Int J Clin Exp Pathol 2017;10(2):2030-2035
www.ijcep.com /ISSN:1936-2625/IJCEP0009456

Original Article

CREPT expression correlates with esophageal squamous cell carcinoma histological grade and clinical outcome

Jiao Liang, Ying Qiu, Yinyin Wang, Fangli Ren, Zhijie Chang

State Key Laboratory of Biomembrane and Membrane Biotechnology, School of Medicine, Tsinghua University, Beijing 100084, China

Received April 22, 2015; Accepted June 26, 2015; Epub February 1, 2017; Published February 15, 2017

Abstract: CREPT is a novel gene that was recently identified to promote tumorigenesis and highly expressed in a variety of tumors and enhances the expression of cyclin D1 by promoting the formation of a chromatin loop. Immunohistochemical analyses were performed to 70 Japanese patients for whom follow-up information was available. The expression of CREPT was significantly higher in the ESCC samples than in the tumor-adjacent samples. CREPT expression positively correlated with tumor size, tumor stage, N-regional lymph node metastasis (all $P<0.05$) and ESCC recurrence ($P=0.001$). The overall mean survival duration of the patients displaying high CREPT expression was shorter than that of those displaying low CREPT expression ($P<0.001$). In addition, the CREPT expression level was found to serve as an independent prognostic indicator of overall survival ($P=0.033$). Our results suggest that CREPT plays an important role in the evolution of ESCC and represents a potential marker of ESCC prognosis.

Keywords: CREPT, biomarker, prognosis, esophageal squamous cell carcinoma, immunohistochemistry

Introduction

Esophageal cancer, a common malignancy of the digestive system [1], is the sixth leading cause of cancer-related death worldwide [2]. Despite recent progress in the development of various therapeutic strategies for esophageal squamous cell carcinoma (ESCC), the 5-year survival rate of ESCC patients remains less than 40% [3, 4]. An improved understanding of the molecular basis of ESCC, including the development of novel biomarkers, is greatly needed to improve the diagnosis, prognosis and treatment of patients with this disease [5, 6].

Cell cycle-related and expression-elevated protein in tumor (CREPT) is a novel gene that was recently identified to promote tumorigenesis via the up-regulation of the expression of genes related to the cell cycle. A previous study demonstrated that CREPT is highly expressed in a variety of tumors and enhances the expression of cyclin D1 by promoting the formation of a chromatin loop. We observed abnormal increases in CREPT expression in digestive system carcinomas, such as gastric, liver, stomach, and colon cancers [7, 8].

In this study, we performed immunohistochemical analysis to investigate the potential role of the expression of CREPT in the clinicopathological characteristics and prognosis of ESCC. The findings of this study contribute to the current understanding of the pathogenesis of this disease.

Materials and methods

Patient samples

This study was approved by the Institutional Review Board of Osaka University. A total of 70 patients with ESCC underwent surgical resection at the Department of Surgery and Oncology of Osaka University (Japan). The median follow-up duration was 14 months (range of 1-94 months). All tumors were staged according to

Table 1. Associations between the clinicopathological characteristics of the ESCC patients and the nuclear expression of CREPT

Characteristic	Total	CREPT		P
	n (%)	Low	High	
Age, years				
<60	15 (21.4)	6 (8.2)	9 (6.8)	1.000
≥60	55 (78.6)	21 (21.2)	34 (33.8)	
Sex				
Male	65 (92.9)	26 (25.1)	39 (39.9)	0.642
Female	5 (0.07)	1 (1.9)	4 (3.1)	
Tumor size				
≤3.0cm	23 (32.9)	15 (8.9)	8 (14.1)	0.002
>3.0cm	47 (67.1)	12 (18.1)	35 (26.9)	
Tumor stage				
Stage I	27 (38.6)	16 (10.4)	11 (16.6)	0.003
Stage II	22 (31.4)	8 (8.5)	14 (13.5)	
Stage III	14 (0.2)	0 (5.4)	14 (8.6)	
Stage IV	7 (0.1)	3 (2.7)	4 (4.3)	
T-primary tumor				
T1	36 (51.4)	19 (13.9)	17 (22.1)	0.023
T2	8 (11.4)	4 (3.1)	4 (4.9)	
T3	26 (37.1)	4 (10.0)	22 (16.0)	
N-regional lymph node metastasis				
N0	37 (52.9)	20 (20.3)	17 (17.7)	0.007
N1	33 (47.1)	7 (12.7)	26 (10.3)	
M-distant metastasis				
M0	65 (92.9)	24 (24.3)	39 (38.7)	1.000
M1	7 (0.1)	3 (2.7)	4 (4.3)	
Grade				
G1	24 (34.3)	11 (9.3)	13 (14.7)	0.176
G2	30 (42.9)	13 (11.6)	17 (18.4)	
G3	16 (22.9)	3 (6.2)	13 (9.8)	

the TNM classification system of The Union for International Cancer Control (UICC). Histological grading of the tumors and diagnosis of ESCC were performed according to the classification system of the World Health Organization. Surgical specimens were fixed in 10% formalin and embedded in paraffin. The paraffin-embedded samples were serially sectioned at a thickness of 4 μm, mounted on slides, and stained with hematoxylin and eosin for histological analysis.

CREPT immunohistochemistry (IHC)

Immunohistochemical staining was performed according to the standard streptavidin-peroxidase-biotin technique (SP technique) [9]. The paraffinized sections (4 μm) were deparaffinized in xylene and then rehydrated using a graded alcohol series. Pretreatment with citrate buffer (10 mM, pH 6.0) was performed by boiling in a hydrated autoclave for 4.5 min. The 4 mm deparaffinized and rehydrated sections were incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. These sections were incubated overnight at 4°C in a 1:500 dilution of the primary monoclonal anti-CREPT antibody 3E10, which has been produced as an engineered antibody and has emerged as a valuable tool for cancer diagnosis [8]. Then, biotinylated immunoglobulin and streptavidin-conjugated peroxidase were added. Finally, 3,3'-diaminobenzidine was used for color development, and hematoxylin was used for counterstaining.

Semiquantitative analysis of the staining of individual tissue array cores was performed by three independent observers in a blinded manner. The frequency of CREPT-positive cells was scored according to the percentage of positive cells as follows: 0%: 0; 1-25%: +1; 26-50%: +2; and >51%: +3. The intensity of CREPT expression was scored as follows: weak: 1; moderate: 2; and strong: 3. The average CREPT expression level of each section was calculated as the intensity multiplied by the frequency and was classified as low (≤2) or high (>2) [7, 10].

Follow-up protocol

Postoperatively, the patients were advised to return for regular visits every 2 months during the first year; every 3 months during the second year; every 6 months during the third, fourth and fifth years; and every 6 months to 1 year thereafter. Telephone interviews with the survivors were conducted every 6 months. The fol-

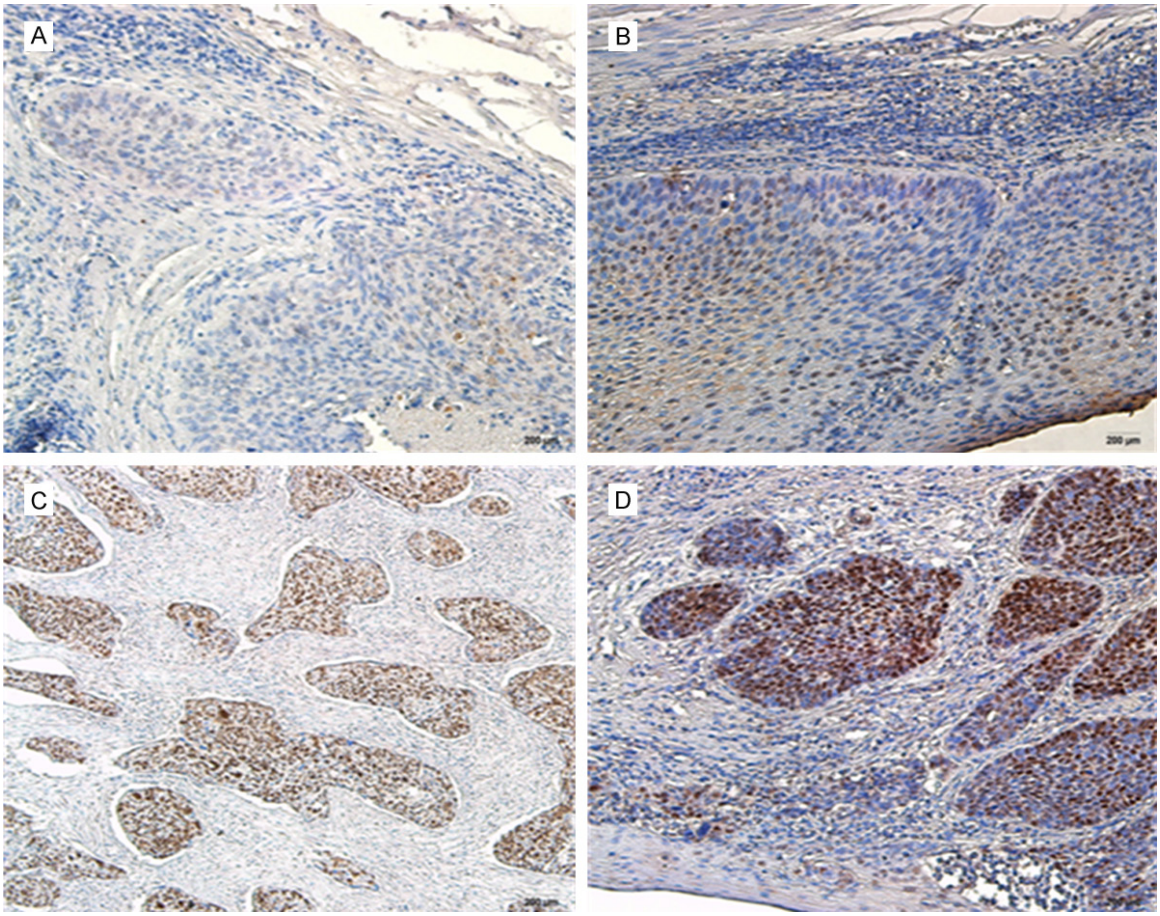

Figure 1. Immunohistochemical analysis of CREPT expression. A. Negative CREPT expression in ESCC-adjacent tissues. B. Weakly positive CREPT immunostaining in ESCC samples. C. Moderately positive CREPT immunostaining in ESCC samples. D. Strong nuclear CREPT expression in ESCC tissue samples (200×).

low-up strategy described above is routinely practiced at our hospitals.

Statistical analysis

All statistical analyses were performed using SPSS version 16.0. The associations between CREPT expression and the clinicopathological characteristics were analyzed using the χ^2 test.

Kaplan-Meier survival curves and the log-rank test were used to analyze overall survival. A Cox regression model was used to analyze the relationships between the potential influencing factors and patient prognosis. The significance of the differences in the cumulative survival curves was evaluated using the log-rank test. Univariate and multivariate regression analyses were performed using a Cox proportional hazards regression model to analyze the independent factors associated with prognosis. The survival rates and the odds ratios are presented with corresponding 95% confidence intervals (CIs). The statistical tests were two-sided, and a 5% level of significance was used.

Results

Patient characteristics

As shown in **Table 1**, the cohort included 70 patients exhibiting clinicopathological characteristics representative of ESCC. A total of 65 patients were male, and 5 patients were female. The mean age of the patients was 64.8 years (range of 40-82 years). The median tumor size was 4.2 cm, with a range of 2 to 12 cm. Based on the UICC criteria, most of the tumors (76%) were graded as well to moderately differentiated, and most of the patients exhibited stage I or stage II disease. Survival data were available for all 70 patients, and the survival duration ranged from 3 to 85 months, with a

Table 2. Univariate and multivariate analyses of the clinicopathological characteristics and CREPT expression as prognostic factors of outcome

Risk factors	Univariate			Multivariate		
	Hazard	95% CI	P	Harzard	95%CI	P
Age	1.561	0.415-5.396	0.482			
Sex	0.454	0.104-1.987	0.295			
Size	9.382	1.248-70.546	0.03			
Tumor stage	1.747	1.165-2.620	0.007			
T-primary tumor	3.259	1.755-6.054	<0.001	9.347	1.366-4.714	0.003
M-Distant metastasis	0.957	0.220-4.166	0.954			
N-Regional lymph nodes	4.543	1.491-13.842	0.008			
G stage	1.597	0.858-2.974	0.14			
CREPT	15.541	2.060-117.258	0.008	9.347	1.200-72.809	0.033

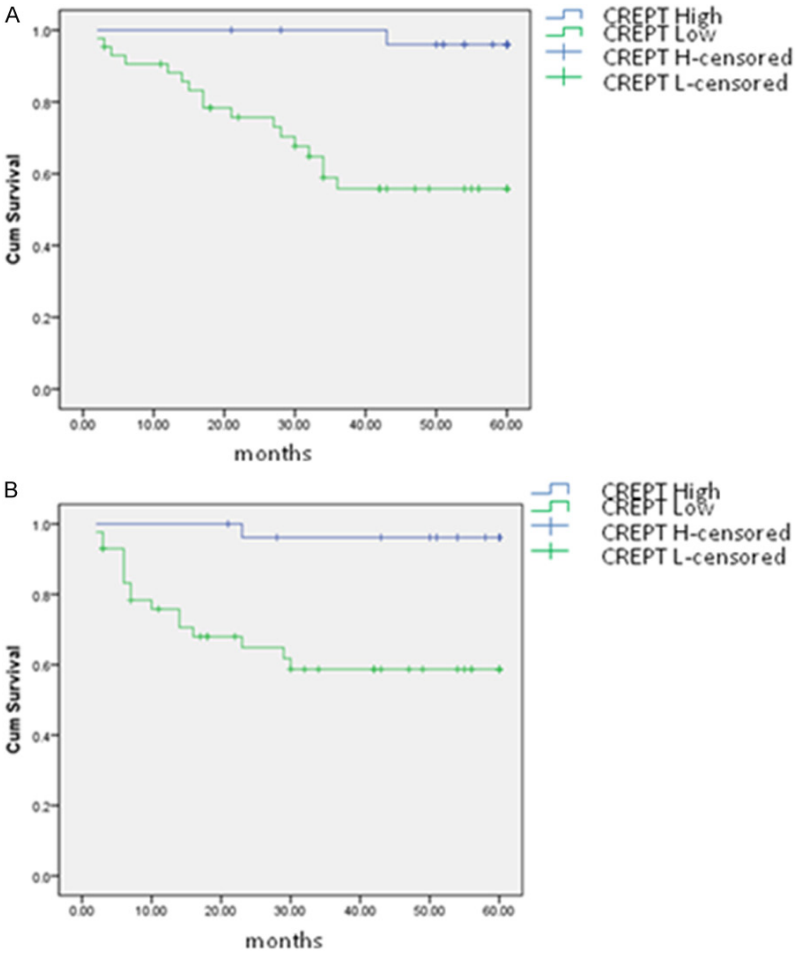

Figure 2. Kaplan-Meier curves for CREPT expression in 70 patients of ESCC. A. High nuclear expression of CREPT in ESCC specimens was associated with poor overall survival. B. High CREPT expression in ESCC specimens displayed a trend toward shorter recurrence-free survival than low CREPT expression.

median of 49.2 months. The 5-year overall survival rate was 38.7%.

CREPT expression in ESCC samples based on IHC

We assessed the CREPT protein levels in ESCC samples via immunohistochemical analysis and found that it was only expressed in the nucleus (**Figure 1**). Of the 70 ESCC samples, 14 (19.5%), 44 (63.4%) and 12 (17%) samples displayed weak, moderate and strong CREPT immunoreactivity, respectively. The tumor-adjacent tissue samples displayed limited to no CREPT immunoreactivity.

Association of CREPT expression with the clinicopathological characteristics

The clinicopathological characteristics and CREPT expression levels of the patients are summarized in **Table 1**. For statistical analysis of CREPT expression, the ESCC specimens were stratified into high-expressing and low-expressing groups. CREPT expression did not significantly correlate with age, gender, or tumor stage. However, the CREPT expression level significantly correlated with

tumor size, tumor stage, early disease stage and N-regional lymph node invasion (P=0.002, 0.003, 0.023 and 0.007, respectively).

CREPT expression correlated with ESCC patient survival and tumor recurrence

Univariate analysis using the log-rank test and Kaplan-Meier plots showed that the CREPT expression level inversely correlated with patient survival (**Figure 2A**, log-rank value =12.680, P<0.001). The median survival duration was 42.54 and 59.32 months for the high and low CREPT-expressing patients, respectively. The 5-year recurrence rate was lower in those with low CREPT expression levels than in those with high CREPT expression levels (**Figure 2B**, log-rank value =11.331, P=0.001).

CREPT protein expression is an independent prognostic indicator of ESCC outcome

To determine whether the CREPT protein level serves as an independent predictor of overall postoperative survival, a Cox proportional hazards model was used for forward selection. Univariate analysis demonstrated that tumor size and stage, the primary tumor stage, CREPT expression and lymph node invasion were significant predictors of poor survival (**Table 2**, P=0.030, 0.007, <0.001, 0.008 and 0.008, respectively). Multivariate analysis revealed that CREPT protein expression remained an independent prognostic indicator of overall survival (P=0.033).

Discussion

Previous studies have reported that CREPT is preferentially expressed in diverse types of human tumors. The overexpression of this protein accelerates tumor growth. CREPT promotes the formation of a chromatin loop and prevents RNAPII from reading through the 3'-end termination site of the cyclin D1 gene, suggesting that CREPT plays an important role in tumorigenesis [7].

Previous studies performed by our group have revealed that CREPT protein expression is elevated in eight different types of cancer, including lung, liver, breast, prostate, stomach, colon, endometrial and cervical cancer [7]. Few studies have been conducted on CREPT protein expression in ESCC. In this study, we observed the overexpression of this protein in ESCC for the first time and demonstrated the clinical relevance of CREPT expression to this type of cancer. High expression of this protein was observed in more than half of the ESCC specimens and was closely associated with the well to moderately differentiated tumor types, UICC stage and tumor size. Based on both univariate and multivariate analyses, high CREPT expression predicted poor overall survival. Multivariate analysis clearly showed that reduced CREPT expression was associated with improved prognosis among the ESCC patients; this result was consistent with a previous report indicating that reduced CREPT expression correlated with prolonged survival among colon cancer and retroperitoneal leiomyosarcoma patients [11]. Analysis of the correlation between CREPT expression and 5-year overall survival and recurrence indicated that low CREPT expression significantly predicted more favorable survival, especially during the early phase after resection.

Additionally, the hypothesis that CREPT may serve as a good indicator of the local aggressiveness and the tumor stage of ESCC is supported by our results that CREPT expression positively associated with the UICC T stage and N-regional lymph node invasion. Consistently, the overexpression or depletion of CREPT in HepG2 or HeLa cells has been shown to induce the acceleration or inhibition of cell proliferation, respectively [7].

In summary, the present findings demonstrate that the high expression of CREPT in ESCC is significantly associated with tumor size, a well to moderately differentiated histopathologic tumor phenotype, early tumor stage according to the UICC staging system, and node lymph metastasis. Moreover, the patients carrying high CREPT-expressing tumors exhibited shorter survival than those carrying low CREPT-expressing tumors, especially during the early phase after resection. CREPT expression is expected to serve as an important index for the assessment of ESCC invasion and metastasis and of ESCC patient prognosis. Furthermore, CREPT may represent a novel therapeutic target for ESCC treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhijie Chang, Tsinghua University, Beijing 100084, China. Tel: (86-10) 62785076; E-mail: zhijiechang@126.com

References

[1] Lu P, Qiao J, He W, Wang J, Jia Y, Sun Y, Tang S, Fu L, Qin Y. Genome-wide gene expression profile analyses identify CTTN as a potential prognostic marker in esophageal cancer. PLoS one 2014; 9: e88918.

[2] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: A Cancer J Clin 2011; 61: 69-90.

[3] Cao HH, Zheng CP, Wang SH, Wu JY, Shen JH, Xu XE, Fu JH, Wu ZY, Li EM, Xu LY. A molecular prognostic model predicts esophageal squamous cell carcinoma prognosis. PLoS One 2014; 9: e106007.

[4] Chen JW, Xie JD, Ling YH, Li P, Yan SM, Xi SY, Luo RZ, Yun JP, Xie D, Cai MY. The prognostic effect of perineural invasion in esophageal squamous cell carcinoma. BMC Cancer 2014; 14: 313.

[5] Yentz S, Wang TD. Molecular imaging for guiding oncologic prognosis and therapy in esophageal adenocarcinoma. Hosp Prac (1995) 2011; 39: 97-106.

[6] Ong CA, Lao-Sirieix P, Fitzgerald RC. Biomarkers in Barrett's esophagus and esophageal adenocarcinoma: predictors of progression and prognosis. World J Gastroenterol 2010; 16: 5669-5681.

[7] Lu D, Wu Y, Wang Y, Ren F, Wang D, Su F, Zhang Y, Yang X, Jin G, Hao X, He D, Zhai Y, Irwin DM, Hu J, Sung JJ, Yu J, Jia B, Chang Z. CREPT accelerates tumorigenesis by regulating the transcription of cell-cycle-related genes. Cancer Cell 2012; 21: 92-104.

[8] Ren F, Wang R, Zhang Y, Liu C, Wang Y, Hu J, Zhang L, Chang Z. Characterization of a Monoclonal Antibody Against CREPT, a Novel Protein Highly Expressed in Tumors. Monoclon Antib Immunodiagn Immunother 2014; 33: 401-408.

[9] Shi ZR, Itzkowitz SH, Kim YS. A comparison of three immunoperoxidase techniques for antigen detection in colorectal carcinoma tissues. J Histochem Cytochem 1988; 36: 317-322.

[10] Zhu M, Xu Y, Mao X, Gao Y, Shao L, Yan F. Overexpression of metastasis-associated in colon cancer-1 associated with poor prognosis in patients with esophageal cancer. Pathol Oncol Res 2013; 19: 749-753.

[11] She Y, Liang J, Chen L, Qiu Y, Liu N, Zhao X, Huang X, Wang Y, Ren F, Chang Z, Li P. CREPT expression correlates with poor prognosis in patients with retroperitoneal leiomyosarcoma. Int J Clin Exp Pathol 2014; 7: 6596-6605.