Original Article
Expression of RKIP, E-cadherin and NF-kB p65 in esophageal squamous cell carcinoma and their correlations

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Abstract: To detect the expression of RKIP, E-cadherin and NF-kB p65 in esophageal squamous cell carcinoma (ESCC) and study their correlations. Steptavidin-peroxidase (S-P) method was employed to detect the expressions of RKIP, E-cadherin and NF-kB p65 in ESCC tissues from 77 cases and paracancerous tissues from 77 cases. The correlations between their expressions and clinicopathological indices and between the expressions of these proteins themselves were analyzed. The expressions of RKIP and E-cadherin in ESCC tissues were obviously lower than those in the paracancerous tissues ($P<0.01$); the expressions in ESCC tissues from cases with lymph node metastasis were lower than those from cases without lymph node metastasis ($P<0.01$); the expression of RKIP was positively correlated with the expression of E-cadherin in ESCC tissues ($P<0.01$). The expression of NF-kB p65 in ESCC tissues was correlated with clinical staging, lymph node metastasis and tumor differentiation ($P<0.01$); the expression of RKIP was negatively correlated with the expression of NF-kB p65 in ESCC tissues ($P<0.05$). Downregulation or depletion of RKIP was related to the onset and progression of ESCC, and facilitated the invasion and metastasis of ESCC by downregulating E-cadherin and upregulating NF-kB p65.

Keywords: Esophageal squamous cell carcinoma (ESCC), RKIP, E-cadherin, NF-kB p65

Introduction

China has a high incidence of esophageal squamous cell carcinoma (ESCC), and the average mortality in Ci County and She County of Hebei Province reaches as high as 142.19/100000 [1]. Because ESCC has no symptoms in early stage, many patients are diagnosed in middle to late stage when the outcomes of radical surgery, chemotherapy and radiotherapy are not satisfactory. The five-year survival of these patients is 15% [2]. Along with the advances in molecular biology of tumors, targeted therapy now represents a new direction for comprehensive treatment of tumors. In the present study, we detected the expressions of RKIP, E-cadherin and NF-kB p65 in ESCC tissues and discussed the correlations between them by using SP method.

Materials and methods

Specimens

ESCC tissues and paracancerous tissues were collected from 77 cases with ESCC receiving surgery at the Fourth Hospital of Hebei Medical University from December 2008 to December 2010. These cases included 59 males and 18 females aged 37-83 years (average, 61.8±8.7 years); 29 cases were aged below 60 years and 48 cases aged ≥60 years. ESCC group showed no significant differences from healthy control in terms of gender and age structure ($P>0.05$); the proportion of subjects that smoked was also not significantly different between the two groups ($P>0.05$). TNM Staging System (6th Edition) revised by Union for International Cancer Control (UICC) in 2002 and developed by American Joint Committee on Cancer (AJCC) was used. According to these criteria, 33 cases were classified as stages I and II, respectively, and 44 cases as stage III and IV respectively. Pathologically, 20 cases were classified as high differentiation, 26 cases classified as moderate differentiation, and 31 cases classified as low differentiation. Forty five cases had lymph node metastasis, and 32 cases had no lymph node metastasis. No cases received preoperative radiotherapy or chemotherapy and the

diagnosis was pathological confirmed. HE staining was performed to determine the composition of tumor tissues and whether there was tumor cell infiltration.

Reagents

Rabbit RKIP polyclonal antibody (Beijing Biosynthesis Biotechnology Co., Ltd.); rabbit anti-human E-cadherin polyclonal antibody (Wuhan Boster Biological Engineering Co., Ltd.); rabbit SP immunochemistry kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.); mouse anti-human NF-kB monoclonal antibody and PV-6000 immunochemistry kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.); DAB reagent (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.); PBS buffer (Tianjin Standard Science Technique Co., Ltd.); citrate liquid (Tianjin Bodi Chemical Co., Ltd.).

Immunohistochemistry

ESCC tissues and paracancerous lesions 5 cm from the tumor were collected intraoperatively and fixed in 10% formalin. After dehydration and embedding, the specimens were cut into about 3-4 μm and baked overnight. Dewaxing and antigen retrieval at high pressure and high temperature were performed. Then the specimens were incubated with primary antibodies at 37°C and then with secondary antibodies. DAB reagent was added for color development, and hematoxylin counterstaining, dehydration and slide sealing were performed. For negative control, PBS buffer was used instead of primary antibodies; for positive control, the sections positive for RKIP, E-cadherin and NF-kB p65 were used.

Criteria for interpreting the results

Positive staining for RKIP was defined as appearance of uniform, brown particles in the cytoplasm. Using Fromowitz's scoring standard [3], 5 high-power fields of view (×400) were randomly selected to count the total tumor cells and positive cells, and the percentage of positive cells was calculated. Positive staining for E-cadherin was defined as appearance of yellowish brown or light yellow particles in the cell membrane and cytoplasm; the percentage of positive cells was calculated according to the

immunochemistry criteria by Ji and Shi [4]: number of positive cells <10% or no positive staining was considered as negative result, and number of positive cells ≥10% as positive result. Positive staining for NF-kB p65 [5] was defined as appearance of brown particles in the nuclei. Ten high-power fields were randomly selected and 1000 tumor cells were counted to calculate the percentage of positive cells: no positive cells or number of positive cells ≤10% was considered as negative; number of positive cells ≥10% was considered as positive. If one tumor tissues had different degree of differentiation, the region showing dominant degree of differentiation was chosen to count the cells [6]: 0 point for number of positive cells ≤25%, 1 point for 26%-50%, 2 points for 51%-75% and 3 points for >75%. Scoring was performed based on the staining intensity presented by the majority of positive cells: 0 point for no staining, 1 point for light brown, 2 points for brown, and 3 points for tan. The above two scores were added up, and "-" for 0 point, "+" for 1-2 points, "++" for 3-4 points, and "+++" for 5-6 points [7]. The result of "++" and "+++" in staining for RKIP, E-cadherin and NF-kB p65 was considered as positive expression; the result of "-" and "+" was considered as negative expression. The sections were reviewed by 3 experienced pathologists and the average scores were taken as the final results.

Statistical analysis

All statistical analyses were performed using SPSS 11.5 software. Data were expressed as frequencies and percentages. Wilcoxon two-sample test was adopted for the comparison of ordered categorical data of the two groups; for comparison of unordered categorical data between two groups or several groups, χ^2 test was used. Correlation analysis between the two ordered categorical variables was carried out using Spearman's rank correlation test, and $P<0.05$ indicated significant differences.

Results

Expression of RKIP, E-cadherin and NF-kB p6 in ESCC tissues and paracancerous tissues

The expressions of RKIP, E-cadherin and NF-kB p6 showed significant differences in ESCC tissues and paracancerous tissues ($P<0.01$); the expressions of RKIP and E-cadherin in ESCC tis-

Table 1. Expressions of RKIP, E-cadherin and NF-kB p65 in ESCC tissues and paracancerous tissues

Cases		RKIP express				E-cadherin express				NF-kB p65 express			
		-	+	++	+++	-	+	++	+++	-	+	++	+++
Cancer tissues	77	33	16	17	11	6	20	29	22	10	25	33	9
Tissue adjacent to carcinoma	77	18	21	29	9	0	9	21	47	63	11	3	0
u values		6.153				4.143				7.344			
p values		<0.001				<0.001				<0.001			

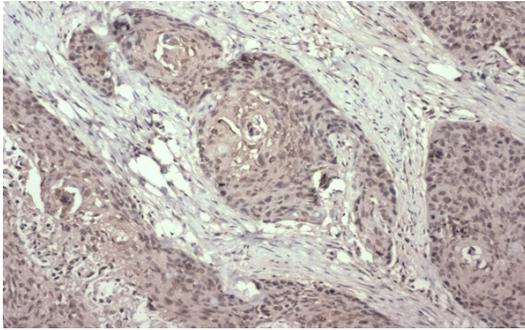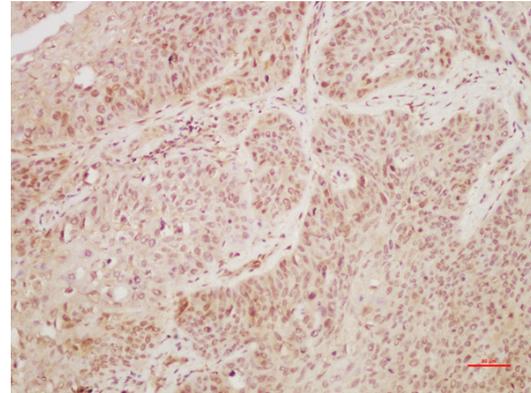**Figure 1.** Positive expression of RKIP in ESCC tissues (IHC×200).

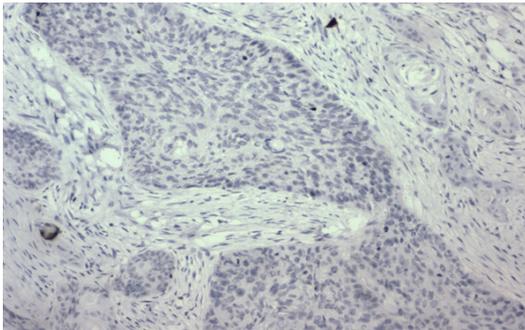**Figure 2.** Negative expression of RKIP in ESCC tissues (IHC×200).

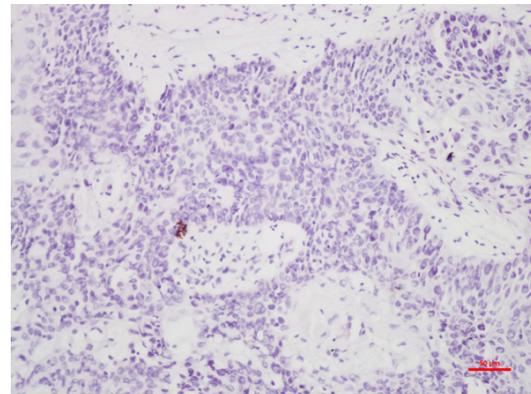**Figure 3.** Positive expression of NF-kp65 in ESCC tissues (IHC×200).

Figure 4. Negative expression of NF-kp65 in ESCC tissues (IHC×200).

sues were considerably lower than those in paracancerous tissues ($P<0.001$); the expression of NF-kB p65 in ESCC tissues was much higher than that in paracancerous tissues ($P<0.001$) (**Table 1**; **Figures 1-6**).

Correlations between expression of RKIP, E-cadherin and NF-kB p65 in ESCC tissues and clinicopathological indices

Positive expressions of RKIP, E-cadherin and NF-kB p65 in ESCC tissues were not correlated with gender and age ($P>0.05$). The positive expression of RKIP in ESCC tissues was not significantly correlated with differentiation degree and clinical staging ($P>0.05$); the positive expressions of E-cadherin and NF-kB p65 in ESCC tissues were both correlated with differentiation degree and clinical staging ($P<0.01$). The expressions of RKIP and E-cadherin in ECSS tissues with lymph node metastasis were obviously lower than those in ESCC tissues without lymph node metastasis ($P<0.01$) (**Table 2**). NF-kB p65 was significantly upregulated in ESCC tissue showing lymph node metastasis compared with ESCC tissues showing no lymph node metastasis ($P<0.01$).
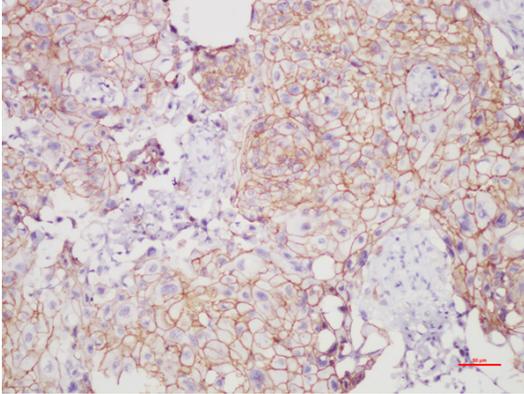
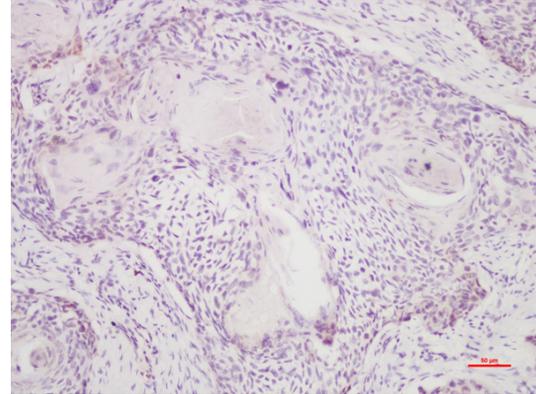**Figure 5.** Positive expression of E-cad in ESCC tissues (IHC×200).

Figure 6. Negative expression of E-cad in ESCC tissues (IHC×200).

Correlations between RKIP expression and the expressions of E-cadherin and NF-kB p65

RKIP expression in ESCC tissues showed a positive linear correlation with E-cadherin expression (rs=0.322, *P*<0.01). RKIP expression was negatively correlated with NF-kB p65 expression in ESCC tissues (rs=-0.324, *P*<0.01) (**Table 3**).

Discussion

As a member of phosphatidylethanolamine-binding protein (PEBP) family, Raf kinase inhibitor protein (RKIP) is a highly conservative and extensively expressed small-molecule cytoplasmic protein [8]. RKIP not only interferes with Raf-1/MEK/ERK signaling pathway, but also inhibits signal transduction by NF-κB and G protein-coupled receptors [9, 10]. Evan T Keller et al. [11] showed in 2005 that RKIP was most highly expressed in normal prostate tissues. With the increase of Gleason score, the expression level of RKIP in prostate cancer tissues was decreased, and no RKIP was detected in metastatic prostate tissues. This indicated that depletion of RKIP was positively correlated with the metastasis of prostate cancer. Similar findings were reported with colorectal cancer: RKIP was lowly expressed in colorectal cancer with lymph node metastasis, and RKIP expression was negatively correlated with tumor recurrence and survival [12, 13]. In mice, upregulation of RKIP was related to the reduction of vascular invasion and inhibited growth of primary tumors [14]. We found that RKIP was obviously downregulated in ESCC tissues compared with paracancerous tissues; RKIP expression in

ESCC tissues with lymph node metastasis was lower than that in ESCC tissues without lymph node metastasis. However, no correlations were found with age, gender, clinical staging and differentiation degree. RIKP was involved in the regulation of metastasis of ESCC, as was found by previous studies. Raf/MEK/ERK signaling pathway is among the most active in MAPK cascade and it can phosphorylate downstream substrate MEK, which in turn activates the ERK downstream target that plays a dominant role. After that, the activated ERK migrates to the nuclei, which enables signal transduction from cell surface to nuclear transcription factors, thereby regulating the transcription [15]. RKIP is the natural inhibitor of this pathway and the depletion of RKIP in ESCC tissues causes the loss of inhibitory action on the pathway. As a result, the transcription and invasiveness of tumor cells are enhanced, and tumor metastasis is promoted.

E-cadherin is a Ca^{2+}-dependent adhesion molecule that mediates adhesion between epidermal cells and the interactions between cells of the same type. While playing a crucial role in maintaining the integrity of epithelial functions, E-cadherin also inhibits tumor metastasis and mediates the adhesion between cells and between cells and extracellular matrix through cytoplasmic catenin and cytoskeletal protein [16]. Abnormalities of functions and structure of E-cadherin can lead to reduced adhesion between tumor cells and hence metastasis. Research shows that abnormal expression of E-cadherin has significant correlates with tumor differentiation, invasion and metastasis [17]. Yu et al. [18] reported that abnormal expres-

Table 2. Correlations between expressions of RKIP, E-cadherin and NF-kB p65 in ESCC tissues and clinicopathological indices

Cases		RKIP express +	RKIP express -	X^2 values	P values	E-radhein express +	E-radhein express -	X^2 values	P values	NF-kB p65 express +	NF-kB p65 express -	X^2 values	P values
Gender													
Male	59	22	37	0.093	>0.05	40	19	0.276	>0.05	34	25	0.967	
Female sex	18	6	12			11	7			8	10		>0.05
Age (years old)													
<60	29	9	20	0.571	>0.05	19	10	0.011	>0.05	16	13	1.440	>0.05
≥60	48	19	29			32	16			33	15		
Clinical stage													
I+II	33	16	17	3.667	>0.05	17	16	5.594	<0.05	20	13	6.731	<0.01
III+IV	44	12	32			34	10			38	6		
Differentiation degree													
High	20	9	11	2.535	>0.05	18	2	27.328	<0.01	10	10	9.787	<0.01
In	26	11	15			7	19			23	3		
Low	31	8	23			26	5			25	6		
lymphatic metastasis													
No	32	17	15	6.648	<0.01	14	18	12.377	<0.01	19	13	7.494	<0.01
Yes	45	11	34			37	8			39	6		

Table 3. Correlations between RKIP expression and the expressions of E-cadherin and NF-kB p65

RKIP express	E-cadherin express -	+	++	+++	NF-kB p65 express -	+	++	+++	Total
-	1	18	22	8	5	20	18	6	49
+	1	4	6	12	9	5	5	4	23
++	0	1	1	3	3	0	0	0	5
Total	2	23	29	23	19	25	23	10	77

sion of E-cadherin was related to clinical staging, differentiation degree and lymph node metastasis of gastric cancer. According to our results, E-cadherin expression in ESCC tissues was decreased obviously compared with paracancerous tissues (P<0.001). E-cadherin expression in ESCC tissues with lymph node metastasis was also lower than that in ESCC tissues without lymph node metastasis. Moreover, E-cadherin expression in ESCC tissues was correlated with clinical staging and differentiation degree. Thus low expression of E-cadherin may play a role in differentiation, invasion and metastasis of ESCC.

NF-kB p65 is widely present in the cytoplasm of cells in resting phase. As a heterodimer composed of p65 and p50, NF-kB p65 remains mainly inactivated in cytoplasm. Viral or bacterial infection, UV radiation or action of cytokines in early stage of infection can cause the activation of NF-kB. Subsequently, innate immune cells produce a large amount of TNFa and IL-6 that are involved in the complex signaling pathways, anti-apoptotic action and the development of tumor cells [19]. NF-kB activation is accompanied by the activation of many factors related to infiltration and metastasis, such as matrix metallopeoteinase (MMP), vascular endothelial growth factors (VEGF), adhesion molecules (ICAM, VCAM) and E-selectin [20]. The present study found that NF-kB p65 expression was significantly different in ESCC tissues and paracancerous tissues: NF-kB p65 was upregulated in ESCC tissues compared with paracancerous tissues; the higher the clinical stage and the lower the differentiation degree, the higher the NF-kB p65 expression was; moreover, NF-kB p65 expression was higher in ESCC tissues with lymph node metastasis than in those without lymph node metastasis. It was inferred that abnormal activation of NF-kB p65 played a role in the occurrence, development and metastasis of ESCC.

Many studies are concerned with the role of RKIP, E-cadherin and NF-kB p65 in gastric cancer, adenocarcinoma of the gastric cardia and colorectal cancer. But few studies are devoted

to the expressions of RKIP, E-cadherin and NF-kB p65 in ESCC and the correlations between them. We found that RKIP expression was positively correlated with E-cadherin expression in ESCC. As RKIP was downregulated, E-cadherin was downregulated as well, leading to reduced adhesion between cells, easy shedding and great invasiveness of tumor cells. RKIP may regulate E-cadherin expression via the following mechanism: binding of RKIP to Raf-1 inhibits Raf-1/MEK/ERK pathway and promotes the inhibition by zinc finger of Snail family on E-cadherin. Zinc finger of Snail family can inhibit the expression of E-cadherin by binding to distal E-box of E-cadherin [21, 22]. Besides, RKIP inhibits Raf and therefore affects E-cadherin expression through TGF-β signaling pathway. Correlation analysis revealed that RKIP expression was negatively correlated with NF-kB p65 expression in ESCC. This implied the role of RKIP in tumor metastasis through the upregulation of NF-kB p65. Inhibition of RKIP expression is associated with the enhanced expression of NF-kB, while overexpression of RKIP reduces the expression of NF-kB 65. This process is independent of MAPK pathway, since RKIP can interact with four kinases, namely, NIK (NF-kB inducing kinase), TAK1 (TGF-β activated kinase-1), IKKα (IkB-kinaseα) and IKKβ (IkB- kinaseβ) that are part of the NF-kB p65-activating pathway, thereby regulating the NF-kB signaling pathway [23]. To sum up, RKIP, E-cadherin and NF-kB p65 are all involved in the occurrence and progression of ESCC, and RKIP is an important regulator of E-cadherin and NF-kB p65 expressions.

Acknowledgements

Disclosure of conflict of interest

None.

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