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Beclin1 enhances cisplatin-induced apoptosis via Bcl-2-modulated autophagy in laryngeal carcinoma cells Hep-2

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To investigate the role of Beclin1 in cisplatin-induced apoptosis in laryngeal carcinoma cells Hep-2 and to explore the potential mechanism. We up-regulated Beclin1 expression in Hep-2 cells. The survival rate and apoptotic rate were evaluated by MTT and flow cytometry (FCM). The Beclin1 overexpression group and the control group were treated with cisplatin for 24 hours. The proliferation and cell apoptosis of laryngeal cancer cell lines were evaluated. The mitochondrial membrane potentials were detected by DiOC6(3). Activities of Caspase-8/9/3 and convention of microtubule-associated protein one light chain 3 (LC3) were detected by western blot. The effect of Bcl-2 overexpression on increased cisplatin-sensitivity and autophagy induced by Beclin1 was investigated using Bcl-2 cDNA transfection. Expression of Beclin1 in Hep-2 cells was meaningfully enhanced by transfection, and the proliferation and the apoptosis were not considerably affected. By cisplatin treatment, the Beclin1 overexpression group showed lower survival rate and higher apoptotic rate than the control group ($p<0.05$). Decrease of mitochondrial membrane potential and increase of activities of Caspase-8, Caspase-9 and Caspase-3 were detected. Beclin1 overexpression increase the convention of LC3, especially after the cisplatin treatment. Overexpression of Bcl-2 decreased the cisplatin-induced apoptosis and inhibited Beclin1-induced autophagy. In conclusion, Beclin1 enhances cisplatin-induced apoptosis in laryngeal carcinoma cells Hep-2 via Bcl-2 modulated autophagy.

Key words: laryngeal carcinoma, Beclin1, cisplatin, autophagy, Bcl-2

Laryngeal carcinoma, classified as squamous cell carcinoma (LSCC) in 90% of the cases, is one of the most common malignant tumors of head and neck region in the world [1]. Despite substantial improvements in the diagnosis and treatment of laryngeal carcinoma during the last decades, no significant increase in the long-term survival rate has been achieved [2]. Five-year survival rate is about 63% in America [3]. New treatment methods that provide more efficient results are highly desirable. Novel targets that can serve as the foundation of biologically based therapeutic strategies are needed.

Autophagy is important for maintaining the stability of cells and some physiological functions of cells, such as meeting the requirement of metabolism and updating some organelles under a physiological or pathological situation [4–6]. Autophagy is rapidly induced during nutritional deficiency, oxidative stress, infection or ischemia-reperfusion [7–8]. It has been found that autophagy has both promoting and suppressive effects in different stages of the cancer development [9–10]. Ongoing studies aim at finding the optimal means to regulate autophagy in cancer prevention and treatment. In our previous study [11], we detected the expression of Beclin1 in laryngeal cancer specimens. The results showed that Beclin1 expression in LSCC was significantly lower compared to the para-carcinoma tissue. The expression of Beclin1 in tumor tissues was significantly lower than in the non-tumor tissues. Reduced Beclin1 expression was significantly correlated with lymph node metastases. Kaplan–Meier survival estimates showed a significant correlation between Beclin1 expression and patient's survival rate. Thus, the expression level of Beclin1 is closely associated with the laryngeal cancer cell's ability of invasion and metastasis. So we detect the influence of Beclin1 expression on cisplatin sensitivity in laryngeal carcinoma cells Hep-2 and explore the potential mechanism.

Materials and methods

Cell lines and reagents. The human laryngeal carcinoma cells Hep-2 were kindly provided by the Department of Otolaryngology and Head and Neck Surgery, Xiangya

Second Hospital of Central South University (Changsha, China). All cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, USA) media supplemented with 10% fetal bovine serum (Hyclone, Logan, USA) and penicillin/streptomycin, and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Cisplatin was purchased from Blue skies biological preparation Co., Ltd. Drugs were dissolved in DMSO in appropriate concentration and stored at −20 °C until used. The following antibodies were purchased from the indicated sources: Rabbit anti-Beclin1 polyclonal antibody, rabbit anti-Caspase-9 polyclonal antibody, rabbit anti-Caspase-8 polyclonal antibody and rabbit anti-Caspase-3 polyclonal antibody (Sigma co., United States), mouse anti-Bcl-2 polyclonal antibody, mouse anti-LC polyclonal antibody (Santa Cruz, CA, United States). Cell viability was determined by using 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazoliumbromide (MTT) assays. Cells (5 000 cells per well) were plated in 96-well plates. After the indicated cisplatin treatment dose (cisplatin doses were from 0 to 10 μg/mL) for 24 hours, cells were incubated for 2–3 hours with 0.5 mg/mL of MTT (Sigma, USA) and lysed with dimethyl sulfoxide (DMSO). The optical density (OD) of each well was measured at 570 nm with a microplate reader. Inhibit rate = $(OD_{control} - OD_{treat})/OD_{control}$.

Apoptosis assessment by Annexin V/PI staining. Levels of cisplatin-mediated apoptosis were determined by using Annexin V/PI staining kit. After the indicated cisplatin treatment or time points, cells were harvested. Cell pellets were suspended in 500 μL binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 2.5 mM $CaCl_2$) at the density of 1×10⁶/mL. Samples were incubated with 1 μL Annexin V-FITC and 5 μL PI for exactly 5 min at room temperature in the dark and then measured on a FACSort flow cytometer (Becton Dickinson, USA). Annexin V-FITC and PI fluorescence were detected in the FL-1 (green) and FL-2 (red) channels respectively, after correction of the spectral overlap between the two channels. Data were analyzed by using CellQuest software.

Beclin1 cDNA transfection. Beclin1-expression vector (pcDNA3.1-Beclin1) and pcDNA3.1 vectors were purchased from Beyotime Institute of Biotechnology China. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer`s instructions. In brief, cells were seeded into wells of a 6-well plate at 10^5 cells/cm^2 and cultured for 24 hours until 60–70% confluence. Then pcDNA3.1-Beclin1 or negative pcDNA3.1 vector (4 μL) was diluted with 100 μL OPTI-MEM (Invitrogen) for 5 min at room temperature. During the incubation period, 5 μL Lipofectamine 2000 (Invitrogen) was diluted in 100 μL OPTI-MEM. These two mixtures were combined, mixed gently, and incubated for 20 min at room temperature for complex formation. This 200 μL mixture was then added to cells. After 48 hours of incubation, transfected cells could be observed by the fluorescence microscope. Transfected cells were selected in cell culture medium containing 700 μg/mL G418 (Invitrogen). After 2 or 3 weeks, mixtures of all the selection resistant cell clones were seeded into wells of a 6-well plate and cultured.

Western blot analysis. Total protein lysates were harvested from tissue specimens and cell lines by lysis buffer (20 mM Na_2PO_4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 100 mM NaF, and 2 mM Na_3VO_4). Proteins (50 μg) were separated by polyacrylamide gel electrophoresis (SDS-PAGE) on a sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Membranes were incubated with the first antibody. After 2 hours, the membranes were washed with Tris Buffered Saline and Tween-20 (TBST) three times and then the membranes were incubated with horseradish peroxidase conjugated rat anti-rabbit secondary antibody. Finally, proteins were detected by enhanced chemiluminescence (ECL) procedure.

Assessment of mitochondrial transmembrane potential. Assessment of mitochondrial transmembrane potential D ym by the cationic lipophilic fluorochrome 3,3'-dihexyloxacarbo-cyanide iodide ($DiOC_6(3)$) (460 ng/mL, Molecular Probes, Eugene, OR, USA) was used to measure the mitochondrial transmembrane potential. Cells were treated for 12 hours with TRAIL (100 ng/mL), then incubated for 30 min at 37.8 °C in complete media with DiOC6, washed twice with PBS and analyzed in a FACSort flow cytometer.

Bcl-2 cDNA transfection. Bcl-2-expression vector (pcDNA3.1-Bcl-2) and pcDNA3.1 vector were purchased from Beyotime Institute of Biotechnology China. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer`s instructions. Transfected cells could be observed by fluorescence microscope.

Statistical analysis. All statistical analyses were performed using Supplementary Power Supply Set (SPSS) version 16.0 software, with the results expressed as mean±SD as appropriate. Results were considered statistically significant at $p<0.05$ obtained with t test.

Results

Beclin1 expression level was enhanced without effect on proliferation and apoptosis. To determine the influence of pcDNA3.1-Beclin1 transfection on Hep-2 cells, western blot was performed to analyze Beclin1 protein level. As shown in Figure 1, the expression of Beclin1 in Hep-2 cells was improved by pcDNA3.1-Beclin1 transfection effectively. PcDNA3.1-Beclin1 transfection group showed a higher expression level of Beclin1, compared to blank vector control group and Hep-2 cells group ($p<0.05$). After comparing the results of 24 hours, 48 hours and 72 hours respectively, we found that Beclin-1 overexpression had no obvious effect on the OD value and the apoptotic rate ($p>0.05$) by MTT and FCM (Figure 2).

Beclin1 overexpression enhanced cisplatin sensitivity. The inhibition rate was determined by MTT after the treat-

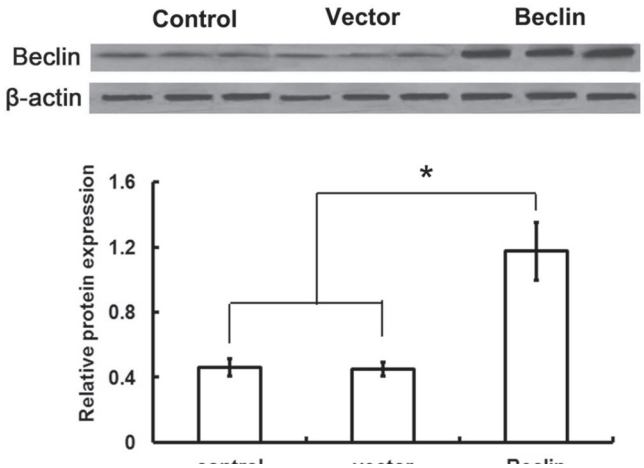

Figure 1. Beclin1 expression level was enhanced. A) Protein expression level of Beclin1 was assessed by western blot after pcDNA3.1-Beclin1 transfection to Hep-2 cells. B) PcDNA3.1-Beclin1 transfection group showed higher relative protein expression level of Beclin1 compared to blank vector control group and Hep-2 cells group ($p<0.05$). (Control: control group, Vector: blank vector group, Beclin: pcDNA3.1-Beclin1 transfection group).

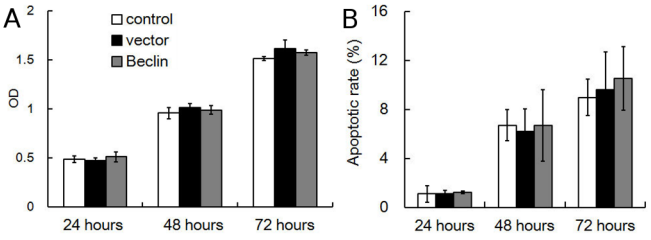

Figure 2. Beclin1 overexpression did not effect the proliferation and apoptosis of laryngeal carcinoma cells. A) The proliferation of Hep-2 cell was assessed by MTT. B) The apoptotic rate was assessed by FCM. Apoptotic ratio was determined by percentage of Annexin V+ PI- cells. OD value and apoptotic rate did not differ among the three cell groups statistically ($p>0.05$).

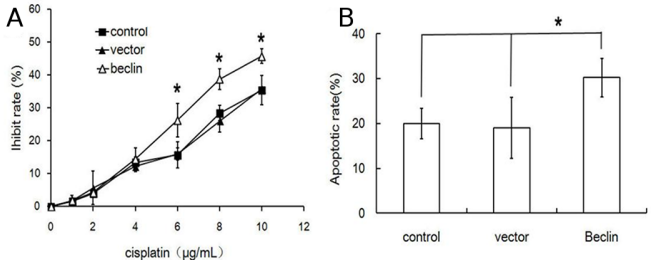

Figure 3. Beclin1 overexpression enhanced cisplatin-induced apoptosis. A) The inhibition rate was assessed by MTT after different concentrations of cisplatin treatment. The inhibition rate of Beclin1 overexpression group was higher than in the two other groups when cells were treated with cisplatin of 6 μg/mL, 8 μg/mL and 10 μg/mL. B) The apoptotic rate of Beclin1 overexpression cells was significantly higher than that in the other groups (*$p<0.05$).

ment with different concentrations of cisplatin (0 μg/mL, 1 μg/mL, 2 μg/mL, 4 μg/mL, 6 μg/mL, 8 μg/mL, 10 μg/mL) for 24 hours. The inhibition rate of Beclin1 in overexpression group was higher than in the two other groups when cells were treated with cisplatin of 6 μg/mL, 8 μg/mL and 10 μg/mL ($p<0.05$). However, in the control group and blank vector group there is no statistical difference ($p>0.05$) (Figure 3A). We further analyzed the apoptosis rate by using FCM to confirm whether the differences in cell death were caused by different apoptotic responses. We assessed the apoptotic rate after the treatment with 10 μg/ml cisplatin for 24 hours. The apoptotic rate was 30.25±4.272% in Beclin1 overexpression cells, which was significantly higher than that in the control group (20±3.367%) and the vector group (19±6.782 %) ($p<0.05$) (Figure 3B). As a result, the Beclin1 overexpression could enhance cisplatin sensitivity in laryngeal carcinoma cells.

Beclin1 overexpression induced Caspase cleavage and mitochondrial depolarization. To elucidate the molecular mechanism responsible for Beclin1-induced cisplatin sensitivity, we examined the effect of Beclin1 on the cisplatin-induced apoptotic pathway. Analysis of Caspase activation showed that the cleaved and mature forms of Caspase-8 (p43/41), Caspase-9 (p35) and Caspase-3 (p17, p10) were generated in cells during 10 μg/ml cisplatin for 24 hours (Figure 4A). Beclin 1 overexpression cells showed more Caspase activation after cisplatin treatment than the other two cell lines. This observation indicated that Beclin1 enhanced cisplatin-induced Caspase cleavage. As shown in Figure 4B, the DiOC6 (3) negative cell rate was lower in blank vector group and control group compared to the Beclin1 overexpression group during cisplatin treatment. The DiOC6 (3) negative cell indicated more mitochondrial depolarization. This result suggested that mitochondrial depolarization was enhanced by Beclin1 overexpression ($p<0.05$). This finding

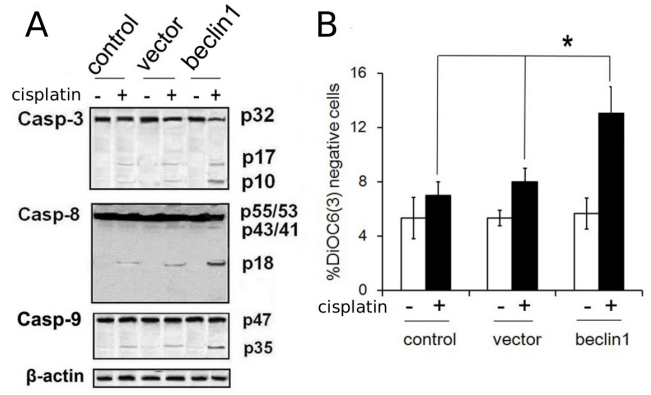

Figure 4. Beclin1 overexpression induced caspase cleavage and mitochondrial depolarization during cisplatin treatment. A) Cleavage of caspases was detected by western blot. Beclin 1 overexpression group showed more caspase cleavage after the cisplatin treatment (10 μg/mL cisplatin, for 24 h treatment) than the two other groups. B) The mitochondrial membrane potential was detected by DiOC6(3). The DiOC6 (3) negative cell rate in Beclin1 overexpression group was higher than that in the two other groups during cisplatin treatment (*$p<0.05$). (control: control group, vector: blank vector group, Beclin1: Beclin1 overexpression group).

suggested that Beclin1, an autophagy gene, could induce the apoptosis of laryngeal carcinoma cancer cells in a mitochondrial-dependent mechanism.

Beclin1 overexpression enhanced cisplatin-induced apoptosis via Bcl-2 modulated autophagy. Beclin1 is an important factor of autophagy [12–13], therefore, we hypothesized that the Beclin1 overexpression enhanced cisplatin-induced apoptosis via autophagy. To evaluate the autophagy induced by Beclin1, we observed the conversion of microtubule-associated protein 1 light chain 3 (LC3) in cells treated by cisplatin via western blot. An attenuation of LC3-I and an increase of LC3-II were observed in Beclin1 overexpression cells. With the cisplatin treatment, conversion of LC3 was detected in all cell groups, and was most obvious in Beclin1 overexpression cells (Figure 5). Bcl-2 binds to Beclin1 and inhibits conventional autophagy pathway in some conditions [14]. Therefore, we verified whether Bcl-2 overexpression inhibits biological behavior of Beclin1. We found that overexpression of Beclin 1 decreases the protein level of Bcl-2 (Figure 6A). As shown in Figure 6B, pcDNA3.1-Bcl-2 transfection decreased apoptotic rate in three groups. Moreover, we found that Bcl-2 overexpression might inhibit cisplatin-induced apoptosis more obviously in Beclin-1 overexpression Hep-2 cells (the p-value in control cell group and vector control group <0.05, the p-value in Beclin1 overexpression group <0.01). Bcl-2 overexpression decreased LC3 conversion in Beclin1 overexpression cells, and more obviously after cisplatin treatment (Figure 6C, D). This result confirms that Beclin1 overexpression enhanced cisplatin-induced apoptosis via Bcl-2 modulated autophagy.

Discussion

Some studies have shown that inducing apoptosis of cancer cells can assist tumor treatment. However, several mutations in apoptosis-induced genes of cancer cells inhibit the normal apoptosis induction. In recent years, some studies have shown that many antitumor drugs can kill tumor cells effectively via overexpression of Beclin1, and that the overexpression of Beclin1 is a promising candidate for cancer therapy via induction of autophagy [15]. Beclin1, the homolog of the yeast Apg6 gene [16], is the first founded operational gene which takes part in autophagy [17]. The Beclin1 protein contains four functional structure domains: Bcl-2–homology-3 (BH3), central coiled coil domain (CCD), evolutionarily conserved domain (ECD) and nuclear output structure domain [18]. These domains can form "Beclin1 Autophagy Complex" by combining with multiple proteins to execute related functions. In addition, Beclin1 involves an interaction with an apoptotic pathway which leads to death of these cancer cells [19]. Therefore, Beclin1 may become a new cancer suppressor gene as a specific gene for autophagy in mammals [20–22], but the mechanism is unclear. In this study, it is for the first time that we have observed the influ-

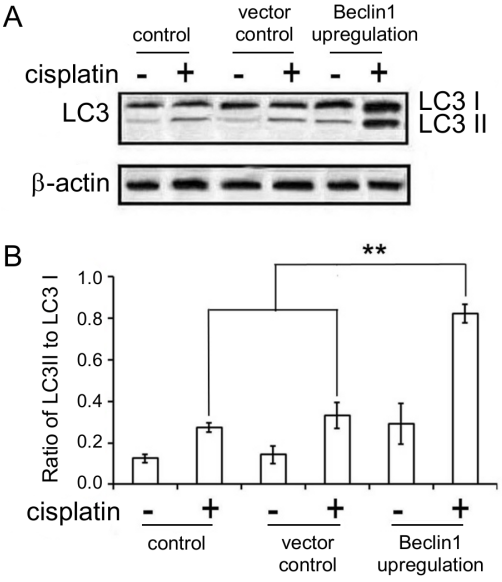

Figure 5. Beclin1 overexpression enhanced cisplatin-induced autophagy. A) The conversion of LC3 in cells treated by cisplatin was detected via western blot. With the cisplatin treatment, an attenuation of LC3-I and increase of LC3-II were observed in three cell groups, and were most obvious in Beclin1 overexpression cells. B) Ratio of LC3 II to LC3 I. The experiments were repeated four times. (p<0.01).**

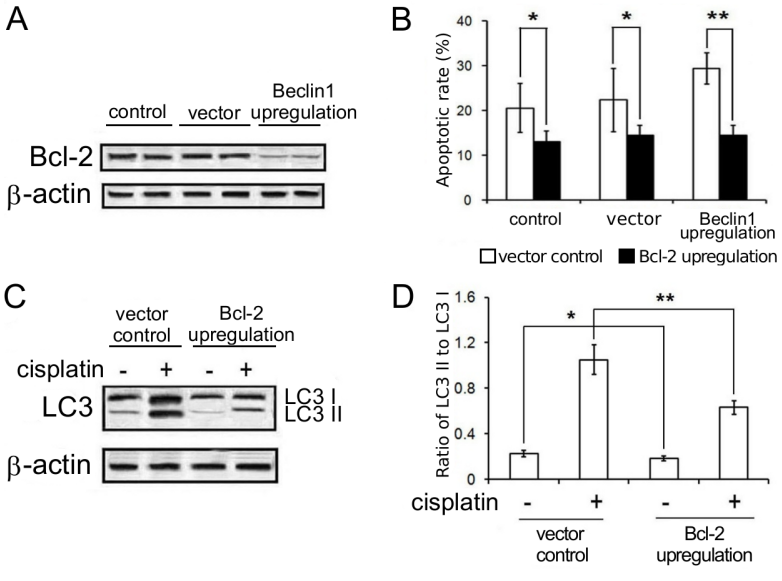

Figure 6. The overexpression of Bcl-2 inhibited the cisplatin-induced autophagy regulated by Beclin1. A) Protein expression level of Bcl-2 was assessed by western blot after Beclin1 upregulation. B) The Bcl-2 overexpression inhibited cisplatin-induced apoptosis in three cell groups and more obviously in Beclin1 overexpression cells. C) Bcl-2 overexpression decreased LC3 conversion in Beclin1 overexpression cells, and more obviously after cisplatin treatment. D) Ratio of LC3 II to LC3 I. (*p<0.05, **p<0.01).

ence of Beclin1 overexpression on cisplatin-sensitivity in laryngeal carcinoma cells Hep-2 and explored the potential mechanism underlying this effect.

We succeeded in transfecting pcDNA3.1-Beclin1 into Hep-2 cells and up-regulated the Beclin1 expression significantly. We found that Beclin1 overexpression had no obvious effect on proliferation and apoptosis of the laryngeal cancer cells. In contrast to the results, Ji Young et al observed that Beclin1 overexpression induced autophagy and apoptosis in lung cancer of mice [15]. The different results may be attributed to different cell lines. Although Beclin1 overexpression did not kill laryngeal carcinoma cells, we detected that Beclin1 overexpression regulated cytotoxicity of chemotherapy. The results showed that Beclin1 overexpression enhanced cisplatin-induced killing effects and apoptosis. Similar to our results, Furuya [12] found that overexpression of Beclin1 could enhance cisplatin-induced apoptosis in human gastric cancer cells. The role of autophagy in cell death is unknown. In contrast to our results, Kang et al [23] suggested that functional autophagy in response to CDDP may lead to cell survival of Hep-2 cells, whereas defective autophagy may contribute to CDDP-induced apoptosis in Hep-2 cells. Thus, modulators of autophagy may be used beneficially as adjunctive therapeutic agents during the treatment of laryngeal cancer with CDDP therapy. In our opinion, the difference may be attributed to different interaction between apoptosis and autophagy and paradoxical function of Beclin1. Apoptotic death was inhibited by autophagy in some cancer cells under conditions of nutrient depletion [24]. Conversely, autophagy was required for apoptosis of malignant cells in other researches [25]. It is difficult to clarify the relationship between autophagy and apoptosis, as both of them are induced by similar activation and impact each other's function [19]. There are also many paradoxical views on the function of Beclin1 as well. In the foregoing indications, Beclin1 is used for promoting cell survival. Paradoxically, Beclin1 might also function as a cancer suppressor under specific conditions. For instance, in contrast to our results, Yang [26] down-regulated Beclin1 expression by Beclin1 silencing, and found that the cisplatin-induced apoptosis was enhanced in human ovarian cancer SKOV3/DDP cells significantly. However, they also found that overexpression of Beclin1 gene up-regulated chemosensitivity to cisplatin, paclitaxel, 5-fluorouracil, and epirubicin by enhancing therapy-induced apoptosis in the cervix squamous carcinoma Caski cells [27].

Some scholars proposed the relationship between autophagy and apoptosis and had obtained further insights into it. Takacs-Vellai [28] found that Beclin1 represents a link between autophagy and apoptosis and supported the view that the two processes act in a concerted manner in the cell death machinery. In our study, we found that Beclin1 overexpression can decrease the mitochondrial membrane potential and increase the caspase cleavage after the cisplatin treatment. The apoptotic signal is transmitted through extrinsic and intrinsic pathways. The extrinsic pathway is activated in a mitochondrial-independent manner, while the intrinsic one in a mitochondrial-dependent manner. Mitochondrial depolarization begins by cleaving Caspase-8, Bid then translocates to mitochondria and activates Bcl-2 family members Bax and Bak. Factors such as cytochrome c are released from mitochondria into the cytosol to trigger activation of Caspase-3 to induce apoptosis [29]. Furuya [12] found that Beclin1 could raise activity of caspases to enhance cisplatin sensitivity in human gastric cancer cells. Yang [13] found that up-regulated Beclin1 expression enhanced cisplatin-induced apoptosis in cervical cancer cells via the mitochondrial-dependent manner. Our findings further confirmed that Beclin1 plays an important status involved in the induction of apoptosis in cancer cells. Moreover, we demonstrate that Beclin1 influences cisplatin-induced apoptosis in laryngeal carcinoma cells by mitochondrial-dependent pathway.

In order to confirm that Beclin1 enhances cisplatin-induced apoptosis via the autophagy pathway, we tested convention of LC3. When autophagy pathway is activated, pro-LC3 (Atg8) is cleaved to form LC3-I, which is then lipoxidized to form LC3-II. It is an important process of autophagy. In our results, Beclin1 overexpression increased convention of LC3, especially after the cisplatin treatment, which suggests that autophagy is the major pathway. Previous researches show that binding of the Bcl-2 to Beclin1 reduces Beclin1's capacity to induce autophagy [30]. However, some other studies show that overexpression of Bcl-2 suppresses the chemotherapy-induced apoptosis rather than the autophagy [31]. In our study, overexpression of Bcl-2 decreased the cisplatin-induced apoptosis in laryngeal carcinoma cells with Beclin1 overexpression. Moreover, overexpression of Bcl-2 inhibited Beclin1-induced LC3 convention. The results suggest that in laryngeal carcinoma Beclin1-induced autophagy is Bcl-2 dependent, and Bcl-2 might be used as a regulator of this process. Under the stimuli of external stress, such as chemotherapy drugs, endoplasmic reticulum produces severe stress in tumor cells, which reduces the activity of Bcl-2 phosphorylation mediated by activating pathway signal molecule c-Jun N-terminal protein kinase1(JNK1). Then, Beclin1/Bcl-2 compound decouples, and also the bond between Bcl-2, Bcl-xL and Bax, Bid weakens. Therefore, Beclin1 and Bax were released to play their own role in promoting autophagy and apoptosis, respectively [32]. Thus, under the intervention of cisplatin, Beclin1 not only participates in autophagy of Hep-2, but also promotes apoptosis of Hep-2 by interacting with positive regulatory factors. In conclusion, up-regulation of Beclin1 can increase the cytotoxicity and apoptosis effect of cisplatin via Bcl-2 modulated autophagy in laryngeal cancer. Study has found that Bcl-2 selective inhibitor enhances breast cancer response in combining therapy with tamoxifen [33]. Furthermore, the development of Bcl_2-BH_4 antagonist may provide a strategy to improve the lung cancer outcome [34]. Next, we plan to use the Beclin 1 activator or the Bcl-2 inhibitor in research for the treatment of laryngeal carcinoma.

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