SPPI promotes ovarian cancer progression via Integrin β1/FAK/AKT signaling pathway

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Objectives: Ovarian cancer is one of the most lethal malignant tumors in women. Secreted phosphoprotein 1 (SPPI) plays an important role in some cancer types. Therefore, the role of SPPI in ovarian cancer was determined and the potential mechanism was elucidated.

Materials and methods: The expression of SPPI in ovarian cancer was determined by immunohistochemistry in ovarian cancer tissues and normal ovarian tissues. Cellular proliferation, migration, and invasion were determined by cell counting kit-8 assay, wound healing assay, and Matrigel invasion assay in SKOV3 and A2780 cells. The protein expression of SPPI, integrin subunit β1 (Integrin β1), focal adhesion kinase (FAK), and phosphorylation protein kinase B (p-AKT) was detected by Western blotting in SKOV3 cells after silencing SPPI. The expression of SPPI was determined in SKOV3 cells after transfecting with miR-181a mimics or inhibitors. The growth of SKOV3 cells in vivo was determined in a nude mouse model of ovarian cancer after silencing SPPI.

Results: The expression of SPPI was higher in epithelial ovarian cancer tissues than in normal ovarian tissues. Silencing SPPI decreased the cell proliferation, migration, and invasion. Ectopic expression of SPPI increased the cell proliferation, migration, and invasion. Silencing SPPI prevented ovarian cancer growth in mice. Silencing SPPI inhibited Integrin β1/FAK/AKT pathway. In agreement, ectopically expressed SPPI activated Integrin β1/FAK/AKT pathway. Also, SPPI was regulated by miR-181a.

Conclusion: SPPI is a biomarker for the prognosis of ovarian cancer. It is also oncogenic and a potential target for ovarian cancer therapy.

Keywords: ovarian cancer, SPPI, Integrin β1, FAK, AKT, miR-181a

Introduction

Ovarian carcinoma is one of the most common gynecologic malignant tumors with a high mortality rate. Despite rapid progress in the chemotheraphy and radiotherapy of ovarian carcinoma, patients with recurrent ovarian cancer are essentially incurable. The 5-year survival rate after diagnosis of ovarian cancer has not improved significantly. The pathogenesis of ovarian cancer remains unclear.

Secreted phosphoprotein 1 (SPPI) is a secreted arginine glycine aspartic acid containing phosphorylated glycoprotein. It has a molecular weight of about 325,000. The human SPPI gene is located on chromosome 4 (4q13) with seven exons and six introns. The protein can be secreted by various cells, such as osteoclasts, macrophages, epithelial cells, and endothelial cells. The expression of SPPI is strongly related to tumor metastasis in gastric cancer and esophageal adenocarcinoma. Previous studies reported that the expression of SPPI is high in numerous tumors, including lung cancer, colon cancer, breast cancer, and prostate cancer. SPPI promotes the progression of cancer through modulation of vascular endothelial growth factor expression and regulation...
of extracellular matrix protein.\textsuperscript{13,14} However, the localization and function of SPP1 in ovarian cancer are not known.

We hypothesized that SPP1 plays an important role in the development of ovarian cancer. Therefore, we intended to detect the expression of SPP1 in ovarian cancer tissue. Then, we studied the effect of SPP1 on ovarian cancer in vitro. Finally, we studied the growth of SKOV3 cells after silencing SPP1 in vivo. In this research, we found an aberrant localization and expression of SPP1 in ovarian cancer tissues. Additionally, we found that SPP1 was involved in the regulation of cellular migration and invasion, and cell proliferation in ovarian cancer. We also found that SPP1 can modulate Integrin β1 (ITGB1)/focal adhesion kinase (FAK)/AKT pathway. Further, we identified SPP1 to be an authentic target of miR-181a.

Materials and methods

The tissue microarray slides including ovarian cancer tissues and normal ovarian tissues (n=55) were purchased from US Biomax Inc. (Rockville, MD, USA). The use of human samples was approved by the Ethics Committee of Chongqing Medical University.

Immunohistochemistry

Immunohistochemistry was performed according to the instructions given in SP kit (ab80436, Abcam Company). The procedure of immunohistochemical staining was in accordance with our previous studies.\textsuperscript{15,16} All the sections were observed by three pathologists using a light microscope.\textsuperscript{17} The intensity of SPP1-positive cells was as follows: 0, 1, 2, and 3 for negative, weak, moderate, and strong intensity. The percentage of SPP1-positive cells was scored as follows: 0 for no cytoplasm expression, 1 for 1%–25% positive tumor cytoplasm, 2 for 26%–50% positive tumor cytoplasm, 3 for 51%–75% positive tumor cytoplasm, and 4 for 76%–100% positive tumor cytoplasm.\textsuperscript{18} The multiplication of the intensity and percentage scores led to the final SPP1 staining score and was defined as follows: staining score <6 was considered as low expression, while staining score of 7 or more was considered as high expression. All values were shown as the mean ± standard error of the mean.

Cell culture, transfection procedure, and reagents

Human ovarian cancer cells lines SKOV3 and A2780 were purchased from Jiangsu KeyGEN BioTECH Corp., Ltd. The cells were cultured in RPM1 1640 medium. The medium was supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in an atmosphere of 5% carbon dioxide at 37°C. The siRNA sequences were obtained from Guangzhou RiboBio Co., Ltd (Guangzhou, China). The following sequences were targeted for human SPP1, ITGB1, FAK, and AKT siRNA, respectively. SPP1-1: 5′-GGAGUGAUAGUGAUGAUGAGA-3′; SPP1-2: 5′-GAAACGUAGAUGAUGAUGAGA-3′; ITGB1: 5′-GGU UACUCUGUGUCAGCUAGG-3′; FAK: 5′-GCAUUAG AUGUAGAAGAGAUG-3′; AKT: 5′-UGUAGUAUGAUG UGUUAAAUA-3′; and negative control (NC) siRNA: 5′-UUCUUCGAAGGUGUACAGGUTT-3′. Lentiviral vector expressing shRNA targeting SPP1 (named LV3-shPP1-1 and LV3-shPP1-2) and SPP1-lentiviral expression vector (named LV5-SPP1) were provided by Guangzhou RiboBio Co., Ltd. miR-181a mimics were synthesized at Guangzhou RiboBio Co., Ltd.

Quantitative real-time polymerase chain reaction (PCR)

Total RNAs were extracted as described previously.\textsuperscript{19} The reverse transcriptase and quantitative PCR reactions were conducted as previously described.\textsuperscript{17} PCR amplifications were started with a 10 min denaturation step at 95°C, followed by 40 amplification cycles (10 sec at 95°C, 20 sec at 60°C, and 10 sec at 72°C). Relative quantification of mRNA was performed using the comparative threshold cycles method. The value was used to plot the gene expression employing the formula 2^{-ΔΔCT}. The primers used for amplifying SPP1, ITGB1, FAK, and AKT and GAPDH were synthesized by Guangzhou Funeng Co., Ltd.

Detection of protein expression by Western blotting

Western blot analysis was performed as previously described.\textsuperscript{20} Three independent experiments in a certain condition were subjected to Western blot analysis. The SPP1 (ab33046), ITGB1 (ab52971), FAK (ab40794), AKT1 (ab81283), and GAPDH (ab8245) antibodies were purchased from Abcam Inc. The antibodies were diluted using 1:1,000. The bands were detected with enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology, Jiangsu, China) and quantified by ImageQuant 3.3 software (Molecular Dynamics).

Cell proliferation assay

Cellular proliferation was determined by cell counting kit-8 assay following the manufacturer’s instructions.
(Beyotime).\textsuperscript{21,22} Cells were seeded into 96-well plates and cultured for an additional 24 h. After treatment with lentiviral vector, 10 μL of the kit reagent was added and then incubated for another 2 h. The OD value was read at 450 nM to obtain the final results.

**Wound healing assay and Matrigel invasion assays**

Cell migration and invasion assay was performed in accordance with our previous studies.\textsuperscript{23,24} Briefly, ovarian cancer cells were cultured in six-well plates to about 80% confluence. The medium was replaced with serum-free medium. After wounding was achieved, the distance between two wounds was measured at 0 and 72 h. Invasion assay was performed as follows. The upper side was coated using Matrigel basement membrane matrix for 2 h at 37°C. The cells were added into the top chamber and then incubated for 48 h. Six percent paraformaldehyde was used to fix the invasive cells. Then, they were stained with 0.5% crystal violet (Beyotime) and counted.

**Dual-luciferase reporter gene assay**

Luciferase reporter gene assay was performed using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the instructions provided by the manufacturer. For SPP1 3’ untranslated region (UTR) luciferase reporter assay, wild-type or mutant reporter constructs (purchased from Genepharma Co., Ltd., Shanghai, China) were co-transfected into SKOV3 cells in 24-well plates with 100 nM miR-181a or 100 nM miR-NC and Renilla plasmid by using Endofectin\textsuperscript{TM}-Plus (GeneCopoeia). Reporter gene assay was performed 48 h post-transfection using the dual-luciferase assay system (Promega). Firefly luciferase activity was normalized for transfection efficiency using the corresponding Renilla luciferase activity. All experiments were performed at least three times.

**Animal experiment**

All animal care and handling procedures followed the guidelines approved by the Institutional Animal Use and Care Committee of Chongqing Medical University. SKOV3 cells were infected with LV3-NC or LV3-shSPP1-1. Six-week-old BALB/c nude mice were grouped into LV3-NC infected group (n=5) and LV3-shSPP1-1 infected group (n=5). The infected SKOV3 cells were injected subcutaneously into the left armpit. Twenty-eight days later, the weight and volume of tumors were calculated.

**Statistical analysis**

All statistical analyses were performed using SPSS 19.0 software. Each experiment was performed in triplicate. Student’s t-test or analysis of variance was used for statistical analysis. The differences were considered significant when \( p<0.05 \).

**Results**

**Increased expression of SPP1 in epithelial ovarian carcinoma tissues**

Using Oncomine analysis, we investigated the mRNA levels of SPP1 genes in ovarian cancers. Three independent data (Adib Ovarian, Yoshihara Ovarian, and Lu Ovarian; \( \text{https://www.oncomine.org/resource/login.html} \)) showed that the mRNA level of SPP1 in ovarian serous adenocarcinoma was higher than in normal ovarian tissues (fold changes were 25.623, 31.013, and 6.844, respectively; \( p=3.12\times10^{-5}, 6.61\times10^{-7}, \) and 0.008, respectively; Figure 1A–C). Then we analyzed the expression of SPP1 in other epithelial cancer histologic types (data derived from Lu Ovarian). The mRNA level of SPP1 in ovarian clear cell adenocarcinoma, ovarian endometrioid adenocarcinoma, and ovarian mucinous adenocarcinoma was higher than in normal ovarian tissues (fold changes were 9.504, 7.185, and 4.496, respectively; \( p=0.003, 0.004, \) and 0.015, respectively; Figure 1D–F).

We next analyzed the location of SPP1 protein in ovarian cancer tissues derived from the human protein atlas. SPP1 was mainly localized in the plasma membrane and cytoplasm (Figure 1G–I). The expression of SPP1 in ovarian serous adenocarcinoma, ovarian mucinous adenocarcinoma, and ovarian endometrioid adenocarcinoma showed moderate staining (Figure 1G–I). The SPP1 in follicle cells was medium staining. However, SPP1 was not detected in ovarian stroma cells (Figure 1J).

We then determined the expression of SPP1 in normal ovarian tissues and ovarian cancer tissues. The clinicopathologic data were obtained from US Biomax Inc. The expression of SPP1 was increased in epithelial ovarian cancer than in normal ovary tissues (\( p<0.05; \) Figure 1K–N; Table 1). Mann–Whitney \( U \) test was used to compare the associations between SPP1 overexpression and clinicopathologic variables of 45 epithelial ovarian cancer samples (Table 1). The associations between SPP1 expression and age were not significant (\( p>0.05; \) Table 1). SPP1 expression was significantly higher in cancer with advanced stages (stage III/IV) as compared with those in the early stages (stage I/II; \( p<0.05 \)). The expression of SPP1 was correlated with tumor grade (grades 2–3 versus 1, \( p<0.05 \)). Evaluation of the SPP1 genes with progression-free
Figure 1 Immunohistochemical analysis of sPP1 expression in epithelial ovarian cancer.

Notes: The mRNA expression of SPP1 in epithelial cancer was investigated. Data were derived from Oncomine database. (A–C) Three independent data (Adib Ovarian, Yoshihara Ovarian, and Lu Ovarian) showed that the mRNA level of SPP1 in ovarian serous adenocarcinoma was higher than in normal ovarian tissues. (D–F; data derived from Lu Ovarian) The mRNA level of SPP1 in ovarian clear cell adenocarcinoma, ovarian endometrioid adenocarcinoma, and ovarian mucinous adenocarcinoma was higher than in normal ovarian tissues. (G–I) The location and expression of SPP1 protein in ovarian cancer tissues was analyzed. The data were derived from the human protein atlas. The expression of SPP1 in ovarian serous adenocarcinoma, ovarian mucinous adenocarcinoma, and ovarian endometrioid adenocarcinoma showed moderate staining. (J) SPP1 in the follicle cells showed medium staining. SPP1 was not detected in ovarian stroma cells. (K–N) The expression pattern of SPP1 was determined in normal ovarian tissues and epithelial ovarian cancer tissue samples using IHC. Original magnification, 200×. SPP1 expression reversely correlated with survival time. Kaplan–Meier survival analysis for the relationship between survival time and SPP1 signature in ovarian cancer was performed by using the online tool (http://kmplot.com/analysis/). (O) PFs curves are plotted for ovarian cancer patients (n=1,436). (P) OS curves are plotted for ovarian cancer patients (n=1,657). (Q) PPS curves are plotted for ovarian cancer patients (n=782). p<0.05 was considered to be a statistically significant difference.

Abbreviations: HR, hazard ratio; OS, overall survival; PFs, progression-free survival; PPS, post-progression survival; SPP1, secreted phosphoprotein 1.
Table 1  Association of SPP1 expression with clinicopathologic characteristics in 55 patients of eOc

<table>
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<th>Characteristics</th>
<th>No of patients (N=55)</th>
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<tr>
<td></td>
<td></td>
<td>Low no (%)</td>
<td>High no (%)</td>
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<tr>
<td>Age (years)</td>
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<td></td>
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<td>15 (62.50)</td>
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<td>≥50</td>
<td>31</td>
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<td>20 (64.52)</td>
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<td>13 (56.52)</td>
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<tr>
<td>III/IV</td>
<td>22</td>
<td>2 (9.09)</td>
<td>20 (90.91)</td>
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<td>3</td>
<td>19</td>
<td>3 (15.79)</td>
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Abbreviations: SPP1, secreted phosphoprotein 1; eOc, epithelial ovarian cancer; FIGO, International Federation of Gynecology and Obstetrics.

To identify the interaction between SPP1 and ITGB1, we designed a co-immunoprecipitation experiment. Cells were co-transfected with Flag-ITGB1 and HA-SPP1. Control group was established simultaneously, and then cells were harvested 24 h later. Anti-HA antibodies were used to pull the interaction protein. Then, they were detected by anti-Flag antibodies using Western blot. Our results indicated that Flag bands could not be detected in cells infected with Flag-ITGB1 or HA-SPP1 only. However, they could be detected in cells that were co-transfected with Flag-ITGB1 and HA-SPP1 (Figure 3B). The Cancer Genome Atlas data (http://gepia.cancer-pku.cn/detail.php) indicated that the expression of SPP1 in ovarian cancer was positively correlated with ITGB1 (R=0.23; p=9.9E–07; Figure 3C).

Previous studies revealed that ITGB1 is involved in the development of ovarian cancer via FAK/AKT pathway. We guessed that SPP1 may regulate the ITGB1/FAK/AKT pathway. ITGB1, FAK, and p-AKT protein levels were decreased in SKOV3 cells when SPP1 was silenced (Figure 3D). However, ectopic SPP1 gene expression in A2780 cells increased the protein level of ITGB1, FAK, and p-AKT (Figure 3E). These data indicated that SPP1 may activate ITGB1/FAK/AKT signaling axis in human epithelial ovarian cancer.

SPP1 regulates cellular proliferation, migration, and invasion

Cellular proliferation, migration, and invasion is an important cause of ovarian cancer progression and recurrence. So, we determined the role of SPP1 in cellular proliferation, migration, and invasion. We firstly determined the expression of SPP1 in four cell lines. Of these, SPP1 expression was higher in the SKOV3 cell line in comparison with the A2780 cell line (Figure 2A). The mRNA and protein levels of SPP1 were reduced in LV3-shSPP1-1- and LV3-shSPP1-2-infected SKOV3 cells compared to LV3-NC-infected SKOV3 cells (Figure 2B). Our data showed that the ability for cell proliferation, migration, and invasion was decreased in SKOV3 cells infected with LV3-shSPP1-1 and LV3-shSPP1-2 compared to that in SKOV3 cells infected with LV3-NC (p<0.05; Figure 2C–E). We also determined the ectopic expression of SPP1 in A2780 cells infected with LV5-SPP1. We found that the ectopic expression of SPP1 in A2780 cells increased the ability of cell proliferation, migration, and invasion, compared to LV-5-GFP-infected cells (p<0.05; Figure 2F–H).

SPP1 regulated ITGB1/FAK/AKT pathway

Bioinformatics analyses (https://string-db.org/) showed that SPP1 may interact with ITGB1 (Figure 3A). In order to identify the interaction between SPP1 and ITGB1, we designed a co-immunoprecipitation experiment. Cells were co-transfected with Flag-ITGB1 and HA-SPP1. Control group was established simultaneously, and then cells were harvested 24 h later. Anti-HA antibodies were used to pull the interaction protein. Then, they were detected by anti-Flag antibodies using Western blot. Our results indicated that Flag bands could not be detected in cells infected with Flag-ITGB1 or HA-SPP1 only. However, they could be detected in cells that were co-transfected with Flag-ITGB1 and HA-SPP1 (Figure 3B). The Cancer Genome Atlas data (http://gepia.cancer-pku.cn/detail.php) indicated that the expression of SPP1 in ovarian cancer was positively correlated with ITGB1 (R=0.23; p=9.9E–07; Figure 3C).

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SPP1-promoted A2780 cellular proliferation, migration, and invasion involves ITGB1/FAK/AKT pathway

We have demonstrated that SPP1 can regulate the ITGB1/FAK/AKT signaling axis. Hence, we presumed that SPP1 regulation of epithelial ovarian cancer proliferation, migration, and invasion involves ITGB1/FAK/AKT pathway. We found that silencing of ITGB1, FAK, or AKT partially inhibited the cellular proliferation, migration, and invasion ability of LV5-SPP1-infected A2780 cells (Figure 4A–C). These results suggest that SPP1 promotes epithelia ovarian cancer cell proliferation, which is mediated by ITGB1/FAK/AKT pathway.

miR-181a regulates SPP1 expression in ovarian cancers

Bioinformatics analyses predicted that SPP1 is a potential target of miR-181a (http://www.targetscan.org/). We also predicted that miR-181a-specific binding site was located within the 3'UTR of SPP1 mRNA (Figure 5A). We observed that miR-181a significantly reduced luciferase activity when SPP1 3'UTR was inserted downstream of luciferase cDNA in the reporter vector (pMIR-SPP1-3'UTR) (Figure 5A).
Figure 2 SPP1 regulates cellular proliferation, migration, and invasion.

Notes: (A) The relative expression of SPP1 mRNA in ovarian cancer cell lines. (B) SPP1 mRNA and protein levels were downregulated on infecting with LV3-shsPP1-1 or LV3-shsPP1-2. (C) Ovarian cancer SKOV3 cells’ migration ability was detected by the wound healing assay. The migration of LV3-shsPP1-1- and LV3-shsPP1-2-infected SKOV3 cells was lower as compared to that of LV3-NC-infected cells. (D) Ovarian cancer SKOV3 cells’ invasion ability was detected by Matrigel invasion assay. The invasion ability of LV3-shsPP1-1- and LV3-shsPP1-2-infected SKOV3 cells was decreased compared to that of LV3-NC-infected cells. (E) Cell proliferation was determined by CCK-8 assay. (F) Ovarian cancer A2780 cells’ migration ability was detected by wound healing assay. The migration ability of LV5-sPP1-infected A2780 cells was increased compared to that of LV3-NC-infected cells. (G) Ovarian cancer A2780 cells’ invasion ability was detected by Matrigel invasion assay. The invasion ability of LV5-sPP1-infected A2780 cells was increased compared to that of LV5-GFP-infected cells. (H) Cell proliferation was determined by CCK-8 assay. Error bars represent standard error. *p<0.05 (C, D, F, G) Magnification 200×.

Abbreviations: CCK-8, cell counting kit-8; NC, negative control; SPP1, secreted phosphoprotein 1.
We also observed that miR-181a mimics reduced the expression of SPP1 (Figure 5B). miR-181a inhibitors increased the expression of SPP1 (Figure 5C).

Silencing SPP1 inhibited the growth of SKOV3 cells in vivo
To determine the effect of SPP1 on the growth of SKOV3 cells in vivo, an animal model was constructed. SKOV3 cells were infected with LV3-NC and LV3-shSPP1-1, which formed tumors in all nude mice. The average volume of tumors was decreased in LV3-shSPP1-1 group compared to that in LV3-NC group (p<0.05; Figure 6A and B). The average weight of tumors was decreased in LV3-shSPP1-1 group compared to that in LV3-NC group (Figure 6C). The expression of ITGB1, FAK, or p-AKT was decreased in tumors derived from LV3-shSPP1-1-infected group compared to that in the LV3-NC group (Figure 6D).

Discussion
In this research, we observed that the expression of SPP1 was increased in epithelial ovarian cancer, compared to normal ovarian tissues. We also found that the expression of SPP1 in ovarian cancer was correlated with tumor stage and histologic grades. Our data revealed that high expression of SPP1 in ovarian cancer promoted cellular migration, invasion, and cell proliferation. These results indicated that SPP1 promoted the progress of ovarian cancer. Our data also demonstrate that SPP1 can regulate ITGB1/FAK/AKT pathway and that miR-181a controls SPP1 by targeting SPP1 3′UTR. Based on the above mentioned results, SPP1 is a potential therapeutic target and a biologic marker of ovarian carcinoma.

The expression of SPP1 is related to tumorigenesis and tumor metastasis. It has been reported that the expression of SPP1 is high in numerous tumors, including lung cancer, colon cancer, breast cancer, and prostate cancer.10–12 SPP1 levels...
were positively correlated with the number of extrapulmonary metastases, indicating that patients with high levels of SPP1 expression had higher risk of extrapulmonary metastasis. In colorectal cancer, SPP1 promotes metastasis by activating the epithelial-mesenchymal transition pathway. In this study, we confirmed that the expression of SPP1 was increased in ovarian cancer. Silencing SPP1 inhibited the malignant behaviors of ovarian cancer. Ectopic expression of SPP1 promoted the malignant behaviors of ovarian cancer. Our data indicated that SPP1 accelerated ovarian cancer progression.

ITGB1 is frequently upregulated in ovarian cancer and promotes ovarian tumorigenesis and cancer progression. ITGB1 regulates many functional activities such as cellular proliferation, adhesion, and invasion. Previous studies have suggested its implication in therapeutic resistance in numerous solid cancer models. FAK is increased in various malignancies including breast, colon, prostate, neck, and ovarian cancer. FAK is an important regulator of survival, proliferation, migration, and invasion, processes that are all involved in tumorigenesis and metastasis. Blocking the ITGB1/FAK/AKT pathway inhibits the metastatic ability of head and neck cancer cells. Activating the ITGB1/FAK/AKT pathway promoted the lymph node metastasis of gastric cancer. In this study, we observed that silencing SPP1 inhibited ITGB1/FAK/AKT pathway. Ectopic expression of SPP1 activated this pathway. This data indicated that SPP1 promoted the progression of ovarian cancer through ITGB1/FAK/AKT pathway.

Several studies report mixed indications of miR-181 as a tumor suppressor. miR-181 inhibited metastasis and proliferative ability of cancer. Concomitant analysis of P-Smad2 and...
miR-181a-5p in surgical samples may be capable of identifying those ovarian cancer patients with poor outcome and little chance of response to Pt-based neoadjuvant chemotherapy.\textsuperscript{47} Our results showed that miR-181 mimics inhibited the expression of SPP1 in SKOV3 cells. We further confirmed miR-181a reduced SPP1 expression by targeting its mRNA 3′UTR. This data indicated that SPP1 is a tumor-promoting gene in ovarian cancer. miR-181a is a potential oncotarget of ovarian cancer.

**Conclusion**

This study confirms that SPP1 is a tumor-promoting gene in ovarian cancer, which is involved in the ITGB1/FAK/AKT signal transduction pathway. The downregulation of SPP1 by miR-181a may be a promising therapeutic target for ovarian cancer.

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**Figure 5** miR-181a regulates SPP1 expression in ovarian cancers.

Notes: (A) Putative binding sites targeted by miR-181a were predicted to be located in the 3′UTR of SPP1 mRNA. SKOV3 cells were co-transfected with miR-181a mimics or control RNA (NC) with luciferase reporter plasmids containing either wild-type (pMir-SPP1-3′UTR) or mutant 3′UTR (pMir-SPP1-3′UTRm) SPP1 genes. Luciferase expression was measured. (B) Ovarian cancer SKOV3 cells were transfected with miR-181a mimics or control RNA (NC). (C) Ovarian cancer SKOV3 cells were transfected with miR-181a inhibitor or control RNA (NC). Error bars represent standard error. *p<0.05.

Abbreviations: NC, negative control; SPP1, secreted phosphoprotein 1; UTR, untranslated region.

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**Figure 6** (Continued)
pathway. These results suggest that SPP1 may be a potential therapeutic target in epithelial ovarian cancer.

Disclosure

The authors report no conflicts of interest in this work.

References


Figure 6: SPP1 regulated tumorigenesis in nude mice model.

Notes: (A–C) Mean tumor volume and weight on day 28 after tumor cell injection. LV3-shSPP1-1- or LV3-NC-infected SKOV3 cells were implanted s.c. into the left armpit. (D) Immunohistochemical analysis of Integrin β1, FAK, and p-AKT expression was performed on tumor xenografts. Representative images are shown (original magnification, ×200). Error bars represent standard error. *p<0.05.

Abbreviations: FAK, focal adhesion kinase; NC, negative control; s.c., subcutaneously; SPP1, secreted phosphoprotein 1.


