Differential expression of androgen, estrogen, and progesterone receptors in benign prostatic hyperplasia

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ABSTRACT

This study aimed to identify the differential expression levels of androgen receptor (AR), estrogen receptors (ERα, ERβ), and progesterone receptor (PGR) between normal prostate and benign prostatic hyperplasia (BPH). The combination of immunohistochemistry, quantitative real-time reverse transcription polymerase chain reaction, and Western blotting assay was used to identify the distribution and differential expression of these receptors at the immunoactive biomarker, transcriptional, and protein levels between 5 normal human prostate tissues and 40 BPH tissues. The results were then validated in a rat model of BPH induced by testosterone propionate and estradiol benzoate. In both human and rat prostate tissues, AR was localized mainly to epithelial and stromal cell nuclei; ERα was distributed mainly to stromal cells, but not exclusively; ERβ was interspersed in the basal layer of epithelium, but sporadically in epithelial and stromal cells; PGR was expressed abundantly in cytoplasm of epithelial and stromal cells. There were decreased expression of ERα and increased expression of PGR, but no difference in the expression of ERβ in the BPH compared to the normal prostate of both human and rat. Increased expression of AR in the BPH compared to the normal prostate of human was observed, however, the expression of AR in the rat prostate tissue was decreased. This study identified the activation of AR and PGR and repression of ERα in BPH, which indicate a promoting role of AR and PGR and an inhibitory role of ERα in the pathogenesis of BPH.

KEY WORDS: Benign prostatic hyperplasia; androgen receptor; estrogen receptor α; estrogen receptor β; progesterone receptor; rat model
DOI: http://dx.doi.org/10.17305/bjbms.2016.1209 *Bosn J Basic Med Sci. 2016;16(3):201-208. © 2016 ABMSFBIH*

INTRODUCTION

Benign prostatic hyperplasia (BPH), common in elderly men, is a pathological condition characterized by nonmalignant enlargement of both epithelial and stromal tissues within the prostate gland [1], which contributes to series of urinary voiding problems commonly known as lower urinary tract symptoms (LUTS) [2]. Autopsy studies performed in different countries revealed a similar age-specific prevalence of pathological BPH, which ranged from 50% of men in the 5th decade to 90% of men over age 80 in Europe, Asia, and America [3]. To date, the exact molecular mechanisms underlying the induction, maintenance, and development of BPH remain unresolved despite research data from a plethora of studies. Only two factors are generally considered essential for BPH, namely, androgens and aging [4].

Because the prostate gland is a sex steroid hormone reactive organ, the regulation of sex steroid hormones, especially androgen and estrogen, is necessary for the gland development, maintenance, and function [4,5]. Multiple researches focused on the regulation of sex steroid hormones have proposed the hormonal theories of BPH etiology. Androgen receptor (AR), estrogen receptor α (ERα), ERβ, and progesterone receptor (PGR) were found expressed in prostate. However, the differential expression levels of these receptors between BPH and normal prostate tissues have been controversial, and the exact roles remain unclear [4,5]. Androgen signaling through AR is reported to play a permissive role in the pathogenesis of BPH [6]. A role of estrogen signaling through ERs in the pathogenesis of BPH is supported by increasing evidence from epidemiological, animal, and *in vitro* studies [4,5,7]. Few researches have revealed the role of PGR in the pathogenesis of BPH [4,8].

Therefore, it is required to design a series of studies focused on the regulation of these receptors in BPH to reveal their exact mechanism. This study has put forward the first step of this program and identified the differential expression levels of these receptors at the immunoactive biomarker,

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Submitted: 18 March 2016/Accepted: 23 April 2016

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transcriptional, and protein levels between BPH and normal prostate tissues, which revealed a promoting role of AR, PGR, and an inhibitory role of ERα in the pathogenesis of BPH.

MATERIALS AND METHODS

Ethic statement

We obtained human tissues from Southwest Hospital, Third Military Medical University PLA, under the procedures approved by the Ethnic Committee for Use of Human Samples of Southwest Hospital.

Patients and tissue samples

We obtained 5 normal prostate tissue samples (aged 28-45 years), of which 3 were from donation after cardiac death caused by traffic accidents and 2 were from the patients scheduled for radical cystectomy for urothelial carcinoma of the bladder. Post-operative biopsies demonstrated that there was no histological hyperplasia, prostate carcinoma, prostatic intraepithelial neoplasia, or metastatic tumor in these 5 samples. In total, 40 prostate tissue samples of BPH (aged 59-83 years) were collected from January to June 2015, of which 36 were from the patients scheduled for transurethral resection of the prostate for BPH exclusively and 4 were from the patients scheduled for radical cystectomy for urothelial carcinoma of the bladder. Post-operative biopsies demonstrating pathologic prostate carcinoma, prostatic intraepithelial neoplasia, or metastatic tumor were excluded from the analysis. Furthermore, the patients with diabetes mellitus, urinary infection, bacterial prostatitis, autoimmune disease, former treatment with 5α-reductase inhibitors, or recurrent BPH were excluded. The transition zone of prostate tissues, without urothelial tissues, was dissected and divided into 6 pieces (>100 mg each). Then, these fresh prostate tissues were immediately washed by cold sterile 0.9% sodium chloride solution during the surgery and then stored in liquid nitrogen until further experiments. The rest of the dissected prostate tissues were fixed using 4% paraformaldehyde (Boster, Wuhan, China) for the pathological diagnosis in the Department of Pathology Southwest Hospital.

Rat model of BPH

In total, 13 specific pathogen-free (SPF) grade male 12-week-old Sprague-Dawley (SD) rats with initial body weight (BW) of 230-270 g were purchased from Third Military Medical University experiment animal centers (Chongqing, China) and were housed in platform for SPF animal experiment maintained at 20-24°C and at a relative humidity of 50-60% with an alternating 12/12 hours light/dark cycle. They were offered a standard laboratory diet and water *ad libitum*. All animal treatment was strictly in accordance with international ethical guidelines and the guide for Care and Use of Laboratory Animals [9] and was approved by the Institutional Animal Care and Use Committee of Third Military Medical University. The rats were divided into BPH group (n = 8) and control group (n = 5). BPH was induced by subcutaneous injection of testosterone propionate (TP, 4 mg/kg, Sigma-Aldrich) and estradiol benzoate (E, 0.04mg/kg, Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich) for 4 weeks following castration in the BPH group, while the rats in the control group were subcutaneously injected with corn oil (1 ml/kg, Sigma-Aldrich) for 4 weeks following sham surgery. The BW was measured once per week. One day after the last treatment, the animals were weighted, anesthetized with phentobarbital (Solabio, Beijing, China) at 100 mg/kg BW injected intraperitoneally. The intact prostate tissue was dissected and removed carefully, and then, the prostate weight (PW) was measured. The prostate index (PI) was calculated as PW/BW×100. One lobe of the ventral prostate was fixed using 4% paraformaldehyde (Boster, Wuhan, China) and embedded in paraffin for histomorphology and immunohistochemistry (IHC) analysis. The other lobe of the ventral prostate was divided into two parts and stored in liquid nitrogen until further analysis.

IHC staining and analysis

Formalin-fixed paraffin-embedded sections of the human and rat prostate tissues were subjected to immunostaining with rabbit anti-AR, anti-PGR, anti-ERα, and anti-ERβ antibodies (1:100 dilutions, Abcom, Shanghai, China). The IHC procedure was performed as described previously [10]. The images acquired by the Olympus BX53 research microscope (Olympus, Tokyo, Japan) were processed by Cell Stem Image software (Olympus, Tokyo, Japan). Semi-quantitative analysis of the IHC images was conducted by Image-Pro Plus Version 6.0 (Media Cybermetics, Maryland, USA), by which integral optical density (IOD) and the area were collected. Then, average optical density (AOD) was calculated as IOD/area, which represented the staining intensity.

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA of tissues was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instruction. For mRNA detection of AR, ERα, ERβ, and PGR reverse transcription was performed using the PrimeScript RT Master Mix (Perfect Real Time, TaKaRa, Dalian, China). Quantitative PCR was performed using SYBR Premix ExTaq II (TliRNaseH Plus; TaKaRa) in a Bio-Rad CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). The mRNA of β-actin was used for normalization. All primers (Table 1) were synthesized by

TABLE 1. Primer sequences for the qRT-PCR analysis

Organism	Target gene product		Primer sequence	Product length
Homo sapiens	AR	F	5'-TACCAGCTCACCAAGCTCCT-3'	105
		R	5'-AAAGTCCACGCTCACCATGT-3'	
	ERα	F	5'-GGCTACATCATCTCGGTTCC-3'	118
		R	5'-AGACTTCAGGGTGCTGGACA-3'	
	ERβ	F	5'-AGTCCCTGGTGTGAAGCAAG-3'	128
		R	5'-TGAGCATCCCTCTTTGAACC-3'	
	PGR	F	5'-AGCCAGAGCCCACAATACAG-3'	102
		R	5'-CCCACAGGTAAGGACACCAT-3'	
	β-actin	F	5'-CAAAGACCTGTACGCCAACAC-3'	218
		R	5'-CATACTCCTGCTTGCTGATCC-3'	
Rattus	AR	F	5'-GGGTGACTTCTCTGCCTCTG-3'	104
		R	5'-CCATCCAAGGTCCCATTTC-3'	
	ERα	F	5'-CTTCTGGAGTGTGCCTGGTT-3'	100
		R	5'-CCAAGAGCAAGTTAGGAGCAA-3'	
	ERβ	F	5'-TCTGGGTGATTGCGAAGAGT-3'	105
		R	5'-TGCCCTTGTTACTGATGTGC-3'	
	PGR	F	5'-CTGCTCTTCTCTCGGTCCTG-3'	131
		R	5'-CTGGGATTTCTGCTTCTTCG-3'	
	β-actin	F	5'-CCCATCTATGAGGGTTACGC-3'	150
		R	5'-TTTAATGTCACGCACGATTTC-3'	

qRT-PCR: Quantitative real-time reverse transcription polymerase chain reaction, AR: Androgen receptor, ERα: Estrogen receptor α, PGR: Progesterone receptor

Sangon Biotech (Shanghai, China). The 2ΔΔCT method was used in the analysis of PCR data.

Western blotting (WB) assay

The prostate tissues were extracted with RIPA buffer (Beyotime, Shanghai, China), and the lysates were analyzed using the standard WB analyses. The β-actin that served as an internal reference was detected with an anti-β-actin rabbit monoclonal antibody (Proteintech, Chicago, USA). The anti-AR, anti-PGR antibodies, and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Abcam (Shanghai, China); the anti-ERα, anti-ERβ antibodies were from Proteintech (Chicago, USA). Bound proteins were visualized using the SuperSignal West Dura Extended Duration Substrate Kit (Thermo Scientific, Beijing, China).

Statistical analysis

All data analysis was performed using GraphPad Prism Version 5.01 for Windows (GraphPad Software, California, USA). Comparisons between the groups were conducted by the independent *t*-test. All tests were two-tailed and $p < 0.05$ was considered statistically significant.

RESULTS

The histomorphological and IHC analysis of human BPH tissues

Histomorphologically, the human BPH tissues showed hyperplasia of epithelial and stromal tissues, which were characterized by hyperplasia nodules, high cylindrical epithelial cells, irregular and dilation of acini with villous projections, back-to-back interacinar space, and ischesis (Figure 1A) [1]. The expression levels and distribution of these receptors in the human prostate were demonstrated by IHC staining (Figure 1A). AR was expressed extensively and localized mainly to the epithelial and stromal cell nuclei of both normal and BPH tissues [11]. ERα and ERβ, the two main subtypes of ERs, were expressed weakly and sporadically in the prostate tissues. ERα was distributed mainly, but not exclusively, to the stromal cells of both normal and BPH tissues as previously reported [4,11-13]. In the BPH tissues, there were potentially increased ERα positive cells in the basal layer of epithelium compared to the normal prostate tissues. ERβ interspersed mainly in the basal layer of epithelium, but sporadically in the epithelial and stromal cells of both normal and BPH tissues [4,12,13]. PGR was expressed abundantly in the epithelial and stromal cell cytoplasm of both normal and BPH tissues [14]. Comparative analysis of the AOD values between the normal and BPH tissues showed significantly increased expression of AR and PGR ($p = 0.0366$ and $p = 0.0490$, respectively) and significantly decreased expression of ERα ($p = 0.0436$) in the BPH tissues (Figure 1B). No significant difference in the AOD values of ERβ was found between the BPH and normal prostate in human ($p > 0.05$).

Rat model of BPH, histomorphology, and IHC analysis

The treatment with TP and E caused hyperplasia of the prostate in castrated male SD rats (Figure 2A). PW and PI

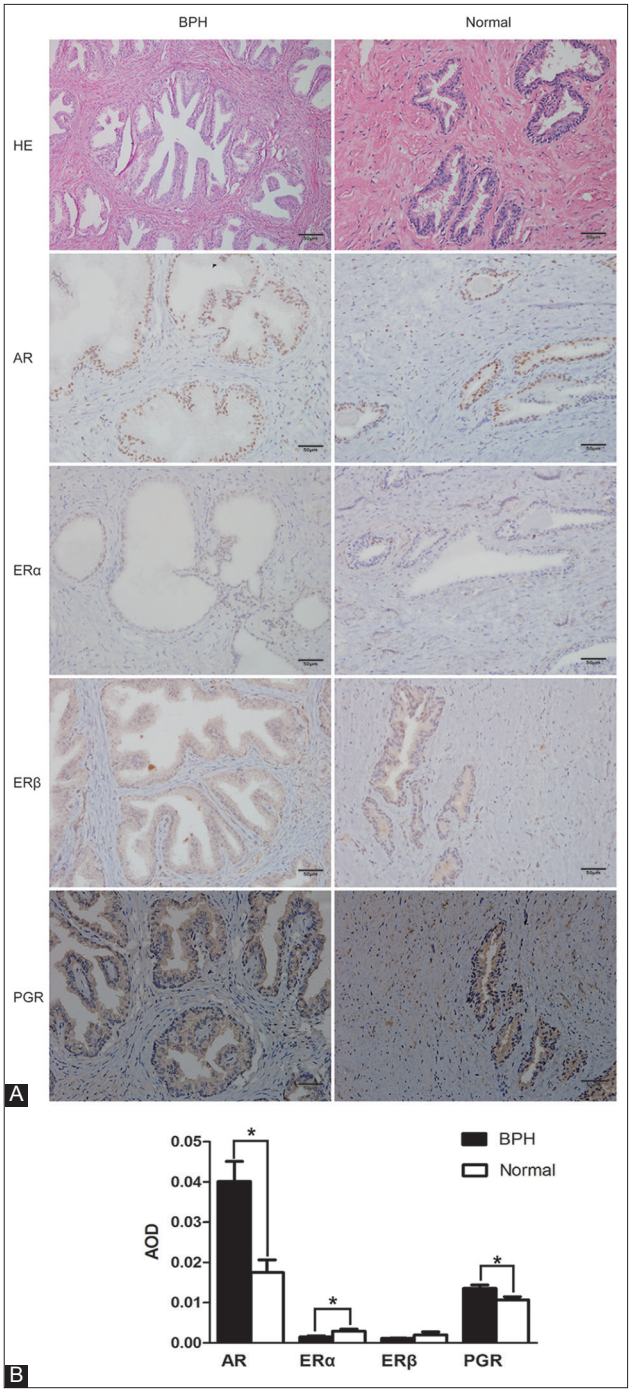

FIGURE 1. Histomorphology and immunohistochemistry (IHC) analysis of human benign prostatic hyperplasia (BPH) and normal prostate tissues in human. (A) Hematoxylin-eosin (HE) staining and IHC staining for androgen receptor (AR), estrogen receptor α (ERα), ERβ, and progesterone receptor (PGR) of BPH and normal prostate tissues, (B) Semi-quantitative analysis of the IHC staining by comparison of average optical density (AOD) between the two groups; *p<0.05.

were increased significantly in the BPH group compared to the control group (Figure 2B, p < 0.0001). According to the previous study, increased PW is an important marker indicating the development of BPH [15]. Histologically, the prostate tissues of the rats in the BPH group showed atypical hyperplasia of epithelial and stromal tissues, which was characterized by irregular acinar shape with villous projections, dilation of acini, high cylindrical epithelial cells, back-to-back interacinar space, and ischesis (Figure 2C) [16]. The IHC staining of the rat prostate tissues demonstrated that the distribution of these receptors was analogous to that of the human prostate (Figure 2C). The comparative analysis of the AOD values between the BPH group and control group revealed a significantly increased expression of PGR (p = 0.0143) and significantly decreased expression of AR and ERα (p = 0.0448 and p = 0.0443, respectively) in the BPH group (Figure 2D). Similar to the human prostate tissues, no significant difference in the AOD values of ERβ was found between the BPH group and control group in the rat model either (p > 0.05).

Differential expression at the transcriptional level

After the analysis of IHC, we resorted to the method of qRT-PCR to identify the differential expression of these receptors at the transcriptional level. In the human BPH tissues, the relative mRNA expression levels of AR and PGR were increased significantly (p = 0.0359 and p = 0.0189, respectively) while the level of ERα was decreased significantly (p < 0.0001) compared to the normal prostate tissues (Figure 3A). In the BPH group of the rat model, the relative mRNA expression levels of AR and ERα were decreased significantly (p = 0.0206 and p = 0.0238, respectively) while the level of PGR was increased significantly (p = 0.0095) compared to the control group (Figure 3B). No significant difference in the ERβ expression at the transcriptional level was found in the BPH compared to the normal prostate of human or rat (p > 0.05).

Differential expression at the protein level

Despite the identification of differential transcriptional expression levels of these receptors, it was still required to identify the differential expression at the protein level using WB assay. The results of WB assay were consistent with that of qRT-PCR. In the human BPH tissues, the protein expression levels of AR and PGR were significantly increased and the expression level of ERα was significantly decreased compared to the normal human prostate tissues (Figure 4A). In the BPH group of the rat model, the protein expression levels of AR and ERα were significantly decreased and the level of PGR was significantly increased compared to the control group (Figure 4B). There was no significant difference in the ERβ expression at the protein level in the BPH compared to the normal prostate in the human or rat.

DISCUSSION

Despite research data from a plethora of studies, the exact etiology or the mechanism of sex steroid receptors in the

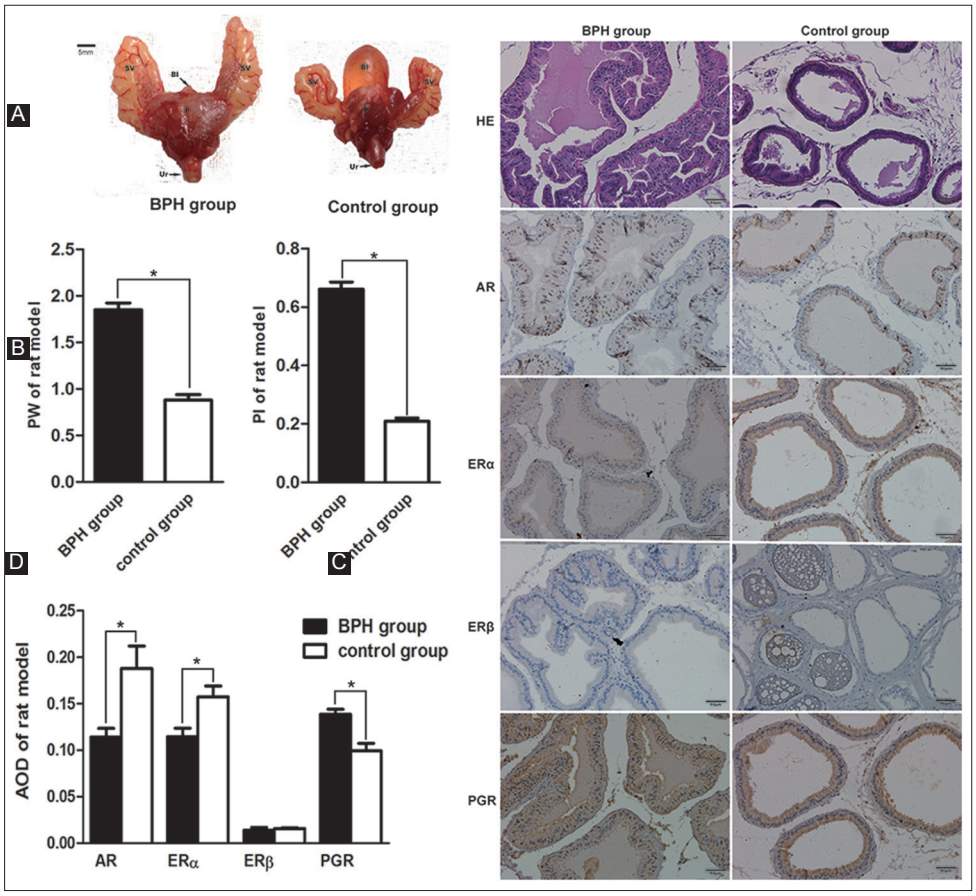

FIGURE 2. Comparative analysis of rat model between benign prostatic hyperplasia (BPH) group and control group. (A) Gross anatomy of the rat prostate: SV - Seminal vesicle, Bl - Bladder, Ur - Urethra, P - Prostate, (B) Comparative analysis of prostate weight (PW) and prostate index (PI) between the two groups; *p<0.05, (C) Hematoxylin-eosin (HE) staining and immunohistochemistry (IHC) staining for androgen receptor (AR), estrogen receptor α (ERα), ERβ, and progesterone receptor (PGR) in the BPH group and control group (right panels and left panels, respectively), (D) Semi-quantitative analysis of the IHC staining by comparison of average optical density (AOD) between the two groups; *p<0.05.

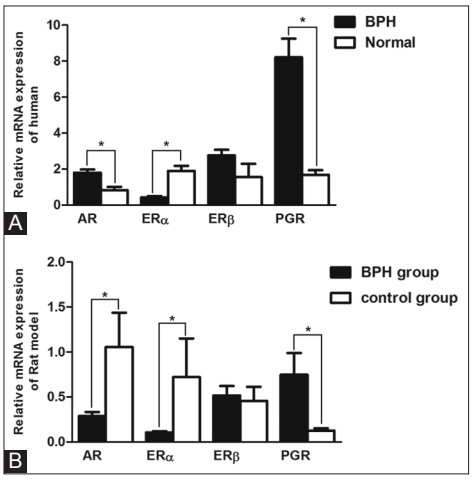

FIGURE 3. The comparison of the relative mRNA expression levels of androgen receptor (AR), estrogen receptor α (ERα), ERβ, and progesterone receptor (PGR) between benign prostatic hyperplasia (BPH) tissues (BPH group) and normal prostate tissues (control group) in the human (A) and rat model (B) using reverse transcription polymerase chain reaction; *p<0.05.

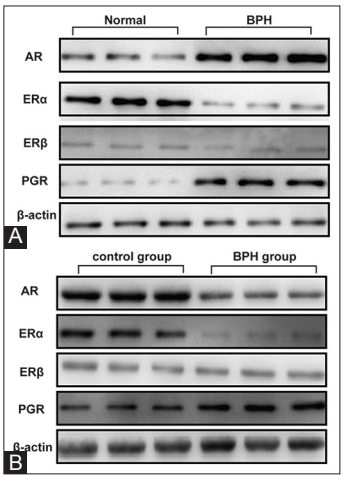

FIGURE 4. The comparison of the expression of androgen receptor (AR), estrogen receptor α (ERα), ERβ, and progesterone receptor (PGR) at the protein level between benign prostatic hyperplasia (BP) tissues (BPH group) and normal prostate tissues (control group) in the human (A) and rat model (B) using Western blotting assay.

pathogenesis of BPH has yet to be elucidated [4,5]. Even to date, no consistent differences in these receptors' expression levels between normal prostate and BPH tissues have been identified [4]. Hence, the first and indispensable step of

elucidating the mechanism of these receptors in BPH is to identify the exact differential expression levels between normal and BPH tissues.

Previous researches practically resorted to the methods of IHC or fluoroimmunoassay to analyze the differences semi-quantitatively. This study combined the classical methods of IHC, qRT-PCR, and WB to identify the distribution and differential expression levels of these receptors at the immunoactive biomarker, transcriptional, and protein levels between normal prostate and BPH tissues. The results from all these methods were consistent. In this study, a significantly increased expression of AR and PGR, decreased expression of ERα, and no significantly different expression of ERβ were identified in the human BPH tissues compared with normal prostate. These results indicated the activation of AR, PGR, and repression of ERα in the human BPH. In the rat model, a significantly increased expression of PGR, decreased expression of ERα, and no significant difference in the expression of ERβ were identified in the BPH group compared to the control group, which indicated the activation of AR and repression of ERα in the rat model of BPH. Although the expression of AR was significantly decreased in the rat model of BPH, AR was definitely activated since the rat model of BPH was induced by a dose that was beyond the physical dosage of exogenous TP. Therefore, AR and PGR may play a promoting role while ERα may play an inhibitory role in the pathogenesis of BPH. These results confirmed the dysregulation of sex steroid receptors in BPH and supported the hypothesis of hormonal theories of BPH etiology [17].

The distribution and expression of AR in BPH were investigated in many studies, but the results were not consistent. Some studies reported that AR was abundantly expressed in the nuclei of epithelium and stroma using IHC [13,18]. Nicholson et al. resorted to the method of multiplexed IHC, which revealed an increased percentage of AR-positive cells and increased AR intensity in both epithelial and stromal cells in BPH compared to normal prostate [11]. However, the study of Hetzl et al. reported that the AR immunoreactivity in BPH was similar to that in normal prostate [19]. Despite the controversy possibly caused by the methodological differences, it is generally accepted that androgen signaling through AR acts permissively for the development of BPH [5]. In the rat model, AR was activated by a dose that was beyond the physical dosage of exogenous TP that can induce hyperplasia of rat prostate [15,16]. However, the elevated serum or intraprostatic androgen levels may not be the causative factor in the activation of AR and etiology of BPH in humans, because the current literature lack conclusive data on serum or intraprostatic androgen levels, DHT in particular. The review by van der Sluis et al. reported that no difference has been shown between DHT concentrations in normal adult prostate and BPH tissue, nor is there a proven difference in androgen levels between histologically distinct regions of the prostate [20]. Furthermore, studies demonstrating that the supplementation of men with androgens does not appear to increase the incident risk of BPH or LUTS indicate that androgens may not influence the prostate growth [21]. Further studies are required to clarify the mechanism of activation of AR and the downstream pathway of AR in BPH.

The prostate is also an important target of estrogens. The most compelling implication of estrogens in the pathogenesis of BPH is that the treatment of male dogs and rats with androgens and estrogens leads to BPH [22], which was also supported by this study. The results from most published reports using animal models of BPH and prostatic primary cultures indicated that, in general, ERα stimulation results in hyperplasia, inflammation, dysplasia [5,23], and that ERβ inhibits the proliferation in prostate [24]. Despite these, neither the differential expression of ERα and ERβ between BPH and normal prostate reached consensus, nor the precise roles of ERα and ERβ in the pathogenesis of BPH are fully understood. Furthermore, the relationship between plasma estrogen levels and the risk of BPH in humans has been identified by some but not all studies [25-27], which makes the role of estrogens in BPH speculative. The semi-quantitative study of Royuela et al. demonstrated increased epithelial immunostaining for both ERα and ERβ in BPH compared to normal prostate [12]. Hetzl et al. reported more intense immunoreactivity of ERα and weak immunoreactivity of ERβ in the epithelium of BPH [19]. Nicholson et al. [11] demonstrated that BPH and normal prostate had a similar percentage of ERα positive cells overall, resulting from an increased expression of ERα in epithelial cells but decreased expression in stromal cells in BPH. However, the research by Zhang et al. [28] revealed that the expression of ERα in BPH was decreased in large prostates compared to small- and medium-sized prostates, while the expression of ERβ did not differ. The present study also revealed that the expression of ERα was significantly decreased in the human BPH and rat model of BPH, indicating that ERα might play an inhibitory role in the pathogenesis of BPH rather than a supposed promoting role. Regarding ERβ, we observed that the difference in the expression at the transcriptional or protein level between the BPH tissues and normal prostatic tissues was not statistically significant, which indicated that ERβ might play little role in the pathogenesis of BPH. Further researches *in vivo* are required to identify the exact role of ERα and ERβ in BPH.

Besides AR and ERs, PGR is also found expressed in both epithelium and stroma of prostate tissues. Previous data on PGR expression, especially those using IHC, were inconclusive [8,29]. To date, little is known about the role and regulation of PGR in the pathogenesis of BPH. The research of Yu et

al. demonstrated that PGR played an inhibitory role in prostate stromal cell proliferation *in vitro* [8], but these results were not confirmed *in vivo*. The research of Mobbs et al. reported that the expression of PGR in the prostate was responsive to estrogen stimulation [30]. However, we found that the expression of PGR in the humans and the rat model of BPH tissues was significantly increased compared to the normal prostatic tissues rather than decreased along with the expression of ERα, which indicated the upregulation of PGR might be related to the activation of AR signaling. These results demonstrated that the upregulation of PGR is probably a promoting-factor in the pathogenesis of BPH rather than an inhibitory-factor.

CONCLUSION

This study identified the activation of AR and PGR and the repression of ERα in BPH, which were further validated in a rat model. These results indicate that AR and PGR may play a promoting role in the pathogenesis of BPH while ERα may play an inhibitory role. Further studies should reveal the exact mechanism of AR regulation in the pathogenesis of BPH.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

ACKNOWLEDGMENTS

This work is supported by the grants of the National Nature Science Foundation of China (No. 81470985 and No. 81270842) and the grants of the Nature Science Foundation for Young Scholars of Gansu province, China (No. 1308RJYA097).

We thank Professor Xiaochu Yan from the Department of Pathology, Southwest Hospital for the assistance in the pathological diagnosis.

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