassie blue G250 staining technique.[6] Total protein extracts (100 ìg) were resolved with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto the nitrocellulose membranes by semi-dry electroblotting (the strength of the electric current in accordance with the membrane surface, 1.5 hour (h)). Membranes were blocked in 5% fat-free milk to inhibit non-specific binding, then incubated with primary antibodies at $4C^0$ overnight. After washing extensively with Tris-buffered saline and Tween 20 (TBST), membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilution) for 1 hour, followed by chemiluminescent detection with enhanced chemiluminescence (ECL) Plus reagents (Amersham Biosciences, Sweden) according to the manufacturer's instructions, using ß-actin as an internal standard.

Evaluation of immunohistochemical and western blot data: Tissue sections were studied by light-microscopy after hematoxylin and eosin (H&E) and immunohistochemical staining. Positive cells were scored for membranous or cytoplasmic expression. We selected randomly 5 high-power fields (20×10) for each specimen. The images of positive stained sections were analyzed by Q550CW image system (Leica, Germany). Subsequently, the expression of EGFR and EGFRvIII were expressed by grayscale value, with the help of the following formula: The final grayscale value = the grayscale value of interstitial substance - the grayscale value of positive cells. Western blot images were acquired by GeneSnap system (Syngene, UK), and densitometry was performed by Gene Tools software (Syngene, UK). The final intensity of expression of each index was calculated using the following formula: Semi-quantitative results of objective band = the OD value of a single objective band/ the OD value of a corresponding internal standard (ß-actin).

Statistical Analysis: Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 13.0. Differences among groups was determined with single factor variance analysis. Comparison between two groups was performed with Student's *t*-test. Significance was accepted at a probability value (*P*) less than 0.05.

RESULTS

Immunohistochemical expression status: Expression of EGFR and EGFRvIII was investigated in pairs of esophageal carcinomas and corresponding adjacent normal tissues. In these, carcinoma cells showed diffuse membrane staining indicating EGFRvIII. The level of expression of EGFRvIII in esophageal cancer tissue was significantly higher than that in the corresponding adjacent normal tissues. EGFR mainly localized in the cytoplasm of tumor cells, either disseminated or in clusters (Figure 1). The image analysis revealed grayscale values for EGFRvIII and EGFR expression in esophageal carcinoma of 22.46 ± 4.21 and 15.42 ± 3.15 respectively, and correspondingly of 5.54 ± 3.01 and 5.03 ± 3.49 for normal esophagus tissues. These differences represented a statistically significant difference ($P < 0.05$).

Western blot analysis: The OD values of EGFR and EGFRvIII expressed in esophageal carcinoma were 1.37±0.41 and 0.83±0.15 respectively. OD values for the corresponding adjacent normal esophageal tissues were 0.21 ± 0.09 and 0.08 ± 0.05, respectively. Paired-sample *t*-tests showed that expression of both proteins was significantly higher in esophageal carcinoma as compared to normal tissues ($P < 0.05$).

Relationship between clinic-pathological characteristics and expression of EGFR and EGFRvIII: Stratifying the patients according to age, invasion degree, tumor size, and growth pattern revealed that the expression of EGFR and EGFRvIII is associated with tumor location, degree of tumor invasion, TNM stage, pathological grade and lymph node metastasis ($P < 0.05$). No associations were found with other clinical parameters of esophageal cancer, including age, tumor size and growth pattern ($P > 0.05$). These results are shown in Table-I, and are in agreement with the result of the Western blot analysis.

Consistency analysis: Using a proximity matrix test for the immunohistochemical and Western blot analysis results, the consistency coefficients of EGFRvIII and EGFR were $r = 0.92$ and $r = 0.66$, respectively, which is considered good consistency.

Correlation analysis: In immunohistochemical staining and Western blot analysis, correlation analysis showed a firm positive linear correlation between EGFR and EGFRvIII ($r = 0.70$, $P < 0.001$ and $r = 0.46$, $P < 0.05$, respectively).

DISCUSSION

Considerable progress has been made in the understanding of the role of EGFR in the regulation of tumor cell progression and invasion.[7,8] In various

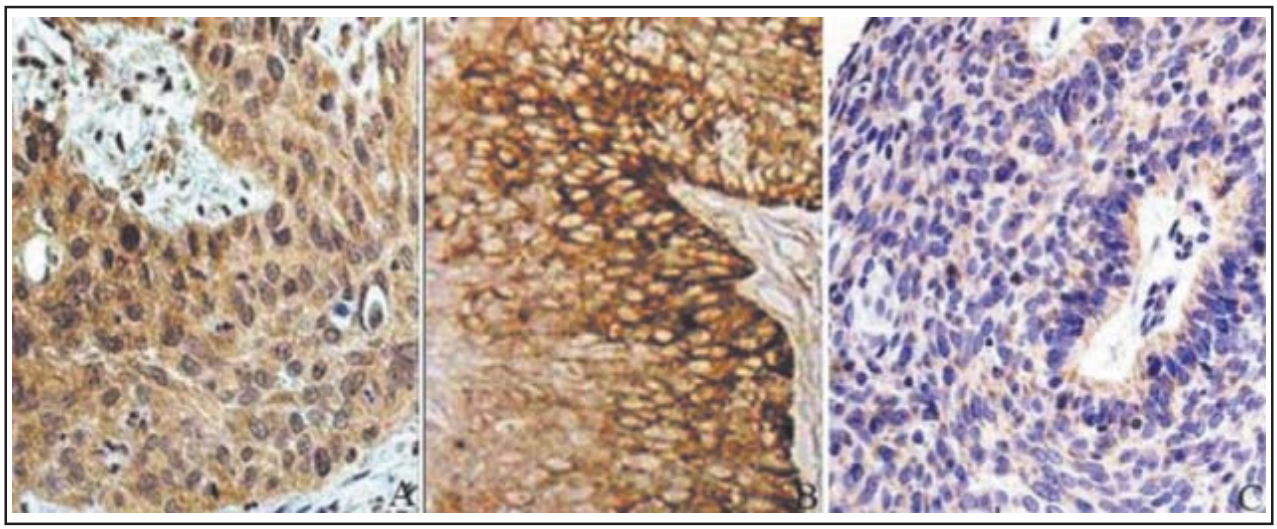

Figure-1: Expression of EGFR and EGFRvIII in esophageal squamous cell carcinoma (200x). The brown color represents positive staining for EGFR and EGFRvIII. The blue color represents the nuclear counterstain. EGFR protein expression was found in the cytoplasm of cancer cells and on the membrane. EGFRvIII protein was predominantly expressed in the cell membrane. (A) EGFR; (B) EGFRvIII; (C) negative control.

kinds of cancer cells, both EGFR and EGFRvIII have been observed to correlate with tumor development and are associated with poor patient survival.[9-14] Earlier studies have shown that over-expression of EGFRvIII in tumor cells results in increased cell proliferation, resistance to apoptosis, and increased cell migration.[15] In addition, EGFRvIII has been associated with poor prognosis and poor survival in cancer patients.[16,17] Consequently, EGFRvIII can be regarded as a suitable marker for early diagnosis of cancer, and may serve as a potential new target for cancer therapies.[18,19] However, the expression of EGFRvIII in human esophageal carcinoma tissues has not been reported until now.

Table-I: The relationship between clinicopathological characteristics and assessment of EGFR and EGFRvIII expression by immunohistochemical staining.

clinical data	*The mean gray value for Immunohistochemistry ($\bar{x}$±s)*					*The mean gray value for Western blot ($\bar{x}$±s)*				
	N	*EGFR*	*P*	*EGFR vIII*	*P*	*N*	*EGFR*	*P*	*EGFR vIII*	*P*
Age			0.63		0.99			0.68		0.95
<50 years	7	13.15±2.99		23.59±3.19		5	1.33±0.39		0.89±0.15	
50~60 years	11	17.30±2.54		22.28±3.16		8	1.51±0.49		0.80±0.12	
ÿ60 years	15	14.47±3.01		22.39±3.29		12	1.33±0.39		0.84±0.15	
Invasion degree			0.04							0.02
Sub mucosa	5	13.26±2.87		18.43±3.06		5	1.45±0.46		0.76±0.14	
muscular	8	15.12±2.90		22.13±3.15		3	1.63±0.50		0.88±0.16	
tunica adventitia	20	17.44±2.67		27.97±3.47		17	1.70±0.50		0.80±0.14	
Tumor size			0.97		0.20			0.93		0.74
<3 cm	13	15.85±3.11		22.46±3.23		10	1.39±0.40		0.89±0.16	
3~5 cm	17	15.08±3.01		22.26±3.31		13	1.39±0.30		0.82±0.16	
<5 cm	3	15.34±2.89		29.85±3.02		2	1.36±0.39		0.78±0.13	
Growth pattern			0.20		0.88			0.41		0.79
medullar	12	17.52±2.99		23.91±3.42		10	1.45±0.41		0.80±0.15	
mushroom	4	11.87±2.90		21.63±3.13		4	1.10±0.39		0.76±0.15	
ulcer	15	14.96±3.10		23.04±3.07		11	1.44±0.41		0.84±0.16	
sclerotic	2	8.53±2.87		15.77±2.97		0				
TNM stage[1,*]			0.03		0.03			0.01		0.01
I-II a	16	12.23±3.00		17.71±3.11		13	1.15±0.40		0.65±0.12	
II b-III	17	18.28±3.15		26.47±4.12		12	1.61±0.41		0.97±0.17	
Growth cite*			0.00		0.00			0.05		0.03
superior	1	33.80±3.20		50.26±4.01		1	1.95±0.42		1.61±0.17	
middle	15	12.04±3.11		18.84±3.40		11	1.21±0.41		0.78±0.15	
inferior	17	17.42±2.99		23.87±3.12		13	1.49±0.42		0.79±0.15	
pathological grade[2,*]										
I	8	11.67±2.84		15.32±2.90		6	1.10±0.40		0.65±0.14	
II	16	13.6±2.76		21.39±3.15		13	1.35±0.41		0.76±0.14	
III	9	22.8±3.09		30.71±3.29		6	1.76±0.41		1.11±0.17	
lymph node metastases*				0.05		0.02			0.02	
0.03										
positive	19	12.48±2.95		18.24±3.01		15	1.20±0.40		0.67±0.14	
negative	14	19.09±3.01		27.44±3.16		10	1.64±0.42		1.02±0.17	

*$p < 0.05$, is considered a significant difference.

1. Tumor stage was classified according to the sixth edition of the tumor–node–metastasis classification of the International Union against Cancer.[24]

2. Pathological grade I: well differentiated, II: moderately differentiated, III: poorly differentiated.

In the present study, EGFR and EGFRvIII expression was detected by using immunocytochemistry and Western blot analysis. Image analysis using Leica's Q550CW image analysis software confirmed a significantly higher EGFR and EGFRvIII expression in esophageal cancer than in normal tissue.

Tumorigenesis is a complicated process involving the deregulation of normal cell proliferation, adhesion, migration and invasion. It subsequently leads to the lethality associated with the metastatic spread of malignant tumors. We found that expression of the two proteins EGFR and EGFRvIII was associated with poorer differentiation, higher TNM grade and a higher rate of lymph node metastasis. Our results confirm those of Kuramochi et al.[20] who reported EGFR expression in primary colorectal cancer and corresponding liver metastases. In addition, the expression of EGFR and EGFRvIII was correlated with each other in esophageal cancer tissues. Based on these observations, we propose that EGFR and EGFRvIII play an important role in the occurrence and development of esophageal carcinoma. Therefore the expressions of EGFR and EGFRvIII can reflect the biological behavior of esophageal cancer cells, and their examination may be helpful for evaluation of lymph node metastases, pathological classification, and prognosis of esophageal cancer.

Targeted cancer therapy has also recently received more attention. A number of studies have proven that there is a relationship between the expression of EGFR or EGFRvIII in lung cancer or colon cancer patients, and the clinical benefit that can be achieved by corresponding targeted therapy.[21-23] In the same way, it is presumed that detection of EGFR or EGFRvIII could be the theoretical basis for the use of EGFR or EGFRvIII inhibitor in the targeted treatment of esophageal carcinoma.

We observed that the results obtained from immunohistochemical analysis were well confirmed by Western blot analysis. Since immunohistochemistry is a simpler and more easily readable and cost effective method, it is suggested that immunohistochemical detection may be of benefit in the diagnosis of esophageal carcinoma.

In summary, detection of the expression of EGFR and EGFRvIII can be used to predict tumor malignancy, and may be used as important predictors for early diagnosis and targeted therapy of esophageal carcinoma.

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Authors Contribution:

Xiaoyi Duan and Suna Zhou are contribut equally to this work.

The contribution of Xiaoyi Duan and Suna Zhou: accomplished the design and process experiment, and written the manuscript.

The contribution of Mingxin Zhang: accomplished the data analysis.

The contribution of Peng Wang and Jia Zhang: made a collection of specimens and clinical data of patients.

The contribution of Jiansheng Wang: gave instructions or directions for us during the whole process.

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