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ORIGINAL ARTICLE

Pim-2 Cooperates with Downstream Factor XIAP to Inhibit Apoptosis and Intensify Malignant Grade in Prostate Cancer

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Abstract To find the exact downstream effector of Pim-2 pathway in prostate cancer cells, and to determine the means by which it affects prostate cancer. XIAP, Pim-2 and p-eIF4B expressions were detected in PCA and BPH tissues. Then the Pim-2 and XIAP expressions were manipulated using transfection or RNAi in prostatic cells. Finally, Pim-2/eIF4B/XIAP levels were examined in PCA tissues with different clinicopathologic features. XIAP was significantly higher in PCA than in BPH tissues. When Pim-2 was transfected into noncancerous prostate epithelial cells RWPE-1, Pim-2, p-eIF4B and XIAP were all significantly increased and the apoptosis rate was significantly decreased. When XIAP was transfected into RWPE-1 cells, XIAP was significantly increased with no impact on Pim-2, p-eIF4B and the apoptosis rate. When Pim-2 SiRNA was transfected into prostate cancer cells PC-3, Pim-2, p-eIF4B and XIAP were significantly decreased and the apoptosis rate was significantly increased. When XIAP SiRNA was transfected into PC-3 cells, XIAP was significantly decreased with no impact on Pim-2, p-eIF4B and apoptosis rate. Pim-2, p-eIF4B and XIAP were all significantly higher in PCA tissues with GS ≥8 than those with GS ≤7. XIAP is the downstream factor of Pim-2 pathway in prostate cancer cells. Pim-2 and XIAP cooperate in inhibiting the apoptosis of prostate cancer cells. The activation of Pim-2 pathway may predict higher GS in prostate cancer.

Keywords Pim-2 · XIAP · Apoptosis · Malignant grade

Introduction

The serine/threonine kinase Pim-2 (Proviral integration site of murine) is a powerful anti-apoptosis factor which plays an important role in the tumorigenesis of several cancers [1]. It has been proved in our previous research that Pim-2 could initiate the anti-apoptosis process in prostate cancer cells through phosphorylating eIF4B (eukaryotic initiation factor 4B), thus promoting the development of prostate cancer [2]. But the downstream effector of eIF4B in Pim-2 pathway has not been illuminated.

EIF4B regulates the translation of several proteins, and different upstream activators determine different translation products [3]. It is now unclear what the downstream product of eIF4B is, when activated by Pim-2 in prostate cancer cells. After reviewing the related literatures, four cytokines were identified as possible candidates, namely Cdc-25, Bcl-2, c-Myc and XIAP (X-linked inhibitor of apoptosis protein), all of which were reported to be regulated by eIF4B, and have been related with prostate cancer [4]. Therefore, in order to illuminate the mechanism how Pim-2 pathway affect prostate caner development and to find out the potential therapeutic

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target for prostate cancer, it is important to characterize the exact downstream product of eIF4B within the Pim-2 pathway, and determine its effect on the development of prostate cancer.

Materials and Methods

Clinical Specimen Collection

All the clinical specimen were obtained in the First Affiliated Hospital of Chongqing Medical University from January 2012 to June 2015. 46 cases of local prostatic cancer (PCA) tissues were obtained from radical prostatectomy surgical specimens. All the tumors were confirmed as prostatic adenocarcinoma and confined within the prostate capsule by pathology with negative margins and absent of lymph node metastasis and seminal vesicle invasion. Included pathologic grades were pT2c and below. Additionally, tissues from 42 cases of metastatic PCA were obtained from prostate biopsy samples. Each was confirmed as adenocarcinoma by pathology. Metastases were determined by the presence of lesions on nuclear bone scans. Finally, 40 cases of benign prostatic hyperplasia (BPH) tissues were collected from transurethral resection of prostate (TURP) surgery and confirmed by pathology. Average age in years for each group was: 67.9 (61~76) for the local PCA. 69.2 (59~81) for the metastatic PCA, and 72.7 (66~79) for BPH patients. There were no significant differences in age amongst the three groups. The clinicopathologic features of the PCA cases are listed in supplementary Table 1.

The collection of all the tissues was based on the informed consent of the patients or their direct relatives. All the procedures were approved by our institutional Ethical Committee of Human Experimentation, and are in accordance with the Helsinki Declaration of 1975.

Cell Culture and Transfection

Prostate cancer cell line PC-3 and noncancerous prostatic epithelial cell line RWPE-1 were purchased from ATCC, and were cultured in RPMI-1640 complete medium (9 ml RPMI-1640 + 1 ml calf serum +100 μl 1% penicillin/streptomycin) with 5% CO_2 at 37 °C.

Pim-2 gene was transfected into RWPE-1. The cDNA for the short form of human Pim-2 protein was cloned from cDNA library of PC-3 cell by reverse transcription polymerase chain reaction (RT-PCR), using the primers as follows: sense: 3′-GATCCGCTTCTTTGGCCAAGTAGTGG TTCAAGAGACCACTACTTGGCCAAAGAAGCTTTTTT G-5′; antisense: 3′-AATTCAAAAAAGCTTCTTTG GCCAAGTAGTGGTCTCTTGAACCACTACTTGGCCAA AGAAGCG-5′. *Eco*RI and *Sal*I digest sites were introduced in the sense and antisense primers, respectively. The cDNA was inserted into the mammalian expression plasmid PCI-neo and then sequenced completely to ensure the correct cloning. Lipofectamine™ reagent (Invitrogen, Carlsbad, CA) was used in the transfection process. Neomycin was used to screen the stable Pim-2 expressing cells. The cells having survived in DMEM with 400 μg/mL neomycin were considered as RWPE-1/Pim-2 cells.

XIAP gene was transfected into another group of RWPE-1 cells using the same method as described above. The primers for XIAP were as follow: sense: 3′ GATCCGCAGGTTGT AGATATATCAGATTCAAGAGATCTGATATATCTACA ACCTGCTTTTTTG; antisense: 3′-AATTCAAAAAAGCA GGTTGTAGATATATCAGATCTCTTGAATCTGATATA TCTACAACCTGCG-5′. Finally, ectopic expression of the Pim-2 and XIAP was verified by western blot separately.

RNAi

Pim-2 SiRNA was transfected into PC-3 cells to silence the Pim-2 expression. The pGenesil-1 vector of the RNA interference eukaryotic expression vector specific to Pim-2 gene was constructed. The Pim-2 SiRNA sequence was designed in a pattern as *Bam HI*-sense DNA-loop (TTCAAGACG)-antisense DNA-stop code-*Hind III*. The sense DNA was designed as: 5′-CTTGTAGGACTATCTGGAT-3′. The SiRNA was synthesized and sequenced by Shanghai Shenggong Com. Ltd. Lipofectamine™ reagent (Invitrogen, Carlsbad, CA, USA) was used in the transfection of SiRNA. Additionally, XIAP SiRNA was purchased from Cell Signaling Technology (Danvers, MA, USA) and transfected into another group of PC-3 cells using Lipofectamine™ reagent (Invitrogen, Carlsbad, CA, USA). Finally, the interference efficiency was verified by western blot.

Western Blot

Antibodies for Pim-2 (sc-13,674), Bcl-2 (SC-509), c-Myc (SC-70469), Cdc-25 (SC-13138) and XIAP (sc-55,550) were purchased from Santa Cruz Biotechnology (CA, USA), and p-eIF4B (Ser-422) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Total protein was extracted from the tissue homogenates and the cell lysates using M-PER Mammalian Protein Extraction Reagent (Pierce). The protein concentration was determined by the Bradford method (Bio-Rad). 10 μl of total protein in 1 × loading buffer of each group was loaded and separated on 10% SDS-PAGE. The protein was transferred to PVDF membrane at 400 mA for 1 h in transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol). Membrane was blocked in Tris-buffered saline-Tween 0.1% (TBST) with 7% skimmed milk powder for 1 h at room temperature. The antibodies were diluted according to protocol (Pim-2 1:800, p-eIF4B 1:1000, Bcl-2 1:800, c-Myc 1:800,

Cdc-25 1:800, XIAP 1:800, GAPDH 1:400). The proper species and diluted HRP-labeled second antibodies were added. Western blotting results were detected by the SuperSignal West Pico Chemiluminescent Substrate (Pierce) with a 30 s exposure time, the films were developed and the results were scanned by Epson scanner.

Cell Apoptosis Assay

Chemotherapeutic agent docetaxel (100 nM) was added in each cell culture medium. After 72 h treatment, cells at logarithmic growth phase were collected, and the concentration was modulated to 10^6/ml. After centrifugalization at 1000 rpm for 5 min, 100 μl 1 × binding buffer was added for resuspension. 5 μl Annexin V-FITC and 10 μl PI were added into each reaction system for staining. After being stored for 20 min away from light at room temperature, 400 μl 1 × binding buffer was added, and the apoptosis rate was assayed by flow cytometry. The results were analyzed by FACScan. The ratio of apoptotic cell number and total cell number was defined as the apoptosis rate. The same assay was repeated three times in each cell group.

Statistical Analysis

All data is presented as mean ± standard deviation (SD), and was analyzed using SPSS 19.0 software package. The comparison between two groups was assessed by Student's t test. *P* value less than 0.05 were considered to be statistically significant.

Results

The Increased Expression of Pim-2, eIF4B and XIAP in Prostate Cancer Tissues

The expression of Cdc-25, Bcl-2, c-Myc and XIAP, as well as Pim-2 and phosphorylated eIF4B (p-eIF4B) were tested in BPH and PCA tissues using western blot (Fig. 1, supplementary Table 2). The Pim-2 and p-eIF4B levels were both significantly higher in the PCA group than in the BPH group ($p < 0.05$). Among the four candidate downstream factors, XIAP level was significantly higher in the PCA group than in the BPH group ($p < 0.05$). But there were no significant differences in the expression levels of Cdc-25, Bcl-2 and c-Myc between the PCA group and the BPH group.

Pim-2 and its Downstream Factor XIAP Cooperate in the Anti-Apoptosis Effect

Pim-2 SiRNA and XIAP SiRNA were separately transfected into PC-3 cells to silence Pim-2 or XIAP expression, respectively. The Pim-2 gene and XIAP gene were separately transfected into RWPE-1 cells to increase Pim-2 or XIAP expression, respectively. Then Pim-2, p-eIF4B and XIAP levels were detected in these cell lines, as well as the apoptotic rate (Figs. 2, 3, 4, supplementary Table 3).

In PC-3 cells, Pim-2, p-eIF4B and XIAP levels were all comparatively high, and the apoptosis rate was comparatively low. In PC-3/Pim-2 SiRNA cells, Pim-2, eIF4B and XIAP levels were all significantly lower than those in PC-3 cells ($p < 0.05$), and the apoptosis rate was increased significantly ($p < 0.05$). In PC-3/XIAP SiRNA cells, XIAP level decreased significantly ($p < 0.05$) without any impact on Pim-2 and p-eIF4B levels, compared to those in PC-3 group, while the apoptosis rate was still significantly higher than that in PC-3 cells ($p < 0.05$).

In RWPE-1 cells, Pim-2, p-eIF4B and XIAP levels were all comparatively low, and the apoptosis rate was comparatively high. In RWPE-1/Pim-2 cells, Pim-2, eIF4B and XIAP levels were all significantly increased ($p < 0.05$), compared with those in RWPE-1 cells. The apoptosis rate was decreased significantly ($p < 0.05$). In RWPE-1/XIAP cells, XIAP level was significantly increased compared with that in RWPE-1 cells ($p < 0.05$), without any impact on Pim-2 and p-eIF4B levels. However, the apoptosis rate was as high as that in RWPE-1 cells with no significant decrease.

Pim-2/eIF4B/XIAP Pathway Is Related to the Gleason Score of Prostate Cancer

Gleason Score (GS), clinical stage and preoperative serum PSA are known as important factors related to the risk categorization of prostate cancer [5]. In order to explore the relationship between Pim-2/eIF4B/XIAP pathway and the clinicopathologic features of prostate cancer, Pim-2, p-eIF4B and XIAP levels in prostate cancer tissues with different clinicopathologic features were detected by western blot (Figs. 5 and 6, supplementary Tables 4 & 5).

In cases with the same clinical stage and PSA, Pim-2 levels were significantly higher in cases with GS ≥8 than those with GS ≤7 (A vs. B, C vs. D, E vs. F, G vs. H, $p < 0.05$). No significant differences were found in Pim-2 levels between groups with different clinical stages (B vs. F, A vs. E, D vs. H, C vs. G, $p > 0.05$), or with different PSA values (B vs. D, F vs. H, A vs. C, E vs. G, $p > 0.05$). The same trend was found in both p-eIF4B and XIAP levels.

Discussion

A relative paucity of research has been done to identify the downstream factor of Pim-2 pathway in prostate cancer. In our study, only XIAP expression level was found to be positively related to Pim-2 in both clinical tissues and cell lines.

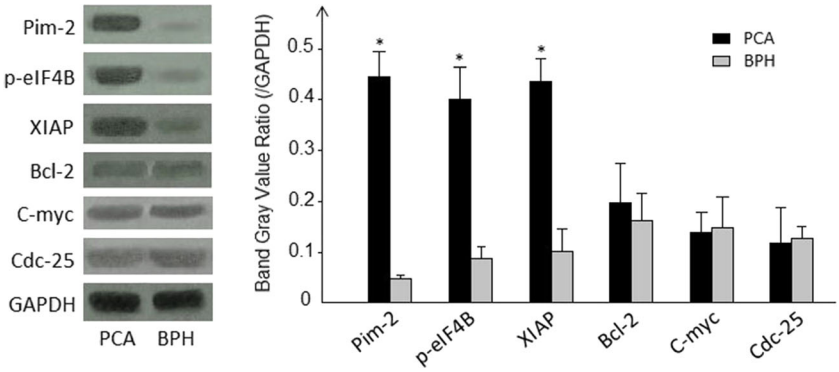

Fig. 1 Comparison of Pim-2, p-eIF4B, Cdc-25, c-Myc, Bcl-2 and XIAP protein levels between PCA tissues and BPH tissues (n = 40). The expression levels of Pim-2, p-eIF4B and XIAP in PCA tissues were significantly higher than the correspondent ones in BPH tissues ($*p < 0.05$). No significantly differences in the expression levels of Cdc-25, c-Myc and Bcl-2 were found between the two groups

Therefore, among the four possible candidate downstream factors, XIAP is most likely to be regulated by Pim-2 in prostate cancer. XIAP is an anti-apoptosis factor which could suppress caspase system induced cell apoptosis [6]. However, research in leukemia cells has found that c-Myc could be regulated by Pim-2 as a downstream effector in preventing leukemia apoptosis, while XIAP has no relation with Pim-2 [7, 8]. Differing anti-apoptotic pathways amongst leukemia and solid tumors may account for these opposite findings. It is reported that Pim-2 is related to several anti-apoptosis signal transduction pathways, in which multiple downstream factors could be involved [7]. More work is needed to further characterize downstream effects of the Pim-2 pathway in different malignancies.

The anti-apoptosis effect of Pim-2/eIF4B/XIAP pathway was a cooperative work of both Pim-2 and XIAP, and neither of the two factors could significantly inhibit the apoptosis of prostate cancer cells by oneself. There is still no definitive explanation of this phenomenon, but some related research has pointed to NF-κB (nuclear factor kappa B). NF-κB is known as a modulating cytokine found broadly in different cells and different signal transduction pathways. Ayala, et al. found that Pim-2 cooperated with NF-κB to promote the survival and growth of cancer cells in perineural invasive prostate cancer [9]. Some other studies also found the anti-apoptotic effect of Pim-2 was associated with NF-κB in leukemia cells, myeloma cells and hepatocellular carcinoma cells [10–12]. Meanwhile, research has also demonstrated the relationship between XIAP and NF-κB in inhibiting prostate cancer cell apoptosis. Park YH, et al. found that simvastatin inhibited NF-κB regulated XIAP expression, which, as effect, inhibited castration resistant prostate cancer cell growth by inducing

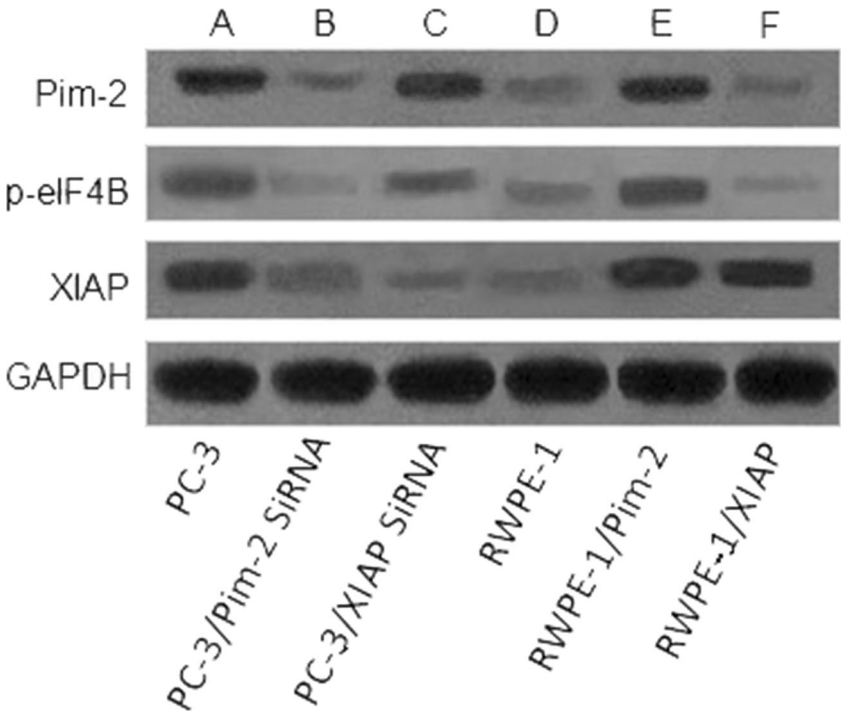

Fig. 2 Pim-2, p-eIF4B and XIAP protein expressions in different prostate cells. (A. PC-3: prostate cancer cell line; B. PC-3/Pim-2 SiRNA: PC-3 cells transfected with Pim-2 SiRNA; C. PC-3/XIAP SiRNA; D. RWPE-1: nontumorous prostatic epithelial cell line; E. RWPE-1/Pim-2: RWPE-1 cells transfected with Pim-2; F. RWPE-1/XIAP: RWPE-1 cells transfected with XIAP)

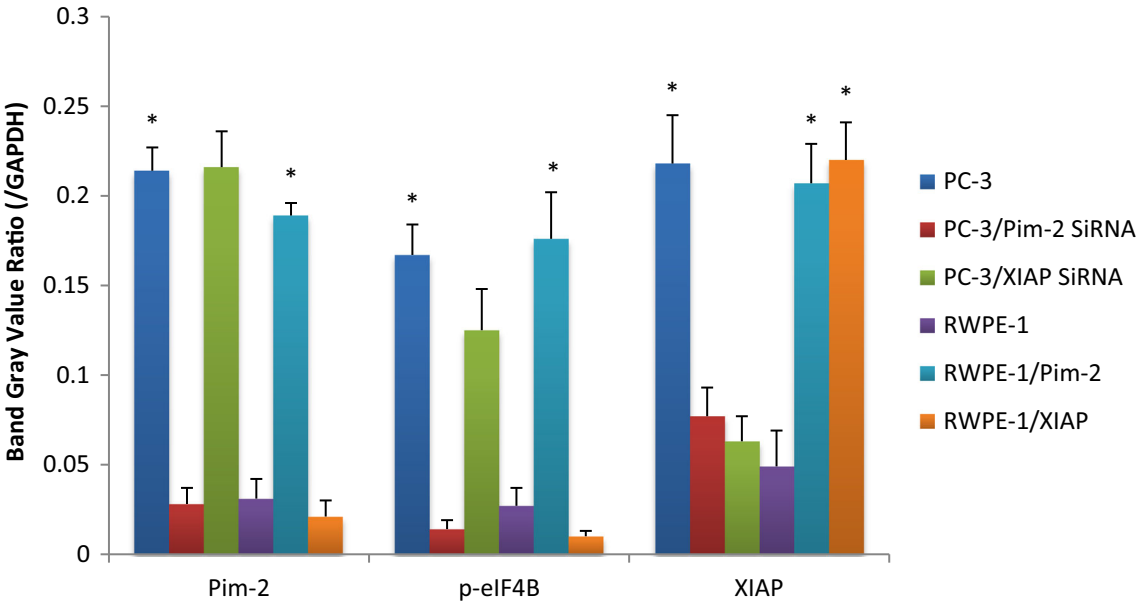

Fig. 3 Comparison of Pim-2, p-eIF4B and XIAP protein expression levels in different prostate cells (n = 3). Pim-2 expression level was significantly decreased in PC-3/Pim-2 SiRNA cells compared to that in PC-3 cells (*p < 0.05). And Pim-2 expression level was significantly increased in RWPE-1/Pim-2 cells than that in RWPE-1 cells (*p < 0.05). The p-eIF4B expression levels always had the same trend with Pim-2 in all the cell goups. XIAP expression levels was significantly lower in PC-3/Pim-2 SiRNA cells and in PC-3/XIAP SiRNA cells, compared with that in PC-3 cells (*p < 0.05). And XIAP expression levels were significantly higher in RWPE-1/Pim-2 cells and RWPE-1/XIAP cells, compared with that in RWPE-1 cells (*p < 0.05)

apoptosis [13]. Kim HR, et al. found that Acacetin exhibited anticancer activity through the suppression of NF-κB signaling in prostate cancer cells, and XIAP was one of the down-regulated effectors [14]. Therefore, NF-κB seems to be a central governor of both Pim-2 and XIAP in the anti-apoptosis pathway. Further study among NF-κB, Pim-2 and XIAP is needed to illuminate the detailed mechanism.

Pim-2/eIF4B/XIAP pathway was also proved to have relationship with Gleason Score of prostate cancer in this study. The activation of Pim-2/eIF4B/XIAP pathway may predict higher Gleason Score. Other researchers have also focused on the relationship between XIAP and the clinicopathological features of prostate cancer. Zhang P, et al. found that XIAP suppression may decrease the metastatic rate of prostate cancer [15]. Others reported that prostate cancer cases with over expression of XIAP were more likely to develop chemotherapy resistance [16, 17]. XIAP has also been found to be related to poor prognosis of many other malignancies [18]. However,

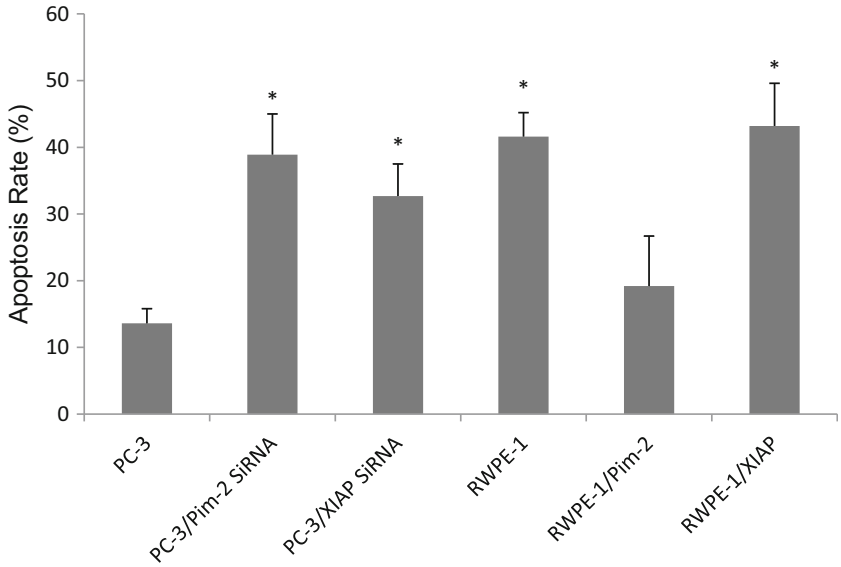

Fig. 4 The apoptosis rate of different prostate cells treated with docetaxel (100 nM) for 72 h (n = 3). The apoptosis rate in PC-3/Pim-2 SiRNA cells and in PC-3/XIAP SiRNA cells were both significantly higher than that in PC-3 cells (*p < 0.05). And the apoptosis rate in RWPE-1/Pim-2 cells was significantly lower than that in RWPE-1 cells and in RWPE-1/XIAP cells (*p < 0.05)

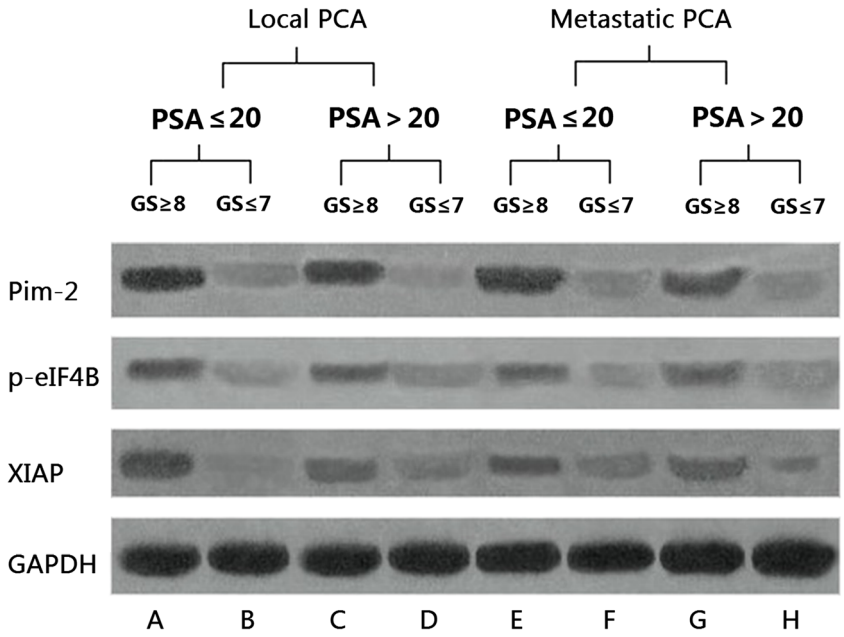

Fig. 5 Pim-2, p-eIF4B and XIAP protein expressions in prostate cancer tissues with different clinicopathologic features. (A. local PCA with PSA ≤20 and GS ≥8; B. local PCA with PSA ≤20 and GS ≤7; C. local PCA with PSA >20 and GS ≥8; D. local PCA with PSA >20 and GS ≤7; E. metastatic PCA with PSA ≤20 and GS ≥8; F. metastatic PCA with PSA ≤20 and GS ≤7; G. metastatic PCA with PSA >20 and GS ≥8; H. metastatic PCA with PSA >20 and GS ≤7)

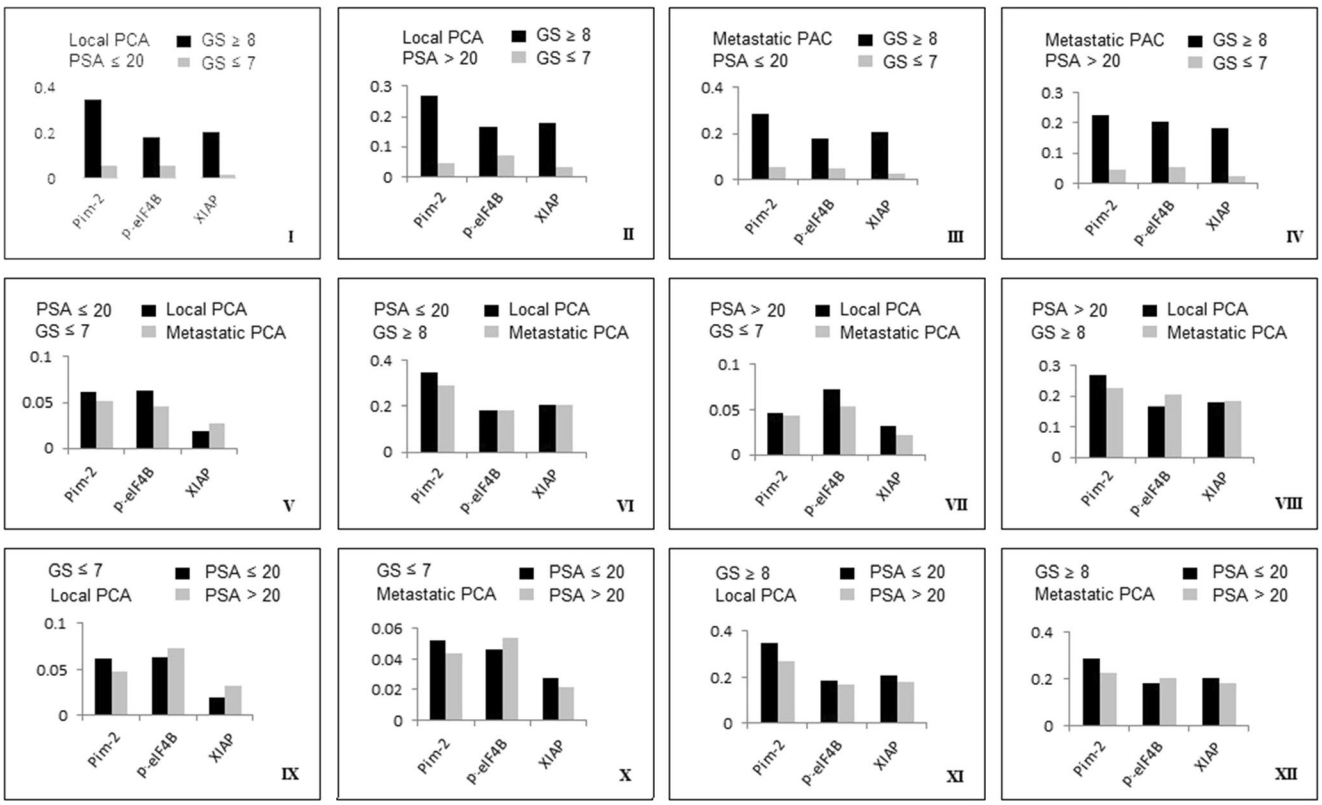

Fig. 6 Comparison of Pim-2, p-eIF4B and XIAP protein expression levels in prostate cancer tissues with different clinicopathologic features ($n = 6$). I~IV: Prostate cancer tissues with different Gleason Scores but same PSA value and clinical stage (M). All the three factors are significantly higher in groups with GS ≥8 than those in groups with GS ≤7 ($p < 0.05$). V~VIII: Prostate cancer tissues with different clinical stage (M) but same PSA value and Gleason Score. No significantly differences in all the three factors between local PCA tissues and metastatic PCA tissues. IX~XII: Prostate cancer tissues with different PSA values but same clinical stage (M) and Gleason Score. No significantly differences in all the three factors between groups with PSA ≤20 and groups with PSA >20

some studies had opposing findings, reporting higher XIAP expression predicted improved prostate cancer related prognosis [19, 20]. Similarly, an animal experiment demonstrated XIAP deficient mice had more aggressive prostate disease [21]. Further, XIAP was also reported as a favorable prognostic factor in other cancers [22, 23]. Therefore, the role of XIAP in the prognosis of prostate cancer remains unclear. Some believe it depend on the specific level of expression of XIAP [24]. Based on our findings, we consider the definite collaborator working with XIAP as the decisive factor to the final effect. That means when XIAP collaborates with Pim-2, they combine to promote prostate cancer, and predict higher Gleason score. However, XIAP may play the role of anti-tumor factor when activated by certain stimulators in other signal transduction pathways.

Conclusions

In conclusion, it was demonstrated in this research that Pim-2/eIF4B/XIAP anti-apoptosis signal transduction pathway may not only promote the tumorigenesis of prostate cancer, but also influence the Gleason score. The activation of the Pim-2/eIF4B/XIAP pathway may predict high Gleason Score, potentially indicating a higher metastatic potential and worse prognosis. The anti-apoptotic effect encouraging growth of prostate cancer cells is a cooperative action of Pim-2 and XIAP. It provides a new therapeutic target for the treatment of prostate cancer, especially for those patients afflicted with poor prognostic disease.

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