Arch Pharm Res Vol 34, No 5, 781-789, 2011
DOI 10.1007/s12272-011-0512-6

Archives of Pharmacal Research
www.springer.com/12272

Inhibition of Invasion and Metastasis of MHCC97H Cells by Expression of Snake Venom Cystatin through Reduction of Proteinases Activity and Epithelial-Mesenchymal Transition

Nanhong Tang[1,2], Qun Xie[1,3], Xiaoqian Wang[2], Xiujin Li[2], Yanlin Chen[1,2], Xu Lin[1], and Jianyin Lin[1]

[1]Key Laboratory of Ministry of Education for Gastrointestinal Cancer, Research Center for Molecular Medicine, Fujian Medical University, Fuzhou 350004, China, [2]Hepatobiliary Surgery Institute, Union Hospital, Fujian Medical University, Fuzhou 350001, China, and [3]Medical College of Putian University, Putian 351100, China

(Received March 11, 2010/Revised September 27, 2010/Accepted October 4, 2010)

Snake venom cystatin (sv-cystatin) is a member of the cystatin family of cysteine protease inhibitors. To further evaluate the possibility of sv-cystatin in cancer therapy, this study examined the effects of sv-cystatin on the invasion and metastasis of liver cancer cells (MHCC97H) *in vitro* and *in vivo* as well as the underlying mechanism. sv-cystatin cDNA was transfected into MHCC97H cells and the anti-invasion and antimetastasis effects of sv-cystatin were determined using migration and matrigel invasion assays and a lung-metastasis mice model. The results suggest that sv-cyst clone (sv-cystatin expression in MHCC97H cells) delayed the invasion and metastasis *in vitro* and *in vivo* compared to the parental, mock and si-sv-cyst clone cells (inhibited sv-cystatin expression by siRNA). The decreased activities of cathepsin B, MMP-2 and MMP-9 and EMT change index including higher E-cadherin, lower N-cadherin and decreased Twist activity were observed in the sv-cyst clone, which contributes to the change in invasion and metastasis ability of MHCC97H cells. This study provides evidence that expression of the sv-cystatin gene in MHCC97H cells inhibits tumor cell invasion and metastasis through the reduction of the proteinases activity and Epithelial-Mesenchymal Transition (EMT), which might contribute to the anticancer research of the sv-cystatin protein.

Key words: Snake venom, Cystatin, Hepatocellular carcinoma, Invasion, Metastasis

Selected by Editors

INTRODUCTION

Tumor invasion and metastasis are usually major causes of cancer death. Although many advances have been made in cancer therapy, most cancer deaths still result from metastatic disease (Deep and Agarwal, 2010). The process of cancer cell metastasis can be summarized as a sequence of events that include the form of tumor embolus in the blood capillary, cell penetrating extracellular matrix (ECM), trapping at a secondary site and growth at the secondry site (Deep and Agarwal, 2010). Cancer cells excrete proteinases including cysteine-cathepsins that breakdown the ECM and account for invasion and metastasis (Mohamed and Sloane, 2006). The activity of cysteine-cathepsins is naturally regulated by cystatins, a group of natural inhibitors (Premzl et al., 2006). Initial studies *in vitro* and *in vivo* have revealed the suppression of tumor cell invasion and metastasis by a stable transfection of human cystatin C and cystatin M cDNA (Shridhar et al., 2004; Sokol et al., 2004).

Snake venom contains a number of proteolytic enzymes with different toxicological functions and special pharmacological effects, which have been used to in clinical therapy for heart and cerebral vessels diseases and cancer (Chiang et al., 1996; Yeh et al., 2001). In 1998, Brillard-Bourdet (Brillard-Bourdet et

Correspondence to: Jianyin Lin, Key Laboratory of Ministry of Education for Gastrointestinal Cancer, Research Center for Molecular Medicine, Fujian Medical University, Fuzhou 350004, China
Tel, Fax: 86-591-83569132
E-mail address: jylin@mail.fjmu.edu.cn

al., 1998) isolated a micromolecule protein, a cystatin-like inhibitor, from the venom of the Taiwan cobra (*Naja naja atra*). A micromolecule cystatin was also isolated and purified from the venom of *Naja naja atra* (sv-cystatin). On the other hand, the natural sv-cystatin protein is quite rare due to the low content in snake venom and complicated biochemical separation. sv-cystatin cDNA was synthesized artificially (Patent Number of China: ZL200510074534X) (Song et al., 2004) based on the 99 amino acids sequence of *Naja naja atra* venom cystatin (Protein Accession No: P81714) and the biased codon usage of *Pichia pastoris* (Lah et al., 1989), and its anti-metastastic effect on mouse melanoma cells and human gastric carcinoma cells was demonstrated by both *in vitro* and *in vivo* (Wan et al., 2005, 2006).

Hepatocellular carcinoma (HCC) is one of the most aggressive malignant tumors, and is associated with a high potential for vascular invasion, metastasis and recurrence, even after surgical resection (Tang, 2005). To further evaluate the possibility of sv-cystatin for cancer therapy, this study examined the effects of sv-cystatin on the invasion and metastasis of MHCC97H cells (high metastasis property) (Tian et al., 1999) *in vitro* and *in vivo* and the underlying mechanism.

MATERIALS AND METHODS

Cell culture and animals

MHCC97H and MHCC97L cells were purchased from Liver Cancer Institute of Fudan University, Shanghai, China and maintained according to their protocols. Huh-7, HepG2, BEL7404, SMMC7721, HL-7702 and NEK293 cells were maintained in the laboratory. Male BALB/c nude mice at the age of 5 to 6 weeks were purchased from the Chinese Academy of Sciences and were cared for in accordance with the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Semiquantitative RT–PCR analysis

The RNA of all the above cell lines were extracted by Trizol (Invitrogen) and further reverse-transcribed. The fragments of cystatin M (364bp), β-actin1 (613bp), cystatin C (261bp) and β-actin2 (500bp) cDNA were amplified by PCR. The primer sequences were as follows: cystatin M: 5'-ATGGGCAGCAACAGCATCTAC-3' (sense) and 5'-GCACTGGCCATTTATTGTGACA-3' (antisense); β-actin1: 5'-GGCATCGTGATGGACTCCG-3' (sense) and 5'-GCTGGAAGGTGGACAGCGA-3' (antisense); cystatin C: 5'-AGGAGGGTGTGCGGCGTG-3' (sense) and 5'-GCCAAGGCACAGCGTAGAT-3' (antisense); β-actin2: 5'-ATGTCACGCACGATTTCCCGC-3' (sense) and 5'-GGCATGGGTCAGAAGGATTCC-3' (antisense).

Establishment of the cell line showing stable sv-cystatin expression

The sv-cystatin cDNA was subcloned into the pBudCE4.1 vector (Invitrogen) MCS region under the CMV promoter from the pUC18-cystatin plasmid (Song et al., 2004). The primer sequence of sv-cystatin-F (5'-ACGCGTCGACAAT GATCCCAGGTGGTTTGTCTC-3') and sv-cystatin-R (5'-GCTCTAGACCAAACTTGGAAACC ACAC-3') contained *Sal*I and *Xba*I sites (underline), respectively. The MHCC97H cells were transfected with either pBudCE4.1/sv-cystatin or the pBudCE4.1 empty vector using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. After 2 weeks, the anti-Zeocin colonies were expanded and maintained in DMEM supplemented with 10% FBS and Zeocin (200 mg/mL, Invitrogen). The pGPU6 siRNA plasmid vector-based gene silence system (Shanghai GenePharma Co., Ltd.) was then employed to transfect the cells with sv-cystatin expression and produces another control cell line. Focusing on the sequence of sv-cystatin, three DNA chains with the following sense and antisense sequences were synthesized: No.1: 5'-GGTTCTGCTAACGCTCACTAC-3' (sense) and 5'-GTAGTGAGCGTTAGCAGAACC-3' (antisense); No.2: 5'-GTGCTAAGACTGCTGGTA AGC-3' (sense) and 5'-GCTTACCAGCAGTCTTAGCAC-3' (antisense); No.3: 5'-GCTGG TAAGCCAAAGGTTTAC-3' (sense) and 5'-GTAAACCTTTGGCTTACCAGC-3' (antisense). The three DNA chains were annealed and ligated into the (*Bbs*I/*Bam*HI) sites of pGPU6. After sequencing, the plasmids were transduced into MHCC97H/sv-cystatin cells to select the most effective siRNA against sv-cystatin expression.

Migration and matrigel invasion assays

For the migration assay, 5×10^4 cells in 500 μL of serum-free DMEM was seeded into the top chamber of the Transwell Culture Insert according to protocol. The migrated cells were counted using an inverted microscope at × 100 magnification. Triplicate experiments were performed for each clone and each time point. For the Matrigel invasion study, the bottom chamber was precoated with 20 μg Matrigel (BD). All other conditions were as described in the migration assays. In addition, the incubation times should be much longer, i.e. 12, 24, 48, and 60 h, because of the barrier function of Matrigel.

Experiments in nude mice

1×10^7 cells in a 0.2 mL volume of 0.9% NaCl were inoculated s.c. into the right flank of nude mice. Each group contained eight mice. At the end of the 6th week, all mice were sacrificed and the lungs were excised, fixed, cut into consecutive 4 μm sections and stained with hematoxyline & eosin. Each metastatic focus in the lung was determined as it appeared at the same site on consecutive sections. Finally, the all determined foci were counted to evaluate metastasis histologically.

Assay of cathepsin B and MMPs activity

1.2×10^6 cells were seeded into 6-well plates in DMEM supplemented with 10% FBS overnight. The cells were then cultured in free serum DMEM for 24 h. The supernatants were collected and the cells were trypsinized and counted for the assay. The cathepsin B activity assay was performed using a Fluorimetric Assay with Methylcoumarylamide Substrates Z-Phe-Arg-NMec according to Barrett's report (Barrett, 1980) with some modifications. The assay of the MMPs activity was performed by Gelatin Zymography according to the Iwai's report (Iwai et al., 2008). The data for the enzymolysis strip volume were analyzed using the software Quantity One (BIO-RAD Inc.)

Electrophoretic mobility band-shift assays (EMSA)

The biotin-labeled Twist double-stranded oligonucleotides (5'-CTGTGGCCGGCAGGTGAACCC TCAGCCA-3') containing the E-box binding site (CANNTG) found in the E-cadherin promoter region and biotin-labeled NF-κB doublestranded oligonucleotides (5'-AGTTGAGGGGACTTTCCCA GGC-3') containing the kB element binding site (GGGRNYYYCC) were used as probes. The binding of the probe was performed and analyzed on 6.5% polyacrylamide gels using an EMSA Kit (Viagene).

Western blotting analysis

The cell extracts (40 μg) were run on Tricine-SDS-PAGE gels (sv-Cystatin protein) or 10% SDS PAGE gels (other proteins) and electro-blotted to PVDF membranes. The rabbit anti-sv-cystatin antibody (Chaoshi), rabbit primary antibodies against human E-cadherin, N-cadherin and β-Tublin (Santa Cruz), goat antibody against Twist (Santa Cruz) were used, and the proteins expression were detected using a Chemiluminescent Western Blot immunodetection Kit (Invitrogen). The data of band analysis was analyzed using the software Quantity One (BIO-RAD Inc.) and the relative amounts of each protein were quantified as the ratios to β-Tublin indicated underneath each gel.

Immunohistochemistry

The fixed cultured cells on sterile 20 mm-coverslips and formalin-fixed tumor sections from nude mice with a thickness of 4 μm were prepared. The primary antibodies (the same as those used in western blot analysis) were applied overnight. A standard avidin-biotin peroxidase technique was applied using a liquid DAB substrate chromogen system.

Statistical analysis

The results are reported as the mean ± S.D. The one-way analysis of variance analysis followed by Fisher's Least Significant Difference post hoc test or Tamhane's T2 test (if equal variance is not assumed) was used to compare the data from different groups. P values < 0.05 were considered significant.

RESULTS

Characterization of cystatin M and C expression in human liver cancer cell lines

As shown in Fig. 1, a low level of cystatin M mRNA expression was detected only in the HL-7702 cell line, which was compared to the NEK293 cell line (positive expression for cystatin M mRNA) and different levels of

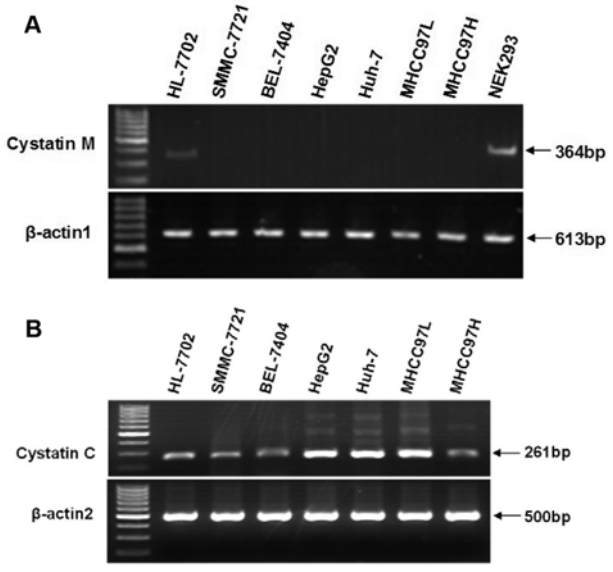

Fig. 1. RT-PCR analysis of cystatin M and cystatin C expression in human liver cancer cell lines. (**A**) Expression of cystatin M mRNA in HL-7702, SMMC7721, BEL7404, HepG2, Huh-7, MHCC97L, MHCC97H and NEK293 cells. (**B**) Expression of cystatin C mRNA in HL-7702, SMMC-7721, BEL-7404, HepG2, Huh-7, MHCC97L and MHCC97H cells.

cystatin C mRNA expression were observed in all cell lines. On the other hand, cystatin C mRNA expression was lower in MHCC97H cells than that in the other liver cancer cell lines. Therefore, the MHCC97H cell line is a better choice for the transfection of sv-cystatin and subsequent studies on the role of sv-cystatin in human liver cancer cells.

Establishment of MHCC97H cell lines stablely transfected by sv-cystatins

The expected 11 kDa sv-cystatin expression was detected only in the sv-cyst1 and sv-cyst2 clones but not in the control clones (parental, mock-1 and mock-2) (Fig. 2A). The same clones were analyzed by immunohistochemistry for sv-cystatin. Clone sv-cyst2 expressed sv-cystatin and clone mock-1 produced no detectable sv-cystatin (Fig. 2B). To examine the function of sv-cystatin further, the stable cell clone sv-cyst2 was transfected with the shRNA vector (si-sv-cyst1, si-sv-cyst2 and si-sv-cyst3). Western blotting showed that the sv-cystatin protein could not be detected in the clone si-sv-cyst3 cells but could be detected at lower levels in the clone si-sv-cyst1 and si-sv-cyst2 cells compare to the parental and clone si-negative cells (Fig. 2C), suggesting that the si-sv-cyst3 shRNA vector is the most effective for reducing sv-cystatin expression in clone sv-cyst2 cells. Therefore, parental, mock-1, sv-cyst2 and si-sv-cyst3 cells were identified and used in all subsequent studies.

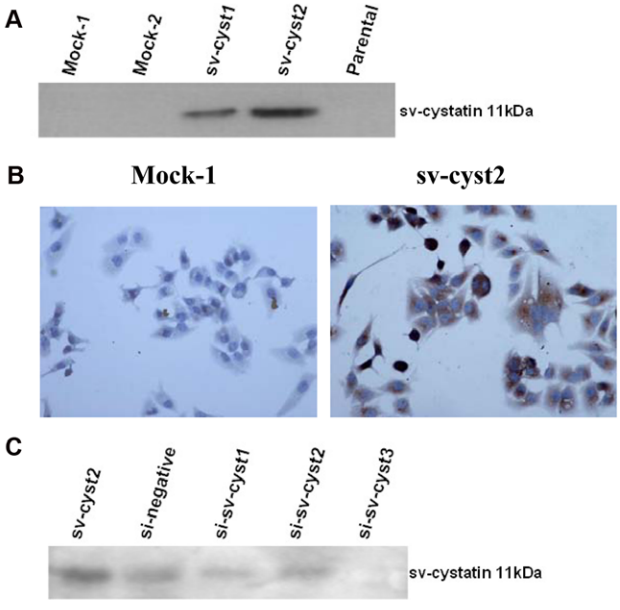

Fig. 2. Characterization of the MHCC97H transfectants. (**A**) Sv-cystatin expression in the mock-1, mock-1, sv-cyst1, sv-cyst2 and parental cells by Westernblot analysis. (**B**) Sv-cystatin expression in the mock-1 and sv-cyst2 cells by immunohistochemistry. (**C**) Effect of different sv-cystatin siRNA on the sv-cystatin expression in sv-cyst2 cells.

Expression of sv-cystatin in MHCC97H cells inhibits invasion and metastasis *in vitro* and *in vivo*

As shown in Fig. 3, the migratory cells accumulated on the bottom surface of the 8 mm filters in a time-dependent manner. At 16 h, migration of the sv-cystatin expressing clone sv-cyst2 was reduced significantly ($p < 0.05$) compared to that of the parental, mock-1 and si-sv-cyst3 control clones (Fig. 3A). At 48-h and 60-h, the invasion of sv-cyst2 cells that expressed sv-cystatin was significantly lower ($p < 0.05$) than the invasion of the parental, mock-1 and si-sv-cyst3 control clones (Fig. 3B). As shown in Fig. 3C, the clone sv-cyst2 cells results in a mean number of tumor colonies (3.13 ± 2.03) with no lung metastasis observed in one nude mouse. In contrast, the parental, clone mock-1 and si-sv-cyst3 cells metastasized to the lungs in all eight nude mice, resulting in a mean number of lung tumor colonies (9.25 ± 2.49, 8.25 ± 1.98 and 7.63 ± 1.41, respectively). The lung metastasis from the clone sv-cyst2 cells was inhibited significantly in the nude mice ($p < 0.05$). Neither liver metastases nor brain metastases were observed in the mice. These results suggest that the expression of sv-cystatin in MHCC97H cells inhibits tumor cell invasion and metastasis *in vitro* and *in vivo*.

The sv-cystatin-induced inhibition of the invasion and metastasis of MHCC97H cells involve in the decreased activity of Cathepsin B, MMP-2 and MMP-9

The invasion and metastasis of liver cancer cells are related to the degradation of the components of the extracellular matrix and basement membrane by different proteinases, such as cathepsin B (Fedorowski et al., 2004) and metalloproteinase (MMPs) (McKenna et al., 2002). The activity of cathepsin B, MMP-2 and MMP-9 secreted by parental, mock-1, sv-cyst2 and si-sv-cyst3 cells were detected to determine if the sv-cystatin induced invasion and metastasis of MHCC97H cells are associated with the reduction of proteinases. As shown in Fig. 4, the cathepsin B activity was reduced by 10.1% in the clone sv-cyst2 cells (379.4 ± 16.5 units) compared to the parental (422.0 ± 15.1 units), mock-1 (426.4 ± 25.0 units) cells and si-sv-cyst3 cells (410.8 ± 28.3 units) ($p < 0.05$) (Fig. 4A). The activity of actMMP-2, proMMP-2, actMMP-9 and proMMP-9 in clone sv-cyst2 cells were significantly lower than those in the parental and mock-1 cells ($p < 0.05$) but the transduction of sv-cystatin siRNA can be play a role

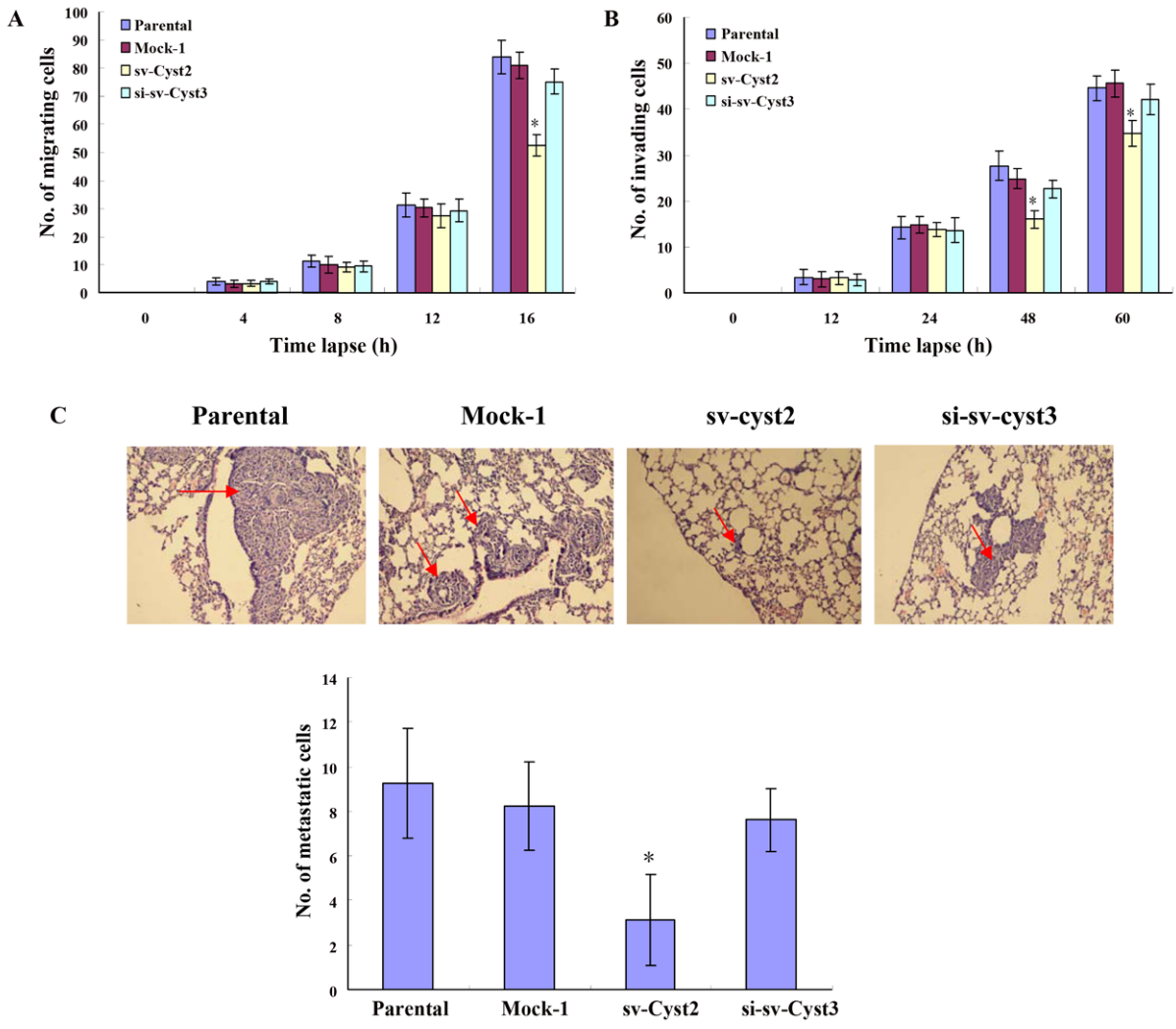

Fig. 3. Expression of sv-cystatin inhibits the migration and invasion of MHCC97H cells *in vitro* and *in vivo*. (**A**) Effect of sv-cystatin on cell migration at each time point (4, 8, 12, and 16 h). (**B**) Effect of sv-cystatin on Matrigel invasion at each time point (12, 24, 48, and 60 h). (**C**) Effect of recombinant sv-cystatin on the metastatic tumor colonies in nude mice 6 week after inoculation (HE staining × 100 → pulmonary metastasis). *indicates a highly significant difference with sv-cyst2 and parental, mock-1 and si-sv-cyst3 cells ($p < 0.05$).

in restoring MMP secretion in MHCC97H cells (colne si-sv-cyst3) (Fig. 4B). This shows that the sv-cystatin induced invasion and metastasis of MHCC97H cells are partly involved in the reduction of cathepsin B, MMP-2 and MMP-9 activity.

The sv-cystatin-induced inhibition of invasion and metastasis of MHCC97H cells correlate with EMT change

EMT is also a key event in the tumor invasion process whereby epithelial cell layers lose polarity and cell-cell contacts, and undergo dramatic remodeling of the cytoskeleton (Yang et al., 2004). Lee et al. (2006b) reported that EMT phenomenon exits in HCC. This study examined whether or not sv-cystatin affects the EMT of MHCC97H cells. As shown in Fig. 5A, an aggregate with a tight cell-cell junction was found in the clone sv-cyst2 cells, but not in the clone si-sv-cyst3 cells (Fig. 5A). This morphological change suggests the inhibition of EMT. In addition, as shown in Fig. 5B, significant upregulated E-cadherin and downregulated N-cadherin expression in the clone sv-cyst2 cells were observed compared to those in the parental, clone mock-1 and si-sv-cyst3 cells. The protein ratio of E-cadherin *vs* β-tublin in clone sv-cyst2 cells was significantly higher than that in the control groups (*$p < 0.05$) but N-cadherin lower (*$p < 0.05$). As shown in Fig. 5C, the higher E-cadherin expression

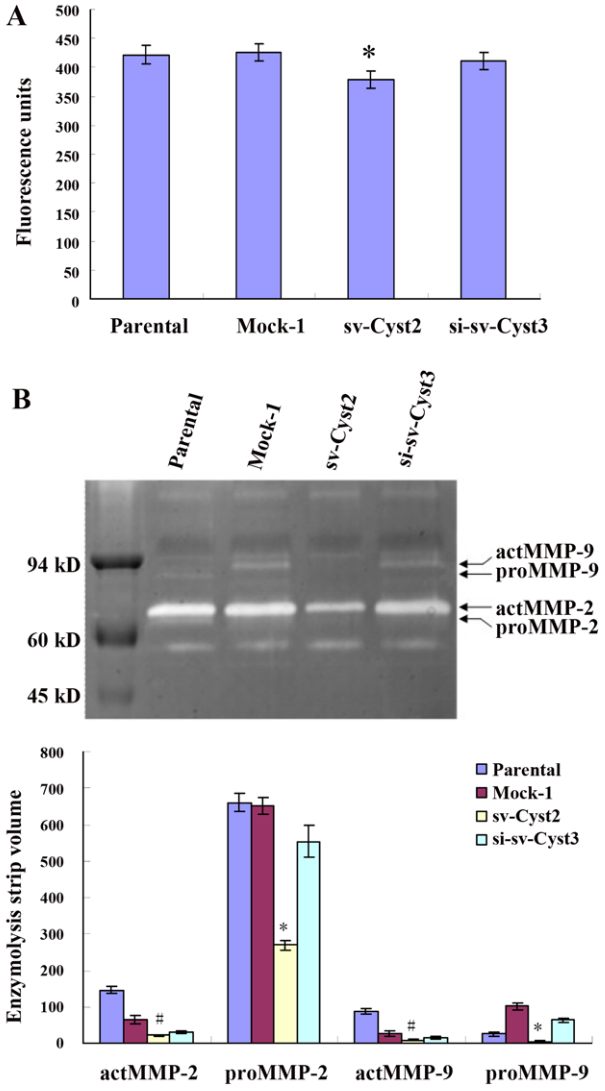

Fig. 4. Expression of sv-cystatin reduces the activity of Cathepsin B, MMP-2 and MMP-9. (**A**) Effect of sv-cystatin on the activity of Cathepsin B. (**B**) Effect of sv-cystatin on the activity of MMP-2 and MMP-9.

level on the cellular membrane of clone sv-cyst2 cells also was observed by immunohistochemistry. These results suggest that the sv-cystatin-induced inhibition of the invasion and metastasis of MHCC97H cells partly correlates with EMT. To clarify the mechanism of EMT induced by sv-cystatin, the transcription factor involved is the primary consideration. Among these transcription factors (including Snail, SIP1, Slug and Twist), Twist was found to play very important roles in regulating the cell EMT (Yang et al., 2004; Lee et al., 2006a). Twist activity by EMSA was lower in the clone sv-cyst2 cells than in the other cells (Fig. 5D), indicating that the interactivation between Twist and E-box in E-cadherin promoter decreased. The NF-κB activity in the parental, mock-1, sv-cyst2 and si-sv-cyst cells were all negative (data not show). This shows that sv-cystatin expression represses the activity of Twist and may contribute to EMT inhibition of MHCC97H cells.

DISCUSSION

Previous studies showed that transfection of the sv-cystatin gene inhibits the invasion and metastasis of mouse melanoma and human gastric carcinoma cells *in vitro* and *in vivo* (Wan et al., 2005, 2006). This study further confirmed that the expression of sv-cystatin in MHCC97H cells (no cystatin M and weak cystatin C mRNA expression) inhibited their invasion and metastasis *in vitro* and *in vivo*, which was examined using the reducing proteinase activity, including cathepsin B, MMP-2 and MMP-9. The activity of cysteine-cathepsins is naturally regulated by cystatins, a group of natural inhibitors (Premzl et al., 2006). Initial studies have revealed the suppression of tumor cell invasion by the stable transfection of human cystatin C, cystatin M and stefins (Shridhar et al., 2004; Sokol et al., 2004; Li et al., 2005). Sv-cystatin, a new cysteine-proteinase inhibitor of the cystatin superfamily, is a type-2 cystatin with a shorter sequence than cystatin M and cystatin C. The amino acid sequence of sv-cystatin shows that it is 25-42% identical to other type-2 cystatins, the most closely related to human cystatin M. sv-cystatin inhibiting cathepsin B might occur via a two-step mechanism, an initial weak interaction followed by a conformational change (Pavlova et al., 2000). In addition, like other type-2 cystatins, another potential physiological target(s) of sv-cystatin could be papain-type cysteine proteases (Mason et al., 2002) or legumin proteases (Alvarez-Fernandez et al., 1999), which can activate the zymogen of matrix metalloproteinase-2 (proMMP-2 or progelatinase A) (Liu et al., 2003). These inhibitions are actually due to a tripartite wedge-shaped structure with very good complementarity to the active site clefts of such enzymes. The three parts of the cystatin polypeptide chain included in the enzyme-binding domain are the N-terminal segment, a central loop-forming segment with the motif Gln-Xaa-Val-Xaa-Gly, and a second C-terminal hairpin loop typically containing a Pro-Trp pair (Alvarez-Fernandez et al., 1999). In particular, the N-terminal segment Gln and central loop-forming motif Gln-Xaa-Val-Xaa-Gly of sv-cystatin are very conservative (Brillard-Bourdet et al., 1998). Overall, these findings suggest that sv-cystatin may be a potent inhibitor of intracellular cathepsin B and MMPs.

The importance of EMT in promoting cancer pro-

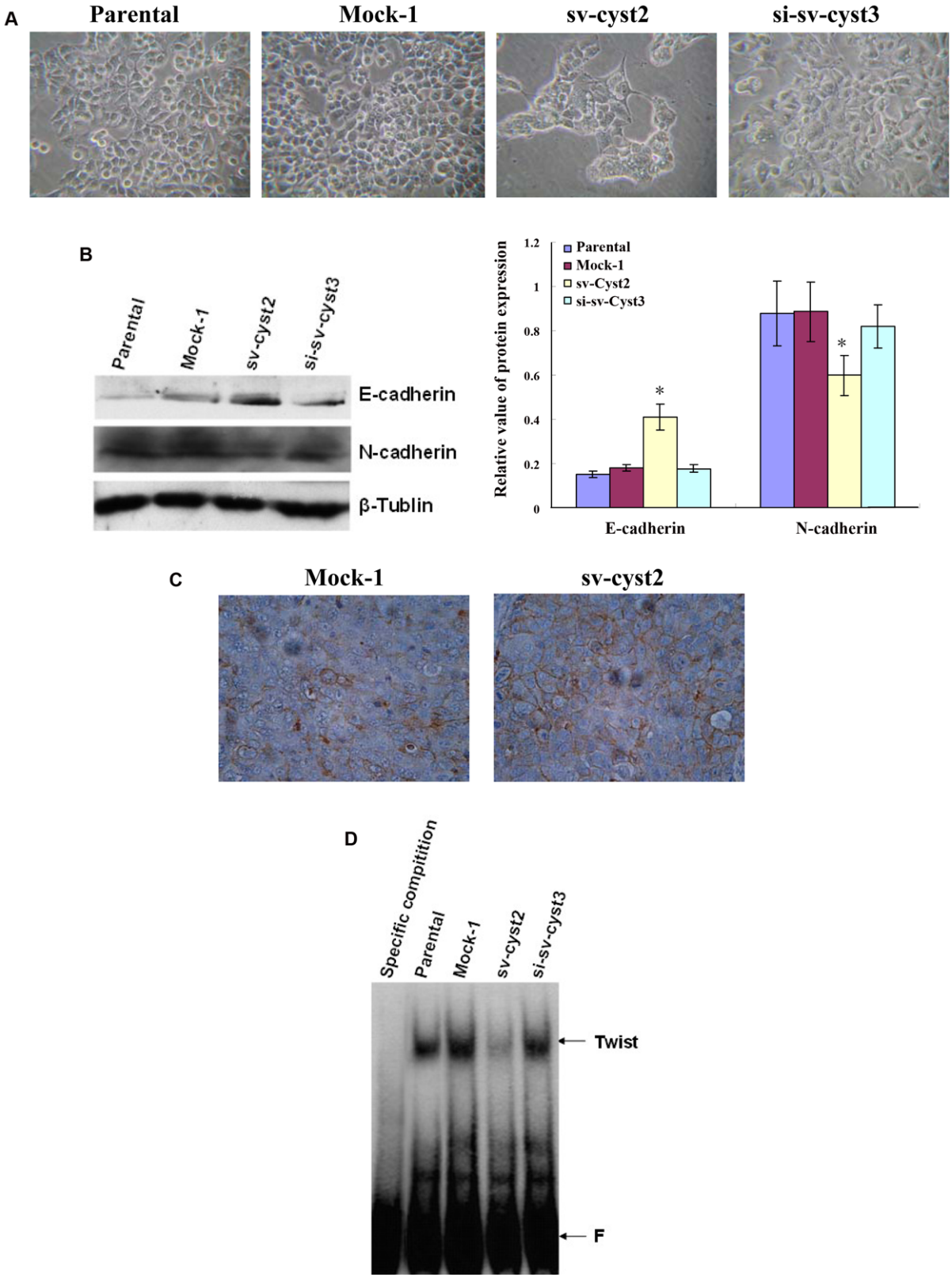

Fig. 5. Expression of sv-cystatin inhibits the EMT of MHCC97H cells. (**A**) Morphology of parental, clone mock-1, sv-cyst2 and si-sv-cyst3 cells (culture for three days). (**B**) Western blotting analysis of E-cadherin and N-cadherin expression. β-tublin was used as the internal loading control. (**C**) Tumor tissues were examined immunohistochemically using the anti-E-cadherin antibody. (**D**) Analysis of the Twist activity by EMSA (F: zone of non-bound free probe).

gression and tumor metastasis is becoming increasingly apparent (Grunert et al., 2003). A hallmark of EMT is the loss of E-cadherin expression, which is a central component of cell-cell adhesion junctions in the maintenance of cell polarity and environment (Hirohashi, 1998), and increased N-cadherin expression (Yang et al., 2007). In HCC, the loss of E-cadherin expression is correlated with tumor invasiveness and metastasis (Zhai et al., 2008). This study found that sv-cystatin expression induces the cells to aggregate with high levels of E-cadherin expression, and sv-cystatin suppresses the EMT of MHCC97H cells. Several EMT-inducing regulators repress E-cadherin transcription via interaction with specific E-boxes of the proximal E-cadherin promoter (Kophengnavong et al., 2000). Recently, the basic helix-loop-helix transcription factor, Twist, a known protein identified in Drosophila melanogaster (Thisse et al., 1987; Peinado and Cano, 2006) as an organizer of the EMT during gastrulation and regulator of mesoderm differentiation, was added to the list of developmental genes with a key role in E-cadherin repression and EMT induction (Lee et al., 2006a). Twist mediates its function by binding to the DNA elements of the NCANNTGN consensus sequence termed E-boxes (Thisse et al., 1987). In this study, a lower Twist activity was also accompanied by increased E-cadherin expression and decreased N-cadherin expression. This suggests that the transduction of sv-cystatin interferes with Twist activation, and dismisses suppression of E-cadherin transcription via an interaction with the specific E-boxes in the promoter, which results in the inhibition of EMT of MHCC97H cells.

In summary, the transfection of human MHCC97H cells with sv-cystatin repressed tumor invasion and metastasis mainly by inhibiting cathepsin B, MMP-2 and MMP-9 activity, as well as EMT. A possible interaction between sv-cystatin and Twist may leads to the loss of Twist activity, which results in EMT regulation. Future studies will examine this mechanism. This study provides evidence that sv-cystatin may play an important role in safeguarding against human liver cancer by reducing the activity of proteinases and EMT, and may contribute to anticancer research of sv-cystatin protein.

ACKNOWLEDGEMENTS

This study was supported by National Natural Science Foundation of China (No.30371747), Key Sci-Tech Research Foundation of Fujian Province (No. 2002Y003), Program for Innovative Sci-Tech Research Team of Universities in Fujian Province (No. FWU-RT001) and Traditional Chinese Medicine Foundation of Fujian Province (No. WZY0614).

REFERENCES

Alvarez-Fernandez, M., Barrett, A. J., Gerhartz, B., Dando, P. M., Ni, J., and Abrahamson, M., Inhibition of mammalian legumain by some cystatins is due to a novel second reactive site. *J. Biol. Chem*., 274, 19195-19203 (1999).

Barrett, A. J., Fluorimetric assays for cathepsin B and cathepsin H with methylcoumarylamide substrates. *Biochem. J*., 187, 909-912 (1980).

Brillard-Bourdet, M., Nguyên, V., Ferrer-di Martino, M., Gauthier, F., and Moreau, T., Purification and characterization of a new cystatin inhibitor from Taiwan cobra (Naja naja atra) venom. *Biochem. J*., 331, 239-244 (1998).

Chiang, H. S., Yang, R. S., and Huang, T. F., Thrombin enhances the adhesion and migration of human colon adenocarcinoma cells via increased beta-3 integrin expression on the tumour cell surface and their inhibition by the snake venom peptide, rhodostomin. *Br. J. Cancer*, 73, 902-908 (1996).

Deep, G. and Agarwal, R., Antimetastatic efficacy of silibinin: molecular mechanisms and therapeutic potential against cancer. *Cancer Metastasis Rev*., 29, 447-463 (2010).

Fedorowski, A., Steciwko, A., and Rabczyñski, J., Serum cathepsin B activity during regression of Morris hepatoma 5123 D. *Med. Sci. Monit*., 10, BR144-BR150 (2004).

Grünert, S., Jechlinger, M., and Beug, H., Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. *Nat. Rev. Mol. Cell Biol*., 4, 657-665 (2003).

Hirohashi, S., Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am. J. Pathol*., 153, 333-339 (1998).

Iwai, S., Nakanishi-Ueda, T., Armstrong, D., and Oguchi, K., Zymographical techniques for detection of matrix metalloproteinases. *Methods Mol. Biol*., 477, 113-121 (2008).

Kophengnavong, T., Michnowicz, J. E., and Blackwell, T. K., Establishment of distinct MyoD, E2A, and twist DNA binding specificities by different basic region-DNA conformations. *Mol. Cell Biol*., 20, 261-272 (2000).

Lah, T. T., Clifford, J. L., Helmer, K. M., Day, N. A., Moin, K., Honn, K. V., Crissman, J. D., and Sloane, B. F., Inhibitory properties of low molecular mass cysteine proteinase inhibitors from human sarcoma. *Biochim. Biophys. Acta*, 993, 63-73 (1989).

Lee, J. M., Dedhar, S., Kalluri, R., and Thompson, E. W., The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J. Cell Biol*., 172, 973-981 (2006a).

Lee, T. K., Poon, R. T., Yuen, A. P., Ling, M. T., Kwok, W. K., Wang, X. H., Wong, Y. C., Guan, X. Y., Man, K., Chau, K. L., and Fan, S. T., Twist overexpression correlates with hepatocellular carcinoma metastasis through induction of

epithelial-mesenchymal transition. *Clin. Cancer Res*., 12, 5369-5376 (2006b).

Li, W., Ding, F., Zhang, L., Liu, Z., Wu, Y., Luo, A., Wu, M., Wang, M., Zhan, Q., and Liu, Z., Overexpression of stefin A in human esophageal squamous cell carcinoma cells inhibits tumor cell growth, angiogenesis, invasion, and metastasis. *Clin Cancer Res*., 11, 8753-8762 (2005).

Liu, C., Sun, C., Huang, H., Janda, K., and Edgington, T., Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. *Cancer Res*., 63, 2957-2964 (2003).

Mason, R. W., Stabley, D. L., Picerno, G. N., Frenck, J., Xing, S., Bertenshaw, G. P., and Sol-Church, K., Evolution of placental proteases. *Biol. Chem*., 383, 1113-1118 (2002).

McKenna, G. J., Chen, Y., Smith, R. M., Meneghetti, A., Ong, C., McMaster, R., Scudamore, C. H., and Chung, S. W., A role for matrix metalloproteinases and tumor host interaction in hepatocellular carcinomas. *Am. J. Surg*., 183, 588-594 (2002).

Mohamed, M. M. and Sloane, B. F. Cysteine cathepsins: multifunctional enzymes in cancer. *Nat. Rev. Cancer*, 6, 764-775 (2006).

Pavlova, A., Krupa, J. C., Mort, J. S., Abrahamson, M., and Björk, I., Cystatin inhibition of cathepsin B requires dislocation of the proteinase occluding loop. Demonstration by release of loop anchoring through mutation of his110. *FEBS Lett*., 487, 156-160 (2000).

Peinado, H. and Cano, A., New potential therapeutic targets to combat epithelial tumor invasion. *Clin. Transl. Oncol*., 8, 851-857 (2006).

Premzl, A., Turk, V., and Kos, J., Intracellular proteolytic activity of cathepsin B is associated with capillary-like tube formation by endothelial cells in vitro. *J Cell Biochem*., 97, 1230-1240 (2006).

Shridhar, R., Zhang, J., Song, J., Booth, B. A., Kevil, C. G., Sotiropoulou, G., Sloane, B. F., and Keppler, D., Cystatin M suppresses the malignant phenotype of human MDA-MB-435S cells. *Oncogene*, 23, 2206-2215 (2004).

Sokol, J. P. and Schiemann, W. P., Cystatin, C. antagonizes transforming growth factor β signaling in normal and cancer cells. *Mol. Cancer Res*., 2, 183-195 (2004).

Song, J., Wan, R., Weng, S. M., Lin, X., and Lin, J. Y., Gene synthesis of cystatin from snake venom and its expression in E. coil. *Fu Jian Yi Ke Da Xue Xue Bao*, 38, 241-245 (2004).

Tang, Z. Y., Hepatocellular carcinoma surgery: review of the past and prospects for the 21st century. *J. Surg. Oncol*., 91, 95-96 (2005).

Thisse, B., el Messal, M., and Perrin-Schmitt, F., The twist gene: isolation of a Drosophila zygotic gene necessary for the establishment of dorsoventral pattern. *Nucleic Acids Res*., 15, 3439-3453 (1987).

Tian, J., Tang, Z. Y., Ye, S. L., Liu, Y. K., Lin, Z. Y., Chen, J., and Xue, Q., New human hepatocellular carcinoma (HCC) cell line with highly metastatic potential (MHCC97) and its expressions of the factors associated with metastasis. *Br. J. Cancer*, 81, 814-821 (1999).

Wan, R., Zheng, H., Song, J., Lin, X., and Lin, J. Y., The studies on effect of snake venom cystatin for invasion and metastasis of the human gastric carcinoma cell line. *Chinese Journal of Clinical Oncology*, 32, 961-965 (2005).

Wan, R., Zheng, H.Y., Song, J., Weng, S. M., Lin, X., and Lin, J. Y., The effect of cysteine protease inhibitor of snake venom (sv-cystatin) on mouse melanoma cells invasion *in vitro* and *in vivo*. *Jie Pou Xue Bao*, 37, 640-645 (2006).

Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R. A., Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*, 117, 927-939 (2004).

Yang, Z., Zhang, X., Gang, H., Li, X., Li, Z., Wang, T., Han, J., Luo, T., Wen, F., and Wu, X., Up-regulation of gastric cancer cell invasion by Twist is accompanied by N-cadherin and fibronectin expression. *Biochem. Biophys. Res. Commun*., 358, 925-930 (2007).

Yeh, C. H., Peng, H. C., Yang, R. S., and Huang, T. F., Rhodostomin, a snake venom disintegrin, inhibits angiogenesis elicited by basic fibroblast growth factor and suppresses tumor growth by a selective alpha(v)beta(3) blockade of endothelial cells. *Mol. Pharmacol*., 59, 1333-1342 (2001).

Zhai, B., Yan, H. X., Liu, S. Q., Chen, L., Wu, M. C., and Wang, H. Y., Reduced expression of E-cadherin/catenin complex in hepatocellular carcinomas. *World J. Gastroenterol*., 14, 5665-5673 (2008).