Asiatic acid attenuates malignancy of human metastatic ovarian cancer cells via inhibition of epithelial-to-mesenchymal transition

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ABSTRACT

Purpose: To investigate the anticancer effects of asiatic acid on human metastatic ovarian cancer cells. Methods: Human metastatic ovarian cancer cell line SKOV-3 was treated with various concentrations of asiatic acid for 24 and 48 h. Cell proliferation, migration, invasion and morphology were analyzed by CCK-8, Transwell and immunoﬂuorescence assays, respectively. Epithelial-to-mesenchymal transition-related gene and protein expressions were analyzed by quantitative polymerase chain reaction (qPCR) and Western blotting. Results: Asiatic acid (10 μM) signiﬁcantly suppressed SKOV-3 cell migration and invasion (both p < 0.01). Moreover, epithelial markers (E-cad and KRT-7/14/19) were elevated, while mesenchymal markers (vimentin, N-cad and ZEB1/2) were suppressed after asiatic acid treatment, at both mRNA and protein levels. Inhibition of epithelial-to-mesenchymal transition was further evidenced by immunofluorescence staining of pan-cytokeratin and F-actin. Conclusion: Asiatic acid attenuates the malignancy of human metastatic ovarian cancer cells via epithelial-to-mesenchymal transition inhibition, and thus, is a therapeutic agent for ovarian cancer management.

Keywords: Asiatic acid, Ovarian cancer, Metastasis, Epithelial-to-mesenchymal transition, Vometin

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INTRODUCTION

Ovarian cancer is a deadly disease accounting for 3 % of cancer while 5 % of cancer-related death primarily in post-menopausal women [1]. With the introduction of cis-platinum and later paclitaxel together with debulking surgery, the cure rate of localized ovarian cancer reached 88 %. However, nearly 65 % of patients presented distant ovarian cancer at diagnosis with a dismal 12-year survival rate of 18 % [2]. Although novel targeted agents such as bevacizumab and olaparib have been applied for ovarian cancer treatment, only improved progression-free survival but not overall survival was observed [3,4]. Thus, there is still an urgent need to find new agents and develop novel therapeutic approaches in ovarian cancer treatment.

Asiatic acid (AA), derived from an annual tropical herb Centella asiatica, is a pentacyclic triterpenoid medicinal compound with various pharmacological effects. Growth suppressive and pro-apoptotic effects of AA have been reported on several cancer cell lines including colon cancer [5], breast cancer [6], melanoma [7], glioblastoma [8,9], multiple myeloma [10] and hepatoma [11]. Moreover, the anticancer potential of AA on ovarian cancer has also been
investigated recently [12]. However, the effect of AA on the migration and invasion of ovarian cancer cells has never been explored. Therefore, the present study was undertaken to investigate the anticancer potential of AA on more metastatic SKOV-3 human ovarian cancer cell line, with special focus on cell migration and invasion.

EXPERIMENTAL

Chemicals and regents

AA (C30H48O5, MW: 488.70, purity ≥ 98%) was purchased from Biopurify Phytochemicals Ltd. (Chengdu, China). Dimethyl sulfoxide (DMSO), streptomycin and penicillin were all products of Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Human metastatic ovarian cancer cell line SKOV-3 was obtained from ATCC (Manassas, VA, USA). Cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 100 μg/mL streptomycin and 100 U/mL penicillin at 37 °C with 5% CO2. AA was dissolved in DMSO and stored at -20 °C, and diluted with DMEM to final concentrations when needed.

Cell viability assay

SKOV-3 cells (5×10^3 cells/well) seeded in 96-well plates were exposed to AA (1, 3, 10 and 30 μM) for 24 and 48 h, respectively. Cell viability was determined by cell counting kit-8 (CCK-8, Dojindo, Tokyo, Japan) according to manufacturer's protocol. The absorbance (450 nm) was determined by a Multiskan Spectrum reader (Thermo Scientific, Boston, MA, USA).

Cell migration assay

SKOV-3 cells (1 × 10^5 cells/well) were inoculated onto the upper chamber of the Transwell insert. After incubation for 24 h with AA (1, 3 and 10 μM), migrated SKOV-3 cells on the lower side of the membrane were fixed with 4% formalin and were further stained with crystal violet. The migrated cells were counted in five random fields under microscope.

Cell invasion assay

The cell invasion assay was performed similar to cell migration assay, with Matrigel (BD Bioscience, Bedford, MA, USA) coated Transwell insert and incubation with AA (1, 3 and 10 μM) for 48 h. Cells migrated across the Matrigel were stained and counted as described above.

Immunofluorescence

The immunofluorescence staining was performed as described previously [13]. In brief, after incubation with AA (10 μM) for 48 h, SKOV-3 cells were stained with anti-pan-cytokeratin antibody (Biologend, San Diego, CA, USA) and phalloidin, and followed by incubation with Alexa-Fluor-488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). Sections were further sealed with Vectashield (Roche Diagnostics, Mannheim, Germany) containing DAPI and were observed under fluorescence microscope (Olympus, Tokyo, Japan).

Quantitative polymerase chain reaction (qPCR) assay

SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) were utilized to perform qPCR. Primers were obtained from Sangon Biotech (Shanghai, China) and were listed in Table. The expression of target genes was analyzed by 2^ΔΔCt method to determine the fold change.

Western blot assay

Western blot was conducted as previously described [14] with specific primary antibodies for vimentin, N-cadherin, zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2, E-cadherin, keratin 7 Table 1: Primer sequences

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>AAA GTG TGG CTA CCA AGA AC</td>
<td>AGC CTC AGA GAG GTC AGC AA</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>CGG CAT CAT CCG TAT CTC TCT GTG</td>
<td>AGC CGT TGG CGT AGT CAA A</td>
</tr>
<tr>
<td>ZEB1</td>
<td>AGA CTA TCT TGA TCC CGG AGG</td>
<td>CCT TCT GAG CTA GTG TCT TGT C</td>
</tr>
<tr>
<td>ZEB2</td>
<td>GCC TTA AGT GTG CCG TAA GGG</td>
<td>TTC TCT GAG TAC GAC CAT AC</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>GAG CCT GAG TCC TGCAGT CC</td>
<td>GTA TTG CTG CTT GCC TCA A</td>
</tr>
<tr>
<td>KRT7</td>
<td>CCG CAA CGG CTC CGG CAT GT</td>
<td>TGG CGT G TG CGA TGG GTT ACT</td>
</tr>
<tr>
<td>KRT14</td>
<td>CCA GGT CTC CTC TGG ATG GCA G</td>
<td>GAT CCT CCA GTG GAG TCT GTG TCC A</td>
</tr>
<tr>
<td>KRT19</td>
<td>TCG CAG CTC AGC ATG AAA GCT</td>
<td>AAA ACC GCT GAT CAC GCT CTG</td>
</tr>
<tr>
<td>ß-Actin</td>
<td>CCA ACC AGG AGA AGA TGA</td>
<td>TCC ATC AGC ATG CCA GTG</td>
</tr>
</tbody>
</table>

ZEB = zinc finger E-box-binding homebox; KRT = keratin
(KRT7), KRT14, KRT19 and β-actin (Cell Signaling Technology, Beverly, MA, USA). The optical density of each protein band was determined by Image J (US NIH, Bethesda, MD, USA).

**Statistical analysis**

All studies were performed in quadruplicate and are reported as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett’s test, with \( p < 0.05 \) considered as statistically significant. All calculations were performed using Prism (GraphPad Software Inc 7.0., La Jolla, CA, USA).

**RESULTS**

**Effect of AA on cell growth**

SKOV-3 cells were exposed to various concentrations of AA (1, 3, 10 and 30 μM) for 24 and 48 h, respectively. Cell growth was determined by CCK-8 assay. As shown in Figure 1, only AA (30 μM) significantly inhibited cell growth at both 24 and 48 h (both \( p < 0.01 \)). Therefore, further studies were conducted using AA (1, 3 and 10 μM) to avoid its cytotoxicity and pro-apoptotic effect, which have been reported in previous research [12].

**Effect of AA on cell migration**

SKOV-3 cells were inoculated into the upper chamber of the Transwell insert and further incubated with various concentrations of AA (1, 3 and 10 μM) for 24 h. As shown in Figure 2, AA (10 μM) could significantly inhibited SKOV-3 cell migration across the membrane \((P<0.01)\), compared to the control group.

**Effect of AA on cell invasion**

SKOV-3 cells were inoculated into the upper chamber of the Transwell insert pre-coated with Matrigel. After 48 h incubation with AA (1, 3 and 10 μM),

![Figure 1: Effect of AA on cell growth. Cells were incubated with various concentrations of AA for 24 and 48 h. Note: AA = asiatic acid; Con = control group; \( ^* p < 0.05 \), \( ^{**} p < 0.01 \)](image1)

![Figure 2: Effect of AA on cell migration. Cells were incubated with various concentrations of AA for 24 h. Migrated cells were stained with crystal violet and counted in five random fields. Note: AA = asiatic acid; Con = control group; \( ^{**} p < 0.01 \)](image2)
there was a concentration-dependent decrease on invaded cell number. However, only AA (10 μM) significantly suppressed SKOV-3 cell invasion, which is similar to previous cell migration results ($P<0.01$, Figure 3).

**Effect of AA on epithelial-to-mesenchymal transition (EMT)**

As shown in Figure 4, SKOV-3 cell in the control group exhibited a more mesenchymal phenotype with elongated morphology and F-actin stain (red), which suggested an EMT was taking place. However, after incubation with AA (10 μM) for 48 h, EMT was greatly inhibited as cells were of a round morphology with wide range stain of cytokeratin (green).

**Effect of AA on EMT-related mRNAs expression**

EMT-related mRNAs expression was verified via qPCR assay. As shown in Figure 5, mesenchymal markers including vimentin, N-cadherin, ZEB1 and ZEB2 were significantly decreased after AA treatment (at 10 μM, $p < 0.01$, $p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively), while epithelial markers involving E-cadherin, KRT7, KRT14 and KRT19 were significantly elevated (at 10 μM, $p < 0.01$, $p < 0.05$, $p < 0.05$ and $p < 0.05$, respectively).

**Figure 3:** Effect of AA on cell invasion. Cells were incubated with various concentrations of AA for 48 h. Invaded cells were stained with crystal violet and counted in five random fields. **Note:** AA = asiatic acid; Con = control group; **$p < 0.01$**

**Figure 4:** Effect of AA on epithelial-to-mesenchymal transition. Cells were treated with AA (10 μM) for 48 h and were further stained with anti-pan-cytokeratin antibody (green) and phalloidin (red). **Note:** AA asiatic acid; Con = control group.
Figure 5: Effect of AA on EMT-related mRNAs expression. The relative fold change was determined by $2^{-\Delta\Delta C_T}$ method. Note: Con = control group; *$p < 0.05$; **$p < 0.01$

Figure 6: Effect of AA on EMT-related proteins expression. Optimal density was determined and quantified using ImageJ software. Note: Con = control group; *$p < 0.05$; **$p < 0.01$

**Effect of AA on EMT-related proteins expression**

EMT-related proteins expression was determined via Western blot. As shown in Figure 6, epithelial markers (E-cad and KRT-7/14/19) were elevated, while mesenchymal markers (vimentin, N-cad and ZEB1/2) were suppressed, indicating the EMT process was significantly suppressed by AA treatment at the concentration of 10 μM.

**DISCUSSION**

As a highly metastatic disease, patients with a stage III/IV ovarian cancer have quite dismal prognosis. Therefore, earlier diagnosis, as well as blocking of metastasis is the main striving direction for both basic and clinical research. Although the pro-apoptotic and cell cycle arrest effects on ovarian cancer cells have been reported recently [12], our present study, for the first time, demonstrated that AA, working at a relative low concentration (10 μM), could inhibit the metastatic malignant behavior of human ovarian cancer cell line SKOV-3. Moreover, this suppressive effect might be related to EMT inhibition.
With the application of trans-vaginal ultrasound and cancer antigen (CA) 125 monitoring [15], and later human epididymis protein 4 plus CA125 [16] and serial CA125 measurements [17], doctors and researchers have been trying their efforts to diagnose more patients with pre-clinical and early stage ovarian cancers. However, due to non-specific symptoms as bloating and pelvic pain, there was usually a four to six months’ delay from symptom onset to diagnosis [18,19]. As a result, distant ovarian cancer accounts for 65% of diagnosis and 90% of ovarian cancer-related death [20].

Therefore, the present study focused on the metastasis of ovarian cancer. We selected a more-metastatic human ovarian cancer cell line SKOV-3 [21] and performed cell viability assay with various AA concentrations (1, 3, 10 and 30 μM). The result is comparable to published data on ovarian cancer cell lines [12] and other tumor cells [11,7,10], that AA (30 μM and above) has a definite pro-apoptotic effect. In our present study, we deliberately used lower AA concentrations in the following assays to testify the inhibitory effect on ovarian cancer metastasis. As lower AA concentrations do not show effects on cell growth, the cells on the lower side of the membrane are exactly the cells migrated or invaded. Transwell insert is frequently used in cell migration and invasion assays, with Matrigel-coated membrane mimicking the extracellular matrix. As evidenced by cell migration and invasion assays, AA (10 μM) could significantly attenuate the malignant invasiveness of SKOV-3 cells.

Besides the normal metastatic pattern of intravasation and extravasation, the mainstream hypothesis suggested a passive dissemination of detached ovarian cancer cells via peritoneal circulation [22]. EMT plays a pivotal role in both passive and hematogenous routes. EMT, by definition, is a cellular process that epithelial cells (SKOV-3 cells) lose their cell polarity and further cell-cell adhesion, turning to a more elongated morphology (mesenchymal phenotype) with enhanced metastatic properties [23]. Therefore, immunofluorescence staining assay was performed to observe the effect of AA on the EMT process of SKOV-3 cells. As a metastatic ovarian cancer cell line, SKOV-3 cell in the control group exhibited a more mesenchymal phenotype with elongated morphology and F-actin stain, which suggested an EMT was taking place. However, after incubation with AA (10 μM) for 48 h, EMT was greatly inhibited as cells were of a round morphology with wide range stain of cytokeratin.

Various signaling pathways, including TGF, FGF, PDGF, Wnt and Notch, are involved in this complicated while precisely regulated process [24]. Among them, E-cadherin and N-cadherin play a prominent role. Loss of E-cadherin and gain of N-cadherin is identified as a key event in EMT and thus cancer progression. AA (10 μM) treatment could reverse the EMT process of SKOV-3 cells, as exhibited by less N-cadherin while more E-cadherin expression, both at mRNA and protein levels. More EMT-related genes and proteins expressions including vimentin, ZEB1/2 and keratin 7/14/19 were observed in our present study, which further support our hypothesis that AA attenuates the metastatic behavior of SKOV-3 cells through EMT suppression. Further investigations on underlying mechanisms and specific signaling pathways are warranted.

CONCLUSION
This study reported, for the first time, that AA inhibits the metastatic malignant behavior of human ovarian cancer cell line SKOV-3 at a relative low concentration, and possibly via EMT suppression, and thus, is a potential therapeutic agent for ovarian cancer management.

DECLARATIONS
Acknowledgement
None declared.

Conflict of Interest
No conflict of interest associated with this work.

Contribution of Authors
The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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REFERENCES