

## Original Article

# Effects of autophagy on the proliferation and apoptosis of clear cell renal carcinoma 786-O cells

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**Abstract:** Objective: Autophagy plays important roles in tumor occurrence and development. The present study aimed to investigate the association between autophagy and apoptosis in clear cell renal carcinoma cells (ccRCCs). Methods: Atg7-overexpressing and -knockdown RCC 786-O cells (pLenti6.3-ATG7 and sh-ATG7-2 lv) were established using lentiviral transfection and interference shRNA. pLenti6.3-GFP and sh-scramb-con lv were used as controls. Cells were cultured in medium with or without the apoptosis inhibitor Z-VAD-FMK. Cell apoptosis were detected by flow cytometry. Cell proliferation was determined by MTT assay. Expression of apoptotic pathway proteins was measured by Western blot. Results: The apoptosis rate of Z-VAD-FMK-treated cells was significantly decreased compared with untreated cells ( $P < 0.05$ ). However, no significant difference in the apoptosis rate was detected among cell groups with different autophagy level. The Z-VAD-FMK treatment induced significant changes in apoptosis rate in all cell groups, but only slightly changed the cell proliferation. When cell apoptosis were inhibited by Z-VAD-FMK, the cell viability in pLenti6.3-ATG7 group was significantly reduced compared with 786-O control ( $P < 0.05$ ), whereas the cell viability in sh-ATG7-2 lv was significantly enhanced ( $P < 0.05$ ) indicating that cell proliferation was closely associated with the level of autophagy. The expression of caspase proteins in pLenti6.3-ATG7 was significantly higher compared with sh-ATG7-2 lv group ( $P < 0.05$ ). Conclusion: autophagy and apoptosis are independent processes of PCD in human ccRCC 786-O cells. Autophagy is the main type of PCD and may be closely associated with apoptosis through the classical death receptor, mitochondria and endoplasmic reticulum apoptosis pathway.

**Keywords:** Renal carcinoma cell (RCC), autophagy, apoptosis, ATG 7, shRNA

## Introduction

Autophagy is a self-degradation process during which unnecessary or dysfunctional cellular components are digested by the lysosomal machinery. Autophagy frequently occurs as an adaptive response to stresses such as nutrition deprivation, hypoxic stress, etc., which maintains cellular energy and thereby promotes cellular survival, whereas it may promote cell death through excessive degradation of cellular, and has been known as type II programmed cell death (PCD) [1].

Studies have suggested that autophagy is related to the occurrence and progression of several diseases including cancer. In recent years, autophagy has therefore become a research hotspot in cancer biology [2, 3]. Autophagy plays a double-edged role in cancer: both tumor suppression and tumor cell survival

[4, 5]. The process of autophagy is strictly controlled by over 30 autophagy-related (Atg) genes [6]. As one of the essential autophagy-related proteins, ATG7 mediates the formation of ATG5-ATG12-ATG16 complex and promotes the formation of autophagic vacuoles. ATG7 is also involved in the phosphatidylethanolamine modification of LC3 during the formation of autophagosome. More importantly, Atg7 knock-out mice are more susceptible to liver cancer compared with healthy mice [7], suggesting that the protein is involved in the autophagy-regulated tumor suppression. Moreover, ATG7 is also essential for the cancer therapy-induced autophagy. Mice with reduced Atg7 expression displays markedly decreased immune responses to chemotherapy [8]. On the other hand, suppressed autophagy by Atg7 silencing will lead to increased sensitivity of tumor cells to chemoradiotherapy, suggesting a cytoprotective effect of therapy-induced autophagy [9].

## Autophagy and apoptosis in ccRCCs

Apoptosis, the type I PCD, is a highly regulated and controlled process that is closely associated with the occurrence and development of cancer. Caspases (cysteine-aspartic proteases) are a family of protease enzymes that are essential in classical apoptotic pathways [10]. As two different forms of programmed cell death, autophagy and apoptosis are both important in cellular growth, as well as the occurrence and progression of various diseases [11]. Nevertheless, the association between autophagy and apoptosis has not been fully clarified. Generally, autophagy can protect cells by preventing them from undergoing apoptosis. However, autophagy can also trigger apoptosis under certain conditions [12]. Therefore, the investigation of their association and related signal pathway is essential for the identification of novel therapeutic targets. This study explored the effect of different autophagy levels on the apoptosis and proliferation of clear cell renal cell carcinoma (ccRCC) 786-O cells in order to clarify the relationship between autophagy and apoptosis.

### Materials and methods

#### Cell culture

Human renal cell carcinoma 786-O cells were purchased from the Chinese Academy of Sciences Library (CCAS, Shanghai) and maintained in high glucose RPMI-1640 medium (HyClone company) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in an incubator with 5% CO<sub>2</sub>. Cells at the exponential phase were used for subsequent experiments.

#### Main reagents and instruments

Thiazolyl blue tetrazolium blue (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma. AnnexinV-FITC/7AAD apoptosis staining kit was purchased from BD Biosciences (San Diego, CA, USA). BCA-100 protein quantification kit was purchased from Dongsheng Biotech. (Shenyang, China). Rabbit anti-human Caspase-3, Caspase-4, Caspase-8 and Caspase-9 polyclonal antibodies were purchased from Proteintech (Chicago, IL, USA). Mouse anti-human  $\beta$ -actin monoclonal antibody, HRP-labeled goat anti-rabbit IgG, and HRP-labeled goat anti-mouse IgG were purchased from TransGen (Beijing, China). ECL luminous color

reagent was purchased from Millipore (Temecula, CA, USA).

#### Construct of recombinant lentivirus

Human autophagy related gene Atg7 was cloned into pLenti6.3-MCS-IRES-GFP vector (Invitrogen, Beijing, China). Empty vector control was also established. Sequence of Atg7 shRNA, 5'-CCGGAAGGAGTCACAGCTCTTCCTTC-TCGAGAAGGAAGAGCTGTGACTCCTTTTTTTG-3', was cloned into pLVshRNA-mCherry(2A)-puro vector (Invitrogen). Sequence of RNA scramble was also cloned to construct vector control. All vectors were respectively transfected into packaging cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacture's instructions. Lentiviruses were purified and concentrated. The obtained virus, empty control, Atg7-knockdown virus, and RNA scramble control was named.

#### Establishment of stable Atg7-overexpressing, Atg7-knockdown cells

To obtain stable transfectants, 786-O cells were transfected with the appropriate lentivirus (10  $\mu$ L) using Lipofectamine 2000 reagent and incubated at 37°C, 5% CO<sub>2</sub>. Medium was replaced every 24 h. After 96 h, cells were selected in complete growth medium containing 4.0  $\mu$ g/mL Blasticidin (Invitrogen) or 2.0  $\mu$ g/mL Puromycin (Invitrogen). The selected Atg7-expressing cells, empty control, Atg7-knockdown cells, and scramble control cells were named as pLenti6.3-ATG7, pLenti6.3-GFP, sh-ATG7-2 lv, and sh-scramb-con lv, respectively.

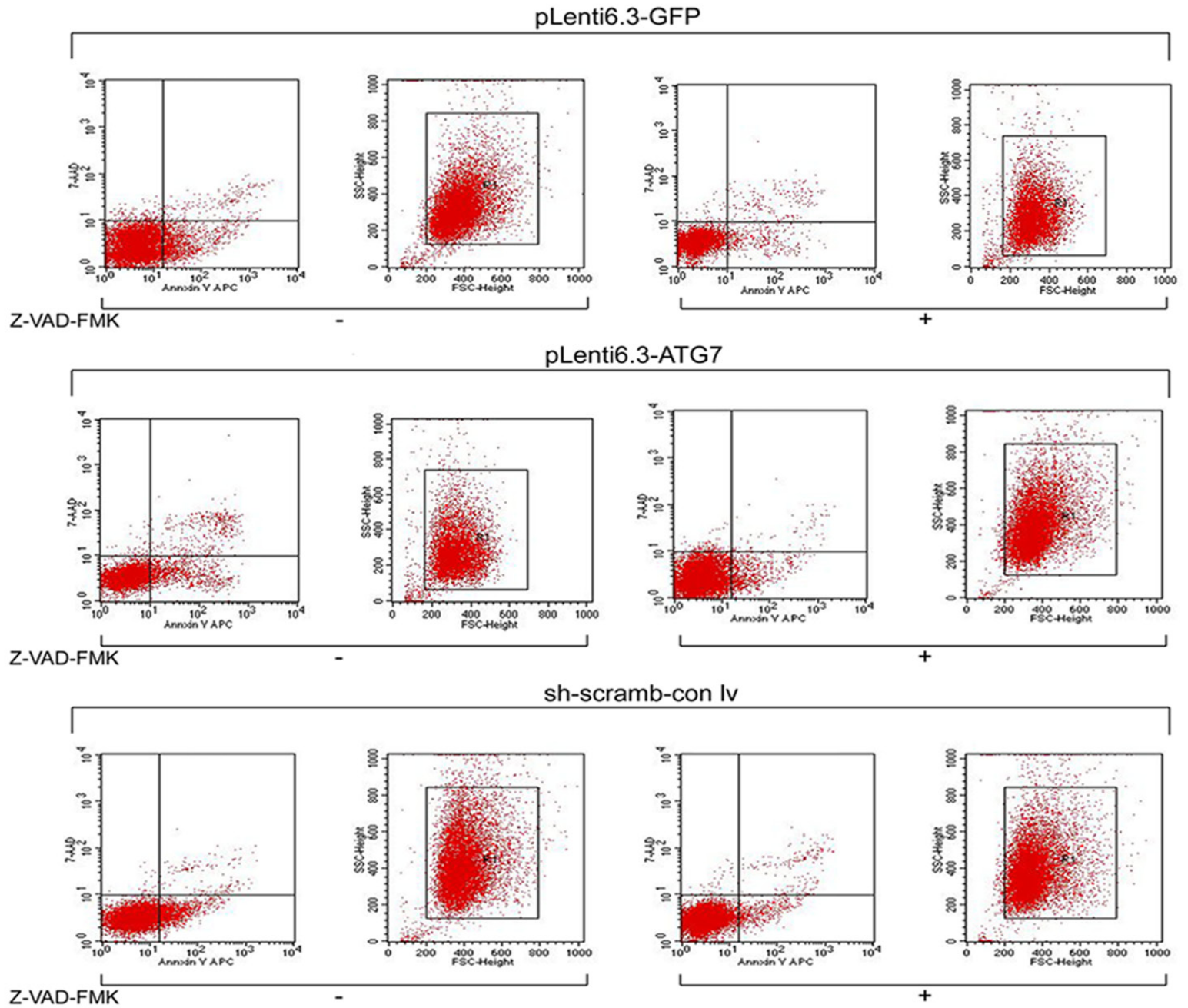
#### Flow cytometry

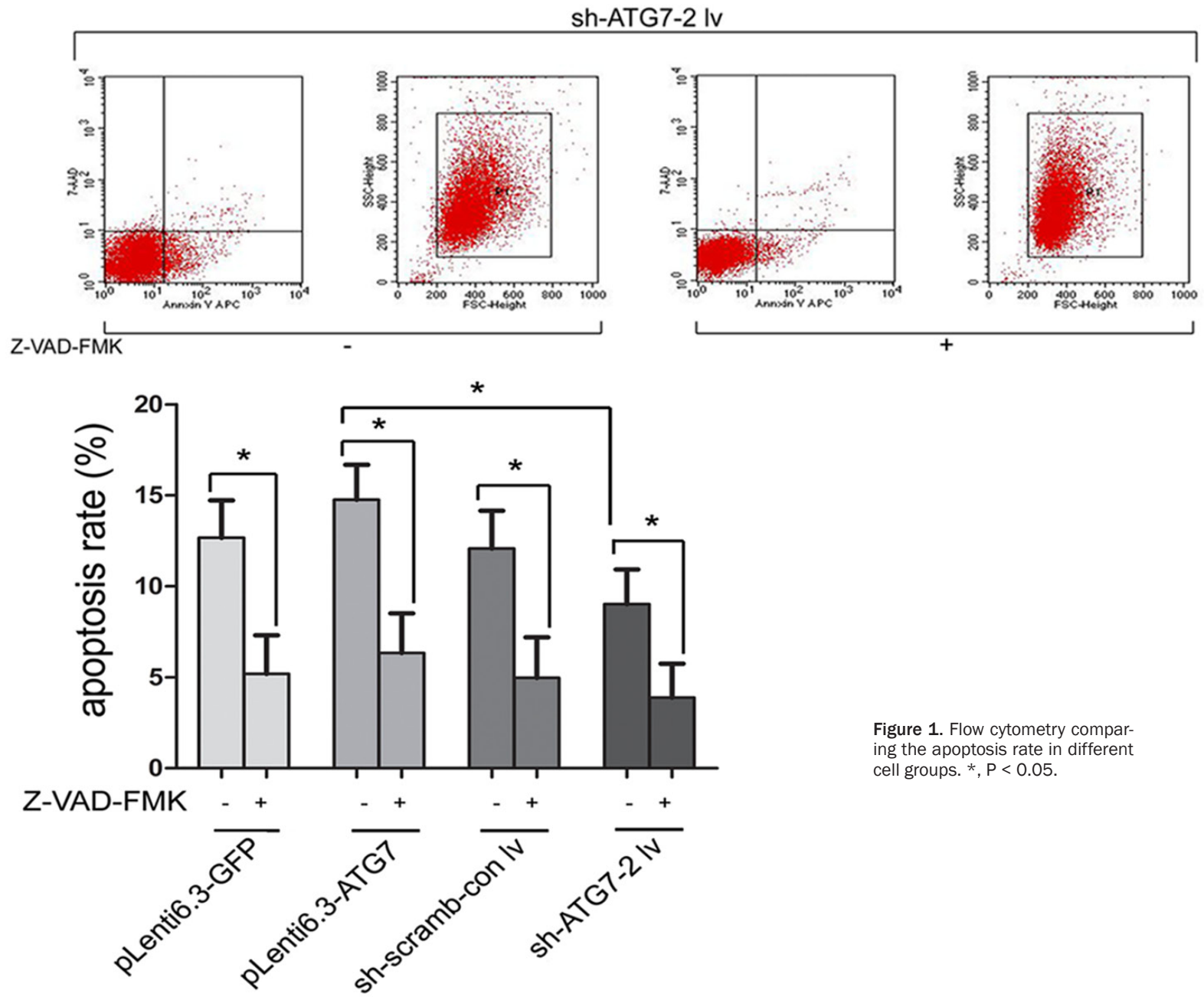
pLenti6.3-ATG7, pLenti6.3-GFP, sh-ATG7-2 lv, and sh-scramb-con lv cells were cultured in complete medium with or without 100  $\mu$ M inhibitor Z-VAD-FMK at 37°C, 5% CO<sub>2</sub> for 24 h. Cells at exponential phase were trypsinized and washed with PBS. Cells were then mixed with binding buffer and treated with 5  $\mu$ l of Annexin V-FITC for 15 min in dark. 7AAD (5  $\mu$ l) was added. Cells were incubated for 5 min and detected by a flow cytometer within 1 h to detect the apoptosis in each group. Triplicate samples were prepared for each group.

#### MTT assay

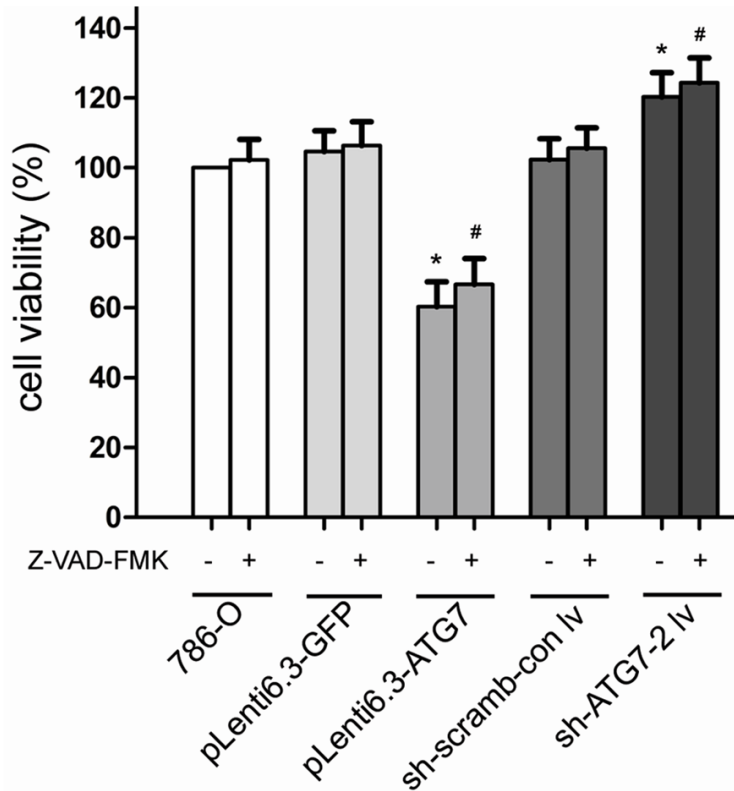
Cell proliferation was determined by MTT assay. Briefly, cells in each group were cultured in

Autophagy and apoptosis in ccRCCs

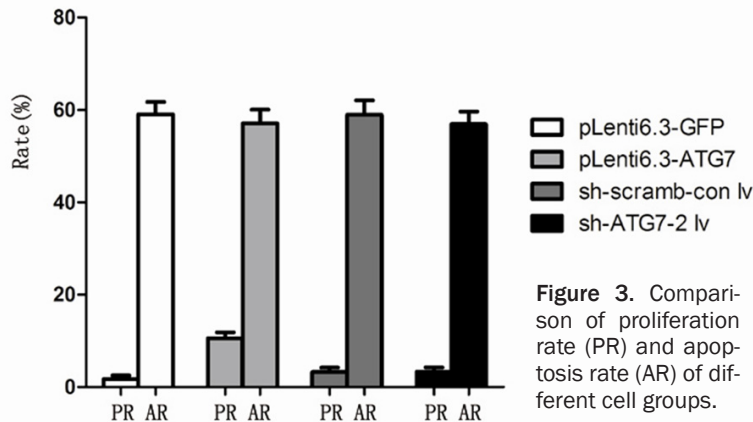




**Figure 1.** Flow cytometry comparing the apoptosis rate in different cell groups. \*, P < 0.05.



**Figure 2.** Comparison of cell viability of different cell groups. \*,  $P < 0.05$  compared with 786-O cells, and #,  $P < 0.05$  compared with 786-O cells treated with Z-VAD-FMK.



**Figure 3.** Comparison of proliferation rate (PR) and apoptosis rate (AR) of different cell groups.

complete medium with or without inhibitor Z-VAD-FMK at 37°C, 5% CO<sub>2</sub> for 24 h and treated with 20 µl of 5 mg/ml MTT for 4 h. The medium was discarded and DMSO was then added to solubilize the formazan crystals. The absorbance (OD) at 590 nm was measured using a microplate autoreader (Bio-Tek Instruments, Vermont, USA). Triplicate samples were prepared for each group.

**Results**

*Effects of autophagy on the apoptosis of 786-O cells*

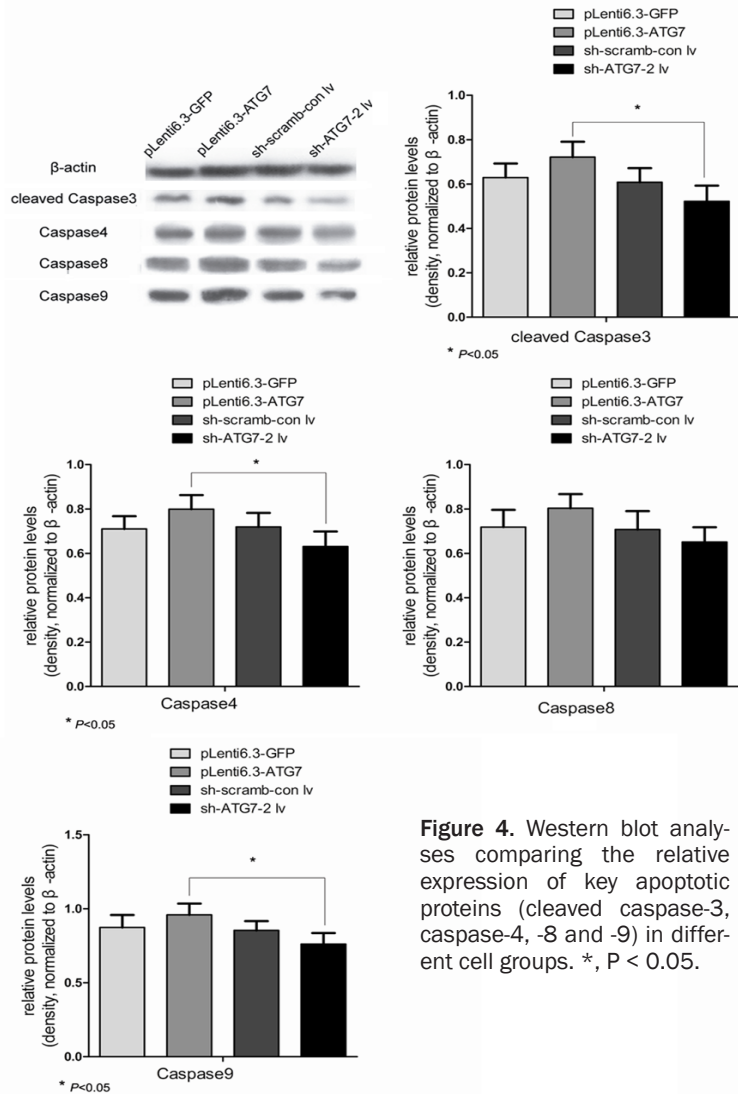
As shown in **Figure 1**, flow cytometry demonstrated that the apoptosis rate of pLenti6.3-ATG7 cultured in medium without apoptosis inhibitor Z-VAD-FMK was significantly higher

*Western blot*

Cells at the exponential phase were collected and mixed with RIPA lysis buffer. Total protein was extracted and quantified using a BCA kit according to the manufacture’s instruction. Equal amounts of total protein (30 µg) were separated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes. The membrane was blocked in TBS buffer containing 5% skim milk at room temperature for 2 h, and incubated with caspase-3, caspase-4, caspase-8 and caspase-9 and β-actin antibodies overnight at overnight at 4°C. The membrane was washed and incubated with HRP-labeled secondary antibodies at 37°C for 1 h. Protein signal was then detected using the ECL detection system. The intensity of bands was detected by a Molecular Imager® ChemiDoc™ XRS System (Bio-Rad Laboratories). The gray value of bands was analyzed by Image Lab 2.0 software (Bio-Rad Laboratories).

*Statistical analysis*

All data were expressed as mean ± standard deviation and analyzed using SPSS 17.0 (IBM SPSS, Chicago, IL, USA). Difference among groups was compared by ANOVA. Difference between groups was compared by t-tests.  $P$  values smaller than 0.05 are considered statistically significant.



**Figure 4.** Western blot analyses comparing the relative expression of key apoptotic proteins (cleaved caspase-3, caspase-4, -8 and -9) in different cell groups. \*, P < 0.05.

than that of sh-ATG7-2 lv cells (P < 0.05). No significant difference in the apoptosis rate was observed among other groups. When cells were treated with Z-VAD-FMK, the apoptosis rate was significantly decreased compared with untreated cells (P < 0.05). However, no significant difference in the apoptosis rate was detected among cell groups with different level of autophagy, suggesting that the level of autophagy did not affect the apoptosis of cells, and autophagy and apoptosis were independent processes of PCD.

*Effects of autophagy on the proliferation of 786-O cells*

Although the apoptosis rate of cells treated with Z-VAD-FMK was significantly decreased

compared with untreated cells (Figure 1), they exhibited similar cell viability (P > 0.05, Figure 2). When cell apoptosis were inhibited by Z-VAD-FMK, the cell viability in pLenti6.3-ATG7 group was significantly reduced compared with 786-O control (P < 0.05), whereas the cell viability in sh-ATG7-2 lv was significantly enhanced (P < 0.05, Figure 2), indicating that cell proliferation was closely associated with the level of autophagy. Moreover, the Z-VAD-FMK treatment induced significant changes in apoptosis rate in all cell groups, but only slightly changed the cell proliferation (Figure 3), further suggesting that the difference in cell viability among different cell groups was caused by different level of autophagy instead of apoptosis, and autophagy was the primary process of PCD in 786-O cells.

*Effects of autophagy on apoptotic signaling pathway proteins*

As shown in Figure 4, the highest expression of caspase3, 4, 8 and 9, the apoptotic signaling pathway proteins, was detected in pLenti6.3-ATG7 group, followed by pLenti6.3-GFP or sh-scramb-con lv groups. The lowest expression of caspase proteins was observed in sh-ATG7-2 lv group. The expression of caspase proteins in pLenti6.3-ATG7 was significantly higher compared with sh-ATG7-2 lv group (P < 0.05). These results suggested that autophagy was closely associated with the apoptotic signaling pathway proteins, and a crosstalk might exist between autophagy and apoptotic pathways.

**Discussion**

Currently, the relationship between autophagy and apoptosis has not been fully clarified. Studies have suggested that autophagy and apoptosis coordinately regulate cell death. They may occur simultaneously in cells. For

instance, TNF-related apoptosis inducing ligand (TRAIL) may induce both autophagy and caspase-dependent apoptosis of human breast epithelial MCF-10A cells [13]. It has also been found that arsenic trioxide induces not only apoptosis but also autophagic cell death in leukemia cell lines [14]. Apoptosis and autophagy are both activated in the treatment of Kaposi's sarcoma [15]. Alternatively, cells start to undergo autophagy or apoptosis once the other form of CPD is deactivated. When apoptosis in neurons is inhibited, autophagy becomes the primary CPD [16]. Nevertheless, studies have also found that autophagy induces the process of apoptosis, and thus is required for the latter. In breast cancer cells, autophagy occurs prior to apoptosis, and the autophagy inhibitor 3-MA can suppress both autophagy and apoptosis [17]. Damage regulated autophagy modulator (DRAM) may promote apoptosis through mediating autophagy, whereas its deactivation suppresses autophagy, leading to reduced apoptosis [18]. In addition, some studies have suggested a mutual antagonism between autophagy and apoptosis. Bauvy et al. have considered depolarized mitochondrial degradation by autophagy as a protective mechanism against apoptosis in human colon cancer HT-29 cells [19]. The inhibition of autophagy can also enhance the sensitivity of breast cancer cells to radiotherapy [20]. In this study, we found that the level of autophagy did not affect the apoptosis of cells, indicating that autophagy and apoptosis were independent processes of PCD in ccRCC 786-O cells. Moreover, the apoptosis rate in pLenti6.3-ATG7 group was significantly increased compared with sh-ATG7-2 lv group ( $P < 0.05$ ), which suggested that some of the cells with high autophagy level might start the process of apoptosis. Autophagy therefore promoted the occurrence of apoptosis.

We further compared the cell viability of cells treated with apoptosis inhibitor Z-VAD-FMK in order to investigate the primary PCD in 786-O cells. It was found that although the apoptosis rate of cells treated with Z-VAD-FMK was significantly decreased compared with untreated cells, they exhibited similar cell viability. The Z-VAD-FMK treatment induced significant changes in apoptosis rate in all cell groups, but only slightly changed the cell proliferation. Moreover, the cell viability in pLenti6.3-ATG7 group was significantly reduced compared with 786-O control, whereas the cell viability in sh-ATG7-2 lv was significantly enhanced. These

results suggested that cell proliferation of 786-O cells was mainly affected by the autophagy level instead of apoptosis, and autophagy was the primary type of PCD in 786-O cells. To our best knowledge, the current study is the first report on the main type of PCD in ccRCC.

Autophagy and apoptosis may occur simultaneously or successively in cells. Therefore, the investigation on the interplay between the two may provide novel therapeutic targets in the treatment of several diseases, especially cancer. There are three classical apoptotic pathways including death receptor, mitochondrial and endoplasmic reticulum pathway [21, 22]. In this study, we further compared the expression of key apoptotic pathway proteins (caspase-3, -4, -8 and -9) in cells with different levels of autophagy. The expression of caspase proteins in pLenti6.3-ATG7 was significantly higher compared with sh-ATG7-2 lv group ( $P < 0.05$ ), indicating a close association between autophagy and these apoptotic signaling pathway proteins, and thereby an interplay between autophagy and apoptosis through the classical apoptotic pathways.

In summary, autophagy and apoptosis are independent processes of PCD in human ccRCC 786-O cells. Autophagy is the main type of PCD and may be closely associated with apoptosis through the classical death receptor, mitochondria and endoplasmic reticulum apoptosis pathway.

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### Disclosure of conflict of interest

None.

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## Autophagy and apoptosis in ccRCCs

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