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Original Article

Expression of esophageal carcinoma related gene 4 (ECRG4) and its clinical significance in prognosis of esophageal carcinoma

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Abstract: This study aimed to analyze the relationship between the expression level of esophageal carcinoma related gene 4 (ECRG4) in esophageal cancer tissues and the occurrence of esophageal carcinoma. 50 cases of esophageal carcinoma tissues and adjacent tissues were collected as study samples. mRNA and protein expression levels of ECRG4 in tumor tissues and adjacent tissues were analyzed by real-time fluorescence quantitative PCR, Western blot and immunohistochemistry. The relationship between the expression level of ECRG4 and the clinical and pathological features and postoperative recurrence and survival was also analyzed. Real-time fluorescent quantitative PCR and Western blot showed that the mRNA and protein levels of ECRG4 in esophageal cancer tissues were significantly down regulated ($P<0.04$). There was ECRG low expression in 74 cases and high expression in 17 cases. The expression level of ECRG4 protein in esophageal carcinoma tissues was closely related to tumor invasion level, TNM staging and lymph node metastasis ($P<0.05$), but not related to gender, age, tumor type and differentiation degree of patients ($P>0.05$). The cumulative recurrence rate of patients of higher ECRG expression was significantly lower than that of patients of lower ECRG4 expression in 5 years after surgery, and the cumulative recurrence rate was 5 years ($P<0.05$). And the cumulative survival rate of patients with high ECRG4 expression was significantly higher than that of patients with low expression of ECRG4 in 5 years after surgery ($P<0.05$). In conclusion, the low expression or no expression of ECRG4 in esophageal cancer tissues was closely related to the degree of tumor invasion level, TNM staging, lymph node metastasis and recurrence and survival after surgery.

Keywords: Esophageal carcinoma, esophageal carcinoma related gene 4 (ECRG4), prognosis, expression

Introduction

Esophageal carcinoma is one of the most common malignant tumors in the clinic, which has the clinical characteristics of strong invasion, high lethal rate and so on. It has become a serious influence on the health of human diseases [1, 2]. The prognosis of patients with esophageal carcinoma is poor. The survival rate is only about 30% in 5 years, so it is very important to enhance the prognosis of patients to improve the life quality and survival time [3, 4]. Human esophageal carcinoma related gene 4 (ECRG4) was expressed by mRNA differential display technology which was a differential expression sequence from the normal esophageal tissues and the high cancer family [5, 6]. ECRG4 protein is found widely in the heart, brain, placenta, liver, skeletal muscle and kidney of normal human body [7]. ECRG4 protein is a tumor suppressor gene. The expression level in esophageal cancer and gastric cancer tissues is significantly down regulated [8, 9]. In tumor tissues, ECRG4 is not only involved in tumor growth and invasion, but also closely related to the variation and apoptosis of tumor cells [10, 11]. Therefore, ECRG4 protein is expected to become a clinical prognostic marker for esophageal cancer. This study analyzed the expression of ECRG protein in esophageal cancer tissues and adjacent tissues, and analyzed the relationship between ECRG4 protein expression level and prognosis recurrence and survival, to provide a theoretical basis for clinical treatment.

Subjects and methods

Clinical data

91 cases of esophageal carcinoma received by our hospital from June 2010 to June 2015 were selected in this study. All patients have received chemotherapy, radiotherapy and other immunotherapy before surgery. The tumor tissues and adjacent tissues were collected as samples of this study. There were 91 male and 41 female, aged 32 to 71. The average age was 50 years (50.7 + 10.52). Clinical stage: 36 cases of stage I, 25 cases of stage II, 18 cases of stage III, 12 cases of stage IV. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Henan provincial People's Hospital. Written informed consent was obtained from all participants.

Fluorescence quantitative PCR

Take 0.1 g esophageal cancer tissues and cancer adjacent tissues, place in 1.5 ml centrifuge tube, add 1 ml of TRNzol solution (TaKaRa, Dalian, China); use homogenate instrument for organize grinding, then add 200 μl of chloroform into the solution, after m. b, centrifuged at 15,000 rpm for 10 min; then supernatant is transferred to an equal volume of isopropanol, put on ice for 30 min, centrifuged at 12000 rpm for 15 min; after precipitation, use 70% ethanol for washing, centrifuged at 8000 rpm for 8 min; discard supernatant and precipitate dissolved in water of DEPC solution treatment for completely dissolving, and determine the RNA concentration. RNA is then transcribed into cDNA by reverse transcription kit (TaKaRa, Dalian, China), which is used as the template for real-time fluorescence quantitative PCR.

According to the ECRG4 mRNA sequence provided by GeneBank, the PCR primers of ECRG4 were designed as follows: ECRG4-F: 5'-AAGCGTGCCAAACGACAGCTGTGGGAC-3', ECRG4: 5'-TTAATAGTCATCATAGTTGACACTGG-3'; β-Actin-F: 5'-GCGGGAAATCGTGCGTGAC-3', β-Actin-R: CGTCATACTCCTGCTTGCTG-3'. Prepare the following reaction system: 2 × SYBR Green universal qPCR Master Mix (TaKaRa, Dalian, China) 10 μl, upstream/downstream primers (10 μmmol/L) are respectively 0.6 μl, 1:100 diluted cDNA 8.8 μl preparation. The total reaction volume is of 20 μl. And then the PCR is made under follow reaction conditions: initial denaturation 95°C, 30 s; denaturation: 95°C, 3 s; annealing and extension 60°C, 30 s; The dissolution curve was constructed. Finally, the data were directly read from the real-time fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA).

Western blot

Take 0.1 g tumor tissues and 0.1 g cancer adjacent tissues from patients, add 300 μl tissue lysates, use the homogenate instrument for complete homogenating, then put it on the ice for 30 min, centrifuged at 15,000 rpm for 15 min, add 5 × Loafing buffer into the supernatant fraction, put in boiling water for 10 min; then make SDS-PAGE and Western blot. After the transmembrane, the PVDF membrane is enclosed by 5% milk, pack the ECRG4 antibody (Abcam, Cambridge, UK) (1:2000 dilution) at a temperature of 4°C for all night, in the next day, wash with PBST for 3 times (5 min for every time). Then, incubate with HRP labeled Goat anti Rabbit secondary antibodies (ZSGB-BIO, Beijing, China) at room temperature for 1 h, wash with PBST for 3 times (5 min for every time), fluorescence was added on film and colored. The expression level of protein was analyzed.

Immunohistochemical analysis

Paraffin embedded cancer tissues and cancer adjacent tissues are prepared into the sample. After the transparent, dewaxing, hydration treatment, the antigen repair buffer liquid containing sodium citrate is dropped in the sample for heating in 56°C of antigen retrieval. After that, use the 1% goats serum to close for 1 h, and then drop 1% BSA prepared Rabbit anti human ECRG4 (Abcam, Cambridge, UK) (1:200 dilution) on the sample, and then packed in 4°C package for all night; the next day, wash with PBS for 3 times (5 min for each time). Then, the HRP labeled Goat anti Rabbit secondary antibodies (ZSGB-BIO, Beijing, China) (1:1000 dilution) is applied to the sample, incubate at room temperature for 2 h, wash with PBS for 3 times (5 min for every time). Then the color liquid DAB is added onto the sample; observe the color change under the microscope (Olympus AX80, Olympus, Tokyo, Japan). On appropriate color reaction, wash off DAB color liquid with water, and stained with hematoxylin eosin nucleus,

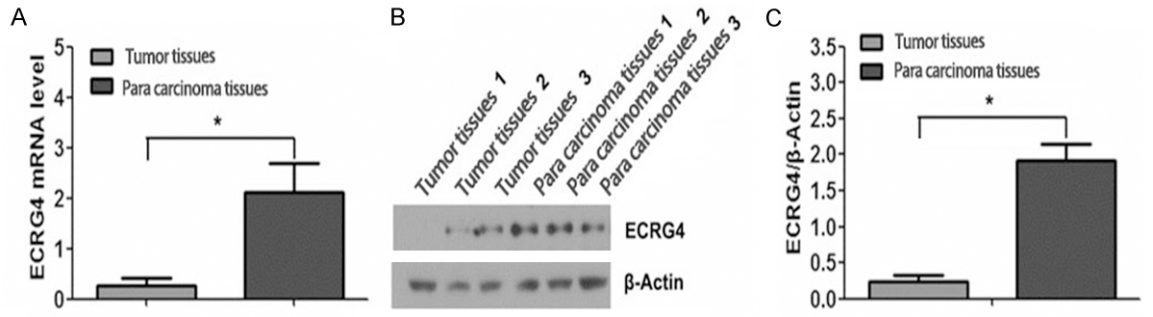

Figure 1. Analysis of ECRG4 mRNA and protein levels in tumor tissues and para carcinoma tissues. A. ECRG4 mRNA expression level by real time PCR; B. ECRG4 protein expression level by Western blot; C. Quantitative analysis showed ECRG4 protein expression level.

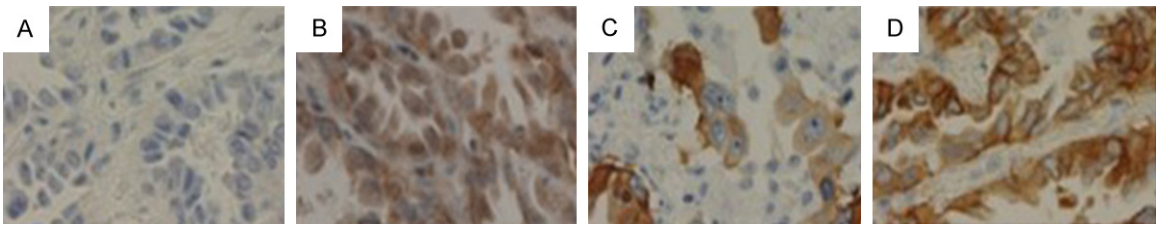

Figure 2. Immunohistochemical analysis for ECRG4 in esophageal carcinoma tissues. A. Negative ECRG4 protein (-); B. Weak positive ECRG4 protein (+); C. Medium positive ECRG4 protein (++); D. Strong positive ECRG4 protein (+++).

wash away excess brazilwood element, use xylene for transparent fixed, then seal with neutral resin. The expression level of ECRG4 protein was observed under microscope.

The expression of ECRG4 protein was quantified after the preparation. ECRG4 protein cytoplasmic and cell membrane showed dark brown. Staining intensity and positive diagnosis adopt Bornman grading standards: (-) for the expression of negative; (+) for the expression level is low, and <25% shows weakly positive; (++) for the cell is medium positive expression, the proportion of positive cells was >25% and < 50%; (+++) for the proportion of positive cells was >50%, which shows strong positive expression.

Statistical analysis

All data were analyzed using SPSS13.0 statistical software (SPSS Inc, Chicago, IL, USA). The measurement data were expressed by X±S, and the data was analyzed by ANOVA. The relationship of ECRG4 protein expression and clinical pathological features was analyzed by square test and Spearman rank correlation analysis. The cumulative survival rate and cumulative recurrence rate were analyzed by Log-Rank. $P<0.05$ showed that the difference had statistical significance.

Results

Comparison of ECRG4 mRNA and protein levels in tumor tissues and para-carcinoma tissues

As shown in **Figure 1**, real-time fluorescent quantitative PCR results showed that the expression level of ECRG4 mRNA in esophageal carcinoma tissues was significantly lower than that in para carcinoma tissues. The difference has statistically significance ($P<0.05$). Western Blot analysis showed that the ECRG4 protein level expression in esophageal carcinoma tissues was lower, but the expression level of the para carcinoma tissues was higher. Quantitative analysis of the 50 cases of tumor tissues in the tumor tissues of the tumor adjacent tissues showed that the expression level of ECRG4 protein in esophageal cancer tissues was significantly lower than that of the adjacent tissues, and the difference has statistical significant ($P<0.05$).

Table 1. Relationship of ECRG4 expression level and clinical pathological characteristics

Clinical pathological characteristics	n	ECRG4 expression				Positive rate (%)	χ^2	P
		-	+	++	+++			
Tissue type								
Para carcinoma tissues	91	0	20	26	45	100	19.321	0.000
Tumor tissues	91	47	27	10	7	48.35		
Sex								
Male	41	17	14	6	4	41.46	1.071	0.815
Female	50	30	13	4	3	40.00		
Age/Years								
<50	33	19	9	3	2	42.42	1.909	0.590
>50	58	28	18	7	5	51.72		
Carcinoma type								
Squamous cell carcinoma	82	43	25	8	6	47.56	1.915	0.601
Adenocarcinoma	9	4	2	2	1	55.56		
Differential level								
G1+G2	63	33	19	7	4	47.61	2.045	0.309
G3+G4	28	14	8	3	3	50.00		
Invasion level (T)								
T1	20	1	12	4	3	95.00	9.183	0.000
T2	25	10	9	4	2	60.00		
T3	30	21	5	2	2	30.00		
T4	16	15	1	0	0	6.67		
TNM staging								
I	36	11	14	7	4	83.33	7.119	0.000
II	25	15	6	2	2	40.00		
III	18	12	4	1	1	33.33		
IV	12	9	3	0	0	25.00		
Lymph node metastasis								
Yes	33	25	4	2	2	24.24	6.991	0.001
No	58	22	23	8	5	62.09		

Relationship between ECRG4 expression level and clinical pathological characteristics

As shown in **Figure 2**, the ECRG4 protein mainly exists in the cytoplasm and cell membrane, and the color is dark brown. By immunohistochemical analysis, in the esophageal cancer tissues, there were 47 cases of (-), 27 cases of (+), 10 cases of (++), 7 cases of (+++); in the para carcinoma tissues, 0 case of (-), 20 cases of (+), 26 cases of (++), 45 cases of (+++). The expression level of ECRG4 protein showed that the expression level of ECRG4 protein in esophageal carcinoma tissues was closely related to tumor invasion level, TNM staging and lymph node metastasis ($P<0.05$), but not related to gender, age, tumor type and differentiation degree of patients ($P>0.05$) (**Table 1**).

Relationship between different ECRG4 expression levels and prognosis of patients

The relationship between the expression level of ECRG4 protein and the cumulative recurrence rate and cumulative survival rate was also analyzed. In this study, (-) and (+) were combined as low expression group; (++) and (+++) were combined as high expression group. The statistical analysis showed that the cumulative recurrence rate of patients of ECRG4 low expression group was 74.32% in 5 years after surgery, while that of the high expression group was 52.94%, which has significant statistical significance ($P<0.05$). The cumulative survival rate of patients of ECRG4 low expression group was 20.71% in 5 years after operation, while that of the high expression group was 76.47%,

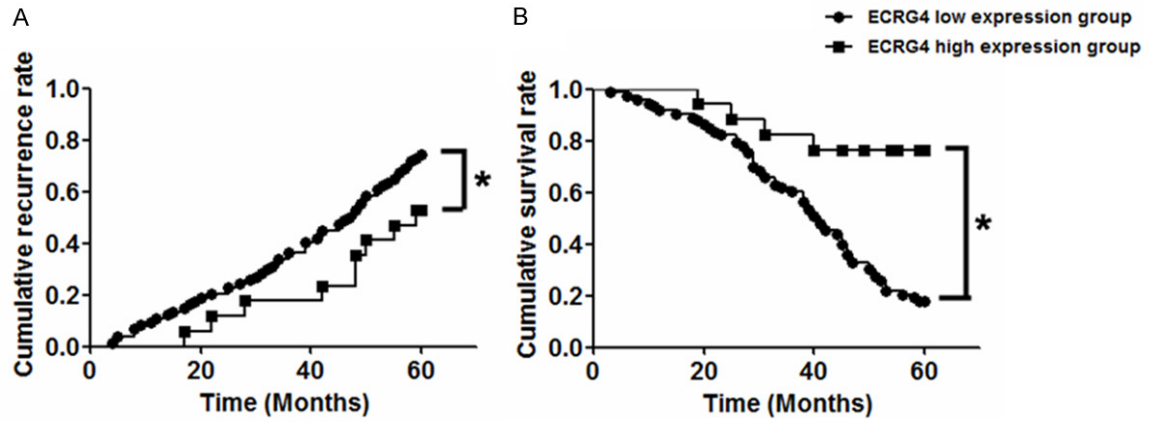

Figure 3. Comparison of cumulative recurrence rate and cumulative survival rate in patients with different ECRG4 expression level. A. Comparison of cumulative recurrence rate in patients with different ECRG4 expression level; B. Comparison of cumulative survival rate in patients with different ECRG4 expression level.

which has significant statistical significance ($P<0.05$) (**Figure 3**).

Discussion

Esophageal carcinoma was one of the common malignant tumors of digestive tract. Its incidence rate and mortality rate ranks second in the digestive tract tumor, after gastric cancer. Esophageal carcinoma was mainly squamous cell carcinoma, accounting for about 90%; adenocarcinoma only accounts about 7%, other types of tumors were rare [12]. The prevention and treatment of esophageal cancer has made great progress with the improvement of medical treatment. However, due to the poor prognosis of esophageal carcinoma, the survival rate was relatively low [13, 14]. To improve the prognosis of patients, it can only rely on the early diagnosis and treatment. Therefore, it was important to find the main index to improve the prognosis of esophageal carcinoma.

Esophageal cancer related gene 4 was cloned by mRNA differential PCR display method. The expression level of mRNA ECRG4 in esophageal carcinoma tissues was significantly lower than that of normal esophageal epithelium mRNA [15]. A study showed that in 63 patients, the mRNA level in esophageal carcinoma tissues was significantly lower than that of para carcinoma tissues. And the ECRG4 was independent of esophageal cancer by multivariate Cox regression analysis [16]. Another chemical analysis showed that ECRG4 protein expression was lower in esophageal cancer tissues [17]. We have analyzed the expression of ECRG4 protein in 91 cases of esophageal carcinoma tissues and adjacent tissues. The results showed that mRNA ECRG4 and protein level were significantly lower than that in the adjacent tissues, and the difference was statistically significant. This result was consistent with the current report [18]. We further analyze the relationship between the expression level of ECRG4 protein and clinical pathological characteristics. The results showed that expression level of ECRG4 protein in esophageal carcinoma tissues was closely related to tumor invasion level, TNM staging and lymph node metastasis ($P<0.05$), but not related to gender, age, tumor type and differentiation degree of patients ($P>0.05$).

Because of the correlation between ECRG4 with the degree of invasion, TNM staging and lymph node metastasis, the tumor invasion, TNM staging and lymph node metastasis were related to the prognosis of the patients with esophageal carcinoma. Therefore, the recurrence and survival of patients with esophageal carcinoma of different ECRG4 expression level after esophageal cancer surgery were analyzed in this paper. The results showed that the cumulative survival rate of patients with low expression of ECRG4 was significantly lower than that of patients with high expression of ECRG4, and the cumulative recurrence rate of patients with low expression of ECRG4 was significantly higher than that with high expression of ECRG4. This result was consistent with the

biological function of ECRG4 protein. ERG4 was considered to be a tumor suppressor factor. And if its expression level was down regulated, it leads to the disorder of local gene regulation, which ultimately leads to tumor occurrence and recurrence.

In conclusion, ECRG4 protein expression in esophageal carcinoma tissues was low, which was closely related to tumor invasion, TNM staging and lymph node metastasis in patients with esophageal carcinoma, and was related to the recurrence and survive rate after surgery. As a result, ECRG4 can be used as a marker for clinical prognosis of esophageal carcinoma after operation.

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Disclosure of conflict of interest

None.

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