Abstract. The present study investigated whether the autophagy inhibitor chloroquine (CQ) can improve the sensitivity of the A549 lung cancer cell line to epirubicin (EPI). The Cell Counting Kit 8 (CCK8) assay was used to determine the EPI IC_{50} in A549 cells treated for 72 h. A549 cells were treated with Western blot analysis was performed to detect the expression level of the autophagy-associated protein, microtubule associated protein 1 light chain 3 β (LC3B), and apoptosis-associated proteins such as cleaved caspase-9 and cleaved caspase-3. CCK8, colony formation, wound healing and Transwell assays were performed to analyze cell proliferation, migration and invasion capacity. Reverse transcription-quantitative PCR (RT-qPCR) was used to analyze the mRNA expression levels of LC3B and beclin-1, and the apoptosis rate was analyzed by flow cytometry. The IC_{50} of EPI was 0.03 µg/ml. The CCK8 results demonstrated that the cell survival rate was lower in CQ + EPI-treated cells when compared with the individual treatment groups. The colony formation results revealed that the number of clones in the EPI + CQ-treated group was reduced compared with EPI or CQ treatment alone. The wound healing assay revealed that migration was reduced in the EPI + CQ-treated group compared with the other treatment groups, and the Transwell results indicated that the number of cells passing through the Matrigel and membrane was lowest in the CQ + EPI treatment group. The mRNA expression levels of LC3B and beclin-1 were increased in the CQ + EPI group by 51.5 and 61.2%, respectively, when compared with the control group. The results indicated that LC3B protein expression was enhanced by EPI in a concentration-dependent manner, and the protein levels of cleaved caspase-3 and cleaved caspase-9 were higher in the combination group than in the EPI alone group. The flow cytometry results demonstrated that the apoptosis rate was highest in the EPI + CQ group. In conclusion, the autophagy inhibitor CQ increased the sensitivity of A549 cells to EPI, and the underlying mechanism of action may be associated with the activation of apoptosis.

Introduction

Lung cancer is the most commonly occurring cancer worldwide (1). Lung cancer is subdivided into small cell lung cancer and non-small cell lung cancer (NSCLC) (2). Surgery is regarded as the most effective treatment for early stage lung cancer; however, radiotherapy, chemotherapy and targeted therapy are often the last option for patients with advanced lung cancer, for whom surgery is no longer an option (2). A combination of anthracyclines and platinum drugs are well-established, safe and effective first-line chemotherapy regimens (3,4). Epirubicin (EPI) is the 4’ epimer of the anthracycline antibiotic doxorubicin, and has been used alone or in combination with other cytotoxic agents for the treatment of a variety of malignancies (5). It has been proposed that EPI functions via several underlying mechanisms: i) Inhibition of DNA replication and transcription; ii) inhibition of topoisomerase II activity; and iii) generation of reactive oxygen species (ROS). Liu et al (6) investigated the mechanism of EPI chemotherapy in the human large cell lung cancer cell line H460. It was revealed that regulation of Glycoprotein 130 (gp130) signaling serves a critical role in epirubicin-based chemotherapy, and the antitumor efficacy of EPI depends on the degradation of gp130. It was also proposed that if gp130 has S-782-A mutation, it will increase the autophagy of lung cancer cells, which may help them survive the crisis. And they found that epirubicin-resistant cells expressed high level of...
of gp130 (6). In a phase III clinical trial, Wachters et al (7) hypothesized that EPI or cisplatin combined with gemcitabine may be used as a first-line treatment for advanced non-small cell lung cancer. However, the long-term use of anti-cancer drugs often results in tumor resistance, which greatly limits the effects of EPI in lung cancer treatment (8). Previous studies have reported that tumor resistance may be associated with increased autophagy (9-13), and inhibiting autophagy may enhance the role of chemotherapeutic drugs. Autophagy is a way for cells to survive crisis; they can produce the energy and substance necessary for cells survival by degrading their subcellular organelles, so as to maintain the homeostasis. In the tumor environment, autophagy can help tumor cells resist radiation or chemotherapy (14,15). Chloroquine (CQ) is a commonly used autophagy inhibitor that functions by disrupting the acidic environment of lysosomes and inhibiting the fusion of autophagosomes with lysosomes (16). A study by Chou et al (17) revealed that cotreatment with CQ enhanced the cytotoxicity of C2-ceramide by 2.4- and 3.4-fold respectively compared with single treatment in the two NSCLC cell lines H460 and H1299; moreover, combination treatment significantly reduced the migration and invasion capability of both cell lines. Furthermore, in vivo analysis demonstrated that a combination of C2-ceramide and CQ resulted in a significant tumor-inhibition efficacy in the zebrafish xenograft model compared with single treatment groups, which suggests that the combination was reliable for lung cancer treatment (17). The present study investigated whether the autophagy inhibitor CQ can improve the sensitivity of the A549 lung cancer cell line to EPI, and attempted to elucidate the mechanisms involved.

Materials and methods

Cell culture. A549 lung cancer cells were donated by Professor Qian HL, Institute of Oncology, Chinese Academy of Medical Sciences (Beijing, China) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Shanghai Shengong Biology Engineering Technology Service, Co., Ltd.) and 100 µg/ml streptomycin. The incubator temperature was 37°C, the CO₂ concentration was 5% and the medium was changed every other day. Cells in the logarithmic growth phase were used for subsequent experiments.

Measurement of the IC₅₀, IC₉₀ and IC₇₀ of EPI. A549 cells in the logarithmic growth phase were seeded in 96-well plates (1,000 cells/well). After 24 h in the aforementioned culture conditions, cells were treated with EPI (200 µl; MedChemExpress) at concentrations of 0, 0.01, 0.02, 0.04, 0.08 and 0.16 µg/ml. At 72 h following treatment, 10% Cell Counting Kit-8 (CCK8) reagent (cat. no. CK04-13; Dojindo Molecular Technologies, Inc.) was added to each well (CQ and EPI were diluted in RPMI-1640 medium). The incubator temperature was 37°C during the 72 h incubation. The OD values of each group were measured using CCK8 according to the previously described protocol. There were three replicates for each experiment and three experimental repeats were performed. The calculated survival rate and inhibition rate were calculated as described previously.

Colony formation assay. A549 cells in the logarithmic growth phase were seeded into 6-well plates at a density of 400 cells/well, and incubated in the aforementioned culture conditions. After 12 h, 500 µl complete RPMI-1640 media, and 10 µM CQ, 0.01 µg/ml EPI or 10 µM CQ + 0.01 µg/ml EPI were added to cells and incubated at 37°C. After 72 h, each drug treatment was replaced with complete RPMI-1640 medium and culture was continued. The experiment was terminated when macroscopic colonies appeared. Colonies were fixed in methanol for 20 min at 25°C and stained with 0.1% crystal violet for 30 min at 25°C and the number of clones was counted. There were three replicates in each experiment and three experimental repeats were performed.

Invasion assay. Matrigel (BD Biosciences), diluted 1:6 (75 µl/well), was plated on the upper chambers of Transwell inserts. A549 cells in the logarithmic growth phase (1.5x10⁴ cells/well) were seeded into the upper chambers containing serum-free medium (cells were serum starved prior to seeding). Additionally, 600 µl RPMI-1640 supplemented with 20% FBS was added to the lower chambers. After 6 h, A549 cells were treated with CQ (10 µM), EPI (IC50) or both (drugs were diluted in serum-free RPMI-1640 media). After 72 h, the chambers were removed, and the invading cells were fixed in methanol for 20 min at 25°C and stained with 0.1% crystal violet for 30 min at 25°C. A cotton swab was used to remove cells in the upper chamber and number of cells passing through the Matrigel and the membrane were counted using a light microscope (Nikon Corp.; magnification, x100). There were three replicates in each experiment and three experimental repeats were performed.

Wound-healing assay. A549 cells in the logarithmic growth phase were seeded at a density of 1x10⁵ cells per well into a 12-well plate and incubated for 24 h at 37°C and 5% CO₂. After 24 h, a vertical scratch-wound was drawn in the center of each well using a sterile tip, and PBS was used to wash off debris. RPMI-1640 supplemented with 2% FBS, and 10 µM CQ, 0.03 µg/ml EPI or 10 µM CQ + 0.03 µg/ml EPI was added to each well (CQ and EPI were diluted in RPMI-1640 containing 2% FBS). After 72 h, the drugs were replaced with RPMI-1640 supplemented with 2% FBS alone. Cell scratches were observed under a light microscope (Nikon; magnification, x100) at 0, 24 and 48 h. There were three replicates in each experiment and three experimental repeats were performed.

Microtubule associated protein 1 light chain 3 α (LC3A) and 3 β (LC3B) protein expression. A549 cells in the logarithmic growth phase were seeded at a density of 1.8x10⁵ cells per well in 6-well plates and incubated overnight at 37°C and...
5% CO₂. The cells were treated with different concentrations of EPI (0.01, 0.03 and 0.08 µg/ml), CQ (10 µM), EPI (0.03 µg/ml) or CQ (10 µM) + EPI (0.03 µg/ml). After 72 h, total proteins were extracted and the protein concentration was determined using the BCA method (Thermo Fisher Scientific, Inc.). Proteins were separated by SDS-PAGE (the separate gel and the concentrated gel were 15 and 5%, respectively). The wet transfer method was performed at 200 mA for 120 min to transfer proteins to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked at 25°C in 5% skimmed milk powder for 1.5 h, and then incubated with the primary antibody Rabbit Anti-MAP1LC3A antibody (cat. no. ab52628; Abcam) or rabbit anti-human LC3B monoclonal antibody (cat. no. 3868; Cell Signaling Technology, Inc.) overnight at 4°C on a shaker. Secondary antibody (horseradish peroxidase-labeled goat anti-rabbit antibody; Bio-Rad, Inc.) overnight at 4°C on a shaker. The experiment was repeated three times. The 2-ΔΔCq analysis method was used to analyze the experimental results (18). The primers sequences are listed in Table I. GAPDH was selected as the reference gene.

Statistical analysis. All experimental data were analyzed using SPSS 17.0 statistical software (SPSS, Inc.) and presented as the mean ± standard deviation. The IC₅₀ was calculated using Probit regression. Multiple comparisons were performed with one-way analysis of variance followed by Tukey's post hoc test. Prior to data analysis, the homogeneity of variance and normality tests was performed using Bartlett's test. P<0.05 was considered to indicate a statistically significant difference.

Results

EPI IC₂₅, IC₅₀ and IC₇₅. The viability of A549 cells was analyzed at 72 h following treatment with EPI at different concentrations (Table II; Fig. 1A). The IC₂₅, IC₅₀ and IC₇₅ concentrations of A549 cells treated with EPI for 72 h were 0.01, 0.03 and 0.08 µg/ml, respectively.

EPI and CQ combination therapy enhances the anti-proliferative effect of EPI in A549 cells. The CCK8 assay demonstrated there was no significant difference in the survival rate of cells treated with CQ alone compared with the control group. The survival rate of EPI-treated cells was decreased to 52.5% compared with the control group (P<0.001). The survival rate of the EPI + CQ group was decreased to 33.3% compared with the control group (P<0.001). The results demonstrated that the combination treatment had a greater effect on cell proliferation compared with EPI or CQ alone (Fig. 1B).

EPI and CQ combination therapy reduces the colony formation ability of A549 lung cancer cells. Colony formation experiments revealed that there were no statistically significant differences between the CQ alone and control groups. The number of colonies in the EPI alone group was decreased to 65.64% compared with the control group (P<0.01; Fig. 2A and B). The number of colonies in the CQ + EPI group was ~71.09% of the number in the EPI alone group (P<0.05; Fig. 2A and B). This indicated that CQ combined with EPI reduced the colony formation ability of A549 cells when compared with EPI treatment alone.

EPI and CQ combination therapy reduces the invasion ability of A549 lung cancer cells. The Transwell assay
results revealed that the number of cells that passed through the Matrigel and membrane was lower in the EPI alone group than in the control group (324±7.937 vs. 574±23.896; P<0.01). The number of cells in the combination group was lower compared with that in the EPI alone group (173±11.240 vs. 324±7.937; P<0.05). There was no significant difference between the CQ alone group and the control group (Fig. 2C and D).

**EPI and CQ combination therapy reduces the migration ability of A549 lung cancer cells.** The results of the wound-healing assay demonstrated that the scratch widths (µm) of the control, CQ alone, EPI alone and the combination groups were 719.7±7.57, 730.7±5.69, 724.0±6.25 and 723.6±6.66, respectively. After 72 h, the results of the wound-healing assay demonstrated that the percentage wound closure of the control, CQ alone, EPI alone and the combination groups were 40.65±0.563, 47.72±2.756, 46.03±1.380 and 24.75±0.661, respectively. And after 96 h, the percentage of wound closure (%) were 85.65±0.892, 82.65±0.595, 73.04±0.375 and 44.53±2.222, respectively, and at 108 h the % values were 100.0±0.000, 100.0±0.000, 80.32±1.558 and 53.76±2.140, respectively. One-way analysis of variance revealed that there was no significant difference between the control group and the CQ alone group (P>0.05); however, there was a significant difference between the EPI alone group and the control group (P<0.001; Fig. 2E and F).

**EPI enhances autophagy in A549 lung cancer cells.** A549 cells were treated with different concentrations (0.01, 0.03 and 0.08 µg/ml) of EPI for 72 h and the expression levels of LC3A and LC3B protein were analyzed. The results demonstrated that the expression ratio of LC3B to LC3A in EPI-treated A549 cells increased in a concentration-dependent manner (Fig. 3). The expression ratio of LC3B to LC3A have previously been used to evaluate the extent of autophagy (19); therefore, these results suggest that EPI enhanced autophagy in A549 cells.

**EPI can induce the mRNA expression of the autophagy-associated genes LC3B and beclin-1.** RT-qPCR revealed the expression of the autophagy-associated gene LC3B was significantly increased in the EPI and combination groups when compared with the control group (P<0.05; Fig. 4A). The expression of beclin-1 was also significantly increased in the EPI and combination groups when compared with the control group (P<0.01; Fig. 4B). Taken together, these results suggest that EPI treatment induced autophagy in A549 cells.

**CQ inhibits autophagy in A549 lung cancer cells.** Western blot analysis demonstrated that there was a significant difference in the expression of LC3B between the control and the CQ alone groups (P<0.001). A significantly difference was also observed between the EPI alone and combination groups (P<0.001; Fig. 4C). This effect was increased following CQ treatment, indicating that CQ inhibited the downstream degradation of autophagy.

**Combination therapy increases apoptosis of A549 lung cancer cells.** Flow cytometry analysis revealed that there was no significant difference in the apoptosis rate between the control group and the CQ alone group; however, the apoptosis rate was significantly increased in the EPI alone group compared with the control group (P<0.01). In addition, the apoptosis rate of the combination group was significantly higher than that of the EPI alone group (P<0.05; Fig. 4D).

**Discussion**

High morbidity and mortality rates make lung cancer a severe threat to human health (1). Chemotherapy serves an important role in the treatment of lung cancer; however, even the most effective chemotherapy has only a 30-50% response rate (20). Drug resistance of tumor cells has become an increasingly prominent setback during chemotherapy, as it severely limits
the efficacy of chemotherapeutic drugs, which in turn has meant that drug resistance has been the focus of an increasing amount of studies (21). Multiple studies have reported that autophagy may be involved in chemotherapeutic resistance developed in lung cancer (20,22-25), and that inhibiting autophagy may reduce resistance and enhance the efficacy of chemotherapeutics. Autophagy protects cells from DNA damage during chemotherapy or targeted therapy, and maintains intracellular environmental stability, which may be one of the mechanisms that contribute to chemotherapy resistance (26,27). Autophagy can promote cell survival under conditions of metabolic stress, exhibiting an oncogenic function (28). During autophagy, the C-terminus of LC3 precursor protein is hydrolyzed by autophagy related (Atg)4 to remove a polypeptide and expose a glycine residue (29). The LC3A protein is then distributed in the cytosol, and Atg7 transports LC3A to Atg3, which covalently binds LC3A and phosphatidylethanolamine to form lipophilic LC3B, which mediates autophagosome membrane elongation. Then autophagosome is formed by closure, and then fused with endosomes or lysosomes to form autolysosomes. Finally, the whole autophagy process is completed by degradation (29).

Figure 1. A549 cell survival rate. (A) Fold change in the survival rate of A549 cells treated with different concentrations of EPI for 72 h. (B) Histogram depicting the survival rate of A549 lung cancer cells. *P<0.01, **P<0.001 vs. control or as indicated. EPI, epirubicin.

Figure 2. Colony formation, Transwell and wound healing assays. (A) Colony formation in each group. (B) Quantification of colony formation in each group. *P<0.05, **P<0.01 vs. control or as indicated. (C) Images of cells passed through the Matrigel and membrane (x400 magnification). (D) Quantification of the number of cells passed through the Matrigel and membrane. *P<0.05, **P<0.01 vs. control group or as indicated. (E) Images from the wound healing assay for each group after 108 h (x100 magnification). (F) Scratch width quantification after 72, 96 and 108 h. *P<0.05, **P<0.01, ***P<0.001 vs. control or as indicated. EPI, epirubicin; CQ, chloroquine.
Figure 3. Effect for EPI on LC3B protein expression. Cells were treated with different concentrations (0.01, 0.03 and 0.08 µg/ml) of EPI for 72 h. (A) Western blot analysis of LC3A and LC3B in each group. (B) Densitometry analysis of the protein expression ratio of LC3B to LC3A in each group. *P<0.05 vs. control group. EPI, epirubicin; LC3A, microtubule associated protein 1 light chain 3 α; LC3B, microtubule associated protein 1 light chain 3 β.

Figure 4. Reverse transcription-quantitative PCR, western blot analysis and cell apoptosis assay. (A) RNA expression levels of LC3B. (B) RNA expression levels of beclin-1. (C) Western blot analysis of LC3A and LC3B in each group, and densitometric analysis of the protein bands (D) Flow cytometry analysis of apoptosis and quantification of the apoptosis rate. *P<0.05, **P<0.01, ***P<0.001 vs. control group or as indicated. EPI, epirubicin; CQ, chloroquine; LC3A, microtubule associated protein 1 light chain 3 α; LC3B, microtubule associated protein 1 light chain 3 β.
The Atg12-Atg5-Atg16 complex is required for this process; therefore, the level of cellular autophagy formation can be assessed using the ratio of LC3B to LC3A proteins, and an increased ratio is indicative of enhanced autophagy formation (19). Increased LC3B protein may indicate an increase in autophagy formation, or it may indicate that the downstream degradation of autophagy is inhibited, and the hydrolysis of LC3B is reduced, therefore resulting in an increase in LC3B protein content (29).

The findings of the present study demonstrated that treatment of A549 lung cancer cells with CQ in conjunction with EPI reduced the proliferation, migration and invasion of A549 cells, suggesting that the combination of an autophagy inhibitor with EPI may enhance the anti-tumor activity. Additionally, this combination of drugs can increase the apoptosis of A549 cells. The results also suggest that the expression of the LC3B protein was higher in the CQ group than in the control group, and LC3B protein expression in the combination group was higher than that in control and EPI groups. This indicates that CQ inhibited cell autophagy. CQ inhibits the fusion of autophagosome and lysosomes, which reduces the degradation of LC3B protein resulting in increased LC3B protein levels (16). This may be the mechanism by which the combination regimen is more effective against A549 cells than their use alone. These results suggest that EPI can induce autophagy of A549 lung cancer cells. When combined with EPI, CQ can reduce EPI-induced autophagy and, thus, enhance the anti-tumor effect of EPI.

In addition, previous studies have demonstrated that inhibition of autophagy can increase mitochondrial aggregation and intracellular damage (35,36), which may be the mechanism by which CQ promotes autophagy-induced apoptosis (31). Autophagy can eliminate damaged mitochondria in cells to maintain intracellular homeostasis (32-34). In summary, EPI treatment of the A549 lung cancer cell line promoted autophagy, and the induction of autophagy was concentration-dependent. The autophagy inhibitor, CQ, combined with EPI increased the ability of EPI to inhibit the proliferation, invasion and migration of A549 cells, exhibiting a synergistic effect. Consequently, it was hypothesized that the mechanism of this synergistic effect may be associated with increased apoptosis. The potential mechanism by which inhibiting autophagy increases apoptosis may be mediated by intracellular aggregation of damaged mitochondria; however, the detailed processes involved require further investigation. With continuous research into this synergistic effect, the use of autophagy inhibitors in combination with traditional chemotherapeutic drugs has the potential to become a novel treatment plan. These findings provide novel ideas for a clinical solution to cancer chemotherapy resistance.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

ALL and JZ were responsible for designing and carrying out the project, and writing and revising the manuscript. SLD, BZ, XM and CYW performed the cell culture, CCK-8 and flow cytometry experiments. YJL was responsible for the overall design of the experimental scheme, technical guidance, data review and paper revision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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