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CC Chemokine Ligand 18 and IGF-Binding Protein 6 as Potential Serum Biomarkers for Prostate Cancer

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Prostate cancer (PCa) is the second leading cause of cancer-related death in men globally. However, there are few sensitive biomarkers for PCa, especially those which can distinguish PCa and benign prostate hyperplasia (BPH). Antibody microarrays allow for high-throughput and high-sensitivity detection of multiple proteins simultaneously, providing a powerful tool for biomarker screening. Here, we selected 46 patients with PCa and 42 controls with BPH, and compared the serum levels of different cytokines in PCa and BPH patients using antibody microarrays. The results indicated that serum levels of macrophage colony-stimulating factor (M-CSF) and CC chemokine ligand 18 (CCL-18) were remarkably higher in PCa patients than those in BPH patients, while serum levels of insulin-like growth factor-binding protein 6 (IGFBP-6) and Fas receptor (Fas), also called tumor necrosis factor receptor superfamily member 6 (TNFRSF6), were significantly lower. M-CSF and Fas/TNFRSF6 have been reported to be associated with PCa pathogenesis, and thus were used as positive controls in the present study. CCL-18 is a chemokine primarily involved in recruitment of the adaptive immune system, while IGFBP-6 has been reported to inhibit proliferation of PCa cells. Serum levels of these four cytokines could distinguish PCa from BPH with high sensitivity and high specificity. Furthermore, the area under the ROC curve (AUC) was above 0.925 and 0.835 for CCL-18 and IGFBP-6, respectively, implying their high diagnostic value. In conclusion, we have identified CCL-18 and IGFBP-6 as new potential serum biomarkers for PCa.

Keywords: benign prostate hyperplasia; biomarker; CC chemokine ligand 18; insulin-like growth factor-binding protein 6; prostate cancer

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Introduction

Prostate cancer (PCa), the most common male cancer, is the second leading cause of cancer-related death in men globally (Jemal et al. 2010). Current diagnosis for PCa includes digital rectal examination (DRE), prostate-specific antigen (PSA) and biopsy (Velonas et al. 2013). PSA is a glycoprotein enzyme secreted by epithelial cells of the prostate gland (Balk et al. 2003). It has been used as a biomarker for PCa screening, and results of PSA test often decide whether patients should undergo a prostate biopsy for PCa detection. However, serum levels of PSA in patients with other prostatic diseases, such as benign prostate hyperplasia (BPH), may also increase, producing high rates of false positive results and leading to over-detection of PCa (Catalona et al. 1994; Heijnsdijk et al. 2009). Therefore, much effort has been made to find more suitable biomarkers for PCa (Makarov et al. 2009).

CC chemokine ligand 18 (CCL-18) belongs to the CC chemokine family. The CC chemokine family contains at least 27 distinct members, and induces the migration of monocytes and other cell types, such as natural killer (NK) cells and dentritic cells (Graves and Jiang 1995; Locati et al. 2005). CCL-18 is produced mainly by dendritic cells, monocytes and macrophages of the innate immune system (Ferrara et al. 2008; Schraufstatter et al. 2012; Bellinghausen et al. 2012). However, this cytokine exerts its effector functions mainly on the adaptive immune system, such as attracting naive T cells and T regulatory cells (Adema et al. 1997; Bellinghausen et al. 2012). Although the relation of CCL-18 to PCa remains unknown, a recent study indicated that CCL-11 was a potential serum marker for PCa (Agarwal et al. 2013).

In biological fluids, insulin-like growth factors (IGFs) are normally bound to their carrier proteins, insulin-like growth factor-binding proteins (IGFBPs). The IGFBP family, which contains six members (IGFBP-1 to IGFBP-6), prolongs the half-life of IGFs and also modulates IGF availability and activity (Hwa et al. 1999). Besides, IGFBP-3 inhibits cell proliferation and induces apoptosis independent of IGFs (Oh et al. 1993, 1995; Rajah et al. 1997). Low serum IGFBP-3 is associated with greater risk of aggressive

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and metastatic PCa (Chan et al. 2002; Mehta et al. 2011). Interestingly, IGFBP-6 inhibits PCa cell proliferation (Koike et al. 2005), and acts as a tumor suppressor gene during nasopharyngeal carcinoma pathogenesis (Kuo et al. 2010), implying that it may be a promising biomarker for PCa like IGFBP-3.

Cytokines play an important role in cancer pathogenesis and act as potential biomarkers for cancer (Dranoff 2004). Antibody microarrays allow for high-throughput and high-sensitivity detection of multiple cytokines simultaneously, and are useful to identify tumor markers. We previously compared the serum cytokine profiling of PCa and BPH patients using antibody microarrays, and identified 19 cytokines which were significantly upregulated or downregulated in PCa patients (Zhang et al. 2010). In this study, the expression of these 19 cytokines in PCa and BPH patients was tested in a much wider population (46 patients with PCa and 42 patients with BPH), compared with our pilot screening. Of these 19 cytokines, macrophage colony-stimulating factor (M-CSF) and CCL-18 represented the top two cytokines which were mostly remarkably increased, while IGFBP-6 and Fas receptor (Fas), also called tumor necrosis factor receptor superfamily member 6 (TNFRSF6), represented the top two cytokines which were mostly remarkably decreased. M-CSF and Fas/TNFRSF6 have been reported to be associated with PCa pathogenesis (Furuya et al. 2001; Ide et al. 2008; Richardsen et al. 2008). Here, we used M-CSF and Fas/TNFRSF6 as positive controls of our studies, and confirmed whether they were suitable biomarkers for PCa. Furthermore, we paid special attention to CCL-18 and IGFBP-6, and explored systemically the potential of these cytokines for differential diagnosis of PCa and BPH.

Methods

Patients

The study was approved by the hospital ethics committee. Patients were from the general hospital of Chinese People's Liberation Army between January 2011 and December 2011, whose serum PSA levels were 4~10 ng/ml. These patients were subjected to prostate biopsy, and classified to the PCa group and the BPH group according to the biopsy results. We selected 46 patients with PCa and 42 patients with BPH for subsequent studies. After prostate biopsy, peripheral venous blood was collected with the agreement of patients. The serum was separated, and subjected to antibody microarray analysis.

Antibody microarray

Protein concentrations of the serum were determined using the KC-430 BCA protein assay kit (KangChen Bio-tech, Shanghai, P.R. China). Serum levels of various cytokines were determined using antibody microarrays (RayBiotech, GA, USA), according to the manufacturer's instructions. Each serum sample was tested in duplicates on the chip, and experiments were repeated twice. Each well of the chip was blocked with 100 μl of 1 × Blocking Buffer for 30 min at room temperature. Then, discard the blocking buffer, add the serum sample containing 50 μg of total proteins (or biotin-labeled protein as the positive control), and incubate for 1 h at room temperature. After washing the wells with 1 × Wash Buffer I for three times, add 50 μl of biotin-labeled antibody mixtures (1:100 dilutions) to each well and incubate for 2 h at room temperature. Wash the wells with 1 × Wash Buffer II for three times, add 50 μl of Alexa-Flour 555-labled streptavidin and incubate for 1 h at room temperature. Detect the fluorescence signals using the Axon 4000B laser scanner (Harlow Scientific, MA, USA) after washing. Raw data were subtracted by the blank data, and then normalized to the positive control. That is to say, the relative level of each cytokine is calculated as follows: (raw data − blank) / (positive control − blank).

Statistical analysis

Differences of various cytokines between PCa and BPH patients were analyzed using student's *t*-test. Receiver operating characteristic (ROC) analysis was carried out to determine sensitivity, specificity and the area under the curve (AUC).

Results

There were 46 patients with PCa and 42 patients with BPH. The mean ages of PCa and BPH patients were 63.5 ± 9.7 and 62.1 ± 11.9, respectively. There were no significant differences in age between the two groups. Furthermore, the average serum level of PSA in PCa patients (5.75 ± 1.13 ng/ml) was also not significantly different from that in BPH patients (5.38 ± 1.24 ng/ml).

Serum samples were obtained from the patients, and subjected to antibody microarray analysis. Compared with the BPH group, the average serum levels of 19 cytokines were significantly changed in the PCa group (Fig. 1). Serum levels of 16 cytokines were upregulated in PCa patients, including macrophage inflammatory protein 1δ (MIP-1δ), CCL-18, interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 16 (IL-16), bone morphogenetic protein 4 (BMP-4), eotaxin-3, interferon γ (IFN-γ), M-CSF, monokine induced by interferon γ (MIG), macrophage inflammatory protein 3α (MIP-3α), neurotrophin 3 (NT-3), stem cell factor (SCF), β-nerve growth factor (β-NGF), CC chemokine ligand 28 (CCL-28) and glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR). Serum levels of 3 cytokines were downregulated in PCa patients, including IGFBP-6, Fas/TNFRSF6, and tumor necrosis factor-related apoptosis-inducing ligand receptor 3 (TRAILR-3).

Of the 16 cytokines upregulated in PCa patients, M-CSF and CCL-18 represented the top two cytokines which were mostly remarkably increased. The mean serum level of M-CSF in PCa patients was more than 2-fold of that in BPH patients (Fig. 2A). The mean serum CCL-18 level was 0.217 in PCa patients, approximately 5-fold of that in BPH patients (Fig. 2B). Of the 3 cytokines downregulated in PCa patients, IGFBP-6 and Fas/TNFRSF6 represented the top two cytokines which were mostly remarkably decreased. The serum levels of both cytokines were reduced by more than 50% in PCa patients, compared with BPH patients (Fig. 2C and D).

Next, we evaluated the possibility of using M-CSF,

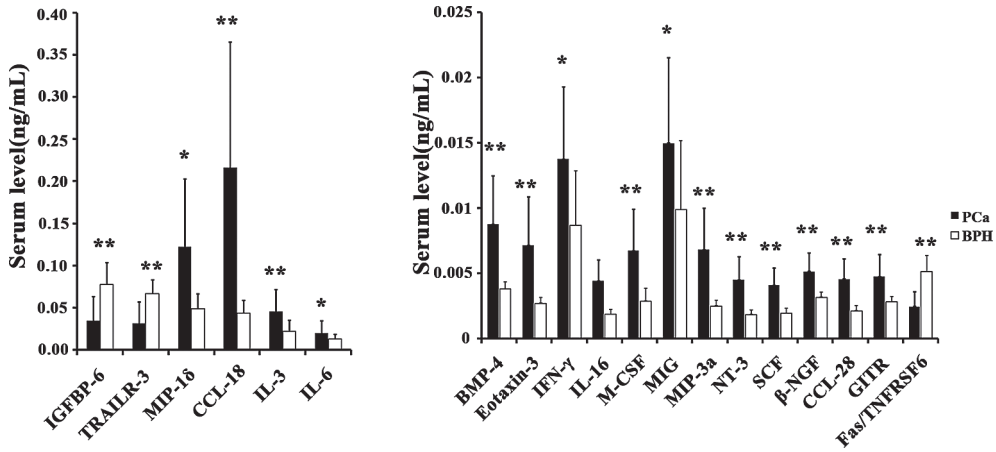

Fig. 1. Serum levels of various cytokines in PCa and BPH patients.
There were 46 patients with PCa and 42 patients with BPH. Serum levels of various cytokines were determined using antibody microarrays. Raw data of antibody microarrays were subtracted by the blank data, and then normalized to the positive control. The mean serum levels of these cytokines in PCa and BPH patients are shown. Differences of various cytokines between PCa and BPH patients were analyzed using student's *t*-test (*$p < 0.05$, **$p < 0.01$).

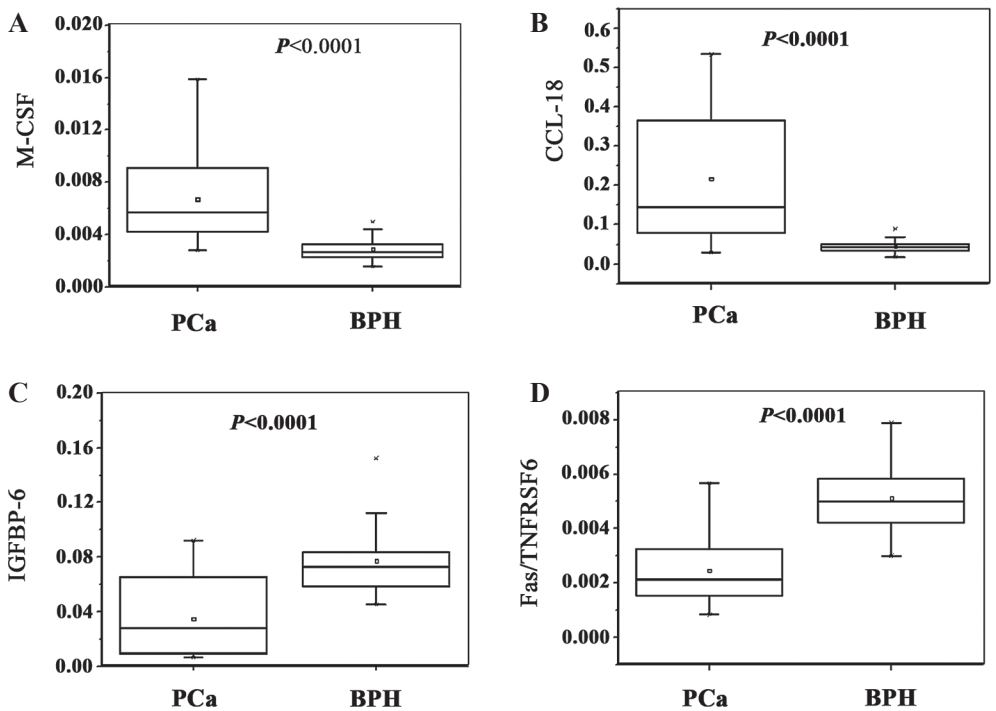

Fig. 2. Serum levels of M-CSF, CCL-18, IGFBP-6 and Fas/TNFRSF6 in PCa patients and BPH patients.
The mean serum levels of M-CSF (A), CCL-18 (B), IGFBP-6 (C) and Fas/TNFRSF6 (D) are shown. Differences of various cytokines between PCa and BPH patients were analyzed using student's *t*-test.

CCL-18, IGFBP-6 and Fas/TNFRSF6 as the biomarkers for PCa. The serum level of M-CSF had a sensitivity of 78.26% and a specificity of 83.33% to distinguish PCa and BPH (cutoff = 0.004), while the serum level of CCL-18 had a sensitivity of 82.16% and a specificity of 83.33% to distinguish PCa and BPH (cutoff = 0.06) (Table 1). On the other hand, PCa and BPH could be differentiated with a sensitivity of 97.62% and a specificity of 67.39% by IGFBP-6 (cutoff = 0.05) (Table 2), and be differentiated with a sensitivity of 85.71% and a specificity of 89.13% by Fas/TNFRSF6 (cutoff = 0.004, Table 2).

Finally, ROC curves were plotted to test M-CSF, CCL-18, IGFBP-6 and Fas/TNFRSF6 for distinguishing between PCa and BPH. AUC reflects the diagnosis value of a biomarker. The ROC curves indicated that M-CSF and CCL-18 could distinguish PCa and BPH in which the AUC was 0.926 and 0.925, respectively (Fig. 3A and B). The AUC for IGFBP-6 and Fas/TNFRSF6 was 0.835 and 0.943, respectively (Fig. 4A and B), suggesting that they are also valuable biomarkers for PCa.

Table 1. The sensitivity and specificity for distinguishing PCa from BPH patients using M-CSF and CCL-18.

	TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV
M-CSF (Cutoff = 0.004)	36	35	7	10	78.26%	83.33%	83.72%	77.78%
CCL-18 (Cutoff = 0.06)	38	35	7	8	82.61%	83.33%	84.44%	81.40%

TP, True positive; TN, True negative; FP, False positive; FN, False negative; PPV, Positive predictive value; NPV, Negative predictive value.

Table 2. The sensitivity and specificity for distinguishing PCa from BPH patients using IGFBP-6 and Fas/TNFRSF-6.

	TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV
IGFBP-6 (Cutoff = 0.05)	41	31	15	1	97.62%	67.39%	73.21%	96.88%
Fas/TNFRSF-6 (Cutoff = 0.004)	36	41	5	6	85.71%	89.13%	87.80%	87.23%

TP, True positive; TN, True negative; FP, False positive; FN, False negative; PPV, Positive predictive value; NPV, Negative predictive value.

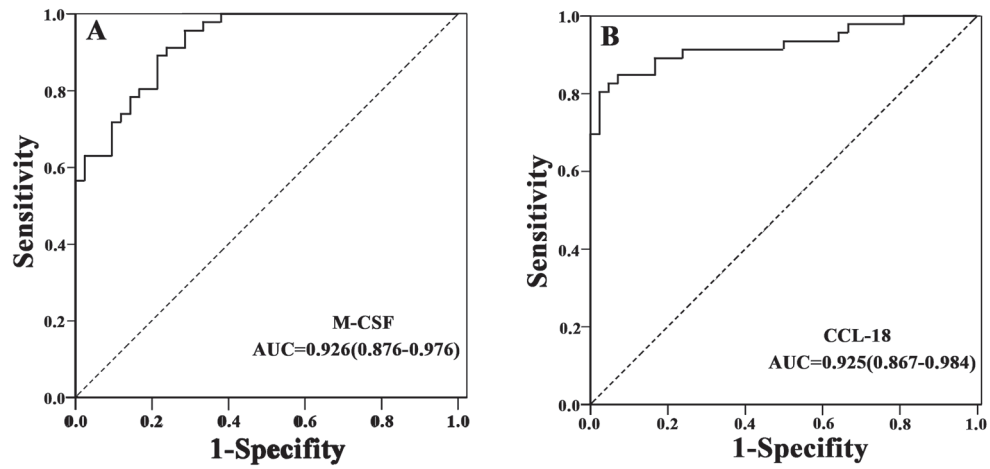

Fig. 3. ROC curves for distinguishing PCa from BPH with M-CSF and CCL-18. Shown are the ROC curves for M-CSF (A) and CCL-18 (B).

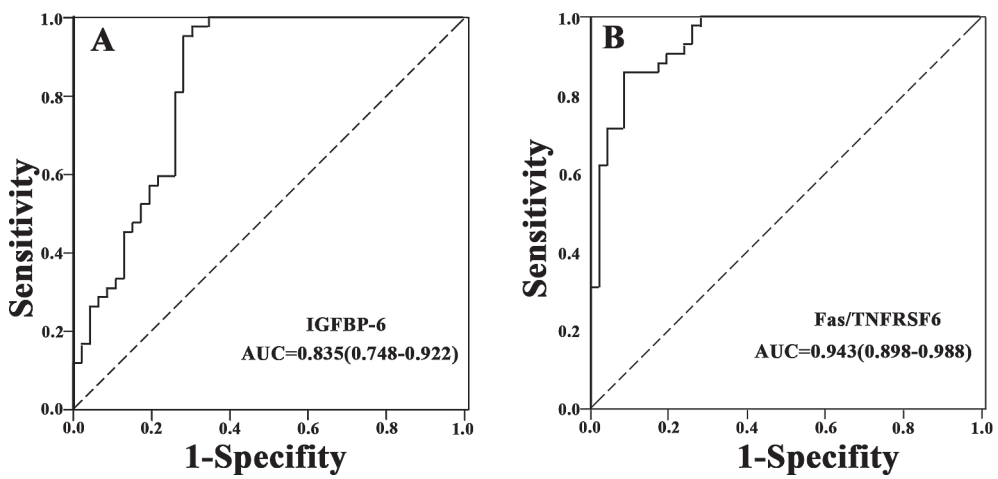

Fig. 4. ROC curves for distinguishing PCa from BPH with IGFBP-6 and Fas/TNFRSF6. Shown are the ROC curves for IGFBP-6 (A) and Fas/TNFRSF6 (B).

Discussion

Serum PSA test has been widely used for early detection of PCa. Because the serum levels of PSA in patients with other prostatic diseases, such as BPH, may also increase, PSA test often leads to over-detection and over-treatment of PCa (Heijnsdijk et al. 2009). The unacceptable low specificity of PSA requires scientists to find more suitable biomarkers for PCa, especially those which can distinguish PCa and BPH.

In this study, we compared the serum levels of different cytokines in PCa and BPH patients using antibody microarrays. Our results indicated that serum levels of M-CSF and CCL-18 were remarkably increased in PCa patients compared with BPH patients, while serum levels of IGFBP-6 and Fas/TNFRSF6 were significantly decreased. Serum levels of these four cytokines can distinguish PCa and BPH with high sensitivity and high specificity. Furthermore, ROC curves showed that the AUC of M-CSF, CCL-18 and Fas/TNFRSF6 was above 0.9, suggesting their high diagnostic value.

M-CSF is produced by numerous cell types, including endothelial cells, fibroblasts, and monocytes-macrophages, and is present in serum and several biological fluids at stable concentrations (Praloran 1991). This cytokine binds to the colony stimulating factor 1 receptor (CSF-1R), stimulates hematopoietic stem cells to differentiate into macrophages or other related cell types (Stanley et al. 1997). Immunohistochemical detection of human prostate tissues showed a high expression of CSF-1R in prostatic intra-epithelial neoplasia or PCa (Ide et al. 2002). Furthermore, the serum level of M-CSF was reported to be increased in metastatic PCa patients, compared with non-metastatic patients (Ide et al. 2008; Richardsen et al. 2008). These results suggest that M-CSF is associated with PCa progression and acts as a potential biomarker to predict the development of metastasis. Our results confirmed that the serum level of M-CSF was elevated in PCa patients.

Fas, also called TNFRSF6, is a death receptor of the Fas ligand (FasL) on cell membrane that induces programmed cell death. Alternative splicing of the *TNFRSF6* mRNA produces a soluble form of Fas, which lacks the transmembrane domain. In contrast with membrane Fas, soluble Fas (sFas) inhibits FasL-induced apoptosis through binding and subsequent inactivation of FasL. The Fas pathway plays a critical role during cancer progression (O'Brien et al. 2005). Fas and FasL polymorphisms contribute to PCa risk in a Chinese population (Shao et al. 2011). Decreased membrane Fas expression was observed in the prostate tissues of some PCa patients (Kim et al. 2009). By contrast, serum levels of sFas are elevated in patients with bladder, breast, ovarian, renal cell, prostate, uterine and gynecological cancers (Mizutani et al. 1998; Ueno et al. 1999; Nonomura et al. 2000; Hefler et al. 2000; Konno et al. 2000; Furuya et al. 2001). However, we showed that the serum level of Fas/TNFRSF6 was decreased in PCa patients, compared with BPH patients. The contradictory results may be due to that PCa patients of different ages and distinct stages were selected in the two studies.

Except for M-CSF and Fas/TNFRSF6, we identified two new potential biomarkers for PCa, IGFBP-6 and CCL-18. Our studies indicated that IGFBP-6 had decreased serum levels in PCa patients, which is reasonable because IGFBP-6 has been reported to inhibit PCa cell proliferation (Koike et al. 2005). As a chemokine primarily involved in recruitment of the adaptive immune system, the function of CCL-18 during PCa pathogenesis remains to be investigated. Besides CCL-18, serum levels of other CC chemokine family members, including MIP-1δ (CCL-15), MIP-3α (CCL-20), Eotaxin-3 (CCL-26) and CCL-28, were also increased in PCa patients, compared with BPH patients. Another study indicated that CCL-11 was also a potential serum marker for PCa (Agarwal et al. 2013).

Antibody microarrays facilitate high-sensitivity detection of multiple proteins simultaneously, and are useful to identify special biomarkers for diagnostic applications. The levels of cytokines can also be determined using the enzyme-linked immunosorbent assay (ELISA). Basically, the principles of antibody microarrays and ELISA are similar, except that absolute values can be obtained with the presence of a standard curve for ELISA. Compared with ELISA, antibody microarrays have many advantages, including high throughput, less sample amount, greater sentitivity and increased range of detection. Actually, antibody microarrays have been widely used in cancer research (Haab 2005), including PCa (Xu et al. 2000; Miller et al. 2003). The antibody chips in this study have been applied in numerous articles, and are scientific and reliable (Kocaoemer et al. 2007; Ye et al. 2008; Bouazza et al. 2009; Sommer et al. 2009; Mamlouk et al. 2012).

In conclusion, we showed that serum levels of M-CSF and CCL-18 were significantly elevated in PCa patients compared with BPH patients, while serum levels of IGFBP-6 and Fas/TNFRSF6 were reduced in PCa patients. These factors act as potential biomarkers for PCa, and may contribute to the correct diagnosis of PCa in the future.

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Conflict of Interest

The authors declare no conflict of interest.

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