Aloin promotes oral squamous cell carcinoma cell apoptosis and autophagy through Akt/mTOR pathway

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RESEARCH ARTICLE

Abstract

Aloin, a natural product, has demonstrated anticancer activity in a variety of cancers, but its role in oral squamous cell carcinoma (OSCC) has not been reported. In this study, we aimed to investigate the effects of Aloin on the biological functions of OSCC cells. Additionally, we evaluated the classical Akt/mTOR signal transduction pathway. CAL-27 OSCC cells were treated with Aloin for *in vitro* experiments. 5-bromo-2'-deoxyuridine (BrdU) and colony formation assays were applied to evaluate cell proliferation potential. Wound scratch and Transwell assays detected cell migration and invasion, respectively. Flow cytometry detected apoptosis. Autophagy was assessed by p62, Beclin-1, light chain 3 (LC3)-I and LC3-II protein expressions through Western blot test. Akt/mTOR signal pathway-related protein levels were also detected by Western blot test. Here, compared with the control group, Aloin treatment memorably reduced the number of BrdU-positive cells and colonies, and undermined the ability of cell migration and invasion. Moreover, Aloin promoted apoptosis and autophagy. Western blot test proved that the protein expression of p62 was down-regulated, while Beclin-1 and LC3-I/LC3-II protein expressions were up-regulated. Further investigations revealed that Aloin inhibited p-Akt and p-mTOR, suggesting that Aloin could block the activation of AKT/mTOR pathway. Our findings demonstrated that Aloin could inhibit the activation of the AKT/mTOR pathway to induce autophagic cell death, which could provide a novel vision for OSCC treatment.

Keywords: oral squamous cell carcinoma; Aloin; proliferation; migration and invasion; apoptosis and autophagy; Akt/mTOR signal transduction pathway

Introduction

Oral squamous cell carcinoma (OSCC), which primarily originates from the mucosal epithelium of the oral cavity, is the most common type of head and neck cancer, accounting for more than 90% cases (Johnson *et al.*, 2020; Tandon, Dadhich, Saluja, Bawane, & Sachdeva, 2017). It is estimated that more than 3 million new cases of OSCC occur worldwide each year, and the number of related deaths is as high as 145,400 (Torre *et al.*, 2015). Surgery

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with auxiliary radiation or chemoradiation therapy is the primary treatments for OSCC (Yang, Chen, Liu, & Yang, 2019). Owing to late diagnosis, drug resistance, adverse effects, and postoperative recurrences, the 5-year survival rate of patients with OSCC is still low (Fridman *et al.*, 2018; Quadri, Tadakamadla, & John, 2019); hence, there is an urgent need to find a suitable treatment.

Natural products (such as traditional Chinese medicine) are established to be an essential source of medicines against cancer and infectious diseases (Sanchez, Gonzalez-Burgos, Iglesias, & Gomez-Serranillos, 2020). Complementary and alternative medicines, such as natural products, have gained more and more attention for treatment of malignant tumors (Kitamura *et al.*, 2020). Aloin is a natural bioactive anthracycline extracted from the leaves of the perennial herb *Aloe vera* (Zhong *et al.*, 2015). Aloin has a wide range of medicinal values, such as neuroprotection (Chang *et al.*, 2016), cardiopulmonary protection (Birari *et al.*, 2020; Lei *et al.*, 2020; McMullen & McMullen, 1989), regulation of immunity (W. Lee *et al.*, 2019) and the regulation of microbial groups (Boudreau *et al.*, 2017; I. C. Lee & Bae, 2021). In addition, Aloin has been reported to have anticancer effects in many types of cancers. Wang *et al.* (2020b) found that Alion could regulate the proliferation and migration of gastric cancer cells through NOX2-reactive oxygen species (ROS)-mediated Akt/mTOR, the signal transducer and activator of transcription-3 (Stat3), and Nuclear Factor kappa B (NF-κB) signals. Sun *et al.* (2020) reported that Aloin reduced the phosphorylation levels of PI3K, AKT and mTOR in liver cancer cells, and promoted liver cancer cell apoptosis and autophagy to enhance the anticancer effect of metformin. However, the role of Aloin in OSCC is still unknown.

Based on the multiple effects of Aloin, we speculated that it also exerts anticancer activity in OSCC. Clarifying the mechanisms related to the development and progression of OSCC would help to improve the level of treatment. In this study, we aimed to explore the effects of Aloin on the functional level of OSCC cells CAL-27, including cell proliferation, cell migration and invasion, cell apoptosis and autophagy. In addition, we also evaluated the classical Akt/mTOR signal transduction pathway, the primary pathway for autophagy regulation (Kim & Guan, 2015). These findings of Aloin might provide a potential strategy for further prognosis for OSCC treatment.

Methods

OSCC cell line

The study adopted CAL-27 cells (Catalog No. CRL-2095; American Type Culture Collection [ATCC], USA) as the research object *in vitro*. This cell line is epithelial, polygonal with a highly granular cytoplasm. CAL-27 cells were kept in Dulbecco's Modified Eagle's Medium (Catalog No. 30-2002, ATCC, USA) supplemented with 10% fetal bovine serum (FBS) (Catalog No. 30-2021; ATCC) in a humid environment at 37°C.

Chemicals

Natural antitumor anthraquinone glycosides Aloin, having purity of 98.32%, was purchased from MedChem Express, USA (Catalog No. No. 123). It was dissolved in phosphate buffered saline (PBS) (Catalog No. D917703; MACKLIN, Shanghai, China) to prepare a 100-mg/mL storage solution.

Aloin treatment

CAL-27 cells were treated with Aloin for 24 h. The cells were divided into four groups according to the treatment dose, namely control group (equal amount of PBS), 100-μg/mL group, 200-μg/mL group, and 400-μg/mL group. Subsequently, the cells were collected to evaluate respective functions (Z. Wang *et al.*, 2020).

5-bromo-2'-deoxyuridine (BrdU) assay

CAL-27 cells were resuspended and around 1×10^6 cells were seeded onto 35-mm petri dishes with built-in glass slides (Catalog No. 150460; Thermo Fisher Scientific, USA) and fixed with 4% paraformaldehyde (Catalog No. P885233; MACKLIN) for 15 min. Then cells were incubated in DNA labeling solution (TUNEL Assay Kit, Catalog No. ab66110; Abcam, UK) for 60 min at 37°C. Cells were incubated with anti-BrdU-Red antibody in dark at room temperature for 30 min. DAPI (4',6-Diamidino-2-phenylindole [double stranded DNA staining], Catalog No. C1002; Beyotime, Shanghai, China) was utilized as a counterstain (blue) for nucleus. Cells were analyzed within 3 h of staining and the five fields of view were randomly selected to acquire pictures by fluorescence microscopy (Leica, TCS SP5II, Germany).

Colony formation assay

Approximately 500 cells were evenly inoculated onto 60-mm petri dishes (Catalog No. 353002; Corning, USA) and cultured for 14 days; the medium was replenished with fresh medium every 24 h. Subsequently, cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.2% crystal violet staining solution (Catalog No. V5265; Sigma-Aldrich, Germany) for 15 min. The images

were acquired and the number of colonies formed (≥50 cells) were counted using GX53 microscope (Olympus Corporation, Japan).

Wound scratch assay

Wound scratch assay was performed to evaluate cell migration. CAL-27 cells were inoculated onto 6-well plates, https://www.sigmaaldrich.cn/CN/zh/product/sigma/cls3516?context=product, (Catalog No. CLS3516; Corning) and cultured to 90–100% monolayer confluency; a sterile pipette was used to make a gap of about 1 mm. Subsequently, the cells were treated with different concentrations of Aloin for 24 h. The images were captured by GX53 microscope.

Cell invasion assay

Transwell assay was performed to evaluate cell invasion. Approximately 5 × 10^4 CAL-27 cells were inoculated onto upper transwell chambers (Catalog No. 356234; BD Bioscience, USA) with 8-μm pore size coated with Matrigel. The upper chambers were added with serum-free medium and the lower chambers were filled with medium supplemented with 10% FBS. In addition, PBS or Aloin with different concentrations was added into the upper chamber medium. After incubation for 24 h, cells in the lower chambers were fixed for 15 min and stained for 30 min with 0.1% crystal violet staining solution. The invasive number of cells was calculated and imaged by GX53 microscope.

Apoptosis

Approximately 1 × 10^5 cells were resuspended with binding solution using Annexin V-FITC/7-AAD Fluorescence Double Stain Cell Apoptosis Detection Kit according to manufacturers' instructions (Procell Life Science & Technology Co. Ltd., Hubei, China). Then these were added with 5-μL annexin V-FITC and 5-μL 7-ADD staining solution followed by incubation at room temperature for 15 min. The apoptosis of cells was analyzed on a flow cytometer (CytoFLEX S Flow Cytometer; Beckman Coulter, Indianapolis, USA).

Western Blot test

Western blot test was performed to detect the protein levels of p62, Beclin-1, LC3-I, LC3-II, AKT, phospho (p)-AKT, mTOR, and p-mTOR. Briefly, the experiment was divided into following three parts: total protein extraction, membrane transfer, and antibody incubation. CAL-27 cells were lysed with RIPA lysis buffer (Catalog No. 20-188; Sigma-Aldrich). Then the concentrations of protein were quantified by a bicinchoninic acid (BCA) kit (Catalog No. P0011; Beyotime). Equal amount of total proteins in each sample was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) Western blotting membranes (Catalog No. FFP28; Beyotime). After being blocked with 5% skimmed milk at room temperature for 1 h, the membranes were incubated with primary antibodies comprised those against p62 (rabbit, 1:1,000, #23214; Cell Signaling Technology [CST], USA), against Beclin-1 (rabbit, 1:2,000, ab207612; Abcam, UK), against LC3-I/LC3-II (rabbit, 1:1,000, #12741; CST), against AKT (rabbit, 1:1,000, ab8933; Abcam, UK), against p-AKT (rabbit, 1:1,000, ab38449; Abcam, USA), against mTOR (rabbit, 1:1,000, #2983; CST, USA), and against p-mTOR (rabbit, 1:1,000, #5536; CST). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (mouse, 1:1,000, ab8245; Abcam, UK) was used as an internal reference. Afterwards, the membranes were incubated with the goat anti-rabbit immunoglobulin G (IgG; 1:3000, ab6721; Abcam, USA) or goat anti-mouse IgG (1:3000, ab6789; Abcam, USA) secondary antibodies. The signals of proteins were tested and collected by enhanced chemiluminescence (ECL) kit (Catalog No. P0018S; Beyotime). The autophagy level was analyzed by p62, Beclin-1, LC3-I, and LC3-II, and Akt/mTOR signal transduction pathway was assessed by the phosphorylation levels of Akt and mTOR.

Statistical methods

All experiments were performed for at least three times, and all values were presented as mean + standard deviation (SD). The GraphPad Prism 8 software (GraphPad, CA, USA) was utilized for data analyzing. One-way analysis of variance (ANOVA) was used with one categorical independent variable in mutigroups with paired *t*-test to analyze data from two groups. For measurements, $P < 0.05$ was considered significant.

Results

Aloin inhibited the proliferation of OSCC cells

First, we evaluated the effect of Aloin on the proliferation of CAL-27 cells. BrdU and colony formation assays are recognized methods for judging cell proliferation. As shown in Figure 1A ($P < 0.05$), the merger graph of BrdU and DAPI illustrated that Aloin could reduce the number of BrdU-positive cells (red) in a dose-dependent manner, which was consistent with statistical results. Moreover, as shown in Figure 1B ($P < 0.05$), Aloin also significantly

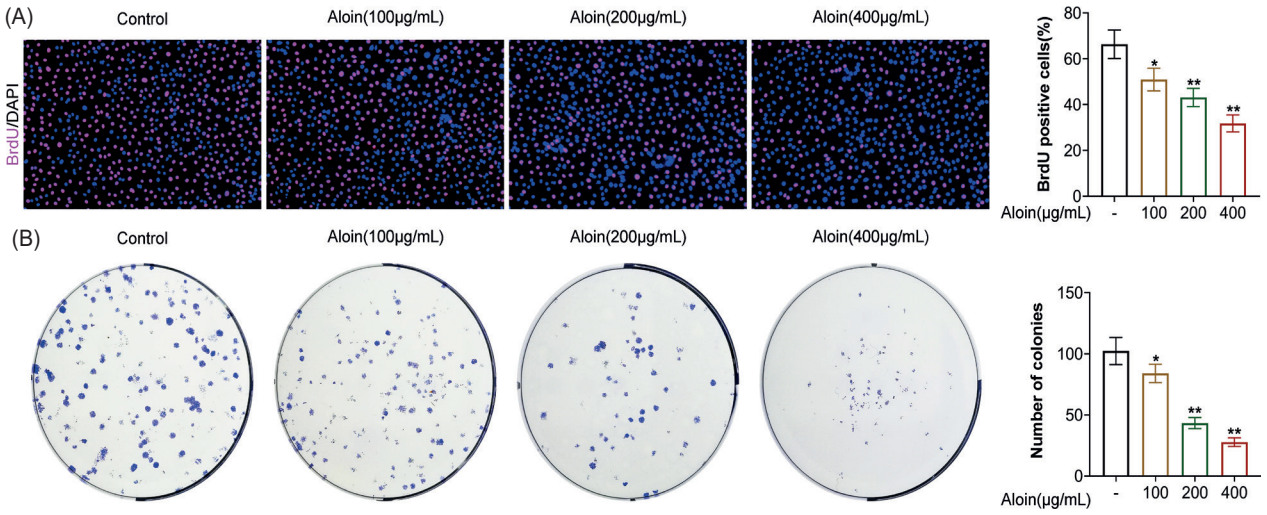

Figure 1. Aloin inhibited the proliferation of CAL-27 oral squamous cell carcinoma (OSCC) cells. CAL-27 cells were treated with Aloin (0, 100, 200 or 400-μg/mL group) for 24 h. (A) BrdU labels newly synthesized DNA in living cells (Red) to assess proliferation. (B) 14-day colony formation experiment were performed, and were captured images and counted the number of colonies formed (≥50 cells) to assess proliferation. *$P < 0.05$, **$P < 0.01$ vs. -.

decreased the number of single colonies in a dose-dependent manner. The evidence indicated that Aloin could effectively inhibit the proliferation of OSCC cancer cells.

Aloin inhibited the migration and invasion of OSCC cells

Migration and invasion are the driving force for the spread of cancer cells (Zhang, Tang, Chen, Du, & Qu, 2020). The results of the Wound Scratch assay demonstrated that the width of wound 24 h after Aloin treatment was greater than that of the control group, suggesting that Aloin weakened the migration ability of CAL-27 cells (Figure 2A, $P < 0.05$). Transwell assay results evidenced that there were markedly fewer invasive cells in Aloin-treated group (Figure 2B, $P < 0.05$), suggesting that Aloin inhibits the invasive ability of CAL-27 cells. Similarly, the inhibitory effect of high concentrations of Aloin was more obvious (Figures 2A and 2B). These results suggested that Aloin could attenuate the migration and invasion of OSCC cells.

Aloin promoted the apoptosis of OSCC cells

In addition to inhibiting proliferation, promoting apoptosis of cancer cells is also an important strategy for cancer treatment. We utilized flow cytometry to detect the rate of cell apoptosis, and the results demonstrated that Aloin promoted cell apoptosis in a dose-dependent manner (Figure 3, $P < 0.01$).

Aloin promoted autophagy in oral squamous cancer cells

Levels of p62, Beclin-1, LC3-I and LC3-II proteins are closely related to autophagy (Wei, Wu, Liu, & Xie, 2020; Xu & Qin, 2019). Compared to the control group, the expressions of p62 and Beclin-1, and LC3-I/LC3-II have all changed (Figure 4, $P < 0.01$). The expression of p62 was down-regulated by Aloin, while Beclin-1 and LC3-I/LC3-II were up-regulated after Aloin treatment (Figure 4, $P < 0.01$). The evidence indicated that Aloin could induce autophagy in OSCC.

Aloin blocked the Akt/mTOR pathway

The Akt/mTOR pathway is the hub of a variety of signals, and is also an important pathway related to autophagy. We detected the phosphorylation levels of Akt and mTOR pathways. As expected, the values of p-Akt/Akt and p-mTOR/mTOR were decreased (Figure 5, $P < 0.01$), suggesting that the Akt/mTOR pathway might be one of the pathways in which Aloin inhibited OSCC cell functioning.

Discussion

In recent years, the importance of drug therapy for malignant tumors has increased with passing years (J. Wang *et al.*, 2020; W. E. Yang *et al.*, 2019). Previous studies have demonstrated that natural plant products could be used

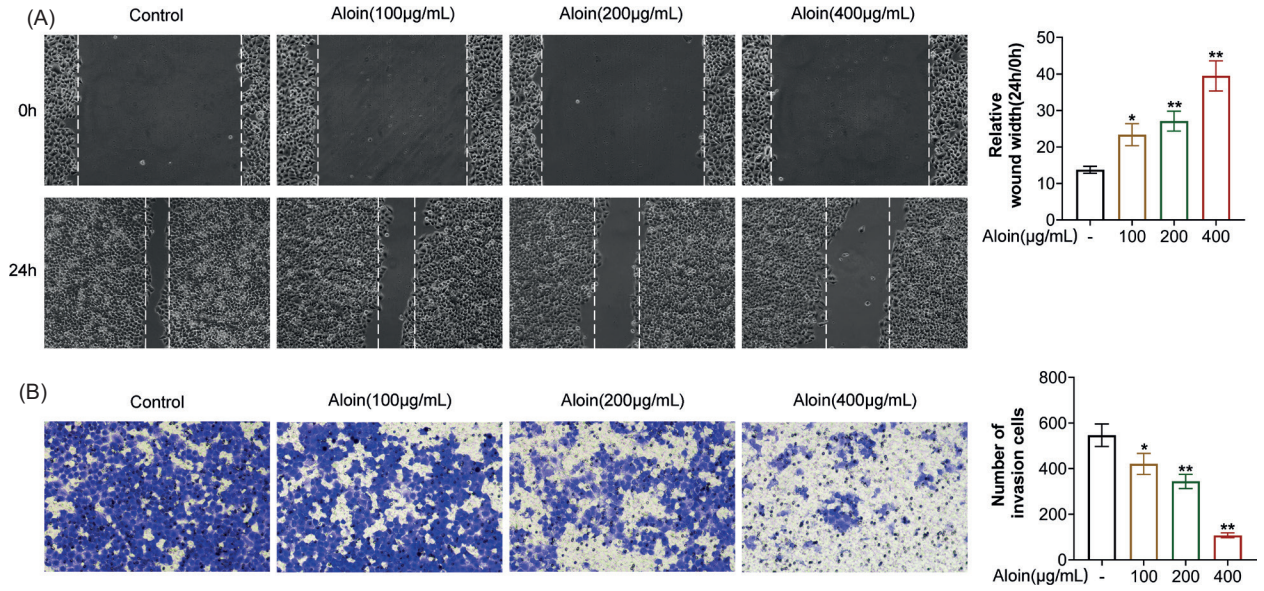

Figure 2. Aloin inhibited the migration and invasion of CAL-27 cells. (A) Wound scratch assay was performed to evaluate cell migration. The cells were treated with different concentrations of Aloin for 24 h. (B) Transwell assay was performed to evaluate cell invasion. *P* < 0.05, *P* < 0.01 vs. -.

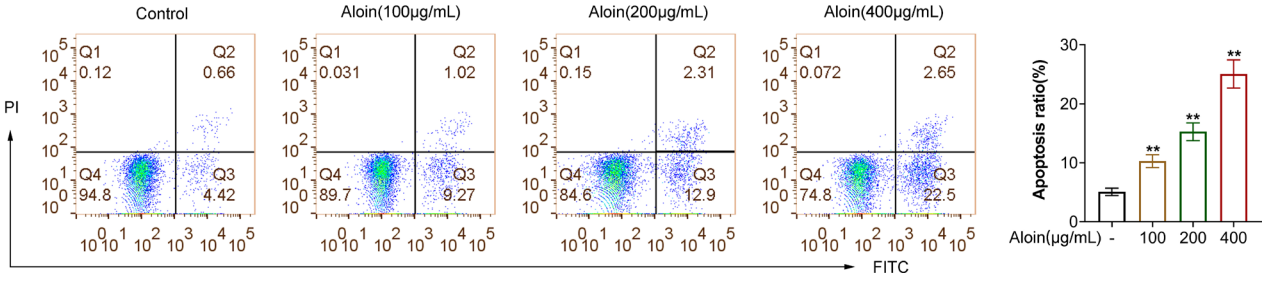

Figure 3. Aloin promoted the apoptosis of CAL-27 cells. Flow cytometry was performed to assess apoptosis. **P < 0.01 vs. -.

as chemopreventive or therapeutic agents for human cancer cells (Khan *et al.*, 2019). As per our knowledge, our study for the first time has investigated the role of Aloin in OSCC. The *in vitro* data have initially revealed that Aloin plays a tumor-suppressor activity in OSCC. It inhibits tumor proliferation, migration, and invasion potential, and stimulates cancer cells' apoptosis and autophagy.

A number of studies have indicated that the disorder of cell proliferation, apoptosis and metastasis is a major inducement for the occurrence and development of cancer, especially tumor metastasis, which is one of the primary problems faced during the clinical treatment of various cancers (Irani, 2016). Lymph node metastasis of OSCC in the neck reduces patient's survival rate by approximately 50% (Sharma, Kim, & Paeng, 2018). Targeted autophagy has been emphasized as a feasible treatment strategy with clinical utility in cancer treatment (Khan *et al.*, 2020). In this study, based on the study of Wang *et al.* (Z. Wang *et al.*, 2020), we selected the concentration range of 100–400 μg/mL Aloin and evaluated the effects of the gradient Aloin on cell proliferation, migration, invasion and apoptosis in CAL-27 cells. Encouragingly, Aloin treatments significantly inhibited the proliferation, migration and invasion potential of cancer cells, and stimulated cancer cells to undergo apoptosis and autophagy. Consistently, the higher the concentration of Aloin, the more pronounced the anticancer effect. The evidence suggested that Aloin is expected to become a potential molecule for the treatment of OSCC. It was reported that high-mobility group protein B1 (HMGB1) could be used as a target for Aloin (Li *et al.*, 2020; Tao *et al.*, 2019). Nevertheless, Aloin still lacks a lot of mechanism research.

Autophagy is a lysosome-dependent cellular degradation process involving the degradation of intracellular components to maintain cell (White, 2012). We utilized Western blot test to detect p62, Beclin-1, LC3-I and

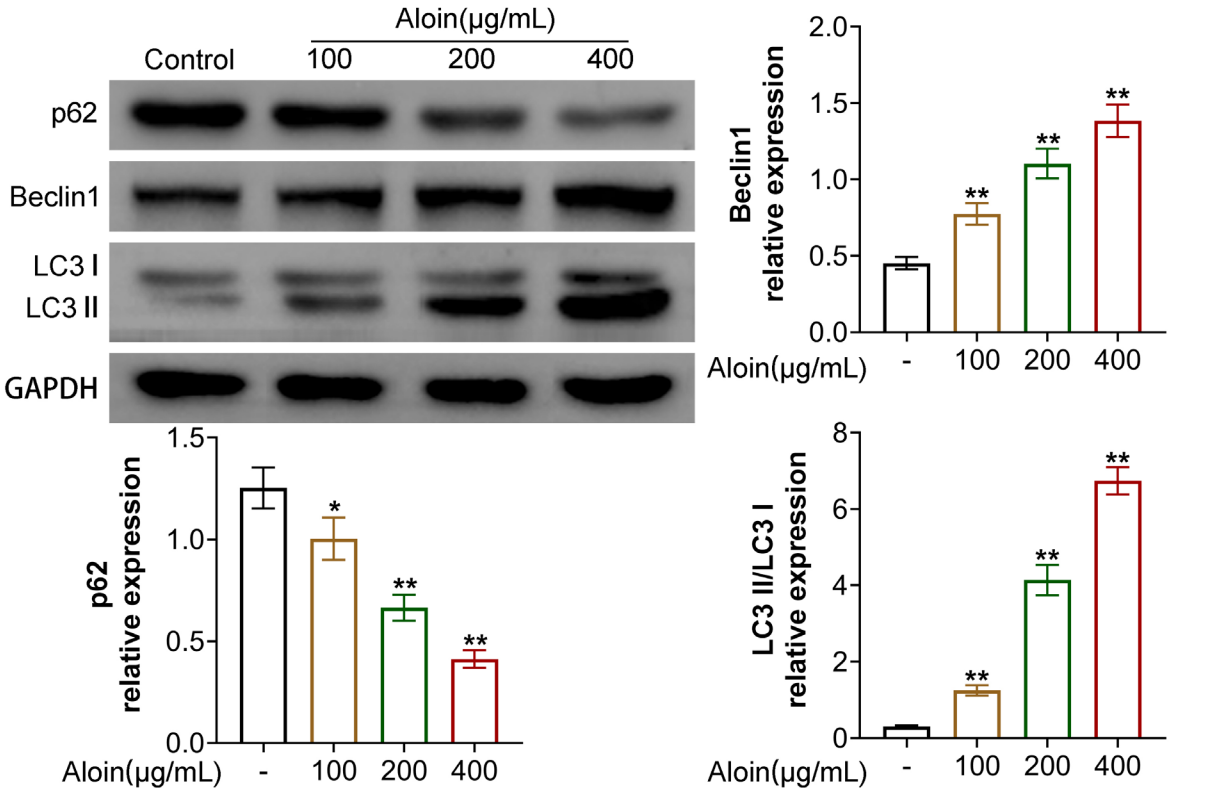

Figure 4. Aloin promoted autophagy of CAL-27 cells. Western blot test was performed to detect the protein expressions of p62, Beclin-1, light chain 3 (LC3)-I and LC3-II to assess autophagy. GAPDH was used as an internal reference. **$P < 0.01$ vs. -.

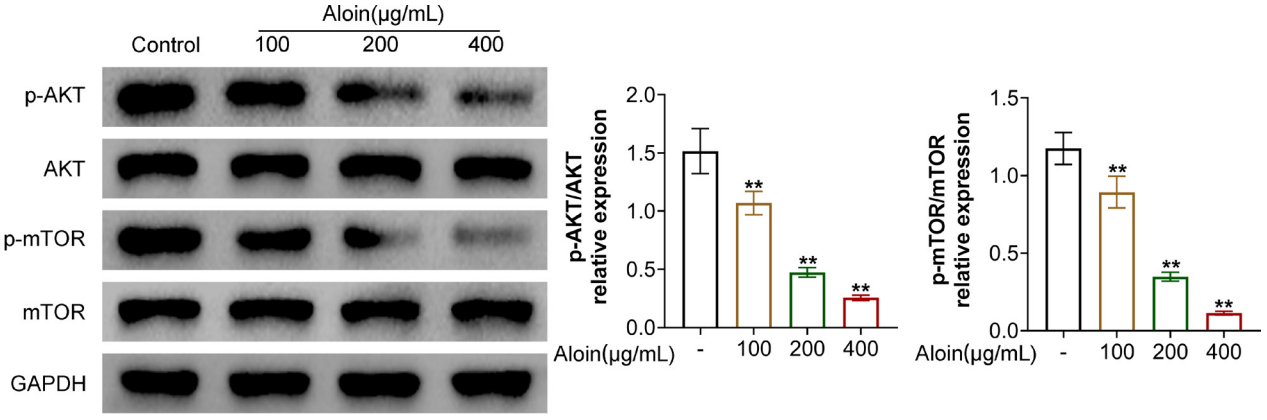

Figure 5. Aloin inhibited the Akt/mTOR pathway. Western blot test was performed to detect the protein expressions of AKT, phosphorylated (p)-AKT, mTOR, and p-mTOR. GAPDH was used as an internal reference. **$P < 0.01$ vs. -.

LC3-II levels to assess autophagy in CAL-27 cells. The multidomain protein p62, also known as sequestosome 1, occupies a central position in autophagy and apoptosis, and is an autophagy receptor and a selective substrate for autophagy (Ichimura, Kominami, Tanaka, & Komatsu, 2008; Islam, Sooro, & Zhang, 2018). In OSCC, high cytoplasmic p62 expression could be a poor prognostic marker for OSCC patients (Liang *et al.*, 2018). Beclin-1, LC3-I and LC3-II are the hallmark proteins of autophagy (Wei *et al.*, 2020; Xu & Qin, 2019). It has been reported that activated Beclin-1 can induce autophagy (Kinarivala, Patel, Boustany, Al-Ahmad, & Trippier, 2017). We found that Aloin treatment caused the up-regulation of Beclin-1 and LC3 I/LC3-II proteins, while it downregulated P62 protein. Therefore, we suggest that Aloin induces autophagy in OSCC.

Unlike apoptosis, autophagy is a mechanism by which cells try to survive. In some cases, autophagy constitutes a stress adaptation that avoids cell death (M Chiara Maiuri, Einat Zalckvar, Adi Kimchi, & Guido Kroemer, 2007). Interestingly, accumulated studies indicate that

there is a complex relationship between autophagy and apoptosis (D'Arcy, 2019; Maiuri, Zalckvar, Kimchi, & Kroemer, 2007). They play seemingly opposite biological roles in response to genotoxic or pharmacological stress but share common regulatory elements, including Akt/mTOR signal pathway (Lin *et al.*, 2019). The classic Akt/mTOR signal transduction pathway is the most common dysregulated cell pathway in human cancers, regulating the development of many cancers, including proliferation, apoptosis, chemoresistance and autophagy (Xia & Xu, 2015). Akt/mTOR pathway negatively regulates the process of autophagy. Western blot test results demonstrated that Aloin treatment significantly reduced the levels of p-AKT and p-mTOR in CAL-27 cells as described previously. Autophagy and apoptosis may regulate each other after treatment to achieve anticancer effects (Adhauliya, Kalappanavar, Ali, & Annigeri, 2016). This finding confirmed that Aloin could inhibit the activation of AKT/mTOR pathway to induce autophagic cell death.

In conclusion, this study strongly demonstrated that Aloin, a natural plant ingredient, is a potential antitumor molecule , which can induce OSCC cell protective autophagy to exert anticancer effects. At present, the study of Aloin is still in its infancy. Further studies toward Aloin in OSCC are required to validate the performances and properties *in vivo* and in real practice.

Competing Interests

The authors state that there are no conflicts of interest to disclose.

Contribution of Authors

Yongxin Hu and Xiaosong Xiang designed the study and supervised data collection. Yuhui Zhang and Zhongqi Tian analyzed and interpreted the data. Lijian Wang prepared the manuscript for publication and reviewed its draft. All authors read and approved the final manuscript.

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