

Expression of P-EGFR and P-Akt protein in esophageal squamous cell carcinoma and its prognosis

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Received June 24, 2016; Accepted March 8, 2017

DOI: 10.3892/ol.2017.6526

Abstract. The phosphorylated epidermal growth factor receptor (P-EGFR) and phosphorylated Akt (P-Akt) protein in esophageal squamous cell carcinoma (ESCC) were studied, and its significance in clinical prognosis of patients was assessed. The expression of P-EGFR and P-Akt protein in 83 cases of ESCC and 83 normal esophageal tissues was determined by immunohistochemical staining. Log-rank test and correlation analysis were used to analyze the prognosis of ESCC. The positive expression of P-EGFR in ESCC was 88% (73/83 cases) compared with 41% in normal esophageal mucosa (34/83 cases) ($P<0.05$). The rate of P-Akt protein expression in ESCC was 90.4% (75/83 cases), compared with 27.7% seen in normal esophageal mucosa (23/83 cases) ($P<0.05$). The expression of P-EGFR and P-Akt protein was positively correlated with lymph node metastasis and degree of differentiation ($P<0.05$) irrespective of sex, age, tumor diameter and TNM stage ($P>0.05$). The expression of P-EGFR was positively correlated with that of P-Akt protein ($r=0.674$, $P<0.01$). P-EGFR expression was negatively correlated with survival time of patients with ESCC ($r=-0.526$, $P<0.01$). The Kaplan-Meier survival curves showed that the cumulative survival rate of P-EGFR-positive cases was significantly lower than that of the P-EGFR-negative cases ($P<0.01$). The expression of P-Akt was negatively correlated with survival in patients with ESCC ($r=-0.473$, $P<0.01$). The Kaplan-Meier survival curves showed that the cumulative survival rate of the P-Akt-positive cases was significantly lower than that of the P-Akt-negative cases ($P<0.01$). In conclusion, P-EGFR

and P-Akt protein expression is closely related to the incidence of ESCC and mediates the development of invasive cancer and metastasis. It is used to determine the prognosis of ESCC, and may represent a new therapeutic target for the disease.

Introduction

Esophageal cancer is a common malignant tumor with an increasing incidence and mortality. Surgery combined with radiotherapy and chemotherapy is indicated for esophageal cancer. However, many patients are diagnosed in advanced stages of esophageal cancer, resulting in poor treatment with poor prognosis (1). Clinical practice suggests (2) that recurrence and metastasis are important factors contributing to the death of patients with esophageal cancer. Epidermal growth factor receptor (EGF) is a tyrosine kinase present on cell surface receptors, which affects several growth factors.

Phosphorylated epidermal growth factor receptor (P-EGFR) activates a variety of intracellular signaling pathways, inducing cell proliferation and survival (3). Akt signaling pathway is an important pathway regulating downstream signaling by EGFR (4). Phosphorylated Akt (p-Akt) activates Akt signaling, and the downstream genes in the signaling pathways. It controls the evolution, invasion, development and apoptosis of cancer cells.

Esophageal squamous cell carcinoma (ESCC) is a common pathological type of esophageal cancer. China has a high proportion of ESCC, prompting studies investigating the genetic mechanisms underlying the disease (5-8). However, the pathogenesis of ESCC is not very clear, and the tumor markers for the diagnosis of ESCC are few in number. Therefore, it is imperative to undertake an in-depth study into the genetic and molecular mechanisms underlying the pathogenesis of ESCC. Development of ESCC markers with strong specificity and high sensitivity, and molecular therapeutic targets has significant clinical implications. In this study, we investigated 83 cases of esophageal squamous carcinoma tissue compared with the corresponding normal esophageal mucosa, from January 2009 to October 2010 at the First Affiliated Hospital of Zhengzhou University. The expression of P-EGFR and p-Akt was detected immunohistochemically using the SP method. Its prognostic value was analyzed to provide a valuable standard of reference for diagnosis and prediction of survival.

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Key words: esophageal carcinoma, phosphorylated epidermal growth factor receptor, phosphorylated Akt, immunohistochemistry, prognosis

Materials and methods

Materials. We studied 83 cases of esophageal squamous carcinoma tissue and the corresponding normal esophageal mucosa, from January 2009 to October 2010 at the First Affiliated Hospital of Zhengzhou University. Patients included 44 males and 39 females. The age ranged from 34 to 68 years, with an average age of 56.2 ± 33.5 years. TNM staging revealed stage II in 23 cases, stage IIIa in 25 cases, stage IIIb in 26 cases, and stage IV in 9 cases. Poor differentiation was seen in 50 cases, moderate in 25 cases, and high in 8 cases. Lymph node metastasis was observed in 43 cases, and none in 40 cases. The tumor diameter was <3 cm in 26 cases, 3-5 cm in 32 cases, and >5 cm in 25 cases. The selected subjects were not treated with neoadjuvant therapy. Seventy patients were followed up over 60 months. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Signed written informed consents were obtained from all participants before the study.

Main reagents. Rabbit monoclonal P-EGFR antibody (dilution, 1:100; cat. no. sc-120) and rabbit polyclonal p-Akt antibody (dilution: 1:100; cat. no. sc-8312) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and xylene (Dongguan Jiabao Petroleum Chemical Co., Ltd., Dongguan, China).

Immunohistochemical assay. Sliced specimens were incubated at 58°C for ~ 3 h, and subjected to xylene treatment for dewaxing. P-EGFR was detected as follows using microwave repair at 650 W for 20 min and cooled to room temperature, washed 3 times using Tween-phosphate-buffered saline (PBS), first at 25°C for 16 h, and washed three times by PBS, followed by DAB for coloration, and counterstained with hematoxylin. After dehydration, it was observed under a light microscope (BX-42; Olympus, Tokyo, Japan). PBS was used as a substitute for the first antibody and as the negative control for P-EGFR detection. The positive film was confirmed by Santa Cruz Biotechnology, Inc. that provided the rabbit anti-human P-EGFR as a positive control group.

The p-Akt was detected using boiling repair at 98°C for 20 min, cooled to room temperature, and washed 3 times with PBS. After incubation with the first antibody at 4°C for 16 h, it was washed three times with PBS, and DAB for coloration, followed by hematoxylin for counterstaining. After dehydration the specimen was visualized under a light microscope. PBS was used as a substitute for the first antibody with P-EGFR as the negative control. The positive film was confirmed by Santa Cruz Biotechnology, Inc. that provided rabbit anti-human p-Akt as a positive control group.

Evaluation criterion. In the cytoplasm, cell membrane and nucleus, the yellow particles were found to be positive cells. Each slice was randomly selected under 5 high power fields, and the number of positive cells in 100 cells was calculated.

Scoring criteria for P-EGFR expression level (9): Absence of staining or $<5\%$ tumor cell staining was scored as (-); 5-19% of tumor cell staining was considered as (+); 20-50% was scored as (++) and $>50\%$ tumor cell staining was determined as (+++).

Scoring criteria for p-Akt expression were (10): No staining or $<30\%$ staining was determined as (-), $>30\%$ tumor cell staining

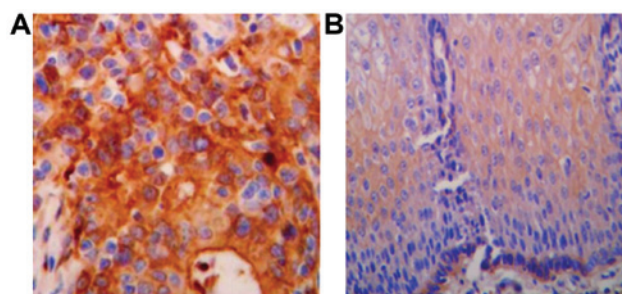


Figure 1. Expression of P-EGFR protein (SP, x400). (A) ESCC; (B) adjacent normal esophageal tissue. P-EGFR, phosphorylated epidermal growth factor receptor; ESCC, esophageal squamous cell carcinoma tissue.

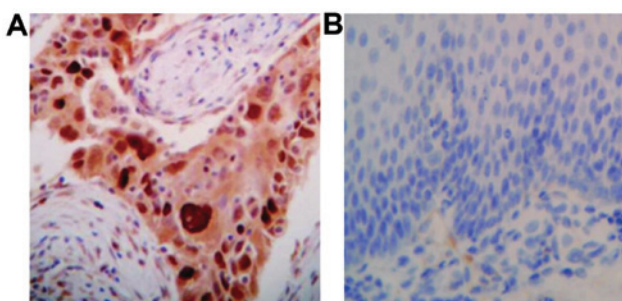


Figure 2. Expression of p-Akt protein (SP, x400). (A) ESCC tissue; (B) adjacent normal esophageal tissue. ESCC, esophageal squamous cell carcinoma tissue.

was determined as positive, yellow as (+), brownish yellow as (++) and brown as (+++). The final results were as follows: (-) denoted negative expression (P-EGFR⁻, p-Akt⁻); and (+), (++) and (+++) represented positive expression (P-EGFR⁺, p-Akt⁺).

Statistical analysis. The experimental data were analyzed by SPSS 21.0 software (IBM, Armonk, NY, USA). The two factors were analyzed by non-parametric Spearman's rank correlation. Kaplan-Meier method was used to analyze the survival based on single factor, and the difference was tested by log-rank test. $P < 0.05$ indicated statistical significance. The survival curve was used for analysis of the follow-up time and the survival condition of the patients.

Results

Expression and distribution of P-EGFR and P-Akt protein. P-EGFR was mainly expressed in the cytoplasm or cell membrane, with the positive expression was represented by brown yellow particles (Fig. 1). The P-Akt protein was expressed in the cytoplasm or nucleus, and the positive expression was visualized as brownish yellow particles (Fig. 2).

The rate of positive expression of P-EGFR in ESCC was 88% (73/83 cases), which was significantly higher than the rate of 41% in the normal esophageal mucosa (34/83 cases) ($P < 0.05$) (Table I).

The rate of P-Akt protein expression in ESCC was 90.4% (75/83 cases), which was significantly higher than in the normal esophageal mucosa (27.7%; 23/83 cases) ($P < 0.05$) (Table II).

Expression of P-EGFR and P-Akt protein and clinical pathology of patients with ESCC. The expression of P-EGFR

Table I. Expression of P-EGFR in esophageal squamous cell carcinoma and adjacent normal esophageal tissues n (%).

Tissue	n	+	++	+++	Total	-
ESCC tissue	83	16 (19.3)	20 (24.1)	37 (44.6)	73 (88)	10 (12)
Adjacent normal esophageal tissue	83	24 (28.9)	6 (7.2)	4 (4.8)	34 (41)	49 (59)
χ^2						16.753
P-value						<0.05

P-EGFR, phosphorylated epidermal growth factor receptor; ESCC, esophageal squamous cell carcinoma.

Table II. Expression of p-Akt in ESCC and adjacent normal esophageal tissues n (%).

Tissue	n	+	++	+++	Total	-
ESCC tissue	83	18 (21.7)	20 (24.1)	37 (44.6)	75 (90.4)	8 (9.6)
Adjacent normal esophageal tissue	83	17 (20.5)	4 (4.8)	2 (2.4)	23 (27.7)	60 (72.3)
χ^2						33.625
P-value						<0.05

P-Akt, phosphorylated Akt; ESCC, esophageal squamous cell carcinoma.

Table III. Pathology of esophageal squamous cell carcinoma: Expression of P-EGFR and P-Akt.

Clinical pathological characteristics	n	P-EGFR-positive cases	χ^2 (P-value)	P-Akt-positive cases	χ^2 (P-value)
Sex					
Male	44	38	0.342 (>0.05)	39	0.351 (>0.05)
Female	39	35		36	
Tumor diameter, cm					
<3	26	23	1.163 (>0.05)	23	2.505 (>0.05)
3-5	32	28		29	
>5	25	22		23	
TNM staging					
II stage	23	21	5.326 (>0.05)	21	1.634 (>0.05)
IIIa stage	25	22		23	
IIIb stage	26	23		24	
IV stage	9	7			
Degree of differentiation					
Low	50	50	9.741 (<0.05)	49	9.266 (>0.05)
Middle	25	19		22	
High	8	4		4	
Lymph node metastasis					
Yes	43	42	8.176 (<0.05)	43	6.282 (<0.05)
No	40	31		32	

P-EGFR, phosphorylated epidermal growth factor receptor; P-Akt, phosphorylated Akt.

and P-Akt protein in patients with ESCC was correlated with lymph node metastasis and degree of differentiation ($P<0.05$), irrespective of sex, age, tumor diameter or TNM stage ($P>0.05$; Table III).

Expression of P-EGFR and P-Akt protein in ESCC. Pearson correlation analysis showed that the expression of P-EGFR was positively correlated with that of P-Akt protein in ESCC ($r=0.674$, $P<0.01$) (Fig. 3).

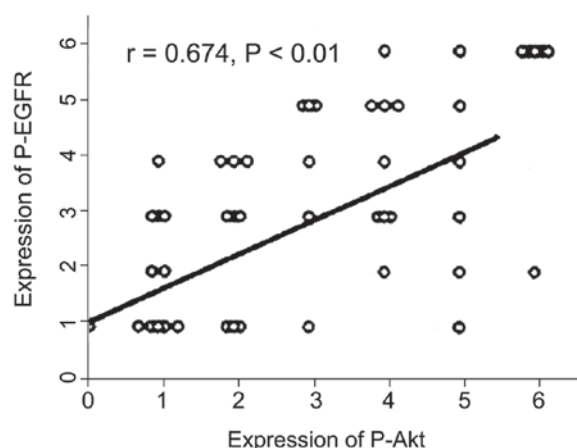


Figure 3. Correlation of P-EGFR and P-Akt expression in ESCC. P-EGFR, phosphorylated epidermal growth factor receptor; ESCC, esophageal squamous cell carcinoma tissue.

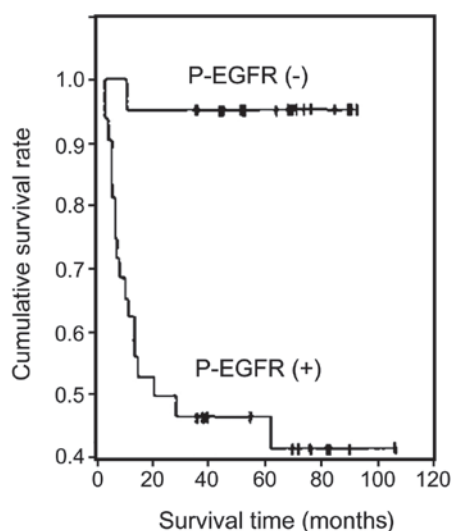


Figure 4. Survival curve of P-EGFR and prognosis of ESCC. P-EGFR, phosphorylated epidermal growth factor receptor; ESCC, esophageal squamous cell carcinoma tissue.

Relationship between P-EGFR and P-Akt expression with survival time in patients with ESCC. P-EGFR expression was negatively correlated with survival time in patients with ESCC ($r = -0.526$, $P < 0.01$). Kaplan-Meier survival curves showed that the cumulative survival rate of the P-EGFR-positive cases was significantly lower than that of the P-EGFR-negative cases ($P < 0.01$) (Fig. 4).

The expression of P-Akt was negatively correlated with survival in patients with ESCC ($r = -0.473$, $P < 0.01$). Kaplan-Meier survival curves showed that the cumulative survival rate of P-Akt-positive cases was significantly lower than that of the P-Akt-negative cases ($P < 0.01$) (Fig. 5).

Discussion

Esophageal cancer is a common malignant tumor of the digestive tract, with a poor prognosis. It is generally divided into squamous cell carcinoma and adenocarcinoma (11). However, there are regional differences in the distribution of

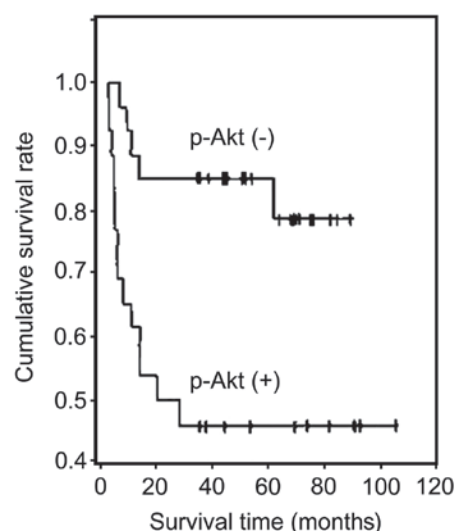


Figure 5. Survival curve of P-Akt and prognosis of ESCC. ESCC, esophageal squamous cell carcinoma tissue.

esophageal cancer: Adenocarcinoma is prevalent in Europe and America, but squamous cell carcinoma occurs predominantly in China. In recent years, several studies have investigated esophageal cancer (12-14). Clinical studies show (15) that patients with ESCC are treated with chemotherapy. Results show that the esophageal cancer is triggered by multiple factors in multiple stages. The developmental course is divided into simple hyperplasia, atypical hyperplasia, carcinoma *in situ* and infiltrative cancer. A series of oncogenes and anti-oncogenes are expressed. N-methyl-N nitrosourea alkyl induces ESCC in rats. Experimental studies confirmed the occurrence of esophageal cancer following long-term exposure to specific carcinogens (16). Therefore, researchers proposed several models, such as nitrosamine carcinogenic model, 4-nitroquinoline-oxide model, ectopic transplantation, ESCC model, and orthotopic transplantation of ESCC. Studies investigated the etiology and development of ESCC. However, the specific regulatory mechanism of ESCC and its pathogenesis remain obscure. The absence of effective clinical treatment resulted in a high incidence of ESCC, poor clinical prognosis, and high mortality rate.

Advances in molecular biology have shed new light on the molecular markers of prognosis in ESCC, including the expression of Fn14, VEGF, NGX6, COX-2, cyclin D1, E-cadherin, and IMP3. The value of prognosis in ESCC is established. Recent studies have indicated that (1,17) a high expression of EGFR is related to prognosis of nasopharyngeal carcinoma. Therefore, we investigated the molecular targeted therapies of cancer. Using EGFR as molecular targets, drugs such as erlotinib and cetuximab have been developed. P-EGFR belongs to the active form of EGFR. Studies have reported that EGFR itself is not an important factor in cancer (such as nasopharyngeal) cell proliferation. Elevated P-EGFR expression plays a key role in the prevalence of cancer, and induces the proliferation of cancer cells. However, researchers investigating gastrointestinal carcinoid and pancreatic cancers detected increased expression of P-EGFR and EGFR proteins. The study also found that pancreatic cancer patients with low or no expression of P-EGFR showed better prognosis than

patients with high expression of P-EGFR (18). Akt is highly activated in tumors suggesting that the growth, differentiation and proliferation of tumor cells, was abnormal. *In vitro* studies suggest that the phosphorylation of Akt residues threonine 308 and serine 473 was closely related to the activation of PI3K/Akt signaling (19). However, the role and clinical significance of P-Akt in the occurrence, development and evolution of tumors in the human body is not very clear.

Cancer specimens derived from pathological archives of immunohistochemical staining revealed gene products in patients with tumor, and retrospective analysis of clinical data is an important approach of clinical investigation. Tumor dissemination in the body caused by cancer metastasis is refractory to surgical treatment. Therefore, it is imperative to understand the factors associated with tumor metastasis, and understand the mechanisms underlying invasive cancer, to predict cancer metastasis and clinical treatment.

In this study, we analyzed P-EGFR and P-Akt expression in ESCC tissues and in the corresponding normal esophageal mucosa immunohistochemically. We found a P-EGFR positive expression rate of 88% in cancer tissues of ESCC, which was significantly higher than the 41% found in normal esophageal mucosa tissues ($P<0.05$). The positive rate of P-Akt protein expression in the cancer tissue of patients with ESCC was 90.4%, which was significantly higher than in the corresponding normal esophageal mucosa tissues, at 27.7% ($P<0.05$). The positive rate of P-Akt and P-EGFR protein expression in ESCC is correlated with lymph node metastasis and differentiation ($P<0.05$) independent of sex, age, tumor diameter and TNM stage ($P>0.05$). The level of P-Akt and P-EGFR expression may be closely correlated with the occurrence and evolution of ESCC.

Our analysis showed that the P-EGFR and P-Akt protein expression in ESCC was positively correlated ($r=0.674$, $P<0.01$). P-EGFR and P-Akt show a synergistic effect in regulating the proliferation and survival of ESCC cells *in vivo*.

We analyzed the follow-up data and survival time. The results suggest that the expression of P-EGFR was negatively correlated with the survival time of patients with ESCC ($r=-0.526$, $P<0.01$). Kaplan-Meier survival curves showed that the cumulative survival rate of the P-EGFR-positive cases was significantly lower than that of the P-EGFR-negative cases ($P<0.01$). The expression of P-Akt was negatively correlated with survival in patients with ESCC ($r=-0.473$, $P<0.01$). Kaplan-Meier survival curves showed that the cumulative survival rate of P-Akt-positive cases was significantly lower than that of the P-Akt-negative cases ($P<0.01$). P-Akt and P-EGFR promoted metastasis of ESCC and shortened the survival of patients with ESCC.

In conclusion, the high expression of P-Akt and P-EGFR is related to lymph node metastasis and differentiation of ESCC. P-Akt and P-EGFR represent markers of ESCC. The combination of P-EGFR and P-Akt levels is helpful in evaluating the severity of ESCC and predicting their survival time.

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