Alisol A Inhibited the Proliferation, Migration, and Invasion of Nasopharyngeal Carcinoma Cells by Inhibiting the Hippo Signaling Pathway

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) occurs frequently in the Southeast Asia countries and southern China. NPC is associated with multiple risk factors, including the Epstein-Barr virus infection, host genetics, and environment. Invasive surgery, radiotherapy, and chemotherapy contribute to the recovery of NPC. Cisplatin is the most commonly used chemotherapeutic drug for NPC, while its acute toxicities are often intolerable. Therefore, the development of new drugs is essential to improve the clinical outcomes of NPC patients.

Natural products are a valuable source of new anti-cancer drugs due to their potential effectiveness and low toxicity. The extracts of Rhizoma Alismatis have multiple pharmacological effects, including anti-hyperlipidemic, anti-hypoglycemic, anti-inflammatory, hepatoprotective, anti-hepatitis B virus, anti-bacterial, and anti-cancer activities. Alisol A is one of the triterpenes isolated from Rhizoma Alismatis. Previous studies have shown that alisol A suppresses the growth of breast cancer cells. However, there has been no report regarding the effect of alisol A on NPC cells.

The Hippo signaling pathway plays a major role in anticancer therapeutics. YAP is a principal member and downstream effector of the Hippo signaling pathway. Molecular docking is used to identify potential protein targets for compound. Based on the molecular docking analysis, we found that alisol A was a potent inhibitor of YAP.

In this study, we used HK1 and C666-1 cells to detect whether alisol A could inhibit cell proliferation, migration, and invasion.

Purpose: Alisol A is a bioactive triterpenoid isolated from the Rhizoma Alismatis. Previous studies have shown that alisol A has anticancer potential. In this study, we explored the effect of alisol A on the growth of nasopharyngeal carcinoma (NPC) cells.

Materials and Methods: MTT assay, colony formation assay, flow cytometry, transwell assay, wound healing assay, and western blotting were used to assess cell viability, proliferation, cell cycle, migration, invasion, and protein expression, respectively, in vitro. AutoDock Vina and Discovery Studio software were used for molecular docking.

Results: Alisol A inhibited the viability, proliferation, migration, and invasion of NPC cells. The molecular docking simulation assay confirmed that alisol A bound to YAP protein. In addition, alisol A promoted the phosphorylation of YAP and suppressed the expression of YAP in NPC cells.

Conclusion: Alisol A inhibited the proliferation, migration, and invasion of NPC cells by inhibiting the Hippo signaling pathway. Alisol A may be a candidate drug for NPC.

Key Words: Alisol A, nasopharyngeal carcinoma, Hippo signaling pathway

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MATERIALS AND METHODS

Cell culture
NPC cell lines (C666-1 and HK1) were purchased from Shanghai Huiying Biotechnology Co., Ltd (Shanghai, China). Cells were cultured in RPMI-1640 containing 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin (HyClone, Logan, UT, USA) at 37°C in a 5% CO2 incubator. Alisol A was purchased from Tauto Biotech Co., Ltd (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay
C666-1 and HK1 cells (2×10^4) were seeded in 96-well plates and treated with alisol A (10, 20, and 40 μM) or cisplatin (DDP, 10 μM) for 24 h. At 24, 48, and 72 h, 20 μL of MTT solution (5 mg/mL) was added to each well of 96-well plates for 4 h at 37°C. Formed formazan crystals were dissolved in 100 μL DMSO. The absorbance was measured at 570 nm by a microplate reader (Bio-Rad, Hercules, CA, USA). Data were expressed as follows: inhibition ratio (%)=[1-(OD_{570\text{treated}}-OD_{570\text{blank}})/(OD_{570\text{control}}-OD_{570\text{blank}})]×100%.

Colony formation assay
C666-1 and HK1 cells (1×10^3) were seeded in 6-well plates. The cells were treated with alisol A (10, 20, and 40 μM) or DDP (10 μM) for 8 days at 37°C. The medium were renewed every 2 days. Colonies were stained with 0.1% crystal violet solution and counted under the inverted microscope. A cell colony should contain ≥50 cell masses.

Cell cycle analysis
C666-1 and HK1 cells (3×10^5) were seeded in 6-well plates and treated with alisol A (10, 20, and 40 μM) for 24 h. After collection and fixation, cells were stained with propidium iodide. Samples were analyzed by a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Wound healing assay
C666-1 and HK1 cells (3×10^5) were seeded into 6-well plates and treated with alisol A (10, 20, and 40 μM) for 24 h. The monolayer cells were scratched by a sterile 10 μL pipette tip. The wound areas were photographed 24 h later using an inverted microscope (Nikon, Melville, NY, USA).

Cell migration and invasion assays
Transwell chambers were coated with or without Matrigel (BD Biosciences). Cells were seeded into the top chamber with 100 μL Serum-free medium. Cells were stained with crystal violet. The number of cells was counted in five randomly fields.

Western blot
Total cells were lysed by lysis buffer on ice. The nuclear and cytoplasmic proteins were extracted and separated using the nuclear protein extraction kit (Beyotime Biotechnology, Jiangsu, China). Then, 20 μg of protein were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated with specific antibodies. Primary antibodies were shown as follows: β-actin (1:2000, 4967), GAPDH (1:2000, 5174), Histone H3 (1:2000, 9715), YAP (1:2000, 4912), p-YAP (ser127) (1:2000, 4911), MMP2 (1:2000, 4022), MMP9 (1:2000, 3852), cyclin D1 (1:2000, 2922), cyclin E1 (1:2000, 20808), CDK2 (1:2000, 2546), CDK4 (1:2000, 12790) (Cell Signaling Technology, Boston, MA, USA). The membranes were incubated with secondary antibody (1:5000, A0545) (Sigma, St. Louis, MO, USA). Finally, protein bands were visualized using an enhanced chemiluminescence detection kit (Cell Signaling Technology) and photographed using a GE Amersham Imager 600 imaging system.

Immunofluorescence
The fixed cells were incubated with YAP (1:300, 14729) (Cell Signaling Technology). Cells were incubated with DAPI and visualized using a confocal microscope (Leica, Wetzlar, Germany).

Molecular docking
The crystal structure of YAP was obtained from the protein data bank (PDB, http://www.rcsb.org/, ID: 4rex). Crystal structure of each protein was selected based on the best resolution available. PubChem (https://pubchem.ncbi.nlm.nih.gov/) was used to obtain the three-dimension (3D) structure of alisol A (MOL000850). AutoDock Vina and Discovery Studio software were used for the docking simulations and calculations. The value of root mean square deviation (RMSD) was calculated using PyMOL. The prediction of binding mode was selected with RMSD below 2.0 Å.

Statistical analysis
All analyses were performed using SPSS 22.0 program (IBM Corp., Armonk, NY, USA). Statistical significance was evaluated by one-way ANOVA and Student’s t-test. A p-value<0.05 was considered statistically significant.

RESULTS
Alisol A inhibits NPC cells proliferation
The two-dimension (2D) and 3D chemical structures of alisol A are shown in Fig. 1A and B. Alisol A significantly inhibited the growth of C666-1 and HK1 cells in a time- and concentration-dependent manner. The inhibition rate of alisol A was less than that of DDP (Fig. 1C). We found that alisol A inhibited the colony formation of C666-1 and HK1 cells in a concentration-dependent manner. The inhibition of DDP on clone formation was stronger than that of alisol A (Fig. 1D).
Alisol A induces cell cycle arrest in G0/G1 phase of NPC cells
As shown in Fig. 2A, the proportion of cells in the G0/G1 phase was distinctly increased, while the proportion of cells in the S phase was distinctly decreased. In addition, we found that alisol A treatment significantly reduced the protein expression of cell cycle-related genes, including cyclin D1, cyclin E1, CDK2, and CDK4 in NPC cells (Fig. 2B). These results indicate that the cell cycle was arrested in the G0/G1 phase.

Alisol A inhibits migration and invasion of NPC cells
The migration and invasion abilities of NPC cells were reduced...
in alisol A-treated cells (Fig. 3A-C). In addition, alisol A treatment significantly reduced the protein expression of MMP2 and MMP9 in NPC cells (Fig. 3D).

**Alisol A attenuates YAP nuclear expression in NPC cells**

The docking interaction between alisol A and YAP was detected by AutoDock Vina software (Fig. 4A). The pink color represented the amino acid breakdown, the yellow dotted line represented the hydrogen bond, and the light green represented the preferential confirmation of molecules (RMSD=0.180 Å, Bond energy=-6.7 Kcal/mol). The interaction between alisol A and YAP was predicted using the Discovery Studio software, and the 2D schematic diagram is shown in Fig. 4B. We further investigated the effects of alisol A on Hippo signaling. As shown in Fig. 4C, YAP protein expression was decreased and p-YAP Ser127 level was increased after alisol A treatment. Alisol A reduced YAP in the nucleus, whereas it increased p-YAP Ser127 in cytoplasm (Fig. 4D).

**DISCUSSION**

Traditional Chinese medicine has been practiced for thousands of years, and is widely accepted as an alternative treatment for cancer.22 Recently, bioactive compounds in Rhizoma Alismatis have been widely elucidated for their antineoplastic effect. Alisol A suppresses the progress of breast cancer.18 Alisol B suppresses the proliferation of breast cancer cells.23 Alisol B 23-acetate induces apoptosis of human lung cancer cells24 and hepatoma cells.25 Our study revealed the anti-cancer activity of alisol A in NPC cells.

Cancer is characterized by uncontrolled cell cycle.26 Cyclin D/E and CDK2, CDK4 are critical for cells entering the S phase from G1.27 Cell cycle arrest is frequently found in NPC.28 Solutealine regulates the expression of cell cycle proteins, including Cyclin D1, Cyclin E1, CDK2, and CDK4 in prostate cancer.29 In this study, the expression of cyclin D1, cyclin E1, CDK2, and CDK4 was significantly down-regulated in alisol A-treated C666-1 and HK1 cells. Similar report has found that alisol A arrests cell cycle at the G0/G1 phase by regulating the expression of CDK4/cyclin D1 and CDK2/cyclin E1 in breast cancer cells.30 Our study showed that alisol A suppresses the prolifera-
tion of C666-1 and HK1 cells in vitro by inducing G0/G1 arrest. MMPs are implicated in the metastatic process of cancer cells. The degradation of extracellular matrix (ECM) is a vital procedure in cell metastasis. MMP2 and MMP9 are involved in the degradation of ECM. Alisol A 24-acetate reduces MMP2 and MMP9 expression, and shortens cell migration distance.

Fig. 3. Alisol A inhibits the migration and invasion of NPC cells. (A) Cell migration was measured by wound healing assay. (B) Cell migration was measured by transwell assay. (C) Cell invasion was measured by transwell assay. (D) The expression of MMP2 and MMP9 was detected by western blot. Data are presented as the mean±SD of three independent experiments (*p<0.05, **p<0.01, ***p<0.001 vs. control group). NPC, nasopharyngeal carcinoma.
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Fig. 4. Alisol A attenuates YAP nuclear expression in NPC cells. (A) The docking interaction between alisol A and YAP was detected by AutoDock Vina software. (B) The docking interaction between alisol A and YAP was detected by Discovery Studio software. (C) The expression of Hippo signaling pathway-related proteins was detected by western blot. (D) YAP expression in cytosol or nuclear fractions was detected by western blot. (E) YAP expression in nuclear fractions was detected by immunofluorescence. Scale bar, 10 µm. Data are presented as the mean±SD of three independent experiments (*p<0.05, **p<0.01, ***p<0.001 vs. control group). NPC, nasopharyngeal carcinoma.
Our results suggest that alisol A treatment inhibits NPC cell migration and invasion by suppressing the expression of MMP2 and MMP9.

YAP is identified as an oncogene in different types of cancers.\textsuperscript{34,35} It is well documented that the YAP-induced promotion of cell proliferation is determined by its nuclear translocation.\textsuperscript{36} YAP expression is decreased following wogonoside treatment in endometrial cancer.\textsuperscript{37} Corosolic acid induces the repression of cancer progress by translocating YAP from the nucleus in hepatocellular carcinoma.\textsuperscript{38} However, YAP protein expression does not change after artemisinin treatment in the whole cell extracts of hepatocellular carcinoma cells.\textsuperscript{39} In this study, alisol A treatment decreased YAP protein expression in the nucleus.

To sum up, our research might provide a novel mechanism of alisol A for the treatment of NPC. Alisol A inhibited the growth, migration, and invasion of NPC cells by suppressing YAP protein expression in the nucleus.

**AVAILABILITY OF DATA AND MATERIAL**

The datasets used and analyzed in the current study are available from the corresponding author upon reasonable request.

**AUTHOR CONTRIBUTIONS**

Conceptualization: Xianghong Chen and Huiqing Liu. Data curation: Xianghong Chen. Formal analysis: Huiqing Liu. Funding acquisition: Xianghong Chen and Huiqing Liu. Investigation: Xianghong Chen and Huiqing Liu. Methodology: Xianghong Chen and Huiqing Liu. Project administration: Huiqing Liu. Resources: Xianghong Chen. Software: Xianghong Chen. Supervision: Huiqing Liu. Validation: Xianghong Chen. Visualization: Xianghong Chen. Writing—original draft: Xianghong Chen. Writing—review & editing: Huiqing Liu. Approval of final manuscript: all authors.

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