Alantolactone inhibits cell autophagy and promotes apoptosis through targeting AP2M1 in acute lymphotic leukemia

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is an aggressive hematopoietic malignancy and most commonly seen in children. Alantolactone (ATL) has been reported to have anti-tumor activities in different types of cancer. This study aimed to evaluate the anti-tumor activity and molecular mechanisms of ATL in ALL.

Methods: The ALL cells were treated with 1, 5 and 10μM of of ALT, and then subjected to MTT assay and RNA sequencing. Flow cytometry, JC-1 staining and immunofluorescence staining assay were employed to measure cell apoptosis and autophagy. Meanwhile, western blot analysis was used to detect apoptosis and autophagy-related proteins. Finally, the effect of ALT on tumor growth was measured in BV173 xenograft nude mouse model.

Results: In this study, we demonstrated that ALT could inhibit the proliferation of ALL cells by inducing apoptosis and inhibiting autophagy. Administration of rapamycin activated autophagy while reversing the effect of ALT on apoptosis. Mechanically, ALT could induce apoptosis and inhibit autophagy by promoting AM2P1 expression. Further, AM2P1 was figured to inhibit beclin1 phosphorylation so that the apartment between beclin1 and bcl-2 was alleviated to participate in the regulation of autophagy and apoptosis in ALL cell.

Conclusions: This study disclosed that Alantolactone can inhibit cell autophagy and promote apoptosis through targeting AP2M1 in acute lymphotic leukemia, indicating a potential therapeutic strategy for ALLtreatment.

Background

Acute lymphoblastic leukemia is the most common leukemia characterized by uncontrolled proliferation of immature lymphoid cells [1, 2]. Over the decades, Despite significant advances to make in the treatment of ALL, about 25% of children and half of the adults are still not sensitive to chemotherapy or relapse [3-5]. So far, the available treatment options for ALL include chemotherapy, antibody therapy and allogeneic bone marrow transplantation depending upon the stage of cancer. Antibody therapy such as anti-CCR4, daclizumab, and alemtuzumab, combined treatment with AZT and IFN, and allogeneic bone marrow transplantation have been suggested to cure ALL. Unfortunately, due to the various limitations, the ideal effect has not yet been achieved [6].

Alantolactone (ALT), a major bioactive sesquiterpene component of Inula helenium has been reported to possess multiple biological and pharmacological activities including antibacterial, antifungal, anti-infammatory and anticancer efects. In recent years, ALT has attracted the attention of researchers due to its potential anticancer activity against various human cancer cells through multiple mechanisms

ATL is a sesquiterpene lactone compound that is primarily obtained from Inula Helenium [7]. ATL has been reported to exert anti-tumor effect on many cancers, including lung cancer, gastric cancer, hepatic

cancer, B-cell acute lymphoblastic leukemia, pancreatic cancer and breast cancer [8-11]. Moreover, ATL has been shown to have synergistic anti-tumor effect with other medicines. For example, Wang J et al. reported that ATL could enhance the sensitivity of lung cancer cells to gemcitabine [12]. Cao et al. stated that ATL might improve the therapeutic efficiency of chemotherapy drug oxaliplatin [13]. Zheng et al. demonstrated that ATL could sensitize human pancreatic cancer cells to EGFR inhibitors [14].

It has been reported that ATL exerts anti-tumor activities through a variety of molecular mechanisms. Firstly, ALT promoted ROS-mediated inhibition of Akt/glycogen synthase kinase (GSK)3β pathway and induction of endoplasmic reticulum (ER) stress [12]. Secondly, ALT regulated p38 MAPK and NF-κB pathways. Thirdly, ALT could inhibit TrxR1 activity and activate ROS-mediated p38 MAPK pathway [15]. Forthly, ALT impaired autophagy-lysosome pathway via targeting TFEB [16]. Finally, ALT enanced the sensitivity of cancer cells to EGFR inhibitors through inhibiting STAT3 signaling [17]. These pathways were overlapping and interacting with each other. However, the role and molecular mechanism of ALT in ALL remains unexplored.

In the present study, we first demonstrated that ALT inhibit the proliferation of ALL cells by modulating autophagy and apoptosis. Inhibition of beclin1 activation by ALT through AP2M1 has revealed an unrecognized mechanism and provided in-depth insight into molecular mechanism of ALT in the treatment of ALL cells as a single agent or in combination with clinical drugs suffering from drug resistance due to induction of AP2M1 activation. The results offer proof that ALT in combination with AP2M1 may be a feasible method for treating ALL.

Methods And Materials

Cell culture and reagents

The human acute lymphoblastic leukaemia cell lines (BV173, NALM6, JM-1, NALM1, RS6 and SUPB15) were purchased from Chinese Academy of Sciences (Shanghai, China) . All of cells were cultured in Dulbecco's altered Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) at 37℃ with 5% CO_2. In addition, the experimental cells were treated with the indicated concentrations, whereas the control cells were treated with an equivalent amount of DMSO. The final concentration of DMSO is 0.1%.

MTT assay

Cell viability analysis was carried out with the MTT assay kit (Sigma-Aldrich, USA). BV173 and NALM6 cells were seeded on 96-well plates and then treated with 1, 5, and 10 μM of Alantolactone for 24h. After that, 10μl MTT was added, and incubated at 37^{o}C for 2 h. The optical density was calculated at 450 nm using a microplate reader. The inhibition rate was calculated. Each experiment was performed at least three times.

RNA sequencing

RNA preparation, library construction and sequencing were performed on the Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA) at the Beijing Genomics Institute (BGI, Shenzhen, China). Statistical analysis was performed, and differentially expressed genes (DEGs) were selected according to the criteria of a fold change ≥ 2, $P < 0.05$ and FDR < 0.05.

Plasmids transfection

The siRNA for AP2M1(si-AP2M1), siRNA for Beclin1 (si-Beclin1), pcDNA3.1 vector overexressing AP2M1 and negative controls were obtained from Genepharma (Shanghai, China). Plasmids were transfected into ALL cells by using Lipofectine 2000 (Invitrogen, USA) according to the manufacturer's instruction.

Cell apoptosis

Upon treatment with different concentrations of Alantolactone, both BV173 and NALM6 cells were harvested and washed with PBS for 3 times. For cell apoptosis analysis, cells were labeled with Annexin V-Fluorescein Isothiocyanate (FITC) /propidium iodide (PI) according to manufacturer's instruction. Finally, cells were observed and photographed with the BD FACS Calibur flow cytometry system (Becton Dickinson, NJ, USA).

Colony formation assay

Cells were seeded in 12-well plates (500 cells/well) and incubated for 14 days and then fixed with 4% paraformaldehyde for 15 minutes. The fixed cells were stained with 0.1% crystal violet, and then photographed with a microscope. The number of visible cell colonies were recorded.

JC-1 staining

JC-1 staining was performed to assess the mitochondrial membrane potential (MMP) using a JC-1 assay kit (Beyotime, Shanghai, China) according to the operating instruction. Images were taken under a fluorescence microscope (Leica, Wetzlar, Germany). The ratio (%) of red/green fluorescence intensity was calculated by Image J software.

Western blot analysis

Total proteins were extracted using RIPA-Buffer supplemented with 10 mM PMSF (Beyotime, Shanghai, China). Bicinchoninic acid assay (BCA) was carried out to quantify protein concentrations. The separation of 40 μg proteins was carried out on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membranes. Then, the membranes were blocked with 5% nonfat milk in Tris-buffered saline and 0.1% Tween 20, and incubated with primary antibodies at 4°C overnight. Together with washing using TBST buffer for 5 times, the membranes were incubated with secondary antibodies for 2 h. Enhanced chemiluminescence (ECL) system kit (Beyotime, Shanghai, China) was employed for bands density. The optical densities (OD) value was analyzed by ImageJ software (NIH, Bethesda, MD, USA).

Immunofluorescence

Tumor tissues were fixed in 4% paraformaldehyde for 24 hours, and then dehydrated in alcohol, embedded in paraffin, and cutted into 5μm sections. The sections were deparaffinized, rehydrated, and then incubated at 96℃ with 0.01 mol/l sodium citrate buffer for the antigen retrieval. Following the incubation in 5% H_2O_2 for 2 hours, the sections were incubated with primary antibodies overnight at 4℃. Immunostaining was carried out with streptavidin-peroxidase and diaminobenzidinef (DAB) following the manufacturer's instructions (Beyotime, Shanghai, China). Finally, the sections were observed and imaged under fluorescence microscope (Leica, Wetzlar, Germany).

Confocal analysis

Following treatment with alantolactone, BV173 cells were transfected with GFP-LC3 vector in 4% paraformaldehyde or 15 minutes, and washed with PBS for 3 times. Subsequently, the cells were permeabilized using 0.05% TritonX-100, and stained with DAPI (Invitrogen, Eugene, OR, USA). In the end, the cells were examined and quantified using the AOBS confocal laser scanning (Leica, Wetzlar, Germany).

Xenograft model

Experiments were performed in BALB/c nu/nu mice (6 weeks old), which were obtained from Charles River Laboratories. A number of 3×10^6 BV173 cells or BV173 cells transfected with AP2M1 siRNA in 100 μl PBS were subcutaneously injected into the posterior flank region of nude mice. The long diameter and short diameter of tumor were measured every 2 days, and calculated using the formula as follows: tumor volume=0.5 × long diameter × short diameter^2. The mice were treated with alantolactone every day since the cell injection. Finally, the mice were sacrificed and excised the tumors.

Statistical analysis

SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) were used to analyze all data for statistical significance. All the variables were presented as mean ± standard deviation (SD). One-way ANOVA followed by Tukey's Post hoc test was used to assess the difference between multiple groups. Differences between two groups were analyzed by the Student’s t-test. $P<0.05$ was considered as statistical significance.

Results

ALT inhibited the proliferation of acute lymphoblastic leukemia cells

To investigate the cytotoxicity of ALT against acute lymphoblastic leukemia cells including BV173, JM-1, NALM1, NALM6, RS6, and SUPB1, MTT assay was carried out to detect the viability of these cells following the treatment with 1, 5 and 10μM of Alantolactone for 24 h. The results showed that both 5 and 10 μM of ALT significantly inhibited the proliferation of BV173, NALM1, NALM6, and RS6 cells, while

10μM of ALT inhibited the proliferation of JM-1 and SUPB1 cells (Figure 1A-F). These data indicate that ALT exerts growth inhibitory effect in ALL cells.

ALT promoted the expression of AP2M1

To elucidate the mechanism underlying the effect of ALT on ALL cells, we screened the expression profile of mRNA using RNA-seq. As showed by the heatmap (Figure 2A), AP2M1 was notably upregulated in ALL cells treated with 5μM of ALT. To confirm this result, qRT-PCR and western blot were performed to detect AP2M1 expression in BV173 and NALM6 cells. As expected, the expressions of AP2M1 mRNA and protein were significantly increased in response to ALT (Figure 2B-D). Further, cell immunofluorescence staining also verified this finding (Figure 2E).

ALT inhibited proliferation and colony formation of ALL cells by targeting AP2M1

We conducted the knockdown of AP2M1 along with ALT treatment to check out whether AP2M1 is involved in the effect of ALT against BV173 and NALM6 cells. As expected, ALT up-regulated the expression of AP2M1 while the transduction of AP2M1 siRNA significantly reduced the expression of AP2M1 (Figure 3A). ALT inhibited the proliferation and colony formation of ALL cells while AP2M1 knockdown could reverse this phenomenon (Figure 3B and C).

ALT inhibited the acute lymphoblastic leukemia growth in vivo

In vivo study was further conducted to investigate the impact of ALT on acute lymphoblastic leukemia. As expected, ALT significantly suppressed the tumor growth and weight in comparison with that in the control group. Importantly, this effect could be reversed by knockdown of AP2M1 in ALL cell (Figure 4 A-C).

ALT induced apoptosis and inhibited autophagy of ALL cells via regulating AP2M1

The mitochondrial membrane potential (MMP) is an important indicator of cell function and health, and its dissipation is considered as an early indicator of cell apoptosis. To evaluate the effect of ALT on cell apoptosis, flow cytometry, JC-1 staining and western blot were used to measure cell apoptotic number, MMP and apoptosis-related proteins. As showed in Figure 5A, treatment with 5μM of ALT for 24 h dramatically increased the apoptosis of BV173 and NALM6 cells, while AP2M1 inhibition remarkably diminished this effect of ALT. Subsequently, JC-1 staining showed that ALT significantly decreased the MMP of ALL cells, which was reversed by AP2M1 knockdown (Figure 5B). Furthermore, western blot showed that ALT treatment substantially elevated the levels of cleavages of caspase-3, bax and cyt-c in cytoplasma while decreased bcl-2 expression, but these effects were attenuated by AP2M1 siRNA (Figure 5E). These results indicate that ALT induced apoptosis of ALL cells via activating AP2M1.

Next, we detected the autophagy of BV173 and NALM6 cells by immunofluorescence staining and western blot. Immunofluorescence assay results revealed that ALT treatment reduced the number of the LC3 fluorescent puncta compared to the control group, while AP2M1 knockdown increased the number of

LC3 puncta in comparison to the ALT group (Figure 5C). Besides, western blot analysis demonstrated that ALT significantly decreased the expression of Beclin1 and LC3II proteins while increased p62 protein expression. Further, we found that ALT-mediated regulation of these autophagy-related proteins were reversed by AP2M1 knockdown, indicating the involvement of AP2M1 in ALT-mediated autophagy regulation (Figure 5D). Above all data support the notion that ALT induced apoptosis and inhibited autophagy of ALL cells via regulating AP2M1.

ALT induced apoptosis of ALL cells via autophagy inhibition

Autophagy and apoptosis are necessary to maintain the cellular homeostasis. In general, autophagy signaling can prevents the induction of apoptosis. To further confirm the relationship between autophagy and apoptosis induced by ALT, BV173 and NALM6 cells were treated with ALT alone or combination with autophagy activator rapamycin. As expected, apamycin could reverse down-regulated beclin1 and LC3II and elevated p62 level induced by ALT in BV173 and NALM6 cells (Figure 6D), further suggesting the involvement of autophagy inhibition in anti-tumor effect of ALT. Interestingly, rapamycin also could abolish the apoptosis promotion of ALT, as demonstrated in Figure 6A-C. Moreover, the finding was confirmed by expression pattern of apoptosis related-proteins (Figure 6E). These findings indicate that ALT could promote cell apoptosis via autophagy inhibition in ALL.

AP2M1 *altered the phosphorylation of bcl-2 and beclin1*

As AP2M1 is is essential for ALT-mediated apoptosis and autophagy of ALL cells, we next examined whether overexpressed AP2M1 affects apoptosis and autophagy. Western blot assay demonstrated that overexpressed AP2M1 inhibited the phosphorylation of beclin1 and elevated the level of bcl-2 (Figure 7A and B). Thus, we speculate that ALT may maintain cellular homeostasis between autophagy and apoptosis by AP2M1/bcl-2/beclin1 signaling pathway, and thereby exert anti-tumor activity in ALL (Figure 7C).

Discussion

In this study, ATL was found to be able to target AP2M1 to inhibit autophagy and promote apoptosis of acute lymphotic leukemia cells. In total, two novelties were made in this study. Firstly, this study provided evidence that ATL exerts an anti-tumor activity via regulating autophagy and apoptosis of ALL cells. More important, this study has shown that AP2M1 protein contributes to the anti-tumor effect of ATL on cancer cells by modulating autophagy.

There are many types of research that show that ATL exerts anti-tumor effects on tumor cells through alone or combined treatment with other anti-tumor agents The mechanisms included regulation of oxygen species-mediated ER stress, ROS response, and other signal pathways such as Akt/GSK3βpathway, p38 MAPK, NF-κB pathway, STAT3 signaling, and Nrf2 signaling. Currently, the function of ALT on autophagy was reported only once in which He et al. found that ATL caused the accumulation of autophagosomes due to impaired autophagic degradation and significantly inhibited

the activity and expression of CTSB/CTSD proteins [18]. Their data demonstrated that ATL, which impaired autophagic degradation, was a pharmacological inhibitor of autophagy in pancreatic cancer cells and markedly enhanced the chemosensitivity of pancreatic cancer cells to oxaliplatin. In this study, we demonstrated that inhibition of autophagy was also the mechanism of ATL to suppress the growth of ALL and this result contributed new proof to the involvement of autophagy in the anti-tumor effects of ATL.

ALT has been shown to contribute to cell apoptosis in numerous cancers. For example, ALT induces gastric cancer BGC-823 cell apoptosis by regulating the AKT signaling pathway [19]. ALT induces apoptosis of breast cancer cells via the p38 MAPK, NFκB, and Nrf2 signaling [20]. Alantolactone also induces apoptosis and enhances the chemosensitivity of A549 lung adenocarcinoma cells to doxorubicin [21]. In this research, it was indicated that ALT also promotes the apoptosis of ALL cells.

Moreover, as suggested in the findings, ALT significantly stimulated apoptosis but at the same time inhibited autophagy of BV173 and NALM6 cells. This function was further confirmed by the use of rapamycin. The results indicated that rapamycin significantly induced autophagy while reversing the effect of ALT on apoptosis.

Necrosis, apoptosis, and autophagy are three types of programmed cell death that contribute critically to cancer cell progression, division, and metastasis [22, 23]. However, the relationship between autophagy and apoptosis remains complicated. In some cases, autophagy promotes cell apoptosis. Paris saponin-induced autophagy promotes acute lymphoblastic leukemia cell apoptosis through the Akt/mTOR signaling pathway [24]. Parthenolide inhibits pancreatic cell progression by autophagy-mediated apoptosis [25]. In other cases, autophagy inhibited the apoptosis process [26]. For example, Rottlerin-stimulated autophagy results in apoptosis in bladder cancer cells [27]. Autophagy inhibition improves heat-stimulated apoptosis in human non-small cell lung cancer cells through ER stress pathways [28].

In order to find out the mechanism underlying the effect of ALT on apoptosis and autophagy, We tried to identify the key factors involved in both apoptosis and autophagy. Therefore, we focused on the beclin1-bcl2 complex and the modification of beclin1 and bcl-2. Among them, the interaction and modification of beclin1 and bcl-2 played a key role in modulating the crosstalk between autophagy and apoptosis [29, 30]. Moreover, previous findings showed that bcl-2 regulated autophagy via beclin1, and bcl-2 phosphorylated resulted in dissociating from beclin1 and induction of autophagy [31]. However, it remained unclear whether the expression, interaction and modification of beclin1 and bcl-2 involved in the crosstalk between ALT-induced autophagy and apoptosis.

In this study, we found that AP2M1 clearly obstructed beclin phosphorylation and the expression of both bcl-2 and beclin1. We further speculated that AP2M1 inhibited beclin1 and bcl-2 by reducing beclin phosphorylation. As a result, AP2M1 inhibited autophagy while promoting ALL apoptosis.

Conclusion

Taken together, we showed that ALT was able to prevent the proliferation of acute lymphoblastic leukemia cells by inducing apoptosis and inhibiting autophagy. The underlying mechanism was involved in the regulation of the AP2M1/beclin1 signal pathway.

Declarations

Ethics approval and consent to participate

All experiments complied with the guiding principles for the care and use of laboratory animals in and were approved by the Committee for Animal Experimentation of the First Affiliated Hospital of Harbin Medical University. All protocols and methods were in accordance with the guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

No conflict of interest.

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Authors' contributions

DY, JW, ZL and YL designed the experiments, performed the research and analyzed the data. YT, WL and MG conducted the cell culture and western blot. TY, FC and JZ did literature research and data analysis. DY and JW wrote and revised the manuscript. YL offered professional advices about the whole research. All authors read and approved the final manuscript.

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Figures

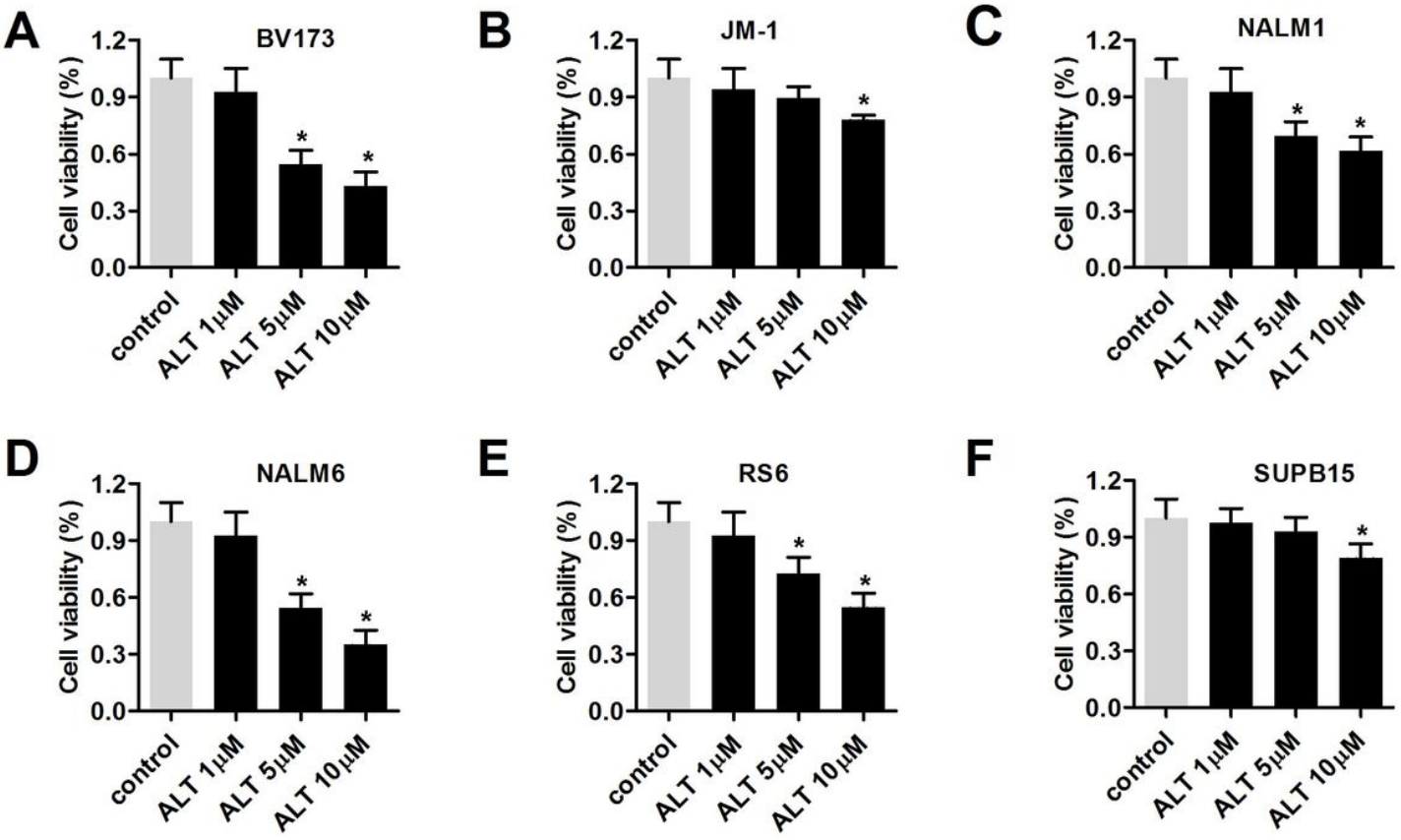

Figure 1

ALT inhibited the proliferation of acute lymphoblastic leukemia cells. (A-F) ALL cell lines including BV173, JM-1, NALM1, NALM6, RS6, and SUPB15 were treated with 1, 5 and 10 μM of ALT for 24 h and MTT was conducted to evaluate the cell viability. Data are expressed as Means ± SD (n=3). *p < 0.05, **p < 0.01 compared with the control groups.

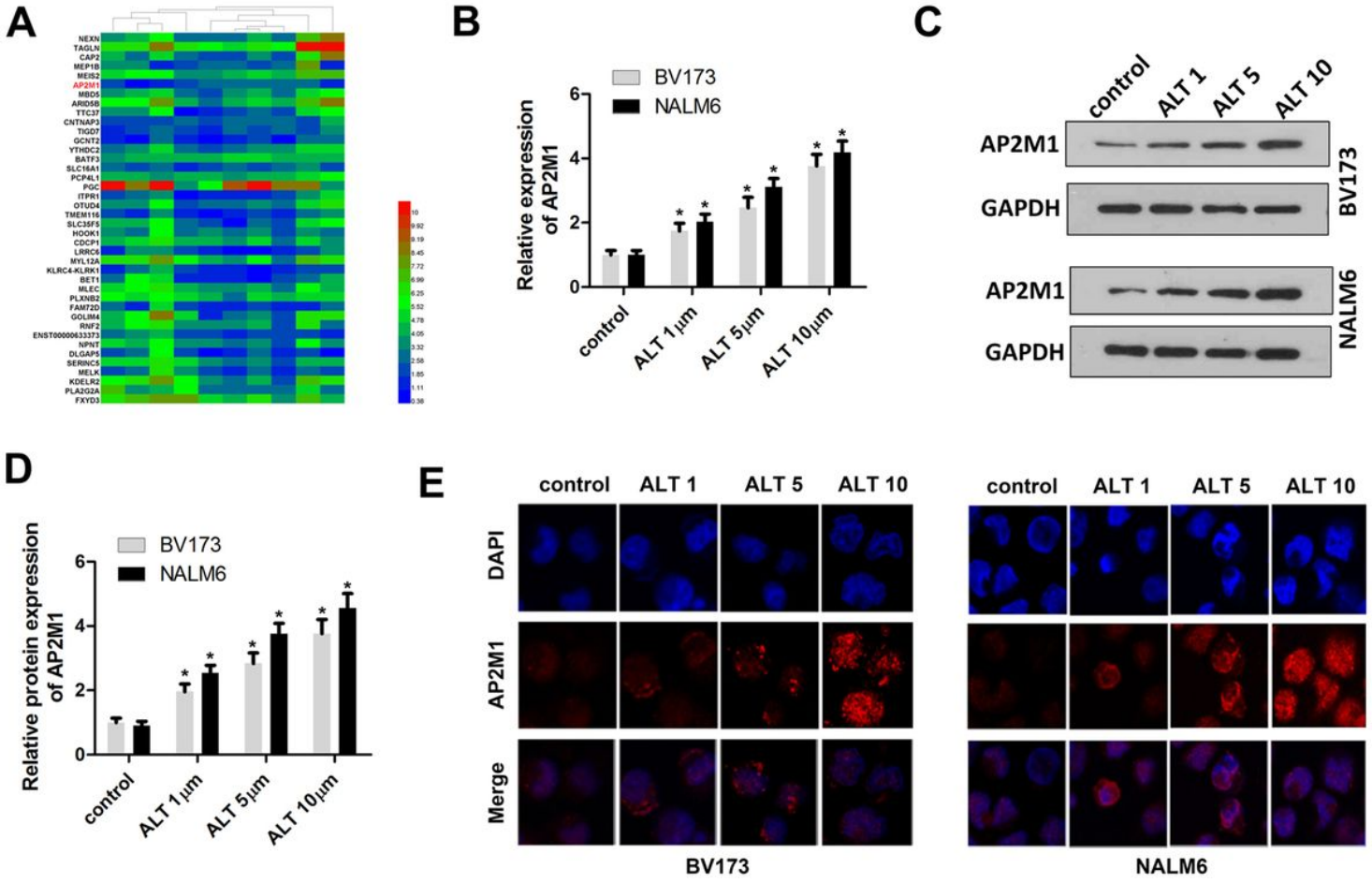

Figure 2

ALT promotes the expression level of AP2M1. (A) RNA-seq was performed to screen the dysregulated gene expression in ALL cell subjected to ALT treatment. After treatment of ALT for different concentrations, (B) qPCR, (C) western blot and (D) immunofluorescence were carried out to evaluate the mRNA and protein expression of AP2M1 in ALL cell *p < 0.05.

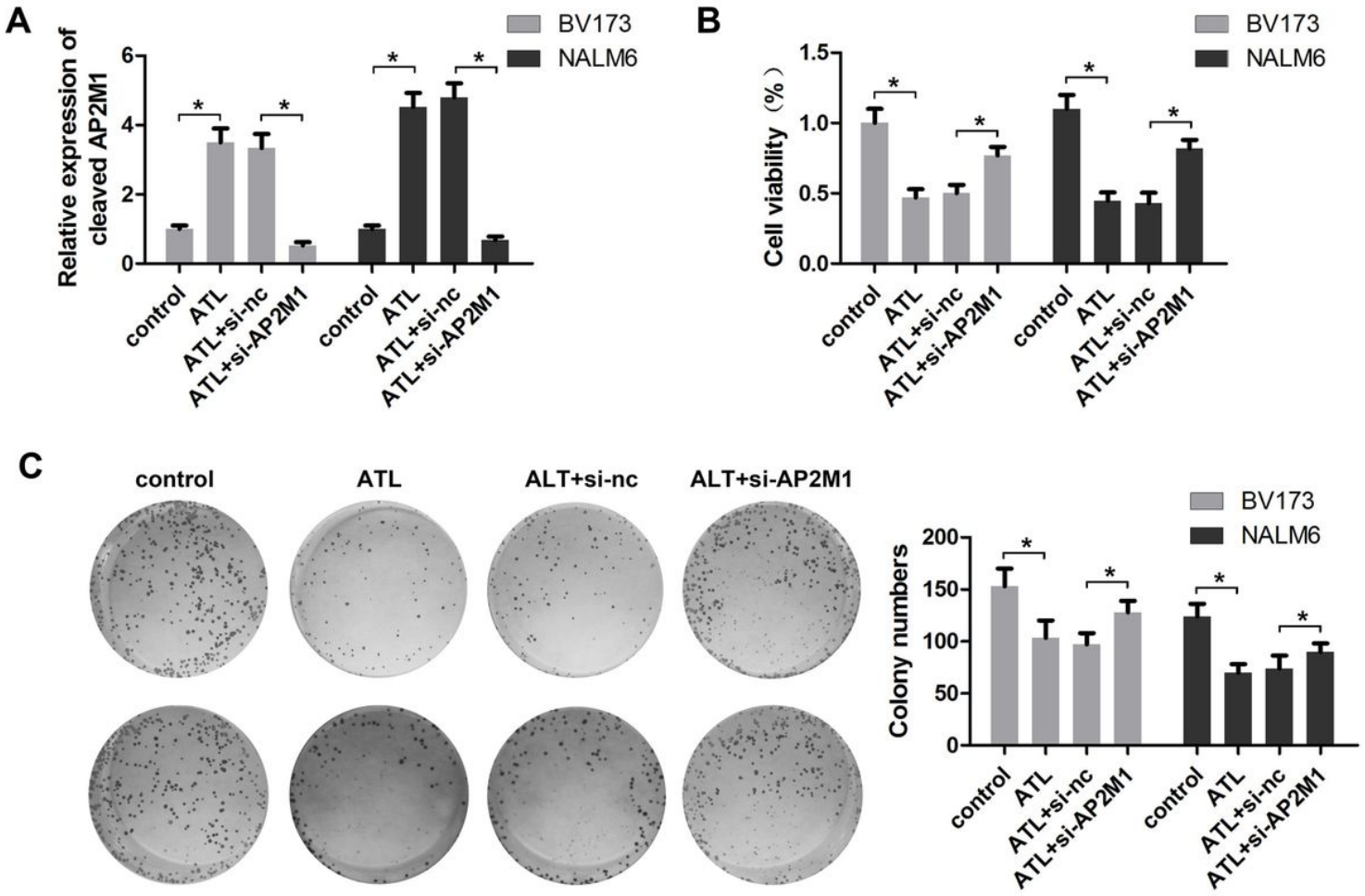

Figure 3

AP2M1 knockdown reversed the inhibitory effect of ALT on ALL cell proliferation. (A) qPCR was performed to evaluate the expression level of AP2M1 after the transfection of si-AP2M1 and the treatment of ALT. (B) After si-AP2M1 transfection and ALT treatment, MTT was used to detect the cell proliferation of ALL cells. (C) Colony formation assay was further conducted to investigate the colony formation ability of ALL cells. *p < 0.05.

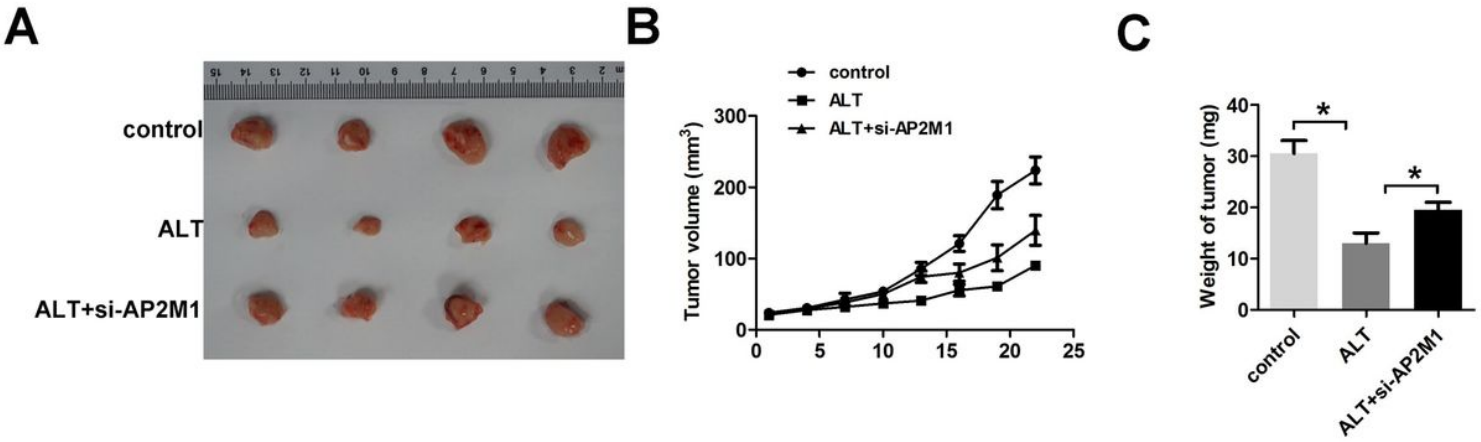

Figure 4

ALT inhibited the acute lymphoblastic leukemia growth in vivo. Control and AP2M1 down-regulated All cells were used to establish a xenograft nude mice model. (A) The subcutaneous xenograft tumors generated from ALL cells in nude mice under different treatment. (B) Tumor growth in nude mice was indicated by the curves represented the trend of the tumor size increase. (C) The weight of the tumors was showed at *p < 0.05

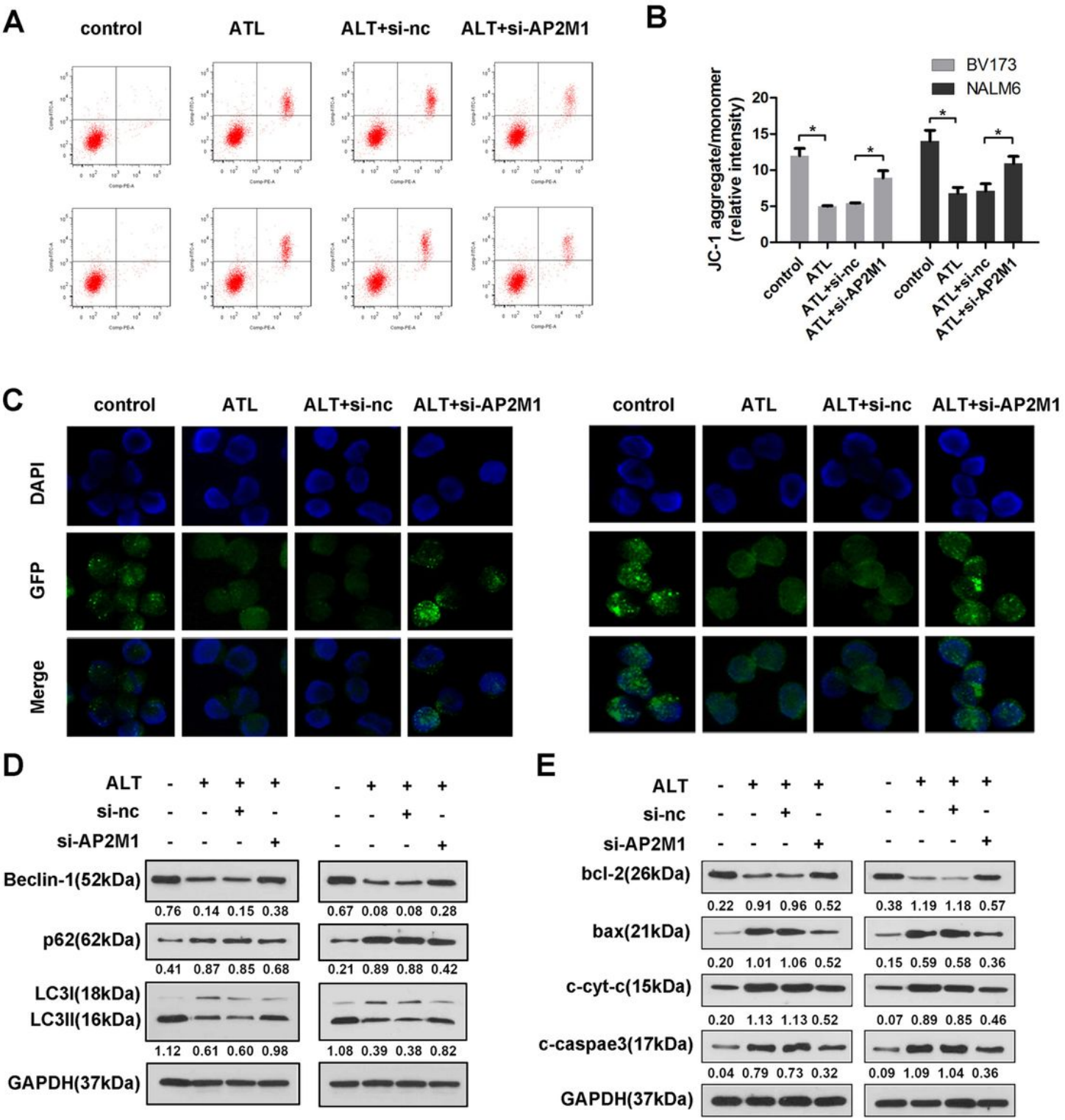

Figure 5

ALT induced apoptosis and inhibited the autophagy of ALL cells. (A) After si-AP2M1 transfection and ALT treatment, Annexin/V-PI staining and flow cytometry were performed to detect the apoptosis of ALL cell under different treatments. (B) JC-1 staining was conducted to detect the MMP of the ALL cell. (C) LC3 fluorescence dots labeled by GFP were observed under the fluorescence microscope. (D) Western blot was carried out to investigate the expression level of proteins at *p < 0.05.

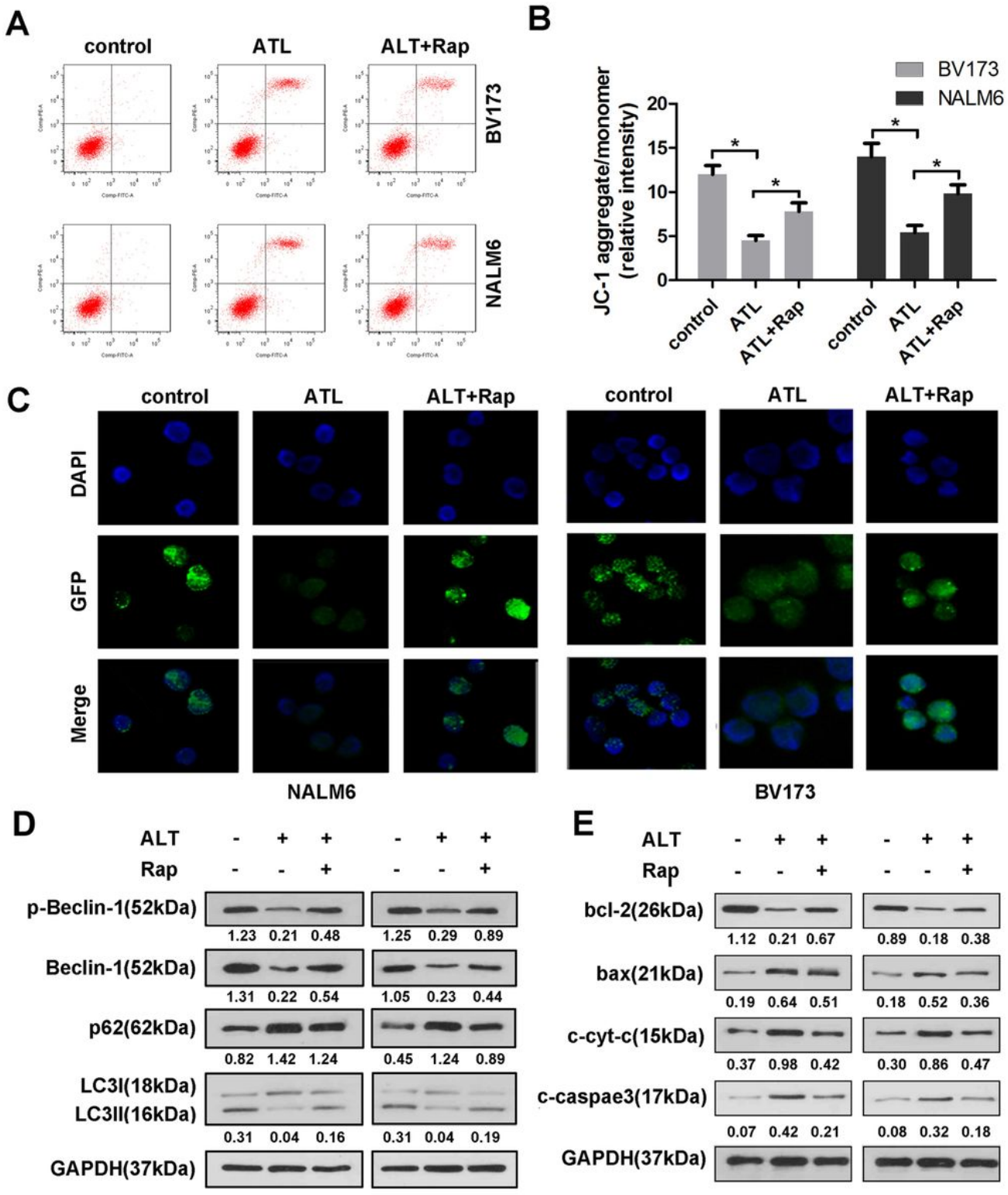

Figure 6

Autophagy induction reversed the effect of ALT on the apoptosis of ALL cells. ALL cells were treated with ALT or rapamycin or their combination for 24 h. (A) Annexin/V-PI staining and flow cytometry were performed to detect the cell apoptosis of ALL cell under different treatments. (B) JC-1 staining was performed to detect the MMP of the ALL cell under different treatments. (C) LC3 fluorescence dots labeled by GFP were observed using under the fluorescence microscope. (D) Western blot was carried out to investigate the expression level of proteins at *p < 0.05.

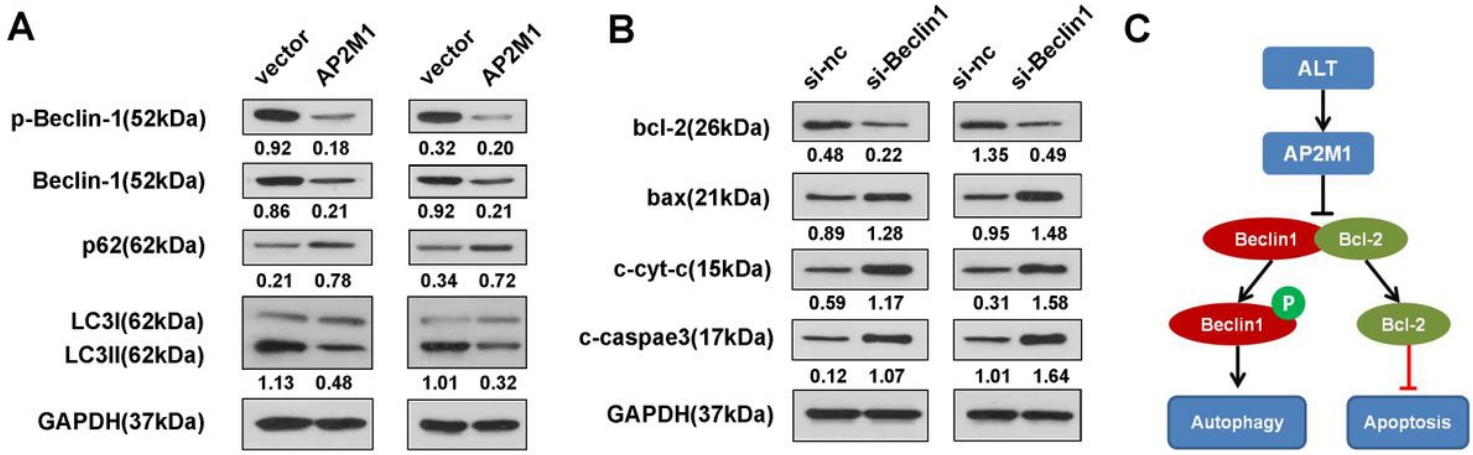

Figure 7

ALT altered the phosphorylation of AP2M1/Beclin1/bcl-2. ALL cells were transfected with pcDNA3.1-AP2M1 or si-Beclin1 for 48 h. Then, western blot was conducted to evaluate the expression level of autophagy (A) and apoptosis-related (B) proteins. (C) The schematic overview of the molecular mechanism underlying the effect of ALT on the apoptosis and autophagy of ALL cells.