Am J Cancer Res 2022;12(8):3843-3856
www.ajcr.us /ISSN:2156-6976/ajcr0143929

Original Article

High cathepsin A protein expression predicts poor prognosis and tumor recurrence of hepatocellular carcinoma patients after curative hepatectomy

Laibang Luo[1*], Xuyang Wang[1*], Huaxiang Wang[2,3*], Chengkai Yang[3], Youfu Zhang[1], Xinchang Li[1], Zhidan Xu[1]

[1]Department of Organ Transplantation, Jiangxi Provincial People's Hospital, The First Affiliated Hospital of Nanchang Medical College, Nanchang 330006, Jiangxi, China; [2]Department of Hepatobiliary and Pancreatic Surgery, Taihe Hospital, Affiliated Hospital of Hubei University of Medicine, Shiyan 442000, Hubei, China; [3]The Fuzong Clinical Medical College of Fujian Medical University, Fuzhou 350025, Fujian, China. [*]Equal contributors.

Received May 5, 2022; Accepted July 19, 2022; Epub August 15, 2022; Published August 30, 2022

Abstract: Cathepsin A (CTSA) is overexpressed in various types of cancer and is linked to poor clinical outcomes. However, the clinical application of CTSA in HCC has not been explored. In this study, we examined the protein level of CTSA in the archived HCC samples from 161 patients by Immunohistochemistry (IHC). The high protein level of CTSA was significantly correlated to the poor clinicopathological parameters, such as TNM stage, serum AFP level, tumor differentiation, liver cirrhosis, Child-Pugh class, vascular invasion, tumor encapsulation, tumor recurrence, and patient death. In addition, multivariate Cox regression analysis indicated that high CTSA expression was an independent prognostic factor of OS and RFS. We also analyzed the area under the curve (AUC) of the time-dependent receiver operating characteristic (ROC) of CTSA expression for 1-, 3-, and 5-year OS and RFS prediction. Furthermore, we constructed a nomogram that exhibited excellent prediction performance, which was validated by the calibration curve and decision curve analysis. Together, our study demonstrated that CTSA protein level is strongly associated with poor clinical outcome of HCC patients and may be used as a potential diagnostic and prognostic biomarker in HCC.

Keywords: Hepatocellular carcinoma, cathepsin A, overall survival, recurrence-free survival, nomogram, decision curve analysis

Introduction

Hepatocellular carcinoma (HCC) accounts for more than 85% of primary liver cancer cases, representing a significant challenge to the economy and health worldwide, especially in East Asia and other low-income countries [1-3]. The 5-year overall survival rate of HCC patients for all stages was only 20% according to the data published by the American Cancer Society in 2021 [4]. With the advancement of imaging technology in screening and diagnosis, an increasing number of HCC can be diagnosed and radically resected by surgery in the early stage; however, the recurrence rate within 5 years was more than 70% [5, 6]. The high recurrence and metastasis rate attributes to the poor prognosis of HCC patients [7, 8]. Therefore, identifying recurrence- and prognosis-related molecular markers is critical for selecting patients with recurrence risk, guiding early treatment, and improving the prognosis of HCC patients [9].

Cathepsins, a group of lysosome-encapsulated cellular proteases, are responsible for the maintenance of cellular homeostasis via degrading many intracellular and extracellular substrates [10]. Abnormal cathepsin activity is often found under many disease conditions, such as chronic obstructive pulmonary disease [11], osteoporosis and arthritis [12], cardiovascular disease [13, 14], obesity [15], and neurodegenerative diseases [16]. Notably, many studies have indicated the role of the aberrant expression of cathepsin in driving tumor growth and metastasis [17, 18]. In particular, Cathepsin A (CTSA), a member of the lysosomal serine protease fam-

ily regulating bioactive peptide functions [19], has been shown to promote cancer development by modulating the p38 MAPK signaling pathway in prostate cancer [20]. In addition, CTSA has been found to be highly correlated with the invasion and metastasis of lung adenocarcinoma [21], laryngeal carcinoma [22], and melanocytic tumors [23]. Our previous bioinformatics study has indicated that CTSA functions as a cancer-promoting gene in HCC, and that its high mRNA expression is associated with poor prognosis [24]. However, the clinical application of CTSA has not been explored.

In the present study, we aimed to determine if the protein level of CTSA could be used as a diagnostic and prognostic marker in patients with HCC. We first investigated the correlation between the CTSA protein expression and the relevant clinicopathological parameters in 161 HCC cases. Then, we investigated the predictive power of CTSA protein expression for prognosis and tumor recurrence using multivariate Cox regression analysis and time-dependent ROC analysis. We further constructed two nomograms based on the immunohistochemical semi-quantitative score of CTSA protein expression and other independent clinical predictors to quantitatively predict 1-, 3-, and 5-year overall survival (OS) and recurrence-free survival (RFS) of HCC patients undergone curative hepatectomy.

Materials and methods

Patient samples

We included 161 HCC specimens collected from HCC patients undergone hepatectomy from January 2012 to May 2014 at the 900 Hospital of the Joint Logistics Team in our study. The specimens were stored as paraffin-embedded blocks. The inclusion criteria of patients were: 1) over the age of 18; 2) had only one tumor lesion and no metastasis; 3) Child-Pugh class A or B; 4) undergone open liver resection; 5) did not receive radiotherapy or chemotherapy before hepatectomy; 6) did not receive target therapy and immunotherapy after hepatectomy; 7) only received a single curative resection; 8) the postoperative histopathological features verified as HCC. The exclusion criteria of patients were: 1) younger than 18 years of age; 2) had more than two tumor lesions or any metastasis; 3) received other types of cancer treatment before surgery; 4) died from non-tumor causes within 1 week after hepatectomy; 5) received repeated surgeries; 6) received laparoscopic hepatectomy or other minimally invasive therapeutics; 7) received relevant extra-hepatic resection.

Clinicopathological parameters and follow-up

We collected the complete clinicopathological data of each HCC patient. The basic clinical features included age at the time of surgery, gender, height, weight, serum α-fetoprotein (AFP) level, HBsAg status, survival status, cancer therapy history, history of alcohol consumption and smoking, and Child-Pugh class. The following information was collected at the time of the procedure and histopathological examination: tumor number, tumor lesion size, the subsegment where the lesion was located, Tumor Node Metastasis (TNM) stage, histopathological differentiation, presence or absence of hepatic vascular invasion, capsule of the tumor, hepatic cirrhosis, Edmonson grade, and adjacent hepatic inflammation of tumor. All the clinicopathological data were acquired from the electronic medical record (EMR) system of the hospital. The pathological diagnosis was evaluated by two independent pathologists. Survival information was obtained from clinic follow-up visit, follow-up by telephone, and the Social Security Death Index. We conducted the last follow-up on May 31, 2018. This study was conducted according to the relevant medical ethics regulations and approved by the Human Research Ethics Committee of the 900 Hospital of the Joint Logistics Team (Fuzhou, China). All participants provided written informed consent prior to surgery and collection of the specimens.

Immunohistochemistry (IHC) assay

The 161 formalin-fixed, paraffin-embedded HCC samples were cut into 4-μm sections followed by standard IHC staining. Briefly, the paraffin sections were deparaffinized and rehydrated, and the antigen retrieval was performed in Tris/Ethylenediaminetetraacetic acid (EDTA) (pH 9.0) by boiling for 20 min. The endogenous peroxidases were inhibited by 3% H_2O_2 for 10 min. The sections were incubated with 10% normal goat serum for 30 min to block nonspecific staining and then incubated with rabbit monoclonal antibody against CTSA

(15,020-1-AP; 1:250; Proteintech, Wuhan, Hubei, China) for 60 min at room temperature. To visualize the staining signal, the sections were incubated with the secondary antibody for 30 min at room temperature and stained with 3, 3'-diaminobenzidine and a substrate-chromogen and hematoxylin. As a negative control, the CTSA antibody was replaced with the same volume of PBS solution.

IHC staining assessment and scoring

The staining results were independently evaluated by two experienced pathologists without any knowledge of the patient background. CTSA protein expression level was determined by a semi-quantitative IHC scoring system based on the total combined scores of the percentage and the intensity of cells staining positive. The percentage of positive cells was scored as 0 (no or <10% positive cells), 1 (11-50% positive cells), and 2 (> 50% cells staining positive). The staining intensity was scored as 0 (no staining or weakly staining), 1 (medium staining), and 2 (strong staining). We calculated the total score of each tissue sample and divided these 161 HCC patients into low-CTSA group (total IHC score of 0, 1, and 2) and high-CTSA group (total IHC score of 3 and 4).

Establishment and validation of the predictive nomogram

The multivariate Cox regression analysis was used to investigate the independent prognostic factors of OS and RFS. Then, all independent risk factors were integrated to establish the nomogram to quantitatively predict 1-, 3-, and 5-year OS and RFS in HCC patients undergone curative hepatectomy. In addition, we plotted the calibration curves of different survival times to discriminate the probabilities predicted by the nomogram and the patients' data. Furthermore, the decision curve analysis (DCA) was employed to assess the predictive performance of the nomogram and the net benefit in HCC patients.

Statistical analysis

The software SPSS 21 (SPSS Inc., Chicago, IL, USA) was employed to perform statistical analysis. The nomogram, calibration curves, and DCA were plotted utilizing the R software (version 4.10) with the "car", "rms", and "ggDCA" R package. The association between CTSA expression level and clinicopathological parameters was compared using Pearson's chi-square test. The Kaplan-Meier method with log-rank test was used for the comparison of patient survival. The Cox regression analysis was used to investigate the prognostic factors of OS and RFS. $P<0.05$ was considered statistically significant.

Results

IHC score of the patients

The IHC staining demonstrated that CTSA was predominantly located in the cytoplasm or cytomembrane, which was consistent with previous studies and the data of the Human Protein Atlas database [25, 26]. The expression of CTSA protein in HCC tissues was significantly higher than that in normal liver tissues. **Figure 1** showed the representative IHC staining images of CTSA protein in adjacent normal liver tissue (**Figure 1A**) and in HCC samples with different IHC scores (0 to 4) (**Figure 1B-F**). At the end of the follow-up, among the 161 patients studied, 97 (60.2%) died, and 83 (51.6) had recurrent. The number of patients with IHC staining score of 0, 1, 2, 3, and 4 was 8, 37, 40, 54, and 22, respectively. We found the percentage of death and recurrence incrementally increased with the increasing IHC staining score (**Figure 1G**, **1H**).

Association between the CTSA expression level and the clinicopathological parameters

Based on the semi-quantitative IHC scoring system, 85 of 161 patients exhibited low CTSA expression (IHC score of ≤2), and 76 patients exhibited high expression (IHC score of 3 and 4). High CTSA expression was positively related to the worse TNM stage ($P=0.002$), high serum AFP level ($P<0.001$), low tumor differentiation ($P=0.010$), hepatic cirrhosis ($P<0.001$), Child-Pugh class B ($P=0.024$), more vascular invasion ($P=0.006$), absence of tumor encapsulation ($P=0.030$), high recurrence rate ($P<0.001$), and high recurrence rate ($P=0.002$). However, high CTSA expression was not related to age, gender, tumor size, tumor location, HBsAg status, Edmonson grade, and adjacent hepatic inflammation of tumors (**Table 1**).

The prognostic significance of CTSA protein expression in the HCC cohort

As shown in the Kaplan-Meier curves, patients with high CTSA IHC score had poor OS (P=

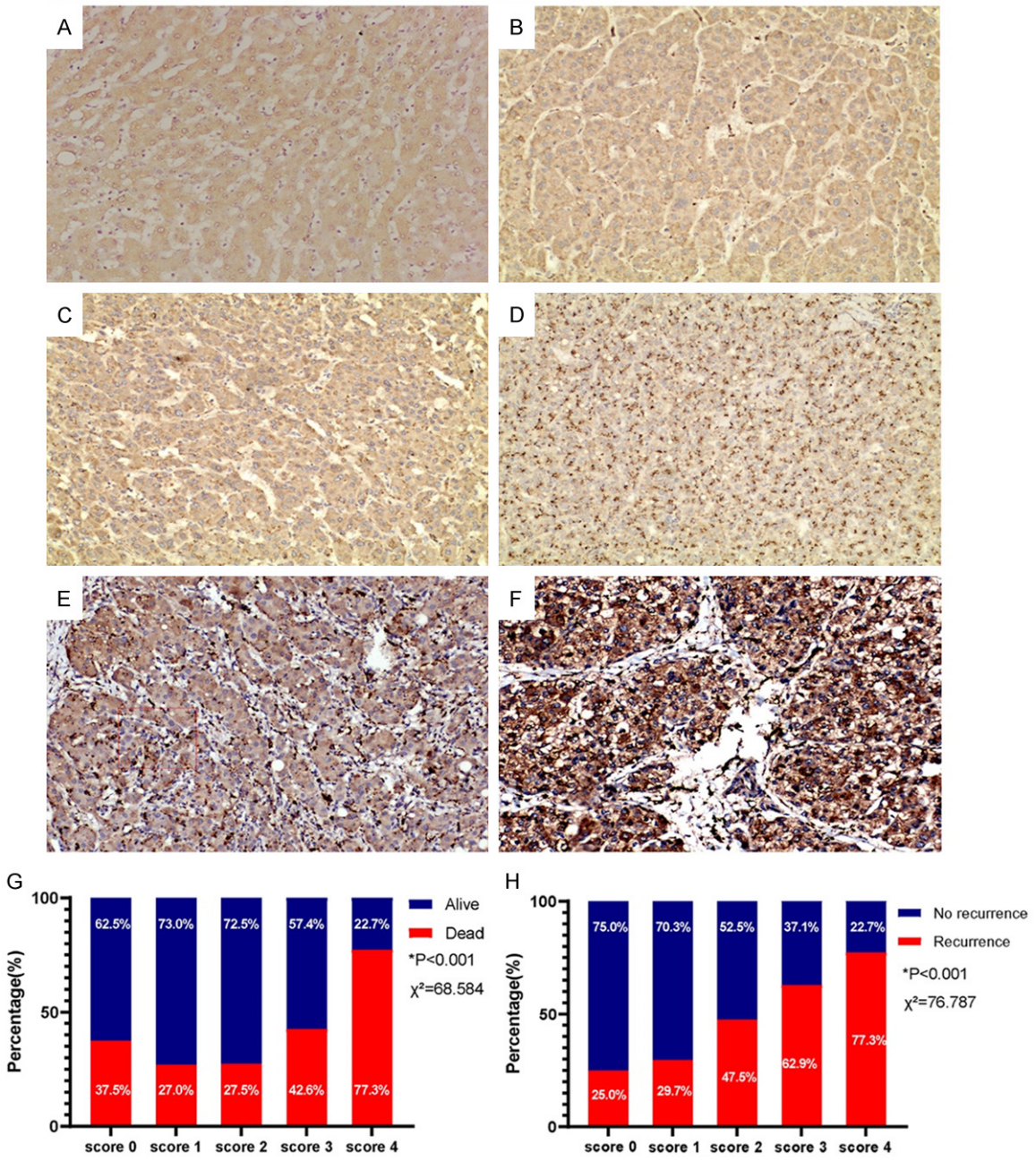

Figure 1. (A) The representative IHC staining images of CTSA protein in adjacent normal liver tissue. (B-F) IHC staining of CTSA in HCC samples with different staining scores (0-4). Representative images of CTSA IHC staining for score of 0 (B), 1 (C), 2 (D), 3 (E), and 4 (F), respectively. Magnification ×200. (G) The percentage of alive and died patients in different CTSA IHC staining score groups. (H) The percentage of recurrence in patients with different CTSA IHC staining score.

0.004) and RFS (P<0.001) than patients with low CTSA IHC score (**Figure 2A**, **2B**). In addition, patients with the IHC score of 4 were predicted to have worse OS than patients of score 3 (P=0.042); however, there was no significant difference in RFS between these two subgroups (**Figure 2C**, **2D**).

Our results from univariate Cox regression analysis indicated that the following features were prognostic factors for OS and/or RFS in the HCC cohort: tumor size (OS: P=0.010, RFS: P=0.012), TNM stage (OS: P=0.002, RFS: P=0.029), serum AFP level (OS: P=0.006, RFS: P=0.039), tumor differentiation (OS: P=0.001,

Table 1. Correlation between CTSA protein expression and clinicopathologic features in 161 patients with hepatocellular carcinoma

Characteristics		N	CTSA level		χ^2	*P-Value
			high (n)	low (n)		
Age (year)	>55	105	52	53	0.651	0.420
	≤55	56	24	32		
Gender	Male	141	65	76	0.557	0.456
	Female	20	11	9		
Tumor size (cm)	>5 cm	86	44	42	1.004	0.316
	≤5 cm	75	32	43		
TNM stage	I/II	101	38	63	9.983	0.002
	III	60	38	22		
Serum AFP level	>400 ng/ml	69	46	23	18.351	<0.001
	≤400 ng/ml	92	30	62		
Tumor location	Left	53	30	23	2.801	0.094
	Right	108	46	62		
Tumor differentiation	Low	20	14	6	9.233	0.010
	Median	106	54	52		
	High	35	8	27		
HBsAg	Positive	74	35	39	0.001	0.983
	Negative	87	41	46		
Liver cirrhosis	Yes	88	58	30	27.245	<0.001
	No	73	18	55		
Edmonson grade	I	28	12	16	0.257	0.612
	II-IV	133	64	69		
Child-Pugh class	A	85	33	52	5.076	0.024
	B	76	43	33		
Vascular invasion	Yes	77	45	32	7.477	0.006
	No	84	31	53		
Adjacent hepatic inflammation	Yes	111	58	53	3.654	0.056
	No	50	18	32		
Tumor encapsulation	Yes	107	44	63	4.737	0.030
	No	54	32	22		
Recurrence	Yes	83	51	32	13.941	<0.001
	No	78	25	53		
Survival status	Alive	64	40	24	9.971	0.002
	Dead	97	36	61		

Abbreviations: CTSA, Cathepsin A; AFP, Alpha fetoprotein; TNM, tumor, node, metastasis. **P*-Value <0.05 were considered statistically significant.

RFS: P<0.001), liver cirrhosis (OS: P=0.039, RFS: P=0.002), Child-Pugh class (OS: P<0.001, RFS: P=0.011), vascular invasion (OS: P< 0.001, RFS: P=0.001), and CTSA protein level (OS: P=0.002, RFS: P<0.001), whereas age, gender, tumor location, HBsAg status, Edmonson grade, adjacent hepatic inflammation of tumor, and tumor encapsulation was not prognostic factors. In addition, the absence of tumor encapsulation was a prognostic factor for RFS (P<0.001) but not for OS (P=0.250) (**Table 2**). The multivariate Cox regression analysis revealed that tumor differentiation (OS: aHR (95% CI) 3.901 (1.176-12.943), P=0.026; RFS: aHR (95% CI) 2.537 (1.109-5.803), P= 0.027), vascular invasion (OS: aHR (95% CI) 2.033 (1.125-3.676), P=0.019; RFS: aHR (95% CI) 2.398 (1.458-3.945), P=0.001), and CTSA protein level (OS: aHR (95% CI) 4.326 (2.416-7.745), P<0.001; RFS: aHR (95% CI) 1.876

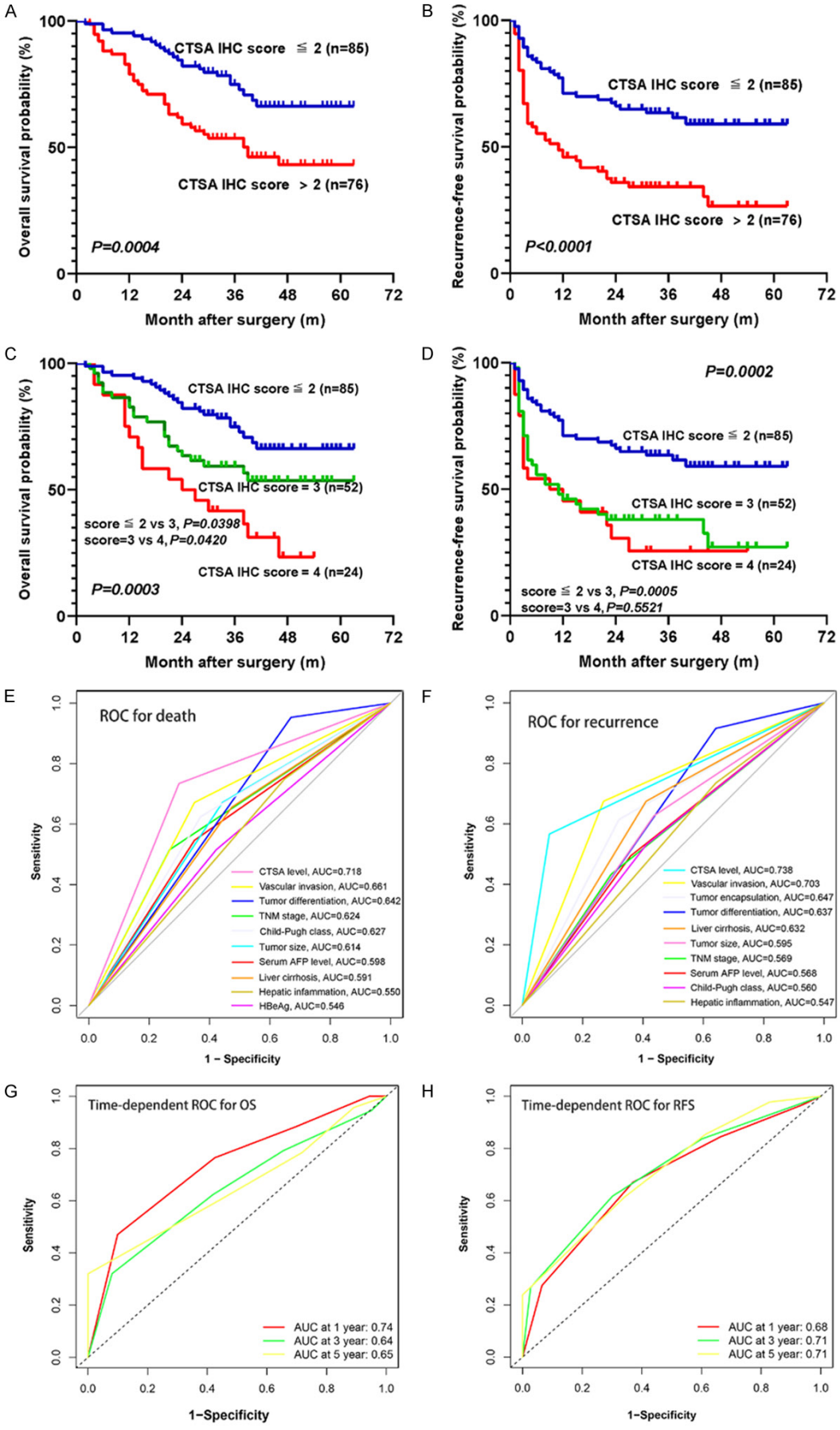
A
Overall survival probability (%)
CTSA IHC score ≦ 2 (n=85)
CTSA IHC score > 2 (n=76)
P=0.0004
Month after surgery (m)
B
Recurrence-free survival probability (%)
CTSA IHC score ≦ 2 (n=85)
CTSA IHC score > 2 (n=76)
P<0.0001
Month after surgery (m)
C
Overall survival probability (%)
CTSA IHC score ≦ 2 (n=85)
CTSA IHC score = 3 (n=52)
score ≦ 2 vs 3, P=0.0398
score=3 vs 4, P=0.0420
CTSA IHC score = 4 (n=24)
P=0.0003
Month after surgery (m)
D
Recurrence-free survival probability (%)
P=0.0002
CTSA IHC score ≦ 2 (n=85)
CTSA IHC score = 3 (n=52)
CTSA IHC score = 4 (n=24)
score ≦ 2 vs 3, P=0.0005
score=3 vs 4, P=0.5521
Month after surgery (m)
E
ROC for death
Sensitivity
1 – Specificity
CTSA level, AUC=0.718
Vascular invasion, AUC=0.661
Tumor differentiation, AUC=0.642
TNM stage, AUC=0.624
Child-Pugh class, AUC=0.627
Tumor size, AUC=0.614
Serum AFP level, AUC=0.598
Liver cirrhosis, AUC=0.591
Hepatic infammation, AUC=0.550
HBeAg, AUC=0.546
F
ROC for recurrence
Sensitivity
1 – Specificity
CTSA level, AUC=0.738
Vascular invasion, AUC=0.703
Tumor encapsulation, AUC=0.647
Tumor differentiation, AUC=0.637
Liver cirrhosis, AUC=0.632
Tumor size, AUC=0.595
TNM stage, AUC=0.569
Serum AFP level, AUC=0.568
Child-Pugh class, AUC=0.560
Hepatic inflammation, AUC=0.547
G
Time-dependent ROC for OS
Sensitivity
1–Specificity
AUC at 1 year: 0.74
AUC at 3 year: 0.64
AUC at 5 year: 0.65
H
Time-dependent ROC for RFS
Sensitivity
1–Specificity
AUC at 1 year: 0.68
AUC at 3 year: 0.71
AUC at 5 year: 0.71

Figure 2. Prognostic value of CTSA protein expression level in patients with HCC. (A, B) Kaplan-Meier curves of OS (A) and RFS (B) for patients with different CTSA expression levels. (C, D) Kaplan-Meier curves of OS (C) and RFS (D) for patients with the IHC score of 3 and 4. (E, F) The death (E) and recurrence (F) prediction ability of the CTSA protein expression level was compared with other clinical parameters by ROC curves in the HCC cohort. (G, H) The time-dependent ROC curves of the nomogram for predicting the OS (G) and RFS (H) at 1-, 3-, and 5-year.

Table 2. Univariate Cox Regression analysis of overall survival and recurrence-free survival in 161 patients with hepatocellular carcinoma

variables		Overall survival	*P-Value	Recurrence-free survival	*P-Value
		HR (95% CI)		HR (95% CI)	
Age (year)	>55 vs. ≤55	0.889 (0.536-1.476)	0.650	0.704 (0.453-1.095)	0.120
Gender	Male vs. female	0.729 (0.315-1.691)	0.462	1.277 (0.693-2.355)	0.433
Tumor size (cm)	>5 vs. ≤5	1.989 (1.178-3.360)	0.010	1.774 (1.135-2.775)	0.012
TNM stage	I/II vs. III	2.136 (1.308-3.490)	0.002	1.624 (1.050-2.512)	0.029
Serum AFP level	>400 vs ≤400	1.990 (1.215-3.260)	0.006	1.575 (1.023-2.426)	0.039
Tumor location	Left vs. right	0.802 (0.481-1.337)	0.397	1.169 (0.731-1.868)	0.515
Tumor differentiation	Hihg vs. median/low	6.748 (2.117-21.513)	0.001	4.225 (1.945-9.178)	<0.001
HBsAg	Positive vs. negative	1.367 (0.837-2.232)	0.212	0.952(0.617-1.469)	0.826
Liver cirrhosis	Yes vs. no	1.721 (1.027-2.884)	0.039	2.050 (1.294-3.249)	0.002
Edmonson grade	I vs. II-IV	0.652 (0.311-1.368)	0.258	0.581 (0.300-1.127)	0.108
Child-Pugh class	A vs. B	5.028 (2.863-8.827)	<0.001	1.767 (1.142-2.734)	0.011
Adjacent hepatic inflammation	Yes vs. no	1.432 (0.813-2.522)	0.214	1.350 (0.828-2.202)	0.228
Vascular invasion	Yes vs. no	2.758 (1.635-4.654)	<0.001	3.338 (1.298-5.313)	<0.001
Tumor encapsulation	Yes vs. no	0.744 (0.450-1.231)	0.250	0.252 (0.162-0.394)	<0.001
CTSA protein level	High vs. low	2.241 (1.350-3.270)	0.002	2.403 (1.540-3.750)	<0.001

Abbreviations: CTSA, CATHEpsin A; HR, HAzard ratio; CI, CONFIDential interval; AFP, ALPha fetoprotein; TNM, TUMor, node, metastasis. *P-Value <0.05 were considered statistically significant.

Table 3. Multivariate Cox Regression analysis of overall survival and recurrence-free survival in 161 patients with hepatocellular carcinoma

variables		Overall survival	*P-Value	Recurrence-free survival	*P-Value
		aHR (95% CI)		aHR (95% CI)	
Tumor size (cm)	>5 vs. ≤5	0.772 (0.365-1.635)	0.499	1.185 (0.633-2.219)	0.595
TNM staging	I/II vs. III	1.339 (0.665-2.697)	0.413	1.108 (0.602-2.040)	0.741
Serum AFP level	>400 vs ≤400	1.219 (0.709-2.095)	0.474	0.983 (0.611-1.583)	0.944
Tumor differentiation	Hihg vs. median/low	3.901 (1.176-12.943)	0.026	2.537 (1.109-5.803)	0.027
Liver cirrhosis	Yes vs. no	1.110 (0.604-2.040)	0.736	1.136 (0.668-1.934)	0.638
Child-Pugh class	A vs. B	1.299 (0.721-2.340)	0.384	1.267 (0.794-2.024)	0.321
Vascular invasion	Yes vs. no	2.033 (1.125-3.676)	0.019	2.398 (1.458-3.945)	0.001
Tumor encapsulation	Yes vs. no			0.249 (1.154-0.402)	<0.001
CTSA protein level	High vs. low	4.326 (2.416-7.745)	<0.001	1.876 (1.103-3.191)	0.020

Abbreviations: CTSA, Cathepsin A; aHR, Adjusted hazard ratio; CI, Confidential interval; AFP, Alpha fetoprotein; TNM, Tumor, node, metastasis. *P-Value <0.05 were considered statistically significant.

(1.103-3.191), P=0.020) were independent prognostic factors for OS and/or RFS (**Table 3**).

Furthermore, the ROC analysis indicated that the prediction value of the CTSA protein level was 0.718 and 0.738 for death and recurrence, respectively, which was better than other clinicopathological parameters in our cohort (**Figure 2E**, **2F**). Moreover, the time-dependent ROC analysis was employed to further assess the prediction performance of the IHC staining score and found that the AUC for 1-, 3-, and 5-year OS prediction was 0.74, 0.64, and 0.65, respectively (**Figure 2G**), and the AUC for 1-, 3-,

and 5-year RFS predictions was 0.68, 0.71, and 0.71, respectively (**Figure 2H**).

The prognostic significance of the CTSA protein level in the subgroups of HCC patients

We further investigated in detail the prognostic significance of the CTSA protein expression in different subgroups of HCC patients, including early-stage (stage I/II), Child-Pugh class A, low AFP level (≤400 ng/ml), and tumors diameter smaller than 5 cm. Our results demonstrated that both the OS and RFS of patients in high CTSA protein expression subgroups (CTSA IHC score >2) were significantly lower than those in low CTSA protein expression subgroups (CTSA IHC score ≤2), such as stage I/II (OS: P=0.0495; RFS: P=0.0318, **Figure 3A**, **3B**), Child-Pugh class A (OS: P=0.0350; RFS: P= 0.0050, **Figure 3C**, **3D**), serum AFP less than 400 ng/ml subgroups (OS: P=0.0356; RFS: P=0.0104, **Figure 3E**, **3F**). In addition, in the subgroup with tumor diameter smaller than 5cm, the high CTSA protein expression (IHC score of >2) was associated with poor RFS (P=0.0461, **Figure 3H**), but was not correlated with OS (P=0.1568, **Figure 3G**).

Establishment and validation of a predictive nomogram for survival

We constructed nomograms incorporating the CTSA IHC staining score and all the clinical independent prediction factors identified from the multivariate Cox regression analysis for predicting 1-, 3-, and 5-year OS and RFS in the HCC cohort. Specifically, the CTSA IHC staining score, vascular invasion, and tumor differentiation were first integrated to construct the predictive nomograms of OS and RFS (**Figure 4A**). Then, the tumor encapsulation was added to the nomogram for RFS prediction (**Figure 5A**). Each independent prognostic factor was given a score on the points scale. By adding up each score to obtain a total score shown on the bottom scale, the nomogram could predict the 1-, 3-, and 5-year survival probability for individual patient. The calibration curve representing the actual and the combined model-predicted 1-, 3-, and 5-year OS and RFS demonstrated the excellent prediction performance of the nomogram (**Figures 4B-D**, **5B-D**). In addition, the DCA curve exhibited the best net benefit of the combined model of all independent prognostic factors compared with the individual factor both for 1-, 3-, and 5-year OS (**Figure 4E-G**) and RFS (**Figure 5E-G**) prediction.

Discussion

HCC is the second-leading cause of cancer-related mortality and poses an immediate economic and health threat worldwide [27]. The poor prognosis of HCC is attributed to the high incidence of recurrence and metastasis after the curative hepatectomy. However, due to the heterogeneity of HCC, traditional prognostic-related features, such as TNM stage, tumor differentiation, tumor size, and Edmonson grade, are not sufficient to accurately predict the prognosis [28]. Therefore, the identification of recurrence- and prognosis-related molecular markers is imperative for stratifying patients with recurrence risk, guiding early treatment, and improving the prognosis of HCC patients.

Cathepsins, a group of lysosome-encapsulated cellular proteases, are involved in virtually all the lysosome-related biological processes, including cellular autophagy, protein degradation, and cellular stress signaling pathway [10, 29]. Importantly, in recent years, members of cathepsin family have been shown to play an important role in the growth and metastasis of multiple human tumors. For example, Cathepsin B is considered as a sellsword of cancer progression and as a mediator of ferroptosis-related cell death [30, 31]. In addition, Gondi, et al. reported that Cathepsin B was a cancer therapy target in breast tumors, melanoma, esophageal squamous, and HCC [32]. CTSA, a well-known member of the serine protease cathepsin family that functions as a promotor for heart failure after myocardial infarction [33], was reported to play a cancer-promoting role in multiple tumor types, such as prostate cancer, lung adenocarcinoma, colorectal cancer, breast ductal carcinoma, and melanocytic tumors [20, 21, 23, 34, 35]. Our previous bioinformatics study also suggested an oncogenic role of CTSA in HCC, as its high mRNA expression was associated with poor prognosis [24]. In consistent with this, a study using quantitative proteomics demonstrated that CTSA protein was significantly upregulated in HCC tissues compared with the adjacent non-tumor tissues; nevertheless, the prognostic significance and clinical application of CTSA were not addressed [36]. In the present study,

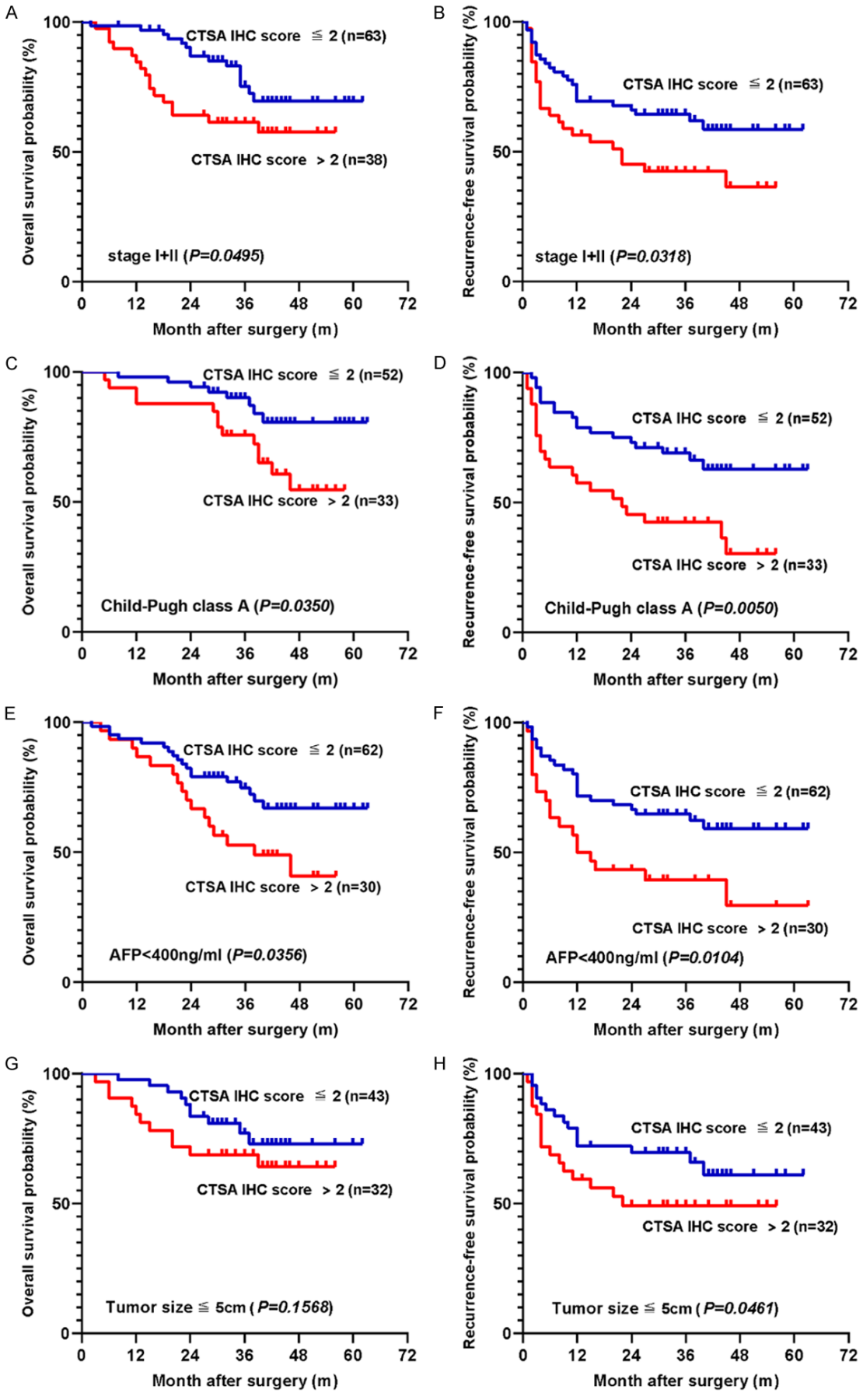
A
Overall survival probability (%)
CTSA IHC score ≦ 2 (n=63)
CTSA IHC score > 2 (n=38)
stage I+II (P=0.0495)
Month after surgery (m)
B
Recurrence-free survival probability (%)
CTSA IHC score ≦ 2 (n=63)
stage I+II (P=0.0318)
Month after surgery (m)
C
Overall survival probability (%)
CTSA IHC score ≦ 2 (n=52)
CTSA IHC score > 2 (n=33)
Child-Pugh class A (P=0.0350)
Month after surgery (m)
D
Recurrence-free survival probability (%)
CTSA IHC score ≦ 2 (n=52)
CTSA IHC score > 2 (n=33)
Child-Pugh class A (P=0.0050)
Month after surgery (m)
E
Overall survival probability (%)
CTSA IHC score ≦ 2 (n=62)
CTSA IHC score > 2 (n=30)
AFP<400ng/ml (P=0.0356)
Month after surgery (m)
F
Recurrence-free survival probability (%)
CTSA IHC score ≦ 2 (n=62)
CTSA IHC score > 2 (n=30)
AFP<400ng/ml (P=0.0104)
Month after surgery (m)
G
Overall survival probability (%)
CTSA IHC score ≦ 2 (n=43)
CTSA IHC score > 2 (n=32)
Tumor size ≦ 5cm (P=0.1568)
Month after surgery (m)
H
Recurrence-free survival probability (%)
CTSA IHC score ≦ 2 (n=43)
CTSA IHC score > 2 (n=32)
Tumor size ≦ 5cm (P=0.0461)
Month after surgery (m)

Figure 3. The prognostic value of the CTSA protein expression in the subgroups of patients with early-stage tumors. Kaplan-Meier analysis of OS and RFS for HCC patients with early-stage disease (A, B), Child-Pugh class-A (C, D), low serum AFP level (E, F), and tumors diameter smaller than 5 cm (G, H).

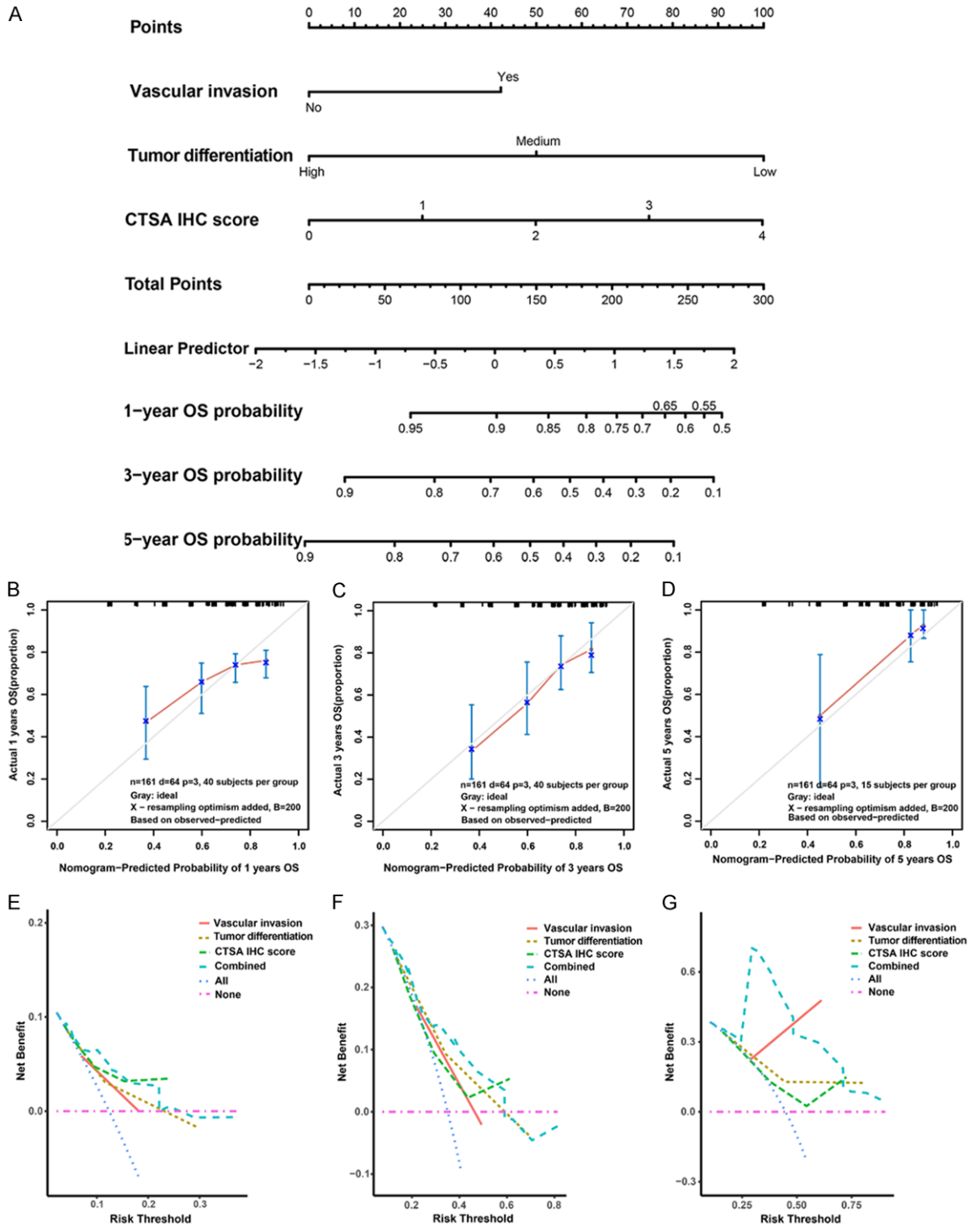

Figure 4. Nomogram, calibration plot, and decision curve analysis (DCA) curves for OS. (A) Nomogram to predict 1-, 3-, and 5-year OS probability. (B-D) The calibration curve for 1- (B), 3- (C), and 5-year OS probability (D) prediction in patients with HCC. (E-G) The net benefit of combined model and individual factors for 1- (E), 3- (F), and 5-year (G) OS probability prediction exhibited in the DCA.

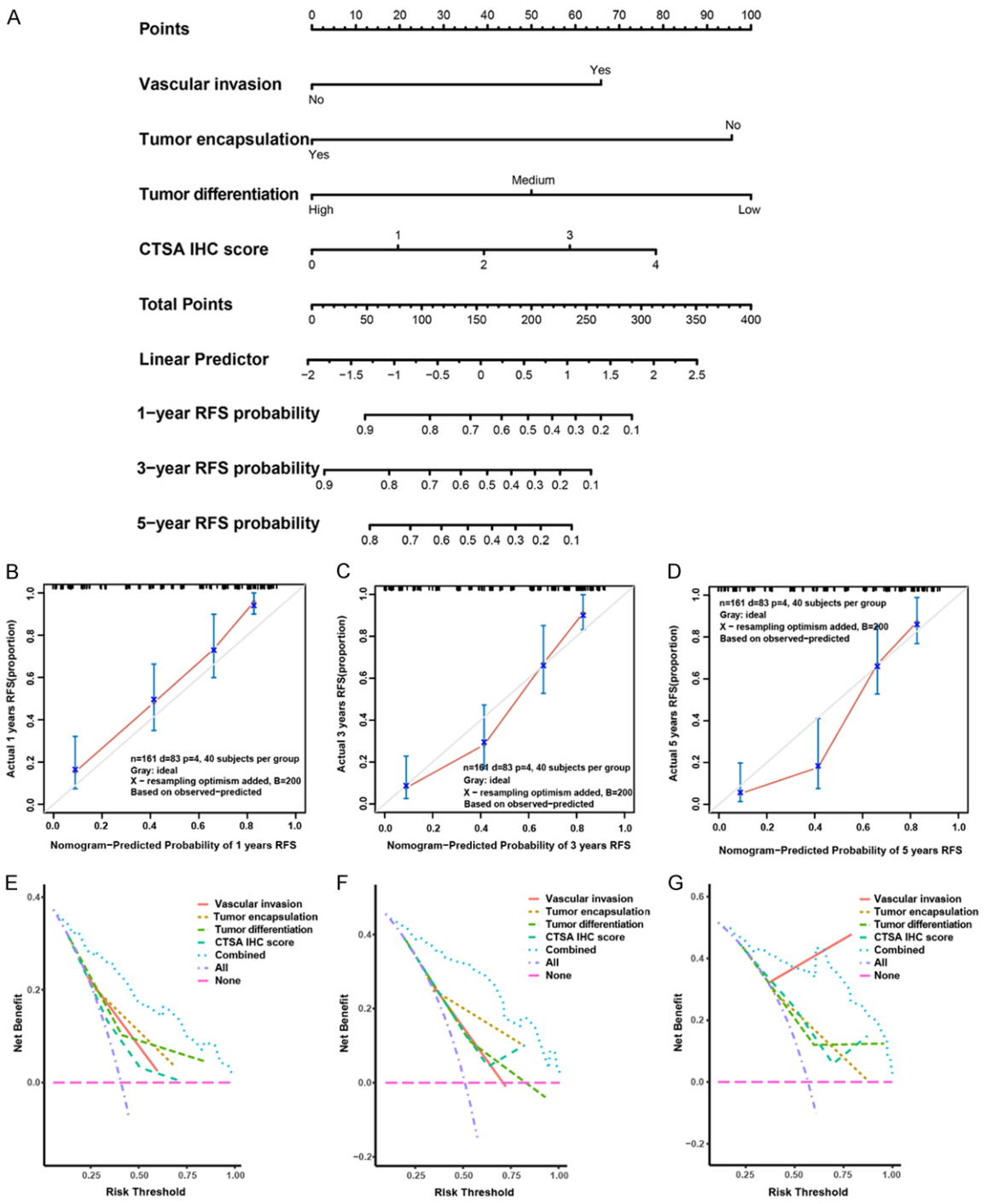

Figure 5. Nomogram, calibration plot, and decision curve analysis (DCA) curves for RFS. (A) Nomogram to predict 1-, 3-, and 5-year RFS probability. (B-D) The calibration curve for 1- (B), 3- (C), and 5-year RFS probability (D) prediction in patients with HCC. (E-G) The net benefit of combined model and individual factors for 1- (E), 3- (F), and 5-year (G) RFS probability prediction exhibited in the DCA.

we aimed to explore if the protein level of CTSA could be used as a diagnostic and prognostic marker in patients with HCC.

By using IHC assay with a semi-quantitative scoring system, we found that a high CTSA IHC staining score could predict the poor clinicopathological outcomes, such as TNM stage, serum AFP level, tumor differentiation, liver cirrhosis, Child-Pugh class, and vascular invasion. In addition, the percentage of death and recurrence incrementally increased with the increas-

ing IHC staining scores. Furthermore, the CTSA protein expression level can also accurately predict the OS and RFS of patients with early stage (stage I/II), Child-Pugh class A, and low AFP level (≤400 ng/ml), indicating its promising application in the prediction of tumor recurrence at early stage. Together, these findings reveal a direct association between the IHC staining score of CTSA and the survival probability of patients. Moreover, the multivariate Cox regression identified high CTSA protein expression as an independent prognostic factor for OS and/or RFS. Finally, the ROC analysis showed that the CTSA protein level was better than other clinicopathological parameters in predicting the death and recurrence. The time-dependent ROC curve exhibited the large AUC for 1-, 3-, and 5-year OS and RFS predictions.

Previous studies have reported that the suppression of CTSA inhibits the growth and invasion of prostate cancer cells by inhibiting the p38 MAPK signaling pathway [20]. In addition, Cathepsin C (CTSC) was reported to promote the proliferation and metastasis of HCC via promoting TNF-α/p38 MAPK Signaling Pathway [37]. Thus, it is reasonable to speculate that high CTSA expression promotes the progression and attributes to the poor prognosis of HCC through enhancing the oncogenic pathways such as p38 MAPK pathway. However, further experiments need to be carried out to prove this hypothesis.

In this study, we also established nomograms by incorporating the CTSA IHC staining score and all the clinical independent prediction factors from our HCC cohort. To the best of our knowledge, this is the first study to establish nomograms based on the CTSA protein expression and other clinicopathologic parameters. The calibration curve exhibited excellent consistency between the actual observations and the predictive probabilities, indicating the reproducibility and reliability of the nomograms. The application of nomograms and the DCA curve enables the early stratification of patients with high risk for recurrence and poor survival and provides the rationale for the efficient clinical decision-making.

This present study had some limitations. First, this was a retrospective and single-center study; only the patients who had undergone curative hepatectomy were included, which might result in the selection bias. Therefore, a prospective study with a larger sample size is required to validate these results. Second, this study only included patients with one tumor lesion and without metastasis, which is not applicable for patients with multiple liver tumors and/or any metastasis. Last, the underlying mechanism of CTSA regulating the tumorigenesis of HCC was not investigated in this study, which should be carried out in the future.

Conclusions

We found high CTSA IHC protein expression level predicted the poor clinicopathological outcomes. In addition, high CTSA protein expression was an independent prognostic factor for OS and/or RFS. Furthermore, we have constructed nomograms incorporating the CTSA IHC staining score and all the clinical independent prediction factors from our HCC cohort, which provides a potential tool for the prediction of recurrence and poor survival of HCC.

Acknowledgements

We would like to acknowledge all the people who have given us help with our article. This work was supported by Jiangxi Province Traditional Chinese Medicine Research Project (No. 2019A199).

Disclosure of conflict of interest

None.

Abbreviations

HCC, Hepatocellular carcinoma; CTSA, Cathepsin A; IHC, Immunohistochemistry; OS, Overall survival; RFS, Recurrence-free survival; ROC, Receiver operating characteristic; AFP, Alpha-fetoprotein; AUC, Area under the curve; TNM, Tumor-node-metastasis; aHR, Adjusted hazard ratio; CI, Confidence interval; DCA, Decision curve analysis; CTSC, Cathepsin C.

Address correspondence to: Zhidan Xu and Xinchang Li, Department of Organ Transplantation, The First Affiliated Hospital of Nanchang Medical College, No. 92 The Aiguo Road, Nanchang 330006, Jiangxi, China. Tel: +86-13597853975; E-mail: xuzhidan1971@163.com (ZDX); Tel: +86-0791-86895550; E-mail: lixinchang1963@163.com (XCL)

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.

[2] Kulik L and El-Serag HB. Epidemiology and management of hepatocellular carcinoma. Gastroenterology 2019; 156: 477-491, e1.

[3] Omata M, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, Tateishi R, Han KH, Chawla YK, Shiina S, Jafri W, Payawal DA, Ohki T, Ogasawara S, Chen PJ, Lesmana CRA, Lesmana LA, Gani RA, Obi S, Dokmeci AK and Sarin SK. Asia-pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. Hepatol Int 2017; 11: 317-370.

[4] Siegel RL, Miller KD, Fuchs HE and Jemal A. Cancer statistics, 2021. CA Cancer J Clin 2021; 71: 7-33.

[5] Tabrizian P, Jibara G, Shrager B, Schwartz M and Roayaie S. Recurrence of hepatocellular cancer after resection: patterns, treatments, and prognosis. Ann Surg 2015; 261: 947-955.

[6] He W, Peng B, Tang Y, Yang J, Zheng Y, Qiu J, Zou R, Shen J, Li B and Yuan Y. Nomogram to predict survival of patients with recurrence of hepatocellular carcinoma after surgery. Clin Gastroenterol Hepatol 2018; 16: 756-764, e10.

[7] Zhou L, Wang SB, Chen SG, Qu Q and Rui JA. Risk factors of recurrence and poor survival in curatively resected hepatocellular carcinoma with microvascular invasion. Adv Clin Exp Med 2020; 29: 887-892.

[8] Long J, Chen P, Lin J, Bai Y, Yang X, Bian J, Lin Y, Wang D, Yang X, Zheng Y, Sang X and Zhao H. DNA methylation-driven genes for constructing diagnostic, prognostic, and recurrence models for hepatocellular carcinoma. Theranostics 2019; 9: 7251-7267.

[9] Lee SC, Tan HT and Chung MC. Prognostic biomarkers for prediction of recurrence of hepatocellular carcinoma: current status and future prospects. World J Gastroenterol 2014; 20: 3112-3124.

[10] Olson OC and Joyce JA. Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. Nat Rev Cancer 2015; 15: 712-729.

[11] Yao RQ, Ren C, Xia ZF and Yao YM. Organelle-specific autophagy in inflammatory diseases: a potential therapeutic target underlying the quality control of multiple organelles. Autophagy 2021; 17: 385-401.

[12] Zhao Q, Jia Y and Xiao Y. Cathepsin K: a therapeutic target for bone diseases. Biochem Biophys Res Commun 2009; 380: 721-723.

[13] Lutgens SP, Cleutjens KB, Daemen MJ and Heeneman S. Cathepsin cysteine proteases in cardiovascular disease. FASEB J 2007; 21: 3029-3041.

[14] Sung HK, Song E, Jahng JWS, Pantopoulos K and Sweeney G. Iron induces insulin resistance in cardiomyocytes via regulation of oxidative stress. Sci Rep 2019; 9: 4668.

[15] Mizunoe Y, Kobayashi M, Hoshino S, Tagawa R, Itagawa R, Hoshino A, Okita N, Sudo Y, Nakagawa Y, Shimano H and Higami Y. Cathepsin B overexpression induces degradation of perilipin 1 to cause lipid metabolism dysfunction in adipocytes. Sci Rep 2020; 10: 634.

[16] Cantres-Rosario YM, Ortiz-Rodriguez SC, Santos-Figueroa AG, Plaud M, Negron K, Cotto B, Langford D and Melendez LM. HIV infection induces extracellular cathepsin B uptake and damage to neurons. Sci Rep 2019; 9: 8006.

[17] Kuester D, Lippert H, Roessner A and Krueger S. The cathepsin family and their role in colorectal cancer. Pathol Res Pract 2008; 204: 491-500.

[18] Yuan L, Liu J, He W, Bao Y, Sheng L, Zou C, Hu B, Ge W, Liu Y, Wang J, Lin B, Li Y and Ma E. Discovery of a novel cathepsin inhibitor with dual autophagy-inducing and metastasis-inhibiting effects on breast cancer cells. Bioorg Chem 2019; 84: 239-253.

[19] Hiraiwa M. Cathepsin A/protective protein: an unusual lysosomal multifunctional protein. Cell Mol Life Sci 1999; 56: 894-907.

[20] Park S, Kwon W, Park JK, Baek SM, Lee SW, Cho GJ, Ha YS, Lee JN, Kwon TG, Kim MO, Ryoo ZY, Han SH, Han JE and Choi SK. Suppression of cathepsin a inhibits growth, migration, and invasion by inhibiting the p38 MAPK signaling pathway in prostate cancer. Arch Biochem Biophys 2020; 688: 108407.

[21] Hu B, Zhu X and Lu J. Cathepsin A knockdown decreases the proliferation and invasion of A549 lung adenocarcinoma cells. Mol Med Rep 2020; 21: 2553-2559.

[22] Li C, Chen L, Wang J, Zhang L, Tang P, Zhai S, Guo W, Yu N, Zhao L, Liu M and Yang S. Expression and clinical significance of cathepsin B and stefin A in laryngeal cancer. Oncol Rep 2011; 26: 869-875.

[23] Kozlowski L, Wojtukiewicz MZ and Ostrowska H. Cathepsin A activity in primary and metastatic human melanocytic tumors. Arch Dermatol Res 2000; 292: 68-71.

[24] Wang H, Xu F, Yang F, Lv L and Jiang Y. Prognostic significance and oncogene function of cathepsin A in hepatocellular carcinoma. Sci Rep 2021; 11: 14611.

[25] Toss MS, Miligy IM, Haj-Ahmad R, Gorringe KL, AlKawaz A, Mittal K, Ellis IO, Green AR and Rakha EA. The prognostic significance of lysosomal protective protein (cathepsin A) in

breast ductal carcinoma in situ. Histopathology 2019; 74: 1025-1035.
[26] Pontén F, Schwenk JM, Asplund A and Edqvist PH. The human protein atlas as a proteomic resource for biomarker discovery. J Intern Med 2011; 270: 428-446.
[27] Yang JD, Hainaut P, Gores GJ, Amadou A, Plymoth A and Roberts LR. A global view of hepatocellular carcinoma: trends, risk, prevention and management. Nat Rev Gastroenterol Hepatol 2019; 16: 589-604.
[28] Zhang X, Bai Y, Xu L, Zhang B, Feng S, Xu L, Zhang H, Xu L, Yang P, Niu T, Zheng S and Liu J. Clinical and morpho-molecular classifiers for prediction of hepatocellular carcinoma prognosis and recurrence after surgical resection. Hepatol Int 2019; 13: 715-725.
[29] Gomez-Sintes R, Ledesma MD and Boya P. Lysosomal cell death mechanisms in aging. Ageing Res Rev 2016; 32: 150-168.
[30] Mijanovic O, Brankovic A, Panin AN, Savchuk S, Timashev P, Ulasov I and Lesniak MS. Cathepsin B: a sellsword of cancer progression. Cancer Lett 2019; 449: 207-214.
[31] Nagakannan P, Islam MI, Conrad M and Eftekharpour E. Cathepsin B is an executioner of ferroptosis. Biochim Biophys Acta Mol Cell Res 2021; 1868: 118928.
[32] Gondi CS and Rao JS. Cathepsin B as a cancer target. Expert Opin Ther Targets 2013; 17: 281-291.
[33] Petrera A, Gassenhuber J, Ruf S, Gunasekaran D, Esser J, Shahinian JH, Hubschle T, Rutten H, Sadowski T and Schilling O. Cathepsin A inhibition attenuates myocardial infarction-induced heart failure on the functional and proteomic levels. J Transl Med 2016; 14: 153.
[34] Ni S, Weng W, Xu M, Wang Q, Tan C, Sun H, Wang L, Huang D, Du X and Sheng W. miR-106b-5p inhibits the invasion and metastasis of colorectal cancer by targeting CTSA. Onco Targets Ther 2018; 11: 3835-3845.
[35] Kim JW, Mahiddine FY and Kim GA. Leptin modulates the metastasis of canine inflammatory mammary adenocarcinoma cells through downregulation of lysosomal protective protein cathepsin A (CTSA). Int J Mol Sci 2020; 21: 8963.
[36] Du Z, Liu X, Wei X, Luo H, Li P, Shi M, Guo B, Cui Y, Su Z, Zeng J, Si A, Cao P and Zhou G. Quantitative proteomics identifies a plasma multiprotein model for detection of hepatocellular carcinoma. Sci Rep 2020; 10: 15552.
[37] Zhang GP, Yue X and Li SQ. Cathepsin C Interacts with TNF-alpha/p38 MAPK signaling pathway to promote proliferation and metastasis in hepatocellular carcinoma. Cancer Res Treat 2020; 52: 10-23.