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# Analysis of the expression of autophagy-related protein in prostate cancer and preliminary investigation of its clinical significance.

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## Abstract

**Objective: To analyze the differences in the expression of Autophagy-related proteins (ATG) in prostate cancer tissues and normal para-carcinoma tissues and to have a preliminary investigation of its clinical significance.**

**Methods: The 90 cases of specimens from the prostate cancer radical operation without the endocrinotherapy from Jan 2013 to Dec 2015 were collected by means of the immunohistochemical staining, the immunohistochemical staining results of the following 8 antibodies in the prostate cancer tissues and normal para-carcinoma tissues were observed: Androgen Receptor (AR), phospho-mammalian Target of Rapamycin (p-mTOR), Beclin-1, Autophagy-related protein5 (ATG5), Autophagy-related protein7 (ATG7), ULK1, p62 and microtubule-associated protein light chain 3B (LC3B). Two pathologists performed the scoring independently according to the same scoring criteria and made a comparative statistical analysis in accordance with the scoring results.**

**Results: The immunohistochemical staining results showed that, the expression of autophagy-related protein p62 (t=2.189, P=0.048), p-mTOR (t=3.956, P<0.001) and AR (t=4.954, P<0.001) in the prostate cancer tissues were significantly up-regulated when compared with the normal para-carcinoma prostate tissues, and the expression of Beclin1 (t=2.855, P=0.005) and ULK1 (t=5.098, P<0.001) was significantly down-regulated when compared with the normal tissues. The difference among ATG5 (t=1.110, P=0.269), ATG7 (t=1.396, P=0.164) and LC3B (t=1.667, P=0.097) in terms of the expression level was of no statistical significance.**

**Conclusion: Cellular autophagy is inhibited in prostate cancer tissues and with the increase in tumor grade and staging, the inhibition degree of cellular autophagy will also escalate, but the specific mechanism is still needed to further investigate.**

**Keywords:** Prostate cancer, Para-carcinoma, Autophagy, Autophagy-related protein, Clinical significance.

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## Introduction

Prostate cancer is one of the common malignant tumors in male urinary system; with its mortality rate ranking number 2 among common male tumors, it has become one of the public concerns worldwide [1]. According to relevant reports, the mortality rate of Chinese male prostate cancer is 2.98/100,000 to 17.69/100,000. Its mortality rate has been on the rise during the recent years and it is one of the urinary malignant tumors that seriously impact male health [2]. Prostate cancer is a non-epithelial malignant tumor. Like most of other non-epithelial malignant tumors, its risk factors and pathogenesis still remain unclear and it is characterized by diversified pathological manifestations, complicated biological behaviours and great differences in prognosis [3,4].

Cellular autophagy, as a degradation process of protein and cell organs mediated by lysosome, participates in the elimination of senile cell organs and damaged proteins, and it is an important mechanism of maintaining the cellular homeostasis [5]. Cellular autophagy plays a dual effect in tumor. Under some circumstances, the tumor cellular autophagy occurs to protect the tumor cells from the damages of external stimulation and further prevent the tumor cells from the impairment of external simulation [6]. In other tumors, the autophagy occurrence promotes the death of tumor cells, and the excessive autophagy of tumor cells can gravely degrade the intracellular important functional proteins and cell organs, resulting in the cell death. As this morphological feature of autophagy related death is significantly different from apoptosis and necrosis, it is defined as type II programmed death [7]. Some studies have revealed that, the dying tumor cells will be accompanied by the high

expression of autophagy during the radiotherapy and chemotherapy [8,9]. The autophagy of tumor cells is a high-profile topic in oncology during the recent years. Currently, lots of scholars have carried out relevant studies on the autophagy of tumor cells, but the autophagy mechanism in prostate cancer tissues and the expression of autophagy-related protein are relatively less studied. Understanding the autophagy status in prostate cancer may lay a basic theoretical foundation to gain an insight on the biology and treatment progress of prostate cancer. Therefore, 90 cases of specimens from the prostate cancer radical operation without the endocrinotherapy from Jan 2013 to Dec 2015 were collected in this study and a comparative analysis was made between the prostate cancer tissues and normal para-carcinoma tissues to find the difference in the expression of autophagy-related proteins for the purpose of further investigating the autophagy in prostate cancer.

## Materials and Methods

### *Materials and devices*

Reagent kits and materials included: Envision two step method kit, pH 8.0 calcium disodium edetate, colour-developing agent diaminobenzidine, AR antibody, universal secondary antibody for rabbits and mice and foetal calf serum; the above reagents and materials were purchased from Guangzhou Genebase Biotechnology Co., Ltd. AR, ATG5, ATG7, Beclin1, LC3B, p62, p-mTOR and ULK1 primary antibodies were purchased from UK abcam. Other reagents and materials such as hydrogen peroxide solution, Harri's haematoxylin solution, alcohol, ammonia water, paraffin, hydrochloric acid, xylene, glass slide, cover glass and paraffin were provided by Clinical Medicine Institute of Hunan Provincial People's Hospital. The main devices included: constant temperature heater (Suzhou Jiangdong Precision Device Co., Ltd.), inverted microscope CKX41 (Japan Olympus), micro sample injector (Germany eppendorf), and Quick-Ray tissue microarrays preparing machine (Korea Unitma).

### *Tissue microarray*

90 cases of specimens from the prostate cancer radical operation without the endocrinotherapy from Jan 2013 to Dec 2015 were collected, the study was approved by the Ethical Committee in our hospital and all the patients were informed and signed the informed consents; the prostate cancer tissue regions and normal para-carcinoma tissue regions that could represent the paraffin specimen tissue characteristics were determined through the HE-stained pathological sections; the pathological resections were placed under the microscope to find the target sampling regions and mark on the paraffin blocks; the 90 cases of paraffin tissues were divided into 10 groups according to the Gleason's score, the tissue microarray was designed, the thickness of each of the 68 sections was set to be 4 μm, add the specific operation methods are similar to those described in the references [10].

### *Immunohistochemical staining and evaluation*

The sections were baked at 70°C for 45 min; the alcohol was removed after the sections were deparaffinated, 3% (volume fraction) $H_2O_2$ was placed, then they were enclosed for 20 min, the sections were placed into the pH 8.0, 1/50 EDTA antigen repair fluid and then they were put into the microwave oven for de-freezing for 20 min to repair the antigen, and the primary antibodies were added for overnight culture at 4°C; EnVision second antibody was added for culture at 37°C for 50 min, DAB was used for color development for 3 min, then counterstaining and differentiation were performed, and finally the sections were sealed. The operation was based on the kit instructions and the specific operation methods were similar to those described in literatures [10,11].

After the preparation of tissue microarray and the immunostaining, associate chief physicians from pathological department finished the scoring independently, both the intensity and quantity of positive staining were taken into consideration, and the specific methods were as follows: 0 point for colorless, 1 point for light yellow, 2 points for brownish yellow, and 3 points for brown. 0 point if there was no positive cell, 1 point if the proportion of positive cells was ≤ 20%, 2 points if the proportion was 20% to 50%, and 3 points if the proportion was ≥ 50% [12] .

### *Statistical analysis*

The experimental results were analyzed according to SPSS19.0 software, the immunohistochemical scoring results were expressed in the form of mean ± standard deviation ($\bar{x} \pm s$), and the t-test was used for the statistical analysis to compare the tumor tissues and normal para-carcinoma tissues to find the difference in expression. The bivariate-related spearman test was used for the statistical analysis to find the inter-antibody connection, the Mann-Whitney U-test of the non-parameter test was used to investigate the relation between the expression difference in autophagy-related protein and the tumor grading and staging, the significance level $\alpha=0.05$ and the two-sided test was used.

## Results

### *Difference of the expression of autophagy-related protein in prostate cancer tissues and normal para-carcinoma tissues*

The tissue microarray staining indicated that, there was no significant difference of the expression of ATG5 and ATG7 in cancer tissues and normal para-carcinoma tissues, LC3B had a slightly lower expression in cancer tissues, but the difference was of no statistical significance. When compared with the normal para-carcinoma tissues, the expression of p62 ($t=2.189$, $P=0.048$), p-mTOR ($t=3.956$, $P<0.001$) and AR ($t=4.954$, $P<0.001$) in prostate cancer tissues increased significantly, showing a statistically significant difference. The staining of Beclin1 ($t=2.855$, $P=0.005$) and ULK1 ($t=5.098$, $P<0.001$) in prostate cancer tissues had a weaker intensity than that in the

normal tissues, showing a statistically significant difference, with details shown in Table 1.

***Table 1.*** *Results of immunohistochemical staining analysis in prostate cancer tissues and para-carcinoma tissues ($\bar{x} \pm s$).*

| Items | Normal | Cancer | t | P |
|---|---|---|---|---|
| ATG5 | 4.85 ± 0.85 | 4.70 ± 0.96 | 1.11 | 0.269 |
| ATG7 | 5.01 ± 0.75 | 4.86 ± 0.69 | 1.396 | 0.164 |
| LC3B | 4.89 ± 0.86 | 5.11 ± 0.91 | 1.667 | 0.097 |
| p62 | 4.80 ± 0.94 | 5.12 ± 1.02 | 2.189 | 0.03 |
| p-mTOR | 4.53 ± 0.68 | 5.08 ± 1.13 | 3.956 | <0.001 |
| AR | 4.38 ± 0.84 | 5.05 ± 0.97 | 4.954 | <0.001 |
| beclin1 | 4.98 ± 1.04 | 4.57 ± 0.88 | 2.855 | 0.005 |
| ULK1 | 5.58 ± 0.52 | 5.12 ± 0.68 | 5.098 | <0.001 |

### *Correlation analysis of autophagy-related protein expression in prostate cancer tissues*

The results of tissue micro assay indicated that, in the prostate cancer tissues, p-mTOR was correlated with the expression of AR (r=0.451, P<0.05) and p62 (r=0.498, P<0.05) and also correlated with the changes in the expression level of LC3B (r=0.627, P<0.05), and it has not been established that the correlation with other autophagy-related proteins is of statistical significance (P>0.05), with details shown in Table 2.

***Table 2.*** *Results of correlation analysis of autophagy-related protein expression in prostate cancer tissues.*

| | P-mTOR | AR | P62 | ATG5 | Beclin1 | ATG7 | ULK1 |
|---|---|---|---|---|---|---|---|
| p-mTOR | - | | | | | | |
| AR | 0.451 | | | | | | |
| | (P<0.05) | - | | | | | |
| P62 | 0.498 | | | | | | |
| | (P<0.05) | 0.156 | | | | | |
| | 0.19 | (P>0.05) | | | | | |
| | (P>0.05) | 0.248 | - | | | | |
| ATG5 | 0.079 | (P>0.05) | | | - | | |
| | (P>0.05) | 0.15 | 0.2 | | | | |
| Beclin1 | 0.088 | (P>0.05) | (P>0.05) | | 0.078 | - | |
| | (P>0.05) | 0.121 | 0.138 | - | (P>0.05) | | |
| | 0.218 | (P>0.05) | (P>0.05) | | 0.097 | 0.116 | - |
| | (P>0.05) | 0.089 | 0.144 | 0.123 | (P>0.05) | (P>0.05) | |
| ATG7 | 0.627 | (P>0.05) | (P>0.05) | (P>0.05) | 0.216 | 0.127 | 0.219 |
| | (P<0.05) | 0.062 | 0.121 | 0.105 | (P>0.05) | (P>0.05) | (P>0.05) |

### *Relationship of difference in autophagy-related protein expression with tumor differentiation and staging*

The results indicated that, p62 (t=5.024, P=0.007) and AR (t=6.531, P=0.002) had a higher expression level in the poorly differentiated cancer than in the highly differentiated cancer, showing a statistically significant difference, and there was no obvious change when compared with other antibodies. During the comparison in the protein expression during the different stages, the statistical analysis indicated: there was a significant difference between the PT2 and PT3 cancers in terms of the expression of p62 (t=2.741, P=0.029), and there was no significant difference in other proteins among the patients with tumors at different stages. In the para-carcinoma tissues, AR (t=2.751, P=0.028) had a higher expression in the PT2 patients than in the PT3 patients, and there was no significant difference in other proteins among the para-carcinoma tissues of different stages, with details shown in Table 3.

***Table 3.*** *Relationship of autophagy-related protein expression with tumor differentiation and staging.*

| Characteristics | P-mTOR | P62 | AR | ATG5 | Beclin1 | ATG7 | ULK1 | LC3B |
|---|---|---|---|---|---|---|---|---|

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Degree of differentiation (carcinoma tissues) | Low | 5.23 ± 0.70 | 5.41 ± 1.15 | 5.50 ± 1.26 | 5.01 ± 1.16 | 4.61 ± 0.99 | 4.79 ± 0.79 | 5.19 ± 0.57 | 5.02 ± 0.93 |
| | Middle | 5.14 ± 0.82 | 5.16 ± 1.01 | 5.22 ± 1.04 | 4.99 ± 1.06 | 4.42 ± 1.08 | 4.96 ± 0.89 | 5.22 ± 0.78 | 4.95 ± 0.87 |
| | High | 5.03 ± 0.75 | 4.9 2 ± 0.94* | 4.93 ± 0.83* | 4.88 ± 0.90 | 4.77 ± 0.91 | 4.99 ± 0.69 | 5.43 ± 0.49 | 4.85 ± 0.85 |
| | F | 1.571 | 5.024 | 6.531 | 0.403 | 2.787 | 1.66 | 3.935 | 0.841 |
| | P | 0.21 | 0.007 | 0.002 | 0.668 | 0.063 | 0.192 | 0.021 | 0.433 |
| PT stage (para-carcinoma tissues) | ≤ PT2 | 5.21 ± 1.09 | 4.95 ± 0.76 | 4.70 ± 0.83 | 4.66 ± 1.06 | 4.24 ± 0.71 | 4.64 ± 0.60 | 4.96 ± 0.75 | 5.32 ± 1.03 |
| | ≥ PT3 | 5.59 ± 1.36 | 6.20 ± 1.32 | 5.14 ± 1.27 | 4.41 ± 1.22 | 4.11 ± 0.52 | 4.51 ± 0.51 | 4.81 ± 0.88 | 5.49 ± 1.21 |
| | t | 2.068 | 2.741 | 2.751 | 1.467 | 1.401 | 1.566 | 1.231 | 1.015 |
| | P | 0.077 | 0.029 | 0.028 | 0.144 | 0.204 | 0.119 | 0.22 | 0.312 |

Note: *compared with low differentiated cancer, P<0.05.

## Discussion

The initiation of autophagy is subject to the regulations of multiple signal pathways, and the phosphoinositide 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) (PI3K-Akt-mTOR) signal pathway plays a vital role in regulating the cellular autophagy [13]. The activity of mTOR complex plays an important role in autophagy; mTOR, called the gate keeper of autophagy, is the most important cell component of down-regulation of autophagy [13]. Therefore, the investigators in this study chose p-mTOR, the active form of mTOR, as an important indicator in detecting the autophagy level. When multiple factors cause an increase in mTOR phosphorylation, autophagy will be inhibited, and the intracellular synthesis effects will increase as a result; vice versa, in case of the de-phosphorylation of mTOR, the autophagy will be up-regulated. The regulation of mTOR on autophagy can be realized through the down-regulation on ATG1, another protein kinase [14,15]. The homologous product of ATG1 in higher eukaryotic cells is ULK1 protein; the increase in mTOR de-phosphorylation caused by external factors will weaken the inhibition on ULK1 and as a result initiate the autophagy. In case of an increase in ULK1 activity, it will act with ATG13 and FIP2000, forming the protein complex, so that multiple proteins can be enrolled to the PSA sites to initiate the assembly of autophagosome [14]. In this study, two proteins, p-mTOR and ULK1, served as the indicators in the initial stage of autophagy detection, and the up-regulation of expression of p-mTOR (P<0.001) in tumor tissues and the down-regulation of expression of ULK1 (P<0.001) in tumor tissues could indicate that the autophagy in the prostate cancer tissues was inhibited from the initial stage.

The formation of autophagosome needs the participation and coordination of multiple cellular components; during the early process, the PI3K kinase complex consisting of phosphatidylinositol 3-phosphate (PI3P), PI3K protein, Vps34, Beclin1 and p150 are relied on during the early process [16]. In addition, the studies on the correlation between the autophagy regulation usibgn the Bcl2-Beclin 1 pathway and the prostate cancer also reveal that Beclin1 plays an important role in the autophagy initial stage, Bcl-2 is located in the cell nuclear membrane, endoplasmic reticulum and mitochondrial outer membrane, and it is considered to be a kind of key protein factor with the dual regulation effects of autophagy and apoptosis [17]. The occurrence of autophagy during the early stage depends on the formation of vacuolar sorting protein 34 (VSP34) and Beclin1 complex [18]. As is shown in literature reports, in the prostate cancer, Bcl-2 can selectively bind with Beclin1 on the endoplasmic reticulum, further hindering the binding between the VSP34 and Beclin1 and inhibiting the occurrence of autophagy [19]. In this study, Beclin1 served as an indicator for the detection of autophagosome formation and the staining analysis was made; Beclin1 (P=0.005) had a down-regulated expression in tumor tissues, indicating that, autophagy was obviously inhibited during the earliest autophagosome forming stage in the tumor tissues.

Due to the specificity of autophagy in substrate selection, we selected p62 for protein detection in this study. During the process of autophagy selecting substrate, the substrate would be recognized by p62 protein, which has a ubiquitin-associated (UBA) domain and a LC3-associated domain and can further specifically transport the ubiquitinated proteins to the autophagosome for degradation [20]. The expression of p62 (P=0.030) was up-regulated in tumor tissues as found in the experiment. The expression level of p62 (P<0.05) was higher in the poorly-differentiated cancer than in the highly-differentiated cancer probably because the autophagy in the prostate cancer tissues with a high malignancy was inhibited to a greater extent. This study also indicated that P62 was also highly expressed in the poorly-differentiated tumors and a tumor of a relatively late stage. The occurrence and development of tumor may be associated with the abnormal stimulation of P62, which is similar to the results of some other studies [3].

Prostate cancer is androgen dependent, and the effect of androgen is mediated by AR. AR is not only expressed in the primary prostate cancer, but also expressed in the prostate cancer patients who fail in the endocrinotherapy and in the patients with recurrent prostate cancer [21,22]. AR plays an important role in the occurrence of autophagy after the prostate cancer castration. Under the same fundamental conditions, we also find that, AR is associated with autophagy. In order to

investigate the specific association between the prostate cancer and autophagy, the investigators of this study chose AR as the specific indicator for the detection of prostate cancer and autophagy. The conclusion of this study indicated that, AR ($P<0.05$) had an up-regulated expression in the prostate cancer tissues when compared with the normal para-carcinoma tissues, and AR ($P<0.05$) had a significantly higher expression in the tumor tissues of poorly-differentiated prostate cancer than that in the tumor tissues of highly-differentiated prostate cancer. The correlation analysis of this study indicated that, the p-mTOR in the cancer tissues had a correlation with the changes in the expression level of AR ($r=0.451$, $P<0.05$), which indicated that AR might play a certain role during the process that the basal autophagy of prostate tumor was inhibited, but specific investigation was needed for the study of specific mechanism.

ATG5, ATG7 and LC3B are proteins involved in the autophagosome formation and the extension of autophagosome membranes to two sides [23]. The comparison of the expression of three proteins in para-carcinoma tissues and tumor tissues indicated no obvious change, indicating that no significant change in the double membrane like structures. Significant up-regulation of expression of p62 that specifically recognized the substrate during the autophagy process indicated that, after the autophagy was inhibited during the two earliest stages, the entire autophagy process had been significantly inhibited, and the autophagy could be down-regulated successfully without the need of down-regulation of the proteins needed during the autophagosome membrane formation [24].

The investigators in this study, by means of the combined method of tissue microarray and immunohistochemistry, preliminarily investigated the difference in the expression of autophagy-related protein in prostate cancer patients and the results indicated that, the differences in autophagy-related protein was somewhat associated with the tumor differentiation and staging and could impact the patient's prognosis. The high-flux large-sample detection of the expression of autophagy-related protein at the protein level effectively avoids the interference of the system error and human factor, the staining intensity and the proportion of positive cells are taken into consideration in the scoring system and relatively accurate scores can be generated, which provide favourable conditions for further studies. However, some errors and deviations may be present due to the limitations in experimental conditions and these results need further confirmation and the clinical significance needs further investigation.

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