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ORIGINAL ARTICLE

Prostate Cancer

Expression of kallikrein-related peptidase 7 is decreased in prostate cancer

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Recent evidence suggests that the human kallikrein 7 (KLK7) is differentially regulated in a variety of tumors. The aim of this study was to determine the expression of kallikrein-related peptidase 7 and KLK7 in our large collection of prostate samples. Between August 2000 and December 2012, 116 patients with histologically confirmed prostate cancer (PCa) and 92 with benign prostate hyperplasia (BPH) were recruited into the study. Using immunohistochemistry, quantitative reverse transcription polymerase chain reaction (RT-PCR) and western blot, kallikrein-related peptidase 7 expression in BPH and PCa tissues was determined at the mRNA and protein levels. The relationships between kallikrein-related peptidase 7 mRNA expression and clinicopathological features were analyzed. A total of 64 of 92 (69.57%) benign cases showed positive staining for KLK7 and 23 of 116 (19.83%) malignant cases showed positive, the difference of KLK7 expression between PCa and BPH was statistically significant ($P < 0.001$). The expression level of kallikrein-related peptidase 7 mRNA was significantly decreased in PCa tissues compared with that in BPH tissues and normal prostate tissue. Kallikrein-related peptidase 7 mRNA exhibited different expression patterns in terms of localization depending on pathological category of PCa. Similarly, our western immunoblot analyses demonstrated that the protein expression levels of KLK7 was lower in PCa than in BPH tissues and normal prostate tissue. Kallikrein-related peptidase 7 and KLK7 expression are down-regulated in PCa and lower expression of kallikrein-related peptidase 7 closely correlates with higher Gleason score and higher prostate-specific antigen level.

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INTRODUCTION

Prostate cancer (PCa) is a heterogeneous disease with an estimated 241 740 new cases and 28 170 deaths related to this disease in the United States in 2012, making PCa the second most frequently diagnosed cancer and the second-leading cause of cancer death in men.[1] The incidence of PCa in China was reported to be 1.6 in 100 000 year^{-1}, and its incidence in China is increasing steadily in recent years.[2] With the development of image technology and new prostate biopsies protocols in recent years, the diagnosis of PCa was improved obviously. The main options for localized PCa are active surveillance, radical prostatectomy, and radiotherapy with or without adjuvant androgen deprivation therapy (ADT).[3] Radical prostatectomy is the standard treatment for organ-confined tumors; however, about 5% of patients initially present with metastatic lesions (most frequent in bone) at the time of primary diagnosis, and surgical removal become infeasible.[4] Even after seemingly complete removal of the tumor, the recurrence occurs in approximately 20%–30% of patients.[5] The incomplete understanding of molecular features of PCa might be one of the reasons for this unsatisfied situation, although recent gene expression studies have significantly improved our knowledge. Therefore, it is important to identify new biomarkers responsible for PCa progression and metastasis to provide effective strategies for the prevention and therapy of this disease.

The human kallikrein (KLK) family represents 15 secreted serine proteases encoded by genes colocalized on chromosome 19q13.4.[6] KLKs maybe causally involved in carcinogenesis, particularly in tumor metastasis and invasion. Most of them have been reported to be potential biomarkers for several carcinomas and other diseases.[7] Determination of the prostate-specific antigen (PSA) also known as kallikrein-related peptidase 3 has improved the detection of PCa. Kallikrein-related peptidase 7, known as a member of the kallikrein gene family, is a proteolytic enzyme important for epithelial cell shedding.[8] Kallikrein-related peptidase 7 is up-regulated in tumor tissues of patients afflicted with cancer of the cervix uteri, colon, pancreas and ovary, while down-regulated in breast cancer and kidney cancer, playing an unfavorable role in prognosis of these pathologies.[9-12] However, there have been few studies reporting the expression of kallikrein-related peptidase 7 in PCa. This study was designed to explore the expression of kallikrein-related peptidase 7 in our large collection of clinical prostatic carcinoma samples, and investigate its clinicopathological significance in PCa.

MATERIALS AND METHODS

Patients and tissue specimens

This study included 116 patients who had undergone radical prostatectomy and bilateral lymphadenectomy at the Department of

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Urology, Ruijin Hospital, between August 2000 and December 2012 and for whom archival tissues were available. No patient was managed preoperatively with either hormonal or radiation therapy, and no secondary cancers were observed. Ninety-two cases of benign prostate hyperplasia (BPH) were obtained from men undergoing suprapubic prostatectomy or transurethral plasmakinetic enucleation of the prostate. Twenty cases of normal prostate tissue were obtained from bladder cancer patients who underwent radical cystoprostatectomy. The stages of cancer for all patients were determined by the American Joint Committee on Cancer 2002 system. The specimens were examined by two-staff pathologists who were blinded to the clinical outcome and follow-up data. The evaluation of the specimen was performed according to the guidelines of the College of American Pathologists. Formalin-fixed, paraffin-embedded tumor tissues from these patients were evaluated. Besides, freshly frozen tissue samples were available. Samples were snap-frozen in liquid nitrogen immediately after surgery and experiments were performed. This study was approved by the Ethics Committee of Ruijin Hospital. All patients provided informed consent.

Immunohistochemistry

Specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and cutted into serial sections at a thickness of 3 μm. Paraffin-embedded tissues were dewaxed in xylene, rehydrated by serial concentrations of ethanol, and then rinsed in phosphate buffer solution (PBS), followed by treated with 3% H_2O_2 to refrain endogenous peroxidase. After being heated in a microwave at 750 W for 15 min to repair the tissue antigen, the sections were incubated with 10% normal goat serum at room temperature for 10 min to block nonspecific reactions. This was followed by a PBS wash and incubation with polyclonal goat anti-hK7 antibody (R and D Systems, Minneapolis, MN, USA) diluted to 1:40 (5 g ml^{-1}) for 12 h at 4°C. The slides were then incubated by anti-mouse EnVisionTM kit (DAKO, USA) for 30 min at room temperature. After a PBS wash, the sections were developed in diaminobenzidine substrate. The sections were then counter-stained in hematoxylin for 2 min and then dehydrated in ethanol and xylene before being mounted. Sections were re-prepared by EnVision immunohistochemical staining. The staining results of ovarian cancer tissue sections which KLK7 positive had already known were regarded as a positive control, PBS instead of primary antibodies was like a negative control.

Evaluation of immunohistochemical results

Digital images of each tissue microarray core were manually scored and displayed according to staining intensity and morphology. Positive hK7 staining was characterized by brown-yellow granules located diffusely in the cell cytoplasm. Lack of any obvious purple-brown or brown-red pigmentation in the cytoplasm of tumor cell was considered negative. For quantitative analyses of expression, five visual fields were randomized selected per section under high-power microscope (×400), and 200 cells were counted in each high-power field. Staining was scored according to the percent of staining tumor cells, including 0: <25%, 1: 25%–49%, 2: 50%–75% and 3: >75%, and as the intensity, including 0: no, 1: weak, 2: moderate, and 3: strong staining. Positive or negative expression was determined according to the combination of these two variables. A total score of >3 was considered positive, and total score of 3 or less was considered negative, as previously reported. The results were scored by two independent pathologists who were blinded to the diagnosis.

Total RNA extraction and cDNA synthesis

Upon collection, the prostate tissues were snap frozen in liquid nitrogen and subsequently kept at −80°C until required. The tissues were pulverized, and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Spectrophotometric methods determined the purity and concentration of RNA. Three micrograms of total RNA were reverse-transcribed into first-strand cDNA using reverse transcription system kit (Promega, Madison, WI, USA) according to the following protocol with the reaction kit. Briefly, samples were preincubated at 70°C for 10 min, cooled on ice then added to the reaction mixture of 10 mmol l^{-1} deoxynucleotide triphosphate mixture, 25 mmol l^{-1} $MgCl_2$, 15 U of avian myeloblastosis virus reverse transcriptase, reverse transcription ×10 buffer, 0.5 U of Rnasin and 0.5 μg oligo-(dT) 15 primer, and scaled up to a final volume of 20 l. The reaction mixture was sequentially incubated at 44°C for 15 min, 99°C for 5 min and 4°C for 5 min. The cDNA was stored at 20°C before use.

Quantitative real-time polymerase chain reaction

Quantitative RT-PCR was performed using SYBR Master Mix (Takara) on an ABI Prism 7900HT (Applied Biosystems). A human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control for sample normalization. Results were presented as the fold expression relative to that of GAPDH. PCR primers were as follows: for human GAPDH, forward 5′-GAGTCAACGGATTTGGTCGT-3′ and reverse 5′-GACAAGCTTCCCGTTCTCAG-3′; for human *KLK7* forward 5′-ACCCTCAGTGCTGGAGAAGA-3′ and reverse 5′-AAGGGTCTCGGTGTACGTTG-3′.

Western blot

Western immunoblot analyses were performed with protein lysates obtained from snap–frozen tissue samples. Protein levels were determined using the BCA Protein Assay Kit (Pierce, USA). About 30 μg of the respective tissue protein were separated by SDS–PAGE (using 10% gels) and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk and then incubated with goat anti-KLK7 polyclonal antibody (1:200; R and D Systems) and actin (1:10,000; MP Biomedicals). Membranes were washed three times for 10 min each with Tris-buffered saline (50 mmol l^{-1} Tris, pH 7.4, 0.9% NaCl) containing 0.05% Tween-20 (TBS-T) and incubated with horseradish peroxidase-conjugated secondary antibodies. Membranes were then washed again three times for 10 min each with TBS-T. Target protein bands were visualized using an enhanced chemiluminescence method. The intensity of the bands was quantified using the Tanon GIS syatem (Tanon, Shanghai, China), and the data were normalized to the actin loading controls. All western immunoblot analyses were performed 3 times.

Statistical analysis

Data analyses were performed using SPSS statistical package 15.0 (SPSS Inc., USA). Patient characteristics were expressed as the mean ± standard deviation (s.d.) for continuous variables, and as the count and percent for discrete variables. Data were analyzed using the Pearson's chi-square test and Fisher's exact test. Statistical significance was taken at the $P < 0.05$ level.

RESULTS

To investigate the clinical significance of kallikrein-related peptidase 7 gene expression in human PCa progression and metastasis, we

analyzed mRNA expression by quantitative real-time PCR in a total of 208 patients, including 92 BPH, 22 prostate intraepithelial neoplasia (PIN), 64 clinically localized PCa and 30 metastatic cases. The patients had a mean age of 63.57 ± 5.36 years at surgery (range 46–80) (**Table 1**).

The expression of kallikrein-related peptidase 7 mRNA normalized to GAPDH mRNA was detected by quantitative real-time PCR (**Figure 1**). The expression level of kallikrein-related peptidase 7 mRNA was significantly decreased in PCa tissues compared with that in BPH tissues and normal prostate tissue ($P < 0.01$). Kallikrein-related peptidase 7 mRNA exhibited different expression patterns in terms of localization depending on pathological category of PCa and metastasis. We stratified localized PCa by the Gleason score into three subgroups, Gleason score <7, =7, and >7. In the localized PCa samples, kallikrein-related peptidase 7 mRNA expression appeared to be associated with lower Gleason score, which reached its predominance in Gleason <7 cases. We further stratified localized PCa by three subcategories on the basis of preoperative PSA levels: <10 ng ml^{-1} as low risk; 10–20 ng ml^{-1} as intermediate risk, and >20 ng ml^{-1} as high risk. The analysis revealed that the low expression of kallikrein-related peptidase 7 mRNA was positively associated with higher PSA level ($P < 0.05$). Next, we analyzed for Gleason score and preoperative PSA simultaneously. The analysis revealed that in the high-risk subcategory (PSA >20 ng ml^{-1}) lower kallikrein-related peptidase 7 mRNA was associated with Gleason >7, and higher kallikrein-related peptidase 7 mRNA were associated with Gleason <7 ($P < 0.05$).

In addition, the difference of kallikrein-related peptidase 7 mRNA expression level between tissues with or not with all types of metastasis (lymphnode, central nervous system or bone) also exists statistical significance ($P < 0.05$). No significant correlation, however, was found between kallikrein-related peptidase 7 mRNA expression and age. Altogether, the kallikrein-related peptidase 7 mRNA expression decreased from PIN to localized and to metastatic PCa.

Immunohistochemical expression of KLK7 was examined in 92 BPH tissues, and 116 PCa tissues. We observed positive staining of KLK7 in BPH epithelium; however, little or no staining of KLK7 was observed in PCa (**Figure 2**). A total of 64 of 92 (69.57%) benign cases showed positive staining for KLK7 and 23 of 116 (19.83%) malignant cases showed positive, the difference of KLK7 expression between PCa and BPH was statistically significant ($P < 0.001$). Western blot analyses NIL the results, and we also found the protein expression levels of KLK7 was lower in PCa than in BPH tissues and normal prostate tissue, and the difference between these groups had statistical significance ($P < 0.001$) (**Figure 3**).

Table 1: Pathological and clinical data for PCa patient

Variable	*n*	*Percentage*
Age (year)		
≤60	36	31.03
>60	80	68.97
Preoperative PSA level (ng ml^{-1})		
<4	23	18.83
4–10	38	32.76
10–20	34	29.31
>20	21	18.10
Pathological category		
PIN	22	18.97
LPCa	75	64.66
Gleason score<7	15	20.00
Gleason score=7	34	45.33
Gleason score>7	26	34.67
MPCa	19	16.38
LN	4	21.05
CNS	1	5.26
Bone	14	73.68

PSA: prostate-specific antigen; PIN: prostatic intraepithelial neoplasia; PCa: prostate cancer; LN: lymph node; CNS: central nervous system; LPCa: localized prostate cancer; MPCa: metastatic prostate cancer

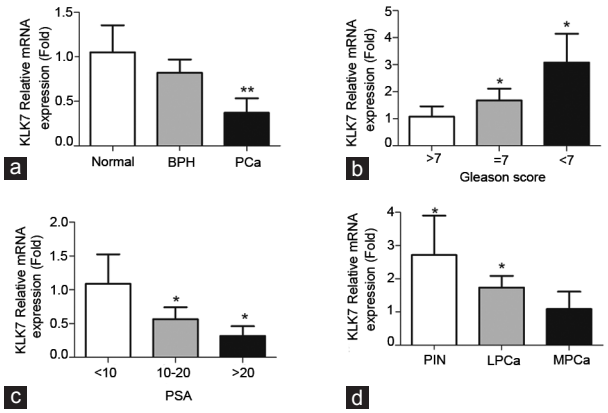

Figure 1: Quantitative real-time polymerase chain reaction showing expression level of *KLK7* mRNA. (**a**) *KLK7* mRNA expression in PCa tissues, BPH tissues and normal prostate tissue (**$P < 0.01$). (**b**) *KLK7* mRNA expression in the three subgroups of localized PCa samples, Gleason score < 7, =7 and > 7 (*$P < 0.05$). (**c**) *KLK7* mRNA expression in the three subgroups of localized PCa samples, PSA levels < 10 ng ml^{-1}, 10–20 ng ml^{-1}, and > 20 ng ml^{-1} (*$P < 0.05$). (**d**) *KLK7* mRNA expression in PIN localized PCa and metastatic PCa (*$P < 0.05$). BPH: benign prostate hyperplasia; PCa: prostate cancer; PIN: prostatic intraepithelial neoplasia; LPCa: localized prostate cancer; MPCa: metastatic prostate cancer; PSA: prostate-specific antigen.

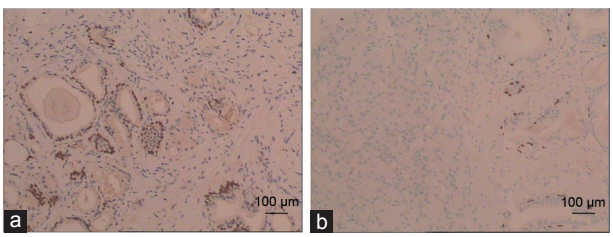

Figure 2: Immunohistochemistry. **(a)** Positive staining of hK7 was observed in the cytoplasm of benign prostate epithelium, scale bar = 100 μm. (**b**) Little or no staining of hK7 was observed in prostate cancer, scale bar = 100 μm.

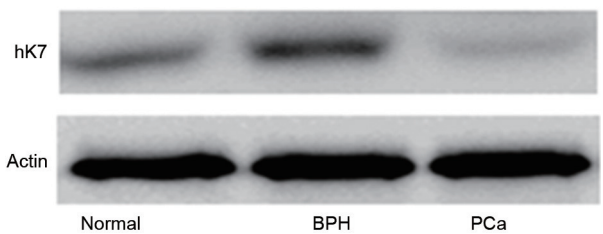

Figure 3: Western blots showing expression of hK7 in PCa tissues, BPH tissues and normal prostate tissue. BPH: benign prostate hyperplasia; PCa: prostate cancer.

DISCUSSION

PCa is one of the leading causes of cancer-related deaths in the worldwide. In China, the incidence of PCa has increased dramatically over the past two decades, most likely due to lifestyle changes and rapidly economic development.[2] In recent years, novel diagnostic and therapeutic technologies have been advanced for early diagnosis and treatment of PCa, but the clinical outcome of PCa patients remains unsatisfactory. Although PSA has been the epoch-making marker for PCa screening and diagnosis, most newly diagnosed Chinese PCa patients already have metastatic disease because PSA and digital rectal examination for PCa screening is not a routine practice in China. Benign prostatic diseases including BPH and acute prostatitis also can present with elevated serum PSA, leading to a higher rate of false positive of diagnosis. Besides, approximately 30% of patients experience recurrence after primary therapy. Recurrent or metastatic PCa progression usually is androgen dependent, so ADT is generally considered first-line therapy at this time point.[13] Unfortunately, castration-resistant PCa invirtually develop within a median of 18–24 months after castration in these patients, and current anticancer treatments are not effective.[14] Thus, exploring possible new biomaker holds great promise for PCa management.

Serine proteases have several functions in tumor development, including cell growth regulation, invasion and angiogenesis. Tumor-associated proteases such as several of the members of the serine protease-type kallikrein-related peptidase family of genes (kallikrein-related peptidase 1–15), located on chromosome 19q13.3–4, exert various physiological functions in tissues and have been implicated in cancer progression.[15] Studies have shown that kallikrein-related peptidases might promote or inhibit cancer cell growth, angiogenesis, invasion and metastasis through activation of growth factors and other proteases, release of angiogenic or antiangiogenic factors, and degradation of extracellular matrix components.[16] Nowadays the tissue kallikrein-related peptidases are considered a rich source of cancer biomarkers. Kallikrein-related peptidases are highly expressed in the prostate suggesting their potential as novel diagnostic markers.[17] Recently, kallikrein-related peptidase 2, kallikrein-related peptidase 4, and kallikrein-related peptidase 11 have been shown to be potential biomarkers in PCa.[18-21]

Several reports describe that kallikrein-related peptidase 7 plays an important role in the normal physiology of the skin, particularly in epidermal homeostasis. The prominent expression of kallikrein-related peptidase 7 in human skin was confirmed in a survey of tissue extracts and biological fluids, and its expression was also detected in other normal tissues, particularly in esophagus and kidney.[21] Recently, kallikrein-related peptidase 7 is has been reported in a number of tumor types and appears of significant prognostic value in various malignant tumors. The presence of KLK7 in serum has been proposed as an unfavorable marker in patients with cancer of the ovary, breast, and uterine cervix. Furthermore, overexpression of KLK7 leads to the development of aggressiveness in ovarian, intracranial, and pancreatic cancer cells.[21,22] However, there are few reports concerning a potential role of kallikrein-related peptidase 7 and KLK7 in PCa.

In this study, we aimed to examine the expression of kallikrein-related peptidase 7 in a large set of prostate samples, including BPH, PIN, localized PCa, and metastatic PCa by immunohistochemistry, quantitative real-time PCR and western blot. Our results show that kallikrein-related peptidase 7 mRNA expression is significantly down-regulated in PCa, compared to BPH and normal prostate tissue. Immunohistochemistry examination showed that positive staining of KLK7 in BPH epithelium; and little or no staining of KLK7 was observed in PCa. Western blot examination showed that protein expression of KLK7 was decreased in PCa. Furthermore, in the localized PCa samples, kallikrein-related peptidase 7 mRNA expression appeared to be associated with lower Gleason score, which reached its predominance in Gleason <7 cases. Similarly, our study demonstrated a close association between kallikrein-related peptidase 7 mRNA expression level and lower PSA level. Furthermore, we analyzed for Gleason score and preoperative PSA simultaneously. We found that the kallikrein-related peptidase 7 mRNA expression level in the high-risk subcategory was associated with lower Gleason, and higher kallikrein-related peptidase 7 mRNA were associated with Gleason <7.

In addition, a significant difference in kallikrein-related peptidase 7 expression was found between tissues with or not with all types of metastasis (lymphnode, central nervous system or bone). No significant correlation, however, was found between kallikrein-related peptidase 7 mRNA expression and age. Recently, Xuan *et al*.[23] reported that the antileukoprotease, a specific inhibitor of KLK7, is frequently decreased in PCa. The mRNA level of kallikrein-related peptidase 7 significantly decreased in PCa compared with that in benign prostate epithelial cells. Significant negative association was found between Gleason grade and both kallikrein-related peptidase 7 and ALP expression.[23] This is in line with our findings. Besides, Jamaspishvili *et al*.[24] confirmed down-regulation of kallikrein-related peptidase 7 in malignant prostate epithelial cells compared to benign/normal prostate epithelial cells. The inhibition of KLK7 of skin cells points to the potential of KLK7 as a target for inhibition or down-regulation in the spread or metastasis of PCa.

We should note that the tissue expression levels of KLK7 might not represent the serum levels of the protein and the level in cells. PSA has been used successfully as a circulating tumor marker for the early detection of PCa. Although levels of PSA and kallikrein-related peptidase 2 are elevated in the serum of PCa patients, their tissue concentration is lower in the PCs tissues. The KLK7-expressing DU145 PCa cells exhibited increased invasiveness and morphological changes. Mo *et al*.[22] demonstrated that KLK7 promotes cancer cell migration and invasiveness and induces epithelial-mesenchymal transition (EMT)-like changes in DU145 prostate tumor cells *in vitro*, suggesting that KLK7 accelerates PCa cells invasion and progression through the induction of EMT.

CONCLUSIONS

We provide strong evidence suggesting that kallikrein-related peptidase 7 and KLK7 expression are down-regulated in PCa and low expression of kallikrein-related peptidase 7 closely correlates with advanced disease stage, which predicts a poorer prognosis. However, further studies will be needed to understand the molecular mechanism of kallikrein-related peptidase 7 involved in PCa progression and prognosis, which may lead to further development of new approaches targeting kallikrein-related peptidase 7 and KLK7 for effective tumor management.

AUTHOR CONTRIBUTIONS

CYZ and YZ designed the experiments, and CYZ carried out the experiments. WBR and JD evaluated the IHC results. CYZ and JD analyzed the data. YZ and ZJS supervised the project. CYZ and JD wrote the manuscript. All authors reviewed and approved the final manuscript.

COMPETING INTERESTS

All authors declare that there are no competing interests.

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