RETRACTED ARTICLE: The anti-osteosarcoma property of ailanthone through regulation of miR-126/VEGF-A axis

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\begin{abstract}
\textbf{Background:} Despite the characters of the resistance of tumour of ailanthone (AIL) were found in various tumour cells, its effect on osteosarcoma is still unclear. Herein, we attempted to see the effects of AIL on an osteosarcoma cell line MG63.

\textbf{Methods:} MG63 cells were treated by AIL, following which CCK-8 assay, BrdU assay, Transwell assay and Western blot were utilized to detect cell proliferation, migration, invasion and apoptosis. miR-126 expression in osteosarcoma tissues and cell lines was measured by qRT-PCR. Further, the target of miR-126 and the downstream signalling for AIL were studied.

\textbf{Results:} Treating MG63 cells with 1.5 \textmu M AIL for 24 h significantly suppressed proliferation, migration, invasion and induced apoptosis. Meanwhile, AIL inhibited PI3K/AKT pathway and up-regulated miR-126 expression. miR-126 of osteosarcoma tissues and cell lines was low expressed, as relative to paracancerous tissues and normal osteoblast. The anti-tumour effects of AIL were attenuated by miR-126 silencing. Further, VEGF-A was a target of miR-126.

\textbf{Conclusions:} This study demonstrated that AIL was effective in inhibiting MG63 cells growth, migration and invasion. The anti-tumour properties may be via up-regulating miR-126 and thereby degradation of VEGF-A.
\end{abstract}

\section*{Introduction}

Osteosarcoma is the fairly commonplace primary bone malignancy along with locally aggressive growth and early metastatic. At present, effective chemotherapeutic agents combined with surgery is the main therapeutic method for osteosarcoma [1]. However, the cross-resistance to anticancer agents is an intractable problem in the treatment of this cancer [2]. Besides, patients presenting with metastatic and recurrent osteosarcoma have a poor prognosis, with less than 20% surviving at 5 years [3,4]. Therefore, finding new therapeutic strategies is likely to shed light on the treatment of osteosarcoma.

\textit{Ailanthus altissima} is a traditional Chinese medicinal plant for relieving fever, pain and bleeding. In recent decades, a great deal of scientific researches have indicated that \textit{Ailanthus altissima} has potent anti-inflammatory, anti-malarial, anti-HIV and anti-microbial properties [5,6]. Ailanthone (AIL) is a glass of quassinoid which acts as one of the main compounds of \textit{Ailanthus altissima}. Multiple studies demonstrated that, AIL exhibits excellent anti-tumour capacities. For the selected examples, AIL has been reported to be effective in inhibiting leukaemia [7], lung cancer [8], breast cancer [9], gastric cancer [10] and so forth. However, whether AIL also exerts anti-tumour property towards osteosarcoma is still unclear.

\textbf{microRNAs (miRNAs) are a kind of sing-stranded non-coding RNAs with lengths of approximately 18–25 bp. Through complementary binding to the 3'UTR of the target gene, miRNAs can degrade or inhibit these genes and thus involve in the control of variety cell biological processes [11]. Countless studies demonstrated that the unusual expression of miRNAs was closely related to osteosarcoma growth, shift, prognosis and other biological behaviours [12–15]. MiR-126 is one of such miRNAs. Circulating miR-126 expression in tissue has potential significance to predict the outcome of osteosarcoma [16,17]. Besides that, \textit{in vitro} data demonstrated that miR-126 functioned as a tumour-stressor in the growth, invasion and migration of osteosarcoma cells [18,19]. These previous studies suggested the pivotal effect of miR-126 on the onset and progression of osteosarcoma. This article desired to release the effect of AIL towards human osteosarcoma cells. Besides, the regulatory role of AIL in miR-126 expression was studied to better understand which molecular AIL exerted its function. This study will provide fundamental data for the improvement of newfangled and effective strategies for osteosarcoma treatment.

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Materials and methods

Patient samples

Osteosarcoma tissues and the paracancerous tissues were collected from 20 patients with osteosarcoma during tumour resection. All patients were diagnosed with osteosarcoma by using X-ray combination with MRI and surgical biopsy. No other therapies were used in these patients before resection. Tumour resection was performed in China-Japan Union Hospital of Jilin University during November 2018 and January 2019. This research was appreciated by the Ethics Committee of China-Japan Union Hospital of Jilin University and written informed consent was obtained from each individual. Tissues were gained and washed twice by phosphate buffer saline (PBS) and contained at −80°C until use.

Cell lines

MG63, U2OS, HOS, Saos-2 and hFOB1.19 were all purchased from ATCC (Manassas, VA). MG63 and HOS cells were cultured in Eagle’s minimum essential medium (ATCC), and U2OS and Saos-2 cells were cultured in the modified McCoy’s 5a Medium (ATCC). hFOB1.19 cells were cultured in Ham’s F12 medium (Sigma-Aldrich, St. Louis, MO). To make complete culture medium, foetal bovine serum was set to the F12 medium (Sigma-Aldrich, St. Louis, MO). AIL was diluted in culture medium, and various concentrations (0.5–2 μM) of AIL were collected to treat cells for 24 h.

Cell transfection

miR-126, mimic, inhibitor and the negative control (NC mimic and NC inhibitor) were from GenePharma (Shanghai, China). MG63 cells were transfected with the 25 nM oligonucleotides by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The final concentration of miR-126 mimic, miR-126 inhibitor, NC mimic and NC inhibitor was 50 nM, 200 nM, 100 nM and 2 μg/mL, respectively. The time transfection lasted for 48 h.

CCK-8 assay

MG63 cells in 96-well plates (5000 cells/well) were treated as indicated. The viability of MG63 cells was measured by adding 10 μL CCK-8 (Dojindo Molecular Technologies, Kyushu, Japan). Absorbance was obtained by a Microplate Reader (Bio-Rad, Hercules, CA) at a wavelength of 450 nm.

BrdU assay

MG63 cells seeded in a 35 mm culture flask (1.5 × 10^5 cells/mL) were treated as indicated. 1 mg/mL BrdU (Sigma-Aldrich, St. Louis, MO) was then added and cultured for 40 min at 37°C. After fixing with methanol, anti-BrdU antibody (Invitrogen, Carlsbad, CA) with dilution of 1:50 was added. At least 1000 cells in each sample were calculated under an inverted fluorescence microscope IX53 (Olympus, Tokyo, Japan).

Transwell assay

MG63 cells migration and invasion were tested by using a 24-well transwell system (Costar, Corning, NY). The pre-treated MG63 cells were suspended in serum free culture medium and placed into the upper layer of the system. The lower layer was placed with the complete culture medium. Twenty-four hour later, the traversed cells were coloured by crystal violet (Beyotime, Shanghai, China) and calculated. Before invasion assay, the insert of the chamber was treated with Matrigel (Millipore, Bedford, MA).

Apoptosis assay

MG63 cells in six-well plates (1 × 10^5 cells/well) were treated as indicated. The apoptosis was tested by using ANNEXIN V-FITC/PI Apoptosis Detection Kit (Solarbio, Shanghai, China). Data were analysed by FlowJo software (Treestar, San Carlos, CA).

Western blot

Proteins in MG63 cells following the indicated treatment were isolated by RIPA buffer (Beyotime, Shanghai, China). Immunoblotting was performed by using the following antibodies: CyclinD1 (#2978), Bax (#5023), Bcl-2 (#4223), cleaved-PARP (#9548), cleaved-Caspase-3 (#9664), PTEN (#9559), PI3K (#4257), p-PI3K (#4228), AKT (#4685), p-AKT (#4060), β-actin (#8457, Cell Signaling Technology, Danvers, MA) and VEGF-A (ab46154, Abcam, Cambridge, MA). After incubation with the secondary antibodies, the positive bands were detected by using BeyoECL Moon (Beyotime, Shanghai, China) and intensity was quantified by using Image Lab™ Software (Bio-Rad, Hercules, CA).

Luciferase reporter assay

The 3′UTR regain of VEGF-A was amplified by PCR and placed into pGL3 reporter vector (Promega, Madison, WI) to form a reporter plasmid VEGF-wt. The predicted binding site in the 3′UTR of VEGF-A which can bind with miR-126 was mutated and inserted to form a control plasmid VEGF-mut. The plasmids were transfected together with miR-126 mimic or NC.
mimic into cell. Fluorescence intensity was measured by Dual-Glo Luciferase assay kit (Promega, Madison, WI).

**Statistics**

Data are performed as mean ± SD. Statistical difference was tested by SPSS 19.0 software (Chicago, IL) and presented as asterisk (*). Student’s t-test and ANOVA followed by Duncan’s procedure were operated to make difference between two or more groups. Statistical difference was placed at \( p < 0.05 \).

**Results**

**MG63 cells were inhibited by treating with AIL**

MG63 cells suffered from different concentrations of AIL for 24 h. As displayed in Figure 1(A), MG63 cell viability was dramatically declined by AIL treatment in a dose-dependent way \( (p < 0.01 \text{ or } p < 0.001) \). It can be seen that, the viability was reduced to about a half when the concentration of AIL was 1.5 \( \mu M \). Hence, 1.5 \( \mu M \) AIL was used for the following experiments. Figure 1(B) shows that BrdU-positive cell rate was also declined when MG63 cells were treated by AIL \( (p < 0.01) \), indicating the anti-proliferating capacity of AIL. This result was coupled with the down-regulated expression of CyclinD1 protein \( (p < 0.001, \text{ Figure 1(C,D)}) \). Besides that, MG63 cells migration and invasion were both repressed by AIL \( (p < 0.05 \text{ and } p < 0.01, \text{ Figure 1(E,F)}) \). Of contrast, apoptotic cell rate was dramatically increased by treating with AIL \( (p < 0.01, \text{ Figure 1(G)}) \). Meanwhile, the expression of Bax was increased, Bcl-2 was declined and PARP and Caspase-3 were cleaved when AIL was applied in MG63 cells \( (p < 0.01 \text{ or } p < 0.001, \text{ Figure 1(H,I)}) \). All the above results illustrated that AIL inhibited proliferation and migration, as well as promoted apoptosis in MG63 cells.

**AIL inhibited PI3K/AKT pathway**

PI3K/AKT has been thought to be one of the most critical oncogenic pathways in human cancer, including osteosarcoma [20]. Thus, we next explored whether AIL can suppress PI3K/AKT pathway. Results in Figure 2(A,B) show that PTEN expression was increased; however, PI3K and AKT phosphorylation levels were declined in MG63 cells by treating with AIL \( (p < 0.05 \text{ or } p < 0.01) \). This described that the activation of PI3K/AKT pathway was suppressed by AIL in MG63 cells.

**AIL up-regulated miR-126 expression, which could directly bind with VEGF-A**

Next, the downstream effectors of which AIL exerted its function were explored. As illustrated in Figure 3(A), miR-126 expression level was up-regulated by AIL \( (p < 0.01) \). However, the expression of VEGF-A was decreased by AIL \( (p < 0.01, \text{ Figure 3(B,C)}) \). The result indicated these two factors are downstream effectors of AIL. Further study found that VEGF-A was a target of miR-126 as the fluorescence intensity was clearly declined by transfection with VEGF-A-wt and miR-126 mimic \( (p < 0.05, \text{ Figure 3(D)}) \). We drew such a conclusion from the above results that AIL up-regulated miR-126 expression, which directly bond with VEGF-A.

**miR-126 was down-regulated in osteosarcoma**

Since miR-126 can be up-regulated by AIL, we studied the level changes of miR-126 in order to see its role in the progression of osteosarcoma. As compared to paracancerous non-tumour tissues, miR-126 was low expressed in osteosarcoma tissues \( (p < 0.01, \text{ Figure 4(A)}) \). Consistently, its expression was lower in osteosarcoma cell lines, like MG63, U2OS, HOS and Saos-2 as relative to normal osteoblast hFOB1.19 \( (p < 0.001 \text{ or } p < 0.001, \text{ Figure 4(B)}) \). Data showed that the level of miR-126 expression was decreased in osteosarcoma.

**AIL inhibited MG63 cells by up-regulating miR-126**

Finally, whether AIL inhibited MG63 cells via miR-126 was tested. For this purpose, miR-126 expression was repressed by inhibitor transfection. Data in Figure 5(A) revealed that, the inhibitor transfection significantly reduced miR-126 level \( (p < 0.01) \). More interestingly, the effects of AIL towards MG63 cells growth, migration and invasion were all relieved when miR-126 was inhibited. As compared to AIL plus NC inhibitor group, the BrdU-positive cell rate \( (p < 0.5, \text{ Figure 5(B)}) \), CyclinD1 expression \( (p < 0.05, \text{ Figure 5(C)}) \), migration \( (p < 0.05, \text{ Figure 5(D)}) \) and invasion \( (p < 0.05, \text{ Figure 5(E)}) \) were increased, while cell apoptosis \( (p < 0.05, \text{ Figure 5(F–H)}) \) was reduced in AIL plus miR-126 inhibitor group. We come to conclusion from above results that AIL possessed anti-osteosarcoma properties through the up-regulation of miR-126.

**Discussion**

Although tumour resection combined with adjuvant chemotherapy has been used to treat osteosarcoma, the prognosis is still dissatisfied because of the rapid proliferation of tumour cells and the distant metastasis [1]. Therefore, inhibition osteosarcoma growth and metastasis has become one of the main approaches for treating osteosarcoma. Herein, studies performed in vitro demonstrated that, AIL was capable of suppressing MG63 cells proliferation, migration, invasion and inducing apoptosis. The data suggested the anti-tumour property of AIL and this property may be associated with the inhibitory effects of AIL on PI3K/AKT pathway. Besides that, miR-126 was found to be a downstream effector of AIL, which was low expressed in osteosarcoma while could be up-regulated by treating AIL. Further, VEGF-A, which is a well-known factor in driving tumorigenesis [21], was found as a target of miR-126.

The anti-tumour characters of AIL have been previously released in other types of cancers, including lung [8], breast [9] and gastric cancers [10]. AIL was able to induce G0/G1 arrest and thus prevent excessive cell division in leukaemia HL-60 cells [6]. In addition, apoptosis of HL-60 cells and gastric cancer SGC-7901 cells was induced by treating AIL [6,10]. This study found that, AIL also has anti-growth impact on
osteosarcoma MG63 cells. The anti-proliferating and pro-apoptotic effects of AIL may be associated with the expression of cell-cycle related protein CyclinD1 and apoptosis-related proteins (Bax, Bcl-2, PARP and Caspase-3). On the other hand, AIL exhibited potent anti-migrating and anti-invasive properties in breast cancer MDA-MB-231 cells and several acute myeloid leukaemia cell lines [7,9]. In this study, the inhibitory effects of AIL towards these two aspects in MG63 cells were also observed. Further, AIL could inhibit PI3K/AKT pathway in MG63 cells, which suggested that AIL controlled MG63 cells growth, migration and invasion via this pathway. Similar findings were discovered in other types of cancer cells which were reported elsewhere [7,8,22,23].

Despite growing number of researches are interested in investigating the anti-tumour capacity of AIL, the molecular mechanisms of AIL are still unclear. Recent paper believed that AIL exerted its function possibly via regulating miRNAs, like miR-449a [7], miR-195 [8], miR-148a [9] and miR-21 [24]. This study for the first time manifested that miR-126 was another downstream effector of AIL. miR-126 was up-regulated by AIL, and the effects of AIL on MG63 cells growth,
migration and invasion were entirely relieved when miR-126 was repressed. Compared with normal tissues, miR-126 was consistently declined in osteosarcoma tissues [18]. This phenomenon was consistent with ours. Besides, the low expressed miR-126 has significance in the development of advanced disease and distant metastasis [18]. Altogether, it seems that AIL inhibited MG63 cells via up-regulating the tumour-suppressor miR-126.

VEGF-A was identified as an endothelial cell-specific mitogen that takes part in almost all aspects of vessel and lymphatic formation. Hence, VEGF-A has long been considered as a specificity factor in the angiogenesis of solid tumours [25,26]. However, the function of VEGF-A in cancer is not limited to angiogenesis [21]. VEGF-A also takes an important contribution for other aspects of cancer onset and progression, like tumour cell proliferation [27], migration [28] and invasion [29]. Herein, VEGF-A was found to be a target of miR-126. This finding partially explained the anti-tumour function of miR-126. Actually, the targeting relationship between miR-126 and VEGF-A has been reported in breast [26] and oesophageal cancers [30]. However, whether AIL inhibited osteosarcoma cells growth, migration and invasion via inhibiting VEGF-A still needs to be further explored.

Overall, this study demonstrated that AIL was effective in inhibiting MG63 cells growth, migration and invasion. The anti-tumour properties may be via up-regulating miR-126 and thereby degradation of VEGF-A.
Figure 5. AIL inhibited MG63 cells by up-regulating miR-126. (A) miR-126 expression in MG63 cells was measured by qRT-PCR, after transfection with miR-126 inhibitor or NC inhibitor. After transfection by miR-126 inhibitor or NC inhibitor for 48 h, the transfected cells were treated by 1.5 μM AIL for 24 h, and then (B) proliferation was measured by BrdU assay, (C) CyclinD1 expression was assessed by Western blot, (D, E) migration and invasion were tested by Transwell assay, (F) apoptosis rate was tested by flow cytometry and (G, H) apoptosis-related protein expression was assessed by Western blot. *, ** and *** stand for $p < .05$, $p < .01$ and $p < .001$, respectively.
Disclosure statement
No potential conflict of interest was reported by the authors.

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