Original Research Article

FOXQ1 promotes proliferation and metastasis of epithelial ovarian cancer via activation of SIRT1/NRF2 signaling pathway

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Abstract

Purpose: To investigate the role of FOXQ1 in the progression of epithelial ovarian cancer and the underlying mechanism.
Methods: Forkhead Box Q1 overexpression was evaluated by quantitative reverse-transcription (qRT-PCR) in clinical epithelial ovarian cancer samples and cell lines. Proliferation, migration, and invasion of cancer cells were determined using CCK8, wound healing and transwell assay.
Results: FOXQ1 depletion inhibited the proliferation, migration, and invasion of `epithelial ovarian cancer cells. Moreover, FOXQ1 overexpression increased the amount of cells in S phase of the cell cycle, and FOXQ1 knockdown arrested cells inG1 phase. Results from ChIP and luciferase reporter assays showed that FOXQ1 was able to bind SIRT1 promoters. In addition, it was involved in sustaining the stability of nuclear factor erythroid derived 2-like 2 (NRF2) by decreasing its acetylation (p < 0.01), which was mediated by SIRT1. The data also demonstrated that NRF2 promotes proliferation, migration, and invasion of cancer cells upon FOXQ1 overexpression.
Conclusion: Forkhead Box Q1 contributes to the progression of epithelial ovarian cancer partly via SIRT1/NRF2 signaling pathway, this highlighting a novel strategy for treating epithelial ovarian cancer.

Keywords: Forkhead Box Q1, Nuclear factor erythroid 2-related factor 2, Epithelial ovarian cancer, Cell proliferation, Metastasis

INTRODUCTION

Ovarian cancer is one of the most common gynecological cancers [1]. High-grade serous epithelial ovarian cancer is highly aggressive with a poor prognosis, often leading to rapid progression and high mortality[2]. Epithelial ovarian cancer is hereditable[3], indicating its occurrence may be due to mutations in specific genes . However, the relationship between the mutated gene and development of the cancer remains unclear. It was assumed that there is a key factor that contributes to the development of epithelial ovarian cancer upon individual gene mutation.

(Forkhead *FOX*box) genes, first discovered in 1989, are involved in embryogenesis, organism

development, cell differentiation, and metabolism[4].

Dysregulation and mutation of *FOX* genes have been associated with cancers via many signaling pathways [5]. The FOXQ1belongs to the FOX transcription factor family that functions to regulate epithelial differentiation and metastasis[6], and is generally upregulated in many cancers such as breast, pancreatic and colorectal[7]. The relationship between FOXQ1 and cancer progression has been studied extensively, especially with regard to the regulation of proliferation, invasion, and metastasis of cancer cells. A previous report showed that knockdown of FOXQ1 inhibited the cell cycle and decreased the invasion of an ovarian cancer cell line [8]. However, it is still not clear how FOXQ1 regulates epithelial ovarian cancer. Epithelial-to-mesenchymal transition is an important event in tumor progression and metastasis [9]. Previous findings have demonstrated that FOXQ1 regulates the progression of cancer through certain signaling pathways related to the epithelial-to-mesenchymal transition and microenvironment[10].

The SIRT1/NRF2 signaling pathway has been reported to be involved in regulating epithelial-to-mesenchymal transition process[11]. Previous studies showed that FOXQ1 upregulated SIRT1 (Sirtuin 1) in esophageal cancer cells[12], suggesting that FOXQ1 might be involved in progression of epithelial ovarian cancer by targeting the SIRT1/NRF2 signaling pathway. However, the relationship between the SIRT1/NRF2 signaling pathway and FOXQ1 has not been fully studied. Therefore, the effect of FOXQ1 on proliferation, migration, and invasion of epithelial cancer cells and the relationship between FOXQ1 and the SIRT1/NRF2 signaling pathway.

EXPERIMENTAL

Clinical specimen collection and cell lines

This study utilized 20 fallopian tube-derived epithelial cell samples collected from 4 healthy patients and 16 patients diagnosed with epithelial ovarian cancer Peking University People's Hospital, Beijing, China from June 19, 2016 to July 19, 2017. The study was approved by the ethics committee of Lanzhou University Second Hospital (no. LZU-F-02704) and all patients signed a consent form approved by the institutional ethics committee [13]. Research protocols involving human specimens was performed in accordance with the guidelines of

Declaration of Helsinki[14]. Following cell lines were used in this study: HOSE (healthy), OVCAR4, SKOV3, COV644 and TOV21G (cancerous, ovarian). OVCAR4 and SKOV3 were purchased from ATCC (Manassas, VA, USA) and cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA; catalog number: 11960044) supplemented with penicillin and streptomycin (1%) and FBS (10%).

The overexpression of FOXQ1 was performed on OVCAR4 cells by transfecting a pCMV6 vector encoded with *FOXQ1* using FuGENE HD (Roche, Basile, Switzerland). The transfected cells were cultured in DMEM supplemented with geneticin (ThermoFisher Scientific, Cheshire, UK) for 6 weeks. Knockdown of FOXQ1 was conducted on COV644 cells by siRNA transfection. The sequences of oligonucleotide used in this study were listed in the following table: The COV644 cells were transfected with siFOXQ1 using FuGENE HD (Roche, Basile, Switzerland).

Name	Sense:5'– 3'	Antisense:5'– 3'
siNC	UUCUCCGAAC GUGUCACGUT T-3'	ACGUGACACG UUCGGAGAATT
siFOXQ1-1	GCACGCAGCA AGCCAUAUATT	UAUAUGGCUUG CUGCGUGCTT
siFOXQ1-2	GCCAAGCAAU UUCUUUAAATT	UUUAAAGAAAU UGCUUGGCTT

Quantitative reverse-transcription-polymerase chain reaction (qRT-PCR)

Total RNA from OVCAR4 and COV644 cells was isolated using the Trizol reagent (Invitrogen Corp, Carlsbad, CA, USA), and then the RNA was reverse transcribed into cDNA using SuperScript reverse transcriptase (ThermoFisher Scientific, Cheshire, UK; catalog number: 18090010). To test the transfection efficiency of FOXQ1, qRT-PCR analysis was performed with the following primers: FOXQ1 forward, 5'-CGCGGACTTTGCACTTTGAA-3' and reverse: 5'-AGCTTTAAGGCACGTTTGATGGAG-3'. The transfection efficiency of FOXQ1 was about 70%.

Western blot assay

OVCAR4 and COV644 cell lysates were extracted at 0°C with a solution containing Tris-HCl (20 mM, pH = 7.5), Triton X-100 (1%) and PMSF (1%) upon overexpression and knockdown of FOXQ1. Total proteins were separated using10% SDS-PAGE and electro-transferred onto PVDF membranes, which were subsequently incubated with anti-FOXQ1 antibody (Abcam, Cambridge, UK; catalog

number: ab51340), anti-Nrf2 antibody (Abcam; catalog no.ab137550), or anti-EID1 antibody (Abcam; catalog no. ab167290) at 4 °C for 16 h. Membranes were then incubated with the anti-goat or anti-mouse secondary antibody at 37 °C for 2 h.

Immunocytochemical analysis

Immunocytochemistry analysis was used to evaluate cell proliferation and invasion. Subsequent to the overexpression or knockdown of FOXQ1 or NRF2 incubation, OVCAR4 and COV644 cells were seeded into 6-well plates, incubated with DAPI (Abcam, Cambridge, UK; catalog no. ab104139) for 30 min, after which the membranes were incubated with DAPI (Abcam, Cambridge, MA) (catalog number: ab104139) for 30 min.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed using ChIP Kit purchased from Upstate Biotechnology (Lake Placid, NY) according to the manufacturer's instructions. The cells were incubated with formaldehyde (1%) for 20 min, followed by 0.125 M glycine for 5 min. Deoxyribonucleic acid fragments (200- to 600-bp) were obtained by sonication and incubated with anti-FOXQ1 antibody (Abcam, Cambridge, UK; catalog no. ab51340) and anti-IgG antibody (NC) (Abcam; catalog no. ab127730) at 4°C overnight. The primers used for amplifying the putative FOXQ1 binding sites of the *SIRT1* promoter were as follows: forward, 5'-TCATAACGCTAGCGAAGCTGTCCG-3'; reverse, 5'-TCCAGTCATTAAACGGGCTAGCAAAC-3'.

Luciferase reporter assay

Interaction between FOXQ1 and SIRT1 was evaluated by the luciferase reporter assay. As previously described, the entire 3′-UTR of human *sirt1* was cloned into the pGL3-promoter vector. The *SIRT1* promoter sequences were mutated at the FOXQ1 binding sites using a QuikChange II Site-Directed Mutagenesis kit (ThermoFisher Scientific, Cheshire, UK; catalog number: A13282). OVCAR4 cells were seeded into 24-well plates and transfected with pGL3-promoter vector using FuGENE HD (Roche, Basile, Switzerland; catalog number: E2311).

Flow cytometry

The DNA content was measured using the Caliber cytometer (Becton Dickinson, San Joes, CA, USA) and cell cycle stage was analyzed with ModFit LT software. The DNA content of the G1 phase was reduced in quantity but the number of cells in this phase was increased. The initiation of DNA replication caused higher DNA content in the S phase. In the G2 phase, the completion of DNA replication led to a further increase in DNA content. OVCAR4 and COV644 cells were collected at 45h post-transfection. Pancreatic enzymes without EDTA were utilized for cell dissociation. The cells were resuspended in alcohol (70%, 4°C) and incubated with the PI/RNase staining buffer solution at 4°C overnight, after which the solution was replaced with PI/RNase staining buffer solution (500 µL).

Cell proliferation assay

OVCAR4 and COV644 cells were seeded into 6-well plates at densities of 2×10^6 cells/well and 1×10^6 cells/well, respectively. After 36 h, the cells were washed with PBS, incubated with DAPI for 30 min, and then observed under a microscope. Cell proliferation was evaluated using the CCK8 assay. Cells were seeded at a density of 1×10^5 cells/well, and incubated with CCK-8 solution (R&D Systems, Minneapolis, MN; catalog number: 1166) at 37 °C for 1.5 h.

Colony formation assay

OVCAR4 and COV644 cells were harvested and resuspended to a concentration of 1×10^6 cells/mL. 4.1 ml of pre-warmed of DMEM medium was mixed with 0.9 mL 4% agar, then the mixture was warmed in 56 °C water bath followed by natural cooling. The single-cell suspension was then diluted to 3×10^4 cells in 3mLof 10 % FBS DMEM and 0.36% agar to form the top layer. The cells were subsequently incubated at 37 °C for 3 weeks. Then the colonies were stained with 0.04 % crystal violet/2 % ethanol in PBS.

Migration and invasion assay

Migration of OVCAR4 and COV644 cells was evaluated using the wound healing assay. OVCAR4 and COV644 cells were seeded into 6-well plates at densities of 2×10^6 and 1×10^6 cells/well. The cells were scratched off at 80 % confluence and washed with PBS. IncuCyte system (Essen Bioscience, Ann Arbor, MI, USA) was used to assess the wound widths.

Invasion of OVCAR4 and COV644 cells was evaluated by the transwell assay. The cells were seeded into 6-well plates and incubated for 36 h. Then, the membranes were incubated with DAPI for 30 min and observed by microscopy.

Statistical analysis

All data were shown as mean ± standard deviation (SD) and analyzed by GraphPad 7.0 and SPSS 18.0. Student's t-test was used to compare two groups. One-way ANOVA and Dunnett test were used to compare multiple groups against control. $P < 0.05$ was considered statistically significant.

RESULTS

FOXQ1 was highly expressed in epithelial ovarian cancer tissues and cell lines

An increased level of mRNA was observed in 16 out of 20 subjects (Figure 1 A). Several epithelial ovarian cancer cell lines (OVCAR4, SKOV3, COV644, and TOV21G) were chosen to verify this observation. Compared with a healthy cell line, HOSE, the four cancer cell lines showed elevated expression of *FOXQ1*mRNA (Figure 1 B), with OVCAR4 and COV644 have the lowest and the highest expression, respectively. The OVCAR4 and COV644 cell lines were used as the cell models to study the effect of FOXQ1 on the development and progression of epithelial ovarian cancer in the following sections.
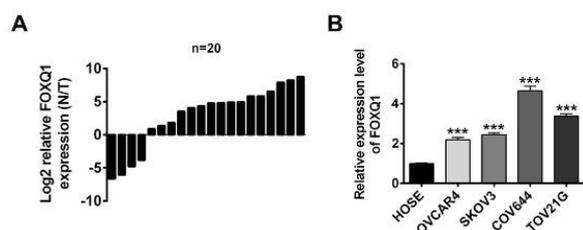

Figure 1: FOXQ1 is overexpressed in epithelial ovarian cancer cells and cell lines. (A) The level of FOXQ1 mRNA in clinical epithelial ovarian cancer samples. (B) Level of FOXQ1 mRNA in epithelial ovarian cancer cell lines; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, versus control

Forkhead Box Q1 enhances the proliferation of ovarian cancer cells FOXQ1 overexpression and knockdown promoted and inhibited cell proliferation, respectively

Transfection of OVCAR4 cells with pCMV6-FoxQ1 plasmid leads to FOXQ1 overexpression as demonstrated by increased FOXQ1 mRNA and protein levels (Figure 2 A and B). Transfection of COV644 cells with siFOXQ1 RNA resulted in FOXQ1 knockdown as indicated by decreased FOXQ1mRNA and protein expression levels. Results from the CCK8 assay showed that FOXQ1 overexpression promoted OVCAR4 proliferation, while FOXQ1 knockdown inhibited COV644 proliferation (Figure 2 C). Moreover,

colony formation assay confirmed that FOXQ1 overexpression increased the proliferation of OVCAR4 cells and COV644 cells, whereas FOXQ1 knockdown caused the opposite effect(Figure 2 D).Consistently, the cell cycle of OVCAR4 and COV644 was also influenced by the expression of FOXQ1 (Figure 2 E). Forkhead Box Q1 overexpression increased the proportion of OVCAR4 cells in the S phase, while FOXQ1 knockdown arrested COV644 cells in the G1 phase. Therefore, these results suggest that FOXQ1 overexpression might contribute to the progression of epithelial ovarian cancer.
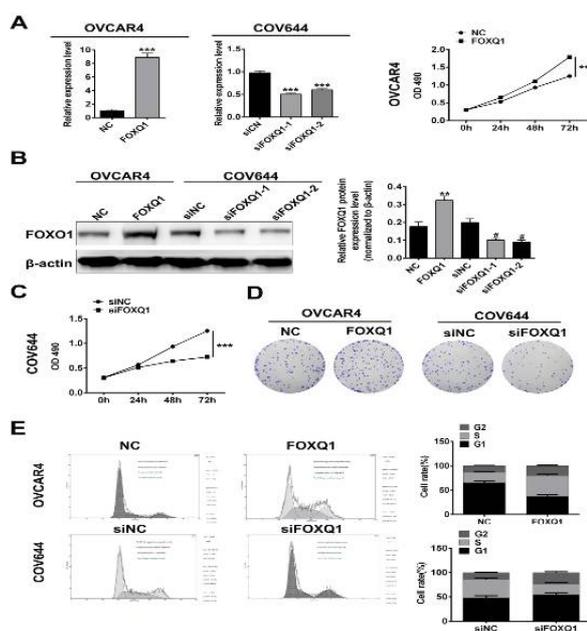
Figure 2: Effect of FOXQ1 on the proliferation of ovarian cancer cells. (A, B) The level of FOXQ1 mRNA (A) and protein (B) were evaluated by qRT-PCR and western blot, respectively.(C - E) The proliferation of ovarian cancer cells was evaluated by CCK8 assay (C), colony-formation assay (D), and flow cytometry (E); *$p<0.05$, **$p<0.01$, ***$p<0.001$, versus control

Forkhead Box Q1 promotes the migration and invasion of ovarian cancer cells

Wound healing and transwell assays were conducted to investigate the migration and invasion of OVCAR4 and COV644 cells. As shown in Figure 3 A, FOXQ1 overexpression markedly promoted the migration of OVCAR4 cells to the edge of the scratch. However, FOXQ1 knockdown in COV644 cells remarkably inhibited cell migration Consistent with this, FOXQ1 overexpression also promoted the invasion of OVCAR4 cells, while FOXQ1 knockdown blocked the invasion of COV644 cells (Figure 3 B). These results indicate that FOXQ1 might be closely associated with the occurrence and progression of epithelial ovarian cancer.
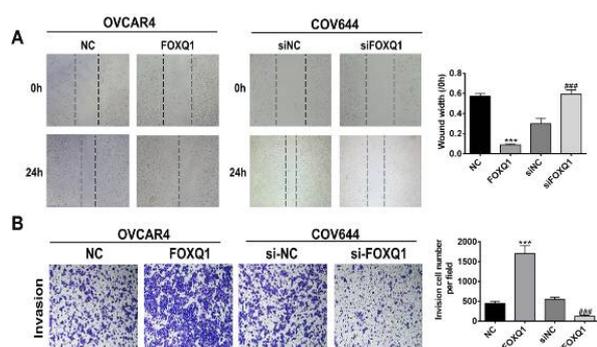
Figure 3: Effect of FOXQ1 on the migratory and invasive capacities of ovarian cancer cells. (A) Migration of OVCAR4 cells was induced by FOXQ1 overexpression (left). Migration of COV644 cells was inhibited by FOXQ1 knockdown (right). (B) Invasion of OVCAR4 cells was induced by FOXQ1 overexpression (left). Invasion of COV644 cells was inhibited by FOXQ1 knockdown (right). N = 5. NC, negative control

FOXQ1 activated SIRT1/NRF2 signaling pathway

To investigate the interaction between the SIRT1/NRF2 signaling pathway and FOXQ1, qRT-PCR and western blot analysis were performed to measure the level of mRNA and protein, respectively, of SIRT1 and NRF2. The results demonstrate that FOXQ1 overexpression in OVCAR4 cells increased *SIRT1* and *NRF2* mRNA levels (Figure 4 A), and FOXQ1 knockdown in COV644 cells decreased their expressions (Figure 4 B). Western blot analysis also confirmed the enhancement of Forkhead Box Q1 overexpression on SIRT1 and NRF2, as well as the inhibitory ability of FOXQ1 knockdown on SIRT1 and NRF2, in both OVCAR4 and COV644 cells (Figure 4 C).

Sirtuin 1 has been reported to decrease NRF2 acetylation to increase its stability. Therefore, FOXQ1 was studied to determine whether it affect *SIRT1* expression at the transcriptional level. The ability of FOXQ1 to interact with and activate the *SIRT1* promoter regions was confirmed with the luciferase reporter assay and ChIP-qPCR. As shown in Figure 4 D, FOXQ1 overexpression in OVCAR4 cells contributed to the enrichment of SIRT1. The *SIRT1* promoter was mutated to evaluate the interaction between FOXQ1 and the *SIRT1* promoter. The luciferase reporter assay demonstrated that FOXQ1 overexpression significantly decreased the activity of the wild-type *SIRT1* promoter in OVCAR4 cells ($p < 0.001$, Figure 4 D).However, this regulatory effect was suppressed when the predicted FOXQ1-binding site in the *SIRT1* promoter was mutated (Figure 4 D).

In addition, western blot analysis was performed to investigate whether FOXQ1 decreased NRF2 acetylation through activation of SIRT1 (Figure4 E). It was found that FOXQ1 overexpression in OVCAR4 cells decreased the level of acetylated lysine. However, FOXQ1 knockdown increased NRF2 acetylation. The result showed that FOXQ1 stabilized NRF2 by abrogating its acetylation. The level of NRF2 protein was evaluate and found to be decreased after 12 h in the normal OVCAR4 cells but 24 h in the FOXQ1-overexpressed OVCAR4 cells.
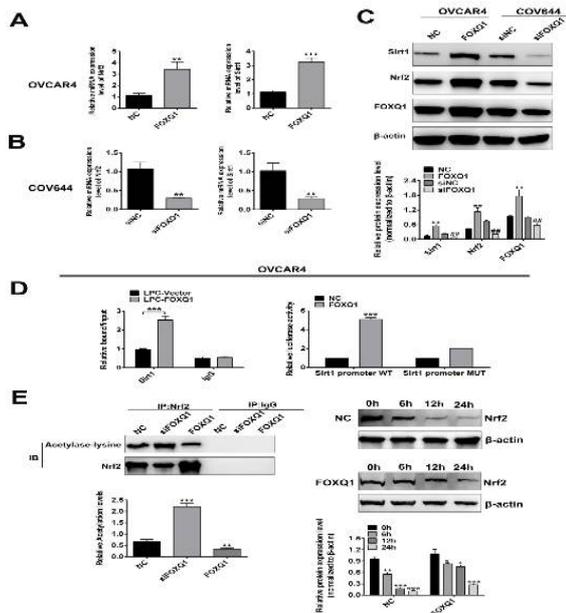

Figure 4: FOXQ1 activated the SIRT1/NRF2 signaling pathway. (A) The level of NRF2 (left) and SIRT1 (right) mRNA was increased by FOXQ1 overexpression. (B) The level of NRF2 and SIRT1 protein was increased by FOXQ1 overexpression. (C) The mRNA levels of *NRF2* (left) and *SIRT1* (right) were decreased by FOXQ1 knockdown. (D) The levels of NRF2 and SIRT1 proteins were decreased by FOXQ1 knockdown. (E) The interaction betweenFOXQ1 and *SIRT1* promoters was increased by FOXQ1 overexpression (left). Mutation of the *SIRT1* promoter decreased the interaction between FOXQ1 and the *SIRT1*promoter. (F) FOXQ1 overexpression influenced the acetylation levels and stability of NRF2; n = 5; *$p<0.05$, ** $p<0.01$, *** $p<0.001$, versus control

FOXQ1 promotes cell proliferation, migration, and invasion by activating the SIRT1/NRF2 signaling pathway

In order to demonstrate the involvement of FOXQ1 in promoting cell proliferation, migration and invasion by activating the SIRT1/NRF2 signaling pathway, we examined these biological processes in three different groups that were established, namely the control group, siFOXQ1 (FOXQ1 knockdown) group, and siFOXQ1+NRF2 (FOXQ1 knockdown with the

addition of NRF2 protein). As shown in Figure 5 A, FOXQ1 knockdown decreased NRF2 levels in COV644 cells. On the other hand, addition of NRF2 reversed this effect. In COV644 cells, FOXQ1 knockdown decreased cell number and proliferation, while the addition of NRF2 increased cell proliferation (Figure 5 B and C). FOXQ1 knockdown inhibited the migration and invasion of COV644 cells. On the other hand, the addition of NRF2 counteracted this effect (Figure 5 D and E). These results demonstrate that NRF2 is downstream of FOXQ1, which promotes cell proliferation, migration, and invasion by upregulating NRF2.
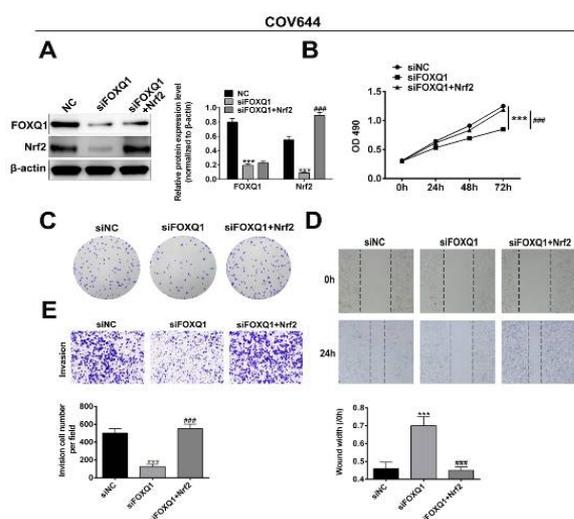
Figure 5: NRF2 promoted cell proliferation, migration and invasion in the absence of FOXQ1. (A) FOXQ1 knockdown decreased NRF2 protein levels, while co-incubation with NRF2 increased FOXQ1 protein levels after knockdown in COV644 cells. (B) NRF2 increased CCK8 activity in COV644 cells upon FOXQ1 knockdown. (C) NRF2 increased COV644 cell number in the absence of FOXQ1. (D) NRF2 promoted the migration of COV644 cells upon FOXQ1 knockdown. (E) NRF2 promoted the invasion of COV644 cells upon FOXQ1 knockdown; (n = 5). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, versus control

DISCUSSION

Data from this study reveal that FOXQ1 is overexpressed and closely related to epithelial ovarian cancer. The results show that regulation of FOXQ1 could influence proliferation, migration and invasion of epithelial ovarian cancer cells. Thus, this study proposed that FOXQ1 is a major factor involved in the pathological processes of epithelial ovarian cancer due to the overexpression of FOXQ1 at all stages and grades. Furthermore, FOXQ1 is a core factor in the network of related signaling pathways.

Among the cell lines used in this study, OVCAR4 and COV644 were derived from human ovarian epithelial serous carcinoma [15], SKOV3 originated from human serous papillary cystadenocarcinoma cell lines, which TOV21G was an ovarian clear cell adenocarcinoma cell line. The chosen cell lines exhibited different expression levels of FOXQ1, indicating that the expression of *FOXQ1*mRNA might be cell type-dependent with different factors regulating it. Considering that the variability of gene expression patterns of cell lines might be related to their physiological or pathological properties, OVCAR4 and COV644 were chosen as the cell models in this study to assess the effect of FOXQ1 expression on epithelial ovarian cancer.

Previous studies showed that the Wnt/β-catenin and TGF-β signaling pathways are involved in the function of FOXQ1 in cancers [6] and that FOXQ1 was a target of the Wnt/β-catenin signaling pathway[16]. A key factor of the Wnt/β-catenin signaling pathway, caudal-related homeodomain transcription 2, was decreased upon FOXQ1 upregulation. In addition, cancer cells incubated with TGF-β increased FOXQ1 mRNA level, suggesting that the TGF-β signaling pathway might target FOXQ1[17]. Nucleus accumbens-associated protein 1also transcriptionally regulated FOXQ1 expression [18]. Therefore, FOXQ1 might mediate crosstalk between the TGF-β and Wnt/β-catenin pathways as well as other important signaling cascades.

NRF2 is a transcription factor responsible for defense and oxidant signaling. For example, NRF2 protects healthy cells from carcinogens [19], but it can also promote cancer development by protecting cancer cells from chronic oxidative stress through anti-oxidative stress responses [20]. Oxidative stress often promotes the migratory and invasive capacities of tumor cells SIRT1/NRF2 signaling pathway has been reported to be crucial for regulating cellular responses upon oxidative stress [21]. In the present work, it was found that FOXQ1 transcriptionally regulated SIRT1 in epithelial ovarian cancer cells, leading to the upregulation of NRF2. Interestingly, NRF2 also promoted proliferation, migration, and invasion of epithelial ovarian cancer cells upon FOXQ1 knockdown. These results suggest that the effect of NRF2 on epithelial ovarian cancer cells was affected by FOXQ1. However, these findings do not exclude the possibility of any beneficial effects resulting from the upregulation of NRF2 by FOXQ1 since the role of FOXQ1 in the absence of NRF2 has not been studied. Nevertheless, FOXQ1 could be a strong regulator of NRF2, thereby participating in the epithelial-to-mesenchymal transition of

epithelial ovarian cancer via the SIRT1/NRF2 signaling pathway [22].

Kelch ECH associating protein 1(Keap1) and Antioxidant Response Elements (ARE) have also been reported to be involved in the anti-oxidative stress response of NRF2 along with SIRT1 [23,24]. Therefore, NRF2 could be the core factor in the anti-oxidative stress signaling network. It has been hypothesized that the FOXQ1/SIRT1/NRF2 signaling pathway is associated with gene mutations in cancer cells, and that the upstream signaling pathway of FOXQ1 is linked with the NRF2-related anti-stresses signaling network. Therefore, by applying nanotechnology that can transfer inhibitors of FOXQ1 to epithelial ovarian cancer, a new cancer therapy may be found. The application of nanotechnology have been shown to be successful in some cancers [25].

CONCLUSION

The findings of this investigation show that FOXQ1 is overexpressed in epithelial ovarian cancer, and promotes the proliferation, migration and invasion of ovarian cancer cells. Therefore, these results add new insight into the regulatory mechanisms of ovarian cancer. Thus FOXQ1 may serve as a potential therapeutic target in the treatment of ovarian cancer.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. YL and RH designed all the experiments and revised the manuscript. JL, WA and RC performed the experiments.

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