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Expression and prognostic influence of NF-κB and EGFR in esophageal cancer

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ABSTRACT. The goal of this study was to investigate the expression profiles of nuclear factor-kappa B (NF-κB) and epidermal growth factor receptor (EGFR) in esophageal cancer and to determine their association with tumor prognosis. This study included 40 esophageal cancer patients [22 men and 18 women; average age = 62.7 ± 3.9 years; tumor-node-metastasis (TNM) staging: 12 patients with stage I, 13 patients with stage II, and 15 patients with stage III disease]. Tumor tissues and tumor-adjacent tissue specimens were collected during radical resections at our hospital. Immunohistochemical staining was used to examine these tissues for NF-κB and EGFR expression. Follow-up of all patients included gathering information such as the 3-year survival rate. We found that NF-κB and EGFR expression was significantly higher in tumor tissues compared to tumor-adjacent normal tissues. Expression was not related to gender or age, but was positively associated with the degree of tumor infiltration. NF-κB and EGFR expression levels gradually increased with higher TNM stage, but this difference was not significant. Follow-up results showed that

patients with higher NF-κB and EGFR levels had a lower survival rate and unfavorable prognosis. In conclusion, we found that NF-κB and EGFR expression was significantly elevated during the occurrence and development of esophageal carcinoma, and expression of these factors appears to be correlated with cancer progression. Higher expression of both genes is associated with an unfavorable prognosis.

Key words: Epidermal growth factor receptor; Esophageal carcinoma; Nuclear factor-kappa B; Tumor prognosis

INTRODUCTION

Esophageal cancer is a commonly occurring malignant tumor of the digestive tract that causes approximately 300,000 deaths worldwide annually. The incidence and mortality rates of this disease vary widely in different countries. China has a high rate of esophageal cancer; approximately 150,000 people die from esophageal carcinoma every year in China, and this disease has a high incidence in men over 40 years old. The most common symptom of esophageal cancer is advancing dysphagia. The pathogenesis of this disease is complicated and includes both internal and external factors. External factors mainly include chemical stimuli, biological pathogen stimuli, insufficient vitamin uptake, and other bad habits, while internal factors include immunosuppression induced by cascade reactions from multiple stages of expression regulation of various genes. It is commonly accepted that esophageal cancer pathogenesis is regulated and influenced by various cytokines (Golledge et al., 2010; Martinez-Pinna et al., 2010; Schwenk et al., 2010; Michel et al., 2011).

Nuclear factor-kappa B (NF-κB), which was first identified in B lymphocyte extracts by Sen and Baltimore, is a nuclear protein factor that can specifically bind to the κB sequence (5'-GGGACTITCC-3') in the enhancer element of the immunoglobulin κ gene (van Kuijk et al., 2010; Ramos-Mozo et al., 2012). NF-κB can be detected in various cell types, and its activation and inhibition are directly correlated with the pathogenesis of multiple diseases. Activated NF-κB specifically binds to certain sequences that are gene expression regulatory elements to impact gene expression, cell proliferation, differentiation, apoptosis, or even the immune system response. Studies have found a close relationship between abnormal NF-κB expression and tumor occurrence. For example, the pathogenesis of colon and lung cancer is accompanied by different degrees of abnormal alteration of NF-κB (O'Hare et al., 2010; Seidel et al., 2010).

Epidermal growth factor receptor (EGFR), which is the specific receptor for epidermal growth factor (EGF), belongs to the family of transmembrane tyrosine kinase growth factor receptors. EGFR is currently a major drug target for cancer treatment because of its critical roles in the pathogenesis and progression of various tumors and its ability to modulate cell proliferation, migration, infiltration, and apoptosis (Chang et al., 2011; Wojtkowiak et al., 2011; Chan et al., 2013).

This study attempted to identify the effect of NF-κB and EGFR expression on the development and prognosis of esophageal carcinoma in order to provide new drug targets for the clinical treatment of esophageal cancer.

MATERIAL AND METHODS

General information

For this study, we selected 40 patients who had undergone radical resection of esophageal carcinoma at The Zhumadian Central Hospital of Henan Province since January 2008 who had complete medical records. The patient group included 22 men and 18 women with an average age of 62.7 ± 3.9 years. Tumor-node-metastasis (TNM) staging results indicated that the patient group included 12 patients with stage I disease, 13 patients with stage II disease, and 15 patients with stage III disease. Pathological examination revealed that 13 patients had highly differentiated tumors, 13 patients had moderately differentiated tumors, and 14 patients had poorly differentiated tumors. No patients in the study received cancer treatment prior to surgery. Pathological tissues and tumor-adjacent tissues (distance > 3 to 5 cm) were collected, immediately fixed in 4% paraformaldehyde, and sent to the Department of Pathology for preparation of paraffin tissue blocks.

Reagents

Goat anti-human NF-κB and EGFR primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Mouse anti-goat secondary antibody was obtained from Hebei Xinle Sci & Tech Co., Ltd. (Shijiazhuang, Hebei, China). An Streptavidin-Peroxidase (SP) reagent kit and DAB chromogenic test kit were purchased from Zhongshan Corp. (Zhongshan, Guangdong, China). All reagents and buffers used for immunohistochemistry (IHC) were prepared in-house following manual instructions.

IHC for NF-κB and EGFR expression

IHC staining was performed to detect expression of NF-κB and EGFR in tumor tissues and tumor-adjacent tissues. In brief, tissues were sectioned into 5-μm-thick slices and were dewaxed and dehydrated following routine procedures. Endogenous peroxidase activity was quenched using 3% H_2O_2, followed by 3 washes in Phosphate buffered saline (PBS) for 5 min. Antigen retrieval was performed in a microwave for 10 min, following which specimens were cooled at room temperature and blocked in normal horse serum. Tissue slices were incubated with primary antibody at 37°C for 1.5 h, biotin-labeled, incubated in secondary antibody for 10 min, and processed in a streptavidin-biotin-H_2O_2 complex for 10 min. Staining was developed using DAB substrates, rinsed in distilled water, counter-stained in hematoxylin, dehydrated, and mounted with coverslips. A negative control group was processed in parallel using PBS instead of a primary antibody. Under microscopic observation, 6 fields were randomly selected for analysis for each tissue slice. Brown granules in the cytoplasm were considered to be positive signals. The strength of the staining signal was classified by color: light yellow was weak staining; brown was moderate staining; chocolate brown was strong staining. Each field was also classified based on staining strength as: negative (no positive cells), weakly positive (less than half of the cells with weak or moderate staining, with <30% positive cells), or positive (more than half of the cells with weak or moderate staining, with >30% positive cells). The nuclei were stained blue, but there were no brown reaction products in the cytoplasm.

Reverse transcriptase polymerase chain reaction analysis of NF-κB and EGFR expression

Total RNA was extracted from tissues and used as a template for cDNA synthesis using a reverse transcription kit. Reverse transcriptase polymerase chain reaction (RT-PCR) primers were designed based on the CDS gene sequences of NF-κB and EGFR obtained from the National Center for Biotechnology Information database. Sequences for all primers used are listed in Table 1. Using β-actin as an internal reference, the expression profiles of NF-κB and EGFR were measured.

Table 1. Polymerase chain reaction primer sequences.

Primer name	Sequence (5'-3')	Expected length (base pairs)
NF-κB-F	ATGGC AGAAG ATGAT CCATA TTTGG	2800
NF-κB-R	CCAAA TATGG ATCAT CTTCT GCCAT	
EGFR-F	ATGCG ACCCT CCGGG ACGGC CGGGG	1200
EGFR-R	CCCCG GCCGT CCCGG AGGGT CGCAT	
β-actin-F	GATCT ACATC AGTGC TATTG ATAAA	800
β-actin-R	TTTAT CAATA GCACT GATGT AGATC	

EGFR = epidermal growth factor receptor; F = forward; NF-κB = nuclear factor-kappa B; R = reverse.

Patient follow-up and data analysis

All patients underwent periodic follow-up after surgery to ascertain 3-year survival rates. We compared these survival rates among groups (TNM stage and tumor differentiation degree). The SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA), was used to analyze all collected data, which are reported as means ± standard error. In-group comparisons were performed using independent sample chi-square tests. Statistical significance was defined as $P < 0.05$.

RESULTS

NF-κB and EGFR expression

Images from IHC staining for NF-κB and EGFR in both tumor tissues and tumor-adjacent tissues are shown in Figure 1. Expression of NF-κB and EGFR was significantly higher in tumor tissues compared to tumor-adjacent normal tissues. Statistical analysis of NF-κB and EGFR expression levels in tissues of different pathological stages and differentiation degrees is shown Table 2. The rate of positivity for NF-κB/EGFR expression was not correlated with tumor stage or differentiation degree, and there were no statistically significant differences between groups ($P > 0.05$). The trends in expression levels of NF-κB and EGFR across different pathological stages and differentiation degrees were similar (Figure 2).

NF-κB and EGFR mRNA expression

We used RT-PCR to quantify mRNA transcript levels for both NF-κB and EGFR. Levels of NF-κB and EGFR mRNA were significantly higher in tumor tissues compared to tumor-adjacent normal tissues (Figure 3).

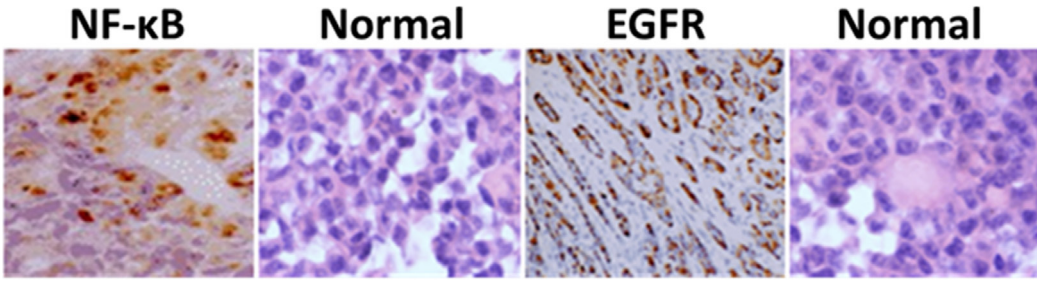

Figure 1. Images of immunohistochemistry staining for NF-κB and EGFR (200X). EGFR = epidermal growth factor receptor; NF-κB = nuclear factor-kappa B.

Table 2. Expressional levels of NF-κB and EGFR.

Group	TNM stage			Differentiation degree		
	Stage I	Stage II	Stage III	High	Moderate	Low
No. of patients	12	13	15	13	13	14
Negative (N, %)	0 (0)	0 (0)	1 (6.7)	0 (0)	0 (0)	1 (7.1)
Positive (N, %)	9 (75)	9 (69.2)	11 (73.3)	11 (84.6)	11 (84.6)	12 (85.8)
Weakly positive (N, %)	3 (25)	4 (30.8)	3 (20)	2 (15.4)	2 (15.4)	1 (7.1)
NF-κB	75.35 ± 3.36	66.48 ± 4.16	64.45 ± 4.35	71.34 ± 3.34	69.76 ± 3.42	67.58 ± 6.06
EGFR	74.40 ± 5.36	67.49 ± 3.56	66.38 ± 3.86	75.38 ± 2.16	73.23 ± 4.72	69.97 ± 3.87

EGFR = epidermal growth factor receptor; NF-κB = nuclear factor-kappa B; TNM = tumor-node-metastasis.

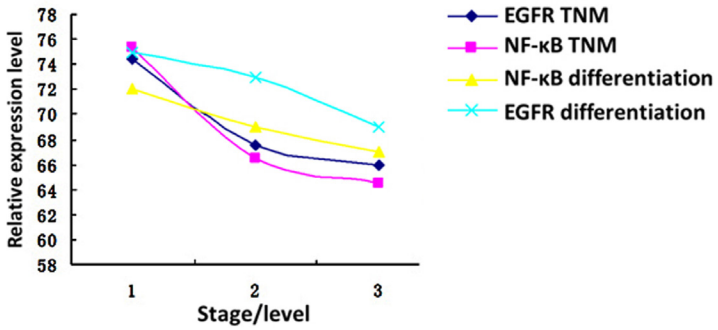

Figure 2. Trends in NF-κB and EGFR expression levels. EGFR = epidermal growth factor receptor; NF-κB = nuclear factor-kappa B.

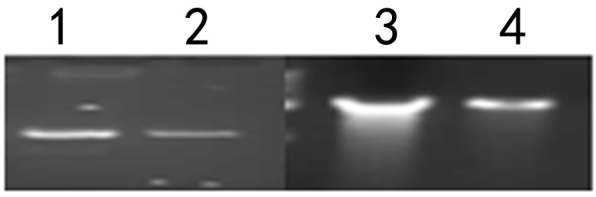

Figure 3. Assessment of mRNA levels of NF-κB and EGFR: 1) NF-κB in tumor tissue; 2) NF-κB in tumor-adjacent normal tissue; 3) EGFR in tumor tissue; 4) EGFR in normal tissue. EGFR = epidermal growth factor receptor; NF-κB = nuclear factor-kappa B.

Statistical analysis of post-surgery follow-up data

All patients were successfully followed-up after surgery. We collected data concerning 3-year survival rates (Table 3). Combined with our expression analysis of NF-κB and EGFR (Table 2), we were able to discern a positive relationship between patient prognosis and NF-κB/EGFR levels. This suggests that there is an interaction or correlation between NF-κB and EGFR expression.

Table 3. Follow-up statistics.

Group	TNM stage			Differentiation degree		
	Stage I	Stage II	Stage III	High	Moderate	Low
No. of patients	12	13	15	13	13	14
No. of survivors	4	3	7	4	5	8
Survival rate (%)	30	23.1	46.7	30.8	38.5	57.1

TNM = tumor-node-metastasis.

DISCUSSION

As previously described, NF-κB can specifically bind certain sequences in the regulatory elements of the immunoglobulin κ gene, affecting its normal genetic regulation and modulating cell proliferation, differentiation, and apoptosis, thereby participating in the pathogenesis of multiple diseases. Researchers have found that NF-κB is involved in the transcriptional regulation of various genes related to apoptosis and proliferation, allowing it to modulate tumor occurrence and development; therefore, NF-κB may warrant further studies as a novel target for tumor prevention and treatment (Hughes et al., 2005; Sudo et al., 2007; Bock et al., 2010; Golledge et al., 2010; Hoare et al., 2011). It is possible that specific inhibition of NF-κB expression can effectively suppress tumor progression and improve patient prognosis (Brand et al., 2013). A study of NF-κB expression profiles in esophageal carcinoma may provide further evidence for its utility as a target in cancer treatment (Sudo et al., 2007; Brand et al., 2013; Fujii et al., 2013).

Studies of EGFR usually focus on the EGF-EGFR signaling pathway. Various studies have shown that the pathogenesis and development of multiple tumors requires modulation of EGF-EGFR pathway function, and this pathway is a major target for cancer therapy. EGFR, which is the specific receptor for EGF, is overexpressed in various tumors and is often associated with unfavorable prognosis (Pretto et al., 2012). High EGFR expression frequently causes abnormal cell differentiation, which impairs cell adhesion, leading to tumor metastasis. Although there is some evidence that EGFR has utility as an index for estimation of esophageal carcinoma prognosis (Chaux et al., 2012), its value as a single index is still uncertain and requires further study. A more accurate method for estimation of esophageal cancer prognosis can only be achieved by combining assessment of EGFR with examination of other genes (Dutta et al., 2011; Godek et al., 2011; Scartozzi et al., 2011; Xu et al., 2012).

This study, which was based on previous knowledge, detected the expression profiles of NF-κB and EGFR in esophageal carcinoma in order to provide support for the use of these factors for clinical treatment and evaluation of prognosis. Our study found that NF-κB and EGFR were highly expressed in esophageal carcinoma tissues, but this expression had little correlation with tumor stage or differentiation degree ($P > 0.05$). Our clinical observations revealed a positive relationship between NF-κB/EGFR expression and the degree of tumor infil-

tration, as well as a common trend in their expression levels in tissues across different stages. Our experimental results identified some correlations between NF-κB and EGFR expression, possibly because of elevated NF-κB levels indirectly induced by EGFR. We also found that high NF-κB or EGFR expression correlated with unfavorable prognosis in esophageal cancer patients. This is consistent with previous studies and suggests a potential function of NF-κB and EGFR expression in esophageal cancer.

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