Original Article
Silence of Beclin1 in oral squamous cell carcinoma cells promotes proliferation, inhibits apoptosis, and enhances chemosensitivity

Xia Wang1, Shu Li2, Shuhua Wu1, Lulu Xie3, Peiyuan Wang3

1Department of Pathology, 2Medical Imaging Research Institute, Binzhou Medical University, Yantai, Shandong, China; 3Medical Imaging Research Institute, Binzhou Medical University, Yantai, Shandong, China

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Abstract: Increasing evidence indicates that autophagy plays an important role in regulating proliferation and apoptosis in several human diseases, including cancer. Beclin1 is the first tumor-suppressor gene in mammals involved in the regulation of autophagy. However, the function of Beclin1 in oral cancer cells is not clear at present. We investigated the effects of Beclin1 on the biological characteristics of oral carcinoma cells by Small interfering RNA (siRNA) technology. We found that Beclin1 silencing promoted proliferation, migration, and invasion of oral squamous cell carcinoma cells, and inhibited apoptosis primarily associated with upregulation of survivin and bcl-2. Further, Beclin1 silencing enhanced chemosensitivity to cisplatin. Thus, the Beclin1 gene is expected to become a new therapeutic target for oral cancer.

Keywords: Beclin1, oral squamous cell carcinoma, chemosensitivity, apoptosis

Introduction
Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the maxillofacial region. Comprehensive treatment based on surgery and chemoradiotherapy is the main therapeutic scheme. Even with advances in cancer treatment, the 5-year survival rate has remained at approximately 50% for the last few decades [1]. Postoperative recurrence and metastasis, and poor chemosensitivity are the main reasons for treatment failure. Thus, it is imminent to explore the mechanism of recurrence and metastasis and how to improve the sensitivity of chemotherapy. The most notable characteristic of malignant tumors is that tumor cells can sustain survival and unlimited growth by self-protecting, evading apoptosis, and avoiding stimulation [2, 3]. Studies have shown that autophagy plays an important role in adapting to low nutrition/hypoxia, tolerating external stimulation, and inducing chemotherapy resistance in tumor cells [4, 5].

Autophagy is considered a self-digestion process, a highly conservative biological behavior of eukaryotic cells, and is an important mechanism to self-protection. Autophagy may maintain the balance of metabolism and survival by recycling and depredating damaged organelles and biological macromolecules in the cytoplasm to alleviate stress [6]. Autophagy is regulated by multiple signaling pathways and genes, of which Beclin1 is the first mammalian tumor suppressor gene to participate in autophagy regulation [7]. While recent studies have shown that Beclin1 plays a dual role in the process cancer process, it is dependent on the type and development stage of the tumor [8, 9]. Our previous study [10] has shown that Beclin1 may inhibit tumor occurrence in oral precancerous lesions by downregulating autophagy, which may remove distorted cells by inducing apoptosis or autophagic death. In infiltrating oral carcinoma, increased expression of Beclin1 may promote the development of cancer by upregulating autophagy. The high expression of Beclin1 is closely related to tumor size (diameter ≥ 25 mm), lymph node metastasis (positive > negative), and the clinical TNM stage (I/II > III/IV).
We aimed to explore the effects of Beclin1 on proliferation, invasion, apoptosis, and chemosensitivity, which is promising to provide a new target for the treatment of OSCC.

**Materials and methods**

**Cell culture and reagents**

The human oral squamous cell lines KB (purchased from the cell banks of the Chinese Academy of Science) and CAL-27 (a gift from Dr. Zhennan Gao of Shandong Provincial Key Laboratory of Oral Tissue Regeneration) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose (Gibco Industries, Inc., South America) with 10% fetal bovine serum (FBS, Gibco Industries, Inc., USA) at 37°C in a humidified atmosphere with 5% CO₂.

Three small interfering RNAs (siRNA) against Beclin1 were designed and synthesized by GenePharma (Shanghai, China): siBECN1-358 5'-GGAGCCAUUUAUUGAAACUTT-3' 5'-AGUUUC-AUAAAUUGCCUCCTT-3'; siBECN1-1052 5'-GUG-GAAUGGAAUGAGOAUUATT-3' 5'-UAAUCUCAUUCGACUUCCACTT-3'; siBECN1-1204 5'-GCGGCUGUUUACUGUUCCTT-3' 5'-AGAACAGUAUAACGGCACTT-3'; Negative control 5'-UUCUCCGAACGUGACACGUTT-3' 5'-ACGUGACACGUUCGGAGAA-3'. KB and CAL-27 cells were seeded in six-well plates and transfected with 100 pmol siRNA using lipfectamine 2000 (Thermo Fisher scientific, Massachusetts, USA). Beclin1 expression was detected using Real-time RCR (qPCR) and western blot to conform transfection efficiency. Of the three siRNAs, we selected the one with the most efficient silence sequence.

**Real-time PCR analysis**

Total RNA was isolated with RNAiso plus reagent (Takara BIO Inc., Dalian, China), and cDNA was generated with a Prime Script™ RT reagent kit with gDNA Eraser (Takara BIO Inc.) according to the manufacturer’s instructions. Quantitative gene expression was performed for Beclin1 and GAPDH (internal control) by LightCycler 480 SYBR Green I Master Mix Reagent Kit and the LightCycler 480 Real-time System (Roche, Co., Germany). Nucleotide sequences of specific primer for genes were as follows: Beclin1 forward 5’-ATGCAGGTGAGCTTCGTGTG-3’ and reverse 5’-GGA-GATTGGTGATGGGATTC-3’; GAPDH forward 5’-GGA-GTGAAAGTCGGAGTC-3’ and reverse 5’-GAA-GATGATATGAGTGATTTC-3’. For each sample, the data was normalized to GAPDH to obtain ΔCT and calculated using the 2^ΔΔCT method. The following formula for gene silencing was used: gene silencing rate (%) = (1-2^-ΔΔCT) × 100%. According to the data, we determined the best efficiency for the silence sequence to perform the following experiments.

**Cell proliferation assay**

Cell proliferation in KB and CAL-27 cells was detected using Cell Counting Kit-8 (CCK-8, Biosharp, Hefei, China). Cells were seeded in a 96-well plate at a density of 5 × 10³ cells/well and incubated for 0, 24, and 48 h, respectively. CCK-8 solution was added to each well according to the manufacturer’s instructions. Cells were incubated for 1 h, and subsequently the optical density was measured using an automatic microplate reader (TECAN, Switzerland) at 450 nm to determine the number of viable cells. Cell viability was calculated based on OD values using the following equation: cell viability = OD_test group /OD_control group × 100%. Experiments were performed in triplicate.

**Colony formation assay**

Cells were seeded into six-well plates (5 × 10³ cells/well) 24 h after transfection and incubated with 10% FBS DMEM for 12 d. We changed the new medium every other 3 to 4 d and when clones were visible on the plates the culture was terminated. Cells were stained with Giemsa (Solarbio, Beijing, China), and counted the colony numbers for more than 50 cells. Five random unduplicated fields were analyzed for each well and each experiment was performed in triplicate. The colony formation rate was calculated as follows: colony formation rate = colony number/seeded cells × 100%.

**Wound scratch assay**

To detect migration ability, we performed the wound scratch assay. 5 × 10³ cells were seeded into six-well plates and cultured for 24 h. We made horizontal lines evenly with a marker pen in advance at the bottom of the well for location. In order to eliminate the effect of cell proliferation, 10 μg/ml of mitomycin C was placed...
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for 1 h before processing. With 20 μl pipette tips perpendicular to where the horizontal lines were scratched, the wells were washed with phosphate buffer saline (PBS) three times to remove the scratched-out cells, and incubated with 1% FBS DMEM. We took pictures after 0, 12, and 24 h, and measured the scratch healing ability of the cells with an Image-Pro Plus 6.0 system (Media Cybernetics, Inc., Rockville, MD, USA). Each experiment was performed in triplicate.

**Cell invasion assay**

Matrigel was coated in the upper chambers, and 5 × 10⁴ cells were resuspended in 200 μl serum-free DMEM medium, which was placed in the upper portion of a Transwell chamber with 8 mm pores (Corning Incorporated, New York, USA). The lower portion of the chamber contained 500 μl of 10% FBS DMEM medium. The cells were cultured at 37°C in 5% CO₂ for 24 h. After erasing the cells in the upper chambers with a swab, cells traversing the Matrigel were stained with 0.1% crystal violet (Solarbio, Beijing, China) according to the manufacturer’s instructions, and photographed under ×100 magnifications for counting using Cytometry. Each experiment was performed in triplicate.

**Apoptosis assay**

Apoptosis was detected by the Annexin V method. In brief, cells were collected with no-Ethylenediaminetetraacetic acid (EDTA) of trypsin digestion 48 h after transfection. A suspension of 1 × 10⁶ cells/ml was made by 1 × Binding Buffer. Next, Annexin V-fluorescein isothiocyanate (FITC) 5 μl was mixed, then 5 μl Propidium Iodide (PI) was added and blended. The mixture was placed at room temperature (20 to 25°C) for 15 min, paying attention to avoid light, and detected using a flow cytometry instrument within 1 h. Each experiment was performed in triplicate.

**Chemosensitivity assay**

Cells were treated with different concentrations of cisplatin for 24 h after transfection. 0, 0.01, 0.03, 0.05, 0.07, and 0.1 mg/ml of cisplatin were used to medicate different groups and cells were stained with Giemsa (Solarbio) to estimate the median lethal dose (LD50). The Annexin V method was used to detect necrosis and apoptosis and the CCK-8 assay was used to detect numbers of viable cells according to the above steps. Each experiment was performed in triplicate.

**Western blot analysis**

Cellular proteins were collected 72 h after transfection and lysed with RIPA lysis buffer and phenylmethanesulfonil fluoride (PMSF) (Beyotime, Shanghai, China). Protein concentration was detected with a BCA Protein Assay Kit (Beyotime) with a loading amount of 30 μg/well. The samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to sodium dodecyl sulfate polyacrylamide gel electrophoresis (PVDF) membranes. Membranes were blocked with 5% nonfat milk in PBST buffer (phosphate-buffered saline, PBS+0.1% Tween 20) for 1-2 h, then incubated with primary antibody overnight at 4°C and secondary antibodies for 1 h at room temperature. Primary antibodies used were as follows: Beclin1, mTOR, LC3B (Abcam, Cambridge, England); E-cadherin, vimentin, survivin, GAPDH (Santa Cruz Biotechnology, Inc. California, USA); bcl-2, and bax (Zhongshan, Beijing, China). Protein bands were visualized by enhanced ECL Chemiluminescence (Beyotime). Integral optical density (IOD) values measured with the Image-Pro Plus 6.0 system was used to calculate the relative expression of the target protein (ratio) with the following equation: ratio = IODtarget protein / IODGAPDH.

**Statistical analysis**

Data were analyzed using SPSS version 19.0 statistical software (SPSS Inc., Chicago, IL, USA). All values are presented as the mean ± standard deviation. The student’s t test or Analysis of Variance (ANOVA) was used to analyze data. A p-value of less than 0.05 was considered statistically significant.

**Results**

**Acquisition of the most efficient silence sequence**

Cellular RNA (at 48 h) and protein (at 72 h) were collected after transfection. According to the results of real-time PCR and western blot, the
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most efficient silence sequence was SiBECN1-1052, with a rate of gene silencing greater than 90% (Figure 1). We performed the following experiments using SiBECN1-1052 as the Beclin1 silencing sequence.

Silence of Beclin1 promotes cell proliferation, migration, and invasion in OSCC cells

To investigate the effects of Beclin1 on cell proliferation, the CCK-8 assay and colony formation assay were performed and a growth curve generated. The proliferation of KB and CAL-27 cells infected with siRNA-Beclin1 significantly improved relative to the negative control group (NC), especially at 48 h after transfection (P < 0.05). Interestingly, the colony formation assay showed that the colony-forming ability of KB and CAL-27 cells was suppressed as downregulation of Beclin1 (P < 0.05; Figure 2A). The wound scratch assay (also called the wound healing assay) was designed to detect cell migration ability. At 12 h after transfection, there was no obvious difference between the SiBECN1 and NC groups; but at 24 h, the cell healing ability in the SiBECN1 group was significantly faster than the NC group (P < 0.05; Figure 2B). To further evaluate the effect of Beclin1 on cell invasion, Transwell chambers coated with Matrigel similar to the structure of the basement membrane were used. The results showed that downregulation of Beclin 1 significantly facilitated invasion through the Matrigel (P < 0.05; Figure 2C). Meanwhile, we detected the expression of E-cadherin and vimentin, which was considered the classical immunophenotype of epithelial-mesenchymal transition (EMT). E-cadherin expression decreased and vimentin increased when Beclin1 was silenced relative to the NC group (P < 0.05; Figure 2D), which may be the substantial mechanism for the involvement of Beclin1 in cell invasion. These results showed that cell proliferation, migration, and invasion activity were dramatically improved when Beclin1 was inhibited.

Apoptosis was inhibited when Beclin1 was silenced and bcl-2 and survivin were involved

In order to investigate whether Beclin1 silencing affects apoptosis and the relationship between autophagy and apoptosis in OSCC cells, we detected apoptosis with flow cytometry, protein expression of autophagy related genes (mTOR, LC3B), and apoptosis related genes (bcl-2, surviving, and bax) by western blot. We
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A

B

C

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found that when Beclin1 was silenced, apoptosis decreased significantly relative to the NC group ($P < 0.05$; Figure 3A). LC3B, considered being an autophagy marker protein, decreased, while mTOR expression had no obvious change. Bcl-2 and survivin, as apoptosis suppressors, increased dramatically ($P < 0.05$), and bax (an apoptosis promoter) had no obvious change (Figure 3B).

**Beclin1 silencing enhanced chemosensitivity in OSCC cells**

The LD50 is a good indicator to explore the chemosensitivity for tumor cells. We used gradient concentrations of Cisplatin (0, 0.01, 0.03, 0.05, 0.07, and 0.1 mg/ml) to treat OSCC cells. The LD50 of the blank control and NC group was 0.05 mg/ml of Cisplatin; while when Beclin1 was silenced, OSCC cells were killed more easily, with a LD50 of 0.03 mg/ml according to the Giemsa stain. Then, we detected viable cells using a CCK-8 kit and found that with the downregulation of Beclin1 cell viability decreased dramatically, especially when treated with 0.03 mg/ml Cisplatin ($P < 0.05$; Figure 4).

We further tested apoptosis with the Annexin V method using flow cytometry, and calculated the ratio of lethal cells (sum of apoptosis and necrosis (Q1+Q2+Q4)) to represent the effects of Cisplatin. The results showed that the lethal cells increased significantly after Beclin1 was downregulated, especially when treated with 0.03 mg/ml Cisplatin, which was in agreement with the results of the CCK-8 assay.

**Discussion**

The notable characteristics of malignant tumors are that tumor cells exert all efforts to maintain homeostasis and unlimited growth by protecting themselves, escaping apoptosis, and avoiding stimulation [2, 3]. Studies have shown that autophagy acting as “self-digestion” can protect tumor cells from external stimuli, low nutrition/hypoxia, and damage from chemotherapy drugs. Autophagy is regulated by varieties of signaling pathways and genes, which includes classical autophagy (also called Beclin1-dependent autophagy) and non-classical autophagy (also called Beclin1-independent autophagy) [11]. Studies have shown that autophagy is a double-edged sword that plays a dual role in cancer, including oral cancer, which depends on the type of tumor and the stage of cancer development [8, 12-14]. This study mainly investigated the roles of Beclin1 on the biological behavior of OSCC cells.

Beclin1 (also called BECN1), located on chromosome 17q21, is the first tumor suppressor gene involved in the regulation of autophagy in mammals, and is a homologous gene of yeast autophagy gene ATG6. Some studies have indi-
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Figure 3. Apoptosis was inhibited when Beclin1 was silenced and apoptosis related proteins were involved. A. Cell apoptosis was detected using flow cytometry. B. Protein expression of autophagy-related genes (mTOR, LC3B) and apoptosis related genes (bcl-2, survivin, and bax) were detected by western blot (*P < 0.05).

cated that a higher activity of autophagy regulated by Beclin1 promotes the progress of malignant tumors, such as endometrioid adenocarcinoma, and colorectal adenocarcinoma [15, 16]. There have been some reports showing that suppression of autophagy promotes the development of tumors, such as breast cancer, gastric cancer, and liver cancer [17-19]. Thus far, the roles of Beclin1 in oral cancer have remained poorly understood. Tang et al. have reported that the dual expression of Beclin1 and ATG5 may be an adverse prognostic indicator for OSCC [20]. Liu et al. considered that overexpression of Beclin1 might inhibit or improve proliferation and invasion of OSCC cells [21]. Our previous study has shown that downregulation in dysplasia might suppress carcinogenesis in oral cancer, while increased expression of Beclin1 might accelerate the development of OSCC for infiltrating cancers [10].

We further investigated the role of Beclin1 using siRNA in OSCC cell lines. We found that with downregulation of Beclin1, LC3B as a marker of autophagy decreased, which showed
the positive regulation of Beclin1 to autophagy. But there was no obvious variation in mTOR, though it is reported that mTOR may inhibit Beclin1. So, there may be no feedback from Beclin1 to mTOR in the regulation of autophagy in oral cancer. The growth curve from the CCK-8 assay showed that cell proliferation was enhanced after 48 h of Beclin1 silencing; but interestingly, its colony forming ability decreased. Colony forming ability is the process of detecting cell division and proliferation of a single cancer cell, which reflects two important characters of cells: population dependence and proliferation. The inconsistent results showed that proliferation of oral cancer cells was not only dependent on self-division ability, but more importantly on the interaction of cells and the surrounding environment (population dependence). This phenomenon suggests that interference of the surrounding environment appears to be more important than only killing cancer cells during oral cancer therapy. We also investigated the effect of Beclin1 on migration and invasion of OSCC cells by the wound healing assay and the Transwell assay. Migration ability was enhanced after Beclin1 silencing, and the scratch healing rate was significantly faster than in the NC group. There were significantly more cells traversing through the basement membrane in the Beclin1 reduced group than the NC group, which has shown that Beclin1 silencing might strengthen the ability of cell invasion. In recent years, EMT has been shown to be an important mechanism for cancer cells to facilitate invasion and metastasis [22, 23]. Epithelial cells transform from a polygon to fusiform shape gradually in morphology and with the temporary absence of immune phenotype, epithelial cells obtain the movement ability phenotype of mesenchymal cells. For example, the cell adhesion protein E-cadherin was weakened or even lost, while vimentin expression was enhanced. Some studies have shown a close relationship between autophagy and EMT, with the functional interaction between the cytoskeleton and mitochondria being a crucial regulatory center at the crossroads between these two biological processes in cancer [24, 25]. Western blot was used to detect the expression of the EMT related immunophenotype in this study; we found that with the loss of Beclin1, E-cadherin expression decreased and vimentin increased, which suggested EMT to be one of the most important mechanisms for Beclin1 silencing to promote migration and invasion in OSCC cells.

In the past, autophagy and apoptosis were considered two ways of programmed cell death. Currently, autophagy is considered a method of cell survival, different from apoptosis in terms of morphology, biochemistry, molecular mechanisms, and biological significance. Studies in recent years have suggested that there was mutual antagonism or promotion between autophagy and apoptosis, even coexisting or occurring in the same cell [26, 27]. Autophagy can prevent diffusion of proapoptotic factors or
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apoptosis-inducing factors separating from the mitochondria to help escape apoptosis of tumor cells. Thus far, the effect on autophagy and apoptosis of Beclin1 in the tumor has been controversial. In this study, we detected the effect of Beclin1 on apoptosis in OSCC cells, and found that the apoptosis rate decreased significantly after Beclin1 silencing. The expression of bcl-2 and survivin protein increased in OSCC cells by western blot, with no obvious change in bax expression. When Beclin1 was silenced, the combination of the BH3 domain with bcl-2 decreased, bcl-2 was separated, strengthening its antiapoptotic function, and inhibiting cell apoptosis. Further, the upregulation of survivin might act synergistically on resistance to apoptosis. Bax might not have interaction with Beclin1.

Poor chemosensitivity is the difficulty in treatment efficacy against malignant tumors. Improving the sensitivity of chemotherapy and overcoming chemotherapy resistance has always been an area of great interest in research. Malignant tumor cells treated by chemotherapy in oral cancer have escaped necrosis through autophagy [8]. Autophagy can maintain the stability of DNA and the internal environment to prevent mutation and inhibit carcinogenesis [12]. On the other hand, autophagy can maintain cell survival and prevent damage from chemotherapy, inducing chemotherapy resistance. Study had shown that after tumor cells are processed with 3-methyladenine (a well-known inhibitor of Beclin1), autophagy marker proteins LC3 and P62 decreased and the cells were led to apoptosis [28]. In this study, OSCC cells were treated by a gradient concentration of cisplatin (0, 0.01, 0.03, 0.05, 0.07, and 0.1 mg/ml); and cells of Beclin1 silencing were more sensitive to cisplatin. The LD50 was downgraded from 0.05 mg/ml to 0.03 mg/ml of cisplatin. With the drop of cisplatin concentration, apoptosis and necrosis rates increased significantly; live cells decreased, suggesting that cisplatin sensitivity of OSCC cells improved due to downregulation of Beclin1. Thus, inhibiting autophagy may serve as a new therapeutic strategy, by directly or indirectly inducing apoptosis or necrosis in tumor cells, especially in chemotherapy-resistant cancer cells including OSCC.

In summary, Beclin1 regulates the biological characteristics of OSCC cells; its effects depend not only on its own characteristics, but even more on tumor stage and the microenvironment. In addition, Beclin1 silencing makes OSCC cells more sensitive to cisplatin. Thus, Beclin1 is expected to become a new therapeutic target for cancer. More research is required to explore the appropriate indications and dosage.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Peiyuan Wang, Medical Imaging Research Institute, Binzhou Medical University, 346# Guanhai Road, Yantai 264003, Shandong, China. Tel: +86-535-6913215; Fax: +86-535-6913016; E-mail: wangpeiyuan1640@163.com; Xia Wang, Department of Pathology, Binzhou Medical University, 346# Guanhai Road, Yantai 264003, Shandong, China. E-mail: wanxia7512@163.com

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