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Original Article

Clinical relevance and implications of autophagy-related proteins in benign prostatic hyperplasia

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Abstract: The role of autophagy in progression of benign prostatic hyperplasia (BPH) is still unclear. This study was designed to evaluate the differential expression and clinical relevance of autophagy-related proteins (LC3A/B and Beclin-1) and apoptosis-related proteins (cleaved caspase-3 and Bcl-2) in stroma and epithelia of BPH tissues. BPH samples were obtained from 60 BPH patients who underwent transurethral resection of prostate (TURP). Tissue samples were analyzed using immunohistochemistry (IHC) and Western blot. The data of clinical parameters of BPH patients, including prostate specific antigen (PSA) value, prostate volume (PV), International Prostate Symptom Score (IPSS), quality of life (QoL), maximum urinary flow rate (Qmax), postvoid residual urine (PVR), and duration of 5α-reductase inhibitor (5-ARI) treatment were recorded and analyzed referring to expression level of LC3A/B and Beclin-1. It was shown that expression of LC3A/B and Beclin-1 in BPH tissues was stronger than that in normal prostate transition zone tissues. Besides, stromal LC3A/B expression level of BPH tissues correlated with PV, Qmax, PVR and duration of 5-ARI treatment, while stromal Beclin-1 expression correlated with PV and Qmax. Values of PV, PVR and duration of 5-ARI treatment were found higher in LC3A/B and Beclin-1 high expression group. Conclusively, our results indicate that autophagy may be induced in BPH tissues especially in prostate stromal cells and patients who received comparatively longer duration of 5-ARI treatment.

Keywords: Benign prostatic hyperplasia (BPH), autophagy, apoptosis, stroma, epithelium

Introduction

Benign prostatic hyperplasia (BPH) is a highly prevalent and age-related urologic disease in men around the world [1, 2]. The most common clinical manifestation of BPH is lower urinary tract symptoms (LUTS), which greatly affecting the daily life of BPH patients and causing a large burden on healthcare resources [1, 3]. Currently, medical therapy and surgical treatment are two main options for BPH treatment [2, 3]. However, the disease may still progress even if definite therapy. Establishing the most appropriate and individualized treatment option and long-term management plan for BPH patients remains a challenge. Thus, great efforts should be made to more deeply understand the basic processes and the specific molecular mechanism associated with BPH in order to find novel and effective therapeutic approaches to prevent BPH progression.

Autophagy is a highly-conserved evolutionary and complex cellular process in the eukaryotic cells, whereby cytoplasmic long half-life proteins and organelles are sequestered within autophagosomes and delivered to the lysosome where the sequestered material is degraded and recycled [4]. Evidence has revealed that autophagy plays important roles in many normal physiological conditions, such as maintaining homeostasis and genome stability, developmental processes and cellular response to stress [5, 6]. Besides, numerous studies demonstrate that autophagy is also involved in the pathological conditions, such as neurodegenerative disease, cancer and infectious diseases [7-10]. Accumulating evidences show that the process of autophagy is regulated by various autophagy specific genes (known as Atg). Microtubule-associated protein light chain 3 (LC3) is routinely used as a biomarker to monitor the level of autophagy [11]. It

Table 1. Patients' clinical parameters

Clinical parameters	BPH patients (n=60)	
	Mean ± SD	Range
Age (year)	72.50 ± 8.27	54-86
PV (ml)	79.40 ± 39.02	25.96-174.00
PSA	10.18 ± 7.51	0.80-36.5
IPSS	19.08 ± 4.85	10-30
QoL	4.1 ± 1.18	2-6
Qmax (ml/s)	9.25 ± 3.46	3.6-20.90
PVR (ml)	95.80 ± 90.68	30.14-560.30

BPH, benign prostatic hyperplasia; SD, standard deviation; PV, prostate volume; PSA, prostate specific antigen; IPSS, international prostate symptom score; QoL, quality of life; Qmax, maximum urinary flow rate; PVR, postvoid residual.

comprises a soluble form localized in the cytoplasm (LC3-I) and a lipidated form in the autophagosomal membrane (LC3-II). During the initiation of autophagy, the autophagosome formation is associated with the conversion from LC3-I to the membrane LC3-II form [12]. Beclin-1 usually forms a complex with a class III phosphoinositide 3-kinase (PI3K) and vacuolar sorting protein 34 (Vps34), which is required for the autophagosome formation [13]. Previous studies suggested that autophagy and autophagy-related proteins may involve in the development of BPH [14-16]. However, the expressions of autophagy-related proteins in stroma of hyperplastic prostate gland and their clinical relevance have not previously been discussed.

In the present study, we hypothesis that autophagy related proteins such as LC3A/B and Beclin-1 could upregulate in BPH tissues especially for patient with long duration of 5α-reductase inhibitor (5-ARI). To this end, we compared the differential expressions of autophagy-related proteins (LC3A/B and Beclin-1) and apoptosis-related proteins (Cleaved Caspase-3 and Bcl-2) both in the stroma and epithelia of BPH tissues and normal prostate transition zone (TZ) tissues. Moreover, we evaluated the association of stromal autophagic biomarker expressions with the clinical parameters of BPH patients.

Materials and methods

Patients and tissue samples

All tissue samples were obtained from surgery between January 2014 and January 2016 at Shanghai General Hospital. BPH tissue samples were obtained from 60 patients who underwent bipolar transurethral resection of prostate (TURP) or open prostatectomy (OP), and detailed information of the patients are shown in **Table 1**. Normal prostate TZ tissue samples were obtained from 30 patients who underwent radical prostatectomy or radical cystectomy. All the collected samples were confirmed by pathology. Patients with prostatitis or prostate cancer in prostate transition zone were excluded. The tissue samples were fixed in formalin and embedded by paraffin after surgical resection. Preoperatively, all BPH patients had a history of receiving 5-ARI with different durations. All BPH cases were preoperatively assessed on serum prostate specific antigen (PSA) value, prostate volume (PV), International Prostate Symptom Score (IPSS), quality of life (QoL), maximum urinary flow rate (Qmax), and postvoid residual urine (PVR). For experiments involving human subjects, study approval was obtained from the institutional review board of Shanghai General Hospital ethics committee. Written informed consent was obtained from all the participants.

Immunohistochemistry (IHC)

IHC staining was performed on 4-μm sections of paraffin-embedded human BPH tissues and normal prostate TZ tissues to determine the expression of autophagy/apoptosis-related proteins using LC3A/B antibody (#12741, dilution 1:1000, Cell Signaling Technology, Danvers, MA, USA), Beclin-1 antibody (ab62472, dilution 1:2000, Abcam, Cambridge, UK), Cleaved Caspase-3 antibody (#9664, dilution 1:2000, Cell Signaling Technology) and Bcl-2 antibody (ab77567, dilution 1:150, Abcam). Briefly, the formalin-fixed and paraffin-embedded tissue samples were cut into 4-μm sections and baked at 65°C for three hours. Then the sections were deparaffinized in xylene twice and rehydrated in graded ethanol. After that, the sections were incubated with 0.3% hydrogen peroxide in methanol for 30 min to quench the activity of endogenous peroxidase and then with 0.3% Triton X-100 (Sangon Biotech, Shanghai, China) for 30 min to increase cell permeability. Thereafter, all the sections were incubated with normal serum to block nonspecific binding. The sections were incubated with primary antibodies overnight at 4°C. Finally, slides were added with diaminobenzidine (DAB, Beyotime, Jiangsu, China) for visualization after three 5-min washing with phosphate buffered saline (PBS) and then counterstained with

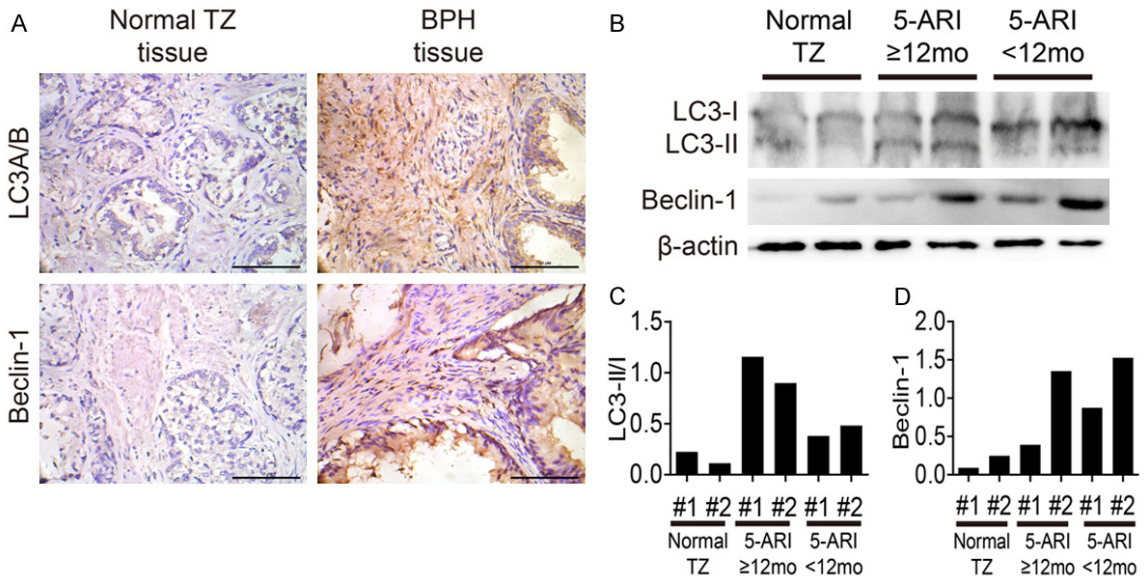

Figure 1. A: Immunohistochemical staining of LC3A/B and Beclin-1 in normal TZ tissues and BPH tissues (Scale bar =10 μm). B-D: Western blot result of LC3 and Beclin-1 expression in normal TZ group and BPH group.

hematoxylin. For negative controls, each primary antibodies was replaced with PBS.

Immunohistochemical scoring

All the tissue slides were reviewed and scored by two pathologists with abundant experience separately who was blinded to the study protocol. Heat induced coagulation layer (about 2 mm) on prostate ablation chip by bipolar TURP was excluded for IHC result inspection. The protein expressions of LC3A/B, Beclin-1, Cleaved Caspase-3 and Bcl-2 were evaluated by the staining intensity of the stromal or epithelial cells. The staining intensity was scored depending on the following criteria: 0 (negative staining), 1 (probably positive staining), 2 (weakly positive staining), 3 (moderately positive staining) and 4 (strongly positive staining). Then we divided the scores into two groups for statistical analysis: low expression group had a score of 0, 1, or 2, while high expression group had a score of 3 or 4.

Western blot

For the protein expression analyses, tissues were lysed with RIPA reagent (Beyotime). Proteins were separated on SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Immunoblots were performed with primary LC3A/B antibody (Cell Signaling Technology), Beclin-1 antibody (Abcam), and β-acting antibody (Sangon Biotech). Later the blots were incubated with HRP conjugated goat-anti-rabbit secondary antibody (Abcam). The signals were detected by ChemiLucent ECL Detection system (Millipore). The results of Western blots were analyzed by the Image J program.

Statistical analysis

All the statistical analyses were done with SPSS software (version 19.0, IBM Corporation, IL, USA). Continuous data were presented as mean ± standard deviation (SD); the categorical data were expressed as n or percentage. T-test was used to analyze differences of clinical parameters between groups. The Chi-square test or Fisher's exact test was used to analyze the correlations between protein expression and clinical parameters. $P < 0.05$ indicated a statistically significant difference.

Results

Autophagy-related proteins upregulate in BPH

In order to evaluate expression levels of LC3A/B and Beclin-1 in normal prostate TZ tissues and BPH tissues, we performed IHC and Western blot analysis. The IHC results are presented in **Figure 1A**, in which we can see that expression levels of both LC3A/B and Beclin-1 are significantly stronger in BPH tissues as compared to

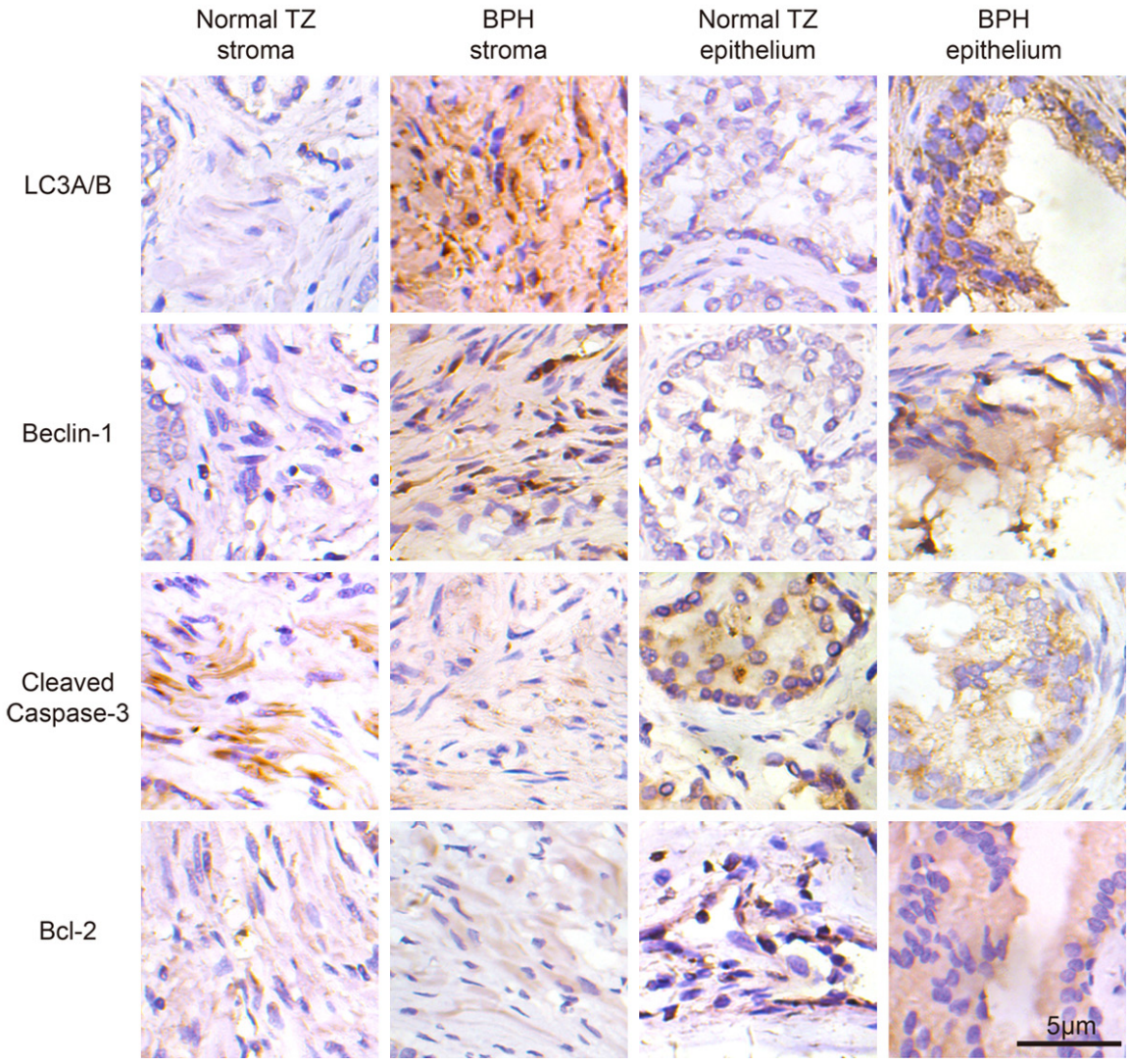

Figure 2. Immunohistochemical staining of autophagy and apoptosis-related proteins in stroma and epithelia of normal prostate TZ tissues and BPH tissues (Scale bar =5 μm).

normal tissues. The results of Western blot further confirmed that enhanced expression of LC3 and Beclin-1 in BPH tissues especially after long duration (≥ 12 months) of 5-ARI usage (**Figure 1B-D**). More importantly, LC3-II/I ratio is notably higher in 5-ARI long duration group compared to that in other groups (**Figure 1C**). These results indicate that autophagy is induced in BPH tissues.

Stromal expression features of autophagy/apoptosis-related proteins in BPH and normal TZ tissue specimens

In order to evaluate expression levels of autophagy-related proteins (LC3A/B, Beclin-1) and apoptosis-related proteins (Cleaved Caspase-3, Bcl-2) in stroma and epithelium between BPH tissues and normal prostate TZ tissues, we scored IHC by the stroma or epithelium compartment separately. As the IHC staining showed in **Figure 2**, protein expression of LC3A/B and Beclin-1 in stromal cells of BPH tissues was significantly higher in comparison to that of the normal TZ group (**Table 2**, LC3A/B $P < 0.001$, Beclin-1 $P=0.045$). Protein expression of Cleaved Caspase-3 in stromal cells of BPH tissues was lower in comparison to that of the normal TZ group (**Table 2**, $P=0.043$). Whereas the staining level of Bcl-2 in stromal cells showed no significant difference between BPH tissues and normal TZ tissues.

Table 2. Comparisons of LC3A/B, Beclin-1, Cleaved Caspase-3 and Bcl-2 proteins expression between BPH tissues and normal TZ tissues. The immunohistochemical staining in stroma and epithelium were compared separately

Items	Normal TZ tissues (n=30)		BPH tissues (n=60)		*P-value*
	Low, n (%)	High, n (%)	Low, n (%)	High, n (%)	
LC3A/B					
Stroma	25 (83.3)	5 (16.7)	13 (21.7)	47 (78.3)	< 0.001
Epithelium	14 (46.7)	16 (53.3)	21 (35.0)	39 (65.0)	0.360
Beclin-1					
Stroma	21 (70.0)	9 (30.0)	28 (46.7)	32 (53.3)	0.045
Epithelium	13 (43.3)	17 (56.7)	9 (15.0)	51 (85.0)	0.005
Cleaved caspase-3					
Stroma	10 (33.3)	20 (66.7)	35 (58.3)	25 (41.7)	0.043
Epithelium	5 (16.7)	25 (83.3)	17 (28.3)	43 (71.7)	0.301
Bcl-2					
Stroma	11 (36.7)	19 (63.3)	15 (25.0)	45 (75.0)	0.325
Epithelium	14 (46.7)	16 (53.3)	28 (46.7)	32 (53.3)	1.000

TZ, transition zone; BPH, benign prostatic hyperplasia.

Epithelial expression features of autophagy/apoptosis-related proteins in BPH and normal TZ tissue specimens

Next, we analyzed expression levels of the proteins in the epithelial compartments of BPH and normal TZ tissue specimens (**Figure 2**). Similarly, LC3A/B, Beclin-1, Cleaved Caspase-3 and Bcl-2 were mainly expressed in the cytoplasm of prostate epithelial cells. The protein expression of Beclin-1 in the epithelia of BPH tissues was significantly higher in comparison to that in the normal TZ group (**Table 2**, *P*= 0.005). However, the expression of LC3A/B, Cleaved Caspase-3 and Bcl-2 in prostate epithelial cells showed no significant difference between the two groups.

Relevance of stromal autophagy/apoptosis-related proteins expression to BPH patients' clinical features

Subsequently, we evaluated the association of autophagy and apoptosis markers expression levels in stromal cells with clinical parameters among BPH patients. It was found that expression level of stromal LC3A/B significantly correlates with clinical parameters such as PV (*P*=0.001), Qmax (*P*=0.016), and PVR (*P*=0.012). More importantly, our data showed that high level expression of LC3A/B was more significant in the stroma of BPH patients after treated with 5-ARI for more than 12 months (*P* < 0.001). Besides, significant correlations were also found between stromal Beclin-1 expression and clinical parameters such as PV (*P*=0.004) and Qmax (*P*=0.001) (**Table 3**). For the apoptosis-related markers expressed in the stroma, statistical analyses indicated that Cleaved Caspase-3 expression level was positively related to PV (*P* < 0.001), PSA (*P*= 0.031), Qmax (*P*=0.006) and PVR (*P*=0.001). Whereas Bcl-2 expression level was only positively related to patients' age (*P*=0.017) (data not shown).

Relevance of epithelial autophagy/apoptosis-related proteins expression to BPH patients' clinical features

Concerning the autophagy-related markers expressed in epithelia of BPH samples, the expression level of LC3A/B was significantly associated with PV (P=0.013), PVR (*P*=0.002) and duration of 5-ARI treatment (*P*=0.001), while the expression level of Beclin-1 was significantly associated with PV (*P*=0.029) and Qmax (*P*=0.024) (data not shown). As for apoptosis-related markers expressed in prostate epithelia, there are significant links between the Cleaved Caspase-3 expression and clinical variables such as PV (*P*=0.046) and PVR (*P* < 0.001), whereas the Bcl-2 expression is only associated with PSA value (*P*=0.007) (data not shown).

Differentiations of clinical parameters between IHC high score group and low score group

Furthermore, we compared the differences of patients' clinical parameters between the high-level cases and low-level cases in the term of autophagy-related proteins LC3A/B and Beclin-1. Our results showed that the PV

Table 3. Relevance of low or high expression levels of autophagy related proteins (LC3A/B and Beclin-1) in prostate stroma to different stratifications of BPH patients' clinical parameters

Clinical parameters	LC3A/B		*P*-value	Bectin-1		*P*-value
	Low, n	High, n		Low, n	High, n	
Age (year)						
< 75 (n=36)	10	26	0.210	15	21	0.342
≥ 75 (n=24)	3	21		13	11	
PV(ml)						
< 50 (n=26)	11	15	0.001	18	8	0.004
≥ 50 (n=34)	2	32		10	24	
PSA (ng/ml)						
≤ 10 (n=38)	9	29	0.751	17	21	0.694
> 10 (n=22)	4	18		11	11	
IPSS						
≤ 19 (n=33)	8	25	0.755	18	15	0.176
≥ 20 (n=27)	5	22		10	17	
Qmax (ml/s)						
≤ 10 (n=41)	5	36	0.016	13	28	0.001
> 10 (n=19)	8	11		15	4	
PVR (ml)						
≤ 50 (n=23)	9	14	0.012	14	9	0.082
> 50 (n=37)	4	33		14	23	
5-ARI (month)						
< 12 (n=13)	8	5	< 0.001	8	5	0.347
≥ 12 (n=47)	5	42		20	27	

PV, prostate volume; PSA, prostate specific antigen; IPSS, international prostate symptom score; Qmax, maximum urinary flow rate; PVR, postvoid residual; 5-ARI, 5α-reductase inhibitor.

was significantly larger in the patients with high level expression of LC3A/B than that in the low-level group ($P < 0.001$). The same result was also noted in the comparison of PVR between the two groups ($P=0.023$). Besides, the 5-ARI treatment duration of LC3A/B high-level group is longer than that of the low-level group ($P=0.001$). Concerning the protein expression of Beclin-1, it was found that the PV, PVR and 5-ARI duration are significantly larger or longer in the high-level group when comparing with that in the low-level group (PV, $P < 0.001$; PVR, $P=0.032$; 5-ARI, $P=0.038$). There were no significant differences in PSA, IPSS, QoL, and Qmax between the two groups concerning the expression of LC3A/B or Beclin-1 (**Table 4**).

Discussion

Since the discovery of autophagy in the 1960s, the knowledge of the autophagic function and the regulation of autophagy exploded largely [17]. It has been suggested that autophagy could affect the cell death procedure responding to various stresses, such as nutrient starvation and hypoxia [18]. Additionally, emerging evidence has shown that autophagy has multiple roles, depending on tissue type, physiological or pathological conditions [4, 19]. Many studies have revealed that autophagy could play an important role in progression of prostate cancer [14, 20, 21], however, the relations between autophagy and BPH have seldom been investigated. In the present study, we found that expression of LC3A/B and Beclin-1 was increased both in stroma and epithelia of BPH tissues in comparison to that of normal prostate TZ tissues, which suggested that autophagy might be involved both in the stromal and epithelial compartments of BPH tissues, Moreover, we analyzed relevance between the expression levels of autophagy-related proteins with clinical features of BPH patients.

It is acknowledged that reduced apoptosis is an apparent feature in development of BPH [22, 23]. In our study, expression of apoptosis-related proteins, Cleaved Caspase-3 and Bcl-2, were also evaluated. Our results showed that expression of Cleaved Caspase-3 was significantly lower in the stromal compartment of BPH tissues, which suggested that apoptosis was inhibited in the stromal cells of BPH. While autophagy-related markers LC3A/B and Beclin-1 are relatively over-expressed in stromal cells of BPH tissues, as well Beclin-1 was found highly expressed in epithelial cells of BPH tissues. Previous studies have suggested that there is a close link between autophagy and apoptosis [18]. Recently, Li *et al*. demonstrated that blockage of autophagy could significantly promoted the apoptosis rate of prostate epithelial cells [16]. Our IHC results may partly support this conclusion. The precise relations between autophagy and apoptosis are still in controversy, while as an age [24] and inflammation [25] related disease that involved in both autophagy and apoptosis, BPH could

Table 4. Comparisons of BPH patients' clinical parameters in the terms of low and high-level (both stroma and epithelium) of autophagy-related proteins

Clinical parameters	LC3A/B, mean ± SD		*P*-value	Beclin-1, mean ± SD		*P*-value
	Low (n=10)	High (n=40)		Low (n=7)	High (n=29)	
PV (ml)	40.27 ± 18.53	89.19 ± 36.64	< 0.001	34.45 ± 7.83	89.76 ± 32.11	< 0.001
PSA(ng/ml)	11.74 ± 10.14	9.79 ± 6.80	0.469	12.42 ± 12.25	8.67 ± 5.33	0.457
IPSS	17.80 ± 3.55	19.40 ± 5.11	0.262	17.86 ± 4.34	18.52 ± 4.84	0.744
QoL	3.70 ± 1.16	4.20 ± 1.18	0.235	4.00 ± 1.15	4.24 ± 1.27	0.650
Qmax (ml/s)	10.17 ± 3.11	9.02 ± 3.54	0.353	10.80 ± 3.12	9.40 ± 3.90	0.384
PVR (ml)	38.33 ± 6.22	110.17 ± 96.25	0.023	36.32 ± 4.45	127.77 ± 106.74	0.032
5-ARI (month)	12.10 ± 9.34	25.30 ± 11.28	0.001	13.57 ± 10.80	23.72 ± 11.21	0.038

SD, standard deviation; PV, prostate volume; PSA, prostate specific antigen; IPSS, international prostate symptom score; QoL, quality of life; Qmax, maximum urinary flow rate; PVR, postvoid residual; 5-ARI, 5α-reductase inhibitor.

provide an appropriate pattern for exploring the interrelations. Thus we deem that reciprocal regulation between autophagy and apoptosis in the process of BPH development needs further investigation.

Hyperplastic prostate tissues are featured not only by over-proliferated epithelial cells but also stromal cells including fibroblasts and myofibroblasts [25, 26]. Hyperplastic stromal cells could occupy more than 70 % of BPH tissues [23]. Besides, stromal-epithelial crosstalk plays essential roles in BPH progression [27-29]. However, autophagy level referring to BPH stroma has not previously been discussed previously. In the present study, we showed that expression of autophagy-related proteins in BPH stromal cells was significantly higher when compared to that in normal TZ group. Importantly, we revealed that autophagy could be induced in stromal cells of BPH patients after treated with 5-ARI for more than 12 months. Zhang *et al*. have demonstrated recently that hypoxia-induced autophagy can promote viability of human prostate stromal cells in vitro [15]. Our results may possibly support this observation, but we believe that autophagy in prostate stromal cells might induced not merely by hypoxia but other factors such as long duration of 5-ARI usage, infection, or senescence, etc.

Theoretically, it may suggest that the therapy of androgen deprivation plus autophagy inhibition may show better effects in the treatment of BPH. Various studies have shown that androgen receptor could play a negative role in regulating autophagy both in prostate cancer and BPH [14, 16, 20, 30]. Li *et al*. have demonstrated that the expression of LC3 protein in benign prostatic epithelial tissues was significantly increased after 5-ARI treatment, due to its effect of androgen ablation [16]. A similar conclusion was also obtained in another study conducted by Liu's team [14]. In accordance with that, we found that protein expression of LC3A/B in both epithelial cells and stromal cells of BPH tissues from patients after a long duration of 5-ARI treatment was up-regulated when compared to the patients with short 5-ARI duration. These data implied that autophagy could be induced after AR ablation.

5-ARI, such as Finasteride or Dutasteride, can inhibit dihydrotestosterone synthesis by targeting 5α-reductase, which use for BPH treatment and can reduce the risk of the disease progression [31-33] {Pirozzi, 2015 #1503}. However, BPH may further progression and most of the patients still need surgical interventions or catheterization in latent state after the failure of medication treatment [34]. Mechanism of BPH progression after a long period 5-ARI treatment remains unclear. Here we found that there is significant correlation between the high expression level of LC3A/B and 5-ARI duration both in the stromal and epithelial cells of BPH tissues. Based on our results, we believe that autophagy may play an essential role in the de novo proliferation of prostate stromal and epithelial cells in BPH progression after receiving long duration of 5-ARI therapy. This finding further suggested that androgen receptor might play a key role in regulating autophagy in the progression of BPH.

In conclusion, our study suggested that expressions of autophagy-related proteins (LC3A/B

and Beclin-1) are up-regulated both in the stroma and epithelium of BPH tissues. Besides, our results indicated that autophagy might be induced in BPH tissues especially in prostate stromal cells after a long duration of 5-ARI treatment. These data may support a novel strategy to target autophagy for therapeutic advantage in suppressing the progression of BPH.

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Disclosure of conflict of interest

None.

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