Vitamin D and DDX4 regulate the proliferation and invasion of ovarian cancer cells

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Abstract. Ovarian cancer is one of the most commonly occurring types of cancer and one of the most common causes of cancer-associated mortality in women. Diagnosis of ovarian cancer at an early stage is difficult due to the lack of specific symptoms. In the present study, it is demonstrated that active vitamin D treatment prohibited the proliferation and invasion of ovarian cancer cells, and the expression level of a germ cell specific marker DEAD (Asp‑Glu‑Ala‑Asp)-box helicase 4 (DDX4), which is overexpressed in ovarian cancer, was downregulated by active vitamin D treatment. Knockdown of DDX4 by siRNA could also suppress the invasive ability of ovarian cancer cells. Therefore, DDX4 may be considered as a diagnostic marker of ovarian cancer, and vitamin D may be a candidate drug for ovarian cancer therapy.

Introduction

Ovarian cancer is one of the most common causes of cancer-associated mortality in women (1). The majority of patients with ovarian cancer are diagnosed at an advanced stage, as there are no reliable symptoms for early diagnosis. Even at advanced stages of disease, signs and symptoms remain nonspecific (2). Upon diagnosis, ovarian cancer has often already metastasized to the uterus, peritoneum or other organs in the pelvic cavity (3,4). Radical surgery and adjuvant chemotherapy are common modes of treatment for ovarian cancer (5,6).

Vitamin D has been reported to inhibit the recurrence and distant metastasis of 19 types of cancer, of which ovarian cancer is one (7,8). Furthermore, a previous study demonstrated that mortality rates of ovarian cancer are lower in areas with higher levels of ultraviolet-B (UVB) radiation (9). An association between vitamin D deficiency and the occurrence of ovarian cancer has been suggested (10).

The germ cell‑specific marker DEAD (Asp‑Glu‑Ala‑Asp)-box helicase 4 (DDX4), which is the human ortholog of the *Drosophila* vasa gene, encodes a member of the DEAD-box family of ATP‑dependent RNA helicases (11). DDX4 is expressed solely in germ cells, including oocytes and spermatocytes, and has been reported to serve a central role in several aspects of germ cell development (11). A previous study demonstrated that DDX4 is overexpressed in ovarian cancer (12).

The present study demonstrated that the proliferative and invasive capacities of ovarian cancer cells were suppressed by active vitamin D. Vitamin D treatment downregulated the expression level of DDX4, and knockdown of DDX4 reduced the invasive ability of ovarian cancer cells.

Materials and methods

Cell culture. The ovarian epithelial carcinoma SKOV3 and OVCAR3 cell lines were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI‑1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology Co., Ltd., Sijiqing, China) and 1% penicillin-streptomycin (Beiyte Institute of Biotechnology, Haimen, China), and incubated at 37°C with 5% CO2. When cultured to 70% confluence, SKOV3 or OVCAR3 cells were treated with active vitamin D (catalog no. D1530; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Small interfering RNA (siRNA) treatment. The siRNAs targeting DDX4 were designed by GenePharma Co., Ltd. (Shanghai, China) and transfected (100 pmol siRNA for each well containing 2x104 cells) into SKOV3 or OVCAR3 cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. RNA was extracted 3 or 5 days after transfection for the following experiments. The sequences

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Abbreviations: DEAD, Asp‑Glu‑Ala‑Asp; DDX4, DEAD‑box helicase 4; CCK‑8, Cell Counting Kit‑8; RT‑qPCR, reverse transcription‑quantitative polymerase chain reaction

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for DDX4-712 (7656; GenePharma Co., Ltd.) were as follows; sense, 5'-GGAAGUGAACGAGGUGGGUUTT-3' and antisense, 5'-AACCACUCGUUCAUUCCCTT-3'. The sequences for DDX4-121 (7654; GenePharma Co., Ltd.) were as follows; sense, 5'-GCAGAAUAUCACCUCUATT-3' and antisense, 5'-UAUGAGGUGUGAUUUUCUGC TT-3'. The sequences for the negative control (7653; GenePharma Co., Ltd.) were as follows: Sense, 5'-UUCUCGGGAACGGUGACGUUTT-3', and antisense, 5'-ACGUGACAGGUUCGGAGAATT-3'.

Cell Counting Kit-8 (CCK-8) assay. The relative cell number was measured using a CCK-8 assay. Briefly, cells were cultured in 96-well plates at a density of 1x10^3 cells/well for 24 h, then treated with 0, 25, 50, 100, 250 or 400 µl/ml active vitamin D for 72 h. Subsequently, 10 µl CCK-8 dye (Beyotime Institute of Biotechnology) was added to each well, according to the manufacturer’s protocol. The plates were read using a microplate reader at a wavelength of 450 nm. Relative cell number was represented by the absorbance value relative to that of the untreated control cells.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from SKOV3 or OVCAR3 cells using TRIzol (Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. cDNA was synthesized using a ReverTraAce qPCR kit (Toyobo Life Science, Osaka, Japan, Japan), according to the manufacturer’s protocol. PCR was performed using an ABI PRISM 7500 system (Thermo Fisher Scientific, Inc.), and the thermocycling conditions were as follows: 95°C for 30 sec, 40 cycles of 95°C of 15 sec and 60°C of 60 sec. The primer sequences for DDX4 are as follows: Forward, CCAGAGGCC TGGATTTGAA, and reverse, GCCAGTATTTCCACA ACGAC. The primer sequences for GAPDH are as follows: Forward, AATCCCATACATCATCTCCA and reverse, AAA TGAGCCCCAGCTTCT. The ΔCq=Cq gene-Cq reference, calculation was adopted to scale the relative levels of gene expression, and 2^ΔΔCq method was used to fold the change of gene expression (13). qPCR was performed in duplicate for 3 independent groups of treated cells.

Western blotting. SKOV3 and OVCAR3 cells extracts were lysed with mammalian protein extraction reagent (CWBIO, Beijing, China) supplemented with 1% protease inhibitors (CWBIO) at 4°C for 30 min. The suspension was then centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was collected and the protein concentration was determined using a BCA protein quantitation kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Proteins (10 µg) were separated by 12% SDS-PAGE and transferred into nitrocellulose membranes (Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% non-fat milk in Tris buffered saline with 0.5% Tween-20 for 1 h at room temperature, then incubated with the following primary antibodies overnight at 4°C: DDX4 (dilution 1:1,000, cat. no. ab13840; Abcam, Cambridge, UK) and GAPDH (dilution 1:1,000, cat. no. AG019-1; Beyotime Institute of Biotechnology, Haimen, China). The membranes were then incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (dilution, 1:1,000, cat. no. SA00001-2; ProteinTech Group, Inc., Chicago, IL, USA) for DDX4 and anti-mouse for GAPDH (dilution 1:1,000, cat. no. AF0006, Beyotime Institute of Biotechnology) for 2 h at room temperature and visualized by chemiluminescence using an eECL western blot kit (cat. no. CW0049; CWBIO) and western enhanced chemiluminescence substrates (cat. nos. 102030838 and 102030839; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The western blotting results were quantified using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Transwell invasion assay. The transwell apparatus was assembled using 8-µm pore Transwell inserts (Corning Incorporated, Corning, NY, USA) in 24-well plates. Each insert was coated with 100 µl Matrigel (diluted in PBS, 1:1). A total of 1x10^5 SKOV3 or OVCAR3 cells were seeded onto the insert and cultured with 250 µl RPMI-1640 medium supplemented with 1% FBS, while the lower chambers contained 500 µl RPMI-1640 supplemented with 10% FBS. Subsequent to incubation for 3 or 5 days, the cells in the upper chambers were removed carefully using cotton swabs, and cells that traversed the Matrigel to the lower surface of the insert were fixed using 4% paraformaldehyde at room temperature for 20 min and stained with eosin (0.5%, R20593, Shanghai Yuan Ye Biological Technology Co., Ltd.) (SKOV3 cells) or hematoxylin (D005, Nanjing Jiancheng Bioengineering Institute, Nanjing China, 0.5%) (OVCAR3 cells) alone, both at room temperature for 20 min. Cells were observed and calculated in five random fields using alight microscope (magnification x100).

Statistical analysis. All statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). The data are presented as the mean ± standard error of the mean. Statistically significant differences between mean values of two groups were identified using unpaired Student’s t-test. Statistically significant differences between mean values of ≥3 groups were identified using analysis of variance and Student-Newman-Keuls post-hoc test. P<0.05 was considered to indicate a statistically significant difference.
Results

Active vitamin D suppresses the proliferation of ovarian cancer cells. To evaluate the effect of active vitamin D on ovarian cancer, SKOV3 or OVCAR3 cells were treated with varying concentrations of biologically active vitamin D for 72 h. The relative cell number was quantified using CCK-8. It was demonstrated that 100 µl/ml active vitamin D was able to suppress the proliferation of ovarian cancer cells.
significantly inhibit the proliferation of SKOV3 and OVCAR3 cells, and a further increase in concentration caused a greater inhibitory effect (Fig. 1).

**Active vitamin D inhibits the invasion of ovarian cancer cells.** Active vitamin D treatment reduced the number of SKOV3 or OVCAR3 cells able to migrate to the lower surface of transwell inserts (Fig. 2A and B), suggesting that vitamin D could partially block the invasion ability of ovarian cancer cells.

**Active vitamin D downregulated the expression of DDX4 in ovarian cancer cells.** It has been established that DDX4 is overexpressed in epithelial ovarian cancer and can be used as an ovarian cancer stem cell marker (14). To assess whether vitamin D affects the expression of DDX4, SKOV3 or OVCAR3 cells were treated with 100 µl/ml active vitamin D for 72 h prior to RT-qPCR and western blot analyses. It was demonstrated that vitamin D treatment downregulated the expression of DDX4 at the mRNA (Fig. 3A) and protein (Fig. 3B) levels.

**DDX4 knockdown inhibits invasion of ovarian cancer cells.**

To investigate whether DDX4 could influence the invasion of ovarian cancer cells, several independent siRNAs targeting DDX4 were transfected into SKOV3 and OVCAR3 cells individually. The knockdown efficiency was evaluated by RT-qPCR, which demonstrated that transfection with siDDX4-121 or siDDX4-712 could significantly reduce DDX4 expression (Fig. 4). siDDX4-121-transfected cells were selected for the transwell assay, due to the greater inhibition of DDX4 expression achieved using this siRNA. Invasive cells were stained 3 or 5 days subsequent to siRNA treatment (Fig. 5A and B). DDX4 knockdown partially inhibited the invasive ability of SKOV3 and OVCAR3 cells (Fig. 5A and B).

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**Figure 4.** DDX4 siRNA reduces the expression level of DDX4. SKOV3 or OVCAR3 cells were transfected with two independent siRNAs against DDX4, and the DDX4 mRNA level was examined by reverse transcription-quantitative polymerase chain reaction, relative to GADPH as an internal control. *P<0.05, compared with blank control group. DDX4, DEAD-box helicase 4; siRNA, small interfering RNA; NC, negative control.

**Figure 5.** DDX4 knockdown inhibited the invasive ability of SKOV3 and OVCAR3 cells. (A) SKOV3 cells or (B) OVCAR3 cells were treated with siDDX4-121 for 3 or 5 days in a transwell assay. **P<0.01, ***P<0.001, compared with control. DDX4, DEAD-box helicase 4; siDDX4, small interfering RNA targeting DDX4.
Discussion
Vitamin D was first recognized for its regulatory function in calcium-phosphorus balance (15). Recently, it has been demonstrated that active vitamin D affects various cellular processes, including proliferation, invasion, differentiation and malignant transformation (8,16-18) in multiple types of cancer (18,19), including male reproductive system carcinomas and prostate cancer (12). It has also been suggested that vitamin D could prevent ovarian cancer progression (8). The present study demonstrated that the proliferative and invasive abilities of SKOV3 and OVCAR3 ovarian cancer cells could be inhibited by active vitamin D. However, the molecular mechanisms of how vitamin D inhibits the proliferation and invasion of ovarian cancer cells remain to be further investigated.

DDX4 is expressed exclusively in the ovaries and testes. The expression level of DDX4 in SKOV3 and OVCAR3 cells was downregulated by active vitamin D at the mRNA and protein level. Knockdown of DDX4 by siRNA partially inhibited the invasion ability of SKOV3 and OVCAR3 cells. It is speculated that vitamin D may inhibit the invasion of ovarian cancer cells through downregulating the expression of DDX4.

In conclusion, vitamin D treatment reduced the proliferation and invasion of ovarian cancer cells. DDX4, which was previously found to be overexpressed in ovarian cancer, was downregulated by vitamin D on the mRNA and protein levels. DDX4 knockdown also inhibited the invasion of ovarian cancer cells. Therefore, the use of vitamin D should be considered as a potential novel therapy for ovarian cancer.

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Availability of data and materials
The analyzed data sets generated during the study are available from the corresponding author upon request.

Authors' contributions
All authors have read and approved the manuscript. ZS performed the experiment and analyzed the data. JX, PW, JT, XS and JL contributed to data collection and analysis. YC, FR, and LX designed the study and wrote the 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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