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Original Paper

SUMO-Specific Protease 2 Suppresses Cell Migration and Invasion through Inhibiting the Expression of MMP13 in Bladder Cancer Cells

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Key Words
SENP2 • Metastasis • MMP13 • Bladder cancer cells

Abstract
Background: SUMO-specific protease 2 (SENP2) is a de-SUMOylation protease family member which has an indispensable role in the regulation of NF-κB transcriptional activation and Wnt signaling. However, whether SENP2 plays a role in tumor metastasis is completely unknown. ***Methods:*** Real-time PCR and Western blot was used to detect the expression of SENP2 in human bladder cancer samples and cell lines. Small interfering RNA (siRNA) was used to silencing the expression of SENP2. Matrigel-coated invasion chambers were used to detect the invasion ability of SENP2 in bladder cancer cells. ***Results:*** SENP2 was down-regulated in bladder cancer samples. SENP2 inhibited bladder cancer cells migration and invasion *in vitro*. Transcriptional analysis of several genes associated with tumor metastasis and invasion demonstrated that SENP2 selectively down-regulated MMP13 in bladder cancer cells. Further analysis indicated that silencing of MMP13 rescued the invasive phenotype in SENP2 expressing T24 cells. ***Conclusion:*** SENP2 functions as a tumor metastasis suppressor in bladder cancer. The effects of SENP2 on bladder cancer invasion are partially mediated by inhibiting the expression of MMP13.

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Introduction

Bladder cancer (BC) is the second most common malignancy of the urinary tract [1]. 90% of BC is urothelial carcinoma (UC), and the majority is papillary low-grade, non-muscle invasive cancers that recur in up to 80% of cases but rarely progress to muscle invasion [2]. In contrast, 10 to 20% of tumors are muscle invasive at diagnosis, and 50% of patients die from metastatic disease [3].

SUMO is a small ubiquitin-like protein that can be covalently attached to proteins through the formation of isopeptide bonds with specific lysine residues of target proteins. The mammalian SUMO protein family includes four members (SUMO-1–4) of which SUMO-2 and SUMO-3 are conjugated in a stress-inducible manner [4]. SUMO conjugated to the target lysine by an enzymatic cascade composed of three enzymes: E1 (Uba2/Aos1), E2 (Ubc9), and E3 ligases. SUMO conjugation starts by formation of a thioester bond with the activating enzyme E1, a heterodimer of Aos1 and Uba2. Aos1/Uba2 transfers SUMO to the single E2-conjugating enzyme Ubc9, which recognizes and SUMOylates the substrate. The Ubc9 substrate recognition is facilitated by specific E3 SUMO ligases [5]. SUMOylation is a dynamic process that is readily reversed by a family of SUMO-specific proteases. In the mammalian, six SUMO-specific proteases have been reported [6]. SENP1 is a nuclear protease that appears to deconjugate a large number of SUMOylated proteins [7]. SENP2 is a nuclear-envelope-associated protease that appears to have activity similar to that of SENP1 when overexpressed [8]. The mouse SENP2 was essential for embryonic cardiac development through regulation of the SUMOylation status of Pc2/CBX4 and loss of SENP2 causes embryonic lethal [9, 10]. SENP2 was also implied a role in neuron system by modulating the dynamics and functional outcome of MEF2A SUMOylation and transcriptional activation [11]. Recent study reported that SENP2 was down-regulated in hepatocellular carcinoma (HCC) tissues and played a role in HCC cells growth control by modulating the stability of beta-catenin [12]. However, the role of SENP2 in other tumors remains to be elucidated.

Here, we show that SENP2 was down-regulated in bladder cancer tissues. SENP2 inhibited bladder cancer cells migration and invasion by regulating the expression of MMP13.

Materials and Methods

Cell Culture

T24 and EJ cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) (Gibco, CA, USA) as well as 100U/ml penicilin and 100μg/ml streptomycin. Cells were placed in a 5% CO_2 and 95% air incubator (20% O_2) at 37°C.

Plasmids

Flag-SENP2WT and Flag-SENP2Mut plasmids were purchased from Addgene and subcloned into pbabe-puro retrovirus vector. MMP13 was amplified from 293T cDNA library and subcloned into pcDNA-3.1 vector. All the plasmids were confirmed by sequencing. All transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

SiRNA and Transfection

siRNA against SENP2 was purchased from Dharmacon (USA). As a negative control, a siRNA sequence targeting green fluorescent protein (non-targeting siRNA) was used. All transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Construction of Stable Cell Line

Viral supernatants were produced in HEK293T cells cotransfected with the pBabe-con or pBabe-Flag-SENP2 constructs and packaging vectors GAG-POL and VSV-G (Clontech). Viral supernatants were collected 48 hours after transfection, filter-sterilized, and stored at -80 °C. For T24 and EJ cells, viral supernatants

were added to the cells with 10 μg/ml Polybrane for 48 h and selected with puromycin (1 μg/ml) for 3 days. Positive polyclonal populations were identified based on Western blot for Flag M2.

RNA isolation and Real-time PCR

Total RNAs from T24 cells were isolated by TRIzol reagent, and reverse transcriptions were performed by Takara RNA PCR kit following the manufacturer's instructions. In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on Light Cycler 480 (Roche, Switzerland). The linearized ΔCt (i.e. 2-ΔCt) was used for comparative purposes. The primer sequences used are available upon request.

Cell migration and invasion assay

Cell migration was assessed using 24-well inserts (Becton Dickinson Labware, USA) with 8-mm pores according to the manufacturer's protocol. After 24 h of incubation, the cells in the upper chamber were removed, and the cells were fixed in ice-cold methanol, stained with Wright–Giemsa solution (Polysciences, USA). Digital images were obtained from the membranes, and cell areas were selected using Scan Scope CS system (Aperio Technologies, USA). The migrating cells were quantified in five randomly selected fields in each membrane, and the average value was defined as a migration or invasion index on three independent membranes. For invasion, the membranes utilized were Matrigel-coated invasion chambers (BD Biosciences, USA) that were pre-hydrated in serum-free medium.

Colony-forming assay

Cells were digested with 0.25% trypsin, pelleted, and resuspended in 1mL fresh media. Trypan blue dye exclusion method was used to determine the cell viability. Cells were planted at a density of 1×10^4/mL cells in six-well dishes and allowed to attach overnight. Two weeks later, cells were fixed by ice-cold methanol and stained with Giemsa staining solution (Polysciences, USA). A population of > 50 cells was counted as one colony.

Western Blot

Cells were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.6, 100 mM DTT, 2% w/v SDS, 20% glycerol). After centrifugation at 10, 000 g for 20 min at 4°C, proteins in the supernatants were quantified and separated by 12% SDS-PAGE, transferred to NC membrane. After blocking with 5% non-fat milk, NC membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies. The signals were detected by Super-Signal West Pico Chemiluminescent Substrate (Pierce, USA) according to manufacturer's instructions.

Statistical Analysis

Data were summarized as mean ± SEM. Statistical significance was analyzed by student's t-test using SPSS 11.0 software (Chicago, IL, US). A value of $P < 0.05$ was considered significant. Statistical significance is displayed as * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

Results

SENP2 is down-regulated in bladder cancer samples

To evaluate the expression of SENP2 in bladder cancer cells, we detected the mRNA level of SENP2 in 20 primary bladder cancer samples and their adjacent normal tissues by real-time quantitative PCR, and relative mRNA levels of SENP2 of tumor tissues and adjacent tissues were analyzed. As shown in Fig. 1A, the mRNA levels of SENP2 were decreased in cancer tissues when compared with the adjacent normal tissues. We then employed western blot with anti-SENP2 antibody to test SENP2 protein expression in those clinic samples including 6 primary bladder cancer samples and their adjacent normal tissues. The results showed that SENP2 was down-regulated in bladder cancer samples (Fig. 1B). To investigate the role of SENP2 in bladder cancer cells, we re-expressed SENP2 in bladder cancer cell T24 and EJ. T24 or EJ cell stable-expressing Flag-SENP2 (SENP2-T24) or vector control (Con-T24)

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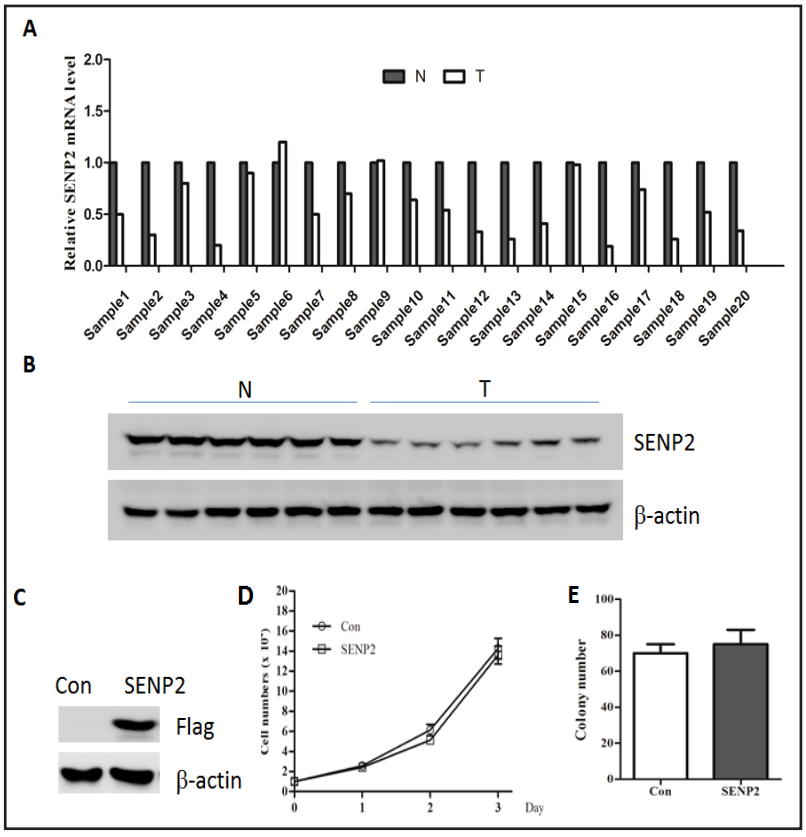

Fig. 1. SENP2 is down-regulated in bladder cancer samples. A: mRNA levels of SENP2 were analyzed by real-time PCR in 20 paired BC tissues (T) or normal tissues (N). B: Protein levels of SENP2 were analyzed by Western Blot in 5 paired BC tissues (T) or normal tissues (N). C: The exogenous expression of Flag-SENP2 was detected by Western Blot with Flag antibody in Con-T24 (Con) or SENP2-T24 (SENP2) cells. β-actin was used as loading control. D: The growth curve of T24 cells stable expression of empty vector (Con) or Flag-SENP2. E: The colony formation numbers of T24 cells stable expression empty vector (Con) or Flag-SENP2.

was selected by G418 and the expression of exogenous SENP2 was detected by western blot with Flag antibody (Fig. 1C and data not shown). Over-expressing of SENP2 did not affect the growth of T24 cells (Fig. 1D). Consistent with this, over-expressing of SENP2 in T24 cells did not affect the number of colony formed in soft-agar (Fig. 1E). Over-expressing of SENP2 also did not affect the growth and colony number of EJ cells (data not show). Taken together, these data suggested that down-regulated SENP2 in bladder cancer samples did not contribute to tumorigenesis.

Stable expression of SENP2 decreases bladder cancer cells migration and invasion

To further investigate the biological role of SENP2, we then asked whether SENP2 affected the migration and invasion of bladder cancer cells. We found that stable expression of SENP2 resulted in decreased cell motility as SENP2-T24 cells demonstrated significantly decreased motility when compared to Con-T24 cells (Fig. 2A). Moreover, using a matrigel-coated Boyden chamber assay, we observed that SENP2-T24 cells had significantly decreased ability to invade as compared to Con-T24 cells, suggesting that SENP2 expression was an impediment to cell invasion (Fig. 2B).

SENP2 modulates the expression of MMP13

To begin to dissect a mechanism of SENP2 -mediated modulation of tumor metastasis, we evaluated the expression of genes involved in cell motility and invasion especially metalloproteases by real-time PCR. Of the twelve genes tested, only one gene, MMP13 was found to be differentially down-regulated in the context of SENP2 expression in T24 cells (Fig. 3A). In consistent with mRNA data, the protein level of MMP13 was also down-regulated in SENP2-T24 cells (Fig. 3B). This phenomenon could be real, because a catalytically inactive SENP2 mutant (SENP2Cat) (R576L, K577M) could not regulate MMP13 (Fig. 3C). To confirm that SENP2 regulates the expression of MMP13, we silenced the expression of SENP2 by two

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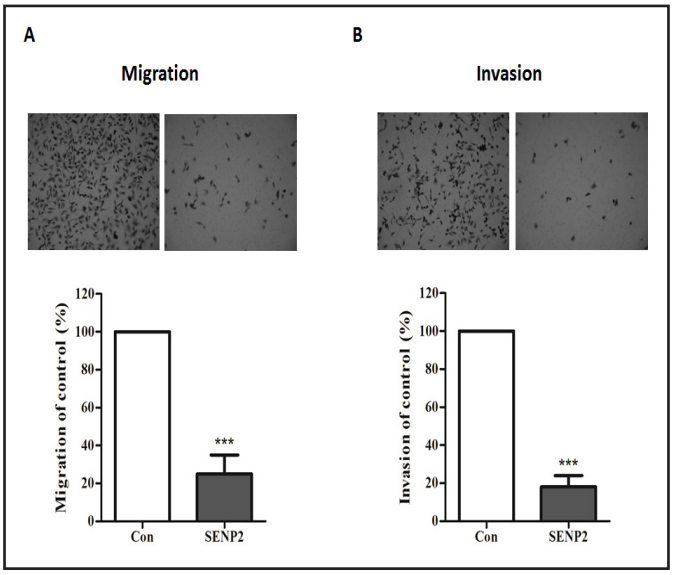

Fig. 2. Stable expression of SENP2 decreases bladder cancer cells migration and invasion. (A-B) T24 cells stable expression of empty vector (Con) or Flag-SENP2 were placed in serum-free culture media and added into the upper compartment of a migration or invasion chamber. After 24 h, cells in the upper chamber were removed and cells that had migrated (A) or invaded (B) onto the lower surface of the membrane were fixed and stained with Wright–Giemsa. The relative-fold migration and invasion values of SENP2-T24 cells was normalised against Con-T24 cells and expressed as percentages of the control, which was assumed to be 100%. Columns, mean of three independent experiments. Bars, s.e.m.

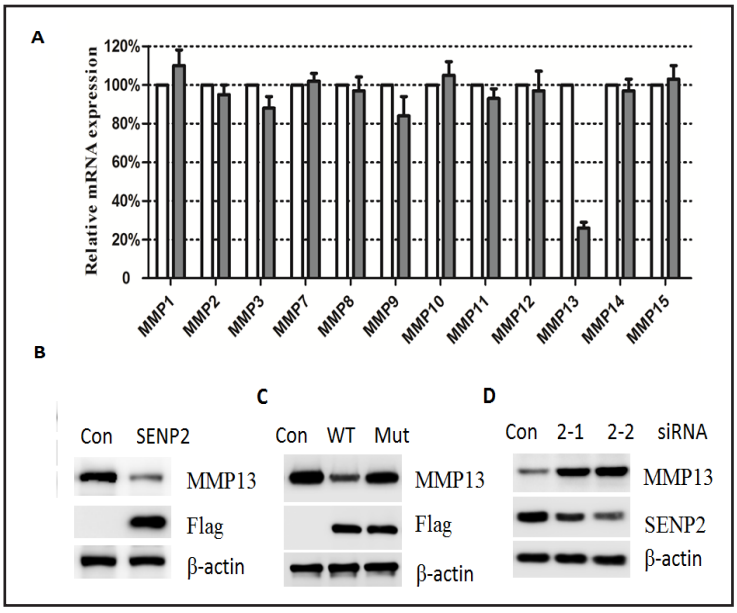

Fig. 3. SENP2 modulates the expression of MMP13. A: mRNA levels of twelve MMP family members were detected by real-time PCR in Con-T24 or SENP2-T24 cells. B: The protein levels of MMP13 were detected by Western Blot in Con-T24 (Con) or SENP2-T24 (SENP2) cells. C: T24 cells were transfected with Flag-SENP2WT or Flag-SENP2 Mut for 36h, cell lysate was subjected to Western Blot with indicated antibodies. D: SENP2 and MMP13 expression was detected by Western Blot in T24 cells transfected with siRNA oligos targeting SENP2 (2-1, 2-2) or scramble siRNA (Con).

siRNAs which target different regions of SENP2 mRNA. Both siRNAs silenced the expression of SENP2 in T24 cells (Fig. 3D). As expected, silencing the expression of SENP2 significantly induced the expression of MMP13 (Fig. 3C). Taken together, these data suggested that SENP2 modulate the expression of MMP13.

Forced MMP13 expression is sufficient to restore cell invasion in SENP2 over-expressed T24 bladder cancer cells

To test whether SENP2 suppresses T24 cells metastasis by inhibiting the expression of MMP13, we reintroduced MMP13 back in the SENP2-T24 cells by retro-virus mediated gene delivery. Both mRNA and protein levels of MMP13 were up-regulated upon infection (Fig. 4A, 4B). Furthermore, MMP13 expression abrogates the effect of SENP2 expression on cell migration and invasion (Fig. 4C), suggesting that MMP13 expression alone is sufficient to overcome the SENP2-induced inhibition of cell migration and invasion.

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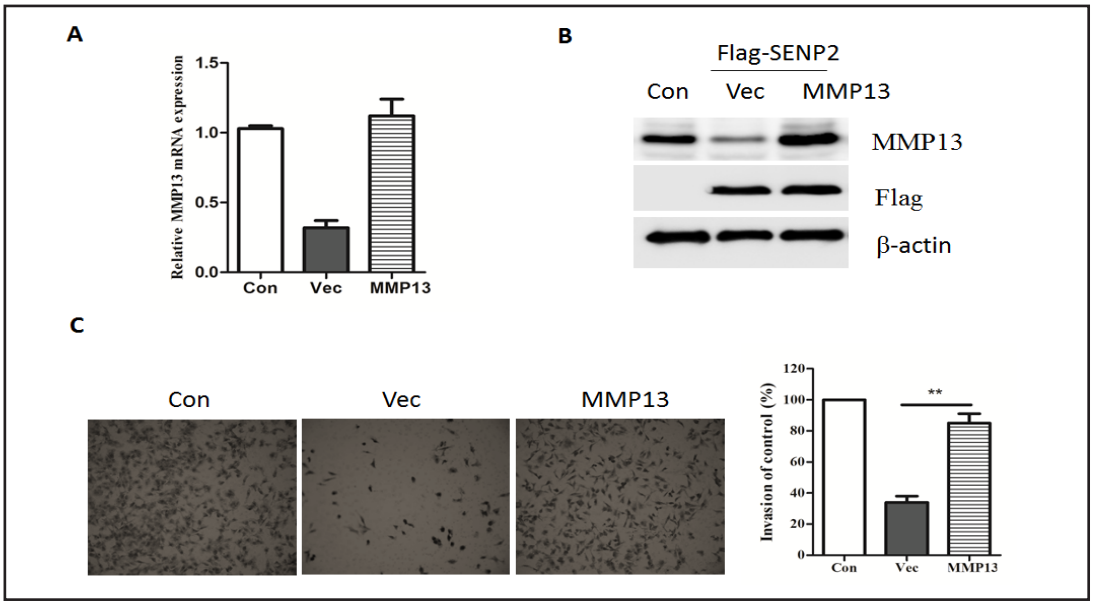

Fig. 4. Forced MMP13 expression is sufficient to restore cell invasion in SENP2 over-expressed T24 bladder cancer cells. A: mRNA levels of MMP13 were analyzed by real-time PCR in Con-T24 cells (Con) and SENP2-T24 cells transfected with empty vector (Vec) or MMP13. B: Protein levels of MMP13 were analyzed by Western blot in Con-T24 cells (Con) and SENP2-T24 cells transfected with empty vector (Vec) or MMP13. C: Con-T24 cells (Con) and SENP2-T24 cells stable expression of empty vector (Vec) or MMP13 were placed in serum-free culture media and added into the upper compartment of a migration or invasion chamber. After 24 h, cells in the upper chamber were removed and cells that had invaded onto the lower surface of the membrane were fixed and stained with Wright–Giemsa. The relative-fold migration and invasion values was normalised against Con-T24 cells (Con) and expressed as percentages of the control, which was assumed to be 100%.

Discussion

Metastasis is the leading perpetrator of tumor-associated deaths, including that of bladder cancer [13]. Although the knowledge of the metastatic process and the players involved continues to be discovered, no curative treatments for metastatic bladder cancer exist. So, identification of novel proteins that could be involved in the metastatic process is crucial for fighting against bladder cancer.

In the present study, we showed that SENP2 is a negative regulator of bladder cancer. Firstly, we found that SENP2 is down-regulated in clinical bladder cancer samples. SENP2 was reported to be down-regulated in HCC samples and played a role in HCC cells growth control by modulating the stability of beta-catenin [12]. However, although SENP2 was also down-regulated in bladder cancer, we did not detect any differences in growth patterns of bladder cancer cells following modulation of SENP2 levels, suggesting that SENP2 could play other roles in bladder cancer rather than growth controlling.

Recently, the SUMOylation E2 enzyme UBC9 and De-SUMOylation enzyme SENP1 has been reported to play a role in cancer metastasis, expending the role of SUMOylation in cancer development [14, 15]. We then test whether SENP2 plays a role in bladder cancer. Using migration or invasion chamber assay, we found that SENP2 could significantly inhibit bladder cancer T24 cells metastasis. Lots of genes were involved in cancer metastasis including MMP2, MMP9 and MMP13 [16]. Using unbiased real-time PCR assay, we found that SENP2 could specifically regulate the expression of MMP13. This finding coupled with our observations that forced expression of MMP13 in SENP2 expressing cells abrogates the effect of SENP2 on cell invasion, suggest that MMP13 is not an innocent bystander, but the

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significant downstream player of SENP2-mediated effects on bladder cancer invasion. Taken together these finding are exciting given that MMP13 is a well-known extracellular matrix degrading enzyme, which is correlated with increased invasive and metastatic bladder cancer.

Given the implications of our important findings, additional studies are warranted to further characterize the role and mechanism of SENP2-mediated effects on bladder cancer metastasis. In addition, the mechanism by which SENP2 is regulated is a significant question that must be answered. The ability to induce the expression of SENP2 through a gene therapy approach, small molecule inducer, or other regulatory mechanisms could be important questions for the prevention or management of bladder metastasis.

Conflict of Interest

All authors have no conflict of interest to declare.

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