Research Paper

Ailanthone Suppresses Liver Cancer Cell Malignant Behavior by Regulating microRNA-489-3p

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Guan *et al.*: Mechanism of Ailanthone in Liver Cancer

Previous literature has validated the clinical effectiveness of ailanthone in repressing the development of different human cancers. Therefore, this project aimed to investigate the role and possible mechanism of ailanthone in liver cancer. Huh-7 cells were treated with various concentrations of ailanthone (low-dose ailanthone, medium-dose ailanthone, high-dose ailanthone), or transfected with microRNA-negative control and microRNA-489-3p mimics. Furthermore, transfected Huh-7 cells were exposed with high-dose ailanthone. Cell counting kit-8, plate clone formation, scratch, and Transwell detected cell proliferation, clone formation, migration, and invasion. Besides, microRNA-489-3p, matrix metalloproteinase-2, and matrix metalloproteinase-9 contents were assessed using quantitative reverse transcription polymerase chain reaction or Western blot. After exposure to various doses of ailanthone, cell proliferation inhibition rate and microRNA-489-3p expression were increased in a dose-dependent manner, whereas cell clone formation, migration, invasive, and matrix metalloproteinase-2, matrix metalloproteinase-9 protein levels were decreased. Beyond that, microRNA-489-3p expression was reduced in liver cancer tissues; its upregulation might block Huh-7 cell growth. MicroRNA-489-3p downregulation might abrogate high-dose ailanthone-mediated cell malignant behaviors inhibition. Ailanthone treatment could hinder Huh-7 cell malignant behaviors through regulating microRNA-489-3p.

Key words: Liver cancer, ailanthone, microRNA-489-3p, cell proliferation, migration, invasion

The most frequent fatal malignancy, liver cancer has been recognized as a global health challenge, with an estimated 1.4 million cases diagnosed in 2040[1]. Substantial progress in treatment options, containing surgical resection, adjuvant or neoadjuvant chemotherapy, have recently acquired some benefits, but liver cancer is prone to metastasis and recurrence even after surgical resection[2,3]. Hence, it is particularly important to seek a novel diagnostic and therapeutic method for early diagnosis of this cancer and to improve the efficiency of treatment. Important role of Traditional Chinese Medicine (TCM) in mitigating the progression of liver cancer has been attracting attention[4,5]. Ailanthone (AIL) represents an important active compounds from famous TCM *Ailanthus altissima*[6]. Numerous studies have suggested that AIL possesses many biological activities, containing anti-inflammatory, anti-microbial, and anti-tumor activities[7]. It has been reported that AIL might exert significant natural anti-cancer activity through modulating different miRNAs and pathways in several human cancers[8,9]. In fact, AIL treatment has been verified to constrain Huh-7 cell growth[10]. However, research on its mechanism of action is still in need of supplementation and improving in liver cancer. At present, extensive laboratory work reveals that microRNAs (miRNAs) might modulate target gene expression at posttranscriptional levels, thereby controlling tumor progression[11]. Some research indicated miR-489-3p might function as a tumor suppressor in several human cancers[12,13]. Apart from that, miR-489 has been verified to repress human liver cancer cell proliferation[14]. Accordingly, we focused on whether AIL might affect Huh-7 cell malignant behavior progression

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via miR-489-3p.

MATERIALS AND METHODS

Reagents:

After signing written informed consent from all participants, 37 liver cancer tissues and matched paracancerous tissue were collected from February 2020 to July 2020. Meanwhile, all patients were diagnosed as liver cancer through pathological diagnosis, and these samples were immediately placed -80° for preservation. Among them, 20 male and 17 female aged between 50 y-68 y (mean=55.35±3.26). Besides, this project was approved *via* the Ethics Committee of our hospital.

AIL (purity >98 %) was provided by SHHEBIO (Shanghai, China). Human liver cancer cell line Huh-7 was acquired from ZQXZBIO (Shanghai, China). Meanwhile, Invitrogen (Carlsbad, California, USA) offered Trizol reagent, reverse transcription and fluorescent quantitative Polymerase Chain Reaction (PCR) reagents, and Lipofectamine™ 2000. RiboBio (Guangzhou, China) provided miR-Negative Control (NC), miR-489-3p mimics, anti-miR-NC, and anti-miR-489-3p. Cell Counting Kit-8 (CCK-8) reagent and Matrigel matrix gel were respectively purchase from Beyotime Biology (Shanghai, China) and Solarbio (Beijing, China). Corning Costar (Corning, United States of America (USA)) provided Transwell. Rabbit anti-human Matrix Metalloproteinase (MMP)-2, MMP-9 antibody, and secondary antibodies were provided by CST (Danvers, Massachusetts, USA).

Method:

Cell treatment and transfection: Based on previously described[15], 1×10^5 Huh-7 cells in Dulbecco's Modified Eagle Medium (DMEM) were exposed with AIL at different concentrations (0.4 µmol/l, 0.8 µmol/l and 1.6 µmol/l) for 24 h, marked respectively as AIL-L, AIL-M, and AIL-H. In parallel, control group was normal cultured Huh-7 cells. For cell transfection, according to Lipofectamine method, we overexpressed miR-489-3p *via* transfecting

miR-NC or miR-489-3p mimics into Huh-7 cells. Transfected Huh-7 cells were culturing with medium containing 1.6 µmol/l AIL for 24 h, generating AIL+anti-miR-NC group or AIL+anti-miR-489-3p group.

CCK-8 assay: In short, cell proliferation inhibition rate was assessed in this experiment. 3×10^3 Huh-7 cells in 96-well plates were subjected to CCK-8 solution incubation for 2 h. Results at 450 nm were monitored based on a microplate reader.

Clone formation: After being cultured for 14 d, 500 Huh-7 cells were subjected to Phosphate Buffered Saline (PBS) washing, 500 µl paraformaldehyde fixtures for 20 min at -20°, and 400 µl crystal violet staining for 15 min at 37°. At last, a microscope was utilized to count the number of cell clone formation (≥50 cells were deemed as 1 clone).

Scratch healing assay: 2×10^5 Huh-7 cells were full-grown; a line was drawn on cell monolayer with the tip of a 200 µl pipette. Subsequently, these samples were observed under a microscope, which was regarded as 0 h at this point. They were placed in the incubator and continued to be cultured for 24 h. ImageJ software was applied to detect the migration distance of the cells in each group and calculate the cell scratch healing rate.

Transwell assay: Collected Huh-7 cells were inoculated in upper chamber (1×10^5 cells/well) which was pre-spread with Matrigel matrix gel dilution, and lower counterpart was added with 600 µl medium containing 10 % Fetal Bovine Serum (FBS), cultured in an incubator for 48 h. After PBS washing, infiltrating cells were fixed by adding 500 µl of 4 % paraformaldehyde, stained, and counted.

qRT-PCR assay: After 1 ml of Trizol reagent addition, total Ribonucleic Acid (RNA) from liver cancer tissues, paracarcinoma tissues, and Huh-7 cells were prepared, which were reverse transcribed to complementary DNA (cDNA). Then, qRT-PCR reaction was implemented, and data was detected *via* applying ABI Step OnePlus real-time fluorescence quantitative PCR instrument.

Western blot assay: After 500 µl Radioimmunoprecipitation Assay (RIPA) lysate addition, extract cellular proteins were prepared. Add 40 µg of protein per well channel for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), transfer the membrane, 5 % skimmed milk closed for 2 h, add primary antibodies; MMP-2 (1:1000), MMP-9 (1:1000), and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (1:2000) dilution, incubate at 4° for 24 h, add the secondary antibody

dilution (1:3000), incubate at 37° for 2 h, apply the Quantity one software to quantify the protein bands.

Statistical analysis:

Statistical Package for the Social Sciences (SPSS) 21.0 analyzed results, which was expression as ($\bar{x}$±s). Data comparison was implemented based on Student's t-test or one-way Analysis of Variance (ANOVA) with Tukey's tests. $p<0.05$ regarded statistically significant.

RESULTS AND DISCUSSION

According to the results displayed in Table 1, cell proliferation inhibition rates were apparently increased with the increase of AIL dose, and colony formation number was reduced.

Results from fig. 1 and Table 2 showed that scratch healing rate ($p<0.05$), invasion number ($p<0.05$), MMP2 and MMP9 ($p<0.05$) were gradually reduced by AIL exposure. As shown in Table 3, miR-489-3p content was clearly declined in liver cancer tissues. According to data shown in Table 4, miR-489-3p content was greatly improved in Huh-7 cells with increasing doses of AIL.

Cell proliferation inhibition rates were augmented in the miR-489-3p group, whereas cell colony formation and invasion number was reduced, and scratch healing rate, and MMP2 and MMP9 protein expression were decreased (fig. 2 and Table 5).

As displayed in fig. 3 and Table 6, proliferation inhibition rates were hindered ($p<0.05$), colony formation and invasion number was enhanced ($p<0.05$), and scratch healing rate and MMP2 and MMP9 content were reinforced ($p<0.05$) in the AIL+anti-miR-489-3p.

Many investigations have revealed that our TCM can exert anti-liver cancer effects *via* regulating several target genes[16,17]. Convincing evidence have presented that miRNAs are abnormally expressed in liver cancer and can regulate tumor development by inhibiting target genes, and may also serve as a potential target for liver cancer therapy[18,19] . Yet, whether miRNAs can be used as potential targets of TCM for liver cancer treatment has not been elucidated.

TABLE 1: EFFECT OF AIL ON HUH-7 CELL PROLIFERATION ($\bar{x}$±s, n=9)

Group	Inhibition rate (%)	Colony number
Control	0.00±0.00	108.71±10.03
AIL-L	23.21±2.22*	83.72±7.17*
AIL-M	39.28±3.82*#	67.93±5.48*#
AIL-H	59.35±5.07*#α	50.64±4.85*#α
F	502.227	106.569
p	0.000	0.000

Note: *p<0.05, #p<0.05 and αp<0.05, *vs.* control, AIL-L and AIL-M

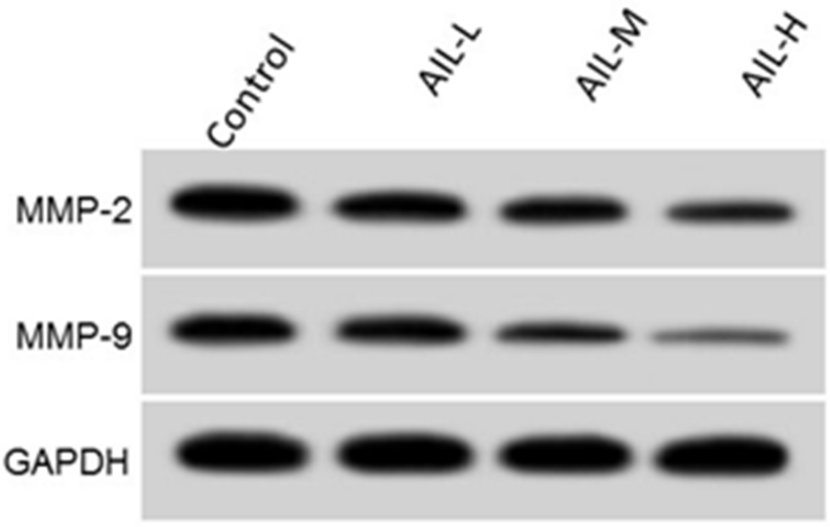

Fig. 1: Effect of AIL-L on invasion and migration-related proteins in Huh-7 cells

TABLE 2: EFFECT OF AIL-L ON HUH-7 CELL METASTASIS (x̄±s, n=9)

Group	Invasion number	Scratch healing rate (%)	MMP-2	MMP-9
Control	124.16±10.56	72.89±5.71	0.77±0.05	0.57±0.05
AIL-L	98.52±6.78*	56.74±5.43*	0.65±0.05*	0.45±0.04*
AIL-M	79.17±6.53*#	40.61±3.18*#	0.52±0.04*#	0.31±0.03*#
AIL-H	57.75±4.29*#&	28.11±2.62*#&	0.34±0.03*#&	0.19±0.02*#&
F	131.616	172.42	162.88	182.222
p	0.000	0.000	0.000	0.000

Note: *p<0.05, #p<0.05 and &p<0.05, *vs.* control, AIL-L and AIL-M

TABLE 3: miR-489-3p CONTENT IN LIVER CANCER (x̄±s, n=37)

Group	miR-489-3p
Paracancerous tissue	1.00±0.11
Liver cancer tissues	0.31±0.03*
t	36.811
p	0.000

Note: *p<0.05, in comparison with paracancerous tissue

TABLE 4: AIL IMPROVED miR-489-3p EXPRESSION (x̄±s, n=9)

Group	miR-489-3p
Control	1.00±0.00
AIL-L	1.79±0.14*
AIL-M	2.66±0.21*#
AIL-H	3.76±0.24*#&
F	516.614
p	0.000

Note: *p<0.05, #p<0.05 and &p<0.05, *vs.* control, AIL-L and AIL-M

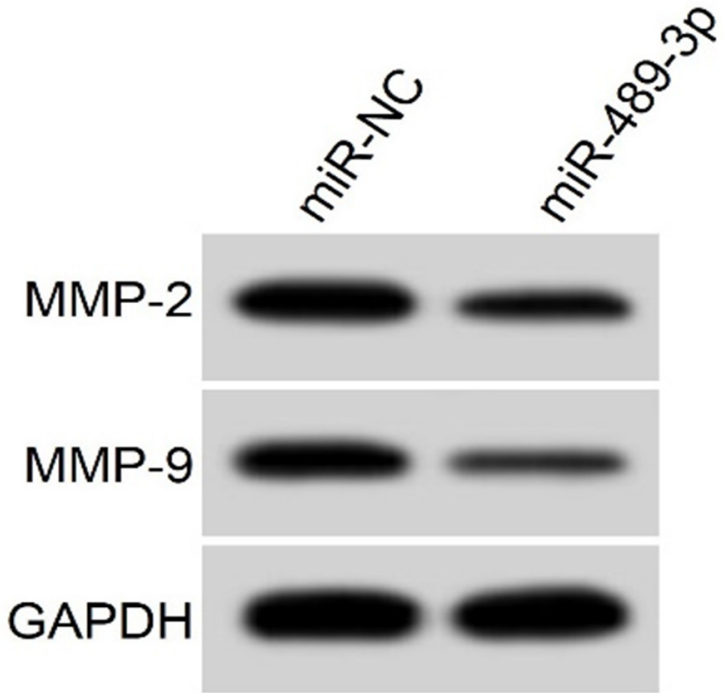

Fig. 2: miR-489-3p overexpression repressed invasion and migration-related proteins in Huh-7 cells

TABLE 5: miR-489-3p OVEREXPRESSION REPRESSED ON HUH-7 CELL GROWTH (x̄±s, n=9)

Group	miR-489-3p	Inhibition rate (%)	Colony number	Invasion number	Scratch healing rate (%)	MMP-2	MMP-9
miR-NC	1.00±0.00	5.58±0.51	105.66±10.15	126.01±11.37	74.44±6.59	0.78±0.06	0.59±0.04
miR-489-3p	3.49±0.28*	49.06±4.06*	59.01±4.97*	63.11±5.78*	34.37±3.36*	0.39±0.04*	0.26±0.02*
t	26.679	31.879	12.038	14.794	16.251	16.225	22.137
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: *p<0.05, *vs.* miR-NC group

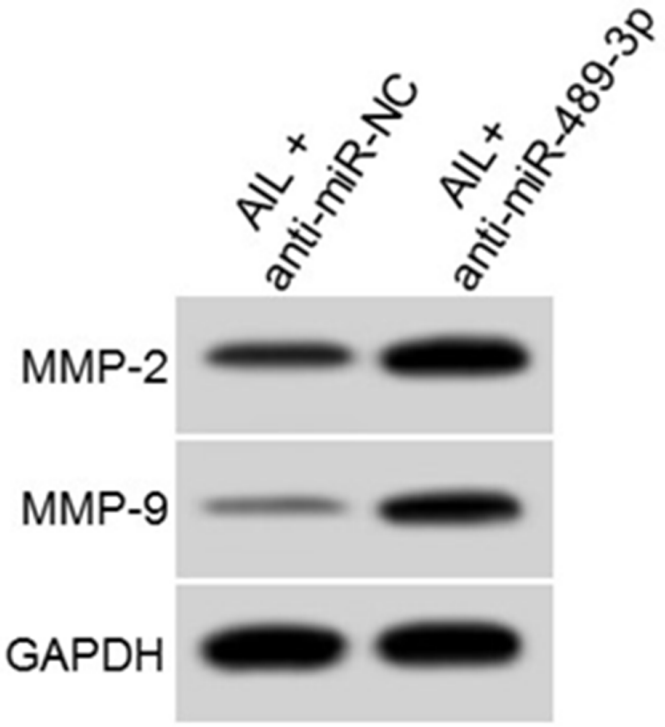

Fig. 3: Interfering miR-489-3p reversed the effect of AIL on invasion and migration-related protein in Huh-7 cells

TABLE 6: miR-489-3p INHIBITOR MIGHT ABOLISH AIL-INDUCED PROLIFERATION, INVASION, AND MIGRATION REPRESSION IN HUH-7 CELLS (x̄±s, n=9)

Group	miR-489-3p	Inhibition rate (%)	Colony number	Invasion number	Scratch healing rate (%)	MMP-2	MMP-9
AIL+anti-miR-NC	1.00±0.00	58.01±4.31	51.65±4.63	55.26±5.17	25.58±2.68	0.32±0.03	0.18±0.02
AIL+anti-miR-489-3p	0.41±0.04*	22.07±2.25*	88.61±7.51*	108.74±11.68*	60.32±5.19*	0.68±0.05*	0.48±0.04*
t	44.25	22.176	12.568	12.561	17.843	18.522	20.125
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: *p<0.05, relative to AIL+anti-miR-NC group

Currently, there is a large number of publications describing the multiple pharmacological properties of AIL, especially the significant anti-tumor effects against a wide range of cancer cells. For example, AIL might promote melanoma cell apoptosis *via* the PI3K/Akt pathway[20]. Of interest, it has been confirmed that AIL might block liver cancer cell growth[10]. Consistent with these findings, Huh-7 cell proliferation increased and clone number decreased by AIL treatment, suggesting that AIL might inhibit liver cancer cell proliferation ability. Previous article uncovered that MMP-2 and MMP-9 belong to the group of MMP, their augment might accelerate cell metastasis *via* degrading the extracellular matrix deposition[21]. Herein, scratch healing rate, invasive number, and MMP-2 and MMP-9 content were reduced after AIL treatment, implying that AIL dwindled liver cancer cell metastasis.

Some studies exhibited miR-489-3p dysregulation is closely linked to different tumor development. For instance, miR-489-3p up-regulation inhibited tumor cell growth and metastasis[22]. Apart from that, overexpressed miR-489-3p might impede bladder cancer cell proliferation and migration[23]. Furthermore, miR-489-3p has been identified to inhibit lung cell proliferation[24]. Here, miR-489-3p content was clearly increased in AIL-triggered Huh-7 cells, suggesting this miRNA participated AIL-mediated liver cancer processes. Functionally, miR-489-3p overexpression might relieve Huh-7 cell development *in vitro*. Moreover, its lack might partially overturn AIL-induced cell growth and metastasis repression. The above results discovered that AIL might repress liver cancer cell malignant behavior *via* increasing miR-489-3p.

Taken together, AIL attenuate liver cancer cell growth and metastasis by promoting miR-489-3p. These findings indicated that miR-489-3p may serve as a potential target for liver cancer treatment by AIL. Yet, whether AIL can exert anti-liver cancer effects *via* modulating other genes needs to be further explored.

Author's contributions:

Ruiyu Guan and Yiyang Yao have contributed equally to this work.

Conflict of interests:

The authors declared no conflict of interests.

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