

Original Article

Breast cancer suppressor candidate 1 overexpression inhibited the proliferation, invasion and migration of SKOV3 cells

Cuiyun Liu¹, Huijuan Liu¹, Yanqiu Du²

¹Department of Pathology, Shouguang People's Hospital, Weifang 262700, Shandong Province, China; ²Department of Mammary Gland, Linyi Cancer Hospital, Linyi 276001, Shandong Province, China

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Abstract: Objective: To investigate the effect of breast cancer suppressor candidate 1 (BCSC-1) overexpression on the biological function of SKOV3 ovarian carcinoma (OC) cells. Methods: Three groups: BCSC-1 group: SKOV3 cells transfected with pcDNA-BCSC-1; empty vector group: cells transfected with pcDNA vector; blank group: cells without any treatment. The effect of BCSC-1 overexpression on the proliferation, invasion, apoptosis, adhesion and migration of SKOV3 cells was examined by MTT, Transwell assay, flow cytometry, *in-vitro* cell adhesion assay and scratch assay, respectively. Tumor models in BAL B/C nude mice were established by injecting untransfected SKOV3 cells, cells transfected with empty plasmid or pcDNA-BCSC-1. Results: After transfection, the mRNA level of BCSC-1 in SKOV3 cells was significantly higher than that of empty vector group and untransfected group ($P < 0.05$). Compared with empty vector group, the growth rate of SKOV3 cells in BCSC-1 group was obviously slower ($P < 0.05$), the invasion rate, adhesion rate and migration distance was decreased significantly, while the apoptosis rate was increased evidently ($P < 0.05$). The growth rate in BCSC-1 group was evidently slower than that of empty vector ($P < 0.05$). No significant difference was detected between blank group and empty vector group. Conclusion: BCSC-1 overexpression can inhibit the proliferation, invasive, adhesive and migratory ability of SKOV3 cells.

Keywords: BCSC-1, SKOV3 cells, proliferation, invasion

Introduction

Ovarian carcinoma (OC) is one of three major malignant tumors of female reproductive system. Ovarian epithelial tumor is currently one of the most common type of OC [1, 2]. In recent years, progress had been made in the chemoradiotherapy of OC. However, its 5-year survival rate was only 25%-30% without significant improvement [3]. Besides, OC was characterized by late awareness, quick development and poor efficacy. Although molecular targeting therapy had played a revolutionary role in the treatment of many malignant tumors, no effective targeting drugs had been used clinically for the treatment of OC up to now [4-6]. Therefore, it is of great significance to investigate the genesis and development mechanism of OC and find out an effective target for improving the efficacy and prognosis of patients with OC.

The malignant biological behavior of tumor is regulated by various genes. Tumor suppressor genes are a class of molecules, which were deleted, mutated or lowly expressed in tumors. The inactivation of tumor suppressor genes usually leads to malignant proliferation, migration, invasion and other biological effects on tumor cells [7, 8]. Human BCSC-1 is located at human chromosome 11q23-q24. Martin et al. believed that BCSC-1 was a cancer suppressor candidate and its deficiency was found in many solid tumors (such as breast cancer, lung cancer) cells [9-12]. Obvious deficiency of BCSC-1 mRNA expression was measured in 80% tumor cell lines (33/41). The malignant biological behavior of cells and tumorigenesis in nude mice could be somewhat inhibited by introducing BCSC-1 into tumors (such as breast cancer, lung cancer and liver cancer) [13-15]. In this study, BCSC-1 recombinant plasmid was trans-

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Table 1. Sequence of the primers and size of the product for amplification

Gene	Primer sequence (5'-3')	Product size (bp)
BCSC-1	F: TGCTTCTGCCCCATTGAAGA	175
	R: ACTGTGCTGGTCCTGTGAC	
β -actin	F: CCTAGAAGCATTGCGGTGG	416
	R: GAGCTACGAGCTGCCTGACG	

ected into SKOV3 cells, to observe the effect of BCSC-1 overexpression on the malignant biological behavior of SKOV3 cells.

Materials and methods

Major materials and reagents

Eukaryotic recombinant plasmid pcDNA3.1/v5-HisB-BCSC-1 was synthesized in this laboratory. Eukaryotic expression plasmid pcDNA3.1/v5-HisB was purchased from Invitrogen; Human SKOV3 OC cells was purchased from Shanghai Institute of Biochemistry and Cell Biology; Matrigel was purchased from BD (USA); Transwell chamber was purchased from Corning Costar (USA?); liposome-based transfection agent Lipotap (Beyotime Institute of Biotechnology); Real-time PCR kit (Takara Bio, Dalian, China); Fetal calf serum (Gibco, USA); PBS (Hyclone, USA); RNA extractant TRIzol and Primer ScriptTM RT Reagent Kit (reverse transcription kit) was purchased from Life (USA); And MTT was purchased from Sigma (USA).

BCSC-1 was overexpressed in SKOV3 cells

SKOV3 cells were inoculated into a 6-well plate at 4×10^5 cells per well. On day 2, when confluence rate reached up to 70%-80%, recombinant plasmid pcDNA3.1/v5-HisB-BCSC-1 and control plasmid pcDNA3.1/v5-HisB were transfected into BCSC-1 group and empty vector group, respectively according to the instructions. SKOV3 cells without any transfection were used as an empty control. Cells in each group were cultured in an incubator with 5% CO₂ under 37°C for 24 h, G418 was then added in the medium at final concentration of 800 μ g/ml for resistance screening. On day 7, when all wild type cells died, the concentration of G418 in empty vector group and BCSC-1 group was adjusted to 400 μ g/ml. After 2 weeks of steady-state culture, positive clones were selected by G418. The expression of BCSC-1 was detected by real-time PCR.

Cellular total RNA was extracted and then synthesized by reverse transcription. The reaction system was added to 20 μ l by supplementing DEPC treated water. The reaction was performed under 42°C for 60 min for complete reaction and under 70°C for 5 min for termination reaction. The above reverse transcripts cDNA was used for PCR amplification. Gene primers were presented in **Table 1**. The reaction system included the following items: premixed solution (2 \times) 12.5 μ l, upstream primer (10 μ mol/L) 1 μ l, downstream primer (10 μ mol/L) 1 μ l and DNA plate (cDNA) 2 μ l. The reaction system was supplemented to 25 μ l by adding dH₂O 8.5 μ l. DNA amplification was conducted by three steps under the following condition: 94°C for 1 min, 94°C for 30 s, 55°C for 30 s, 72°C for 45 s; 40 cycles; 72°C for 10 min. The expression level of mRNA was calculated by relative quantification, namely, computing by substituting Ct value of genes to be detected and β -actin into $2^{-\Delta\Delta Ct}$. Two duplicate tubes were set in this assay. Experiments were repeated for three times.

MTT assay

Cells during logarithmic phase of each group were treated by trypsinization. After centrifugation, cell density was adjusted to 2.5×10^3 cells/ml in DMEM culture medium. In a 96-well plate, 200 μ l cell suspension was inoculated into each well and was cultured for 7 days continuously. MTT assay was conducted every 24 h. After 20 μ l MTT solution was added into each well, the plate was cultured in an incubator with 5% CO₂ at 37°C for 4 h. Then, 100 μ l triad solution [10% SDS, 5% (V/V) isobutanol and 0.01 mol/L HCl] was added in each well. After culture overnight, the OD value at 492 nm was measured by using a microplate reader. Three duplicate wells were set in each group of cells.

Transwell assay

Matrigel was diluted by DMEM containing 1% fetal calf serum at the ratio of 1:8. Then, 60 μ l dilution was laid on the transwell chamber, which was put into a incubator at 37°C for 4 h for clotting. Cells in each group were starved for 12 h and then received trypsinization. After centrifugation, cells were re-suspended by DMEM with 1% fetal calf serum, cell density was adjusted to 5×10^5 cells/ml. 100 μ l cell suspension was added in each chamber and 600 μ l DMEM with 20% fetal calf serum was added

BCSC-1 overexpression inhibited SKOV3 cells

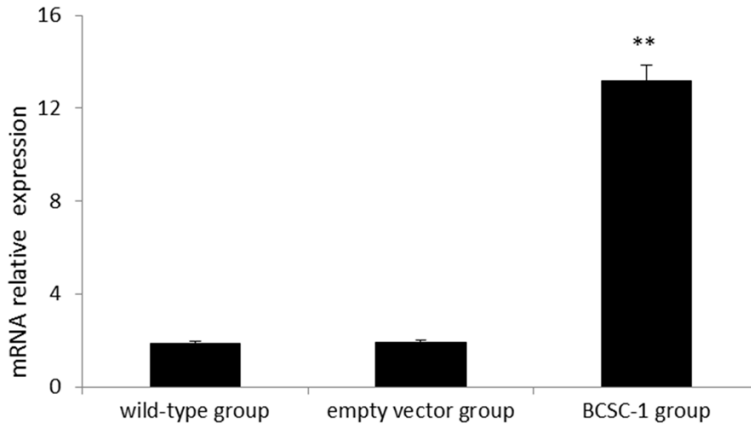


Figure 1. The expressions of BCSC-1 in SKOV3 cells with or without pcDNA3.1/v5-HisB-BCSC-1 transfection. ** $P < 0.01$ vs empty vector group.

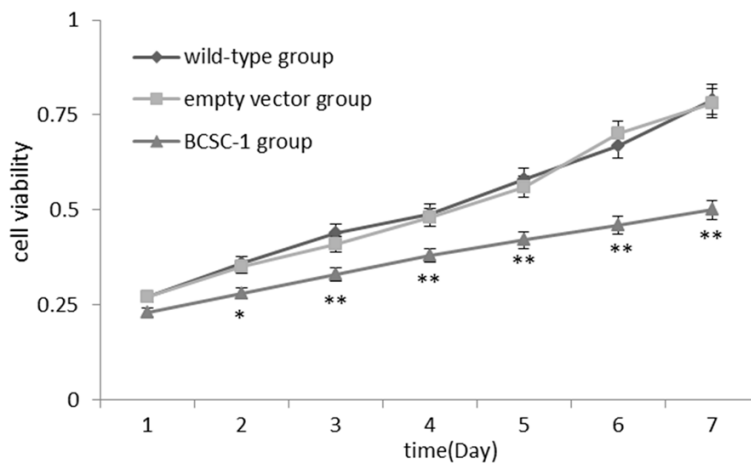


Figure 2. Proliferation of SKOV3 cells was inhibited by BCSC-1 overexpression in vitro. ** $P < 0.01$ vs empty vector group.

in lower ones. After culturing in an incubator with 5% CO₂ at 37°C for 24 h, media in these chambers were discarded. Supernatant cells were wiped off with a cotton swab. After being washed with PBS twice and fixed with pre-cooled methanol for 10 min, methanol was removed. After 0.1% crystal violet staining for 20 min, five visual fields (Amplified 100 times) were taken randomly for cell counting under an optical microscope.

Flow cytometry

Cells during logarithmic phase were inoculated into a 6-well plate, ensuring about 1×10^6 cells were inoculated in each well. After cells were treated with different drugs, 5 μ l FITC-Annexin V was added and the mixture was incubated in the dark for 15 min. Then, 5 μ l PI was added

and the mixture was incubated in the dark for another 10 min. After cells were filtered with a 300 mesh sieve, flow cytometry was conducted. 10000 cells were collected in each specimen.

Adhesion assay

In a 96-well plate, 25 μ l matrigel was added in each well. The plate was cultured in an incubator at 37°C overnight. In each well, 20 μ l serum-free DMEM with 2% BSA was added. After blocking at 37°C for 1 h, plate was washed by PBS twice and reserved for use. Cells during logarithmic phase in each group were prepared into cell suspension at the density of 5×10^5 cells/ml, 100 μ l of which was inoculated in each well of the above-mentioned plate, and cultured in an incubator with 5% CO₂ at 37°C for 1 h, media and non-adherent cells were then discarded. 20 μ l MTT solution was added in each well. After continuous culture for 4 h, 100 μ l triad solution was added in each well and the mixture was cultured overnight. At last, the OD value at 492 nm was measured with a

microplate reader. For each group of cells, three duplicate wells were set. Cell adhesion rate (%) = $D_{\text{Assay}}/D_{\text{Blank}} \times 100\%$.

Scratch assay

Cells during logarithmic phase were underwent trypsinization and centrifugation. Cells were resuspended with complete DMEM and prepared into cell suspension at the density of 2×10^5 cells/ml. In a 6-well plate, 2 ml of this suspension was inoculated into each well. When cell confluence reached up to about 80%, the original medium was changed into serum-free DMEM. After culture for 24 h, a scratch was made on the monolayer by using a 200 μ l sterile pipette Tip. At 0 and 24 h, cell migration was observed under an optical microscope and migration distance was measured by the cam-

BCSC-1 overexpression inhibited SKOV3 cells

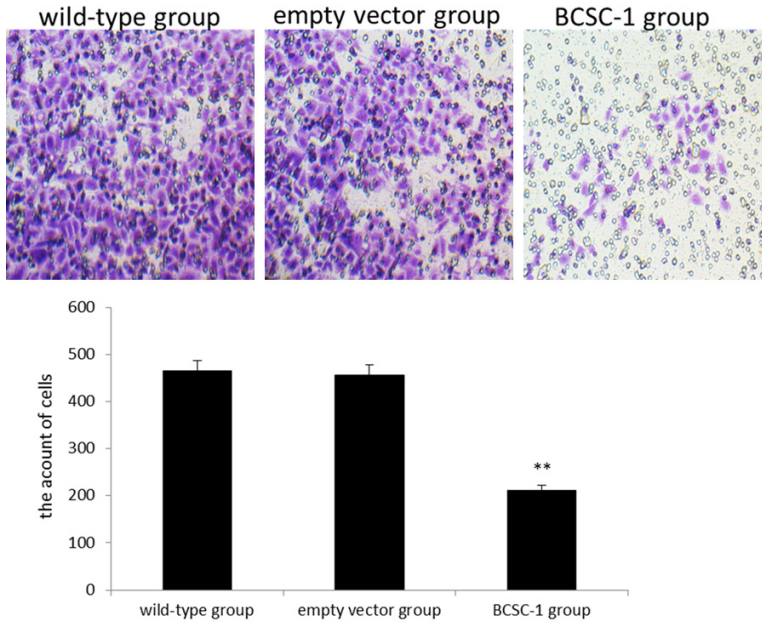


Figure 3. Invasion ability of SKOV3 cells was inhibited by the overexpression of BCSC-1 ($\times 200$). Transwell assay of SKOV3 cells with or without pcDNA3.1/v5-HisB-BCSC-1 transfection; histogram indicated statistic analysis of cell numbers in each group. ** $P < 0.01$ vs empty vector group.

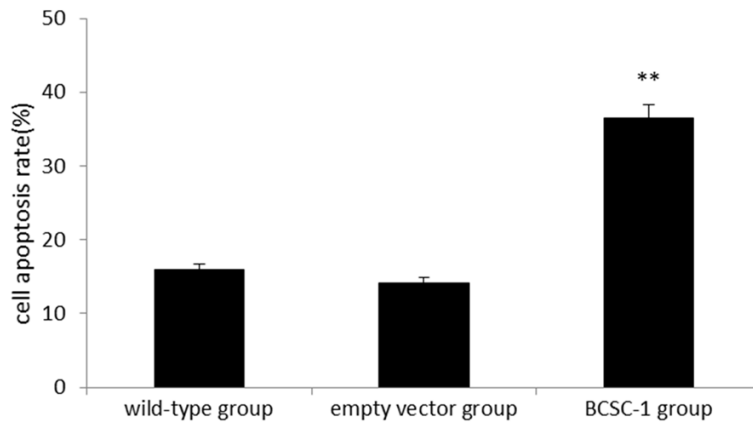


Figure 4. Cell apoptosis was analyzed by flow cytometry in SKOV3 cells transfected with or without pcDNA3.1/v5-HisB-BCSC-1. ** $P < 0.01$ vs empty vector group.

era system of a NIS-Elements D3.2 electron microscope.

In vivo anti-tumor assay

SKOV3 cells during logarithmic phase were collected and resuspended in a serum-free DMEM medium and prepared into suspension at the density of 3×10^6 tumor cells per 0.1 ml. On an operation desk disinfected by ultraviolet light, 0.2 ml cell suspension was injected subcutaneously into the middle part of armpit of BALB/c

nude mice carefully by a 1 ml sterile syringe. The growth of subcutaneously transplanted tumor and nude mice was observed and the transplantation could not be considered as successful until the diameter of tumor reached up to 3-4 mm. 32 tumor-bearing nude mice were randomized into three groups as follows: Control group receiving subcutaneous injection of normal saline 0.2 ml/d for 5 weeks; Empty vector group receiving subcutaneous injection of empty vectors [(50 μ g/mouse, 0.2 ml once daily] for 5 weeks; BCSC-1 group receiving subcutaneous injection of BCSC-1 plasmid [(50 μ g/mouse, 0.2 ml once daily] for 5 weeks.

From day 15 after tumor plantation, the length (a) and width (b) of tumor were measured with a vernier caliper every 2-3 days. Tumor volume was calculated ($V = \pi ab^2/6$) and a tumor growth curve was drawn. On day 30 after administration (day 45 after plantation), nude mice were killed by anesthetics and tumor weight was measured on an electronic scale.

Statistical treatment

Measurement data was expressed as $x \pm s$. Statistical software SPSS17.0 was used. Comparisons among groups were analyzed by one-way analysis of variance (ANOVA),

with a post hoc Student-Newman-Keuls test (SNK) to determine where the differences lie. $P < 0.05$ indicated that significant difference.

Results

BCSC-1 was overexpressed successfully in SKOV cells

Cellular total RNA in each group was extracted, cDNA was synthesized by reverse transcription and the mRNA level of intracellular BCSC-1 was

BCSC-1 overexpression inhibited SKOV3 cells

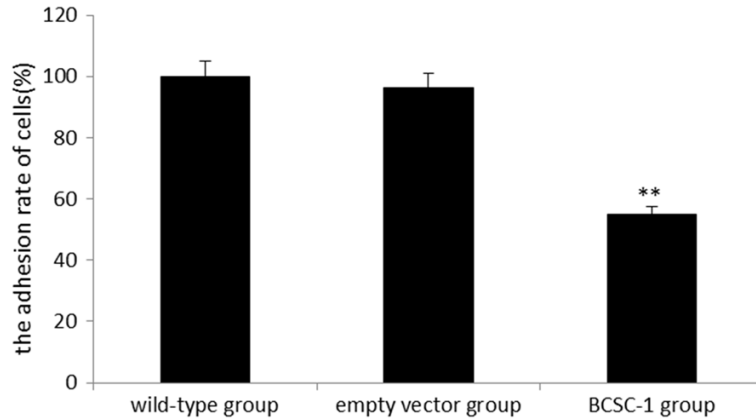


Figure 5. Cell adhesion was analyzed by MTT in cells transfected with or without pcDNA3.1/v5-HisB-BCSC-1. ****** $P < 0.01$ vs empty vector group.

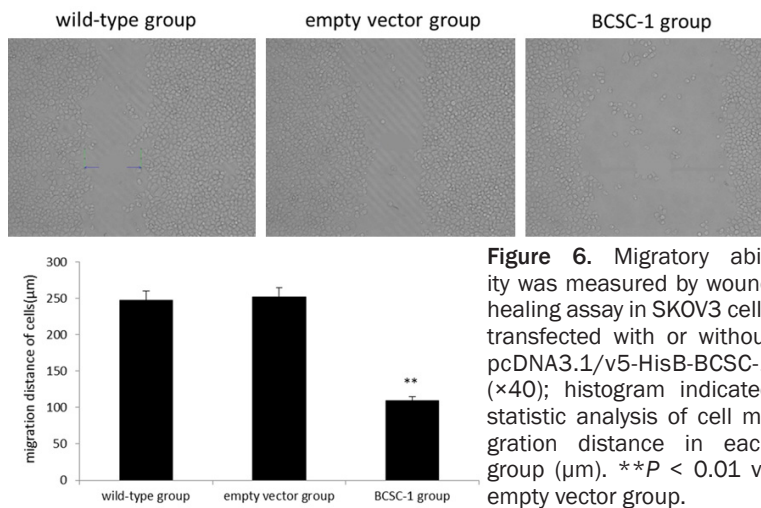


Figure 6. Migratory ability was measured by wound healing assay in SKOV3 cells transfected with or without pcDNA3.1/v5-HisB-BCSC-1 ($\times 40$); histogram indicated statistic analysis of cell migration distance in each group (μm). ****** $P < 0.01$ vs empty vector group.

detected by Real-time PCR. Results (**Figure 1**) showed that the expression of BCSC-1 in SKOV3 cells in the wild-type group and empty vector group had no significant difference ($P > 0.05$). Compared with empty vector group, the expression of BCSC-1 in SKOV3 cells in BCSC-1 group increased evidently [(13.18 ± 0.66) vs (1.90 ± 0.32) , $P < 0.01$].

The proliferation of SKOV3 cells was inhibited by BCSC-1 overexpression

Results (**Figure 2**) showed that cell proliferation in empty vector group and wild-type group had no significant difference from day 1 to day 7 ($P > 0.05$). From day 3, cell survival rate in BCSC-1 group was significantly lower than that of empty vector group ($P < 0.01$) and the difference became more evident from day 3 to day 7.

The invasion of SKOV3 cells was inhibited by BCSC-1 overexpression

Results of Transwell assay (**Figure 3**) showed that the number of invasive SKOV3 cells in BCSC-1 group was significantly fewer than that of empty vector group [(212.12 ± 11.07) vs (455.31 ± 14.05) cells, $P < 0.01$].

The apoptosis of SKOV3 cells was induced by BCSC-1 overexpression

The apoptosis rate of SKOV3 cells in BCSC-1 group was significantly higher than that of empty vector group [$(36.55 \pm 2.81)\%$ vs $(14.19 \pm 0.78)\%$, $P < 0.01$], while no significant difference was found between empty vector group and wild-type group ($P > 0.05$) (**Figure 4**). These results suggested that BCSC-1 overexpression could promote the apoptosis of SKOV3 OC cells.

The adhesive ability of SKOV3 cells was inhibited by BCSC-1 overexpression

Results of MTT assay (**Figure 5**) showed that the adhesion rate of cells in BCSC-1 group was obviously lower than that of empty vector group and wild-type group [$(54.78 \pm 0.74)\%$ vs $(96.19 \pm 0.55)\%$, $P < 0.01$].

The migration of SKOV3 cells was inhibited by BCSC-1 overexpression

Results (**Figure 6**) showed that there were different degrees of migration in three groups after scratch for 24 h. The migration distance of SKOV3 cells with overexpressed BCSC-1 was obviously less than that of empty vector group [(109.62 ± 9.37) vs (251.76 ± 10.39) μm , $P < 0.01$].

Tumor growth in nude mice was inhibited by BCSC-1 overexpression

Tumor growth curve of each group was shown in **Figure 7A**. Six days after subcutaneous

BCSC-1 overexpression inhibited SKOV3 cells

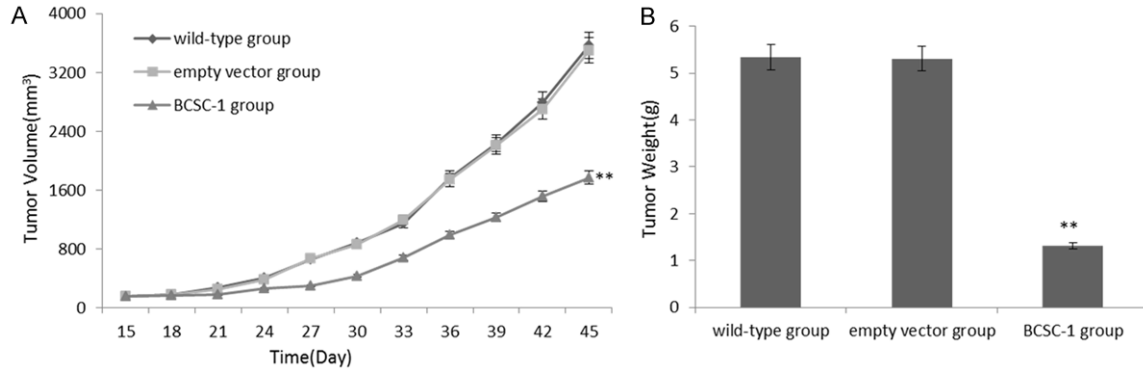


Figure 7. Effects of BCSC-1 over-expression on the *in vivo* tumor growth. 32 tumor-bearing nude mice were randomized into three groups as follows: Control group receiving subcutaneous injection of normal saline 0.2 ml/d for 5 weeks; Empty vector group receiving subcutaneous injection of empty vectors for 5 weeks; BCSC-1 group receiving subcutaneous injection of BCSC-1 plasmid for 5 weeks. A: Tumor volume (mm³) in BALB/C nude mice with subcutaneous inoculation of SKOV3 cells transfected with or without pcDNA3.1/v5-HisB-BCSC-1. B: At 30 days after inoculation, the mice were sacrificed and dissected, tumor weight was measured., ** $P < 0.01$ vs empty vector group.

tumor cell implantation, tumor body could be observed in all group. So the tumor formation rate was 100%. The tumor growth rate in BCSC-1 group was relatively slower than that of wild-type group from day 15 ($P < 0.01$). The weight of implanted tumor in BCSC-1 group (Figure 7B) was evidently lower than that of wild-type group ($P < 0.01$).

Discussion

BCSC-1, one of tumor suppressor genes found in recent years, could encode proteins with 786 amino acids. When introduced into nasopharyngeal carcinoma cell CNE-2L2 and small cell lung cancer cell NCI-H446 with endogeneous low-level expression, BCSC-1 could inhibit various biological behaviors such as malignant proliferation [16-18]. However, further studies need to be performed to explore whether BCSC-1 has inhibitory effect on tumor cells with endogeneous low-level expression. Malignant proliferation, adhesion, invasion and migration were both biological features of malignant tumors and important mechanisms, which led to death [19, 20]. In this study, BCSC-1 was transfected into SKOV3 OC cells to investigate the effect of BCSC-1 on the proliferative, migratory, invasive and adhesive ability of SKOV3 cells. Results proved that, compared with SKOV3 cells transfected with empty vectors, the *in vitro* proliferative, extracellular adhesive, invasive and migratory ability was inhibited to some extent in SKOV3 cells transfected with pcDNA-BCSC-1.

The results indicate that exogenous BCSC-1 had an inhibitory effect on OC.

However, the mechanism of BCSC-1 was rarely known at present. It was demonstrated in a literature [21] that, BCSC-1 overexpression in nasopharyngeal carcinoma cell CNE-2L2 could induce decreased tumorigenesis and migration, with increased adhesive ability and cell cycle arrest. Furthermore, Anghel et al. [22] suggested that BCSC-1 could also inhibit *in vitro* proliferation of melanoma cells. In Mewo cells, BCSC-1 could interact with SOX10 directly and thus down-regulating the expression of microphthalmia-associated transcription factors (MITF). These results was speculated to explain that BCSC-1 inhibit the proliferation of melanoma cells. It was reported [23] that the inhibitory effect of BCSC-1 on the proliferation and invasion of tumor cells was probably associated with OPN changes. Studies [24, 25] confirmed that tumor cells (liver cancer, gastric cancer, prostatic cancer et al.) could synthesize a large number of OPN, which promoted cell chemotaxis, adhesion and migration, through the interaction between RGD sequences associated with cell adhesion and extracellular matrix and membrane molecules. Therefore, it is speculated that BCSC-1 overexpression can inhibit the invasion, adhesion and migration of SKOV cells by down-regulating the expression of OPN.

In conclusion, our results indicated that BCSC-1 overexpression inhibited the proliferative,

invasive, migratory and adhesive ability in SKOV3 cells. BCSC-1 is a potential tumor suppressor gene in OC.

Disclosure of conflict of interest

None.

Address correspondence to: Yanqiu Du, Department of Mammary Gland, Linyi Cancer Hospital, Linyi 276001, Shandong Province, China. E-mail: yaolizrmyy@163.com

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