



Dual inhibition of autophagy and PI3K/mTOR pathway as a potential therapeutic strategy against laryngeal squamous cell carcinoma

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Background: New and effective chemotherapy or targeted therapy strategies are needed against laryngeal squamous cell carcinoma (LSCC). We aimed to explore the antitumor effect of dual PI3K/mTOR inhibitor combined with autophagy suppression on LSCC and its underlying mechanism.

Methods: Hep-2 and AMC-HN-8 cell lines were treated with the Akt inhibitor LY294002, mTOR inhibitor rapamycin, and dual inhibitor NVP-BEZ235 separately. The biological characteristics of *in vitro* proliferation, cell cycle, apoptosis, migration, invasion, and autophagy were analyzed, and the expression levels of PI3K/Akt/mTOR pathway-related proteins were also measured. The *in vivo* effects of NVP-BEZ235 combined with inhibition of autophagy using pharmacological inhibitor was further assessed.

Results: Compared with Akt or mTOR inhibitor, NVP-BEZ235 had the most significant biological effects on LSCC cells. When combined with various autophagy inhibitors, along with siRNA against ATG7, NVP-BEZ235 showed a synergic antitumor effect in LSCC through increasing cell apoptosis and death both *in vitro* and *vivo*.

Conclusions: NVP-BEZ235 exerted potent antitumor effects on LSCC, especially when combined with the autophagy inhibitor both *in vitro* and *vivo*, providing convincing experimental data for new molecular targeted therapy for LSCC.

Keywords: Laryngeal squamous cell carcinoma (LSCC); PI3K/mTOR inhibitors; autophagy

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Introduction

Laryngeal squamous cell carcinoma (LSCC) is the second most prevalent head and neck squamous cell carcinoma (HNSCC) throughout the world, and its incidence has significantly increased in the past decade (1). Despite advances in the treatments, 5-year survival rate of LSCC has decreased from 66% to 63% over the past 40 years (2). This may be related to the popularization of the concept of preserving laryngeal function during this period. In China, approximately 40% of LSCC patients are diagnosed at an advanced stage and total laryngectomy is usually required according to the National Comprehensive Cancer Network (NCCN) treatment guidelines (3). For patients with LSCC, radical surgical excision is of paramount importance for achieving a favorable prognosis, but it inevitably affects their vocalization and swallowing to a great extent. Considering that the postoperative life quality and overall survival rate of patients with LSCC remain unsatisfactory, new chemotherapy strategies or targeted therapy are urgently required (4).

The mammalian target of rapamycin (mTOR) is overactivated in many malignancies and plays a crucial part in PI3K/Akt/mTOR pathway (5), which can promote cell growth and proliferation. Moreover, PI3K/Akt/mTOR pathway seemed to be a prognostic factor and a predictor of response to radiotherapy and chemotherapy in clinical practice (6). Since its critical influence on tumor activity, regulation of this signaling pathway has become a potential target for LSCC intervention.

Furthermore, recent studies have indicated that autophagy inhibition could enhance anticancer therapies in various malignancies (7,8), suggesting that autophagy is also emerging as a promising therapeutic target in oncotherapy. Considering that the PI3K/Akt/mTOR pathway is also involved in the regulation of autophagy, along with the possible limitation of monotherapy with PI3K or mTOR inhibitor, a combination of dual PI3K/mTOR inhibitor and autophagy inhibitor has become a novel research direction.

To identify a potential therapy strategy for LSCC, we compared the effects of three PI3K/mTOR signaling pathway inhibitors (LY294002, rapamycin, NVP-BEZ235) against LSCC cells *in vitro*, and the synergistic effect of the combined application of dual PI3K/mTOR inhibitor and autophagy inhibitor was evaluated *in vivo*. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-21-2325/rc>).

Methods

Cell lines

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Two LSCC cell lines, AMC-HN-8 and Hep-2, were acquired from the Stem Cell Bank of the Chinese Academy of Science. Both cells were cultured in RPMI-1640 medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 1% penicillin-streptomycin (Genom Biotechnology, China), and maintained in 5% CO₂ at 37 °C. In this experiment, cells were cultured 24 hours in advance and underwent subsequent treatment.

Reagents

Antibodies against caspase-8 (SAB5701263), caspase-3 (SAB5700914), caspase-9 (SAB5700785), cyclin-D1 (SAB140551), cyclin-E2 (SAB4503557), phospho-Akt (05-1003), Akt (SAB4500797), phospho-P70S6K (SAB4301562), P70S6K (SAB2500736), phospho-4E-BP1 (SAB4301339), 4E-BP1 (SAB4500736), phospho-S6RP (SAB5700395), S6RP (SAB4300484), LC3-II (L8918), LC3-I (L8918), and GAPDH (G9545) were purchased from Sigma-Aldrich, USA. PI3K/mTOR inhibitors LY294002 (S1105), rapamycin (S1039), NVP-BEZ235 (S1009), and autophagy inhibitors 3-Methyladenine (3-MA, S2767) and Chloroquine Phosphate (CQ, S4157) were purchased from Selleck, USA.

MTT detection for the calculation of drug IC50

Hep-2 and AMC-HN-8 cells were cultured in 1640 medium containing 10% FBS until the cells grew to 80–90% and digested with trypsinization. The cell suspension concentration was adjusted for 10,000 cells per well in 96-well plates. After cultured for 24 hours, cells were treated with different concentrations of LY294002 (1, 5, 10, 20, 40, 80, 160 μM), rapamycin (1, 5, 10, 20, 40, 80, 160 μM), and NVP-BEZ235 (0.1, 0.5, 1, 2, 4, 8, 16 μM) for 72 h. DMSO served as the control group. The culture medium was not replaced during the period. Twenty microliters of MTT (5 mg/mL) were added to each well later, and all plates were further incubated for 4 h. Later, 150 μL of DMSO was added, and the OD490 values were measured. The inhibition rate was selected as the y-coordinate and the drug concentration (lnX) as the x-coordinate. The fitting curve formula $Y = A * \ln X + B$ was used to calculate the semi-

inhibition rate of cells, $IC_{50} = \exp [(0.5 - b)/a]$. All assays were performed in three replicates, and each experiment was repeated three times.

Western blotting

Hep-2 and AMC-HN-8 cells were cultured in 1640 medium containing 10% FBS until the number of cells reached 5×10^6 – 5×10^7 . Hep-2 cells were treated with or without LY294002 (7.5 μ M), rapamycin (15 nM), and NVP-BEZ235 (1.5 μ M), while AMC-HN-8 cells were treated with or without LY294002 (6 μ M), rapamycin (15 nM), and NVP-BEZ235 (0.75 μ M). The culture medium was not replaced for 72 hours. Whole cell protein lysates were obtained later using RIPA lysis buffer solution (Beyotime, China). Equivalent quantities of cell lysate protein (25 μ g) were exposed to SDS-PAGE and transferred to a PVDF membrane (Millipore, USA), which was blocked with 5% nonfat milk for 1 hour at room temperature and incubated with antibodies at 4 °C overnight. Dilutions of the primary antibodies are as follows: caspase-8 (1:500), caspase-3 (1:500), caspase-9 (1:500), cyclin-D1 (1:1,000), cyclin-E2 (1:1,000), phospho-Akt (1:500), Akt (1:500), phospho-P70S6K (1:500), P70S6K (1:500), phospho-4E-BP1 (1:500), 4E-BP1 (1:500), phospho-S6RP (1:500), S6RP (1:500), LC3-II (1:500), LC3-I (1:500), and GAPDH (1:1,000). Dilution of the secondary antibodies was 1:5000. Diluted primary antibodies were generally repeated no more than three times, while the secondary antibodies were used only once. The protein bands were visualized by using an enhanced chemiluminescent substrate (Millipore, USA). ImageJ software was used to analyze relative protein levels and normalize the internal reference protein GAPDH.

Apoptosis assay

An Annexin V-FITC/PI Detection Kit (Invitrogen, USA) was used to examine cell apoptosis according to the manufacturer's instructions. Referring to the above-described processing method, the treated LSCC cells were collected and stained with Annexin V-FITC and PI. After 30 min of incubation in the dark, the cell apoptosis ratio was detected using FCM (Becton Dickinson, USA).

The TUNEL kit (Roche, Switzerland) was used to detect apoptotic cells in tumors according to the manufacturer's instructions. The proportion of TUNEL-positive cells showing a brown color on each section was counted to determine the occurrence of apoptosis.

Cell cycle assay

The cell cycle was examined using the propidium iodide (PI) technique. After treatment with LY294002, rapamycin, or NVP-BEZ235 for 72 h without a replacement of culture medium, the LSCC cells were starved for 24 h in RPMI-1640 without serum and the above inhibitors. Single cell suspensions were then fixed in 75% ethanol overnight at –20 °C, washed twice with PBS, and incubated with PI/RNase staining buffer (BD Biosciences). Samples were tested on a flow cytometer (BD Biosciences) and analyzed with FlowJo software (FlowJo LLC).

Invasion and migration assay with a trans-well chamber

The invasive and migratory capabilities of Hep-2 and AMC-HN-8 cells following treatment with the three inhibitors were evaluated by trans-well assay. For invasion, 100 μ L Matrigel (Becton Dickinson, USA) was added to the bottom of each upper chamber in advance and dried for 4 h at 37 °C to a gelatinous form. Then, for both experiments, 5×10^5 /mL cells were placed in the upper chambers with LY294002, rapamycin, NVP-BEZ235, DMSO separately, and incubated for 24 h at 37 °C. The lower chambers were filled with a culture medium with 600 μ L of 20% FBS. The culture medium was not replaced during the period. Cells in the chambers were later fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, China). Five random fields were selected to count, and the results were averaged and plotted.

ATG7 siRNA Interference

We transfected cells with siRNA oligos against ATG7 with the purpose of genetically inhibit autophagy. The sequences of ATG7 were as follows: Atg7-1 5'-GATCCGGTGCTG GTTTCCTTGCTTAACTCGAGTTAAGCAAGGAAA CCAGCACCTTTTTG-3' and antisense 5'-AATTCAAAA AGGTGCTGGTTCCTTGCTTAACTCGAGTTAAG CAAGGAAACCAGCACCG-3'; Atg7-2 5'-GATCCGCAG CCTCTCTATGAGTTTGACTCGAGTCAAACATCATA GAGAGGCTGCTTTTTG-3' and antisense 5'-AATTCA AAAAGCAGCCTCTCTATGAGTTTGACTCGAGTC AAACATCAGAGAGGCTGCG-3'.

A total of 1×10^5 AMC-HN-8 and Hep-2 cells were seeded in 6-well plates overnight before transfection. Cells were transfected with siRNA oligonucleotides at 4 μ g/250 μ L medium with 10 μ L Lipofectamine 2000

(Invitrogen, USA) each well according to the manufacturer's instructions.

Tumorigenicity assay in nude mice

Experiments were performed under a project license (No. KJ2008-01) granted by Ethics Committee of the EENT Hospital, Fudan University, in compliance with Fudan University guidelines for the care and use of animals. All male 6- to 8-week-old BALB/c nude mice (SLAC Laboratory Animal Co., Ltd, Shanghai) were inoculated with 6×10^6 Hep-2 cells before being randomly divided into four groups (n=5 each) as follows: (I) control group; (II) NVP-BEZ235 group, oral administration of NVP-BEZ235 (30 mg/kg/d); (III) CQ group, intraperitoneal (i.p.) administration of CQ (50 mg/kg/d); (IV) NVP-BEZ235+CQ group, mice were treated with NVP-BEZ235 (30 mg/kg/d, p.o.) and CQ (50 mg/kg/d, i.p.) simultaneously. The tumor sizes were measured once a week with calipers. All surviving mice were sacrificed 28 days later to collect tumors for further detection. Tumor volumes were calculated: tumor volume = $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$. Tumor cell apoptosis of each group were analyzed.

Statistical analysis

Data analysis was accomplished using SPSS 22.0 software. Differences between groups were assessed by the Student's *t*-test (two-tailed), and the results are shown as means \pm SEM. $P < 0.05$ was considered statistically significant.

Results

Dual PI3K/mTOR inhibitor NVP-BEZ235 leads to cell cycle arrest and apoptosis in LSCC cell lines

To clarify the role of PI3K/Akt/mTOR pathway in LSCC, Hep-2 and AMC-HN-8 cell lines were treated with PI3K inhibitor LY294002, mTOR inhibitor rapamycin, and dual PI3K/mTOR inhibitor NVP-BEZ235 separately. Different drug concentrations were applied to two LSCC cell lines according to the results of MTT assay (Figure S1). Cell apoptosis and the cell cycle were then assessed. NVP-BEZ235 elicited significant blockage at G2/M phase, and the protein levels of cyclin D1 and cyclin E2 also decreased in both cell lines than LY294002 and rapamycin (Figure 1), which suggested its better effect on the cell cycle as a dual

PI3K/mTOR inhibitor. Treatment with all three inhibitors resulted in significant apoptosis in Hep-2 and AMC-HN-8, especially with NVP-BEZ235 (Figure 2A,2B), whereas the protein level of caspases 3, 8, and 9 increased after inhibitor treatment (Figure 2C-2E).

NVP-BEZ235 shows more potent and dual blocking effects for mTORC1 and mTORC2

We next tested the activation of AKT, p70S6K, 4EBP1, and S6RP in the two cell lines after the treatment with the inhibitors. NVP-BEZ235 modulated not only mTORC1 targets p-70S6K, p-4EBP1 and p-S6RP, but also mTORC2 targets p-Akt (Figure 3). To evaluate antitumor effects, we further manipulated the migration and invasion test by transwell chamber assay in Hep-2 and AMC-HN-8 treated with LY294002, rapamycin, NVP-BEZ235, or DMSO (as control). All three inhibitors reduced cell migration and invasion *in vitro* (Figure 4); however, NVP-BEZ235 elicited the most significant effect.

Considering the above assessment, NVP-BEZ235 had a better block effect on PI3K/Akt/mTOR pathway as a dual PI3K/mTOR inhibitor on both LSCC cell lines than LY294002 and rapamycin.

Synergic antitumor effect of combination NVP-BEZ235 with autophagy inhibition in vitro

As NVP-BEZ235 can regulate cell autophagy in many tumors (9,10), we investigated the regulatory effects of all three inhibitors on autophagy in LSCC cell lines. Autophagy control was observed using a confocal laser scanning microscope after acridine orange staining (Figure S2) and verified by LC3, a protein embedded in autophagosomes that became lipids during active autophagy. LC3II/LC3I ratio was obtained using western blotting analysis (Figure 5). According to the analyses, the trend of LC3I and LC3II change was consistent in the two cells. Although some of the changes were not statistically significant, the rise of LC3II/LC3I ratio in both cell lines suggested that NVP-BEZ235 is more effective in promoting autophagy.

Furthermore, we tested the combination of NVP-BEZ235 with autophagy inhibitors, 3-methyladenine (3-MA), chloroquine (CQ) and ATG7-SiRNA. 3-MA inhibits the initiation of autophagy, CQ inhibits the fusion of autophagosomes and lysosomes; and ATG7-SiRNA, is a genetic silencing of Atg7, which inhibits the early phase

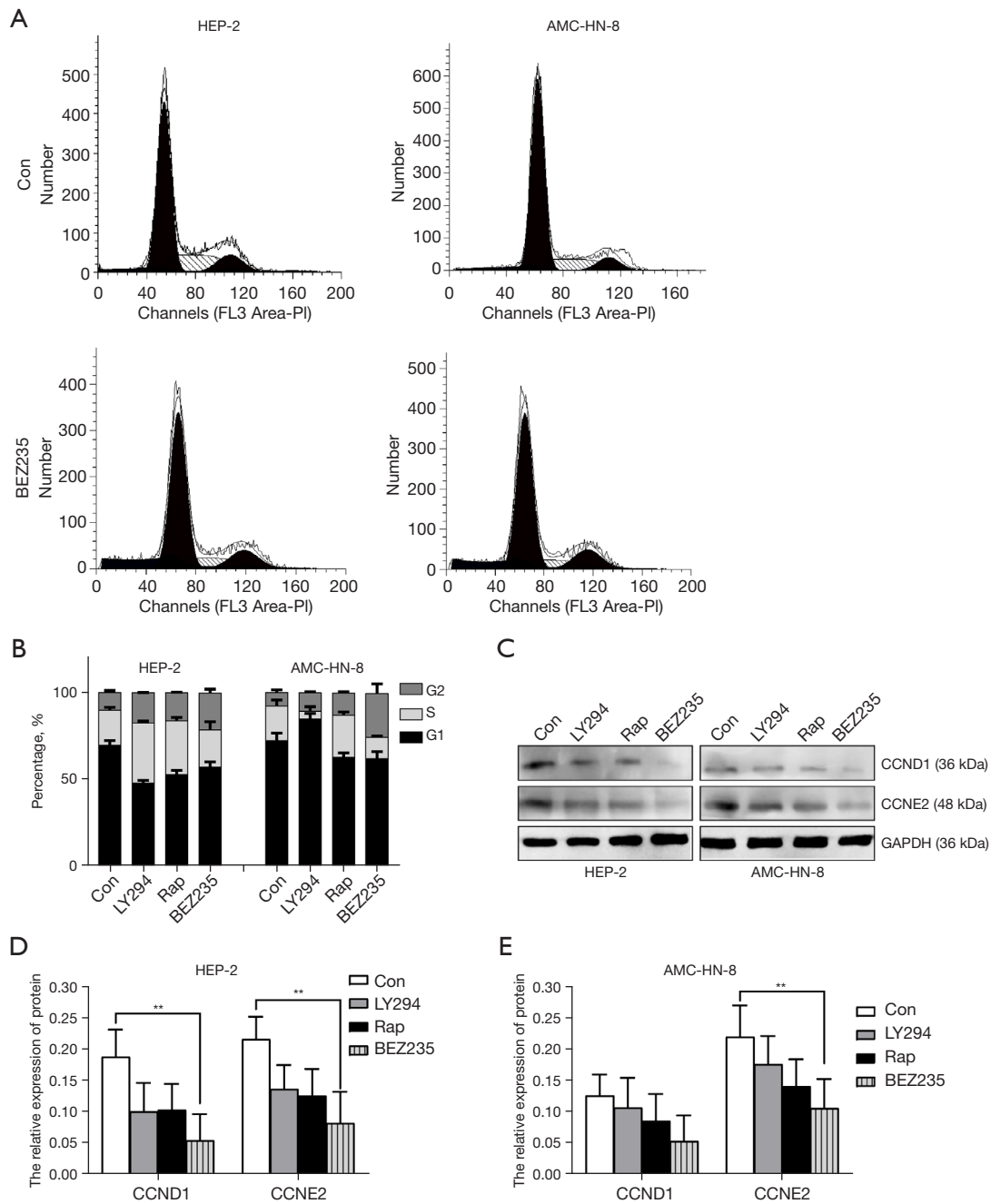


Figure 1 Cell cycle is affected by inhibitors of PI3K/mTOR pathway. (A) FCM analysis of cultured Hep-2 and AMC-HN-8 cells. (B) Cell cycle analysis of the Hep-2 and AMC-HN-8 cell lines. Cells in G2/M phase were increased by NVP-BE235 in both cell lines. Columns, means of duplicate measurements; bars, \pm SD. (C) Western blotting analysis of the expression levels of cyclin D1 and cyclin E2 in two cell lines treated with three different PI3K/mTOR inhibitors. Cyclin D1 and cyclin E2 decreased significantly after treatment with NVP-BE235. (D,E) The expression levels were evaluated by ImageJ and analyzed with 2way ANOVA; bars, \pm SD; **, $P < 0.001$. FCM, flow cytometric.

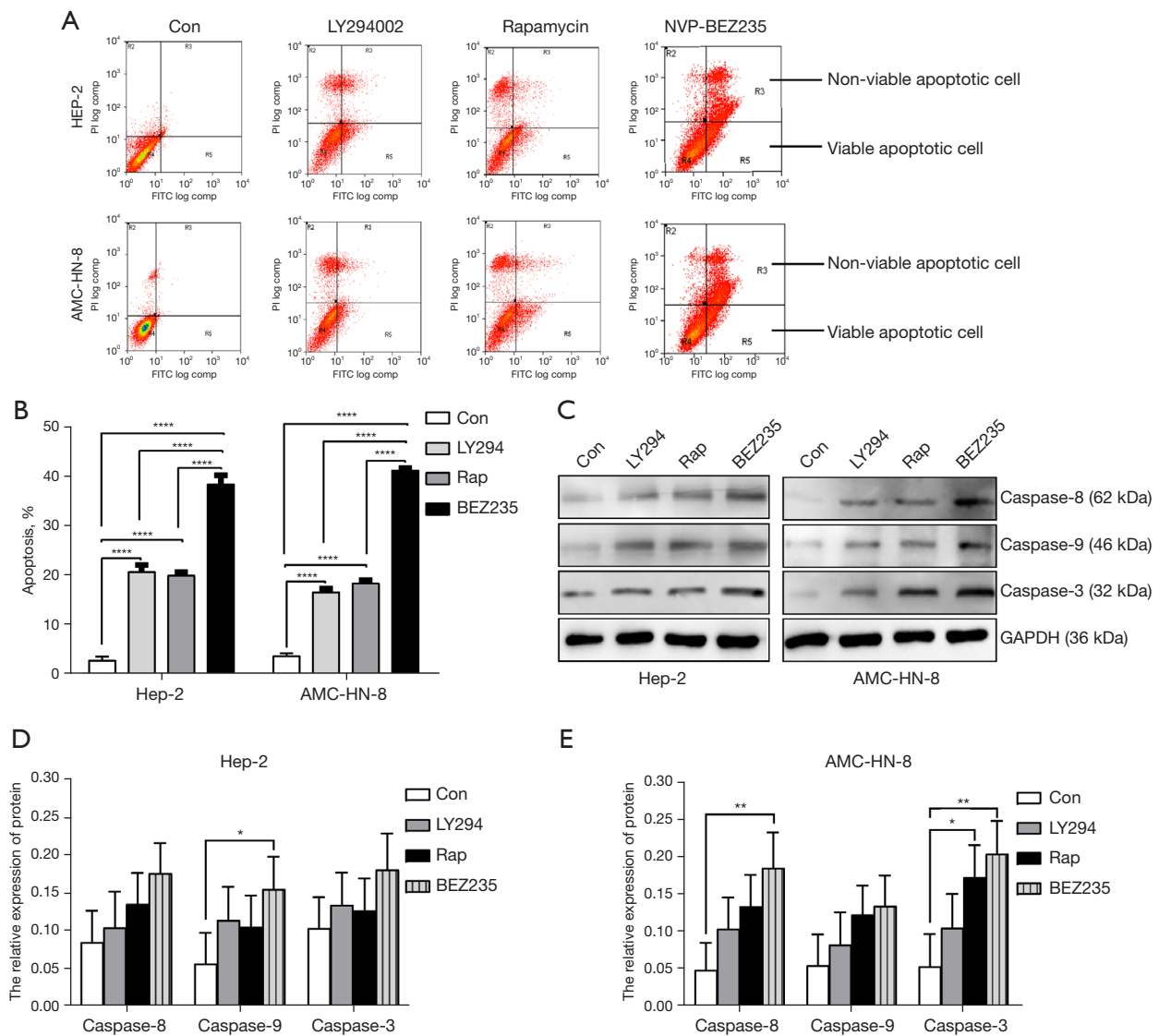


Figure 2 Cell apoptosis is increased by inhibitors of PI3K/mTOR pathway. (A) FCM analysis of the cell apoptosis of cultured Hep-2 and AMC-HN-8 cell lines. (B) Cell apoptosis was increased by all three inhibitors in both cell lines; ****, P<0.0001. The apoptosis increase was similar with Ly294002 and rapamycin but was more pronounced with NVP-BE2235; ****, P<0.0001. Columns, means of duplicate measurements; bars, \pm SD. (C) Caspases 8, 9, and 3 expression in the Hep-2 and AMC-HN-8 cell lines increased after NVP-BE2235 treatment. (D,E) The expression levels were evaluated by ImageJ and analyzed with 2way ANOVA; bars, \pm SD; *, P<0.05; **, P<0.001. FCM, flow cytometric.

of autophagy. The results showed that cell death was significantly increased when NVP-BE2235 was combined with autophagy inhibitors (Figure 5), although LC3II/LC3I ratio and the expression level of caspase 3 were not significantly changed by the combination. This result strongly indicates that the combination of NVP-BE2235 and autophagy inhibitors has a synergistic antitumor effect

through increasing LSCC cell apoptosis and death.

Synergistic antitumor effect of combination NVP-BE2235 with autophagy inhibition in vivo

We then tested the antitumor efficacy of the combination of NVP-BE2235 with CQ *in vivo*. BALB/c mice bearing

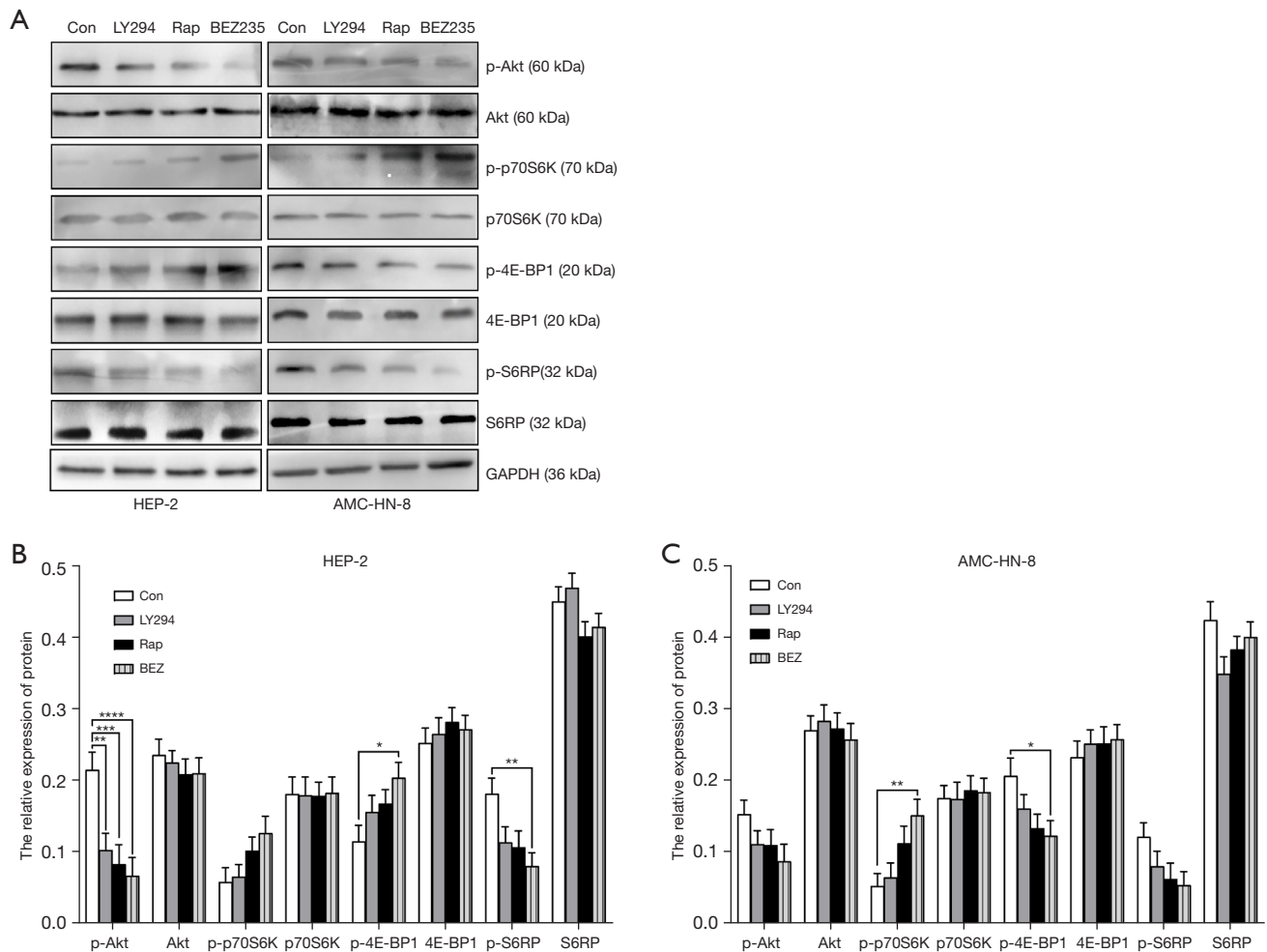


Figure 3 Protein expression and phosphorylation of members of the PI3K/Akt/mTOR pathway of Hep-2 and AMC-HN-8 cell lines, assessed by western blotting. (A) Expression of molecules in PI3K/mTOR signaling pathway in the Hep-2 and AMC-HN-8 cell lines after treated with inhibitors. (B,C) The expression levels were evaluated by ImageJ and analyzed with 2way ANOVA; bars, \pm SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Hep-2 xenograft tumors were treated with NVP-BE235, CQ, NVP-BE235+CQ, and physiological saline solution as a control for 4 weeks after tumor cell injection. First, we tested the drug toxicity of all groups by evaluating the changes in animal weight, behavior, and appearance. The tumor size was then measured weekly before the animals were sacrificed. The final tumor masses were dissected after the last measurement. Combination of NVP-BE235 and CQ demonstrated synergistic effect against autophagy, indicated by reduced tumor size and final tumor weight (Figure 6). The interaction effect was elicited by the interaction index ($P < 0.0001$). Based on TUNEL assay of fragmented DNA of apoptotic cells in final tumor masses,

the most apoptotic cells were found in the group treated with NVP-BE235 and CQ.

Discussion

In this study, the dual PI3K/mTOR inhibitor NVP-BE235 revealed a better effect on the cell cycle, cell apoptosis, migration, and invasion in LSCC. The expression changes in downstream molecules of PI3K/Akt/mTOR signaling pathway further explained its underlying mechanism. We demonstrated that the combination of NVP-BE235 and autophagy inhibitors has a synergic antitumor effect by promoting cell apoptosis and death both *in vitro* and *vivo*.

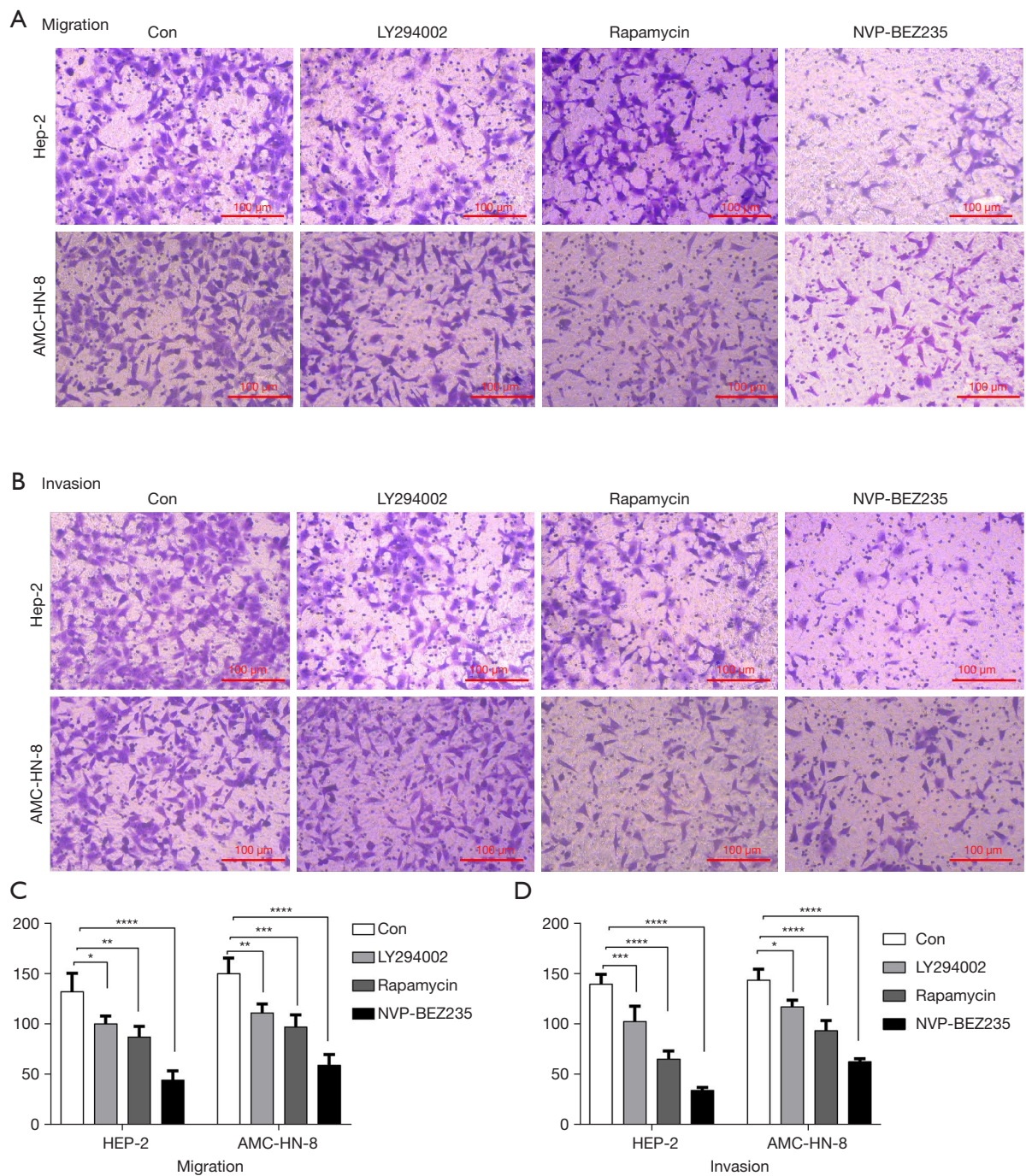


Figure 4 Cell migration and invasion is modulated by PI3K/mTOR inhibitors. (A,B) Representative images of the Transwell migration and invasion assay at 24 h in different treatment groups (LY294002, rapamycin, NVP-BEZ235 and DMSO as control group) in Hep-2 and AMC-HN-8 cell lines (HE stain). (C,D) Cell quantification showed a significant decrease in migration and invasion by all three inhibitors in both cell lines, and NVP-BEZ235 showed a stronger effect. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; scale bar = 100 μm). HE, haematoxylin and eosin.

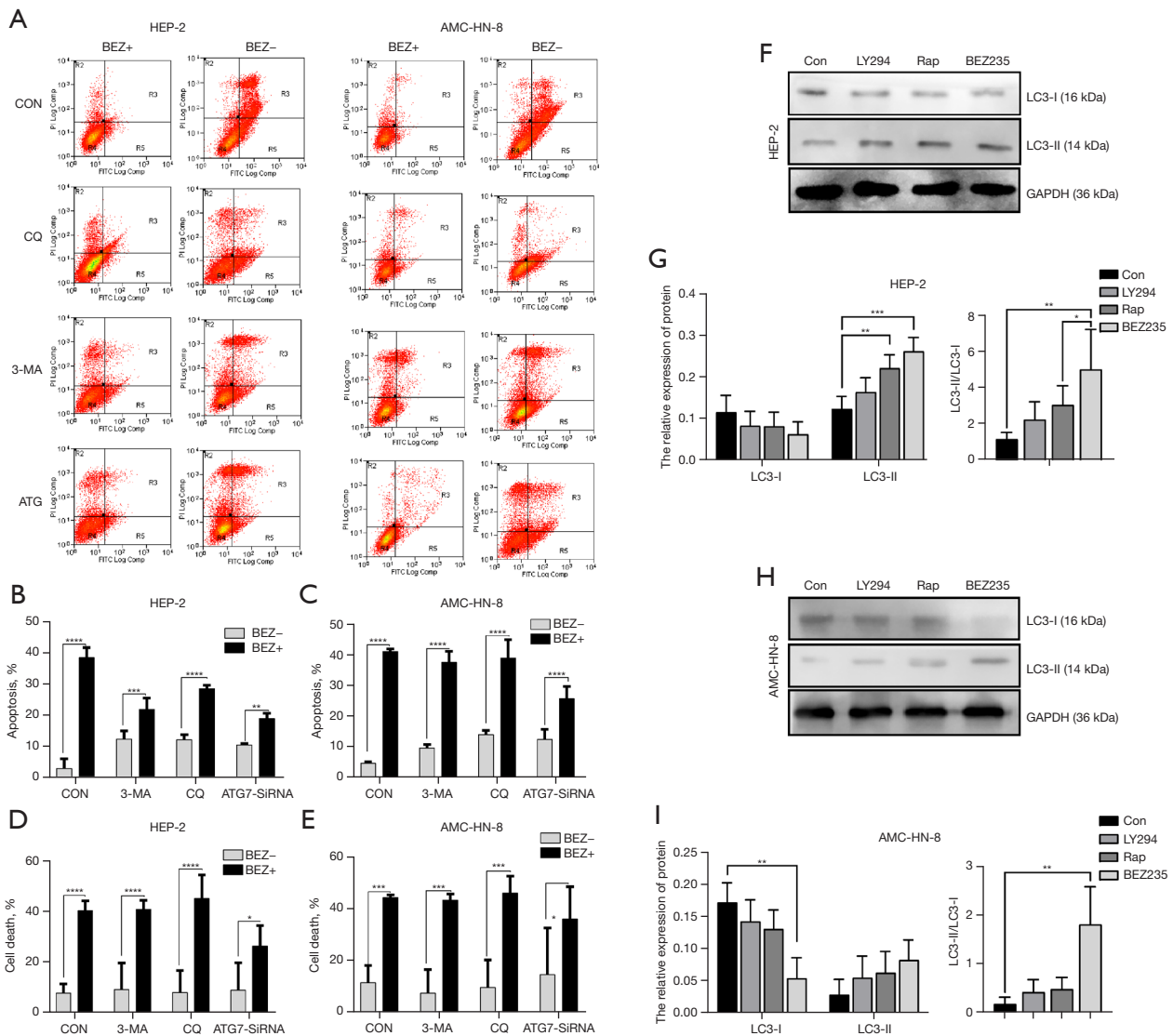


Figure 5 Cell autophagy and of PI3K/Akt/mTOR pathway inhibition. (A) FCM analyses of the cell apoptosis of cultured Hep-2 cells or AMC-HN-8 cells. (B,C) Cell apoptosis increased in both cell lines treated with a combination of NVP-BE235 and autophagy inhibitors. Columns, means of duplicate measurements; bars \pm SD. (D,E) Cell death increased in both cell lines treated with a combination of NVP-BE235 and autophagy inhibitors. (F,H) Western blotting of LC3II and LC3I expression in two cell lines treated with three PI3K inhibitors. (G,I) Western blotting analysis showed LC3II/I ratio decreased significantly following treatment of both cell lines with Ly294002, rapamycin, and NVP-BE235. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. FCM, flow cytometric.

Since PI3K/Akt/mTOR signaling pathway plays a crucial role in tumorigenesis by strikingly promoting cell growth and proliferation (11,12), inhibition of this pathway is also postulated as a therapeutic option in LSCC (13). The mTOR protein serves as an integral part in this signaling complex, which is involved in two major complexes: mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2)

(14). Although discovered much later than mTORC1, mTORC2 is emerging as a promising candidate target in oncotherapy (15). Studies have proved that mTORC2 can directly regulate Akt phosphorylation via activating a critical regulatory site required for maximal Akt kinase activity, which enables Akt to become a crucial downstream protein of the mTORC2 signal pathway (16). Due to multiple

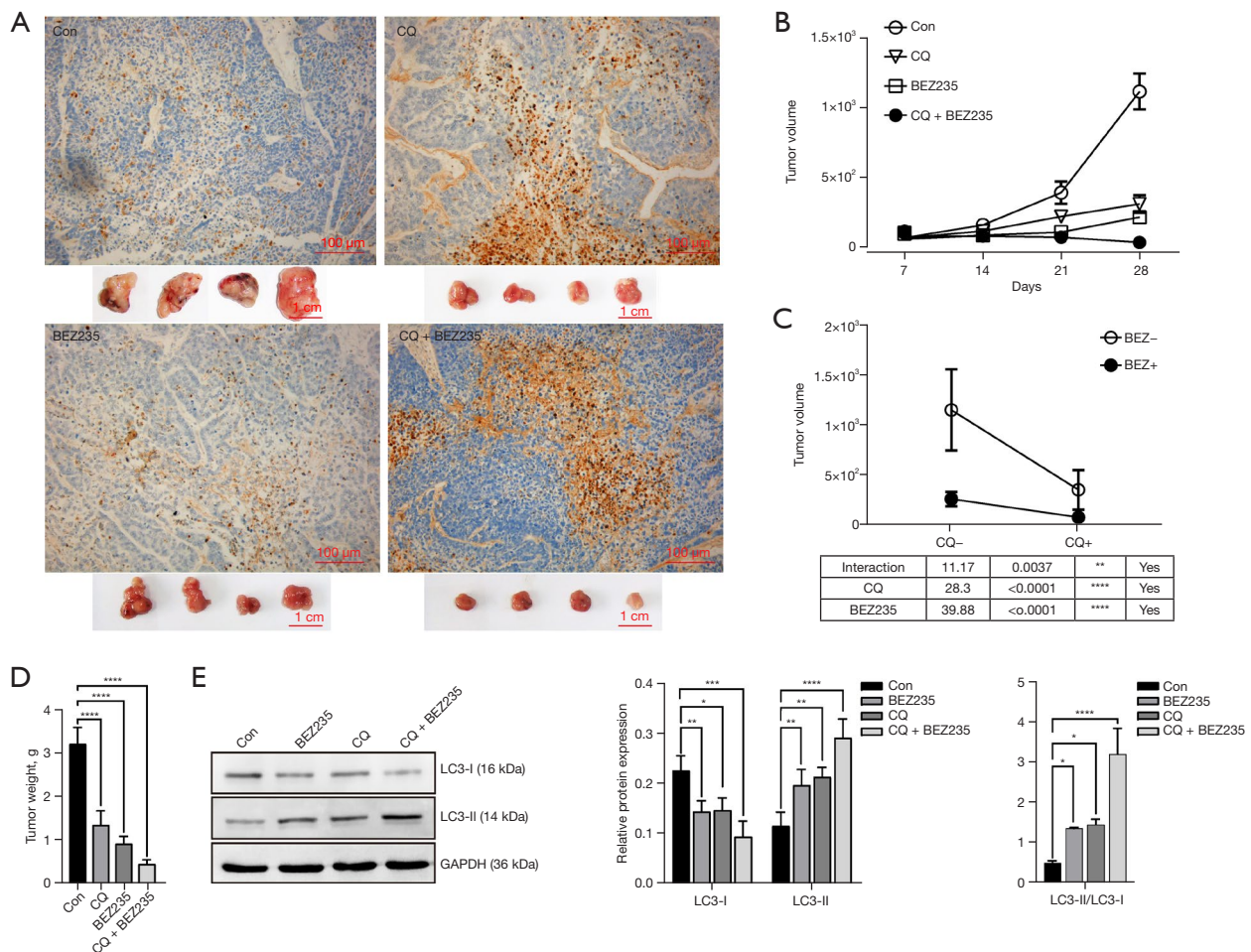


Figure 6 Anti-tumor efficacy of the combination of NVP-BEZ235 with autophagy inhibitor *in vivo*. (A) Tumors obtained from mice and their immunohistochemical results. (B,C) Tumor volumes increased with days, and their analysis results. Points, mean of four replicate determinations; bars \pm SD. (D) Differences in tumor weights between the four groups. Points and columns, mean of four replicate determinations; bars \pm SD. (E) Western blotting testing for LC3II and LC3I expression of tumors from the four groups and the corresponding analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

resistance mechanisms, such as the feedback activation of the PI3K/Akt signaling network and the insensitivity of the mTORC1 complex, inhibition of the mTORC1 pathway has met with limited success in many studies (17,18), which is also verified in our experiment. Compared with Akt inhibitor LY294002 and mTORC1 inhibitor rapamycin, dual PI3K/mTOR inhibitor NVP-BEZ235 showed better anticancer effects in arresting the cell cycle, promoting cell apoptosis, and inhibiting cell invasion and metastasis by suppressing mTORC1 and mTORC2 at the same time. Changes in downstream proteins, such as p-Akt, p-S6RP, and p-p70S6K, also supported the anticancer effect of NVP-BEZ235 and its corresponding mechanism in two

LSCC cell lines.

However, an inhibition of mTOR pathway, especially an inhibition of phosphorylation of mTOR, can also induced autophagy in head and neck squamous cell carcinomas (19). Through degradation of damaged proteins and organelles, autophagy can protect tumor cells from stressful conditions, thus mediating resistance to anticancer therapies such as radiation, chemotherapy, and targeted treatments (20,21). In our study, an increase of LC3II/I ratio was found in both LSCC cell lines treated with PI3K or mTOR inhibitors, among which the dual inhibitor NVP-BEZ235 is the most significant, supporting its induction of cell autophagy. Based on accumulating evidence that inhibition of autophagy

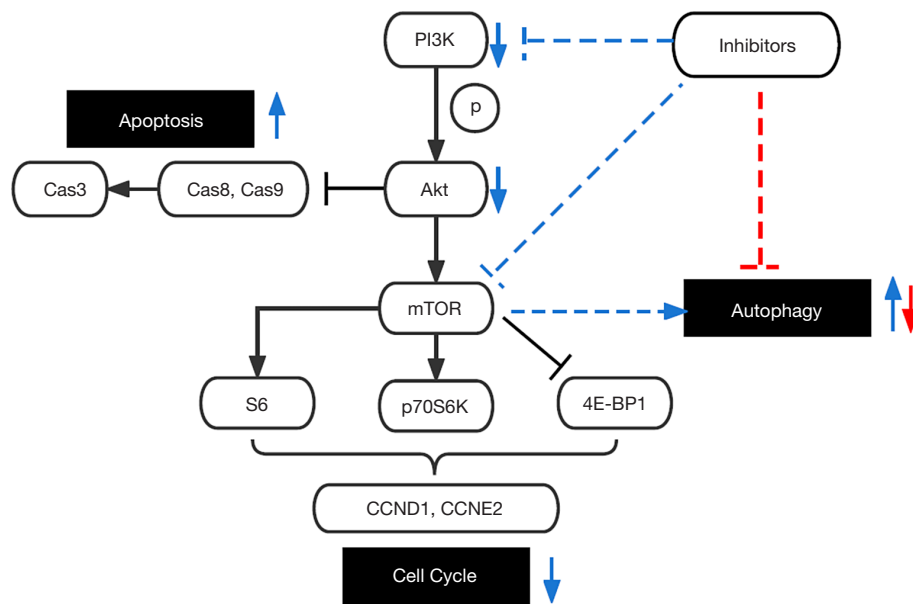


Figure 7 Mechanism of the anti-tumor effect following the treatment of PI3K/mTOR and autophagy inhibitors. Inhibitors of PI3K/mTOR pathway resulted in decreased Akt and mTOR phosphorylation, subsequently regulates downstream molecules and influence cell apoptosis, autophagy (blue arrow). The combination of autophagy inhibitors weakens the promoting effect of autophagy caused by the inhibition of mTOR, therefore exerts synergistic antitumor effect.

can enhance the efficiency of antitumor drugs (22), we combined NVP-BEZ235 with autophagy inhibitors for further verification.

Surprisingly, the combination of NVP-BEZ235 with two autophagy inhibitors (3-MA, CQ) showed a synergistic antitumor effect in both cell lines. LSCC cells that inhibited autophagy in the early phase by silencing Atg7 also had increased cell apoptosis and death, further indicating that inhibition of autophagy can effectively improve the antitumor effect of PI3K/Akt inhibitors. We then combined NVP-BEZ235 with the less toxic autophagy inhibitor CQ to test their antitumor efficacy *in vivo*. The increased proportion of apoptotic cells in the tumor, along with the obviously decreased tumor size and tumor weight, were all consistent with those of our previous cell experiments.

In conclusion, we have proved the remarkable antitumor effect of dual PI3K/Akt inhibitor NVP-BEZ235 compared to other PI3K or Akt inhibitors in LSCC cells, which may be achieved by inhibiting mTORC1 and mTORC2 simultaneously. Combined treatment with CQ, which could inhibit autophagy induced by NVP-BEZ235, further promoted tumor cell apoptosis and death both *in vitro* and *in vivo*, indicating their potential synergistic antitumor effect in LSCC (Figure 7). Based on the current

trend of combination therapy in cancer treatment (23), our experiments have solved the limitations of PI3K/Akt inhibitors to some extent, suggesting that combined application of autophagy inhibitors could be an effective way to overcome resistance of LSCC to PI3K/Akt inhibitors, while providing a promising therapy with the combined application of targeted drugs in LSCC.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-21-2325/rc>

Data Sharing Statement: Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-21-2325/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tc.amegroups.com/article/view/10.21037/tcr-21-2325/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Experiments were performed under a project license (No. KJ2008-01) granted by Ethics Committee of the EENT Hospital, Fudan University, in compliance with Fudan University guidelines for the care and use of animals.

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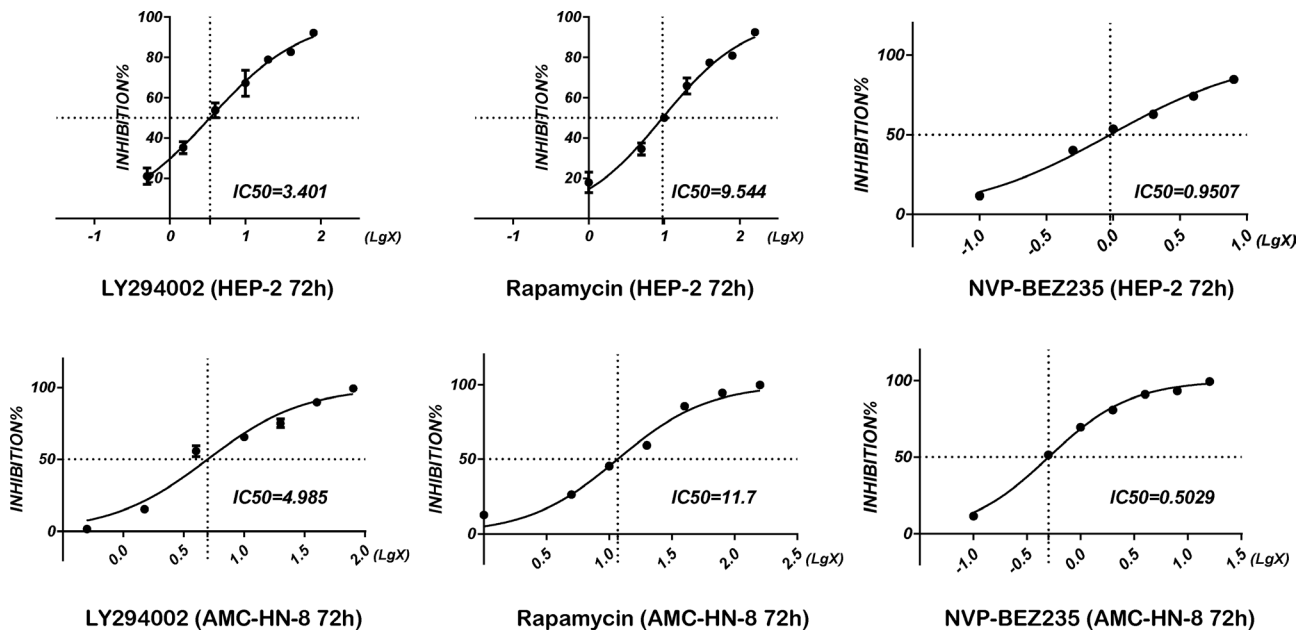


Figure S1 MTT assay in HEP-2 and AMC-HN-8 cell lines after treatment with different concentrations of LY294002 (1, 5, 10, 20, 40, 80, 160 μ M), rapamycin (1, 5, 10, 20, 40, 80, 160 μ M), and NVP-BE2235 (0.1, 0.5, 1, 2, 4, 8, 16 μ M) for 72 h.

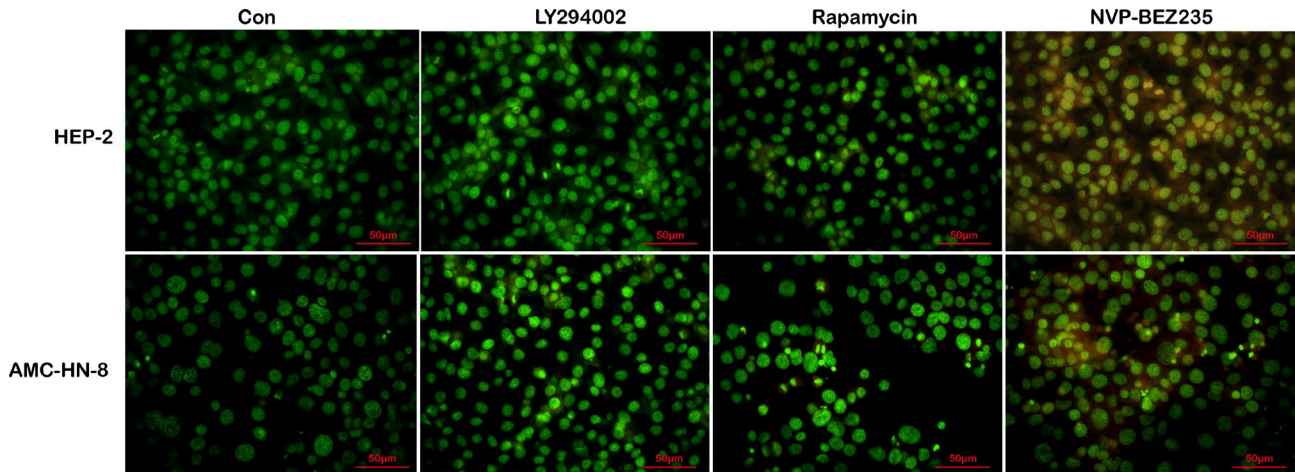


Figure S2 Fluorescence micrographs of acridine orange-stained HEP-2 and AMC-HN-8 cell lines after culture in the absence (control) and presence of three different inhibitors.