Integrin αvβ3 is required for cathepsin B-induced hepatocellular carcinoma progression

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Abstract. The cysteine protease cathepsin B (Cat B) is important in the progression of tumor cells, however, the function and molecular mechanisms of Cat B in hepatocellular carcinoma (HCC) remain to be elucidated. Our previous study demonstrated that integrin αvβ3 regulated the biological behavior of HCC. The present study demonstrated that Cat B was also important in cell proliferation and apoptosis in HCC. Notably, Cat B was observed to activate the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway to promote HCC proliferation. Furthermore, inhibition of integrin αvβ3 significantly prevented Cat B-induced activation of PI3K/Akt and the progression of HCC. Thus, the results of the present study suggested the presence of a Cat B/integrin αvβ3/PI3K/Akt axis in the regulation of the progression of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth and sixth most prevalent neoplasm in males and females, respectively, and is the second most frequent cause of cancer-associated mortality in China, following lung cancer (1). Due to the characteristics of this type of cancer and limitations in its diagnosis, the majority of the patients are already in the mid-late stages at the time of diagnosis and are no longer able to have surgery. Therefore, dissecting the molecular mechanisms involved in the survival and growth of HCC cells is essential for the development of targeted therapies to reduce patient mortality.

It is known that the invasion and metastasis of tumor cells are commonly associated with proteases, particularly cysteine protease. There are 11 and 19 members of the cysteine cathepsin family in humans and mice, respectively (2). Cathepsin B (Cat B) is a cysteine protease of ~40-45 kD. There is increasing evidence that Cat B is frequently overexpressed in a variety of tumor tissues (3,4) and is involved in distinct tumorigenic processes, including angiogenesis, invasion through extracellular matrices and metastasis (5-9). However, whether Cat B is involved in the progression of HCC remains to be elucidated.

In the present study, Cat B promoted the proliferation and inhibited the apoptosis of HCC cell lines and was identified as an upstream regulator of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. Furthermore, the data suggested that integrin αvβ3 was essential in the signal transduction of Cat B into the PI3K/Akt signaling pathway in HCC.

Materials and methods

Tissue samples and cell culture. A total of eight pairs of HCC tissues and their adjacent normal tissues were obtained from patients who had undergone surgery at the Department of General Surgery, Qianfoshan Hospital, Shandong University (Jinan, China). The present study was approved by the Hospital Institutional Review Board of Qianfoshan Hospital, Shandong University. Written informed consent was obtained from the patient's family. The HepG2, SMMC-7721 and BEL-7402 cell lines were obtained from Shandong Province Key Laboratory of General Surgery Center (Jinan, China). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St Louis, MO, USA) at 37˚C in 5% CO_2.

Transfection. The BEL-7402 cells were transfected with either pcDNA or pcDNA-Cat B using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. The HepG2 and SMMC-7721 cells were transfected with either 40 nM Cat B or control siRNA (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 80% confluence using Geneporter 2 transfection reagent (Genlantis, San Diego, CA, USA) according to the manufacturer's instructions.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. A SYBR RT-PCR kit (Takara Bio, Inc., Shiga, Japan) was used for RT-qPCR analysis. The

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following specific primers were used for RT-qPCR assays: Cat B, forward 5'-CCAGG GAGCA AGACAGAGAC-3' and reverse 5'-GAGAC TGGCG TTCTC CAAAG-3' and β-actin, forward 5'-GGCCT CCAAG GAGTA AGACC-3' and reverse 5'-AGGGG TCTAC ATGGC AACTG-3'. Data from each sample were normalized to the expression of β-actin.

Tumor growth assay. Male, 4-week-old BALB/c nude mice were purchased from the Shanghai Laboratory Animal Company (Shanghai, China). HepG2 cells ($1x10^5$) with stable knock down of Cat B and the negative controls were injected subcutaneously under the front legs of the nude mice. The mice were observed over 5 weeks for tumor formation. Subsequently, the mice were sacrificed and, following tumor removal, the wet weights of each tumor were determined.

Western blotting. To prepare the proteins, the treated cell monolayers were rinsed twice using cold phosphate-buffered saline (PBS) and then scraped and transferred into cell lysis buffer containing 50 mM Tris-HCL (pH 7.5), 150 mM NaCl and 0.5% Nonidet P40 (Sigma) with Roche complete EDTA-free protease inhibitor cocktail (Sigma). These suspensions were then centrifuged using a Beckman centrifuge at 10,000 x g for 10 min and the protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal quantities of the proteins were separated using SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) for western blot analysis using primary antibodies against human Cat B (Abcam, Cambridge, UK), phosphorylated Akt (Ser473), Akt (Cell Signaling Technology, Inc., Danvers, MA, USA), β-actin and secondary polyclonal goat anti-rabbit antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc.). Following washing with tris-buffered saline with Tween 20 (Beyotime Institute of Biotechnology, Haimen, China), bound antibodies were detected using enhanced chemiluminescence (Thermo Fisher Scientific) according to the manufacturer's instructions.

Cell counting kit-8. Cell viability was assessed using a Cell counting kit-8 (Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Apoptosis. An annexin-V assay was performed using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). Briefly, $2x10^5$ cells were collected, washed twice with cold PBS, resuspended in 100 μl binding buffer containing Hepes (10 mM), NaOH (pH 7.4), NaCl (140 mM) and $CaCl_2$ (2.5 mM) and incubated with Annexin V-FITC at room temperature for 10 min. This was followed by the addition of 6 μl propidium iodide (PI; 20 μg/ml) for an additional 5 mins. The fluorescent intensities were determined using flow cytometry (Beckman Coulter, Miami, FL, USA).

Statistical analysis. All data are expressed as the mean ± standard deviation of three or four experiments. Analysis was performed using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cat B is upregulated in HCC tissues and cell lines. The expression of Cat B was compared between the HCC tissues and the adjacent normal tissues using RT-qPCR analysis. The results demonstrated that the expression of Cat B was markedly increased in cancer tissues (Fig. 1A). The upregulation of Cat B was also confirmed by representative western blot analysis using the proteins extracted from the tissues (Fig. 1B). Furthermore, several HCC cell lines were selected to detect the expression of Cat B and the results revealed that the expression of Cat B was highest in the HepG2 cells and lowest in the BEL-7402 cells (Fig. 1C). Taken together, these data suggested that Cat B was overexpressed in the HCC tissues and cell lines.

Overexpression of Cat B promotes HCC cell proliferation. To determine the effect of Cat B on HCC cell growth, the BEL-7402 cells were transfected with either the pcDNA3-Cat B or pcDNA3-vector (mock; Fig. 2A). Cell growth was significantly increased in the Cat B-overexpressed cells compared with their corresponding controls (Fig. 2B). In addition, the cells overexpressing Cat B had a high rate of proliferation (Fig. 2C) and the Annexin V/PI analysis of the Cat B-overexpressed cells revealed a significant decrease in the rate of apoptosis (Fig. 2D).

Cat B-knockdown inhibits HCC cell proliferation. The upregulation of Cat B observed in the present study prompted further investigation to determine the effect of its knock down on HCC cell growth. The HepG2 cells were infected with either a lentivirus targeting Cat B (shRNA-Cat B) or a negative control (shRNA-Ctrl). As shown in Fig. 3A-B, endogenous Cat B was significantly downregulated by shRNA-Cat B. Consequently, cell growth was significantly decreased in the cells transfected with shRNA-Cat B compared with shRNA-Con (Fig. 3C). These cells also had a lower rate of proliferation (Fig. 3D).

Cat B-knockdown inhibits HCC growth in vivo. To further determine the role of Cat B knockdown, HepG2 cells with stable knock down of Cat B or negative controls were injected subcutaneously under the front legs of the nude mice and tumor growth was closely monitored for 4 weeks. The results demonstrated that the tumor size and weight were markedly reduced following Cat B knockdown compared with the control (Fig. 4A-B), suggesting that Cat B inhibition suppresses HCC growth *in vivo*.

Integrin αvβ3 is essential for the progression of Cat B-induced HCC. It has been demonstrated that Cat B promotes tumor progression, not only by proteolytic function, but also by a series of signal transduction pathways (10,11). Modulation of the PI3K signaling pathway is important in tumor growth. Therefore, in the present study, this intracellular signaling pathway was analyzed in HCC cells by manipulating the levels of Cat B. As shown in Fig. 5A and B, the total level of Akt level was unaltered despite the varying levels of Cat B. However, the phosphorylation of Akt was markedly increased in the cells overexpressing Cat B compared with the control vector-transfected cells (Fig. 5A). By contrast, the

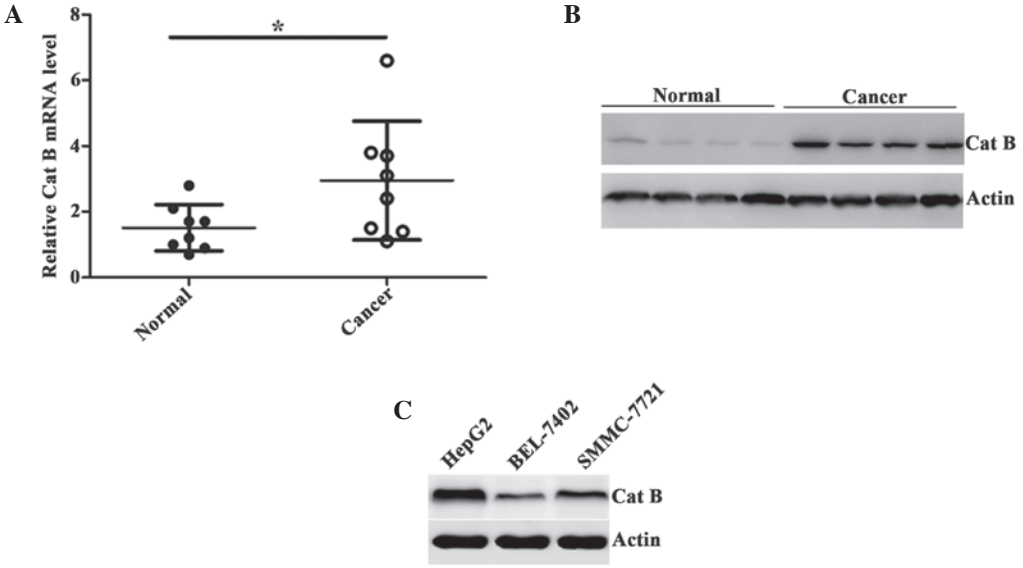

Figure 1. Cat B is upregulated in HCC tissues and cell lines. (A) Expression of Cat B was determined by reverse transcription quantitative polymerase chain reaction in human HCC tissues and adjacent noncancerous tissues. *P<0.05, compared with normal tissues (n=8). (B) Expression of Cat B was determined using western blot analysis in human HCC tissues and adjacent noncancerous tissues. (C) Expression of Cat B was determined using western blot analysis in HepG2, SMMC-7721 and BEL-7402 cell lines. Cat B, cathepsin B; HCC, hepatocellular carcinoma.

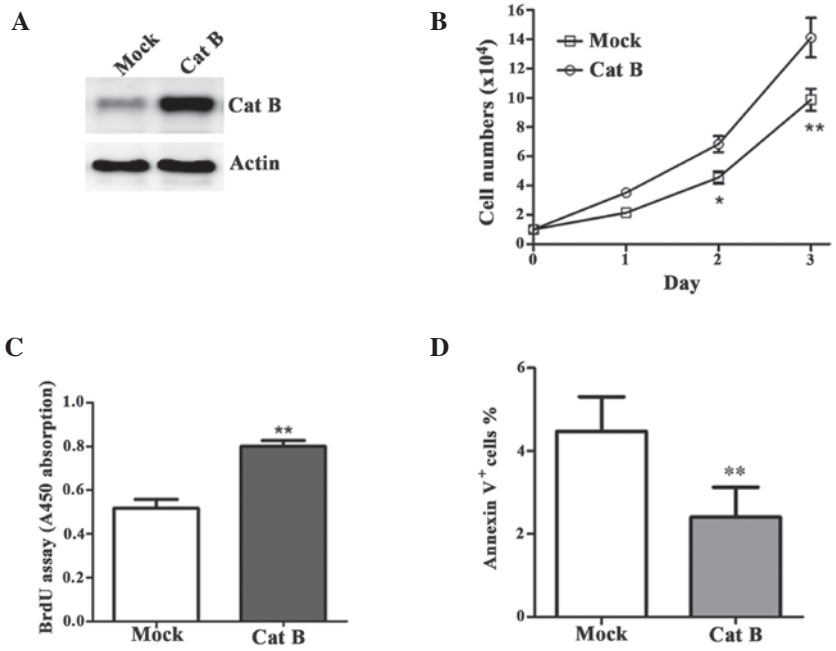

Figure 2. Overexpression of Cat B promotes HCC cell proliferation. (A) BEL-7402 cells were transfected with pcDNA3-Cat B or pcDNA3-vector (mock). Expression of Cat B was determined by western blot analysis. (B) Growth of the BEL-7402 cells transfected with pcDNA3-Cat B or pcDNA3-vector (mock). (C) A BrdU assay was used to detect the proliferative potential of BEL-7402 cells transfected with pcDNA3-Cat B or pcDNA3-vector (mock). (D) Apoptotic rate was determined in BEL-7402 cells transfected with pcDNA3-Cat B or pcDNA3-vector (mock). **P<0.05, compared with Mock. HCC, hepatocellular carcinoma; Cat B, Cathepsin B; BrdU, bromodeoxyuridine.

phosphorylation of Akt was markedly decreased in the shRNA-Cat B cells compared with the shRNA-control cells (Fig. 5B).

Our previous study demonstrated that the expression of integrin αvβ3 was higher than normal in primary liver cancer and that antisense integrin αvβ3 suppresses the growth of subcutaneously implanted HCC by inhibiting tumor angiogenesis (12). Additionally, it has been suggested that the interaction between integrins and extracellular components is important in tumor differentiation and progression (13). Therefore, the present study hypothesized that Cat B was involved in the progression of hepatoma through integrin αvβ3 to ultimately affect the phosphorylation of Akt. To verify this hypothesis, BEL-7402 cells overexpressing pcDNA3-Cat B were pretreated with either an integrin αvβ3 inhibitory antibody or with an isotype-matched control, immunoglobulin G1. The main

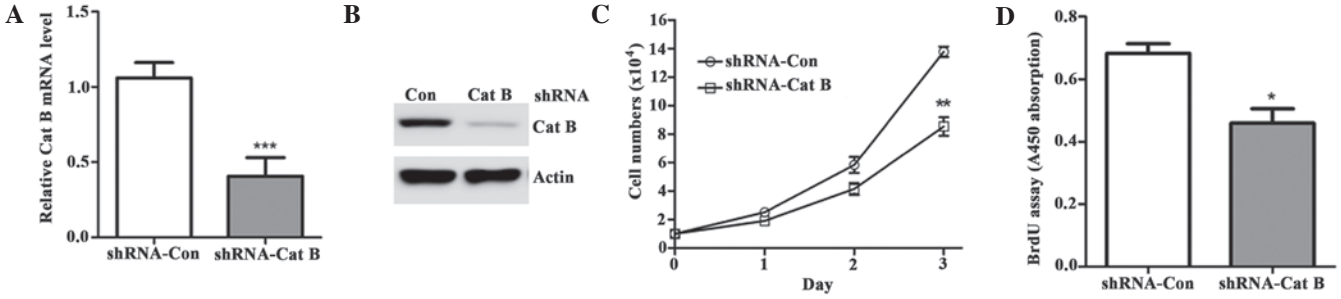

Figure 3. Cat B knockdown inhibits HCC cell proliferation. (A) Expression of Cat B was determined by reverse transcription quantitative polymerase chain reaction in HepG2 cells infected with shRNA-Cat B or shRNA-Con. (B) Expression of Cat B was determined by western blot analysis in HepG2 cells infected with shRNA-Cat B or shRNA-Con. (C) Growth of HepG2 cells infected with shRNA-Cat B or shRNA-Con. (D) BrdU assay to determine the proliferative potential of HepG2 cells infected with shRNA-Cat B or shRNA-Con. Cat B, cathepsin B; HCC, hepatocellular carcinoma; shRNA-Cat B, lentivirus targeting Cat B; shRNA-Con, negative control; BrdU, bromodeoxyuridine.

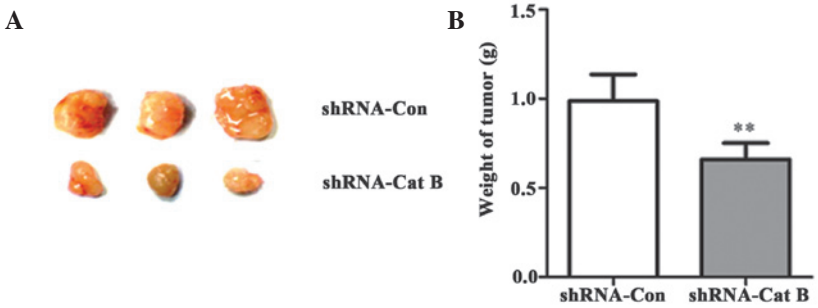

Figure 4. Cat B knockdown inhibits the growth of hepatocellular carcinoma *in vivo*. (A) Representative images of HepG2 cells stably infected with shRNA-Cat B or shRNA-Con and injected into nude mice (n=5 in each group) for assessment of tumorigenesis. (B) Tumor weights of HepG2 cells stably infected with shRNA-Cat B or shRNA-Con and injected into nude mice (n=5 in each group) for assessment of tumorigenesis. shRNA-Cat B, lentivirus targeting Cat B; shRNA-Con, negative control.

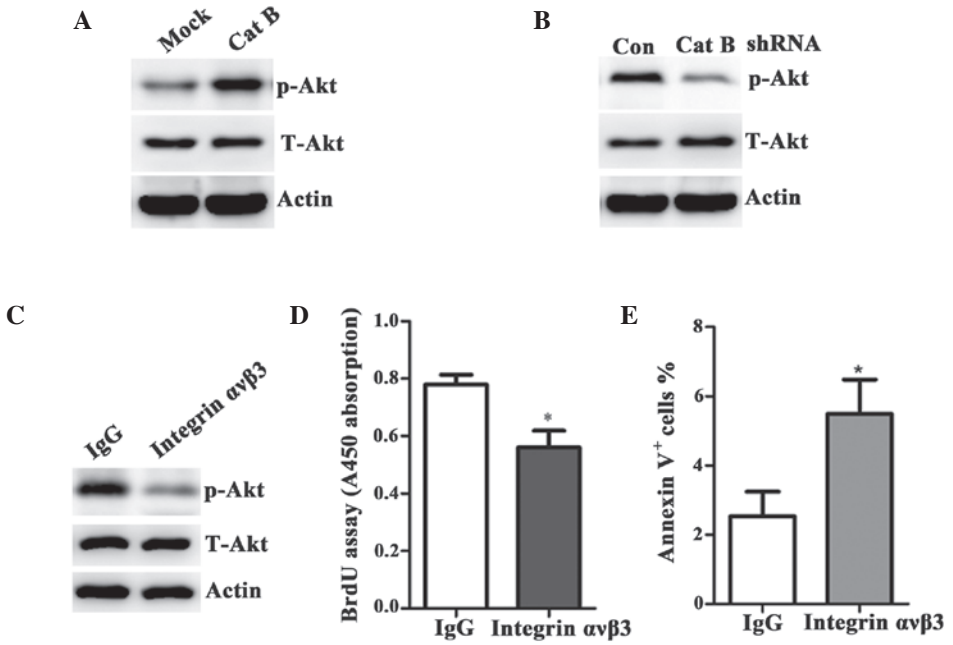

Figure 5. Integrin αvβ3 is essential for Cat B-induced hepatocellular carcinoma progression. (A) Expression levels of T-Akt and p-Akt were determined by western blot analysis in BEL-7402 cells transfected with pcDNA3-Cat B or pcDNA3-vector (mock). (B) Expression levels of T-Akt and p-Akt were determined by western blot analysis in HepG2 cells infected with shRNA-Cat B or shRNA-Con. (C) BEL-7402 cells overexpressing pcDNA3-Cat B were pretreated with integrin αvβ3 inhibiting antibody or isotype-matched control IgG1. Expression levels of T-Akt and p-Akt were determined by western blot analysis. (D) BEL-7402 cells overexpressing pcDNA3-Cat B were pretreated with integrin αvβ3 inhibiting antibody or isotype-matched control IgG1. BrdU assay was used to assess the proliferative potential. (E) BEL-7402 cells overexpressing pcDNA3-Cat B were pretreated with integrin αvβ3 inhibiting antibody or isotype-matched control IgG1. The apoptotic rate was then determined. T-Akt, total Akt; p-Akt, phosphorylated Akt; shRNA-Cat B, lentivirus targeting Cat B; shRNA-Con, negative control; IgG, immunoglobulin G; BrdU, bromodeoxyuridine.

components of the PI3K signaling pathway were then detected by western blot analysis. Inhibiting integrin αvβ3 significantly prevented the Cat B-overexpressing phosphorylation of Akt (Fig. 5C), limited Cat B-induced proliferation (Fig. 5D) and led to the apoptosis of Cat B-overexpressing cells (Fig. 5E). Taken together, these results demonstrated that Cat B regulated HCC growth by integrin αvβ3 and subsequently by the PI3K/Akt signaling pathway.

Discussion

Deregulation of cysteine cathepsins functions, including Cat B and Cat L, is associated with a number of disease states, including cancer (5,6,14). Cat B is a 40-45 kD cysteine protease, composed of a dimer of disulfide-linked heavy and light chains that are produced from a single protein precursor. It is initially synthesized on the rough endoplasmic reticulum, further modified through glycosylation and sulfation in the Golgi complex and recognized by the mannose-6-phosphate receptor into lysosomes. Accumulating studies have demonstrated that Cat B is important in a several types of tumor. For example, Withana *et al* observed that Cat B inhibition limited bone metastasis in breast cancer (15) and Wu *et al* found that Cat B may be a potential biomarker in cervical cancer (16). The present study demonstrated that Cat B is overexpressed in HCC. Notably, Cat B was observed to promote the progression of HCC, including the enhancement of cell proliferation and the inhibition of cell apoptosis.

Akt is an important effector kinase, which relays signaling downstream of the PI3K pathway (17). It regulates a variety of cellular processes, including cell growth, survival, differentiation, migration and invasion. Akt activation inhibits cell apoptosis and promotes cancerous growth and invasion (18). Upon ligand binding to receptor tyrosine kinases or G protein-coupled receptors, the activation of Akt is initiated by membrane recruitment, via interaction of its pleckstrin homology domain with the phospholipid phosphatidylinositol (3,4,5)-trisphosphate. Subsequent to membrane recruitment, Akt is sequentially phosphorylated at threonine 308 by pyruvate dehydrogenase lipoamide kinase isozyme-1 and at serine 473 by mammalian target of rapamycin complex 2 (19,20). Following this, the phosphorylated Akt translocates from the plasma membrane to the intracellular compartments, including the cytoplasm and nucleus where it leads to the phosphorylation of its substrates. In the present study, phosphorylated Akt notably increased in the cells overexpressing Cat B compared with the control vector-transfected cells. By contrast, phosphorylated Akt decreased markedly in the cells with RNA interference-mediated knock down of Cat B compared with the control RNAi-transfected cells. Thus, these data indicated that Cat B regulated the progression of HCC through the PI3K/Akt signaling pathway.

Cat B is an extracellular molecule and it has been observed that the interaction of Cat B with urokinase plasminogen activator (uPA), activates the uPA receptor (uPAR) to promote cancer cell invasion and migration, as well as angiogenesis (21,22). It is well established that uPAR has no intracellular domain, therefore, it may exert its signaling capacity through interactions with other components of the plasma membrane, including G protein-coupled receptors, receptor tyrosine kinases and integrins (13). The present study confirmed that integrin αvβ3 is essential in the signal transduction of Cat B into PI3K/Akt. In conclusion, the results demonstrated that Cat B positively regulated the progression of HCC, which included accelerating cell growth and inhibiting cell apoptosis. Furthermore, the present study demonstrated that Cat B promoted the progression of HCC via the PI3K/Akt signaling pathway and found that integrin αvβ3 was essential for the activation of Cat B-mediated PI3K/Akt activation and the progression of HCC. Therefore, the present study identified the effect of and the mechanism underlying Cat B in HCC, providing new potential for HCC therapy in the future.

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