

Original Article

Expression of hepsin in prostate cancer and its biological implications

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Abstract: As one common urinary/reproductive system malignant tumor in males, the incidence of prostate cancer is increasing globally. Hepsin is one transmembrane protein and exerts critical function for cellular integrity. Previous study has suggested the pivotal role of Hepsin in infiltration and invasion of tumor cells. This study thus investigated the expression profile of Hepsin in prostate cancer and its biological significance. A total of 40 prostate cancer patients were from January 2013 to December 2014 in our hospital were recruited in this study, in parallel with 10 cases of benign prostatic hyperplasia (BPH) patients. Tissue samples were collected during surgery. *In situ* hybridization (ISH) and RT-PCR were performed to detect Hepsin expression level. RNA interference (RNAi) technique was employed to inhibit the expression of Hepsin in prostate cancer cell line 22RV1. MTT assay was further used to evaluate cell proliferation while Transwell approach was applied for cell invasiveness. Hepsin expression was significantly elevated in prostate cancer tissues compared to BPH tissues ($P < 0.05$). With advanced stage of cancer, Hepsin expression level was further elevated. When Hepsin expression was knocked-down in 22RV1 cells, the invasion ability was decreased by 45.5% but not for cell proliferation. The expression level of Hepsin had no significant effects on prostate cancer cell proliferation but significantly modulated migration and invasion of tumor cells.

Keywords: Prostate cancer, hepsin, RNA interference, cell proliferation, cell invasion

Introduction

Prostate cancer is one malignant tumor derived from epithelial cells of male prostate tissues [1]. It has a relatively higher incidence in Western countries, and is becoming more popular in China [2]. The exact pathogenesis mechanism of prostate cancer is still unclear, but may include various factors including genetic, age, diet habit and sexual activity [3]. With progression of biological behavior and molecular mechanism of prostate cancer cells, a series of membrane protein has critical role on the occurrence of prostate cancer and biological activity [4].

Hepsin protein is a type II transmembrane serine proteinase, and is composed of three domains including intracellular domain, transmembrane domain and extracellular domain with protein hydrolysis activity [5]. Many studies showed that Hepsin is closely correlated

with multiple cellular activities including intracellular adhesion, transmembrane signal transduction and degradation of extracellular matrix [6]. Studies have found the up-regulation of Hepsin in various malignant tumors such as liver cancer and ovarian cancer, and the close correlation between Hepsin and tumor cell activities such as metastasis and invasion [7]. Other scholars had found up-regulation of Hepsin in prostate cancer tissues by DNA microarray analysis, thus proposing the involvement of Hepsin in occurrence and progression of prostate cancer [8]. Therefore, this study aimed to investigate the expression of Hepsin in prostate cancer tissues and its implication in proliferation/invasion of cancer cells.

Materials and methods

Study objects

A total of 40 cases of prostate cancer patients (age: 48~64 years, average age: 55.1±

Table 1. Primer sequence of RT-PCR

Target gene		Sequence	Product length
β-tublin	F	5'-TGTCCTGATGGCGAGTGTTC-3'	454 bp
	R	5'-CCTGTTGGCCATAGTACTGC-3'	
Hepsin	F	5'-TGCATTGACAACGAGGC-3'	282 bp
	R	5'-CTGTCTTGACATTGTTG-3'	

Table 2. Nucleotide sequence for RNA interference

Sequence name	Sequence
Hepsin-RNAi	5'-AGCGGTGGTCCCTTTGTGTGTGAGGACAG-3'
Scramble-RNAi	5'-TGTAGGTGACGGGTCTTGCGTGCCTAAG-3'

6.5 years) from January 2013 to December 2014 were recruited in The Third Affiliated Hospital of Kunming Medical University. All patients were primary cancer cases, without any chemo-/radio-/immune-therapy before admitting. Based on Whitmore-Jewett standard, there were 15 cases at A+B stages, 11 stage C and 13 stage D patients. Another 10 patients (age: 45~63 years, average age: 52.1±3.5 years) who received surgical resection of benign prostatic hyperplasia (BPH) during the same time period were recruited as the control group. Prostate tissues collected during the surgery were fixed in normal formaldehyde and embedded in paraffin. This study has been pre-approved by the ethical committee of The Third Affiliated Hospital of Kunming Medical University and has obtained written consents from all participants.

In situ hybridization (ISH)

Based on mRNA sequence of Hepsin (Genebank access No. M18930), anti-sense RNA probe in ISH was designed as 5'-ATCCCACC-ACCGGGATCACCTCGGG-3'. All paraffin-embedded tissues samples were detected for mRNA expression using LNA-Hepsin-Probe using ISH test kit (Boster, China). In brief, paraffin slices were dewaxed by routine procedures and re-hydrated. The activity of hydrogen peroxidase was blocked, followed by proteinase K digestion. After rinsing in 0.5M PBS (pH7.4), pre-hybridization buffer was added at 63~65°C for 3~4 hours incubation. Hybridization buffer containing probes were then added for incubation at 65°C for 12~16 hours. After that slices were

rinsed in SSC solution and were blocked in buffer. Rabbit anti-DIG antibody working solution was added for 60-min incubation at 37°C. After rinsing in PBS and alkaline phosphatase buffer, developing reagent was added for dark incubation. The reaction was quenched in PBS. Tissues were finally fixed in 4% paraformaldehyde for 2 hours, and were rinsed in PBS. After mounting coverslips, slices were observed under a microscope. Five high magnification fields (X400) were randomly selected in each slide for calculating the average number of positive cells, which were deduced as blue-purple granules inside cells. If more than 40% of total cells showed positive staining the sample was judged as positive expression for Hepsin as previously described [9].

RT-PCR

Tissues samples were embedded in paraffin for preparing tissues slices. Primers were designed based on gene sequence of Hepsin (Genebank access No. M189830) as shown in **Table 1**. One-step RNA extraction kit Trizol (Invitrogen, US) was used to extract total RNA from tissues. Using BPH tissue as the control, RT-PCR assay was performed using test kit (TianGen, China). Reverse transcription was firstly performed under 37°C for 2 hours. cDNA products were then used in PCR under the following conditions: 95°C for 5 min, followed by 40 cycles each containing 95°C denature for 1 min, 63.1°C annealing for 30 sec and 72°C elongation for 3 min. PCR products were separated by 1% agarose gel electrophoresis. Hepsin relative expression level was then calculated by analyzing images [10].

RNA interference

Based on mRNA sequence of Hepsin gene, we selected a 29-bp sequence with 50% GC contents of mRNA sequence. By Blast alignment against other gene mRNA sequence in human genome database, we ruled out any homology sequence and obtained Hepsin-RNAi with high specificity. A randomized sequence of Hepsin-RNAi oligonucleotide was synthesized as negative controlled scramble-RNAi [11]. RNAi sequences were shown in **Table 2** and were synthesized by Sangon (China).

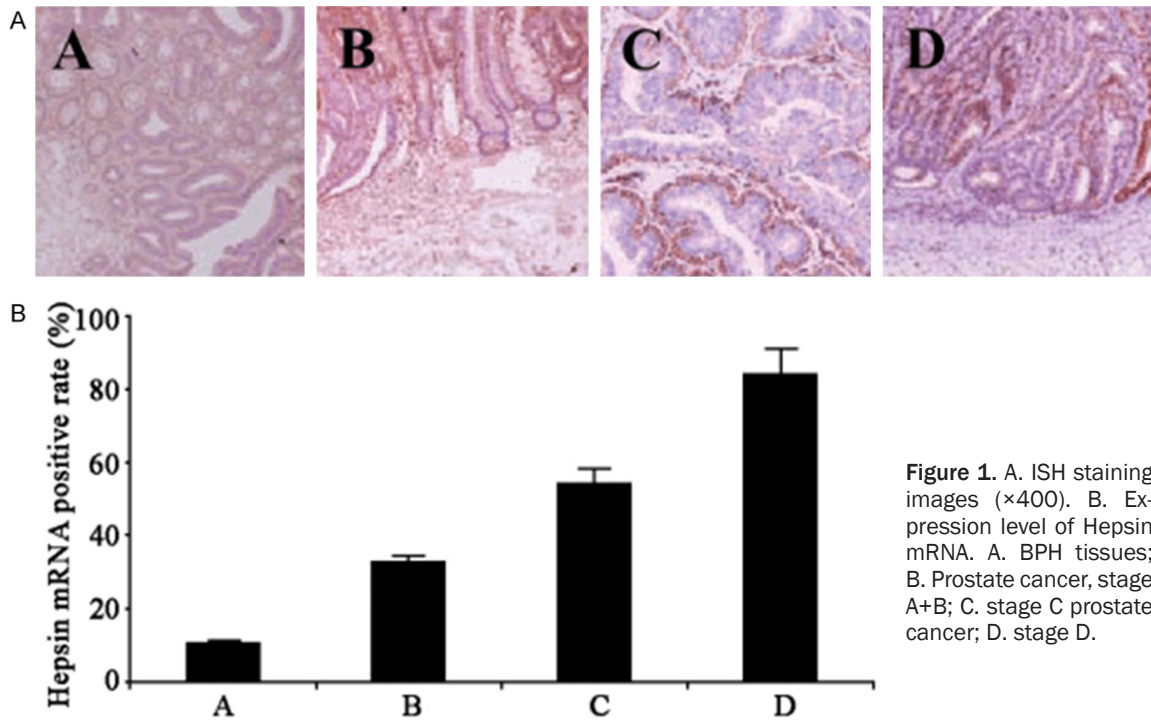


Figure 1. A. ISH staining images ($\times 400$). B. Expression level of Hepsin mRNA. A. BPH tissues; B. Prostate cancer, stage A+B; C. stage C prostate cancer; D. stage D.

Cell transfection

Prostate cancer cell line 22RV1 was purchased from Cell Bank of Chinese Academy of Sciences. After resuscitation, cells were cultured to reach log-phase, and were digested by trypsin and counted. Diluted cells were seeded into 96-well plate for 24-hour incubation before transfection. Liposome INTERFER in transfection kit (Polyplus transfection, US) was employed following manual instruction [12]. Relative expression level of Hepsin was determined by Western blotting.

Western blotting

Cells at log-phase were collected and lysed in pre-cooled lysis buffer for extracting total proteins, which were then separated in SDS-PAGE using 15% separation gel and 5% condensation gel. Total proteins after gel electrophoresis was then transferred to PVDF membrane, which was blocked in 5% defatted milk powder for 1 hour at 37°C. After rinsing in TBST, primary antibody working solution (mouse anti-human Hepsin, mouse anti-human β -tubulin) were added for 4°C overnight incubation. Excess antibody was rinsed by TBST, followed by the addition of DAB chromogenic substrates and

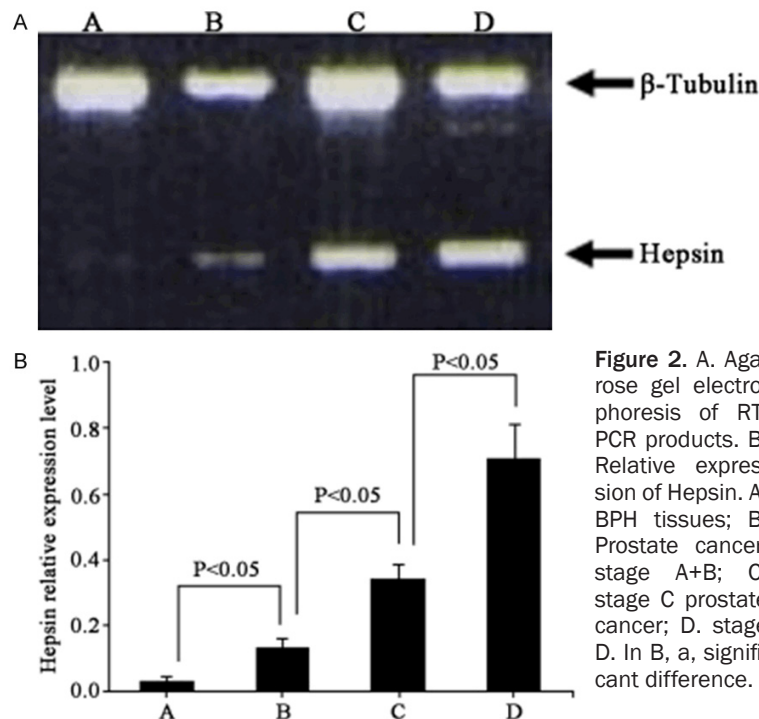
dark development for 10 min. Western blotting images were captured and analyzed by gel imaging software to detect the integrity density for all bands. Using internal reference protein, relative protein level of Hepsin was calculated.

MTT assay

Those 22RV1 cells with successful transfection were cultured until log phase, and were digested in trypsin. After centrifugation, supernatants were discarded and cells were suspended. Equal volumes (6 μ L) of cell suspensions and trypan blue dye were mixed and added into cell counter chamber with coverslips. Under microscope cells were counted. Cell suspensions were then seeded into 24-well plate for 12-hour culture in a CO₂-incubator at 37°C. 70 μ L MTT solution was then added into each well for further 3-hour incubation. DMSO was then added into the mixture until resolving of solids. A570 value was then measured under a microplate reader [13].

Transwell assay for cell migration and invasion

Following manual instruction, 60 μ L matrix gel (5 mg/mL, BD, US) was firstly added into the upper chamber of Transwell, which was dried at



30 min, followed by 10% acetic acid washing. OD₅₇₀ values were measured by microplate reader in triplicates [14].

Statistical analysis

SPSS 19.0 software was used to process all collected data, of which measurement data were presented as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used for analysis using significant level at 0.05. A statistical significance was defined when P<0.05.

Results

ISH staining

By ISH staining we measured the expression profile of Hepsin in both prostate cancer and BPH tissues. As shown in **Figure 1**, specific hybridization probe can bind with Hepsin mRNA in tissues of BPH and different stages of prostate cancer. With elevated mRNA expression level, the staining intensity was even higher. **Figure 1A** showed that staining intensity in prostate cancer for Hepsin was significantly higher than BPH tissues. By calculating positive rate in different tissues (**Figure 1B**), we found higher positive rate for Hepsin in prostate cancer with advanced grade. Therefore, the expres-

sion level of Hepsin was increased with higher clinical stage.

RT-PCR

Using RT-PCR in conjunction with agarose gel electrophoresis, we confirmed the results from ISH (**Figure 2A**). The relative expression level of Hepsin was calculated by fluorescent intensity (**Figure 2B**). In BPH tissues, Hepsin had depressed expression. Whilst in prostate can-

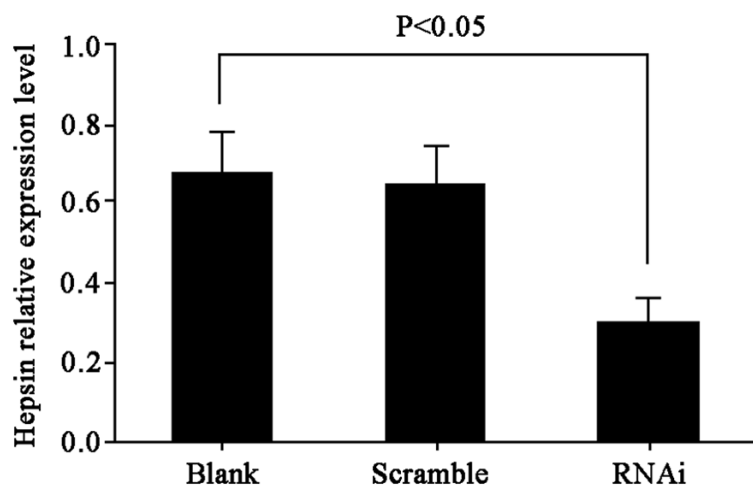


Figure 3. Relative level of Hepsin.

4°C. Cells at log-phase were digested by trypsin, followed by washing and re-suspension in serum-free medium. Cell concentrations were adjusted to 1×10^6 per mL and were added into the upper chamber (200 μ L each). In the lower chamber, 600 μ L medium containing 10% FBS was added. The whole chamber was incubated in a CO₂-incubator at 37°C for 24 hours. Remaining matrix gel and cells in the upper chamber were gently removed. Cells in the lower chamber were stained by crystal violet for

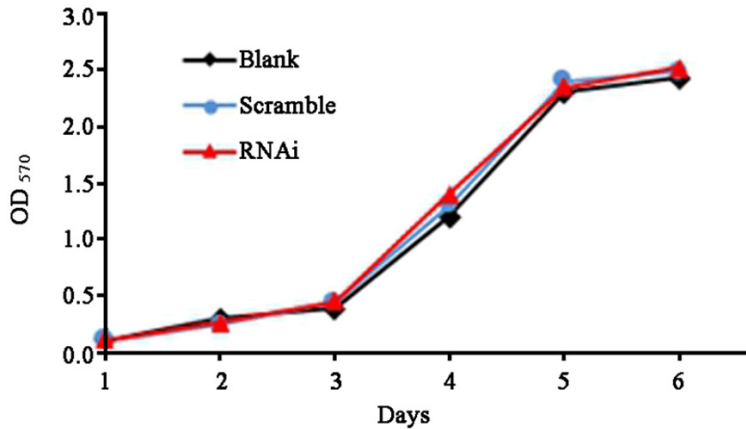


Figure 4. MTT for cell proliferation.

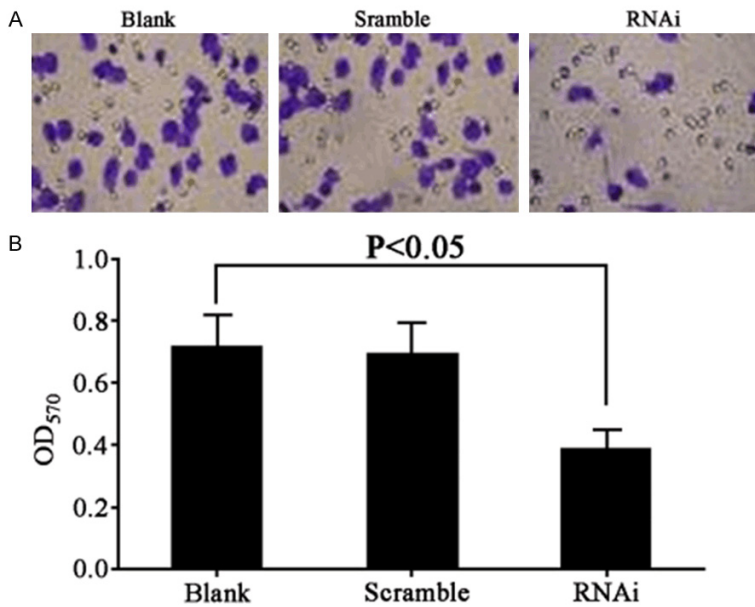


Figure 5. A Transwell image after stable transfection. B Cell invasion ability after 14-hour charging.

cer tissues, Hepsin had higher expression level. Furthermore, Hepsin was up-regulated with advancement of clinical stage ($P<0.05$), as consistent with ISH.

RNA interference

Using exogenous oligonucleotide sequence Hepsin-RNAi and scramble-RNAi to transfect human prostate cancer line 22RVA1, which were detected for Hepsin expression by Western blotting. The relative level of Hepsin was decreased by 55.9% in RNAi treated (Figure 3). Therefore the oligonucleotide was successfully

transfected and RNAi was accomplished.

MTT assay

After successful transfection, MTT approach was used to process cell proliferation rate. Using untransfected group as the blank control and those cells transfected with Scramble-RNAi sequences as negative control, cells were counted at differentiation time points. Although having slightly decreased 22RV1 cells viability, in general, the proliferation was not significant discussed (Figure 4, $P>0.05$).

Transwell for cell invasion

Using Transwell assay, we successfully detected the cell invasion ability in 14 hours, with the help from non-transfected and Scramble-RNA based cells. Those 22RV1 cells transfected with Hepsin-RNAi had decreased Hepsin expression level and invasion ability ($P<0.05$, Figure 5A). The invasion ability of all cells was listed in Figure 5B. By measuring OD₅₇₀, we found that Hepsin-RNAi treated 22RV1 cells had lower invasion by 45.5%.

Discussion

This study utilized both ISH and RT-PCR to detect the expression of Hepsin in prostate cancer tissues compared to BPH tissues, and found elevated Hepsin expression in cancer tissues. Moreover, the expression level of BPH was further enhanced with elevated clinical stage of prostate cancer. These results suggested that Hepsin expression level might be correlated with occurrence/progression of prostate cancer and clinical features.

The over-expression of Hepsin in mouse prostate cancer cell, however, did not facilitate the proliferation ability, indicating that Hepsin was

not an oncogene [15]. By analyzing expression level of Hepsin in prostate cancer tissues, scholars found over-expression of Hepsin in near 90% of all prostate cancer tissues. By further gene polymorphism study, it is proposed that such polymorphism may up-regulate Hepsin gene expression. As most of such polymorphism sites locate in introns of Hepsin genes, they may regulate gene expression via modulating mRNA splicing patterns [16]. However, no direct evidences have been obtained regarding such views.

Hepsin has been found to be correlated with invasion/migration of various tumors including prostate cancer, liver cancer and breast cancer [8, 11, 17]. Therefore we employed RNA interference technique to inhibit the expression of Hepsin in prostate cancer cell line 22RV1. We found that as Hepsin level was suppressed, no significant change of cell proliferation has occurred, while the migration and invasion ability has been down-regulated by 45.5%, as consistent with the hypothesis that Hepsin participates in the invasion process of prostate cancer cells. The mechanism of Hepsin during tumor cell migration and invasion, however, remained inconclusive.

Tumor migration is one complicated process, among which the invasion of basal membrane by tumor cells is necessary. Such invasion normally involves four major steps: the weakening of cell-to-cell adhesion, attachment of tumor cells on basal membrane, degradation of extracellular matrix and migration of cells out from basal membrane [18]. During the process of extracellular matrix degradation, proteinase induced the hydrolysis of various protein components including laminin, fibronectin, proteoglycan and collagen [19]. Hepsin protein is a type II transmembrane serine proteinase, and is composed of three domains including intracellular domain, transmembrane domain and extracellular domain with protein hydrolysis activity [5]. Current study has demonstrated that Hepsin can activate urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) to activated plasminogen/plasmin hydrolysis reaction to eventually break the basal membrane structure for accelerating tumor cell invasion and migration [20]. In addition, Hepsin can degrade LN-332, which is one cellular matrix molecule for cell-to-cell adhesion, for enhancing tumor cell invasion and migration [21].

Major weakness and limitation of this study included insufficient number of research objects, impairing the power to reflect the correlation between Hepsin expression level and clinical stages of prostate cancer. Moreover, the relationship between Hepsin and related genes of extracellular matrix has not been illustrated. In summary, this study demonstrated the up-regulation of Hepsin in prostate cancer tissues, in addition to the correlation between Hepsin expression and migration/invasion of prostate cancer cells, thus providing evidences for the diagnosis and treatment of prostate cancer.

Disclosure of conflict of interest

None.

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