Original Article
Ardipusillosidelinhibits the growth, invasion and epithelial-to-mesenchymal transitionof gastric cancer cells through the JAK/STAT3 signaling pathway

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Abstract: Ardipusilloside I (ADS-I) is the major triterpenoidsaponinisolated from ArdisiapusillaA.DC (Myrsinaceae), a Chinese medicinal herb also known as Jiu-Jie-Long. It has been shown to have potent antitumor activity in various cancer types. However, the role of ADS-I in gastric cancer remains to be unclear. Therefore, in this study, we investigated the effects of ADS-I on gastric cancer cell proliferation, invasion and epithelial-to-mesenchymal transition (EMT), and the underlying mechanism was also explored. Our results showed that ADS-I inhibits gastric cancer cell proliferation, invasion and EMT. Furthermore, ADS-I significantly decreased the phosphorylation levels of the JAK1, JAK2 and STAT3 proteins, as well as the expression of STAT3-dependent tumor-associated genes in gastric cancer cells. Taken together, the present study suggests that ADS-I inhibits the growth, invasion and EMT of gastric cancer cells through the JAK/STAT3 signaling pathway. Therefore, ADS-Imay represent a chemopreventive and/or therapeutic agent in the prevention of gastric cancer.

Keywords: Ardipusilloside I (ADS-I), gastric cancer, invasion, epithelial-to-mesenchymal transition (EMT)

Introduction

Gastric cancer is the fourth most commonly diagnosed malignancy and the second most common cause of cancer-related mortality worldwide [1]. Although the range of therapeutic strategies available for treatment of this disease has improved over the past decades, the 5-year survival of gastric cancer patients is currently estimated at 30% [2]. This is largely attributed to a lack of complete understanding of the exact cause and mechanisms for this malignancy. Therefore, further understanding of the molecular mechanisms of cancer progression and the development of new therapeutic tools based on these mechanisms are required.

Saponins, plant glycosides which occur in various plants, have been reported to possess anticancer properties. They have been found to induce apoptosis and inhibit cell growth, cell cycle progression, invasion, and metastasis in cancer cells [3]. Ardipusilloside I (ADS-I) is the major triterpenoidsaponinisolated from ArdisiapusillaA.DC (Myrsinaceae), a Chinese medicinal herb also known as Jiu-Jie-Long [4]. It has been shown to have potent antitumor activity with low toxicity in various cancer types. Xiong *et al.* reported revealed that ADS-I inhibited the growth of human glioblastoma cells, and induced apoptosis through the FasL/Fas signaling pathway [5]. ADS-I also inhibits the growth and induces apoptosis inhepatocellular carcinoma [6], mucoepidermoid carcinoma [7] and cervical adenocarcinoma [8]. However, the role of ADS-I in gastric cancer remains to be unclear. Therefore, in this study, we investigated the effects of ADS-I on gastric cancer cell proliferation, invasion and epithelial-to-mesenchymal transition (EMT). This study provides the first direct evidence that ADS-I inhibits gastric cancer cell proliferation, invasion and EMT through suppressing JAK/STAT3 signaling pathway.

Materials and methods

Cell culture and reagents

Human gastric carcinoma cell lines (TSGH and N87) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI 1640 medium (Abcam, Cambridge, UK) containing 10% fetal bovine serum (Abcam, Cambridge, UK), penicillin (100 units/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), and sodium pyruvate (1 mM). All cells were incubated at 37°C in a 5% CO_2 atmosphere. ADS-I (>98% pure) was provided by Prof. Tang (Department of Pharmacy, Xijing Hospital, Xi'an, PR China).

Cell proliferation assay

The proliferation of cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells ($1×10^4$ cells/well) were seeded in 96-well culture plates. After 24 h incubation, cells were treated with different concentrations of ADS-I (0, 10, 20 and 40 µM) for 24 h, 48 h, 72 h and 96 h. Then, 10 µL of 5 mg/mL MTT (Sigma, St. Louis, MO, USA) was added to each well. After incubation for 4 h at 37°C in the dark, the supernatant was discarded, and 100 µL DMSO was added to solubilize the formazan crystals. After mixing, absorbance was measured at 570 nm with an ELISA reader (Bio-Rad, CA, USA). Cell viability was expressed as the percentage of value against that of the solvent-treated control group.

Cell invasion assay

Cell invasion was detected using modified Boyden chambers with filter inserts for 24-well dishes containing 8-µm pores (Millipore, Boston, MA, USA), pre-coated with Matrigel (BD, San Jose, CA, USA). Cells were treated with different concentrations of ADS-I (0, 10, 20 and 40 µM) and plated into 100 µL of RPMI in the upper chamber. Medium containing 20% FBS was placed in the lower chamber as a chemoattractant. After 48 h in culture, the non-invading cells were removed from the upper surface of the membrane using cotton tipped swabs, and then the cells on the underside of the filters were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. The invaded cells were viewed and counted under a microscope (Olympus, Tokyo, Japan). Each

clone was plated in triplicate in each experiment and each experiment was repeated at least three times.

RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA from the treated cells was extracted with TRIzol RNA-extraction reagent (Gibco, USA). Approximately 5 µg total RNA for each sample were reverse transcribed into first strand cDNA. The cDNA was used as a template in quantitative real-time PCR (qRT-PCR) amplifications performed using TaqMan PCR Master Mix and an ICycler system. Primers for TaqMan PCR were obtained from Applied Biosystems' predesigned TaqMan Gene Assays (Ki67, Mm01278617_m1; matrix metalloproteinase 9 (MMP-9), Mm00600163_m1; Snail, Mm01249564_g1; Twist, Mm00442036_m1). Amplification cycles consisted of 94°C for 3 min, then 33 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min, followed by 72°C for 15 min. β-actin was used as a control for normalizing gene expression. Experiments were performed independently at least three times. Relative quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Cells were homogenized and lysed with RIPA lysis buffer (100 mM NaCl, 50 Mm Tris-HCl pH 7.5, 1% TritonX-100, 1 mM EDTA, 10 mM β-glycerophosphate, 2 mM sodium vanadate and protease inhibitor). The protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). For western blotting, proteins were separated by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Whatman Schleicher & Schuell, Middlesex, UK). After blocking, the target proteins were probed with primary antibodies [p-JAK1, JAK1, p-JAK2, JAK2, p-STAT3 and STAT3 (Cell Signaling Technology, Beverly, MA), E-cadherin, N-cadherin and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA)]. The membrane was labeled with secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the protein bands were visualized using an enhanced chemiluminescence reagent (Amersham Biosciences, Little Chalfont, UK). The signals were quantified by densitometry using Sion Image software (Scion Corporation, Frederick, MD, USA).
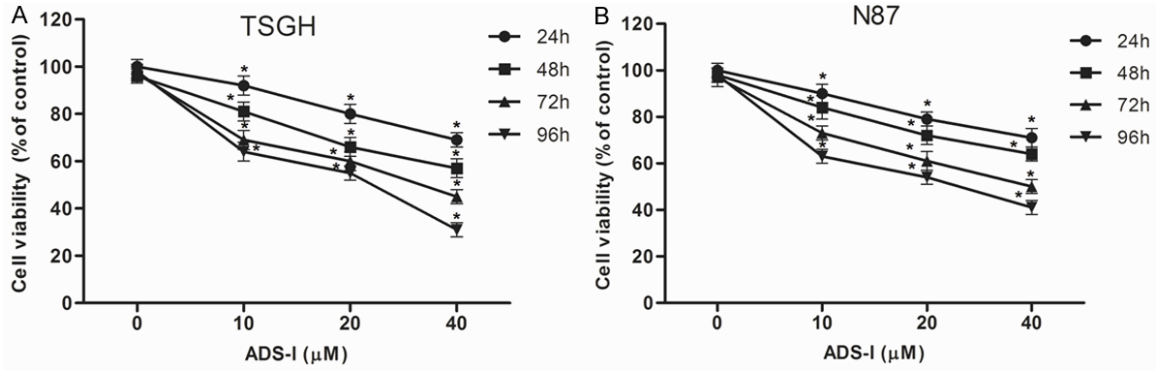
Figure 1. Ardipusilloside I treatment inhibits the proliferation of TSGH and N87 cells. TSGH and N87 cells were treated with different concentrations of ADS-I (10, 20 and 40 μM) for 24 h, 48 h, 72 h and 96 h, and cell proliferation was determined by MTT assay. A. ADS-I inhibited proliferation of TSGH cells in a concentration-dependent manner. B. ADS-I inhibited proliferation of N87 cells in a concentration-dependent manner Data are mean ± SD. All experiments were repeated at least three times. *$P<0.05$ compared with control group.
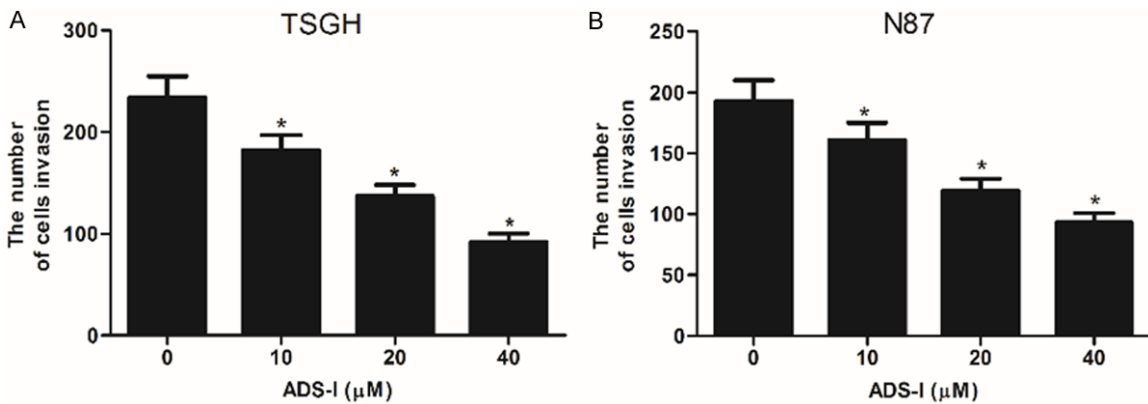

Figure 2. Ardipusilloside I treatment inhibits the invasion of TSGH and N87 cells. Invasion was evaluated using Matrigel Boyden chambers. The number of migration cells through the Matrigel-coated membrane was decreased in TSGH cells (A) and N87 cells (B) treated with apigenin. Data are mean ± SD. All experiments were repeated at least three times. *$P<0.05$ compared with control group.

Statistical analysis

Results are expressed as means ± SD. Differences between two groups were analyzed by Student's t test, while differences between multiple groups were analyzed by ANOVA. Data were considered to be statistically significant when $P<0.05$.

Results

ADS-I inhibits gastric cancer cell proliferation

The inhibitory effect of ADS-I on cell proliferation of TSGH and N87 cells was evaluated by MTT assay. As shown in **Figure 1**, ADS-Isignificantly inhibited the proliferation of TSGH (**Figure 1A**) and N87 cells (**Figure 1B**) in a dose-dependent manner, and TSGH cells are more

sensitive to ADS-I-induced cell growth inhibition than N87 cells. The viabilities of TSGH and N87 cells decreased to 32.3% and 41.7% after 96 h of treatment with 40 μM ADS-I.

ADS-I inhibits gastric cell invasion

We further explored the effects ADS-I on gastric cell invasion. As shown in **Figure 2**, ADS-I led to significantly decreased invasion (ADS-I groups, 182 ± 15, 137 ± 11 and 92 ± 8 cells per field; control group, 234 ± 21 cells per field) of TSGH cells (**Figure 2A**). Similarly, ADS-I also resulted in decreased invasion (ADS-I groups, 161 ± 14, 119 ± 10 and 93 ± 7 cells per field; control group, 193 ± 17 cells per field) of N87 cells (**Figure 2B**).
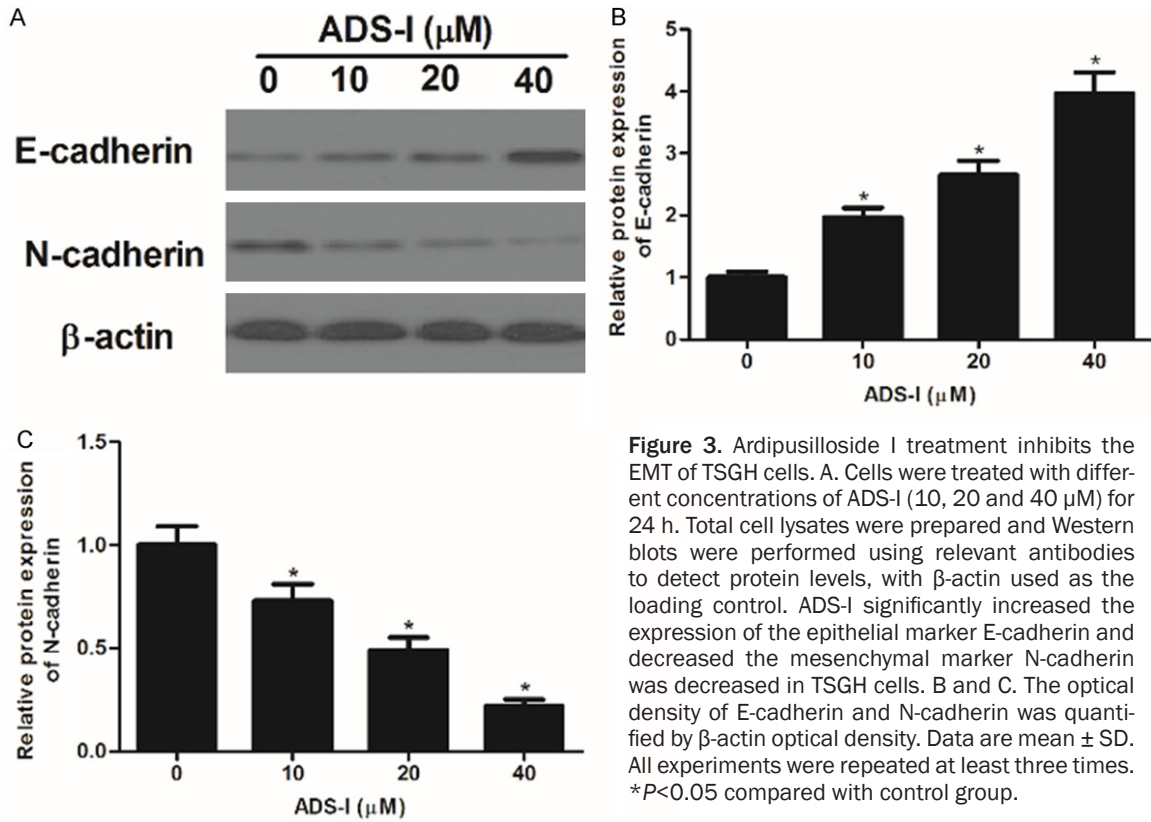
Figure 3. Ardipusilloside I treatment inhibits the EMT of TSGH cells. A. Cells were treated with different concentrations of ADS-I (10, 20 and 40 μM) for 24 h. Total cell lysates were prepared and Western blots were performed using relevant antibodies to detect protein levels, with β-actin used as the loading control. ADS-I significantly increased the expression of the epithelial marker E-cadherin and decreased the mesenchymal marker N-cadherin was decreased in TSGH cells. B and C. The optical density of E-cadherin and N-cadherin was quantified by β-actin optical density. Data are mean ± SD. All experiments were repeated at least three times. *P<0.05 compared with control group.
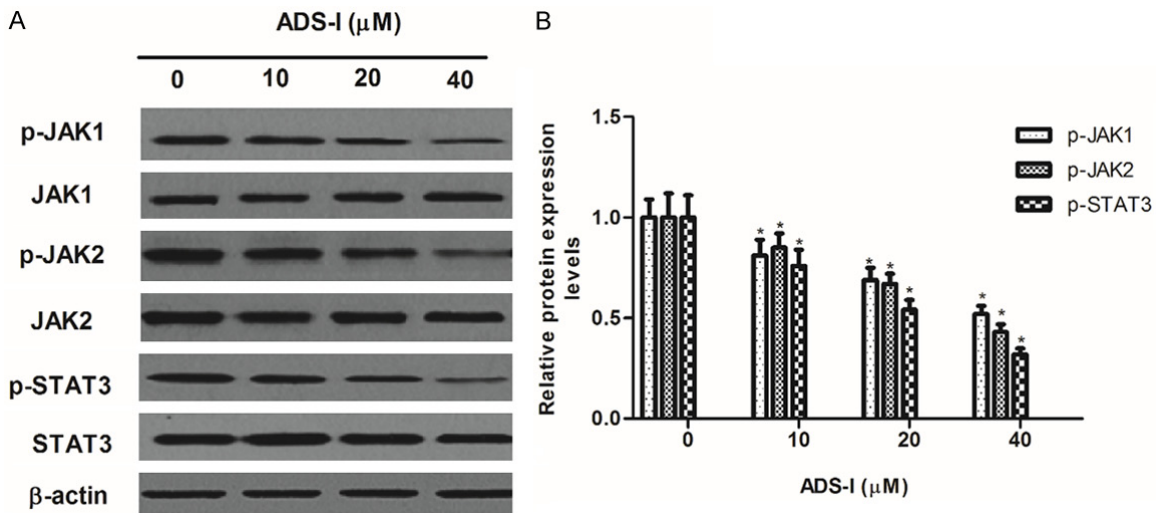

Figure 4. Ardipusilloside I inhibits JAK/STAT3 signaling pathway in TSGH cells. (A) Cells were treated with different concentrations of ADS-I (10, 20 and 40 μM) for 24 h. Total cell lysates were prepared and Western blots were performed using relevant antibodies to detect protein levels, with β-actin used as the loading control. ADS-I significantly decreased the phosphorylation levels of the JAK1, JAK2 and STAT3 proteins (B), The optical density of each protein was quantified by β-actin optical density. Data are mean ± SD. All experiments were repeated at least three times. *P<0.05 compared with control group.

ADS-I inhibits EMT

EMT is characterized by the loss of cell-cell adhesion and the increase of cell motility, and it is a key process in cancer progression and metastasis [9, 10]. So, we investigated the effect of ADS-I on the expression of EMT-related markers. As shown in **Figure 3**, the expression
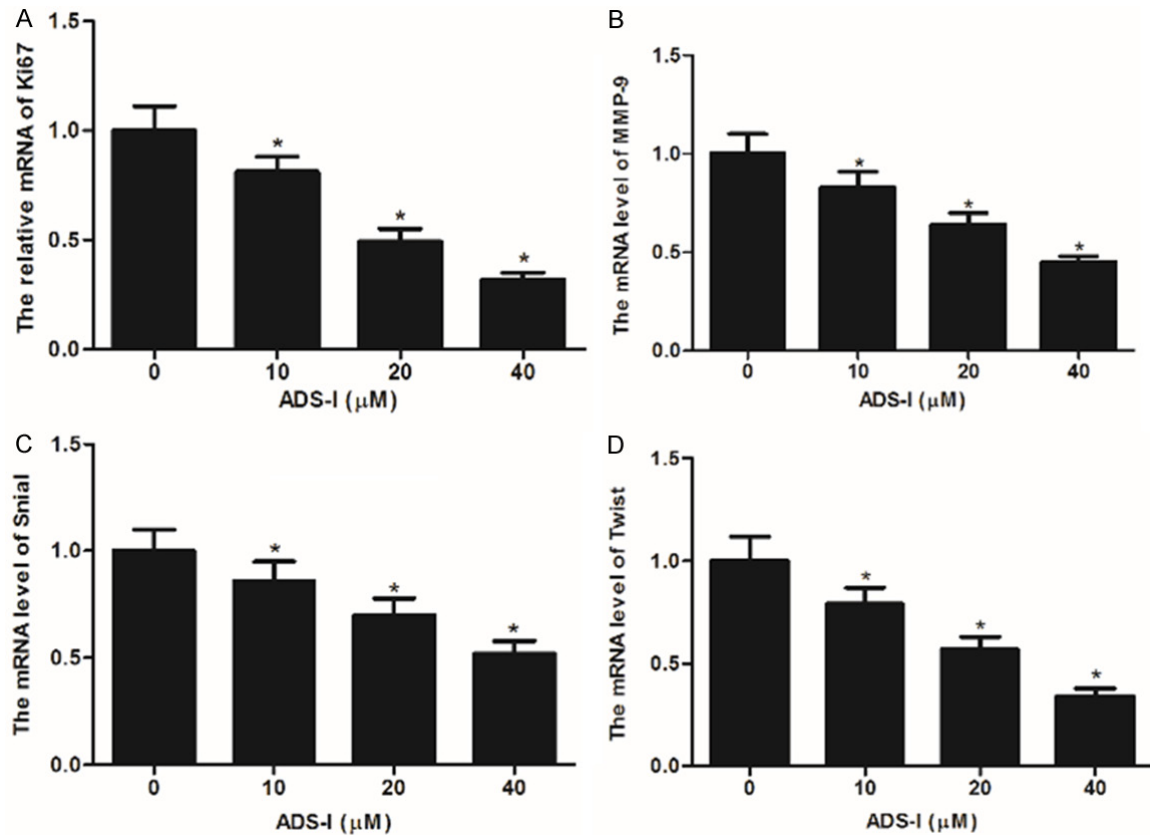
Figure 5. Ardipusilloside I modulates STAT3 target gene expression in TSGH cells. Cells were treated with different concentrations of ADS-I (10, 20 and 40 μM) for 24 h. Total RNA was extracted and reverse transcribed. mRNA expression levels of Ki67 (A), MMP-9 (B), Snail (C) and Twist (D) was detected by qRT-PCR. The fold changes of mRNA were presented after normalization with control group. Data are mean ± SD. All experiments were repeated at least three times. *$P < 0.05$ compared with control group.

of the epithelial marker E-cadherin was increased at the protein level, while protein expression of the mesenchymal marker N-cadherin was decreased in TSGH cells.

ADS-I inhibits JAK2/STAT3 signaling in gastric cells

Given that STAT3 is constitutively activated in gastric cancer, we investigated whether ADS-I might influence the JAK/STAT3 pathway. As shown in **Figure 4**, ADS-I treatment dose-dependently down-regulated the active tyrosine-phosphorylated forms of JAK1 and JAK2. Likewise, ADS-I also inhibited the expression of phosphorylation ofthe downstream JAK substrate STAT3 in gastric cancer cells.

ADS-I regulates expression of STAT3-dependent tumor-associated genes

Because STAT3 target genes play important roles in tumor growth, migration, and inva-

sion, we investigated the effect of ADS-I on the expression of STAT3-dependent tumor-associated genes. As shown in **Figure 5**, as compared with the control group, the cell proliferation marker Ki67 was markedly reduced in ADS-I-treated cells. In addition, ADS-I treatment also inhibited the mRNA expression of invasion-relevant enzyme MMP-9, and the transcription factors Snail and Twist, were also reduced after ADS-I treatment.

Discussion

The present study verified that ADS-I inhibits gastric cancer cell proliferation, invasion and EMT. To explore the underlying mechanisms, we found that ADS-I significantly decreased the phosphorylation levels of the JAK and STAT3 proteins, as well as the expression of STAT3-dependent tumor-associated genes in gastric cancer cells.

Previous studies found that triterpenoid saponins possess anti-tumor activity, including

gastric cancer. For example, one study showed that a triterpenoidsaponin from Adenophoratriphylla var. japonica suppresses the growth of human gastric cancer cells [11]. Another study reported that Raddeanin A, one of the triterpenoidsaponins in herbal medicine Anemone raddeana Regel, induces human gastric cancer cells apoptosis and inhibits their invasion in vitro [12]. Consistent with these results, in the present study, we found that ADS-I inhibits gastric cancer cell proliferation and invasion.

EMT, a biological process occurs in various types of cancers including gastric cancer, is associated with cancer cell migration and invasion [13]. EMT mainly experiences the following steps: dissociation of adhesions between epithelial cells, loss of the apical-basolateral polarity, reorganization of the actin cytoskeleton, and increases of cell motility [14]. Downregulation of E-cadherin, an epithelial marker, is a hallmark of EMT. The loss of E-cadherin is accompanied by the upregulation of mesenchymal markers, such as N-cadherin and vimentin. In this study, we found that ADS-I increased the expression of E-cadherin epithelial markers and decreased the expression of mesenchymal marker N-cadherin in TSGH cells.

The JAK/STAT3 pathway plays an important role in human cancers [15-18]. JAK family proteins include JAK1, JAK2, JAK3 and tyrosine kinase2. Members of the JAK family of protein tyrosine kinases phosphorylate and activate cytoplasmic STAT proteins [19]. A growing body of evidence indicates that aberrant STAT signaling, and in particular STAT3 initiated cascades, participate in the development and progression of human cancers [20-22]. Recently, one study showed thatOPB-31121, a novel STAT3 inhibitor, down-regulated JAK2 and gp130 expression and inhibited JAK2 phosphorylation which leads to inhibition of STAT3 phosphorylation, and decreased cell proliferation in both gastric cancer cells and in a xenograft model [23]. In addition, Yu *et al*. reported that fucoxanthin inhibits proliferation and promotes apoptosis in human gastric adenocarcinoma MGC-803 cells via JAK/STAT signal pathway [24]. In line with these results, in this study, we found that ADS-I significantly decreased the phosphorylation levels of the JAK and STAT3 proteins. These results suggest that ADS-I inhibits the growth and invasion of gastric cancer cells through the JAK/STAT3 signaling pathway.

It has been reported that STAT3 directly and indirectly upregulates the expression of genes that are required for uncontrolled proliferation and invasion of tumor cells [13, 25]. In this study, we showed that ADS-I down-regulated the expression of STAT3-regulated gene products involved in cell proliferation (Ki67), migration (MMP-9) and EMT (Snail and Twist).

In conclusion, the present study suggests that ADS-I inhibits the growth, invasion and EMT of gastric cancer cells through the JAK/STAT3 signaling pathway. Therefore, ADS-Imay represent a chemopreventive and/or therapeutic agent in the prevention of gastric cancer.

Acknowledgements

Disclosure of conflict of interest

None.

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