

Overexpression of *Beclin 1* inhibits proliferation and promotes apoptosis of human laryngeal squamous carcinoma cell Hep-2

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Objective: *Beclin1* was previously found to be downregulated in human laryngeal cancer (LC) tissues, and it results in poor prognosis. This study aimed to further confirm the antitumor effects of *Beclin1* in LC cell line Hep-2.

Materials and methods: *Beclin 1* was overexpressed in Hep-2 cells using liposomal transfection and confirmed using reverse transcription polymerase chain reaction and Western blotting. Then, cell proliferation and apoptosis were determined in control (untransfected), empty vector transfected, and *Beclin1* overexpressed groups using MTT and flow cytometry procedure, respectively.

Results: The expression of the *Beclin1* gene in Hep-2 cells was significantly increased after vector transfection compared with control (1.173 ± 0.046 vs 0.453 ± 0.016 , $P<0.01$) and empty vector (1.173 ± 0.046 vs 0.440 ± 0.021 , $P<0.01$). Overexpression of *Beclin1* inhibited proliferation at 4 days (0.619 ± 0.051 vs 0.891 ± 0.081 and 0.619 ± 0.051 vs 0.878 ± 0.105 , $P<0.01$), 5 days (0.684 ± 0.078 vs 1.127 ± 0.094 and 0.684 ± 0.078 vs 1.162 ± 0.117 , $P<0.01$), and 6 days (0.725 ± 0.069 vs 1.168 ± 0.103 and 0.725 ± 0.069 vs 1.194 ± 0.097 , $P<0.01$) and promoted apoptosis ($14.48\%\pm 1.42\%$ vs $4.07\%\pm 0.66\%$ and $14.48\%\pm 1.42\%$ vs $4.39\%\pm 0.80\%$, $P<0.01$) in Hep-2 cells in comparison with the control and empty vector groups, respectively.

Conclusion: *Beclin1* may be an underlying target for the treatment of LC. This study has provided some experimental basis for the gene therapy of LC.

Keywords: laryngeal cancer, antitumor effect, cell proliferation, cell apoptosis

Introduction

Laryngeal cancer (LC) is a common head and neck cancer characterized by hoarseness, sore throat, and persistent cough, and the incidence is increasing.^{1,2} Radiotherapy and surgery are the most common therapies used recently for the treatment of LC.^{3,4} However, surgery is always associated with the loss of language function, and patients undergoing radiotherapy treatment are prone to relapse. Therefore, it is urgent to explore a new therapy for LC.

Autophagy is a highly conserved catabolic process involving the lysosomal transport of proteins and organelles.⁵ Disorders of this system are associated with many human diseases and the clearance of cancer cell carcasses.^{6,7} *Beclin1* is the encoding gene of autophagy-associated protein Beclin 1, which is a component of the PI3K complex involved in the vesicular transport. Previous studies have shown the important role of *Beclin1* in cancers such as breast cancer,⁸ CaSki cervical cancer,⁹ lung cancer,¹⁰ MIA PaCa-2 pancreatic cancer,¹¹ and tongue squamous cell carcinoma.¹² A recent study has also revealed that the expression of *Beclin1* is significantly decreased in laryngeal

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squamous cell carcinoma than that in non-tumor tissues and is obviously associated with the lymph node metastasis and poor survival rate, suggesting that Beclin 1 may be a potential target for the treatment of LC.¹³ However, the role of *Beclin1* in cells was not verified by experiment.

To further reveal its biofunction, Beclin 1 was overexpressed in a human LC cell line hep-2, and the effects of Beclin 1 on cell proliferation and apoptosis were determined in this study, so that it could provide a theoretical basis for the mechanism of *Beclin 1* in LC.

Materials and methods

Construction of pIRES2-AcGFP-*Beclin 1* expression vector and cell transfection

The amplification of *Beclin1* gene was performed by polymerase chain reaction (PCR) using the full-length *Beclin1* cDNA clone (pCMVsPORT6-*Beclin1* from Funeng Genes Co., Ltd, Guangzhou, China) as the template, and then electrophoretic detection was performed. The amplified product was digested with SacI and EcoRI (Promega Corporation, Fitchburg, WI, USA) and purified using the DNA gel recovery kit (Dongsheng Biotech Co., Ltd., Guangzhou, China) according to the manufacturer's instructions. The target gene *Beclin1* was ligated to the expression vector pIRES2-AcGFP (Clontech Laboratories, Inc., Mountain View, CA, USA) and transformed into competent DH5 α , and then the cloned recombinants were screened. The recombinant plasmid pIRES2-AcGFP-*Beclin1* was extracted with a small-scale plasmid extraction kit from Shanghai Labaide. Finally, the extracted plasmid was transfected into human laryngeal carcinoma Hep-2 cell line (Nanjing KeyGen Biotech, Nanjing, Jiangsu, China) using Lipo2000 according to the manufacturer's protocol. Hep-2 cells transfected with pIRES2-AcGFP-*Beclin1* were considered as the *Beclin1* group and Hep-2 cells transfected with pIRES2-AcGFP as the empty vector group, while the control group consisted of the Hep-2 cells not transfected with pIRES2-AcGFP.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Primers of genes were synthesized in Shanghai Biological Engineering Co., Ltd (Shanghai, China) as follows: *Beclin1*, forward sequence: 5'-ACAGAGCTCATGGAAGGGTCTAAGACGTCC-3' and reverse sequence: 5'-TACGAATTCTCATTTGTTATAAAATTGTG-3'; and *GAPDH*, forward sequence: 5'-ACCACAGTCCATGCCATCAC-3' and reverse sequence: 5'-TCCACCACCCTGTTGCTGTA-3'. Total mRNA was extracted from cells using an mRNA rapid extraction kit (Shanghai Jie Rui Bioengineering Co., Ltd).

Then, mRNA was reverse transcribed into cDNA in 30 μ L transcription reaction system by AMV with the universal OLIGO hexamer as the reverse transcription primer. The PCR program for *Beclin1* and *GAPDH* was designed as follows: 94°C for 3 min, 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s, and finally extension at 72°C for 7 min. The PCR products of *Beclin1* gene and internal controls were detected by electrophoresis in agarose gel.

Western blotting

After culturing for 48 h, the cell culture medium was discarded and washed twice with PBS. Then, the cells were collected and lysed with WIP cell lysate (Beijing Boosen Biotechnology Company, Beijing, China) at 4°C for 15 min. Following this, lysates were centrifuged at 4°C for 10 min, and the concentration of supernatant of each protein sample was determined using the bicinchoninic acid protein concentration assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The collected protein samples were boiled with sodium dodecyl sulfate polyacrylamide gel electrophoresis protein loading buffer (5 \times) for 3–5 min and then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Thereafter, the gel was equilibrated in transfer buffer for 15 min and transferred using Bio-Rad's standard wet transfer device. After the transfer, the protein membrane was put into the Western washing solution for 1–2 min and blocked with Western blocking solution at room temperature for 1 h. Following this, the membrane was incubated with a mouse anti-human Beclin1 antibody (1:200; Santa Cruz Biotechnology Inc., Dallas, TX, USA) and incubated at room temperature for 1 h. Then, the membrane was incubated with a horseradish peroxidase-conjugated anti-mouse IgG antibody (1:3,000; Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Subsequently, the membrane was rinsed four times with the Western wash solution for 5–10 min and visualized using the enhanced chemiluminescence method. The images were saved using the imaging scanning analysis system (Syngene, Cambridge, UK).

MTT assay

The culture medium was discarded, and the cells were washed twice with PBS. Then, 80 μ L of fresh RPIM-1640 medium and 20 μ L of 0.5% MTT solution (dissolved in PBS) were added to each well and cultured with cells for 5 h. The supernatant was discarded and 150 μ L of dimethyl sulfoxide was added to each well shaking for 10 min at dark condition. The OD of the absorbance at 570 nm was measured by enzyme-linked immunosorbent assay. The growth curve was plotted based on the average of the OD values.

Flow cytometry analysis

In this assay, sequence of *Beclin1* was inserted into pcDNA3.1 vector and transfected into hep-2 cells using Lipo2000. Then, the cells were divided into control group (treated only with Lipo2000), empty vector group (transfected with vector), and *Beclin1* groups. After transfecting for 48 h, the cells were harvested in 10 mL centrifuge tube and washed with PBS for two times after cooling. Then, the supernatant was discarded and the cells' concentration was adjusted to 1×10^6 /mL with PBS. After centrifuging for 5 min at 1,000 rpm, the supernatant was removed and the cells were washed once with the incubation buffer followed by centrifugation for 5 min at 1,000 rpm. Subsequently, the cells were resuspended in 100 μ L of the labeling solution and incubated for 15 min at room temperature in the dark, and then centrifuged for 5 min at 1,000 rpm. The precipitated cells were collected and washed once with the incubation buffer. The cells were incubated with the fluorescent solution at 4°C for 20 min in dark. The apoptotic rate was detected using flow cytometry with the wavelength of the excitation light at 488 nm. The filter with a wavelength >560 nm was used to detect the propidium iodide.

Statistical analyses

All statistical analyses were performed with SPSS18.0 software (SPSS Inc., Chicago, IL, USA). The results were analyzed by one-way analysis of variance and were

expressed as mean \pm SD. $P < 0.01$ was considered statistically significant.

Results

Construction of pIRES2-AcGFP-*Beclin1* vector

In order to identify the extraction of target genes, *Beclin1* gene was amplified using specific primers. The results of electrophoresis showed a bright band at 1,353 bp, which was consistent with the full length of *Beclin1* gene (Figure 1A). To confirm the recombination of target *Beclin1* gene and the expression vector pIRES2-AcGFP, the vector was digested with *SacI* and *EcoRI* and detected by agarose gel electrophoresis. The results showed that there was a bright band at about 1,000 bp of the *Beclin1* group (Figure 1B). Because the expression vector had a green fluorescent protein AcGFP marker, the transfection efficacy was also determined by the color of cells under a microscope (Olympus Corporation, Tokyo, Japan) at a wavelength of 450–490 nm. As a result, green-colored cells were identified in both *Beclin1* group and empty vector group (Figure 1C), indicating that gene transfection screening was successful.

Expression of *Beclin1* mRNA in Hep-2 cells

RT-PCR was used to detect the relative mRNA expression of *Beclin1* in Hep-2 cell line. The results showed that the relative mRNA expression of *Beclin1* was significantly higher in the

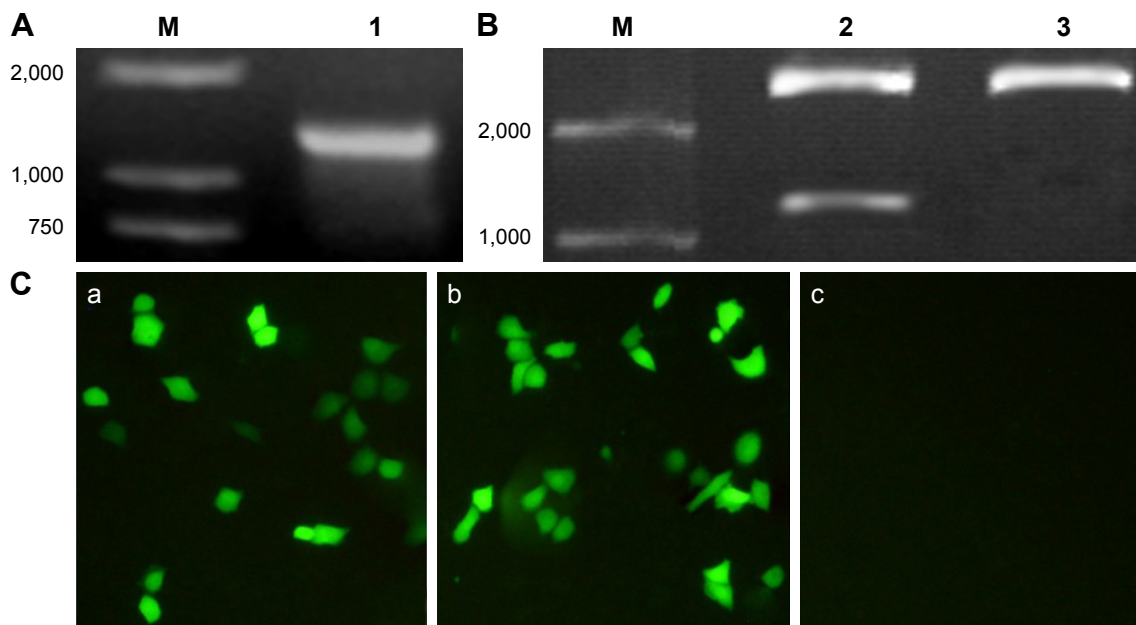


Figure 1 The amplification and digestion of *Beclin1*, and its expression in Hep-2 cells.

Notes: (A) The amplification of *Beclin1*. M: molecular weight marker; (1) targeted gene *Beclin1*. (B) *SacI* and *EcoRI* double digestion results of clone recombinants: (2) positive clone recombinant pIRES2-AcGFP-*Beclin1*; (3) control sub-cloning vector pIRES2-AcGFP. (C) Expression of *Beclin1* expression vector in Hep-2 cells: (a) Hep-2 cells transfected with *Beclin1* expression vector pIRES2-AcGFP-*Beclin1*; (b) Hep-2 cells transfected with empty vector pIRES2-AcGFP; and (c) Hep-2 cells not transfected.

Beclin1 group than in the empty vector group (1.173 ± 0.046 vs 0.453 ± 0.016 , $P<0.01$) and the control group (1.173 ± 0.046 vs 0.440 ± 0.021 , $P<0.01$). The results showed that the expression of *Beclin1* mRNA in Hep-2 cells could be enhanced by transfection of *Beclin1* into Hep-2 cells (Figure 2A and B).

Protein expression of Beclin1 in Hep-2 cells

Western blot was performed to verify whether Beclin1 protein can be successfully expressed in transfected cells. The result showed an obvious lighter imprinted band at ~60 kDa in the Beclin1 group than in the control and empty vector groups (Figure 2C). This finding indicated that the Beclin1 expression vector pIRES2-AcGFP-*Beclin1* was successfully transfected into Hep-2 cells.

Overexpression of *Beclin1* significantly inhibits the growth of Hep-2 cells

To understand the effect of autophagy gene *Beclin1* on the proliferation of Hep-2 cells, the OD values of these three groups were measured by MTT assay. At 4 days, the OD

values of Beclin1 group, empty vector group, and control group were 0.619 ± 0.051 , 0.891 ± 0.081 , 0.878 ± 0.105 , respectively. Besides, the OD values of Beclin1 group, empty vector group, and control group were 0.684 ± 0.078 , 1.127 ± 0.094 , 1.162 ± 0.117 , respectively, at 5 days. Moreover, the OD value of Beclin1 group at 6 days was also obviously lower than those of empty vector group and control group (0.725 ± 0.069 vs 1.168 ± 0.103 , 0.725 ± 0.069 vs 1.194 ± 0.097 , respectively). The results showed that the proliferation of cells in the Beclin1 group was significantly attenuated compared with that in the empty vector and control groups from 4 to 6 days ($P<0.01$). However, there was no significant difference among these three groups before 3 days (Figure 3A). Then, the cell growth at different culture time points was observed under a microscope. Microscopic observation showed that there were no significant differences in cell growth among these three groups within 3 days (Figure 3B). However, after 3 days of cell culture, the growth rate of the Beclin1 group was significantly inhibited compared with those of empty vector and control groups, but no difference was identified between the control and empty vector groups ($P>0.05$). At the same time, the numbers of aging (cell morphology rounded, intracellular granules) and dead (cell roundness, floating) cells were obviously increased in the Beclin1 group compared with the control and empty vector groups (Figure 3C).

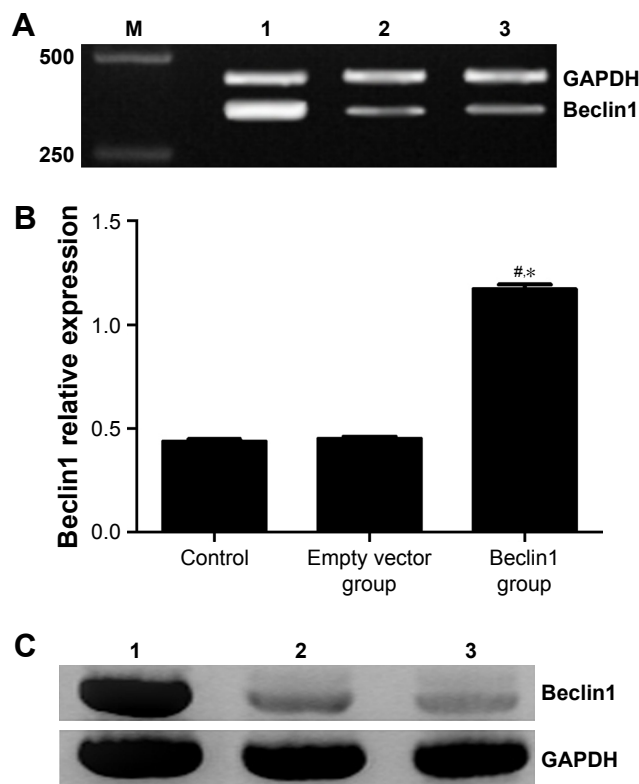


Figure 2 Analysis of *Beclin1* expression.

Notes: (A) Electrophoresis test results. (B) The relative expression of *Beclin1* mRNA in each group. (C) Beclin1 protein expression. M: molecular weight marker; (1) Hep-2 cells transfected with *Beclin1* expression vector pIRES2-AcGFP-*Beclin1*; (2) Hep-2 cells transfected with empty vector pIRES2-AcGFP; and (3) Hep-2 cells not transfected. GAPDH was used to ensure equal loading. The results are representative of at least five independent experiments. [#] $P<0.01$ vs control group; ^{*} $P<0.01$ vs empty vector group. Student's *t*-test was used to analyze the data.

Overexpression of *Beclin1* increases the apoptosis of Hep-2 cell lines

To determine the autophagy effect of *Beclin1* on apoptosis of human laryngeal carcinoma Hep-2 cell lines, the apoptotic cells were stained with annexin V-fluorescein isothiocyanate, and the apoptosis rate was determined by flow cytometry. The apoptosis rate of the Beclin1 group was significantly higher than those of the empty vector group ($14.48\%\pm 1.42\%$ vs $4.07\%\pm 0.66\%$, $P<0.01$) and the control group ($14.48\%\pm 1.42\%$ vs $4.39\%\pm 0.80\%$, $P<0.01$; Figure 4). This evidence indicated that the overexpression of *Beclin1* in Hep-2 cells could increase the apoptosis of Hep-2 cells.

Discussion

Although studies have shown that low expression of *Beclin1* has a close relationship to poor prognosis of LC, in-depth investigation of the mechanism is rarely reported till now. Therefore, this study aimed to demonstrate the central role of *Beclin1* in LC in vitro. Our results showed that overexpression of *Beclin1* significantly suppressed cell proliferation, but obviously increased cell apoptosis in Hep-2 cells, which were consistent with previous studies.^{12,14-16}

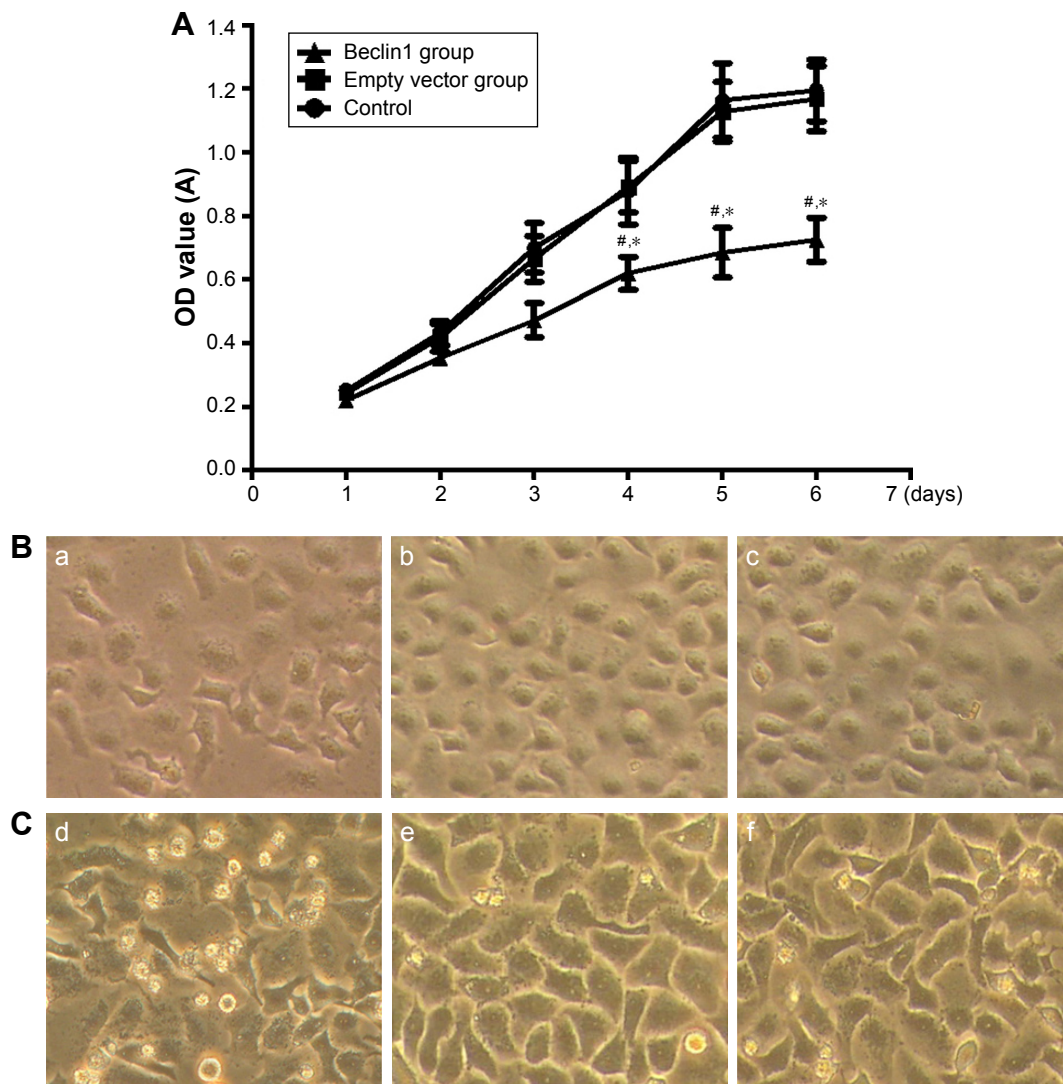


Figure 3 Effects of *Beclin1* on the growth of Hep-2 cells.

Notes: (A) The effect of *Beclin1* on the survival of Hep-2 cells within 7 days through tetramethyl-azo-zole-blue. (B) The cells observed under a microscope after 3 days of culture. (C) The cells observed under a microscope after 6 days of culture. $^{*}P < 0.01$ vs control group; $^{*}P < 0.01$ vs empty vector group. The results are representative of at least five independent experiments. Student's *t*-test was used to analyze the data. (a) Hep-2 cells transfected with *Beclin1* expression vector pIRES2-AcGFP-*Beclin1*; (b) Hep-2 cells transfected with empty vector pIRES2-AcGFP; (c) Hep-2 cells not transfected. (d) Hep-2 cells transfected with *Beclin1* expression vector pIRES2-AcGFP-*Beclin1*; (e) Hep-2 cells transfected with empty vector pIRES2-AcGFP; and (f) Hep-2 cells not transfected. Magnification 400 \times .

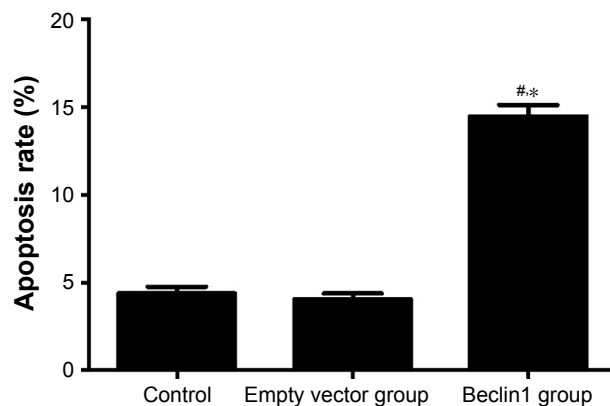


Figure 4 Apoptosis rate analysis through flow cytometry.

Notes: The results are representative of at least five independent experiments. $^{*}P < 0.01$ vs control group; $^{*}P < 0.01$ vs empty vector group. Student's *t*-test was used to analyze the data.

Autophagy is closely related to the development of cancer. However, the role of autophagy in cancer remains controversial; for example, a study has reported enhancement of autophagy in cancer cell metastasis,¹⁷ but the inhibition is suggested by another study.¹⁶ There are many claims about the mechanism of *Beclin1* in cancers. For example, *Beclin1* regulates the cell growth in breast and lung cancers,^{18,19} inhibits cell proliferation, invasion, and migration in cervical cancer and tongue squamous cell carcinoma,^{12,16} and induces apoptosis in lung and pancreatic cancers.^{10,11} Although several studies have reported *Beclin1* in many cancers,^{19,20} the investigation of *Beclin1* in LC is rarely reported.

Autophagy and apoptosis are crucial cellular house-keeping and tissue survival mechanisms, and *Beclin1* is a

platform protein that mediates the crosstalk between apoptosis and autophagy.²¹ A previous study demonstrated that *Beclin1* regulates the autophagic activity by complexing with the type III PI3K. RNase L cleavage products promote autophagy switch to apoptosis by caspase-mediated cleavage of Beclin1.²² Liu et al have documented that CHOP mediates ASPP2-induced autophagic apoptosis by releasing Bcl-2 from Beclin1 and promoting nuclear location of Bcl-2 in hepatoma.²³ These findings indicated that Beclin1 plays a crucial role in regulating autophagy and apoptosis. Caspase-mediated cleavage of Beclin1 inactivates Beclin1-induced autophagy and promotes apoptosis by increasing the release of proapoptotic factors from the mitochondria.²⁴ Autophagy inhibits apoptosis by phagocytosing apoptotic pro-caspases such as caspase-8.²⁵ *Beclin1* was previously reported as an important gene in the prognosis of LC.¹³ In this study, we found that overexpression of *Beclin1* increased the apoptosis of Hep-2 cells in vitro. Therefore, we deduced that abnormal overexpressed Beclin1 in Hep-2 might trigger some underlying mechanism that could cleave Beclin1 to promote the apoptosis of Hep-2 cell. In fact, autophagy and apoptosis exhibit certain inhibition and have common stimuli and signaling pathways.^{26,27} As a mediator between these two cell activities, Beclin 1 performs a double-sided regulation of autophagy. On the one hand, autophagy renders the cells chemically resistant by contributing to glycolysis, oxidative metabolism, and cell adhesion-dependent growth, and thereby helps in cell proliferation.²⁸ On the contrary, *Beclin1* plays an antiapoptotic role in many stresses interacting with Bcl-2.^{29,30} For example, caspase-3 and caspase-8 could co-work to cleave Beclin1 into C-terminal orienting to mitochondria to inhibit cells autophagy and N-terminal.^{26,27,31} In this study, the growth of Hep-2 cells was not significantly changed within 3 days, while the proliferation was significantly decreased in 4–6 days after transfection of *Beclin1*. Considering this, we suspect that promotion of apoptosis by *Beclin 1* might be the potential explanation for the decrease in proliferation of Hep-2 cells. Taken together, *Beclin1* might perform a critical role in the survival of Hep-2 cells and can be used as a target of gene therapy for LC.

There were some limitations in this study. For example, although *Beclin1* gene significantly inhibited cell proliferation and increased apoptosis of cells in vitro, whether this phenomenon exists in vivo is still uncertain. Therefore, future studies are required to be performed in in vivo conditions. Besides, the specific mechanism was not studied. Future research would discuss whether caspase-3 and caspase-8 are involved in the autophagy process.

Conclusion

The results of this study show that the overexpression of *Beclin1* in Hep-2 cells significantly inhibited cell proliferation and increased the rate of apoptosis in vitro. *Beclin1* might be used as a target of gene therapy for LC.

Disclosure

The authors report no conflicts of interest in this work.

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