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Cathepsin L is involved in proliferation and invasion of breast cancer cells

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Cathepsin L(CTSL), a lysosomal endopeptidase was found overexpressed in Breast cancer (BC). The purpose of this work was to investigate the possible role of CTSL in the development of BC. RNA interference(RNAi) with a CTSL small hairpin RNAs(CTSL-shRNA) and plasmid with CTSL were used to identify the effects of CTSL on malignant behaviors of BC. MCF-7 and SKBR-3 were selected as cell models in vitro and in vivo. The results showed that down-regulation of CTSL can significantly inhibit the proliferative and invasive ability of MCF-7 cell, while up-regulation of CTSL in SKBR-3 cells had opposite effects. Comparing to parental BC cells, CTSL knockdown cells exhibited attenuated capacities in developing tumor in nude mice, furthermore, the growth of these xenografts were dramatically regressed. In conclusion, our findings suggest that CTSL contributes to the proliferation and metastasis of BC and might be a potent molecular target for BC treatment.

Key words: breast cancer, cathepsin L, invasion, proliferation

Approximately 232,340 new cases of invasive breast cancer and 39,620 breast cancer deaths occurred among US women in 2013. One in eight women in the United States will develop breast cancer in her life time [1, 2]. Recent advances in breast cancer detection and treatment have decreased the mortality rate of breast cancer, but rely largely on detection of the disease at early stages. A lack of knowledge regarding the molecular mechanisms underlying breast tumor progression to invasive and then metastatic disease limits our ability to treat advanced disease. The identification of factors that promote metastasis is essential for the development of new breast cancer therapies and a further reduction in breast cancer mortality.

Tumor development, neovascularization, and metastatic spread depend on the ability of cancer cells to invade tissue barriers in a process involving degradation of the extracellular matrix (ECM) and basement membrane structures [3]. Basement membranes underlie epithelial and endothelial cell layers and form a structural network of characteristic proteins and polysaccharides. Additionally, expression of many ECM remodeling enzymes is often deregulated in human cancers. For example, heparanases, sulfatases, and matrix metalloproteinases (MMPs) are frequently overexpressed in various cancers. CTSL, a lysosomal endopeptidase expressed in most eukaryotic cells, is a member of the papain-like family of cysteine proteinases. Although commonly recognized as a lysosomal protease, CTSL is also secreted. This broad-spectrum protease is potent in degrading several extracellular proteins (laminins, fibronectin, collagens I and IV, elastin, and other structural proteins of basement membranes) as well as serum proteins and cytoplasmic proteins [4, 5]. CTSL plays a major role in antigen processing, tumor invasion and metastasis, bone resorption, and turnover of intracellular and secreted proteins involved in growth regulation, such as transforming growth factor-β (TGF-β),basic fibroblast growth factor (bFGF) and epidermal growth factor receptors (EGFR) [6-12]. Increased CTSL level was found in multiple tumor types and associated with short survival of several cancers [13-17].

Previous study has demonstrated that CTSL is overexpressed in breast cancer cells and considered to play a role in bone degradation mediated by metastatic breast cancer[18]. Furthermore, CTSL is overexpressed in breast cancer tissues inversely correlated with patients' survival [19].However, it

remains largely unknown about whether inhibition of CTSL expression can affect on the biological function of breast cancer cells. This study analyzed the effects CTSL on cultured BC cells, suggesting that expression of CTSL affects the growth and invasion of BC cells.

Materials and methods

Cell lines and reagents. The human breast cancer cell lines MCF-7, SKBR-3, MDA-MB-453 and MDA-MB-231cellswere obtained from the American Type Culture Collection, and Human Breast Epithelial cells MCF-10A was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Science. These cells were cultured in Dulbeccos'smodified Eagles medium (DMEM) (Hyclone laboratories Inc, South Logan, Utah, USA) supplemented with 10% fetal calf serum (FCS), Invitrogen, Grand Island, NY, USA), 100U/mL penicillin, and 100U/mL streptomycin (Hyclone laboratories Inc, South Logan, Utah, USA). Cell cultures were performed at 37°C in humidified air with 5% CO_2.

RNA isolation, qRT-PCR. Total cellular RNA was isolated from four BC cell lines and Human Breast Epithelial cells MCF-10Ausing Trizol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions and quantified using UV spectrophotometer. RNA (2μg) was reverse transcribed using an access reverse transcription system (Promega, Madison, Madison, USA) according to the standard protocol. In brief, reaction mixtures (total volume, 20μl) containing 500ng cDNA were amplified at a final concentration of 250nM using 10μl of 2× Brilliant SYBR Green QPCR Master Mix kit (Stratagene, Foster City, California, USA). The primers were: CTSL-forward: 5'-CTGGTGGTTGGCTACGGATT-3';CTSL-reverse: 5'-CTCCGGTCTTTGGCCATCTT-3'(GenBank:NC_000009.12); β-actin-forward:5'-TAAGAAGCTGCTGTGCTACG-3'; β-actin-reverse:5'-GACTCGTCATACTCCTGCTT-3'(GenBank:NM_001101).Morever, Thermal cycling conditions were as follows: 94°C for 5 minutes and 45 cycles at 94°C for 30 seconds, followed by 60°C for 30 seconds and 72°C for 45 seconds. Experiments were performed in triplicate in the same reaction. Target genes andβ-actin gene were amplified in the same reaction. The results of relative quantitative were analyzed by comparison of $2^{-\Delta\Delta Ct}$.

Vector construction and transfection. The pcDNA3.0 vector (Cyagen,Guangzhou,China) was used to generate pcDNA-CTSL. The CTSL shRNA Plasmid was purchased from Santa Cruz Biotechnology (Cat. No:sc-29939-SH). Vector transfection was performed according to the instructions, SKBR-3 cells were transfected with pcDNA expressing CTSL or empty vector and MCF-7 were used to knock-down the expression of CTSL. SKBR-3 cells expressing CTSL or empty vector were selected for 14 days with G418 after transfection. MCF-7 transfected with CTSL-shRNA was selected for 14 days with puromycin (Sigma, St. Louis, MO USA) after transfection.

Western blot. Cell samples were lysed in a lysis buffer (Beyotime, China) after collection from a 100 mm dish and disruption, respectively. Proteins(50μg) were resolved on 10% SDS-PAGE and transferred to PVDF membranes. Western blot analysis was performed using anti-CTSL antibody, with anti-β-actin as control. The blocking steps and dilutions for the assessment of all proteins were made in5% bovine serum albumin. After incubation with horseradish peroxidase-conjugated antibodies (Amersham Pharmacia, Fairfield,Connecticut,USA), labeled proteins were detected with an ECL-Plus detection system (Amersham Pharma cia,Fairfield,Connecticut,USA).The anti-CTSL antibody, anti-total-PI3K, anti-p-PI3K,anti-total Akt, anti-p-Akt anti-total-mTOR and anti-p-mTOR antibodies were from Abcam(Cambridge,England). The anti-β-actin antibody was from Santa Cruz(Dallas, Texas,U.S.A).

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-Bromide reduction (MTT) assay. Cells were seeded into 96-well plates at 2000 cells/well. Each sample had four replicates. The cells were incubated with 0.2% MTT for 4h at 37°C, 100μl DMSO/well was added to the culture cells to dissolve the crystals, and cells were counted everyday by reading the absorbance at 490 nm.

Cell motility assay. 1×10^5cells in 0.5 ml serum-free medium were placed in the upper chamber, whereas the lower chamber was loaded with 0.8 ml medium containing 10% FBS. The total number of cells that migrated into the lower chamber was stained with 0.5% crystal violet and counted after 24 h of incubation at 37°C with 5% CO2.

Cell invasion assay. Upper chambers of 24-well transwell plate (Corning Incorporated, New York city,NY,USA) were coated with 50% Matrigel (BD Biosciences, Franklin, New Jersey,USA) in phosphate-buffered saline. Cells were incubated in the upper chamber. After 24 hours incubation, invaded cells were stained with 0.5% crystal violet, examined by bright field microscopy(OLYMPUS cx31,TOKYO,Japan), and photographed. Invasion rate was quantified by counting the invaded cells in five random fields per chamber under the fluorescence microscope(OLYMPUS IX71,TOKYO,Japan). Data summarized three independent experiments.

Tumorigenicity assay. MCF-7/shCTSL and MCF-7/NC (5×10^6cells) were injected subcutaneously in the flank of 6- to 7-week-old nude mice. Tumor growth was evaluated for 3 weeks after injection and every 7 days thereafter. Volume was determined by measuring the largest(a) and the smallest(b) axisusing a caliper, and calculated as $V=0.5ab^2$. Mice handling and experimental procedures followed institutional guidelines. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Nanfang Hospital of Southern Medical University.

Statistical analysis. Unless otherwise stated, all data were shown as mean ±standard error of the mean (SEM). Statistical significance ($P<0.05$) was determined by t test or analysis of variance (ANOVA) followed by assessment of differences using SPSS 16.0 software (SPSS Inc.,Chicago, IL).

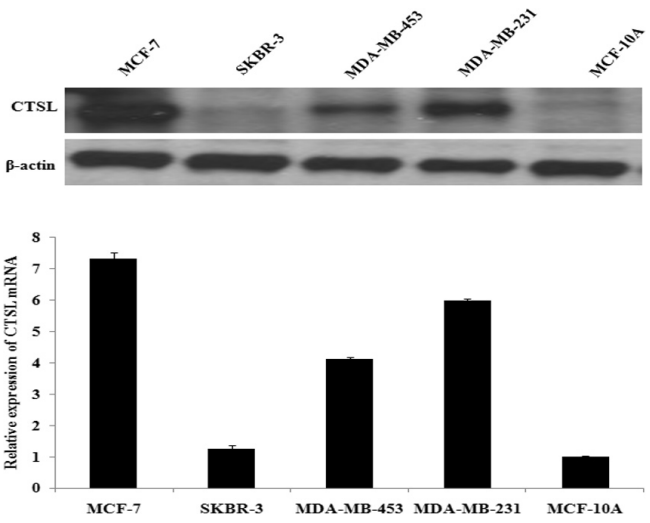

Figure 1. CTSL expression in four human BC cell lines with different metastasis potential.(A). CTSL protein expression levels in MCF-7, SKBR-3, MDA-MB-453 and MDA-MB-231and MCF-10Acell lines were determined by Western blot. Breast cancer cell lines expressed highlevel of CTSL protein compared to normal breast Epithelial Cells. (B). Quantification of CTSL mRNA expression in different breast cancer cells related to normal breast Epithelial Cells as detected by qRT-PCR. Breast cancer cell linesshowed high expression of CTSLmRNA compared to normal breast Epithelial Cells. The expression of β-actin protein and mRNA was also examined and served as controls for sample loading. The figure (A) or (B) shows a representative result of three independent experiments.

Results

Expression of CTSL in four breast cancer cell lines. To investigate the expression of CTSL in breast carcinoma cells, we performed QRT-PCR and Western blotting detection of MCF-7, SKBR-3, MDA-MB-453, MDA-MB-231 and MCF-10A. The mRNA and protein levels of CTSL of five cell lines were shown in Fig.1. The data showed that CTSL expression of human breast cancer cell lines are higher than that of human breast epithelial cells .Additionally,MCF-7 and SKBR-3 expressed highest and lowest level of CTSL mRNA and protein among four breast cancer cell lines respectively. Thus, MCF-7 and SKBR-3 were chosen for further study on the biological function of CTSL.

The vector stably expression CTSL-shRNA caused effective and specific down-regulation of CTSL expression. The knock-down efficiencies of different CTSL specific shRNAs in MCF-7 cells were first evaluated using qRT-PCR. Relative CTSL mRNA levels in individual stable transfectants were normalized against mRNA levels of an internal control gene, β-actin, performed in the same run. As shown in Figure 2A and 2B, cells transfected with CTSL-shRNA showed a significantly reduced level of CTSL protein when compared with that of vector control and negative transfectants, respectively. The above results demonstrated that expression of CTSL could be down-regulated specifically and effectively by specific CTSL-shRNA, and that different shRNA showed

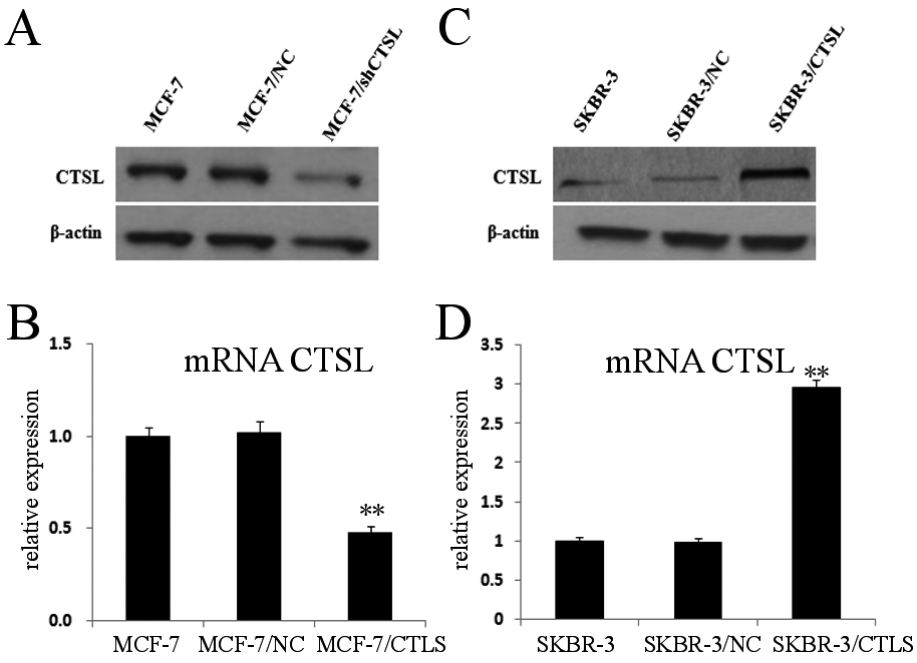

Figure 2. CTSL expression inMCF-7 and SKBR-3. (A).CTSL specific shRNAs resulted in the reduction of CTSL protein in MCF-7 cells. Levels of CTSL protein were assessed by Western blotting. (B).CTSL specific shRNAs resulted in the reduction of CTSL mRNA in MCF-7 cells. Levels of CTSLmRNA were assessed by Real-time PCR. (C). Protein of SKBR-3 cells stably transfected with pcDNA-CTSLincreased most significant comparing to SKBR-3/NCcells and SKBR-3 cells.(D). mRNA of SKBR-3 cells stably transfected with pcDNA-CTSLalso increased most significant comparing to SKBR-3/NC-cells and SKBR-3 cells.The expression of β-actin mRNA and protein was also examined and served as control for sample loading. (P<0.01)**

striking differences in silencing efficiency.Thus,MCF-7 cells transfected with CTSL-shRNA was namedMCF-7/shCTSL, and MCF-7 cells transfected with negative control vector was named MCF-7/NC.

CTSL overexpression in breast cancer cells. SKBR-3 cells transfected with pcDNA3.0-CTSL plasmid displayed a significant increase in the expression levels of CTSL as compared with vector control (Figure 2C and 2D).The overexpression of CTSL was confirmed by performing western blot analysis and qPCR.Thus, SKBR-3 cells transfected with pcDNA3.0-CTSL was named SKBR-3/CTSL, and SKBR-3 cells transfected with negative control vector was named SKBR-3/NC.

Effect of CTSL on cell proliferation of breast cancer cells. The proliferation activity of tumor cell is important in invasion/metastasis of tumor. Having establishedMCF-7/shCTSL cells and SKBR-3/CTSL cells, we immediately examined cell proliferation activity of the transfected cells using MTT assay. As known in Figure 3, the growth of MCF-7 cells in vitro was markedly inhibited after the transfection of CTSL-shRNA($P<0.05$), and over-expression of CTSLpromotedSKBR-3 cell proliferation ($P<0.05$). This result indicates a positive relation between the expression of CTSL and the rate of BC cells growth.

Effect of CTSL on migration and invasion of breast cancer cells. MCF-7/shCTSL displayed remarkable diminishments in

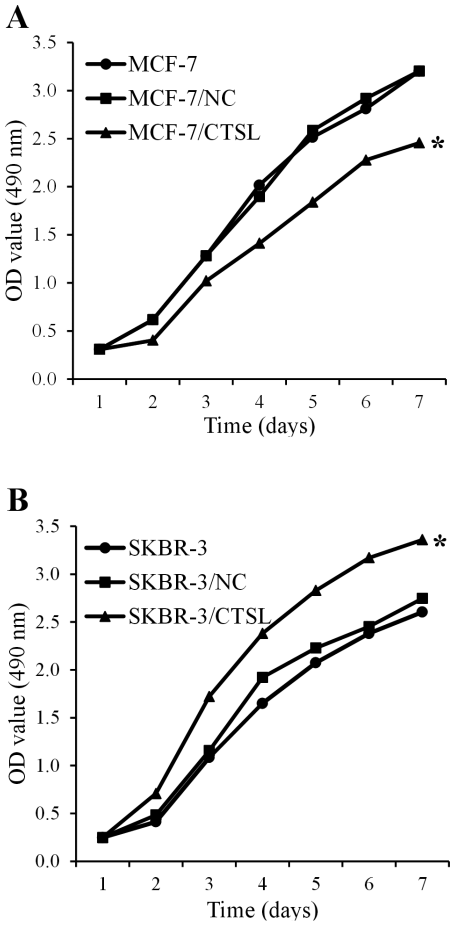

Figure 3. Effect of CTSL on cell proliferationin vitro.(A) MTT assay ofMCF-7 cells with down-regulated CTSL. MCF-7/shCTSL showed remarkable inhibition of cell proliferation when compared with both the MCF-7/NC and MCF-7 (*P<0.05).(B)MTT assay of SKBR-3 cells with over-expression ofCTSL.SKBR-3/CTSL showed remarkable promotion of cell proliferation when compared with both the SKBR-3/NC and SKBR-3 (*P<0.05).The number of viable cell was assessed using MTT assay at d1,d2,d3,d4,d5,d6 and d7 respectively. Each sample was tested in triplicates and the results were reported as OD readings (mean±SD). Values represent menas±SD of at least three independent experiments.

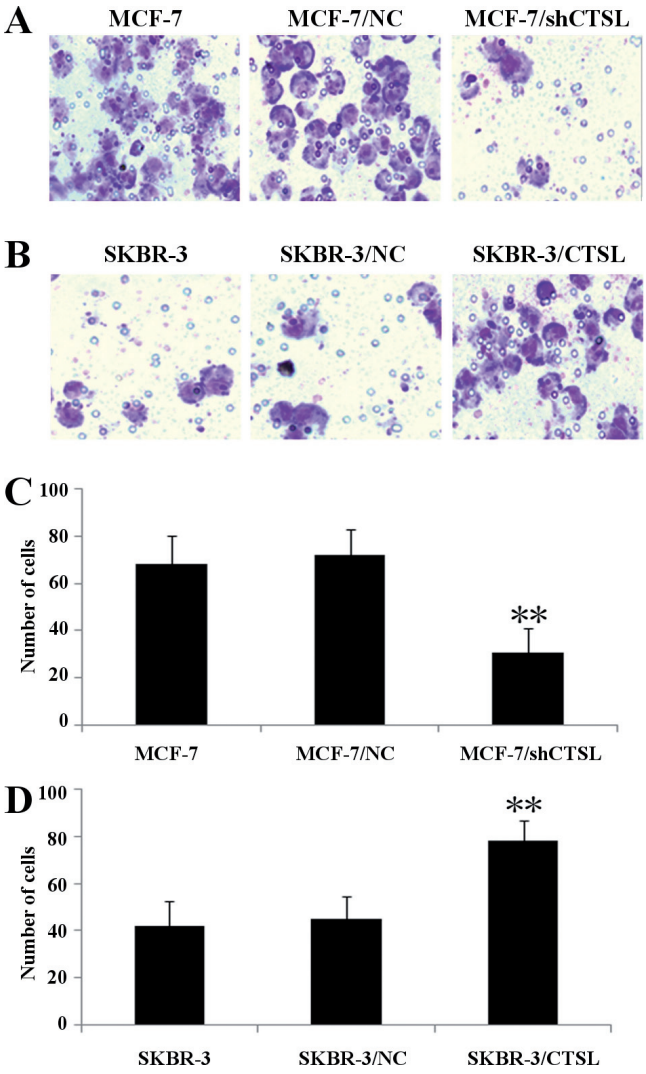

Figure 4. Effect of CTSL on the invasive potential of BC cells.(A).Effect of CTSL knockdown on the invasive potential of MCF-7 cells. (B).Effect of CTSLoverexpession on the invasive potential of SKBR-3 cells. (C) Quantitative Transwellinvasionassay of MCF-7 cells. (D) Quantitative Transwellinvasionassay of SKBR-3 cells.The Y-axis represents the number of migrative cells. The data were the average numbers of cells thatmigrated in a representative experiment, measured in triplicate and presented as the mean ± SD. Magnification, x200 (P<0.01).**

invasion ability as comparing with either MCF-7/NCorMCF-7- (P<0.01, respectively).As shown in Fig. 4Aand Fig. 4C, migration cells ofMCF-7/shCTSLwere31.2±8.2, while MCF-7and MCF-7/NC groups were68.7±11.5and72.4±10.3.Inhibition of CTSL cause significantly attenuated invasion of MCF-7 cells. In addition,SKBR-3/CTSL showed much higher invasion activities than either SKBR-3orSKBR-3/NC (P<0.01, respectively) (Fig. 4B and Fig. 4D). The migration cells ofSKBR-3/CTSL,SKBR-3 and SKBR-3/NC groups were78.1±9.6,42.8±7.3 and 45.7±8.5 respectively. The results indicated that CTSL was associated with the invasion ability of BC cells.

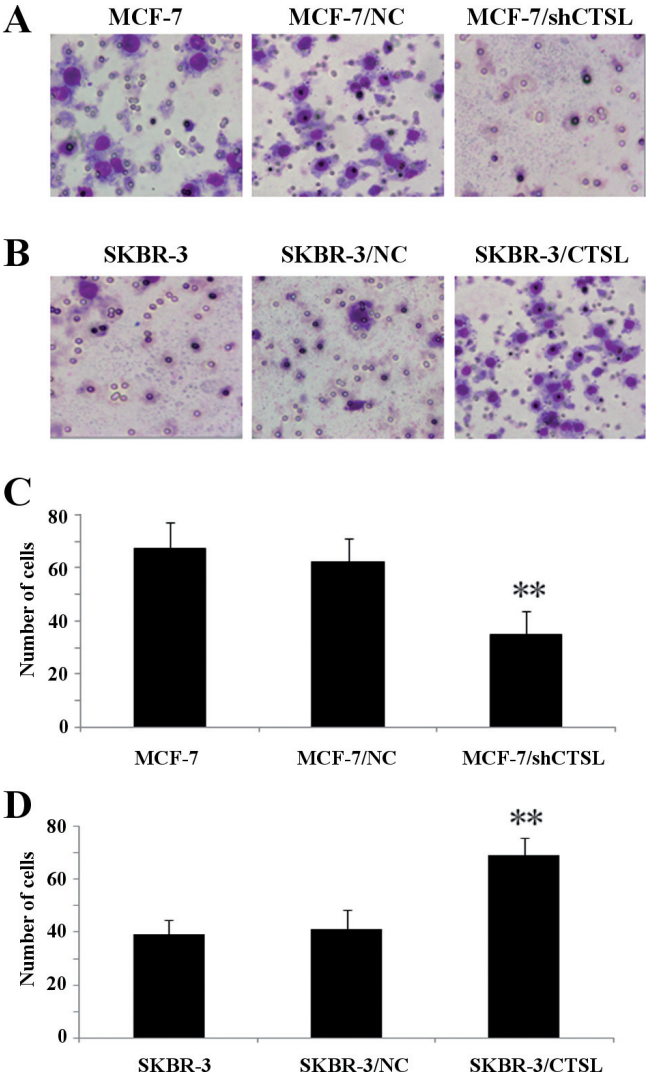

Figure 5. Effect of CTSL on the migration abilities of BC cells.(A).Effect of CTSL knockdown on the migration abilities of MCF-7 cells. (B).Effect of CTSLoverexpession on the migration abilities of SKBR-3 cells. (C) Quantitative Transwell migration assay of MCF-7 cells. (D) Quantitative Transwell migration assay of SKBR-3 cells.The Y-axis represents the number of migrative cells. The data were the average numbers of cells thatmigrated in a representative experiment, measured in triplicate and presented as the mean ± SD. Magnification, x200 (P<0.01).**

Cell motility assay showed that the MCF-7 cells transfected with shCTSL, but not with NC-shRNA, presented an impaired migration capacity (Fig. 5Aand Fig. 5C). In addition, cancer cells transfected with CTSL, but not with control vector, exhibited markedly increased abilities in migration (Fig. 5B and Fig. 5D).

Effects of CTSL on expression of PI3K/Akt/mTOR transduction. As shown in Figure 6,western blot was applied to detect the expression of components of PI3K transduction in BC cell lines. Protein levels of p-PI3K, p-Akt and p-mTOR in MCF-7/shCTSL were lower than both control groups (MCF-7 and MCF-7/NC), and in SKBR-3/CTSL were higher than both control groups (SKBR-3 and SKBR-3/NC).

CTSL gene silencing suppresses cell proliferation in vivo. The effect of CTSL on in vivo tumor growth was assessed by the subcutaneous injection of MCF-7/shCTSL and MCF-7/NC cells for 21 days. As shown in Figure 7, a remarkable reduction of tumor size of groups MCF-7/shCTSL was observed as compared with that of the control group(P<0.05). By day 21 after cell injection, the average tumor weight (n=4) of the groups MCF-7/shCTSL and MCF-7/NC was 1.32±0.09 and 2.33±0.11g,respectively(<0.05),indicating knockdown of CTSL in BC cells reduced their tumorigenic potential.

Discussion

Here, we describe that overexpression of CTSL playsan important role in the progression of BC cells. These findings suggest that CTSL is an important contributor to the proliferation, invasion and migration of BC cells.

The first observed function of CTSL in cancer progression was its ability to promote cancer metastasis [20]. Early

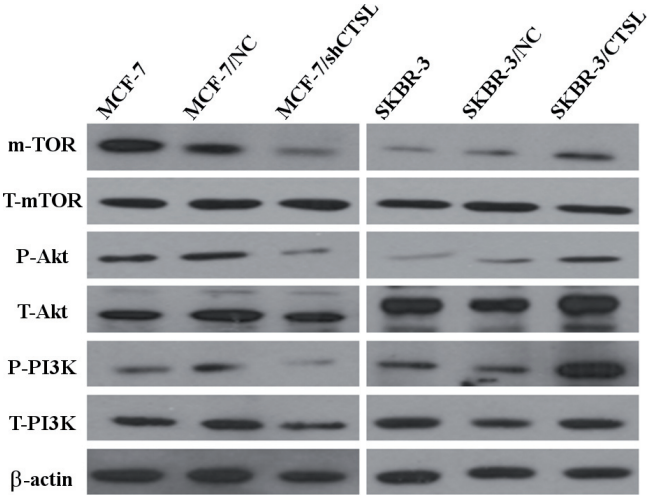

Figure 6. Effect of CTSLon the PI3K pathway. Totalcellular proteins were collected and subjected to Western blot analysis. Stable expression of CTSLincreased the protein level of p-PI3K, p-Akt and p-mTOR.Down-regulation of CTSL showed the opposite effects

experimental studies revealed that metastatic capability of tumor cells was correlated to CTSL activity. Subpopulations from murine B16 melanoma cell lines with high metastatic potential were found to express higher levels of CTSL when compared to low-metastatic counterparts [21]. The invasive ability of brain tumor cells was markedly reduced by full-length antisense cDNA of CTSL. Moreover, the finding that CTSL contribute to anti-apoptosis is also well accepted. Enhanced susceptibility to spontaneous and anti-Fas-induced apoptosis of CTSL-deficient A549 lung cells was reported, with a possible mechanism involving altered Cathepsin D processing by CTSL [22]. However, by far, little has been known about whether CTSL is involved in BC progression. Thus, in this study, we investigate the role of CTSL in the development of BC.

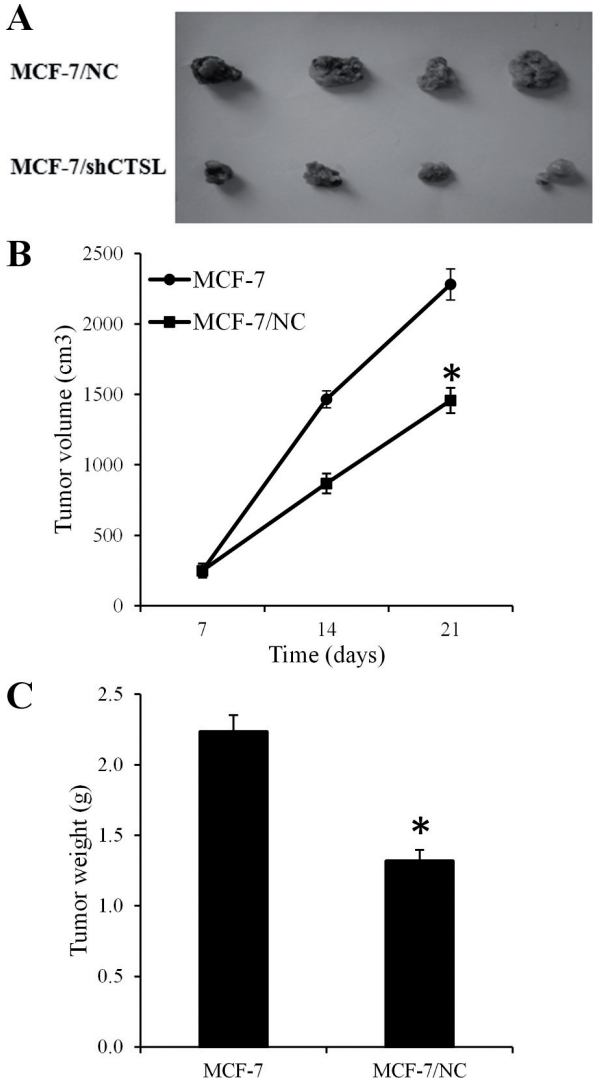

Figure 7. Knockdown of CTSL in the MCF-7cells retards tumorigenicity in nude mice. MCF-7/shCTSL and the control cells (MCF-7/NC) were injected subcutaneously into nude mice at the right flanks. (A). On the 21st days after injection and the tumors were dissected and weighed (n=4). (B). MCF-7/shCTSL showed a significant decrement in growth rate, as compared to MCF-7/NC (*P<0.05). (C). Average tumor weight (n=4) of the groups MCF-7/NCand MCF-7/shCTSL(*P<0.05).

In spite of the potential significance in BC, function of CTSL in BC has not been detailed [18, 19]. Evidence of its oncogenic activity in BC is still lacking. To understand the functions of CTSL, endogenous CTSL expression in an BC cell line (MCF-7) was down-regulated by shRNA. Cell properties of the CTSL-depleted cells were then analyzed and compared with the control cells. The results showed that CTSL knockdown stable clones displayed suppressed cell proliferation. Besides, migration and invasion of cells were largely impeded upon CTSL depletion. Additionally, over-expression of CTSL promoted the aggressive behaviors of SKBR-3 cells. Our study has also provided with the first validation about the oncogenic capacity of CTSL in vivo. MCF-7 with high level of CTSL expression displayed increased ability forming tumors in nude mice. All these studies confirmed our findings that CTSL exerts oncogenic effect on MCF-7 cells.

The above results indicated a possible reason for the poorer outcomes observed in BC patients with CTSL overexpression.

PI3K/Akt/mTOR is one of the three major signaling pathways that is vital in cancer. mTOR is a key kinase downstream of PI3K/AKT, which regulates tumor cell proliferation, growth, survival and angiogenesis [23, 24]. Our results indicate that the anti-proliferative effects of CTSL in BC cells are partially mediated by PI3K signaling. Suppression of CTSL in BC cells inhibited cell proliferation accompanied by decreased p-PI3K, p-Akt and p-mTOR expression. Moreover, overexpression of CTSL enhanced cell growth of SKBR-3 cells by increasing p-PI3K, p-Akt and p-mTOR levels.

However, we noted that even after CTSL knock-down, BC cells still possessed the capabilities of invasion and metastasis. We can not exclude that other factors, such as uPA and MMPs, also influence these characteristics of BC, which warrants further investigation [25, 26].

In the present study we found that down-regulation of CTSL by shRNA inhibits BC cells growth and migration, and overexpression of CTSL by cDNA transfection promotes BC cells proliferation and motility. Combined with previous reports that interference with CTSL expression additionally regulates at least two major signaling pathways (Wnt/β-catenin and TGF-β) in cancers [15], we suggest that interfering with CTSL expression and function exert broad-spectrum biologic effects that might be beneficial for the treatment of BC. Further studies on the mechanisms by which CTSL regulates multiple signaling pathways may reveal other significance of intervention of this important target.

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