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Ailanthone up-regulates miR-449a to restrain acute myeloid leukemia cells growth, migration and invasion

Yang Zhang[a,b,1], Chunzhi Zhang[c,1], Dejin Min[d,*]

[a] Department of Hematology, Jining No.1 People's Hospital, Jining 272000, Shandong, China.
[b] Affiliated Jining No.1 People's Hospital of Jining Medical University, Jining Medical University, Jining 272067, Shandong, China
[c] Department of Clinical Laboratory, Jining No.1 People's Hospital, Jining 272000, Shandong, China
[d] Department of Hepatobiliary Surgery, Jining No.1 People's Hospital, Jining 272000, Shandong, China

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ABSTRACT

Background: Ailanthone (AIL) is a quassinoid isolated from traditional Chinese herbal medicine *Ailanthus altissima*. The anti-tumor activities of AIL have been reported in various solid tumors. This study aimed to reveal the *in vitro* effect of AIL on acute myeloid leukemia (AML) cells.

Methods: The effects of AIL on five AML cell lines (KG1, HL60, U-937, THP-1 and OCI-AML2) as well as myeloid progenitor cells were evaluated by performing CCK-8 assay, flow cytometry, Transwell assay and Western blotting. KG1 and HL60 cells were transfected with miR-449a inhibitor or its negative control, and then were treated by AIL. The above mentioned assays were performed again to study the involvement of miR-449a in AIL's function.

Results: AIL dose-dependently inhibited the viability of AML cells and myeloid progenitor cells. The IC50 value of AIL towards KG1 and HL60 cells was 0.58 and 0.57 μM, respectively. AIL with concentration of 0.5 μM significantly induced the apoptosis of AML cells rather than myeloid progenitor cells. Meanwhile, 0.5 μM AIL significantly reduced migration and invasion of AML cells. miR-449a was highly expressed in response to the treatment of 0.5 μM AIL. Besides this, the anti-tumor activities of AIL in AML cells were attenuated by miR-449a silence. Further, the blockage of Notch and PI3K/AKT signaling pathways induced by AIL was reversed by miR-449a silence.

Conclusion: AIL restrained AML cells growth, migration and invasion through up-regulation of miR-449a, and deactivation of Notch and PI3K/AKT signaling pathways.

1. Introduction

Acute myeloid leukemia (AML) is characterized by a clonal proliferation of immature myeloid precursor cells (Boffo et al., 2018), which impairs the production of normal blood cells (Marcucci et al., 2011). Acute promyelocytic leukemia and acute monocytic leukemia are two subtypes of AML. While the pathogenesis of AML is still unclear, scientists around the world believed that other blood disorders, chemical exposures, radiation, and genetics like GATA2 deficiency are risk factors of AML. To date, different therapies are applied for treating AML (Medinger et al., 2016), and the five-year survival rate of AML is significantly improved (Dohner et al., 2015). Hematopoietic stem cell transplantation (HSCT) has been traditionally considered as the best strategy in this setting, but the therapy is depending on the fitness of patients and the availability of the stem cell donor (Medinger et al., 2016). Therefore, it has great significance for developing novel and effective treating strategies for AML.

microRNAs (miRNAs) are a group of small non-coding RNAs with length of 18–25 nucleotides. Emerging evidence showed that abnormal expression of miRNAs contributes to the initiation and progression of AML (De Luca et al., 2017; Elhamamsy et al., 2017; Wang et al., 2017). Thus, miRNAs have been mentioned as promising biomarkers for AML diagnosis and prognosis, as well we therapeutic targets for AML (Trino and Lamorte, 2018). miR-449a is a member of miR-449 family that is frequently down-regulated in many solid tumors, like osteosarcoma (Chen et al., 2015), non-small cell lung cancer (Luo et al., 2013), and gastric cancer (Li et al., 2015). Furthermore, miR-449a was capable of modulating the expression of tumorigenesis-associated genes, such as Flot2 (Li et al., 2015) and SOX4 (Sandbothe et al., 2017), which suggested miR-449a as a tumor suppressor. It seems that miR-449a has

* Corresponding author at: Department of Hepatobiliary Surgery, Jining No.1 People's Hospital, No.6 Jiankang Road, Jining 272000, Shandong, China.
E-mail address: mindejin123@sina.com (D. Min).
[1] These authors contributed equally to this work.

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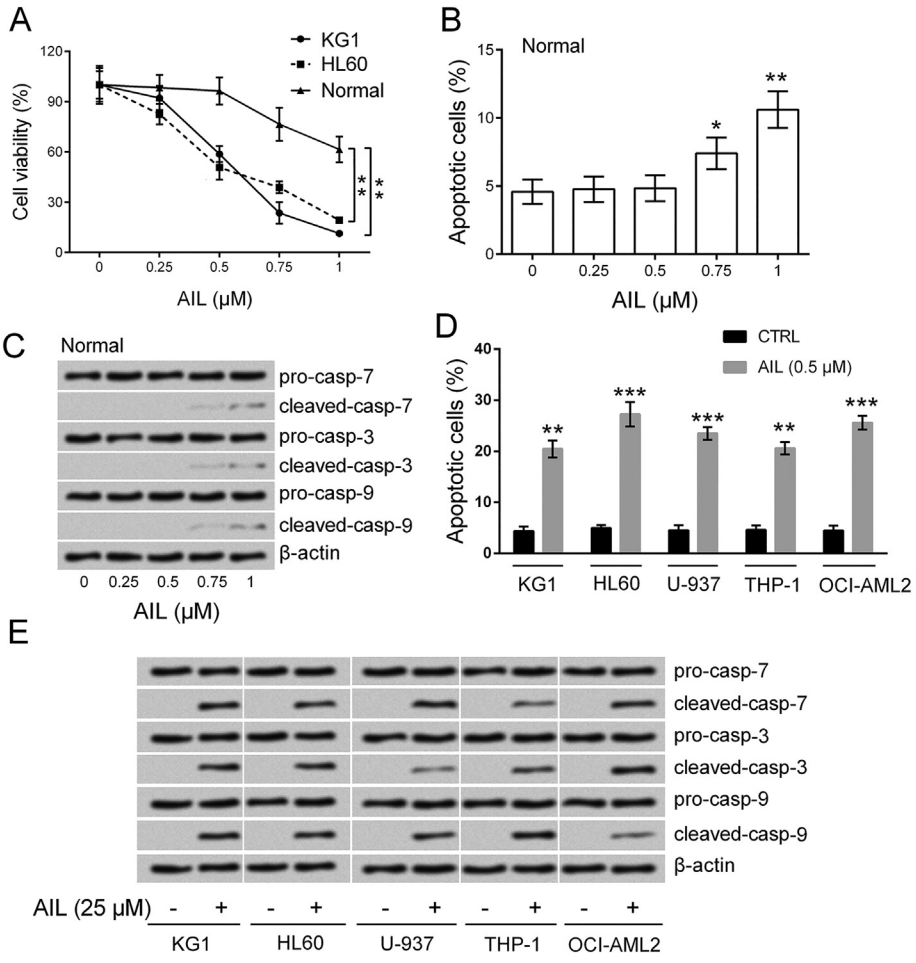

Fig. 1. Effect of ailanthone (AIL) on acute myeloid leukemia (AML) cells growth. (A) Viability of AML cell lines (KG1 and HL60) and normal myeloid progenitor cells was detected by CCK-8 assay, after treating with various doses of AIL for 48 h. (B) Apoptotic cells rate and (C) expression changes of caspases in normal myeloid progenitor cells were detected by flow cytometry and Western blot respectively, after treating with various doses of AIL for 48 h. (D) Apoptotic cells rate and (E) expression changes of caspases in five AML cell lines (KG1, HL60, U-937, THP-1 and OCI-AML2) were detected by flow cytometry and Western blot respectively, after treating with 0.5 μM of AIL for 48 h. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ when compared to the control (CTRL) group.

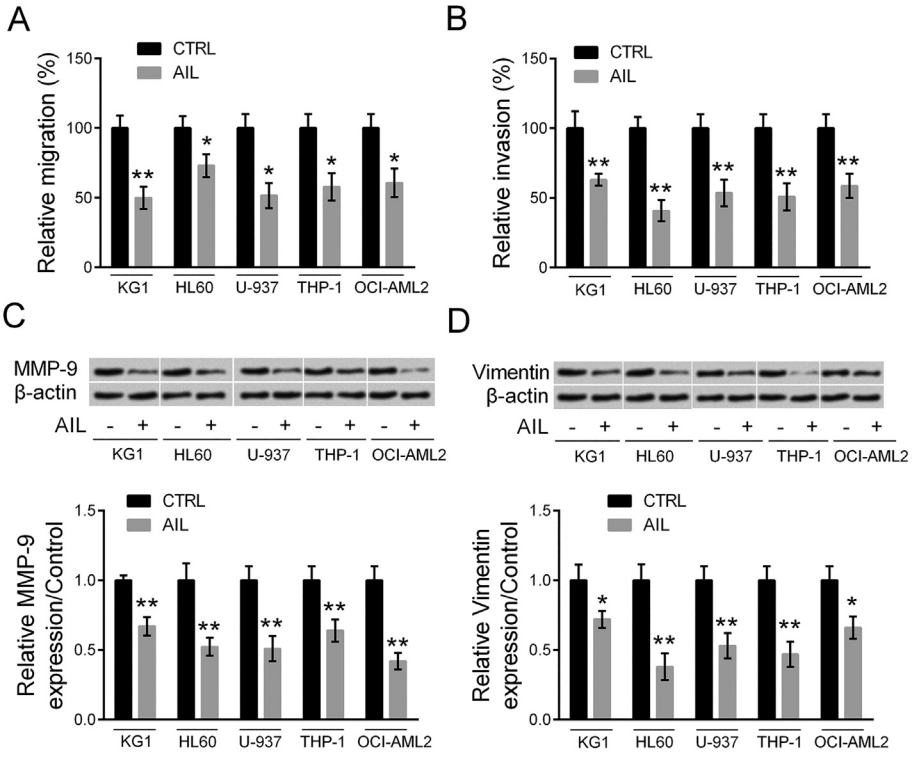

Fig. 2. Effect of ailanthone (AIL) on acute myeloid leukemia (AML) cells migration and invasion. AML cell lines (KG1, HL60, U-937, THP-1 and OCI-AML2) were treated by 0.5 μM of AIL for 48 h. After which, (A) the relative migration, and (B) relative invasion were determined by Transwell assay; protein levels of (C) MMP-9 and (D) Vimentin were detected by Western blot. $^{*}P < .05$ and $^{**}P < .01$ when compared to the control (CTRL) group.

potentials for use as therapeutic drug or drug target for treating AML.

Notch and PI3K/AKT signaling pathways have been reported as two downstream signaling of miR-449a (Cheng et al., 2018; Liu et al., 2018). In hematopoietic system, Notch is pivotal in determining the fate of stem, progenitor and mature cells (Lobry et al., 2014). The activation of Notch signaling is associated with a poor prognosis of AML (Xu et al., 2011), and targeting Nocth1 has been considered as a novel strategy for AML treatment (Ono et al., 2016). Meanwhile, sustained activation of PI3K/AKT signaling in different types of leukemia has been identified (Kubota et al., 2004; Samuels et al., 2004; Xu et al., 2003). PI3K/AKT signaling is believed to promote AML cells proliferation, migration and invasion (Kubota et al., 2004; Xu et al., 2003).

Ailanthone (AIL) is a quassinoid isolated from traditional Chinese herbal medicine *Ailanthus altissima* (Zhuo et al., 2015). AIL has been reported as an active compound possesses a wide range of pharmacological activities, such as antiplasmodial (Okunade et al., 2003), anti-inflammation, anti-HIV, and anti-ulcer activities (Kundu and Laskar, 2010). Moreover, the anti-tumor activities of AIL has been found in various types of cancers, including breast cancer (Wang et al., 2018), vestibular schwannoma (Yang et al., 2018), non-small cell lung cancer (Ni et al., 2017), gastric cancer (Chen et al., 2017), prostate cancer (Peng et al., 2017), and hepatocellular carcinoma (Zhuo et al., 2015). *In vitro* and *in vivo* studies revealed that two AIL derivatives showed cytotoxic activity towards P388 leukemia mice and L1210 cultured cells, and the cytotoxic activity was close to that of bruceantin and vincristine (Kato et al., 1988).

This study aimed to reveal the anti-tumor activities of AIL towards AML cell lines *in vitro*. Besides, whether AIL inhibited AML cells through regulating miR-449a and its downstream signaling was studied. This study may help to enlarge our understanding of AIL.

2. Materials and methods

2.1. Cell culture

The following cell lines were purchased from ATCC (Manassas, VA): AML cell line KG1 derived from a 59-year-old male patient (Catalog No.: CCL-246), acute promyelocytic leukemia cell line HL60 derived from a 36-year-old female patient (Catalog No.: CCL-240), acute monocytic leukemia cell lines U-937 (Catalog No.: CRL-1593.2) and THP-1 (Catalog No.: TIB-202) derived from a 37-year-old male patient and a 1-year-infant male patient respectively. OCI-AML2, a cell line derived from a 65-year-old male patient with AML, was purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). KG1 and HL60 cells were routinely cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY). U-937 cells were cultured in RPMI-1640 medium (Invitrogen) with 10% FBS. THP-1 cells were cultured the same as U-937 cells, except adding 0.05 mM 2-mercaptoethanol (Gibco) into culture medium. OCI-AML2 cells were cultured in α-MEM medium (Gibco) supplemented with 20% FBS. All cells were maintained at 37 °C in a humidified 5% CO_2 incubator.

Myeloid progenitor cells were isolated from the femurs of C57/BL6 mice as described previously (Shah et al., 2011). The cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS. Murine recombinant GM-CSF, IL-3 and SCF all from Sangon Biotech (Shanghai, China) were added into culture medium with concentrations of 10 ng/ml, 10 ng/ml and 100 ng/ml, respectively.

AIL (HPLC ≥98%) was purchased from Baomanbio (Shanghai, China). AIL was dissolved in culture medium, and the cells were treated by 0.25, 0.5, 0.75 or 1 μM of AIL for 48 h.

2.2. Cell viability assessment

Cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After 12 h of incubation at 37 °C for adhesion, cells were treated by various doses of AIL for 48 h. The culture medium was removed, the cells were washed twice with phosphate buffer saline (PBS), and then 10 μl CCK-8 solution (Dojindo Molecular Technologies, Kyushu, Japan) was added. The plates were incubated at 37 °C for 1 h, and then the absorbance of each well was determined by a Microplate Reader (Bio-Rad, Hercules, CA) at 450 nm.

2.3. Apoptosis assay

Cells were seeded in 6-well plates at a density of 5×10^5 cells/well. After 12 h of incubation at 37 °C for adhesion, cells were treated by AIL for 48 h. Then the cell apoptosis was analyzed by using Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). Briefly, the cells were collected by trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO), and were resuspended in 200 μl Binding Buffer containing 5 μl Annexin V-FITC. After 30 min of incubation at room temperature in a dark room, 10 μl PI and 300 μl PBS was added, and the samples were analyzed by the FACS can (Beckman Coulter, Fullerton, CA). The rate of apoptotic cells (Annexin V-FITC-positive and PI-negative) was calculated by FlowJo software (TreeStar, Ashland, OR).

2.4. Transwell assay

A 24-well Boyden chamber with 8.0-μm pore filter (Costar-Corning, NY) was used to assess cell migration. The matrigel-coated Boyden chamber was used in invasion assessment. In brief, cells were seeded in the upper side of the chamber with non-serum culture medium. The lower chamber was filled with 600 μl complete culture medium. The cells were treated by 0.5 μM AIL for 48 h, then the traversed cells (the cells in the lower chamber) was stained with crystal violet and counted microscopically. Five fields were randomly selected for calculation of relative migration or invasion.

2.5. miRNA transfection

miR-449a inhibitor (5'-ACCAGCUAACAAUACACUGCCA-3′) and its scrambled control (NC, 5'-UCACAACCUCCUAGAAAGAGUAGA-3′) were synthesized by GenePharma Co. (Shanghai, China). Cells in 6-well plates were transfected with 200 nM miR-449a inhibitor or NC for 48 h under non-serum and non-antibiotic conditions. Lipofectamine 3000 reagent (Invitrogen) was used in transfection following the manufacturer's protocol.

2.6. qRT-PCR

Total miRNAs in cell were extracted by miRNeasy Mini Kit (Qiagen, Shenzhen, China). Reverse transcription was performed with the specific reverse transcription primer for hsa-miR-449a (5'-GTCGTATCCA GTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACCAGC-3′) under the catalysis of PrimeScript Reverse Transcriptase (Takara, Dalian, China). qRT-PCR was performed by using Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) with the specific primer for hsa-miR-449a (Forward: 5'-GGGTGGCAGTGTATTGTTA-3′, and Reverse: 5'-CAGTGCGTGTCGTGG AGT-3′). Human U6 was used as an internal control. Data were analyzed with the $2^{-\Delta\Delta Ct}$ method.

2.7. Western blot

The protein in cell was extracted by RIPA lysis buffer (Beyotime), and the purity and concentration of protein in the extracts was measured by BCA™ Protein Assay Kit (Pierce, Appleton, WI). The protein was separated by the SDS-PAGE, and was transferred onto PVDF membranes (Millipore, MA). The protein loaded membranes were incubated with 5% non-fat milk for 1 h at room temperature, following by incubation with the primary antibodies at 4 °C overnight. Anti-caspase-

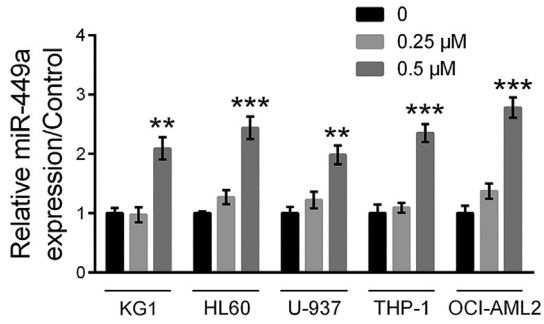

Fig. 3. Effect of ailanthone (AIL) on miR-449a expression. qRT-PCR analysis of miR-449a expression in five AML cell lines (KG1, HL60, U-937, THP-1 and OCI-AML2), after treated by 0.5 μM of AIL for 48 h. $^{**}P < .01$ and $^{***}P < .001$ when compared to the 0 μM group.

7 (ab32522), anti-caspase-3 (ab208161), anti-caspase-9 (ab25758), anti-MMP-9 (ab73734), anti-Vimentin (ab137321), anti-Notch1 (ab65297), anti-Notch2 (ab8926), anti-t-PI3K (ab180967), anti-p-PI3K (ab182651), anti-t-AKT (ab227100), anti-p-AKT (ab81283), and anti-β-actin (ab8227) antibodies were all purchased from Abcam (Cambridge, MA). The membranes were then incubated with goat anti-rabbit (ab7090) and goat anti-mouse (ab97040, Abcam) IgG for 1 h at room temperature, and the bands were developed by the Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA), and the intensity was quantified by ImageJ 1.49 software (National Institutes of Health, Bethesda, MD).

2.8. Statistics

Unless the data from Western blot analysis, all other data were presented as mean ± SD from three independent experiments. Statistical analyses were done by SPSS 20 software (IBM, New York, US). The statistical difference between groups was analyzed by ANOVA. $P < .05$ indicates significant differences.

3. Results

3.1. AIL induces apoptosis of AML cells

To start with, the viability of an AML cell line KG1 and an acute promyelocytic leukemia cell line HL60 was measured following the stimulation of various doses of AIL. After 48 h of stimulation, the viability of these two cell lines was found to be inhibited significantly as compared to normal myeloid progenitor cells ($P < .01$, Fig. 1A). Besides this, high doses of AIL possessed a stronger inhibitory effects on cell viability as compared to low doses. And the viability of normal myeloid progenitor cells was also declined by AIL with doses higher than 0.5 μM. Then, whether AIL-induced viability lose in myeloid progenitor cells through inducing apoptosis was tested. Results in Fig. 1B showed that, the percentage of apoptotic cells was significantly increased in 0.75 and 1 μM AIL groups ($P < .05$ and $P < .01$). But, no such increases were observed in AIL groups treated with doses lower than 0.75 μM. In line with this phenomenon, the caspase-7, −3 and − 9 were clearly cleaved when the doses of AIL increased to 0.75 μM (Fig. 1C). Considering the IC50 values of AIL towards KG1 and HL60 cells are 0.58 and 0.57 μM respectively, and 0.5 μM of AIL could not impact normal myeloid progenitor cells viability and apoptosis, 0.5 μM was selected as an AIL-stimulating condition for the following experiments. Next, the effects of 0.5 μM AIL on the apoptosis of AML cell lines were investigated. KG1, HL60, OCI-AML2, as well as two acute monocytic leukemia cell lines U-937 and THP-1 were utilized in this process. As shown in Fig. 1D, the apoptotic cell rate was significantly increased by AIL treatment in all these cell lines ($P < .01$ or $P < .001$). Western blot results shown that, treating these cell lines with AIL remarkably increased the cleavage of caspases, including caspase-7, −3 and − 9 (Fig. 1E). These data collectively suggested the pro-apoptotic effects of AIL on AML cells, and AML cells were more sensitive to AIL as compared to normal myeloid progenitor cells.

3.2. AIL represses the migration and invasion of AML cells

Transwell assay was conducted to assess the migratory and invasive capacity of AML cells. As shown in Fig. 2A-2B, the relative migration and invasion were significantly reduced in AIL group, as compared to

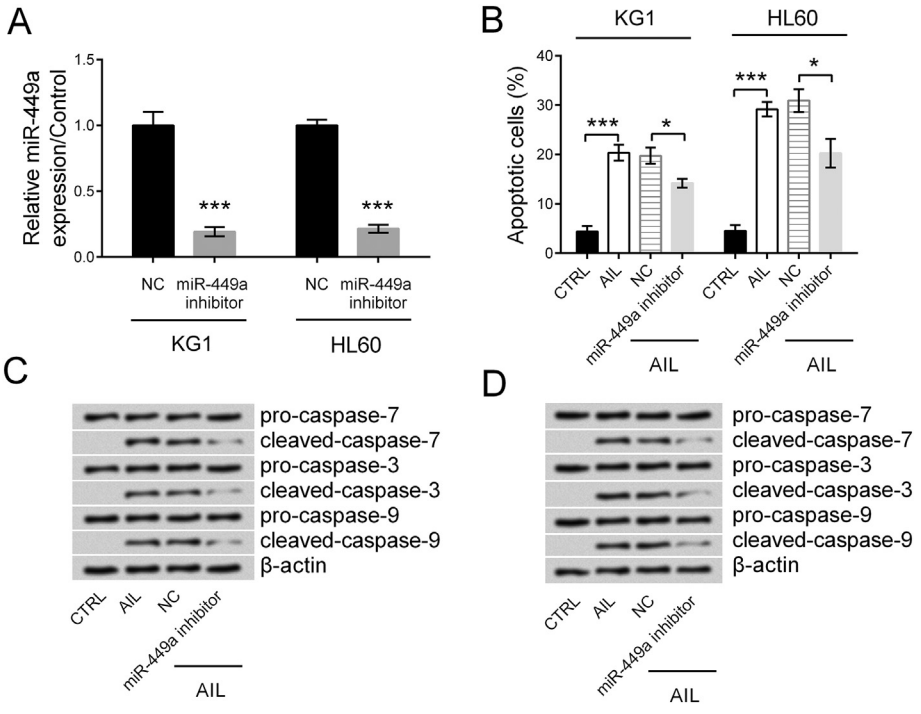

Fig. 4. Effect of miR-449a silence on ailanthone (AIL)-induced growth inhibition in KG1 and HL60 cells. (A) qRT-PCR analysis of miR-449a expression in KG1 and HL60 cells, after transfected with miR-449a inhibitor or its negative control (NC). $^{***}P < .001$ when compared to the NC group. (B) The rate of apoptotic cells, and (C-D) expression changes of caspases were detected by flow cytometry and Western blot respectively, after cells were transfected with miR-449a inhibitor or NC, and then treated by 0.5 μM of AIL for 48 h. $^{*}P < .05$ and $^{***}P < .001$ when compared to the indicated group.

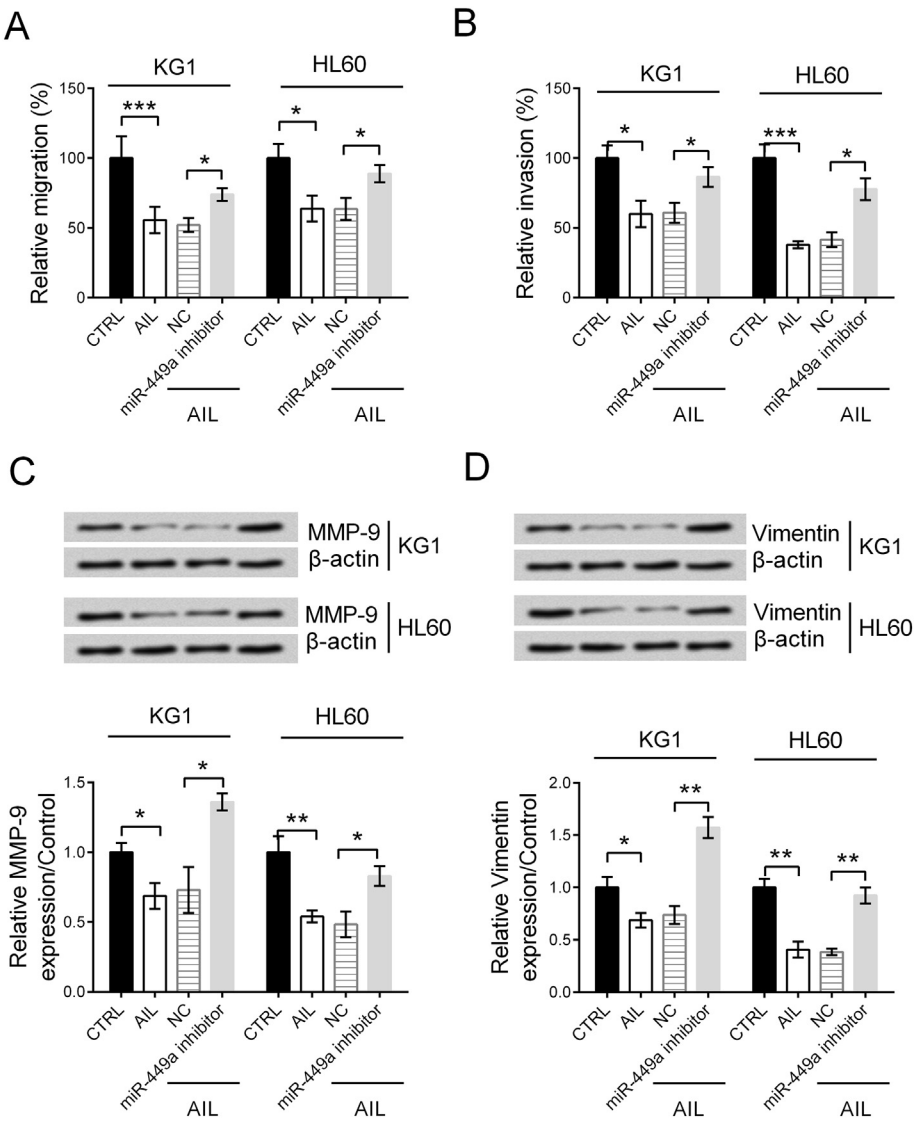

Fig. 5. Effect of miR-449a silence on ailanthone (AIL)-induced migration and invasion impairment in KG1 and HL60 cells. KG1 and HL60 cells were transfected with miR-449a inhibitor or NC, and then treated by 0.5 μM of AIL for 48 h. After which, (A) the relative migration, and (B) relative invasion were determined by Transwell assay; and protein levels of (C) MMP-9 and (D) Vimentin were detected by Western blot. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ when compared to the indicated group.

the control group ($P < .05$ or $P < .01$). By performing Western blotting, we found that the protein levels of MMP-9 and Vimentin were both significantly down-regulated in AIL group, as compared to the control group ($P < .05$ or $P < .01$, Fig. 2C-2D). The results hint us that, AIL was able to repress the migratory and invasive capacities of AML cells.

3.3. AIL up-regulates the expression of miR-449a

Next, the regulatory effects of AIL on the expression of miR-449a were studied. To this end, AML cells were subjected to 0.25 or 0.5 μM AIL for 48 h, after which the expression changes of miR-449a were detected by qRT-PCR. Data in Fig. 3 showed that 0.5 μM, rather than 0.25 μM AIL significantly up-regulated miR-449a expression ($P < .01$ or $P < .001$).

3.4. AIL induces apoptosis of AML cells by up-regulation of miR-449a

To explore whether miR-449a was implicated in AIL-induced apoptosis in AML cells, miR-449a inhibitor was transfected into KG1 and HL60 cells. qRT-PCR data in Fig. 4A showed that miR-449a expression was significantly reduced by inhibitor transfection as compared to the NC transfection ($P < .001$). Fig. 4B showed that, AIL-induced the increases of apoptosis rate were significantly ameliorated by miR-449a inhibitor ($P < .05$). Also, Fig. 4C-D showed that, AIL-induced the cleavage of caspase-7, -3 and -9 was remarkably ameliorated by miR-449a inhibitor.

3.5. AIL represses the migration and invasion of AML cells by up-regulation of miR-449a

Next, the importance of miR-449a in AIL-modulated migration and invasion in AML cells was investigated. Fig. 5A-B showed that, the repressed migration and invasion made by AIL were attenuated by miR-449a inhibitor ($P < .05$). Western blot results indicated that AIL-induced down-regulations of MMP-9 and Vimentin were reversed by miR-449a inhibitor ($P < .05$ or $P < .01$, Fig. 5C-D).

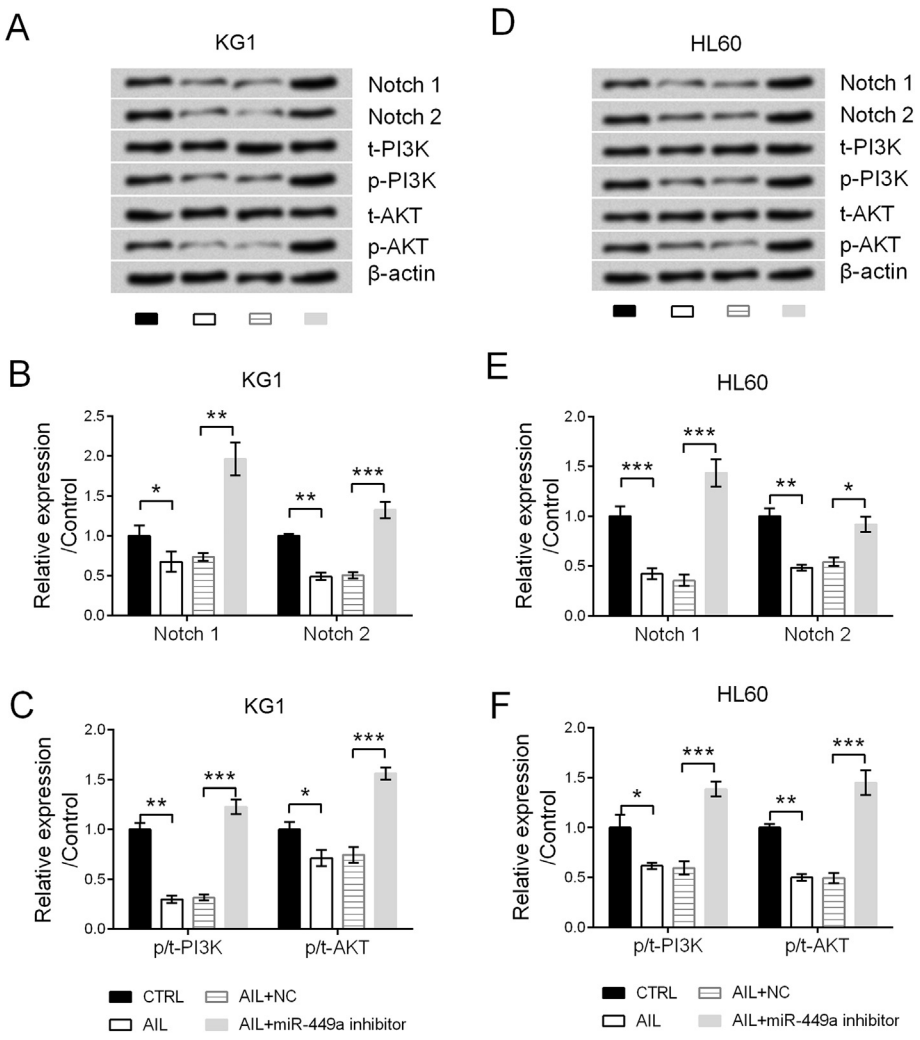

Fig. 6. Effect of miR-449a silence and ailanthone (AIL) treatment on the activation of Notch and PI3K/AKT signaling pathways. KG1 and HL60 cells were transfected with miR-449a inhibitor or NC, and then treated by 0.5 μM of AIL for 48 h. After which, the protein expression changes of Notch1, Notch2, PI3K, and AKT in (A-C) KG1 and (D-F) HL60 cells were measured by Western blot. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ when compared to the indicated group.

3.6. AIL inhibits Notch and PI3K/AKT signaling pathways via up-regulation of miR-449a

Finally, we focused on Notch and PI3K/AKT signaling pathways, to explore the underlying mechanisms of which AIL impacted AML cells apoptosis, migration and invasion. As shown in Fig. 6A-F, protein levels of Notch1, Notch2, p-PI3K, and p-AKT were much lower in AIL group, as compared to the control group ($P < .05$, $P < .01$ or $P < .001$). Meanwhile, when compared to the AIL + NC group, the protein levels of Notch1, Notch2, p-PI3K, and p-AKT were significantly higher in the AIL + miR-449a inhibitor group ($P < .05$ or $P < .001$).

4. Discussion

In this study, we detected the anti-tumor activities of AIL in five AML cell lines, and found that 0.5 μM AIL induced cell apoptosis, and reduced migration and invasion in AML cells. We additionally found that, miR-449a was highly expressed in response to the treatment of 0.5 μM AIL. The anti-tumor activities of AIL in KG1 and HL60 cells were attenuated by miR-449a silence. Furthermore, the blockage of Notch and PI3K/AKT signaling pathways induced by AIL was reversed by miR-449a silence.

AML is a common cancer characterized by the rapid proliferation of immature myeloid progenitors (Boffo et al., 2018). Apoptosis is a kind of mechanism of programmed cell death, and has been recommended and privileged as a strategy for clearing tumor cells (He et al., 2017). Herein, we demonstrated that AIL significantly induced apoptosis of five AML cell lines (KG1, HL60, U-937, THP-1 and OCI-AML2), indicating AIL might be a promising agent for AML treatment by controlling apoptosis. Besides, our data suggested that AIL led to apoptosis *via* intrinsic pathway, as the activation of caspase-9, and the following activation of caspase-3 and -7.

By performing CCK-8 assay, the viability of normal myeloid progenitor cells was also found to be declined by AIL with doses higher than 0.5 μM. But, AIL with concentration of 0.5 μM did not significantly impact the viability or apoptosis of myeloid progenitor cells. Thus, it seems that AML cells were more sensitive to AIL than normal myeloid progenitor cells. The cancer-specific pro-apoptotic effect of AIL enables AIL in inhibiting AML cells without inducing excessive death to normal cells.

Migration and invasion are two essential processes towards the progression of cancer. MMPs are critical regulators in tumor metastasis that contribute to the degradation of extracellular matrix (ECM) components and allow cells to traverse the protein fibers of the ECM to reach distant target sites (Nalla et al., 2010). It has been reported that endogenous or exogenous binding of MMP-9 inhibited B-cell chronic lymphocytic leukemia cells migration (Redondo-Munoz et al., 2008). Vimentin is a cytoskeletal protein that belongs to the family of

intermediate filaments (Lazarides, 1982). Vimentin promotes cell migration which is a co-ordinated process involving the activity of the migration effector PI3K (Bouamrani et al., 2010). Our current study found that AIL repressed AML cells migration and invasion. In addition, the results from Western blot suggested that AIL inhibited AML cells migration and invasion possibly *via* down-regulation of MMP-9 and Vimentin.

With better understanding of AML, more and more recent evidence show that miRNAs hold key regulatory functions in AML (Gerloff et al., 2015; Whitman et al., 2010). In accordance with this, various miRNAs have been shown to impact treatment efficiency towards AML therapy (Zebisch et al., 2016). Herein, we found that miR-449a, a widely reported tumor suppressive miRNA (Li et al., 2015; Sandbothe et al., 2017), was significantly up-regulated in response to AIL treatment in AML cells. As previous data have suggested that miR-449a can induce apoptosis, cell cycle arrest, and metastasis impairment in several types of cancer cells (Chen et al., 2015; Wu et al., 2018), we inferred that AIL-related cell death might be associated with the elevated expression of miR-449a. Our further experiments clearly showed that, the anti-tumor activities of AIL were attenuated by miR-449a silence. These data indicated that the anti-AML functions of AIL might be related to the up-regulation of miR-449a induced by AIL treatment.

In order to further reveal the underlying mechanisms of which AIL inhibited AML cells, the downstream signaling of miR-449a was studied. Our group found that AIL significantly inhibited the activation of Notch and PI3K/AKT signaling pathways. Besides this, the inhibitory effects of AIL on the activation of these two signaling were reversed when miR-449a was silenced. These findings collectively suggested that, AIL conferred its anti-tumor function possibly through up-regulating miR-449a, and thereby deactivating Notch and PI3K/AKT signaling pathways. Further *in vivo* investigations are required to validate the cross-talk among AIL, miR-449a, and Notch and PI3K/AKT signaling pathways.

To conclude, this study showed that AIL restrained AML cells growth, migration and invasion. The anti-AML functions of AIL were through up-regulation of miR-449a, and deactivation of Notch and PI3K/AKT signaling pathways.

Conflict of interest

Authors declare that there is no conflict of interests.

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