

# Clinical significance of serum cathepsin B and cystatin C levels and their ratio in the prognosis of patients with esophageal cancer

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**Objective:** The main purpose of this study was to analyze the serum cathepsin B (CTSB) and cystatin C (CysC) levels in patients with esophageal carcinoma and their correlation with the clinical indices and prognosis.

**Methods:** The serum levels of CTSB and CysC from 56 patients with esophageal carcinoma and 30 healthy donors were determined preoperatively by using enzyme-linked immunosorbent assay. The correlation between CTSB and CysC was evaluated by Spearman correlation coefficient test. Kaplan–Meier survival curves were plotted, while the survival rates were compared using the log-rank test. Univariate and multivariate analyses of prognostic factors for survival were performed using the Cox proportional hazard regression model with a 95% confidence interval.

**Results:** CTSB ( $38.35 \pm 4.3$  ng/mL) and CysC ( $703.96 \pm 23.6$  ng/mL) levels were significantly higher in the sera of the patients than in controls. A significant correlation was observed between CTSB and CysC ( $r=0.754$ ,  $P<0.001$ ). The levels of CTSB and CysC/CTSB in the patient serum significantly correlated with the T status. CysC/CTSB ratio was also found to be significantly correlated with lymph node metastasis. None of the parameters were observed to be related to CysC, including age, gender, pathologic type, tumor differentiation and tumor invasion depth. Kaplan–Meier analysis showed that patients with higher levels of CysC/CTSB and negative lymph node metastasis experienced significantly longer overall survival time, whereas patients with higher CTSB levels tended to live shorter, although the difference was not statistically significant ( $P=0.081$ ).

**Conclusion:** Serum CTSB and CysC levels are of diagnostic significance in esophageal cancer. The ratio of serum CysC/CTSB is prognostic for the survival of esophageal carcinoma patients.

**Keywords:** esophageal squamous cell carcinoma, cathepsin B, cystatin C, diagnostic value, prognosis

## Introduction

Esophageal cancer is the eighth most common cancer worldwide, which is of low survival rate and rapid progression.<sup>1,2</sup> Over 80% of the deaths from esophageal cancer occur in developing and less-developed countries.<sup>3,4</sup> People's Republic of China has a high incidence of esophageal cancer. In spite of the constant progress in treatment, the 5-year survival rate of esophageal cancer patients is still  $<20\%$ .<sup>5</sup> Most patients with esophageal cancer are unresectable in time due to lack of effective diagnostic approaches. Serum markers are of great importance for early diagnosis and prognosis in esophageal cancer patients. Therefore, it is urgent to find new reliable markers.

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Proteases were regarded as playing vital roles in a diverse range of processes including inflammation, cell migration, angiogenesis, apoptosis, tumor invasion and metastasis.<sup>6–8</sup> Cathepsin B (CTSB), a member of lysosomal cysteine protease, has a structure analogous to the papaya enzyme papain and correlates with the general protein turnover in lysosomes.<sup>9,10</sup> A large body of literature revealed that increased activity of CTSB could enhance metastasis or the malignancy of various cancer cells.<sup>11,12</sup> Besides, many genomic and proteomic analyses showed that CTSB expression was markedly upregulated in numerous types of cancer, such as breast, gastric, thyroid, ovarian and colorectal cancers, and higher levels of CTSB were significantly related to poorer prognosis.<sup>13–17</sup>

The activity of CTSB is regulated mainly by its endogenous cysteine protease inhibitors, the cystatins, which are divided into three families. Cystatin C (CysC) is a nonglycosylated 13 kDa basic protein, which is composed of 120 amino acids. It is regarded as a housekeeping gene and is strongly inhibited by the activities of cathepsins B, K and L. CysC is present in all human body fluids including the cerebrospinal fluid and semen, while its concentration could be detected by enzyme-linked immunosorbent assay and immunohistochemical methods. CysC levels were found to be higher, similar, or lower than normal controls in different types of cancers.<sup>18,19</sup> CysC overexpression was found to inhibit the invasion and metastasis of tumor cells significantly.<sup>20,21</sup> Thus, CTSB and CysC could be potential diagnostic biologic markers for cancers.

Our previous study showed that in 56 surgically resected specimens of esophageal carcinoma patients, the protein and mRNA levels of both CTSB and CysC were markedly higher in the cancer tissues than those in the adjacent normal epithelial tissues, and CysC overexpression could inhibit the invasion of EC9706 cells *in vitro*. However, it is still unclear whether CTSB and CysC serve as diagnostic markers of esophageal carcinoma. In this study, we aimed to determine CTSB and CysC levels in the sera of esophageal carcinoma patients and controls and study their correlation with the clinicopathologic characteristics and prognosis of esophageal cancer patients.

## Materials and methods

### Ethics statement

This research was approved by the Ethical Committee of the First Affiliated Hospital of Zhengzhou University. A written informed consent was obtained from all participants.

### Serum and tissue samples

Fifty-six cases of esophageal carcinoma (31 males and 25 females) with a median age of 59.5 years (ranging from

36 to 77 years), who underwent tumor resection at the First Affiliated Hospital of Zhengzhou University from March 2009 to May 2011 were enrolled randomly in this study. Tumor histology was classified according to the World Health Organization classification. None of the patients had received radiotherapy or chemotherapy. The clinical and histopathologic features of the 56 patients were recorded, including age, pathologic type, differentiation grade, depth of invasion and the lymph node metastasis (LNM). The control sera were obtained from 30 healthy volunteers who came for check-up at the First Affiliated Hospital of Zhengzhou University, which consisted of 18 males and 12 females, with a median age of 60.4 years (40–79 years).

Blood samples were collected 1–3 days before surgery from the subjects. Morning fasting venous blood samples (3 mL) were obtained from all patients and healthy controls. The sera were centrifuged at  $3,000\times g$  for 10 min within 1 h and then stored at  $-80^{\circ}\text{C}$  until required.

Specimens of cancer tissues and matched adjacent normal esophageal tissues of these 56 patients were obtained during the operation. These tissues were placed in liquid nitrogen immediately and then were given routine paraffin embedding.

### Measurement of CTSB and CysC levels in the sera

Serum CTSB and CysC levels were measured using enzyme-linked immunosorbent assay kits for CTSB and CysC (Yanjin Biological, Shanghai, People's Republic of China), respectively. The sera were diluted at 1:20 and the assays were performed according to the manufacturer's instructions. All samples were analyzed simultaneously. First, we added different concentrations of standard diluent (50  $\mu\text{L}$ ) to the standard wells and then added 40  $\mu\text{L}$  of sample dilutions and 10  $\mu\text{L}$  of testing samples to the testing sample wells. After 100  $\mu\text{L}$  of horseradish peroxidase-conjugate reagent was added to each well, it was covered with a plate cover. Then the plates were incubated at  $37^{\circ}\text{C}$  for 60 min. Then we removed the cover and washed the plate. After the washing was completed, substrates A and B were added to each well at 50  $\mu\text{L}$ , respectively. After incubation in dark at  $37^{\circ}\text{C}$  for 15 min, stop reagent was added to each well. The concentrations were measured by reading the absorbance values at 450 nm, which were subsequently calculated using standard curves and expressed as ng/mL. Each test was repeated three times.

### Follow-up

All 56 patients were followed up. We adopted overall survival (OS), which was defined as the time from the diagnosed date

till the date of death or of the latest follow-up. Patients who did not die of esophageal cancer were ruled out. The longest follow-up period was 60 months.

## Statistical analysis

All data were expressed as mean  $\pm$  standard deviation (SD) and analyzed by using the statistical software Statistical Package for the Social Sciences 17.0. The intergroup differences were assessed using Mann–Whitney *U* test and Kruskal–Wallis tests. The correlation between CTSC and CysC was evaluated by Spearman correlation coefficient. Kaplan–Meier survival curves were plotted, while the survival rates were compared using the log-rank test. Univariate and multivariate analyses of prognostic factors for survival were performed using the Cox proportional hazard regression model with a 95% confidence interval. In all tests, two-sided *P*-values  $<0.05$  were regarded statistically significant.

## Results

### Serum CTSC and CysC levels were significantly upregulated in esophageal cancer patients

Serum CTSC, CysC and CysC/CTSC levels of patients with esophageal carcinoma and healthy controls are listed as mean with quartile deviation and median (min–max) in Table 1. Serum CTSC levels of the patients ( $38.35 \pm 4.3$  ng/mL) were significantly higher ( $P < 0.01$ ) than those of the control group ( $22.35 \pm 1.8$  ng/mL). Serum CysC levels of the patients group ( $703.96 \pm 23.6$  ng/mL) were also significantly higher ( $P < 0.01$ ) than those of the controls ( $501.97 \pm 11.5$  ng/mL). As for the ratio of CysC/CTSC, although no significant difference was observed, the patients displayed a lower ratio than that of the control group ( $P = 0.093$ ). In addition, a significant correlation between CTSC and CysC ( $r = 0.754$ ,  $P < 0.001$ ) was demonstrated by using Pearson correlation analysis.

### Correlation of CTSC and CysC levels with clinicopathologic features

The association between CTSC, CysC and CysC/CTSC levels and the clinicopathologic features are shown in Table 2.

CTSC and CysC/CTSC levels significantly correlated with the clinical T stage. CTSC levels in group T3+T4 were higher than in group T1+T2 ( $P = 0.008$ ), and CysC/CTSC levels in group T3+T4 were markedly lower than in group T1+T2 ( $P = 0.001$ ). Besides, CysC/CTSC levels were significantly lower in the group with metastasis of lymph node than those of the negative group ( $P = 0.001$ ). They showed no correlation with age, gender, pathologic type or tumor differentiation.

### Correlation of CTSC and CysC levels with OS

We used the median values of CTSC, CysC and CysC/CTSC as the cutoff points to respectively divide the patients into low- and high-level groups. Kaplan–Meier curve together with log-rank test was used to find the factors influencing OS. The results showed that the median OS time of patients with higher CysC/CTSC ratio (median OS, 38.5 months) was significantly longer than that of the low ratio group (median OS, 26.2 months,  $P = 0.001$ ; Figure 1). However, it was very interesting to see that, when adjusted for T stage, the difference in OS was statistically significant only in the T3+T4 stage group ( $P = 0.007$ , Figure 2) rather than in the T1+T2 group ( $P = 0.727$ , Figure 3). The median OS period of patients with positive LNM (median OS, 27 months) was found to be significantly shorter than that of those with negative LNM (median OS, 38.1 months,  $P = 0.01$ ; Figure 4). Besides, patients in low-level CTSC group (median OS, 35.9 months) were found to live longer than those in high-level CTSC group (median OS, 28.5 months), although the difference was not statistically significant ( $P = 0.081$ , Figure 5). However, levels of CysC were not associated with the survival of esophageal cancer patients. Likewise, there was no significant correlation between the OS of cases with esophageal cancer and their age, gender, pathologic type, tumor differentiation and tumor invasion depth (Table 2).

### CysC/CTSC was an independent prognostic factor in esophageal cancer patients

As shown in Table 3, univariate and multivariate Cox regression analyses were used to evaluate the proportional

**Table 1** Serum CTSC, CysC and CysC/CTSC levels in esophageal carcinoma patients and healthy population

Indexes	Patients (N=56)		Controls (n=30)		P-value
	Mean ( $\pm$ SD)	Median (min–max)	Mean ( $\pm$ SD)	Median (min–max)	
CTSC (ng/mL)	$38.35 \pm 4.3$	38.14 (20.97–62.72)	$22.35 \pm 1.8$	21.39 (13.21–48.37)	0.001
CysC (ng/mL)	$703.96 \pm 23.6$	698.72 (440.51–1,424.48)	$501.97 \pm 11.5$	492.63 (325.25–1,136.74)	0.001
CysC/CTSC	$18.71 \pm 2.96$	18.85 (14.87–24.27)	$22.21 \pm 3.75$	21.73 (15.26–28.21)	0.093

**Abbreviations:** CTSC, cathepsin B; CysC, cystatin C; SD, standard deviation; min, minimum; max, maximum.

**Table 2** The correlation between clinicopathologic characteristics and serum CTSC or serum CysC

Characteristic	n	CTSB		CysC		CysC/CTSB	
		Mean $\pm$ SD	P-value	Mean $\pm$ SD	P-value	Mean $\pm$ SD	P-value
Age (years)			0.412 <sup>a</sup>		0.428 <sup>a</sup>		0.510 <sup>a</sup>
$\geq 58.5$	28	39.17 $\pm$ 2.51		725.34 $\pm$ 9.19		18.59 $\pm$ 2.65	
<58.5	28	37.44 $\pm$ 3.26		681.55 $\pm$ 13.39		18.08 $\pm$ 1.89	
Gender			0.341 <sup>a</sup>		0.212 <sup>a</sup>		0.635 <sup>a</sup>
Male	31	39.46 $\pm$ 2.34		733.89 $\pm$ 11.81		18.55 $\pm$ 2.71	
Female	25	37.24 $\pm$ 4.51		673.61 $\pm$ 8.67		17.66 $\pm$ 1.94	
Pathologic type			0.631 <sup>a</sup>		0.621 <sup>a</sup>		0.378 <sup>a</sup>
SCC	50	38.13 $\pm$ 2.61		701.44 $\pm$ 16.62		18.42 $\pm$ 2.46	
ADC	6	39.65 $\pm$ 3.64		723.74 $\pm$ 13.74		18.22 $\pm$ 2.07	
Grade			0.459 <sup>b</sup>		0.771 <sup>b</sup>		0.169 <sup>b</sup>
G1	11	37.57 $\pm$ 6.98		702.34 $\pm$ 13.69		18.71 $\pm$ 2.39	
G2	25	39.36 $\pm$ 2.99		716.35 $\pm$ 9.00		18.18 $\pm$ 2.28	
G3	20	38.32 $\pm$ 5.21		688.98 $\pm$ 12.33		18.10 $\pm$ 1.88	
T status			0.008 <sup>a</sup>		0.459 <sup>a</sup>		0.001 <sup>a</sup>
T1 + T2	24	29.35 $\pm$ 3.38		693.18 $\pm$ 14.62		22.57 $\pm$ 2.73	
T3 + T4	32	45.12 $\pm$ 4.56		711.40 $\pm$ 9.88		16.28 $\pm$ 1.87	
Lymph node metastasis			0.871 <sup>a</sup>		0.239 <sup>a</sup>		0.001 <sup>a</sup>
No	20	38.81 $\pm$ 4.71		729.14 $\pm$ 8.32		19.36 $\pm$ 2.28	
Yes	36	38.17 $\pm$ 2.86		687.58 $\pm$ 11.89		15.28 $\pm$ 1.64	

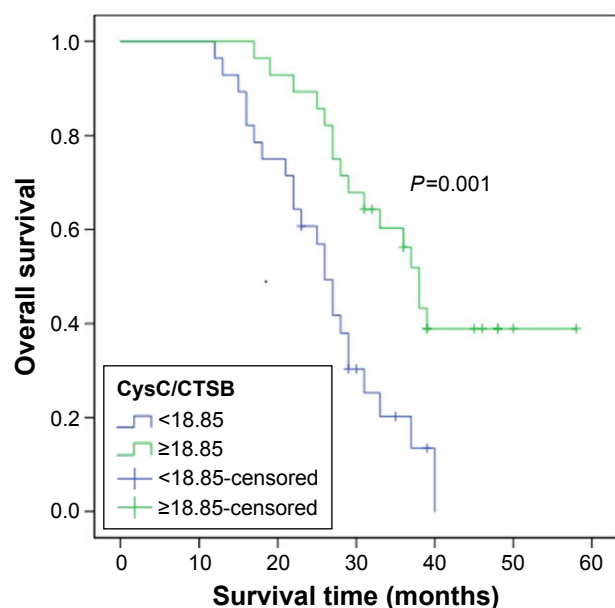
Notes: <sup>a</sup>Mann–Whitney U test, <sup>b</sup>Kruskal–Wallis test.

Abbreviations: ADC, adenocarcinoma; CTSB, cathepsin B; CysC, cystatin C; IQ, interquartile; SCC, squamous cell carcinoma; SD, standard deviation.

hazards of the indices in the OS time, including age, gender, pathologic type, grade, T stage, LNM, CysC/CTSB ratio, CTSB level and CysC level. The results showed that CysC/CTSB and LNM could be regarded as independent prognostic factors in esophageal cancer patients.

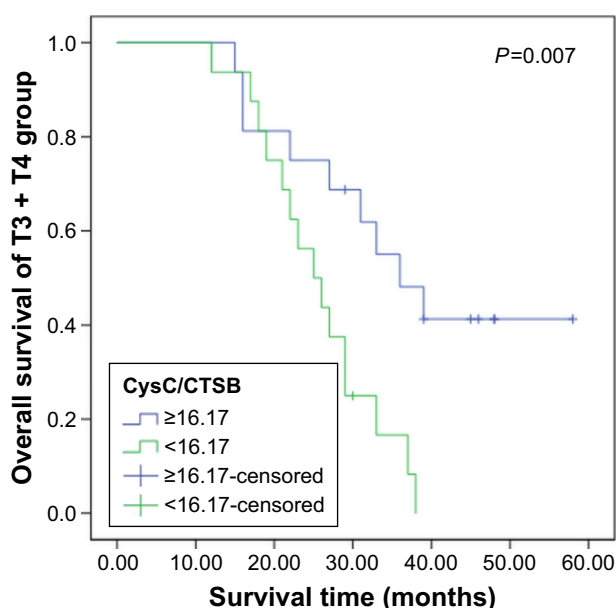
## Discussion

Elevated levels of CTSB have been observed in primary and metastatic tumor tissues of various types of cancers. A two-dimensional electrophoresis assay of the protein patterns of thyroid tissues revealed that CTSB was



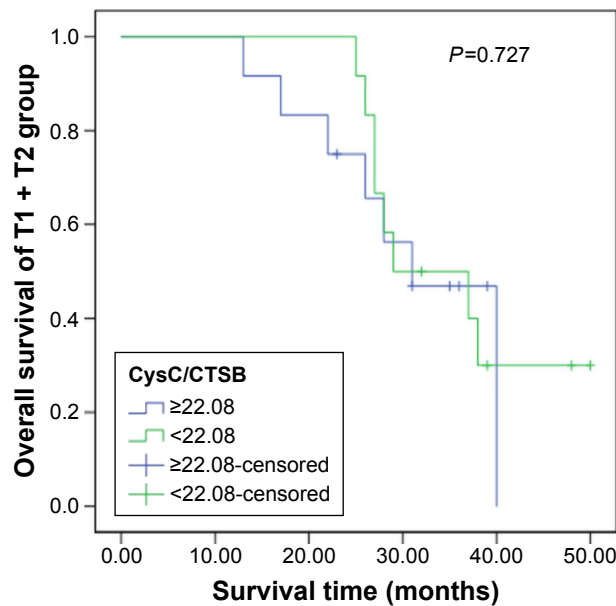
**Figure 1** Kaplan–Meier curve of overall survival for patients with low and high levels of CysC/CTSB.

Abbreviations: CTSB, cathepsin B; CysC, cystatin C.



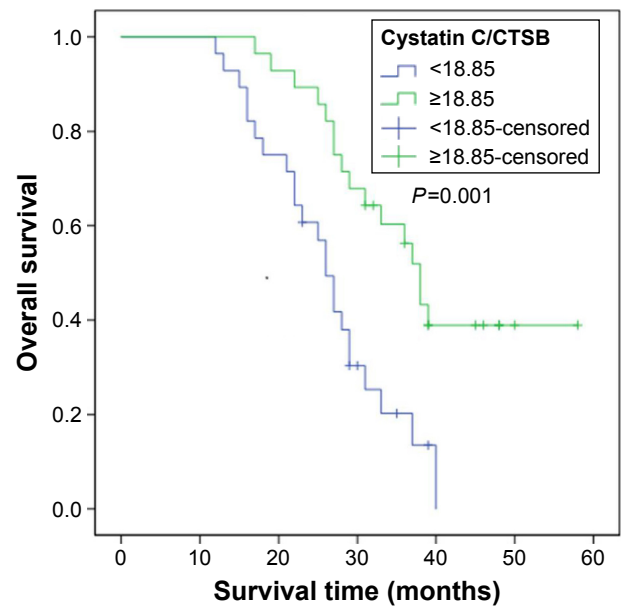
**Figure 2** Kaplan–Meier curve of overall survival for patients in T3 + T4 group with low and high levels of CysC/CTSB.

Abbreviations: CTSB, cathepsin B; CysC, cystatin C.



**Figure 3** Kaplan-Meier curve of overall survival for patients in T1 + T2 group with low and high levels of CysC/CTSB.

**Abbreviations:** CTSB, cathepsin B; CysC, cystatin C.



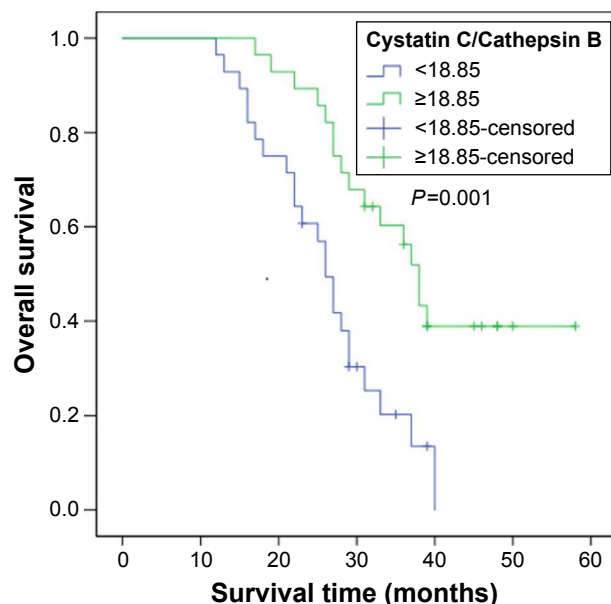
**Figure 5** Kaplan-Meier curve of overall survival for patients with low and high levels of CTSB.

**Abbreviation:** CTSB, cathepsin B.

significantly upregulated in neoplastic diseases compared with nonneoplastic diseases.<sup>15</sup> The expression of CTSB was upregulated in more than a half of gastric cancer patients, while the serum level of CTSB was higher in patients with advanced stage cancer than in healthy controls.<sup>14</sup> Besides, CTSB expression was positively correlated to lymph node metastasis, poor differentiation and remote metastasis. Tan et al showed that the serum level of CTSB was considerably

higher in nasopharyngeal carcinoma patients than in controls and was correlated with the TNM score.<sup>22</sup> Moreover, CTSB was highly upregulated in human esophageal squamous cell carcinoma (ESCC), suggesting it plays a key role in the regulation of ESCC initiation and progression.<sup>23,24</sup> In the present study, we observed a significant increase of CTSB levels in the sera of patients with esophageal cancer compared with that of the healthy controls. Furthermore, CTSB levels were positively correlated with increasing tumor invasion depth, which is consistent with the findings listed earlier. It suggests that CTSB might be involved in the occurrence and progression of esophageal cancer. We speculate that CTSB may serve as a candidate biomarker for esophageal cancer.

CysC overexpression could inhibit both invasion and metastasis of cancer cells. Numerous studies about CysC have shown that its expression levels vary in different types of cancers. CysC showed significantly higher expression in prostate cancer as well as in non-small-cell lung cancer.<sup>25,26</sup> In contrast, the expression of CysC was down-regulated in breast cancer and large granular lymphocytic leukemia.<sup>27,28</sup> As for the CysC levels in sera, patients with cancers such as lung, gastric, breast and ovarian were found to have much higher levels than controls. In this study, we observed a significant increase of serum CysC levels in patients with esophageal cancer. In addition, we demonstrated a significant correlation between CTSB



**Figure 4** Kaplan-Meier curve of overall survival for patients with positive lymph node metastasis versus negative lymph node metastasis.



**Table 3** Univariate and multivariate analyses of CysC, CTSB and other potential factors for EC prognosis

Characteristics	n	Overall survival (months)	Univariate analysis		Multivariate analysis	
			P-value	RR	P-value	RR
Age (years)						
≥58.5	28	30.4	0.159	1.491	0.267	1.522
<58.5	28	33.3				
Gender						
Male	31	34.7	0.108	1.109	0.134	2.043
Female	25	29.1				
Pathologic type						
SCC	50	31.7	0.318	1.455	0.204	1.517
ADC	6	28.1				
Grade						
G1 + G2	36	34.4	0.175	1.074	0.127	1.353
G3	20	27.5				
T stage						
T1 + T2	24	36.1	0.458	1.373	0.671	1.564
T3 + T4	32	28.9				
LNM						
No	20	38.1	0.01	1.357	0.007	1.606
Yes	36	27				
CysC/CTSB						
<18.85	26	38.5	0.001	2.253	0.001	2.411
≥18.85	30	26.2				
CTSB						
≥38.14	27	35.9	0.081	0.969	0.065	1.518
<38.14	29	28.5				
CysC						
<698.72	28	33.6	0.152	1.177	0.183	0.923
≥698.72	28	29.3				

**Abbreviations:** CTSB, cathepsin B; CysC, cystatin C; EC, esophageal cancer; LNM, lymph node metastasis; RR, relative risk.

and CysC. The exact mechanism for this increase has not been studied so far. One putative cause is that increased CTSB could result in the upregulation of CysC expression by positive feedback regulation in order to inhibit the increased activity of CTSB.<sup>29</sup> On the other hand, our previous study indicated that expression of CysC had a reverse relationship with the apoptosis of esophageal carcinoma. It was speculated that elevated CysC might protect esophageal cancer cells against CTSB-mediated apoptosis. Further studies are still needed to clarify the dual effect of CysC on different tumor types. In addition, no significant correlations were observed between CysC levels and the patient's age, gender, pathologic type, grade, tumor invasion depth and lymph node status in our study. Konduri et al observed an inverse correlation between CysC and the tumor grade of glioma.<sup>19</sup> Saleh et al found that the expression of CysC correlated with the differentiation and invasion of tumor cells in colorectal cancer.<sup>30</sup> However, CysA, but not CysC, was one of the differentiation-associated genes regulated by c-Jun in the progression of ESCC.<sup>31</sup> The inconsistency of our findings

with those above may be mainly due to the following reasons: limited sample size, different detection method and distinct types of tumors.

Monsouvanh et al found that the CTSB/CysC ratio of cholangiocarcinoma patients was considerably higher than that of the healthy group.<sup>32</sup> Another study showed that the serum levels of CTSB/CysC complexes were much lower in patients with lung cancer than in the patients with benign lung diseases or healthy controls.<sup>33</sup> In this study, we found that the CysC/CTSB ratio of the esophageal cancer group was significantly lower than that of the control group, and its level significantly correlated with the T stage and LNM. A separate study on another inhibitor for cysteine proteinases, named stefin A, also showed the correlation between its ratio with CTSB and the aggressive potential of prostate carcinoma.<sup>34</sup> These findings suggest that the imbalance between CTSB and CysC may contribute to tumorigenesis. Thus, the ratio of CysC to CTSB is more meaningful and significant than the absolute levels.

Analysis of the correlation between the variables and the prognosis of esophageal patients revealed that CysC to

CTSB ratio as well as the status of lymph node possessed significant prognostic value. Patients with higher CysC/CTSB ratio exhibited significantly longer survival time. This is not consistent with the findings of another study which revealed that the ratio of cathepsins and CysC in the ovarian cyst fluid was not a significant predictor of survival for patients with epithelial ovarian cancer.<sup>35</sup> In addition, we found that patients with lower CSTB levels tend to live longer than those with higher CSTB levels, although the difference was not statistically significant. CSTB levels were found to be associated with survival in pancreatic adenocarcinoma, breast cancer, acute myeloid leukemia, oral squamous cell carcinoma and hepatocellular carcinoma.<sup>36–40</sup> This remains to be clarified by using larger samples in the future.

Generally, serum CTSB and CysC levels were markedly increased in patients with esophageal cancer. Serum CTSB and CysC concentrations are of diagnostic significance in esophageal cancer. The ratio of serum CysC/CTSB is prognostic for the survival for esophageal carcinoma patients.

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## Disclosure

The authors report no conflicts of interest in this work.

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