Cathepsin S is aberrantly overexpressed in human hepatocellular carcinoma

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Received April 24, 2009; Accepted June 25, 2009

DOI: 10.3892/mmr_00000161

Abstract. Several lysosomal cathepsins have been implicated in a number of diseases, from arthritis to cancer. A recent member of the cathepsin family, cathepsin S (Cat S) has been associated with several types of cancer in humans. However, to date, no report has linked Cat S to human hepatocellular carcinoma (HCC). Here, we investigated the expression of Cat S in human normal and HCC livers using immunohistochemistry and Western blot analysis. The results showed that no expression or very low levels of Cat S expression were detected in the hepatocytes of normal livers. In contrast, a significant increase in Cat S expression was detected in the cancerous hepatocytes in 34 of the total 63 HCC livers (54%; P<0.01). The Cat S-positive rate was significantly higher in the HCC nodule than in the peri-nodular region (P<0.01). Nevertheless, the Cat S-positive rate in the peri-HCC region was still significantly higher than that in the normal liver tissue (P<0.01). Elevated Cat S expression in HCC was positively correlated with the presence of portal vein tumor thrombus (P<0.01), extra-hepatic metastasis (P<0.05) and the degree of de-differentiation (P<0.01), but was not correlated with age, the presence of hepatitis B virus surface antigen and cirrhosis, the level of serum α-fetoprotein, the number of tumor nodules, the tumor size and the clinical stage (P>0.05). Aberrant overexpression of Cat S in the cancerous hepatocytes may be one of the key events involved in HCC tumorigenesis, invasion and metastasis.

Introduction

Cathepsin S (Cat S) is a rather recent addition to the lysosomal cysteine protease family (Cat B, C, F, H, K, L, O, S, V, W and X). Similar to other members of this family, Cat S can degrade a number of extracellular matrix (ECM) proteins, including fibronectin, laminin, collagens and elastin (1,2). However, Cat S as a secreted enzyme distinguishes itself from other cathepsins by its relatively limited tissue distribution and catalytical activity in a broad range of pH (4.5-8.0). In addition, Cat S is known to actively participate in antigen presentation in both professional and non-professional antigen-presenting cells in a tissue-specific manner (reviewed in ref. 3) (Vasiljeva *et al*). The unique properties of Cat S in affecting the ECM microenvironment as well as inflammation and the immune response, indicate that Cat S may play an active role in tumorigenesis, angiogenesis, invasion and metastasis. It was demonstrated that angiogenesis and the growth of cancerous pancreatic cells was reduced in a mouse Cat S knock-out model (4,5). In addition, Cat S was found to be overexpressed in endothelial cells in a late-stage liver tumor in a genetic mouse model of HCC (6), suggesting a role for Cat S in angiogenesis and cancer metastasis. In humans, Cat S has been implicated in several types of cancers, including lung cancer (7), prostate cancer (8) and astrocytoma (9). Based on this new evidence, Cat S was proposed as a potential drug target for cancer therapy (3,10).

HCC is the fifth most common cancer in the world and is characterized by poor diagnosis and prognosis. To the best of our knowledge, no Cat S data have been reported in conjunction with human HCC. Therfore, in the present study, we investigated and compared expression of Cat S in normal and HCC tissues.

Materials and methods

Human patient samples. Samples from 63 HCC patients (58 males, 5 females, average age 45 years) were obtained from the pathological archives at the First Affiliated Hospital of Guangxi Medical University (Nanning, China) during the period of January 2005 to February 2008. Of these, 7, 10, 37 and 9 samples were classified as grade I, II, III and IV, respectively, according to the 1997 TNM staging system (5th edition). Specifically, 17 of the HCC patients had portal vein tumor thrombus (PVTT), 7 with metastasis outside the liver and 48 with poor cell differentiation. A more detailed pathological description can be found in Table I.

Immunohistochemistry. Paraffin sections were prepared from tissue blocks and subsequently dewaxed, rehydrated and incubated in 3% H_2O_2 in methanol for 5 min to block endogenous peroxidase activity. After rinsing with phosphate buffer solutiion (PBS) three times, the sections were subjected to a

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Key words: cathepsin S, hepatocytes, hepatocellular carcinoma

Table I. Cat S-positive hepatocytes and their association with hepatocellular carcinoma pathology.

Pathology	Total no. of cases	No. of Cat S-positive cases	Percentage	P-value[a]
Normal	11	0	0	
HCC				
Nodule	63	34	54.0	P<0.01
Peri-nodule	63	19	30.2	
HBsAg				
Yes	55	30	54.6	P>0.05
No	8	4	50.0	
PVTT				
Yes	17	14	82.4	P<0.01
No	46	20	43.5	
Extra-hepatic metastasis				
Yes	7	7	100.0	P<0.05
No	56	27	48.2	
Tumor size (cm)				
≥5	44	27	61.4	P>0.05
<5	19	7	36.8	
No. of tumor nodules				
Multiple	11	8	72.7	P>0.05
Single	52	26	50.0	
AFP (μg/l)				
≥400	54	30	55.6	P>0.05
<400	9	4	44.4	
Liver cirrhosis				
Yes	45	23	51.1	P>0.05
No	18	11	61.1	
Age (years)				
>35	53	30	56.6	P>0.05
≤35	10	4	40.0	
TNM stage				
I-II	17	7	41.2	P>0.05
III-IV	46	27	58.7	
HCC differentiation				
Medium/high	15	3	20.0	P<0.01
Low	48	31	64.6	

HCC, hepatocellular carcinoma; HBsAg, hepatitis B virus surface antigen; PVTT, portal vein tumor thrombus; AFP, α-fetoprotein. [a]χ^2 test.

microwave antigen retrieval procedure in 0.05 M Tris buffer (pH 9.0). Sections were initially incubated with PBS containing 0.1% (v/v) Triton X-100 and 10% non-immune goat serum to block non-specific binding, and then with a goat anti-human Cat S antibody (BioVision, CA, USA), which was finally stained with a biotinylated rabbit anti-goat IgG and visualized with the streptavidin-biotin-peroxidase complex. Negative controls using only secondary antibodies were also performed, but the results are not shown. The slides were counter-stained with hematoxylin for nuclear staining. To assess the results of immunohistochemistry, 100 cells randomly selected from each of the three regions (normal, peri-HCC nodule and HCC nodule) were examined under x400 magnification (Nikon bright field) by two experienced pathologists. Cells stained positive for Cat S (yellowish/brownish staining) were counted and recorded in a double-blind manner. To ensure conservative estimates, only HCC tissues with ≥10 hepatocytes stained with yellowish/brownish color were classified as Cat S-positive in Table I.

Dual-color immunofluorescence. Formalin-fixed and paraffin-embedded tissue slides from normal or HCC males from our

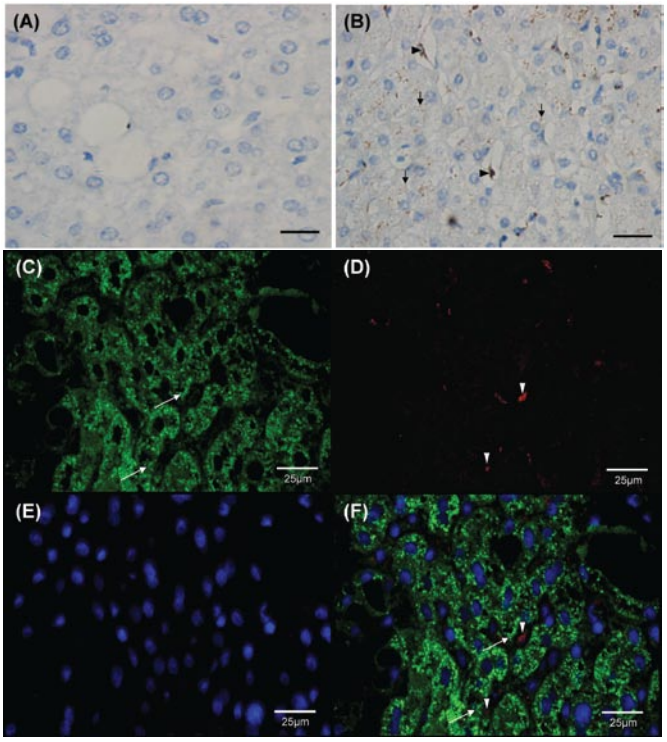


Figure 1. Representative cathepsin S immunohistochemistry in normal livers. (A) Absence of Cat S staining in the hepatocytes of a normal liver. (B) Some hepatocytes have spotted Cat S staining (arrows). In contrast, some smaller non-parenchymal cells, presumably Kupffer cells, have intense Cat S staining (arrowheads). (C) The tissue section was stained for hepatocyte-specific antigen (HSA) and visualized in green. (D) The same section was also stained for Cat S and visualized in red. Basal expression of Cat S was noted. (E) DAPI staining for visualization of nuclei (blue). (F) The merged image of C, D and E shows that the Cat S staining (red, arrowheads) does not co-localize with the HSA staining (green, arrows) of the hepatocytes, indicating that Cat S is mostly expressed in non-parenchymal cells in normal liver. Scale bar, 20 μm for A and B; scale bar, 25 μm for C-F.

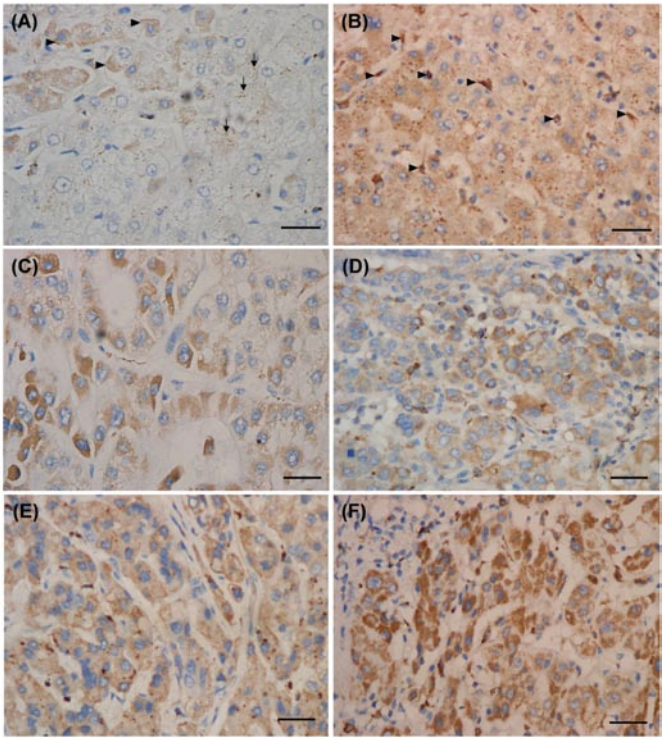


Figure 2. Cathepsin S immunohistochemistry in peri-HCC and HCC nodules. (A) Diffuse Cat S staining is visible in the cytoplasm of some hepatocytes (arrowheads) in the peri-HCC nodule area. In the same region, some hepatocytes have a spotted Cat S staining pattern (arrows). (B) Widespread intense Cat S staining is noted in the hepatocytes of an HCC nodule. A number of smaller non-parenchymal cells positive for Cat S (arrowheads) are also visible in the same region. Representative Cat S staining in additional HCC patients without PVTT and extra-hepatic metastasis, but with medium/high differentiation (C); with PVTT and poor differentiation but without extra-hepatic metastasis (D and E); or with PVTT and extra-hepatic metastasis and with poor differentiation (F). Scale bar, 20 μm.

hospital archives and from ProSci, Inc. (Poway, CA, USA) were used for dual-color immunofluorescence (IF). Tissue sections were deparaffinized with xylene, rehydrated in a series of descending ethanol concentrations and microwaved in antigen retrieval buffer (Tris-EDTA, pH 9.0). The sections were then permeabilized with 0.5% Triton X-100 and blocked in PBS containing 10% horse serum. The slides were incubated with goat anti-human Cat S antibody (AF1183, R&D Systems; 1:50) overnight at 4˚C and rinsed with PBS three times. Mouse anti-human HSA (hepatocyte-specific-antigen, sc-58693; 1:100), rabbit anti-human CD31 (sc-8306; 1:50) and rabbit anti-human CD68 (sc-9139; 1:100) (all from Santa Cruz) were then added to the sections and incubated overnight at 4˚C for the labeling of hepatocytes, endothelial cells and Kupffer cells, respectively. After rinsing with PBS three times, the slides were incubated with secondary antibodies (donkey anti-goat Alexa Fluo 594 (1:200) together with donkey anti-mouse or anti-rabbit Alexa Fluor 488 (1:200) (Invitrogen) for 1 h at room temperature. The slides were rinsed with PBS three times and mounted with mounting media containing DAPI (H-1200, Vector Laboratories). The tissues were examined and photographed using a Leica confocal microscope (Model TCS SP2).

SDS-PAGE and Western blotting. Total liver proteins (10 μg) were loaded in each lane of a 12% polyacrylamide gel for electrophoresis. The proteins were then transferred onto a PVDF membrane, and Cat S was detected with a goat anti-Cat S antibody (BioVision Research) diluted 1:500 at 4˚C overnight. The antibody for detecting GAPDH was purchased from Alpha Diagnostics (USA). Signal visualization was conducted using the ECL chemiluminescence system (Pierce, IL, USA). The protein band intensity was quantified using a Bio-Rad Gel Doc densitometer equipped with Quantity One software (Bio-Rad).

Statistics. All results as listed in Table I were analyzed and statistically tested by the χ^2 method by using the SPSS 13.0 package for PC (SPSS Inc., Chicago, IL, USA).

Results

Minimal Cat S expression in the hepatocytes of normal livers. To examine the Cat S expression level and pattern in normal liver tissue, we conducted immunohistochemistry on paraffin sections from the pathological archives prepared using an antibody against human Cat S. As illustrated in a representative image in Fig. 1A, no Cat S staining was detected in any of

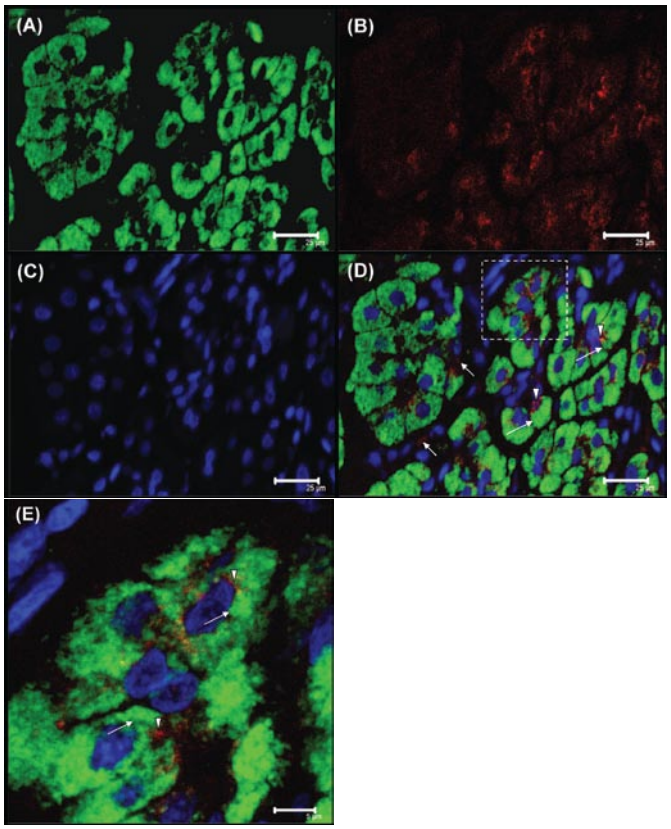


Figure 3. Dual-color fluorescent immunohistochemistry of hepatocytes in a HCC nodule. (A) The tissue section was stained for hepatocyte-specific antigen (HSA) and visualized in green. (B) The same section was also stained for Cat S, which was visualized in red. (C) DAPI staining was used for the visualization of nuclei (blue). (D) The merged image of A, B and C. An elevated level of Cat S (red, arrowheads) was localized to the peri-nuclear region in the same hepatocytes labeled with HSA (green, long arrows). Some Cat S immunoreactivity was also noted in non-parenchymal cells (short arrows). (E) The boxed area in D is shown at a higher magnification to demonstrate peri-nuclear localization. Scale bar, 25 μm for Fig. 3A-D; scale bar, 5 μm for Fig. 3E.

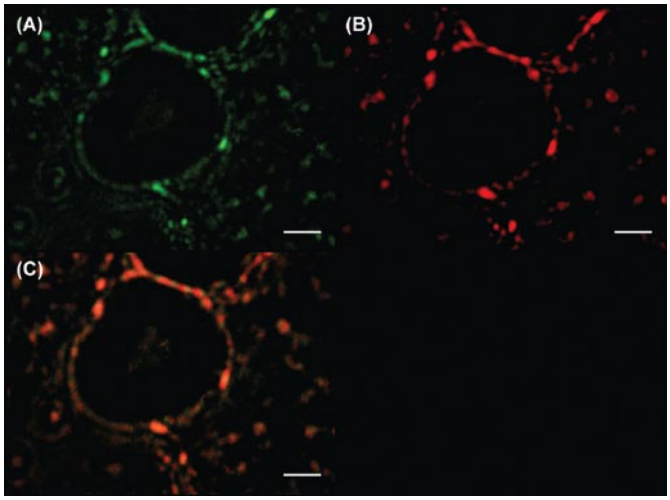


Figure 4. Representative image of dual-color immunofluorescence staining of sinusoidal endothelial cells. A highly malignant HCC liver with PVTT, extra-hepatic metastasis and poor cellular differentiation was stained for CD31 (red) and Cat S (green). Cat S (A) is highly expressed in the sinusoidal endothelial cells, which are labeled by CD31 (B). (C) The merged image of A and B. No Cat S signal was detected in the similar region of the normal liver (data not shown). Scale bar, 20 μm.

the hepatocytes (with large multinuclei) in 9 of the 11 normal livers. Basal Cat S immunoreactivity was noted in 2 of the 11 normal livers. In these 2 cases, the Cat S staining appeared spotted and intracellular (presumably lysosomal) inside the hepatocytes (Fig. 1B). The light and sparse Cat S staining observed in Fig. 1B was regarded as negative in Table I. On the other hand, we routinely observed intense Cat S immunoreactivity in a small number of smaller (non-parenchymal) cells in the normal livers (arrowheads in Fig. 1B).

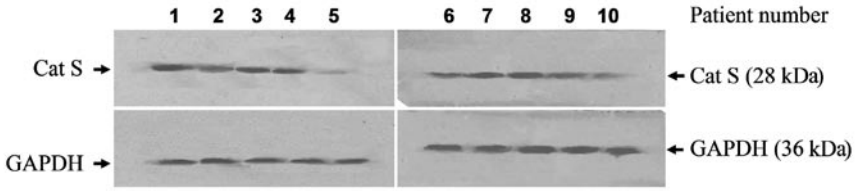
In addition to Cat S immunohistochemistry, we performed dual-color IF using antibodies against HSA (green) and Cat S (red), respectively, to confirm that hepatocytes (arrows in Fig. 1C) in the normal liver do not express a significant amount of Cat S (arrowheads in Fig. 1D). The merged image (Fig. 1F) confirmed that the Cat S signal did not co-localize with the hepatocytes, but resided instead in certain non-parenchymal cells (arrowheads in Fig. 1F). A separate dual-color IF staining indeed verified that most (if not all) of the Cat S signals in the normal liver originated from the CD68-positive Kupffer cells (staining not shown).

Aberrant overexpression of Cat S in HCC hepatocytes. In contrast to the basal staining pattern in normal hepatocytes (Fig. 1), several hepatocytes from the peri-HCC nodular region displayed a more intense, though diffuse, Cat S staining throughout the cytoplasm (arrowheads in Fig. 2A), while other hepatocytes retained the spotted and/or diffuse Cat S staining pattern (arrows in Fig. 2A). A similar staining pattern, although with a much higher intensity, was observed in almost all the hepatocytes within the HCC nodule (Fig. 2B). In addition, an increase in the number of activated KCs was noted in the HCC (arrowheads in Fig. 2B) compared to the normal (arrowheads in Fig. 1B) liver. Cat S staining for four additional HCC livers with different pathologies is shown in Fig. 2C-F, confirming our initial observation in Fig. 2B.

To verify that Cat S protein expression was increased in the hepatocytes of HCC livers, we performed dual-color IF, using antibodies against HSA (which recognizes normal, benign and malignant hepatocytes) and Cat S. Fig. 3B clearly shows that most of the Cat S signals (Fig. 3B) in the HCC cases were co-localized with hepatocytes (Fig. 3A), as noted in the merged image (Fig. 3D and E). We also noted that some of the Cat S signals came from non-parenchymal cells (short arrows in Fig. 3D).

Aberrant overexpression of Cat S in HCC endothelial cells. Ryschich *et al* (6) reported a significant increase in Cat S expression in endothelial cells of a murine HCC model at a late stage. To investigate whether a similar situation exists in human HCC, we performed dual-color IF using antibodies against CD31 and Cat S. We found Cat S expression in the endothelial cells of all 63 HCC cases. High Cat S expression appeared to be associated with certain HCC pathologies, such as PVTT, extra-hepatic metastasis and poor cell differentiation (Fig. 4). In sharp contrast, no obvious Cat S expression was

A.



B.

Patient number	1	2	3	4	5 (normal)	6	7	8	9	10 (normal)
Relative intensity	23.06	22.32	24.56	22.52	10.89	22.12	23.28	25.98	22.35	14.38

Figure 5. Detection of cathepsin S in normal and HCC livers using Western blot analysis. (A) Total protein extracted from 2 normal and 8 HCC livers were blotted onto two separate membranes and quantified using GAPDH as a loading control. (B) Densitometric analysis of the bands corresponding to mature Cat S normalized using GAPDH. The Cat S level was ~1.8 fold higher in the HCC livers compared to the normal livers.

observed in the endothelial cells of the 11 normal livers using the current dual-color IF method (data not shown).

Higher level of Cat S protein in the HCC liver. To estimate the Cat S protein levels in the normal and the HCC livers, respectively, we performed immunoblotting of Cat S using total cellular proteins extracted from a total of 10 livers (2 normal and 8 HCCs). As shown in Fig. 5, individual HCC livers had higher Cat S levels than the 2 normal control livers. In this small number of samples, the normalized Cat S level in the HCC liver was ~1.8-fold higher than that in the normal liver. With the present experimental setting, we could not quantify the relative contribution of each cell type to the Cat S elevation. However, it is likely that the hepatocytes and endothelial cells were the major Cat S producers in the HCC liver, based on our combined immunohistochemistry and Western blot data.

Correlation of Cat S overexpression in hepatocytes with tumor pathology. At the cohort level, no overexpression of Cat S in hepatocytes was detected in any of the 11 normal livers. In contrast, a very intense Cat S immunoreactivity was found in the tumor nodules in 34 of the total 63 HCC livers (54%). Among the 34 Cat S-positive livers, a less intense Cat S staining was also observed in the peri-nodular region in 19 HCC livers. Detailed analysis of an additional 10 physiopathological parameters revealed that overexpression of CatS in cancerous hepatocytes was significantly associated with PVTT, extra-hepatic metastasis and de-differentiation status, but not with hepatitis B virus surface antigen, tumor size, number of tumor nodules, α-fetoprotein, cirrhosis, age and tumor stage (Table I).

Discussion

Overexpression of cathepsin S has been reported in several types of human cancer in the lung (7), prostate (8), brain (9) and pancreas (4). In the present study, we showed for the first time that Cat S was not expressed or expressed at a very low basal level in the hepatocytes of normal human livers, but was greatly elevated in cancerous hepatocytes in a significant percentage of human hepatocellular carcinoma livers. In general, whether the increase in Cat S observed in various cancers, including HCC, was the cause or the result of tumor formation and progression is unclear. Nevertheless, our observations raise intriguing questions about the possible role of the up-regulation of Cat S in the liver parenchyma for tumorigenesis. One of the possible consequences could be the escape of cancerous hepatocytes from immune surveillance. It has been shown that hepatocytes are capable of presenting antigens to $CD4^+$ T cells to modulate an immune response (11,12). Intuitively, one would interpret an increase in Cat S in an antigen-presenting cell as an increase in antigen presentation. However, a recent report (13) revealed, in dendritic cells, a more complex interplay between HCC-promoting interleukin-6 (14) and Cat S. In fact, the activation of the IL-6/STAT3 pathway resulted in an increase in Cat S expression, which in turn suppressed MHC class II expression and attenuated T cell activation. We speculate that IL-6 promoted the up-regulation of Cat S. The resulting immunosuppression, as observed in the dendritic cells, may occur in cancerous hepatocytes as a way of masking tumor antigens in order to escape immune surveillance, consequently allowing the propagation of cancerous cells.

Aside from modulating immune response, Cat S produced in hepatocytes localized to the membrane surface (15) or was secreted to remodel the extracellular matrix and therefore augment tumorigenesis. However, we do not have convincing evidence from the present study to pinpoint the localization of Cat S (Fig. 3D and E). Whether Cat S was secreted into the extracellular space in the HCC livers, which is a likely event, requires further investigation and confirmation. If Cat S is proven to be secreted into the tumor microenvironment, the elevated activity of a proton pump, as observed in cancer cells (10), would help to acidify the local extracellular space and to degrade the ECM components by Cat S. This, on the other hand, could enhance angiogenesis, tumor cell proliferation and invasion. A previous report (8) described that, in prostate cancer, the highest Cat S expression was noted in the area of transitional metaplasia or the invasive front of the progressing tumor mass. However, we did not observe a higher level of Cat S in the invasive front in the HCC livers. On the contrary,

we saw a higher level of Cat S inside the HCC nodules than in the peri-HCC regions, which could be attributed to the neovascularization of the HCC nodules.

The present study also provided the first clinical evidence that Cat S is aberrantly overexpressed in the endothelial cells of HCCs, which is in line with a previous pre-clinical finding, that Cat S mRNA was greatly elevated by 74-fold in endothelial cells in a late stage tumor of a murine HCC model (6). Both pre-clinical and clinical findings certainly substantiate the role of Cat S in tumor angiogenesis and metastasis (5). By jointly examining hepatocytes, endothelial cells and Kupffer cells, a comprehensive Cat S-targeted intervention strategy, focusing on distinct stages such as tumorigenesis, invasion, angiogenesis and metastasis, can be derived to more effectively combat HCC.

Acknowledgements

This work was supported by the Institute of Bioengineering and Nanotechnology (Biomedical Research Council, Agency for Science, Technology and Research, Singapore), and by a research development grant (no. 0836008 to J.X.) from the Guangxi Science and Technology Commission, China. The authors thank Dr Saravana Kumar for assistance with the illustrations.

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