CBX3 promotes ovarian cancer progression by regulating p53/p21-mediated glucose metabolism via inhibiting NCOR2

Keywords
ovarian cancer, glucose metabolism, CBX3, NCOR2, p53/p21

Abstract
Introduction
Chromobox protein homolog 3 (CBX3) has been reported to regulate a variety of cellular biological functions and play an oncogenic role in various tumor. Nevertheless, the role of CBX3 remains vague in ovarian cancer. This research aimed to assess the role and potential regulatory mechanism of CBX3 in ovarian cancer.

Material and methods
The CBX3 expression was determined by qRT-PCR and western blotting in ovarian cancer tissues and cell lines. Cell proliferation, cycle and apoptosis were detected by using CCK-8 assay and flow cytometry. Transwell and wound healing assay were used to determine cell invasion and migration. Furthermore, the modulation of CBX3 on NCOR2 expression and p53/p21-mediated glycolysis was confirmed.

Results
The expression of CBX3 was significant elevated in ovarian cancer tissues and cell lines. CBX3 knockdown inhibited cell proliferation, invasion and migration, while promoted G1/S phase blockade and cell apoptosis. Mechanism analysis verified that CBX3 downregulation increased NCOR2 expression and blocked subsequent p53/p21-mediated glucose metabolism. NCOR2 silencing and p53/p21 inhibitor treatment reversed the inhibitory effects of CBX3 knockdown on ovarian cancer cellular function.

Conclusions
We revealed that CBX3 promoted ovarian cancer progression by promoting p53/p21-mediated glucose metabolism via inhibiting NCOR2. These results provide a theoretical basis for the diagnosis and treatment of ovarian cancer.

Explanation letter
Dear editors and reviewers:
Thank you for your letter and for the reviewers’ comments concerning our manuscript entitled “CBX3 promotes ovarian cancer progression by promoting p53/p21-mediated glucose metabolism via inhibiting NCOR2 (AMS-13391-2021-02)”. Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made response which we hope meet with approval. The main responds to the reviewer’s comments are as following:

Review 1:
The authors present a study which aims to show that CBX3 promotes ovarian cancer progression by promoting p53/p21-mediated glucose metabolism via inhibiting NCOR2. It is a well-designed study and well written manuscript. However, the authors should mention about the limitations of their study and the clinical implications of their findings in a separate paragraph of the discussion part. Moreover, they should avoid reiterating the word “promoting” in the title.
Response: We are grateful for the suggestion. We discussed the limitations and clinical implications of
our study in a separate paragraph of the discussion section (See lines 265-266). Besides, the title was revised in our revised manuscript.

Review 2:
Please correct the sentence 'CBX3 promoted ovarian cancer progression by promoting p53/p21-mediated glycolysis via inhibiting NCOR2.'
Response: We agree with the comment. We modified this sentence in our revised manuscript.

We appreciate Editors/Reviewers' warm work earnestly, and hope that the responses will meet with approval.
Once again, thank you for your comments and suggestions.
Yours sincerely
Chunfang Ha

Response to reviewers.docx
CBX3 promotes ovarian cancer progression by regulating p53/p21-mediated glucose metabolism via inhibiting NCOR2

Running title: CBX3 aggravated ovarian cancer progression

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Abstract

Instruction:

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The expression of CBX3 was significant elevated in ovarian cancer tissues and cell lines. CBX3 knockdown inhibited cell proliferation, invasion and migration, while promoted G1/S phase blockade and cell apoptosis. Mechanism analysis verified that CBX3 downregulation increased NCOR2 expression and blocked subsequent p53/p21-mediated glycolysis. NCOR2 silencing and p53/p21 inhibitor treatment reversed the inhibitory effects of CBX3 knockdown on ovarian cancer cellular function.

Conclusion:

CBX3 promoted ovarian cancer progression by promoting p53/p21-mediated glycolysis via inhibiting NCOR2.

Key words: CBX3; NCOR2; p53/p21; glycolysis; ovarian cancer
Introduction

Ovarian cancer (OC) is a common gynecological malignancies in the world, and its mortality rate ranks forefront among gynecological tumors [1]. Emerging evidences have proved that molecular targeted therapy may be one of the effective treatment methods. However, the molecular mechanism of OC still remains unclear, which limits the progress of clinical treatment. Therefore, identification of novel biomarkers and elucidation of their mechanisms are critical for the diagnosis and therapy of patients with ovarian cancer.

Chromobox protein homolog 3 (CBX3; also called HP1γ), a member of heterochromatin protein 1 family, is involved in regulation of a variety of cellular biological functions [2]. Multiple previous studies have reported that CBX3 abnormal expressed and regulated cellular functions in a variety cancer disease [3-5]. In pancreatic cancer, CBX3 also has been reported to be associated with glycolysis [6]. A recent study suggested that the abnormally high expression of CBX3 was found in ovarian cancer tissues [7]. However, the precise role and regulatory mechanism of CBX3 in ovarian cancer remain unclear.

Alteration of glucose metabolism is a faster but less efficient process than mitochondrial oxidative phosphorylation in ATP production and considered as a hallmark of cancer [8, 9]. In tumor microenvironment, most cancer cells showed increase of glucose consumption and lactate secretion, which promotes cancer environmental adaptability and cell survival [10]. Subsequently, the decreased potential
of hydrogen (PH) caused by elevated glucose uptake and lactate production promotes epithelial-to-mesenchymal transition (EMT), ultimately facilitating cancer cell migration and invasion [11]. Alteration of glucose metabolism in cancer cells is driven by inactivation of tumor suppressors, including p53 [12].

In the present study, the role of CBX3 on the cell growth and glycolysis was investigated, the putative regulatory mechanism of CBX3 in ovarian cancer was further explored. The results provide a novel biomarker for the diagnosis and therapy of patients with ovarian cancer.

Materials and methods

Ovarian cancer samples

The 30 pairs of epithelial OC tumor tissue and corresponding adjacent non-tumor tissue (about 3 cm away from the tumor tissues) samples were obtained from OC patients undergoing surgery at the General Hospital of Ningxia Medical university. All samples were histopathologically diagnosed and confirmed by a pathological evaluation. The informed consents were obtained from all patients. The ethics approval was given by General Hospital of Ningxia Medical university (KYLL-2022-0271) All procedures conformed to the declaration of Helsinki.

Cell culture and transfection

Human normal ovarian epithelial cell line IOSE80 and OC cell lines (ES2, SKOV3, OVCAR3, A2780, HEY) were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China) and kept in RPMI-1640 medium
supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., MA, USA), penicillin (100 μg/mL) and streptomycin (100 μg/mL) (Thermo Fisher Scientific, Inc., MA, USA) with 5% CO₂ at 37°C.

All indicated siRNA (siCBX3, siNCOR2), pcDNA3.1 (pcNCOR2) and respective negative control (siCtrl, pcCtrl) were synthesized and constructed by Shanghai Genepharma Company (China). The transfection into SKOV3 cells were performed using a Lipofectamine™ 2000 (Invitrogen, Waltham, USA). All the transfected cells were collected at 48 h post-transfection for the following experiments.

qRT-PCR

RNA was extracted from tissue samples and cultured cells using Trizol reagent (TaKaRa, Shiga, Japan). The reverse transcription of extracted RNA was synthesized using a Primer Script RT Reagent Kit (TaKaRa, Shiga, Japan). PCR amplification was performed using SYBR Premix Ex Taq™ (TaKaRa, Shiga, Japan). The total volume of the reaction mixture was 20 μL, which contained 1 μL of each primer, 2 μL of cDNA and 10 μL of SYBR Premix Ex Taq™. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as an internal standard. Relative expression levels were calculated according to the 2^ΔΔCt method.

Western blot analysis

Protein was extracted and quantified using a BCA kit (Pierce, Shanghai, China). Then, protein samples were separated on SDS-PAGE electrophoresis and transferred onto PVDF (Millipore, Billerica, MA, USA) membranes. After being blocking with 5%
nonfat milk, the membranes were incubated with primary antibodies CBX3 (1:1000), Cyclin A (1:1000), Cyclin B1 (1:2000), Cyclin D1 (1:1000), Cyclin E (1:1000), Bcl-2 (1:1000), BAX (1:1000), Caspase-3 (1:500), E-cadherin (1:10000), N-cadherin (1:100), Vimentin (1:1000), NCOR2 (1:1000), p53 (1:1000), p21 (1:1000), p-p53 (1:1000) at 4°C overnight separately. Rabbit anti-human GADPH (1:2500) as internal controls.

Subsequently, the membranes were washed three times and incubated with horseradish peroxidase-labeled secondary antibody (Sigma, St. Louis, MO, USA) for 1 h. Proteins were visualized by an enhanced chemiluminescence detection system (ECL, Thermo Fisher Scientific, USA). The blots were quantitatively analyzed using Image Lab 3.0 (Bio-Rad, USA).

**CCK-8 assay**

CCK-8 reagents (Dojindo, Japan) were used to detect cell proliferation according to the manufacturer’s protocol. Cells (5×10^3 cells/well) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum for 24 h. Then, cells were washed and starved for 24 h. After 48 h incubation, each well was added into 10 μL CCK-8 to culture for 2 h. Finally, the plates were placed to a microplate reader (Thermo Fisher Scientific, USA) for absorbance determination for 450 nm.

**Flow cytometry analysis**

The cell cycle and cell apoptosis were detected using a cell cycle kit (US Everbright Inc) and an Annexin V fluorescein isothiocyanate kit (FITC; BD, USA). FC-500 flow cytometer (Beckman Coulter, Brea, USA) was used to evaluate the cell cycle
distribution and percentage of apoptotic cells.

**Wound healing assay**

Cells were incubated at 37°C with 5% CO\(_2\) until they reached 90% confluence. After starvation, cells (1×10\(^6\) cells/well) were wounded with sterile 200 μL pipette tips, and the injury line were marked. Then, the disconnected cells were washed with PBS, while the remaining cells were incubated with RPMI-1640 medium supplemented with 10% FBS. Micrographs were acquired at 0 and 24 h.

**Invasion assay**

Cell invasion assays were performed using Transwell chambers (8 μm pore size) (BD, USA). Cells (1×10\(^5\) cells/well) in serum-free medium were seeded in the upper chamber coated with Matrigel (1:6; BD, USA) before. Medium supplemented with 20% FBS was added to the lower chambers, and cells were cultured in a thermostatic incubator with 5% CO\(_2\) at 37°C for 24 h. Finally, invaded cells were stained with 0.5% crystal violet and counted under a microscope (Olympus Crop., Tokyo, Japan).

**Detection of ATP level and oxygen consumption**

ATP Determination Kit (Thermo Fisher Scientific) was used to assess the ATP production. Briefly, ovarian cancer cells were lysed, centrifuged and filtered. Then, treated samples were mixed with ATP reaction. Glomax 20/20 (Promega, USA) luminometer was used to determine the ATP production. Extracellular Oxygen Consumption Assay (Abcam) was introduced to determine oxygen consumption.

**Measurement of glucose consumption and lactate production**
After transection, ovarian cancer cells were harvested and centrifuged to collect the supernatant of cells. Glucose and Lactate assay kit (BioVision, Milpitas, CA, USA) was used to detect the amount of glucose and lactate.

**Measurement of Glucose-6-Phosphate (G6P) levels**

G6P assay kit with WST-8 (Beyotime, Shanghai, China) were used to evaluate the level of G6P in cells according to manufacturer’s instructions.

**Statistical analysis**

The results are presented as mean ± SEM of at least three independent experiments. Statistical analyses were performed by GraphPad Prism software version 8.2 (La Jolla, CA, USA). The results were coincided with the normal distribution, the pairwise comparisons were performed using Student’s t-test and the comparisons among multiple groups were performed by one-way ANOVA analysis test. $P<0.05$ (*) indicates statistically significant differences.

**Results**

**CBX3 expression was increased in OC tissues and cell lines**

The results showed a significant up-regulation of CBX3 in OC tissues compared with the adjacent non-tumor tissues (Figure 1A). Consistently, the increased expressions of CBX3 mRNA and protein were also detected in OC cell lines compared with IOSE80 cell lines (Figure 1B and C). In addition, CBX3 presented the highest expression level in SKOV3 cell lines, so SKOV3 cell line was used in subsequent experiments.

**Knockdown of CBX3 suppressed OC cells proliferation while promoted G1/S**
As expected, the mRNA and protein expressions of CBX3 were reduced in si-CBX3s transfected cells (Figure 2A and B). Knockdown of CBX3 conspicuously suppressed cell proliferation in SKOV3 cells (Figure 2C). As well as, the inhibition of CBX3 was exhibited cell arrested at G1/S phase, resulting in the decreased of cell numbers at S phase, accompanied with lower expressions of Cyclin A, Cyclin B1, Cyclin D1 and Cyclin E (Figure 2D and E). Furthermore, CBX3 silencing promoted cell apoptosis as evidenced with elevated Bax and cleaved caspase 3 expressions and reduced Bcl-2 expression (Figure 2F and G).

**Knockdown of CBX3 suppressed OC cells invasion and migration**

Down-regulation of CBX3 significantly suppressed cell invasion of SKOV3 cells (Figure 3A). Besides, wound healing assay showed that knockdown of CBX3 also dramatically inhibited cell migration (Figure 3B). In addition, epithelial-mesenchymal transition (EMT) is widely recognized as a marker of tumor cell metastasis. CBX3 silencing markedly increased the expression of epithelial regulator, E-cadherin, and decreased the expressions of mesenchymal regulators, N-cadherin and Vimentin (Figure 3C and D).

**Knockdown of CBX3 suppressed glycolysis of OC cells**

The results showed that CBX3 knockdown markedly suppressed glucose upstate, lactate production and G6P level in SKOV3 cells (Figure 4A-C). Besides, the results showed that downregulation of CBX3 significantly inhibited ATP production and O₂
consumption (Figure 4D and E).

CBX3 blocked the activation of p53/p21 pathway by inhibiting NCOR2

Notably, we found that NCOR2 was downregulated in OC tissues and cell lines (Figure 5A-C). Meanwhile, CBX3 knockdown promoted the expression of NCOR2 (Figure 5B and C). Correlation analysis showed that CBX3 negatively correlated with NCOR2 expression in OC tissues (Figure 5D). Subsequently, we transfected siNCOR2 and pcNCOR2 to reduce and boost the expression of NCOR2 in OC cells (Figure 5E and F). The protein expressions of p53, p21 and p-p53 were increased after transfecting siCBX3-1 or pcNCOR2. These results were rescued by co-transfecting with siCBX3-1 and siNCOR2 in SKOV3 cells (Figure 5G).

CBX3 mediated glycolysis by blocking p53/p21 via inhibiting NCOR2

The results showed that CBX3 knockdown facilitated ATP production and hindered glucose uptake, lactate production and G6P level, these effects were abolished by NCOR2 knockdown and pifithrin-α (PFT-α; p53/p21 inhibitor) treatment (Figure 6A-D). Moreover, the up-regulation of NCOR2 also promoted ATP production and reduced glucose uptake, lactate production and G6P level, which were restored by inactivating the p53/p21 pathway (Figure 6A-D).

CBX3 regulated OC progression by blocking p53/p21 pathway via inhibiting NCOR2

The results showed that knockdown of CBX3 markedly inhibited cell proliferation and cell cycle, and promoted cell apoptosis, which were reversed by siNCOR2 and PFT-
α (Figure 7A-E). Furthermore, knockdown of NCOR2 and treatment with PFT-α significantly rescued effects on cell invasion and migration concomitant with the increased E-cadherin expression and decreased N-cadherin and Vimentin expression in CBX3-downregulated OC cells (Figure 7F-H). Similarly, PFT-α also alleviated the impacts of NCOR2 overexpression on OC cell proliferation, cycle, apoptosis, invasion and migration (Figure 7A-H).

Discussion

Ovarian cancer is considered as the most lethal gynecological malignancy owing to lack of early diagnosis and effective treatment [13]. Therefore, understand the molecular mechanism of ovarian cancer initiation and development is vital. Previous studies have reported that elevated expression of CBX3 was closely related to the progression of various cancers. CBX3 was reported to promote colorectal cancer proliferation via targeting p21 [3]. In gastric cancer, knocking down CBX3 resulted in inhibition of proliferation, invasion and cell cycle arresting at G1 phase [14]. Moreover, CBX3 was considered as a novel therapeutic strategy in pancreatic adenocarcinoma [4]. In this study, the result showed that CBX3 was upregulated in OC tissues and cell lines. Xu and colleagues demonstrated that CBX3 protein was forced expressed by detecting with immunohistochemistry in ovarian carcinoma tissues, which is consistent with these findings [7]. Importantly, we found that knockdown of CBX3 suppressed cell proliferation, invasion and migration, also led to cell cycle arrest at G1/S phase and promoted cell apoptosis. These findings suggest that CBX3 is involved in the
Altering glucose metabolism is considered as a hallmark of cancer. In cancer, aerobic glycolysis was activated thereby promoting tumor growth and metastasis [9]. A recent report suggested that enhancing the expression of MIEF2 promoted ovarian cancer cellular functions through activating aerobic glycolysis [15]. In gastric cancer, the activated aerobic glycolysis was inhibited by H19 knockdown, thus inhibiting the malignant progression of tumors [16]. Similarly, the present study revealed that glycolysis was activated in OC cells. CBX3 knockdown inhibited cancer cellular function by suppressing glycolysis.

Furthermore, the results suggested that the NCOR2 expression was increased by CBX3 knockdown and negatively related with the CBX3 expression, and the similar result was also found in lung adenocarcinoma [17]. NCOR2 is a nuclear corepressor and involved in the pathogenesis of cancer. Studies have shown that decreased NCOR2 facilitated tumor cell growth, invasion and migration in hepatocellular carcinoma [18]. In addition, the elevated expression of NCOR2 inhibited cell growth, chemical resistance and promotes cell apoptosis in head and neck squamous cell carcinoma [19]. Only one study suggested that NCOR2 was significantly associated with ovarian cancer with microarray analysis [20]. However, the role and function of NCOR2 in ovarian cancer are still vague. We found that NCOR2 was down-regulated in both ovarian cancer tissues and cell lines and CBX3 promoted tumorigenesis by inhibiting expression of NCOR2 in ovarian cancer. Moreover, NOCR2 has been identified as a vital factor in
the maintenance of metabolic signaling [21], suggesting that NCOR2 may be associated with glycolysis in cancer. In this study, the inhibited glycolysis by CBX3 knockdown was reversed by the inhibition of NCOR2.

P53, an important tumor suppressor gene, is involved in the regulation of a variety of cellular processes in cancer progression, including cell cycle, DNA repair, apoptosis and autophagy [22]. p21 is a downstream target of p53, which binds to the p21 promoter and activates its transcription [23]. In addition, p21 is a negative regulator of the cell cycle, and regulates various physiological activities, such as cell growth, differentiation, DNA repair, and senescence [24]. An increasing number of studies demonstrated that inhibition of the p53/p21 pathway promoted cell proliferation and tumorigenesis, and suppressed cell senescence in colorectal cancer [25, 26]. Besides, the p53/p21 signaling pathway was also involved in regulating the proliferation, migration and invasion of pancreatic ductal adenocarcinoma and endometrial cancer cells [27, 28]. In ovarian cancer, USP39 knockdown induced cell cycle arrest was also found to be associated with the p53/p21 signaling pathway [29]. In this study, the results revealed that CBX3 regulated ovarian cancer progression by inactivating p53/p21 pathway via inhibiting NCOR2. Emerging studies also demonstrated that p53 and p21 acts as a tumor suppressor by regulating the Warburg effect. The activated reprograming of glucose metabolism in a p53-dependent manner facilitated human colon adenocarcinoma cell growth [30]. Additionally, p21, targeted by miR-512-5p, mediates non-small cell lung cancer cells apoptosis and glycolysis [31]. Consistently, the inactivation of p53/p21
promoted the glycolysis inhibited by CBX3 knockdown and NCOR2 overexpression.

In the present study, we explored the role and molecular mechanism of CBX3 in OC, as well as its regulatory effect on glycolysis in OC. These findings provide a novel and potential biomarker for the treatment of ovarian cancer. Furthermore, blocking glycolysis may represent a new form for the treatment of OC, however, the specific mechanism of the glycolysis in OC is currently limited. The study found that CBX3 regulates glycolysis in ovarian cancer, but more in vivo and clinical research is required to further explore the specific mechanism underlying these effects.

Conclusions

CBX3 was highly expressed in OC tissues and cell lines and was negatively correlated with NCOR2. Furthermore, CBX3 promoted the viability, migration, and invasion of OC cells through activated p53/p21-mediated glycolysis via inhibiting NCOR2. Therefore, CBX3 may be a novel target for the prognosis and treatment of OC.

Declaration of Conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Data availability

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Authors’ Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by HC, CLH, DL, FW and CFH. The first draft of the manuscript was written by HC and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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A. Relative mRNA expression of CBX3

B. Relative mRNA expression of CBX3

C. Relative protein expression of CBX3

n=30

* p < 0.05

** p < 0.01

CBX3

GAPDH

SOE, E2, SKOV3, OVCAR3, A2780, HEY
Figure A: Cell invasion (fields)

Figure B: Cell migration (fields)

Figure C: Western blot analysis of E-cadherin, N-cadherin, and Vimentin with GAPDH as a control.