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Low expression level of HMBOX1 in high-grade serous ovarian cancer accelerates cell proliferation by inhibiting cell apoptosis

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ABSTRACT

Homeobox-containing 1 (HMBOX1) has been described as a transcription factor involved in the occurrence of some tumors, but its roles in ovarian cancer have never been reported. Here we aimed to investigate the roles of HMBOX1 on high-grade serous ovarian carcinoma (HGSOC). In this present study, HMBOX1 expression was decreased in HGSOC tissues and ovarian cancer cell lines (HO8910 and A2780) compared with ovarian surface epithelial tissues or normal human ovarian surface epithelial cell line (HOSEpiC). The cell proliferation of HOSEpiC was weaker than ovarian cancer cell lines. By altering the expression of HMBOX1 in A2780 and HOSEpiC, we demonstrated that HMBOX1 inhibited the cell proliferation and promoted the cell apoptosis. Furthermore, our study revealed that HMBOX1 downregulated the expression of anti-apoptotic proteins (Bcl-2, Bcl-xL), raised the expression of pro-apoptotic-regulated proteins (Bad, Bax), apoptotic executionior (Caspase3), and P53. In conclusion, HMBOX1 played important roles in occurrence of HGSOC through regulation of proliferation and apoptosis, which implied that HMBOX1 might serve as a new therapeutic target for HGSOC.

1. Introduction

Ovarian cancer is one of the most common types of malignant tumors of female genital organs [1]. Due to widespread intraperitoneal metastasis at early stage and lack of effective methods for early diagnosis, its mortality is still the highest in gynecological cancers, and the 5 year survival rate is poor after diagnosis [2]. According to the new WHO Classification of Ovarian Cancer, Ovarian Cancer can be mainly divided into the six subtypes: serous, mucinous, seromucinous, endometrioid, clear cell and Brenner (transitional) tumors. Among these subtypes, serous tumors are the most common ones, which are further divided into low-grade and high-grade serous ovarian carcinomas (LGSOC and HGSOC, respectively) [3]. HGSOC are the most common ovarian carcinomas and most patients are diagnosed with advanced-stage disease (approximately 80%); tumors confined to the ovary at diagnosis are distinctly uncommon (<10%) [4]. To clarify the mechanism of the occurrence and development of HGSOC and to hunt for new therapeutic targets have become a valuable and essential task in gynecologic oncology research.

Homeobox-containing genes play important roles in regulation of embryonic development and cell proliferation [5]. In recent years, it is found that some homeobox genes are also involved in the occurrence and progression of many tumors [6]. Currently, the relationship between hepatocyte nuclear factor (HNF) subfamily genes of the homeobox genes and cancers has gradually abstracted more and more attention [7–9]. HMBOX1 is a member of the HNF subfamily. It is highly conserved and has high homology with other HNF molecules [10]. In cancer research, it is reported that the expression of HMBOX1 is up- or downregulated in some tumors, and HMBOX1 induces the expression of some molecules related with tumor and immune functions signal pathways, including P53,

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changed in hepatocarcinoma cell lines. It suggests that HMBOX1 may be correlated with tumorigenesis, but whether HMBOX1 is related to the regulation of ovarian cancer has not been reported.

In this research, we reported for the first time that the expression of HMBOX1 in patient samples with HGSOC and in ovarian cancer cell lines (HO8910 and A2780) were downregulated, and we discovered that cells with higher level expression of HMBOX1 tended to present weaker proliferative ability. By altering the expression of HMBOX1 in A2780 or HOSEpiC cell lines, we observed the changes of some apoptosis-regulated genes after overexpression or knockdown of HMBOX1. It implies that HMBOX1 decrease the proliferation of ovarian cancer cells by the regulation of apoptosis-associated proteins.

2. Material and methods

2.1. Clinical tissues samples

A total of 253 cases of Specimens were used for immunohistochemical analysis (90), *Western blotting* (52) and *real-time PCR* (111). All the tissue specimens were collected from patients at Qilu Hospital from 2010 to 2016. Our study was approved by the Ethics Committee of Qilu Hospital. All specimens were handled and made anonymous according to the ethical and legal standards.

2.2. Cell culture

The human normal OSE cell line (HOSEpiC) (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) and the ovarian cancer cell lines (A2780 and HO8910) (Gynecology Oncology Key Laboratory, Qilu Hospital, Shandong University) were cultured in RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (Gibco, Sydney, Australia) and 1% penicillin/streptomycin in a humidified atmosphere with 5% CO_2 maintained at 37 °C.

2.3. Real-time quantitative reverse transcription PCR

RNA was reverse transcribed into cDNA with the Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, MA, USA) and then was amplified and detected with SYBR® Premix Ex Taq™ (Takara Bio Inc., Otsu, Japan) in an ABI StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Primers (Shanghai GenePharma Co., Shanghai, China) were shown in Table 1. Data were analyzed according to relative gene expression using the $2^{-\Delta\Delta CT}$ (Livak) method.

2.4. Western blotting

Protein (30 mg) was separated by 12% SDS polyacrylamide gel electrophoresis and then electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were probed with anti-HMBOX1 (Abcam, Cambridge, UK), anti-Bcl-1, anti-Bcl-xL, anti-Bax, anti-Bad, anti-Bcl-2, anti-caspase-3 (Santa Cruz Biotechnology, CA, USA) and β-actin (Cell Signaling Technology, Danvers, MA, USA) was measured as an internal control. Immunoblots were visualized by the Immobilon Western Horseradish peroxidase substrate (Millipore, Billerica, MA, USA). The protein band intensity was calculated by the Image J software (National Institutes of Health, Bethesda, MD, USA).

2.5. Immunohistochemistry

The avidin-biotin-peroxidase complex (SP9001 rabbit SP test kit, ZSJQ Biotech Company, Beijing, China) method was performed for immunohistochemical staining analysis according to manufacturer's protocol. Rabbit anti-human HMBOX1 polyclonal antibody was applied at 1:600 dilution. Negative control experiments were performed by using isotype control antibodies. The semi-quantitative analysis was performed with Image Pro-Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

2.6. Cell proliferation assay

Cell viability was assessed by MTT. Approximately 2.0×10^3 cells were seeded in 96-well plate. Cells were stained with sterile MTT dye (5 mg/ml; Sigma Aldrich, St. Louis, MO, USA) at 37 °C for 4 h following the manufacturer's instructions every 24 h. Then we removed the culture medium and added dimethyl sulfoxide for 10 min and the absorbance of wells was detected at 490 nm wavelength. Then we evaluated cell proliferation and drew up the cell proliferation curve for 72 or 96 h.

2.7. Colony formation assay

Cells were seeded at a very low density in plates to determine their clonogenicity for about 2 weeks. After methanol fixation and crystal violet staining, the colonies can be visualized (colonies $\geq$ 50 cells). Colonies were counted and the colony-forming efficiency was calculated.

2.8. Overexpression of the HMBOX1 gene in A2780 cell lines

A2780 cells were divided into two groups and transfected by Adenovirus-HMBOX1 (HMBOX1+) and Adenovirus-negative control (NC) with green fluorescent protein (GFP) respectively (cat. no. VH847005; ViGene Biosciences, Shandong, China). Cells were cultured in 6-well plates to reach 60% confluency, and transfected the Adenovirus expression vectors with MOI = 400. Then we performed experiments with the cells 2–7 days after transfection.

2.9. Silencing of the HMBOX1 gene in HOSEpiC cell line

The small interfering RNA sequences targeting human HMBOX1 (HMBOX1 si-RNA1, si-RNA2, si-RNA3) and its negative control sequence (si-NC) (Table 1) were designed by GenePharma Co., Ltd. (Shanghai, China). Cells were cultured in 6-well plates to reach 60% confluency, and transfected with 50 nM of the si-RNAs using

Table 1
Primers used in QRT-PCR analysis and the small interfering RNA sequences.

	Forward	Reverse
HMBOX1	5′-AACCCTGGCGCTACACTAAG-3′	5′-TCCTTTCTCCAGGTA AATCGAC-3′
β-actin	5′-TCATCACCATTGGC AATGAG-3′	5′-CACTGTGTTGGCGTACAGGT-3′
si-RNA1	5′-CCAACUCGCUACCAUGCAATT-3′	5′-UUGCAUGGUAGCGAGUUGGTT-3′
si-RNA2	5′-GGAUCUCUCAUUGGCUGUUTT-3′	5′-AACAGCCAAUGAGAGAUCCTT-3′
si-RNA3	5′-CCAAUAGAGGACCCUGAAUTT-3′	5′-AUUCAGGGUCCUCUAUUGGTT-3′
si-NC	5′-UUCUCCGAACGUGUCACGUTT-3′	5′-ACGUGACACGUUCGGAGAATT-3′

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