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Original Article

High expression of Capn4 is associated with metastasis and poor prognosis in esophageal squamous cell carcinoma

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Abstract: Calpain small subunit1 (Capn4) is present in various cancer types and is implicated in tumor metastasis. However, the role of Capn4 in esophageal squamous cell cancer (ESCC) has not been elucidated. In this study, immunohistochemistry was conducted to detect Capn4 expression and localization in 155 ESCC tissues and in 35 adjacent normal esophageal mucosal tissues. Following knockdown of Capn4 in esophageal cancer cells using RNA interference, we detected the migration and invasion ability of cells. The role of Capn4 on prognosis was evaluated using univariate and multivariate analysis in 155 ESCC patients. The immunohistochemistry results showed that Capn4 was highly expressed in ESCC tissues compared with normal peritumor esophageal tissues, and Capn4 overexpression was significantly related to tumor size (P = 0.027), tumor invasion depth (P = 0.019), and lymph node metastasis (P = 0.011). Knockdown of Capn4 drastically reduced migration and invasion of ESCC cells. Based on the results of the univariate analysis, patients with higher Capn4 had a poor prognosis. Through multivariate analysis, we found that Capn4 was an independent prognostic factor for overall survival in ESCC. These findings suggest that Capn4 overexpression contributes to the aggressive progression of ESCC and functions as a prognostic marker for patients with ESCC.

Keywords: ESCC, Capn4, immunohistochemistry, siRNA, prognosis

Introduction

Esophageal cancer, the eighth most common malignant tumor, is one of the deadliest cancers worldwide [1], and the average five-year survival rate is approximately 15%-25% [2, 3]. Esophageal squamous cell cancer (ESCC) is a dominant histological subtype of esophageal cancer [4]. Commonly, ESCC is treated with surgical resection, but the prognosis remains poor because of early lymph node metastasis and invasion into adjacent organs. Therefore, it is urgent that we investigate the molecular mechanisms of ESCC and identify novel biomarkers in order to develop effective therapeutic strategies.

Calpains are Ca^{2+}-dependent cysteine proteases which can selectively hydrolyze specific substrate, and are involved in many biological functions, such as cell migration, invasion, proliferation [5], differentiation [6], and apoptosis [7, 8]. To date, fourteen calpain isoforms have been identified in humans, and most of these isoforms are found at focal adhesions [9, 10]. As a small regulatory subunit, calpain small subunit1 (Capn4), also known as Capn4s1, maintains calpain stability and activity. Accumulating evidence has proven the important role of abnormal calpain expression during tumorigenesis. For example, calpain-4 is detected to be highly expressed in gastric cancer [11]. Increased calpain-1 or calpain-2 occurs in breast cancer cell lines and inhibition of calpain-1/2 results in increased cell death in breast cancer cell lines [12]. Additionally, Capn4 has been found to be overexpressed in nasopharyngeal carcinoma (NPC) [13], clear cell renal cell carcinoma [14], and intrahepatic chol-

Table 1. Correlation between Capn4 expression and clinicopathological features of ESCC

Clinicopathological factors	Cases (n = 155)	Capn4 expression		x^2	*P* value
		Low (n = 50)	High (n = 105)		
Gender				0.356	0.551
Male	126	42 (33.3%)	84 (66.7%)		
Female	29	8 (27.6%)	21 (72.4%)		
Age (year)				0.931	0.335
≤68	75	27 (36.0%)	48 (64.0%)		
>68	80	23 (28.8%)	57 (71.3%)		
Smoking history				0.804	0.370
No	51	14 (27.5%)	37 (72.5%)		
Yes	104	36 (34.6%)	68 (65.4%)		
Tumor location				3.541	0.170
Upper	6	2 (33.3%)	4 (66.7%)		
Middle	127	37 (29.1%)	90 (70.9%)		
Lower	22	11 (50.0%)	11 (50.0%)		
Tumor size (cm)				4.918	0.027*
<3.5	64	27 (42.2%)	37 (57.8%)		
≥3.5	91	23 (25.3%)	68 (74.7%)		
Histological grade				3.140	0.208
G1	10	2 (20.0%)	8 (80.0%)		
G2	119	36 (30.3%)	83 (69.7%)		
G3	26	12 (46.2%)	14 (53.8%)		
Depth of tumor invasion				7.941	0.019*
T1-T2	25	14 (56.0%)	11 (44.0%)		
T3	82	24 (29.3%)	58 (70.7%)		
T4	48	12 (25.0%)	36 (75.0%)		
Lymph node metastasis				6.418	0.011*
Negative	89	36 (40.4%)	53 (59.6%)		
Positive	66	14 (21.2%)	52 (78.7%)		

*p value <0.05 was regarded as statistically significant.

angiocarcinoma (ICC) [15]. Furthermore, additional studies have illustrated that Capn4 contributes to tumor progression by promoting local tumor invasion and distant metastasis [15]. Aberrant calpain expression plays an important character in tumorigenesis and Capn4 is essential in maintaining calpain activity and stability. Therefore, it is significant to identify the role of Capn4 in ESCC.

In this study, we detected the Capn4 expression in ESCC using immunohistochemistry. Capn4 was downregulated in CaEs-17 cells using RNA interference to evaluate the metastatic ability of Capn4 in esophageal cancer. Furthermore, the prognostic role of Capn4 was assessed using the Kaplan-Meier method and multivariate analysis in 155 ESCC patients.

Materials and methods

Cell line

The esophageal cancer cell line CaEs-17 was provided by the cell library at the China Academy of Sciences and cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum in an incubator with 5% CO_2 at 37°C.

Patients and tissue samples

A total of 155 ESCC specimens [126 males (81.3%) and 29 females (18.7%); median age of 68 years (39-87 years) and 35 peritumor esophageal mucosa tissues (more than 5 cm away from the tumor border) were from patients undergoing curative esophagectomy at the

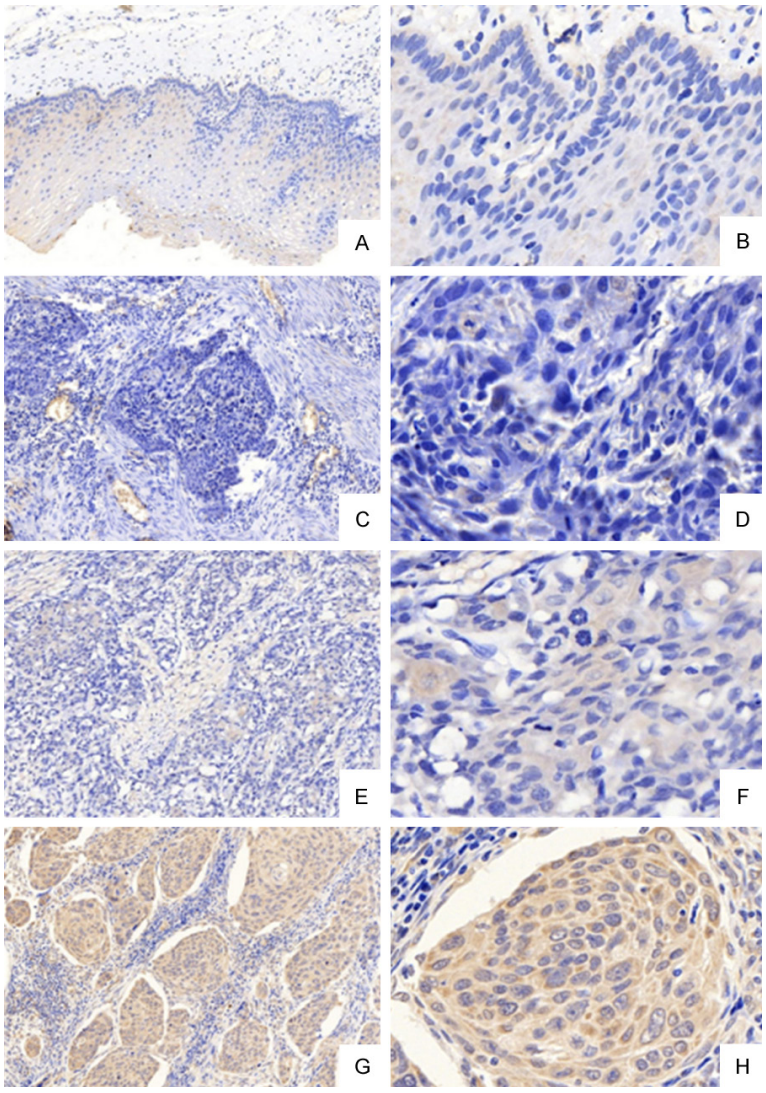

Figure 1. Capn4 immunohistochemistry in ESCC and adjacent normal mucosal tissues. A and B: Capn4 expression in peritumor normal tissues. C and D: Negative expression of Capn4 in ESCC. E and F: Low expression of Capn4 in ESCC. G and H: High expression of Capn4 in ESCC. Magnification: 200× (left); 400× (right). ESCC, Esophageal squamous cell cancer.

Tianjin Medical University Cancer Institute and Hospital from January 2005 to September 2006. None of the patients received preoperative therapies. Histological grading was determined by criteria based on the World Health Organization classification of esophageal tumors. Tumor stage of patients was classified according to the seventh edition of the TNM classification system of the AJCC (American Joint Committee on Cancer). Patient clinicopathological features are presented in **Table 1**.

Ethical approval was obtained from the Tianjin Medical University Cancer Institute and Hospital Ethics Committee. Moreover, written informed consent was obtained from the 155 patients. Follow up assessments were initially conducted by phone interview, every 3-6 months in the first two years and annually thereafter, until death or September 30, 2016. The median follow up was 30 months (3-107 months).

Immunohistochemistry analysis

Formalin-fixed and paraffin-embedded tissue samples were sectioned at a thickness of 4 μm. Each slice was deparaffinized by dimethylbenzene, dehydrated in ethanol, and heated in a microwave oven to perform heat-mediated antigen retrieval. Endogenous peroxidase activity was destroyed using 3% H_2O_2 at room temperature. The sections were then incubated with polyclonal rabbit anti-Capn4 antibody (1:100, Proteintech Group, Inc. China) overnight at 4°C. Biotinylated immunoglobulin was selected as a secondary antibody. The section was stained with diaminobenzidine and counterstained with hematoxylin. The sections treated with PBS instead of primary antibody were selected as a negative control. Each image was captured using an Olympus DP70 optical microscope (Tokyo. JPN).

The sections were assessed by two independent pathologists, blindly, and any disagreements were resolved through discussion. Capn4 expression level was evaluated based on the ratio of positive cells and the staining intensity. The proportion of positive cells was scored as follows: 0 for 5% positive cells; 1 for 6%-25% positive cells; 2 for 26%-50% positive cells; 3 for 51%-75% positive cells; and 4 for 76%-100% positive cells. The staining intensity was graded as follows: 0 (negative staining); 1 (weak staining); 2 (moderate staining); and 3 (strong staining). The final score was obtained by multiplying the ratio of positive cells score

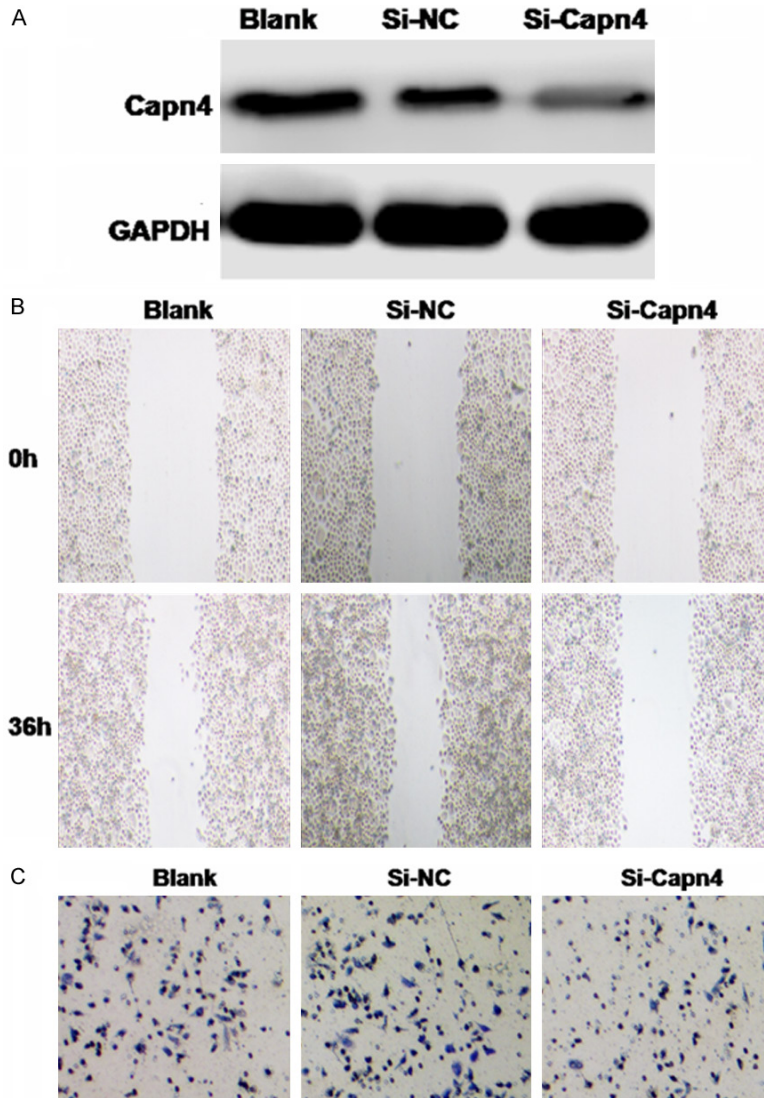

Figure 2. Functional analysis of CaEs-17 cells transfected with si-Capn4 in vitro. A: Western blot detection of Capn4 expression in the blank group, si-NC group, and si-Capn4 group. GAPDH was used as an internal control. $P<0.05$ versus blank and si-NC groups. B: CaEs-17 cells in the si-Capn4 group migrated slowly at 36 h in comparison to the cells in the blank group and the si-NC group in the wound assay. C: Transwell also revealed that the down-regulation of Capn4 was accompanied by impaired invasiveness of CaEs-17 cells. siRNA, small interfering RNA; si-NC, non-silencing control siRNA; si-Capn4, Capn4 siRNA.

and the intensity score, and was stratified into two categories: low expression (0-7 score) and high expression (8-12 score).

Small interfering RNA (siRNA) transfection

The siRNA targeting Capn4 (si-Capn4) and non-silencing siRNA control (si-NC) were purchased from Guangzhou RiboBio Co., Ltd (China). CaEs-17 was transfected by Lipofectamine 2000 (Invitrogen). The stably transfected cells were verified and used for analysis 48 h after transfection.

Western blot

Western blotting was carried out to determine Capn4 protein expression in CaEs-17 cells. The cells were lysed at 48 h after transfection. After being extracted and quantitated, equal amounts of the protein were separated on SDS-PAGE gels and transferred onto PVDF membranes. These membranes were incubated with 5% milk and blotted with anti-Capn4 antibody (1:1000; Proteintech Group, Inc. China) and anti-GAPDH antibody (1:5000; Proteintech Group, Inc. China) at 4°C overnight. Horseradish peroxidase-conjugated IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was selected as a secondary antibody. Immune bands were observed using ECL reagents and visualized with Bio-Rad Gel Doc 2000 (Hercules, CA, USA). Procedures were performed three times.

Wound healing assay

Wound healing was carried out to detect the migration ability of CaEs-17 cells. A linear wound was manufactured using a 200-μL pipette tip when the cells grew to 80%-90% density. The remaining cells were washed three times with phosphate buffer saline and then incubated in serum-free medium at 37°C. At times 0 h and 36 h after wounding, the width of the wound was photographed and compared.

Transwell invasion assay

Transwell assay was conducted to observe the invasion ability of CaEs-17 cells by using 24-well

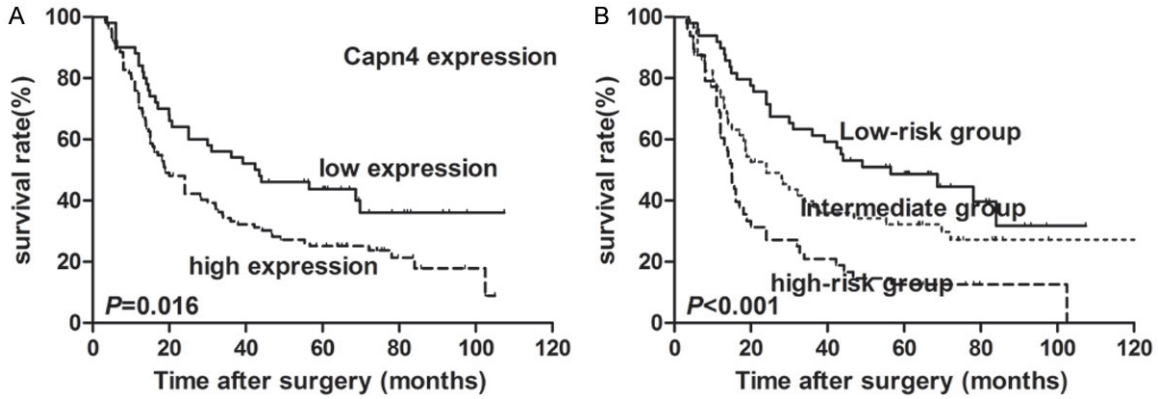

Figure 3. Prognosis assessment through Kaplan-Meier analysis and log-rank tests. A: Survival analysis was performed for capn4 expression in a cohort of 105 high expression patients and 50 low expression patients. B: Combined effects of lymph node metastasis, tumor invasion depth, and Capn4 expression on OS. OS, overall survival.

transwell plates (8-μm pore size, Corning Inc., Corning, NY, USA), and 1×10^5 cells were seeded in the top chambers pre-coated with Matrigel. A conditioned medium was added to the bottom chambers as a chemoattractant. After 48 h of incubation at 37°C, the invaded cells on the underside of the membrane were fixed in methanol and stained with 0.1% crystal violet. The number of invading cells was counted under a microscope.

Statistical analysis

SPSS 17.0 was used to perform data analysis. The correlation between Capn4 expression and clinical features was evaluated by a Chi-square test. The survival curves were plotted with the Kaplan-Meier method and differences between survival curves were compared by the log-rank test. Cox regression was used for univariate and multivariate analysis. *P* values <0.05 were considered statistically significant.

Results

Capn4 was overexpressed in ESCC

Immunohistochemistry was carried out to detect the protein expression level of Capn4 in 155 primary ESCC and in 35 peritumor normal tissues. The staining results indicated that the Capn4 was primarily expressed in the cytoplasm of cells. Of the 155 cancer lesions, 105 (67.7%) yielded high Capn4 expression. Of the 35 cases with adjacent normal tissues, 14 (40.0%) also exhibited high Capn4 expression. Capn4 level is significantly higher in ESCC tissues compared with peritumor tissues (P = 0.002) (**Figure 1**).

Capn4 protein expression by Western blot

The si-Capn4 and si-NC were transfected into CaEs-17 cells to elucidate the role of Capn4 in ESCC, and Western blot was performed to determine Capn4 expression. Expression level of Capn4 was effectively decreased in the si-Capn4 group compared with those in the blank group and the si-NC group (*P*<0.05, **Figure 2A**).

Capn4 promotes migration and invasion of ESCC cells

Transwell and wound healing assays were performed to assess the effects of Capn4 on invasion and migration ability in ESCC cells. The invasive and metastatic ability of si-Capn4-transfected CaEs-17 cells was remarkably lower than those of the blank group and si-NC-transfected cells (**Figure 2B** and **2C**).

High expression of Capn4 was correlated with poor prognosis in ESCC

Of the 155 patients, all had complete follow up data. As shown in **Table 1**, the upregulation of Capn4 was strongly associated with tumor size (P = 0.027), tumor invasion depth (P = 0.019), and lymph node metastasis (P = 0.011). Conversely, we did not find the association of Capn4 expression with other clinical features, including gender, age, smoking history, tumor location, and histological grade.

Table 2. Univariate and multivariate survival analyses of patients with ESCC

Variables	5-YSR (%)	Univariate analysis		Multivariate analysis	
		x^2 value	*P* value	HR value	*P* value
Gender		0.099	0.753		
Male	31.6				
Female	29.3				
Age (year)		4.933	0.026*	1.274	0.224
≤68	40.0				
>68	23.1				
Smoking history		1.545	0.214		
No	40.8				
Yes	26.4				
Tumor location		3.354	0.187		
Upper	44.4				
Middle	28.2				
Lower	46.4				
Tumor size (cm)		4.273	0.039*	1.384	0.107
<3.5	38.4				
≥3.5	26.2				
Histological grade		6.324	0.042*	1.450	0.060
G1	66.7				
G2	31.7				
G3	16.7				
Depth of tumor invasion		8.426	0.015*	1.383	0.036*
T1-T2	52.0				
T3	29.7				
T4	22.6				
Lymph node metastasis		11.128	0.001*	1.868	0.005*
Negative	40.6				
Positive	18.5				
Capn4 expression		5.835	0.016*	1.683	0.023*
Low	43.7				
High	25.2				

5-YSR = 5-year survival rates; HR = Hazard ratio; *p value <0.05 was regarded as statistically significant.

Table 3. Combined effect of lymph node metastasis, depth of tumor invasion, and Capn4 expression on prognosis

Group	Cases (n = 155)	5-YSR (%)	x^2 value	*P* value
Low-risk	49	48.6	19.315	<0.001*
Intermediate-risk	57	32.2		
high-risk	49	12.5		

5-YSR = 5-year survival rates; *p value <0.05 was regarded as statistically significant.

In the 155 patients, high Capn4 expression was inversely related to the patient's overall survival (OS) (**Figure 3A**). The findings indicated that ESCC patients with low Capn4 expression had significantly higher five-year survival rates than those with high Capn4 expression (43.7% vs. 25.2%, respectively). In addition, univariate analysis demonstrated that age (P = 0.026), tumor size (P = 0.039), histological grade (P = 0.042), tumor invasion depth (P = 0.015), lymph node metastasis (P = 0.001), and Capn4 expression (P = 0.016) were strongly associated with OS. Multivariate survival analysis with significant variables in univariate analysis revealed that Capn4 overexpression (P = 0.023) was an independent prognostic factor of ESCC (**Table 2**).

Combined effects of lymph node metastasis, tumor invasion depth, and Capn4 expression on prognosis

Multivariate analysis revealed three risk factors, namely, lymph node metastasis (positive), tumor invasion depth (T3 or T4), and high Capn4 expression. To identify the greatest risk factor affecting prognosis, we categorized the patients as follows: low risk group (n = 49 patients) with zero or one risk factor; intermediate risk group (n = 57 patients) with two risk factors; and high risk group (n = 49 patients) with three risk factors. The five-year survival rates of the patients in the three groups were 48.6%, 32.2%, and 12.5% (P< 0.001), respectively. The prognosis of the patients with high risk factors was poorer than that of the patients with intermediate and low risk factors (**Table 3** and **Figure 3B**).

Discussion

ESCC is the most common type of esophageal cancer, which has a high incidence and mortality rate. Early esophageal cancer is asymptomatic and most patients do not realize they have it until progressive dysphagia and weight loss occurs. Additionally, the rich submucosal lymphatics of the esophagus accelerate early spread of cancer cells [16]. The usual treatment for EC is surgery. However, the presence of regional lymph node metastasis in patients disqualifies them from surgery [17]. These clinical facts demonstrate that early detection is crucial to treatment.

Capn4, a small regulatory subunit of the calpain family, forms a heterodimer with an 80-kDa large catalytic subunit and regulates a wide spectrum of biological functions. Detailed physiological mechanism of Capn4 in tumorigenesis and tumor progression remains unclear. To date, accumulated evidence supports the use of Calpains, including Capn4, which affect cancer progression through promoting epithelial-mesenchymal transition (EMT). Recently, Capn4 has been found to be highly expressed in ICC and NPC, and high expression of Capn4 is associated with poor patient outcome [13, 15]. In this study, the findings suggested that Capn4 was overexpressed in ESCC tissues and that its expression was strongly associated with lymph node metastasis and tumor invasion depth. Therefore, we conclude that Capn4 overexpression is associated with the progression of ESCC.

Tumor development and progression are associated with metastasis of tumor cells. ESCC will have early lymph node metastasis and invasion into adjacent organs because of rich submucosal lymphatics. In this study, we revealed that knockdown of Capn4 by siRNA decreased CaEs-17 cells invasion and migration. Initial research implied that Capn4 promotes migration and invasion of tumor cells through activation of the EMT pathway, in which the cancer cells in the primary site are transformed from epithelial features to mesenchymal characteristics. The knockdown of Capn4 increased the expression of E-cadherin and decreased the expression of β-catenin, N-cadherin and vimentin in human melanoma cells [18, 19]. Additionally, Zheng et al. demonstrated that downregulation of Capn4 by siRNA led to decreased expression of MMP2, Snail, and Vimentin, but increased expression of E-cadherin in NPC cells [13, 20]. This suggested that Capn4 may be involved in EMT-mediated cell migration, and overexpression of Capn4 may be associated with ESCC metastasis.

To investigate the effect of Capn4 on prognosis, we conducted univariate and multivariate analysis and found that the five-year survival rate of patients with low Capn4 expression was higher than that in patients with high tumor expression ($P = 0.016$). Multivariate analysis of Capn4 expression and other parameters indicated that Capn4 expression was an independent indicator of prognosis in this study. This result suggested that Capn4 might play a vital role in the aggressive progression of ESCC and might serve as a novel marker to predict the prognosis for ESCC patients.

In summary, the study has shown that Capn4 is overexpressed in ESCC tissues and Capn4 high expression is significantly correlated with poor prognosis. Capn4 may thus serve as a potential therapeutic target of ESCC.

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Disclosure of conflict of interest

None.

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