Silencing of Cathepsin B suppresses the proliferation and invasion of endometrial cancer

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Abstract. The molecular mechanism involved in the metastasis of endometrial cancer (EC) remains unclear. The lysosomal cysteine protease Cathepsin B has been implicated in the progression of various human tumors. In the present study, we assessed the expression of Cathepsin B and its functions in EC. Immunohistochemistry was used to examine Cathepsin B expression in 76 paraffin-embedded endometrial tumor tissues. Lentiviral packing short hairpin RNA (shRNA) was transfected into HEC-1A cells to build a stable Cathepsin B knockdown cell line. The cellular levels of Cathepsin B mRNA and protein were detected by real-time PCR and western immunoblotting. The functions of Cathepsin B in EC cells were measured by MTT, migration and invasion assays. In additon, tumorigenicity assays were established in nude mice to study tumor growth *in vivo*. The results of our study showed that Cathepsin B was overexpressed in EC tissues compared with normal endometrium and endometrial atypical hyperplasia. Depletion of Cathepsin B *in vitro* inhibited cell proliferation, migration and invasion. Tumor formation assays confirmed that suppression of Cathepsin B inhibited the proliferation potential of HEC-1A cells *in vivo*, demonstrated by lower proliferation rates. These results suggest that Cathepsin B may act as an oncogene in EC, with the potential to provide a new therapeutic target for treating endometrial malignancy.

Introduction

Endometrial cancer (EC) is the most common gynecological malignancy affecting women in the Western world. It was predicted that 47,130 new cases would be diagnosed in the USA in 2012 resulting in 8,010 disease-related deaths (1). Localized disease is preferentially treated surgically by hysterectomy with or without adjuvant treatment. This results in 5-year survival rates of approximately 96%. However, 30% of all EC cases remain undiagnosed until regional or distant metastasis are present, resulting in much poorer prognosis and survival (2). Thus, there is a need to improve our understanding of the molecular and cellular mechanisms responsible for the development of EC, and to develop novel therapeutic strategies to prevent EC progression.

The tumor microenvironment plays an important role in the development and progression of EC. The extracellular matrix (ECM) is extensively remodeled or digested by matrix metalloproteinases and other proteases produced by cancer and stromal cells. The process is mediated by a number of different proteases that catalyze the degradation of the basement membrane that otherwise would confine the spread of the solid primary tumor. These enzymes include cysteine, serine and aspartic proteases (3), matrix metalloproteinases (4), vascular endothelial growth factors (5) and kallikreins (6).

Cathepsins are believed to play a housekeeping role in terminal protein degradation within the lysosome. Previous evidence indicates that extracellular cathepsins play a central role in cancer metastasis by altering ECM remodeling and facilitating invasion (3,7,8).

Cathepsin B is a lysosomal cysteine protease which belongs to a family of 11 cysteine proteases (Cathepsins B, C, H, F, K, L, O, S, V, W and X/Z) (9). In benign cells, it is synthesized as a pre-pro-enzyme that is mainly stored in the lysosome (10). In malignant cells, Cathepsin B is secreted from the lysosomes and contributes to degradation of the basement membrane of the cell, thereby facilitating invasion (11). Accumulating evidence has implicated Cathepsin B in human breast (12), lung (13), colorectal (14) and ovarian (15) cancer.

It has recently been reported that increased Cathepsin B acts as an unfavorable and independent tumor marker for EC (16). However, little information is available about the precise function of Cathepsin B in EC carcinogenesis. In the present study, we investigated the association between Cathepsin B expression and clinical outcome in patients with EC. We also investigated the role of Cathepsin B in promoting EC carcinogenesis *in vitro* and *in vivo*.

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Table I. Relationship between Cathepsin B expression and clinicopathological factors in endometrial cancer.

Variable	No. of patients (%)	Cathepsin B expression		P-value
		Negative	Positive	
Total	76 (100)	11	65	
Age (years)				
≤50	15 (19.7)	3	12	0.446
>50	61 (80.3)	8	53	
FIGO stage				
I	60 (78.9)	6	54	0.091
II	6 (7.8)	2	4	
III	8 (10.5)	3	5	
IV	2 (2.8)	0	2	
Grade (endometrioid, n=64)				
G1	30 (46.8)	5	25	0.867
G2	24 (37.5)	4	20	
G3	10 (15.7)	1	9	
Histological type				
Endometrioid	64 (84.2)	10	54	1.000
Non-endometrioid	12 (15.8)	1	11	
Myometrial invasion				
<1/2	61 (80.2)	7	54	0.212
≥1/2	15 (19.8)	4	11	
Lymph node metastasis				
No	67 (89.1)	11	56	0.342
Yes	9 (10.9)	0	9	
Lymphovascular space involvement				
No	58 (76.3)	10	48	0.442
Yes	18 (23.7)	1	17	
ER expression				
Negative	17 (22.4)	3	14	0.702
Positive	59 (77.6)	8	51	
PR expression				
Negative	13 (17.1)	3	10	0.388
Positive	63 (82.9)	8	55	

Materials and methods

Patients and samples. Samples were collected from 76 patients diagnosed with uterine EC at the International Peace Maternity and Child Health Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, between August 2009 and April 2011. None of the patients had undergone hormone therapy, radiotherapy, or chemotherapy prior to surgery. All cases were classified and graded according to the criteria of the International Federation of Obstetrics and Gynecology (FIGO 2009) (17). The characteristics of all EC tissue samples are provided in Table I.

Twenty normal endometrial samples were obtained from patients undergoing hysterectomy for other conditions such as myoma or adenomyosis. Fifteen endometrial atypical hyperplasia (EAH) tissues were collected from patients undergoing hysteroscopic examination for irregular bleeding.

The study was approved by the Ethics Committee of the Medical Faculty of Shanghai Jiao Tong University. Informed consent was obtained from all patients.

Immunohistochemistry. Tissue sections (4 μm) were processed for hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC) as previously described (18). Rabbit monoclonal antibodies Cathepsin B (3547-1) were purchased from Epitomics (Burlingame, CA, USA).

Cathepsin B expression was evaluated in terms of staining intensity, scored as 0 (negative), 1 (weak), 2 (medium), or 3 (strong). The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%), according to the percentage of the positively stained areas in relation to the whole tumor area. The sum of the intensity score and extent scores was used as the final staining score (0-7) (19).

Table II. shRNA oligo sequences.

Oligonucleotide	Sequences
shRNA-CTSB#1	F: CCGGGGATCACTGTGGAATCGAATTCAAGAGATTCGATTCCACAGTGATCCTTTTTTG
shRNA-CTSB#1	R: AATTCAAAAAAGGATCACTGTGGAATCGAATCTCTTGAATTCGATTCCACAGTGATCC
shRNA-CTSB#2	F: CCGGCCACATTTGTCACAGAAATTTCAAGAGAATTTCTGTGACAAATGTGGTTTTTTG
shRNA-CTSB#2	R: AATTCAAAAAACCACATTTGTCACAGAAATTCTCTTGAAATTTCTGTGACAAATGTGG
shRNA-CTSB#3	F: CCGGCCAACACGTCACCGGAGAGATCTCGAGATCTCTCCGGTGACGTGTTGGTTTTTTG
shRNA-CTSB#3	R:AATTCAAAAAACCAACACGTCACCGGAGAGATCTCGAGATCTCTCCGGTGACGTGTTGG
shRNA-CTSB#4	F: CCGGGCTGGTCAACTATGTCAACAACTCGAGTTGTTGACATAGTTGACCAGCTTTTTTG
shRNA-CTSB#4	R: AATTCAAAAAAGCTGGTCAACTATGTCAACAACTCGAGTTGTTGACATAGTTGACCAGC
shRNA-NT	F: CCGGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTG
shRNA-NT	R: AATTCAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAA

F, forward; R, reverse.

The results were assessed by two pathologists who were blinded to details regarding patient background.

The formalin-fixed, paraffin-embedded sections of xenografted tumors from nude mice were analyzed using standard avidin-biotin immunohistochemical techniques following exposure to anti-Ki-67 antibody and anti-PCNA antibody (1:100; Wuhan Boster Bio-Engineering Co., Wuhan, China) according to the manufacturer's instructions. Labeled cell nuclei in tumor sections were regarded as positive.

Vector construction. Four short hairpin RNA (shRNA) oligonucleotides targeting Cathepsin B and one non-target oligonucleotide were designed and inserted into lentiviral vector pMAGic 4.0 at the sites of *Age*I (R0552S; New England Biolabs UK Ltd., UK) and *Eco*RI (R0101S; New England Biolabs UK). The sequences used are shown in Table II.

Cell culture and lentiviral infection. The human EC cell line HEC-1A was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences and cultured with DMEM/F12 supplemented with 10% FBS. To generate EC cell lines expressing shRNAs, HEC-1A cells were infected with viral supernatant containing non-target (NT) or CTSB-specific shRNA lentiviral particles, in the presence of polybrene (6 μg/ml). Cells were treated with puromycin (2 μg/ml) to generate stable Cathepsin B knockdown clones.

Real-time PCR analysis. Total RNAs were extracted using TRIzol reagent (Invitrogen, Life Technologies, Shanghai, China). For Cathepsin B mRNA detection, RNAs were reverse transcribed according to the manufacturer's protocol (Takara, Dalian, China). Real-time PCR analysis was performed using SYBR-Green (Takara) on an ABI Prism 700 thermal cycler (Applied Biosystems, Foster City, CA, USA).

Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ formula. The following primers were used: Cathepsin B, 5'-CTG TCG GAT GAG CTG GTC AAC-3' (sense) and 5'-TCG GTA AAC ATA ACT CTC TGG GG-3' (antisense); GAPDH, 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' (sense) and 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3' (antisense).

Western blot analysis. Cells were washed with PBS once and harvested in 10% SDS. The extracted proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were first blocked with 5% BSA in TBST and then probed with the indicated primary antibodies at room temperature for 1.5 h. After washing three times, the membranes were incubated with the appropriate peroxidase-conjugated secondary antibodies for 1 h. The signals were detected using an enhanced chemiluminescence kit (GE Healthcare). The antibodies used were Cathepsin B (1:1,000; Epitomics), GAPDH (1:1,000; Epitomics) and peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell proliferation assay. Cells were plated into 96-well plates including three control wells with medium alone that provided blanks for absorbance readings. After 48 or 72 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reagent was added to each well (including the control wells) according to the manufacturer's protocol (Sigma, St. Louis, MO, USA). After exposure for 24, 48, 72, 96 and 120 h the cells were incubated for 4 h after which the medium was discarded. DMSO (150 μl) was added to all wells and absorbance was measured in 490 nm (Bio-Rad Laboratories).

Migration and invasion assays. Cell migration and invasive ability was examined using 24-well Transwell plates with 8.0 μm pore, polycarbonate membrane inserts. The experiments were performed according to the manufacturer's protocol (Corning).

For the invasion assay, the upper side of the membranes was coated with 100 μg Matrigel (BD), while the migration assay was not. Then, $1x10^5$ cells per well (200 μl/chamber) were seeded into the top chamber in serum-free media; 600 μl of complete medium was added to the lower chamber. Cells that invaded through the surface of the membrane were fixed with methanol and stained with crystal violet after 48 or 72 h.

Non-invasive cells were scraped from the top of the Transwell plate with a cotton swab. Cells from five random microscope field per filter were selected for counting.

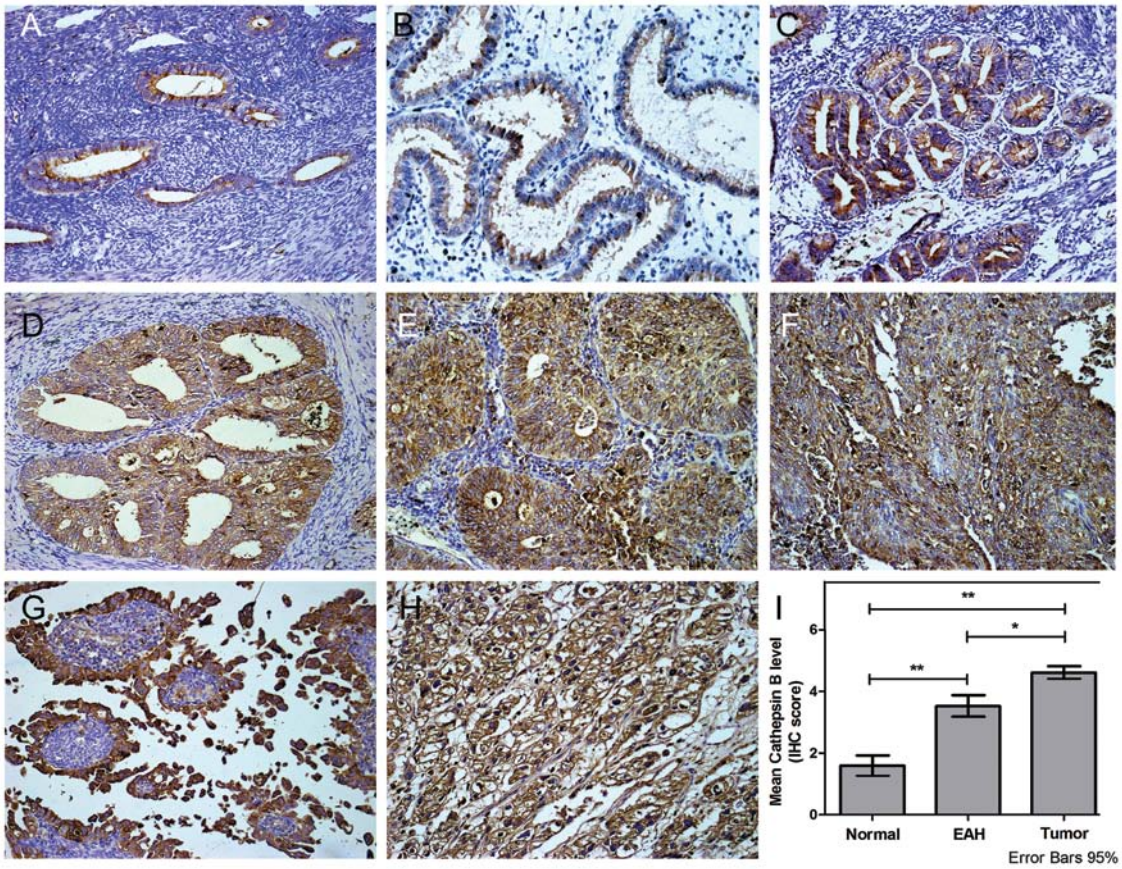

Figure 1. Immunohistochemical analysis of Cathepsin B in normal endometrium, endometrial atypical hyperplasia (EAH) and endometrial cancer (EC). (A and B) Weak expression of Cathepsin B in the proliferative and secretory phase of normal endometrium. (C) Moderate expression of Cathepsin B in EAH. (D-H) Strong expression of Cathepsin B in cytoplasm and cell membrane of endometrioid adenocarcinoma G1 (D) endometrioid adenocarcinoma G2 (E), endometrioid adenocarcinoma G3 (F), uterine papillary serous carcinoma (G) and endometrial clear cell carcinoma (H) (magnification, x200). (I) Statistical results of immunostaining score in normal endometrium, EAH and EC (*P<0.05, **P<0.01).

Xenograft tumor formation assays. Eighteen female BALB/c nude mice (5 weeks of age) were obtained from the Chinese Academy of Sciences, Shanghai, China. The mice were housed under a laminar flow hood in an isolated room using protocols approved by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine. HEC-1A and two other stable HEC-1A derived cell-lines (HEC-1A NT and HEC-1A sh-CTSB) were harvested and resuspended at a density of $5x10^6$ cells/200 μl of sterile saline.

Six mice per group were subcutaneously injected with different EC cell lines in the subdermal space on the medial side of the neck. Tumor volume was measured weekly for 4 weeks, until the end of the experiment. Tumor volume was calculated using the formula: largest diameter x smallest diameter^2 x 0.5. Tumor weight was determined after the animals were sacrificed at the end of the xenograft experiments.

Statistical analysis. Statistical analyses were performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). Data are represented as means and standard deviations (±SD). Numerical data were analyzed using unpaired Student's t-tests. One-way analysis of variance (ANOVA) was used for multiple comparisons. Chi-square tests were used to compare the categorical data. Values of P<0.05 were considered to indicate statistically significant differences.

Results

Cathepsin B is highly expressed in EC tissues. The expression of Cathepsin B protein in EC cells was analyzed by IHC. Diffuse positive Cathepsin B immunostaining was observed throughout the cytoplasm and occasionally on the cell membrane. Extracellular localization was also noted. Moderate and weak Cathepsin B immunoreactivity was seen in EAH and normal endometrial tissues (Fig. 1A-C), while strong immunoreactivity was observed in type I (Fig. 1D-F) and type II EC (Fig. 1G-H) cancer.

Of the 76 tumor samples analyzed, 64 tumors showed cytoplasmic and cell membrane expression of Cathepsin B (IHC score ≥4). In all cases this was significantly higher than that in normal endometrium or EAH tissue (Fig. 1I). No significant association was found between patient age, FIGO staging, pathological grade, histological type, myometrial invasion, lymph node metastasis, lymphovascular space involvement or expression of estrogen (ER) or progesterone receptor (PR) (P>0.05; Table I).

Cathepsin B expression is suppressed by RNA interference. RNA interference oligonucleotides (shRNA-CTSB#1, shRNA-CTSB#2, shRNA-CTSB#3 and shRNA-CTSB#4) which targeted Cathepsin B and non-target shRNA-NT were

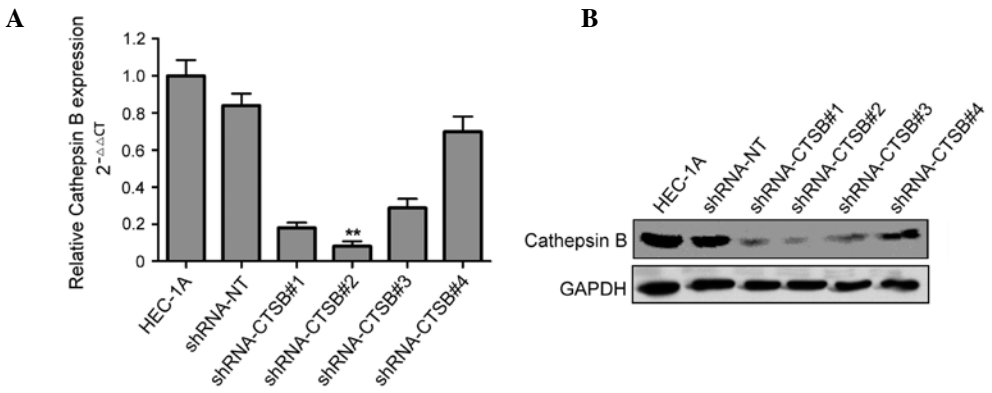

Figure 2. Cathepsin B suppression by RNA interference. (A) The expression of Cathepsin B was analyzed by qRT-PCR in HEC-1A cells infected by different Cathepsin B shRNA oligonucleotides. shRNA-CTSB#2 resulted in maximum inhibition compared with both parent cells and shRNA-NT (**P<0.01). In these experiments GAPDH was used as an internal control. (B) Protein levels of Cathepsin B were analyzed by western blot analysis with GAPDH as an internal control.

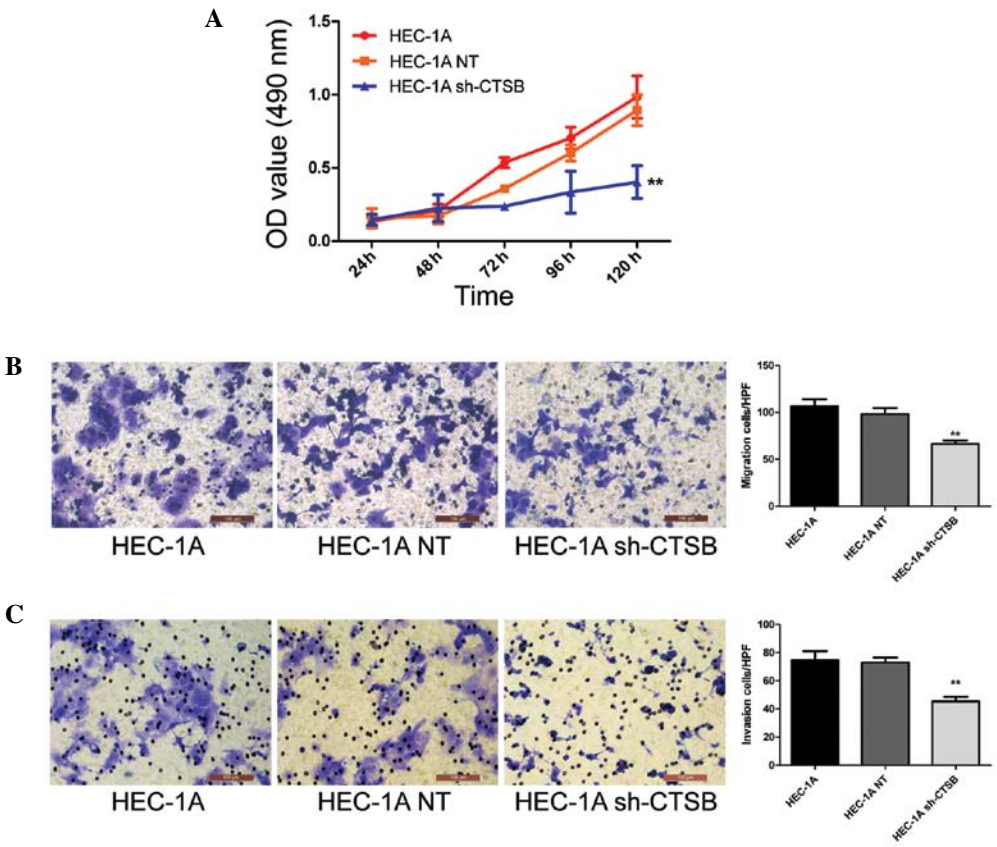

Figure 3. Suppression of Cathepsin B inhibits the proliferation, migration and invasion of EC cell lines. (A) The growth of HEC-1A, HEC-1A NT and HEC-1A sh-CTSB cells was analyzed by MTT assay. Growth was significantly inhibited in cells with Cathepsin B knockdown (**P<0.01). (B and C) Cell migration and invasion were tested with Transwell assays with and without Matrigel. Cells in the HEC-1A sh-CTSB group showed a much lower penetration rate than cells in the HEC-1A and HEC-1A NT groups (**P<0.01). In these experiments, cells were counted with a microscope in five high-powered fields (magnification, x200). Bars show means ± SD. All experiments were repeated in triplicate.

synthesized and built into a lentiviral vector. HEC-1A cells were infected by viral supernatant, enabling stable cell lines to be established.

Western blot analysis and qRT-PCR indicated that both mRNA and protein levels of Cathepsin B were suppressed by Cathepsin B shRNA (Fig. 2A and B). Among four shRNA oligonucleotides which were designed to target Cathepsin B, shRNA-CTSB#2 showed maximum inhibition efficiency at both mRNA and protein levels and was chosen for subsequent studies.

Suppression of Cathepsin B inhibits the proliferation, migration and invasion of EC cell lines. To explore whether the suppression of Cathepsin B inhibits the growth of EC cell lines, HEC-1A, HEC-1A NT and HEC-1A sh-CTSB cells were seeded into 96-well plates. MTT assays performed every 24 h

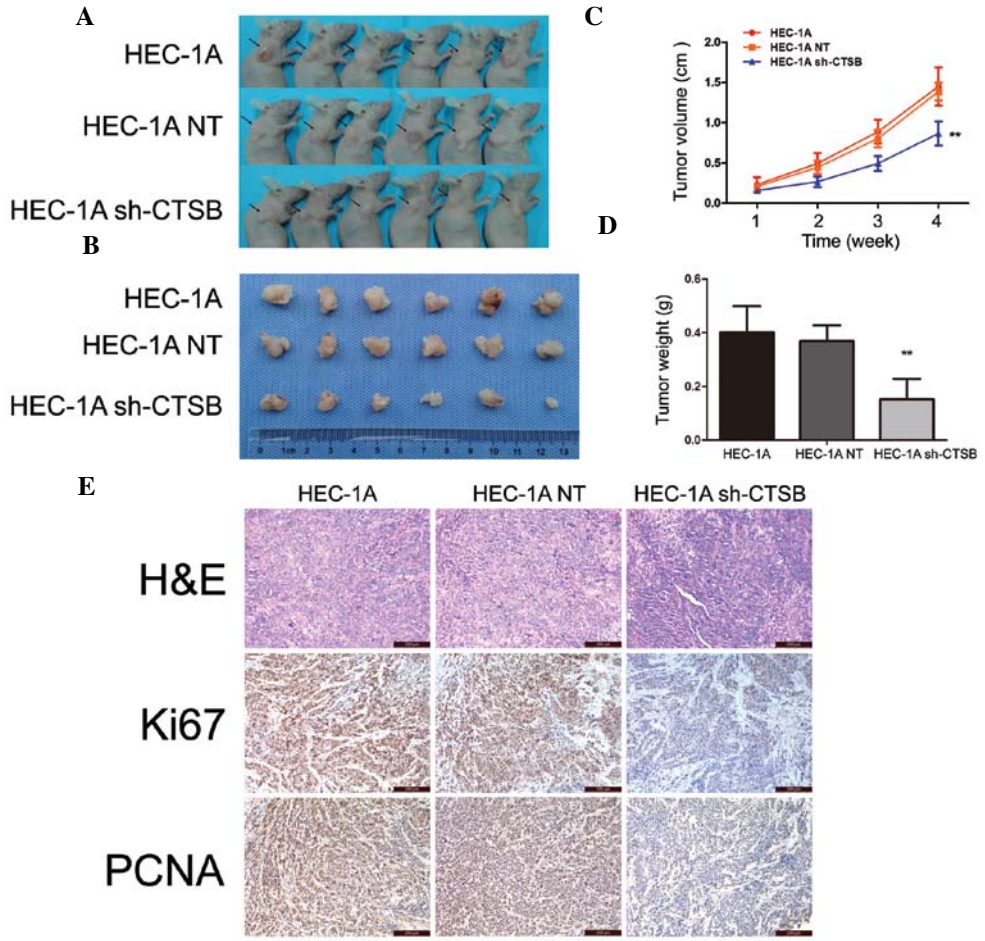

Figure 4. Suppression of Cathepsin B inhibits tumor growth *in vivo*. (A) Tumor burden in mice from different groups is indicated by black arrows. (B and C) After 4 weeks, tumor volume in the HEC-1A sh-CTSB group was lower than in both the HEC-1A and HEC-1A NT groups. (D) Tumor weight in the HEC-1A sh-CTSB group was lower than in the HEC-1A and HEC-1A NT groups (**P<0.01). The data are presented as means ± SD. (E) H&E staining and immunohistochemistry showing Ki-67 and PCNA expression levels in tumor xenograft tissues. Both Ki-67 and PCNA expression were lower in the HEC-1A sh-CTSB group than in the HEC-1A and HEC-1A NT groups (magnification, x100).

demonstrated that HEC-1A sh-CTSB inhibited cell proliferation compared with both HEC-1A and HEC-1A NT cells (P<0.01) (Fig. 3A). We also performed Transwell migration and invasion assays to investigate the effects of Cathepsin B on the migratory and invasive behaviors of EC cells *in vitro*. The results showed that cells in the HEC-1A sh-CTSB group had a much lower penetration rate than cells in the HEC-1A and HEC-1A NT groups (P<0.01) (Fig. 3B and C). No significant differences were seen between the HEC-1A and HEC-1A NT groups.

Suppression of Cathepsin B inhibits tumor growth in vivo. Animal studies were conducted to evaluate the effect of Cathepsin B on tumor growth in nude mice. HEC-1A, HEC-1A NT and HEC-1A sh-CTSB cells ($5x10^6$ cells/200 μl of sterile saline) were subcutaneously injected in the subdermal space on the medial side of the neck. After 4 weeks, tumor volume was smaller in the HEC-1A sh-CTSB group than in the HEC-1A and HEC-1A NT groups (P<0.01) (Fig. 4A-C). Tumor weight determined after sacrificing the animals at 4 weeks showed the same results as tumor volume (P<0.01) (Fig. 4D). In addition, nuclear expression of Ki-67 and PCNA was lower in the HEC-1A sh-CTSB group than in the HEC-1A and HEC-1A NT groups (Fig. 4E).

Discussion

Endometrial cancer (EC) spreads by direct extension through the myometrium, by exfoliation of cells that are shed through the fallopian tubes or by lymphatic and/or hematogenous dissemination (20). The most common route of EC spread is the direct extension of the tumor to the myometrium (21). Localized invasion and metastasis of EC results from several interdependent processes involving proteolytic enzymes (22). Among several cancer invasion-related characteristics, release of tumor-derived proteases is thought to break the basement membrane and extracellular matrix, thereby promoting cancer cell invasion into surrounding normal tissues and facilitating distant metastasis through lymphovascular channels (23).

In the present study, we showed that Cathepsin B is highly expressed in human EC. Using preclinical models, we demonstrated specific knockdown of Cathepsin B in the EC cell line HEC-1A led to inhibition of tumor growth and invasion *in vitro* and *in vivo*. These results suggest that Cathepsin B may be a useful diagnostic biomarker of metastatic potential in EC.

Previous studies have demonstrated that Cathepsin B immunostaining in EC is associated with cancer progres-

sion. Immunohistochemical studies (24) reported that the malignant endometrium displays higher Cathepsin B activity than benign tissue samples. A recent study (16) examined Cathepsin B protein levels in 64 paraffin-embedded endometrial tumor tissues, and found 27 of the 46 tumors (42.2%) were Cathepsin B positive.

In the present study, we found that the normal endometrium produces weak levels of Cathepsin B (mean IHC score <2), whereas extensive expression was detected in 76 cases of EC (mean IHC score >4). Previous studies have reported that Cathepsin B positivity is significantly associated with the FIGO stage of the disease as well as cervical and stromal invasion (16). However, our results showed no evidence of a relationship between high Cathepsin B levels and clinicopathological factors in EC. Differences in racial background, and sample sizes between the two studies may explain these apparently discrepant findings. Thus, further investigation is required to determine whether Cathepsin B is an independent prognostic factor for EC.

Cathepsin B has been associated with enhanced malignant potential (such as proliferation, migration and invasion) in several different types of cancer (12,18,25). However, its precise role in the carcinogenesis of EC remains uncertain. In the present study, we investigated the malignant characteristics of Cathepsin B in the EC cell line HEC-1A using shRNA transfection. We showed that inhibition of Cathepsin B significantly decreased the proliferation of cancer cells, and that suppression of Cathepsin B significantly attenuated migration and invasive activity of HEC-1A cells. These findings are in agreement with those reported for other types of tumors (26,27). Taken together, these results suggest Cathepsin B has metastatic potential in EC cells.

The tumor microenvironment is known to modulate the expression of cathepsin B in tumor cells and in other cell types (such as stromal fibroblasts) associated with tumors (28). Our *in vivo* experiments indicated that tumor volume and weight were significantly reduced by suppression of Cathepsin B. This finding is consistent with a recent study of Cathepsin B in breast cancer (29). We also demonstrated lower proliferation indexes (Ki-67 and PCNA) in xenograft tumor tissues from the Cathepsin B knockdown group. This finding is consistent with our results *in vitro*, and suggests that Cathepsin B overexpression may facilitate tumor growth, while reduced expression may suppress EC growth and development.

It has previously been shown that cathepsin D converts pro-cathepsin B into active cathepsin B (30). Overexpression of cathepsin D has been reported in adenomatous hyperplasia, but not in endometrial adenocarcinomas, and thus it has been regarded as a possible index for malignant transformation (3). Additional evidence suggests that Cathepsin B can be activated by other proteases, including cathepsin G, urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA) and elastase, all of which interact with Cathepsin B in the role of modulating tumor invasion (31,32). Other investigators have shown that Cathepsin B undergoes auto-activation under certain conditions, adding to the possible mechanisms that might regulate cathepsin B (33). Thus, further studies are required to delineate the regulation of Cathepsin B functions and to elucidate the mechanisms that underlie its oncogene activities in EC.

In summary, our results show that Cathepsin B expression is higher in EC than in normal endometrium and that knockdown of Cathepsin B inhibits the proliferation, migration and invasion of EC cell lines both *in vitro* and *in vivo*. High expression of Cathepsin B, therefore, appears to play an important role in tumorigenesis and progression of EC. These findings indicate that Cathepsin B has potential as a new molecular target for EC therapy.

Acknowledgements

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