53BP1 inhibits the migration and regulates the chemotherapy resistance of ovarian cancer cells

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Abstract. The major problems faced during the treatment of ovarian cancer are metastasis and the development of intrinsic or acquired drug resistance. The present study assessed whether tumor protein p53 binding protein 1 (53BP1) regulated migration and modulated chemotherapy resistance in SKOV3 cells and identified proteins associated with the molecular mechanisms underlying this coordinate regulation. SKOV3 cells were transfected using a 53BP1-expressing vector, which induced 53BP1 overexpression. The migration of the transfected cells was observed using a Transwell assay. The expression of matrix metalloproteinase (MMP)-2 and MMP-9 were assayed using gelatin zymography. In addition, the effects of 53BP1 on the chemosensitivity of SKOV3 cells to cisplatin were evaluated using MTT and western blot assays. Compared with the control, the average number of migrating SKOV3/pLPC-53BP1 cells was decreased from 230±58 to 45±12 ($P<0.05$) and the protein expression of MMP-9 was significantly inhibited. However, the chemosensitivity of SKOV3/pLPC-53BP1 to cisplatin decreased significantly: Cisplatin half maximal inhibitory concentration (IC_{50}) for SKOV3/pLPC-53BP1=7.58±0.51 μg/ml; cisplatin IC_{50} for control=2.98±0.27 μg/ml ($P<0.01$). Decreased chemosensitivity to cisplatin may be associated with increased expression of phosphorylated-protein kinase B and cyclin dependent kinase 2 and with decreased expression of p21 and the B cell lymphoma (Bcl)-2 associated X/Bcl-2 ratio. The results of the present study demonstrated that 53BP1 may inhibit migration but upregulate chemoresistance to cisplatin in SKOV3 cells.

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Introduction

In addition to the implementation of tumor cytoreductive surgery, the majority of patients with ovarian cancer require regular chemotherapy to remove residual small lesions. Though initially sensitive to chemotherapy, up to 80% of patients with ovarian cancer produce endogenous or acquired chemoresistance (1). The recurrence and metastasis of ovarian cancer results in poor prognosis. The 5-year survival rate for patients with advanced ovarian cancer is 34-39% (2).

Previous studies have demonstrated that epithelial ovarian cancer treatment improves with platinum-based combination chemotherapy compared with nonplatinum-based chemotherapy (3-5). Cisplatin is commonly used by gynecologists to kill tumor cells by inducing DNA damage, promoting cell cycle arrest and increasing the rate of apoptosis (6,7). Previous studies have reported the following mechanisms underlying chemotherapy resistance: Certain tumor cells exhibit enhanced DNA repair functions and a weakened apoptotic process, with the transformation to epithelial stroma facilitating the secretion of matrix protein enzymes, including matrix metalloproteinase (MMP)-2 and MMP-9, which enable these tumor cells to break through the basement membrane, metastasize and invade. Therefore, chemoresistance and invasion are associated in tumor cells (8-12).

Tumor protein p53 binding protein 1 (53BP1), an adaptor/mediator protein, is a cytologic marker of endogenous doublestranded DNA damage (13-15). 53BP1 is primarily associated with DNA damage response activation, which maintains the stability of genetic information within the cell. However, Lai *et al* (11) studied the clinical and biological significance of 53BP1 and demonstrated that increased expression of 53BP1 was associated with increased resistance to cisplatin and worsened prognosis in lung adenocarcinomas. To the best of our knowledge, no research has focused on the effects of 53BP1 on migration and chemoresistance in ovarian cancer. The present study assessed the modulation of 53BP1 expression level in the ovarian cancer cells SKOV3, and recorded alterations in cell metastasis and chemotherapy sensitivity.

Materials and methods

Reagents. Lipofectamine 2000, RPMI-1640 medium and puromycin were purchased from Invitrogen; Thermo

Fisher Scientific, Inc. (Waltham, MA, USA). N-Myc-proto-oncogene (MYC)-53BP1-wild type (WT) pLPC-Puro plasmids were obtained from Addgene, Inc. (Cambridge, MA, USA). The N-MYC-WT pLPC-Pruo plasmid was obtained from the Laboratory of Cell Biology and Genetics of Rockefeller University (New York, NY, USA). The enhanced chemiluminescence (ECL) kit used for western blot analysis and the gel electrophoresis device were acquired from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA). The antibodies against protein kinase B (Akt; 1:1,000; cat. no. 9272), phosphorylated (p)-Akt (1:1,000; cat. no. 4060), B cell lymphoma (Bcl)-2 (1:1,000; cat. no. 2872), Bcl-2 associated X (Bax; 1:1,000; cat. no. 2772), p21 (1:1,000; cat. no. 2947), cyclin dependent kinase 2 (CDK2; 1:1,000; cat. no. 2546) and β-actin (1:1,000; cat. no. 4970) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Cisplatin was purchased from Sigma-Aldrich; Merck KGaA.

Cell culture and transfection. The human ovarian cancer cell line SKOV3 was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Tianjin Haoyang Biological Products Technology Co., Ltd., Tianjin, China) in accordance with the supplier's protocol. The cells were transfected with N-MYC-proto-oncogene-53BP1-WT pLPC-Puro and N-MYC-WT pLPC-Pruo using Lipofectamine 2000 according to the manufacturer's protocol. Endogenous 53BP1 overexpression was induced in SKOV3 cells using N-MYC-53BP1 WT PLPC-Puro plasmids. Cells were selected for overexpression in complete RPMI-1640 medium containing 1.5 μg/ml puromycin at 37˚C. Cells that survived for 10 days were named SKOV3/pLPC-53BP1 (as the experimental group) or SKOV3/pLPC-vector (as the control group). The efficiency of transfection was confirmed using western blot analysis as subsequently described.

Transwell migration assay. SKOV3/pLPC-53BP1 and SKOV3/pLPC-vector cells ($1x10^7$ cells/ml, 100 μl) were resuspended in RPMI-1640 medium without FBS and placed into the coated membrane of the upper chamber of Transwell plates. RPMI-1640 supplemented with 20% FBS was used as an attractant in the lower chamber. Following incubation at 37˚C for 24 h, migratory cells located on the lower side of the chamber were fixed in methanol (at room temperature for 15 min) and then stained with 0.2% crystal violet (at room temperature for 15 min). The stained cell images were captured using a light microscope and 10 random fields at magnification, x10 were counted. Results represented the average of triplicate samples from three independent experiments.

Gelatin zymography analysis. Gelatinolytic activity was induced in the cell culture supernatant of SKOV3/pLPC-53BP1 and SKOV3/pLPC-vector cells via SDS-PAGE with a 10% gel containing 1 mg/ml gelatin (Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation in renaturation buffer (2.5% Triton X-100 at room temperature for 45 min) and development buffer (pH 7.6, Tris. HCl 6.06 g, $CaCl_2$ 0.56 g, NaCl 11.688 g, $ZnCl_2$ 0.136 mg, ddH_2O up to 1,000 ml) at 37˚C for 18 h, the gels were stained using Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Inc.), and destained in a mixture of acetic acid and methanol at room temperature every 5 min until a colorless enzyme band showed. Relative quantities of protein were measured using a densitometer (ImageJ software v1.48, National Institutes of Health, Bethesda, ML, USA). All procedures were performed in duplicate.

Drug sensitivity assay. An MTT assay was performed to assess the sensitivity of SKOV3/pLPC-53BP1 cells to cisplatin; SKOV3/pLPC-vector cells served as the control. Cells were seeded onto 96-well plates in RPMI-1640 medium with 10% FBS ($4.0x10^3$ cells/well; final volume=200 μl). Following attachment to the plates, cells were exposed to 0, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 or 25.6 μg/ml cisplatin for 72 h at 37˚C in a 5% CO_2 incubator. Subsequently, 20 μl MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to each well and wells were incubated for a further 4 h at 37˚C. The medium was then removed and 150 μl dimethyl sulfoxide was added to each well. Absorbance of each well at 490 nm was read using a microplate reader. The half maximal inhibitory concentration (IC_{50}) of each drug was estimated from the relative survival curves. Independent experiments were performed three times in 5 duplicate wells. Mitochondrial activity, which may reflect cellular growth and viability, was evaluated by measuring optical density (OD) at 490 nm on a microtiter plate reader. The relative survival rate was calculated as follows:

$$\text{Relative survival rate (\%)}=(OD_{treated}-OD_{zero\ setting})/(OD_{control}-OD_{zero\ setting})\ x100\%.$$

Western blot analysis. The SKOV3/pLPC-53BP1 cells that were maintained in 0, 0.4, 0.8 or 1.6 μg/ml cisplatin for 72 h were harvested. Whole-cell lysates were prepared by incubating cells in RIPA buffer (Shennengbocai, Shanghai, China; 1% NP-40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate, 1 mM sodium vandate) containing protease inhibitors (1 mM phenylmethane sulfonyl fluoride and 1 mM sodium fluoride) on ice for 30 min. Cell lysates were centrifuged at 8,000 x g for 15 min at 4˚C. The supernatant was subsequently collected and the protein concentration was measured using a BCA Protein Assay kit (Merck KGaA). A total of 50 μg proteins were separated using 8% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked using 5% non-fat milk for 1 h at room temperature and then incubated with primary antibodies, including Akt, p-Akt, Bcl-2, Bax, p21, CDK2 and β-actin overnight at 4˚C. The membranes were then washed three times with TBST and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. no. 5210-0174; Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) at room temperature for 2 h. Bands were detected with an enhanced chemiluminescence detection system (cat. no. NEL102001EA; PerkinElmer Inc., Waltham, MA, USA). ImageJ software (version 1.48; National Institutes of Health, Bethesda, ML, USA) was used for the densitometric analysis of western blotting.

Statistical analysis. Data were expressed as the mean ± standard deviation. Statistical comparisons between groups of normally

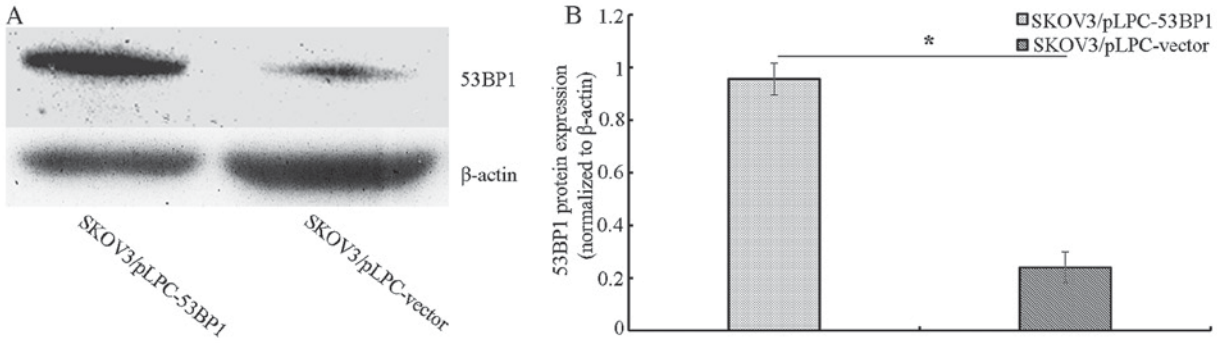

Figure 1. Protein expression of 53BP1 in modified ovarian cancer cells. (A) 53BP1 expression in SKOV3/pLPC-53BP1 and SKOV3/pLPC-vector cells was evaluated using western blot analysis. β-actin served as a loading control. (B) Relative expression of 53BP1 protein in ovarian cancer cells. Data were represented as the mean ± standard deviation. β-actin was a normalization control. *P<0.05 vs. control. 53BP1, tumor protein p53 binding protein 1.

distributed data were performed using the Student's t-test via SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Evaluation of 53BP1 protein expression in ovarian cancer cells following transfection with 53BP1-overexpressing plasmids. Stable transfected cell lines were generated by expanding the resistant colonies, including SKOV3/pLPC-53BP1 and SKOV3/pLPC-vector transfected cell lines. The expression of 53BP1 in these cell lines was assessed using western blot analysis. SKOV3/pLPC-53BP1 cells exhibited increased 53BP1 expression compared with SKOV3/pLPC-vector cells (Fig. 1A and B).

53BP1 ectopic expression decreases migration in SKOV3-derived cell lines. To determine the effect of 53BP1 on SKOV3 migration, Transwell assays were performed. SKOV3/pLPC-53BP1 cells exhibited a significant decrease in migration compared with SKOV3/pLPC-vector cells (P<0.05). The number of migratory SKOV3/pLPC-53BP1 cells was 45.00±12.00, compared with 230.00±58.00 migratory SKOV3/pLPC-vector cells (P<0.05; Fig. 2A-C). Therefore, the present study demonstrated that 53BP1 significantly decreased migration in SKOV3 cells.

53BP1 decreases MMP-associated proteolytic activity. Using gelatin zymography, the effect of 53BP1 on MMPs was evaluated. SKOV3/pLPC-vector cells (gray value=18.17±2.13) exhibited increased MMP-9 activity compared with SKOV3/pLPC-53BP1 cells (gray value=7.94±1.12; Fig. 2D-F). However, the two types of cell exhibited no difference in the activity of MMP-2.

Evaluation of cisplatin-associated antitumor activity using an MTT assay. The cytotoxicity of cisplatin in SKOV3/pLPC-53BP1 and SKOV3/pLPC-vector cells was evaluated using an MTT assay. SKOV3/pLPC-vector cells (IC_{50}=2.98±0.27 μg/ml) were demonstrated to be more sensitive to 0.4-6.4 μg/ml cisplatin compared with SKOV3/pLPC-53BP cells (IC_{50}=17.58±0.51 μg/ml; Fig. 3A).

Western blot analysis. When culturing cells without cisplatin, increased 53BP1 expression was associated with the upregulation of proteins associated with the inhibition of apoptosis, including Bax, p21 and the Bax/Bcl-2 ratio, However, with the downregulation of proliferation-promoting proteins, including p-Akt, Bcl-2 and CDK2 (Fig. 3B-F), SKOV3/pLPC-53BP1 cells exposed to 0.4-1.6 μg/ml cisplatin exhibited a decrease in the protein expression of Bax/Bcl-2 and p21 compared with SKOV3/pLPC-vector cells (Fig. 3B, D and F, respectively). For treatment with 0.8-1.6 μg/ml cisplatin, the expression of p-Akt and CDK2 was increased in SKOV3/pLPC-53BP1 compared with SKOV3/pLPC-vector cells (Fig. 3C and E, respectively).

Discussion

Despite the progress in cancer treatment and the understanding of tumor biology, ovarian cancer remains one of the most lethal gynecologic malignancies (16). Further study is required to resolve aggressive behavior and drug resistance in ovarian cancer, which are major challenges to developing more effective therapies. The BRCA1 C-Terminal domain-containing 53BP1, a p53-binding protein, is associated with the regulation of the cell cycle and DNA damage response (17-19). Previous studies have demonstrated that following knockdown of 53BP1, mice exhibited growth retardation, radiotherapy sensitivity and tumor susceptibility (20,21). Furthermore, 53BP1 may attenuate the expression of p-Akt and Bcl-2, increase the protein expression of Bax and the Bax/Bcl-2 ratio, and induce cell cycle arrest and apoptosis, resulting in decreased proliferation, in SKOV3 cells (22). To further assess the effects of 53BP1 on SKOV3 cells, the present study evaluated migration and chemosensitivity to cisplatin in 53BP1-overexpressing SKOV3 cells.

During metastasis, cancer cells receive signals from the tumor microenvironment and undergo an epithelial-mesenchymal transition, which decreases tumor polarity and cell-cell adhesion (23). Furthermore, cancer cells may secret proteinases to degrade multiple components of the extracellular matrix and thereby enhance migration and metastasis (24). The extracellular matrix and basement membrane are barriers inhibiting metastasis. The degradation of the extracellular matrix by metastatic cancer cells is associated with multiple proteolytic enzymes, including MMPs and cathepsins (25,26). Typically, increased cancer aggression is associated with increased MMP secretion by the cancer (27).

In the present study, MMP-9 expression was downregulated by 53BP1 overexpression compared with the control, resulting in the inhibition of migration in SKOV3 cells (Fig. 2). However,

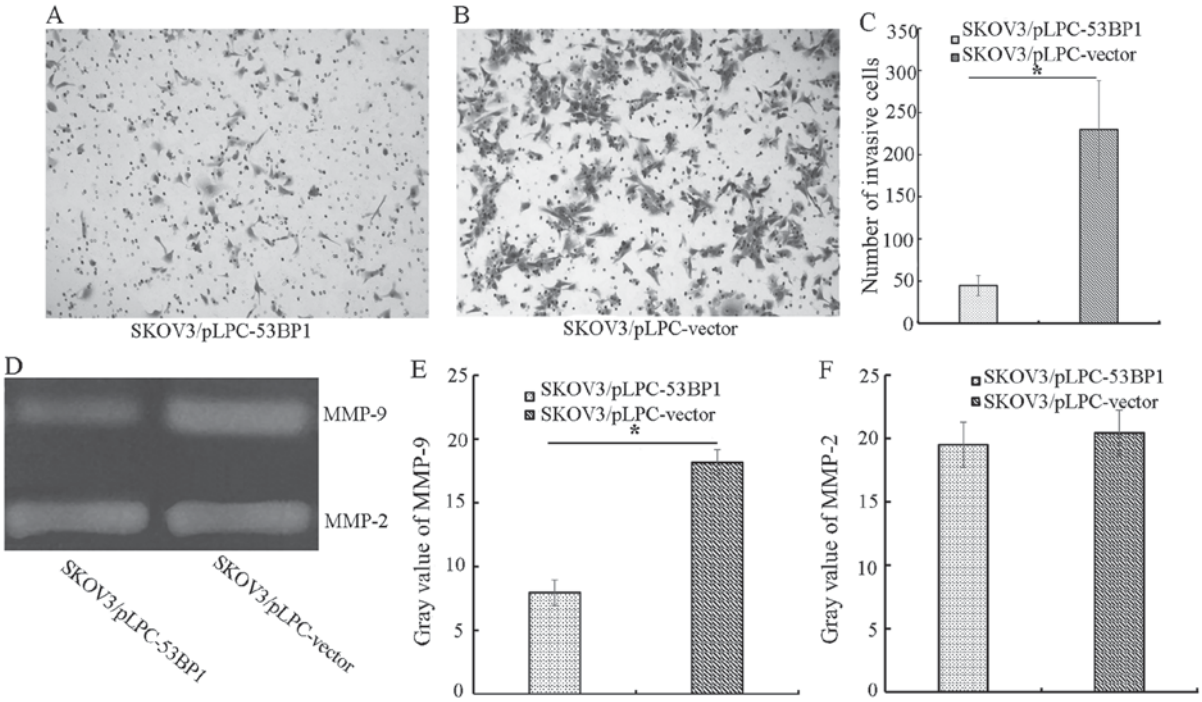

Figure 2. Transwell assay reveals that cell migration is inhibited by 53BP1 overexpression. (A) Representative image of migratory SKOV3/pLPC-53BP1 cells (magnification, x10). (B) Representative image of migratory SKOV3/pLPC-vector cells (magnification, x10). (C) Compared with migratory SKOV3/pLPC-vector cells, the mean number of migratory SKOV3/pLPC-53BP1 cells was decreased from 230±58 to 45±12 (P<0.05). (D) MMP-9 and MMP-2 activity were assessed using gelatin zymography. (E) Compared with that in SKOV3/pLPC-vector cells, the protein expression of MMP-9 in SKOV3/pLPC-53BP1 cells was significantly inhibited and (F) the protein expression of MMP-2 was not significantly altered. Data were represented as the mean ± standard deviation. *P<0.05 vs. control. 53BP1, tumor protein p53 binding protein 1; MMP, matrix metalloproteinase.

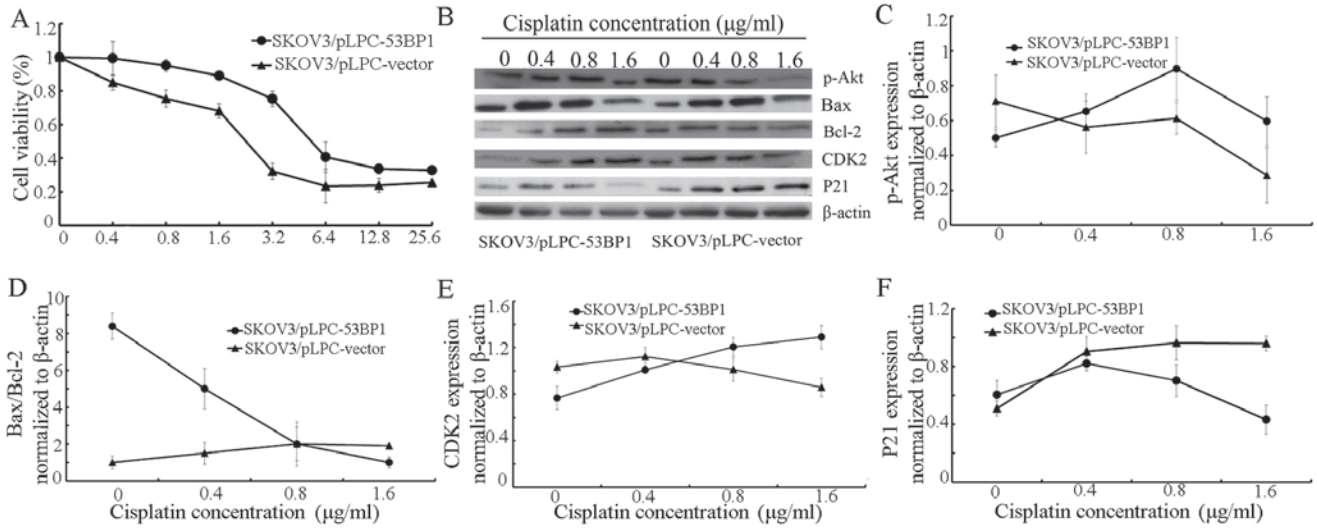

Figure 3. Expression of 53BP1 is associated with cisplatin chemoresistance. (A) Cell viability was assayed following treatment with an increasing concentration of cisplatin for 72 h. (B) Expression of p-Akt, Bax, Bcl-2, CDK2 and p21 in SKOV3/pLPC-53BP1 and SKOV3/pLPC-vector cells following treatment with 0, 0.4, 0.8, 1.6 μg/ml cisplatin was assessed using western blot analysis. β-actin served as a loading control. The relative expression of (C) p-Akt, (D) Bax/Bcl-2, (E) CDK2 and (F) p21 in SKOV3/pLPC-53BP1 and SKOV3/pLPC-vector cells. β-actin was a normalization control. Data were represented as the mean ± standard deviation. 53BP1, tumor protein p53 binding protein 1; p-Akt, phosphorylated protein kinase B; Bax, Bcl-2 associated X; Bcl-2, B cell lymphoma-2; CDK2, cyclin dependent kinase 2.

no statistically significant difference in MMP-2 expression was demonstrated between groups. These results suggested that 53BP1 decreased the metastatic potential of SKOV3 cells by inducing the downregulation of MMP-9. Further study is required to determine the 53BP1-associated pathway that facilitated MMP-9 attenuation.

Certain tumor invasion-regulating genes are associated with numerous characteristics of tumor biology, including drug sensitivity and the induction of angiogenesis (28). Drug resistance remains a substantial challenge to successfully treating ovarian cancer. Cisplatin is an effective chemotherapeutic agent for the treatment of ovarian cancer. The present study evaluated the

effect of 53BP1 on the chemosensitivity of SKOV3 cells to cisplatin. SKOV3 cells overexpressing 53BP1 and cultured without cisplatin exhibited decreased viability compared with control cells cultured without cisplatin, a result consistent with those of a previous study (22). However, increased 53BP1 expression was associated with increased cisplatin resistance in SKOV3 cells. With particular respect to the 0.4-1.6 μg/ml cisplatin treatment, 53BP1 expression was associated with resistance to cisplatin.

Resistance to cisplatin is associated with multiple complex system properties, and the underlying mechanism remains to be fully understood. Previously, Bcl-2, p-Akt, p21, Bax and CDK2 have been demonstrated to be associated with chemotherapy resistance in multiple types of tumor (22,29). The present study therefore proposed that these proteins may also be associated with 53BP1-induced chemotherapy resistance in SKOV3 cells. The present study demonstrated that treatment with 0.4-1.6 μg/ml cisplatin resulted in a decreasing trend in the protein expression of Bax/Bcl-2 and p21; an increasing tendency in the expression of p-Akt and CDK2 in SKOV3/pLPC-53BP1 was also observed at 0.4-1.6 μg/ml cisplatin, compared with SKOV3/pLPC-vector cells. The Akt signaling pathway is associated with Bax/Bcl-2-mediated cell survival and the phosphorylation of the Thr145 and Ser146 residues of p21, which renders the protein incapable of entering the nucleus from the cytoplasm (30). Increased active CDK2 expression promotes p21 degradation and thereby facilitates p21 repression. Thus, decreased expression of p21, a tumor suppressor, may combine with increased CDK2 expression to promote cell survival (31).

However, a consensus on the effects of 53BP1 on cellular responses to chemotherapeutic agents has yet to be reached, with multiple studies having demonstrated increased or decreased drug sensitivity with 53BP1 overexpression (11,32,33). These conflicting reports suggest that the association between 53BP1 and chemosensitivity may be more complex than previously assumed.

To conclude, the present study revealed that 53BP1 suppressed SKOV3 cell migration by decreasing MMP-9 expression. However, the present study also suggested that 53BP1 promoted cisplatin chemoresistance, a function associated with decreased Bax/Bcl-2 and p21 expression and increased p-Akt and CDK2 expression. Although the mechanism underlying 53BP1-induced chemoresistance remains to be understood, a potential underlying mechanism for future studies to evaluate is DNA repair.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

BK designed the study. SH performed experiments, participated in statistical analysis and drafted the manuscript. SH, XL, YZ and QY assisted with experiments and manuscript preparation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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