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Original Article

Studying the mRNA and protein expression pattern of apoptosis and autophagy-related genes in renal cell carcinoma

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Abstract: Objective: To explore the function of autophagy and apoptosis genes (Bax, Bcl-2, Caspase-3, Caspase-8, Caspase-9, ATG2b, ATG3, ATG4a and ATG4b) in renal cell carcinoma. Methods: we analyzed the autophagy and apoptosis-related proteins expressions in human renal cell carcinomafrom the human protein atlas, applied the RT-PCR and WB to detect the mRNA and protein expressions of autophagy and apoptosis-related genes. Results: The immunohistochemistry atlas showed the autophagy and apoptosis genes all appeared an obvious change, the mRNAand protein expressions of autophagy and apoptosis genes including Bax, Caspase 3, Caspase 8, Caspase 9, ATG2b and ATG3 significantly increased, while the mRNA and protein expressions of autophagy and apoptosis genes including Bcl-2, ATG4a and ATG4b significantly decreased. Conclusion: In summary, there is significant difference in the expression of autophagy and apoptosis-related genes, indicating the participation of apoptosis and autophagy in renal cell carcinoma.

Keywords: Autophagy, apoptosis, renal cell carcinoma

Introduction

Renal cell carcinoma is the most common kidney cancer, accounting for 90% of all kidney cancers [1]. The incidence of renal cell carcinoma in men and women is about 1.5:1, and the peak age is about 60-70 years old [2]. Etiology researchers believe that the incidence of renal cell carcinoma is related with smoking, obesity and hypertension [3].

Autophagy is an intracellular process captured by autophagos and transported to lysosomal degradation processes [4]. Autophagy is often activated by cell starvation and provides an architectural module needed to synthesize macromolecules by endogenous methods and maintain cell and mammalian activity [5]. In the case of nutrient uptake, autophagy can maintain the ability of cell biosynthesis. Since energy loss caused by cell damage can also activate autophagy, it can interfere with the accumulation of organellesto maintain cell survival. Cell autophagy caused by intracellular waste removal and recycling can also reduce oxidative stress caused by cell damage and can improve the ability of cells to stimulate the regulation. Neonatal insufficiency caused by hypoxia, lack of nutrition and lack of growth factors due to the stress in the tumor is very common. Autophagy can be activated in cells of the hypoxic region to support tumor growth [6]. Although autophagy is important for normal cells, it can also be used as a target for tumor therapy.

Autophagy is a very complex process that invovles a variety of genes and proteins in the strict control. We referred to these genes collectively as the autophagy-related gene (ATG) family [7]. Autophagy activation depends on the regulation of ATG family. ATG3 is an important regulatory gene for ATG8 binding system, in which the main role of E2-homologous enzyme is involved in the regulation of autophagy [8]. ATG4B is an important molecule to regulate the level of autophagy in mammalian cells via cutting autophagy light chain tubulin precursor forma-

Table 1. Primer pairs of genes

Gene	Primer pairs	
Bax	FORWARD	GCCTCTGGATCTTCACTTGG
	REVERSE	GTCTGGGCATAAGTGCCAAT
Bcl-2	FORWARD	CTGGTCCAAGAGGATTTCCA
	REVERSE	TCATTGCCTTGCACGTAGAG
Caspase-3	FORWARD	AATTGCCTCCACACCTTCAC
	REVERSE	TCACCAAGCTGCTCATCAAC
Caspase-8	FORWARD	AGACCAGTCCTGTGGCTGAT
	REVERSE	GCGGTCTTTGACGTAGGAAG
Caspase-9	FORWARD	AAAGCCCCATCATTCTCCTT
	REVERSE	CACCAGACTCGGCACAATC
ATG2b	FORWARD	TGCCACAACGAGAAGAATGA
	REVERSE	TGCTCCCAGATGAAGGTGAT
ATG3	FORWARD	GCATAGACCTGCTCATCAAGC
	REVERSE	TTCCGTTCCACTCCTTTTTG
ATG4a	FORWARD	GTTCCTCCAGTCCGAGAGT
	REVERSE	CGTGAGAAGGTCCGAGTT
ATG4b	FORWARD	CAGAGGAAGAAGGGACACCA
	REVERSE	TTGTATTGCCCCGTGCTAGT

tion of free cytoplasmic LC3 [9]. ATG4B can also be cleaved by LC3-II to be esterified, and ATG4B significantly affects cell autophagy by regulation of the LC3 system [10]. In addition, study also found that ATG4A, ATG4B, ATG4C had different cleavage activity from ATG8 homologues, and ATG4B has the best activity followed by ATG4A and ATG4C [11].

Apoptosis is autonomously ordered by gene-controlled cell death, and apoptosis is closely related to the occurrence of malignant tumors [12]. The activation of Caspase is an important part of the apoptosis process [13]. Caspase family is divided into two categories, one category is for the implementation of apoptosis. A example of such category is caspase 3, which directly degrade intracellular, functional proteins and cause apoptosis. However, it can't be activated by self-catalyzation or self-editing [14]. The other category is the promoter including caspase 8 and 9. They can cause caspase cascade reaction through the self-editing activation after receiving the signal. Caspase 3, is a key protease in mammalian cell apoptosis and can directly cleave a number of important structural and functional proteins as the ultimate executor of apoptotic death [15].

In this study, we analyzed the mRNA and protein levels of autophagy and apoptosis-related genes to explore the function of autophagy and apoptosis genes in renal cell carcinoma.

Materials and methods

Tissue samples

Tissue samples were collected from 20 patients with renal cell carcinoma as a test set. In each case, natural death human renal tissue was included as a control. All of the patients were given written informed consentand the study was approved by the Ethics Committee.

Analysis of autophagy and apoptosis related-protein expression in human renal cell carcinoma

Autophagy and apoptosis related genes protein expression in renal cell carcinomas and normal tissues was determined from the human protein atlas (www.proteinatlas.org).

Real-time RT-PCR

The total RNA was reverse transcribed (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. The concentration and purity of the total RNA were determined using spectrophotometer at 260/280 nm. The complementary DNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, MA, USA) following the manufacturer's protocol. Gene expression levels were measured by performing RT-PCR using Light Cycler ® 480 System (Roche, Basel, Swit) and Fast Universal SYBR Green Master (Roche, Basel, Swit). After normalization with reference to expression of GAPDH, the relative expression levels of hsa-miR-3613-3p and core genes were calculated by the $2^{-\Delta\Delta Ct}$ methods. The primers were showed in **Table 1**.

Western blot analysis

Protein extracts were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions on 15% gels. Separated proteins were then transferred to nitrocellulose membranes using tank transfer for 1.5 h. The membranes were blocked with 5% skim milk for 18-24 h and incubated overnight at 4°C with diluted primary antibody, followed by a horseradish peroxidase (HRP) conjugated secondary antibody against rabbit IgG (1:2000, Santa

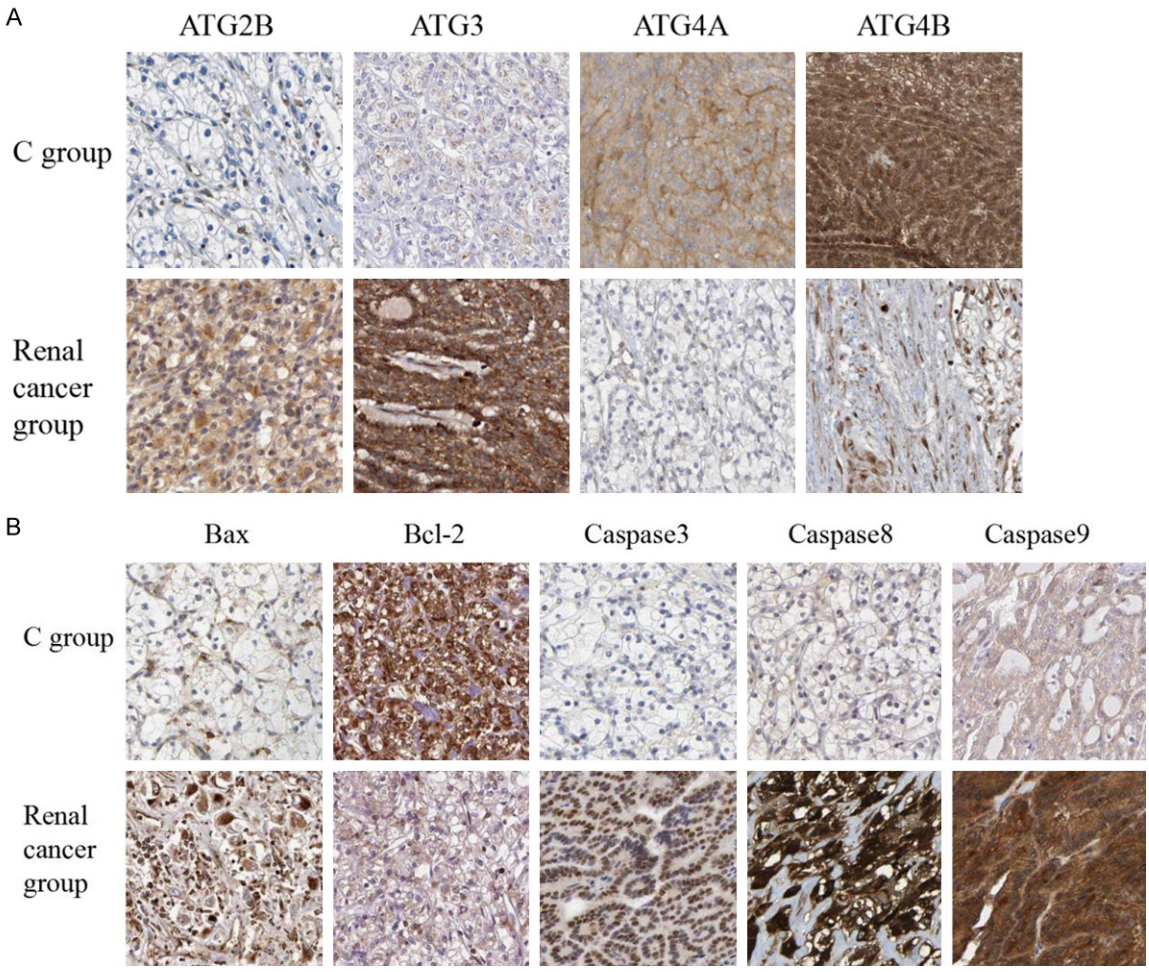

Figure 1. A. Immunohistochemistry of autophagy related genes including normal and cancer tissues (200×). B. Immunohistochemistry of apoptosis related genes including normal and cancer tissues (200×).

Cruz Biotechnology, USA). The signal was detected using an enhanced chemiluminescence system (Cheml Scope5300, Clinx Science Instruments, Shanghai, China).

Statistical analysis

All statistical parameters were calculated using GraphPad Prism 7.0 software. Values are expressed as the mean ± S.D. Comparisons of two groups were performed using Student's t-tests. $P < 0.05$ was considered statistically significant.

Results

Analysis of autophagy and apoptosis-related protein expression in human renal cell carcinoma

To determine the protein expressions of autophagy and apoptosis genes, we first analyzed the protein expression in clinical specimens from the human protein atlas. We found that Bax, Caspase 3. Caspase 8, Caspase 9, ATG2b and ATG3 had the strong expression in renal cell carcinoma tissues, and weak expression in normal tissues. Bcl-2, ATG4a and ATG4b had weak expression in renal cell carcinoma tissues, and strong expression in normal tissues (**Figure 1A, 1B**).

The expression of autophagy and apoptosis mRNAs in tissues

The mRNA expressions of apoptosis related genes wereshowed in **Figure 2A**. As the results showed, the mRNA expressions of Bax increased to 144% compared to the control group (C group) ($P < 0.05$), the mRNA expressions of Bcl-2 decreased to 78% compared to the C group ($P < 0.05$), the mRNA expressions of Caspase 3 increased to 139% compared to

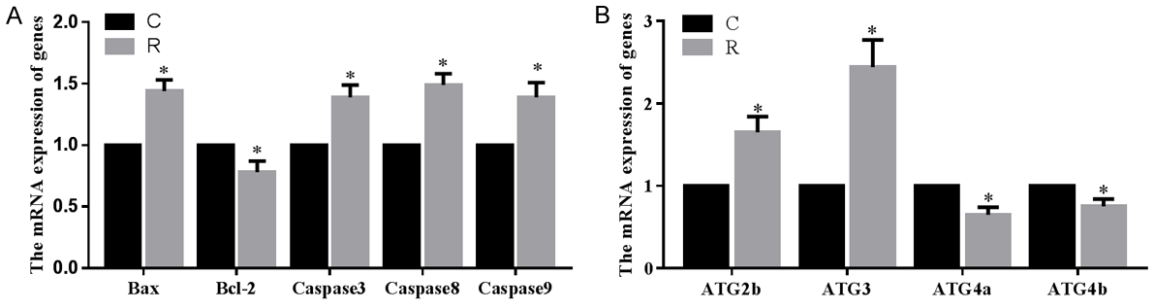

Figure 2. A. The mRNA expression of apoptosis related mRNAs in tissue.*P < 0.05. B. The mRNA expression of autophagy-related mRNAs in tissue. *P < 0.05.

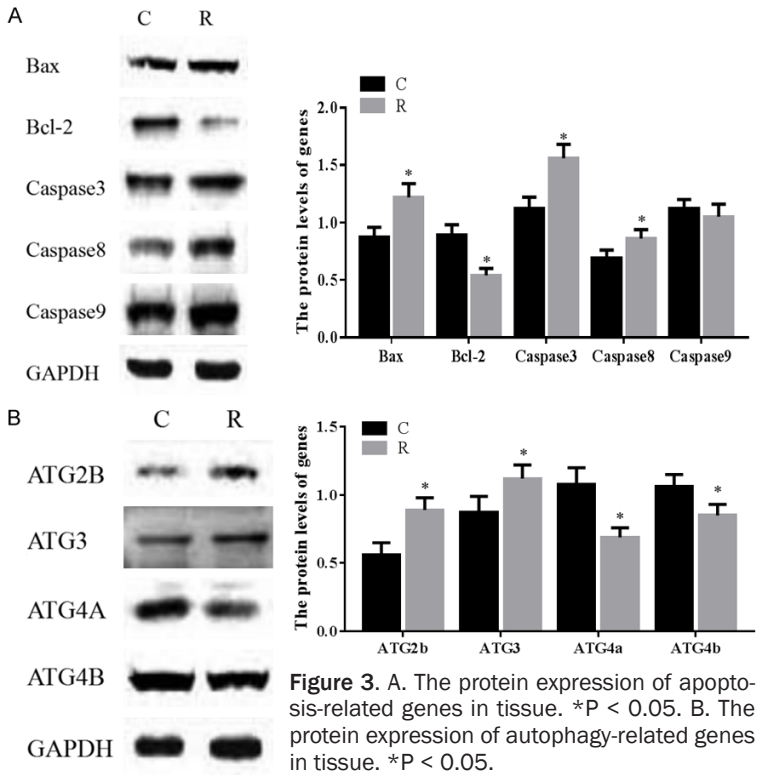

Figure 3. A. The protein expression of apoptosis-related genes in tissue. *P < 0.05. B. The protein expression of autophagy-related genes in tissue. *P < 0.05.

the C group ($P < 0.05$), the mRNA expressions of Caspase 8 increased to 149% compared to the C group ($P < 0.05$), the mRNA expressions of Caspase 9 increased to 149% compared to the C group ($P < 0.05$).

The mRNA expressions of autophagy related genes wereshowed in **Figure 2B**. As the results showed, the mRNA expressions of ATG2b increased to 165% compared to the C group, the mRNA expressions of ATG3 increased to 244% compared to the C group ($P < 0.05$), the mRNA expressions of ATG4a decreased to 65% compared to the C group ($P < 0.05$), the mRNA expressions of ATG4b decreased to 75% compared to the C group ($P < 0.05$).

The expression of autophagy and apoptosis-related proteins in tissues

The protein levels of apoptosis related genes were showed in **Figure 3A**. As the results showed, the protein levels of Bax, Bcl-2, Caspase 3 and Caspase 8 increased significantly compared to control group (all $P < 0.05$).

The protein levels of autophagyrelated genes were showed in **Figure 3B**. As the results showed, the protein levels of ATG2b and ATG3 significantly increased while the level of ATG4a and ATG4b decreased significantly compare to control group (all $P < 0.05$).

Discussion

In renal cell carcinoma, autophagy level was negatively correlated with tumor stage and grade, suggesting that autophagy can induce cell death [16]. Several issues still need further investigation: Does autophagy play a key role in the development and progression of renal cell carcinoma? What is the relationship between autophagy and apoptosis? Does autophagy dominates the death of renal cell carcinoma? What is the effect of autophagy on the proliferation of renal cell carcinoma? Based on this, the choosing an effective and specific target for

the regulation of renal cell death is the purpose of this study.

Autophagy is a cellular defense process that respond to altered environmental stimuli [17]. Autophagy is divided into microautophagy, macroautophagy and molecular chaperone-mediated autophagy [18]. When macroautophagy occurs, autophagosomes participate in the entire autophagy process as an important biomarker. Macroautophagy is a conservative bulk protein degradation pathway responsible for the turnover of related proteins, dealing with excessive or damaged organelles and removing aggregated tendencies [19].

Autophagy is the process of degrading organism or macromolecule material by using lysosomes under the regulation of ATG. Since the ATG are the main components of the autophagy process and the regulator, any change of ATG in the autophagosome formation process will lead to autophagic formation disorder. ATG and other cofactors can run through the autophagy process and participate in a variety of cancer and neurodegenerative diseases such as the development process [20].

In the case of physiology, autophagy is maintained at the basal level and can respond quickly to various stimuli and extracellular signals [21]. More and more evidence proved that ATGs play a very important role in toxicology research. The protein cysteine residue can increase the autophagosome formation by encoding the necessary ATGs such as ATG4. Scott et al. found that cell death happened when overexpressed the ATG1 in cells [22]. In this study, the mRNA and protein levels of autophagy-related genes significantly changed, which also indicate the important role of autophagy related genes in renal cell carcinoma.

Apoptosis and necrotic cell suicide are different, the process by a variety of gene regulation, involving a series of gene changes. Caspase family is a key component in the process of apoptosis and activation or abnormal expression of caspase can cause the occurrence of apoptosis. The proliferation of tumor cells in renal cell carcinoma is related to the decrease of tumor apoptosis, tumor cells down-regulate the apoptosis genes through complex mechanisms, promote the malignant proliferation of tumor cells in renal cell carcinoma. Apoptosis can be induced by activating the order of caspase family. Caspase family is a key enzyme downstream of apoptosis. Caspase 3 is an important member of the family and apoptotic effector molecule [23]. Most of the factors that trigger apoptosis through the Caspase 3-mediated signal transduction pathway lead to apoptosis. Meanwhile, in this study, the expression of caspase 3 and caspase 9 significantly increased. This also indicated apoptosis happened in the renal cell carcinoma process.

Caspase family is widely present in the body cells, mainly in the form of zymogen. In the stimulation of the apoptotic signal, caspase can be activated. Caspase 9 is located in the upstream of the caspase cascade reaction; it can be self-activated with the participation of other protein cofactors and activate downstream Caspase 3, initiate Caspase cascade reaction and induce apoptosis. Caspase 3 is the most important member of the caspase family, and most of the factors that trigger apoptosis will eventually lead to the occurrence of apoptosis by Caspase 3-mediated signal transduction pathways [24]. The expression of caspase 3 and caspase 9 decreased also indicate the importance of apoptosis in the occurrence and development of renal cell carcinoma. Thus, further reveal the mechanism of apoptosis in renal cell carcinoma have a certain clinical significance.

In summary, there is significant difference in the expression of autophagy and apoptosis-related genes, indicating the participation of apoptosis and autophagy in renal cell carcinoma.

Disclosure of conflict of interest

None.

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