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Original Research Article

Effect of WWOX on epithelial-mesenchymal transition in ovarian cancer cells in vivo, and its association with Elf5/Snail-1 signal pathway

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Abstract

Purpose: *To investigate how WW domain-containing oxidoreductase (WWOX) gene affects epithelial-mesenchymal transition (EMT) of ovarian cancer (OC) cells, and the implication of Elf5/Snail-1 pathway in the process.*

Methods: *This study employed female BALB/c nude mice aged 6 weeks. The expression levels of E-cadherin, β-Catenin, N-cadherin, Vimentin, Snail1 and Elf5 were assayed using qRT-PCR. The protein levels of WW1, WW2, E-cad, β-Cat, N-cad, vimentin, Snail-1 and Elf5 were determined using western blotting (WB). Invasion changes in cells in each group were analyzed by Transwell invasion test while WWOX and Elf5 were evaluated by GST pull-down test; furthermore, WWOX and ELF5 were evaluated by co-immunoprecipitation (COIP).*

Results: *Relative to control group, tumor volume and weight of WWOX high-expression mice were significantly decreased, while those of WWOX low-expression group were increased ($p < 0.05$). The levels of E-cad, Elf5 and β-catenin increased, but those of N-cad, Snail-1 and vimentin decreased in WWOX high expression group. In the WWOX low-expression group, the levels of E-cad, Elf5 and β-cat were down-regulated, while those of N-cad, Snail-1 and vimentin were up-regulated ($p < 0.05$). The expression of E-cad, Elf5 and β-cat declined; on the other hand, those of N-Cad, Snail-1 and vimentin were elevated after mutation of WW1 domain in WWOX gene ($p < 0.05$).*

Conclusion: *Based on the in vivo results, the role of WWOX/Elf5/Snail1 signal route in the epithelial-mesenchymal transition of ovarian cancer, and its related mechanisms have been clarified. This provides a new target for gene therapy of ovarian cancer.*

Keywords: *WWOX gene, Elf5, Snail1, Ovarian cancer, BALB/c nude mice*

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INTRODUCTION

Ovarian cancer (OC) is among the three malignancies of female reproductive system. It is characterized by sudden onset and complex pathogenesis, which imply that it is difficult to make early diagnosis of OC. The mortality linked to OC ranks first among gynecological malignancies, thereby posing a great threat to women's health [1]. WW domain-containing

oxidoreductase (WWOX) gene is an oxidoreductase containing WW domain. The WW domain-containing oxidoreductase (WWOX) gene plays a wide and important regulatory role in steroid metabolism, growth metabolism and tumor progression [2].

It is known that Epithelial-mesenchymal transition (EMT) is a key step in the development and progression of OC. It refers to the process through which epithelial cells acquire some specific characteristics of mesenchymal cells under some special physiological or pathological conditions. In other words, it refers to the loss of epithelial cell surface specific markers, intercellular adhesion and polarity, remodeling of epithelial cell skeleton and the acquisition of mesenchymal-like phenotypic characteristics. These changes not only increase the invasiveness of tumor cells, but also contribute to the aggravation of tumor malignant degree [3,4]. In a previous study, it was confirmed that WWOX gene affected EMT ability of OC cells by regulating Elf5/Snail-1 pathway at clinical level [5], and *in vitro* cell level [6]. This study aimed to verify the above assertions *in vivo*, and also to investigate the mechanism of action involved.

EXPERIMENTAL

Materials

The Ovarian cancer cell line SKOV3 was supplied by Shanghai Cell Bank, Chinese Academy of Sciences. Over expression WWOX (OeWWOX) lentiviral vector, WWOX-siRNA lentiviral vector, Elf5-siRNA lentiviral vector, His-DsbA-WWOX; GST-Elf5, His-DsbA-WW1, GST-E3, pcDNA3.1-GFP-WWOX^{WW1Mutant}, pcDNA3.1-GFP-WWOX^{WW2Mutant}; HA-WWOX, HA-WW1, HA-WW2, Flag-Elf5, Flag-E1, Flag-E2, and Flag-E3 plasmid vectors were constructed and preserved by the Gynecological Oncology Laboratory of The Affiliated Hospital of Xuzhou Medical University. Roswell Park Memorial Institute-1640 (RPMI-1640) cell culture medium and fetal bovine serum (FBS) (GIBCO, USA; Lipofectamine 2000 was supplied by Invitrogen, USA.

Anti-Flag mouse monoclonal antibody, anti-HA rabbit monoclonal antibody, E-cadherin, N-cadherin, Vimentin, Snail1, Elf5, and GAPDH antibody were obtained from Proteintech. Matrigel matrix glue was purchased from Biosciences, U.S.A. Transwell chamber was obtained from Chemicon. TRIzol RNA extraction reagent was obtained from Life technologies, USA. Reverse transcription kit was obtained from Vazyme Biotech, Nanjing. Real-time fluorescence quantitative PCR kit was bought from CWBIO, Beijing. Cell culture dish (6-well plate) was purchased from Corning, U.S.A.

Cell culture

Human OC cell line SKOV3 was cultured in RPMI-1640 culture medium containing 1 % double antibody (penicillin + streptomycin) and 10% FBS in an incubator at 37 ℃ and 5 % CO_2. Different lentiviral plasmids constructed in our laboratory were transferred into SKOV3 cells for stable passage. This study was approved by the ethics committee of Xuzhou Municipal Hospital Affiliated to Xuzhou Medical University.

Animal grouping

The SKOV3 cells carrying lentiviral plasmid were inoculated into 6-week-old female BALB/c nude mice to establish subcutaneous transplantation tumor model of OC. The mice were divided into 4 groups, 5 in each group: A: control group; B: WWOX high expression group (oeWWOX group); C: WWOX low expression group (siWWOX group); D: WWOX high expression+Elf5-siRNA group (oeWWOX+siElf5 group). The nude mice were sacrificed after 5 weeks. Plasmid complementation Deoxyribonucleicacid 3.1 (pcDNA3.1)-GFP-WWOX^{WW1Mutant} and pcDNA3.1-GFP-WWOX^{WW2Mutant} were transfected into human OC SKOV3 cells (with empty plasmid and untransfected cells as control). The cells were divided into 4 groups: WW1 Mutant group, WW2 Mutant group, empty plasmid group, blank control group. Hemagglutinin-WWOX (HA-WWOX), HA-WW1, HA-WW2, Flag-Elf5, Flag-E1, Flag-E2 and Flag-E3 plasmid vectors constructed in the early stage were transfected into human OC SKOV3 cells, which were divided into groups according to the names of transfection plasmids.

Assessment of mRNA expression of target genes

Trizol was added to culture dish 1 milliliter (mL) every 10 cm^2, the total RNA of cells was extracted according to the instruction of the RNA extraction kit, and was inverted into cDNA by reverse transcription kit. Each group of cells was set up in three multiple wells. The resultant cDNA was regarded as template, and the primers synthesized in Table 1 and GAPDH were used as internal reference according to the method provided by the real-time fluorescence quantitative Polymerase Chain Reaction (PCR) kit instructions. The Polymerase Chain Reaction (PCR) conditions were as follows: 95 ℃ for 10

min, 95 ℃ for 15 s; 60 ℃ for 1 min, with 40 cycles in total. After three repeated independent experiments, the relative mRNA expression of each gene was expressed by $2^{-\Delta\Delta Ct}$ method, and then it was statistically analyzed (Table I).

Table 1: Primer sequences for real-time PCR

Gene name	Primer sequence
GAPDH-Foward(F)	CGCTGAGTACGTGGAGTC
GAPDH-Reverse(R)	GCTGATGATCTTGAGGTGTTGTC
WWOX-F	TCATTGTGGTCTCCTCAGAGTCCC
WWOX-R	AGCCAGCATCGCCCAATAGTC
Snail1-F	CTTCTCCTCTACTTCAGTCTCTTCC
Snail1-R	TGAGGTATTCCTTGTTGCAGTATTT
Elf5-F	CTTGTTCCCTATCTTCCCATT
Elf5-R	AAGCCTCCAAAGTTCTCATCT
E-cadherin-F	CTTCCATGACAGACCCCTTAA
E-cadherin-R	AGAACGCATTTGCCACTACAC
N-cadherin-F	TGTTGGGTGAAGGGGTGCTTG
N-cadherin-R	CACCAGGTTCATCTGTTGC
V-cadherin-F	AGGGGAGTCITCATCATCTGRRGC
V-cadherin-R	ACGGACACGGGACTCATCA

Western blotting

Changes in protein expression and the activities of target genes were assayed with Western blotting. Total protein was extracted from cells from different treatment groups, and the protein was quantified using BCA protein assay kit. Then, total protein from each group was blended and mixed with 5 × loading buffer, denatured in water bath, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, they were wet-transferred to PVDF membrane which was sealed for 2 h with 10% skimmed milk powder, and incubated with different target strips in corresponding primary antibodies at 4 ℃ overnight. Thereafter, the membrane was washed thrice with TBST solution (each wash for 5 min) followed by incubation with HRP-conjugated secondary antibody for 2 h at indoor temperature. Next, the membrane was washed thrice with TBST solution for 10 min and then developed with chemiluminescence reagent. The experimental strips were scanned and stored with Odyssey infrared fluorescence scanner.

Transwell invasion experiment

Matrigel was evenly spread in the upper chamber of the invasion chamber and kept overnight at room temperature. Cells in each group were transferred into serum-free culture medium 24 h before the experiment, suspended with fetal bovine serum-free culture medium, and were counted after dilution. Transwell chamber was put into 24-well plate, while 800 μL of RPMI-1640 culture medium containing 20 % FBS was put in the lower chamber. Then, 200 μL of cell suspension (SKOV3 density = 1×10^5 cells/well) was collected from the upper chamber and put into the incubator for 36 h. Using a cotton swab, cells on the inner surface of the chamber were wiped off and fixed for 30 min with 95 % methanol, stained for 20 min with crystal violet, and rinsed twice with PBS buffer. The cells that passed through the membrane were counted under a microscope after natural air drying. Each group of cells was set in triplicate wells, and five visual fields were randomly selected for counting.

Protein sedimentation test (GST knock-down)

A total of 20 μL of Glutathione-Sepharose 4B particles attached to with GST-Elf5 and GST proteins (containing about 1.5 μg of each target protein), and 1 μg His-DsbA-WWOX were added to 500 μL of 1 × binding buffer (PBS pH 7.3, containing 1 mmol/L DTT, 1 g/L NP-40 and protease inhibitor), and incubated at 4 ℃. His-DsbA protein was used as negative control. The sepharose particles were washed 5 times with binding buffer, each wash for 10 min. The supernatant was discarded and unbound protein was eluted. Altogether 1 × loading buffer was added to the eluted sepharose particles and was bathed for 5 min in boiling water during SDS-PAGE electrophoresis. Western blot analysis was performed with anti-His tag antibody. The remaining target areas were measured using the same method.

COIP

Hemagglutinin-WWOX (HA-WWOX), Flag-Elf5 and empty vector used for transfection were extracted with endotoxin-free plasmid extraction kit. The specific operation steps were in line with the kit manufacturer's instructions. Srempe Kld Ovarian 3 (SKOV3) cells were co-transfected with HA-WWOX and Flag-Elf5. After 24 - 36 h, the cells were lysed with NP-40 lysate, and the total lysate was harvested. A total of 30 μL was used as the WB control (lysate). Protein content was quantified according to the instructions on BCA protein quantification kit. Altogether, 1 mg cell total protein and 1 μg IP antibody (anti-HA, rabbit origin) was added and shaken for 3 h at 4 ℃. Then, 40 microliter (μL) proteinA/G-agarose was added and shaken at 4 ℃ overnight (over 8 h). followed by centrifugation at 3 000 rpm for 5 min at 4 ℃.

The supernatant was discarded, and the agarose precipitate was collected. Then, 1 mL cell lysate was used to wash the precipitate. It was then shaken for 10 min at 4 ℃, centrifuged 5 min at

3000 rpm at 4 ℃, and the sediment was repeatedly rinsed. The washed agarose precipitate was resuspended in 50 μL 1 × loading buffer, boiled for 5 min at 100 ℃, and centrifuged at 4 ℃ for 5 min at 3000 rpm. The supernatant was IP. Then, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the supernatant from antibody precipitation and on the reserved cell lysate at the same time. Then, the protein bands were transferred to nitrocellulose membrane, and WB was performed. The cell lysate and IP supernatant were incubated with HA-labelled antibody and FLAG-labelled antibody simultaneously. The remaining target areas were detected using the same method.

Statistical analysis

Data are presented as mean ± SD, and were subjected to statistical analysis with SPSS 16.0 software. Statistical chart was drawn with Graphpad Prism 6. Comparison between two groups was made with *t*-test, while comparison amongst three groups and multiple groups was made using ANOVA. Differences were assumed statistically significant at $p < 0.05$.

RESULTS

The BALB/c nude mouse OC subcutaneous transplantation tumor model was successfully established using OC SKOV3 cells carrying different gene vectors. The changes in volume and weight of tumor tissues in each group are shown in Table 2.

Table 2: Volume and weight changes in subcutaneous transplantation tumors in each group of mice

Group	Tumor volume (mm^3)	Tumor weight (g)
Control	489.3 ± 36.71	2.31 ± 0.26
oeWWOX	335.7 ± 21.52	1.49 ± 0.48
siWWOX	602.1 ± 19.37	3.07 ± 0.56
oeWWOX + siE1f5	501.3 ± 28.62	2.43 ± 0.31

Values are mean ± SD

Compared with the control group, the tumor volume and weight In the WWOX high expression group were markedly reduced ($p < 0.05$), while those in the WWOX low expression group were markedly increased markedly ($p < 0.05$). There were no marked changes in tumor volume and weight in WWOX high expression + Elf5-siRNA group.

Changes in expressions genes and transcription factors in tumor tissues are shown in Figure 1.

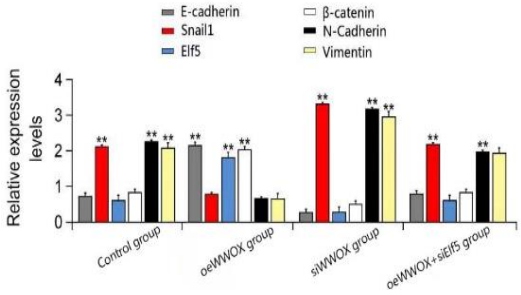

Figure 1: Changes in expression of related genes and transcription factors in tumor tissues of each group

Results from Real-time Quantitative Polymerase Chain Reaction (qRT-PCR) revealed increased expression levels of E-cad, Elf5 and β-cat increased, while those of N-cad, Snail-1 and vimentin decreased in the WWOX high expression group. In the WWOX low expression group, the levels of E-cad, Elf5 and β-cat were reduced, while those of N-cad, Snail-1 and vimentin were accentuated. However, levels of E-cad, Elf5, β-cat, N-Cad, Snail-1 and vimentin in the WWOX high-expression+Elf5-siRNA group were comparable with control values. After mutation of WW1 domain in WWOX gene, there were changes in expressions of related genes and transcription factors and cell invasion changed, as shown in Figure 2.

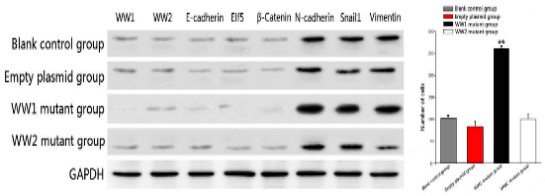

Figure 2: Expression levels of genes, transcription factors and cell invasion after mutation of WW1 domain in WWOX gene

The expression of E-cad, Elf5 and β-cat were reduced, and the expression of N-Cad, Snail-1 and vimentin were up-regulated after mutation of WW1 domain in WWOX gene. The invasion of cells was markedly enhanced. The results from GST knock-down test are shown in Figure 3.

GST-Elf5 purified protein or GST protein was mixed with purified His-DsbA-WWOX. Then GST knock-down test was conducted. His-DsbA was used as control. The results indicated that His-DsbA-WWOX bound GST-Elf5, but not GST. Moreover, GST-Elf5 did not bind His-DsbA, which indicated that WWOX was specifically bound to Elf5 *in vitro*. Similar results were obtained for WW1 and E3.

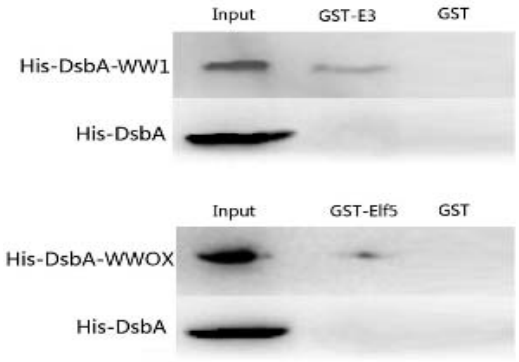

Figure 3: *In vitro* interactions of WOX and Elf5, and WW1 and E3. Results of GST known-down test showing specific binding interaction between of WWOX and Elf5, and WW1 and E3 *in vitro*

COIP results are shown in Figure 4. The results showed that Flag-Elf5 could be precipitated from the cell lysate co-transfected with HA-WWOX and Flag-Elf5 by anti-FLAG antibody. The IP lysate co-transfected with HA empty vector and pcdna3.1-Flag-Elf5 showed no signal with anti-FLAG antibody. E74-like factor 5 (Elf5) bound to WWOX, but not to HA tag, indicating that WWOX and Elf5 had specific interaction *in vivo*. Similar results were obtained for WW1 and E3.

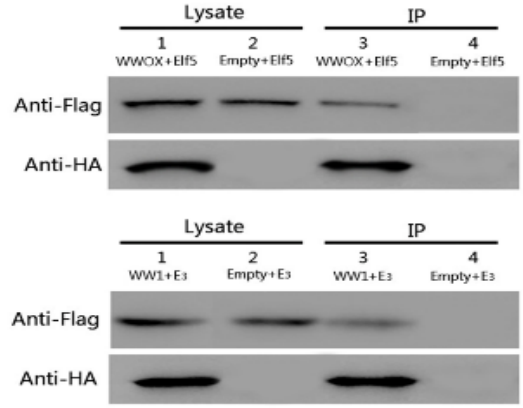

Figure 4: Interactions between WOX and Elf5, and between WW1 and E3 *in vivo.* Co-immunoprecipitation (COIP) test showed that WWOX and Elf5 interacted with each other; ditto for WW1 and E3, in SKOV3 cells. 1: Cell lysate of experimental group (co-transfected HA-WWOX and Flag-Elf5); 2: control group (co-transfected HA/pCDNA3.1-Flag-Elf5) cell lysate; 3: IP lysate of experimental group; 4: IP lysate of control group

DISCUSSION

Invasion, metastasis and postoperative recurrence of OC are the main causes of death in most OC patients. It is of great significance to study and clarify the specific mechanism involved in the invasion and metastasis of OC, in order to improve the clinical treatment effect and survival rate of OC patients [7]. There are two WW domains (WW1 and WW2) at the terminal amino acid position of WWOX gene. The WW1 domain combines with proline-rich domain, which is vital role in various cell signalling routes, thereby affecting their biological behaviour [8]. The WW domain-containing oxidoreductase (WWOX) is an important tumor suppressor gene which is relevant to the development of various cancers e.g., breast cancer, prostate cancer and lung cancer [9]. Current investigations have revealed WWOX gene is involved the pathogenesis of OC, and it has continued to receive a lot of attention, although its specific role in OC development and progression and its molecular biological mechanism are still not clear [10]. Epithelial-mesenchymal transition (EMT) plays an important role in OC. The main molecular characteristics of EMT are as follows: epithelial markers, such as E-cad and β-cat are down-regulated, while the expressions of interstitial phenotypic markers, such as vimentin, Fibronectin and N-cad are up-regulated. Down-regulation of E-cad expression (mainly regulated by Snail-1) is the most important symbol of change of in EMT [11]. It combines with the E-BOX promoter of E-cad, thereby inhibiting the transcription of E-cadherin, leading to EMT [12]. Snail-1 is a transcription factor containing basic helix-loop-helix motif in *Drosophila melanogaster*, mouse and humans, and belongs to the Snail family.So far, studies have demonstrated that Snail family members are involved in EMT [13,14]. Indeed, Snail, as a transcription factor that regulates EMT of tumor cells, is at the centere of the cascade reaction of signal pathway, and its expression is regulated by upstream transcription factor Elf5 [15].

The E74-like factor 5 (Elf5) is a member of E twenty-six (Ets) transcription factor family with epithelial specificity [16]. It plays a key role in the process of cell development, differentiation and apoptosis, and regulates cell proliferation and tumorigenesis. It is located in the short arm 13-15 region of human chromosome 11, which is prone to loss of heterozygosity in cancer. It contains two recognizable domains, E1 and E2, a motif that all ETS transcription factors contain, which can bind to TTCC core sequence, as well as an E3 domain which participates in protein-protein interaction. The E3 domain is rich in proline and it has protein binding function [17].

In previous studies, it was confirmed that WWOX gene affected EMT ability of OC cells by regulating Elf5/Snail1 pathway. In order to further confirm the above research results from *in vivo*

experiments, the lentiviral vectors of different expression types constructed in the early stage were transferred into OC SKOV3 cells, and a subcutaneous transplantation tumor model of OC with 6-week-old female BALB/c nude mice was established. The results showed that the tumor volume and weight of the WWOX high expression group were markedly reduced, while those of the WWOX low expression group increased. There was no marked change in tumor volume and weight in the WWOX high expression+Elf5-siRNA group. In addition, the expressions of E-cadherin, Elf5, and β-catenin increased, while those of N-Cad, Snail-1 and vimentin decreased in the WWOX high expression group.

The levels of E-cadherin, Elf5 and β-catenin in the WWOX low-expression group were decreased, while those of N-Cad, Snail-1 and vimentin increased. However, the levels of E-cad, Elf5, β-cat, N-Cad, Snail-1 and vimentin in the WWOX high- expression+Elf5-siRNA group were comparable with control values. The above *in vivo* experimental results further confirmed the conclusions in clinical and *in vitro* experiments.

To further explore the mechanism involved in the regulation of Elf5 by WWOX, mutant plasmids of WW1 and WW2 domains were constructed and transferred into SKOV3 cells. The expression of E-cad, Elf5 and β-cat were reduced, but those of N-Cad, Snail-1 and vimentin were accentuated after WWOX gene mutation. The invasion of cells was obviously enhanced, indicating that WW1 domain was the functional region where WWOX played a part. This was further confirmed using Glutathione-S-transferase (GST) knock-down and COIP experiments. Structure domain with double w1 (WW1) and E3 interacted with each other *in vivo* and *in vitro*.

CONCLUSION

The findings of this study show that WWOX gene affects the EMT potential of OC by regulating the Elf5/Snail-1 pathway. Furthermore, WWOX gene exerts its regulatory effect on EMT of OC cells through the combination of WW1 domain and E3 domain of Elf5. These findings provide a solid scientific basis for the development of a therapeutic strategy for the management of OC.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Chunhuan Song and Yuchen Yan conceived the study and drafted the manuscript. Yongli Liu and Lisha Fang were responsible for cell culture and experimental grouping. Qiang Li, Yang Xu and Hongchao Yan performed Transwell invasion experiment and protein sedimentation test. All authors read and approved the final manuscript.

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