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Original Article

Elevated CTSL2 expression is associated with an adverse prognosis in hepatocellular carcinoma

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Abstract: Objective: Cathepsin V, also known as CTSL2, plays an important role in tumor development and progression. This study was designed to investigate the clinical significance of CTSL2 expression in hepatocellular carcinoma (HCC) and the relationship between CTSL2 expression and prognosis. Methods: Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) were performed to determine the levels of CTSL2 mRNA and protein, respectively, in tumor tissue and matched non-tumor (NT) tissue. Moreover, the relationship between CTSL2 expression and hepatocellular carcinoma's clinicopathological features and survival was evaluated in HCC tissue. Results: The levels of CTSL2 mRNA and protein were increased in HCC tissue. Moreover, for HCC patients, a high level of CTSL2 protein was significantly correlated with tumor number ($P = 0.008$), pathological grade ($P = 0.001$), vascular invasion ($P = 0.001$), T ($P = 0.001$), and TNM stage ($P = 0.006$). A Kaplan-Meier analysis showed that elevated CTSL2 expression was correlated with shorter disease-free survival (DFS) ($P < 0.001$) and overall survival (OS) ($P < 0.001$). Furthermore, a multivariate analysis showed that CTSL2 expression was an independent prognostic factor for DFS ($P = 0.032$) and OS ($P = 0.025$). Conclusion: This study showed that abnormal CTSL2 expression may contribute to HCC progression and that elevated CTSL2 expression is associated with an adverse prognosis in HCC.

Keywords: CTSL2, hepatocellular carcinoma (HCC), PCR, IHC, prognosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the second leading cause of cancer-related deaths [1]. In the US, 33,000 new HCC cases and 23,000 HCC-related deaths were reported in 2014 [2]. HCC is the fourth most common malignancy in China, and in 2015, approximately 422,100 patients died from HCC [3]. The development and progression of HCC are related to hepatitis B and hepatitis C virus infection, alcohol-induced damage, and non-alcoholic fatty liver [4-8]. Currently, common treatments for HCC include surgical resection and sorafenib, an anti-angiogenic multikinase inhibitor [9, 10]. Due to postoperative recurrence and metastasis, the five-year survival rate of HCC patients has remained unchanged over the past several years [11-13]. The current treatment options only moderately improve the survival rate, and sorafenib improves survival by only a few months [14]. It is important to discover novel markers to confirm an early recurrence and an adverse prognosis of HCC.

Cathepsins (CTSs) are a large family of proteins that are highly expressed in various human cancers and that are associated with cancer invasion and metastasis [15]. Cathepsin V (also known as CTSL2) is a lysosomal cysteine protease that is specifically expressed in the thymus, testis, and corneal epithelium. It is also involved in the development of keratoconus [16-19]. Studies have shown that CTSL2 expression may be related to tumor metastasis [20], as CTSL2 is often overexpressed in various human cancers [18, 21]. Moreover, the mRNA level of CTSL2 is significantly increased in endometrial cancer, especially in G3 tumors; thus, CTSL2 may be involved in the progression of endometrial cancer [22]. Haider et al. [23] used a high-density oligonucleotide microarray to determine the expression of 412,000 genes in surgically resected specimens of human squamous cell carcinoma (SCC). The results showed that

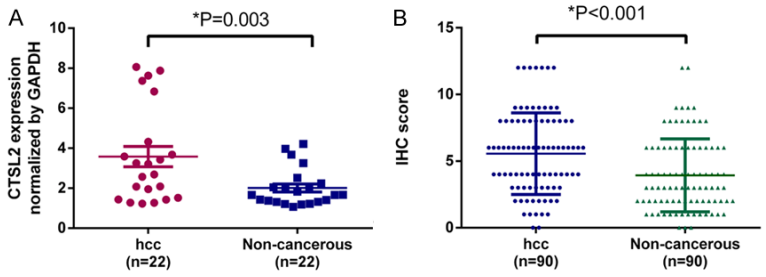

Figure 1. A: CTSL2 expression in hepatocellular carcinoma (HCC) tissues and tumor adjacent non-cancerous tissues. qRT-PCR demonstrated that the expression of CTSL2 in HCC tissues (3.58 ± 0.508) was significantly higher than in matched non-cancerous tissues (2.01 ± 0.201), when normalized to the GAPDH internal control. *P < 0.05. B: The IHC score of CTSL2 was indicated by Dot distribution graph. Data are mean ± SD (Wilcoxon signed-rank test).

CTSL2 expression was significantly increased in SCC. In addition, CTSL2 is expressed physiologically in thymic epithelial cells, and its expression is increased in some thymomas and cases of thymic carcinoma, which suggests that CTSL2 may potentially be used as an auxiliary diagnostic and prognostic marker for thymic epithelial tumors [24]. In addition, CTSL2, CTSL, CTSK, and CTSS are considered potential drug targets [25, 26]. Therefore, it is important and relevant to investigate whether CTSL2 has a similar effect in HCC and whether CTSL2 may be used as a novel biomarker for HCC diagnosis and treatment.

In this study, qRT-PCR was performed using fresh HCC tissue, and IHC was performed using an HCC tissue microarray (TMA) to investigate the relationship between CTSL2 expression and the clinicopathological features of HCC patients and to evaluate the prognostic value of CTSL2.

Materials and methods

Patients and tissue samples

Twenty-two paired fresh HCC tumor and nontumorous tissue samples were collected immediately after surgery resection from the People's Hospital of Jurong Affiliated with Jiangsu University. Archival tissue samples (90 formalin-fixed, paraffin-embedded HCC tissues and 90 matched tumor-adjacent normal tissues) were obtained to construct tissue microarrays (TMA) from the People's Hospital of Jurong Affiliated with Jiangsu University between June 2007 and July 2012. Representative and important clinical data, such as age, gender, tumor size, tumor capsule, tumor number, hepatitis B virus (HBV) infection, and liver cirrhosis, pathological grade, vascular invasion and TNM stage, were collected for further analyses. The TNM stages were defined according to the 2010 AJCC staging system for HCC. Ethical approval for this study was granted by the Medical Ethics Committee of each local hospital. All patients signed an informed consent.

qRT-PCR analysis

The reagents used and the detailed procedure of qRT-PCR were performed as before [27, 28]. The primers for CTSL2 were as follows: forward primer 5'-TCGCGTCCTCAAGGCAATC-3' and reverse primer 5'-CACAGTTGCGACTGCTTTCAT-3'. The GAPDH was employed as internal control, and the primers for GAPDH were as follows: forward primer 5'-GCCTCAAGATCATCAGCAAT-3' and reverse primer 5'-GGACTGTGGTCATGAGTCCT-3'. Expression data were normalized to the geometric mean of the GAPDH housekeeping gene and calculated by using the comparative Ct (2-ΔΔCt) method.

TMA construction and IHC analysis

Construction of liver cancer tissue microarrays as well as immunostaining was performed according to standard protocols described elsewhere [29, 30]. TMA sections were incubated with mouse monoclonal anti-cathepsin V antibody (1:200, ab24508, Abcam, Cambridge, MA, USA) in phosphate-buffered saline (PBS) and then incubated with horseradish peroxidase conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) after washing. Negative controls were included by replacement of the primary antibody with PBS. We multiplied the percentage score by the staining intensity score. The percentage of positively stained cells was scored as "0" (0%), "1" (1%-25%), "2" (26%-50%), "3" (51%-75%), or "4" (76%-100%). The intensity was scored as "0" (negative staining), "1" (weak staining), "2" (moderate staining), or "3" (strong staining). For each case, 1000 cells were randomly selected and scored. The scores were independently decided by 2 pathologists. The median IHC

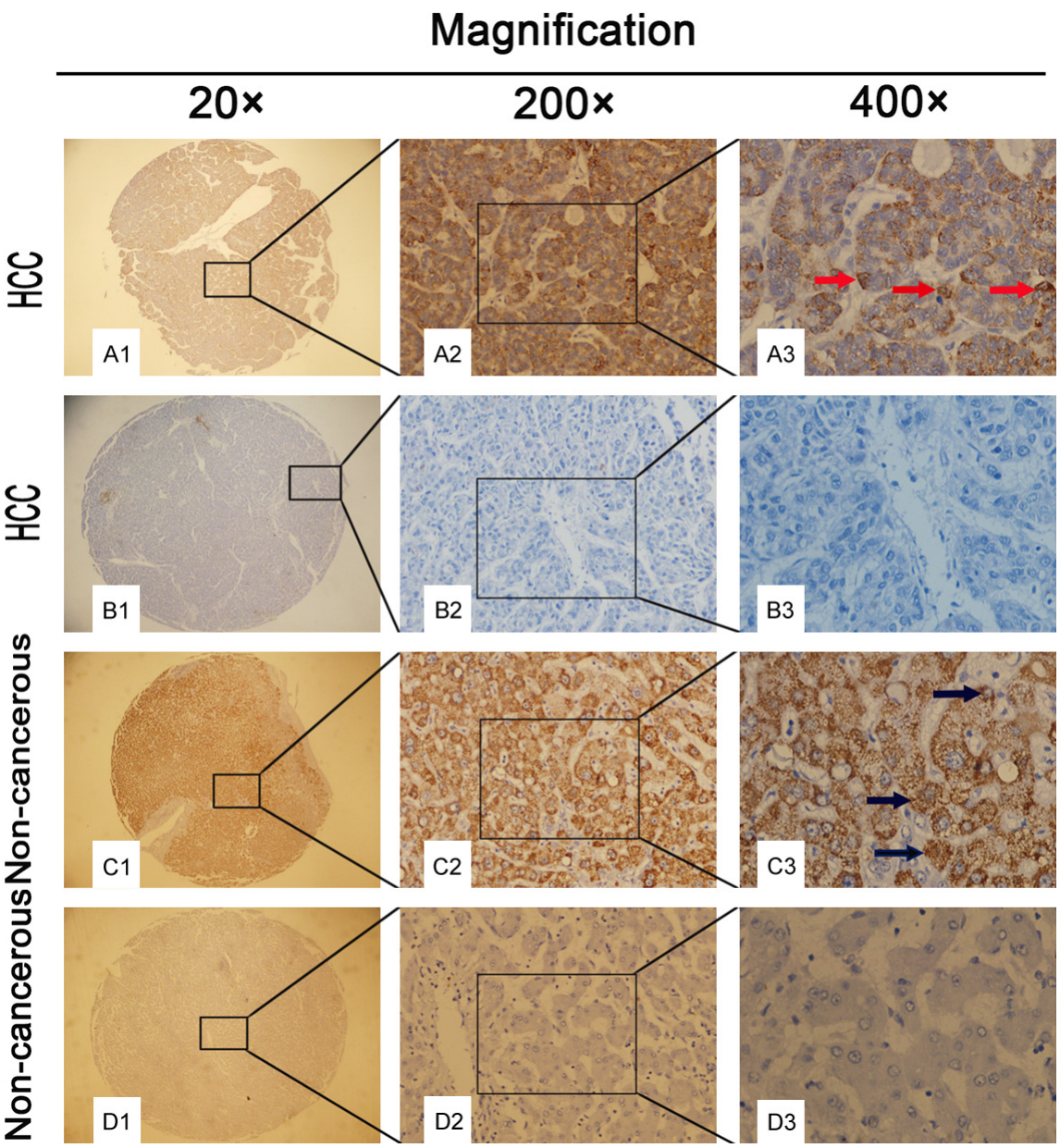

Figure 2. Representative types of CTSL2 protein expression in HCC tissue samples and corresponding non-cancerous tissue samples. A1-A3. High cytoplasmic and membranous expression of CTSL2 in HCC tissue samples. Red arrows show the positive staining in the cytoplasm and cytomembrane of cancer cells. B1-B3. Low expression of CTSL2 in HCC tissue samples. C1-C3. High expression of CTSL2 in non-cancerous tissue samples. Dark blue arrows show the positive staining in the cytoplasms and cytomembranes of non-cancerous cells. D1-D3. Low expression of NDRG3 in non-cancerous tissue sample. Original magnification: × 20 in A1-D1; × 200 in A2-D2; × 400 in A3-D3.

score (4.5) was chosen as the cut-off value to define groups of high and low expression.

Statistical analysis

The CTSL2 mRNA expression in fresh HCC tissues and the IHC score in TMA relative to the matched non-cancerous tissues were analyzed with the Wilcoxon signed rank nonparametric test. The significance of CTSL2 protein expression in clinical data from HCC patients was calculated by the chi-square test. Both univariate and multivariate analyses were performed with Cox proportional hazards regression models to identify important factors that were associated with disease-free and overall survival status. The Kaplan-Meier method was utilized to analyze the relationship between CTSL2 expression and the outcome of HCC patients. The significance level for statistical analysis was set at

Table 1. Correlation between Clinicopathological Features and CTSL2 Expression

Groups	No.	CTSL2		χ^2	*p* value
		+	%		
Total	90	48	53.3		
Gender					
Male	80	42	51.2	0.20	0.654
Female	10	6	60.0		
Age (years)					
≥ 60	22	14	63.6	1.24	0.265
< 60	68	34	50.0		
Tumor size (cm)					
> 5	28	15	53.6	0.01	0.976
≤ 5	62	33	53.2		
Tumor encapsulation					
None	47	30	63.8	5.75	0.057
Complete	42	17	40.5		
Insufficient data	1	1			
Tumor number					
Multiple	11	10	90.9	7.11	0.008*
Solitary	79	38	48.1		
Hepatitis B virus infection					
Yes	70	36	51.4	1.14	0.567
No	19	11	57.9		
Insufficient data	1	1			
Liver cirrhosis					
Yes	80	45	56.3	4.65	0.098
No	9	2	22.2		
Insufficient data	1	1			
Pathological grade					
Grade 1-2	43	14	32.6	14.28	0.001*
Grade 3	47	34	72.3		
Vascular invasion					
Present	21	19	90.5	27.7	0.001*
Absent	58	19	32.8		
Insufficient data	11	10			
T					
T1	58	19	32.8	27.9	0.001*
T2	28	25	89.3		
T3	4	4	100		
TNM stage					
Stage I	58	24	41.4	10.2	0.006*
Stage II	29	21	72.4		
Stage III	3	3	100		

*$p < 0.05$.

$P < 0.05$. All statistical analyses were conducted by utilizing STATA 14.0 (Stata Corporation, College Station, TX, USA) and SPSS 18.0 (SPSS Inc, Chicago, IL,USA).

Results

qRT-PCR detection of the mRNA level of CTSL2 in HCC tissue

To determine the mRNA level of CTSL2 in HCC, qRT-PCR was performed in 22 pairs of HCC tissue and matching adjacent tissue. The results showed that the mRNA level of CTSL2 was significantly higher (mean: 1.8-fold) in tumor tissue than in normal non-tumor tissue ($P = 0.003$) (**Figure 1A**).

IHC detection of CTSL2 expression in HCC tissue

The expression of CTSL2 was further analyzed in a TMA that contained 90 HCC cases and a TMA that contained matching adjacent tissue. Compared with non-tumor tissues, the HCC tissues showed significantly higher levels of CTSL2 protein ($P < 0.001$) (**Figure 1B**). IHC showed that CTSL2 was primarily localized in the cytoplasm and cell membranes and that its expression was usually increased in HCC tissue (**Figure 2**). The red arrows indicate positive staining in the cytoplasm and cell membranes of cancer cells. The expression of CTSL2 was increased in 68.9% (62/90) of HCC samples relative to normal tissues. According to the median IHC staining score (4.5), the patients were divided into two groups: the high CTSL2 expression group and the low CTSL2 expression group, and 53.3% (48/90) of the patients were in the high expression group.

Correlation between CTSL2 expression and the clinical features of HCC patients

We analyzed the correlation between CTSL2 expression and the clinical features of HCC in order to determine the clinical significance of CTSL2 in HCC. High CTSL2 expression was closely related to tumor number ($P = 0.008$), Pathological grade ($P = 0.001$), Vascular invasion ($P = 0.001$), T ($P = 0.001$), and TNM stage ($P = 0.006$). No significant correlation was observed between CTSL2 and other clinicopathological features, including age, gender, tumor size, tumor capsule,

Table 2. Univariate and Multivariate Analyses of the Clinicopathological Features and CTSL2 in relation to the DFS of HCC Patients (n = 90)

	Univariate analysis			Multivariate analysis		
	HR	p > |z|	95% CI	HR	p > |z|	95% CI
CTSL2 expression						
High versus Low	3.95	0.001*	1.693-9.197	3.06	0.032*	1.102-8.476
Gender						
Male versus Female	3.57	0.210	0.487-26.20			
Age (years)						
≥ 60 versus < 60	1.40	0.391	0.646-3.051			
Tumour size (cm)						
> 5 versus ≤ 5	2.07	0.047*	1.009-4.229	1.88	0.099	0.887-4.000
Tumor encapsulation						
None versus Complete	1.79	0.122	0.856-3.738			
Tumor number						
Multiple versus Solitary	1.81	0.226	0.693-4.718			
Hepatitis B virus infection						
Yes versus No	0.96	0.930	0.415-2.237			
Liver cirrhosis						
Yes versus No	1.68	0.476	0.402-7.062			
Pathological grade						
Grade 1 and 2 versus Grade 3	0.31	0.004*	0.138-0.693	0.50	0.118	0.207-1.194
Vascular invasion						
Present versus Absent	1.75	0.165	0.794-3.859			
T						
T1 versus T2 versus T3	0.51	0.022*	0.289-0.908	0.79	0.823	0.100-6.236
TNM stage						
Stage I versus Stage II versus Stage III	0.49	0.027*	0.262-0.920	0.78	0.832	0.081-7.558

*$p < 0.05$.

Table 3. Univariate and Multivariate Analyses of the Clinicopathological Features and CTSL2 in relation to the OS of HCC Patients (n = 90)

	Univariate analysis			Multivariate analysis		
	HR	p > |z|	95% CI	HR	p > |z|	95% CI
CTSL2 expression						
High versus Low	4.04	0.001*	1.744-9.373	3.10	0.025*	1.151-8.361
Gender						
Male versus Female	1.92	0.371	0.459-8.052			
Age (years)						
≥ 60 versus < 60	1.30	0.508	0.600-2.807			
Tumour size (cm)						
> 5 versus ≤ 5	1.98	0.056	0.983-3.999			
Tumor encapsulation						
None versus Complete	2.00	0.066	0.956-4.181			
Tumor number						
Multiple versus Solitary	1.44	0.354	0.667-3.105			
Hepatitis B virus infection						
Yes versus No	0.97	0.936	0.412-2.263			
Liver cirrhosis						

Yes versus No	1.76	0.439	0.420-7.388			
Pathological grade						
Grade 1 and 2 versus Grade 3	0.33	0.006*	0.151-0.723	0.48	0.083	0.213-1.100
Vascular invasion						
Present versus Absent	1.71	0.181	0.778-3.779			
T						
T1 versus T2 versus T3	0.53	0.018*	0.310-0.897	0.80	0.831	0.107-6.048
TNM stage						
Stage I versus Stage II versus Stage III	0.52	0.022*	0.293-0.910	0.82	0.859	0.091-7.365

*$p < 0.05$.

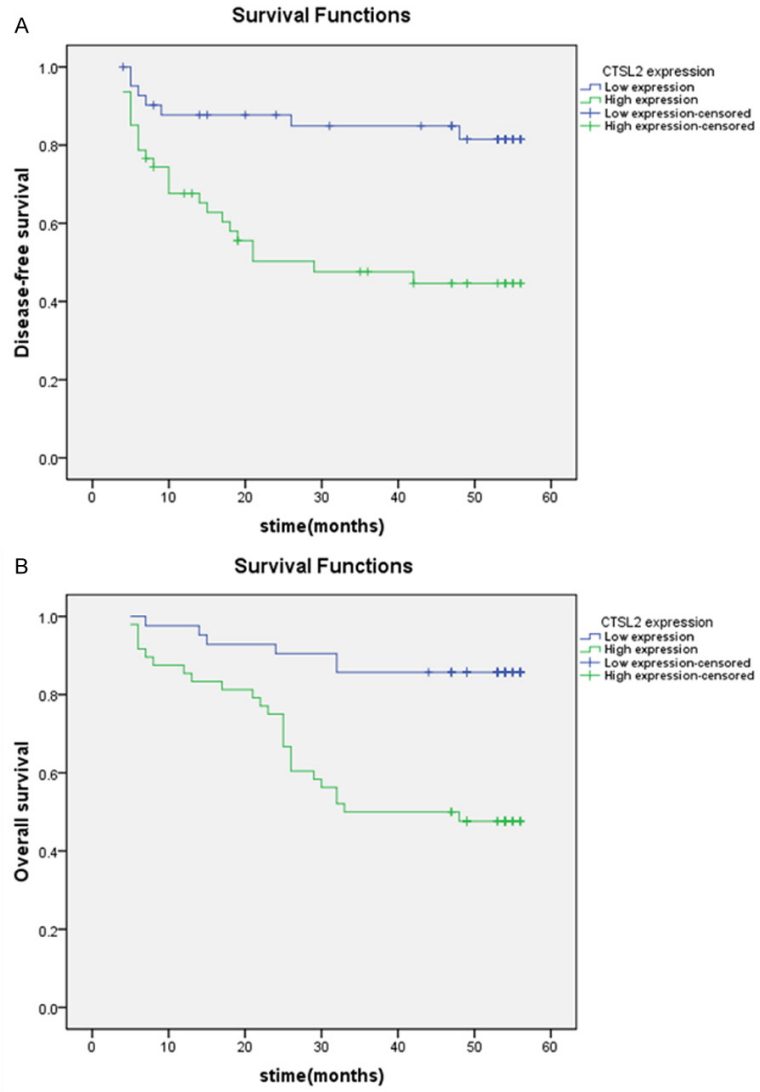

Figure 3. A Kaplan-Meier analysis revealed significant differences in OS and DFS rates between the high CTSL2 expression group and the low CTSL2 expression group in this HCC cohort (log-rank test). High CTSL2 expression was associated with an adverse outcome in HCC patients. The DFS rate was significantly lower in patients with high CTSL2 expression than in patients without high CTSL2 expression (A). The OS rate was significantly lower in patients with high CTSL2 expression than in patients without high CTSL2 expression (B).

tumor number, HBV infection, and liver cirrhosis (**Table 1**).

Univariate and multivariate analysis of prognostic variables in HCC

A univariate analysis showed that several factors were associated with both DFS and OS of HCC patients including CTSL2 expression, pathological grade, T, and TNM stage. In addition, tumor size also affected disease-free survival, but not overall survival (**Tables 2** and **3**). A multivariate analysis using the Cox regression model showed that CTSL2 expression was an independent prognostic factor for DFS and OS (**Tables 2** and **3**).

The relationship between CTSL2 expression and survival

A Kaplan-Meier survival analysis was performed to determine the prognostic value of CTSL2 in HCC. High CTSL2 expression was associated with an adverse prognosis and a shorter DFS (**Figure 3A**). In addition, CTSL2 expression was associated with a lower OS rate in HCC patients (**Figure 3B**).

Discussion

Cathepsins were first discovered in 1955 [31] and were

first cloned in 1998 [18]. At present, there are 11 known human cathepsins in this protein family [32]. CTSL2 (cathepsin L2), which is encoded by *CTSL2*, is 78% homologous with human *CTSL* [19] and shares a 60% homology with CTSK, CTSL, and CTSS [33]. The expression and role of the cathepsin family in human cancers has an important clinical significance. Studies have shown that in cancer tissue, CTSs play a role in tissue remodeling, cell proliferation, angiogenesis, cancer progression, and metastasis [15]. An immunofluorescence assay showed that CTSL2 is present in lysosomes and in the Golgi apparatus [34], and its intracellular activity is believed to play a role in cancer progression [35]. CTSL2, along with other CTSs, degrades the extracellular matrix during tumor progression [36]. Studies have shown that CTSL2 expression is increased in endometrial cancer and that its expression is highly positively correlated with the expression of Ki-67 (which regulates cell growth), cyclin B1, MYB proto-oncogene like 2 (MYBL2), p21/WAF, and human epidermal growth factor receptor 2 (HER2) tyrosine kinase [22]. In addition, CTSL2 elicits cell-type specific responses. Joie et al. [37] showed that CTSL2 silencing elicits specific responses in different types of tumor cells. The results showed that CTSL2, if inhibited on the cancer microarray, inhibited the growth of MCF7 cells but stimulated the growth of SKBR-3 cells. This unique effect may be mediated by cytosolic CTSs, which play a role in the initiation of apoptosis [38]. Moreover, CTSL2 is a direct target of E2F transcription factor 1 (E2F1) and induces E2F1-dependent apoptosis. It is also an important molecular determinant of cell death during cancer therapy [39]. CTSL2 has been shown to be a novel and useful human cancer biomarker. However, its relationship with the clinicopathological features of HCC, especially its prognostic role in HCC, has not been studied. More research is therefore needed to investigate the potential of CTSL2 as a candidate for targeted HCC therapy.

This study demonstrated for the first time that CTSL2 expression was an independent predictor of HCC. We first performed qRT-PCR to analyze the mRNA level of CTSL2 in fresh HCC tissues and matching non-tumor tissues. The results showed that the level of CTSL2 mRNA was significantly higher in HCC tissue samples than in non-tumor tissue samples. Moreover, we constructed an HCC TMA and performed IHC to further confirm that CTSL2 protein expression was significantly higher in HCC than in non-tumor tissues. Skrzypczak et al. [22] showed that the level of CTSL2 mRNA is increased in endometrial cancer. These results are consistent with and support the findings of this study. Furthermore, this study showed that some clinical features including Tumor number, Pathological grade, Vascular invasion, T, and TNM stage are related to CTSL2 protein expression.

For the survival analysis, Cox proportional hazards regression models, in which the effect of covariates is determined by multiplying the hazard function by a function of the explanatory covariates, are widely applied in the analysis of time-to-event data, with censoring and covariates [40]. In this study, we first performed a univariate analysis to determine potential important factors for the prognosis of HCC patients; we then performed a multivariate analysis to determine the reliability and accuracy of the prognostic factors detected in the univariate analysis. Finally, we screened a valid prognostic factor (CTSL2 expression for DFS and OS). The results suggested that high CTSL2 expression was associated with an adverse prognosis and shortened DFS and OS in HCC patients. These results were consistent with the findings of previous studies on breast cancer [20]. A Kaplan-Meier analysis also showed that high CTSL2 expression significantly shortened the OS and DFS of HCC patients.

To date, no adequate or thorough research has been conducted to investigate the role of CTSL2 in cancer. High CTS activity and anomalous CTS localization largely promote cancer progression, proliferation, and invasion. Growing numbers of studies on the CTS family have shown that different members of these proteins play key roles in the development of cancer and that CTS is considered a highly relevant clinical target [41, 42]. In preclinical models, inhibitors of CTS reduced tumor burden and suppressed tumor invasion, and researchers are currently developing CTS inhibitors for cancer clinical trials [41-43].

This study has some limitations. For example, we did not collect data on TNM Stage IV or lymph node metastasis of HCC patients, which may cause certain bias. For future research, we

will further improve the study design in by improving the clinical data collection.

In short, this study concluded for the first time that CTSL2 may be a prognostic factor for HCC, which may provide a promising therapeutic strategy for HCC. A larger number of clinical HCC samples are needed to validate these results and to investigate the potential mechanisms of CTSL2 in HCC.

Disclosure of conflict of interest

None.

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