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Original Article

Expression of cathepsin B in human hepatocellular carcinoma and its prognostic significance

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Abstract: The objective of this study was to analyze the expression of cathepsin B (CTSB) and its clinicopathological significance in patients with hepatocellular carcinoma (HCC). The expression of CTSB mRNA was analyzed in Oncomine database. The expression of CTSB protein in 90 specimens of HCC and paired non-tumor tissue microarray (TMA) was determined by immunohistochemistry. Associations of CTSB expression with the clinicopathological features were analyzed, and prognosis of HCC patients was evaluated. Unlike the expression pattern in most other cancers, the status of CTSB mRNA and protein in HCC tissues is much lower than that in paracarcinoma tissues or normal liver tissues. In our research, we used IHC analysis to reveal low expression levels of CTSB in 55 of 90 (61.11%) hepatic carcinoma and 31 of 90 (34.4%) paracarcinoma specimens. Down-expression of CTSB was significantly associated with survival ($P = 0.001$) and tumor grade ($P = 0.007$). In addition, Multivariate analysis also suggested that CTSB expression was an independent prognostic marker for survival in patients with HCC (HR 0.514, 95% CI 0.281-0.942; $P = 0.031$). To sum up, the down-expression of CTSB may be an independent prognostic factor for HCC patients.

Keywords: Hepatocellular carcinoma, cathepsin B, prognostic marker

Introduction

Hepatocellular carcinoma (HCC) is a major health problem in that it is the fifth most common cancer in the world and the third most frequent cause of cancer-related deaths [1]. Moreover, it is also considered as a common malignant disease. At present time, patients with advanced HCC continue to show a poor outcome, despite recent progress in anticancer therapy [2-4]. Though the way of diagnosis and treatment have advanced quickly, the disease remains a major health concern due to the infiltrative nature of these tumors, their resistance to chemotherapy, their high rate of recurrence, and our limited understanding of the mechanisms underlying initiation and progression of the disease [5]. Consequently, this research tempts to find a new biomarker for diagnosing HCC.

Lysosomes and cathepsins play a major role in tumor cell death. Cathepsin B is a cysteine protease primarily involved in the degradation or processing of lysosomal proteins [6-8]. It participates in innumerous physiological and pathological processes. It degrades structural proteins and enzymes in the cell, degrades the main elements of the basement membrane, and hormones [9-11]. CTSB was observed to activate the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway to promote HCC proliferation [12]. Over expression of cathepsin B has been observed in malignant tumors, and specifically in the cells at the invasive edge of these tumors [13]. It also was found overexpression in brain tumors, especially high-grade gliomas [14, 15].

Patients and methods

Patients

The ethics committee at the First Hospital of Guangzhou Medical College approved this study, and all the patients gave written informed

Table 1. Correlation of CTSB expression with clinicopathologic features

Characteristics	Total (n = 90)	CTSB		χ^2 value	*P* value
		Negative (n = 55)	Positive (n = 35)		
Gender				3.247	0.072
Female	9 (10%)	3 (33.3%)	6 (66.7%)		
Male	81 (90%)	52 (64.2%)	29 (35.8%)		
Survival				10.341	0.001
Survival	33 (36.7%)	13 (39.4%)	20 (60.6%)		
Death	57 (63.3%)	42 (73.7%)	15 (26.3%)		
Age (years)				0.664	0.415
<60	66 (73.3%)	42 (63.6%)	24 (36.4%)		
≥60	24 (26.7%)	13 (54.2%)	11 (45.8%)		
HepB				0.003	0.958
Negative	85 (94.4%)	52 (61.2%)	33 (38.8%)		
Positive	5 (5.6%)	3 (60%)	2 (40%)		
Grade				10.066	0.007
1	3 (3.3%)	3 (100%)	0 (0%)		
2	54 (60%)	26 (48.1%)	28 (51.9%)		
3	33 (36.7%)	26 (78.8%)	7 (21.2%)		
Tumor size				0.894	0.344
Small	51 (56.7%)	29 (56.9%)	22 (43.1%)		
Large	39 (43.3%)	26 (66.7%)	13 (33.3%)		
T stage				4.942	0.176
1	11 (12.2%)	4 (36.4%)	7 (63.6%)		
2	29 (32.2%)	16 (55.2%)	13 (44.8%)		
3	47 (52.2%)	33 (70.2%)	14 (29.8%)		
4	3 (3.3%)	2 (66.7%)	1 (33.3%)		
N stage				0.644	0.422
0	89 (98.9%)	54 (60.7%)	35 (39.3%)		
1	1 (1.1%)	1 (100%)	0 (0%)		
M stage				0.644	0.422
0	89 (98.9%)	54 (60.7%)	35 (39.3%)		
1	1 (1.1%)	1 (100%)	0 (0%)		
AJCC stage				5.716	0.126
I	11 (12.2%)	4 (36.4%)	7 (63.6%)		
II	29 (32.2%)	16 (55.2%)	13 (44.8%)		
III	48 (53.3%)	33 (68.8%)	15 (31.3%)		
IV	2 (2.2%)	2 (100%)	0 (0%)		

HBV, hepatitis B virus; AJCC, American Joint Committee on Cancer.

consent on the use of clinical specimens for medical research. 90 paraffin-embedded specimens of HCC and paracarcinoma were collected respectively from October 2007 to September 2013 for immunohistochemical (IHC) assay. The demographic features and clinicopathologic data were detailed in **Table 1**. All the tissues were collected from 90 patients, including 81 males and 9 females; the median age of patients was 52 years (range 25-73 years); until September 2013, 33 (36.7%) patients were still surviving. The follow-up time of the hepatic cancer cohort ranged from 4 to 78 months, and the median follow-up time was 33 months. Both tumor and adjacent non-tumor tissue (the adjacent non-tumor tissue was near the tumor) were collected after operation.

Tissue microarray (TMA) construction

Formalin-fixed, paraffin-embedded specimens were used to construct tissue microarray slides (Shanghai Biochip Company, Ltd., Shanghai, China). A representative tissue area was first selected on an H&E-stained slide for preparation of the TMA sections. Then the selected area was punched out using a biopsy needle, and a 3-mm tissue core was transferred to a recipient block. Two tissue cores of hepatic tumor and an additional normal liver tissue core were deposited in a paraffin block using a semi-automated tissue arrayer.

Immunohistochemistry

Immunohistochemical (IHC) analysis was performed to study altered protein expression in 90 human hepatic cancer tissues. Firstly, 4-μm-thick paraffin sections were baked for 1 h at 65°C, deparaffinized with xylenes and rehydrated through graded ethanol series to distilled water. Next, these sections were submerged in sodium citrate buffer and heated for antigenic retrieval. Then the sections were blocked by the endogenous peroxidase with 0.3% H_2O_2 for 15 min at ambient temperature. After this, normal goat serum was used as a negative control and all sections were incubated with rabbit polyclonal

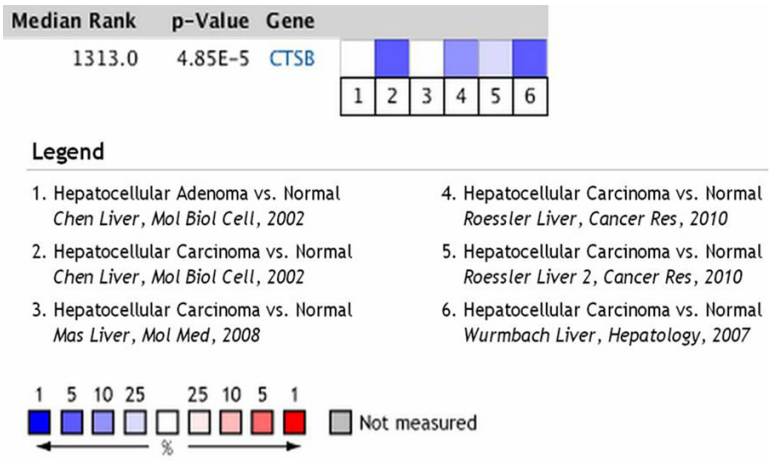

Figure 1. *CTSB* expression is up-regulated in human hepatocellular carcinomas in Oncomine database. Oncomine heat map of *CTSB* gene expression in clinical hepatocellular carcinoma samples compared with the normal liver tissues (www.oncomine.org). In the meta-analysis, *CTSB* expression is significantly lover than the corresponding normal tissues in hepatocellular carcinoma with a median rank of 1313.0 and a *p* value of 4.85E-5.

anti-cathepsin B antibody (1:100) overnight at 4°C. After washing through sterile phosphate-buffered saline, the sections were incubated with a biotinylated anti-rabbit secondary antibody followed by further incubation with streptavidin-horseradish peroxidase at 37°C for 30 min. Finally, diaminobenzidine (DAB) was used for color reaction which is conductive to microscopic observation.

After fixing by formalin, paraffin-embedded sections were reviewed and evaluated separately by two independent observers. The scores given by the two independent investigators were averaged and based on both the proportion of positively stained tumor cells and the intensity of staining. The percentage of stained cells was evaluated as follows: 1 (<25% positive tumor cells), 2 (25-50% positive tumor cells), 3 (50-75% positive tumor cells), and 4 (>75% positive tumor cells). The intensity of staining was graded according to the following criteria: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). By multiplication of both values, a final score ranging from 0 to 12 was obtained. CTSB over-expression was defined as final score more than zero.

Statistical analysis

Cathepsin B positive rates in tumor and matched adjacent non-tumor tissues were compared through chi-square test. Clinicopathologic features (**Table 1**) in Cathepsin B-positive patients and cathepsin B-negative patients were compared using the chi-square tests for categorical variables, and the Student's t-test for continuous data. Overall survival (OS) is a common term used to describe the chances of survival and cure in cancer Patients who were alive and OS were censored at the date of last follow-up. Kaplan-Meier method was used to analyze OS of patients, and comparisons were analyzed by log-rank test. Cox's proportional hazards model was used for multivariate analysis. Then, adjusted hazard ratios (HRs) and their 95% confidence intervals (CIs) were calculated. All the statistical analyses were evaluated using the SPSS18.0 for Windows (SPSS Inc. Chicago, IL, USA). All statistical tests were two-sided, and a *p* value of less than 0.05 was considered statistically significant.

Results

CTSB is down-regulated in HCC patients

In order to determine whether the CTSB expression levels were differentially between human liver carcinoma and normal liver samples, we firstly analyzed the *CTSB* mRNA expression pattern in Oncomine database (www.oncomine.org), in the meta-analysis, *CTSB* expression significantly lower than the corresponding normal tissues in HCC with a median rank of 1313.0 and a *p* value of 4.85E-5 (**Figure 1**). To confirm this result, we obtained paired HCC tissues and adjacent normal liver tissues for Immunohistochemical staining analysis. The typical image result as shown in **Figure 2**, the expression level of CTSB is significantly higher in HCC tissues compared to the adjacent non-tumor tissues. Furthermore, the Immunohistochemical staining of 90 HCC samples results showed that the negative rate of CTSB was 61.1% (55/90) in tumor tissues but 34.4% (31/90) in adjacent non-tumor tissues. CTSB was predominantly present in the cytoplasm of

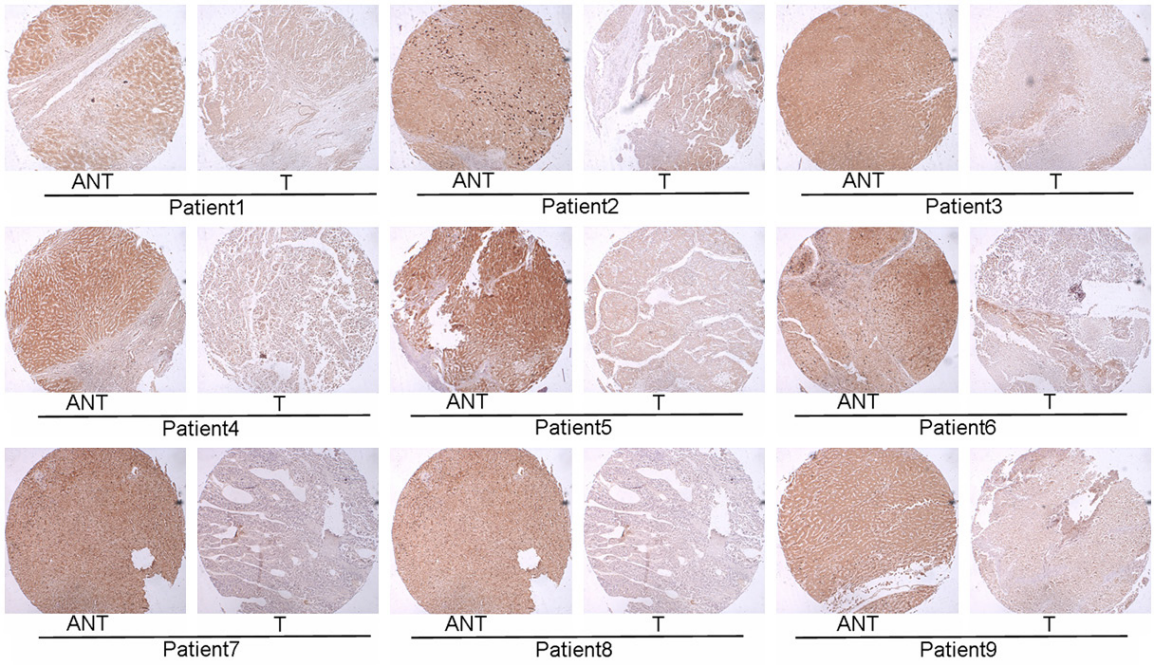

Figure 2. Immunohistochemical assay of *CTSB* protein expression in pairs of matched hepatocellular carcinoma tissues. T, gastric carcinoma tissues, ANT, matched adjacent non-tumor gastric tissues (×200).

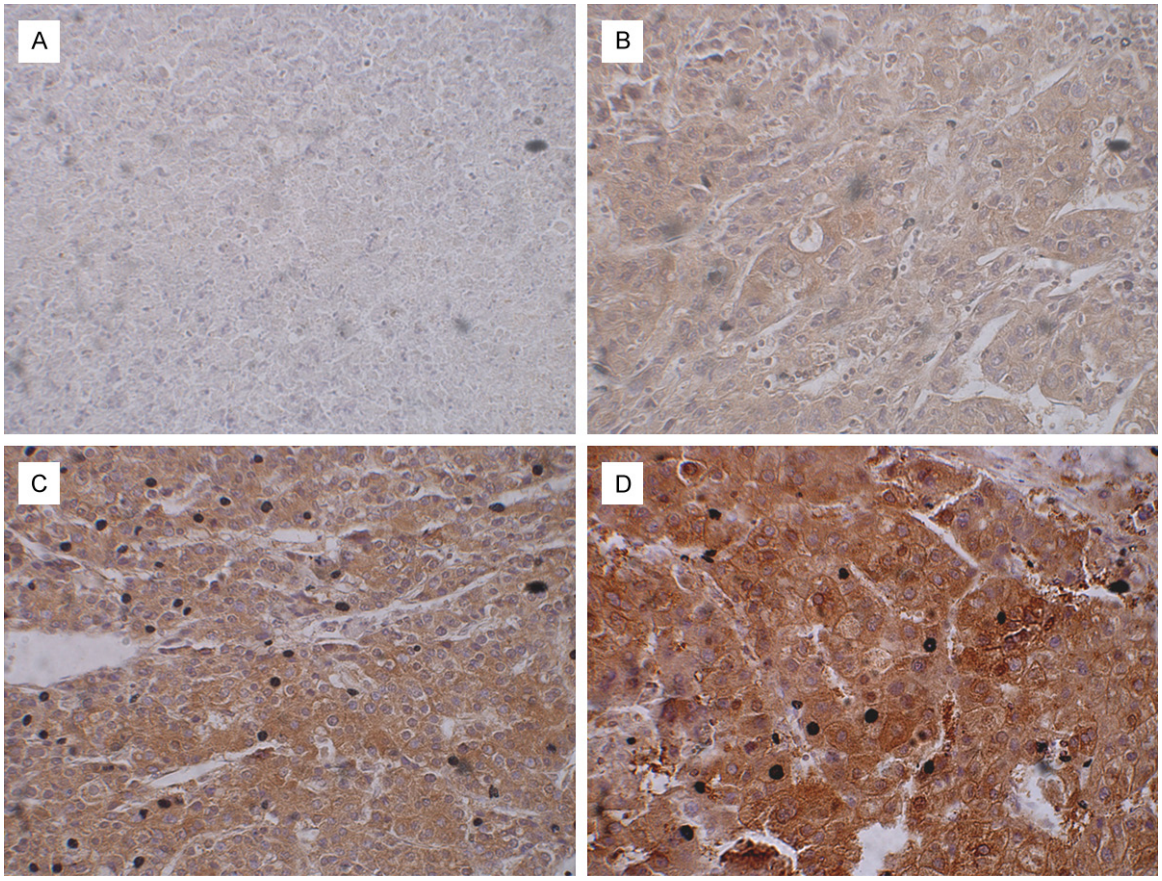

Figure 3. Expression of CTSB protein by immunohistochemistry. CTSB expression was mainly localized in the cytoplasm of tumor cells. Negative expression of CTSB (A), low (B), medium (C) and high (D) expression of CTSB in HCC tissues (×400).

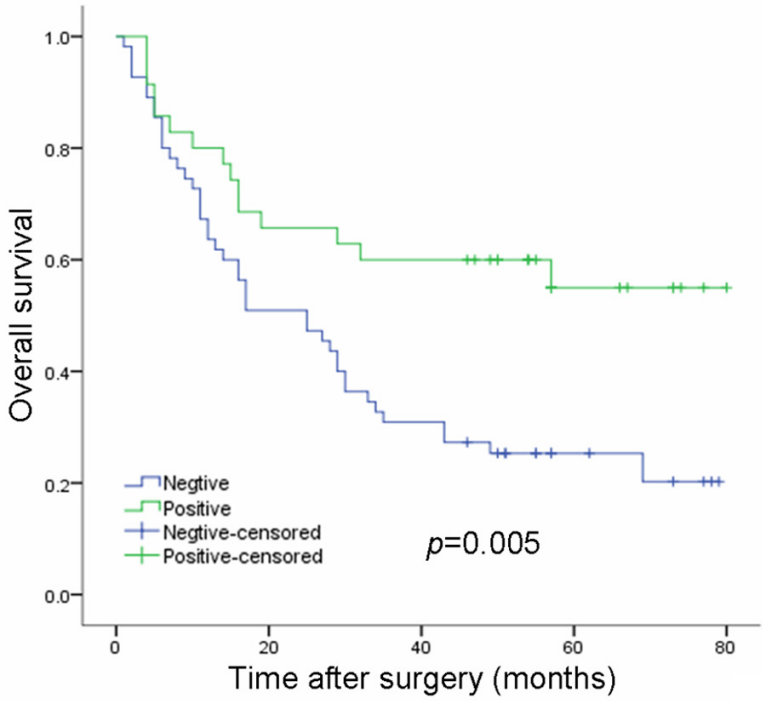

Figure 4. Correlation of CTSB expression with patient overall survival in hepatocellular carcinoma. Lover CTSB expression was correlated with unfavorable prognosis (P = 0.005).

tumor cells; CTSB protein staining was weak in the HCC cells than the non-cancerous adjacent tissues (**Figures 2**, **3**). These results showed that CTSB was decreased in HCC tumor tissues.

CTSB correlates with clinicopathological features of HCC

For a better understanding of the potential roles of CTSB playing in HCC development and progression, patients were grouped according to CTSB expression. The results showed CTSB expression was significantly associated with survival situation (P = 0.001) and tumor grade (P = 0.007). However, there was no correlation of CTSB expression with other clinical features, such as age, gender, and hepatitis (**Table 1**).

Lower-expression of CTSB in HCC patients correlates with worse overall survival

In this study, ninety HCC patients were followed up for 5 years after surgery. These patients were divided into two groups according to the expression of CTSB, which included the positive group (n = 35) and the negative group (n = 55). The patients with positive CTSB expression were more likely to have better OS according to the overall survival analysis (P = 0.005) (**Figure 4**). In multivariate analysis (**Table 2**), factors associated with worse OS were T stage 3 (HR 3.385; 95% CI 1.179-9.715; P = 0.023), T stage 4 (HR 9.002; 95% CI 1.94-41.772; P = 0.005), M stage1 (HR 18.915; 95% CI 1.947-183.796; P = 0.011), and CTSB expression (HR 0.514, 95% CI (0.281-0.942); P = 0.031). These results indicated that CTSB expression may be an independent prognostic marker for HCC patients.

Discussion

Hepatocellular carcinoma (HCC) is a major health problem in that it is the fifth most common cancer in the world and the third most frequent cause of cancer-related deaths [1]. On the one hand, it represents the major histological subtype and it is one of the most common malignant human tumors. On the other hand, HCC has been reported to show a more aggressive behavior, compared with conventional HCC without stemness-related marker expression. Therefore, it is important to develop a suitable marker in order to facilitate its diagnosis [16-18].

As is known, cathepsin B belongs to a class of cysteine that participates in numerous physiological and pathological processes. It degrades structural proteins and enzymes in the cell, degrades the main elements of the basement membrane, and activates proenzymes, hormones, and growth factors involved in the induction phase and the executive apoptosis proteinases [9-11]. Besides, overexpression of cathepsin B was found in brain tumors, especially high-grade gliomas, and it was also correlated with invasive cancer phenotype and poor prognosis due to its contribution to the change of the extracellular matrix [14].

Based on the previous studies, we investigated the expression of CTSB in HCC and its prognos-

Table 2. Univariate and multivariate analysis of the effect of covariates on OS for all patients

Factor	Univariate		Multivariate	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender				
Male	Reference			
Female	0.798 (0.318-1.999)	0.630	-	-
Age				
<60	Reference			
≥60	1.534 (0.825-2.852)	0.176	-	-
HepB				
Negative	Reference			
Positive	0.358 (0.141-0.907)	0.030	-	-
Grade				
1	Reference			
2	0.792 (0.186-3.363)	0.752	-	-
3	0.756 (0.442-1.292)	0.306	-	-
Tumor size				
Small	Reference			
Large	0.484 (0.286-0.821)	0.007	-	-
T stage				
1	Reference			
2	0.115 (0.025-0.529)	0.005	1.397 (0.453-4.304)	0.560
3	0.174 (0.048-0.627)	0.007	3.385 (1.179-9.715)	0.023
4	0.467 (0.142-1.536)	0.210	9.002 (1.94-41.772)	0.005
N stage				
0	Reference			
1	0.357 (0.049-2.629)	0.312	-	-
M stage				
0	Reference			
1	0.034 (0.004-0.327)	0.003	18.915 (1.947-183.796)	0.011
CTSB expression				
Negative	Reference			
Positive	2.275 (1.258-4.114)	0.007	0.514 (0.281-0.942)	0.031
AJCC stage				
I	Reference			
II	0.070 (0.012-0.399)	0.003	-	-
III	0.106 (0.023-0.490)	0.004	-	-
IV	0.287 (0.067-1.221)	0.091	-	-

HBV, hepatitis B virus; AJCC, American Joint Committee on Cancer.

tic values in predicting tumor recurrence following curative resection in the present study. Our results clearly showed that down-regulated at protein levels in HCC tissues compared with paired normal tissues. Furthermore, IHC analysis showed that the expression of CTSB was negative in 55 of 90 (61.1%) HCC specimens but 31/90 (34.4%) in adjacent non-tumor tissues. The meta-analysis result in oncomine database also confirms this result in mRNA levels. However, some similar research in brain cancer, lung cancer, laryngeal cancer, breast cancer, colon cancer or pituitary adenomas found that the over-expressed CTSB occurred and associated with worse outcome [18-23]. Interestingly, compared with other cancer tissues, the expression pattern of CTSB is different in the hepatic carcinoma. The difference in

the expression of CTSB between tumor and non-tumor tissues indicates that CTSB may be a key regulator in hepatic carcinoma.

In our research, CTSB down-expression in HCC was highly corrected with survival (P = 0.001) and tumor grade (P = 0.007). What can be found is that the prognosis of HCC was significantly associated with HepB (P = 0.030), Tumor size (P = 0.007), T stage (T1: P = 0.005, T2: P = 0.007), M stage (P = 0.003), CTSB expression (P = 0.007), AJCC stage (II: P = 0.003, III: P = 0.004). Therefore, it was found that CTSB down-expression was associated with worse outcome, and it was an independent prognosis factor for HCC patients after curative resection (HR 0.514, 95% CI 0.281-0.942; P = 0.031). These results suggested CTSB could be a new biomarker for HCC and a potential therapeutic target.

In conclusion, CTSB was down-regulated expression in HCC and correlates with HCC patients' survival and tumor grade. Most importantly, patients in the high CTSB expression group had a 60.6% cumulative 6-year survival rate, which was significantly higher than that of patients with low CTSB expression levels (39.4%). This finding indicates the possibility of using low expression levels of CTSB as a predictor for prognosis and survival. It was the first time that we found CTSB is down-expressed in the tumor, which was different from many scientists' result that CTSB was over-expressed in lots of tumor such as brain tumor, breast carcinoma, etc. So, to our knowledge, this is the first report to identify down-expression of CTSB as an independent prognostic factor for HCC patients. Further studies are warranted to reveal the molecular mechanism of CTSB in the development and progression of HCC.

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Disclosure of conflict of interest

None.

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