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Original Article

Expression of A20 protein in prostate cancer tissues and its relationship with postoperative recurrence and survival

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Abstract: This study aimed to investigate the expression of A20 protein in prostate cancer tissues and its relationship with postoperative recurrence and survival. 86 patients with prostate cancer received surgery were collected as observation group, while 20 cases of benign prostatic hyperplasia were selected as control group in our hospital during June 2012 to June 2015. RT-PCR and immunohistochemistry were used to detect A20 mRNA and protein levels in both prostate cancer tissues and prostatic tissues respectively. Next patients in observation group were divided into A20 positive group and A20 negative group according to A20 gene expression. Finally, we analyzed the association of A20 gene expression and the prognosis of patients with prostate cancer. Compared with normal prostatic tissues, A20 mRNA and protein levels significantly were increased in prostate cancer tissues ($P<0.05$), and there are 59 cases presenting A20 negative expression while 27 cases showing A20 positive expression in 86 cases of observation group. A20 expression rate in positive patients was positively correlated with the degree of tumor differentiation, and negatively correlated with tumor invasion depth, lymph node metastasis and clinical stage. 1-, 2- and 3-year cumulative recurrence rate of patients with A20 negative expression was significantly higher than that of A20 positive patients ($P<0.05$). 1-, 2- and 3- year cumulative survival rate in patients with A20 negative expression was significantly lower than that of A20 positive patients ($P<0.05$). In conclusion, A20 mRNA and protein positive expression in prostate cancer tissues are significant higher than those in control group, which indicates that A20 mRNA and protein expression is closely related to the pathologic features and prognosis of prostate cancer, so it can be an important auxiliary marker for the treatment of prostate cancer.

Keywords: Prostate cancer, A20, recurrence rate, survival rate, prognosis

Introduction

Prostate cancer (PC) is one of the most common carcinomas in urogenital system. In North America and Europe, it possesses the highest incidence in men and exceeded the incidence of lung cancer. Moreover, its incidence in China, Japan, India and other Asian countries is much lower than that in Europe and America, but it appears to be increasing in recent years [1, 2]. In 2007, the Shanghai Municipal Center for Disease Control & Prevention reported that PC's incidence ranks the fifth in male with malignant tumors. Due to the aging of the population, patients with prostate cancer are mainly elderly men, so the incidence of this disease increases year by year, and it has been a serious threat to men's life and health. Due to few symptoms existing in early clinic stage, more than 50-80% patients present advanced PC until diagnosis, so radical surgery of timing was lost [3]. Endocrine therapy is the main method for advanced prostate cancer, and it can defer the progression of prostate cancer, but result in side effects and poor quality of life. However, almost all patients after treatment have gradually developed castration-resistant prostate cancer and has poor prognosis [4, 5]. Therefore, it is essential to elucidate PC's pathogenesis for early diagnosis, early treatment and prognosis

of prostate cancer. A20, as a TNF-inducible primary response gene, is one of important regulatory genes for body's inflammatory response and apoptosis, and its product, a zinc finger protein, has antiapoptotic function and inhibitory effects on NF-κB activity in several cancer cells [6, 7]. Some studies showed that abnormal expression of A20 existed in tumor tissues or cells, and A20-mediated apoptosis might be correlated with patients' prognosis as well as the initiation and development of tumor [8]. Moreover, reports on A20 expression in prostate cancer were few at home and abroad, so this study analyzed the expression of A20 gene in prostate cancer tissues and its relationship with postoperative recurrence and survival, which aimed to provide some guidance for improving the treatment plan of prostate cancer.

Subjects and methods

General data

86 patients with prostate cancer received surgery in our hospital were collected as observation group during June 2012 to June 2015. All patients had not received preoperative treatments for cancer, such as chemotherapy, radiotherapy or other anti-tumor therapy, and the prostatic tissues were surgically removed and collected after diagnosed with PC by histopathological examination. These patients were in the 56-82 age range with the average of 69.78±8.63 years old, and were composed of 39 cases with poorly-differentiation and 47 well-differentiation according to tissue typing, as well as 48 cases in T1-T2 and 38 cases in T3-T4 in accordance with the infiltration. Meanwhile 20 cases of benign prostatic hyperplasia were selected as control group, and these patients were in the 55-80 age range with the average of 67.25±8.18 years old. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Tongji Hospital, Tongji University School of Medicine. Written informed consent was obtained from all participants.

RT-PCR

0.1 g prostatic tissues were drawn from the individuals in the two groups, and total RNA was extracted using total RNA extraction kit (TianGen Bio, Beijing, China). Two kinds of samples were fully homogenized in Trizol. Following addition of 200 μl chloroform, above mixture was well blended with a slight concussion, and after standing for 5 min, it was centrifuged with 12000 rpm for 15 min at 4°C. The colorless supernatant in aqueous phase was collected and placed into RNase-free EP tube (0.5 ml), and following addition of an equivalent volume of isopropanol, the mixture was placed at room temperature for 10 min; then it was centrifuged with 12000 rpm for 15 min at 4°C. Next the supernatant was removed, and the precipitate was washed twice with precooling 75% anhydrous ethanol, and dissolved with an appropriate amount of RNase-free water for obtaining RNA. Finally, the purity and content of RNA were detected and calculated. Then reverse transcription PCR kit (TianGen Bio, Beijing, China) was applied to perform reverse transcription from RNA to cDNA, which was seen as a template for PCR, and the procedures was carried out according to manufacturer's instructions. With β-actin as an internal reference, primers for A20 were designed according to the gene sequences provided by GenBank. A20-F: 5'-CGTTCAGGACACAGACTTGG-3', A20-R: 5'-ATTCCAGTTCCGAGTATCATAGCA-3'; β-actin-F: 5'-CGTGCGTGACATTAAAGAG-3', β-actin-R: 5'-TTGCCGATAGTGATGACCT-3'. Then above primers were diluted into 10 μmol/L. After condition optimization was performed for the annealing temperature and specificity of primers, PCR reaction system with total volume 20 μL was prepared for RT-PCR using PCR amplification instrument (TaKaRa, Dalian, China). And the suitable PCR procedures included one preliminary denaturation at 95°C for 2 min, 35 cycles each involving denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 min. Finally, the specificity of the products was determined, solubility curve was made and the readings on the instrument were recorded for analyzing A20 gene expression in the two groups followed by calculation of the average values.

Immunohistochemical detection

The tissue samples were paraffin-embedded, consecutively cut at 4 μm thickness sections, and then were adhered to the poly-lysine-treated glass slides. Next, above slides were placed in cabinet drier for 2 h, and they were immersed

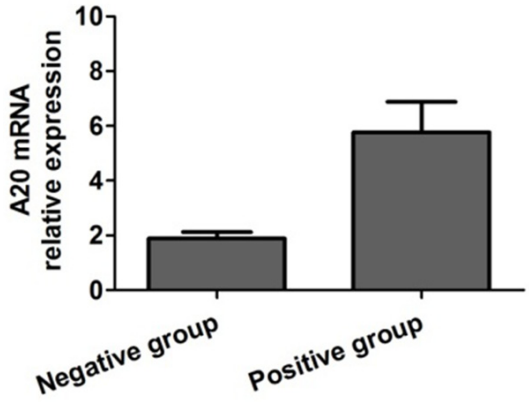

Figure 1. RT-PCR analysis of A20 mRNA relative expression in positive group and negative group.

in Xylene for 5 min/time with a total of 3 times, then accordingly soaked in dehydrated alcohol for 5 min, 95% ethanol for 5 min, 75% ethanol for 5 min, and finally washed with PBS for 3 min/time with a total of 3 times. These slides were then placed in 10 mM sodium citrate buffer (pH 6.0), heated for 10 min, and cooled for 30 min, with two replicates for this procedure. Following a good wash with PBS for 3 min/time with a total of 3 times, the slides were incubated in 3% H_2O_2 solution for 10 min to block endogenous catalase activity at room temperature. Finally, they were rinsed with PBS for 3 min/time with a total of 3 times. After A20 rabbit-anti-human polyclonal antibody (I: 100; Santa Cruz Bio, California, USA) was added, the slides were incubated at 4°C overnight. Then they were washed with PBS for 3 min/time with a total of 3 times. Next 10% goat anti-rabbit IgG secondary antibody (Santa Cruz Bio, California, USA) was added dropwise, the slides were incubated for 1 hour at room temperature. Then the slides were washed with PBS for 3 min/time with a total of 3 times, and diaminobenzidine (DAB) was used as the chromogen; next they were counterstained with hematoxylin and differentiated with 0.1% hydrochloric acid alcohol. Following return to blue in PBS, the slides were thoroughly rinsed with tap water, counterstained with hematoxylin, then gradient dehydrated in ethanol with xylene as clearing medium and mounted by neutral resin.

Observation indexes

Observation under the microscope showed that A20 gene expression mainly existed in the cytoplasm, little in the nucleus, and none in the cell membrane. Therefore, we identified brown-yellow or brown granules appearing in the cytoplasm as positive cells according to immunohistochemical results and then analyzed staining intensity and positive rate of cells. 5 low-power fields in each slide were randomly selected, and 100 targeted cells were counted in each field at high magnification. Finally, average value of positive cells in 5 fields was calculated as the result of the determination. Standards for evaluation: no positive cell was scored 0, positive cells ≤10% was 1 point, 11%-50% was 2 points, 51%-75% was 3 points, and >75% was 4 points. Staining intensity: no staining was evaluated as 0, faint yellow was 1 point, brown was 2 points; deep yellow was 3 points. Above both point were multiplied as the total score (4 points as the demarcation), and total point <4 points was defined as A20 negative expression, while ≥4 points was A20 positive expression. Finally, patients in observation group were divided into positive group and negative group according to the results of immunohistochemical score, and patients' prognosis was evaluated using the recurrence rate and the 1, 2, 3-year survival rates.

Statistical analysis

The data in this study was analyzed by SPSS 17.0 software. Measurement data from multiple groups was compared using analysis of variance and expressed as X±S. Comparison of data between the two groups was analyzed using LSD methods. $P<0.05$ was considered statistically significant.

Results

Comparison of A20 mRNA levels in prostate cancer tissues and prostatic tissues

As shown in **Figure 1**, results from RT-PCR analysis for A20 mRNA level in observation group and control group showed that the average level of A20 mRNA in prostate cancer tissues was significantly higher than that in prostatic tissues, and the difference was statistically significant ($P<0.05$; **Figure 1**).

Comparison of A20 protein level in prostate cancer tissues and prostatic tissues

As shown in **Figure 2**, immunohistochemical results showed that A20 gene expression mainly existed in the cytoplasm, little in the nucleus, and none in the cell membrane. Immuno-

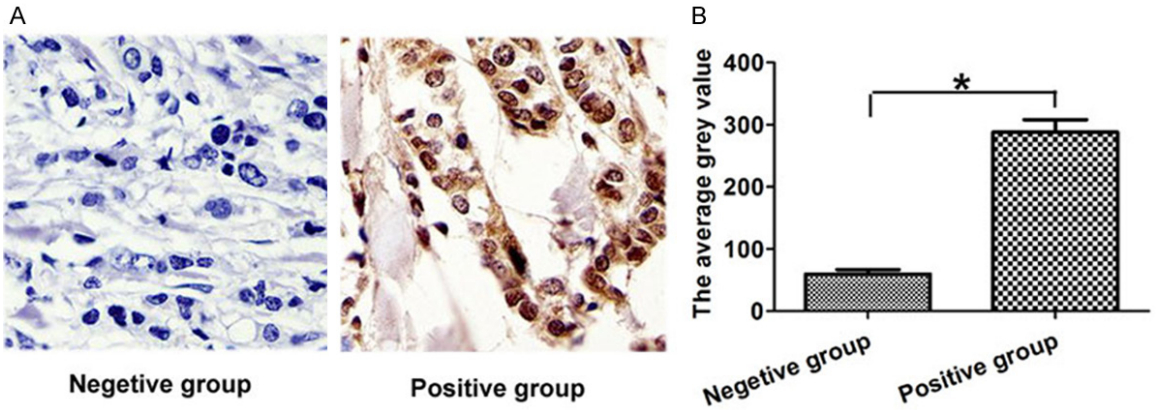

Figure 2. A20 protein levels in positive group and negative group. A. Immunohistochemical analysis of A20 protein levels in positive group and negative group; B. Quantitative analysis of A20 protein levels in positive group and negative group.

Table 1. The correlation of A20 expression with clinical pathological features of patients

Pathological features	Age		Clinical stage		Tissue typing		Lymphatic metastasis		Infiltration	
	≤65 years old	65 years old	II	III	Poor differentiation	Well differentiation	Positive	Negative	T1-T2	T3-T4
Cases (n)	61	25	41	45	46	40	39	47	43	43
Positive cases (n)	42	17	30	29	28	31	24	35	27	32
Proportion (%)	68.9	68.0	73.2	64.4	60.9	77.5	61.5	74.5	62.8	74.4
X^2	0.481		5.212		6.364		5.895		5.654	
P value	0.416		0.056		0.031		0.032		0.041	

histochemical assessment results showed that normal prostatic tissue presented a very low A20 gene expression and even non-expression, while its expression in prostate cancer was significantly up-regulated, and the difference was statistically significant (P<0.05; **Figure 2**). Therefore, according to A20 expression, 83 patients in observation group included 59 patients presenting A20 negative expression and 27 cases appearing A20 positive expression.

Correlation of A20 protein with pathological characteristics of patients

As shown in **Table 1**, the results showed that A20 gene expression was correlated with the degree of tumor differentiation, tumor invasion depth, lymph node metastasis and clinical stage but not with age (P<0.05).

Correlation of A20 gene expression with patients' postoperative recurrence and survival rate

As shown in **Figure 3**, 83 patients in positive and negative group were followed up for more than 36 months after treatment, and a total of 13 patients in negative group relapsed while 16 patients in positive group relapsed. Kaplan-Meier curves showed that 1, 2 and 3-year cumulative recurrence rate of patients with A20 negative expression was 29.63% (8 cases), 37.04% (10 cases) and 48.15% (13 cases), while positive patients' was 10.17% (6 cases), 23.73% (14 cases), and 27.12% (16 cases) respectively. Log-Rank test showed that A20-negative patients had significant higher cumulative recurrence rate than positive patients and the difference was statistically significant (P<0.05). Moreover, survival statistics results showed 1, 2 and 3-year cumulative survival rates of patients with A20 negative expression was 85.19% (23 cases), 70.37% (19 cases), 51.85% (14 cases), while positive patients' was 91.53% (54 cases), 84.75% (50 cases), 71.19% (42 cases) respectively. Log-Rank test showed that A20-negative patients had significant lower cumulative survival rates than positive patients and the difference was statistically significant (P<0.05).

Discussion

Prostate cancer refers to the malignant tumor that occurs in prostatic epithelium, and its inci-

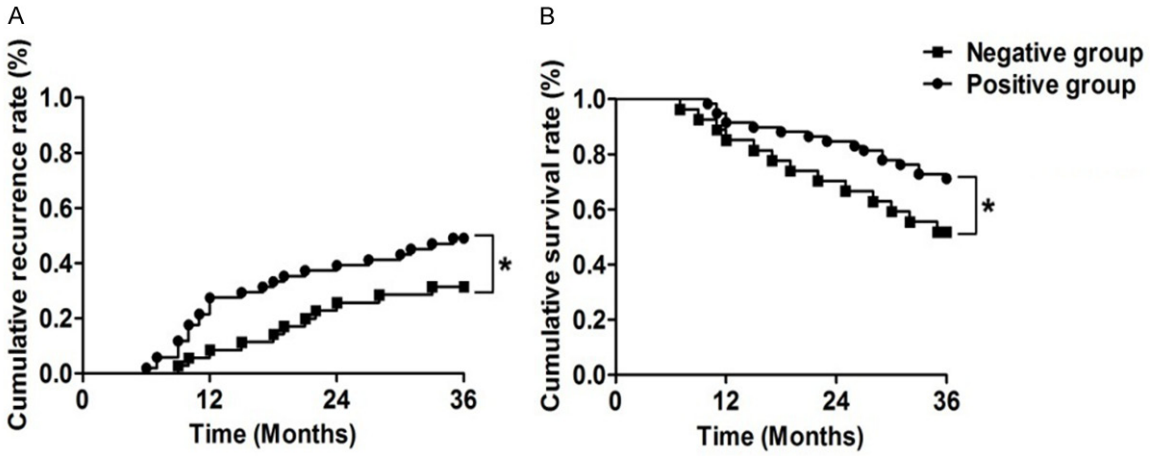

Figure 3. Comparison of 1, 2, 3-year cumulative recurrence and survival rate in negative group and positive group. A. Curves of cumulative recurrence rate; B. Curves of cumulative survival rate. *: P<0.05.

dence ranks the second and the mortality ranks the sixth in all male malignancies [9, 10]. Although the incidence of prostate cancer in China is relatively low, yet with the intensification of the aging of Chinese society, enhancement of national health-conscious and improvement on tumor detection technology, accompanied by the changes in people's living environment and habits, its incidence has increased year by year and ranked the third in male urinary tract tumors. Therefore, in recent years, studies on the molecular mechanisms, clinical diagnosis, treatment and prognosis of prostate cancer have become one of the key research areas of urology [11].

Many domestic and foreign research data showed that chronic inflammation played an important role in the development and progression of a variety of malignancies, among which included prostate cancer. Nuclear factor-kappaB (NF-κB) is considered to be an important regulator of inflammation and immune responses. Studies confirmed that NF-κB activation was in an abnormal state and inhibition of the activity of NF-κB could suppress the progression of multiple tumors [12, 13]. A20, as a TNF-inducible primary response gene, was found in human umbilical vein endothelial cell for the first time. A20 was supposed to have anti-apoptotic function, and its stable expression can inhibit TNF-induced apoptosis in many cell lines, thus achieving cytoprotective effect [14-16]. Studies also showed that inflammation was closely related to tumor development, progression, invasion and metastasis. Meanwhile, A20 is also a inflammatory response gene, and its overexpression can directly regulate the expression of NF-κB in negative feedback, which will limit the inflammatory reaction to a certain extent. Therefore, A20 gene is a typical inhibitor of NF-κB signaling pathway, and it can inhibit TNF-α-activated NF-κB signaling pathway using A20 bifunctional ubiquitin editing enzyme [17-23].

This study was carried out to detect A20 gene expression in prostate cancer and prostatic tissues and the results showed that A20 mRNA and its protein levels in patient with prostate cancer were significantly higher than those in patients with benign prostatic hyperplasia, which indicated that signaling pathways of A20 gene in prostate cancer was in active state, which resulted in the increased levels of A20 protein. In addition, the study also analyzed the correlation of A20 gene expression and the degree of tumor differentiation, tumor invasion depth, lymph node metastasis and clinical stage, and the results showed that A20 expression rate in positive patients was positively correlated with the degree of tumor differentiation, and negatively correlated with tumor invasion depth, lymph node metastasis and clinical stage. Meanwhile, 1, 2 and 3-year cumulative recurrence rate of patients with A20 negative expression was significantly higher than positive patients, and negative patients' cumulative survival rate was significantly lower than positive patients.

In conclusion, A20 gene expression is up-regulated in prostate cancer tissues and is closely related to the clinicopathological features and prognosis, which can be considered as a reference for clinical diagnosis and treatment plan of patients with prostate cancer.

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Disclosure of conflict of interest

None.

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