

Potential use of chymotrypsin-like proteasomal activity as a biomarker for prostate cancer

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Abstract. Although it is the most widely used biomarker for prostate cancer, the use of prostate specific antigen (PSA) is controversial due to its limitations in specificity and sensitivity. The proteasome is a complex associated with cell proliferation and apoptosis, and the abnormality of these processes may lead to tumor occurrence. Previous studies have reported that proteasomal activity is associated with cancer progression and can be used in risk stratification. The purpose of the present study was thus to investigate the feasibility of proteasome activity as a biomarker for prostate cancer. Proteasome activity *in vitro* and *in vivo* was detected, along with the expression of the substrate proteins NF- κ B inhibitor- α (I κ B- α), Bcl-2-associated X (Bax) and p27. Chymotrypsin-like proteasomal activity was elevated by 70% *in vitro* and 23% *in vivo*, and the expression levels of the proteasome substrate proteins I κ B- α , Bax and p27 were decreased in prostate cancer cells and prostate tumor xenografts compared with normal mouse prostate tissue. In conclusion, proteasomal chymotrypsin-like activity maybe a potential biomarker for prostate cancer, and may be suitable to supplement PSA in clinical application for prostate cancer diagnosis.

Introduction

Prostate cancer is the most commonly diagnosed malignancy in men in western countries (1). As the second most common cause for cancer-associated mortality among men, prostate cancer caused ~27,540 deaths in the United States (2015) (2).

In addition, the incidence and mortality for prostate cancer have been evidently increasing in Asia, including in China, in recent years (3).

Although novel therapies for prostate cancer with proven survival benefits have been developed relatively recently (4,5), the overall increase in survival rate has been negligible. A major clinical challenge in prostate cancer is the insufficient power of the currently available diagnostic tests. Since the introduction of serum prostate specific antigen (PSA) screening >30 years ago, prostate cancer diagnosis and management have been guided by this biomarker; it remains the most commonly used tumor marker for prostate cancer diagnosis, postoperative monitoring and prognosis evaluation. PSA is a protein secreted by the epithelial cells of the prostate; an increase in serum PSA is often identified in prostate cancer patients (6,7). However, PSA is susceptible to various confounding factors, including benign prostatic hyperplasia, prostatitis and urethral surgery, which may influence the clinical reliability (8,9). The deficiencies of PSA, including the lack of specificity and sensitivity, may lead to false-positive or false-negative results. In view of this, identifying a biomarker with an improved diagnostic and prognostic potential for prostate cancer assessment may be of great significance.

Refinements to PSA measurements have been proposed, including early PSA, benign PSA, free PSA, dynamic PSA parameters (including PSA velocity and PSA doubling time), PSA density (PSA to prostate volume ratio) and age-specific PSA level (1). Although these alternative applications of PSA may improve the diagnosis accuracy of prostate cancer to a certain extent, their relatively complicated implementation and the same confounding issues as usually affect tPSA continue to limit their application (10).

The proteasome is a multicatalytic proteinase complex responsible for the degradation of the majority of intracellular proteins, including the proteins required for cell cycle regulation and apoptosis (11). The 26S proteasome is comprised of two 19S regulatory subunits and a 20S core. In the proteasome degradation system, the target protein is recognized by the 19S subunits following ubiquitination, and can then access the 20S core for further degradation. The 20S subunit is a multicatalytic threonine protease with three types of enzymatic activity, described as chymotrypsin-like, trypsin-like and caspase-like activities. As part of the ubiquitin-proteasome pathway, the

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proteasome plays a vital role in the degradation of proteins from a broad range of cellular pathways, and contributes to the pathology of a number of human diseases, including cancer, in which regulatory proteins may be stabilized due to decreased degradation, or lost due to accelerated degradation (12). The proteasome degrades a range of endogenous proteins associated with cancer, including transcription factors, cyclins, Bcl-2-associated X (Bax), p53, p27 and inhibitor of NF κ B- α (I κ B- α), and it has become an important potential target for cancer therapy.

The majority of studies regarding this topic have concentrated on the proteasome inhibitor for tumor treatment; research on the proteasome for disease diagnosis is insufficient. Stoeckner *et al* (13) reported that in 20 tumor patients (including those with breast, gastric, kidney, colon, testicular, liver and lung cancer), the proteasome 20S serum level was significantly elevated compared with controls, indicating serum proteasome could be applied in tumor diagnostics. Our previous study demonstrated that a proteasome inhibitor could affect the proliferation and apoptosis of prostate cancer cells by inhibiting chymotrypsin-like activity, thus influencing the expression of the target proteins I κ B- α , Bax and p27 (14). Therefore, in the present study, the chymotrypsin-like, trypsin-like and caspase-like proteasomal activity in cultured LNCaP cells and tumor-bearing nude mice was assessed, in addition to the expression of the proteasomal substrates I κ B- α , Bax and p27, in order to analyze the feasibility of proteasomal activity as a candidate biomarker for prostate cancer.

Materials and methods

Materials. LNCaP human prostate cells were provided by Ryder Guangzhou Lian Kang Biological Technology Co., Ltd. (Guangzhou, China). A Prostate Epithelial Cell Medium BulletKit™ was purchased from Lonza Group, Ltd. (Basel, Switzerland). The keratinocyte-serum free medium was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and PBS was from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). RPMI-1640, penicillin and streptomycin were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Fetal bovine serum (FBS) was purchased from Hyclone (GE Healthcare Life Sciences). Mouse monoclonal antibodies against Bax (cat. no. sc-23959) and p27 (cat. no. sc-1641), and rabbit polyclonal antibodies against inhibitor of nuclear factor (NF)- κ B- α (I κ B- α ; cat. no. sc-203) and GAPDH (FL-335; cat. no. sc-25778) were both from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Suc-LLVY-AMC, Z-LLE-NA, and BZVGR-AMC was all from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany).

Cell culture. LNCaP cells were grown in RPMI-1640 supplemented with 10% FBS, 100 U/ml of penicillin and 100 μ g/ml of streptomycin, and were maintained at 37°C and 5% CO₂ for 1 week. Prostate epithelial cells were obtained from prostate tissue by conventional tissue culture methods: A human prostate tissue specimen was obtained from men undergoing robotic radical prostatectomy, and was sliced into 1 mm³ blocks and placed in a T25 flask coated with collagen. The tissue blocks were cultured at 37°C with 5% CO₂ for ~1 week in prostate epithelial cell medium, and the medium was

changed every 3 days. The cells were digested and collected when the primary monolayer cells covered the surface of the flask, and they were passaged with keratinocyte-serum free medium. The first generation of cells were used for further experiments to ensure the integrity of the prostate epithelial cells. Cells were observed for morphological changes and photographed under a phase contrast inverted microscope (magnification, x100; Olympus Corporation, Tokyo, Japan). The use of human tissue was approved by the Research Ethics Committee of Guangzhou First People's Hospital, Guangzhou Medical University (Guangdong, China), and written informed consent from the patient was acquired prior to the use of the tissue in research.

Human prostate tumor xenograft experiments. Animal experiments in the present study were performed in accordance with the guidelines outlined by the Institute for Laboratory Animal Research in Guangzhou Medical University (Guangzhou, China). A total of 30 male BALB/C-nu mice aged 4-6 weeks, (mean body weight, 20 g) were purchased from Guangdong Medical Laboratory Animal Center (Foshan, China) and housed in accordance to a protocol described previously (15). Mice were randomly divided into two groups (15 mice/group). In the experimental group, 5x10⁵ LNCaP cells suspended in 0.2 ml PBS were inoculated into the left flank of each mouse. Mice in the control group were injected with 0.2 ml PBS. Tumor sizes were measured every 3 days using calipers, and tumor volumes were calculated according to the standard formula: Width² x length x 0.52. At 4 weeks, the mice were sacrificed. Blood and tumor tissues were taken for further analysis.

Proteasome activities assay. 100 μ l suspension LNCaP cells (1.0x10⁵ cells/ml) and prostate epithelial cells (1.0x10⁵ cells/ml) were plated in a 96-well plate and cultured for 24 h. Then 1 μ l of proteasome activity assay buffer containing 4 mM a fluorogenic peptide substrate, including Suc-LLVY-AMC for detecting chymotrypsin-like activity, Z-LLE-NA for detecting caspase-like activity or BZVGR-AMC for detecting trypsin-like activity, was added to the wells. Following a 2 h incubation, the fluorescence intensity was measured by a microplate reader with the excitation wavelength of 380 nm and the emission wavelength of 460 nm.

For the serum assay, 1 μ l containing 4 mM of a fluorescent substrate was added to 100 μ l of mouse serum and incubated at 37°C for 2 h. The proteasome activity was then detected as the fluorescence intensity by the microplate reader, with the excitation and emission wavelengths of 380 and 460 nm, respectively.

Western blot analysis. Cells were harvested, washed with PBS twice and lysed in cell lysis buffer (50 mM Tris-HCl; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.5% Na-deoxycholate; 0.1% SDS; 1 mM PMSF; 10 μ l/ml protease inhibitor cocktail P8340 provided by Sigma-Aldrich; Merck KGaA) for 30 min at 4°C. Then the cells were scraped off and transferred into a 1.5 ml centrifuge tube. The lysates were centrifuged at 14,000 x g for 5 min at 4°C. The DC™ Protein Assay kit II (cat. no. 5000112; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to determine the protein concentration

with the Bradford assay method, as follows: Standards from 0 to 4 mg/ml protein were prepared; 20 μ l of reagent S from the kit was added to 1 ml reagent A and mixed thoroughly. Then 5 μ l of each standard and sample solution was added into 25 μ l of the S/A mixture in separate microtiter plate wells. Next, 200 μ l of reagent B was added, and the wells were mixed thoroughly using a micro plate mixer. Following incubation at room temperature for 15 min, the absorbance was measured at 750 nm in a plate reader. Then 50 μ g of cell lysates were separated using SDS-PAGE (10% gel) and electrophoretically transferred to a PVDF membrane, followed by western blotting using the aforementioned specific antibodies to I κ B- α , Bax, p27 and GAPDH, as previously described (16). Images were then visualized using an enhanced chemiluminescence kit (cat. no. 32106; Pierce, Rockford, IL USA) and quantified using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA). Benign gross prostate tissues from the mice in the control group were obtained, as described by a previous protocol (17). Western blotting using the tumor and benign prostate tissue samples from the mice was then performed as for the cultured cancer cells.

Statistical analysis. SPSS (version 18; SPSS, Inc., Chicago, IL, USA) was used for data analysis. Student's t-test was applied to evaluate the differences between the experimental and control groups. Data were expressed as the mean \pm standard deviation (SD), and results from at least three independent experiments were used for statistical analysis. All statistical tests were two-sided. $P < 0.01$ was considered to indicate a statistically significant difference.

Results

Isolation of prostate epithelial cells from prostate tissues. Prostate epithelial cells were obtained from prostate tissue by conventional tissue culture methods. On observation, cells were identified to be well adhered to the collagen surface with a good growth state (Fig. 1A). The prostate epithelial cells exhibited epithelium-like morphology and enhanced cell viability, over-spreading the bottom of the flask at the 16th day. Subsequent to the formation of a cell monolayer, cells were passaged with Keratinocyte-SFM medium (Fig. 1B). The first generation of cells was used for further experiment.

Establishment of a prostate cancer model in mice. At 4 days after the inoculation of LNCaP cells into nude mice, the formation of tumors started to be observed. At the end of the experiment (day 29), the mean size of the tumors was 350 mm³ (Fig. 2). The mice were then sacrificed, and blood and tumor tissues were collected for further assays. Thus, a mouse model for prostate cancer was established.

Proteasomal chymotrypsin-like activity of LNCaP cells is elevated, whereas Bax, I κ B- α and p27 protein levels are decreased. Previous reports have demonstrated an elevated proteasome level in patients with myeloid hematopoietic malignancies (18-20), solid tumors (19) and autoimmune diseases (21). To determine the proteasomal activity variation at acellular level, the proteasomal chymotrypsin-like, caspase-like and trypsin-like activities were measured in

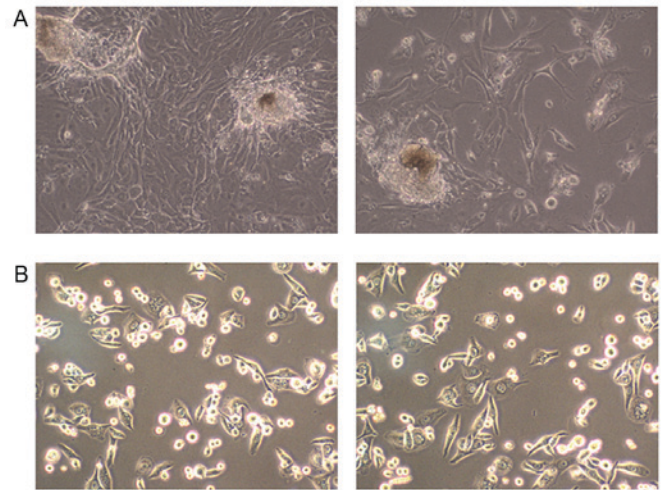


Figure 1. Microscopy images of prostate epithelial cells obtained from human prostate tissue by conventional tissue culture methods. (A) Cells were isolated from prostate tissues in prostate epithelial cell medium and incubated to form a cell monolayer. (B) Prostate epithelial cells were harvested from the monolayer and subcultivated in keratinocyte-serum free medium. Original magnification, x100.

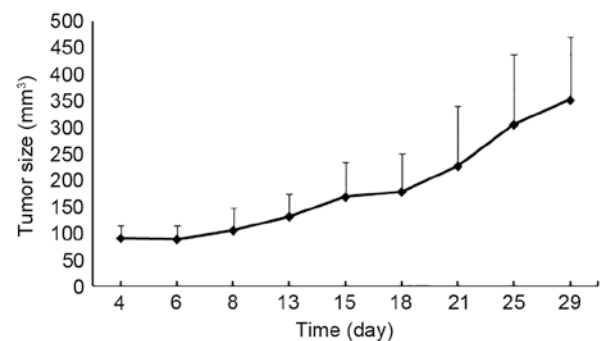


Figure 2. Size of tumors in LNCaP cell-inoculated mice. The growth of tumors was observed from day 4. The mean size of the tumors reached 350 mm³ at the end of the experiment. n=15, data are presented as mean \pm standard deviation (SD).

LNCaP prostate cancer and normal epithelial prostate cells four times. The proteasomal chymotrypsin-like activity was elevated by $\sim 70\%$ in LNCaP cells compared with prostate epithelial cells ($3,286 \pm 259.01$ vs. $1,080 \pm 100.13$; $P < 0.01$). Caspase-like activity was decreased in LNCaP cells ($P < 0.01$), whereas trypsin-like proteasomal activity was not significantly altered (Fig. 3A). The expression levels of three of the most important proteasomal target proteins, Bax, I κ B- α , and p27 (22,23), were then assessed by western blotting. The results indicated all three of the proteins were markedly reduced in LNCaP cells when compared with PE cells ($P < 0.01$; Fig. 3B and C), which was consistent with a previous study (14).

Chymotrypsin-like activity is increased in xenografts compared with normal mouse prostate tissue, accompanied by a reduced Bax, I κ B- α and p27 protein level. In order to clarify whether the chymotrypsin-like proteasomal activity was also elevated *in vivo*, the serum of the mice with or without LNCaP prostate cancer cell xenografts was collected for proteasome activity assays. The chymotrypsin-like

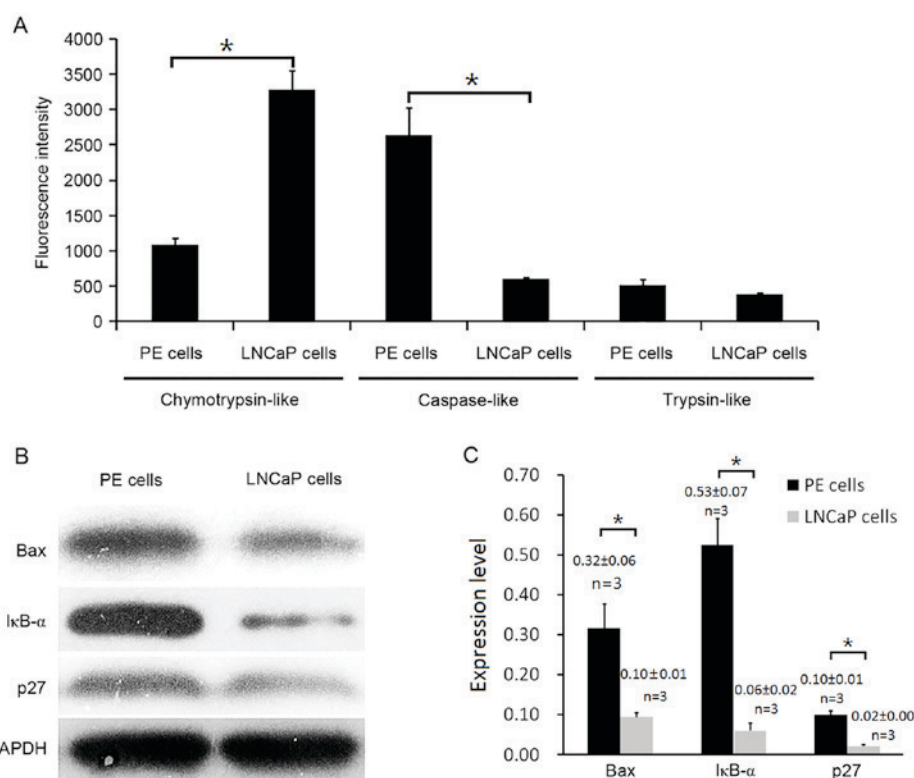


Figure 3. Proteasomal activity *in vitro*. (A) Chymotrypsin-like, caspase-like and trypsin-like activity of the proteasome was measured in LNCaP and prostate epithelial cells. Chymotrypsin-like and caspase-like activities were significantly different between the LNCaP and prostate epithelial cells. Data are presented as means \pm standard deviation. * $P < 0.01$. (B) Representative images of western blotting to determine the expression of Bax, IκB-α and p27 in LNCaP cells compared with prostate epithelial cells, with GAPDH as a loading control. (C) Quantified western blotting results. Each bar represents the mean of three independent experiments; data are expressed as mean \pm standard deviation (SD). * $P < 0.01$. Bax, Bcl-2-associated X; IκB-α, NF-κB inhibitor-α.

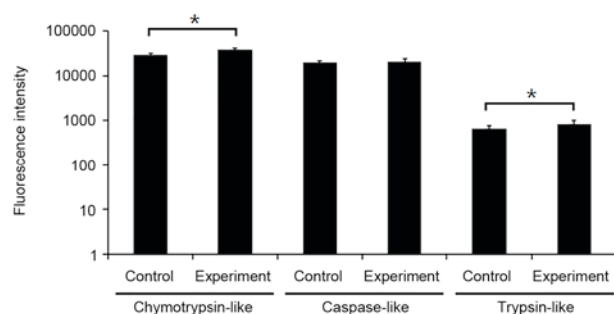


Figure 4. Chymotrypsin-like, caspase-like and trypsin-like proteasomal activity in mouse serum. Chymotrypsin-like and trypsin-like activities were statistically different between xenograft-bearing mice and control mice, whereas caspase-like activity was equivalent. Data are presented as means \pm standard deviation. * $P < 0.01$.

activity of the serum in tumor bearing mice was increased by 23% ($37,344.67 \pm 2,719.64$ vs. $28,845.87 \pm 1,880.47$; $P < 0.01$) compared with the control mice, which was in accord with the *in vitro* results. In addition, the trypsin-like activity was elevated by 21% (824.53 ± 164.87 vs. 648 ± 97.5 , $P < 0.01$; Fig. 4) in the experimental mice, whereas the caspase-like activity of the two groups was at a similar level (Fig. 4). Furthermore, the protein levels of Bax, IκB-α and p27 were analyzed in three pairs of tumor bearing and normal mice using western blot analysis. Decreased levels of Bax, IκB-α and p27 protein were observed in the mouse tumor tissue compared with the prostate tissue from the control mice ($P < 0.01$; Fig. 5A and B).

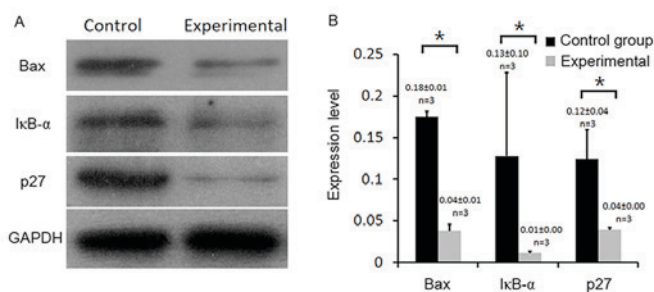


Figure 5. Expression of Bax, IκB-α, and p27 protein *in vivo*. (A) Representative images of western blotting to determine the expression of Bax, IκB-α and p27 in the mouse tumor tissue and the prostate tissue from the control mice. Tumor tissue was removed on day 29 for analysis by western blotting. Decreased Bax, IκB-α, and p27 protein levels were observed in the tumor tissues, with GAPDH as a loading control. (B) Quantified western blotting results. Three mice were selected from each group for this assay. Each bar represents the mean of three independent experiments; data were expressed as the mean \pm standard deviation (SD). * $P < 0.01$. Bax, Bcl-2-associated X; IκB-α, NF-κB inhibitor-α.

Discussion

Prostate cancer is a worldwide concern with a rising incidence and mortality rate (3,24,25); PSA has been used as a biomarker for prostate cancer since the 1980s, although its use remains controversial due to its lack of specificity. Previous studies have reported that increased chymotrypsin-like proteasomal activity is associated with various types of tumor,

including acute myeloid leukemia (26) and melanoma (13). In the present study, the chymotrypsin-like proteasomal activity was significantly elevated ($P < 0.01$) in prostate cancer cells and tumor-bearing mice compared with normal epithelial prostate cells and control mice, suggesting that chymotrypsin-like proteasomal activity may be a candidate biomarker to supplement PSA in the diagnosis of prostate cancer. As the inherent limitations of PSA may cause over-diagnosis, leading to the over-treatment of prostate cancer and causing psychological distress, loss of bodily function, pain and suffering for patients (27), improving the sensitivity and specificity of the detection of prostate cancer is critical. The chymotrypsin-like proteasomal activity assay is relatively simple and reproducible, and can be performed on peripheral blood plasma. Therefore, achymotrypsin-like activity assay together with PSA detection could potentially enhance the accuracy of PSA for prostate cancer diagnosis, ultimately reducing the pain and burden for patients.

Although previous studies (18,19,26) have demonstrated that plasma proteasomes may act as a biomarker in several types of tumor, the origin of the proteasomal activity is has not been identified. Deng *et al* (28) hypothesized that the aggressiveness of prostate cancer could be a mechanism; highly malignant cells escape more frequently into the circulation system, and the chymotrypsin-like proteasomal activity in the serum could become elevated with the increase of these cells and their tumor-specific products. This hypothesis is in accord with the increased chymotrypsin-like activity in the LnCaP cell medium and the tumor-bearing mice serum in the present study. In addition, the caspase-like and trypsin-like activity at both the cell culture and serum level were altered, as the caspase-like activity was significantly decreased in the cell experiments, whereas the trypsin-like activity was elevated in the tumor-bearing mouse serum, implying that the activity of the proteasomal pathway maybe altered during, or as a result of, tumorigenesis and progression.

In the present study, the expression of proteasome target proteins was also assessed, including p27, which functions in cell cycle progression; Bax, which is associated with apoptosis; and I κ B- α , which is part of the nuclear factor (NF)- κ B pathway (29). The expression levels of all three substrates were decreased in prostate cancer cells and xenograft tumor tissue in the present study. The elevated chymotrypsin-like activity may have induced the degradation of these tumor suppressor proteins, ultimately leading to a greater resistance to apoptosis, and more aggressive cancer behavior. As an inhibitor of NF- κ B, the degradation of I κ B- α by the proteasome facilitates the translocation of NF- κ B into the nucleus and thus promotes cell survival (30), resulting in the reduced effectiveness of anticancer therapy (31,32). A relatively low level of p27 is frequently detected in human tumors, and the degradation of p27 by the proteasome may result in uncontrolled cell division, ultimately leading to transformation and tumor development (33). The degradation of Bax has been identified in aggressive human prostate cancer, which corresponds with the results of the present study (34). As a Bcl-2 family member, Bax is a critical molecule upstream of intrinsic cellular apoptosis; its degradation contributes to maintaining cancer cell survival (35). It has been reported that the inhibition of chymotrypsin-like proteasome activity may

lead to the accumulation of I κ B- α , Bax and p27 in different types of cancer cell and tumor models (14), followed by the induction of cell death. Therefore, proteasome inhibitors that target chymotrypsin-like activity may be a potential strategy for prostate cancer treatment.

There are some limitations to the present study. Firstly, this research detected the proteasomal chymotrypsin-like activity in cell culture and xenografts, and not in clinical samples from prostate cancer patients. As a proof of concept, the present study provided some data to suggest that chymotrypsin-like activity is a potential candidate biomarker for prostate cancer, and may build a foundation for future study. Further work to validate the data of the present study for patients with prostate cancer will be required. Secondly, proteasomal chymotrypsin-like activity is not a specific marker for prostate cancer; it is likely to be increased in various types of carcinoma (36-38). Therefore, elevated chymotrypsin-like activity cannot independently indicate the occurrence of prostate cancer; however, it may improve the accuracy of prostate cancer diagnosis when used in conjunction with increased PSA level, thus decreasing the chances of over-diagnosis and over-treatment for patients with suspected prostate cancer.

Ma *et al* (26) reported that the chymotrypsin-like activity in plasma may provide a powerful biomarker for the risk stratification of acute myeloid leukemia and advanced-stage myelodysplastic syndrome, which provides a novel perspective on the application of chymotrypsin-like activity as a cancer biomarker. Further studies will be required to verify whether this serological test may serve as a prognostic factor to detect disease progression in patients with prostate cancer.

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Availability of data and materials

The authors confirm that all data underlying the findings are fully available without restriction.

Authors' contributions

Conceived and designed the experiments: PT. Performed the experiments: XW and WZ. Analyzed the data: KX and PF. Contributed reagents/materials/analysis tools: PT. Contributed to the writing of the study: XW and PT.

Ethics approval and consent to participate

The use of human tissue was approved by the Research Ethics Committee of Guangzhou First People's Hospital, Guangzhou Medical University (Guangdong, China), and written informed consent from the patient was acquired prior to the use of the tissue in research.

Consent for publication

Written informed consent from the patient was acquired prior to the use of the tissue in research.

Competing interests

The authors declare that they have no competing interests.

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