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Ailanthone Promotes Human Vestibular Schwannoma Cell Apoptosis and Autophagy by Downregulation of miR-21

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Ailanthone (AIL) is a quassinoid isolated from the traditional Chinese medicinal herb *Ailanthus altissima.* The antitumor activities of AIL have been reported in several cancers. The purpose of the present study was to explore the effect of AIL on vestibular schwannomas (VSs). Various concentrations of AIL (0–1 μM) were used to treat human primary VS cells, and then cell viability, proliferation, apoptosis, and autophagy were assessed. Expression of miR-21 in VS cells was altered by miRNA transfection. The functional actions of AIL on miR-21 dysregulated cells were also assessed. AIL significantly reduced the viability of VS cells, and the IC_{50} value was 0.48±0.023 μM. In response to 0.6 μM AIL, $BrdU^+$ cell rate and cyclin D1 expression were reduced, apoptotic cell rate was increased, caspase 3 and caspase 9 were cleaved, Beclin-1 and LC3-II were accumulated, and p62 was downregulated. miR-21 was lowly expressed in AIL-treated cells, and AIL-induced apoptosis and autophagy were attenuated by miR-21 overexpression. In addition, AIL downregulated Ras and Raf and deactivated MEK, ERK, mTOR, and p70S6K, while the downregulation and deactivation induced by AIL were reversed by miR-21 overexpression. To conclude, AIL inhibited VS cell proliferation and induced apoptosis and autophagy. The antitumor activities of AIL in VS cells were realized possibly via downregulation of miR-21 and blocking the Ras/Raf/MEK/ERK and mTOR pathways.

Key words: Vestibular schwannoma (VS); Ailanthone (AIL); miR-21; Apoptosis; Autophagy

INTRODUCTION

Vestibular schwannomas (VSs) are benign skull base tumors that originate from myelin-forming schwann cells that surround the vestibular branches of the eighth (auditory) cranial nerve[1]. The incidence of VS is 1–20 cases per million persons per year and is reported to be rising almost certainly due to increasing diagnosis in the magnetic resonance imaging era[2]. Currently, VSs are managed by observation, surgical resection, or radiotherapy. Microsurgery is the main choice for treating large tumors with radiological or neurological signs of brainstem compression[3]. However, there exist risks of neurological sequelae such as hearing loss and facial nerve palsy[4,5]. Thus, novel management strategies with less risk and side effects need to be explored.

Ailanthone (AIL), a kind of water-soluble quassinoid, is isolated from the well-known traditional Chinese medicinal herb *Ailanthus altissima*[6]. Recently, several studies have focused on the antitumor activities of AIL. Chen et al. have reported that AIL inhibited the proliferation of human gastric cancer SGC-7901 cells by inducing G_2/M phase cell cycle arrest and apoptosis[7]. AIL has been suggested to be a new potential drug candidate for prostate cancer, as AIL possesses good bioavailability, high solubility, lack of cytochrome P450 inhibition, low hepatotoxicity, as well as inhibiting effects on the growth and metastasis of castration-resistant prostate cancer cells[8,9]. In vitro and in vivo investigations elucidated that AIL also acts as a natural anti-hepatocellular carcinoma (HCC) component, as AIL inhibited the tumor growth without marked toxicity[10]. However, the potential effect of AIL on VS has not been determined.

MicroRNAs (miRNAs) are short, noncoding RNAs that posttranscriptionally regulate the expression of target genes[11]. Numerous evidence has demonstrated that altered profiles of miRNAs are related to carcinogenesis and tumor progression via oncogenic or tumor-suppressive properties[12,13]. miR-21 is an abundantly expressed miRNA in mammalian cells, and a high level of miR-21 expression is associated with several types of cancer[14,15]. By using microarray technology, miR-21 has been found to be upregulated in VS when compared

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to healthy tissues[16]. Functional investigation indicated that anti-miR-21 decreased VS cell proliferation in response to platelet-derived growth factor stimulation and increased apoptosis[17].

In this study, we aimed to investigate the effect of AIL on VS cell apoptosis and autophagy and explored whether miR-21 was involved in the underlying mechanism by which AIL impacted VS cells. The findings of this study provided in vitro evidence that AIL may have potential use in the clinical treatment of VS.

MATERIALS AND METHODS

VS Collection and Primary VS Cultures

VSs were collected from patients undergoing microsurgery in Linyi People's Hospital between April 2015 and December 2016. Freshly harvested human specimens of VSs were washed three times with saline solution and then snap frozen in liquid nitrogen until use. This study was approved by the ethics committee of our local institution. Informed consent was obtained from each participant. Primary human VS cultures were prepared as previously described[18,19]. In brief, VSs were cut into 1-mm^3 fragments, digested with 0.25% trypsin and 0.1% collagenase for 30–40 min at 37°C, and dissociated by trituration through fire-polished Pasteur pipettes. Following centrifugation at 300×*g* for 5 min, the supernatant was discarded. The cells were resuspended in culture plates and cultured in DMEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) and N2 additives (Sigma-Aldrich, St. Louis, MO, USA). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

AIL Treatment

AIL with purity ≥98% was purchased from Shanghai Solarbio Co., Ltd. (Shanghai, P.R. China). AIL was dissolved in complete culture medium of various concentrations, and VS cells were treated for 48 h. Culture medium without AIL served as the control group. The chemical structure of AIL is shown in Figure 1.

CCK-8 Assay

Cell viability was detected using cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kyushu, Japan). VS cells (5×10^3 cells/well) were seeded in 96-well plates, and then AIL at various concentrations (0.2, 0.4, 0.6, 0.8, and 1 μM) was added. Following incubation at 37°C for 48 h, the culture medium was removed, and 10 μl of CCK-8 solution was added into each well. The plates were placed in a humidified incubator for 2 h. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA). The cells without AIL treatment were 100% viable.

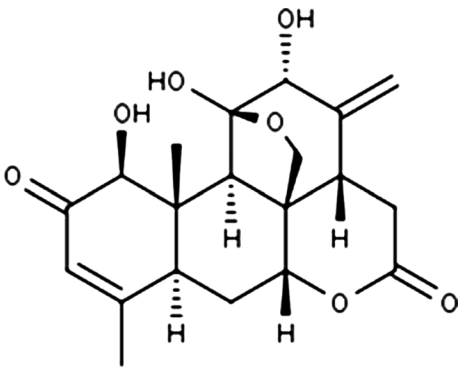

Figure 1. The chemical structure of ailanthone (AIL).

BrdU Staining Assay

Cell proliferation was detected using bromodeoxyuridine (BrdU) assay kit (Roche Diagnostics GmbH, Germany). VS cells (5×10^5 cells/well) were seeded in six-well plates followed by treatment with 0.6 μM AIL for 48 h. Then 10 μM BrdU solution was added into each well, and the cultures were placed in a humidified incubator for 4 h. BrdU integration was detected by labeling with 100 μl of anti-BrdU-POD monoclonal antibody and incubating at room temperature for 30 min. A tetramethyl-benzidine (TMD) substrate solution was used to develop the color for 15 min in the dark. The reaction product was inactivated with 1 M sulfuric acid. Immune complexes were detected by reading absorbance at 490 nm (OD_{490}).

Apoptosis Assay

Cell apoptosis was analyzed using Annexin-V-FITC Apoptosis Detection Kit (Solarbio). After treating with 0.6 μM AIL for 48 h, 1×10^5 VS cells were collected from each sample. The cells were suspended in 300 μl of binding buffer containing 10 μl of FITC-annexin V, and the samples were then incubated in the dark at room temperature for 30 min. Subsequently, 5 μl of propidium iodide (PI) and 200 ml of ice-cold PBS were added, and the samples were immediately analyzed under a FACSscan (Beckman Coulter, Fullerton, CA, USA). $FITC^+$ and PI^- were recognized as an apoptotic cell.

miRNA Transfection

For miR-21 overexpression, miR-21 mimic was transfected into VS cells using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The sequences for miR-21 mimic were UAGCUUAUCAGACUGAUGUUGA (sense) and AAC AUCAGUCUGAUAAGCUAUU (antisense). Scrambled sequences were used as its negative control (NC). Both miR-21 mimic and NC were synthesized by GenePharma

Co. (Shanghai, P.R. China). At 48 h of transfection, cells were collected for use in the following experiments.

qRT-PCR

To test for miR-21 expression, miRNA in VS cells was extracted using an miRNeasy Mini Kit (Qiagen, Shenzhen, P.R. China). Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), and qRT-PCR was performed by the TaqMan Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems). U6 served as an internal control for normalizing miR-21 expression. To test for expression of cyclin D1, Beclin-1, and p62, total mRNA in VS cells was extracted by TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Reverse transcription was performed using the Transcriptor First-Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). qRT-PCR was performed using Fast Start Universal SYBR Green Master (ROX) (Roche). GAPDH served as an internal control for normalizing the expressions of cyclin D1, Beclin-1, and p62. Data were analyzed with the $2^{-\Delta\Delta}$Ct method.

Western Blot

Total protein was isolated from VS cells using RIPA lysis buffer (Beyotime, Shanghai, P.R. China). Protein concentration was qualified by the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The whole-cell extracts were resolved over sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat milk for 1 h at room temperature and then probed by the primary antibodies for detection of cyclin D1 (ab21699), caspase 3 (ab13586), caspase 9 (ab32539), LC3B (ab51520), Beclin-1 (ab114071), p62 (ab56416), Ras (ab221163), Raf (ab196901), t-MEK (ab32517), p-MEK (ab30622), t-ERK (ab224313), p-ERK (ab214362), t-mTOR (ab87 540), p-mTOR (ab84400), t-p70S6K (ab47504), p-p70 S6K (ab2571), and β-actin (ab8226). After rinsing with TBST two times, the membranes were incubated with the secondary antibodies for 1 h at room temperature. Blots were visualized by the enhanced chemiluminescence (ECL) method. The intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, P.R. China).

Statistical Analysis

All data were represented as mean±SD from three independent experiments. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The *p* values were calculated using a one-way analysis of variance (ANOVA). A value of $p<0.05$ was considered to indicate a statistically significant result.

RESULTS

AIL Inhibits VS Cell Proliferation

VS cells were treated with different concentrations of AIL (0, 0.2, 0.4, 0.6, 0.8, and 1 μM), and then cell viability was detected. As the results show in Figure 2A, viability of VS cells was reduced by the addition of AIL in a dose-dependent manner; higher concentrations of AIL possessed lower viability. The IC_{50} value of AIL in VS cells was 0.48±0.023 μM, and 0.6 μM was selected as the AIL concentration used in the following investigations. Effect of AIL on VS cell proliferation was then detected by BrdU staining. Data displayed in Figure 2B show that $BrdU^+$ cell rate was significantly reduced by the addition of 0.6 μM AIL ($p<0.001$). qRT-PCR and Western blot analyses were performed to assess the expression changes of cyclin D1. Results showed that both the mRNA and protein expressions of cyclin D1 were remarkably reduced by AIL administration (Fig. 2C and D).

AIL Promotes Apoptosis and Autophagy of VS Cells

Effects of AIL on the apoptosis and autophagy of VS cells were evaluated by detection of apoptotic cell rate and the expressions of apoptosis- and autophagy-related factors. We found that AIL significantly increased apoptotic cell rate ($p<0.001$) (Fig. 3A) and increased the protein expression levels of cleaved caspase 3 and caspase 9 (Fig. 3B). AIL upregulated the mRNA level of Beclin-1 ($p<0.001$) and downregulated the mRNA level of p62 ($p<0.001$) (Fig. 3C). In addition, AIL upregulated the protein level of Beclin-1 and downregulated the protein levels of LC3-II and p62 (Fig. 3D).

AIL Promotes Apoptosis and Autophagy of VS Cells in an miR-21-Dependent Manner

It has been reported that miR-21 in VS contributes to tumor growth[17]. We investigated whether miR-21 is also involved in the mechanism by which AIL suppressed VS cell growth. qRT-PCR analytical results displayed in Figure 4A showed that AIL significantly reduced the miR-21 level in VS cells ($p<0.01$), indicating miR-21 was negatively regulated by AIL. miR-21 in VS cells was overexpressed by transfection with the miR-21 mimic, and changes in cell apoptosis and autophagy were assessed. Figure 4B shows that miR-21 expression was significantly increased by miR-21 mimic transfection ($p<0.001$). Results shown in Figure 5A and B indicated that AIL induced the increase in apoptotic cell rate ($p<0.01$), and upregulations in cleaved caspase 3 and caspase 9 were all alleviated when miR-21 was overexpressed in VS cells. Moreover, AIL induced upregulation of

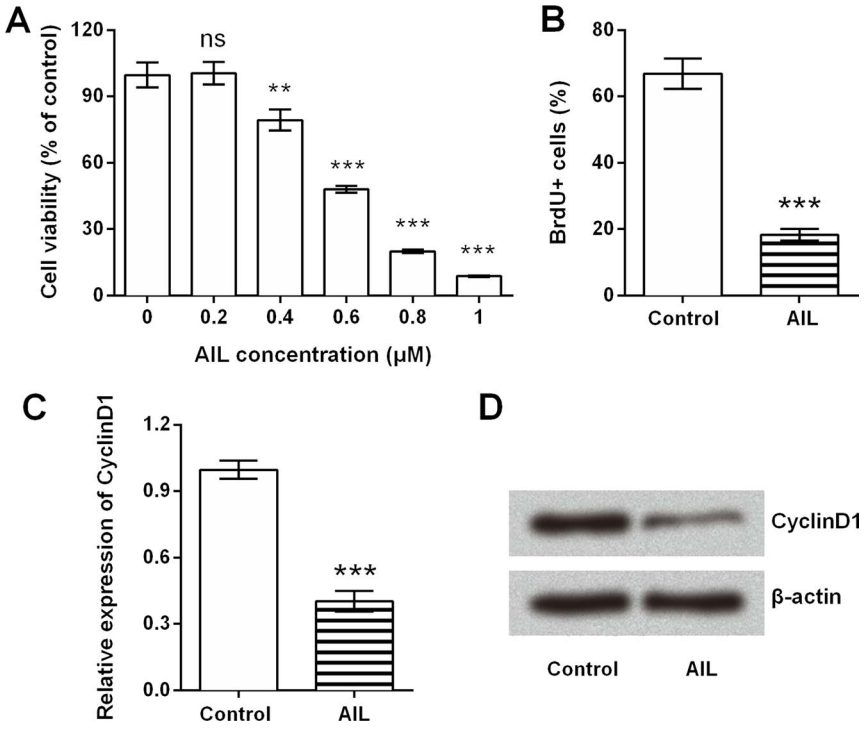

Figure 2. Antiproliferation effect of AIL on vestibular schwannoma (VS) cells. (A) Viability of VS cells after administration with various concentrations of AIL. (B) Bromodeoxyuridine-positive ($BrdU^+$) cell rate of cells after treatment with 0.6 μM AIL. (C) mRNA level of cyclin D1 in cells that were treated with 0.6 μM AIL. (D) Protein level of cyclin D1 in AIL-treated cells. ns, no significance. $^{**}p<0.01$; $^{***}p<0.001$.

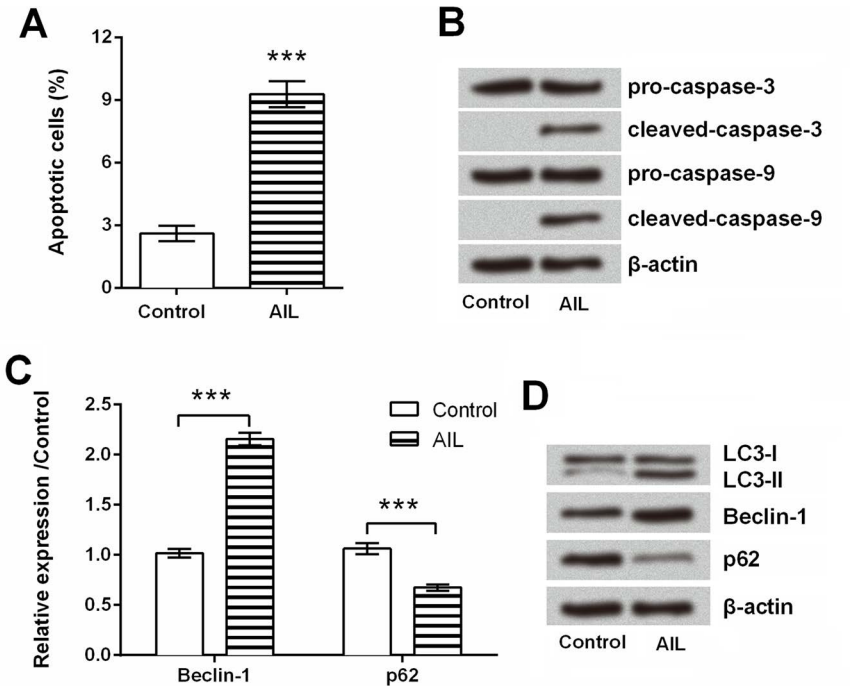

Figure 3. Induction of apoptosis and autophagy in AIL-pretreated cells. (A) Apoptotic cell rate of VS cells after AIL administration. (B) Protein expressions of apoptosis-related factors. (C) mRNA level expressions of autophagy-related factors. (D) Protein level expressions of autophagy-related factors. $^{***}p<0.001$.

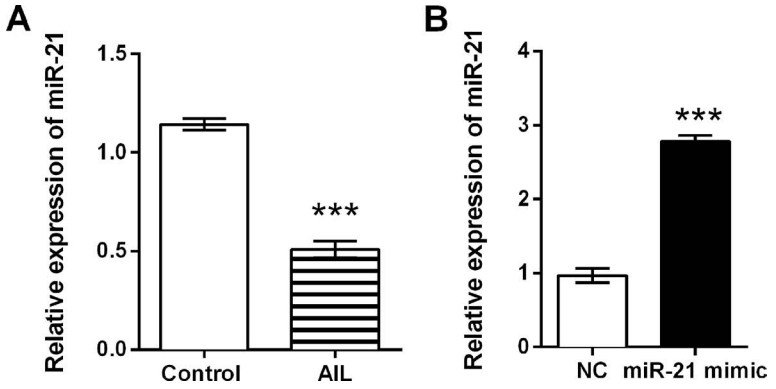

Figure 4. Downregulated expression of miR-21 in AIL-pretreated cells. (A) Expression of miR-21 in VS cells pretreated with AIL. (B) Expression of miR-21 in cells after transfection with the miR-21 mimic or NC (scrambled control). ***$p<0.001$.

Beclin-1, and downregulations of LC3-II and p62 were also alleviated by miR-21 overexpression (Fig. 5C and D).

AIL Blocks the Ras/Raf/MEK/ERK and mTOR Pathways in an miR-21-Dependent Manner

To further explore the possible mechanism via which AIL induced apoptosis and autophagy in VS cells, we focused on the Ras/Raf/MEK/ERK and mTOR signaling pathways. Western blot analytical results showed that AIL reduced the protein expressions of Ras, Raf, p-MEK, p-ERK, p-mTOR, and p-p70S6K, whereas the downregulations induced by AIL were all reversed by miR-21 overexpression (Fig. 6A–D).

DISCUSSION

It has been reported that AIL possesses certain antitumor properties against several cancers, including gastric cancer, prostate cancer, and HCC with less side

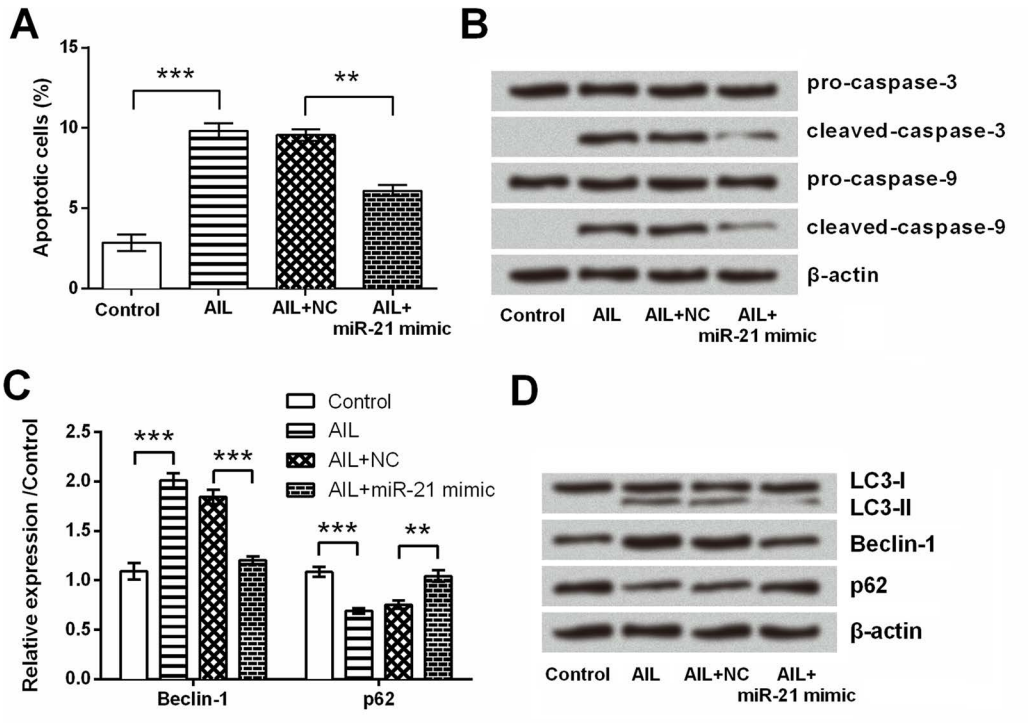

Figure 5. AIL induces apoptosis and autophagy in an miR-21 dependent manner. (A) Apoptotic cell rate after VS cells were pretreated with AIL, AIL plus NC, and AIL plus miR-21 mimic. The cells without any treatment served as control. (B) Protein expressions of apoptosis-related factors. (C) mRNA level expressions of autophagy-related factors. (D) Protein level expressions of autophagy-related factors. **$p<0.01$; ***$p<0.001$.

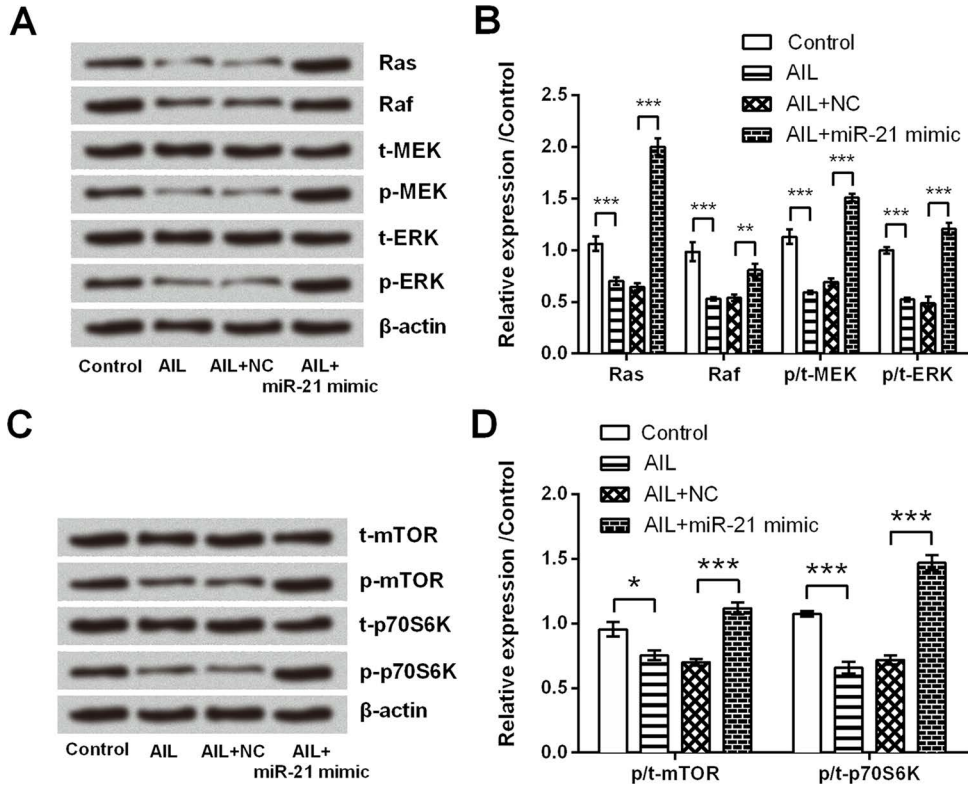

Figure 6. AIL blocks Ras/Raf/MEK/ERK and mTOR pathways in an miR-21-dependent manner. (A) Protein expressions of Ras, Raf, MEK, and ERK in cells pretreated with AIL, AIL plus NC, and AIL plus miR-21 mimic. The cells without any treatment served as control. (B) Quantitative result of Ras, Raf, MEK, and ERK expressions based on Western blot analysis. (C) Protein expressions of mTOR and p70S6K in cells. (D) Quantitative result of mTOR and p70S6K expressions based on Western blot analysis. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

effects[7–10]. The antitumor activity of AIL in VS and its underlying mechanism of action have not been determined. Our data suggested that a 0.6-μM concentration of AIL inhibited VS cell proliferation and induced apoptosis and autophagy. More interestingly, AIL administration downregulated the expression of miR-21, and AIL induced cell apoptosis and autophagy in an miR-21-dependent manner. Furthermore, AIL exhibited blocking actions on the Ras/Raf/MEK/ERK and mTOR signaling pathways; this blocking possibly results from the downregulation of miR-21 expression.

The inhibitory effects of AIL on the growth of several human cancer cells has been previously reported. He et al. indicated that AIL potently inhibited the growth of several prostate cancer cell lines including LNCaP, c4-2b, 22RV1, and LAPC4[8]. Zhuo et al. demonstrated that AIL inhibited the growth of HepG2, Hep3B, and Huh7 cells; the IC_{50} values were 0.628 ± 0.047, 0.544 ± 0.031, and 0.350 ± 0.016 μM, respectively[10]. In our study, for the first time, we found that AIL exerted a growth inhibitory effect on primary VS cells, and the IC_{50} value was 0.48 ± 0.023 μM. It seems that a low concentration of AIL exerted excellent antitumor activity via inhibition of tumor cell proliferation. This hypothesis was further verified by the detection of cyclin D1 expression in this study, as both mRNA and protein levels of cyclin D1 were downregulated after AIL administration. Cyclin D1 is a critical target of proliferative signals in the G_1 phase that promotes cell cycle progression[20].

Induction of programmed cell death (PCD) has become a hot topic in the field of cancer treatment. Previous studies have revealed that AIL improved tumor cell apoptosis (type I PCD) by inducing mitochondrial membrane depolarization and caspase 3 activation[7,10,21]. Consistent with these studies, our data indicated that AIL induced VS cell apoptosis via activation of caspase 3 and caspase 9. However, to our knowledge, no literature has reported the role of AIL in tumor cell autophagy (type II PCD). The conversion of LC3-I to LC3-II, accumulation of Beclin-1, and clearance of p62 are considered hallmarks of the autophagic flux[22]. Our data showed that AIL remarkably increased LC3-II and Beclin-1 expressions, whereas it decreased p62 expression, indicating autophagy was induced by AIL administration. AIL inhibited VS cells not only via induction of apoptosis but also via autophagy.

Among all of the miRNAs, miR-21 has gained considerable attention due to its oncogenic role in malignant tumors, including lung cancer[23], breast cancer[24], oral carcinoma[25], prostate cancer[26], glioma[27], and HCC[28], among others. In this study, we found that miR-21 was downregulated in AIL-pretreated VS cells. Functional analytical results showed that AIL-induced apoptosis and autophagy were attenuated by miR-21 overexpression, implying that AIL induced apoptosis and autophagy in VS cells at least partially via regulating miR-21.

The Ras/Raf/MEK/ERK and mTOR signaling pathways contribute core effects in regulating cell proliferation, differentiation, and survival in the signaling networks[29]. In activated Jurkat cells, miR-21 has been predicted as a regulator of ERK[30]. miR-21 drives tumorigenesis through inhibition of negative regulators of the Ras/MEK/ERK pathway[31]. Studies have also suggested that miR-21 modulated tumor cell autophagy via the mTOR pathway[32,33]. We found that both Ras/Raf/MEK/ERK and mTOR signaling pathways were deactivated in response to AIL administration and were reactivated when miR-21 was overexpressed. Our data suggested that AIL blocked the Ras/Raf/MEK/ERK and mTOR pathways in an miR-21-dependent manner and provided us a new viewpoint that AIL induced VS cell apoptosis and autophagy via the downregulation of miR-21 and thereby deactivation of these two pathways. Further investigations are required to confirm this hypothesis.

To sum up, this study suggested that AIL inhibited VS cell proliferation and induced apoptosis and autophagy. Oncogenic gene miR-21 was downregulated by AIL administration, and the antitumor activities of AIL in VS cells were possibly realized via downregulation of miR-21 and blocking of the Ras/Raf/MEK/ERK and mTOR pathways.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

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