B7-H3 is related to tumor progression in ovarian cancer

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Abstract. B7-H3, a co-stimulatory molecule, has been found expressed in ovarian cancer, but its role and mechanism is not clear. In this study, we further verified the expression of B7-H3 in ovarian carcinoma and normal epithelial ovarian tissues. Three ovarian cancer cell lines, A2780, SKOV3 and HO8910 were selected to explore the effects of B7-H3 on proliferation, apoptosis, migration and invasion. We found that B7-H3 was mainly located in the cytoplasm of ovarian cancer cells as determined by immunofluorescence staining. The ability of cell invasion, migration, proliferation decreased after silencing B7-H3 whereas the apoptosis increased, which was related to the upregulation of Bax, caspase-8, cleaved caspase-8 and the downregulation of Bcl-2, Bcl-xl, matrix metalloproteinase-2 (MMP2) by western blotting. In addition, B7-H3 enhanced the HO8910 cell capacities in invasion, migration and proliferation. Expression of the phosphorylation signal transducer and activator of transcription 3 (pStat3) molecules and their upstream molecules phosphorylation Janus kinase 2 (pJak2) were significantly increased. In order to investigate whether B7-H3 plays a role in this pathway, we treated the overexpressed HO8910 cells with AG490 (inhibitors of Jak2). Our findings revealed that B7-H3 affect ovarian cancer progression through the Jak2/Stat3 pathway, indicating that B7-H3 has the potential to be a useful prognostic marker.

Introduction

Ovarian cancer is the most lethal gynecological malignancy and the fifth most common cause of cancer death among women, 90% is epithelial ovarian cancer (EOC) (1). The early diagnosis of ovarian cancer is not perfect so that the majority of patients are diagnosed with advanced disease, and the five-year survival rate for EOC is approximately 30% (2). Therefore it is necessary to find specific diagnostic methods and therapeutic approaches for ovarian cancer.

B7-H3 is encoded by the CD276 gene, including two main isoforms, named 4Ig-B7-H3 and 2Ig-B7-H3, which were first identified in 2001 (3,4). A newly found member of B7/CD28 family, and was identified as an accessory co-stimulatory molecule after initial antigen priming in cooperation with a putative counter receptor (5). Recently, a number of articles have reported that B7-H3 is highly expressed in many cancers, including colorectal cancer (6), hepatocellular carcinoma (7), lung cancer (8), gastric cancer (9), breast cancer (10) and prostate cancer (11). In addition, B7-H3 has been shown to be associated with poor prognosis by the Jak2/Stat3 pathway. Although the expression of B7-H3 in ovarian cancer was mentioned in a document, it was verified only at the tissue level that the high expression of B7-H3 in ovarian cancer and B7-H3-positive tumor vasculature is associated with high-grade serous histological subtype, increased recurrence and reduced survival (12). There is no study on the biological behavior and mechanism of the cells. Thus in this study, we show how B7-H3 affects the biological behavior of the cells and its mechanism.

Materials and methods

Patients and tissue samples. Ovarian cancer tissue samples were collected from 41 patients who underwent surgery from 2015 to 2016 in Qilu Hospital of Shandong University. In addition, 31 normal ovarian tissues were collected to use as control. None of the patients received preoperative chemotherapy or radiotherapy before the surgery. All patients were confirmed by pathological diagnosis. The present study was approved by the Ethics Committee of Qilu Hospital.

Cell lines and cell culture. The human ovarian cancer cell lines A2780, SKOV3, and HO8910 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). A2780 and HO8910 were cultured in the medium of Roswell Park Memorial Institute (RPMI)-1640 (Hyclone Laboratories, Logan, UT, USA), while SKOV3 in the medium of Micro-5A (Gibco-Invitrogen, Grand Island, NY, USA). All of them were supplemented with 10% fetal bovine serum (FBS; Gibco, Sydney, Australia) and incubated at 37°C in a humidified incubator supplemented with 5% CO2.
**Antibodies and reagents.** Rabbit anti-human Jak2, Phospho-Jak2 (Tyr1007), signal transducer and activator of transcription 3 (Stat3), phospho-Stat3 (Tyr705), Bcl-2 and Bax antibodies were purchased from Abcam (Cambridge, MA, USA). Mouse anti-human Bcl-xl, caspase-8 and MMP-2 were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit anti-human B7-H3, cleaved caspase-8 and the GAPDH were purchased from Cell Signaling Technology (CST) (Danvers, MA, USA). All of the above are monoclonal antibodies. Tryphostins AG490 was from Abcam and prepared at a concentration of 100 mmol/l stock solution in dimethyl sulfoxide (DMSO).

**Immunohistochemical analysis.** Polink-2 Plus Polymer HRP Detection System (ZSGB-BIO, Beijing, China) was used. Paraffin tissues were cut into 4-µm sections. Section were de-paraffinized in xylene, re-hydrated through a graded ethanol series, then repaired by citric acid (microwave boiling method). H2O2 (3%) was incubated for 10 min to block endogenous peroxidase activity. PBS (phosphate-buffered saline) was used for washing three times for 3 min each. After blocking endogenous peroxidase activity, the slides were incubated with primary antibody overnight at 4°C. The next day, sections were washed in PBS three times for 5 min each. Secondary antibody was applied for 30 min at 37°C. DAB was used for coloring. Finally slides were counterstained with water rinsing, staining, dehydration, transparent and mounting. Quantification was recorded as follows: <10% positive cells, 0; 10-25%, 1; 26-50%, 2; >50% positive cells, 3. Sections with a final score of 0-1 was classified as negative, and ≥2 was considered positive.

**Immunofluorescence staining.** To find the B7-H3 expression location, the A2780 and SKOV3 cells were seeded in a 24-well plate at 37°C overnight. Then, after three washes with PBS, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.2% Triton 150-200 µl in PBS for 10 min. After that, the B7-H3 (1:200 dilution) antibody was incubated at 4°C for overnight and secondary antibody (1:200) for 1 h. Finally, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) for 5 min in the dark at room temperature, and permeabilized with 0.2% Triton 150-200 µl in PBS for 10 min. After that, the B7-H3 (1:200 dilution) antibody was incubated at 4°C for overnight and secondary antibody (1:200) for 1 h. Finally, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) for 5 min in the dark at room temperature. The fluorescence images were observed using a fluorescence microscope (Olympus, Tokyo, Japan).

**Silencing and overexpression of B7-H3.** Silencing or over-expression of B7-H3 sequences were designed by GenePharma Co, Ltd. (Shanghai, China). The cells were seeded in 6-well plates, 24 h after the cell attachment, the virus liquid was mixed in the culture medium. The cells were screened with puromycin dihydrochloride (2 µg/ml; Amresco, Solon, OH, USA) 72 h later. After screening for 5-7 days, the cell line with stable overexpression of B7-H3 (HO8910-B7-H3-EGFP) and their control cell lines (HO8910-NC) were obtained. The A2780 and SKOV3 cells were seeded in a 6-well plate with 3x10^9/ml cell per well, 24 h later, cells were transfected with 50 nM of the Sh-B7-H3 or control sequences using Lipofectamine-2000 (Invitrogen Life Technologies). The transfected cells were harvested 4 h post-transfection for the follow-up experiments, with A2780-NC, A2780-sh-B7-H3, SKOV3-NC and SKOV3-sh-B7-H3.

**Western blot assay.** The cells were washed 3 times with PBS and then lysed on ice for approximately 30 min. The pyrolysis solution is composed of radio immunoprecipitation assay buffer (RIPA), phenylmethylsulfonyl fluoride (PMSF) and NaF. The cells were then lysed with ultrasound. Cells were centrifuged at 12000 rpm at 4°C, then supernatant were drained, loading buffer was added and heated for 5 min in metal bath. The protein concentrations were measured by using the BCA Protein Assay kit (Beyotime, Jiangsu, China). Total protein (30-50 µg) was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels (Invitrogen) and transferred to PVDF membranes (ImmobilonP; Millipore, Bedford, MA, USA). After blocking with 5% skim milk for 2 h, the membranes were cut into strips and incubated with the indicated primary antibodies overnight at 4°C. The next day, the membranes were washed 3 times with TBS-T (20 mM Tris, pH 7.4, 137 mM NaCl, 0.05% Tween-20) and then indicated with secondary antibodies at room temperature for 1-2 h. Ultimately, the immunoreactive protein bands were detected.

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<th>Positive</th>
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Values in bold, P<0.05.
by enhanced chemiluminescence (ECL) using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Logan, UT, USA). The results were analyzed by ImageJ software (NIH, Bethesda, MD, USA).

**Cell proliferation assay.** The cell proliferation was evaluated by MTT assay. In brief, cells were seeded in 96-well plates at 2000 cells per well. At indicated time-points, the cells in the 96-well plate were incubated with 20 µl MTT at 37˚C for 4 h. The cell growth was monitored every 24 h for up to 5 days. Absorbance was measured for each well at a wavelength of 490 nm using a microplate reader (Infinite 2000; Tecan, Männedorf, Switzerland).

**Cell migration and invasion assays.** For the *in vitro* migration and invasion assays, 3-5x10^4 cells of the A2780, HO8910 or SKOV3 derived cell cultures, in serum-free RPMI and Micro-5A medium, were seeded into the top of 8 µm pore size transwell chambers (Costar, Cambridge, MA, USA), which contained 100 µl of Matrigel (1:8 dilution in serum-free medium; Corning Inc., Corning, NY, USA) or not. Then the chambers were put into the 24-well plates which included culture medium with 10% serum. After incubation at 37˚C for 24 h in a 5% CO₂ atmosphere, the cells were stained with 0.1% crystal violet and then washed 3 times with PBS. The images were captured by the Olympus IX51 inverted microscope. The number of migrating and invading cells on the chambers were counted in 5 random fields per chamber at x200 magnification of each group.

**Cell cycle and apoptosis assays.** To analyze the effect of B7-H3 on the different phases of the cell cycle and apoptosis, flow cytometry was used. Cells were harvested from each sample then fixed with cold 75% ethanol at 4˚C overnight. The cells were washed 3 times and stained for 30 min in propidium iodide (PI) staining solution in the dark. The cell cycle was detected by FACSCalibur flow cytometer (both from BD Biosciences, Franklin Lakes, NJ, USA) and analyzed by ModFit LT software. For apoptosis, cells were harvested and processed as described in the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit I manual (BD Transduction Laboratories, BD Biosciences) and analyzed by flow cytometry (BD LSR II).

**Statistical analysis.** The data were analyzed using GraphPad Prism version 5.01 (GraphPad Software Inc., San Diego, CA, USA). The experiment was performed a minimum of three times, and all data are shown as means with standard deviations (SDs). The data were analyzed for statistical significance using an unpaired Student’s t-test or a Chi-squared test. *P*-value at <0.05 difference was considered to be statistically significant.

**Results**

**B7-H3 expression in clinical specimens and the location of B7-H3 in ovarian cancer cell lines.** In order to find out the expression of B7-H3 in ovarian cancer and the location of B7-H3, we used immunohistochemistry and immunofluorescence methods. Immunohistochemistry was performed to detect the expression of B7-H3 in 41 cases of ovarian cancer tissues and 31 cases of normal ovarian tissues. Although no expression or low expression of B7-H3 can be found in ovarian cancer and there are some weak expression in normal tissues

![Figure 1. B7-H3 expression in ovarian tissues and its expression location. B7-H3 immunostaining in ovarian cancer tissues (A) Weak positive, moderate positive, strong positive (magnification, x200). (B) B7-H3 was stained in the cytoplasm of cells, while DAPI was stained in the nucleus (magnification, x200).](image-url)
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The results showed that the expression of B7-H3 in ovarian cancer was significantly higher than that in normal tissues (P<0.001) (Table I). Immunohistochemistry revealed that B7-H3 was expressed in the cytoplasm of the tissue, and a few were expressed in the interstitial tissue. In order to clarify the localization of B7-H3, immunofluorescence was utilized and the results showed that B7-H3 was mostly expressed in the cytoplasm of ovarian cancer cells (Fig. 1B).

B7-H3 expression in relation to patient clinicopathological factors. The relationship between clinicopathological factors and B7-H3 expression in patients with ovarian cancer is shown in Table I. Our data suggest that B7-H3 expression is associated with distant metastasis of ovarian cancer (P=0.041), whereas it is not correlated with patient age (P=0.195), tumor size (P=0.903), tumor histology type (P=0.920), differentiation degree (P=1.000), clinical stage (P=0.057), and vascular invasion (P=0.300). However, in this study the number of cases is too small, therefore, in order to further clarify the relationship between B7-H3 and the clinicopathological characteristics of patients the number of samples should be increased.

Silencing B7-H3 weakens cell proliferation. To characterize the role of B7-H3 in A2780 and SKOV3 cell growth we measured the cell proliferation rate in vitro by MTT assay (11). The experiment was divided into two groups: NC group and sh-B7-H3 group. The results suggest that the cell viability of sh-B7-H3 group was weakened at 3, 4 and 5 days compared with the NC group (Fig. 2A and B). Similar results were also found in the cell cycle (Fig. 3C and D). Compared with the control group, A2780 and SKOV3 cell lines that interfered with B7-H3 decreased the S and G2/M phases of the cell cycle, which was a period of cell proliferation ability. Thus, B7-H3 molecule expressed in ovarian cancer cells might play an important role in regulating the colony formation ability.

Silencing B7-H3 increases cell apoptosis. To verify the effect of B7-H3 on cell apoptosis after B7-H3 interference, we performed flow cytometry. The extent of apoptosis was investigated by measuring the amount of Annexin V stained cells, a marker for early stage apoptosis. The amount of all reagent-positive cells, which reflect the late stage apoptosis, were also measured. We found that when B7-H3 affected the early stage apoptosis of A2780 and SKOV3 cells were increased, and the late stage apoptosis although increased, was P>0.05 (Fig. 3A and B). Therefore, B7-H3 mainly promotes apoptosis of cells through the early stage apoptosis. In addition, we observed

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Figure 2. Effect of silencing B7-H3 on cell proliferation. (A and B) The cell viability of sh-B7-H3 group and NC group was tested at 24 h, 2, 3, 4 and 5 days by MTT assay. (C and D) A flow cytometry was used to detect the cell cycle of cells treated with stable silencing and the percentage of the S and G1/G2 phases in the cell cycle is used to indicate cell proliferation; *P<0.05, **P<0.01, ***P<0.001 compared with the control groups.
the changes of apoptosis related proteins by the western blot method with the B7-H3 knockout. The results showed that

Figure 3. Effect of B7-H3 on cell apoptosis. (A and B) Knockdown of B7-H3 by siRNA induced apoptosis of the A2780 cell line and SKOV3 cell line. (C and D) Early apoptosis was used to express the apoptosis rate that is shown in (A and B).

Figure 4. The changes of related protein molecules after silencing of B7-H3. (A and B) Western blot analysis for B7-H3 and after silencing B7-H3 the expression of anti-apoptotic proteins Bcl-2 and Bcl-xl and the pro-apoptotic protein Bax and caspase-8, cleaved caspase-8 were measured by western blotting. (C and D) Quantification of the protein expression levels as shown in (E and F); *P<0.05, **P<0.01, ***P<0.001 compared with the control groups.

with the silence of B7-H3 the expression of apoptosis regulator proteins of the Bcl-2 family, including the anti-apoptotic
Figure 5. Silencing B7-H3 effects cell migration and invasion. (A and B) Silencing B7-H3 reduces the migration and invasion potential of A2780 and SKOV3 cell lines. (C and D) Quantification of the images of (A and B); *P<0.05, **P<0.01, ***P<0.001 compared with the control groups.

Figure 6. Effect of overexpression of B7-H3 on HO8910 cell line invasion, migration and proliferation. (A) Overexpression of B7-H3 in the HO8910 cell line increased the growth of cells that was analyzed by flow cytometry. (B) Quantification of the cell cycle. (C and E) Overexpression of B7-H3 effect on A2780 and SKOV3 cell migration and invasion by transwell chamber assay. (D and F) Quantification of the images of (D and E) (magnification, x200); *P<0.05, **P<0.01, ***P<0.001 compared with the control groups.
proteins Bcl-2 and Bcl-xl decreased, while expression of the pro-apoptotic protein Bax, caspase-8 and cleaved caspase-8 increased (Fig. 4A and B). The expression of B7-H3 in ovarian cancer cells is closely related to apoptosis related molecules, which suggests that silencing B7-H3 may promote apoptosis of ovarian cancer cells.

Silencing B7-H3 reduced the migration and invasion potential of ovarian cancer cells. We next further assessed the influence of B7-H3 on ovarian cancer cell migration and invasion by transwell assays. As shown in the results, compared with the control groups, the number of cells in migration passing through the chamber was significantly reduced in A2780-sh-B7-H3 group and SKOV3-sh-B7-H3 group (Fig. 5A and B). The same phenomenon was observed in the cell invasion test. Silencing B7-H3 can impede cell migration and inhibit cell invasion via downregulating the expression of MMP-2 (13). Thus, we measured the invasion related proteins by western blotting and found that MMP-2 were lower in A2780-sh-B7-H3 and SKOV3-sh-B7-H3 cells than in the NC groups (Fig. 4A and B). Based on these results, we come to the conclusion that the downregulation of B7-H3 expression could suppress cellular migration and invasion in human ovarian cancer cells.

Overexpression of B7-H3 enhances ovarian cancer cell invasion, migration and proliferation in vitro. We described above the effect of silencing B7-H3 in ovarian cancer cells. In order to further verify the role of B7-H3 in ovarian cancer cells, HO8910 cell line was overexpressed by lentivirus. Using cell cycle assay, we observed that the growth of HO8910-B7-H3-EGFP was increased compared to NC cells (Fig. 6A and B). Besides, we performed transwell assay and the result showed that overexpression of B7-H3 significantly increased the ability of cell migration and invasion (Fig. 6C and E). The expression of related protein MMP-2 was elevated with the over expression of B7-H3 (Fig. 7A). The expression of B7-H3 and the anti-apoptotic proteins were positively correlated in CRC cell lines (6). Therefore, we found that the expression of protein Bcl-2, Bcl-xl added while Bax, caspase-8, cleaved caspase-8 increased (Fig. 4A and B). The expression of B7-H3 in ovarian cancer cells is closely related to apoptosis related molecules, which suggests that silencing B7-H3 may promote apoptosis of ovarian cancer cells.
caspase-8 reduced by upregulation of B7-H3 expression (Fig. 7A). To summarize, overexpression of B7-H3 inhibits apoptosis and enhanced cell invasion, migration and proliferation in ovarian cancer cells.

**Overexpression of B7-H3 affects the Jak2-Stat3 pathway.** The role of B7-H3 in ovarian cancer cells is described above, therefore, we investigated which signaling pathway was involved in this process. The Jak2/Stat3 pathway has been reported to be the key in cell migration, invasion and metastasis, and inhibition of Jak2/Stat3 signaling induced CRC cell apoptosis, cell arrest and reduced tumor cell invasion (14-16). Thus, it was analyzed whether this pathway could be affected by B7-H3 in ovarian cancer. HO8910-B7-H3-EGFP cells were treated with AG490 with a concentration of 100 nmol/l. After 48 h, the protein expression of related molecules in Jak2/Stat3 pathway was detected by western blotting. As shown in the chart, the phosphorylation levels of Jak2 and Stat3 increased with the overexpression of B7-H3. However, when the cells were treated with AG490, the expression decreased correspondingly. Furthermore, anti-apoptotic proteins Bcl-2 and Bcl-xl increased while expression of the pro-apoptotic protein Bax was reduced following upregulated expression of B7-H3 and after joining the AG490 the results were exactly the opposite (Fig. 7B). These findings indicated that B7-H3 may participate and influence the Jak2/Stat3 pathway in ovarian cancer cells. However, whether B7-H3 affects this pathway through activation or other ways, or not, needs further experiment.

**Discussion**

B7-H3, as an immunoregulatory molecule, playing different roles in different types of human cancers (17). The role of B7-H3 in tumor immunity is complicated as both T cell co-stimulatory and co-inhibitory effects have been shown (18). In this study, we provide evidence that B7-H3 may be associated with tumor progression in ovarian cancer. In other words, B7-H3 probably plays a negative regulatory role in ovarian cancer.

In the past studies, only few scattered stromal cells in non-neoplastic ovarian tissues expressed B7-H3 (3,19,20). Only one recent investigation showed that most ovarian cancers express B7-H3 (12). In this study, we further demonstrated that B7-H3 is highly expressed in patients with ovarian cancer, mainly in adenocarcinoma, but has no expression or low expression in normal epithelial ovarian tissues. The correlation between B7-H3 and the age, clinical stage, prognosis and other factors of patients with ovarian cancer was analyzed. The data suggest that B7-H3 expression is only associated with distant metastasis of ovarian cancer. We also found that B7-H3 was mainly expressed in the cytoplasm of ovarian cancer cells. To date, there are no published studies looking at the ability of B7-H3 to affect the growth of human ovarian cancer cell lines in vitro (21). Therefore, this study provides the basis how B7-H3 affects the biological behavior of ovarian cancer cell lines. Our study demonstrated that B7-H3 is closely associated with tumor progression in ovarian cancer cells. Silencing B7-H3 can attenuate the A270, SKOV3 cell lines of proliferation, migration and invasion and increase cell apoptosis, and induce changes in related molecules. On the contrary, overexpression of B7-H3 can increase the proliferation, migration and invasion of HO8910 cell line, and decrease the rate of apoptosis. The changes of protein molecules suggest that B7-H3 may be a potential therapeutic target for ovarian cancer.

The Jak2-Stat3 pathway plays a significant role in biological function on various human cancers. Zhang et al confirmed that the Jak2-Stat3 signaling pathway played an important role in regulating the anti-apoptotic ability of B7-H3 (6). Silenced B7-H3 expression suppresses migration and invasion of HCC cells via Jak2/Stat3/Slug signaling pathway, which was proved by Kang et al (5). Stat3 regulates cellular differentiation, proliferation, migration and survival as a cytoplasmic transcription factor (22-25), which was regulated by its upstream molecule Jak2. Jak2-Stat3 are reported to be the downstream signaling pathways that adjust the ability of B7-H3 to regulate the expression of Bcl-2, Bcl-xl, Bax and other molecules (6). In the present study, we found that after overexpression of B7-H3 the phosphorylation levels of Jak2 and Stat3 increased. However, after treatment with AG490, which is the inhibitor of Jak2, the expression of anti-apoptotic proteins Bcl-2 and Bcl-xl and pro-apoptotic protein Bax also changed. The data indicate that B7-H3 enhanced proliferation and reduces apoptosis probably by influencing Jak2-Stat3 pathway. But how B7-H3 specifically influenced this pathway, needs further experiments to verify the mechanism.

In conclusion, results of this study identified that B7-H3 is related to tumor progression in ovarian cancer and probably can be used as an indicator in clinic in the future. The limitations are that we did not conduct animal experiments in this research due to the lack of experimental funds and the limitations of laboratory conditions. We consider this aspect well worth studying. As mentioned above, the specific role of B7-H3 and its mechanism need further studies.

**Acknowledgements**

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