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Original Article

CREPT is a novel predictor of the response to adjuvant therapy or concurrent chemoradiotherapy in esophageal squamous cell carcinoma

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Abstract: CREPT has been shown to be highly expressed in most tumors and is associated with a poor prognosis, but the histologic characteristics of CREPT expression and its impact on clinical outcomes in esophageal squamous cell carcinoma (ESCC) are unclear. Therefore, we retroactively evaluated tissue microarrays (TMA) from 300 surgical cases, including 300 ESCC tissues and 161 adjacent non-tumor tissues, and pretreatment tumor biopsies from 113 concurrent chemoradiotherapy (CCRT) cases by immunohistochemistry (IHC). Notably, CREPT was increasingly expressed from non-cancerous tissues to atypical hyperplasia to tumor tissues ($P < 0.01$). Furthermore, patients were divided into low CREPT (≤ 8 scores) and high CREPT (> 8 scores) groups. Patients with high CREPT expressions had a worse overall survival (OS) (5-year OS: 40.9% vs. 50.1%, $P=0.040$) and disease-free survival (DFS) (5-year DFS: 29.5 vs. 43.0%; $P=0.020$) than those with low expressions. Nevertheless, only in the high CREPT subgroup did adjuvant therapy (AT) prolong the OS (5-year OS: 53.8 vs. 28.9%; $P=0.020$), especially for adjuvant radiotherapy (ART) (5-year OS: 85.7 vs. 28.9%; $P=0.037$; 5-year DFS: 85.7 vs. 22.3%; $P=0.020$). Surprisingly, high CREPT expressions endowed CCRT-treated patients with higher complete response rates (50% vs. 26%; $P=0.018$) and a favorable OS (3-year OS: 54.3 vs. 28.1%; $P=0.046$) compared to low expression. Overall, our findings indicate that CREPT is highly expressed in ESCC tissue compared with non-cancerous tissue and this feature is associated with a poor prognosis. Otherwise, patients with high CREPT expression were more sensitive to AT and CCRT. Moreover, CREPT could be a predictive immunohistochemical biomarker used to guide individualized clinical treatment.

Keywords: Esophageal squamous cell carcinoma, concurrent chemoradiotherapy, adjuvant therapy, CREPT, clinical individualized treatment

Introduction

Esophageal cancer ranks as the sixth leading cause of cancer-related death and the eighth most common cancer globally [1]. Esophageal squamous cell carcinoma (ESCC) accounts for about 90% of the 456,000 newly diagnosed cancer cases every year [2]. Surgical resection using various techniques is the standard treatment for early stage esophageal cancer. However, most patients with ESCC are identified at the advanced stages of the disease, when only 15-20% of patients have a successful surgical resection [3], and those patients most likely received adjuvant therapy (AT) [4]. The RTOG-8501 trial revealed that the overall 5-year survival was 26% in radiotherapy combined with chemotherapy compared with 0% following radiotherapy alone [5]. In cases who do not want surgery or for whom surgery is not possible for technical or medical reasons, concurrent chemoradiotherapy (CCRT) is now ac-

cepted as the standard of care in ESCC patients. Unfortunately, patients have different clinical outcomes after AT or CCRT. What's more, extensive treatment might result in a crucial decline in health-related quality of life yet still have a poor prognosis [6]. Therefore, the precise AT or CCRT are recommended treatment strategies for esophageal cancer.

Cell cycle-related and expression-elevated protein in tumor (CREPT; also named RPRD1B) is overexpressed in a variety of tumors and associated with a poor prognosis [7-9]. It accelerates tumor growth by binding to the cyclin D1 promoter and interacting with RNA polymerase II to regulate cyclin D1 expression [7, 10]. Meanwhile, the transcription of several other cell cycle-related genes including CDK2, CDK4, CDK6 and cyclin-E are induced by CREPT, which eventually accelerates the cell cycle and promotes cell proliferation. [7] In addition, CREPT facilitates cancer growth by promoting the Wnt/β-catenin pathway by interacting with p300 [11, 12].

Cancer cells are more likely to be damaged by drugs or radiation when cells undergo the cell cycle. DNA is considerably exposed during cell division, and this exposure is widely exploited in cancer therapy. In colorectal cancer patients treated with 5-FU-based adjuvant chemotherapy (ACT), a high expression of CREPT correlates with longer survival [13].

However, the level of CREPT expression in ESCC is unknown, and the relationships between CREPT and AT or CCRT in patients with ESCC are unclear. We performed a retrospective study to determine the potential impact of CREPT and its sensitivity to AT and CCRT.

Materials and methods

Patients and specimens

Three hundred patients who underwent surgery from 2007 to 2010 and one hundred thirteen patients who underwent CCRT from 2014 to 2016 with histologically-proven ESCC qualified for the study. All enrolled patients were staged according to the 7th edition of the American Joint Committee on Cancer (AJCC) system. Patients who died from unrelated causes or who had another active cancer were excluded. We obtained formalin-fixed paraffin-embedded (FFPE) specimens from the Department of Pathology of Shantou Central Hospital, including 300 tumor tissues and 161 paired adjacent non-tumor tissues of surgical patients and 113 pretreatment tumor biopsies of CCRT patients. The study was approved by the ethics committee of the Shantou Central Hospital and the ethics committee of the Medical College of Shantou University, and we obtained prior consent from all patients.

Treatments and follow-up

All surgical patients were treated with surgery only or AT after surgery. All CCRT patients received 50-70 Gy radiotherapy, 5 days per week in once-daily fractions and at 1.8 Gy or 2.0 Gy per fraction, and they received concurrent chemotherapy. The patients were assessed every 3 months during the first year, every 6 months during the following 2 years, and every year thereafter until death.

Tissue microarrays (TMAs) and immunohistochemistry (IHC)

TMA construction has been described previously [14]. The TMAs contained tumor and adjacent non-tumor tissues. In preparation for immunohistochemical staining, 3 μm-thick unstained sections were baked overnight at 56°C. Monoclonal mouse anti-human CREPT (1:50) was used for IHC [7]. IHC was performed according to a two-step protocol (PV9000 Polymer Detection System, ZSGB-BIO, Beijing, China) as previously described [15]. The immunohistochemical staining was examined in a blinded manner without access to any clinical data.

A positive reaction for CREPT was defined as showing a brown signal in the cell nucleus. Each separate tissue core was scored on the basis of the intensity and the area of positive staining. The intensity grades of the positive staining were: 0, negative; 1, weak staining; 2, moderate staining; 3, strong staining. The percentages of positive cells were scored as: 0, 0-5%; 1, 6-25%; 2, 26-50%; 3, 51-75%; 4, > 75%. The final scores were achieved by multiplying the two scores above, and the results ranged from 0 to 12. For the statistical analysis, with the tumor tissues, we used X-tile soft-

Table 1. Baseline patient characteristics of the analyzed population (surgery)

Characteristics	No. of patients (%)
Specimens	300
Median age, years (range)	58 (39-88)
Age (years)	
≤ 58	153 (51)
> 58	147 (49)
Gender	
Male	235 (78)
Female	65 (22)
pTNM-stage	
I	25 (8)
II	144 (48)
III	131 (44)
Treatment	
S	168 (56)
S + ACT	58 (20)
S + ART	42 (14)
S + ACTRT	28 (9)
Others#	4 (1)

Note: All patients underwent surgical treatment. Abbreviations: S: surgery; ACT: adjuvant chemotherapy; ART: adjuvant radiotherapy; ACTRT: adjuvant chemoradiotherapy. #Neoadjuvant chemotherapy (2 cases) and neoadjuvant chemoradiotherapy (2 cases).

Table 2. Baseline patient characteristics of the analyzed population (CCRT)

Characteristics	No. of patients (%)
Specimens	113
Median age, years (range)	64 (46-85)
Age (years)	
≤ 64	57 (50)
> 64	56 (50)
Gender	
Male	91 (81)
Female	22 (19)
cTNM-stage	
II	19 (17)
III	37 (33)
IV	57 (50)
Response	
CR	36 (32)
PR	67 (59)
SD	8 (7)
PD	2 (2)

Note: All patients underwent concurrent chemoradiotherapy. Abbreviations: CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

ware to divide the CREPT scores into two subgroups [16]; scores of 0-8 were defined as having a low-expression, and scores of 9-12 were defined as having a high-expression.

Endpoints and statistics

The primary endpoint was overall survival (OS). The secondary endpoints were disease-free survival (DFS) and local relapse-free survival (LRFS). OS was calculated from the date of surgery/beginning of CCRT until either death from ESCC or the last follow-up, and DFS was defined as the date of surgery until the appearance of radiological evidence for recurrence or metastasis. LRFS was defined as the time from the initiation of CCRT to the first documentation of local recurrence or death. The differences in the survival rates between the groups were analyzed using the Kaplan Meier log-rank test. Pearson's chi-squared test for categorical variables was used to compare the patients' baseline characteristics and the CREPT expression/clinical responses. A correlation analysis was performed using Spearman's rank correlation coefficient. Comparisons of CREPT expressions between tumor and adjacent non-tumor tissues were performed using Student's *t*-test. Univariate and multivariate analyses were performed using the Cox proportional hazard model. A two-sided *p*-value less than 0.05 was considered statistically significant. The statistical analyses were performed using SPSS 19.0 software.

Results

IHC characteristics of CREPT

The baseline patient characteristics are summarized in **Tables 1** and **2**. We examined 300 ESCC tissues and 161 adjacent non-tumor tissues, including 51 simple hyperplasia and 112 dysplasia epithelia, by IHC. CREPT-positive immunostaining could be observed in the three types of tissues and was localized predominantly in the cell nuclei. Negative, and weak to strongly positive signals were seen in the tumor tissues and in the epithelial, hyperplastic and dysplastic tissue. Representative images of different staining scores are shown in **Figure 1A**.

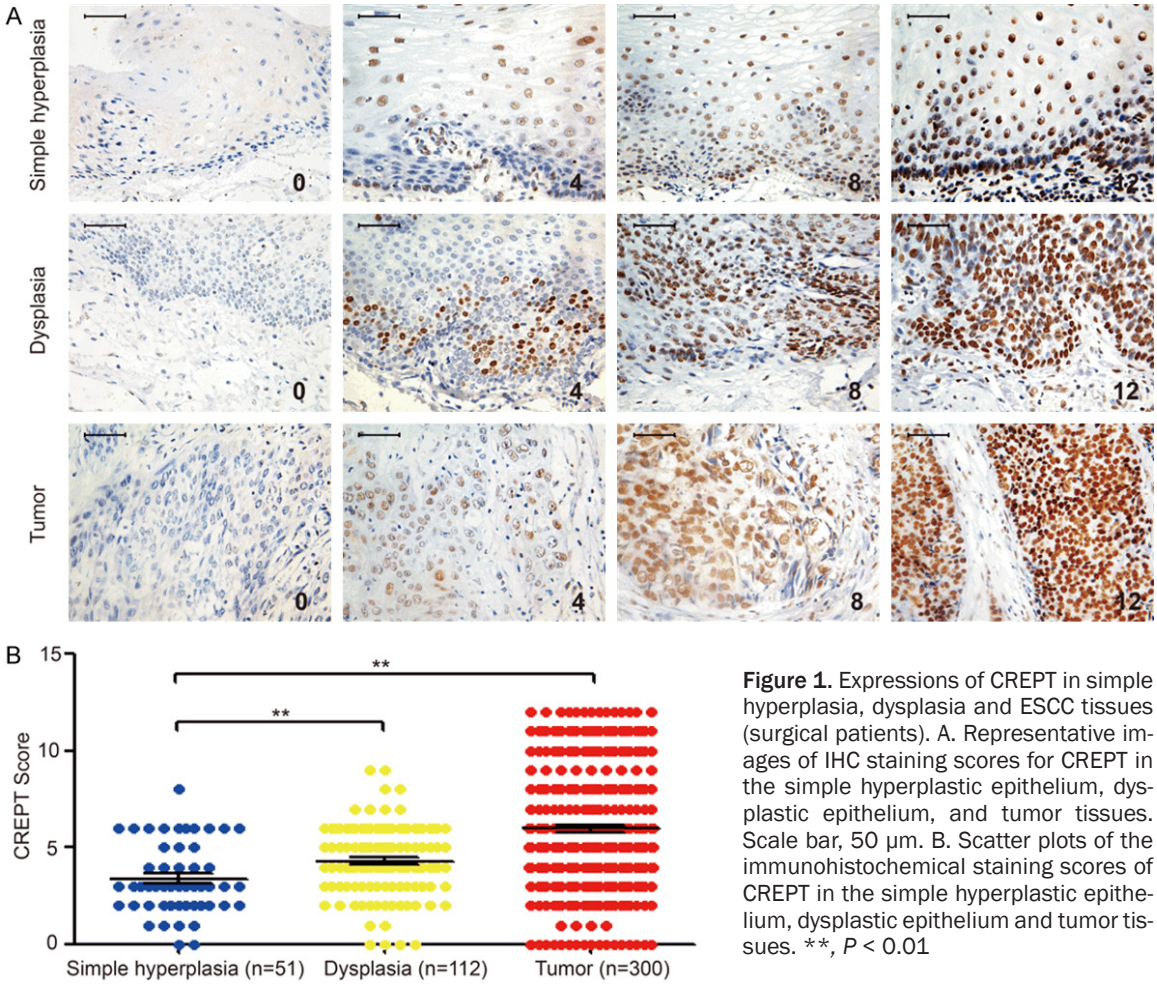


Figure 1. Expressions of CREPT in simple hyperplasia, dysplasia and ESCC tissues (surgical patients). A. Representative images of IHC staining scores for CREPT in the simple hyperplastic epithelium, dysplastic epithelium, and tumor tissues. Scale bar, 50 μm. B. Scatter plots of the immunohistochemical staining scores of CREPT in the simple hyperplastic epithelium, dysplastic epithelium and tumor tissues. **, $P < 0.01$

Comparing the expression levels of CREPT in the three kinds of epithelia, we found that there was a statistically significant difference between simple hyperplasia and ESCC, and dysplasia and ESCC (**Figure 1B**; $P < 0.01$), supporting the conclusion that CREPT is up-regulated in ESCC tissues. These results are similar to the reports of other tumors [7].

Patients with high CREPT expressions and treated with surgery have poor OS and DFS

Next, we identified the impact of high CREPT expression on survival in all surgical patients. All the patients were followed up for 0.6 to 84 months, with a median time of 33.8 months. CREPT was highly expressed in 72 (24%) patients and expressed at low levels in 228 (76%) patients. Kaplan-Meier curves revealed that, compared to patients with low CREPT expressions, patients with high CREPT expressions had a worse OS (5-year OS: 40.9% vs. 50.1%, P=0.040; **Figure 2A**) and DFS (5-year DFS: 29.5 vs. 43.0%, P=0.020; **Figure 2B**). Thus, patients with higher CREPT expressions had poor prognoses. Furthermore, we found there were no correlations between CREPT expressions and the clinicopathological characteristics (Table S1). Therefore, all the factors were included in our univariate and multivariate Cox regression analyses, including age, gender, pTNM-stage, therapies, and CREPT expression. The expressions of CREPT (high vs. low, HR, 1.380, 95% CI, 1.000-1.905, P=0.050), pTNM-stage (II vs. I, HR, 2.329, 95% CI, 1.073-5.056, P=0.033; III vs. I, HR, 4.563, 95% CI, 2.109-9.874, $P < 0.001$) and age (> 58 vs. ≤ 58, HR, 1.435, 95% CI, 1.066-1.932, P=0.017) were independent predictors of DFS for patients with resectable tumors (**Table 3**).

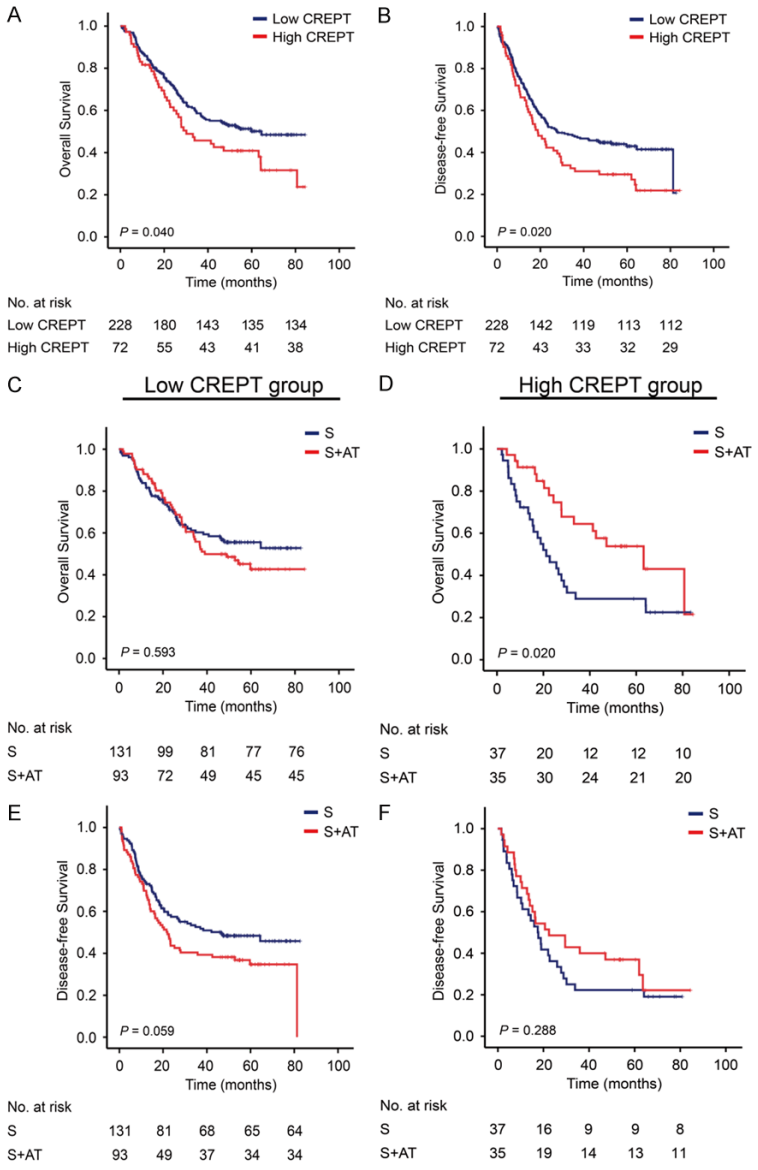


Figure 2. Kaplan-Meier survival curves of overall survival and disease-free survival according to CREPT expression. A and B. Overall survival and disease-free survival in all 300 surgical patients with ESCC. C-F. Overall survival and disease-free survival of patients who received adjuvant therapy after surgery (S + AT group), and of the patients who did not (the S group) in the low and high CREPT-expressing subgroups.

High CREPT expression correlates with better survival in patients treated with adjuvant radiotherapy and chemotherapy

Among the 300 patients, 168 (56%) patients had surgery only (S), and 128 (43%) patients had AT, including ACT, ART, and adjuvant chemoradiotherapy (ACTRT) (neoadjuvant chemotherapy or chemoradiotherapy were excluded in the stratified analysis because of the low number of cases). Therapies were not a predictor according to the univariate and multivariate Cox regression analyses (**Table 3**). However, when we investigated the impact of the therapeutic options on the OS and DFS in the low and high CREPT subgroups, we found that only in the high CREPT subgroup did AT prolong the OS (5-year OS: 53.8 vs. 28.9%; *P*=0.020; **Figure 2D**). Furthermore, we divided the AT into subgroups (**Figure 3**). Interestingly, ART provided the most benefit to patients after surgery in the high CREPT subgroup, based on the OS (5-year OS: 85.7 vs. 28.9%; *P*=0.037) and DFS (5-year DFS: 85.7 vs. 22.3%; *P*=0.020), and ACT showed a similar trend for OS (5-year OS: 48.8 vs. 28.9%; *P*=0.095). The impact was not observed in the low CREPT subgroup. Moreover, there was no statistical significance between ACTRT and S in the high CREPT subgroup. Conversely, in low CREPT group, quite remarkably, patients who underwent ACTRT had a shorter OS (5-year OS: 15.7 vs. 55.5%; *P*=0.027) and DFS (5-year DFS: 15.7 vs. 48.4%, *P*= 0.022) than patients who did not undergo ACTRT.

High CREPT expression correlates with improved OS and complete response rates in patients treated with CCRT

To validate the above conclusion, we enrolled 113 patients with ESCC who had been treated with CCRT. Among all 113 patients, 36 patients (32%) achieved a complete response (CR), 67 (59%) achieved a partial response (PR), 8 (7%) had stable disease (SD), and 2 (2%) had progressive disease (PD). The median follow-up time was 17.8 months (range, 1.9 months-51.0 months). Compared with the other three efficacy responses, patients with CR had the best OS

Table 3. Univariate and multivariate analysis of factors associated with overall survival (OS) and disease-free survival (DFS)

Variables	Univariate analysis				Multivariate analysis			
	OS		DFS		OS		DFS	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age (> 58 *vs.* ≤ 58)	1.419 (1.024 to 1.965)	0.035	1.256 (0.937 to 1.684)	0.127	1.550 (1.102 to 2.181)	0.012	1.435 (1.066 to 1.932)	0.017
Gender (Female *vs.* Male)	0.809 (0.557 to 1.174)	0.264	0.994 (0.699 to 1.412)	0.971				
pTNM-stage		0.000		0.000		0.000		0.000
II *vs.* I	1.645 (0.751 to 3.605)	0.214	2.299 (1.059 to 4.993)	0.035	1.864 (0.847 to 4.101)	0.122	2.329 (1.073 to 5.056)	0.033
III *vs.* I	3.446 (1.589 to 7.472)	0.002	4.382 (2.029 to 9.463)	0.000	4.481 (2.036 to 9.865)	0.010	4.563 (2.109 to 9.874)	0.000
Treatment		0.221		0.221		0.081		
S + ACT *vs.* S	0.786 (0.499 to 1.239)	0.300	1.190 (0.817 to 1.733)	0.364	0.634 (0.398 to 1.010)	0.055		
S + ART *vs.* S	0.831 (0.504 to 1.369)	0.467	0.996 (0.632 to 1.571)	0.987	0.688 (0.409 to 1.156)	0.158		
S + ACTRT *vs.* S	1.441 (0.891 to 2.331)	0.136	1.572 (0.996 to 2.481)	0.052	1.294 (0.794 to 2.111)	0.301		
Others[#] *vs.* S	0.380 (0.053 to 2.735)	0.337	0.325 (0.045 to 2.330)	0.263	0.399 (0.055 to 2.907)	0.364		
CREPT (High *vs.* Low)[a]	1.452 (1.016 to 2.074)	0.041	1.460 (1.060 to 2.012)	0.021			1.380 (1.000 to 1.905)	0.050

Note: Multivariate analysis, Cox proportional hazards regression model. $P < 0.05$ was considered significant. [a]low, score ≤ 8; high, score > 8. Abbreviations: OS: overall survival; DFS: disease-free survival; S: surgery; ACT: adjuvant chemotherapy; ART: adjuvant radiotherapy; ACTRT: adjuvant chemoradiotherapy. [#]Neoadjuvant chemotherapy and neoadjuvant chemoradiotherapy.

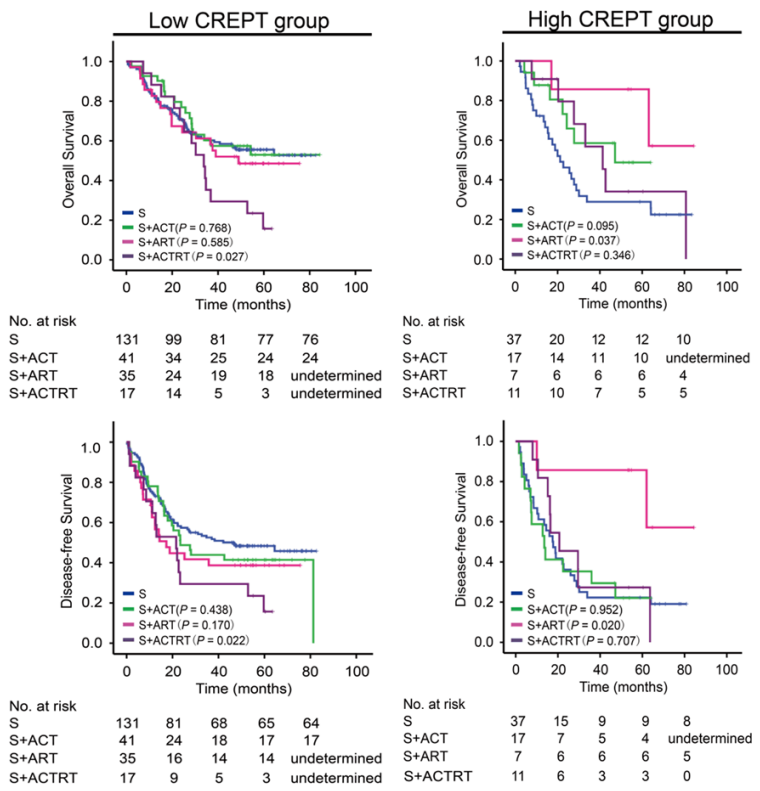


Figure 3. Kaplan-Meier survival curves of overall survival and disease-free survival for various adjuvant therapies in the low and high CREPT-expressing subgroups. S, surgery only; S + ACT, adjuvant chemotherapy after surgery; S + ART, adjuvant radiotherapy after surgery; S + ACTRT, adjuvant chemoradiotherapy after surgery.

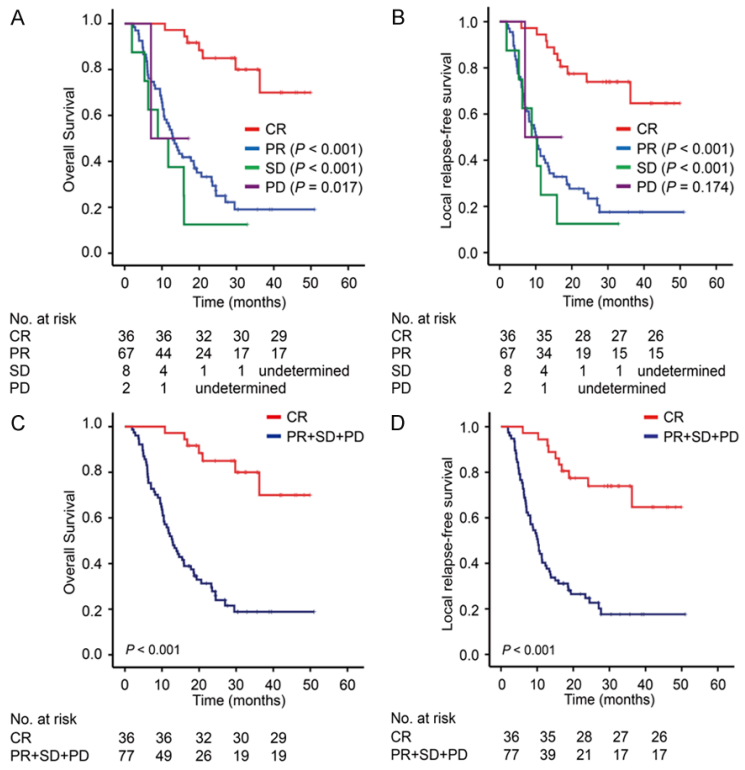


Figure 4. Kaplan-Meier survival curves of overall survival (A and C) and local relapse-free survival (B and D) for various responses in ESCC patients treated with concurrent chemoradiotherapy. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

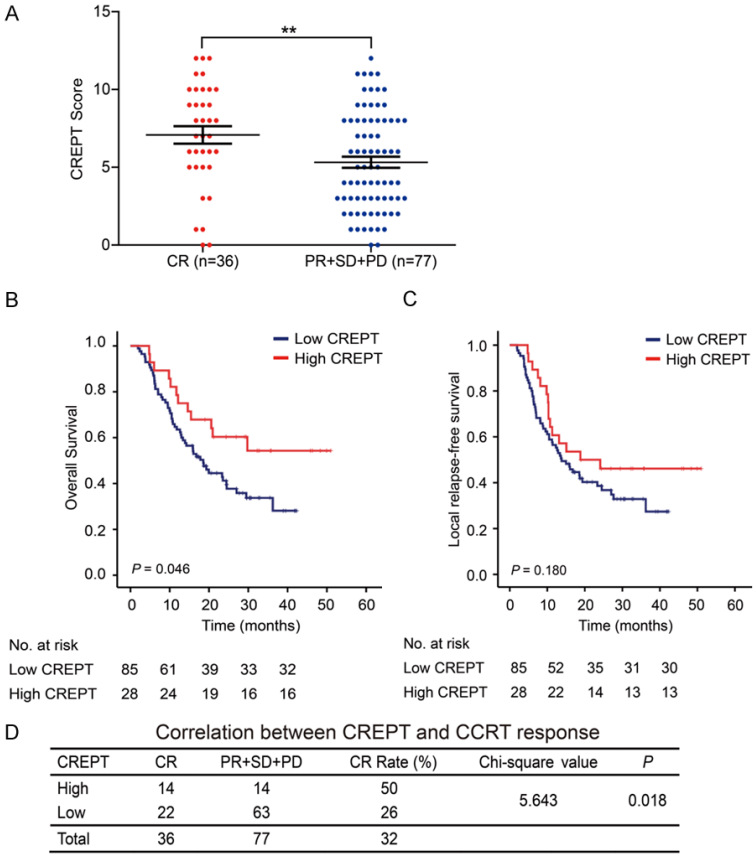
(**Figure 4A**) and LRFS (**Figure 4B**). In view of the above results, we defined the CR group as the responsive group, and the other three groups were categorized as the nonresponsive groups. The Kaplan-Meier curves showed that survival was in accordance with the above (**Figure 4C**, **4D**). A correlation analysis found no significant correlation between efficacy responses and gender or age, but efficacy did correlate with clinical stage (**Table 4**).

We then explored the correlation between CREPT expression and the survival and clinical responses after CCRT. Immunohistochemistry showed that CREPT expression was high in 28 (25%) patients and low in 85 (75%) patients. These percentages are consistent with those of the surgical patients. The level of CREPT expression was not related to gender, age or clinical stage (Table S2). Interestingly, patients with high CREPT expressions had favorable OS (3-year OS: 54.3% vs. 28.1%; *P*=0.046; **Figure 5B**), with the same trend in LRFS (3-year OS: 46.2% vs. 27.4%; *P*=0.180; **Figure 5C**). Moreover, among the patients in the high expression group, 14 (50%) patients achieved CR, compared to only 22 (26%) in the low expression group. The difference in the CR rate between the two groups was statistically significant (*P*=0.018) (**Figure 5D**). Taken together, we conclude that hi-

Table 4. Correlation between clinical characteristics and chemoradiotherapy response in ESCC patients

Variables	No.	Response		Chi-square value	*P**
		CR	PR + SD + PD		
Age (years)				0.004	0.949
≤ 64	57	18	39		
> 64	56	18	38		
Gender				1.031	0.310
Male	91	27	64		
Female	22	9	13		
cTNM-stage				16.923	0.000
II	19	9	10		
III	37	19	18		
IV	57	8	49		

Note: *Pearson's χ^2 test; $P < 0.05$ was considered significant. Abbreviations: CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.



D

Correlation between CREPT and CCRT response

CREPT	CR	PR+SD+PD	CR Rate (%)	Chi-square value	*P*
High	14	14	50	5.643	0.018
Low	22	63	26		
Total	36	77	32		

Figure 5. The correlation between the expressions of CREPT and the therapeutic response in ESCC patients treated with concurrent chemoradiotherapy. A. Scatter plots of the immunohistochemical staining score of CREPT in various responses. B and C. Kaplan-Meier survival curves of overall survival and local relapse-free survival according to CREPT expression. D. Complete response rates in the low and high CREPT-expressing subgroups.

gh CREPT expressions are associated with better outcomes in ESCC patients after treatment with CCRT.

Discussion

In this study, we demonstrated that CREPT is increasingly expressed from non-cancerous tissue to atypical hyperplasia to tumor tissue. Moreover, patients treated with surgery alone and with high CREPT expressions had shorter survival times. However, interestingly, surgery followed by adjuvant therapy conferred better OS in the high CREPT subgroup, especially for adjuvant radiotherapy and chemotherapy. As for patients with adjuvant chemoradiotherapy, we did not observe this benefit, possibly because patients could not tolerate radiation damage combined with the toxicity of chemotherapy after surgery. A recent study showed that neoadjuvant chemoradiotherapy followed by surgery improves survival over surgery alone among patients with locally advanced ESCC [17]. Meanwhile, CCRT is an effective treatment in cases who refuse surgery or for whom surgery is not possible for technical or medical reasons. Therefore, we further explored the impact of CREPT expression on predicting the clinical response after CCRT. Surprisingly, patients with high CREPT expressions achieved better OS and higher CR rates. These results provided a novel prospective to individualized clinical treatment with an acceptable economic expense and current available technology.

Takahashi et al. reported that pretreatment whole-body total

lesion glycolysis (TLG_{WB}) and metabolic tumor volume (MTV_{WB}) are predictors in patients with esophageal cancer treated with CCRT [18], but this method is not suitable for most patients due to the cost of 18F-fluorodeoxyglucose positron emission tomography (FDG-PET), and due to the fact that most cases occur in developing countries [2]. On the contrary, immunohistochemistry on tumor tissues using biomarkers is a currently used, effective, and inexpensive means to predict treatment outcomes. Although we analyzed only one biomarker, our research systematically shows the expression of CREPT in hyperplasia, dysplasia and ESCC tissues and indicates a high CREPT expression is associated with a good prognosis in adjuvant radiotherapy and chemotherapy settings. This was further verified for CCRT patients. On the one hand, high CREPT expression accelerates tumor cell cycle progression and increases the risk of malignancy, which leads to poor prognosis [19]; on the other hand, tumors with a large population of cycling cells are more likely to be damaged by chemotherapy and radiotherapy. CREPT is thus a logical and appropriate predictor for the outcomes of esophageal cancer. In the future, patients with high CREPT expressions will tend to have better survival when treated with surgery followed by adjuvant radiotherapy or chemotherapy. Even if they refuse to undergo surgery, CCRT would be a viable option.

Although our study has profound prospects for clinical application, this study has several limitations. Firstly, this study was a single-center retrospective study, and the conclusions should be validated in a multicenter prospective study. Secondly, we did not stratify the chemotherapy regimens. In fact, regardless of whether fluoropyrimidine-, taxane- or platinum-based chemotherapy regimens are used, these agents exert their cytotoxicity and radiosensitizing effect by interacting with DNA, RNA, or tubulin to affect the cell cycle [20-25]. Therefore, we ignored the differences among the regimens because CREPT drives the initiation of the cell cycle. Thirdly, we also need to further research the influence of CREPT on neoadjuvant chemoradiotherapy.

In conclusion, CREPT is a predictor of therapeutic outcome in ESCC patients treated with surgery or CCRT. Patients with high CREPT expressions are more sensitive to adjuvant therapy or CCRT.

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Disclosure of conflict of interest

None.

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Table S1. Correlation between CREPT expression and clinicopathological characteristics in ESCC (surgery)

Variables	No.	CREPT[a]		Chi-square value	Spearman correlation coefficient	*P**
		Low	High			
Age (years)				0.380	-0.036	0.538
≤ 58	153	114	39			
> 58	147	114	33			
Gender				0.620	-0.045	0.431
Male	65	47	18			
Female	235	181	54			
pTNM-stage				3.197	0.099	0.202
I	25	20	5			
II	144	115	29			
III	131	93	38			
Treatment				7.345	0.049	0.119
S	168	131	37			
S + ACT	58	41	17			
S + ART	42	35	7			
S + ACTRT	28	17	11			
Others[#]	4	4	0			

Note: *Pearson's χ^2 test; $P < 0.05$ was considered significant. [a]Low, score ≤ 8; high, score > 8. [#]Neoadjuvant chemotherapy and neoadjuvant chemoradiotherapy.

Table S2. Correlation between CREPT expression and clinicopathological characteristics in ESCC patients (CCRT)

Variables	No.	CREPT[a]		Chi-square value	Spearman correlation coefficient	*P**
		Low	High			
Age (years)				0.146	-0.036	0.703
≤ 64	57	42	15			
> 64	56	43	13			
Gender				0.091	0.028	0.763
Male	91	69	22			
Female	22	16	6			
cTNM-stage				1.877	-0.126	0.391
II	19	13	6			
III	37	26	11			
IV	57	46	11			

Note: *Pearson's χ^2 test; $P < 0.05$ was considered significant. [a]Low, score ≤ 8; high, score > 8.