FBP1 promotes ovarian cancer development through the acceleration of cell cycle transition and metastasis

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Abstract. Epithelial ovarian cancer (EOC) is the fifth most common malignancy in women, with a 5-year mortality of >70% in North America. As the symptoms are often not observed until the cancer has spread extensively, few women are diagnosed at an early stage of disease. Large-scale gene expression analyses have identified molecular subtypes within high-grade ovarian cancer with variable survival rates and drug resistance. The understanding of gene expression, the mechanisms underlying cancer processes and drug resistances have facilitated the development of targeted therapies. The far-upstream element (Fuse)-binding protein 1 (FBP1) is overexpressed in a number of malignancies such as hepatocellular carcinoma, and has been identified as an oncoprotein. In our early studies, FBP1 was demonstrated to physically interact with p53 and suppresses p53 transcription activity. In the present study, FBP1 expression increased as ovarian cancer developed. Among ovarian normal, adenoma and carcinoma tissues, the highest FBP1 expression was identified in carcinoma tissues. Furthermore FBP1 did not influence the apoptosis of ovarian carcinoma cells, yet enhanced cell cycle transition and metastasis. Therefore, it was hypothesized that FBP1 promotes ovarian cancer development through the acceleration of cell cycle transition and metastasis, and FBP1 is a novel potential biological marker for epithelial ovarian cancer diagnosis.

Introduction

Epithelial ovarian cancer (EOC) is the fifth most common malignancy in women, with a 5-year mortality of >70% in North

America (1). Combined surgery and cytotoxic therapy have previously been demonstrated to produce a favorable clinical response in 50-80% of patients (2). The platinum-paclitaxel combination regimen is utilized as the principal chemotherapy for advanced EOC, and yields response rates of >80% and complete response rates of 40-60% (3-7). Owing to the symptoms often not being observed until the cancer has spread extensively, <25% of women are diagnosed at an early stage of disease (2). Large-scale gene expression analyses have identified molecular subtypes within high-grade ovarian cancer with variable survival rates and drug resistance (8,9). These variations in gene expression may influence the outcomes of different treatment types. The understanding of gene expression, the mechanisms underlying cancer processes and drug resistances have facilitated the development of targeted therapies, such as small-molecule inhibitors. These agents target the proteins associating with cell viability/death, metastasis and angiogenesis. Previous studies have demonstrated breast cancer 1/2 (BRCA1/2) and DNA repair proteins, as markers for platinum treatment (8-10). Ribonucleotide reductase subunit M1 (RRM1) and DNA topoisomerase II-α (TOP2A) have been used as markers to predict the response to gemcitabine and anthracyclines for patients with ovarian cancer (11-13).

The far-upstream element (Fuse)-binding protein 1 (FBP1) has been identified as an anti-apoptotic and pro-proliferative oncoprotein that is overexpressed in a number of malignancies, including hepatocellular carcinoma (HCC) (14,15), non small-cell lung cancer (16) and colorectal cancer (17). Importantly, nuclear enrichment of FBP1 is associated with poor overall survival of patients with liver cancer (14). FBP1 functions as a transcriptional regulator by binding to the single-stranded DNA element (fuse) and interacting with the basal transcriptional machinery (18,19). The study of Rabenhorst et al (15) previously demonstrated that FBP1 serves a role in hematopoietic development and/or homeostasis. In our early studies, FBP1 was demonstrated to physically interact with p53 and suppress p53 transcription activity under radiation-induced cellular stress and facilitates hepatitis C virus replication in hepatoma cells (20,21). Silence of FBP1 increases the sensitivity of ovarian cancer cells to carboplatin (22).

Since FBP1 is always overexpressed in certain malignancies, the present study aimed to clarify the association between

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FBP1 expression and EOC development. We hypothesized that FBP1 enhances EOC development and that FBP1 is a novel potential biological marker for EOC diagnosis. The study also attempted to analyze the potential mechanisms underlying the promotion of FBP1 in EOC development.

Materials and methods

Patients and samples collection. The present study was conducted after informed consent was obtained from all subjects and the protocol was approved by the Medical Ethics Committee of Guangzhou Red Cross hospital of Medical College, Jinan University (Guangzhou, China). For immuno-histochemical analysis, a total of 58 ovarian specimens were obtained from the Department of Pathology, Guangzhou Red Cross Hospital from January 2010 to June 2015 with a median age of 47.6 from 17.0-76.0 years old and assigned into three groups, including normal epithelial ovarian tissues (14 samples), epithelial ovarian adenoma tissues (25 samples) and epithelial ovarian cancer tissues (19 samples). None of the patients received preoperative therapies such as radia-tion, chemotherapy, or immunotherapy. All tissues were fixed with 10% formalin and then embedded in paraffin prior to sectioning.

Antibodies and reagents. The antibodies used for immuno-histochemistry and western blot analysis were as follows: GAPDH (catalog no. 5174; 1:2,000) and FBP1 (cat. no. 72736; 1:1,000) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Ki-67 (cat. no. 550609; 1:200) was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Cyclin D1 (cat. no. sc-70899; 1:500), cyclin E (cat. no. sc-377100; 1:500), c-Myc (cat. no. sc-398624; 1:500), p21 (cat. no. sc-817; 1:500), p27 (cat. no. sc-1641; 1:500) and matrix metalloproteinase-2 (MMP-2; cat. no. sc-13594; 1:500) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and L-glutamine, were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The CellTiter 96® AQueous One Solution Reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] was purchased from Promega Corporation (Madison, WI, USA). Penicillin and streptomycin sulfate were purchased from GE Healthcare Life Sciences (Logan, UT, USA).

Cell culture and FBP1 knockdown cell construction. The human ovarian cancer SKOV3 cells (Chinese Academy of Sciences Cell Bank) were cultured in DMEM with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2 in a humidified incubator.

FBP1-knockdown lentiviral particles (sc-43760) and control lentiviral particles (cat. no. sc-108080) were obtained from Santa Cruz Biotechnology, Inc. SKOV3 cells were plated in complete DMEM containing 10% (v/v) FBS until they reached 70% confluence. Subsequently, cells were transfected with 20 μl of either control lentiviral particles or FBP1-knockdown lentiviral particles ($1x10^6$ IFU) in serum-free medium according to the manufacturer's protocol. The trans-fection medium was replaced by fresh complete DMEM after

6 h and the cells were incubated for another 48 h at 37°C. Cells were collected after screening with 2.0 μg/ml puromycin for ~2 weeks and termed FBP1-knockdown (FBP1-KD) and FBP1 control (FBP1-C) SKOV3 cells, respectively.

Cell viability and plate colony formation assays. FBP1-C and FBP1-KD SKOV3 cells were plated into a 96-well plate at a density of $1x10^4$ cells/well and cultures for 24 h. Cell viability was measured using MTS in accordance with the manufacturer's (Promega Corporation) protocol, and the absorbance at the wavelength of 490 nm was read in an automated plate reader (Bio Tek Instruments Inc., Winooski, VT, USA). The experiments were repeated at least three times.

FBP1-C and FBP1-KD SKOV3 cells ($2.0x10^4$ cells/plate) were cultured in 5 ml DMEM supplemented with 10% FBS in 10-cm plates. After 14 days, colonies were washed with PBS, fixed with 10% methanol at 4°C for 30 min and stained with 1% crystal violet for 30 min. The colony formation images were captured by camera.

Immunohistochemical staining. Formalin-fixed paraffin embedded ovarian tissue sections (5-μm thick) were depa-raffinized (using 100% of turpentine oil, three times for 10 min/time) and hydrated consecutively (100% of ethanol for 3 min, 95% of ethanol for 3 min, 90% of ethanol for 3 min, 85% of ethanol for 3 min, 80% of ethanol for 3 min, 75% of ethanol for 3 min and H_2O for 3 min). The sections were incubated with citrate buffer (pH 6.0) to retrieve the antigen using a microwave cooker. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide (H_2O_2). The sections were blocked with 10% BSA (Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 30 min at room temperature prior to incubation with rabbit anti-human FBP1 or Ki-67 antibody (diluted 1:500) overnight at 4°C, and then incubated with HRP-conjugated secondary antibody (cat. no. 8114; 1:200; Cell Signaling Technology) for 1 h at room temperature. After washing with PBS, the reaction was visualized by incubating the sections with DAB and hematoxylin for 5 min at room temperature. The negative control section was incubated with blocking reagent alone in the absence of primary antibody. Images were captured by an x40 objective and image processing and analyses were performed using the Image-Pro Plus 6.0 software (Media Cybernetics, Shanghai, China). The intensity of the immunohistochemical reaction was expressed as integral optical density (IOD) of DAB brown reaction products. The results of 5 separate measurements for each of sample were expressed as mean ± standard deviation (SD).

Western blot analysis. Cells were lysed for western blot-ting in modified RIPA buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-Cl [pH 8.0], 0.1% SDS) supplemented with PMSF (1 mM) protease and phosphatase inhibitor. Following rapid homogenization, the homogenates were incubated in ice for 30 min and centrifuged at 12,000 x g for 15 min at 4°C. Protein concentrations were determined by bicincho-ninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.). The protein samples (30-50 μg/lane) were resolved by SDS-PAGE (10-12% gel) and transferred to polyvinylidene

difluoride filter membranes (Merck KGaA, Darmstadt, Germany). Following transfer, membranes were blocked with 5% skimmed milk in TBS-Tween (0.05 M Tris, 0.15 M NaCl, pH 7.5, 0.2% Tween-20) for 1 h at room temperature and then incubated at 4°C overnight with primary antibodies of GAPDH, FBP1 (Cell Signaling Technology), Cyclin D1, Cyclin E, c-Myc, p21, p27 and MMP-2 (Santa Cruz). Membranes were washed with TBST for three times and then incubated with anti-rabbit/mouse HRP-labeled secondary antibodies for 1 h at room temperature, respectively, and detected with ECL-Plus detection systems (Pierce; Thermo Fisher Scientific, Inc.). Relative abundance was quantified by densitometry using Quantity One 4.6.7 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell cycle and cell apoptosis analysis. Flow cytometry was used for the analysis of cell cycle distribution and apoptosis. For cell cycle distribution, FBP1-C and FBP1-KD SKOV3 cells were collected and fixed in 70% (v/v) ethanol over-night at 4°C, and then incubated with 1 mg/ml RNase A for 30 min at 37°C. Cells were stained with 50 mg/ml propidium iodide (PI) (BD Biosciences) in PBS containing 1% Triton X-100. The data were acquired using a BD FACSCAN flow cytometer (FACSAria II, BD Biosciences) and analyzed using the BD CellQuest Pro software (BD Biosciences). For apoptosis, FBP1-C and FBP1-KD SKOV3 cells were firstly incubated with Annexin V-fluorescein isothiocyanate (FITC) (Beijing Biosea Biotechnology Co., Ltd., Beijing, China) for 30 min at 4°C in the dark, and then incubated with PI for 5 min. Analysis was immediately performed by FACSAria II (BD Biosciences) according to the manufac-turer's instructions.

Wound healing and Transwell migration assays. FBP1-C and FBP1-KD SKOV3 cells were seeded on 6-well plates at a density of $2x10^5$ cells/well. The cells were scratched using a sterile 100 μl micropipette tip and washed twice with PBS. The cells were cultured with complete DMEM with 10% FBS for an additional 48 h. Images were captured by a microscope at the 0-, 24- and 48-h time-points.

The Transwell inserts (8 μm pore size, Corning Incorporated, Corning, NY, USA) were coated with 0.1% gelatin for 30 min at 37°C. The bottom chambers of Transwell were filled with DMEM with 10% FBS, and the top chambers were seeded $1x10^5$ cells per well (pretreated with mitomycin C) in 200 μl DMEM (FBS free). After 24 h of incubation at 37°C, the migrated cells were stained with 1% crystal violet for 30 min at room temperature. Images were captured using an Olympus inverted microscope (Olympus Corporation, Tokyo, Japan) at x200 magnification.

Statistical analysis. The data were derived from at least three independent experiments. Values reported are mean ± SD. The data were analyzed using one-way analysis of variance and Student-Newman-Keuls post-hoc test by the SPSS 17.0 statistical package from SPSS Inc. (Chicago, IL, USA). The SPSS non-parametric Spearman's rank correlation was used to evaluate the correlation between FBP1 expression and the cell proliferation marker Ki-67. P<0.05 was considered to indicate a statistically significant difference.

Results

FBP1 overexpression in ovarian tissues. To identify whether FBP1 is associated with the development of ovarian cancer, FBP1 expression was analyzed in epithelial ovarian normal, adenoma and carcinoma tissues by immunohistochemistry. As shown in Fig. 1A-a/b/c, FBP1 expression was identified in all three groups. FBP1 expression increased as cancer develop-ment and highest FBP1 expression was found in epithelial ovarian carcinoma tissues. A significant higher expression of FBP1 was identified in adenoma than in normal, or in carcinoma than in adenoma, or in carcinoma than in normal tissues (Fig. 1B). The expression of Ki-67, a key marker used to show cell proliferation, was also examined in all three groups. Mimicking the changes identified in FBP1, Ki-67 expres-sion was increased in cancer development and the highest Ki-67 expression was found in epithelial ovarian cancer tissues. However, Ki-67 expression was very low in normal and adenoma tissues (Fig. 1C). Furthermore, the correlation between the expression of FBP1 and Ki-67 was analyzed by Spearman's rank correlation. The correlation coefficient was 0.782 at the 0.01 P-value level. This meant that a significant positive correlation was present between FBP1 and Ki-67. These data indicated that FBP1 is tightly associated with EOC development and cell proliferation.

Knockdown of FBP1 influenced cell proliferation. SKOV3 cells were transfected with control lentiviral particles or FBP1 knockdown lentiviral particles obtained from Santa Cruz, Biotechnology, Inc. screened with puromycin (2.0 μg/ml) for 2 weeks and polyclonal control or FBP1 knockdown SKOV3 cells were generated and named as FBP1-C and FBP-KD SKOV3 cells. As shown in Fig. 2A, the proliferation of FBP1-KD cells was significantly slower than that of the FBP1-C cells according to MTS assay. The colonies of FBP1-KD were significantly smaller than that of FBP1-C (Fig. 2B). In FBP1-C and FBP1-KD cells, the total number of apoptotic cells was ~5.0%, as analyzed by Annexin-V-FITC/PI flow cytometry (Fig. 2C and D). As shown in Fig. 2E, the expres-sion of FBP1 in FBP1-KD cells was ~15% of that in FBP1-C cells; this meant that the knockdown of FBP1 was successful. This data implied that that FBP1-knockdown did not influence apoptosis in SKOV3 cells. Based on these data, we believe that FBP1-knockdown influences cell proliferation; however, it does not influence apoptosis.

Knockdown of FBP1 inhibited cell cycle transition. Since FBP1 knockdown influenced cell proliferation, the effect of FBP1 on cell cycle progression was examined by flow cytometry. As shown in Fig. 2D, the percentage of cells in G1 phase was increased from 53.01% in FBP1-C cells to 72.86% in FBP1-KD cells. In contrast, the percentage of cells in S and G2 phase was decreased from 22.01 and 24.86% in FBP1-C cells to 13.16 and 13.98% in FBP1-KD cells. These data implied that FBP1 knockdown significantly inhibited cell cycle transition from G1 to S and G2 phase. Additionally, the expressions of proteins associated with cell cycle progression were analyzed. The proteins promoting cell cycle progression, including c-Myc, cyclin D1/E, were inhibited in FBP1-KD cells. However, proteins inhibiting cell cycle progression, such
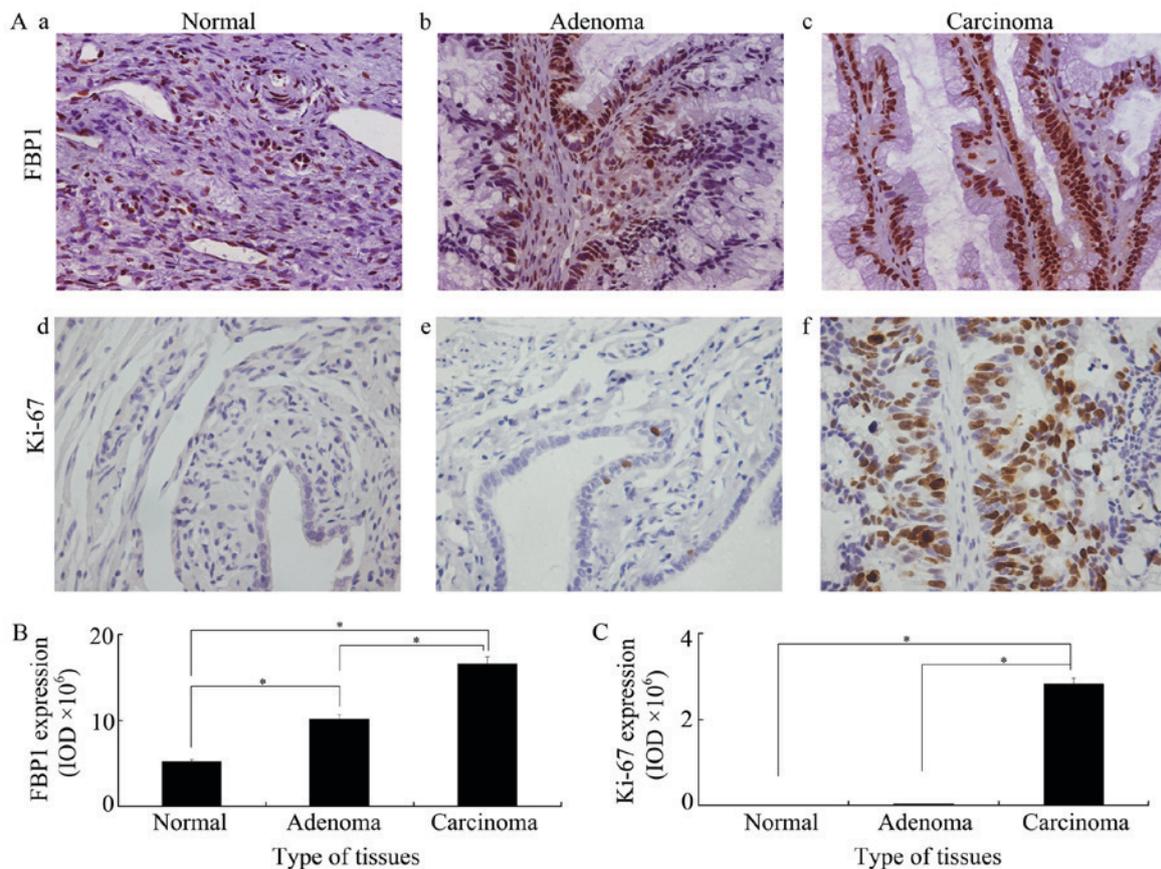
Figure 1. FBP1 and Ki-67 expressions in epithelial ovarian tissues. (A) FBP1 and Ki-67 expressions in (a and d) epithelial ovarian normal tissues, (b and e) adenoma tissues and (c and f) carcinoma tissues. Paraffin sections (5 mm) were deparaffinized and immunostaining was performed using anti-FBP1 or anti-Ki-67 antibodies. The antigen-antibody reactions were visualized with DAB and the images were captured by a x40 objective. Quantitative analysis of (B) FBP1 and (C) Ki-67 expression in epithelial ovarian normal, adenoma and carcinoma tissues by Image-Pro Plus 6 software. The intensity of DAB brown reactions was expressed as IOD. An analysis of variance with Student-Newman-Keuls post-hoc test was used to analyze statistical significance between groups. *P<0.05 was considered to be statistically significance. IOD, integral optical density.

as p21 and p27, were increased in FBP1-KD cells (Fig. 2E). These data demonstrated that FBP1 influences cell cycle transition by adjusting the expression of cell cycle associated proteins.

FBP1 was associated with cell migration and metastasis. Metastasis is a key process that is required for cancer development (23). An improved understanding of the genetic evolution of metastasis can provide insight into the biology of cancer and contribute to the development of novel therapeutics. Therefore, the influence of FBP1 on ovarian cell migration and invasion was investigated. In the wound-healing assay, the migration of FBP1 knockdown SKOV3 cells was slower than that of FBP1 control cells (Fig. 3A). At 24 or 48 h later, the migration distance of FBP1-C cells was significant higher than that of FBP1-KD cells (Fig. 3B). Additionally, the results from the Transwell assay also revealed that FBP1 knockdown inhibited cell migration to the bottom chambers (Fig. 3C).

As MMPs are key regulators of cellular matrix degradation and promote cell migration (24), the expression of MMP-2 in FBP1-C and FBP1-KD SKOV3 cells was investigated and it was demonstrated that FBP1 knockdown inhibited the expression of MMP-2 (Fig. 3D). These data implied that FBP1 could promote cell migration and facilitate cell metastasis.

Discussion

In 2015, ovarian cancer was present in 1.2 million women and resulted in 0.16 million mortalities worldwide (24). The evolution of surgical techniques and chemotherapy regimens over the past three decades has resulted in improvements in the survival of patients with ovarian cancer (3). Despite high initial response rates for patients with chemotherapy, the majority of patients ultimately suffer from recurrence due to chemotherapy resistance (3). Therefore, novel approaches for the diagnosis and the treatment of ovarian cancer are urgently required. Improvements in the understanding of cancer biology and the underlying mechanisms governing the cancer processes have facilitated the development of targeted therapies, including small-molecule inhibitors and monoclonal antibodies (3). Certain ovarian cancers may be caused by mutations, such as the BRCA1 or BRCA2 mutation (25,26) and dysregulation in the mitogen-activated protein kinase (MAPK) pathway (27). However, the majority of ovarian cancers can be attributed to a growing number of somatic aberrations (28). In the present study, the progression of ovarian cancer was positively associated with FBP1 expression. As the epithelial ovarian cancer progressed, the expression of FBP1 increased significantly. Furthermore, FBP1 was demonstrated to not only enhance cell proliferation but also promote cell metastasis; despite
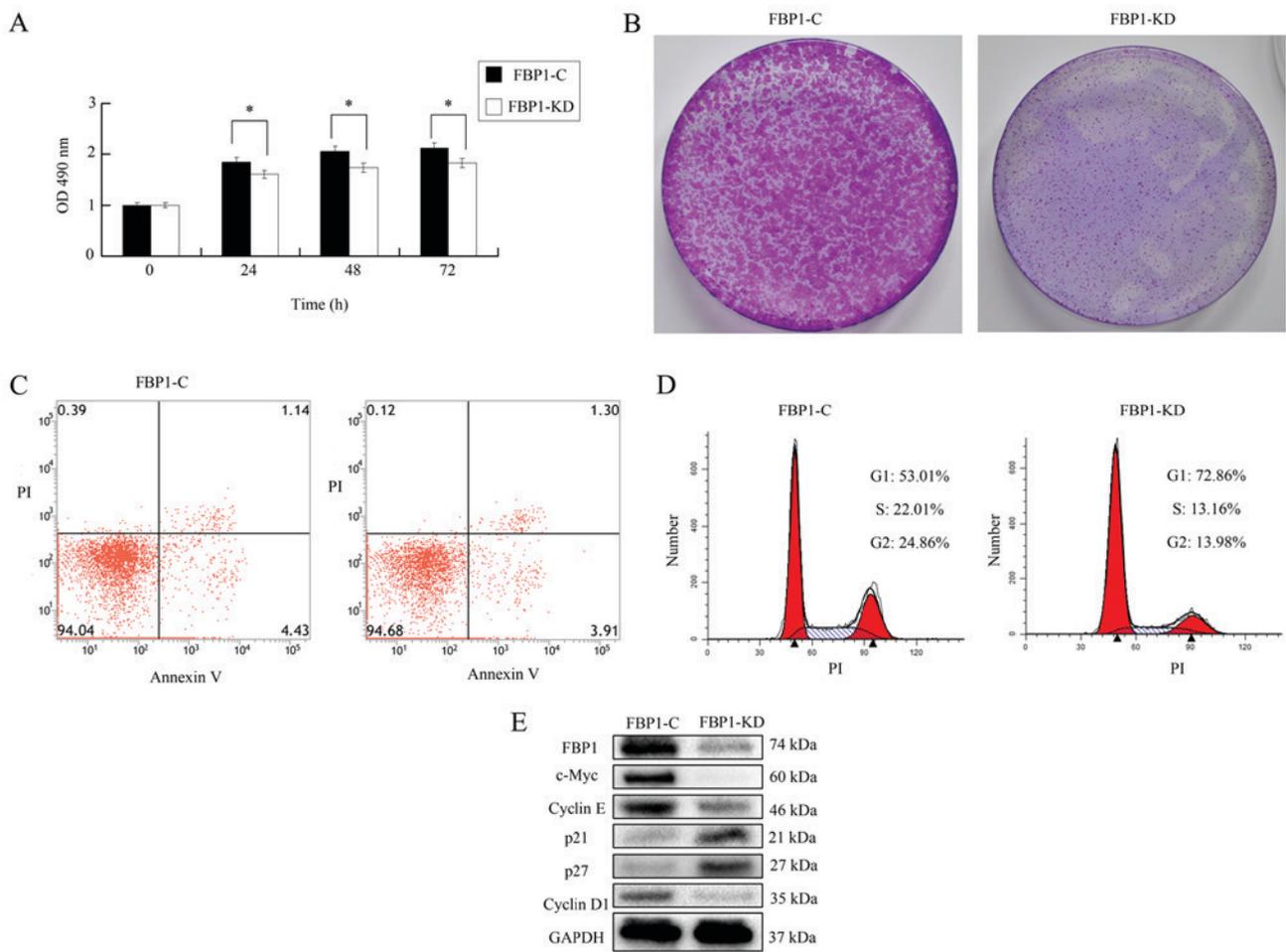
Figure 2. The knockdown of FBP1 inhibited cell proliferation and cell cycle transition. (A) FBP1 knockdown decreased cell proliferation examined by MTS assay. (B) FBP1 knockdown decreased colony formation. (C) FBP1 knockdown did not influence apoptotic occurrence in FBP1-C and FBP1-KD SKOV3 cells as analyzed by flow cytometry. (D) FBP1 knockdown inhibited cell cycle transition analyzed by flow cytometry. (E) The expression of cell cycle associating proteins in FBP1-C and FBP1-KD SKOV3 cells analyzed by western blotting. GAPDH acted as a loading control. Student's t-test was used to test statistical significance between groups. *P<0.05 vs. FBP1-C. OD, optical density; FBP1-C, FBP1 normal control SKOV3 cells; FBP1-KD, FBP1 knockdown SKOV3 cells.
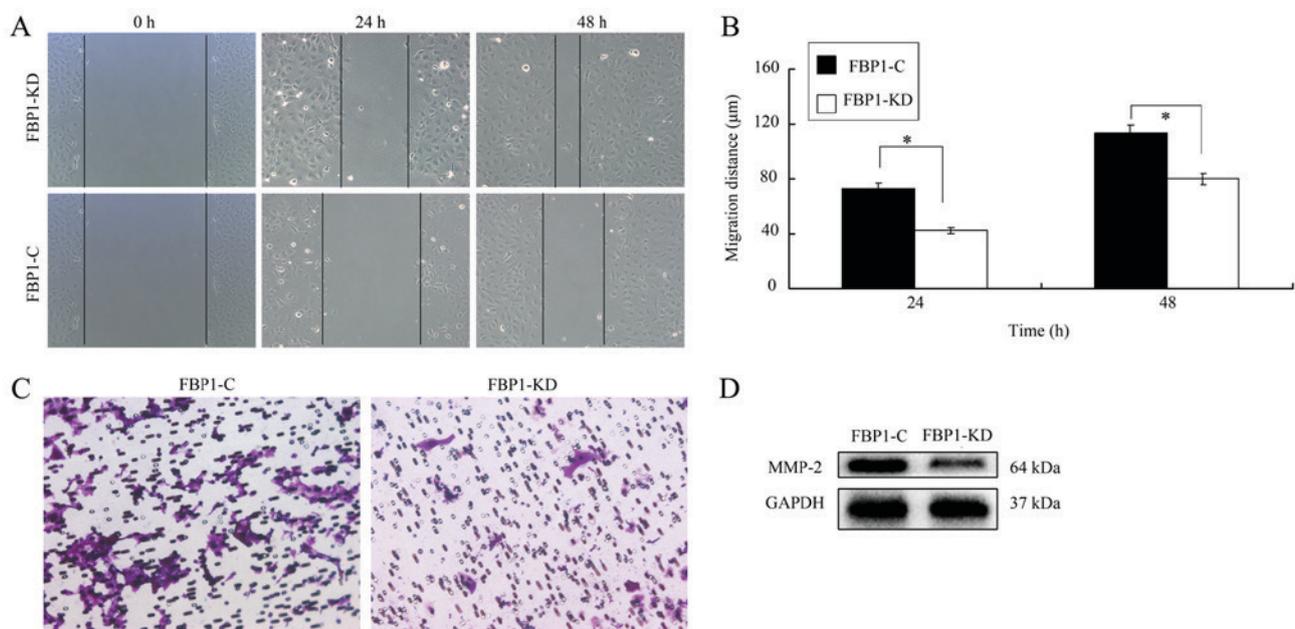

Figure 3. The knockdown of FBP1 inhibited cell metastasis. (A) Knockdown of FBP1 inhibited cell migration analyzed by wound healing experiment. (B) Migratory distance of the wound-healing assay (C) Knockdown of FBP1 inhibited cell migration as analyzed by the Transwell assay. (D) MMP-2 expression in FBP1-C and FBP1-KD SKOV3 cells. Student's t-test was used to test statistical significance between groups. *P<0.05 vs. FBP1-C.

FBP1 not affecting the occurrence of apoptosis. Therefore, we hypothesized that FBP1 is a potential target for novel treatment development and may serve as a diagnostic marker for ovarian cancer early screen. In our previous studies, FBP1 was demonstrated as a potential target of platinum and knockdown of FBP1 increased the sensitivity of ovarian cancer cells to carboplatin (22).

Decreased cell viability may be induced by cell cycle transition suppression and cell death activation. In the colony formation experiment of the present study, the knockdown of FBP1 was demonstrated to significantly inhibit colony formation and an increased number of cells were restrained in G1 phase and fewer cells were restrained in the S and G2 phases due to FBP1 knockdown. The cell cycle transition inhibiting proteins, such as p21 and p27, were induced by FBP1 knockdown. Conversely, cell cycle transition promoting proteins, including cyclin D1/E, c-Myc, were suppressed by FBP1 knockdown, and no influence of FBP1 knockdown on apoptosis was identified. When combined with the results of a previous study in which caspase 3 was not demonstrated to be activated in ovarian cancer cells (22), the results of the present study suggest that FBP1 knockdown significantly suppresses cell cycle transition; however, does not affect cell death in ovarian cancer cells.

Metastasis, the process by which a localized cancer becomes a systemic disease, is a complex, multistep process that requires cancer cells to detach from the primary tumor, travel, survive and proliferate in distant organs (29,30). Metastatic tumors can occur in the early and late stages in the primary tumor (29,30). An improved understanding of the genetic evolution of metastasis may provide insight into the biology of cancer and contribute to the development of novel therapeutics. MMPs have been traditionally considered to regulate a number of processes such as cell migration and metastasis (31,32). MMPs are generally expressed at low levels; however, can be upregulated during tissue remodeling, inflammation, wound healing, and cancer progression (33,34). It has been reported that the expression of MMP-2 was significantly higher in advanced ovarian cancer than in their benign or premalignant counterparts (35). In the present study, the knockdown of FBP1 was demonstrated to inhibit the migration of EOC cells, metastasis and the expression of MMP-2; an important member of the MMP family. Suggesting that FBP1 knockdown decreases the migration and metastasis through the inhibition of MMP expression.

The diagnosis of ovarian cancer has been challenging due to a lack of biomarkers in the early stage of disease, and the majority of patients eventually experience a median progression-free survival of 18 months (36). The current gold-standard chemotherapy, a platinum-paclitaxel combination regimen, is still far from optimum even though it is a significant improvement from previously administered treatments (3). A previous study demonstrated that FBP1 is a target protein of platinum-based treatment (37). In the present study, the progression of ovarian cancer was demonstrated to be positively associated with FBP1 expression. FBP1 enhanced cell cycle transition and metastasis, leading to the conclusion that FBP1 is a potential diagnostic marker for ovarian cancer in the early stage of disease.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Authors' contributions

XX, ZL performed initial experimental design. XX, JZ, WC and SQ performed the experiments. XH, LD and WL prepared the tissue samples. XX and ZL wrote the paper. ZZ and XL made contirbutions to the experimental design and the manuscript.

Ethics approval and consent to participate

This study was conducted after ethics approval was obtained from the Medical Ethics Committee of Guangzhou Red Cross hospital of Medical College, Jinan University (Guangzhou, China). All contributors consented to participate.

Consent for publication

All authors read and approved the final manuscript.

Competing interests

The authors declared that they have no competing interests.

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