

Anthocyanins from *Lycium ruthenicum* Murray inhibit HCC tumor angiogenesis and promote autophagic apoptosis through AMPK/PI3K/AKT/mTOR pathway

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Abstract

Purpose

Angiogenesis is a crucial factor in the initiation, progression and metastasis of tumor. The exploration for novel anti-angiogenic agents from natural sources has garnered significant attention. Our previous study confirmed the inhibitory effects of anthocyanins obtained from *Lycium ruthenicum* Murray (ALR) on the growth and metastasis of HepG2 cells, yet its potential to suppress neovascularization in hepatocellular carcinoma (HCC) remains unknown. This research aimed to assess the anti-angiogenic effect of ALR in HCC and elucidate its underlying mechanism.

Methods

Cell viability, colony formation, migration, invasion, and tubule formation were performed to evaluate the effect of ALR on EA.hy926 cells. Transcriptomics sequencing was performed to analyze the functional enrichment and pathway prediction of differentially expressed genes (DEGs). The formation of autophagosomes and autophagy-related proteins level were observed by AO staining, MDC staining, GFP-LC3-Adenovirus infection and western blot analysis. The relationship between autophagy and cell proliferation, angiogenesis, as well as AMPK/PI3K/AKT/mTOR pathway were analyzed in EA.hy926 cells treated with ALR in the presence or absence of 3-Methyladenine (3-MA, autophagy inhibitor). The transplanted hepatocellular carcinoma (HCC) model was established using HepG2 cells, and the therapeutic effect and underlying mechanism of ALR on HCC were assessed by H&E staining, TUNEL assay, immunofluorescence and immunohistochemistry analysis.

Results

Our findings demonstrated that ALR significantly suppressed the viability, proliferation, migration, invasion and angiogenesis of EA.hy926 cells in a dose-dependent pattern. The transcriptomic sequencing results revealed a close association between ALR inhibition of angiogenesis and autophagy, primarily enriched in the PI3K/AKT, AMPK and mTOR signaling pathways. *In vitro* outcomes further validated that ALR significantly augmented the presence of autophagosomes, enhanced the levels of Beclin-1, LC3-II/LC3-I, and p-AMPK while concurrently suppressing the levels of p62, p-PI3K, p-AKT, and p-mTOR. Notably, these effects were effectively counteracted using 3-Methyladenine. *In vivo* xenograft results demonstrated that ALR significantly inhibited the growth of HCC tumors. Mechanistically, ALR activated autophagy to suppress the expression of CD31, VEGF, and VEGFR2 in tumor tissues. Additionally, ALR induced autophagy-dependent apoptosis by up-regulating the levels of Bax, Cleaved caspase-3, and Cleaved caspase-9 while down-regulating the Bcl-2 level. Furthermore, it was discovered that ALR regulates autophagy by activating AMPK and suppressing the activity of PI3K/AKT/mTOR axis.

Conclusion

In combination, our data suggest that ALR executes its anti-HCC activity by inhibiting tumor angiogenesis and promoting autophagic apoptosis through regulation of the AMPK/PI3K/AKT/mTOR signaling cascade. The utilization of ALR as a natural adjuvant against tumors may hold immense potential in the foreseeable future.

1 Introduction

Hepatocellular carcinoma (HCC) has become one of the major cancers threatening human health due to its high incidence and fatality rates (Sung et al. 2021). Despite the current advances in the clinical management of HCC, the outlook remains relatively unfavorable as a result of its strong inclination towards tumor recurrence and metastasis (Ganesan and Kulik 2023). The process of tumorigenesis and metastasis is often accompanied by neovascularization. Angiogenesis is tightly controlled under normal conditions, however, abnormal angiogenesis is an important regulator of tumor development risk (Latacz et al. 2020). Therefore, inhibition of tumor neovascularization emerges as a hopeful approach for anticancer treatment.

Extensive researches have proven that natural products exhibit potent inhibitory effects on tumor angiogenesis, such as curcumin and astragaloside (Ai et al. 2022). *Lycium ruthenicum* Murray, commonly known as black goji, is a perennial thorny shrub primarily planted in the northwestern regions of China, specifically Ningxia and Qinghai provinces (Yun et al. 2022). Its fruits, which have a dark violet-black appearance, have been widely used in China for over 2000 years as health food and traditional Chinese medicine (Wang et al. 2018a). Multiple studies have provided evidence supporting the various biological properties of black goji, including its anti-inflammatory, anti-oxidant, and anti-tumor properties, among which the main bioactive ingredient is anthocyanins (Zeng et al. 2023; Han et al. 2022). Anthocyanins are a type of flavonoid compounds that can be found in various plants and play a pivotal role in the prevention and control of cancer (Chen et al. 2022). For example, the extraction of anthocyanins from blackberries, mulberries, grapes, and black soybeans have demonstrated promising potential in terms of their anticancer activity (Zeng et al. 2023). Mechanistically speaking, anthocyanins are primarily attributed to their ability to induce apoptosis and autophagy processes within cells (Long et al. 2018). Apoptosis and autophagy are both programmed cell death processes, but their ultimate outcomes are different. Apoptosis leads to cell demise, while autophagy plays a dual role in determining cell fate. Moderate autophagy maintains cellular homeostasis and promotes cell survival, whereas excessive autophagy can negatively impact vital cellular components and processes, ultimately triggering apoptosis or necrosis (Cao et al. 2021). Studies have shown that numerous anticancer agents are participated in the mechanisms of autophagic cell death (Miller and Thorburn 2021). Our previous research has also shown that ALR effectively inhibited the proliferation and metastasis of HepG2 cells by inducing autophagic apoptosis (Fan et al. 2022). In addition, dysregulation of autophagy has been

observed to be closely associated with angiogenesis in different types of cancers (Schaaf et al. 2019). Nevertheless, it is currently unclear whether ALR has an impact on neoangiogenesis in HCC tumors.

This research was conducted to explore the potential anti-angiogenic properties of ALR on both *in vitro* EA.hy926 cells and *in vivo* hepatoma xenograft models, and then further to investigate the underlying regulatory mechanisms involving angiogenesis. Our study demonstrated that ALR can effectively inhibit HCC tumor angiogenesis and promote autophagic cell apoptosis by regulating the AMPK/PI3K/AKT/mTOR signaling cascade.

2 Materials and Methods

2.1 ALR and cells

ALR (contains 51.6% anthocyanins, 3.31% ash, 4.58% moisture, pesticide residues < 1 ppm and heavy metal < 10 ppm) was purchased from Xi'an Realin Biotechnology Co., Ltd in Shanxi, China. Human hepatocellular carcinoma cell lines (HepG2, Shanghai institute of cell biology) and human umbilical vein endothelial fusion cell lines (EA.hy926, ATCC institute) were propagated in DMEM medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin solution at a temperature of 37 °C with a CO₂ concentration of 5%. The ALR was dissolved in serum-free DMEM medium, filtered using a sterile syringe filter with a pore size of 0.22 µm, and used immediately.

2.2 Antibodies and reagents

The antibodies against LC3B from Abmart (Shanghai, China); PI3K, Cleaved caspase-3/9, Bcl-2 and Bax from Proteintech (Wuhan, China); p62, Beclin-1, p-AMPK, AMPK, p-AKT, AKT, p-mTOR, mTOR, CD31 and HRP-labeled mouse/rabbit IgG from Abcam (Cambridge, USA); VEGF and VEGFR2 from Santa Cruz (California, USA); p-PI3K from ZEN BIO (Chengdu, China); β-Actin and FITC/TRITC-labeled mouse/rabbit IgG from ZSGB-BIO (Beijing, China). TUNEL red fluorescence in situ apoptosis detection kit from Vazyme (Nanjing, China). Chloroquine (CQ) from MCE (New Jersey, USA). 3-methyladenine (3-MA) from APExBIO (Houston, USA).

2.3 Cell viability assay

EA.hy926 cells were incubated in 96-well plates (7.0×10^3 cells/well) overnight and then exposed to different concentrations of ALR solution for an additional 24 h, with or without the presence of 3-MA (2.5 mM). After that, each well received 10% CCK-8 solution (Beyotime, Shanghai, China) and was cultured at a temperature of 37°C for about 30 min. The optical density (OD) values were then recorded at a wavelength of 450 nm by a microplate reader (Multiskan GO, Thermo, USA). Finally, the calculation for cell viability (%) was performed as: $(OD_{\text{treatment}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}}) \times 100\%$.

2.4 Colony formation assay

EA.hy926 cells were subjected to ALR solution at varying concentrations for a duration of 24 h. Subsequently, the cells were cultivated in 6-well plates (1.0×10^3 cells/well) and continuously cultured in DMEM media supplemented with 0.5% FBS and 1% penicillin/streptomycin for around two weeks until visible colonies formed. Afterwards, the colonies were treated using 4% paraformaldehyde and tinted by 0.1% crystal violet before photographs were taken in natural light.

2.5 Cell scratch assay

EA.hy926 cells were subjected to ALR solution at different concentrations for a duration of 24 h. Once the cells fully filled the bottom surface of 6-well plates, each well was gently scraped using a pipette tip to form an area devoid of cells, then DMEM containing 1% FBS was used as the new medium. The pictures were photographed immediately following the scraping and again after incubation for 24 h. The wound closure area was calculated by employing Image J software (NIH, USA).

2.6 Cell invasion assays

EA.hy926 cells were subjected to varying concentrations of ALR solution for 24 h, then resuspended at a density of 2.0×10^4 cells/well in the upper chamber of 24-well transwell system which precoated with Matrigel (0.5 mg/mL). The bottom chamber was filled with DMEM complete medium, while the upper chamber was supplied with FBS-free DMEM medium. After 24 h, the upper chamber's cells were cleaned using cotton swabs, whereas the bottom chamber's invading cells were immobilized using 4% paraformaldehyde and colored using 0.1% crystal violet. Afterwards, images were taken by an inverted microscope for further analysis.

2.7 Tubule formation assay

EA.hy926 cells were subjected to varying concentrations of ALR solution for 24 h, with or without the presence of 3-MA (2.5 mM). Then, the cells were resuspended onto Matrigel-coated 96-well plates (3.5×10^4 cells/well) and cultured at a temperature of 37°C for 4 h. The tubule network formation was visualized by an inverted fluorescence microscope (100x), and quantification was carried out by the Image J software by calculating the total length of the master segments in 5 randomly selected fields.

2.8 RNA sequencing

The total RNA from EA.hy926 cells, with or without ALR treatment for 24 h, was isolated using TRIzol reagent (Thermo, USA) according to the manufacturer's guidelines, and the quality and concentration were measured through a NanoDrop 2000C spectrophotometer (Thermo, USA). The RNA sequencing was performed and analyzed by Biomarker Technologies Corporation (Beijing, China).

2.9 Analysis of functional annotations and enrichment pathways

Functional annotation analysis was conducted using PANTHER (www.pantherdb.org). Briefly, the distribution of differentially expressed genes (DEGs) in biological process (BP), cellular component (CC)

and molecular function (MF) were examined via Gene Ontology (GO) enrichment analysis. The enriched pathways of the DEGs were predicted using Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.ad.jp/kegg/pathway.html>) database.

2.10 Acridine orange (AO), monodansylcadaverine (MDC), mGFP-LC3-adenovirus staining

EA.hy926 cells were grown in 12-well plates (5.0×10^4 cells/well) overnight, and then exposed to different concentrations of ALR solution for 24 h, with or without the presence of 3-MA (2.5 mM). Subsequently, the cells were subjected to incubation at 37°C with either 5 μ L AO solution (Sangon, Shanghai, China) or 20 μ L MDC solution (Solarbio, Beijing, China) for 15 min. Alternatively, EGFP-LC3-Adenovirus (HanBio, Shanghai, China) was added to the cells at an MOI of 100 and incubated for 24 h at a temperature of 27 °C. Finally, an inverted fluorescence microscope was used to capture images.

2.11 Western blot assay

EA.hy926 cells were subjected to varying concentrations of ALR solution for 24 h, with or without the presence of 3-MA (2.5 mM). Next, the overall protein concentration was determined using the BCA kit (KeyGen, Nanjing, China) after collecting and lysing samples. Subsequently, a same quantity of proteins was run through 12% SDS-PAGE gels and transferred onto a PVDF membrane. After that, the bands were subjected to blocking using skim milk for 1 h, followed by overnight incubation at 4°C with primary antibodies. Afterwards, the HRP-labeled mouse/rabbit IgG were applied for 1 h. The bands were then exposed to ECL solution (Thermo, USA) in a light-protected environment for 1 min before being analyzed and quantified using the gel imaging system (Bio-Rad, USA) and Image J software (NIH, USA), respectively.

2.12 Hepatocellular carcinoma xenograft mouse model

Female BALB/c nude mice, with an age of 5 weeks, were procured from Weitong Lihua (Beijing, China), and fed in a specialized facility with strict pathogen control measures at the Experimental Animal Center of Ningxia Medical University. After one week of animal feeding observation, HepG2 cells (2.0×10^6 cells) suspended in 100 μ L PBS were subcutaneously injected into the right dorsal side of each mouse. The mice were assigned to four groups randomly (five mice/group) when the tumor volume rose to about 60 mm³, and subsequently treated with normal saline, ALR (administered intragastrically 200 mg/kg/day for 12 days), CQ (administered intraperitoneally 25 mg/kg/day for 12 days), and a combination of ALR and CQ (same dose and administration method as before), respectively. The tumor volume was measured by dial caliper every three days following administration, while the weight of mice was recorded daily until day 12.

2.13 The H&E staining, immunofluorescence, immunohistochemistry and TUNEL

All mice were humanely euthanized on day 12 post-administration, followed by excision of tumor tissues, fixation in 4% paraformaldehyde, and embedding in paraffin. The *H&E*, immunohistochemistry and immunofluorescence assays were performed according to ZSGB-BIO's standard protocol (Beijing, China). Briefly, primary antibodies including Beclin-1, LC3B, p62, Bax, Bcl-2, Cleaved caspase-3/9, CD31, VEGF, VEGFR2, p-AKT, p-AMPK, p-mTOR and p-PI3K were incubated overnight at 4°C for immunofluorescence or immunohistochemistry staining, then the secondary antibodies specific to each species were incubated at room temperature for 1 h. TUNEL assay for tissue apoptosis was performed according to Vazyme's standard protocol (Nanjing, China). The photographs were obtained utilizing a fluorescence microscope (Ni-U, Nikon, Japan). The quantification of positive expression in each section was conducted by calculating the integral optical density (IOD)/Area through Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA).

2.14 Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) employing SPSS 26.0 software (IBM, NY, USA). The findings were obtained from at least three separate trials, and expressed in terms of the average value $\pm$ standard deviation. Differences were deemed statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

3 Results

3.1 ALR inhibits the cell proliferation, migration and angiogenesis in EA.hy926 cells

Our prior research has confirmed that ALR effectively suppresses the proliferation and migration of HepG2 cells (Fan et al. 2022), but whether it also affects the function of vascular endothelial cells remains unclear. Therefore, we examined the viability, proliferation, metastatic potency, and angiogenic changes in ALR-treated EA.hy926 cells through the assays of CCK-8, colony formation, cell scratch, invasion and tubule formation. The CCK-8 results (Fig. 1A) indicated that the viability of EA.hy926 cells was dose-dependently inhibited. Notably, a significant inhibitory effect was observed at an ALR concentration of 500 $\mu\text{g/mL}$ ($p < 0.01$). Thus, we selected 500 $\mu\text{g/mL}$ and 1000 $\mu\text{g/mL}$ of ALR for subsequent tests. Furthermore, the proliferation capacity, migratory and invasive potential as well as the number of intact tubular network structures of EA.hy926 cells exhibited a significant dose-dependent reduction following 24 h of ALR treatment, as depicted in Fig. 1B-D. The aforementioned results indicate that ALR possesses the capability to inhibit cellular proliferation, migration and angiogenesis in EA.hy926 cells.

3.2 Transcriptomic analysis of the function and pathway enrichment of DEGs

To elucidate the potential molecular mechanism of ALR inhibiting angiogenesis in EA.hy926 cells, we performed the transcriptomic sequencing on ALR-treated and untreated EA.hy926 cells, screened for DEGs, functional enrichment and signal pathways prediction. The RNA sequencing results showed that a significant number of genes, specifically 1706 in total exhibited differential expression, with 893 up-regulated and 813 down-regulated, meeting the criteria of fold change (FC) being equal to or greater than 1.5 and *P* value less than 0.05 (Fig. 2A). The annotation and functional analysis of the obtained DEGs through the GO database showed that the BP associated with the DEGs was primarily involved in the 'autophagy' and the 'angiogenesis', while in the category of CC and MF, the 'ruffle membrane', 'phagocytic cup', 'histone acetyltransferase activity' and 'protein kinase regulator activity' terms were the most enriched as depicted in Fig. 2B. To determine whether the DEGs were involved in specific pathways, the KEGG database annotation results revealed (Fig. 2C) that the signaling pathways related to autophagy and angiogenesis were PI3K-AKT (5.46%), AMPK (2.03%), mTOR (1.56%) and VEGF (0.94%). The mammalian target of rapamycin (mTOR) has been identified as the downstream target of phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), and AMP-activated protein kinase (AMPK), and inhibition of mTOR can promote the occurrence of autophagy (Fukuda and Wada-Hiraike 2022; Wang and Zhang 2019). Therefore, we further analyzed the association between these pathway differential genes through the KEGG database. Figure 2D showed that AMPK and PI3K-AKT signaling pathways can affect the expression changes of mTOR-related genes, thereby triggering changes in autophagy-related genes.

3.3 ALR triggers autophagy in EA.hy926 cells

To verify the transcriptomic prediction that ALR triggers autophagy in EA.hy926 cells, a series of experiments were conducted to address this inquiry. As shown in Fig. 3A, the number of autophagic vacuoles labeled with both AO and MDC (Orange-red fluorescence and blue fluorescence, respectively), as well as GFP-LC3-adenovirus-labeled autophagosomes (green spots), exhibited a dose-dependent increase with escalating concentrations of ALR when compared to the control group. After inhibiting the formation of autophagosomes in the early stages of autophagy by administering 3-MA, a reduction in the population of fluorescent cells was observed in the combined group compared to the ALR group (Fig. 3B), indicating that autophagic flux occurrence can be induced by ALR in EA.hy926 cells. We further conducted western blot to examine the expression changes of autophagy-related biomarkers. As shown in Fig. 3C, ALR treatment significantly up-regulated the expression of Beclin-1 and LC3-II/LC3-I proteins, while simultaneously down-regulating p62 protein abundance. When combined with 3-MA, these protein levels were found to be reversed (Fig. 3D). These findings indicate that ALR can trigger autophagy in EA.hy926 cells.

3.4 Autophagy inhibition impedes the cell viability and angiogenesis in ALR-treated EA.hy926 cells

To explore the potential role of autophagy in the inhibitory effects of ALR on EA.hy926 cell proliferation and angiogenesis, we further examined the impact of ALR on cellular viability and angiogenic function following 3-MA intervention using CCK-8 and tubule formation assays. As depicted in Fig. 4A and B, the

combination of ALR and 3-MA resulted in a reversal of cell viability and angiogenesis compared to ALR alone. Moreover, a similar experimental result was observed, wherein the combined group antagonized the expression level of CD31, a protein associated with angiogenesis, compared to the ALR group alone (Fig. 4C). These findings indicate that interfering with autophagy impairs the cell viability and angiogenesis triggered by ALR in EA.hy926 cells.

3.5 ALR induces autophagy through activation of AMPK and suppression of PI3K/AKT/mTOR pathways in EA.hy926 cells

To further investigate the underlying molecular regulatory mechanism responsible for initiating autophagy induction through ALR, we conducted western blot analysis to verify the expression changes of AMPK/PI3K/AKT/mTOR cascade based on the transcriptomic prediction results. As shown in Fig. 5A, with the increase of ALR concentration, a significant increase in phosphorylated p-AMPK expression, while a dose-dependent decrease was noticed in the expressions of phosphorylated p-PI3K, p-AKT, and p-mTOR when contrasted with the control group. When the combination of ALR and 3-MA, the levels of phosphorylated AMPK/PI3K/AKT/mTOR were all reversed compared to those observed in ALR alone (Fig. 5B). These findings suggest that autophagy can be induced by ALR in EA.hy926 cells through the activation of AMPK and suppression of PI3K/AKT/mTOR signaling.

3.6 ALR inhibits tumor growth by inducing autophagy in tumor tissues

To further evaluate the *in vivo* anti-tumor efficacy of ALR and elucidate the role of autophagy, a xenograft model of HCC was established in nude mice to assess the effect of ALR with or without the autophagy inhibitor CQ on tumor growth. As illustrated in Fig. 6A-C, the ALR group exhibited significantly smaller tumor size, weight, and volume in comparison to the other three groups, while these parameters were obviously reversed in the ALR + CQ group as compared to the ALR group. We also observed that the administration of ALR did not result in any significant changes in the weight of mice (Fig. 6D), indicating that ALR was safe. We further examined the tumor tissue for histopathological changes using *H&E* staining. As depicted in Fig. 6E, the ALR group exhibited a large amount of tumor tissue necrosis, whereas the ALR + CQ group demonstrated an obvious reduction in the extent of necrotic area when compared to the ALR group. The aforementioned findings suggest that ALR possesses potential inhibitory effects on HCC transplant tumor growth by inducing autophagy.

3.7 ALR inhibits tumor angiogenesis by inducing autophagy in tumor tissues

To further confirm that ALR induces autophagy in tumor tissues, we evaluated the expression levels of p62, Beclin-1, and LC3-II in tumor specimens. The results (Fig. 7A) demonstrated a significant increase of Beclin-1 and LC3-II levels following ALR treatment, accompanied by a notable decrease in p62 expression. Furthermore, co-treatment with ALR and CQ resulted in further elevation the levels of Beclin-1, LC3-II and

p62. This may be attributed to the ability of CQ, acting as a late-stage autophagy inhibitor, to impede autophagosome-lysosome fusion and thus hinder degradation of these proteins. Moreover, in order to further investigate whether ALR inhibits tumor growth by suppressing neovascularization, we conducted immunofluorescence staining to examine the alterations in proteins associated with angiogenesis. The findings indicated that ALR significantly decreased the expression levels of CD31, VEGF and VEGFR2 in tumor tissues. However, the combination of ALR and CQ exhibited an opposite trend compared to ALR alone (Fig. 7B). These results indicate that ALR exerts anti-tumor effects by promoting autophagy-mediated inhibition of angiogenesis.

3.8 ALR promotes tumor apoptosis by inducing autophagy in tumor tissues

Several studies found that autophagy activation is concomitant with tissue cell apoptosis, yet their relationship is sometimes contradictory (D'Arcy 2019). Thus, to clarify the correlation between ALR-induced autophagy and apoptosis in tumor tissues, we first examined the apoptosis using the TUNEL assay. Figure 8A demonstrated a noticeable rise in the number of red fluorescent apoptotic cells within the ALR group, while this number in the ALR + CQ group was notably less than in the ALR group. Subsequently, we further detected the expression changes in proteins associated with apoptosis through immunofluorescence assay. The results (Fig. 8B) showed a noteworthy reduction in Bcl-2 levels and a significant elevation in Bax, cleaved caspase-3, and cleaved caspase-9 protein levels within the ALR group when compared to the control group. Interestingly, ALR + CQ group exhibited a significant ability to reverse the protein expressions mentioned above when compared to the ALR group. Taken together, these results confirm that ALR has the ability to trigger autophagy-dependent apoptosis in tumor tissues by regulating the caspase-3 and caspase-9 signaling pathways.

3.9 ALR induces autophagy through activation of AMPK and suppression of PI3K/AKT/mTOR pathways in tumor tissues

To further elucidate the *in vivo* regulatory mechanism of ALR-induced autophagy, the AMPK/PI3K/AKT/mTOR signaling in tumor tissues were examined through immunohistochemistry staining. As depicted in Fig. 9, the positive expression rate of p-AMPK was significantly higher, while the levels of p-PI3K, p-AKT and p-mTOR were significantly lower within the ALR group than in the control group. When ALR combined with CQ, compared with ALR alone, there was an obvious reversal trend in the expression of the aforementioned proteins. These findings indicate that ALR induces autophagy by regulating the AMPK/PI3K/AKT/mTOR signaling axis in HCC tumors.

4 Discussion

HCC, recognized as one of the most lethal forms of cancer in humans, constitutes around 75%~85% of primary liver cancer (Sung et al. 2021). In spite of the extensive utilization of novel therapeutic strategies, such as targeted therapy and immunotherapy, in clinical settings, the therapeutic effect remains suboptimal due to the high metastatic potential of HCC (Ganesan and Kulik 2023). Hence, it is crucial to

discover innovative substances that can hinder the progression and metastasis of HCC. Natural products serve as a crucial reservoir for the discovery of novel drugs, with approximately 80% of clinical anticancer medications either originating from natural sources or being derived from natural product analogues (Naeem et al. 2022). Anthocyanins, naturally occurring flavonoid pigments found in a variety of fruits and vegetables, have been extensively investigated for their potential biological properties (Chen et al. 2022). *Lycium ruthenicum* Murray is an optimal botanical resource for anthocyanins extraction due to its high content of these compounds (Zeng et al. 2023). Our previous studies have provided evidence that ALR effectively hinders the growth and spread of HepG2 cells, while also promoting cell death and arresting the G2/M phase cycle (Fan et al. 2022). However, as far as we know, there is currently no available information on the impact of ALR on angiogenesis in HCC. Here, we tested the anti-angiogenic properties of ALR and its potential regulatory mechanisms.

Tsakiroglou et al. found that anthocyanins derived from blueberries decreased the overall tubular formation of HUVECs cells (Tsakiroglou et al. 2021). Our results also confirmed that ALR exerts a significant inhibitory effect on the growth, migration and angiogenesis of EA.hy926 cells (Fig. 1). Together with our previous studies, we found that ALR exhibits dual functionality as an inhibitor of hepatoma cells and vascular endothelial cells. The regulation of tumor angiogenesis involves a complex interplay between different factors that either promote or inhibit blood vessel formation (Huang and Bao 2004). Once this equilibrium is disrupted, tumor cells enhance the secretion of pro-angiogenic factors to promote neovascularization and accelerate metastasis (Parmar and Apte 2021). VEGF and VEGFR2 are major pro-angiogenic factors and also action targets of many anti-angiogenic drugs for cancer treatment, such as bevacizumab and lamuzumab (Olejarz et al. 2020). To further explore the mechanism of ALR inhibiting angiogenesis, we screened for differentially expressed genes and functional enrichment analysis by transcriptomic sequencing. Our results found that ALR inhibition of angiogenesis is closely associated with autophagy (Fig. 2A, B). Autophagy serves as a self-defense mechanism enabling cells to adjust to diverse stimuli and uphold cellular homeostasis (Schaaf et al. 2019). During the process of autophagy, Beclin-1 plays a vital role in initiating autophagy and facilitating the formation of autophagosomes, while LC3-I undergoes conversion to LC3-II and associates with the membrane during the autophagosome formation (Kabeya et al. 2000). p62 interacts with LC3, promoting its integration into the autophagosomes and subsequent gradual degradation within the autophagolysosome (Lamark et al. 2017). The phenomenon of autophagy has been found in diverse cancer cells upon exposure to various natural compounds, including but not limited to quercetin and resveratrol (Reyes-Farias and Carrasco-Pozo 2019; Li et al. 2022). However, based on our current knowledge, this work presents initial evidence suggesting that ALR has the potential to trigger autophagy in EA.hy926 cells. Our findings demonstrated that autophagic vacuoles and autophagosomes exhibited a dose-dependent aggregation, accompanied by an up-regulation in the levels of Beclin-1 and LC3-II/LC3-I, as well as a decrease in the p62 expression (Fig. 3A-C). Suppression of autophagy *in vitro* by 3-MA led to the reversal of both ALR-induced autophagy morphology and the levels of autophagy-related proteins (Fig. 3B and 3D). The results from the transplantation of hepatoma in mice further support the role of ALR in inducing autophagy, as evidenced by the increased expression of Beclin-1 and LC3-II, along with the decreased expression of p62 (Fig. 7A).

Surprisingly, administration of CQ, an inhibitor of autophagy, did not lead to a reversal in the levels of LC3II, p62 and Beclin-1 proteins (Fig. 7A). This phenomenon may be attributed to the utilization of CQ disrupts the acidic environment of lysosomes and thus impedes the merging process of autophagosomes with lysosomes, resulting in the accumulation of these proteins. Taken together, these findings suggest that ALR possesses the capability to trigger autophagy in EA.hy926 cells and mouse HCC transplanted tumors.

Autophagy plays a dualistic role in the modulation of tumorigenesis, promoting or inhibiting tumor development (Li et al. 2020). Chen et al. found that delphinidin, a primary anthocyanidin monomer, exerts an augmented against breast cancer tumor effect by inhibiting autophagy, suggesting a potential role of delphinidin in inducing protective autophagy (Chen et al. 2018). However, our results (Fig. 6) demonstrated that ALR significantly inhibited the growth of HCC, and the inhibitory ability was attenuated after suppression of autophagy, suggesting that ALR-induced autophagy plays a cytotoxic role. Accumulating evidences indicated that autophagy is also involved in regulating tumor angiogenesis (Schaaf et al. 2019). Our study revealed that inhibition of autophagy antagonized the viability, tube formation ability and CD31 protein expression of ALR-treated EA.hy926 cells (Fig. 4), and the *in vivo* results also confirmed that ALR inhibited VEGF, VEGFR2 and CD31 expression which was subsequently restored upon autophagy inhibition (Fig. 7B). Collectively, these findings present evidence that ALR hampers tumor vascularization by activating autophagy, thereby impeding tumor growth. Similar studies have also demonstrated that curcumol, a natural product, can regulate autophagy in LSEC cells to exert anti-angiogenic effects (Gao et al. 2021).

Emerging evidence suggests a close interplay between autophagy and apoptosis, wherein inhibition of autophagy can potentiate apoptosis in cancer cells; however, it is noteworthy that autophagy may also exert pro-apoptotic effects in certain situations (Sorice 2022). Apoptosis is primarily mediated by caspase-dependent and mitochondria-dependent pathways (Estaquier et al. 2012). Caspase-9 acts as an initiator of apoptosis, while caspase-3 serves as the executor. The activation of caspase-3 by caspase-9 leads to direct degradation of intracellular proteins and induction of apoptosis (Unnisa et al. 2023). Mitochondrial-mediated apoptosis is closely associated with the Bcl-2 family, where Bcl-2 functions as a negative regulator inhibiting pro-apoptotic protein activity, whereas Bax exerts pro-apoptotic effects (Bock and Tait 2020). Our results indicated that the administration of ALR led to a notable rise in the presence of apoptotic cells in tumor tissues (Fig. 8A). Additionally, it was observed that ALR suppressed the expression of Bcl-2 while enhancing Cleaved caspase-3, Cleaved caspase-9, and Bax levels within these tissues (Fig. 8B). These effects were reversed upon autophagy inhibition, suggesting that ALR-induced autophagy is a pro-apoptotic mechanism. This finding aligns with prior study that has demonstrated the ability of crocin to induce autophagy-dependent apoptosis in HCC cells (Yao et al. 2018). Notably, down-regulation of Bcl-2 induced by ALR in tumor tissues may promote both autophagy and apoptosis since it negatively regulates Beclin-1 protein involved in autophagy regulation (Xu and Qin 2019).

The occurrence of autophagy is a complex process involving multiple signaling pathways, in which AMPK and AKT/mTOR pathways have been extensively studied and reported (Cui et al. 2020). We also

confirmed a close correlation between the AMPK/PI3K/AKT/mTOR signaling axis and both angiogenesis and autophagy in ALR-induced EA.hy926 cells by transcriptomics and KEGG database analysis (Fig. 2C, D). The PI3K/AKT/mTOR cascade is of significant importance in the control of neovascularization, and its inhibition can effectively suppress angiogenesis by down-regulating VEGF expression (Karar and Maity 2011). Moreover, this pathway has been demonstrated in negative modulation of autophagy (Aoki and Fujishita 2017). mTOR functions as a cellular detector that positively regulates cellular nutrition, energy levels, and stress signals. In situations of stress like nutrient deprivation and low oxygen levels, the PI3K/AKT/mTOR cascade is suppressed, thereby triggering autophagy (Xu et al. 2020). Several herbal compounds, including tetrandrine, onjisaponin B, neferine, and hederagenin have been found to trigger autophagy through the suppression of mTOR activity (Liu et al. 2021; Wang et al. 2020). On the other hand, AMPK acts as another cellular detector that exerts a negative control on cellular energy levels and facilitates the transition from anabolism to catabolism upon energy stress (Jiang et al. 2021). Studies have demonstrated that AMPK expression is down-regulated in HCC patients, leading to a worse prognosis (Chen et al. 2017). As an oncogenic factor, AMPK is crucially involved in the regulation of diverse cellular mechanisms, such as cell cycle, apoptosis, autophagy, and glucose degradation (Jiang et al. 2019). Upon stimulation, AMPK can induce autophagy by directly inhibiting mTOR phosphorylation (Wang et al. 2018b). In our investigation, the treatment of ALR in EA.hy926 cells resulted in the AMPK activation, while simultaneously inhibiting the PI3K/AKT/mTOR axis activity (Fig. 5). These findings align with the observed outcomes *in vivo* (Fig. 9), indicating that ALR modulates the AMPK/PI3K/AKT/mTOR signaling axis to induce autophagy.

5 Conclusions

In conclusion, our study, summarized in Fig. 10, originally reveal that ALR exerts anti-HCC effects by inhibiting angiogenesis. The underlying mechanism involves the induction of autophagic apoptosis through regulation of the AMPK/PI3K/AKT/mTOR signaling cascade. Our research suggest that ALR holds promise as a naturally derived dietary supplement for the prevention of tumors or as a complementary treatment alongside conventional medications in tumor management.

Declarations

Author contribution statement

Conceptualization: Hongli Fan and Zhisheng Wang; methodology: Tingting Wei, Jiajie Cheng, Xue Cao, and Zhisheng Wang; investigation: Tingting Wei, Jiajie Cheng, and Shuqin Ding; data curation: Tingting Wei and Jiajie Cheng; animal experiment: Tingting Wei, Jiajie Cheng, Fei Kang and Fangye Li; writing—original draft preparation: Tingting Wei, Jiajie Cheng and Hongli Fan; writing—review and editing: Zhisheng Wang; supervision: Zhisheng Wang. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest The authors declare no competing interests.

Ethics statement The animal study protocol was approved by the Ethical and Welfare Committee of Ningxia Medical University (IACUC-NYLAC-2022-065).

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Figures

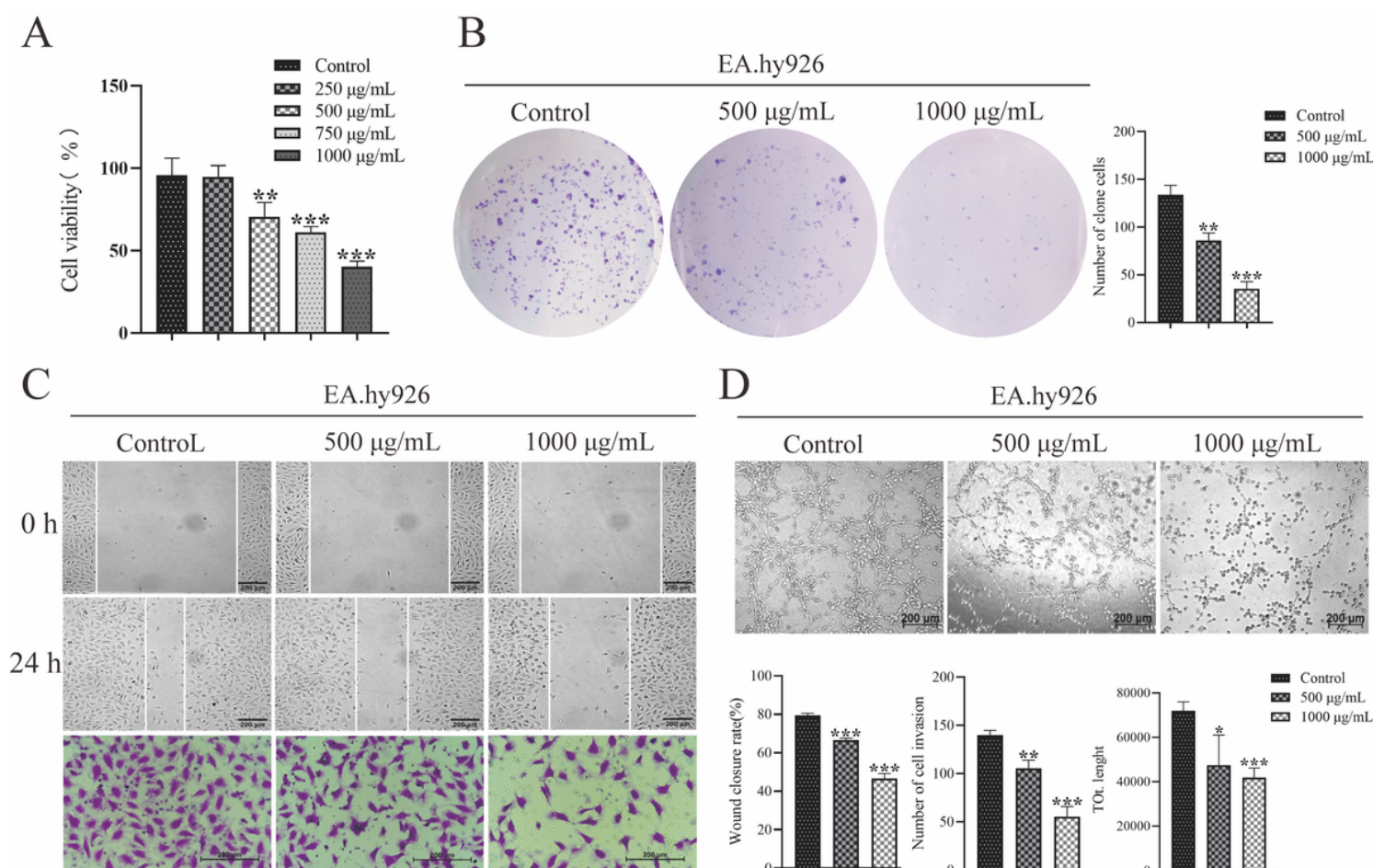


Figure 1

ALR inhibits the cell proliferation, migration and angiogenesis in EA.hy926 cells. The EA.hy926 cells were subjected to varying concentrations of ALR for 24 h. (A) Cell viability was evaluated by employing the CCK-8 assay. (B) Cell proliferation was detected through the colony formation assay. (C) Cell migratory and invasive ability were evaluated by scratch and transwell assays. (D) Tubular network structures were observed by tubule formation assay. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. control group.

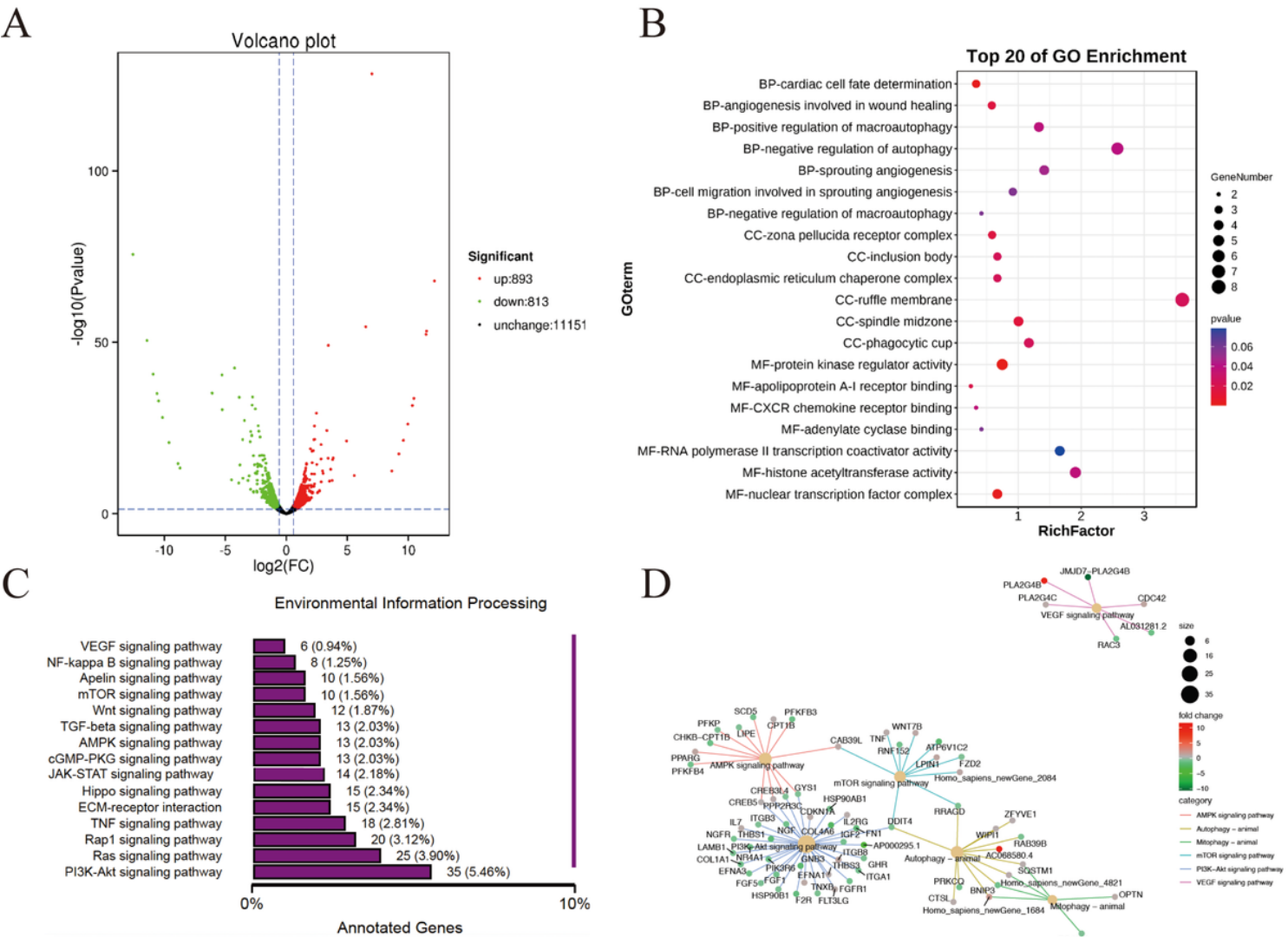


Figure 2

Transcriptomic analysis of the function and pathway enrichment of DEGs. Total RNA was isolated using TRIzol reagent from EA.hy926 cells treated with or without ALR for 24 h, and sequencing, functional annotation and pathway enrichment analysis were performed. (A) Volcano plot of DEGs. (B) GO database analyzed the BP, CC and MF of DEGs. (C) KEGG database predicted the enriched signal pathways. (D) KEGG analyzed the relationship between the main enriched pathway-related differentially genes. Abbreviations: DEGs, differentially expressed genes; GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, kyoto encyclopedia of genes and genomes.

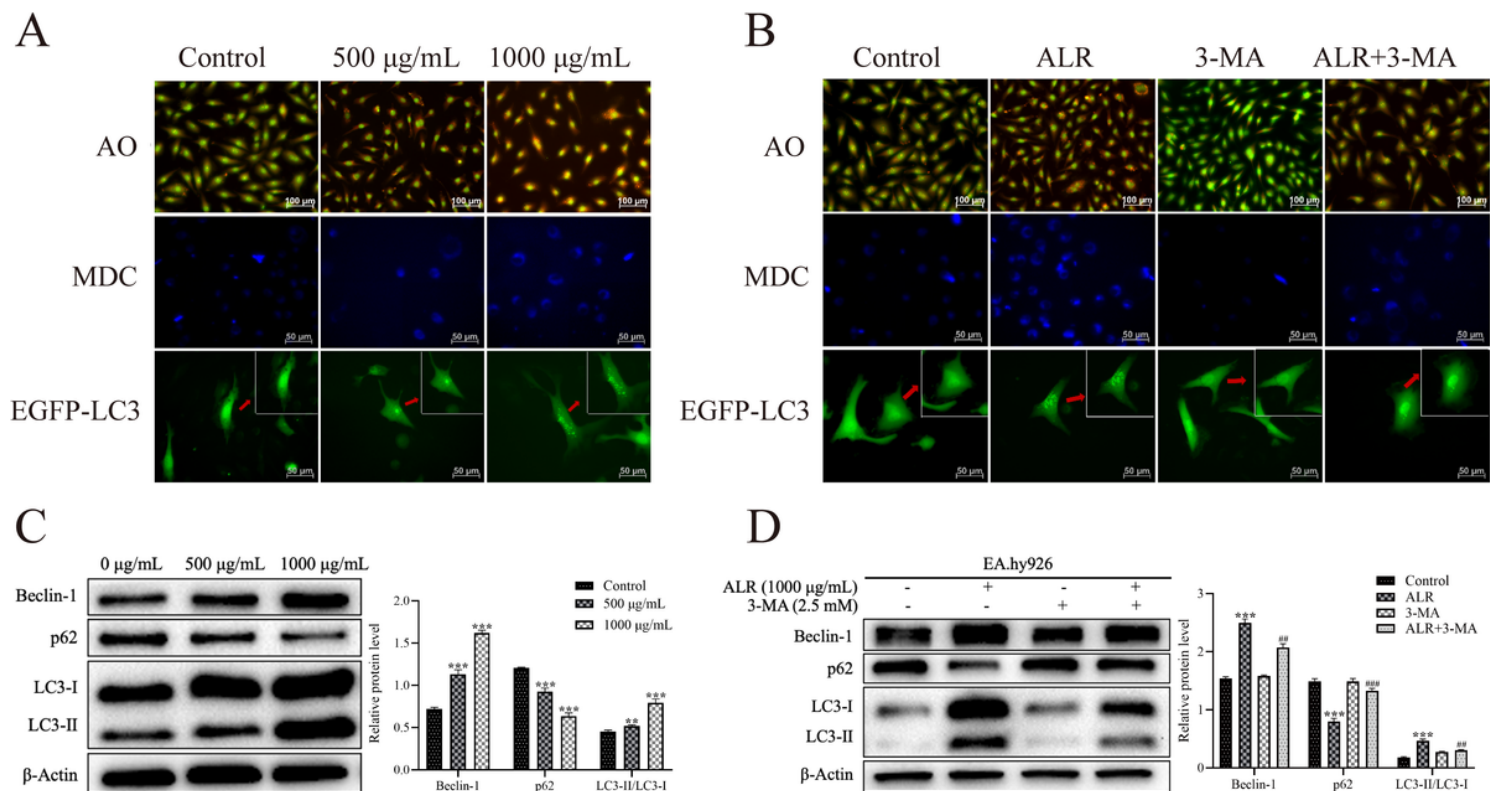


Figure 3

ALR triggers autophagy in EA.hy926 cells. The EA.hy926 cells were exposed to ALR for 24 h in the absence (**A**) or presence (**B**) of 3-MA intervention and subsequently subjected to AO, MDC, and EGFP-LC3-adenovirus staining. The autophagy-related protein levels in EA.hy926 cells treated with ALR for 24 h in the absence (**C**) or presence (**D**) of 3-MA intervention were analyzed using western blot, with β -actin served as a loading control. $**p < 0.01$ and $***p < 0.001$ vs. control group. $##p < 0.01$ and $###p < 0.001$ vs. ALR group.

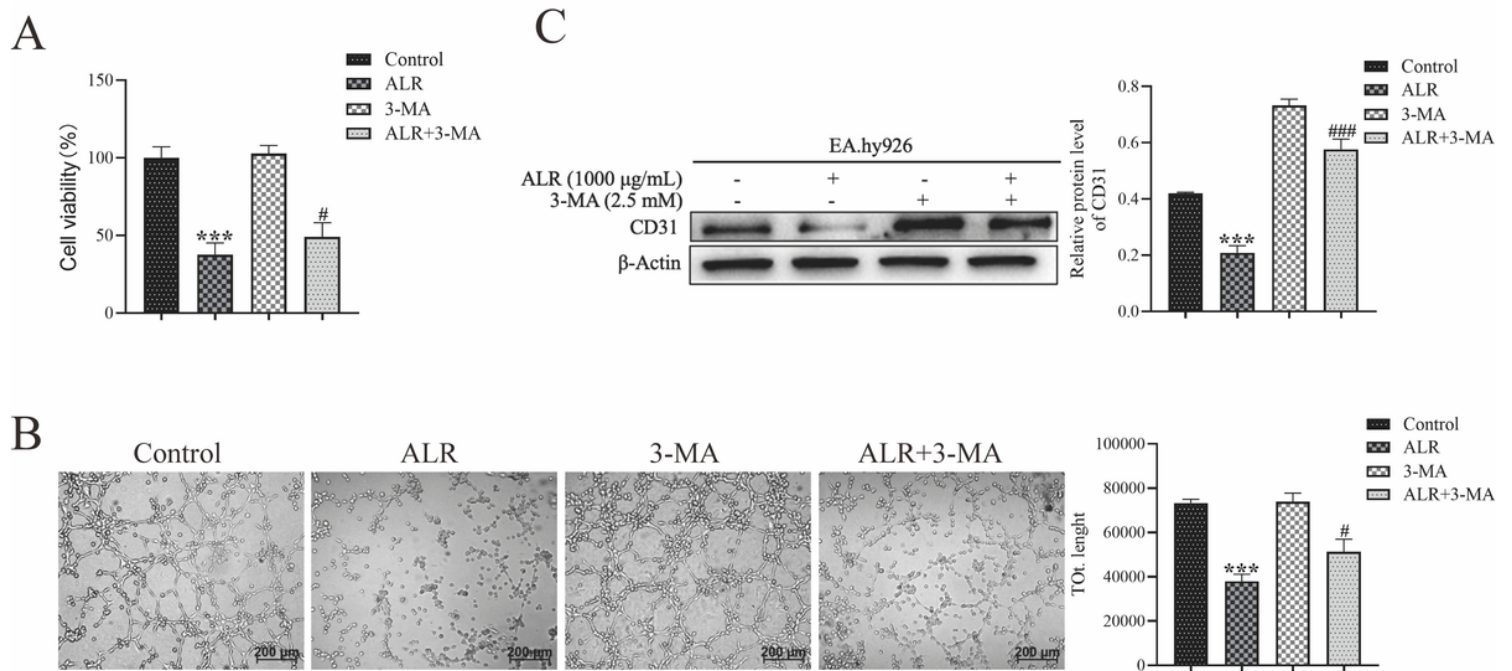


Figure 4

Autophagy inhibition impedes the cell viability and angiogenesis in ALR-treated EA.hy926 cells. EA.hy926 cells were subjected to ALR (1000 $\mu\text{g/mL}$) for 24 h with or without the presence of 3-MA. **(A)** Cell viability was observed through the CCK-8 assay. **(B)** Tubular network structures were analyzed by tube formation assay. **(C)** The protein level of CD31 was determined by western blot assay, with β -actin employed as a loading control. *** $p < 0.001$ vs. control group, # $p < 0.05$ and ### $p < 0.001$ vs. ALR group.

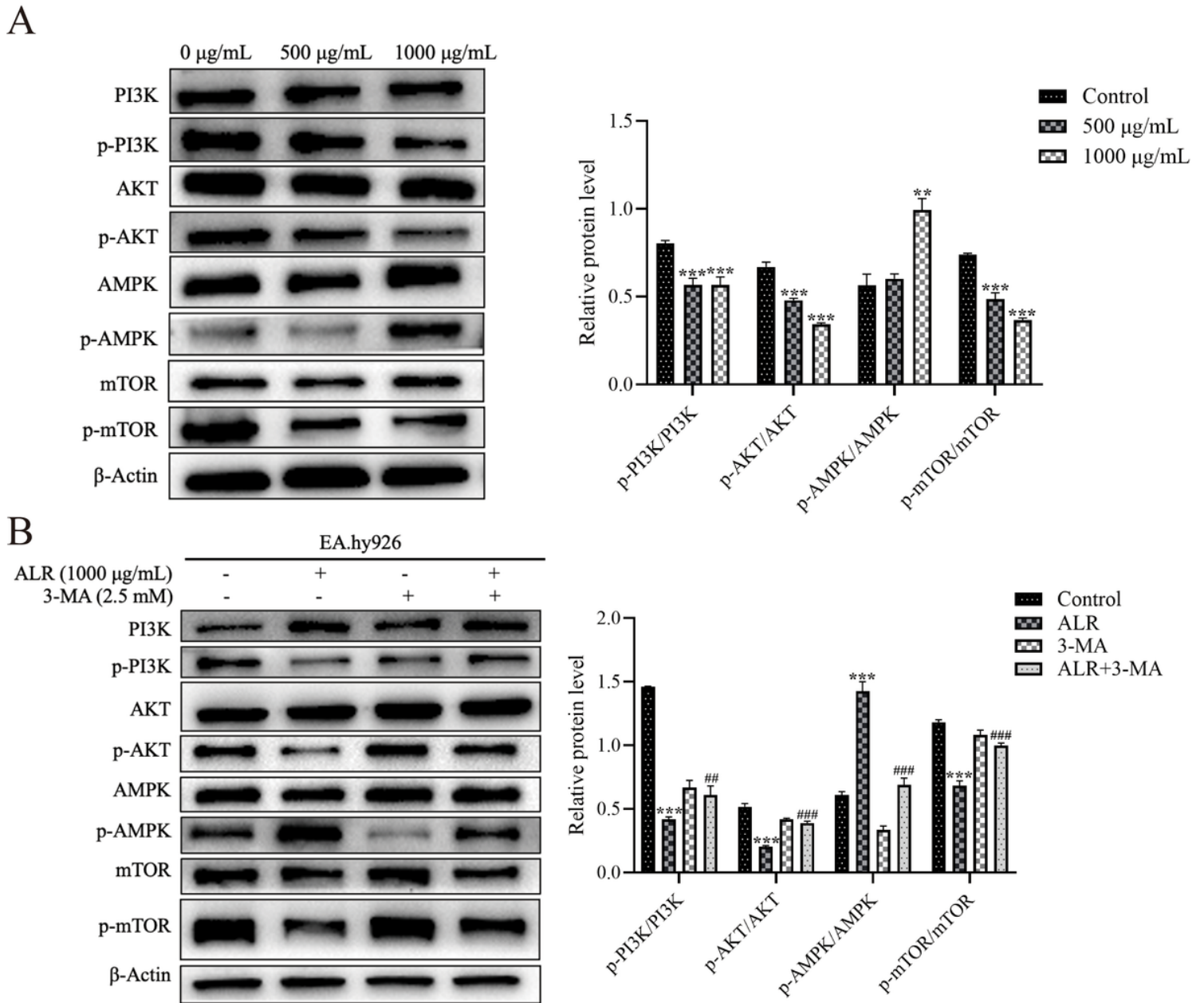


Figure 5

ALR induces autophagy through activation of AMPK and suppression of PI3K/AKT/mTOR pathways in EA.hy926 cells. The pathway-related proteins levels were analyzed by western blot in EA.hy926 cells treated with ALR for 24 h without (A) or with (B) of 3-MA, with β -actin employed as a loading control. $**p < 0.01$ and $***p < 0.001$ vs. control group, $##p < 0.01$ and $###p < 0.001$ vs. ALR group.

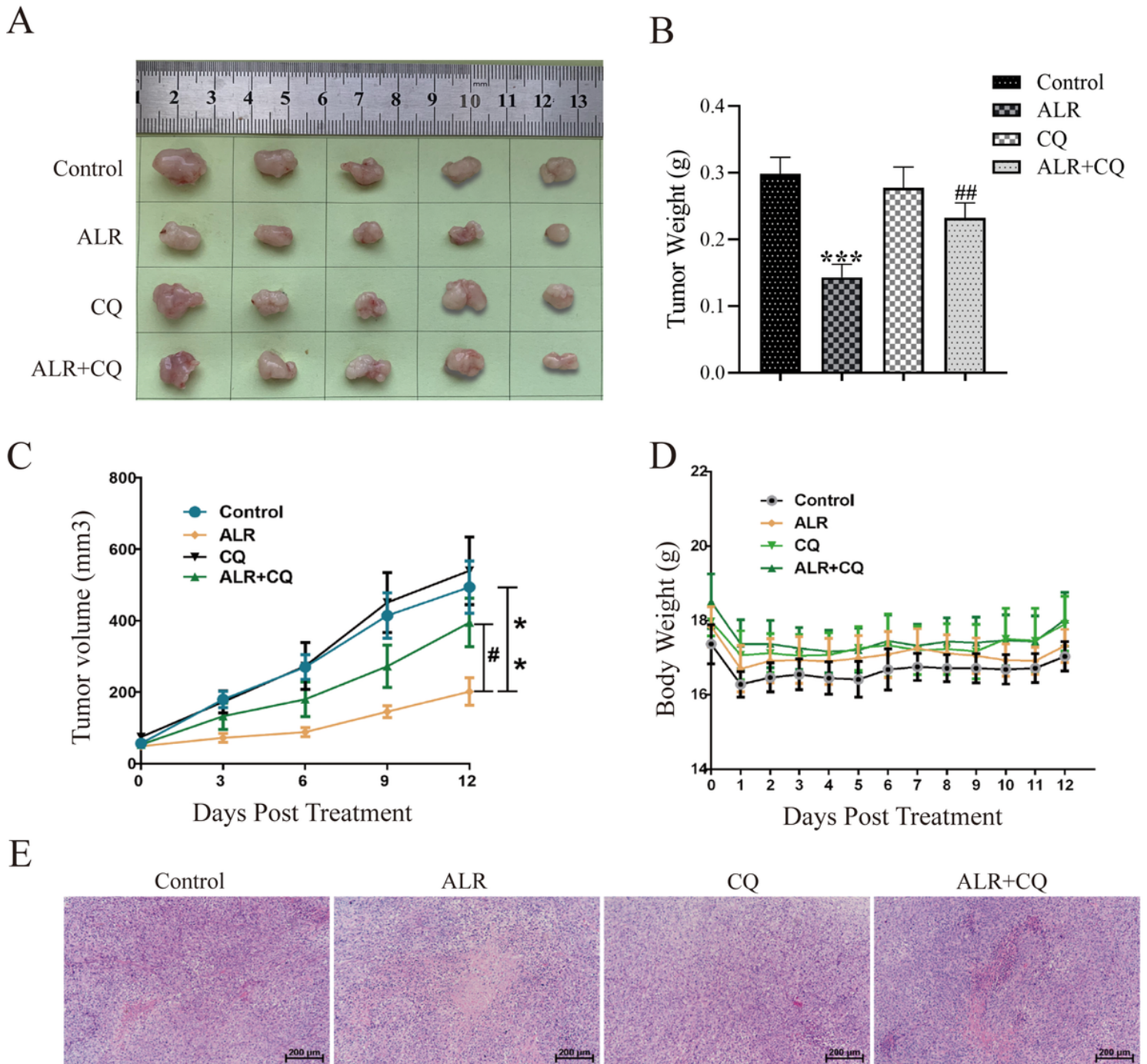


Figure 6

ALR inhibits tumor growth by inducing autophagy in tumor tissues. (A) Tumor anatomical map of nude mice; Statistical chart of tumor weight (B), volume (C) and body weight of mice (D); (E) The histopathological changes of tumor tissues were examined by *H&E* staining. ** $p < 0.01$ and *** $p < 0.001$ vs. control group, # $p < 0.05$ and ## $p < 0.01$ vs. ALR group.

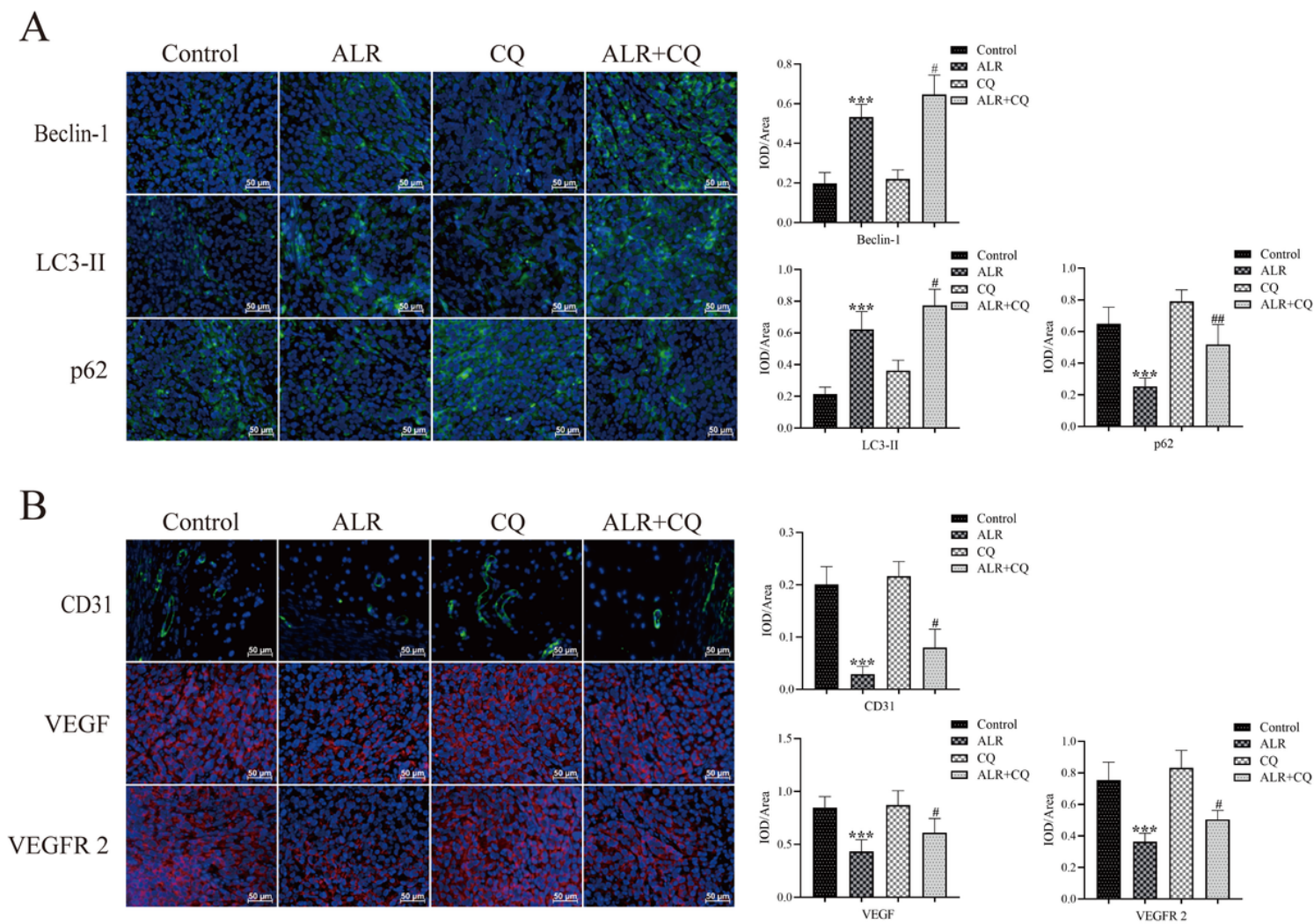


Figure 7

ALR inhibits tumor angiogenesis by inducing autophagy in tumor tissues. The levels of autophagy-related markers (A) and angiogenesis-related factors (B) were detected in tumor tissues by means of immunofluorescence analysis. *** $p < 0.001$ vs. control group, # $p < 0.05$ and ## $p < 0.01$ vs. ALR group.

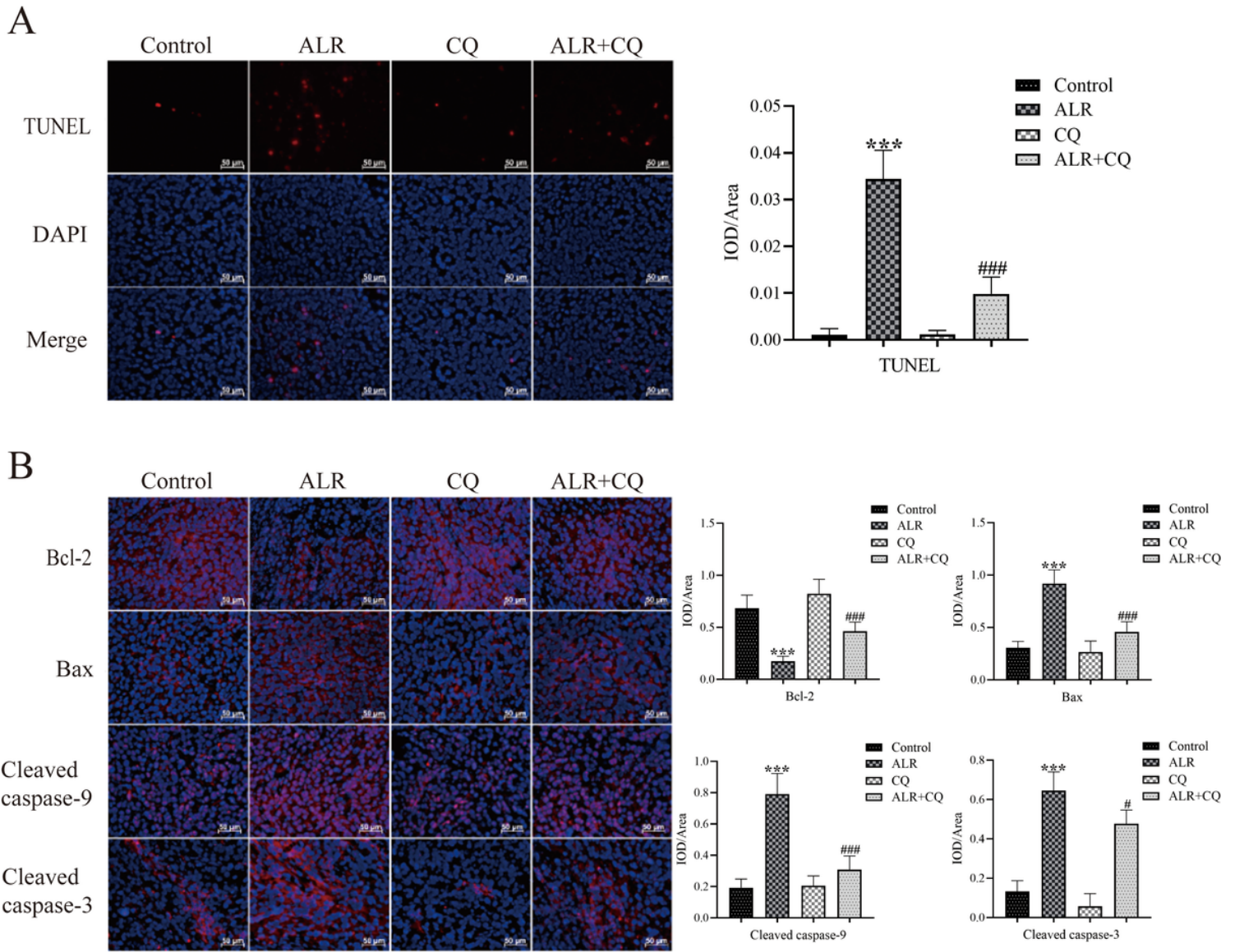


Figure 8

ALR promotes tumor apoptosis by inducing autophagy in tumor tissues. **(A)** The TUNEL assay was utilized to detect the tumor tissue apoptosis. **(B)** The immunofluorescence assay was employed to assess the expression levels of apoptosis-related proteins. *** $p < 0.001$ vs. control group, # $p < 0.05$ and ### $p < 0.001$ vs. ALR group.

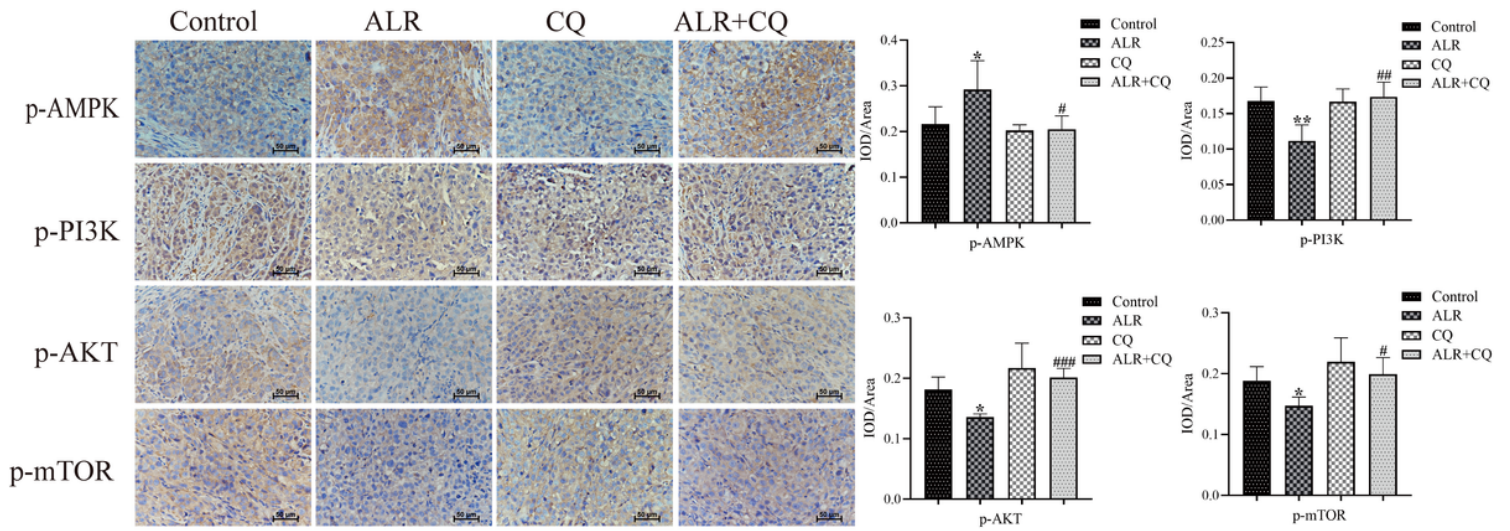


Figure 9

ALR induces autophagy through activation of AMPK and suppression of PI3K/AKT/mTOR pathways in tumor tissues. Immunohistochemical analysis was performed to determine the levels of p-AMPK, p-PI3K, p-AKT and p-mTOR expression. * $P < 0.05$ and ** $p < 0.01$ vs. control group, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. ALR group.

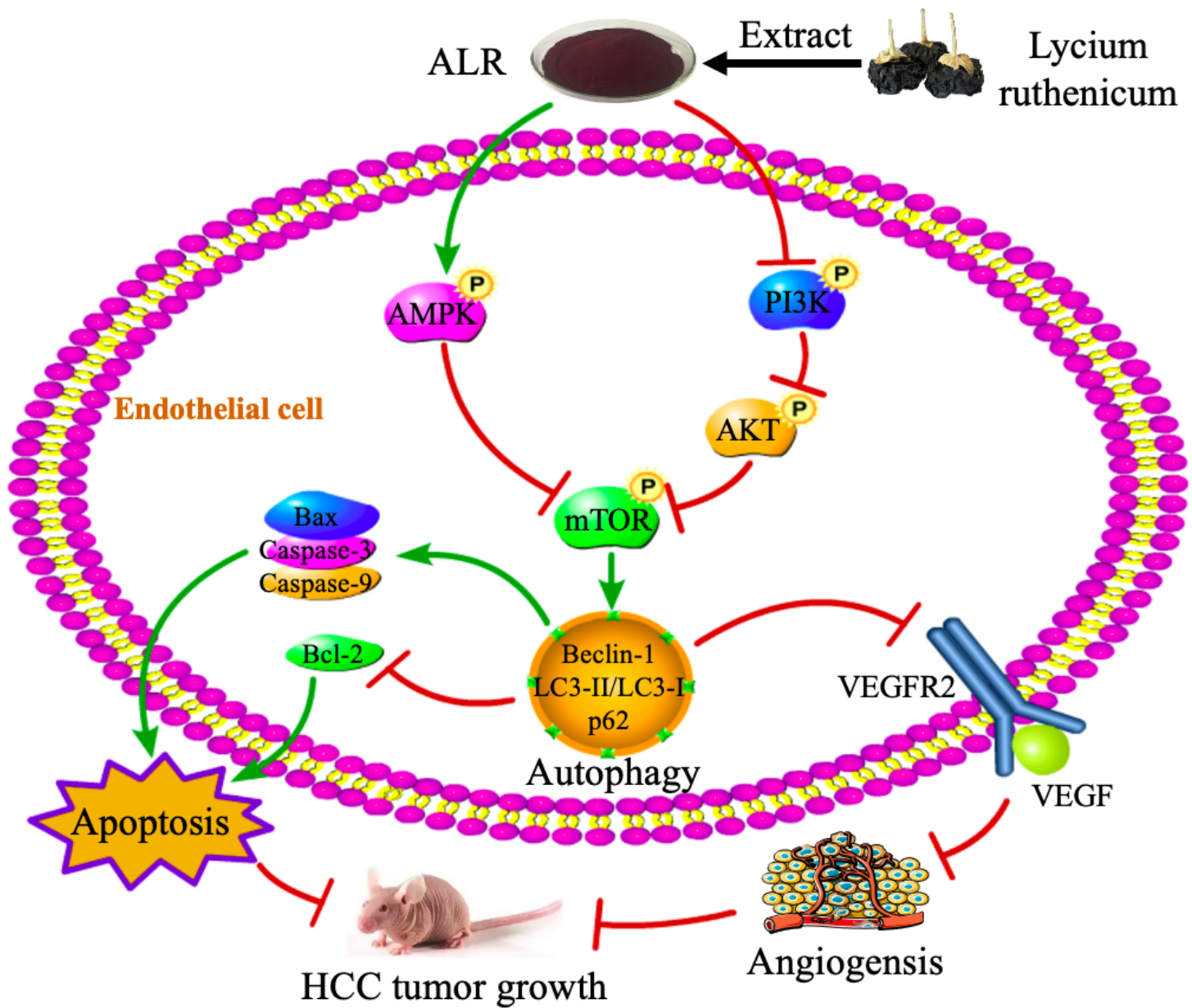


Figure 10

Schematic illustration of the inhibitory mechanism of ALR on tumor angiogenesis in HCC.