Oxysterol-Binding Protein 2 Promotes Pancreatic Ductal Adenocarcinoma Progression Through Epithelial-Mesenchymal Transition Process

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Research Article

Keywords: epithelial-mesenchymal transition, Oxysterol-binding protein 2, pancreatic ductal adenocarcinoma, migration, invasion, proliferation, apoptosis

Posted Date: September 9th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-832603/v1>

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Version of Record: A version of this preprint was published at Frontiers in Oncology on January 20th, 2022. See the published version at <https://doi.org/10.3389/fonc.2021.762233>.

Abstract

Oxysterol-binding protein 2 (OSBP2) is crucial for the promotion of growth and development of cancers, however, its effects in pancreatic ductal adenocarcinoma (PDAC) were still unclear. Here we report the evidence that OSBP2 works as an efficient tumor-associated protein to lead to PDAC extremely malignant characters. We discovered that raised OSBP2 expression in primary tumors was associated with shorter survival in PDAC patients. Therefore, we used immunohistochemistry to analysis the levels of OSBP2 expression in PDAC tissues and adjacent para-cancer tissues. We used wound-healing assay and transwell assay to evaluate the effects of OSBP2 on PDAC cells' (ASPC-1 and BXP-3) migration and invasion, respectively. Using CCK-8 and Annexin V / PI double staining, we evaluated the effects of OSBP2 on PDAC cells' proliferation and apoptosis, respectively. We also explored the effect of OSBP2 on chemosensitivity (gemcitabine and 5-Fluorouracil). And we further validated these findings in vivo of mice. At last, we used western blot to analysis the effect of OSBP2 on PDAC cells' phenotype. We proved that OSBP2 overexpression promoted PDAC cells' migration, invasion, proliferation and chemotherapy resistance, decreased apoptosis. OSBP2 overexpression downregulated E-cadherin, and upregulated the levels of N-cadherin, vimentin, Snail, Slug, ZEB1 and β -Catenin. Taken collectively, our findings indicated that OSBP2 exhibited overexpression in PDAC, and upregulation of OSBP2 may promote the progression of PDAC. OSBP2 may have potential diagnostic and therapeutic values in PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a serious health problem worldwide. PDAC has a very poor prognosis, with a five-year survival rate of less than 5 percent and is the eighth most common cause of cancer death globally¹⁻³. In China, PDAC patients' median survival time is only 7.8 months, with 30.0% of patients receiving effective radical operations and only 9.8% of patients undergoing synthetical curative treatment^{4,5}. However, the causes of PDAC remain unclear. Although tremendous efforts have been made and great progress has been made in this field over the last few decades, PDAC patients' survival exhibit no obvious improvement^{6,7}. An aggressive biological phenotype which has early local invasion and metastasis features causes the low survival rates^{8,9}. Therefore, we need to do more to elucidate the molecular mechanisms behind the occurrence, development, treatment resistance and metastasis of this deadly disease.

Cancer cells' invasion and metastasis courses are very complex involving many factors, extracellular matrix plays essential roles in the initiation and maintenance of tumor metastasis¹⁰⁻¹². The most critical relevant mechanisms mediating the metastatic cascade is epithelial-mesenchymal transition (EMT) process in cancer cells, which is also a primary step in the induction of tumor cells' migration and invasion^{13,14}. EMT plays important roles in embryonic development and in the differentiation of multiple tissues and organs as well as in the metastasis of cancer cells¹⁵⁻¹⁷. That process is characterized by a loss of cell to cell or extracellular matrix contacts through the inhibition of epithelial markers including E-cadherin expression, and acquisition of mesenchymal features, such as up-regulation of the

mesenchymal markers N-cadherin and vimentin^{18,19}. Cells undergoing EMT process are endowed with increased migratory and invasive properties²⁰. A large number of research have demonstrated that EMT process is involved in the development and progression of PDAC^{21–23}.

Twelve mammalian genes constitute oxysterol-binding protein (OSBP) and OSBP-related proteins (ORPs), which is characterized by a conserved OSBP homology domain (OHD) that binds sterols and lipids, as well as the pleckstrin homology domain and two phenylalanines in an acidic tract motif that mediate interaction with organelle membranes²⁴. A great number of studies demonstrated that some OSBP/ORP members participate in progress of cancer. However, how OSBP/ORP family members influence cancer cells' initiation and progression and the mechanism behind these processes remain unclear. OSBP2, which is also named OSBP4, is routinely expressed in the human brain, heart and testis, but not in other human tissues and mouse tissues^{25,26}. ORP4 plays an important role in human malignant tumor cells' proliferation and survival²⁷ and is a target of the natural anti-proliferative steroidal saponin OSW-1²⁸, and published studies show that OSBP2 plays a critical role in the control of oncogenic cell growth. At present, no evidence showed the relationship between PDAC and OSBP2.

In summary, we first demonstrated that OSBP2 may work as a tumor-associated protein which significantly affect the malignant behaviors of PDAC. The malignant behaviors of PDAC could be significantly enhanced when OSBP2 overexpression, while down-regulation of OSBP2 could decrease the migration and invasion abilities of PDAC cells in vitro and in vivo. Also, we proved that OSBP2 was involved in the EMT process of PDAC.

Results

Overexpression of OSBP2 is correlated with shorter survival in PDAC

The relationship between the clinicopathological information of PDAC and the expression of OSBP2 was revealed in Table I. Our data demonstrated that the expression of OSBP2 in PDAC tissues was related to nerve invasion ($P=0.05$) and pathological grading ($P=0.05$). But OSBP2 expression was not related to age, sex, metastasis of lymph node and AJCC stage. IHC staining results showed that the expression of OSBP2 was higher in PDAC tissues than in adjacent para-cancer tissues (Fig.1A). We used the Kaplan-Meier curve to analyze the relationship between the expression of OSBP2 in PDAC and the total survival of patients. Our data revealed that overexpression of OSBP2 was related to the shorter survival of these patients (Fig.1B). Therefore, we further investigated the effect of OSBP2 expression on the biological behavior of PDAC cells.

OSBP2 promotes the migration and invasion of PDAC cells

We studied the role of OSBP2 in PDAC cells' mobility. So, we inhibited OSBP2 expression in ASPC-1 cells using a specific siRNA and performed wound-healing assay, which revealed that OSBP2 inhibition resulted in reduced wound closure compared with the control ASPC-1 cells ($P=0.05$; Fig. 2A). We next

transfected BXPc-3 cells with a construct to achieve OSBP2 overexpression. Then, wound-healing assay was performed, which revealed that OSBP2 overexpression led to increased cell migration compared with empty vector-treated BXPc-3 cells ($P=0.05$; Fig. 2B). The transwell assay were employed to further evaluate the impact of OSBP2 on PDAC cells' invasion, which showed that PDAC cells' migration and invasion was suppressed by OSBP2 inhibition in ASPC-1 cells ($P=0.01$; Fig. 2C) and increased by OSBP2 overexpression in BXPc-3 cells ($P=0.01$; Fig. 2D).

OSBP2 decreases the apoptosis of PDAC cells

We investigated the effect of OSBP2 on PDAC cells' apoptosis. In ASPC-1 cell line which was transfected with OSBP2 siRNA, OSBP2 inhibition increased the number of apoptotic cells significantly and decreased the number of tumor cells compared with the control ASPC-1 cells (Fig. 3A). On the contrary, in BXPc-3 cell line that transfected with a construct to achieve OSBP2 overexpression, OSBP2 overexpression decreased the number of apoptotic cells dramatically and increased the number of tumor cells compared with empty vector-treated BXPc-3 cells (Fig. 3B).

OSBP2 promotes the proliferation of PDAC cells

We performed CCK-8 assay to explore the effect of OSBP2 on PDAC cell proliferation. Our data showed that the OD (optical density) value of si-OSBP2-infected ASPC-1 cells was lower than ASPC-1 cells at different time points (Fig.4). And that the OD value of OSBP2 overexpression BXPc-3 cells was higher than BXPc-3 cells at different time points (Fig.4). It indicated that OSBP2 inhibition suppressed PDAC cells proliferation in ASPC-1 cell line and OSBP2 overexpression promoted PDAC cells proliferation in BXPc-3 cell line.

OSBP2 increases the resistance of PDAC cells to 5-FU and GEM drugs

Cells from different treatment groups (ASPC-1, ASPC-1+si-OSBP2, BXPc-3, BXPc-3+ OSBP2) were exposed to different concentrations (0.000,0.010,0.100,1.000,10.000,100.000,1000.000,2000.000 μ mol/L) of 5-FU and GEM. After 72 hours, the OD value of different groups was examined. Our data showed that as the concentration of the drug increases, the rate of inhibition of cells by drugs is increasing (Fig.5A and 5B). And that OSBP2 inhibition decreased the resistance of ASPC-1 cells to 5-FU and GEM, OSBP2 overexpression increased the resistance of BXPc-3 cells to 5-FU and GEM (Fig.5A and 5B).

OSBP2 promotes the proliferation of PDAC cells in vivo

We examined the effect of OSBP2 on tumor growth using transplanted tumor mouse models. To this aim, OSBP2-interference ASPC-1 cells, ASPC-1 cells, BXPc-3 cells and OSBP2 overexpression BXPc-3 cells were subcutaneously inoculated into the right posterior back of BALB/c nude. Four weeks after tumor formation, we monitored the growth of transplanted tumors by removing the tumor nodules. We observed that OSBP2 inhibition suppressed the growth of ASPC-1-engrafted tumors and OSBP2 overexpression accelerated the growth of BXPc-3-engrafted tumors (Fig. 6A). In agreement with the tumor volumes, the weight of tumor of injecting with OSBP2-interference ASPC-1 cell were significantly lower than the control

tumor and the weight of tumor of injecting with OSBP2 overexpression BXPC-3 cells were significantly higher than the control tumor (Fig. 6B). Moreover, we performed hematoxylin and eosin (H&E) and OSBP2 staining of randomly selected mouse tumors. We observed that OSBP2 inhibition resulted in downregulation of OSBP2 expression and OSBP2 promotion resulted in upregulation of OSBP2 expression (Fig. 6C and 6D).

OSBP2 promotes the metastasis of PDAC cells in vivo

We further conducted metastasis assay in vivo. Thus, OSBP2-interference ASPC-1 cells, ASPC-1 cells, BXPC-3 cells and OSBP2 overexpression BXPC-3 cells were injected into tail vein of NOD/SCID mice, respectively. Eight weeks after injection, the animals were sacrificed, and metastasis progression was monitored. Next, the both lungs were dissected for histologic analysis, which revealed that the wet weight of the both lungs of injecting with OSBP2-interference ASPC-1 cells was lighter than injecting with ASPC-1 cells (Fig. 7A). And the wet weight of the both lungs of injecting with OSBP2 overexpression BXPC-3 cells was heavier than injecting with BXPC-3 cells (Fig. 7A). Hematoxylin and eosin (H&E) and OSBP2 staining of randomly selected mouse lungs showed that OSBP2 inhibition resulted in downregulation of OSBP2 expression and OSBP2 promotion resulted in upregulation of OSBP2 expression (Fig. 7B and 7C).

OSBP2 promotes epithelial–mesenchymal transition (EMT) in PDAC cells

Western blot analysis showed that OSBP2 inhibition led to the increase of E-cadherin expression and the reduction of N-cadherin, Vimentin, Snail, β -Catenin, Slug and ZEB1 expression in ASPC-1 cells (Fig.8A). On the contrary, OSBP2 overexpression resulted in the reduction of E-cadherin expression and the increase of N-cadherin, Vimentin, Snail, β -Catenin, Slug and ZEB1 expression in BXPC-3 cells (Fig.8B). The results of the above showed that OSBP2 overexpression promoted epithelial–mesenchymal transition process in PDAC cells.

Discussion

At present, therapies for managing pancreatic cancer patients are largely ineffective. PDAC generally predicts a poor prognosis, in part because the symptoms often do not appear obviously until the disease is too advanced for surgical treatment^{2,29,30}. The major reason accounting for the extremely high mortality rate of PDAC is the invasive and metastatic phenotype of the cancer cells³¹. Early detection and intervention remain as the greatest challenges in the treatment of this malignant tumor. Understanding the molecular pathogenesis of PDAC is the mainstream of cancer research. In this study, we first demonstrated that OSBP2 could positively promote PDAC cell migration and invasion in vitro and in vivo probably through EMT process.

The domain of OSBP and ORPs are conserved, high affinity lipid and apt to bind to sterol. These domains of OSBP and ORPs together with the domains of the PH and FFAT participate in sterol/lipid transfer and signaling transduction between organelle membranes³². Diffusion and metastasis are characteristics of malignant tumors. The level of various OSBPs in tumors cells is different from normal cells on mRNA or

protein level, indicating the important role of these proteins in tumorigenesis. ORP inhibitors (named ORPphilins), acting as anti-proliferative agents, are crucial for the inhibition of tumor growth and are capable of attenuating OSBP or ORP4L^{28,33}. The effects of OSBPs on tumor migration and invasion in various cancer types were revealed by previous studies^{34,35}. But, at present, no data demonstrate OSBP2 expression and function in pancreatic cancer. In our research, we initially found that OSBP2 was expressed not only in PDAC cell lines but also on PDAC tissues. The expression of OSBP2 on PDAC tissues was obviously higher than para-carcinoma tissues, more importantly the patients with OSBP2 high expression had worse prognosis, which could demonstrate that OSBP2 could work as the potential tumor marker for PDAC patients.

PDAC has the lowest 5-year relative survival rate (8%) among tumors, and this rate will dwindle to 3% when tumor is diagnosed in the terminal stage³⁶. Surgery has efficient therapeutics values in this type of cancer, whereas patients that have indications for surgery is less than 20%^{37,38}. Gemcitabine is still the first-line chemotherapeutics for patients who are not eligible for radical operation, and 5-FU also is often used as first choice, but chemotherapy has limited efficacy for treating metastatic PC^{39,40}. We first demonstrate that OSBP2 could obviously affect PDAC patients' chemosensitivity, when OSBP2 is selectively down-regulated, the apoptotic rates of PDAC cells undergoing GEM and 5-Fu chemotherapeutics are significantly increased. Some research show that there exists a connection between OSBP family members and malignant tumor cells' proliferation and metastasis. Our in vitro and in vivo experiments show that OSBP2 not only promote PDAC cells' proliferation, but also enhance the invasion and metastasis process. Above mentioned preliminary results imply that OSBP2 play a key role in the neoplastic process.

To improve the survival and dismal prognosis of patients, new effective therapeutic strategies are urgently required for PDAC treatment. Previous studies reveal that epithelial to mesenchymal transition (EMT) is accountable for tumor cells' early-stage dissemination and reduces invasion and metastasis of PDAC^{13,41,42}. Previous research showed that OSBP family could regulate the subcellular distribution of ORP–VAPA complexes and their impacts on organelle structure, and then affect malignant tumor cells invasion and metastasis, potentially through the change of vimentin distribution⁴³. Our research explored the effect of OSBP2 on PDAC cells' phenotypic transformation by selectively knockdown and overexpression of OSBP2. We demonstrated that OSBP2 knockdown and overexpression could significantly change PDAC cells' EMT-related makers' expression for the first time. When OSBP2 was selectively interfered, the EMT-related mesenchymal markers N-cadherin and Vimentin were obviously decreased. The related EMT-TF (transcription factor) β -catenin, ZEB1, Slug and Snail expressions were obviously decreased⁴⁴. So, we hypothesis that OSBP2 overexpression could be recognized as a potential inducer of EMT process.

Bleak prognosis urgently pleas for new therapeutic programs. However, the understanding of tumor biology and taxonomy about PDAC are far from fully elucidated to improve the treatment effects of available therapies. Here, we provided relevant evidence in these aspects. According to our prior data, our

research indicated that OSBP2 is a hallmark of malignant level, offered strong evidence that it regulates the epithelial-mesenchymal transition through novel mechanisms and showed its potential as a marker for pancreatic cancer patients' stratification.

Materials And Methods

Cell culture and reagents

We purchased human PDAC cell lines ASPC-1, BXPC-3 from the American Type Culture Collection (ATCC). We cultured the cells in complete growth medium according to the manufacturer's instructions. We cultured the cells in an incubator with temperature at 37°C and concentration of CO₂ at 5%. Every 3 months, Mycoplasma-free testing was performed for depollution. (Last verified negative date, May 1, 2020). The experiment used cell lines within ten generations after thawing in this research.

The following reagents were used: We purchased cell culture media RPMI-1640 from Gibco. Fetal bovine serum was obtained from Biological Industries. Pancreatic enzyme and EDTA were acquired from Sigma. Phosphate buffered saline (PBS) were from DHeLix.

Tissue specimens

We obtained 100 cases of PDAC specimens from patients who underwent pancreatic surgical resection, and they were informed consent before pancreatic operation. None of the patients received radiotherapy or chemotherapy before surgery. Both normal and tumor samples obtained from resected tissues were immediately frozen at -80°C. Both tumor and adjacent nontumor tissues were sampled respectively, with approximate 1 cm³ size of each specimen, and were proved by pathological examination. This study was approved by the Ethics Committee of The Ethics Committee of First Affiliated Hospital of Zhengzhou. Zhengzhou University authorized this study(KY-2021-0382). The study was carried out in compliance with the ARRIVE guidelines.

OSBP2 knockdown and overexpression in PDAC cells

We cloned full-length human OSBP2 cDNA into pcDNA3.1 plasmid (invitrogen Co.,Ltd. China) to induce OSBP2 stable expression. Jima Genomics Co., Ltd designed the siRNAs for OSBP2(si-RNA-OSBP2). In short, 80% confluent cells were seeded at a density of 5×10⁵ cells/well in 6-well plates in medium which contains 10% FBS. When the cells were 80% confluent, they were transfected with 50 nmol/L siRNAs in Lipofectamine 3000 (invitrogen Co., Ltd. China) for 48 hours. Puromycin treatment was used to select stably transfected cells. We used western blot to evaluate the level of OSBP2 expression.

Mouse strains

The Laboratory Animal Center of Zhengzhou University provided female BALB/c nude mice and NOD/SCID mice for us. Procedures relating to the care and use of mice in this study were conducted in accordance with National Institute of Health (NIH) guidelines, with the animals housed in an

association for assessment and accreditation of Laboratory Animal Care accredited facility. All animal studies were carried out in accordance with approved Institutional Care and Use Committee protocols for Zhengzhou University. And the Zhengzhou university approved the study protocol.

In vivo subcutaneous injection

Female BALB/c nude mice ages 4 to 6 weeks were maintained in a barrier facility on high-efficiency particulate air-filtered racks. Twenty mice were divided into four groups (5 mice/group): one group was injected with mixed populations from OSBP2-interference ASPC-1 cells and the other group with the control ASPC-1 cells; one group was injected with mixed populations from OSBP2-overexpression BXPC-3 and the other group with the control BXPC-3 cells. The number of cells injected in each group was 10^7 . The cells were inoculated subcutaneously on the right posterior back of the nude mice. Tumor nodules were observed weekly, and the length of the tumor were measured. Four weeks later, all tumor nodules were collected and fixed with 4% paraformaldehyde for subsequent experiments. For the sacrifice of the mice, the euthanasia box was used to contain the nude mouse and 20%-30% CO₂ was infused into the box. It was ensured that the nude mouse did not move, had no breathing and had dilated pupils. The carbon dioxide was turned off, followed by observation for 2 min to confirm that the nude mouse was dead.

In vivo caudal vein injection

Female NOD/SCID mice ages 3 to 4 weeks were maintained in a barrier facility on high-efficiency particulate air-filtered racks. Twenty mice were divided into four groups (5 mice/group): one group was injected with mixed populations from OSBP2-interference ASPC-1 cells and the other group with the control ASPC-1 cells; one group was injected with mixed populations from OSBP2-overexpression BXPC-3 and the other group with the control BXPC-3 cells. The number of cells injected in each group was 10^7 . The cells were injected into the corresponding groups through tail vein and observed for 8 weeks. Eight weeks later, all of the mice were sacrificed, lung tissues were taken out of each group of mice after death, the wet weight of both lungs was weighed, and fixed with 4% paraformaldehyde for subsequent experiments. For the sacrifice of the mice was as mentioned above

Western blot

We extracted total cell proteins by using lysis buffer with 1% phenylmethanesulfonyl fluoride and quantified using a Bicinchoninic Acid Protein Assay Kit (wanleibio Co., Ltd., China). We used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to denature and separate 20 µg protein. Then, the protein was transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). During the course of immunoblotting, the membranes were cultured in Tris-buffered saline-Tween-20 (TBS-T) with 5% skim milk powder (Yili Co., Ltd., China). Then, we incubated it with the corresponding primary antibody overnight at 4°C. The next day, we rinsed the membrane 4 times with TBS-T for 5 minutes/per time. Then, the membranes were cultured with horseradish peroxidase conjugated secondary antibody for 45 minutes at 37 followed by enhanced chemiluminescent reagent (ECL; EMD Millipore) At

last, the cell membranes were cultured at 37 ° C with horseradish peroxidase-bound secondary antibodies for 45 min, followed by enhanced chemiluminescence reagents (ECL; EMD Millipore). The proteins E-Cadherin(cat.no.3195), N-Cadherin (cat.no.13116), Vimentin(cat.no.5741), Snail(cat.no.3897), β -Catenin(cat.no.8480), Slug(cat.no.9585), ZEB1

(cat.no.3396) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). β -actin was purchased from wanleibio. All antibodies were used in accordance with the manufacturer's instructions. The first antibody was diluted at the ratio of 1:1000 and the second antibody was diluted at the ratio of 1:5000 according to the manufacturer's instructions.

Proliferation assay

The cells of various treatment groups (ASPC-1 $\oslash$ OSBP2-interference ASPC-1; BXPC-3,OSBP2-overexpression BXPC-3) were cultured until the density was about 90%—we washed them once with PBS (DHeLix Co., Ltd., China) and disposed of the supernatant. Then, we added 0.25% appropriate volume trypsin (Sigma Co., Ltd., USA) digested cells, and when the cells became round, adding complete medium to terminate the reaction. Next, we blow all the cells in the cell culture plate with the tip of a 5ml gun, and collected the mixture into a 15ml test tube, and centrifuged at 88g for 3min. The supernatant was removed, 1ml of complete medium was added, and the cells were counted. Cells were seeded into 96-well plates according to experimental groups, and each well was seeded with 5×10^3 cells. Then, CCK-8 test was performed after cultured in an incubator at 37°C and 5% CO₂ for 0h, 24h, 48h and 72h, respectively. The supernatant was discarded, then added 100 μ L complete medium and 10 μ L CCK-8 to each well, then cultured in incubator at 37°C and 5% CO₂ for 2h. The optical density value at 450nm was measured on an enzyme standard instrument (BIOTEK Co., Ltd., USA).

Cytotoxicity assay

Cells were seeded at low density (5000 cells per well) in 96-well plates and treated with different concentrations (0-2000 μ mol/L) of 5-FU or gemcitabine, respectively. After 72 hours incubation, we used CCK-8 test to measure the optical density value at 450nm, as mentioned above.

Transwell assay

We used transwell chambers (Corning Inc. USA) to evaluate cell migration and invasion. Matrigel was applied in the invasion assay. Cells were suspended in medium without fetal bovine serum (FBS). Then we added 6×10^3 cells of various treatment groups respectively to the upper chamber pre-coated with Matrigel (Corning Inc. USA), and added 800 μ l medium with 30% FBS to the lower chamber. The transwell chamber was washed twice with PBS, fixed with 4% paraformaldehyde at room temperature for 20min, stained with 0.5% crystal violet solution for 5min, and rinsed with distilled water. The cells migrated to the sublayer of the microporous membrane were counted under an inverted microscope (200 $\times$) (Motic Electric Group Co., Ltd., Xiamen, China).

Wound healing assay

The cells of various treatment groups (ASPC-1, OSBP2-interference ASPC-1; BXPC-3, OSBP2-overexpression BXPC-3) were cultured until the density was fused. Before the experiment, the medium was changed to serum-free medium and 1 µg/ mL mitomycin C was added to treat for 1 h. Cells in each group were scratched by 200 µL pipette tip, washed cell surface with serum-free medium once, removed cellular debris, observed the cells under the microscope and took photos in groups, noted down the positions of the cells in the photos, and prepared them for subsequent photos. The culture medium without serum was used instead. Cells in each group were placed in an incubator at 37°C and 5%CO₂ for 48 h and then photographed.

Apoptosis assay

The Annexin V-FITC/PI Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA) was used to access cell apoptosis. We carried out the test according to the manufacturer's instructions. In brief, we used ice-cold PBS to wash these cells. Then, Annexin V-FITC and PI solutions was used to culture them for 15 min in the dark. Next, we used FACScan flow cytometry to study them for apoptosis. At last, we summarized the data as the mean ± standard error (SD).

Immunohistochemistry

The fixed tissue was sectioned into 5 µm-thick sections. After heating to 60°C for 2 h, the specimens were deparaffinized using xylene at room temperature and rehydrated in a descending graded series of ethanol (95, 85 and 75%). For IHC staining, endogenous peroxidases were inactivated by 3% hydrogen peroxide for 15 minutes. Tissues were stained with primary antibodies for 12 hours at 4°C according to the manufacturer's instructions, then, stained with secondary antibodies. The staining results were observed and imaged through microscopy (OLUMPUS Co., Ltd., Japan) at a magnification of 400.

H&E staining

The fixed tissue was sectioned into 5 µm-thick sections. After heating to 60°C for 1 h, the specimens were deparaffinized using xylene at room temperature and rehydrated in a descending graded series of ethanol (95, 85 and 75%). Subsequently, the samples were stained with hematoxylin for 5 min and eosin for 3 min both at room temperature. Then, after dehydration, transparent and sealing piece, images were captured using a microscope (x200; OLUMPUS Co., Ltd., Japan).

Microarray analysis

PDAC tissues and adjacent para-cancer tissues for the tissue microarray (TMA) were derived from the First Affiliated Hospital of Zhengzhou University. Firstly, an experienced pathologist is responsible for selecting the 1 mm core area, then, we used a TMA Grand master machine (3DHISTECH) to drill and place on recipient blocks. Each primary tumor included three cores and metastatic lymph nodes contained 1–3 cores. We coded and placed the cores on the recipient randomly. Relevant clinical

information comes from hospital cases. To compare staining intensity and survival, at least two histological core scores are required per patient. In the process of tissue staining analysis, the clinical information was blinded.

Statistical analysis

We used SPSS16.0(IBM) to conduct statistical analysis in this study. Mean $\pm$ SD was used to represent the data. All in vitro experiments were conducted independently in triplicate. Comparisons between treatment groups were done by Student t test. Statistical analysis was conducted with either Microsoft Excel or Origin Labs. The Kaplan–Meier method was used to compare OS among patients in different groups. The log-rank test was used to estimate differences in survival. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. $P < 0.05$ was considered statistically significant.

Declarations

Acknowledgements

We sincerely express our gratitude to the Laboratory Animal Center staff of Zhengzhou University.

Funding

The present study was supported by The Key Scientific Research Project Plan of Henan University (grant no. 20A320037)

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SH performed experiments, contributed to analysis and interpretation of the data, and was a major contributor in writing the manuscript. XDZ and KL performed experiments, and contributed in drafting and revising the manuscript. LJ contributed in the data analysis and revision of the manuscript. RFL and JHJ contributed to the conception and design of the study, the data analysis and interpretation, and the writing and revision of the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The Ethics Committee of the First Affiliated Hospital of Zhengzhou University and Zhengzhou University approved our study, and all participants signed written informed consent. The present study was

conducted in accordance with the ethical standards of the Helsinki Declaration and the ARRIVE guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Tables

Table I. Relationship between the expression level of OSbp2 and the clinicopathological parameters of pancreatic cancer patients.

Clinical parameters	n	OSBP2 expression		P-value
		High [n (%)]	Low [n (%)]	
Sex				0.284
Male	63	49 (77.8)	14(22.2)	
Female	37	32(86.5)	5(13.5)	
Age(years)				0.135
<60	47	41(87.2)	6(12.8)	
≥60	53	40(75.5)	13(24.5)	
Nerve invasion				0.011
Yes	58	52(89.7)	6(10.3)	
No	42	29(69.0)	13(31.0)	
Pathological grading				0.018
I	12	6(50.0)	6(50.0)	
II	78	66(84.6)	12(15.4)	
III	10	9(90.0)	1(10.0)	
AJCC stage				1.000
I-II	98	79(80.6)	19(19.4)	
III-IV	2	2(100)	0(0)	
Lymph node metastasis				0.427
Absent (N0)	55	43(78.2)	12(21.8)	
Present (N1-3)	45	38(84.4)	7(15.6)	

All data are expressed as the number of patients (%). P-values were calculated using SPSS 17.0 using a Chi-square test. P-values <0.05 were considered to indicate statistical significance. OSBP2, Oxysterol-binding protein 2; AJCC stage, American cancer Joint Committee stage

Figures

Figure 1

OSBP2 overexpression in PDAC correlates with shorter survival. (A). Expression of OSBP2 in pancreatic cancer tissues and paired adjacent para-cancer tissues (Scale bars, 100 μ m). (B). Association between tumor OSBP2 expression levels and overall survival in 100 PDAC patients ($P = 0.034$ by log-rank test). OSBP2, Oxysterol-binding protein 2; PDAC, pancreatic ductal adenocarcinoma

Figure 2

Effect of OSBP2 on PDAC cell migration and invasion. (A). Low-expression of OSBP2 inhibited ASPC-1 cell migration ($P \leq 0.05$). (B). Overexpression of OSBP2 promoted BXPC-3 cell migration ($P \leq 0.05$). (C). Low expression of OSBP2 inhibited ASPC-1 cell invasion ($P \leq 0.01$). (D). Overexpression of OSBP2 promoted BXPC-3 cell invasion ($P \leq 0.01$). OSBP2, Oxysterol-binding protein 2; PDAC, pancreatic ductal adenocarcinoma; Si-OSBP2, si-RNA-OSBP2; EV, empty vector.

A

ASPC-1

B

BXPC-3

Figure 3

Effect of OSBP2 on PDAC cell apoptosis. (A). Low-expression of OSBP2 increased tumor cell apoptosis in ASPC-1 cell line. (B). Overexpression of OSBP2 decreased tumor cell apoptosis in BXPC-3 cell line. OSBP2, Oxysterol-binding protein 2; PDAC, pancreatic ductal adenocarcinoma; Si-OSBP2, si-RNA-OSBP2; EV, empty vector.

Figure 4

Effect of OSBP2 on PDAC cell proliferation. Low-expression of OSBP2 decreased tumor cell proliferation in ASPC-1 cell line ($P \leq 0.01$). Overexpression of OSBP2 increased tumor cell proliferation in BXPC-3 cell line ($P \leq 0.01$). OSBP2, Oxysterol-binding protein 2; PDAC, pancreatic ductal adenocarcinoma; EV, empty vector.

Figure 5

Effect of OSBP2 on the resistance of 5-FU and GEM. (A). OSBP2 low-expression decreased the resistance of ASPC-1 cell to 5-FU ($P < 0.01$) and OSBP2 overexpression increased the resistance of BXPC-3 cell to 5-FU ($P < 0.01$). (B). 2 low-expression decreased the resistance of ASPC-1 cell to GEM ($P < 0.01$) and OSBP2 overexpression increased the resistance of BXPC-3 cell to GEM ($P < 0.01$). OSBP2, Oxysterol-binding protein 2; 5-FU, 5-Fluorouracil; GEM, gemcitabine.

Figure 6

OSBP2 promotes the proliferation of PDAC cells in vivo. (A). OSBP2 inhibition decreased the tumor formation capacity of ASPC-1 cells and OSBP2 overexpression increased the tumor formation capacity of BXPC-3 cells as observed in xenograft models. (B). Photographs of dissected tumors from nude mice and the tumor weights calculated at the end of the experiment are shown in the right panel. (C) and (D). Pathologic analysis of tissue sections from recipient mice at 4 weeks postinjection. H&E (magnification, $\times 200$) and OSBP2 staining (magnification, $\times 200$) were performed. Normalization of OSBP2 expression is shown in the right. OSBP2, Oxysterol-binding protein 2; PDAC, pancreatic ductal adenocarcinoma; sh, shRNA; pC, pcDNA3.1 plasmid.

Figure 7

OSBP2 promotes PDAC cell invasion in vivo. (A). Photographs of dissected tumors from SCID mice and the lung weights calculated at the end of the experiment. (B) and (C). Pathologic analysis of tissue sections from recipient mice at 8 weeks postinjection. H&E (magnification, $\times 200$) and OSBP2 staining (magnification, $\times 200$) were performed. Normalization of OSBP2 expression is shown in the right. OSBP2, Oxysterol-binding protein 2; PDAC, pancreatic ductal adenocarcinoma; sh, shRNA; pC, pcDNA3.1 plasmid.

Figure 8

Western blot. (A). Western blot revealed upregulated E-cadherin, and downregulated N-cadherin, vimentin, and Snail following inhibition of OSBP2 in ASPC-1 cells. (B). Western blot revealed upregulated N-cadherin, vimentin, and Snail, and downregulated E-cadherin following overexpression of OSBP2 in BXP-3 cells. OSBP2, Oxysterol-binding protein 2.