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Original Article

Pathological characteristics of CD40/CD40L and NF-κB proteins related to inflammation in benign prostatic hyperplasia tissue: a retrospective study of 120 cases

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Abstract: This paper aims to investigate the pathological characteristics of CD40/CD40L and NF-κB proteins related to inflammation in benign prostatic hyperplasia (BPH) tissue, as well as the outcomes of inflammation. A total of 120 BPH samples were obtained, and clinical data were gathered. The prevalence of BPH-associated inflammation was 91.7%, while inflammation infiltrates were more likely to be mild, multifocal, and stromal. Patients were divided into grades 0, 1, 2, and 3 according to the grade of inflammation. Serum prostate specific antigen (PSA) levels, prostate volumes, and International Prostate Symptom Score (IPSS) were higher in grade 1, 2, and 3 patients (increasing with the grade) than those in grade 0 patients. In addition, the present study demonstrated that CD40 and CD40L were mainly expressed in prostate epithelial cell membranes and some areas of the cytoplasm, whereas NF-κB proteins were mainly expressed in the cytoplasm and nuclei of glandular epithelial cells and prostatic stromal cells but less likely expressed in normal tissues. In the BPH group associated with inflammation, the positive expression rates of these proteins obviously gradually increased along with an increase in the degree of inflammation. BPH-associated with inflammation is a common condition that is associated with higher prostatic volumes, PSA levels, and IPSS compared with BPH alone. Moreover, CD40/CD40L and NF-κB expressions in BPH tissues were associated with the degree of inflammation and may play a role in BPH.

Keywords: Benign prostatic hyperplasia, CD40/CD40L, inflammation, NF-κB, transurethral resection of prostate

Introduction

Benign prostatic hyperplasia (BPH) is one of the most common urological diseases in the elderly male population worldwide, the presence of which increases the economic burden [1]. BPH is histologically defined as an enlargement of the prostate gland by stromal and epithelial cells and mainly affects the transition zone of the gland. Its incidence steeply increases as men age [2]. A study has shown that over 70% of men aged 60 years and over and 90% of those aged 70 years have pathological BPH [3]. Therefore, BPH is the most common benign tumor in men.

Although the two important factors associated with the development of BPH were advanced age and the presence of functioning testes, the exact etiology and pathogenesis of BPH still remains unclear. In recent years, another crucial factor has also been proposed. Given that inflammatory factors and their signaling pathways play an important role in many diseases, some research suggests that inflammation may represent a critical mechanism in the pathogenesis and progression of BPH [4-6]. CD40/CD40L is an important immunological signaling system that can promote inflammation by activating the nuclear factor-kappa (NF-κB) pathway [7-10].

Though it remains unclear whether this signaling pathway is involved in inflammation-enhanced BPH formation, a few studies worldwide have examined whether these expressions in the BPH are associated with inflammasome activation. In this study, we explored the expression of CD40, CD40L, and NF-κB proteins in BPH tissue. This study also analyzed the corre-

lation between the expression of the aforementioned proteins and clinical and pathologic features of BPH using pathological technique.

Material and methods

Patient selection

We included 120 patients who were diagnosed with BPH and scheduled for transurethral resection of the prostate (TURP) from November 2014 to June 2016 at West China Hospital of Sichuan University (Chengdu, China). Patients who had prostatitis, urinary tract infections, urinary stones, and prior prostate surgery were excluded. We also used a survey to exclude patients who had complaints of suprapubic or perineal pain. Patients catheterized before the operation were also excluded. Moreover, cases of prostate cancer revealed through postoperative pathological diagnosis were also excluded from the evaluation.

Patients characteristics and preoperative outcome assessment

The patient's clinical data were acquired mainly from electronic medical records of West China Hospital of Sichuan University. Basic information, such as age and serum prostate specific antigen (PSA) levels, were collected and recorded. The International Prostate Symptom Score (IPSS, a total score of 0-35 with seven problems about incomplete emptying, frequency, intermittency, urgency, weak urinary stream, straining and nocturia) was determined using questionnaires before TURP [11]. Urinalysis was performed before TURP and was considered abnormal when white blood cell counts exceeded 5 per high-power field [12].

Clinical specimen collection

During TURP, BPH tissues were collected using the resectoscope sheath in sterile conditions, and then the samples were repeatedly cleansed with sterile normal saline. Subsequently, more than 30 mg of tissue specimens from each individual was placed in a numbered eppendorf tube and independently immersed in a 10% formalin solution using a glass container for hematoxylin and eosin (HE) staining and immunofluorescence and histochemistry analyses.

Histopathological examination and immunohistochemistry

In the laboratory, BPH tissue specimens were immediately isolated and fixed in a new 10% buffered formalin solution for 24 h. Thereafter, these specimens were dehydrated using graded ethanol, embedded in paraffin, and then sectioned (4 μm). HE staining was implemented based on a standard protocol to evaluate inflammation in BPH tissue. In addition, immunohistochemical (IHC) and immunofluorescence (IF) stainings were used to measure the expression of CD40 (ab58391; 1:100 dilution; Abcam, USA), CD40L (ab65854; 1:100 dilution; Abcam, USA), NF-κB p65 (ab32536; 1:400 dilution; Abcam, USA), and NF-κB p50 (ab31410; 1:400 dilution; Abcam, USA) related to the inflammation in BPH tissue. The Envision immunohistochemical staining kit (K5007; ready-to-use; DAKO, Denmark) was used for IHC detection. Alexa Fluor 488 goat anti-rabbit IgG (A11034; 1:1000 dilution; Life Technologies, USA) was used for IF detection. IHC photographs were captured using a light microscope (BX51; Olympus, Tokyo, Japan), whereas IF photographs were captured using a fluorescence microscope (DM4000B; Leica, Germany).

Histopathological classification for chronic prostatic inflammation

The pathologic appearances of BPH tissue in pathological sections were observed through HE staining. Based on the histopathological classification system of prostatic tissue inflammation recommended by the North American Chronic Prostatitis Collaborative Research Network and the International Prostatitis Collaborative Network [13], we classified histologic inflammation in these pathological sections according to anatomical location, extent, and grade. The BPH group without inflammation was regarded as grade 0, whereas mild, moderate, and severe inflammations were regarded as grades 1, 2, and 3, respectively.

Evaluation of the immunohistochemical staining results

Positive stainings of CD40, CD40L, and NF-κB (p65/p50) were all indicated as brown granules in the cytomembrane, cytoplasm, or nucleus. Each section was observed through microscopic examination (×400). From each section, five

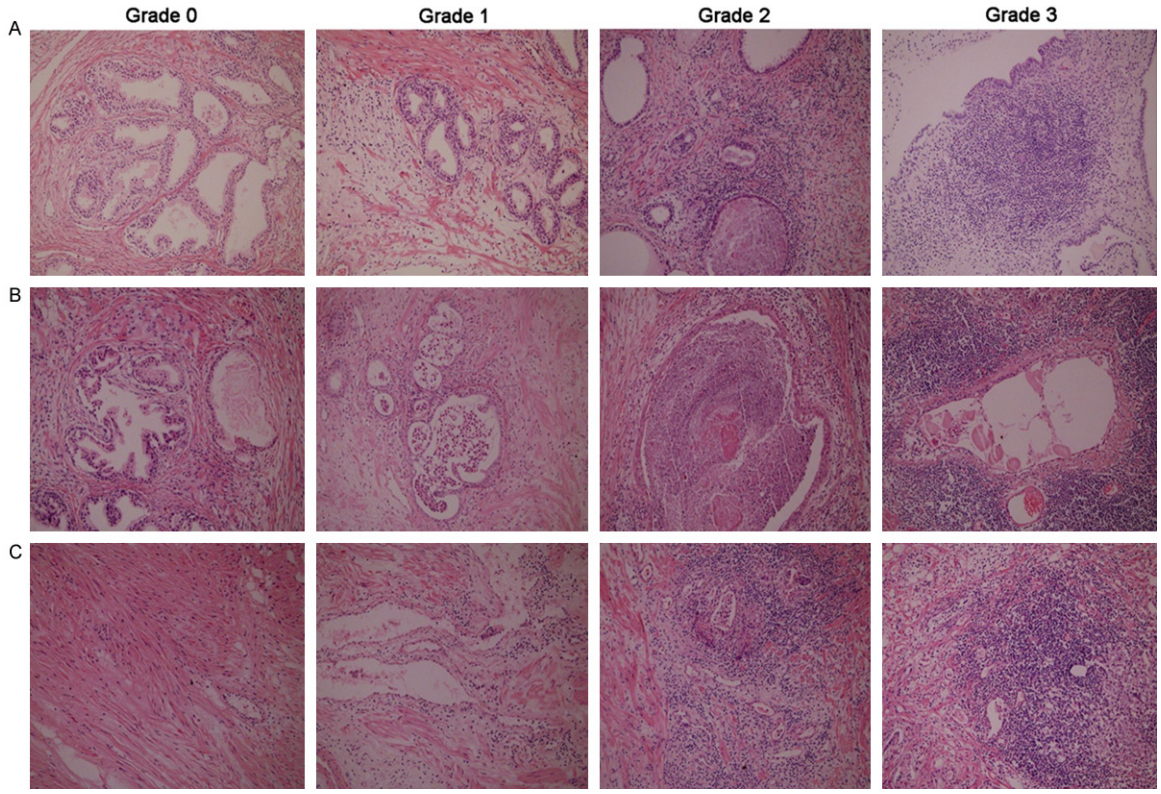

Figure 1. The pathologic appearances of the pathological sections in prostate tissue were observed with hematoxylin and eosin (HE) staining. A: Periglandular; B: Glandular; C: Stromal (HE×300).

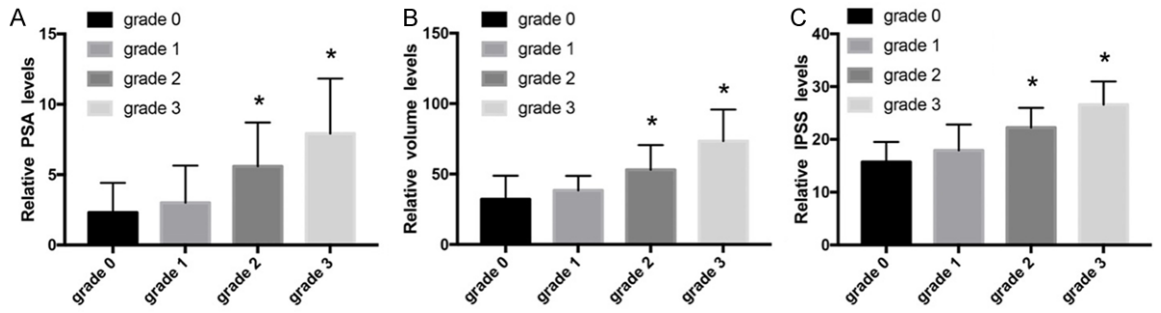

Figure 2. The relative levels compare grade 1, grade 2, and grade 3 groups with grade 0 group. A: PSA level; B: Prostate volume; C: IPSS score. *P < 0.05.

noncontiguous visual fields were randomly selected, and the score for each visual field depended on the percentage of its positive cells and their staining intensity. For the percentage of positive cells, < 10%, 11%-50%, 51%-75%, and > 75% were recorded as 0, 1, 2, 3, and 4 points, respectively. For staining intensity, non-stained, light yellow, yellowish brown, and brown were recorded as 0, 1, 2, and 3 points, respectively. The product of these two scores (percentage and intensity scores) was regarded as the visual field score, while the average score of five visual fields was regarded as the final score for that section. Finally, >3 points was recorded as positive staining (+); otherwise, negative staining was indicated. The evaluation of staining was performed by two independent pathologists who were blinded to the clinicopathological data.

Statistical analysis

All statistical analyses were performed using SPSS (Statistical Package for Social Science,

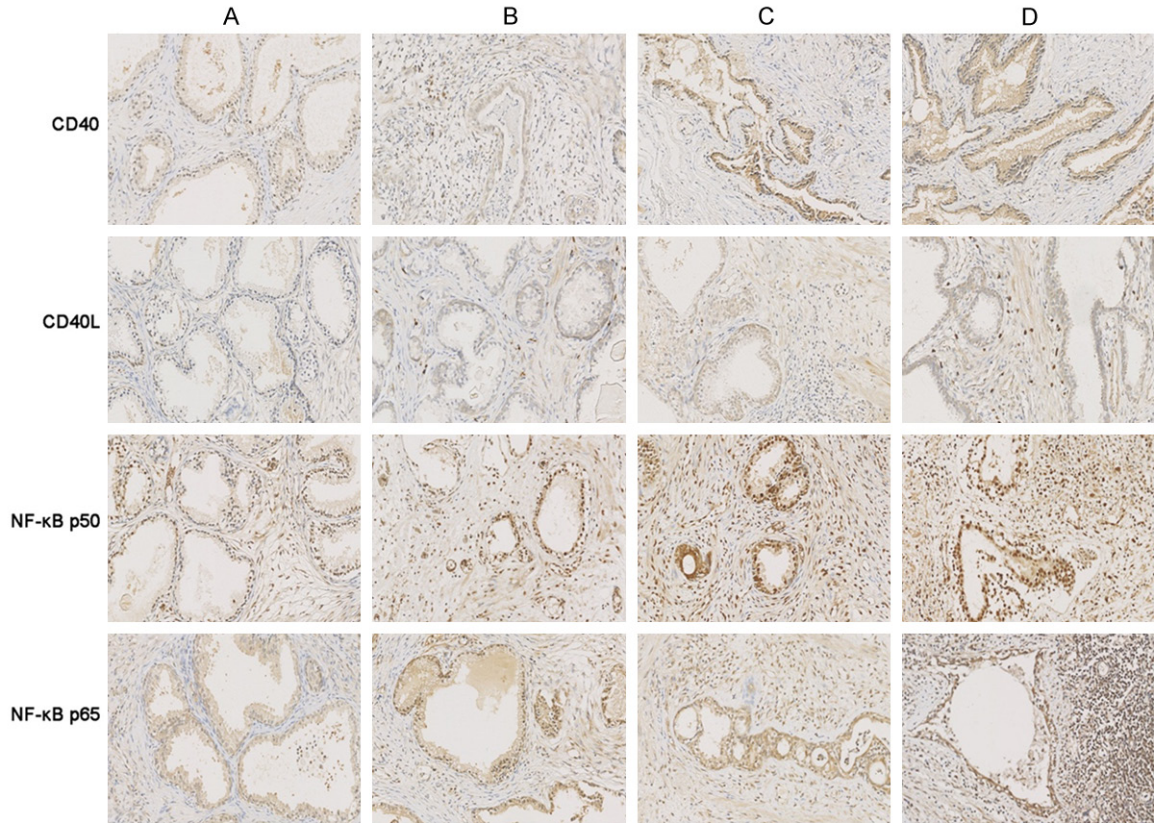

Figure 3. Immunohistochemistry staining was adopted to observe the positive expression (brown) of CD40, CD40L, and NF-κB (p65 and p50) in the BPH tissue. A: Grade 0 (BPH alone); B: Grade 1; C: Grade 2; D: Grade 3 (×300).

Chicago, IL, USA) version 18.0. Non-normal distributions (age) were expressed as median and range. Normal distributions of continuous variables (prostate volume, PSA level, and IPSS score) were expressed as mean ± standard deviation, and comparing statistical differences between the two groups were independently compared using independent samples t-test. Differences in dichotomized variables (CD40, CD40L, and NF-κB) were tested for statistical significance using the chi-square test as appropriate. *P* values < 0.05 were considered statistically significant.

Results

Tissue inflammation characteristics of BPH patients

All 120 patients comprising the study group underwent TURP and had complete clinical data. HE staining observation showed that a total of 110 cases (91.7%) were diagnosed with both BPH and tissue inflammation, whereas the other 10 cases (8.3%) were diagnosed with BPH alone.

After BPH pathological sections, tissue inflammation was classified according to its anatomical location, extent, and grade. Based on the histopathological classification system, inflammatory infiltrates were classified as glandular in 23 patients (20.9%), periglandular in 36 patients (32.7%), and stromal in 51 patients (46.4%).

Based on the extent of inflammatory infiltration, multifocal region infiltration occurred in 65 patients (59.1%), whereas focal and diffuse region infiltration occurred in 34 (30.9%) and 11 patients (10.0%), respectively. In addition, 51 patients (46.4%) were considered to have mild-grade prostatic inflammation, which accounted for majority of the patients. This was followed by moderate- and severe-grade tissue inflammations, which were observed in 37 (33.6%) and 22 (2.0%) patients, respectively. Through histopathological analysis, patients

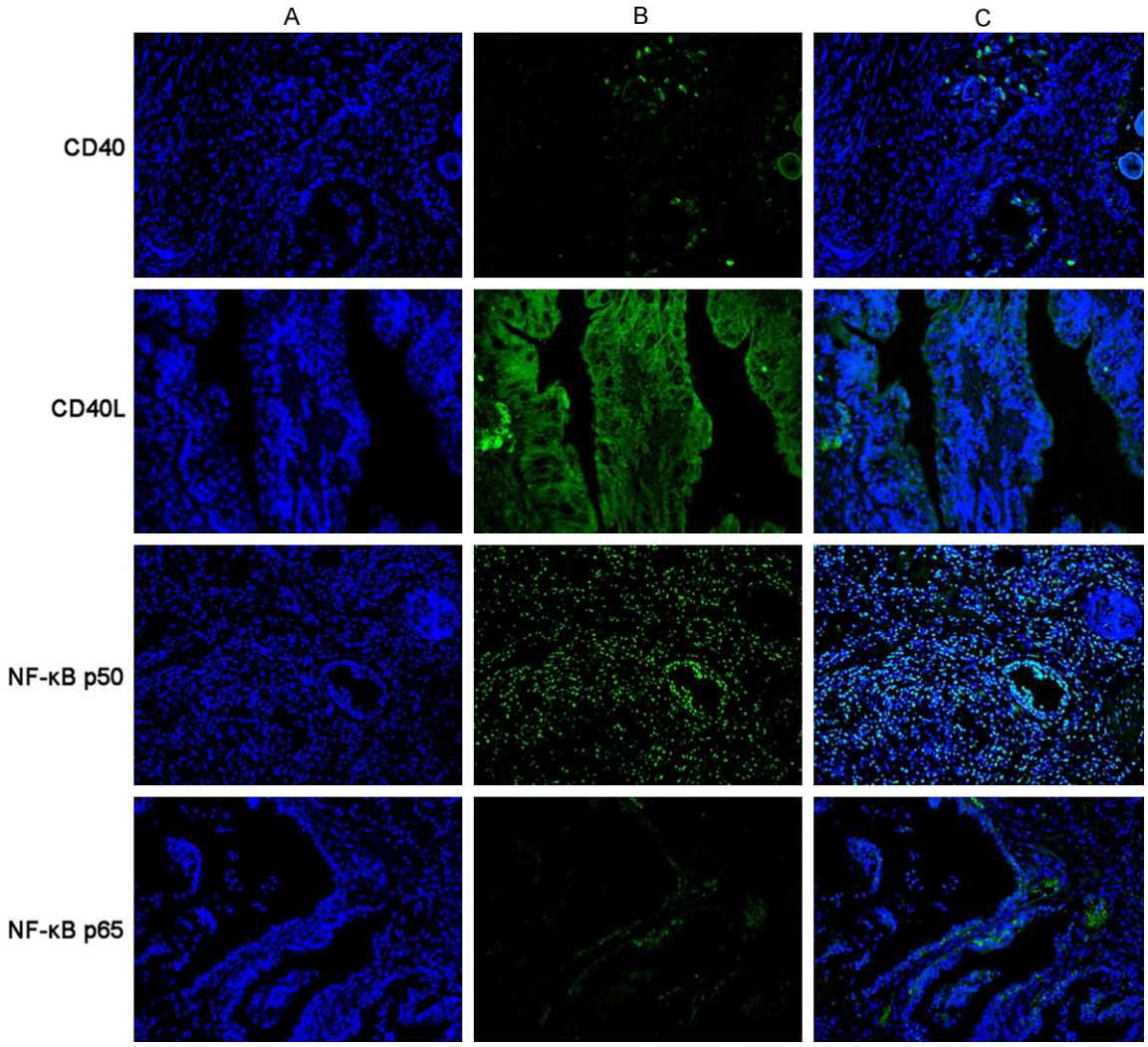

Figure 4. Immunofluorescence showed the expression of CD40, CD40L and NF-κB (p65 and p50) in BPH tissue specimens associated with inflammation tissue. A: DPAI, Blue; B: Antibodies, Green; C: Overlay.

having BPH-associated with inflammation were more likely to develop mild, multifocal, and stromal prostatic inflammation in this study. The results of the pathological characteristics are shown in **Figure 1**.

General information

Overall, the age of all the included patients was 53 to 84 years (median, 67 years). The mean prostate volume was 48.8 ± 20.8 cm^3, the mean serum PSA level was 4.64 ± 3.57 ng/ml, and the mean IPSS was 20.6 ± 5.6. Patients were then divided into four groups according to the grade of inflammation (grades 0, 1, 2, and 3).

The serum PSA level increased to 5.59 ± 3.12 in the grade 2 group (P = 0.003) and to 7.92 ± 3.91 in the grade 3 group (P < 0.001), when compared with that in the grade 0 group (2.29 ± 2.12). Simple trends were found for prostate volume (53.09 ± 17.51, P = 0.001 and 73.44 ± 22.37, P < 0.001) and IPSS (22.24 ± 3.74, P < 0.001 and 26.59 ± 4.40, P < 0.001) in the grade 2 and grade 3 groups, when compared with the grade 0 group (32.01 ± 16.79 and 15.70 ± 3.83) respectively, although differences between the grade 1 and grade 0 groups were not statistically significant (P = 0.423, P = 0.109, and P = 0.182 respectively). The results are shown in **Figure 2**.

Expression characteristics of the proteins in BPH tissue

We differentiated paraffin section specimens using immunohistochemistry and immunofluo-

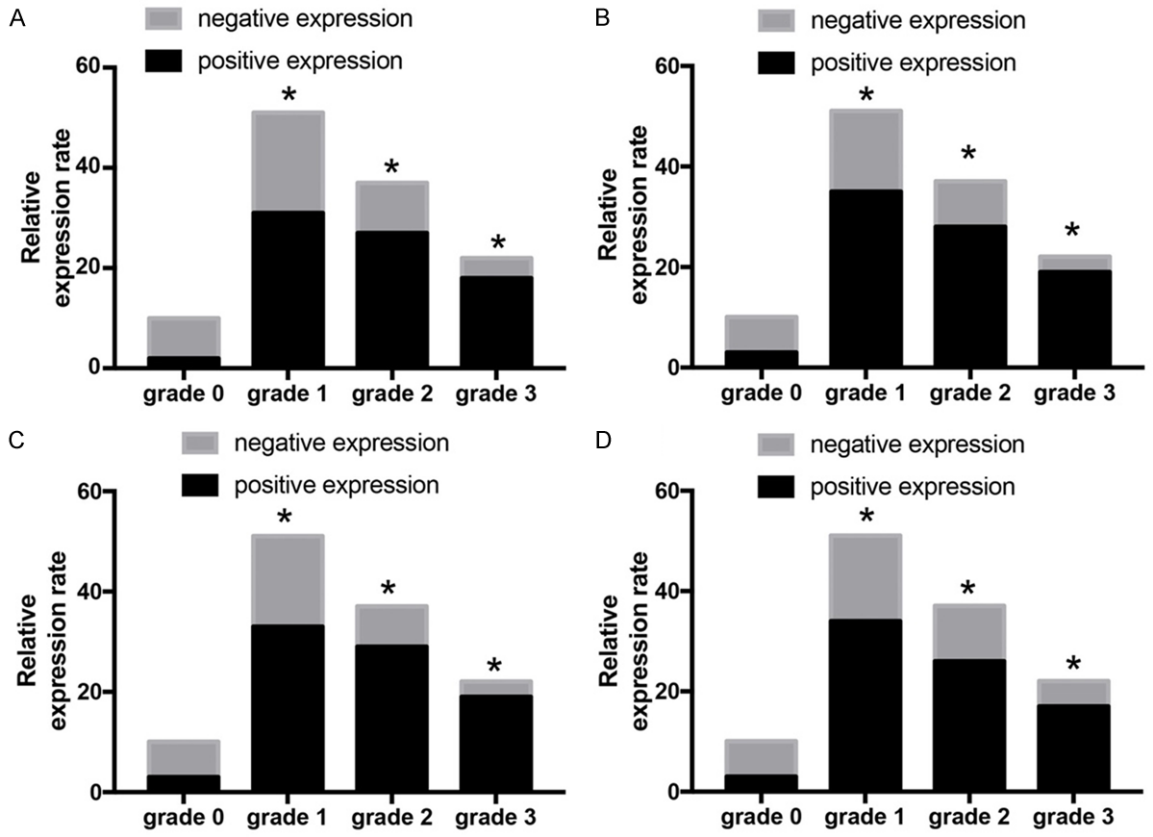

Figure 5. The positive expression rate of protein between grade 1 group, grade 2 group and grade 3 groups compared with grade 0 group, respectively. A: CD40; B: CD40L; C: NF-κB p65; D: NF-κB p50. *P < 0.05.

rescence. The results showed that CD40 and CD40L were mainly expressed mainly in the prostate epithelial cell membranes and some areas of the cytoplasm, as well as secondarily in the prostatic stromal cells, in BPH tissue. In addition, NF-κB (p65 and p50) were mainly expressed in the cytoplasm/nuclei of glandular epithelial cells and prostatic stromal cells. **Figures 3** and **4** independently show the histopathological characteristics of proteins by immunohistochemistry and immunofluorescence.

The positive expression rates of CD40 protein were higher in grade 1 (60.8%), grade 2 (81.8%), and grade 3 (73.0%) groups than in the grade 0 group (20.0%), the difference being statistically significant (P = 0.018, P = 0.002, and P = 0.001 respectively). Simple trends were found for CD40L (68.6%, P = 0.021; 75.7%, P = 0.007; and 86.4%, P = 0.001), NF-κB p65 (66.0%, P = 0.034; 78.4%, P = 0.004; and 86.4%, P = 0.001), and NF-κB p50 (66.7%, P = 0.03; 70.3%, P = 0.02; and 77.3%, P = 0.01) in grade 1, grade 2, and grade 3 groups compared with those in the grade 0 group (30%, 30%, and 30%), respectively. The results are shown in **Figure 5**.

Discussion

BPH is one of the most common diseases in aging men and has greater prevalence rates as the population ages. Approximately 20% of the men aged ≥ 65 years in the United States are estimated to be affected by 2030 [14]. Few studies have been performed on the clinical and pathological features of BPH and relationship between CD40/CD40L and NF-κB related to inflammation in BPH. To better understand this characteristics, we performed a series of experiments. To our knowledge, this is the first comprehensive analysis on BPH tissue in this field.

Currently, several research works have shown that BPH is an immune-mediated inflammatory

disease and that chronic inflammation has an important role in the pathogenesis of this disease [4, 15, 16]. Inflammation has been rated as an etiologic factor in the development of BPH [17]. Due to the significance of this role, inflammation has been clinically studied in the last decade. Some studies have confirmed that the risk of urinary retention due to BPH was significantly higher in men with prostate inflammation than in those without [17]. In addition, BPH-associated inflammation can cause significant deterioration of lower urinary tract symptoms and also lead to PSA elevation [18]. Nickel et al., who performed prostate biopsies for 8224 men, showed that 77.4% of the research samples had inflammation, identifying a special feature wherein more severe inflammation was associated with higher IPSS [19]. Our previous retrospective study indicated that 95.6% of the BPH biopsy specimens had inflammation [20]. Zlotta et al. [21], who studied the prevalence of inflammation and BPH in Asian and Caucasian men (n = 320), found that inflammation occurred in more than 70% during autopsy. Furthermore, individuals with inflammation were 6.8 times more likely to have a higher BPH score than those without. In the present study, we found that 91.7% of the BPH samples had tissue inflammatory infiltration. The current results have confirmed our previous findings and those of other studies. Our results clearly suggest that PSA levels and IPSS were significantly greater in the BPH-associated with inflammation than in BPH without inflammation. Moreover, these values gradually increased typically with an increasing degree of inflammation.

In addition, several clinical studies have demonstrated the role of inflammation in BPH. Roehrborn et al. showed an interesting discovery regarding inflammation in these patients from the Medical Therapy of Prostatic Symptoms trial [22]. They discovered that patients with evidence of inflammation had larger glands (44.1 vs. 36.8 ml; P = 0.0002) compared to those without inflammation. Similar data were found by Di Silverio et al., who also demonstrated a statistically significant correlation between inflammation and prostate volume [23]. The present study showed that patients who had BPH and increasing inflammatory grade had obviously larger prostate volumes than those with BPH alone.

Bacterial infections, hormones, urine reflux, and dietary factors have been considered to cause inflammation in the prostate. Further insights were provided by Gandaglia et al. [16], who studied the role of inflammation in the pathogenesis and progression of BPH. The authors hold that inflammatory infiltration leads to tissue damage and inflammatory response, as well as the chronic process of wound healing, which might lead to a persistent and continuous stimulation of epithelial and stromal prostatic tissues, silently resulting in BPH. In addition, it has also been speculated that the prostate is an immune organ and BPH is an immune-mediated inflammatory disease. T-cell activity and associated autoimmune reactions may be induced by stromal and epithelial cell proliferation. Although the pathogenesis of BPH is still not fully understood, this study strongly demonstrated that inflammatory infiltrates have been routinely studied in prostate tissue specimens from patients with BPH. In addition, the present study also demonstrated that inflammatory grade was closely related to PSA levels, prostate volume, and IPSS.

The molecular and cellular mechanisms involving stromal and epithelial components of the prostate that lead to BPH remain unclear. Nunzio et al. [24]. Emphasized that understanding the prostatic inflammation pathways is a very important area in clinical and basic research of BPH and may contribute to the identification of new therapeutic targets and strategies for decreasing the risk of BPH. CD40, a type I transmembrane glycoprotein receptor of the tumor necrosis factor gene superfamily, is expressed in different kinds of cells, such as in smooth muscle cells, endothelial cells, epithelial cells, fibroblasts, B cells, and monocytes [25]. CD40L, a type II transmembranic protein, is also expressed in multifarious populations, such as in epithelial cells, endothelial cells, smooth muscle cells, leukocytes, and so on [25]. Studies have demonstrated that the interaction between CD40/CD40L enhances their expression and release mainly through the stimulation of NF-κB [25]. The NF-κB family consists of five members: NF-κB1 (p105/p50), NF-κB2 (p100/p52), c-Rel, RelA (p65), and RelB. The activation of NF-κB dimers produce secondary inflammatory mediators that are mostly associated with NF-κB p50/p65 [26].

Pace et al. [27] enrolled 15 consecutive patients affected with BPH while controlling the investigation of specific plasma markers of inflammation. They found that in systemic blood samples, CD40L was higher in BPH (4.25 ± 0.65 ng/ml) than in the control group (2.31 ± 0.20 ng/ml; $P < 0.05$). Penna et al. demonstrated that human stromal prostatic cells obtained from BPH tissue can express co-stimulatory molecules of CD40, which could be forcefully upregulated by inflammatory stimuli [28]. Our present study first showed that CD40 and CD40L were mainly expressed in prostate epithelial cell membranes and some areas of the cytoplasm of BPH tissue. In addition, the present study confirmed that NF-κB was mainly expressed in the cytoplasm and nuclei of glandular epithelial cells and prostatic stromal cells of BPH tissue. Most importantly, the expression rate of CD40, CD40L, and NF-κB in the BPH group associated with inflammation obviously gradually increased with increasing degree of inflammation.

Chatzigeorgiou et al. [29] claimed that the CD40/CD40L pathway plays a pivotal role in mediating the body's inflammatory response and may also be implicated in the pathogenesis and progression of many inflammatory and autoimmune disease. Several studies have shown that the blocking of the CD40/CD40L signaling pathway has been the object of intensive study in cellular systems or animal models as a potential therapeutic mechanism for inflammatory, autoimmune, and other diseases [30, 31]. On the other hand, studies have shown that the direct transcriptional regulation of the CD40 gene seems to depend on transcription factors, such as NF-κB [32, 33]. In addition, many studies have confirmed that NF-κB induces the activation of inflammatory pathways, which is the most important transcription factor activated by CD40/CD40L signaling [34]. In summary, the present study showed that CD40, CD40L, transcription factors such as NF-κB (p65 and p50), inflammation, and BPH are closely related. BPH-associated with inflammation is a common condition that is associated with higher PSA levels, prostatic volume, and IPSS than with BPH alone. CD40/CD40L and the NF-κB signaling pathway may be factors affecting BPH and inflammation that cannot be overlooked. Nevertheless, further work is necessary to take this research another step forward.

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Disclosure of conflict of interest

None.

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