Long non-coding RNA *ASAP1-IT1* suppresses ovarian cancer progression by regulating Hippo/YAP signaling

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Received July 3, 2020; Accepted December 11, 2020

DOI: 10.3892/ijmm.2021.4877

Abstract. Long non-coding RNAs (lncRNAs) are a class of non-protein coding transcripts that are involved in the regulation of gene expression in mammalian cells. Transcriptional co-activator Yes associated protein 1 (YAP1) plays a key role in the progression of ovarian cancer. However, the regulation of Hippo/YAP signaling in ovarian cancer remains elusive. In the present study, the expression levels of lncRNA *ASAP1-IT1* were investigated. The analysis indicated that lncRNA *ASAP1-IT1* expression was downregulated in ovarian tumor samples and ovarian cancer cells. The overexpression of *ASAP1-IT1* inhibited ovarian cancer cell proliferation and induced cell apoptosis. Bioinformatics analysis predicted that miR-2278, a previously reported upregulated miRNA in ovarian tumors, may bind to *ASAP1-IT1*. Dual luciferase assay confirmed the direct regulatory association between *ASAP1-IT1* and miR-2278. In addition, the data demonstrated that large tumor suppressor 2 (LATS2) was a target gene of miR-2278, whose expression was upregulated by *ASAP1-IT1* in ovarian cancer cells. By regulating the expression of LATS2, *ASAP1-IT1* induced the downregulation of YAP1 expression in ovarian cancer cells. Moreover, the silencing of LATS2 attenuated the inhibition of cell proliferation and the apoptosis induced by *ASAP1-IT1* overexpression in ovarian cancer cells. The association among the expression levels of *ASAP1-IT1*, miR-2278 and LATS2 was observed in specimens obtained from patients with ovarian cancer. Taken together, the data presented herein demonstrate that *ASAP1-IT1* functions as a potential tumor suppressor lncRNA by upregulating LATS2 expression in ovarian cancer.

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Key words: *ASAP1-IT1*, ovarian cancer, microRNA-2278, Hippo/YAP signaling, large tumor suppressor 2

Introduction

Ovarian cancer is the 7th most common form of cancer encountered in women worldwide, with an estimated 295,000 new cases and 185,000-related deaths in 2018 (1). Epithelial ovarian cancer is the most prevalent type of ovarian cancer, which can be further classified into endometrioid, clear-cell, mucinous, high-grade and low-grade serous carcinomas (2). Despite advancements being made in the understanding of ovarian cancer pathology and the application of novel early diagnostic and treatment strategies for patients with ovarian cancer, the majority of the patients are diagnosed at an advanced stage of the disease and the 5-year overall survival rate has been estimated to <50% (3). Therefore, the identification of novel targets for the diagnosis and treatment of patients with ovarian cancer is of utmost importance.

Long non-coding RNAs (lncRNAs) are single-stranded RNA molecules that are ≥200 nucleotides in length (4). According to the competing endogenous RNA hypothesis, certain lncRNAs contain miRNA response elements and can compete for binding to miRNAs with mRNAs, resulting in the regulation of gene expression (5). In doing so, lncRNAs are implicated in almost all physiological processes (6). Recent studies have indicated that the aberrant expression of lncRNAs is involved in cancer initiation and development, and notably in cancer cell proliferation and apoptosis (7). For example, lncRNA *DNM3OS* has been shown to be involved in the epithelial-to-mesenchymal transition process and to promote ovarian cancer cell migration and invasion (8). lncRNA *ABHD11-AS1* has been shown to facilitate ovarian cancer cell proliferation, migration and invasion, and inhibit cell apoptosis via the upregulation of RhoC expression (9). lncRNA *ASAP1-IT1* is an intronic transcript of the ASAP1 gene (10). A previous study indicated that *ASAP1-IT1* expression was decreased in high-grade ovarian tumors compared with the corresponding expression noted in low-grade tumors (11). A high expression of *ASAP1-IT1* has been shown to be associated with the optimal prognosis of patients with ovarian cancer (11). However, the mechanisms through which *ASAP1-IT1* contributes to ovarian cancer progression remain unknown.

Cell number and cell size are tightly controlled by the Hippo pathway (12). The dysregulation of the Hippo pathway leads to the overgrowth of cells and resistance to cell apoptosis (13).

Accumulating evidence has suggested that the overexpression of Yes associated protein 1 (YAP1), which is the downstream effector of the Hippo pathway, contributes to cancer development and initiation in different organs, including the colon, bladder, breast and ovaries (14-17). The higher expression of YAP1 has been observed in ovarian cancer tissues and cells, and is considered critical for cancer cell proliferation and survival (18). Among several tumor suppressive kinases of the Hippo pathway, the loss of large tumor suppressor 2 (LATS2) expression has been considered a major cause of YAP1 overexpression in cancer cells (19,20). Decreased expression of LATS2 was also reported in ovarian cancer (21). However, the regulation of LATS2/YAP1 signaling by lncRNAs in ovarian cancer remains elusive.

In the present study, the overexpression of *ASAP1-IT1* was found to inhibit cell proliferation and induce apoptosis by sponging miR-2278. Concomitantly, it was able to increase LATS2 expression in ovarian cancer. The findings presented herein indicate that the *ASAP1-IT1*/miR-2278/LATS2 axis may play a key role in ovarian cancer.

Materials and methods

Clinical samples. Consecutive patients with ovarian cancer (n=58) who were treated at the China-Japan Union Hospital of Jilin University between January, 2015 and January, 2017, were used in the present study. The healthy tissues (n=58) were those 2 cm away from the tumors. The clinical stage and metastatic status of the cancer was determined by surgical evaluation, whereas histopathological analysis was conducted by 2 gynecological pathologists to assess cancer type and grade, independently. The extracted tissue samples were stored at -80˚C. All the participants in the present study provided written informed consent prior to the surgery and the study conduct. Ethical approval was also provided for their participation by the Ethical Committee of The China-Japan Union Hospital of Jilin University.

Cell lines and cell culture. The human ovarian surface epithelial cell line HOSEpiC was purchased from ScienCell. The human ovarian serous cystadenocarcinoma cell line, SKOV3, and the human ovarian serous adenocarcinoma cell line, OVCAR3, were obtained from the American Type Culture Collection (ATCC). These cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) and supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The cells were maintained in an incubator at 37˚C with 5% CO_2.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the HOSEpiC, OVCAR3, SKOV3 cells and tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. RNA was reverse transcribed into first-stranded cDNA using the RevertAid RT Reverse Transcription kit (cat. no. K1691, Thermo Fisher Scientific, Inc.). RT-qPCR was carried out with TB Green® Premix Ex Taq™. The reaction conditions included the following steps: Step 1: 95˚C for 30 sec; step 2: 35 cycles of 95˚C for 10 sec and 60˚C for 30 sec. GAPDH was used as the internal control for lncRNA and mRNA, while U6 was used as the control for miRNA expression. The relative expression of each gene was calculated with the $2^{-\Delta\Delta Cq}$ method (22). The primer sequences are listed in Table I.

Overexpression of ASAP1-IT1. The full length of *ASAP1-IT1* was amplified from HOSEpiC cDNA, digested by restriction endonucleases *Eco*RI (NEB) and *Xho*I (NEB) and ligated into the pcDNA3.1 plasmid (Invitrogen; Thermo Fisher Scientific). The primer sequences were as follows: *ASAP1-IT1* forward, 5'-GGGTACCCAAATGGGAAAAAAA-3' and reverse, 5'-GGAATTCCAAAGACTATCACA-3'. For overexpression experiments, 2 μg pcDNA3.1-*ASAP1-IT1* were transfected into the OVCAR3 and SKOV3 cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The cells were allowed to grow for 48 h and RT-qPCR was performed to confirm the overexpression of *ASAP1-IT1* as described above.

Overexpression and downregulation of miR-2278. miR-NC mimic, miR-2278 mimic, miR-NC inhibitor and miR-2278 inhibitor were purchased from Suzhou GenePharma Co., Ltd. For transfection, 50 nM miR-NC mimic (5'-GUGGAUUUU CCUCUAUGAUUU-3'), miR-2278 mimic (5'-GAGAGC AGUGUGUGUUGCCUGG-3'), miR-NC inhibitor (5'-UUC UCCGAACGUGUCACGUUU-3') or miR-2278 inhibitor (5'-CCAGGCAACACACACUGCUCUC-3') were transfected into $1x10^6$ OVCAR3 or SKOV3 cells in 6-well plates using Lipofectamine 300 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 48 h of cell growth, the cells were harvested and the transfection efficiency was determined by RT-qPCR as described above.

Small interference RNA mediated silencing of LATS2. Control siRNA (5'-UUCUCCGAACGUGUCACGUTT-3') and LATS2 siRNA (5'-UACCAUAAAUACAAUCUUCTT-3') were synthesized and purchased from Suzhou GenePharma Co., Ltd. A total of 50 nM control siRNA or LATS2 siRNA were transfected into $1x10^6$ OVCAR3 or SKOV3 cells in 6-well plates using Lipofectamine 300 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, cells were harvested and the transfection efficiency was determined by western blot analysis as described below.

Cell proliferation assay. The proliferative ability of the OVCAR3 and SKOV3 cells was detected using the CCK-8 kit (Dojindo Molecular Technologies, Inc.). Briefly, 10 μl CCK-8 solution were added into the culture medium and sustained for 1 h at 37˚C. The absorbance at 450 nM of the medium was measured using a microplate reader (iMark™, Bio-Rad Laboratories, Inc.).

Cell apoptosis assay. The percentage of apoptotic cells was detected by flow cytometry. OVCAR3 and SKOV3 cells were incubated for 48 h at 37˚C following transfection, harvested and stained with propidium iodide (PI) and Annexin-V provided by the Annexin-V Apoptosis Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The stained cells were examined by flow cytometry on a BD FACSVerse flow cytometer (BD Biosciences). PI^+/Annexin-V^+ and PI-/Annexin-V^+ cells were considered as apoptotic cells.

Table I. Sequences of primer used for RT-qPCR.

Primer	Sequence
LATS2-forward	5'-ACTTTTCCTGCCACGACTTATTC-3'
LATS2-reverse	5'-GATGGCTGTTTTAACCCCTCA-3'
YAP1-forward	5'-TAGCCCTGCGTAGCCAGTTA-3'
YAP1-reverse	5'-TCATGCTTAGTCCACTGTCTGT-3'
SGK1-forward	5'-CATATTATGTCGGAGCGGAATGT-3'
SGK1-reverse	5'-TGTCAGCAGTCTGGAAAGAGA-3'
FGF2-forward	5'-AGAAGAGCGACCCTCACATCA-3'
FGF2-reverse	5'-CGGTTAGCACACACTCCTTTG-3'
ETV5-forward	5'-TCAGCAAGTCCCTTTTATGGTC-3'
ETV5-reverse	5'-GCTCTTCAGAATCGTGAGCCA-3'
ASAP1-IT1-forward	5'-TCTGGTCCAAAAAGATTTCTGA-3'
ASAP1-IT1-reverse	5'-CTTTGCAGAAAGCTTTTACCATA-3'
GAPDH-forward	5'-ACAACTTTGGTATCGTGGAAGG-3'
GAPDH-reverse	5'-GCCATCACGCCACAGTTTC-3'
Stem-loop primer	5'-CTCAACTGGTGTCGTGGAGTCGGCAATT CAGTTGAGCCAGGA-3'
miR-2278-forward	5'-GCCGAGGAGAGCAGTGTGTGTT-3'
miR-2278-reverse	5'-CTCAACTGGTGTCGTGGA-3'
U6-forward	5'-CTCGCTTCGGCAGCACA-3'
U6-reverse	5'-AACGCTTCACGAATTTGCGT-3'

Bioinformatics analysis. The sequence of *ASAP1-IT1* was entered into the online miRDB software (http://mirdb.org/) (23) to predict potential binding miRNAs. The potential target genes of miR-2278 were predicted with the TargetScan software (http://www.targetscan.org/vert_72/) (24).

Western blot analysis. Tissues and cells were subjected to protein extraction using RIPA lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The lysates were quantified with a BCA protein assay kit (Thermo Fisher Scientific, Inc.). A total of 20 μg proteins were loaded into each lane of the 8% SDS-PAGE gel and electrophoresis was performed. The proteins were transferred to a PVDF membrane. Subsequently, the membrane was blocked in 5% non-fat milk for 1 h at room temperature and incubated with primary and secondary antibodies at room temperature for 1 h at 37˚C. The blots were developed using the ECL Western blot substrate (Pierce; Thermo Fisher Scientific, Inc.). The results were quantified using ImageJ software (version 1.8, National Institutes of Health). The antibodies used were as follows: YAP1 (cat. no. 14074, 1:1,000), p-YAP1 (Ser127l; cat. no. 13008, 1:1,000), LATS2 (cat. no. 5888, 1:1,000) and GAPDH (cat. no. 97166, 1:10,000) antibodies were purchased from Cell Signaling Technology, Inc; p-LATS2 (T1079 + T1041; cat. no. ab111344, 1:1,000) antibody was bought from Abcam. HRP-conjugated secondary antibodies against mouse (cat. no. L3032-2, 1:5,000) and rabbit (cat. no. L3012-2, 1:5,000) were products of Signalway Antibody LLC.

Dual luciferase reporter assay. The full length of *ASAP1-IT1* was sub-cloned from pcDNA3.1 into the pmirGLO plasmid (Promega Corporation). The 3'UTR of LATS2 was amplified from HOSEpiC cDNA, digested with restriction endonucleases *Nhe*I (NEB) and *Sal*I (NEB) and then ligated into the pmirGLO plasmid. Point mutations were introduced into the putative binding site for miR-2278 on *ASAP1-IT1* and LATS2 3'UTR with a QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Inc.) following the manufacturer's protocol. OVCAR3 and SKOV3 cells were seeded at a density of $5x10^5$ cells in 24-well plates and transfected with 1 μg pmirGLO-*ASAP1-IT1*-WT, pmirGLO-*ASAP1-IT1*-Mut, pmirGLO-LATS2 3'UTR-WT or pmirGLO-LATS2 3'UTR-Mut. The cells were incubated for 48 h following transfection and subsequently collected for the determination of the relative luciferase activity, which was detected with a Dual Luciferase Reporter System (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis. Graphpad Prism 6 was used to analyze the data and the data are presented as the means ± SD. Differences between 2 groups were compared with the Student's t-test and those between multiple groups were compared with one-way ANOVA followed by a Tukey's post hoc test. The log rank test and Kaplan-Meier curve were used for survival analysis and the graphs were prepared using Graphpad Prism 6. The association between ASAP1-IT1, miR-2278 and LATS2 expression with the clinicopathological characteristics of 58 ovarian tumors was examined using the Chi-squared test. The correlation between the differences in the mRNA expression levels of *ASAP1-IT1*, miR-2278 and *LATS2* were analyzed by the Pearson's correlation analysis. A P-value <0.05 ($P<0.05$) was considered to indicate a statistically significant difference.

Table II. Association of ASAP1-IT1, miR-2278 and LATS2 expression with the clinicopathological characteristics of 58 patients with ovarian tumors.

Clinicopathological features	Total number	ASAP1-IT1 expression			miR-2278 expression			LATS2 expression		
		Low	High	P-value	Low	High	P-value	Low	High	P-value
Pathological type				0.395			0.155			0.777
Serous carcinoma	40	22	18		17	23		19	21	
Other pathology types	18	7	11		12	6		10	8	
Age, years				0.999			0.292			0.599
>50	31	16	15		18	13		14	17	
≤50	27	13	14		11	16		15	12	
FIGO stage				0.019			0.003			0.248
I-II	17	4	13		14	3		11	6	
III-IV	41	25	16		15	26		18	23	
Differentiation status				0.021			0.747			0.331
Well	12	2	10		5	7		8	4	
Moderate and poor	46	27	19		24	22		21	25	
Lymph node metastasis				0.229			0.549			0.229
Negative	15	5	10		6	9		10	5	
Positive	43	24	19		23	20		19	24	

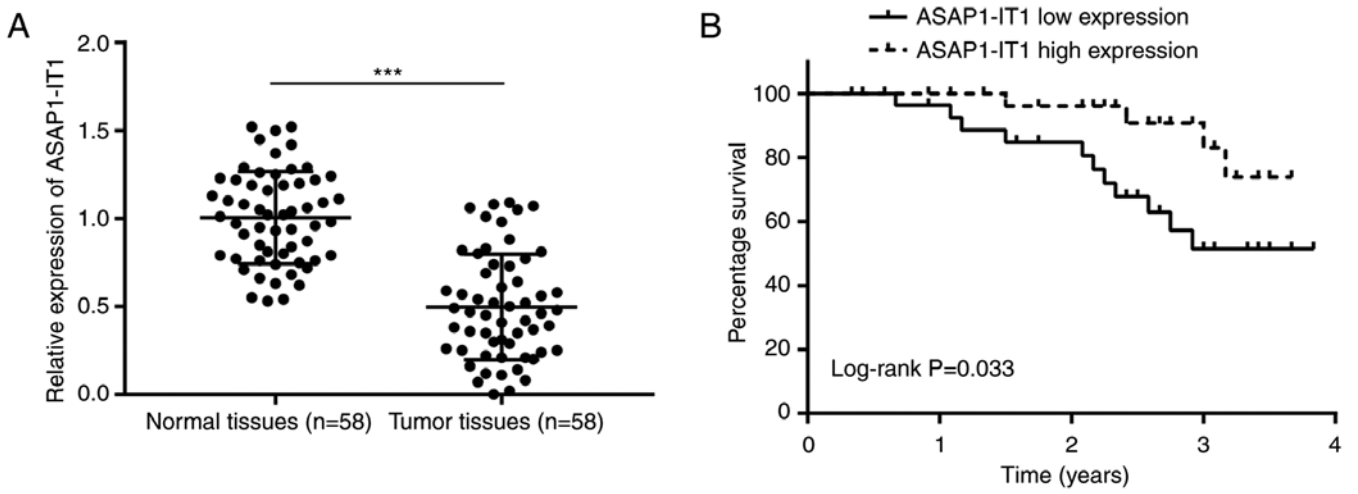

Figure 1. *ASAP1-IT1* overexpression is associated with a good prognosis of patients with ovarian cancer. (A) RT-qPCR results showing that *ASAP1-IT1* was downregulated in ovarian tumors compared to healthy ovary tissues obtained from 58 patients with ovarian cancer. (B) Kaplan-Meier analysis showing that a high expression of *ASAP1-IT1* was associated with the prolonged overall survival of patients with ovarian cancer. ***P<0.001.

Results

High ASAP1-IT1 levels predict the optimal prognosis of patients with ovarian cancer. To examine the role of *ASAP1-IT1* in ovarian cancer, 58 pairs of ovarian tumors were collected and matched with healthy tissues from the patients. The expression levels of *ASAP1-IT1* were downregulated in ovarian tumor tissues compared with the non-cancer specimens (Fig. 1A). No significant associations were observed between *ASAP1-IT1* expression and the clinical parameters of pathological type, patient age and lymph node metastasis (Table II). However, disease stage and differentiation status were associated with *ASAP1-IT1* expression (Table II). Subsequently, the patients were divided into the *ASAP1-IT1* high and low expression groups according to the median expression of *ASAP1-IT1*. The results of Kaplan-Meier analysis indicated that a high expression of *ASAP1-IT1* was associated with the optimal prognosis of patients with ovarian cancer, whereas the 3-year survival rate was estimated at 85 and 45% in the high and low expression groups, respectively (Fig. 1B). These results highlighted the potential involvement of *ASAP1-IT1* in the progression of ovarian cancer.

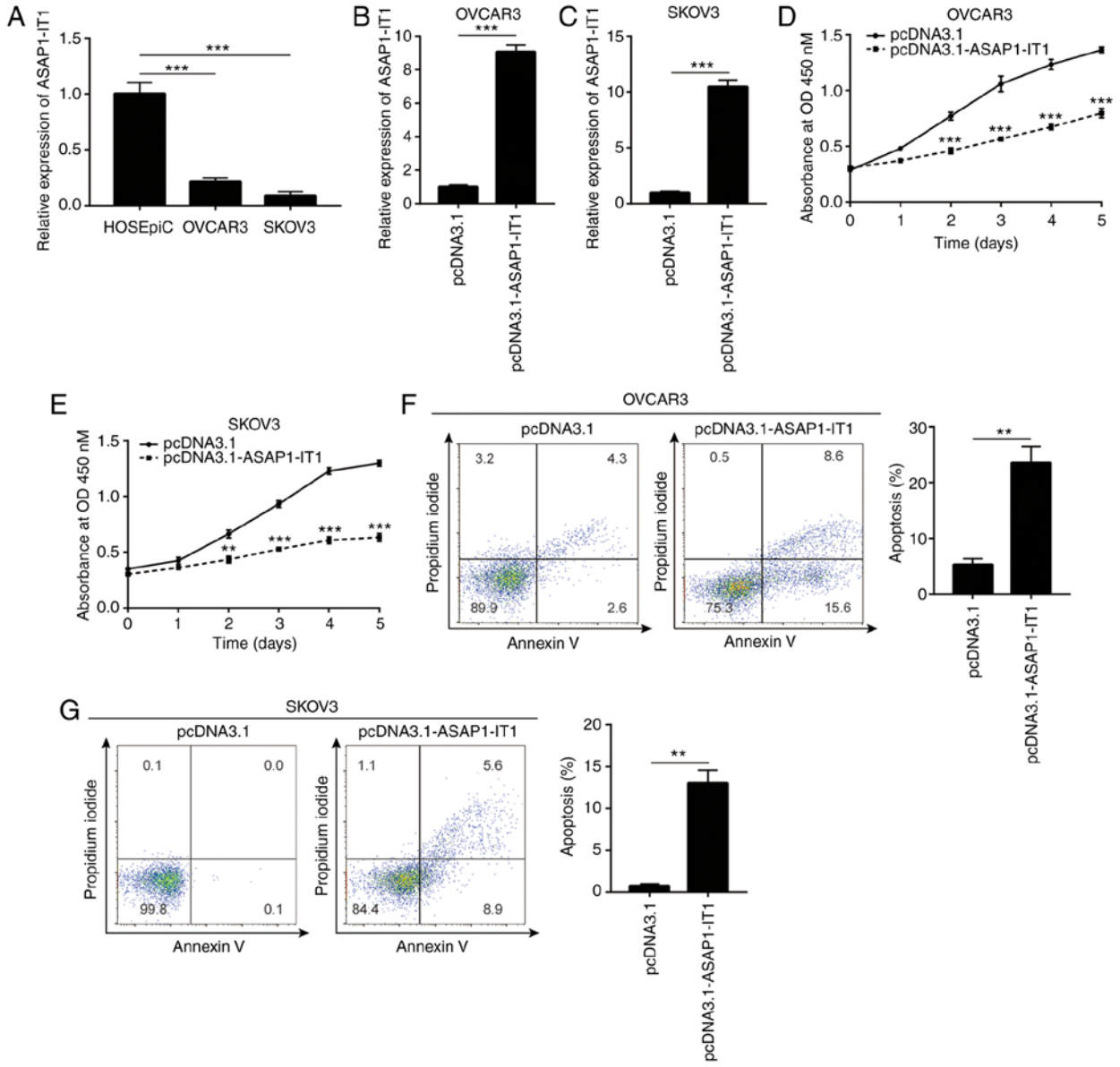

Figure 2. Overexpression of *ASAP1-IT1* inhibits ovarian cancer cell proliferation and induces cell apoptosis. (A) RT-qPCR showing that *ASAP1-IT1* was downregulated in ovarian cancer cells (OVCAR3 and SKOV3) compared to normal ovarian cells (HOSEpiC). (B) Transfection with recombinant *ASAP1-IT1* increased *ASAP1-IT1* expression in OVCAR3 cells. (C) Transfection with recombinant *ASAP1-IT1* increased *ASAP1-IT1* expression in SKOV3 cells. (D) Overexpression of *ASAP1-IT1* decreased the proliferative ability of the OVCAR3 cells. (E) Overexpression of *ASAP1-IT1* decreased the proliferative ability of the SKOV3 cells. (F) Overexpression of *ASAP1-IT1* induced the apoptosis if OVCAR3 cells. (G) Overexpression of *ASAP1-IT1* induced the apoptosis of SKOV3 cells. **P<0.01; ***P<0.001.

Overexpression of ASAP1-IT1 inhibits ovarian cancer cell proliferation and induces apoptosis. To investigate the biological function of *ASAP1-IT1* in ovarian cancer cells, RT-qPCR was conducted to detect *ASAP1-IT1* expression in normal ovarian cells and ovarian cancer cells. It was found that *ASAP1-IT1* expression was decreased in OVCAR3 and SKOV3 cells, which are 2 ovarian cancer cell lines, compared with the corresponding expression noted in the HOSEpiC cell line (Fig. 2A). The vector containing the full length of *ASAP1-IT1* was transfected into the OVCAR3 and SKOV3 cells in order to increase *ASAP1-IT1* expression (Fig. 2B and C). The proliferative ability of the OVCAR3 and SKOV3 cells was detected by CCK-8 assay and the data indicated that it was significantly inhibited following *ASAP1-IT1* overexpression (Fig. 2D and E). Moreover, flow cytometric analysis indicated an increase in the number of apoptotic cells observed in the OVCAR3 and SKOV3 pcDNA3.1-*ASAP1-IT1*-transfected cell lines (Fig. 2F and G). These data suggested that *ASAP1-IT1* was involved in ovarian cancer cell proliferation and survival.

ASAP1-IT1 inactivates YAP1 signaling in ovarian cancer cells. Hippo/YAP1 signaling is critical for ovarian cancer cell proliferation and survival (17). In the present study, western blot analysis was used to detect the expression levels of LATS2, which is the major kinase component of the Hippo pathway (19). Moreover, the expression of YAP1 was detected in ovarian cancer cells following *ASAP1-IT1* overexpression. *ASAP1-IT1* overexpression increased LATS2 and p-LATS2 expression, and decreased YAP1 protein expression in the OVCAR3 and SKOV3 cells (Fig. 3A and B). An increase in

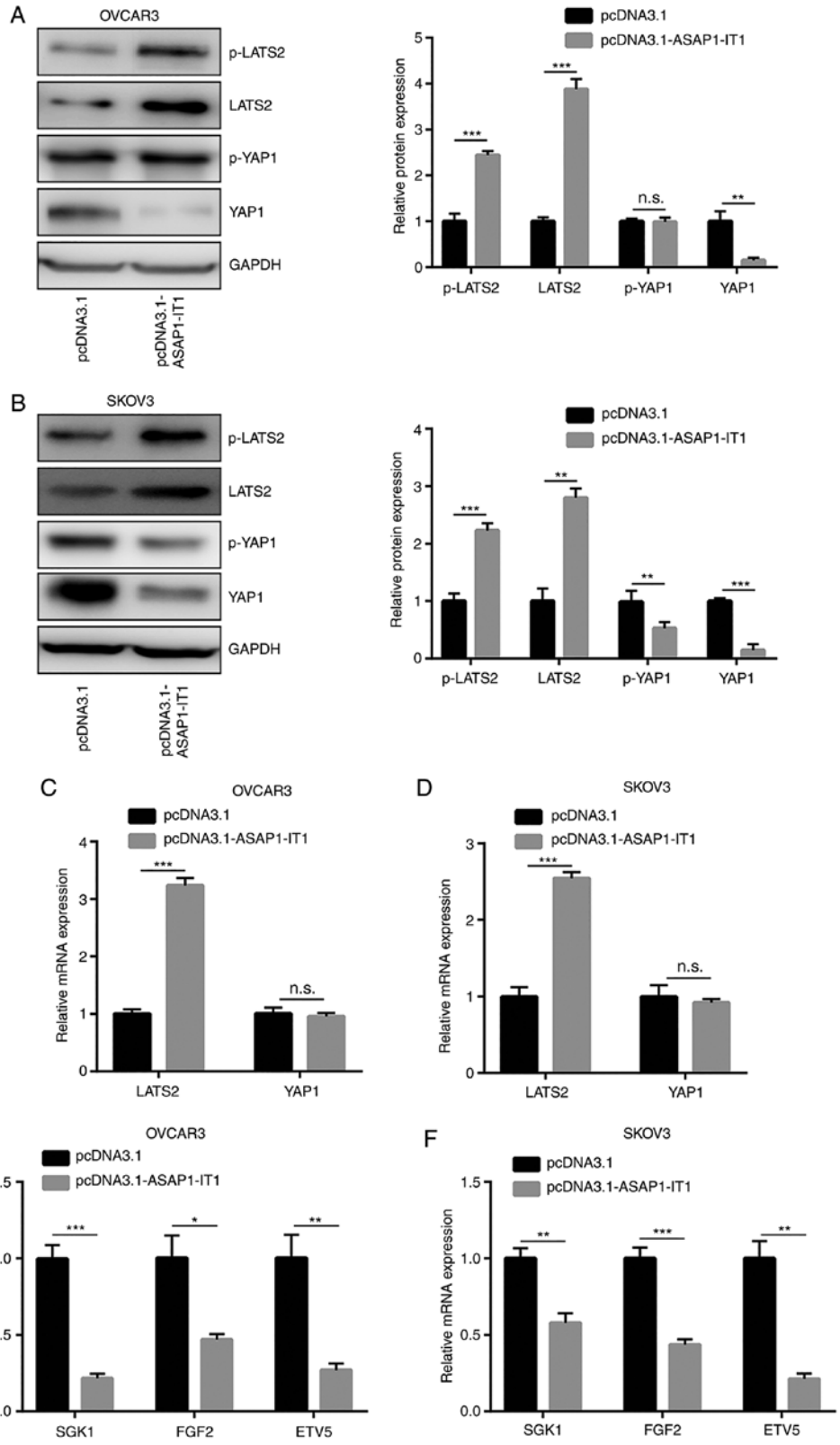

Figure 3. *ASAP1-IT1* overexpression increases LATS2 expression and inactivates YAP1 signaling in ovarian cancer cells. (A) Western blot analysis showing that the overexpression of *ASAP1-IT1* elevated LATS2 and p-LATS2, and decreased YAP1 expression in OVCAR3 cells. (B) Western blot analysis showing that the overexpression of *ASAP1-IT1* elevated LATS2 and p-LATS2, and decreased YAP1 and p-YAP1 expression in SKOV3 cells. (C) RT-qPCR showing that *ASAP1-IT1* overexpression upregulated *LATS2* mRNA expression in OVCAR3 cells. (D) RT-qPCR showing that *ASAP1-IT1* overexpression upregulated *LATS2* mRNA expression in SKOV3 cells. (E) Overexpression of *ASAP1-IT1* decreased *SGK1*, *FGF2* and *ETV5* mRNA expression in OVCAR3 cells. (F) Overexpression of *ASAP1-IT1* decreased *SGK1*, *FGF2* and *ETV5* mRNA expression in SKOV3 cells. *P<0.05; **P<0.01; ***P<0.001; n.s., not significant.

the LATS2 mRNA levels was also observed (Fig. 3C and D). In addition, the mRNA expression levels of *SGK1*, *FGF2* and *ETV5*, which are 3 conserved YAP1 downstream genes involved in ovarian cancer cell apoptosis (25-28), were

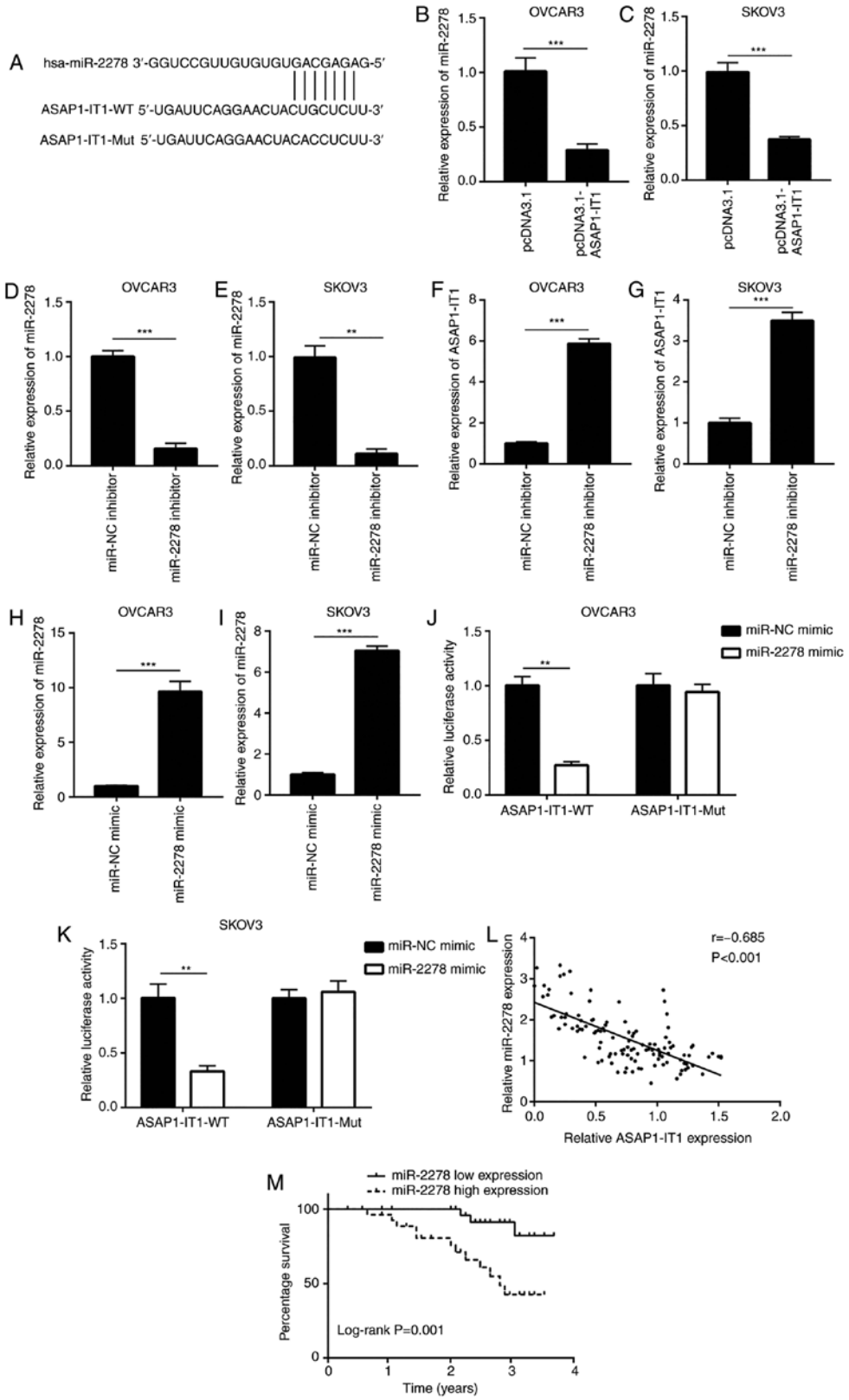

Figure 4. *ASAP1-IT1* sponges miR-2278 in ovarian cancer cells. (A) Sequence alignment of miR-2278 and sequence of *ASAP1-IT1* containing miRNA responsive element. Two site mutations were introduced to miRNA responsive element in *ASAP1-IT1*. (B) Overexpression of *ASAP1-IT1* decreased miR-2278 expression in OVCAR3 cells. (C) Overexpression of *ASAP1-IT1* decreased miR-2278 expression in SKOV3 cells. (D) Transfection with miR-2278 inhibitor decreased miR-2278 expression in OVCAR3 cells. (E) Transfection with miR-2278 inhibitor decreased miR-2278 expression in SKOV3 cells. (F) Downregulation of miR-2278 increased *ASAP1-IT1* expression in OVCAR3 cells. (G) Downregulation of miR-2278 increased *ASAP1-IT1* expression in SKOV3 cells. (H) Transfection with miR-2278 mimic increased miR-2278 expression in OVCAR3 cells. (I) Transfection with miR-2278 mimic increased miR-2278 expression in SKOV3 cells. (J) Dual luciferase reporter assay showing that the overexpression of miR-2278 reduced the relative luciferase activity of *ASAP1-IT1*-WT in OVCAR3 cells. (K) Dual luciferase reporter assay revealed that the overexpression of miR-2278 reduced the relative luciferase activity of *ASAP1-IT1*-WT in SKOV3 cells. (L) There was a negative correlation between *ASAP1-IT1* expression and miR-2278 expression in specimen from 58 patients with ovarian cancer. (M) Kaplan-Meier analysis showing that a high expression of miR-2278 was associated with a short overall survival of patients with ovarian cancer. **P<0.01; ***P<0.001.

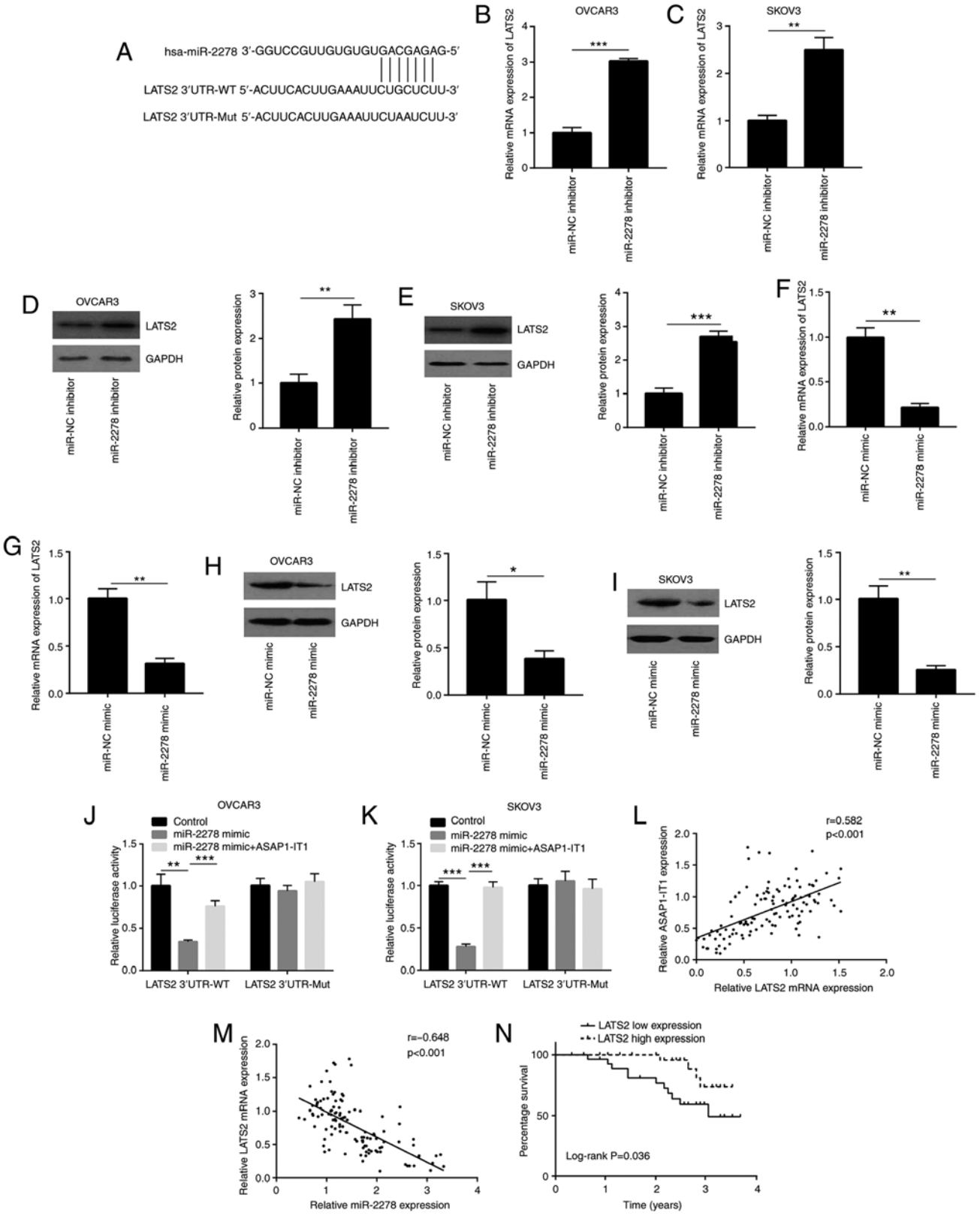

Figure 5. LATS2 is a target gene of miR-2278 in ovarian cancer cells. (A) There was a putative binding site for miR-2278 on the 3'UTR of *LATS2* mRNA. (B) Downregulation of miR-2278 increased *LATS2* mRNA expression in OVCAR3 cells. (C) Downregulation of miR-2278 increased *LATS2* mRNA expression in SKOV3 cells. (D) Downregulation of miR-2278 increased LATS2 protein expression in OVCAR3 cells. (E) Downregulation of miR-2278 increased LATS2 protein expression in SKOV3 cells. (F) Overexpression of miR-2278 decreased *LATS2* mRNA expression in OVCAR3 cells. (G) Overexpression of miR-2278 decreased *LATS2* mRNA expression in SKOV3 cells. (H) Overexpression of miR-2278 decreased LATS2 protein expression in OVCAR3 cells. (I) Overexpression of miR-2278 decreased LATS2 protein expression in SKOV3 cells. (J) Overexpression of miR-2278 decreased relative luciferase activity of *LATS2* 3'UTR-WT, which was reversed after *ASAP1-IT1* overexpression in OVCAR3 cells. (K) Overexpression of miR-2278 decreased relative luciferase activity of *LATS2* 3'UTR-WT which was reversed following *ASAP1-IT1* overexpression in SKOV3 cells. (L) There was a positive correlation between *ASAP1-IT1* expression and *LATS2* mRNA expression in specimens from 58 patients of ovarian cancer. (M) There was a negative correlation between miR-2278 expression and *LATS2* mRNA expression in specimens from 58 patients of ovarian cancer. (N) Kaplan-Meier analysis showing that a high expression of *LATS2* was associated with a prolonged overall survival of patients with ovarian cancer. *P<0.05; **P<0.01; ***P<0.001.

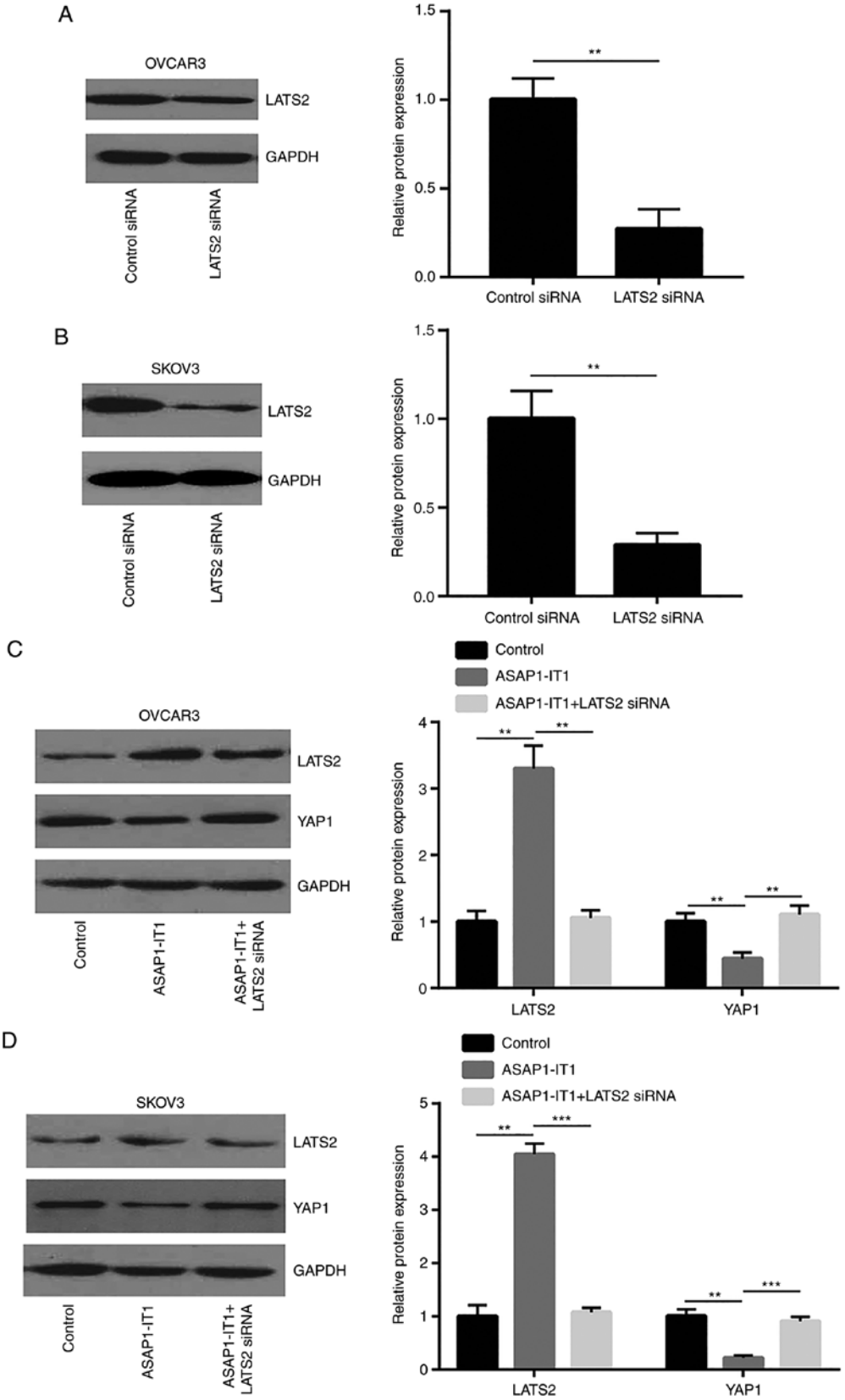

Figure 6. *ASAP1-IT1* downregulates YAP1 expression via the upregulation of LATS2. (A) Transfection with LATS2 siRNA decreased LATS2 protein expression in OVCAR3 cells. (B) Transfection with LATS2 siRNA decreased LATS2 protein expression in SKOV3 cells. (C) LATS2 silencing reversed the downregulation of YAP1 protein expression induced by *ASAP1-IT1* overexpression in OVCAR3 cells. (D) LATS2 silencing reversed the downregulation of YAP1 protein expression induced by *ASAP1-IT1* overexpression in SKOV3 cells. **P<0.01; ***P<0.001.

decreased in the OVCAR3 and SKOV3 cells (Fig. 3E and F). These data indicated that *ASAP1-IT1* upregulated LATS2 expression and inactivated YAP1 signaling in ovarian cancer cells.

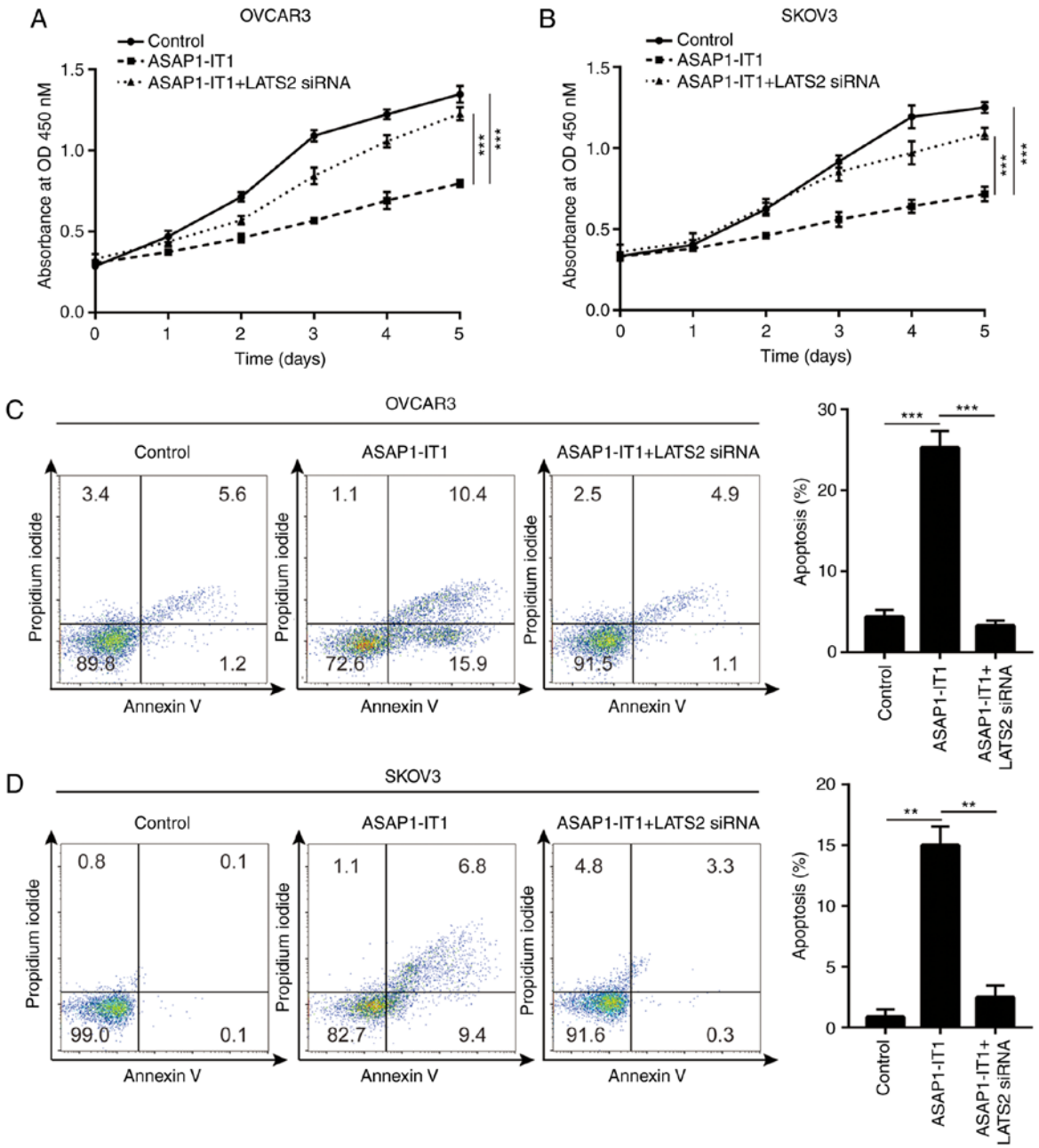

Figure 7. Silencing of LATS2 reverses the biological effects of *ASAP1-IT1* overexpression on ovarian cancer cells. (A) Knockdown of LATS2 attenuated the inhibition of OVCAR3 cell proliferation induced by *ASAP1-IT1* overexpression. (B) Knockdown of LATS2 attenuated the inhibition of SKOV3 cell proliferation induced by *ASAP1-IT1* overexpression. (C) Knockdown of LATS2 attenuated the apoptosis of OVCAR3 cells induced by *ASAP1-IT1* overexpression. (D) Knockdown of LATS2 attenuated the apoptosis of SKOV3 cells induced *by ASAP1-IT1* overexpression. **P<0.01; ***P<0.001.

ASAP1-IT1 sponges miR-2278 in ovarian cancer cells. Bioinformatic analysis was used to predict potential miRNAs that may interact with *ASAP1-IT1*. Among the candidate miRNAs, the *ASAP1-IT1* sequence harbored the miRNA responsive element for miR-2278 (Fig. 4A). The expression of the latter has been shown to be upregulated in the serum from patients with ovarian cancer (29). It is interesting to note that in the present study, high expression levels of miR-2278 were associated with an advanced FIGO tumor stage (Table II). The overexpression of *ASAP1-IT1* decreased miR-2278 expression in OVCAR3 and SKOV3 cells (Fig. 4B and C). Subsequently, miR-2278 inhibitor was used to decrease miR-2278 expression in OVCAR3 and SKOV3 cells (Fig. 4D and E). The downregulation of miR-2278 increased *ASAP1-IT1* expression in these cells (Fig. 4F and G). In addition, transfection with miR-2278 mimic increased miR-2278 expression in the OVCAR3 and SKOV3 cells (Fig. 4H and I). In the OVCAR3 cells, the overexpression of miR-2278 decreased the relative luciferase activity of the *ASAP1-IT1*-WT cells, whereas this effect was not noted in the *ASAP1-IT1*-Mut cells that harbored mutations in the putative binding site (Fig. 4J). Similar results were observed in SKOV3 cells (Fig. 4K), suggesting a direct interaction between *ASAP1-IT1* and miR-2278. Furthermore, a significant negative correlation between *ASAP1-IT1* and miR-2278 expression was noted in the clinical samples (Fig. 4L). In contrast to the *ASAP1-IT1* cells, a high expression

of miR-2278 was associated with a poor prognosis of patients with ovarian cancer (Fig. 4M).

ASAP1-IT1 upregulates LATS2 expression via sponging miR-2278. Bioinformatics analysis indicated a putative binding site of miR-2278 in the 3'UTR of *LATS2* mRNA (Fig. 5A), suggesting that *ASAP1-IT1* may regulate LATS2 via miR-2278. The downregulation of miR-2278 increased *LATS2* mRNA expression in OVCAR3 and SKOV3 cells (Fig. 5B and C). In addition, western blot analysis confirmed an elevation of LATS2 protein expression in OVCAR3 and SKOV3 cells transfected with the miR-2278 inhibitor (Fig. 5D and E). In contrast to these observations, the overexpression of miR-2278 decreased *LATS2* mRNA (Fig. 5F and G) and protein expression levels (Fig. 5H and I). Dual luciferase reporter assay indicated that miR-2278 reduced the relative luciferase activity of the plasmid containing the *LATS2* 3'UTR sequence, which was reversed when the *ASAP1-IT1* overexpression plasmid was transfected into OVCAR3 and SKOV3 cells (Fig. 5J and K). Furthermore, a positive correlation was noted between *LATS2* mRNA and *ASAP1-IT1* expression in the clinical samples (Fig. 5L). In contrast to these observations, the miR-2278 expression negatively correlated with the *LATS2* mRNA levels in these samples (Fig. 5M). *LATS2* expression was also negatively associated with the overall survival status of the patients (Fig. 5N). However, *LATS2* mRNA expression was not associated with the parameters pathological type, age, FIGO stage, differentiation status and lymph node metastasis (Table II). The data indicated that miR-2278 directly suppressed LATS2 expression and suggested that *ASAP1-IT1* may inactivate YAP1 signaling by binding to miR-2278.

ASAP1-IT1 inactivates YAP1 signaling via the upregulation of LATS2 expression. To explore whether *ASAP1-IT1* can downregulate YAP1 via LATS2, LATS2 siRNA was used to silence LATS2 expression in OVCAR3 and SKOV3 cells (Fig. 6A and B). The silencing of LATS2 attenuated the downregulation of YAP1 protein expression induced by *ASAP1-IT1* overexpression in OVCAR3 and SKOV3 cells (Fig. 6C and D). The Hippo pathway suppressed YAP1 activity via the phosphorylation of YAP1 to induce its translocation from the nucleus to the cytoplasm. Taken together, these data indicated that *ASAP1-IT1* inactivated YAP1 signaling via the upregulation of LATS2 expression.

ASAP1-IT1 suppresses ovarian cancer progression via the regulation of LATS2 expression. The results of cell proliferation assay indicated that the silencing of LATS2 attenuated the growth inhibitory effects of *ASAP1-IT1* overexpression on the OVCAR3 and SKOV3 cells (Fig. 7A and B). Furthermore, LATS2 silencing inhibited the apoptosis of OVCAR3 and SKOV3 cells induced by *ASAP1-IT1* overexpression (Fig. 7C and D), suggesting that LATS2 is essential for the tumor suppressive role of *ASAP1-IT1* in ovarian cancer.

Discussion

Accumulating evidence has indicated that lncRNAs are mainly involved in the initiation and development of ovarian cancer by controlling signaling networks and promoting sustained cancer cell proliferation and resistance to cell apoptosis (18,30,31). The inactivation of uncontrolled cell growth signaling and the induction of cancer cell death are critical for improving the prognosis of patients with ovarian cancer (32). Therefore, investigations into the biological roles and molecular mechanisms of lncRNAs is crucial for providing insight into the treatment of ovarian cancer.

Due to the oncogenic potential of ASAP1 (33), *ASAP1-IT1*, the intronic transcript of ASAP1 (11), has gained considerable attention in cancer research over the past years. The aberrant upregulation of *ASAP1-IT1* expression has been observed in several cancer types, including bladder cancer (10), non-small cell lung cancer (34) and cholangiocarcinoma (35). In non-small cell lung cancer cells, *ASAP1-IT1* facilitates cell proliferation, migration and invasion via the activation of the PI3K/AKT pathway (34). *ASAP1-IT1* positively regulates Hedgehog signaling in cholangiocarcinoma cells, leading to an enhanced cell proliferation and migration (35). In contrast to these observations, *ASAP1-IT1* expression is downregulated in ovarian cancer and is associated with the optimal prognosis of patients with this disease (11). Moreover, the results demonstrated that the upregulation of *ASAP1-IT1* expression in ovarian tumors was associated with optimal prognosis. In addition, high expression levels of ASAP1-IT were associated with early-stage tumors and an optimal differentiation status. The overexpression of *ASAP1-IT1* significantly inhibited ovarian cancer cell proliferation and induced cell apoptosis. The data suggested the involvement of *ASAP1-IT1* in ovarian cancer progression.

The molecular mechanisms of *ASAP1-IT1* in ovarian cancer cells have not yet been previously examined, at least to the best of our knowledge. The transcription co-activator, YAP1, plays a pivotal role in ovarian cancer initiation, proliferation and survival (36). The loss of LATS2 is essential for the malignant transformation of ovarian cells via the regulation of YAP1 expression (37). The present study indicated that *ASAP1-IT1* upregulated LATS2 mRNA and protein expression, while it suppressed YAP1 protein expression. This suggested that *ASAP1-IT1* may promote LATS2 mRNA stability, activate the Hippo pathway, destabilize YAP1 protein and inactivate YAP1 signaling. By using bioinformatic analysis, several miRNAs were predicted to interact with *ASAP1-IT1*. Among the list of miRNAs, miR-2278 was a highly ranked potential target of *ASAP1-IT1*. According to the microarray data derived from the sera of 168 high-grade serous ovarian carcinoma patients and 65 healthy controls, miR-2278 has been shown to be one of the top upregulated miRNAs identified (29). Currently, the association between miR-2278 and LATS2 has not yet been reported in any cell type, at least to the best of our knowledge. Bioinformatics analysis indicated that miR-2278 may target *LATS2*. By using RT-qPCR, western blot analysis and dual luciferase reporter assays, the data of the present study confirmed that *LATS2* was a target of miR-2278 and that *ASAP1-IT1* upregulated LATS2 expression. The Hippo pathway phosphorylates YAP1, leading to translocation of YAP1 from the nucleus to the cytoplasm, which is required for subsequent degradation (19). In the present study, *ASAP1-IT1* overexpression induced the translocation of YAP1, which was reversed following LATS2 the silencing in OVCAR3 cells. Furthermore, silencing of LATS2 attenuated the biological function of *ASAP1-IT1* in ovarian cancer cells. Previous studies have indicated that YAP1 is directly regulated by

miRNA/lncRNA in ovarian cancer (17,18). The findings of the present study suggested that *ASAP1-IT1* inhibited ovarian cancer progression by sponging miR-2278 and inducing the upregulation of LATS2 expression, the activation of the Hippo pathway and the inactivation of YAP1.

Taken together, the present study revealed that the *ASAP1-IT1*/miR-2278/LATS2 axis was involved in the regulation of ovarian cancer progression. Due to the complexity of signaling transduction in cancer cells, the current findings will be further extended in future studies in order to examine the molecular mechanisms of *ASAP1-IT1* in ovarian cancer.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

They are available at special request.

Authors' contributions

KW and YZ contributed to the experiment design and the performance of the experiments. KW and YBH collected the specimens and clinical information of the patients. CY supervised the study, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in the present study involving human participants were supervised and approved by the Ethical Committee of The China-Japan Union Hospital of Jilin University. Written informed consent for the publication of all data was obtained from all patients prior to participate the current study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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