

Lentiviral vector mediated-ASAP1 expression promotes epithelial to mesenchymal transition in ovarian cancer cells

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Abstract. Ovarian cancer is one of the most common malignancies in women and has a high mortality rate due to metastatic progression and tumor recurrence. ASAP1 (ArfGAP with SH3 Domain, Ankyrin Repeat and PH Domain 1) is an ADP-ribosylation factor GTPase-activating protein, which is involved in tumor metastasis. However, the role of ASAP1 in ovarian cancer is completely unknown. The present study reported that ASAP1 was highly expressed in ovarian carcinoma, and expression positively-correlated with overall poor survival and prognosis of patients. Lentiviral vector mediated ASAP1 expression promoted cell migration and invasion in ovarian cancer cell lines SKOV3 and OVCAR3. In addition, ASAP1 promoted cell proliferation, survival and inhibited chemotherapy drug paclitaxel-induced cell apoptosis. Furthermore, ASAP1 expression promoted epithelial to mesenchymal transition (EMT) by upregulating the mesenchymal cell markers N-cadherin and vimentin, and downregulating epithelial cell marker E-cadherin in the ovarian cancer cell lines. The data indicate for the first time that ASAP1 exhibits an oncogenic role by promoting EMT in ovarian cancer cells.

Introduction

Ovarian cancer is one of the most common gynecological malignancies with a high mortality rate (1). In 2017, 22,440

new cases and 14,080 deaths of ovarian cancer were estimated to occur in the United States (2). At the time of diagnosis, the majority of ovarian cancer patients have already progressed to advanced disease, due to the lack of early clinical symptoms, which results in high mortality (3). However, the molecular mechanisms underlying the tumor metastasis and chemoresistance in ovarian cancer are not well understood. The epithelial-mesenchymal transition (EMT) contributes to the tumor metastasis and chemoresistance (4). EMT is a biological process accompanied by the loss of cell adherence junctions and apical-basal polarity, and acquisition of the mesenchymal phenotype to increase cell motility and invasiveness (5). EMT marker gene expression is altered during the EMT process with downregulation of epithelial markers such as E-cadherin and upregulation of mesenchymal markers, including Snail and 2, N-cadherin, vimentin, Zeb 1/2, and Twist1/2 (6,7).

ASAP1 (ArfGAP with SH3 Domain, Ankyrin Repeat and PH Domain 1) protein has N-terminal BAR, PH, ARF GAP, ankyrin repeat, proline-rich, and C-terminal SH3 domains (8). ASAP1 expression is upregulated in a variety of cancers as compared to normal tissue, and correlates with poor survival and prognosis in colorectal, and neck and head cancer patients (9,10). In breast cancer ASAP1 expression contributes to tumor invasion and metastasis (11). ASAP1 is upregulated in ovarian cancer and associated with poor patient survival and prognosis (12). However, the function of ASAP1 in ovarian cancer has not been investigated.

In the present study, we investigated the role of ASAP1 in ovarian cancer cells using lentiviral vector mediated overexpression. We demonstrated that ASAP1 was highly expressed in ovarian cancer and associated with patient poor survival based on analysis of expression of ASAP1 in clinical specimen from database. To define the function of ASAP1 in ovarian cancer cells, we overexpressed ASAP1 in both ovarian cancer SKOV3 and OVCAR3 cells and found that overexpression of ASAP1 promoted cell proliferation, migration, invasion and colony formation, and reduces sensitivity to chemotherapy drug paclitaxel. Moreover, overexpression of ASAP1 promoted EMT, suggesting that ASAP1 plays an oncogenic role in ovarian cancer cells and potentially contributes to ovarian tumor metastasis.

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Materials and methods

Cell culture. The ovarian cancer cell lines (SKOV3 and OVCAR3) were obtained from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). HEK293FT cells were purchased from Invitrogen and cultured in DMEM medium with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1% glutamine, 1% nonessential amino acid, and 1 μ g/ml geneticin.

Lentiviral vector production. The lentiviral ASAP1 expression vector was purchased from Applied Biological Materials Inc., (Richmond, Canada). ASAP1 cDNA was driven by EF1 α promoter, while luciferase cDNA driven by EF1 α promoter in the same lentiviral vector served as a control. Lentivirus was packaged in HEK293FT cells as we published previously (13). Stable cell lines were generated by transducing the SKOV3 and OVCAR3 cells with the lentiviral ASAP1 or control vector (without insert), and selected with 3 μ g/ml puromycin.

Cell migration assay. The transwell migration assays were performed using a modified chamber system of BDFalcon™; BD Biosciences (San Jose, CA, USA). These chambers were inserted into a 24-well plate with 3×10^4 of cells in 300 μ l serum-free DMEM added to the upper chamber. DMEM supplemented with 10% FBS was added into the lower chamber as a chemoattractant, and cells were incubated for 24 h. The medium and non-migrated cells in the upper chamber were removed, whereas the migrated cells on the lower side of the membranes were fixed with methanol and stained with hematoxylin-eosin (H&E). Pictures were taken at 10x magnification, and transmigrated cells from at least three different fields were counted.

Cell invasion assay. SKOV3 and OVCAR3 cells (5×10^5 per well) transduced with ASAP1 and control lentiviral vectors were seeded in serum-free DMEM onto inserts pre-coated with Matrigel (BDBioCoat™; BD BioSciences). DMEM supplemented with 10% FBS was added to the bottom chamber, and cells were incubated for 24 h. The upper chamber containing the non-invasive cells were removed, and the invasive cells on the lower side of the membranes were fixed with methanol and stained with H&E. Images were taken at 10x magnification, and invasive cells from at least three different fields were counted.

Cell proliferation assay. Cell proliferation at different time points was examined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Assay kit purchased from ATCC (Manassas, VA, USA) following manufacturer's instructions. Ovarian cancer SKOV3 or OVCAR3 cells were plated (8×10^3 cells/well) in 96-well plates. 10 μ l of MTT reagent (10 mg/ml) was added to each well and incubated for 4 h. The reaction was terminated by adding 100 μ l lysis reagent, and incubated at 22°C in the dark for 2 h. The absorbance was measured at 570 nm wavelength on a Bio-Rad Laboratories, Inc., (Hercules, CA, USA) plate reader.

Cell clonogenic assay. SKOV3 and OVCAR3 cells transduced with lentiviral ASAP1 or control vector were plated into 6-well plates (200 cells/well) in triplicate and then stained with 0.1% Crystal Violet following culture for two weeks. Cell colonies were counted as described previously (14).

Soft agar colony formation assay. SKOV3 (1×10^4) and OVCAR3 (5×10^4) cells were trypsinized and resuspended in growth medium containing 0.35% agarose (BD Biosciences), and then plated onto a solid layer of 0.7% agarose in growth medium in 6-well plates. Fresh medium (500 μ l) was added on alternate days for two weeks. After 12 h incubation with 20 μ l of MTT reagent (10 mg/ml), stained cell colonies were imaged using a Nikon Ti inverted microscope (Nikon Corporation, Tokyo, Japan).

Cell apoptosis. Stable SKOV3 and OVCAR3 ovarian cancer cell lines were established with lentiviral ASAP1 expression and control vectors. Cell apoptosis was measured using a caspase3/7 activity assay kit (Promega Corporation, Madison, WI, USA) following treatment with the chemotherapy drug paclitaxel at the indicated doses for 24 h. Cell apoptosis was also detected in both SKOV3 and OVCAR3 cells using western blot by examining cleaved-PARP and cleaved-caspase3.

Immunofluorescence. To detect ASAP1 expression in ovarian cancer tissue, sections from three different paraffin embedded human serous carcinoma specimens were antigen-retrieved and incubated with blocking buffer (5% normal goat serum, 3% bovine serum albumin, and 0.1% Triton-X 100 in PBS) for 1 h. Primary antibodies to ASAP1 (1:200; Rockland; Atlanta, GA, USA), and PCNA (1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were incubated with the sections at 4°C overnight. After three rinsing for 5 min with PBST, Alexa 594 or 488 conjugated goat anti-rabbit or mouse antibodies were applied (1:200 dilution; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Cell nuclei were counterstained with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). Images were taken using a Nikon Ti inverted fluorescence microscope (Nikon Corporation).

Western blot analysis. Ovarian cancer cells were collected in RIPA buffer (Thermo Fisher Scientific, Inc.) containing 1% Halt Proteinase Inhibitor Cocktail (Thermo Fisher Scientific, Inc.). An equal amount of protein (80 μ g/lane) was loaded onto 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 1 h, and incubated with primary antibodies against ASAP1 (Rockland; Atlanta, GA, USA), E-cadherin, N-cadherin, β -catenin, vimentin, Snai2, Cleaved-PARP, Cleaved-caspase3 (Cell Signaling Technology, Inc., Danvers, MA, USA) and GAPDH (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) overnight at 4°C. After three rinses with PBST buffer for 5 min, the membranes were incubated with secondary antibodies at room temperature for 1 h. The membranes were incubated with chemiluminescence solution for 2 mins and exposed on X-ray film.

Statistical analysis. Significant differences were determined in at least two independent experiments performed in triplicate

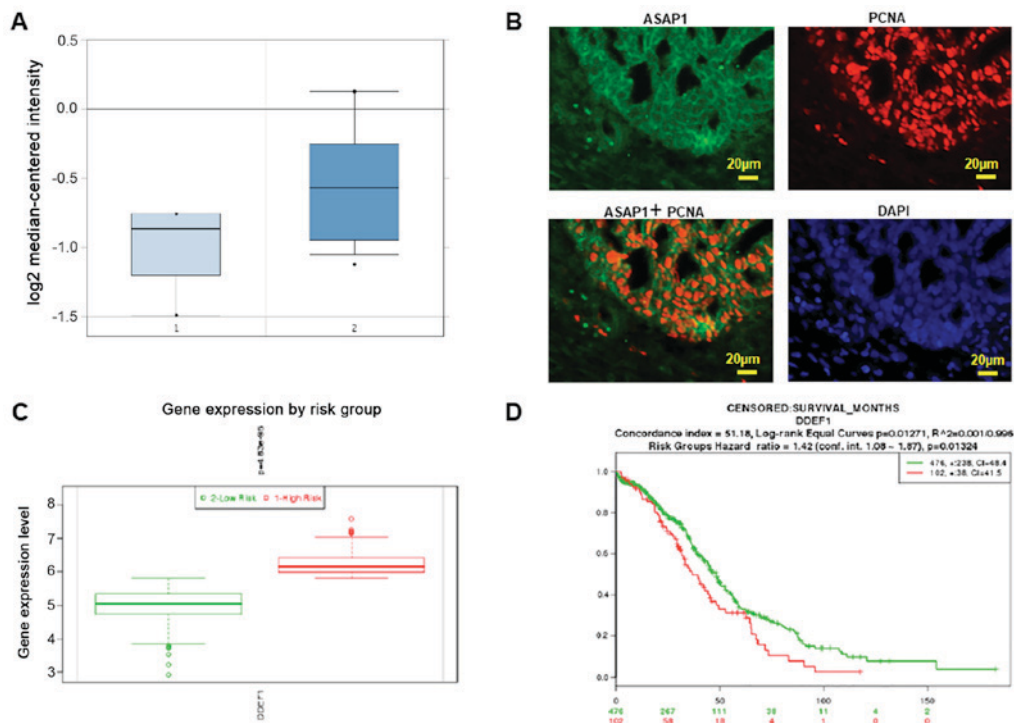


Figure 1. ASAP1 is highly expressed in ovarian cancer cells and associated with overall poor patient survival. (A) ASAP1 expression was significantly higher in ovarian tumor (2) compared to normal control (1) based on TCGA database ($P=4.49\text{e-}4$). (B) A representative immunofluorescent staining of ASAP1 and PCNA expression in human serous carcinoma section was shown. ASAP1 was stained in cytoplasm and membrane (green) and tumor area was stained with PCNA antibody (Red) in cell nuclei. (C) ASAP1 expression was significantly higher in high risk ovarian cancer patients compared with the low risk group based on TCGA dataset. (D) High risk group ovarian cancer patients have low survival compared to low risk group ($P=0.01324$).

and presented as means \pm SD using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ASAP1 was upregulated in ovarian carcinoma and associated with poor patient survival and prognosis. To examine ASAP1 expression in ovarian cancer, we analyzed TCGA dataset on human ovarian serous carcinoma using the Oncomine database. ASAP1 (DDEF1) expression was significantly upregulated in 586 tumor specimens compared to expression in 8 normal tissue specimens ($P=4.49\text{e-}4$) (Fig. 1A). ASAP1 expression was examined by immunofluorescent staining of sections from three different paraffin embedded ovarian serous carcinoma specimen and ASAP1 were strongly stained in the cytoplasm and membrane. The cell proliferation marker PCNA was stained in the nuclei of tumor cells as shown in Fig. 1B. ASAP1 expression was then analyzed in 568 tumor and 8 normal ovaries in TCGA database according to risk groups using SurvExpress program. ASAP1 expression was found to be significantly higher in the high risk group as compared to the low risk group ($P=4.83\text{e-}95$) (Fig. 1C). Moreover, ovarian cancer patients in the high risk group had significantly poorer overall survival compared to the low risk group ($P=0.01324$) (Fig. 1D).

Overexpression of ASAP1 promoted EMT in ovarian cancer cells. Since ASAP1 was highly expressed in ovarian cancer and associated with poor patient survival, we hypothesized that ASAP1 may promote tumorigenesis in ovarian cancer

cells. To test this hypothesis, we overexpressed ASAP1 in ovarian cancer SKOV3 and OVCAR3 cells by lentiviral transduction. The expression level of ASAP1 was increased by approximately two-fold in SKOV3 cells compared to control as detected by western blotting. Since ASAP1 was found to be associated with tumor metastasis and chemoresistance in other types of cancer, we examined the effect of ASAP1 overexpression on expression of EMT markers in ovarian cancer cells. Overexpression of ASAP1 increased the expression of mesenchymal cell markers including N-cadherin and Vimentin, while decreasing epithelia cell marker E-cadherin expression in SKOV3 cells (Fig. 2A). Moreover, SKOV3 cells expressing ASAP1 had a mesenchymal phenotype with a spindle-like fibroblast morphology, while control cells had a cobblestone-like appearance of epithelial cells (Fig. 2B). Similarly, overexpression of ASAP1 promoted EMT in OVCAR3 cells compared to empty vector transduced control cells (Fig. 2C). However, overexpression of ASAP1 only led to a modest mesenchymal phenotype in OVCAR3 cells compared to control cells transduced with empty vector (Fig. 2D). Our results indicated that overexpression of ASAP1 promoted EMT in both ovarian cancer SKOV3 and OVCAR3 cells.

Overexpression of ASAP1 promoted cell migration and invasion in ovarian cancer cells. ASAP1 expression promoted EMT in ovarian cancer cells, suggesting that ASAP1 may contribute to tumor metastasis. Therefore, we examined the effect of ASAP1 expression on cell migration and invasion in both SKOV3 and OVCAR3 cells. As shown in Fig. 3A and B, ASAP1 expression significantly promoted cell migration

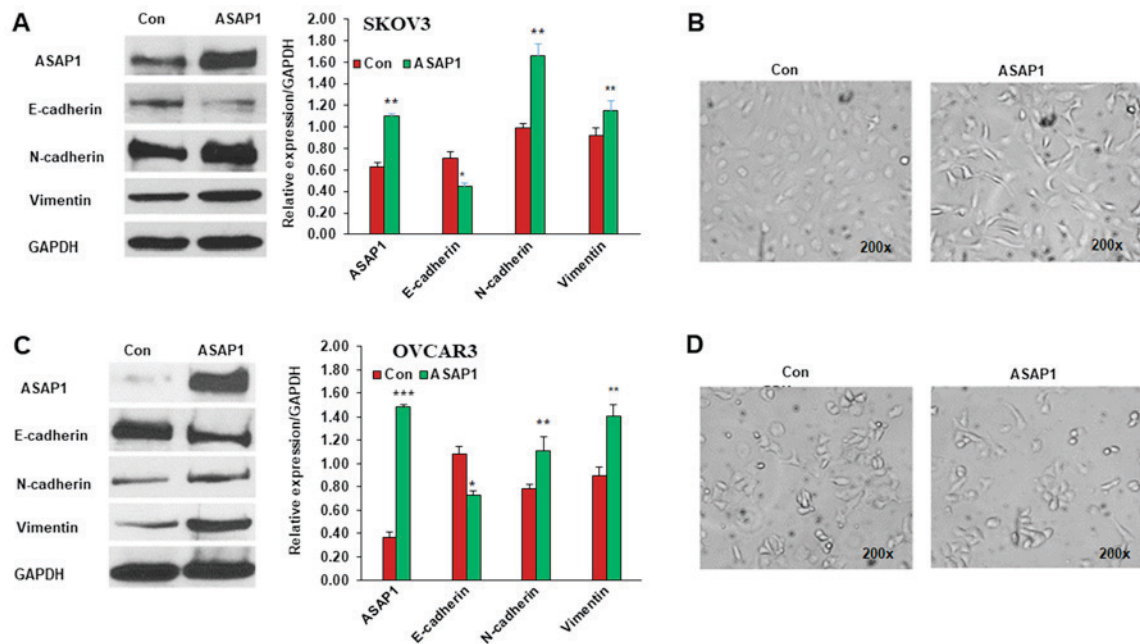


Figure 2. Overexpression of ASAP1 promoted the EMT in ovarian cancer cells. (A) EMT marker genes E-cadherin, N-cadherin, β -catenin, vimentin and Snai2 were examined in ASAP1-expressing and control SKOV3 cells by western blot. One representative western blot was presented from three similar independent experiments. Band intensity was compared between ASAP1 overexpression and control group (* $P < 0.05$, ** $P < 0.01$). (B) SKOV3 cell morphology in ASAP1 expressing and control cells. (C) EMT markers were detected by western blot and compared in ASAP1 expressing and control OVCAR3 cells (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (D) OVCAR3 cell morphology in ASAP1 expressing and control cells.

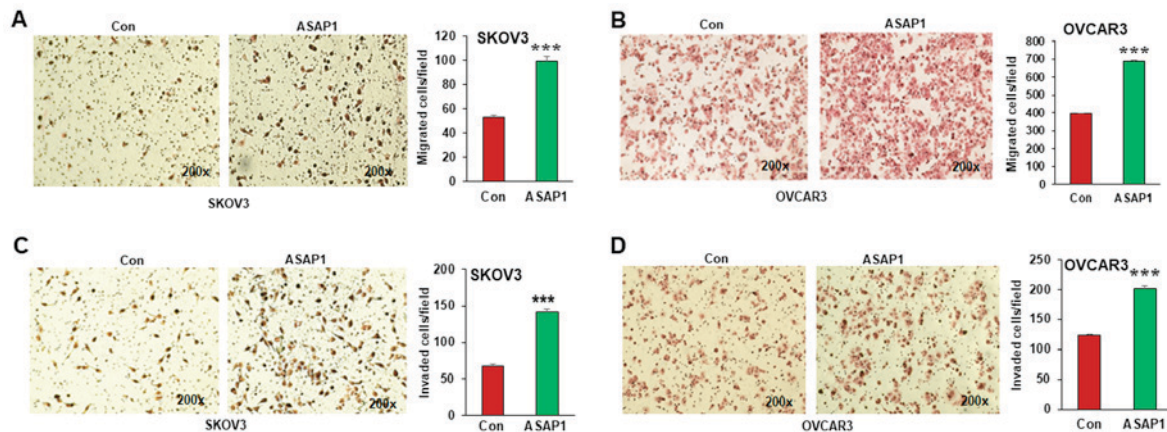


Figure 3. Overexpression of ASAP1 promoted cell migration and invasion in ovarian cancer cells. (A, B) Transwell migration assays were performed in ASAP1-expressing and control SKOV3 and OVCAR3 cells. Transmigrated cells were hematoxylin-eosin (H&E) stained and counted. Migrated cells in ASAP1 overexpressing SKOV3 and OVCAR3 cells were compared with empty vector transduced control cells (*** $P < 0.001$). (C, D) Invasion assays were performed using Matrigel-coated transwell plates with SKOV3 and OVCAR3 cells transduced by ASAP1 and luciferase control lentiviral vectors. The number of invading cells were counted after H&E staining. The invaded cells in ASAP1 overexpressing SKOV3 and OVCAR3 cells were compared to that in empty vector transduced control cells (*** $P < 0.001$).

in both SKOV3 and OVCAR3 cells on transwell plates. We also examined cell invasion of ovarian cancer cells using matrigel coated transwell plates. Overexpression of ASAP1 significantly promoted cell invasion in both SKOV3 (Fig. 3C) and OVCAR3 (Fig. 3D) cells.

Overexpression of ASAP1 promoted ovarian cancer cell proliferation and survival. To determine whether ASAP1 plays a role on cell proliferation, we performed MTT assays on ASAP1 expressing and control ovarian cancer cells at different time points (1, 2 and 3 days). Overexpressing ASAP1

significantly increased cell proliferation in both SKOV3 and OVCAR3 cells (Fig. 4A and B). To determine cell survival, we performed cell clonogenic assays and counted cell colonies in ASAP1 expressing and control SKOV3 and OVCAR3 cells. Overexpression of ASAP1 significantly promoted cell survival in both cell lines (Fig. 4C). In addition, to examine the role of ASAP1 in tumorigenesis, we examined the anchorage-independent cell growth by soft agar assays, which is an *in vitro* method to detect tumor cell malignancy. Overexpression of ASAP1 significantly increased the number of colonies formed in soft agar as compared to control cells (Fig. 4D).

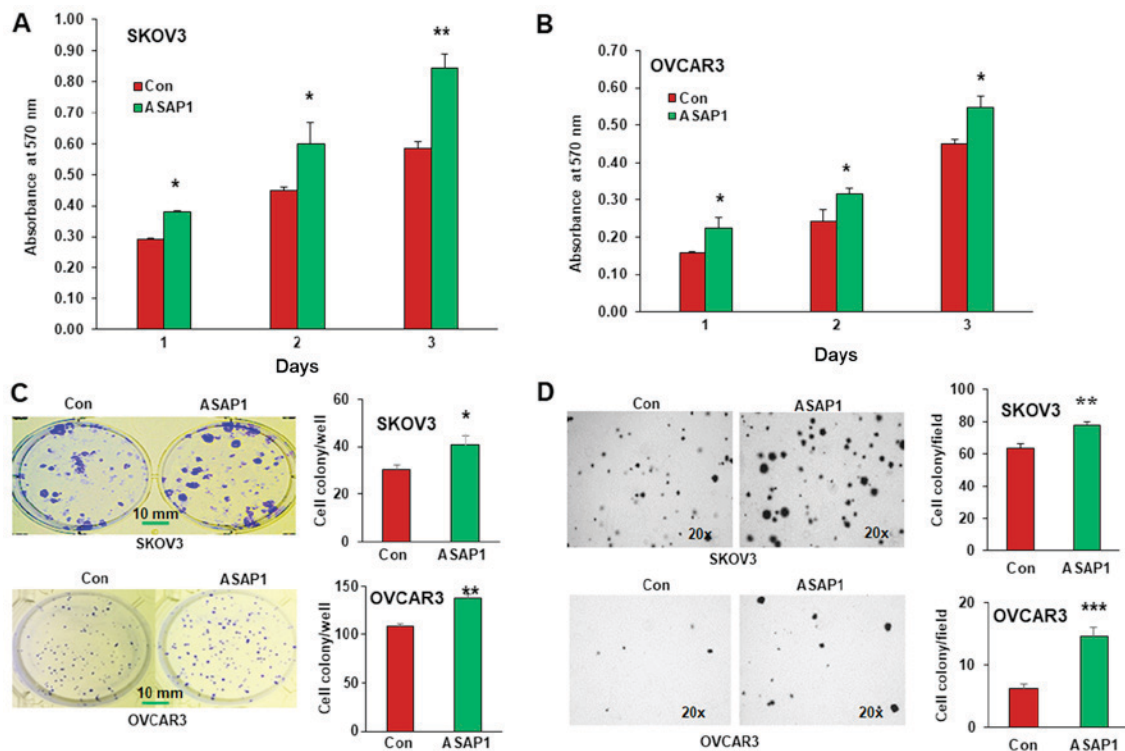


Figure 4. ASAP1 increased colony formation of ovarian cancer cells in both soft-agar and monolayer culture conditions. (A, B) Cell proliferation in ASAP1 expressing SKOV3 and OVCAR3 cells was detected by MTT assay and compared to empty vector transduced controls at different time points (* $P < 0.05$, ** $P < 0.01$). (C) Cell colonies in ASAP1 overexpressing SKOV3 and OVCAR3 cells was compared to empty vector transduced control cells (* $P < 0.05$, ** $P < 0.01$). (D) Anchorage-independent growth in soft agar was performed with ASAP1-expressing and control SKOV3 and OVCAR3 cells and cell colonies were counted per field under microscopy and compared between ASAP1 overexpressing and empty vector transduced control cells (* $P < 0.01$, *** $P < 0.001$).

Overexpression of ASAP1 inhibited apoptosis induced by chemotherapy drug paclitaxel in ovarian cancer cells. To determine the role of ASAP1 in cell apoptosis, we treated ovarian cancer cells with different doses of the chemotherapy drug paclitaxel, and cell apoptosis was examined by measuring Caspase3/7 activity. Overexpression of ASAP1 led to 1.7-fold decrease of apoptosis in ASAP1 expressing SKOV3 cells compared to control (Fig. 5A). Furthermore, ASAP1 expression inhibited cell apoptosis induced by paclitaxel by 1.6-fold when cells were treated with 20 and 40 nM paclitaxel, respectively (Fig. 5A). Overexpression of ASAP1 in OVCAR3 cells led to 1.4-fold decrease in apoptosis when cells were treated with 20 or 40 nM paclitaxel (Fig. 5B). Cell apoptosis was also examined by detecting cleaved-PARP and cleaved-caspase3 using western blot, ASAP1 expression decreased paclitaxel induced cell apoptosis in both SKOV3 (Fig. 5C) and OVCAR3 cells (Fig. 5D). These data indicate that ASAP1 expression in ovarian cancer cells promoted chemoresistance.

Discussion

In the present study, for the first time we showed that endogenous ASAP1 was highly expressed in ovarian cancer compared to normal ovaries, and ASAP1 expression was associated with overall poor patient survival by analyzing TCGA database, suggesting that ASAP1 is a potential biomarker for diagnosis and prognosis of ovarian cancer patients. Our finding was consistent with previous studies that ASAP1 was also highly expressed in several other cancers including melanoma (15),

colorectal cancer (9), head and neck carcinoma (10), and breast cancer (11). Interestingly, ASAP1 expression was shown to correlate with the poor prognosis of ovarian cancer patients (12). ASAP1 was endogenously expressed in both SKOV3 and OVCAR3, although we are not able to compare it with normal human ovarian epithelial cells. However, ASAP1 was not detectable in normal mammary epithelial cells (11). Our studies indicated that ASAP1 expression was associated with poor survival and prognosis in ovarian cancer patients. However, further studies are required to understand how ASAP1 expression is correlated with the different types of ovarian cancer, as well as disease grade and stage.

Although we used a more invasive SKOV3 and less invasive OVCAR3 cells for our studies, morphologically, SKOV3 showed a mesenchymal while OVCAR3 displayed an epithelial phenotype. We used gain of function approach to define the role of ASAP1 in ovarian cancer cells. For the first time we have shown that ASAP1 functions as an oncogene by promoting EMT in ovarian cancer cells. Overexpression of ASAP1 significantly altered EMT marker gene expression in both SKOV3 and OVCAR3 cells compared to control cells transduced with empty vector (Fig. 2A and C). In OVCAR3 cells, we did not observe a robust EMT phenotype switch following ASAP1 expression as we observed in SKOV3 cells. This was not surprise since OVCAR3 cells are well-differentiated epithelial cells and non-invasive (16). Overexpression of ASAP1 in OVCAR3 cells was not sufficient to fully convert epithelial to mesenchymal phenotypes, but only a partial phenotypic switch. In this case, it may require additional factors for a full phenotypic switch.

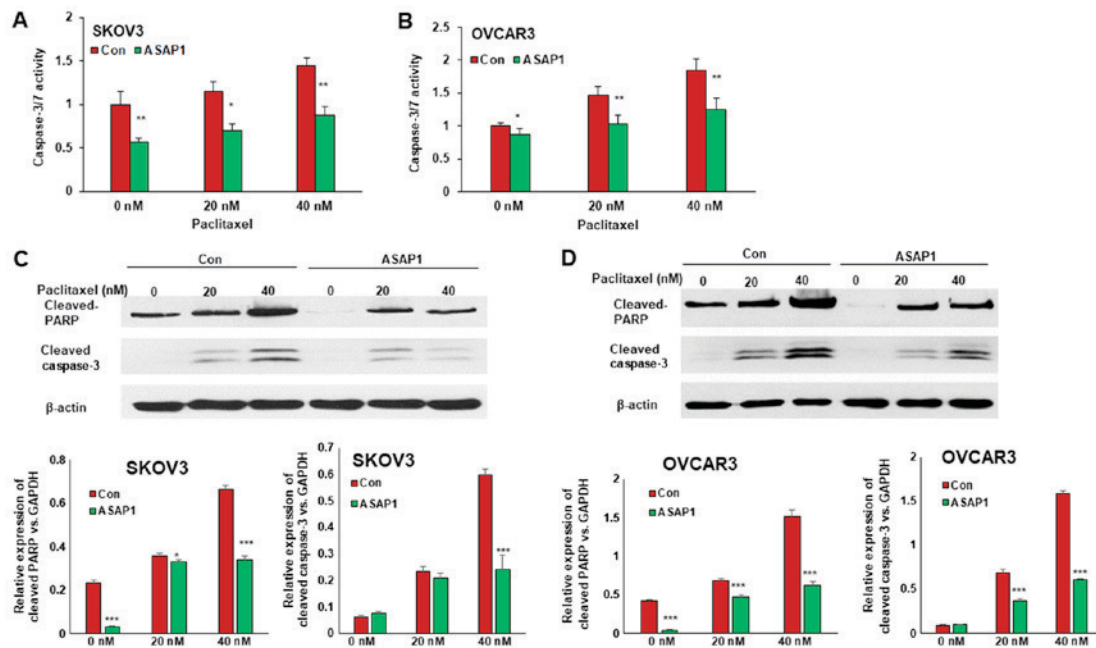


Figure 5. Overexpression of ASAP1 inhibited cellular apoptosis induced by chemotherapy drug paclitaxel in ovarian cancer cells. (A, B) Paclitaxel induced cell apoptosis was detected by determining caspase3/7 activity and compared in SKOV3 (A) and OVCAR3 (B) cells transduced with ASAP1 expressing with control vectors, respectively (* $P < 0.05$, ** $P < 0.01$). (C, D) Apoptosis in SKOV3 (C) and OVCAR3 (D) cells transduced with ASAP1 and control lentiviral vectors was examined by detecting cleaved-PARP and Caspase3 using western blot and band density was compared in ASAP1 overexpressing to empty vector transduced control cells (* $P < 0.05$, *** $P < 0.001$). One representative western blot was presented from three similar independent experiments.

Extensive studies demonstrated that EMT was associated with tumor metastasis and chemoresistance in various human cancers. We showed that overexpression of ASAP1 promoted EMT in both SKOV3 and OVCAR3 cells, indicating that ASAP1 may contribute to tumor metastasis and chemoresistance in ovarian cancer cells. Although it is not clear how ASAP1 contributes to EMT in ovarian cancer cells, it was previously found that GEP100 activated an Arf6 pathway with ASAP1 serving as an effector via receptor tyrosine kinases (RTKs), thus activated beta1 integrins and disrupted E-cadherin-based adhesion and promoted EMT in breast cancer (17). Arf6-ASAP1-EPB41L5 axis is another pathway to contribute to ASAP1-mediated EMT (18). ASAP1 was also shown to bind the SH3 domain of several members of the src family through its proline-rich domain and contributed to tumor metastasis in colorectal cancer (9). In addition, the non-coding endogenous small miRNAs also play a role in regulating EMT by directly targeting EMT marker genes or through indirect regulation. We previously showed that miR-203 inhibited EMT by targeting mesenchymal marker Snail in ovarian cancer cells (19). Interestingly, ASAP1 was identified as a target gene of miR-203 and overexpression of miR-203 led to inhibition of EMT in prostate cancer cells (20). Although we did not test whether miR-203 directly regulated ASAP1 and thereby inhibited EMT in ovarian cancer cells, it was highly possible that ASAP1 mediated EMT was also regulated by miR-203 in ovarian cancer cells. ASAP1 expression promoted EMT in ovarian cancer cells, which may lead to tumor metastasis and chemoresistance. We showed that overexpression of ASAP1 promoted cell proliferation, migration and invasion in both SKOV3 and OVCAR3 cells. Although the overexpression level of ASAP1 in OVCAR3 cells is higher than that in SKOV3 cells, the migration and

invasion were similar in both cell lines (Fig. 3), which may be caused by more invasive SKOV3 compared with less invasive OVCAR3 cells (16).

Therefore, ASAP1 may promote ovarian tumor metastasis. ASAP1 was shown to promote tumor metastasis in breast cancer (11), colorectal (9) and prostate cancer (21). The role of ASAP1 on ovarian tumor metastasis is presently being tested in our lab using an orthotopic ovarian cancer mouse model. Moreover, ASAP1 expression can be inhibited with a small inhibitor UCS15A by disrupting ASAP1 binding with cortactin, thus blocking breast tumor metastasis (11). ASAP1 expression inhibited apoptosis in both SKOV3 and OVCAR3 cells induced by the chemotherapy drug paclitaxel as we showed by two different assays including luciferase reporter gene assay (Fig. 5A and B) and western blot (Fig. 5C and D). The luciferase reporter gene assay for apoptosis was more sensitive than western blot (22), thus we observed the significant difference of apoptosis at 20 nM by measuring caspase3/7 activity. Thus, inhibition of ASAP1 expression using small molecule inhibitor like UCS15A may provide a novel approach in treating ovarian cancer patients by inhibiting cell invasion and enhancing the efficacy of chemotherapy drugs.

In summary, our studies revealed that the high ASAP1 expression was associated with poor ovarian patient survival and prognosis. Overexpression of ASAP1 promoted ovarian cancer cell proliferation, migration and invasion, and enhanced chemoresistance by promoting EMT. This study is a part of our long-term work to define the molecular mechanisms underlying ASAP1 function in ovarian cancer. We have already generated ASAP1 knockout cell lines using CRISPR/Cas9 and they will be used to address its function in ovarian tumor metastasis as our further studies. Targeting ASAP1 using small molecule

inhibitors may inhibit tumor metastasis and enhance the efficacy of chemotherapy drugs by inhibiting EMT, thus provide a novel target for ovarian cancer therapy.

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