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Original Research Article

FOXN3 attenuates doxorubicin resistance of bladder urothelial carcinoma via SIRT6/PI3K/AKT/mTOR pathway

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Abstract

Purpose: *To investigate the effect of forkhead box N3 (FOXN3) protein on doxorubicin (DOX) resistance of urothelial carcinoma (BLCA).*
Methods: *Bioinformatics prediction and immunoblotting were used to evaluate FOXN3 expression in BLCA tissues and cells. The FOXN3 overexpression was achieved by cell transfection. The effects of FOXN3 on DOX resistance and cell apoptosis were determined by immunoblotting, DOX resistance assay, and flow cytometry, while immunoblotting was applied to evaluate SIRT6/PI3K/AKT/mTOR signaling activity. Finally, SIRT6 overexpression and exogenous addition of a PI3K/AKT activator were used to investigate the molecular mechanism by which FOXN3 regulates DOX resistance phenotype.*
Results: *The FOXN3 was downregulated in DOX-resistant BLCA tissues and cells while its overexpression attenuated doxorubicin resistance ($p < 0.01$). Furthermore, apoptotic cell ratio increased from 7.54 to 26.83 % in J82/DOX cells and from 6.31 to 17.89 % in T24/DOX cells ($p < 0.01$) after FOXN3 overexpression. Moreover, FOXN3 upregulation inhibited sirtuin 6 (SIRT6) expression and inactivated PI3K/AKT/mTOR signaling pathway. Both SIRT6 overexpression and PI3K/AKT activation abrogated the FOXN3-mediated inhibition of DOX resistance in BLCA cells.*
Conclusion: *The FOXN3 attenuates the DOX resistance of BLCA through SIRT6/PI3K/AKT/mTOR pathway, thus providing a promising therapeutic strategy for the management of BLCA.*

Keywords: *Forkhead Box N3, Doxorubicin resistance, Bladder urothelial carcinoma, Sirtuin 6*

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INTRODUCTION

Bladder cancer, particularly bladder urothelial carcinoma (BLCA), is the most frequently diagnosed urological malignancy in men [1]. Surgical resection followed by chemotherapy is the routine therapeutic strategy for BLCA. Although numerous advances in therapeutic strategies associated with chemo-resistance have been extensively reported recently, resistance to chemotherapeutic drugs invariably develops, and a subset of patients eventually undergo progression [2]. Therefore, developing a mechanism-based strategy to resolve drug resistance during BLCA treatment is crucial.

Doxorubicin (DOX) has a wide antitumor spectrum but is prone to drug resistance

compared with other anticancer agents [3]. Kwatra *et al* found that the efficacy of DOX was highly influenced by the activity of efflux pump P-glycoprotein (P-gp) [4]. However, identifying effective molecular targets for DOX resistance remains challenging. Forkhead box N3 (FOXN3) belongs to the forkhead box transcription factor family, which shares a homological domain in the DNA-binding structure, also called a winged helix [5]. Previous studies have reported that FOXN3 plays a vital role in organ development, and FOXN3 inactivation in mice leads to growth retardation and craniofacial defects. Moreover, FOXN3 cannot inhibit proliferation and protein synthesis [6]. At the molecular level, FOXN3 acts as a transcriptional factor regulating various downstream targets, including sirtuin 6 (SIRT6), AKT signaling, and Wnt/β-catenin signaling [7]. Thus, FOXN3 function in human carcinogenesis warrants further investigation. This study aimed to investigate the role of FOXN3 in regulating DOX resistance using BLCA cell lines and the underlying mechanisms.

METHODS

Bioinformatics analysis

UALCAN (http://ualcan.path.uab.edu) is a website that provides customizable functionalities based on the data from The Cancer Genome Atlas (TCGA; http://tcga-data.nci.nih.gov/tcga/). In this study, UALCAN was used to compare the FOXN3 expression levels between BLCA specimens and paracancerous normal tissues. In addition, GEPIA (http://gepia.cancer-pku.cn/index.html) and TIMER (https://cistrome.shinyapps.io/timer) were utilized to analyze FOXN3 expression levels in BLCA specimens and paracancerous normal tissues.

Cell culture and transfection

Human BLAC cell lines T24 and J82 were obtained from the Chinese Academy of Sciences (Shanghai, China). The T24 and J82 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Hyclone, Logan, UT) with 10 % fetal bovine serum (Gibco, MA, USA) under a 5 % CO_2 atmosphere at 37 °C. DOX-resistant BLCA cell lines (J82/DOX and T24/DOX) were established following a previously published study [8]. Briefly, J82 and T24 cells were exposed to increasing concentrations of DOX (Sigma-Aldrich, MO, USA), and then 0.5 mg/L of DOX was added to the culture medium to maintain the DOX resistance phenotype. Immunoglobulin-G (IGF-1) was obtained from Abcam (ab270062; Cambridge, UK). The overexpression vectors pcDNA3.1-FOXN3 (pcDNA-FOXN3) and pcDNA3.1-SIRT6 (pcDNA-SIRT6) and the vehicle control (pcDNA) were constructed by GenePharma (Shanghai, China). Transient transfection was performed using Lipofectamine™ 3000 (Thermo Fisher Scientific, USA) following the manufacturer's instructions.

CCK-8 assay

The Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) assay was used to assess DOX resistance. The BLCA cells were pre-exposed to various concentrations of DOX (0.01, 0.1, 1, and 10 mg/L) for 48 h in a 96-well plate. Next, the cells were incubated with 20 µL of CCK-8 solution at 37 °C for 2 h. After that, the content of the 96-well plate was mixed on an orbital shaker for 3 min for homogeneous distribution of color. Finally, the absorbance was read at 450 nm in a microplate reader (Tecan, Switzerland). The IC_{50} value was defined as the DOX concentration resulting in 50 % inhibition of cell viability based on the relative dose-response survival curve. Each assay was repeated at least three times.

Immunoblotting

The total protein of BLCA cells was isolated using a western blot-specific lysis buffer (Beyotime, Shanghai, China) containing protease and phosphatase inhibitor cocktails (Pierce, Rockford, USA). The protein samples were separated using 10 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and electro-transferred onto polyvinylidene fluoride membranes (PVDF; Millipore). The membranes were then blocked with 5 % bovine serum albumin and incubated with the indicated primary antibodies (Abcam, Cambridge, UK) overnight at 4 °C: FOXN3 (1:500; cat. no. ab129453), P-gp (1:500; cat. no. ab168337), cleaved-caspase 3 (1:500; cat. no. 2302), Bax (1:1,000; cat. no. 32503), Bcl-2, (1:2,000; cat. no. 182858), and GAPDH (1:3,000; cat. no. 37168). Next, the membranes were further incubated with a secondary antibody conjugated to horseradish peroxidase (Abcam, Cambridge, UK; 1:5,000, cat. no. 6721) at room temperature for 1 h. The protein signal was detected using enhanced chemiluminescence (Bio-Rad), and an Alphalmager 2000 Imaging System (Alpha Innotech, San Leandro, USA) was used to quantify the band density.

Colony formation assay

A total of 1,000 BLCA cells were inoculated in 6-well plates, and the cells were incubated at 37 °C for two weeks. The cells were stained with 0.4 %

dissolved crystal violet after fixation with 4 % paraformaldehyde for 1 h. The images were obtained using an optical camera, and the number of colonies was counted.

Apoptosis assay

The apoptotic ratio of BLCA cells was assessed using flow cytometry with an Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, UK; cat. no. 14085). After pcDNA transfection, BLCA cells were plated in six-well plates (2×10^6 cells per well) and cultured in a complete medium for 24 h. Next, the cells were harvested, rinsed with pre-cooled phosphate-buffered saline (PBS), centrifuged, and resuspended in 400 µL of working buffer to a final concentration of 6×10^5 cells/mL. Furthermore, samples were stained with propidium iodide (PI) and Annexin V-FITC solution at the same time for 25 min in the dark. Finally, apoptosis profiling was performed using a flow cytometer (BD Biosciences, San Jose, USA) and FACS software. The apoptotic ratio (%) was calculated using CellQuest software (BD Biosciences).

Statistical analysis

The data are presented as means ± standard deviation (SD). Each assay was repeated at least three times. Student's *t*-test or one-way analysis of variance (ANOVA) was utilized to compare results from different groups. $P < 0.05$ was considered statistically significant. All data analyses were performed using SPSS 22.0 software (SPSS Inc., Chicago, IL).

RESULTS

FOXN3 is downregulated in BLCA tissues and DOX-resistant BLCA cells

Using the UALCAN, GEPIA, and TIMER websites, FOXN3 expression was significantly downregulated in BLCA clinical tissues compared with that in cancer-adjacent normal tissues (Figure 1 A). To further elucidate the association between FOXN3 and DOX resistance phenotype, two DOX-resistant BLCA cell lines, J82/DOX and T24/DOX, were established via a long-term and stepwise exposure to DOX. Drug effect curves of DOX demonstrated that the IC_{50} values of J82/DOX and T24/DOX BLCA cells were significantly higher than those in J82 and T24 BLCA cells (Figure 1 B). P-glycoprotein (P-gp) is a resistance marker that plays an essential role in the efficiency of chemotherapeutical agents, particularly DOX. Therefore, P-gp expression in BLAC cells and DOX-resistant BLCA cells was next investigated via immunoblotting. As expected, P-gp protein expression in J82/DOX and T24/DOX cells was much higher than that in J82 and T24 cells, collectively indicating that the DOX resistance phenotype was successfully established (Figure 1 C). Finally, FOXN3 protein expression in normal urothelial cells (HCV-29), BLCA cells (J82 and T24), and DOX-resistant BLCA cells (J82/DOX and T24/DOX) was verified using immunoblotting. The FOXN3 expression in BLCA cells was significantly lower than that in normal urothelial cells, a finding that was consistent with the bioinformatics results (Figure 1 D). The FOXN3 expression in DOX-resistant cells was further decreased compared with that in BLCA cells. These data indicate that FOXN3 is downregulated in BLCA tissues and DOX-resistant BLCA cells.

FOXN3 overexpression attenuates DOX resistance

To confirm the essential role of FOXN3 in the DOX-resistant phenotype of BLCA cells, FOXN3 in DOX-resistant cell lines was overexpressed using Lipofectamine-mediated transfection. Immunoblotting showed that transfection with pcDNA-FOXN3 vectors effectively upregulated FOXN3 expression in both BLCA cell lines (Figure 2 A). Compared with the pcDNA control, the IC_{50} of DOX in DOX-resistant BLCA cells with upregulated FOXN3 expression was significantly decreased following transfection (Figure 2 B). Consistently, the protein expression of P-gp was also attenuated in DOX-resistant J82 and T24 cells with FOXN3 overexpression (Figure 2 C). Furthermore, the colony formation capacity of DOX-resistant BLCA cells with/without FOXN3 overexpression was assessed. As expected, FOXN3 overexpression diminished the colony formation capacity of J82/DOX and T24/DOX BLCA cells (Figure 2 D). These results indicate that FOXN3 overexpression attenuates DOX resistance in DOX-resistant BLCA J82 and T24 cells.

FOXN3 overexpression enhances apoptosis

Escape from apoptosis is a hallmark of the DOX-resistant phenotype. Therefore, the effect of FOXN3 overexpression on apoptosis was investigated using the flow cytometry assay. The FOXN3 upregulation significantly enhanced cell apoptosis in both DOX-resistant J82 and T24 cells, as evidenced by the increased number of Annexin V-FITC$^+$/PI$^+$ cells (Figure 3 A). Bax, Bcl-2, and cleaved-caspase 3 are classic apoptosis-associated biomarkers. FOXN3 overexpression increased pro-apoptosis protein (Bas and cleaved-caspase 3) expression (Figure 3 B) but

also attenuated anti-apoptosis protein (Bcl-2) expression in both cell lines (Figure 4). Thus, FOXN3 overexpression enhances apoptosis in DOX-resistant J82 and T24 cells.

FOXN3 overexpression inhibits the SIRT6/PI3K/AKT/mTOR pathway

Activation of the SIRT6/PI3K/AKT/mTOR pathway in BLCA J82/DOX and T24/DOX cells with/without FOXN3 overexpression was further evaluated using immunoblotting (Figure 5). FOXN3 upregulation decreased SIRT6 expression and the phosphorylation level of PI3K, AKT, and mTOR in both cell lines. This result implied that FOXN3 overexpression inhibits the SIRT6/PI3K/AKT/mTOR pathway in DOX-resistant BLCA cells.

FOXN3 inhibits DOX resistance through the SIRT6/PI3K/AKT/mTOR pathway

To investigate whether the SIRT6/PI3K/AKT/mTOR pathway was essential for the FOXN3-induced inhibition of DOX resistance, pcDNA-SIRT6 and IGF-1 (a PI3K/AKT activator) was used to upregulate SIRT6 and PI3K/AKT signaling in J82/DOX and T24/DOX cells. As anticipated, both SIRT6 overexpression and IGF-1 treatment increased the IC_{50} values of DOX and P-gp protein expression in DOX-resistant J82 and T24 cells transfected with pcDNA-FOXN3 (Figure 6). Moreover, SIRT6 overexpression and IGF-1 treatment also reversed the FOXN3-induced inhibition of colony formation capacity in DOX-resistant J82 and T24 cells, as evidenced by the increased colony number. More importantly, the apoptotic ratios of J82/DOX and T24/DOX cells transfected with pcDNA-FOXN3 were also decreased by SIRT6 overexpression and IGF-1 treatment (Figure 7). Collectively, these data indicated that FOXN3 inhibits DOX resistance in BLCA cells through the SIRT6/PI3K/AKT/mTOR pathway.

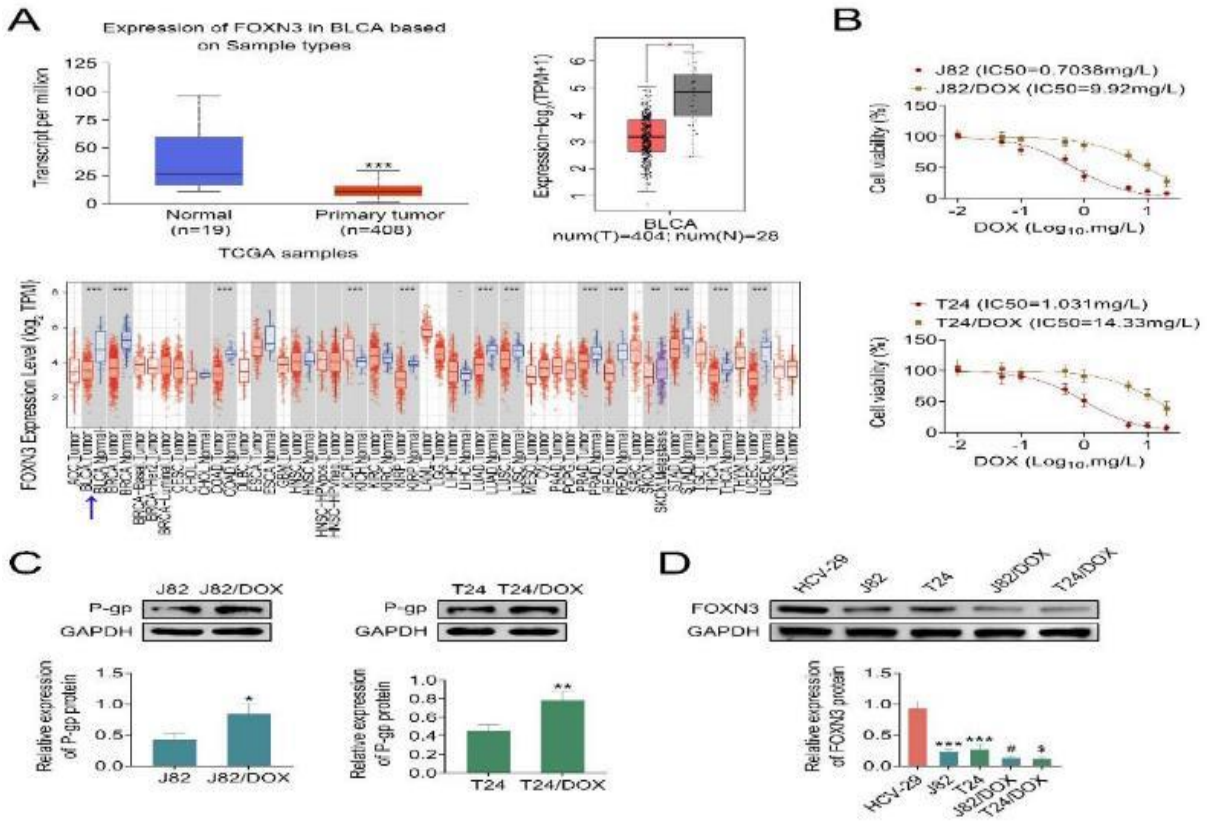

Figure 1: FOXN3 is downregulated in BLCA tissues and DOX-resistant BLCA cells. (A) Bioinformatic analysis of FOXN3 expression in BLCA and normal tissues using UALCAN, GEPIA, and TIMER websites. (B) Drug effect curves of DOX for BLCA cells (J82 and T24) and DOX-resistant BLCA cells (J82/DOX and T24/DOX). (C) Immunoblotting of P-gp protein expression in BLCA cells (J82 and T24) and DOX-resistant BLCA cells (J82/DOX and T24/DOX). *$P < 0.05$, **$p < 0.01$. (D) Immunoblotting of FOXN3 protein expression in normal urothelial cells (HCV-29), BLCA cells (J82 and T24), and DOX-resistant BLCA cells (J82/DOX and T24/DOX). ***$P < 0.001$ compared with the HCV-29 cells, $^{\#}p < 0.05$ compared with the J82 cells, $^{\$}p < 0.05$ compared with the T24 cells

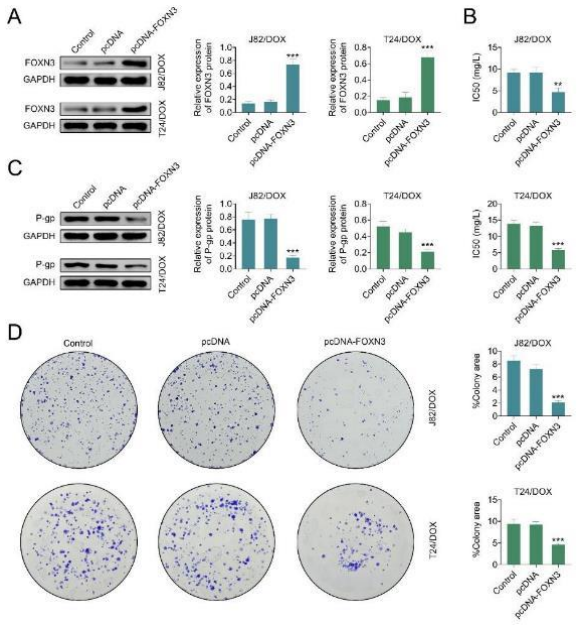

Figure 2: FOXN3 overexpression attenuates DOX resistance in BLCA J82/DOX and T24/DOX cells. (A) Immunoblotting of FOXN3 protein expressions in J82/DOX and T24/DOX cells with/without FOXN3 overexpression. (B) The CCK-8 assay showed the IC_{50} value of DOX in J82/DOX and T24/DOX cells with/without FOXN3 overexpression. (C) Immunoblotting of P-gp protein expression in J82/DOX and T24/DOX cells with/without FOXN3 overexpression. (D) Colony formation assay showed the proliferation capacity of J82/DOX and T24/DOX cells with/without FOXN3 overexpression. ***P* < 0.01 and ****p* < 0.001 compared with the control group

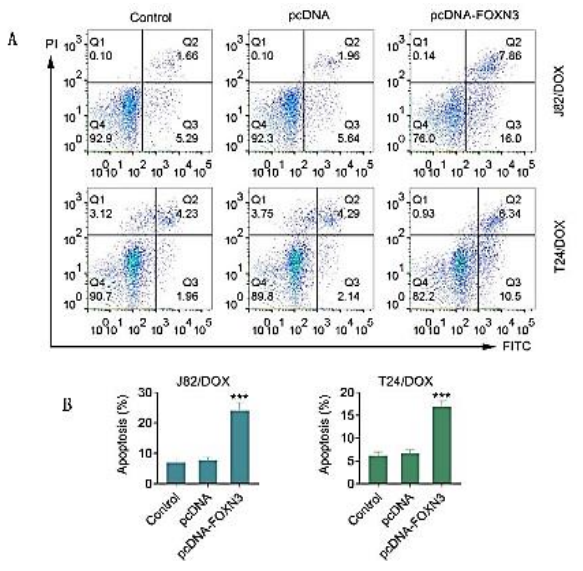

Figure 3: (A) FOXN3 overexpression enhances apoptosis in BLCA J82/DOX and T24/DOX cells. (B) Flow cytometry assay of the apoptotic ratio of J82/DOX and T24/DOX cells with/without FOXN3 overexpression. ***P* < 0.01 and ****p* < 0.001 compared with the control group

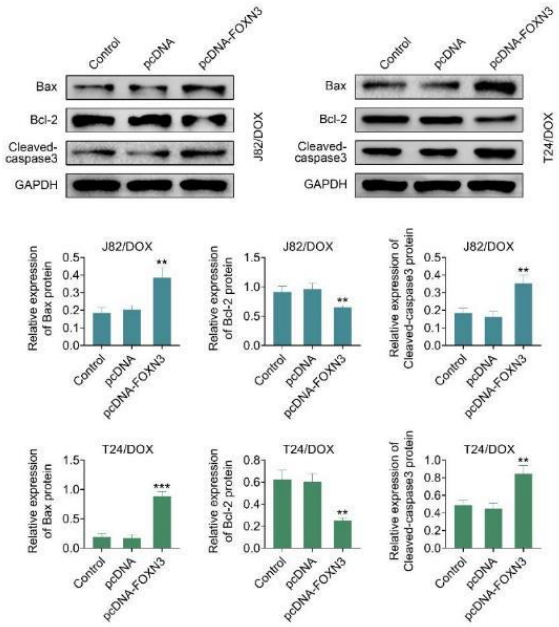

Figure 4: FOXN3 overexpression activates the apoptosis signaling pathway in BLCA J82/DOX and T24/DOX cells. Immunoblotting of apoptosis-specific protein (Bax, Bcl-2, and Cleaved-caspase 3) expression in J82/DOX and T24/DOX cells with/without FOXN3 overexpression. ***P* < 0.01 and ****p* < 0.001 compared with the control group

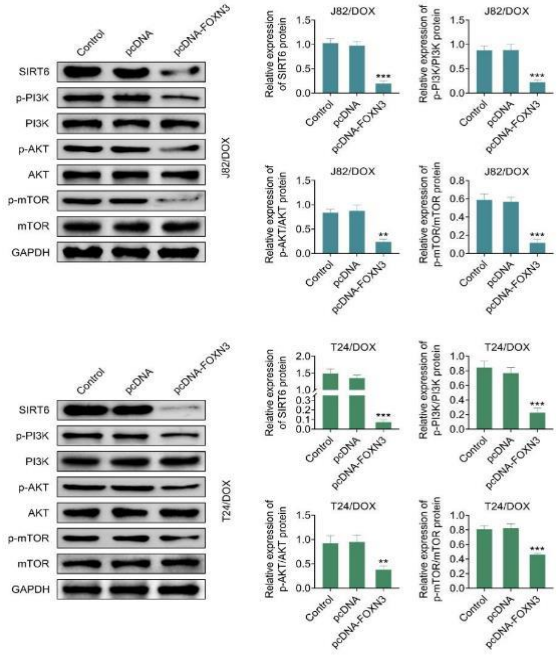

Figure 5: FOXN3 overexpression inhibits the SIRT6/PI3K/AKT/mTOR pathway in BLCA J82/DOX and T24/DOX cells. Immunoblot assay of the protein expression of SIRT6, p-PI3K, PI3K, p-AKT, AKT, p-mTOR, mTOR, and GAPDH in J82/DOX and T24/DOX cells with/without FOXN3 overexpression. ** *p* < 0.01 and *** *p* < 0.001 compared with the control group

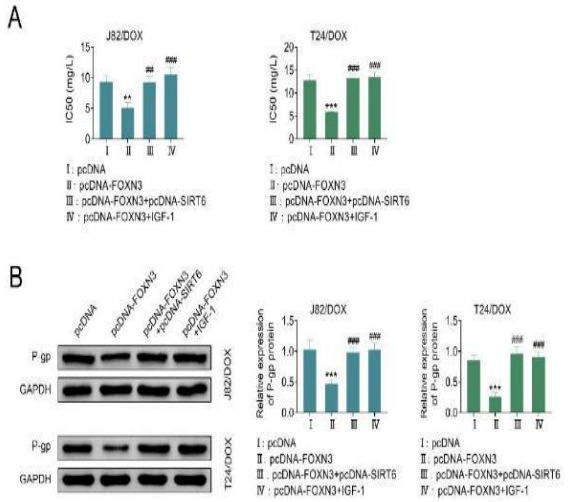

Figure 6: FOXN3 inhibits DOX resistance in J82/DOX and T24/DOX cells. (A) The IC_{50} values of DOX in J82/DOX and T24/DOX cells were detected using the CCK-8 assay. Ⅰ: cells transfected with pcDNA; Ⅱ: cells transfected with pcDNA-FOXN3 alone; Ⅲ: cells transfected with pcDNA-FOXN3 and pcDNA-SIRT6; Ⅳ: cells transfected with pcDNA-FOXN3 and also treated with IGF-1 (a PI3K/AKT activator). (B) P-gp protein expression in J82/DOX and T24/DOX cells was evaluated using immunoblotting. $^{**}P < 0.01$ and $^{***}p < 0.001$ compared with the pcDNA group. $^{\#\#}P < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the pcDNA-FOXN3 group

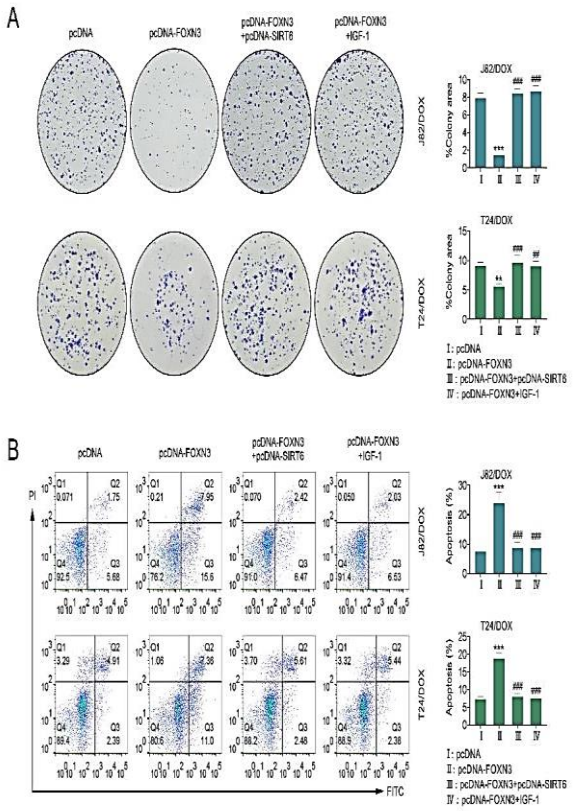

Figure 7: FOXN3 inhibits DOX resistance in J82/DOX and T24/DOX cells using the SIRT6/PI3K/AKT/mTOR pathway. (A) The proliferation capacity of J82/DOX and T24/DOX cells was detected using the colony formation assay. Ⅰ: cells transfected with pcDNA; Ⅱ: cells transfected with pcDNA-FOXN3 alone; Ⅲ: cells transfected with pcDNA-FOXN3 and pcDNA-SIRT6; Ⅳ: cells transfected with pcDNA-FOXN3 and also treated with IGF-1 (a PI3K/AKT activator). (B) The apoptotic ratio of J82/DOX and T24/DOX cells was calculated using flow cytometry. $^{**}P < 0.01$ and $^{***}p < 0.001$ compared with the pcDNA group. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the pcDNA-FOXN3 group

DISCUSSION

DOX is a common chemotherapeutic agent used for BLCA treatment [11]. However, chemo-resistance severely hinders its clinical application. In the present study, FOXN3 expression was decreased in BLCA tissues and cells and FOXN3 upregulation attenuated DOX resistance and promoted apoptosis in BLCA cells by inhibiting the SIRT6//PI3K/AKT/mTOR signal pathway. Thus, FOXN3 could be a promising target to resolve DOX resistance during BLCA treatment.

FOXN3 is both a transcriptional repressor and an activator [12]. It functions by interacting directly with a distinct transcriptional complex to regulate downstream gene expression. Herein, FOXN3 overexpression enhanced the vulnerability to DOX of BLCA cells by inhibiting SIRT6 expression and PI3K/AKT/mTOR signaling activation. The SIRT6 is a lifespan checkpoint controller responsible for cellular proliferation and survival [13]. Its aberrant expression leads to tumorigenesis. Wentao Xue *et al* reported that FOXN3 serves as a tumor suppressor in osteosarcoma via transcriptional inhibition of SIRT6 expression [14].

Wang *et al* [15] demonstrated that FOXN3 inhibited human glioma cell proliferation and invasion by modulating AKT activation. Thus, FOXN3 offers an ideal protein target for the development of broad-spectrum anticancer agents to facilitate the effectiveness of DOX treatment. Identifying the direct transcriptional complex interacting with FOXN3 in BLCA cells warrants further study.

Insensitivity to chemotherapeutic drugs and escape from apoptosis are predominant challenges in the clinical treatment of various cancers [16]. In the present study, FOXN3 overexpression significantly attenuated P-gp protein expression in both DOX-resistant J82 and T24 cells. Drug efflux is a crucial resistance mechanism that rapidly decreases intracellular

drug concentration. P-gp is a major efflux transporter that affects the pharmacokinetics of various chemotherapeutic agents [17]. Thus, FOXN3 is speculated to decrease the IC_{50} value of DOX by increasing the DOX concentration in the cytosol by suppressing drug efflux.

Previous studies have shown that the intrinsic mitochondrial pathway plays a critical role in the mechanism of cell apoptosis [18]. In agreement with this theory, FOXN3 overexpression significantly reduced Bcl-2 expression and upregulated Bax expression. Activation of intracellular caspase is a key step in the initiation of apoptosis. The FOXN3 overexpression also induced BLCA cell apoptosis by activating caspase 3, as evidenced by increasing cleaved-caspase expression. Therefore, FOXN3 functions as a reliable tumor suppressor by targeting both drug resistance and apoptosis signal pathways.

The present study has some limitations. First, how FOXN3 inhibits SIRT6 expression at the transcriptional level remains unclear. Second, no animal experiments were performed to verify the results *in vivo*. Future studies are warranted to elucidate the detailed molecular mechanisms of FOXN3 in chemo-resistance.

CONCLUSION

The FOXN3 attenuates DOX resistance and induces apoptosis of BLCA cells by activating SIRT6/PI3K/AKT/mTOR pathway, thereby diminishing DOX efflux and increasing drug concentration in the cytosol. These findings deepen the understanding of the pathological mechanism of BLCA as well as FOXN3's role as a novel protein target for the potential drug development for the treatment of BLCA.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest 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. All the authors contributed to the study's conception and design. Material preparation and experiments were performed by Yinan Han and Shengxing Wang. Data collection and analysis were performed by Rurui Xia, Jinhhuo Chen, and Bangfen Zhou. The first draft of the manuscript was written by Yinan Han, and all the authors commented on previous versions of the manuscript. All the authors read and approved the final manuscript.

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