

Inhibition of autophagy enhanced chemosensitivity in cisplatin resistant hypopharyngeal squamous carcinoma cells

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Abstract

Background:

Hypopharyngeal carcinoma is characterized by high degree of malignancy. The most common pathological type is squamous cell carcinoma (HSCC). It has been confirmed that high autophagy level promotes the development of hypopharyngeal cancer in recent years. Clinical researches have reported that high autophagy level often caused insensitivity to chemotherapy, a common phenomenon that greatly reduces therapeutic effect in cisplatin-resistant tumor cell lines. Therefore, exploring internal mechanisms of autophagy on cisplatin resistant HSCC is necessary for founding theoretical basis for synergistic antitumor drugs by interfering with autophagy.

Methods:

Part I: Cisplatin-resistant FaDu cell line was established and cultured. Cell counting kit-8 was used to detect drug resistance. Inverted microscope was used to observe the morphological changes at different concentrations, then the survival rate was calculated. After MDC staining, the autophagic vacuoles were observed by fluorescence microscopy. The expression of Beclin1 from each group was confirmed by RT-PCR and Western blot method.

Part II: Beclin1 was knocked down by plasmid transfection, autophagy inhibitor 3-MA was applied for cisplatin-resistant cells intervention. Cell cycle was detected using flow cytometry assay, apoptosis with necrosis was detected by staining with propidium iodide (PI). CCK-8 was used to observe the cell survival rate in each group. The expression of autophagy-related gene Beclin1,LC3I,LC3II,Atg-5 and P62 in each group was verified by Western blot analysis.

Results:

Cisplatin-resistant FaDu cell line can be stably constructed by cisplatin intervention. Compared with normal group, autophagy and its related protein Beclin1 expression was enhanced in cisplatin resistant FaDu cells. Autophagy inhibition group showed significant cell cycle changes, mainly manifested by G1 arrest, increased apoptosis rate and significantly decreased survival rate at 24h level. Furthermore, Western blot showed that expression of Beclin1, lc3i, lc3ii, atg-5 protein decreased significantly after the inhibitor used, while the expression of p62 up-regulated, which also confirmed autophagy flow was blocked.

Conclusion:

Our work confirmed high autophagy level is important for the cisplatin-resistance of HSCC and insensitivity to chemotherapy. The use of 3-MA and Beclin 1 inhibition can significantly reduce autophagy level of cisplatin-resistant FaDu cells, arresting its cell cycle, promote apoptosis and reverse the multidrug resistance condition. These results provide the experimental basis for overcoming multidrug resistance through combination chemotherapy.

#These authors contributed equally to this work.

Background

Squamous cell carcinoma, the main pathological types of hypopharyngeal carcinoma, representing approximately 97% of such cases.^[1] It has been reported that the 5-year survival rate for this disease is estimated between 30% – 40%^[2]. There are many treatment methods, among them, Currently, chemotherapy is the main treatment for hypopharyngeal squamous cell carcinoma(HSCC), especially for the late stage and elder patients. The major challenge for HSCC chemotherapy is drug resistance, which is easy to occur in the course of chemotherapy and greatly affects the prognosis^[3]. Therefore, intervention of drug resistance and improved sensitivity to chemotherapy is an existing research gap for in-depth studies on hypopharyngeal cancer^[4].

In the mechanism of tumor drug resistance, apoptosis-resistance has been proved to be a key reason of chemotherapy insensitivity^[5]. Several studies have shown that apoptosis-resistance is linked to autophagy^[6]. As a process of material catabolism in cells, autophagy^[7] is a form of programmed cell death. Changes in autophagy level is related to the occurrence and development of human tumor, this affects tumor cell apoptosis, angiogenesis and anti-tumor treatment^[8].

Beclin1 is an autophagic activator. It has been reported that Beclin1 interacts with anti-apoptotic Bcl-2 family members through a BH3 domain^[9],which hinders Beclin 1's participation in the assembly of preautophage and the binding of Bcl-2 with apoptotic protein, resulting in the reduction of autophagy level and the promotion of apoptosis^[10, 11]. Furthermore, chemotherapy has been proved to induce autophagy in several cancer cells^[12]. The sensitivity of tumor to chemotherapy drugs is reduced by the activation of protective autophagy phenomenon, which leads to the emergence of drug resistance in tumor cells^[13].

To sum up, we raised three questions in our research:

Is there a high autophagy level in drug resistant hypopharyngeal squamous cell carcinoma?

Can the use of autophagy inhibitors and the reduction of the expression of Beclin 1 reverse the drug resistance of hypopharyngeal squamous cell carcinoma cells?

Do autophagy inhibitors work through Beclin1 signaling pathway?

In this study, we constructed a stable cisplatin-resistant FaDu cell models(Human hypopharyngeal squamous cell carcinoma cells)using cisplatin. Observed autophagic bodies and detected the expression of Beclin1 gene to determine the autophagic level. Based on the above experimental data, plasmids transfected (siRNA) method was used to construct Beclin1 knocked down group, and 3-MA was used to intervene cisplatin-resistant cell lines to construct inhibitor group. Cell cycle, apoptosis and cell survival rate were analyzed to determine the effect of intervention on cisplatin-resistant cells. We further detected

the expressions of various autophagic proteins to clarify the internal mechanism. Taken together, our research indicates that inhibition of autophagy in drug resistance of hypopharyngeal cancer might be a crucial means to reverse chemotherapy insensitivity, Beclin 1 plays an important role in the induction of autophagy in cisplatin-resistant cells and can be a therapeutic target that cannot be ignored.

Methods

Construction of drug resistance FaDu cell model

FaDu cell line from human hypopharyngeal squamous cell carcinoma was obtained from Shanghai Branch of Chinese Academy of Sciences. Cells were cultured in DMEM medium composed of 10% fetal bovine serum and 1% double antibody (penicillin-streptomycin mixture) in 37°C and 5% CO₂ incubator. FaDu cell line of cisplatin resistant hypopharyngeal squamous cell carcinoma was induced and cultured by gradually increasing the concentration and intermittent action^[14]. The initial cisplatin intervention concentration was 0.1 µg/mL, the concentration was doubled after every two weeks in a gradient of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 µg/mL concentration. FaDu cell line of hypopharyngeal squamous cell carcinoma resistant to cisplatin was obtained at the intervention concentration of 1.0 µg/mL.

Drug Resistance Validation

CCK-8 kit was used to determine cell drug resistance condition. FaDu cells in logarithmic growth phase were prepared into cell suspension with a density of 3 × 10⁴/ml, and blank control group was established. Cells in logarithmic growth phase were prepared into cell suspension with a density of 3 × 10⁴/ml and blank control group was established. After overnight culture at 37°C, conventional FaDu cell lines and cisplatin-resistant groups were treated with 0/2.5/5/10 µmol cisplatin, respectively. 450 nm wavelength absorbance was measured and recorded by a microplate reader at 0 h/24 h/48 h/72 h timepoint.

Observation Of Autophagosomes By Mdc Staining

Cells from each group were seeded on 24-well plates with a density of 3 × 10⁴/ml. After 1-day culturing, the autophagic vacuoles were subjected to MDC staining and observed under electron microscopy. Autophagic vesicles that emitted green fluorescence were noted.

Knockdown Of Target Genes

The Beclin1 gene was knocked down using plasmid transfection method. The transfection sequence was site: 865–883; Sense: 5'-GCUCAGUAUCAGAAUUU-3' and Antisense: 5'-AUUCUCUCUCUGAUACUGAGCUU-3'. After transfection, mRNA expression of Beclin1 was detected by Real-time PCR. RT-PCR amplified primer

sequences were: Primer F 5'AGGGATGGAAGGGTCTAAG 3',Primer R 5'GGGCTGTGGTAAGTAATGG 3'. Data were analyzed using BI Prism 7300 SDS Software.

Cell Cycle Detection

Cell cycle was detected by flow cytometry. Cells were digested with trypsin, centrifuged(1000 RPM for 15 minutes), resuspended. Thereafter, fixation was done with absolute ethanol, stained with PI (Propidium Iodide) after careful removal of RNA with RNase A solution. Red fluorescence was observed at 488 nm excitation wavelength by flow cytometry within 24 hours and light scattering was detected. Cell cycle analysis was performed using FLOWJO software.

Cell Apoptosis

Apoptosis was also detected using flow cytometry method. Cells were seeded in 6-well plates, cells were seeded in 6-well plates and collected after 24 h grouping treatment. After PBS washing, the cells were digested with trypsin and counted. 50,000-100,000 resuspended cells were taken for centrifuged, discarded the supernatant then resuspended with 195 L Annexin V-FITC binding solution, 5 L Annexin V-FITC was added, incubated at 4° C in dark for 15 min. PI staining was performed and tube without AnnexinV-FITC and PI was used as a negative control. Annexin V-FITC was observed as green fluorescence and propidium iodide (PI) was red fluorescence.

Cell Survival

Cell survival was monitored using CCK-8 kit. Grouped cells were grown in 96-well plates and cell survival was measured after 24 hours following the manufacturer's protocol. CCK-8 and serum-free medium were mixed at a volume ratio of 1:10 and incubated in a 5% CO₂ at 37 °C for 1 hour. Absorbance at 450 nm wavelength (OD value) was measured by a microplate reader.

Western Blotting Assay

Total proteins were extracted from FaDu cells using One Step Animal Tissue/Cell Active Protein Extraction buffer (RIPA; Thermo Fisher Scientific). The cells were seeded in 6-well plates and incubated overnight in 37°C and 5% CO₂ incubator, thereafter, siRNA method was used to establish the knockdown group. All groups were starved for 48 hours in FBS-free medium to achieve synchronization. Cells cultured in drug-free medium represented the control group. Samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Electrophoresis System. The proteins were transferred to a nitrocellulose membrane gel electrophoresis separation. After blocking with 5% skim milk for 1 hour, Tris-buffered saline with Tween 20 (TBST) was used to wash the membranes. Overnight incubation of the membranes with a 1:1000 dilution of rabbit primary antibodies: Beclin1, Icf3i,

Ic3ii, atg-5, p62 and GAPDH (Abcam) was done at 4 °C. This followed another incubation with a 1:10000 dilution of Sheep anti-rabbit HRP labeled secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 hour. A Bio-Rad ChemiDoc MP was used to expose the membranes, and analysis was performed using Image Lab software (Bio-Rad).

Statistical analysis

All experiments were repeated in triplicate and the results were presented as the mean \pm standard deviation (SD). All statistical analyses were performed using SPSS version 21.0 (IBM, Armonk, NY, USA) and GraphPad Prism 7 (La Jolla, CA, USA). Statistically significant differences were determined at $P < 0.05$.

Results

Increasing concentration gradient induced cisplatin resistance in FaDu cells

Stable cisplatin resistant FaDu cell model formed the basis of this experiment. In order to determine the drug resistance of cisplatin hypopharyngeal squamous cell carcinoma FaDu cell line, we adopted CCK-8 assays to calculate the cell survival rate after cisplatin intervention. The survival rate of cisplatin resistant FaDu cells at 24 h, 48 h and 72 h were higher than those of conventional FaDu cells. Furthermore, intervention respectively with 2.5, 5, 10 μ M cisplatin, consistent with above results, drug resistant FaDu cells survival rate is still higher than the conventional control group. These data indicate that increasing concentration gradient method successfully induced cisplatin resistant FaDu cell lines.

Active Autophagy In Drug Resistant Fadu Cells

To determine whether the level of autophagy is increased in cisplatin-resistant cells, we detected the expression of autophagy-specific factor Beclin 1 using Western-blot method. Beclin1 expression was significantly higher in cisplatin resistant FaDu cells as compared to conventional FaDu cells. This implied that high autophagic activity existed in cisplatin resistant FaDu cells. Further, Autophagic vesicles between the two groups was observed by MDC fluorescence staining. The number of autophagic vesicles in cisplatin-resistant cells increased significantly and the fluorescence staining was more obvious.

Effect of 3-MA and Beclin 1 siRNA transfection on autophagy

In this experiment, autophagy inhibitor 3-Ma was used for intervention and Beclin 1 siRNA gene transfection was used to knock down Beclin1 expression with the aim of inhibiting autophagy. To verify a successful Beclin1 knocked down, expression of Beclin1 mRNA in cells was detected by RT-PCR, whereas expression level of Beclin1 protein was detected by Western blot. Acquired data confirmed that the knockdown efficiency of Beclin1 was about 84%.

To verify the specific effects of these two interventions on autophagy, MDC staining was used to compare autophagy in each group. The autophagic vacuoles stained by fluorescence were more significant in the control group compared to 3-MA group and the Beclin1-siRNA group. Fewer autophagic vacuoles were observed in the Beclin1-siRNA group than in the 3-MA group. These results indicate that 3-MA intervention and siRNA gene transfection effectively inhibited the high autophagy level of cisplatin-resistant FaDu cells.

Inhibition of autophagy decreases survival of cisplatin-resistant cells

The CCK-8 assay was used to detect cell survival after intervention. Compared with each group at 24 h, the survival rate of cisplatin-resistant cells in Beclin1-siRNA group and 3-MA group decreased significantly compared with the control group, and the survival rate of Beclin1-siRNA group was even lower compared with the 3-MA group. These results indicate that inhibition of autophagy can effectively reduce cell resistance and increase the mortality of tumor cells.

Inhibition of autophagy causes cell cycle arrest and increased apoptosis

To explore the mechanism of increased cell death in drug resistant FaDu cells, we adopted flow cytometry to study cell cycle changes and apoptosis in each group of cells. After 24 hours of intervention, the proportion of Beclin1-siRNA group and 3-MA group in G0/G1 phase increased while the number of cells in S phase and G2/M phase decreased compared with control group. This was more pronounced in the Beclin1-siRNA group. These data indicated that autophagy inhibition caused G0/G1 arrest of cell cycle. To further determine apoptosis condition, the early and late stage apoptosis of cells were increased after the intervention of Beclin1-siRNA and 3-MA, which was more significant in the Beclin1-siRNA group. Similar results were obtained using flow cytometric analysis of necrotic cells. The above data confirmed that inhibiting autophagy increased the sensitivity of drug resistant FaDu cells, by arresting the cell cycle, promoting cell apoptosis and necrosis.

3-ma Inhibits Autophagy Partly Through Beclin1

Western blot method was used to investigate the protein expression of each group to help elucidate the autophagy of cisplatin-resistant group, study the intrinsic working mechanism of beclin1 and further explore the drug mechanism of 3-MA. Results showed that the expression of LC3-1, LC3-2, ATG12-ATG5 decreased significantly, however in the expression increased in P62 in the absence of Beclin1 when compared with the control group. This phenomenon may be caused by the accumulation of P62 as a result of blocking the initial stage and flow of autophagy. By using 3-MA, protein expression of Beclin1 and above was also significantly inhibited. Western blot results indicate that Beclin1 might be a key factor in regulating autophagy in drug resistant FaDu cells, 3-MA could inhibit Beclin1 expression and play a role in inhibiting autophagy.

Discussion

HSCC accounts for 95% of the pathological classification of hypopharyngeal cancer. It is the sixth most common malignant tumor worldwide^[15]. HSCC lesions occur in concealed areas for example, piriform fossa and posterior sections of pharyngeal wall. In addition, the rich lymphatic network is a predisposing factor to the occurrence of lymphatic metastasis in its early stage. Poor prognosis has been observed in most patients diagnosed in the middle and late stage at the time of initial diagnosis.^[16] At present, hypopharyngectomy, radiotherapy and chemotherapy are the main clinical treatment methods, among which, chemotherapy plays a pivotal role in delaying the recurrence of tumors and prolonging the survival of patients^[17].

Among various HSCC chemotherapy regimens including induction chemotherapy and combined chemotherapy, chemoresistance is regarded as the most critical factor that causes unsuccessful treatment. Current studies suggest that series of factors for example, apoptosis, cell cycle arrest, drug transport, DNA damage and autophagy promotes intrinsic and acquired resistance^[18] of HSCC. Tumor resistance to chemotherapeutics, like tumorigenesis, is a multifactorial, multistep process. Among these factors, autophagy is considered by most researchers to play a key role in regulating drug resistance in multiple tumors^[10, 19, 20]. So, is there a link between autophagy and HSCC resistance as well?

In order to verify this interpretation, We constructed a stable cisplatin-resistant FaDu cell model, inhibiting autophagy by reducing the expression of autophagy factor Beclin1 and using inhibitor 3-MA. In this way, we found that the level of autophagy was significantly increased in drug resistant FaDu cells, inhibition of autophagy led to G0/G1 phase arrest, promoted cell apoptosis.

Autophagy is an evolutionarily conserved catabolic process which is widespread within eukaryotic cells^[21]. The role of autophagy is very complex^[21], In the study of lymphocytic leukemia^[22, 23] and multiple myeloma^[24, 25], arsenic trioxide has been adopted to up-regulate the expression of Beclin1, a key initiating molecule of autophagy, which induce autophagic cell death (type II programmed death), thereby alleviating the disease^[26]. However, more studies have shown that the increased autophagy under this

condition is regarded as a protective mechanism of tumor cells against foreign interventions, thus, resulting in drug resistance^[27].

A report by Yorimitsu^[28] *et al.* revealed that the intrinsic mechanism is endoplasmic reticulum stress induced by chemotherapy can lead to increased autophagy, so that the misfolded protein accumulated in the endoplasmic reticulum cavity can be removed. Furthermore, interfering with the expression of autophagy-related genes accelerates the death of chemo-resistant tumor cells^[29]. By using some autophagy inhibitors such as chloroquine^[30] and 3-methyladenine^[31] will effectively induces apoptosis in cisplatin-resistant tumor cells. To sum up, in anti-tumor therapy, if we can inhibit the high level of autophagy caused by chemotherapy in some way, initiate apoptosis-related signaling pathways, we can improve the treatment efficiency by promoting the death and chemo sensitization of cisplatin-resistant tumor cells.

Consistent with the above conclusions, our experiments showed that knocking down Beclin1 and using 3-MA to inhibit autophagy effectively lead to cell cycle G0/G1 arrest, promoted cell apoptosis of all phase in cisplatin-resistant FaDu cells. Demonstrated that increased autophagy in HSCC leads to the occurrence of cisplatin resistance, inhibition of autophagy and its initiating factor Beclin1 effectively promote the death of cisplatin-resistant tumor cells, render them more sensitive to cisplatin chemotherapy.

This research however, present several limitations. Even though apoptosis and autophagic cell death are both programmed cell death modes, great differences exist between them. Some studies suggests that during cellular stress response, several abnormal proteins are formed within the cells, this is accompanied by mitochondrial swelling and release of apoptotic factors such as cytochrome C^[32]. At this stage, cells activate autophagy to remove the damaged components, so as to avoid the release of apoptosis factors into the cytoplasm. In the process, there are typical node molecules for example, Bcl-2 and JNK-1, which needs further study to establish their role. Also, in order to clarify the mechanism of 3-MA, there is need to carry out in-depth study on the role of inherent cell signaling pathway in inhibiting autophagy. Researchers, therefore, needs to further explore the mechanism of autophagy in drug resistant FaDu cells and develop targeted drugs based on autophagy genes. This will be significant in developing multi drug combination therapy and the improving the therapeutic potential of HSCC.

Conclusion

The present study aims at to explore whether autophagy is active in drug resistant HSCC and, more specifically, examined whether inhibition of autophagy can reverse drug resistance and increase chemosensitivity. In order to achieve this, we constructed cisplatin resistant FaDu cells using a concentration gradient intervention method. Found that the expression of Beclin1 and the number of autophagy vesicles increased significantly in the resistant FaDu cells, which verified the previous hypothesis. Furthermore, we knocked down Beclin1 and adopted 3-MA, an autophagy inhibitor, to act on cisplatin-resistant FaDu cells. Results showed that after autophagy inhibition, the survival rate of cisplatin-resistant cells decreased, cell cycle G0 / G1 phase arrested and apoptosis rate increased.

Observed significant reduction in the number of autophagy vesicles confirms the decline in autophagy levels. These data indicated that inhibition of autophagy can lead to increasement of cell death rate of cisplatin-resistant cells at the same concentration of cisplatin, which means chemotherapy sensitivity was recovered. Furthermore, by analyzing the expression of downstream factors lc3-1, lc3-2, atg12-atg5 and P62, results suggest that Beclin 1 plays an important role in the development of autophagy in cisplatin resistant FaDu cells. 3-MA, as an autophagy inhibitor, may achieve its function partially by inhibiting Beclin 1. Taken together, we suggest that in cisplatin induced chemotherapy resistance, autophagy inhibit is a very effective method to regain chemosensitivity. It should be used combined with chemotherapy drugs for increasing the treatment efficacy. Beclin1 might be a potential lead research target that regulates autophagy and chemotherapy resistance.

Abbreviations

HSCC, Hypopharyngeal squamous cell carcinoma ; BSA, bovine serum albumin; CCK, Cell Counting Kit; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FCM, flow cytometry; MDC, Dansylcadaverine; IHC, immunohistochemistry; PBS, phosphate-buffered saline; PI, propidium iodide;

Declarations

Availability of data and materials

The data and materials are stored in the Key Laboratory of ENT of Shanghai General Hospital and can be requested from the first author and corresponding author.

Ethical approval and consent to participate

All experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, approved by the Animal Ethics Committee of Nanjing Medical University. During the experiments, all animals were handled humanely and with care.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

JZ and WM designed and experiments and drafted the manuscript. WM, LYY, ZWY, and JD performed the experiments and analyzed and interpreted the data. GLW, SY helped with data collection and analysis. PD coordinated the research group and participated in the experimental design. All authors were involved in critically revising the manuscript.

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Figures

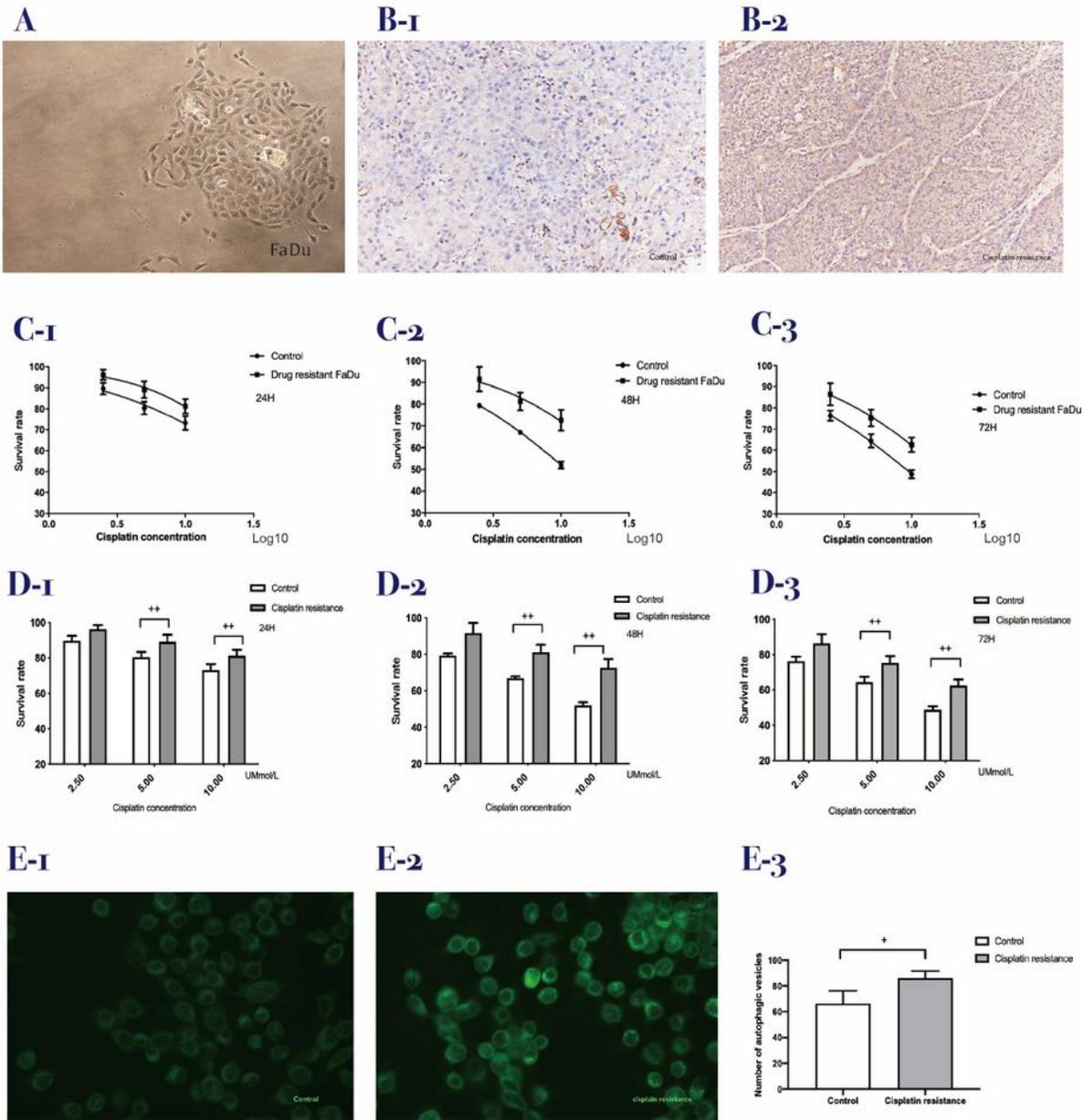


Figure 1

Cisplatin induced drug resistance and autophagy in FaDu cells. A: FaDu cells were cultured in medium with increasing cisplatin concentration, cisplatin concentration doubled 1 time in 2 weeks. B1-B2: Immunohistochemical staining showed that the positive expression of Beclin 1 increased after cisplatin resistance. C1-C3: CCK-8 kit was used to detect the cell survival rate in 24 hours, 48 hours and 72 hours, respectively. The survival rate of cisplatin resistant cells after chemotherapy was higher than that of the

control group with a dose effect relationship. +, $p < 0.05$, ++, $p < 0.01$, compared with control group. D1-D3: Statistical analysis of survival rate of each group. E1-E2: Autophagic vesicles in each group stained with MDC. Compared with the control group, the number of autophagic vesicles stained fluorescent green in cisplatin resistant group significantly increased. E3: Statistical comparison of the number of autophagic vesicles between the two groups. Data represents the results of at least three independent experiments, \pm SE. $p < 0.05$ was considered significant.

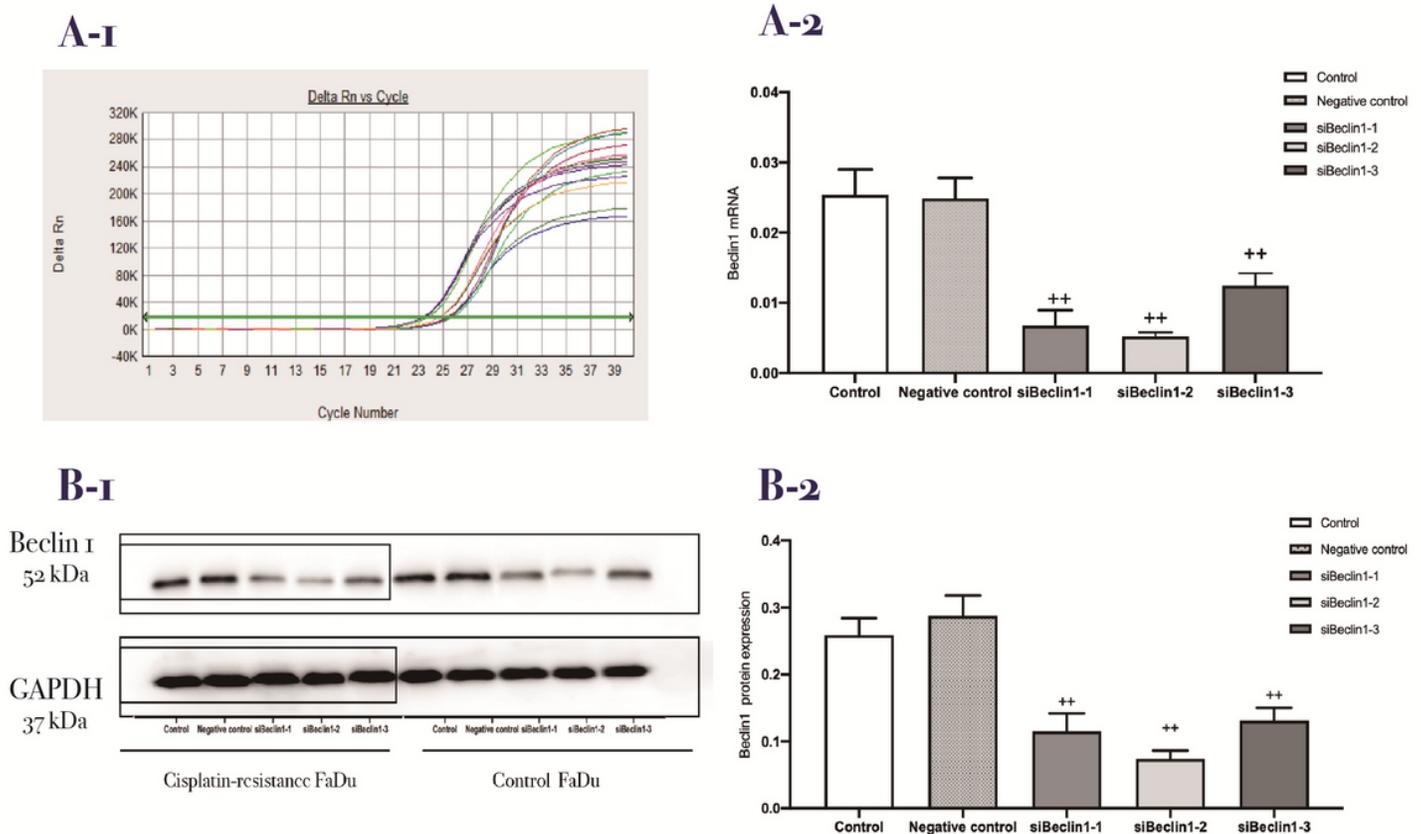


Figure 2

Successful Knock-down of Beclin 1 gene by plasmid transfection. A: Verification of knock down efficiency of three targets by PCR analysis. A-1: Beclin 1 amplification curve. A-2: Statistical analysis of Beclin1 mRNA expression in each group. SiBeclin1-2 was the most effective target. +, $p < 0.05$, ++, $p < 0.01$, compared with control group. B: B-1: Western blot analysis of protein expression of Beclin 1 protein in cisplatin resistant FaDu cells and normal FaDu cells. We cropped cisplatin resistant FaDu bands for further analysis. B-2: Statistical analysis of Beclin1 protein expression. SiBeclin1-2 was the most effective target. Data represents the results of three independent experiments, \pm SE. $p < 0.05$ was considered significant.

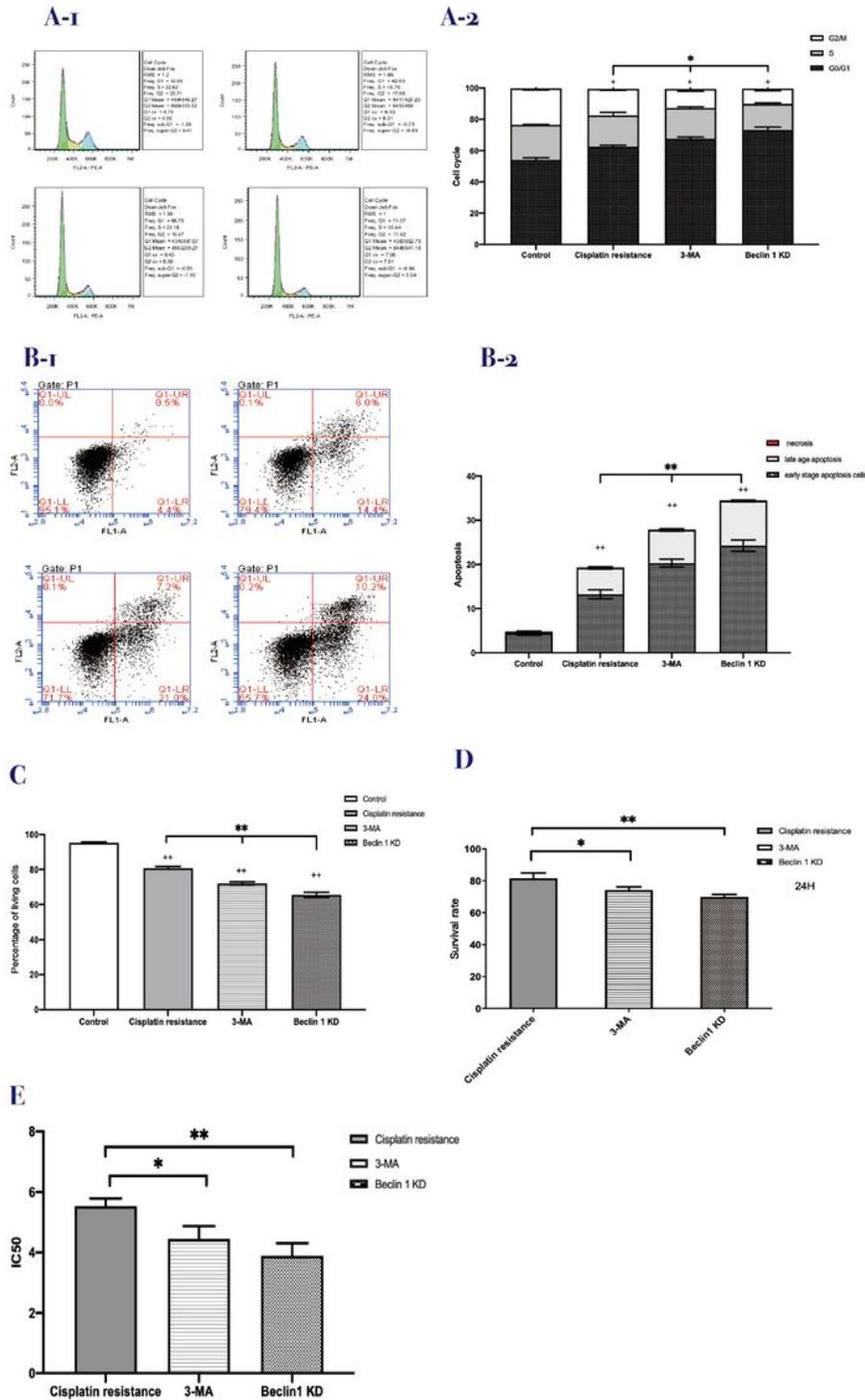
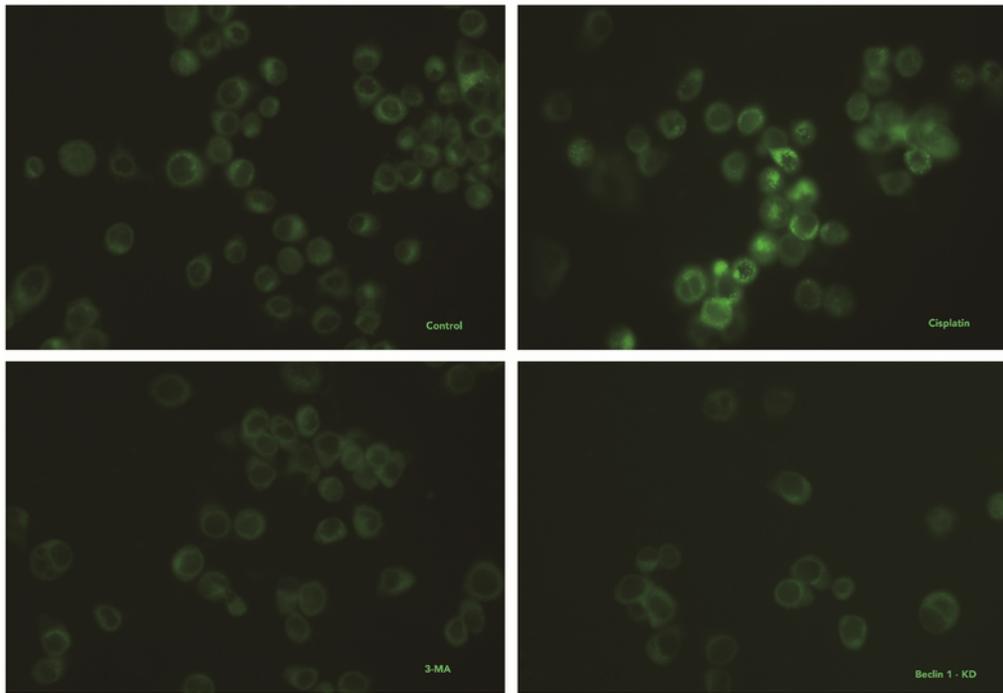


Figure 3

Autophagy inhibition decreases cell viability by promoting apoptosis and necrosis. **A** **A-1**: Analysis of cell cycle by flow cytometry. **A-2**: Statistical analysis of cell cycle. Using 3-mA and Beclin 1 knockdown to inhibit autophagy resulted in G0 / G1 phase arrest and the proportion increased significantly. +, $p < 0.05$, ++, $p < 0.01$, compared with control group, *, $p < 0.05$, **, $p < 0.01$, compared with cisplatin-resistance group. **B** **B-1**: Analysis of cell apoptosis and necrosis by flow cytometry. **A-2**: Statistical analysis of cell

apoptosis. Using 3-mA and Beclin 1 knockdown to inhibit autophagy resulted in both early and late stage apoptosis increased. +, $p < 0.05$, ++, $p < 0.01$, compared with control group, *, $p < 0.05$, **, $p < 0.01$, compared with cisplatin-resistance group. C: Statistical analysis of living cell percentage. Consist with apoptotic rates, autophagy inhibition decreases living cell percentages. D: Compartment of cell survival rate of each group. Autophagy inhibition decreases cell survival. Beclin1 Knockdown group showed significant lower survival rate. *, $p < 0.05$, **, $p < 0.01$, compared with cisplatin-resistance group. E: Analysis of IC50 showed that after intervention, cisplatin resistance was reversed, this tendency is more obvious in Beclin 1 knockdown group. *, $p < 0.05$, **, $p < 0.01$, compared with cisplatin-resistance group. Data represents the results of three independent experiments, \pm SE. $p < 0.05$ was considered significant.

A



B

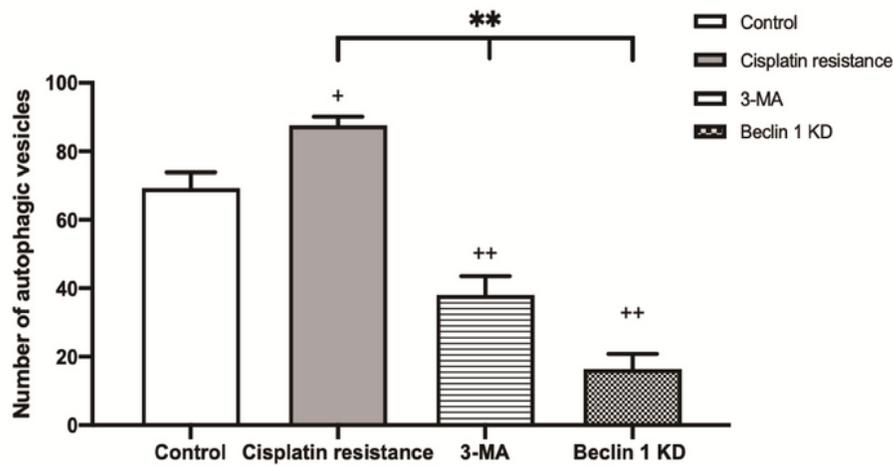


Figure 4

The number of autophagic vesicles decreased after using 3-MA and Beclin 1 knockdown. A: Autophagic vesicles in each group stained with MDC. Using 3-MA and Beclin 1 knockdown significantly reduced the number of autophagic vesicles increased due to cisplatin resistance. B: Statistical analysis of autophagic vesicles number. $p < 0.05$, ++, $p < 0.01$, compared with control group, *, $p < 0.05$, **, $p < 0.01$, compared with

cisplatin-resistance group. Data represents the results of three independent experiments, \pm SE. $p < 0.05$ was considered significant.

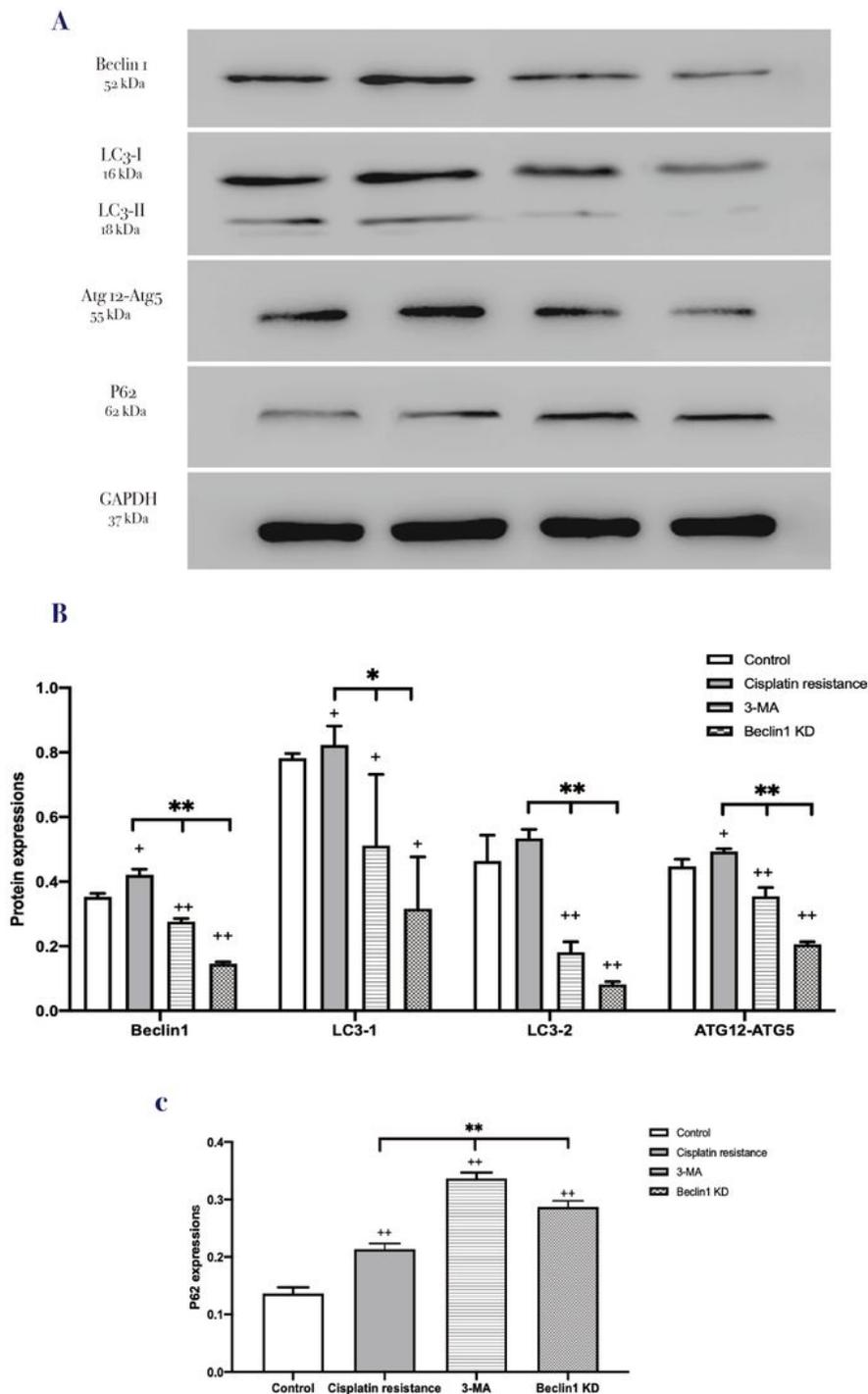


Figure 5

Western blot analysis of protein expression of autophagy related factors. GAPDH was served as the internal reference. B: Statistical analysis and comparison of protein expression in each group. Beclin1, lc3i, lc3ii, atg-5 expression analysis showed that 3-MA and Beclin 1 knockdown efficiently decreased

protein expression induced by cisplatin resistance. C: While the expression of P62 increased compared to control group. $p < 0.05$, ++, $p < 0.01$, compared with control group, *, $p < 0.05$, **, $p < 0.01$, compared with cisplatin-resistance group. Data represents the results of three independent experiments, \pm SE. $p < 0.05$ was considered significant.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [P62.tif](#)
- [LC3.tif](#)
- [GAPDHKD.tif](#)
- [ATG12ATG5.tif](#)
- [GAPDH.tif](#)
- [Beclin1.tif](#)
- [Beclin1KD.tif](#)