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Pim-3 is a Critical Risk Factor in Development and Prognosis of Prostate Cancer

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Pim-3 kinase is a highly homologous serine/threonine kinase that is overexpressed in hematological malignancies and solid tumors. Few studies have been conducted to define the role of Pim-3 in solid tumors, especially in prostate cancer. The aim of this study was to define the role of Pim-3 in development and prognosis of prostate cancer.

Material/Methods: We collected specimens from 160 patients with prostate cancer, as well as 100 patients with benign prostatic hyperplasia. Realtime polymerase chain reaction was used for the assessment of Pim-3 expression at the RNA level and Western blot was used to quantify the Pim-3 protein synthesis in 3 different cell lines.

Results: We found that Pim-3 mRNA expression in prostate cancer tissue was significantly higher than that in benign prostatic hyperplasia tissue ($p<0.05$). Accordingly, the protein level expression of Pim-3 in prostate cancer cell lines was also significantly higher than that in control cells. In addition, the expression status of Pim-3 mRNA was significantly associated with pathological parameters such as pre-surgery prostate specific antigen, Gleason score, pathological stage, and lymphoid metastasis. High expression of Pim-3 also significantly decreased the survival rate of patients after surgery.

Conclusions: Pim-3 expression is an important risk factor for prostate cancer; we are the first team to report Pim-3 as a valuable biomarker in Chinese.

MeSH Keywords: Molecular Biology • Prostatic Neoplasms • Proto-Oncogene Proteins c-pim-1

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Background

Prostate cancer (PCa) is a common urological malignancy, and is the second leading cause of cancer-specific deaths among men throughout the world [1]. With the development of prostate-specific antigen (PSA) serum level screening and transrectal ultrasound-guided prostate biopsy, the detection rate and incidence of PCa is obviously increased [2]. Most tumor-related deaths are caused by tumor metastasis, and no curative therapy is currently available for this advanced condition [3]. Ability to identify advanced PCa *vs.* low-risk localized PCa at an early stage would be valuable for development of treatment strategies. Molecular biomarkers will be helpful for the determination of PCa properties and status.

The Pim kinases are a family of highly homologous serine/threonine kinases, including Pim-1, Pim-2, and Pim-3, which were originally found in Moloney-murine leukemia virus infection as a proviral insertion site [4]. The Pim kinases have been reported to be overexpressed in hematological malignancies and solid tumors. Pim kinases are constitutively active and they can increase tumor cell growth and survival *in vitro* and *in vivo* by the regulation of apoptosis, the cell cycle, and migration, which makes them interesting targets for anti-cancer drug discovery. It was demonstrated in a mouse model *PIM-1* and *PIM-2* are oncogenes. Pim-1 and Pim-2 increase was mainly found in hematologic malignancies and PCa [5,6]. Overexpression of Pim-1 can selectively inhibit cell and tumor growth in a cell line-dependent manner. Pim-1 overexpression can lead to significant increase of cellular senescence with the increase of p53 and p53 activated genes, which suggests that the most profound effect of Pim-1 on tumorigenesis is through the p53-p21 pathway. Pim-1, Pim-2, and Pim-3 are related kinases. Pim-3 plays a role in many cellular processes, including cell proliferation, protein synthesis, and survival. *PIM-3* silence can promote cell apoptosis. Pim-3 is expressed in multiple normal organs and is overexpressed particularly in tumor tissues of endoderm-derived organs such as the liver, pancreas, and colon [7,8]. Li et al. suggested Pim-3 can promote growth and angiogenesis of human pancreatic cancer cells *in vivo* in an orthotopic nude mouse model. Further, Pim-3 kinase inhibitor inhibited the proliferation of human pancreatic cancer cells injected into nude mice [9].

The expression and role of Pim-3 in PCa remain unclear. In this study we explored the expression of Pim-3 in PCa tissue and cell lines to assess whether Pim-3 is a risk factor for PCa development and prognosis.

Table 1. The clinical pathological parameters for patients enrolled in this study.

Pathological parameters		N (%)
Preoperative PSA (ng/ml)	<4	5 (3.1%)
	4–10	61 (38.1%)
	>10	94 (58.8%)
Gleason score	<7	81 (50.6%)
	7	37 (23.1%)
	>7	42 (26.3%)
Pathological stage	T2	71 (44.4%)
	T3	89 (55.6%)
Lymphoid metastasis	–	139 (86.9%)
	+	21 (13.1%)
Surgical margin status	–	143 (89.4%)
	+	17 (10.6%)
Biochemical recurrence	–	92 (57.5%)
	+	68 (42.5%)
Age	Pca	69 (48–83)
	BPH	72 (43–86)

Material and Methods

Subjects and tissue samples

PCa specimens were collected from 160 patients with an average age of 69 years (range 48–83) who accepted prostatectomy as a final treatment, from January 2000 to June 2012. Subjects were diagnosed with presurgery biopsies or pathological assessment post-surgery by 2 senior pathologists. Each tumor in a PCa patient was graded and staged by the Gleason and TNM systems. None of the patients had undergone radiation therapy, chemotherapy, or endocrinological therapy before surgery and none had any other type of tumor. We collected 100 benign prostatic hyperplasia (*BPH*) specimens from patients with an average age of 72 years (range 43–86) with electric or open surgery prostate tissue at the same period as controls. Only patients diagnosed as having *BPH* without family tumor history were enrolled. The samples of untreated prostate gland were taken after total prostatectomy and kept at –70°C after snap freezing in liquid nitrogen to avoid degradation of RNA during years of storage. The following biochemical and pathological parameters for PCa patients were recorded (Table 1): preoperative serum prostate specific antigen (PSA), Gleason score, pathological stage, surgical margin status, lymphoid metastasis status, and biochemical recurrence status. The biochemical recurrence means 2 successive

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values of serum PSA level ≥0.2 ng/ml. The survival status of PCa patients was maximally followed up to 120 months post-surgery. Overall survival was defined as the period between surgical treatment and death or the time of the last follow-up. Informed consent was obtained from all patients or their family.

Cell culture

PC-3 and LNCaP PCa cell lines were obtained from the American Type Culture Collection (Rockville, MD). Normal prostate cell line RWPE-1 was frozen and maintained in our lab. All cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37° in 5% CO_2. Cells were sub-cultured or harvested at 90% confluence for total protein extraction.

RNA extraction and Q-PCR

The tissues were snap frozen in liquid nitrogen and stored at –70° until RNA extraction. The tissue total RNA was extracted using a PureLink™ Mini Kit from the Invitrogen Corporation, following the manufacturer's instructions. RNA concentration and OD260/OD280 ratio were measured with an Eppendorf biophotometer, then the total RNA was used for the first-strand cDNA synthesis with 2×NI-RT Master Mix. The RT-PCR primers were designed following the general rules and synthesized by Invitrogen Biotechnology Corporation in Shanghai. The primer sequence for *PIM-3* was Forward: AAGGACGAAAATCTGCTTGTGG and Reverse: CGAAGTCGGTGTAGTCCGTG, respectively, and the PCR product was 100 base pairs. The primer sequence for reference gene GAPDH was Forward: GGAGCGAGATCCCTCCAAAAT and Reverse: GGCTGTTGTCATACTTCTCATGG, respectively, and the PCR product was 197 base pairs. Comparative C_T method was used for analysis of Q-PCR data [10].

Western blot

More than 10^6 cells were used for total protein extraction. A Bradford protein assay kit (Beijing Bio-Med Co. Ltd.) was used to measure the protein concentration following the manufacturer's instructions. Under reducing conditions, 100 µg of total protein was subjected to SDS/PAGE in 12% Bis-Tris-polyacrylamide gels. After electrophoresis, the proteins were transferred to PVDF membranes and the blots were blocked with 5% non-fat milk in TBST buffer prior to incubation with the appropriate primary antibodies. The primary antibodies were prepared in TBST and incubated at 30°C for 2 h. The primary antibodies used to detect Pim-3 and β-tubulin (Wuhan Boster Biotechnology Co. Ltd.) were used at 1:200 dilution and 1:500 dilution, respectively. The blots were then incubated with HRP-conjugated secondary antibody at a 1:3000 dilution (VECTOR laboratories) for 2 h at room temperature. After a final washing, the membrane was visualized with an enhanced chemiluminescence reagent and exposed to X-ray films. The image was scanned and band intensity was estimated using Image J software. Band gray value ratio for Pim-3 was calculated by taking the gray value of reference β-tubulin as a standard (1.0). An average of 6 replicates were used for quantification of the Western blot results.

Statistical analyses

Chi-square analysis was used for the comparison of frequency distribution of the PCa patient subgroup according to the expression status of Pim-3. Quantitative variables including relative Pim-3 mRNA levels were analyzed with Student's t-test and expressed as mean±S.D. The post-surgery survival rate was analyzed with Kaplan-Meier method and the log rank test was used to assess the significance of differences between survival curves. The cases with missing data were entirely excluded in further analysis. The significance threshold was set at $P \leq 0.05$. All statistical analyses were conducted with the SPSS 18.0 software package.

Results

Pim-3 mRNA expression in PCa and *BPH* tissue samples.

We sought to define the association of Pim-3 expression with PCa development. Firstly, the Pim-3 mRNA expression in PCa and *BPH* samples was assessed with real-time quantitative PCR, the expression level of GAPDH was taken as an internal reference, and the Pim-3 mRNA expression in *BPH* was corrected to standard 1.0 according to the average of its relative expression. After correction, the relative Pim-3 mRNA level was 1.0±0.31 and 2.05±0.38 in *BPH* and PCa samples, respectively. The relative expression of Pim-3 mRNA in PCa was significantly higher, about double that in *BPH* ($p<0.05$), as indicated in Figure 1.

Pim-3 protein expression in PCa and control cell lines

We explored Pim-3 protein expression in cell lines by Western blot analysis. Two PCa cell lines, LNCaP and PC-3, and 1 normal prostate epithelial cell line, RWPE-1, were used (Figure 2). β-tubulin was used as an internal control. Band intensity gray value ratio was evaluated through Image J software. The gray value ratio for RWPE-1 was corrected to 1.0. Compared with RWPE-1, the Pim-3 protein expression in LNCaP and PC-3 almost doubled, at 2.09±0.18 and 1.92±0.16, respectively, and the difference was statistically significant ($p<0.05$)

The association between Pim-3 mRNA expression and clinical pathological parameters

To confirm the role of Pim-3 expression in the development of PCa, we also assessed the association between Pim-3 expression

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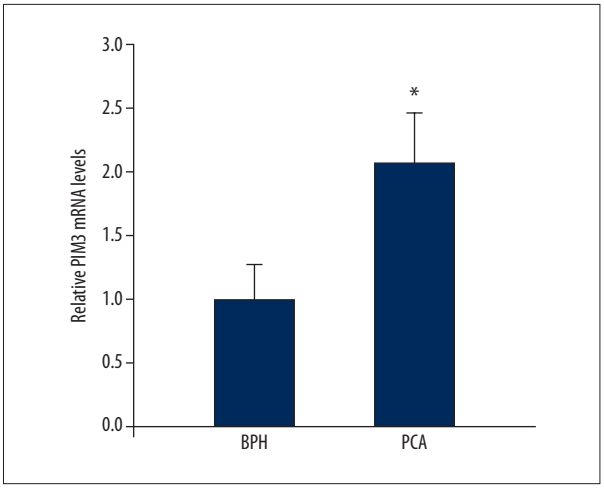

Figure 1. The relative expression of Pim-3 mRNA in BPH and PCa specimens. Pim-3 mRNA was significantly increased in PCa samples compared with *BPH* ($p<0.05$).

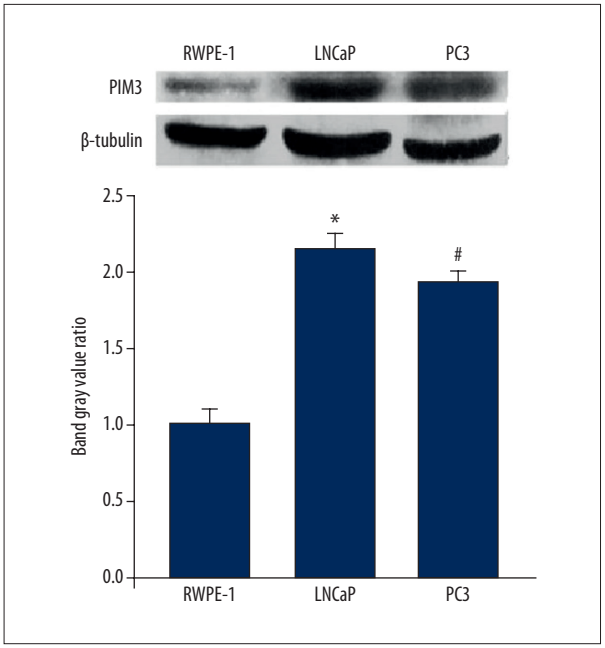

Figure 2. Pim-3 expression in different cell lines. The Pim-3 protein expression in LNCaP and PC3, which were derived from PCa, were significantly increased compared with normal cell line RWPE-1. Upper: Western blot; Lower: band gray value ratio by Image J. * ($p<0.01$) # ($p<0.05$).

and clinicopathological variables. The PCa patients were divided into 2 subgroups according their Pim-3 mRNA expression status. The Pim-3 high group included the patients whose Pim-3 mRNA level was higher than the average for all patients and the Pim-3 low group included the patients whose Pim-3 mRNA level was lower than the average for all patients. We finally enrolled 75 patients in the Pim-3 high group and 85 patients in

Table 2. The association between PIM3 mRNA expression and clinical pathological parameters.

Pathological parameters		N	PIM3 high		PIM3 low		*P* value
Preoperative PSA (ng/ml)	<4	5	2	(40.0%)	3	(60.0%)	0.002
	4–10	61	18	(29.5%)	43	(70.5%)	
	>10	94	55	(58.5%)	43	(41.5%)	
Gleason score	<7	81	25	(30.9%)	56	(69.1%)	<0.001
	7	37	20	(54.1%)	17	(45.9%)	
	>7	42	30	(71.4%)	12	(28.6%)	
Pathological stage	T2	71	26	(36.6%)	45	(63.4%)	0.021
	T3	89	49	(55.1%)	40	(44.9%)	
Lymphoid metastasis	–	139	60	(43.2%)	79	(56.8%)	0.016
	+		15	(71.4%)	6	(28.6%)	
Surgical margin status	–	1	66	(46.2%)	77	(53.8%)	0.944
	+	17	9	(52.9%)	8	(47.1%)	
Biochemical recurrence	–	92	35	(38.0%)	57	(62.0%)	0.015
	+	68	39	(57.4%)	29	(42.6%)	
Age	<70	87	40	(46.0%)	47	(54.0%)	0.804
	≥70	73	35	(47.9%)	38	(52.1%)	

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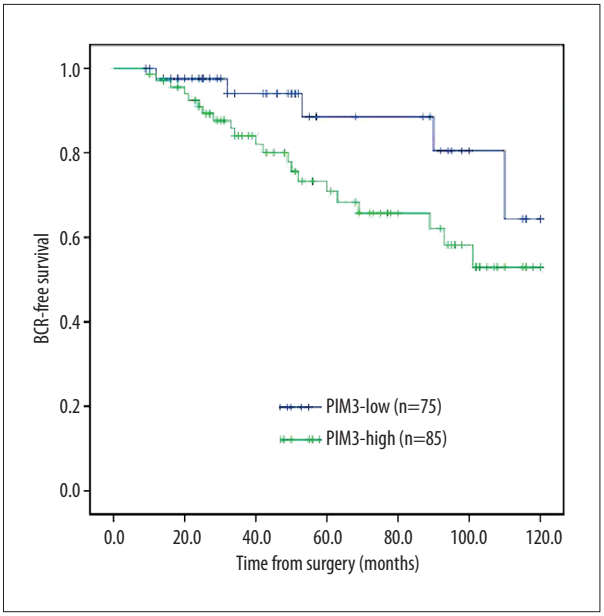

Figure 3. Kaplan-Meier survival curves depict the BCR-free survival curve of PCa patients with different levels of Pim-3 expression. High expression of Pim-3 was correlated with poor survival of PCa patients (p=0.042).

the Pim-3 low group. We found Pim-3 expression status was significantly associated with presurgery PSA, Gleason score (p<0.01), pathological stage, lymphoid metastasis and biochemical recurrence (p<0.05). Details are presented in Table 2.

Survival of PCa was significantly associated with the Pim-3 kinase expression

Because it was associated with the development of PCa and its pathological variables, we infer that Pim-3 expression may be correlated with the outcome or prognosis of PCa patients. To assess the association between Pim-3 expression and biochemical recurrence (BCR)-free survival rate of patients after surgery, Kaplan-Meier method was used and the log rank test was used to assess the significance of differences between survival curves. Our results suggest that high expression level of Pim-3 kinase is associated with a significantly reduced BCR-free survival rate of post-surgery patients (p=0.042), as indicated in Figure 3.

Discussion

PCa is a serious men's health problem all over the world. Prognosis varies greatly by type and stage. Although PSA was developed as a sensitive marker for PCa in the 1980s, a recent study reported that systematic serum PSA provides no tumor-specific motility benefit *vs.* opportunistic screening [11]. A specific molecular biomarker would be helpful to determine the condition or status of PCa and the development of treatment strategy. The Pim kinases are a family of highly homologous serine/threonine kinases reported to be overexpressed in hematological malignancies and solid tumors, and their abnormal expression may be an indicator of advanced malignancies.

In this study we explored the expression of Pim-3 kinase in PCa and its association with the pathological parameters of PCa patients. We found the expression of Pim-3 was significantly increased in PCa patients compared to samples from *BPH* patients. The protein expression in PCa cell lines and non-tumor control prostate cells was in accordance with the RNA expression in tissue specimens. Our results suggest the expression status of Pim-3 kinase is significantly associated with most of the pathological parameters, including preoperative PSA, Gleason score, and pathological stage. In addition, the increase of Pim-3 expression was significantly associated with a decrease of BCR-free survival rate of patients after surgery.

To the best of our knowledge, the present study is the first to explore Pim-3 kinase expression in PCa. Previously, Pim-1 and Pim-2 were reported to be expressed in PCa and play a part in tumor development [12–14]. As a member of the Pim kinase family, similar to Pim-1 and Pim-2, it is reasonable that Pim-3 also plays an important role in PCa. Our study provides some substantial evidence supporting the role of Pim-3 in PCa. We enrolled 160 PCa and 100 *BPH* patients in this study, all confirmed by pathological assessment, with sufficient statistical power to reveal any association between Pim-3 expression and development of PCa.

Although not reported in PCa, Pim-3 was reported to play a critical role in the development of pancreatic cancer [9,15–18]. Similar to Pim-1 and Pim-2, Pim-3 lacks regulatory domains and is constitutively active once it is expressed. Its expression is regulated at transcriptional and post-transcriptional levels by transcription factors and post-translational modifiers. Pim-3 can promote growth and angiogenesis of human pancreatic cancer cells in an orthotopic mouse model *in vivo*. A Pim-3 inhibitor can inhibit the proliferation of human pancreatic cancer cells injected into nude mice free of major adverse effects. Therefore, Pim-3 kinase may be a good target for developing drugs targeting pancreatic cancer. Xu et al. found suppression of Pim-3 led to decreases in anchorage-dependent growth, invasion through Matrigel, and chemoresistance to gemcitabine as measured by caspase-3 activity. They further demonstrated that Pim-3 and Pim-1 play overlapping but non-identical roles in gemcitabine sensitivity of pancreatic cancer cells [18]. Besides its role in pancreatic cancer, Wu et al. suggested Pim-3 cannot initiate but can accelerate HCC development when induced with a hepatocarcinogen by using a mouse model expressing Pim-3 transgene, specifically in the liver [19].

The mechanism by which Pim-3 kinase exerts its effects and the regulation of its expression and stability have received much

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research attention. Liu et al. suggested Pim-3 can regulate the phosphorylation of Bad Ser^{112} and determine the proliferation or apoptosis of cells. Furthermore, Pim-3 overexpression upregulated the intratumoral levels of $pSTAT3^{Try705}$, $pSurvivin^{Thr34}$, HGF, EGF, FGF-2, and VEGF, suggesting that Pim-3 kinase can promote angiogenesis in human pancreatic cancer [9]. Wang et al. also demonstrated Pim-3 can promote tumor growth and angiogenesis by stimulating the VEGF pathway in a human pancreatic cancer orthotopic nude mouse model [17].

Zhang et al. found that translationally controlled tumor protein (TCTP/TPT1) can regulate Pim-3 kinase through a mechanism involving the ubiquitin-proteasome degradation system. RNAi-mediated TCTP ablation leads to Pim-3 instability and subsequent degradation, and tumor growth *in vitro* and *in vivo* was inhibited by cell-cycle blockage and apoptosis promotion, with supporting evidence obtained in pancreatic adenocarcinoma specimens [16]. Yang et al. reported Pim-3 plays a role in TNF-α-induced angiogenesis. TNF-α can increase Pim-3 mRNA expression through TNFR1 and TNF-α can promote stability of Pim-3 mRNA in endothelial cells [20]. Forshell et al. reported Pim-3 kinase was directly regulated by c-myc via binding to a conserved E-box in *PIM-3* gene. In addition, inhibition of Pim kinases in myc-induced lymphoma leads to cell death that seems to be independent of caspases, indicating that human lymphomas that rely on Pim-3 kinase expression may be effectively treated with Pim kinases inhibitor [21]. Li et al. found that transcription factor Ets-1 can induce aberrant Pim-3 expression and subsequently prevent apoptosis in human pancreatic cancer cells [22].

In recent years there have been few reports on the association of Pim-3 with the development and prognosis of PCa. The present investigation was a pilot study of the role of Pim-3 kinase in PCa and it has some limitations. Firstly, different durations of tissue sample cold-storage can lead to variation of RNA degradation, which may have biased our pim-3 expression assessment. Further, we neglected the PCa patients with medium Pim-3 expression and manually subgrouped them into high or low expression groups by taking the average expression of all PCa as a boundary, which may have introduced some confounding effects to the association study with pathological parameters. Secondly, it is unknown if this association can be generalized to populations outside China; further research is needed to confirm this association in different populations. Finally, we did not explore the molecular mechanism by which Pim-3 is associated with PCa. Does Pim-3 play a role in PCa similar to its role in human pancreatic cancer? Does it follow a pathway and regulating system in PCa similar to that in other types of cancer? Much work is needed to answer these questions. Also when determining the association between Pim-3 expression and pathological parameters, because the present study had few patients enrolled in some subgroups, our findings lack sufficient statistical power. For example, there were only 5 patients with pre-surgery PSA less than 4 ng/ml, so it is necessary to find more patients with PSA less than 4 ng/ml to provide more supporting evidence for this association.

Conclusions

We found Pim-3 kinase was aberrantly expressed in PCa specimens and PCa cell lines at the mRNA and protein levels, and this aberrant Pim-3 expression was associated with the pathological parameters of PCa patients. The high Pim-3 kinase expression was an independent risk factor for the development of PCa, and it was also significantly associated with the BCR-free survival rate of post-surgery PCa patients, suggesting a critical role of Pim-3 for the development of PCa, but the molecular mechanism of this role needs further exploration.

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Conflicts of interest

None.

Compliance with ethical standards

This study was approved by the Human Ethics Committee of Tianjin Medical University.

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