Autophagy suppresses the proliferation of renal carcinoma cell

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Abstract. – OBJECTIVE: To investigate the effects of autophagy on the proliferation of renal carcinoma (RCCs).
MATERIALS AND METHODS: Autophagy-related protein 7 (Atg7)-overexpressing and knockdown RCC cell lines were established using lentiviral transfection and shRNA interference, respectively. (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) was used to determine the Cell growth rate, and western blot was used to determine the expression of protein. In order to establish xenograft animal models, stable transfected cells were injected into nude mice. After that those mice were used to detect the effect of autophagy on the growth of RCC in vivo.
RESULTS: Atg7 overexpression could up-regulate the level of LC3II in RCC cell lines, while Atg7-knockdown suppressed the expression of light chain 3 II (LC3II) in RCC cell lines. Atg7-overexpression cells exhibited a decreased growth profile, while suppressing the expression of Atg7 could accelerate the growth of RCC formed tumors.
CONCLUSIONS: Our data indicated that autophagy could suppress the growth of RCC cells in vivo and in vitro, and autophagy appeared to be a new therapeutic target for treating advanced renal cell carcinoma.

Key Words: Autophagy, Renal carcinoma, Proliferation, Atg7, Tumor.

Introduction

Renal cell carcinoma, (RCC), appears to be the most common type of malignant kidney tumors. Renal cell carcinoma is derived from renal tubular epithelial cell. Renal clear cell carcinoma (ccRCC) is reported to be the most type of RCC. Radical nephrectomy is an effective method for the treatment of early stage ccRCC, but 30% of cases treated with radical nephrectomy will continue to progress1, which in turn increased the incidence of advanced renal cell carcinoma. ccRCCs are insensitive to the standard cancer therapies (chemotherapy and radiotherapy), which means that ccRCC are intractable. The treatment efficiency of advanced renal cell carcinoma is only about 10%2. Exploring new mechanism for proliferation of ccRCC is of great significance in treatment of ccRCC. Autophagy, which a process involved in cell survival or death, is different from apoptosis.

The homeostasis of eukaryotic cells makes autophagy a complicated procedure. With the appearance of stimuli-responses (lack of oxygen or food), defective organelles will be separated from cytoplasm. Then, the separated defective organelles will be encircled by an autophagosome, which is a double-membrane vesicle3,4. Autophagosomes combine with lysosomes to form autolysosomes, which results in a degradation by hydrolases5. Various genes can participate in autophagy named, so they were called Autophagy-Related Genes (ATGs). The formation of an isolated membrane is started by the ULK1 complex (which is the mammalian homologue of the yeast gene ATG1)6. And the nucleation as well as assembly of the initial membrane depend on the complex formed by Beclin1 (ATG6 in yeast) and a family of class III phosphatidylinositol 3-kinases (PI3Ks) in the trans-Golgi network (TGN)7,8. Two systems are required to use ubiquitin-like conjugation for amplification and blocking of autophagosomes: one is ATG5-ATG12 complex, and the other is is microtubule-associated protein light chain 3 (ATG8 referred to the yeast homologue; and MAP-LC3 here alluded to LC3). The ATG5-ATG12 complex acts as an essential part in...
stimulation and localization of LC3 double membrane in the conjugation reaction\textsuperscript{9}. In the ATG4 dependent course, Glycine residues at the c-terminal region of LC3 is exposed, then LC3 I will be processed by ATG7 and ATG3 to produce LC3 II\textsuperscript{10}. LC3 II can be found on inner and outer surface of the growing autophagosomal membrane, which made LC3 II different from other ATG proteins. The LC3 II on inner surface is measurable when the content of the autolysosome is degraded\textsuperscript{10}. LC3 II and Beclin1 are commonly used markers for the ongoing autophagy\textsuperscript{11}. Autophagy has paradoxical effect. During adverse stimuli, cells can degrade some pathogens and degenerate or damage organelles by autophagy to recycle energy so as to escape from apoptosis or necrosis\textsuperscript{12}. On the other hand, the excessive activation of autophagy in cells leads to clearance of essential ingredients, which causes cell death, another form of programmed cell death is apoptosis\textsuperscript{13}. Autophagy plays various roles in various types of tumors in different phases\textsuperscript{14}. In our former study, we found autophagy was suppressed in ccRCCs and low-level autophagy was associated with the high-level of ccRCCs, suggesting that autophagy defects might be very important in the proliferation of ccRCC. However, not many studies on the effect of autophagy in proliferation of ccRCC have been reported. This study, which aimed to verify the role of autophagy in proliferation of ccRCC, is a continuation of our former study.

\textbf{Materials and Methods}

\section*{Agent}
Roswell Park Memorial Institute-1640 medium (RPMI-1640) and Dulbecco’s Modified Eagle Medium (DMEM), and fetal bovine serum (FBS) were from Hyclone (Logan, UT, USA) and from Transgen (Beijing, China), respectively. Antibody against human Atg7 was purchased from Epitomics (Burlingame, USA), LC3 was obtained from Sigma-Aldrich (St. Louis, MO, USA), and \textbeta;-actin was purchased from TransGen (Beijing, China). The study was approved by the Ethics Committee of Second Affiliated Hospital, School of Medicine, Xi’an Jiaotong University.

\section*{Cell Lines and Culture}
786-O, 769-P, OS-RC-2, ACHN human RCC cell lines and HK-2 human renal tubular epithelial cells were obtained from Committee on Type Culture Collection of Chinese Academy of Sciences. All cell lines were kept in high glucose DMEM or RPMI-1640 with the supplementation of 10% heat inactivated fetal calf serum, 100 units/mL penicillin, as well as 100 mg/mL streptomycin. The cell lines were incubated in a humidified incubator (37\degree C, 5\% CO\textsubscript{2}).

\section*{Establishment of Stable Atg7-overexpressing, Atg7-knockdown Cells}
Autophagy related gene Atg7 was cloned into pLenti6.3-MCS-IRES-GFP vector (Invitrogen, Carlsbad, CA, USA) to establish stable Atg7-overexpression cell. Empty vector control was also used as a control. Sequence of Atg7 shRNA was 5’-CCGGAAGGAGTCACAGCTCTTTCTCTTTCTTTTGG-3’. Atg7 shRNA was cloned into the pLVshRNA-mCherry(2A)-puro vector (Inovogen, Beijing, China). Based on the manufacturer’s protocol, we used Lipofectamine 2000 reagent (Invitrogen) to perform transfection. To obtain stable transfecants, we selected transfected cells in the whole growth medium that contains 4.0 µg/mL Blasticidin (Invitrogen, Carlsbad, CA, USA) or 2.0 µg/mL Puromycin (Invitrogen, Beijing, China). We verified all clones we chose by Western blot and fluorescence microscope was used for observation.

\section*{MTT Assay}
3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation experiment was applied to determine cell growth rate. We seeded the cells in 96-well plates in the medium contained C/D FBS and the MTT (0.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added. Then the cells were incubated for 4 h, and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystalloid. We used the Microplate Autoreader (Bio-Tek Instruments, Winooski, VT, USA) to measure the absorbance (O.D.) at 590 nm. Independent experiments were repeated in triplicate.

\section*{Western Blot}
We used radioimmunoprecipitation assay (RIPA) buffer to prepare the whole cell lysate in order to prevent the protein from inhibition. We separated equal quantities of lysates (30 µg) by using 12\% SDS-PAGE and then we transferred lysates to a nitrocellulose membrane. The membrane was blocked with 5\% skim milk for 1 h in Tris-buffered saline (TBS) under room tem-
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Autophagy suppression of renal carcinoma cell proliferation, the membrane was then cultured with primary antibodies under 4°C. After washing with Tris-buffered saline-tween 20 (TBST) the membrane was then cultured with secondary antibody along with horseradish peroxidase under room temperature for 1 h, and the protein bands were visualized using the ECL reagents (Millipore, Billerica, MA, USA). β-actin served as endogenous control.

**Xenograft Animal Model**

*In vivo* study was conducted in athymic BALB/c nude mice (2-3 weeks old) to determine the tumor transplantation and growth rate of the tumor. EC xenografts were included by subcutaneous injection of 200 µL cell suspension (5×10^6) cultured in serum-free RPMI 1640 or DMEM with the supplementation of Matrigel (1:1, v/v; at BD Biosciences, Franklin Lakes, NJ, USA) into right flank of mice. After injection, mice having tumors were observed and sacrificed after 4 weeks. After measuring the tumor volume, primary tumors tissues were collected, and were then fixed with 4% paraformaldehyde, followed by paraffin blots, and the tumor tissues were finally stained with haematoxylin and eosin (HE).

**Statistical Analysis**

SPSS software version 13.0 (Version X; IBM, Armonk, NY, USA) was used for all data analyses. *p* < 0.05 was considered to be statistically significant.

**Results**

**Levels of Autophagy in RCC Cell Lines**

The protein levels of Atg7 and LC3II in human RCC cell lines including 786-O, 769-P, OS-RC-2, ACHN as well as HK-2 human renal tubular epithelial cell line were detected by western blot (Figure 1). We found that levels of Atg7 and LC3II in all RCC cell lines were lower than those in HK-2 cell line, which suggested that autophagy in RCC cells was suppressed.

**Establishment of Atg7-overexpression and -knockdown RCC Cell Lines**

According to the level of autophagy, we up-regulated the expression of Atg7 gene in OS-RC-2 cell line and down-regulated it in 786-O cell line. To establish Atg7-overexpression in OS-RC-2 cells, we transfected Atg7-expression carrier and control carrier into OS-RC-2 cells. Then, we used Blasticidin (4.0 µg/mL) to select target cells (Blasticidin-resistant cells) for 4 weeks. Western blot was used to determine the expression of Atg7. The target cells which were the ones with over-expression of Atg7 were named OS-RC-2/Atg7 and the control cells in blank transfection were named OS-RC-2/Con (Figure 2). We established Atg7-knockdown in 786-O cells with the similar method. The cells with decreased expression of Atg7 were named 786-O/sh-Atg7 and the control cells in blank transfection were named 786-O/sh-Con (Figure 2). All transfected cell lines were observed by fluorescence microscopy to examine the transfection efficiency (Figure 3).

**Regulating Atg7 Could Affect the Level of Autophagy in RCC Cell Lines**

To detect the level of autophagy after regulating Atg7, Western blot was performed to de-

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tect LC3II protein levels in all cell lines. LC3II protein level in OS-RC-2/Atg7 was significantly higher than that in OS-RC-2, while LC3II protein level in 786-O/sh-Atg7 was lower than that in 786-O (Figure 2). The results suggested that regulating Atg7 could affect the level of autophagy in RCC cell lines.

**Autophagy Suppressed in vivo as Well as in vitro Growth of the RCC Cells**

As shown in Figure 4A, OS-RC-2/Atg7 cells exhibited a decreased growth profile compared to the parental OS-RC-2 or control OS-RC-2/Con cells which displayed no significant growth over 72 hours. In contrast, 786-O/sh-Atg7 cells grew faster than parental 786-O or control 786-O/sh-Con cells, which had no significant difference from each other in growth course of over 72 hours.

Moreover, we also detected tumor formed by these cells and in vivo tumor growth in animal model. Data suggested that abnormal autophagy expression decreased incidence of tumor and slowed down tumor growth in OS-RC-2/Atg7 cells. In contrast, parental OS-RC-2 cells formed tumors only 1 week after injection. Similar results were observed in 786-O/sh-Atg7 cells. However, 786-O cells formed tumors much slower. After 8 weeks, all the xenograft mice received euthanasia and all the tumors were resected (Figure 5a). Tumor volume of OS-RC-2/Atg7 cells was smaller than the tumor volume of OS-RC-2 cells, while tumor volume of 786-O/sh-Atg7 cells was bigger than that of 786-O cells (Figure 5b). The results of H&E staining were shown in Figure 5c.

**Discussion**

Autophagy is a highly conserved metabolic process which was first reported in 1960s. We found autophagy was suppressed in human RCCs in our previous studies15. In this study we measured the levels of autophagy in a few RCC cell lines, and we found they were lower than those in human renal tubular epithelial cell line. Thus, we hypothesized that low level of autophagy may affect the outgrowth of RCC. To prove this theory, we investigated the effects of autophagy on the growth capability of RCC cells. We chose 786-O as well as OS-RC-2 cell lines for the further experiments, in which the levels of autophagy were highest and lowest, respectively. Our research demonstrated that low level of autophagy was critical for the outgrowth of RCC cells in different lines. Firstly, Atg7-overexpression dramatically reduced the growth of OS-RC-2 cells, whereas Atg7-knockdown cells exhibited an increased growth. Secondly, we obtained consistent results from the xenograft animal model. OS-RC-2/Atg7 cells exhibited smaller tumor volume and lower tumor growth in relation to parental OS-RC-2 cells. Similarly, 786-O/sh-Atg7 cells exhibited bigger tumor volume and accelerated tumor growth compared with 786-O cells. Both in vitro and in vivo experiments indicated that autophagy defect might be necessary for growth of RCC cells.

Autophagy appears at basal rates in most of the cells. Autophagy is in general considered as an important homeostatic mechanism that provides survival benefits to cells in certain situations. Moreover, autophagy could interact with apoptosis to induce cell death in several pathological circumstances. Autophagy, under certain pathological conditions, can kill cells without the
Autophagy suppresses the proliferation of renal carcinoma cell involvement of apoptosis\textsuperscript{16}. To better investigate this phenomenon, many researches put attention on the relation between cancer and autophagy. Lately, studies on autophagy in different kinds of tumors have been reported. Autophagy was decreased in liver cancer. Autophagy defects were common in disease with low disease-free survival and overall survival rate; this suggested

\textbf{Figure 3.} Stable transfected cells observed by fluorescence microscope. The magnification of all figures is ×200. \textit{A, C, E, G,} were observed in white light; \textit{B, D,} were observed in blue light; \textit{F, H,} were observed in green light. \textit{A, B,} were OS-RC-2/Con\textit{} cells; \textit{C, D,} were OS-RC-2/Atg7 cells; \textit{E, F,} were 786-O/sh-Con cells; \textit{G, H,} were 786-O/sh-Atg7 cells.
that the autophagy defect is correlated to the aggressive cancer phenotype along with a poor prognosis. Oh et al. demonstrated that MCF7 breast cancer cell growth was promoted by down-regulating autophagy. Another study found out a molecule can cause the reduced tumor growth of the VHL-deficient RCC cells through autophagy. This research indicated that upregulated autophagy could cause cell death of VHL-deficient RCC cells. There was a study which showed that autophagy defect could be a therapeutic way to inhibit EGFR tyrosine kinase. With the used of autophagy inducer (rapamycin), these cells can partially regain their sensitivity to EGFR tyrosine kinase inhibition. This indicated that activation of autophagy may cause cell death in these types of cancer. All these researches indicated that autophagy defect is important for the growth of cancer cells. Coincidentally, our report showed the similar results. How autophagy induced cell death is still not well understood, but some researchers believed that high-level autophagy can destroy organelles as well as parts of the cytoplasm, resulting in autophagic cell death. In addition, autophagy may possibly kill cells by selectively degradating crucial proteins in cells.

**Figure 4.** Effect of autophagy on *in vitro* growth. a and b, MTT assays.

**Figure 5.** Effect of PrlZ on *in vivo* tumor growth in mice. A, Representative tumors isolated from OS-RC-2-, OS-RC-2/Atg7, 786-O- and 786-O/sh-Atg7-injected mice. B, Tumor growth assay. The right flank of the mice was subcutaneously injected with OS-RC-2, OS-RC-2/Atg7, 786-O, or 786-O/sh-Atg7 cells. Tumor volume was measured weekly. C, Histology from OS-RC-2, OS-RC-2/Atg7, 786-O, and 786-O/sh-Atg7 tumor are stained with HE and photographed under light microscopy (×200).
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However, the effect of autophagy on tumor growth is still controversial, and the effects are different in different types of tumors. For example, in melanoma, endometrial adenocarcinomas and colorectal cancer, autophagy was related to metastasis, proliferation, as well as poor outcome. Another paper indicated that autophagy might be required by the pancreatic cancers for tumor growth. All the studies suggested a converse result that autophagy may promote metastasis by enhancing the adaption of tumor cell to environmental stresses, such as anoikis, during proliferation and metastasis. In a word, autophagy is a double-edged sword for cancer in both proliferation and metastasis. The role of autophagy may be related to the basal level of autophagy in tumor and the tumor types.

Conclusions

We demonstrated that autophagy defect plays a role in the growth of RCC cells. Thus, autophagy is a potential factor in the suppression of RCC. So, autophagy might be a target for RCC therapy.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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