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Silencing cathepsin S gene expression inhibits growth, invasion and angiogenesis of human hepatocellular carcinoma *in vitro*

Qi Fan [a,1], Xuedi Wang [a,1], Hanguang Zhang [a], Chuanwei Li [a], Junhua Fan [b], Jing Xu [a,*]

[a] Department of Hepatobiliary and Vascular Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, China
[b] Department of Gastroenterology, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, China

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ABSTRACT

Cathepsin S (Cat S) plays an important role in tumor invasion and metastasis by its ability to degrade extracellular matrix (ECM). Our previous study suggested there could be a potential association between Cat S and hepatocellular carcinoma (HCC) metastasis. The present study was designed to determine the role of Cat S in HCC cell growth, invasion and angiogenesis, using RNA interference technology. Small interfering RNA (siRNA) sequences for the Cat S gene were synthesized and transfected into human HCC cell line MHCC97-H. The Cat S gene targeted siRNA-mediated knockdown of Cat S expression, leading to potent suppression of MHCC97-H cell proliferation, invasion and angiogenesis. These data suggest that Cat S might be a potential target for HCC therapy.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer death worldwide [1]. With improvement of surveillance programs and advances in imaging techniques, HCC can be diagnosed at an earlier stage and curative treatments can be used [2–4]. Surgical resection, liver transplantation, and ablation by radiofrequency or ethanol injection are now conventional therapies at early disease stages, with 5-year survival rates of 50–70% [5]. However, the outcomes are still hampered by a high incidence of recurrence and metastasis after curative resection and liver transplantation [6]. Therefore, there is an urgent need to elucidate the mechanisms of invasion and metastasis, and to search for new targets for treatment of HCC.

The recurrence and metastasis of HCC is a multistep process, and includes invasion of the extracellular matrix (ECM), intravasation, translocation via the blood vessels, migration to and invasion of a secondary site, and finally, formation of metastatic nodules [7]. In this process, many biomarkers have been shown to have potential predictive significance, such as vascular endothelial growth factor (VEGF) [8], matrix metalloproteinase-2 (MMP-2) [7] and Rac1 [9]. Although much progress has been made in this field, we know little about the mechanisms underlying HCC invasion and metastasis, and further research is needed.

Cathepsin S (Cat S) is one of the members of the cathepsin family (Cat B, C, F, H, K, L, O, S, V, W and X) that can be found in a variety of cell types [10]. As a cysteine protease, Cat S can degrade ECM elements such as laminin, fibronectin, elastin and collagen [11,12]. Previous studies have shown that Cat S can regulate the inflammation and immune response in a variety of pathological processes, and is closely connected with rheumatoid arthritis, Alzheimer's disease, bronchial asthma, obesity, diabetes, dyslipidemia and cardiovascular disease [13]. In addition, Cat S has been demonstrated to be involved in multiple types of cancer such as lung, prostate and gastric cancer and astrocytoma [14–17]. Moreover, recent studies have confirmed that Cat S plays a key role in tumorigenesis, angiogenesis, invasion and metastasis [18]. Our previous work has indicated that Cat S is aberrantly overexpressed in the endothelial cells of HCC, and the expression level is correlated with portal vein embolus, extrahepatic metastasis, and degree of differentiation [19]. However, little is known about the function of Cat S in HCC growth, invasion and angiogenesis. Thus, the present study aimed to confirm the expression of Cat S in human HCC cell lines and illustrate the function of Cat S in HCC growth, invasion and angiogenesis *in vitro*.

2. Materials and methods

2.1. Cell culture

The HCC cell lines MHCC97-L and MHCC97-H [20], which have low and high metastatic potential, respectively, were obtained from the Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China). Human umbilical vein endothelial cells (HUVECs) were purchased from the Cell Collection of the Chinese

* Corresponding author. Fax: +86 771 5359801.
E-mail address: jxuapr@yahoo.com.cn (J. Xu).
[1] These authors contributed equally and should be considered as first authors.

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Table 1
Three pairs of Cat S gene targeted siRNA and a pair of negative control siRNA.

Name	Paired siRNA fragment
siRNA-770	5′GCUGCCACAUGUUCAAAGUTT3′ 5′ACUUUGAACAUGUGGCAGCTT3′
siRNA-669	5′CGGCUUUCCAGUACAUCAUTT3′ 5′AUGAUGUACUGGAAAGCCGTT3′
siRNA-1011	5′GCCACAACUUUGGUGAAGATT3′ 5′UCUUCACCAAAGUUGUGGCTT3′
siRNA-NC	5′ UUCUCCGAACGUGUCACGU3′ 5′ACGUGACACGUUCGGAGAA3′

Academy of Sciences (Shanghai, China). MHCC97-L, MHCC97-H and HUVECs were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone). The normal human hepatic cell line L02 was purchased from the Cell Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 (Hyclone) supplemented with 10% FBS (Hyclone). The cell lines were cultured at 37 °C in a humidified incubator with 5% CO_2.

2.2. Small interfering RNA (siRNA) design and transfection

Three pairs of Cat-S-gene-targeted siRNA sequences and a pair of negative control siRNA sequences were synthesized based on the siRNA design guidelines by Shanghai GenePharma Company (Shanghai, China) (Table 1). MHCC97-H cells were plated onto six-well plates and grown to 50–60% confluence before transfection. siRNAs were transfected into MHCC97-H cells at a final concentration of 20 nM with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.3. RNA extraction and real-time quantitative PCR

Total RNA was isolated 24 h post-transfection with Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of total RNA were determined by a spectrophotometer (Bio-Rad, Hercules, CA, USA). Reverse transcription was performed using PrimeScript RT Master Mix (TaKaRa, Dalian, China). cDNA was stored at −20 °C. Primers for real-time PCR were as follows: Cat S: forward: 5′-GCCTGATTCTGTGGACTGG-3′,reverse: 5′-GATGTACTGGAAAGCCGTTGT-3′; GAPDH: forward: 5′-GGAGTCCACTGGCGTCTTC-3′, reverse: 5′-GCTGATGATCTTGAGGCTGTTG-3′. Real-time quantitative PCR was performed using Platinum SYBR Green qPCR Super Mix (Invitrogen) according to the manufacturer's instructions. PCR conditions were 3 min at 94 °C, 40 cycles of 20 s at 94 °C, 20 s at 60 °C, and 25 s at 72 °C. Amplification of the single product in PCR was confirmed by monitoring the melting curve from 65 to 95 °C. Each real-time PCR was done in triplicate. The relative expression level of mRNA in each sample was normalized to its GAPDH content. The relative expression levels of mRNA were calculated as $2^{-\Delta\Delta Ct}$.

2.4. Western blotting

After 48 h transfection, cells were washed twice with ice-cold PBS, and incubated with Cell Lysis Solution (beyotime, Shanghai, China) on ice for 30 min. Lysates were clarified by centrifugation (16,000*g* for 10 min at 4 °C) and total protein concentration was determined by the bicinchoninic acid (BCA) kit (Thermo Scientific, Rockford, IL, USA). The supernatants were mixed 1:4 with SDS buffer containing 50 mM DTT. The samples were heated in boiling water for 10 min and then subjected to SDS–PAGE. Samples that had the same amount of total proteins were then loaded onto 12% separation gels with a 5% stacking gel, and run at 90 V for 100 min. After electrophoresis, blotting was performed for 120 min at 250 mA. After completion of the protein transfer, the ready PVDF membranes (Millipore, Billerica, MA, USA) were incubated for 2 h at room temperature in TBST buffer, containing 1% BSA powder to block the nonspecific protein binding sites, and then incubated with specific primary antibodies (anti-Cat S and anti-MMP-2 monoclonal antibodies; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C with mild shaking. Membranes were then washed with TBST three times for 10 min and incubated for 2 h with secondary antibodies (rabbit anti-mouse; R&D Systems, Minneapolis, MN, USA) in 1% BSA–TBST with gentle shaking. Final detection was performed by ECL detection reagents (Thermo Scientific) according to the manufacturer's instructions. The computer-assisted densitometry program was used to detect the gray degree of the protein bands.

2.5. Cell proliferation assay

Cell proliferation was measured using the MTT assay. MHCC97-H cells were plated in sextuplicate in 96-well plates at an initial cell density of 5×10^4 cells/ml after 24 h transfection. After 0, 24, 48, 72 and 96 h incubation in 5% CO_2 at 37 °C, 10 μl MTT (Sigma, St Louis, MO, USA) (5 mg/ml in PBS) was added and cells were incubated for a further 4 h. One hundred microliters of SDS–isobutanol–HCl solution (10% SDS, 5% isobutanol, and 12 μM HCl) were added to each well and incubated overnight. Absorbance of each well was measured at 570 nm using a microplate reader (Bio-Rad).

2.6. Assessment of apoptosis with flow cytometry

The Annexin V-FITC Apoptosis Kit (Invitrogen) was used to analyze quantitatively apoptosis of MHCC97-H cells, according to the manufacturer's instructions. After 48 h transfection, the cells were washed twice with cold PBS and resuspended in annexin-binding buffer. Annexin V–FITC and propidium iodide (PI) were added and the tubes were incubated at room temperature for 15 min in the dark. After the incubation period, annexin-binding buffer was added, and the stained cells were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) as soon as possible.

2.7. Cell invasion assay

The Transwell chambers (Corning, Corning, NY, USA) with 8.0-μm pore polycarbonate membrane insert were used to assay invasion activity of MHCC97-H cells. For the invasion assays, the polycarbonate membrane was coated with Matrigel (BD). After 24 h transfection, 5×10^5 cells were suspended in 100 μl serum-free DMEM and placed into the upper compartment of the Transwell chambers. The lower chamber was filled with 600 μl DMEM supplemented with 10%FBS. After 24 h incubation, cells were removed from the upper surface of the polycarbonate membrane with a cotton swab. The invaded cells on the lower surface were fixed with 75% alcohol and stained with 1% crystal violet for 15 min. After air-drying at room temperature, the invaded cells were counted at 200× magnification from four randomly selected fields, with an inverted phase contrast microscope (Leica, Solms, Germany). The mean cell number of four randomly selected fields was used for statistical analysis.

2.8. Wound healing assays

The wound healing assays were used to measure the migration ability of MHCC97-H cells. MHCC97-H cells were plated in six-well plates at an initial density of 4×10^5/ml and grown to 70–80% confluence before transfection. A wound was made by scratching with a 100-μl micropipette tip. Images were taken at 0 and 48 h after

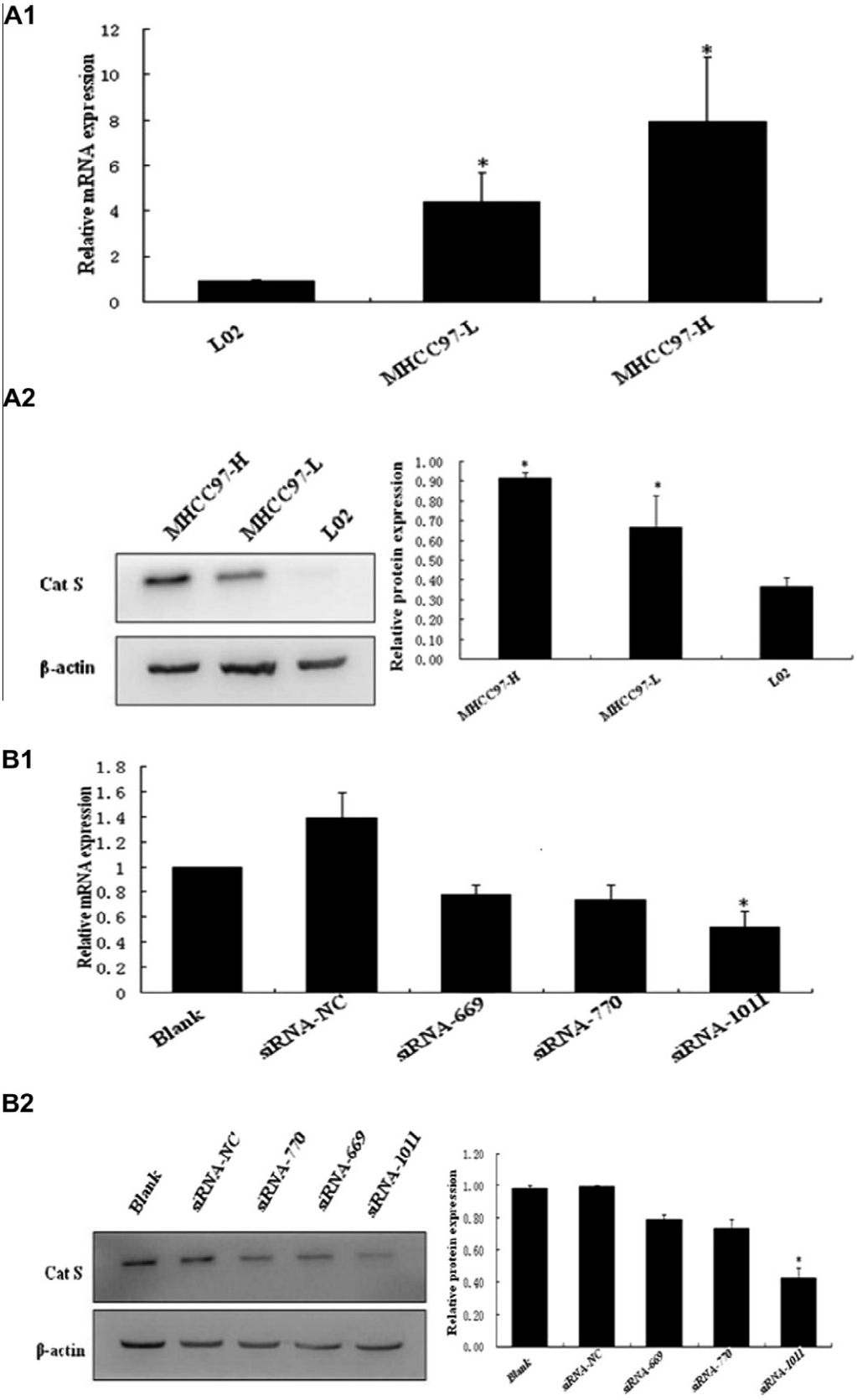

Fig. 1. The expression of Cat S. (A) Expression of Cat S in different HCC cell lines. (A1) Real-time quantitative PCR was performed to detect Cat S mRNA levels with GAPDH mRNA as an endogenous control. $^{*}P < 0.05$ compared with the L02 cell line. (A2) Western blot analysis was performed to detect Cat S protein levels. β-Actin was used as a loading control. $^{*}P < 0.05$ compared with the L02 cell line. (B) Knockdown of Cat S by siRNA at the mRNA and protein levels. (B1) Real-time quantitative PCR was performed to analysis the interfering effects of three candidate sequences and a negative control sequence at the mRNA level. siRNA-1011 significantly inhibited Cat S expression in MHCC97-H cells. $^{*}P < 0.05$ compared with the siRNA-NC and blank control. (B2) Western blotting was used to detect the interfering effects at the protein level and obtain consistent results. $^{*}P < 0.05$ compared with the siRNA-NC and blank control.

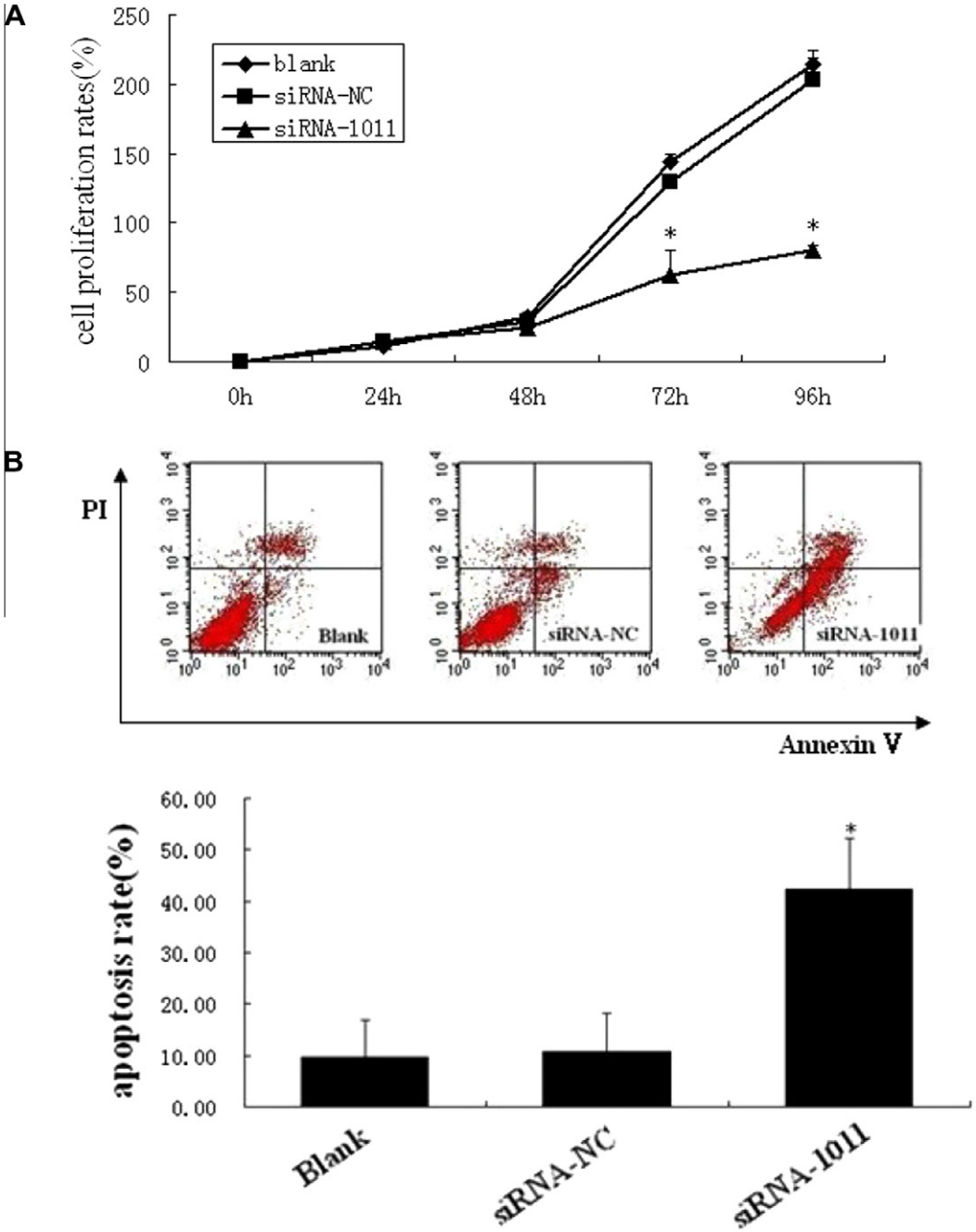

Fig. 2. Effect of Cat S knockdown on proliferation and apoptosis of MHCC97-H cells. (A) Effect of Cat S knockdown on proliferation of MHCC97-H cells as measured by the MTT assay. $^{*}P < 0.05$ versus the siRNA-NC and blank control. (B) Knockdown of Cat S induced apoptosis in MHCC97-H cells. Apoptosis rate of MHCC97-H cells significantly increased after transfection with siRNA-1011. $^{*}P < 0.05$ versus the siRNA-NC and blank control.

scratching using an inverted phase contrast microscope (Leica). The distance migrated by the cells was determined by measuring the scratch wound width at 48 h and subtracting it from that at 0 h. The values obtained were then expressed as percentage migration, setting the wound width at 0 h as 100% [21,22].

2.9. Enzyme-linked immunosorbent assay (ELISA)

The MHCC97-H cells were plated onto 24-well plates and grown to 50–60% confluence before transfection. The cell media were collected after 48 h transfection, and secretion of VEGF was determined by ELISA (R&D Systems) according to the manufacturer's instructions.

2.10. Tube formation assay

Conditioned media were collected from transfected MHCC97-H cells and filtrated with a 0.22-μm filter(Millipore), and then stored at −70 °C for subsequent use. For the tube formation assays, the 24-well plates were coated with Matrigel (BD). HUVECs were harvested and diluted (4×10^4 cells) in 200 μl DMEM supplemented with 10% FBS, then seeded on Matrigel-coated 24-well plates in triplicate at 37 °C for 1 h. Cell culture medium was then replaced by 700 μl conditioned medium. After incubation at 24 h, tube formation images were captured with an inverted phase contrast microscope (Leica). The level of the tube formation was quantified by measuring the length of the tubes in four randomly chosen

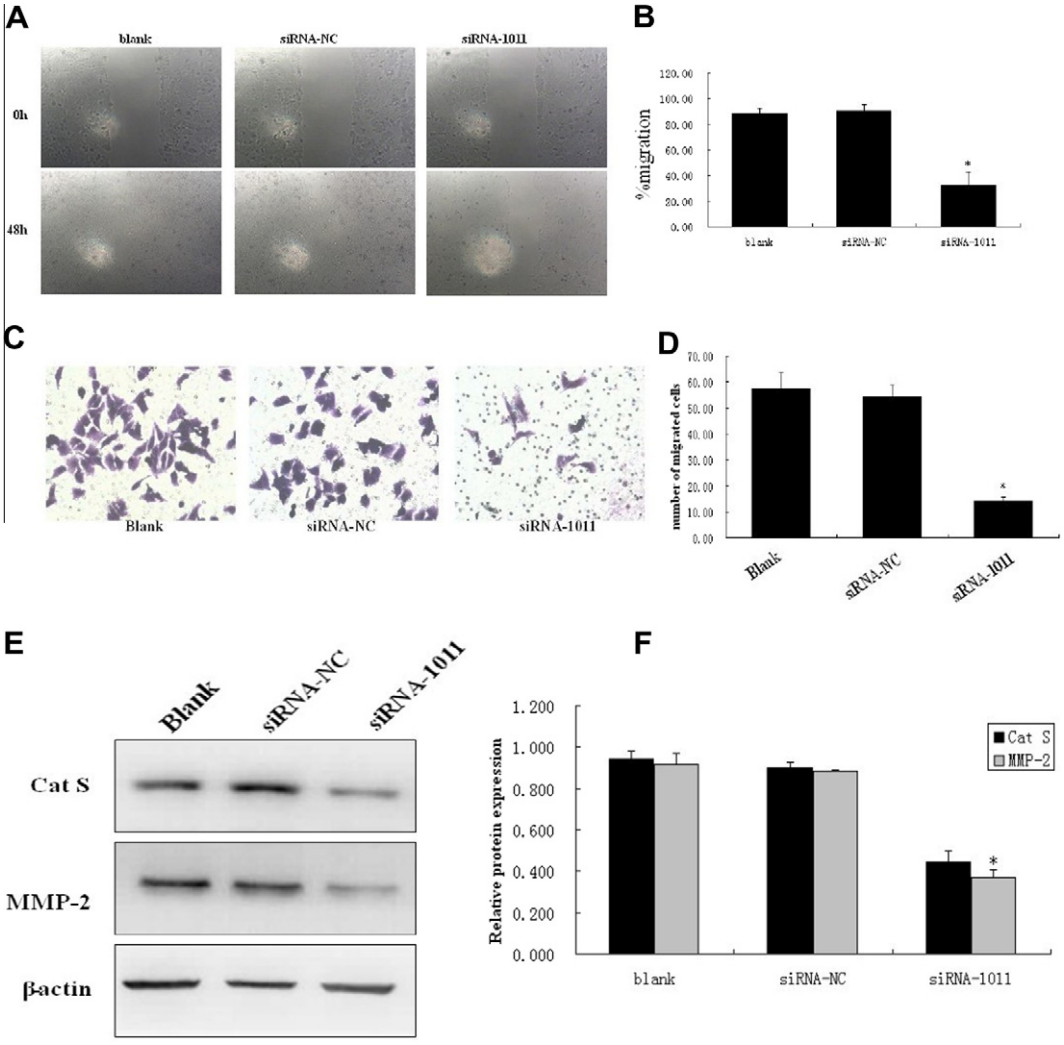

Fig. 3. Effect of Cat S knockdown on migration and invasion of MHCC97-H cells. (A) Images were taken at 0 and 24 h after scratching (200×). (B) Migration was quantified and expressed as a percentage. $^{*}P < 0.05$ versus siRNA-NC and blank control. (C) Knockdown of Cat S inhibited invasive ability of MHCC97-H cells. Blue-stained cells are those migrating through the Matrigel to the lower surface of the polycarbonate membrane. (D) The cells were counted and photographed under an inverted phase contrast microscope at 200× magnification. The number of migrated cells was significantly decreased after transfection with siRNA-1011. $^{*}P < 0.05$ versus siRNA-NC and blank control. (E) Western blotting was performed to examined MMP-2 protein expression levels after 48 h transfection. (F) Quantification showed that knockdown of Cat S significantly inhibited MMP-2 protein expression in the siRNA-1011 group. $^{*}P < 0.05$ compared with siRNA-NC and blank control.

fields from each well using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA) [23,24].

2.11. Statistical analysis

All of the experiments were repeated at least three times and the data were expressed as the mean ± SD. Comparisons between two groups were done using Student's *t* test. Multiple comparisons were determined by a one-way ANOVA. The experimental data were deal with SPSS 16.0 software. $P < 0.05$ was considered significant.

3. Results

3.1. Cat S was overexpressed in human HCC cell line MHCC97-H

To examine Cat S expression in HCC cell lines with different metastatic potential, real-time quantitative PCR and western blotting were performed. Cat S mRNA and protein levels in MHCC97-L and MHCC97-H cell lines were higher than those in normal hepatic cell line L02. Among the three cell lines analyzed, MHCC97-H cells had the highest Cat S expression (Fig. 1A). Therefore, we chose the MHCC97H cell line for our study.

3.2. Silencing of Cat S gene in MHCC97-H cells by RNA interference (RNAi)

Three pairs of Cat-S-gene-targeted siRNAs or a pair of negative control siRNAs were transfected into MHCC97-H cells. Real-time quantitative PCR and western blotting were performed to evaluate the interference effects of the three candidate sequences and a missense sequence (the negative control sequence) to downregulate Cat S in MHCC97-H cells. As shown in Fig. 1B, Cat S expression significantly inhibited transfection with siRNA-1011 compared with siRNA-NC and the blank control. Quantification analysis showed that the siRNA reduced Cat S mRNA levels by 48% (siRNA-1011), 26% (siRNA-770) and 22% (siRNA-669) compared with the blank control. Thus, siRNA-1011 was used to transfect MHCC97-H cells in the next experiments.

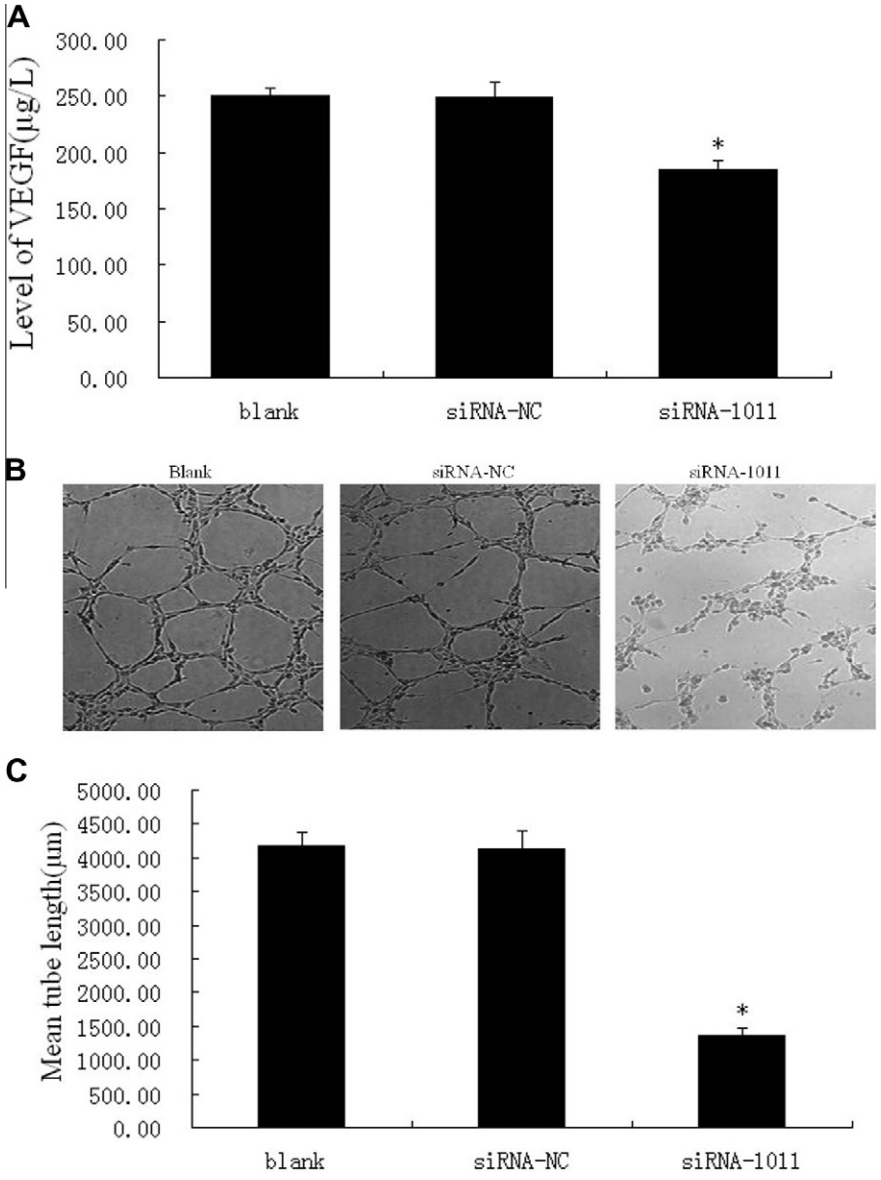

Fig. 4. Knockdown of Cat S inhibits HCC-associated angiogenesis. (A) ELISA showed that VEGF secretion was significantly suppressed in siRNA-1011-transfected cells. $^{*}P < 0.05$ compared with siRNA-NC-transfected cells and blank control. (B) Effects of conditioned media on HUVEC tube formation. Images were captured with an inverted phase contrast microscope at 200× magnification. (C) Quantitative measurements indicated that siRNA-1011-transfection-conditioned medium caused a significant decrease in mean tube length compared with siRNA-NC-transfection-conditioned medium and blank control ($^{*}P < 0.05$).

3.3. Knockdown of Cat S inhibits proliferation of MHCC97-H cells

The MTT assay was performed to examine the effect of Cat S silencing on proliferation of MHCC97-H cells. As shown in Fig. 2A, proliferation was significantly inhibited at 48 and 72 h after transfection with siRNA-1011.

3.4. Knockdown of Cat S induces apoptosis in MHCC97-H cells

The annexin V–FITC/PI double staining assay was used to analyze quantitatively apoptosis of MHCC97-H cells. After transfection with siRNA-1011 for 48 h, the apoptosis rate of MHCC97-H cells was 42.22 ± 9.94%, which was significantly higher than that with siRNA-NC (10.71 ± 7.58%) and blank control (9.75 ± 7.11%) (Fig. 2B).

3.5. Knockdown of Cat S inhibits MHCC97-H cells invasion and migration

The Transwell assay was used to determine the impact of knockdown of Cat S on MHCC97-H cell invasion. As shown in Fig. 3C and D, the number of MHCC97-H cells invading through Matrigel was significantly decreased after transfection with siRNA-1011, compared with that in siRNA-NC-transfected cells and the blank control. The wound healing assay showed a significant reduction in motility of the siRNA-1011-transfected cells compared with the siRNA-NC-transfected cells and the blank controls (Fig. 3A and B). Western blotting was performed to determine the MMP-2 protein expression levels after 48 h transfection. As shown in Fig. 3E and F, expression of MMP-2 was significantly inhibited in MHCC97-H cells infected with siRNA-1011 compared with siRNA-NC and the blank controls.

3.6. Knockdown of Cat S inhibits HCC-associated angiogenesis

MHCC97-H cell medium was collected after 48 h transfection, and the VEGF level was determined by ELISA. The results showed that, compared with the siRNA-NC-transfected cells and the blank controls, VEGF secretion was markedly suppressed in the siRNA-1011-transfected cells (Fig. 4A). As seen in Fig. 4B and C, blank control and siRNA-NC-transfection-conditioned medium induced HUVEC tube formation, and culture with siRNA-1011-transfection-conditioned medium resulted in actively restrained tube formation.

4. Discussion

Although several previous studies have reported that Cat S plays a part in the development of cancer by promotion of cell invasion, metastasis and tumor angiogenesis, it is unclear whether and how Cat S contributes to the development of HCC. In the present study, we showed that both Cat S mRNA and protein were expressed at a high level in human HCC cells with high metastatic potential. These different levels in HCC cells with different metastatic potential indicated that Cat S expression correlated with metastatic capacity. This result was consistent with our previous study about the expression of Cat S in HCC tissues [19].

In this study, we evaluated the effects of Cat S on the growth, invasion and angiogenesis of MHCC97-H cells, using RNAi technology. We showed that the Cat S-target siRNA inhibited expression of Cat S in human MHCC97-H cells, leading to suppression of cancer cell proliferation, invasion and angiogenesis, as well as inducing apoptosis.

In recent years, it has been found that the cancer proteomics is important for classification, establishment of diagnostic markers, and selection of therapeutic targets [25]. Previous evidence has demonstrated that secretomes are required for the processes of proliferation, differentiation, invasion, metastasis and angiogenesis of cancer [26]. It is well known that cathepsins promote cancer invasion and metastasis through degradation of the ECM [27]. Like most of the members of the cathepsin family, Cat S is vital for cancer invasion, metastasis and angiogenesis [27]. Fernandez et al. have shown that Cat S is expressed in neoplastic prostatic cells from preinvasive to invasive and clinically detectable stages, suggesting a putative role in local invasion [28]. Flannery et al. have found that CatS expression is upregulated in grade IV astrocytoma cells and provides a potential role for invasion [14]. In a mouse model of hepatocellular carcinogenesis, Cat S is the major protease specifically overexpressed during vessel sprouting [29]. Cat S has been demonstrated to play novel roles in gastric cancer cell migration and invasion; putatively via a network of proteins associated with cell migration, invasion, or metastasis [17]. Given its role in tumor development and progression, Cat S has been put forward as a potential therapeutic target for pharmaceutical intervention and Cat S inhibitors have been proposed as anticancer agents [13,27]. Many selective inhibitors have been designed to suppress Cat S expression [20–32]. Although some Cat S inhibitors are already in clinical trials, the efficacy and safety of these compounds still need to be assessed.

For many years, MMPs, including MMP-2, were the main investigative focus in the field of proteases and cancer [27]. MMPs promote tumor invasion through degradation of the ECM [7]. MMP-2 can degrade the tumor basement membrane to promote tumor cell escape, and is closely related to HCC invasion and metastasis [33,34]. It is well known that cathepsins facilitate cancer invasion, metastasis and angiogenesis through ECM digestion [23]. In the present study, we examined the MMP-2 protein expression levels after transfection by siRNA. The results showed that knockdown of Cat S significantly inhibited MMP-2 protein expression in MHCC97-H cells. The effect of Cat S in regulation of MHCC97-H cell invasion may be related to regulation of MMP-2 expression.

In summary, it is believed that the present study shows for the first time that Cat S is highly expressed in HCC cells with high metastatic potential. Moreover, our results indicate that Cat S has an important role in HCC cell proliferation, invasion and angiogenesis. These data suggest that Cat S might be a potential target for HCC therapy.

Acknowledgments

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