FOXO3a-ROS Pathway is Involved in Androgen-Induced Proliferation of Prostate Cancer Cell

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Abstract

Background: Although FOXO3a can inhibit the cell proliferation of prostate cancer, its relationship with reactive oxygen species (ROS) in prostate cancer(PCa) has not been reported.

Methods: We analyzed the correlation between the expression of FOXO3a and the antioxidant enzyme catalase in prostate cancer through the UALCAN and GEPIA databases. We also constructed a PPI network of FOXO3a via the STRING database. The mRNA and protein expression of FOXO3a and catalase in LNCaP cells after DHT treatment were detected by qRT-PCR and western blot analysis. The effects of FOXO3a on catalase expression were tested by over-expression and siRNA interference respectively. At the same time, the catalase activity and ROS level in LNCaP cells after DHT treatment were detected. The changes of cell proliferation and ROS in LNCaP by antioxidant were also analyzed.

Results: We found that the catalase expression was down-regulated and has positively correlation with FOXO3a in PCa by public databases. The results of qRT-PCR and western blot showed that the mRNA and protein expression of FOXO3a and catalase were significantly reduced after DHT treatment in the LNCaP cells. Over-expression and knockdown of FOXO3a can also induce the change of catalase expression. DHT treatment can inhibit catalase activity and increase ROS level. We found that antioxidant treatment reduced DHT-induced proliferation and ROS production.

Conclusions: Our data show that the mechanisms by which DHT promotes PCa cell proliferation is that FOXO3a suppresses catalase expression and activates ROS signaling.

Background

Prostate cancer (PCa) has exceeded lung cancer and bronchus cancer as the first pathogenic cancer among men in America[1]. Similarly, its incidence has been steadily increasing in China[2]. Although prostate cancer can be effectively treated by radiotherapy, chemotherapy or androgen deprivation therapy, resistance to these therapies such as castration treatment can also develop tumor metastasis and recurrence, and lead to the progression of castration-resistant prostate cancer(CRPC)[3]. Therefore, understanding the molecular mechanisms leading to CRPC and identifying related molecular pathways are important in developing more effective treatments for CRPC.

Forkhead box O transcription factors (FOXO) regulate multiple cellular processes, including cell cycle arrest, cell death, DNA damage repair, stress resistance and metabolism[4]. Emerging evidence has revealed a negative correlation between FOXO expression and prostate cancer, indicating that it acts as an inhibitor of prostate cancer[5]. In PCa cells, numerous therapies can induce cell growth arrest by suppressing the activity of FOXO3a (FOXO3) transcription factors, which is one of the FOXO proteins[6–8]. Other studies have shown that oxidative stress is associated with the progression of prostate cancer from androgen-dependent to androgen-independent[9]. FOXO3a is an important molecule in oxidative stress of cellular response[10]. It can respond to oxidative stress which regulates cellular processes, such as cell growth and apoptosis. FOXO3a also regulates detoxification ability of cells to reactive oxygen

species (ROS) and enhances the ability to resist stress[11]. Therefore, the FOXO3a-ROS pathway may play an important role in the generation of oxidative stress and the progression of prostate cancer. In the current study, we have identified a novel mechanism through which reducing the expression of FOXO3a can activate the ROS signaling to promote cell proliferation. Our observations found that DHT treatment promoted cell proliferation via inhibiting the expression of FOXO3a and then we demonstrated that FOXO3a could inhibit the catalase activity, which in turn increased ROS levels of PCa cell.

Methods

UALCAN Database Analysis

The expression of FOXO3a and catalase in prostate cancer tissue were analyzed using UALCAN (http://ualcan.path.uab.edu/)[12]. Statistical significance of each comparison performed was provided in tabular form.

GEPIA Database Analysis

GEPIA (http://gepia.cancer-pku.cn/index.html)[13]was used to analyze the correlation between FOXO3a expression and catalase expression in PCa. GEPIA is an interactive web to analyze the RNA sequencing expression, including 9736 tumors and 8587 normal samples from TCGA and the GTEx projects. The correlation module generated the expression scatter plots between a pair of user-defined genes in PCa, together with the estimated statistical significance and the spearman's correlation. The x-axis represents the expression level of FOXO3a, and the y-axis represents the expression level of catalase.

Protein-protein interaction (PPI) network analysis

STRING (https://string-db.org/) was used to construct the PPI network. FOXO3a (protein name) and Homo sapiens (organism) were chosen.

Culture of prostate cancer cell

Human prostate cancer cell LNCaP was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured in the medium (Roswell Park Memorial Institute (RPMI)1640 medium, HyClone) supplemented with 10% heated-inactivated fetal bovine serum (FBS, HyClone), 100 U/ml penicillin and 100μg/ml streptomycin. The cell was grown at 37°C incubators in a humidified 5% CO_2 atmosphere.

In vitro cell proliferation assays

5×10^3 cells/well in 100μl RPMI1640 medium supplemented with 10% FBS were seeded into 96-well plates and incubated at 37°C with 5% CO_2 for 24h. Then cells were cultured with DHT (1, 10, 50, 100nm) (Sigma-Aldrich Chemicals) or DHT(10nM) and the ROS scavenger Tiron (1mM) (4,5-dihydroxy-1,3-benzenedisulfonic acid, disodium salt monohydrate; Sigma-Aldrich Chemicals) for another 48h. The cell proliferation was measured by adding 10μl CCK8 (DojinDo, Japan) into the wells and following 4h

incubation at 37°C. Then OD values well were measured by multifunctional chemiluminescence detector (Berto, Germany) at the absorbance of 450nm. Growth curves were made according to the OD value of each well.

Recombinant plasmid vector-mediated FOXO3a over-expression and siRNA transfection

Recombinant plasmid vector containing FOXO3a over-expressing fragment and control vectors containing corresponding empty fragment were purchased from GeneChem (Shanghai, China). The recombinant plasmid vector transfection was performed according to the protocol provided by the company. For silencing FOXO3a expression, cells were transiently transfected with 100pM of FOXO3a-specific siRNA and negative control siRNA(NC) which were designed and synthesized by GenePharma (Shanghai, China). Sequences of FOXO3a siRNAs were as follows: siRNA1 5'-GCUGUCUCCAUGGACAAUATT-3'; siRNA2 5'- GCACAGAGUUGGAUGAA GUTT-3'; negative control siRNA: sense 5'-UUCUCCGAACGUGUCACGUTT-3', antisense 5'ACGUGACACGUUCGGAGAATT-3'. Cells were using Lipofectamine 2000 transfection reagent (Invitrogen 11668-019) following the manufacturer's protocol.

In brief, after the cells (2×10^5 cells/well in 1ml RPMI1640 medium supplemented with 10% FBS) were seeded into 6-well plates and incubated at 37°C with 5% CO_2 for 24h, 2μg DNA of recombinant plasmid vector and 100pM of siRNA were added to each well and the cells were incubated at 37°C with 5% CO_2 for 6h. The medium was replaced with fresh RPMI1640 medium containing 10% FBS. After 24h of transfection, 10nM of DHT were added to each well and the cells were incubated at 37°C with 5% CO_2 for 48h. Quantitative real-time-PCR (qRT-PCR) was performed to assess the gene expression of FOXO3a and catalase.

RNA isolation and qRT-PCR

Total RNA of LNCaP cells were isolated by the TRIzol reagent (Takara Biotechnology Co., Dalian, China), according to the manufacturer's protocol. Total RNA was reversely transcribed into cDNA according to the PrimeScript RT reagent kit (Takara, Dalian, China) by C1000 thermal cycler (Bio-Rad Laboratories, Inc., USA). Following the instructions on the SYBR Premix Ex Taq kit (Takara, Dalian, China), the qRT-PCR was performed using the obtained cDNA in real-time quantitative instruments (CFX96, Bio-Rad Laboratories, Inc., USA). PCR conditions were as follows: 1 cycle of 95°C for 30 s, followed by 40 cycles of a two-step cycling program (95°C for 5 s; 60°C for 30 s). The mRNA expression was normalized to the expression of Actin mRNA and calculated using the $2^{-\Delta\Delta Ct}$ method. Specific primers for FOXO3a, catalase and Actin were: FOXO3a forward: CTACGAGTGGATGGTGCGTT and reverse: TCTTGCCAGTTCCCTCATTC; catalase forward: 5-AGATGCAGCACTGGAAGGA-3 and reverse: 5-CACGGGGCCCTACTGTAATA-3; Actin forward: GCACAGAGCCTCGCCTT and reverse: GTTGTCGACGACGAGCG, respectively.

Protein extraction and western blot analysis

The protein expression of FOXO3a and catalase were detected by western blot. The cell lysis buffer (Beyotime Biotechnology, China) were used to extract the total proteins of cells. The bicinchoninic acid

(BCA) kit (Beyotime Biotechnology, China) was used to quantify the concentration of extracted protein. 30μg protein of each cell sample were electrophoresed by 10% SDS-PAGE and then transferred to PVDF membranes (0.22μm, Millipore). After being blocked with blocking buffer (LI-COR, USA) (Do not add Tween to the blocking step), the PVDF membranes were incubated overnight at 4°C with the primary antibody (anti-FOXO3a antibody, Cell Signaling Technology; anti-catalase antibody, Santa Cruz Biotechnology). Then membranes were washed for three times by TBST (5min each), and incubated with the dye-labeled secondary antibody (IRDye800 or IRDye700, LI-COR, USA) at 37°C for 1h. Protein expression was visualized with Odyssey CLx (LI-COR, USA). β-actin (TA-09, ZSGB-BIO, Beijing, China) served as a loading control, and band intensity was quantified using the Image Studio Software.

Catalase activity detection

Catalase activity was detected using a catalase analysis kit (Beyotime Biotechnology, China) according to the manufacturer's instructions. The cell lysates were treated with excess hydrogen peroxide for an indicated time, and then the remaining hydrogen peroxide (not decomposed by catalase) was coupled with a substrate that on treatment with peroxidase produced N-4-antipyryl-3-chloro-5-sulfonate-p-benzoquinonemonoimine, which has an absorption maximum at 520nm and was quantified spectrophotometrically. Catalase activity was then calculated from the assay results.

ROS detection

5×10^3 cells in 100μl medium were seeded into 96-well plates per well and incubated at 37°C with 5% CO_2 for 24h. Then cells were cultured with DHT(10nM) or/and Tiron (1mM) for another 48h, washed two times with RPMI1640 medium, received DCFH-DA (Sigma-Aldrich Chemicals), and were incubated for 30min at 37°C incubators. Fluorescence intensity was measured at 485nm for excitation and 535nm for emission. ROS production was measured by the increased absorbance in 2h.

Statistical Analysis

Data analyses were performed using GraphPad Prism7.0. A two-tailed Student’s t-test was used to compare mean values. $P < 0.05$ was considered statistically significant.

Results

The mRNA expression levels of FOXO3a and catalase in prostate cancer

To determine the differences of FOXO3a and catalase expression in prostate cancer and normal tissues, the FOXO3a and catalase mRNA levels in prostate cancer and normal tissues were analyzed using the RNA-seq data of prostate cancer from TCGA by UALCAN database. This analysis showed that the catalase expression was significantly reduced in prostate cancer compared with normal tissues (Fig. 1B). Although the FOXO3a expression was lower in prostate cancer, there was no significant difference between prostate cancer and normal tissues (Fig. 1A).

The correlation between FOXO3a and catalase

To further explore the correlation between FOXO3a and catalase in PCa, we evaluated the correlation by the GEPIA database. As shown in Fig. 2A, the expression of FOXO3a was strongly positively correlated with catalase in PCa (p 0.001). To studying the function of FOXO3a, a PPI network was constructed using the STRING database. A total of 40 FOXO3a-interacting proteins were included in the PPI network complex by filtering, and the resulting PPI network contained 41 nodes (Fig. 2B). The edges represent the known and predicted PPIs. As shown in Fig. 2B, FOXO3a has an interaction with catalase (CAT).

DHT treatment promoted proliferation in LNCaP cells

To detect the effects of DHT on cell viability, LNCaP cells were treated with increasing concentrations of DHT for 48h, and cell viability was evaluated by the CCK8 assay. As shown in Fig. 3A, a significant increase in the proliferation effect was detected in a dose-dependent manner after treatment with 1nM, 10nM, 50nM, 100nM of DHT. The proliferative effect reached about 16%, 30%, 35%, and 41% after 48h exposure time.

DHT treatment downregulated the expression of FOXO3a and catalase in the LNCaP cells

Previous studies have demonstrated that FOXO3a is down-regulated in prostate cancer[6]. Indeed, the results of qRT-PCR and western blotting showed that FOXO3a mRNA (Fig. 3B) and protein (Fig. 3C and Fig. 3D) levels were significantly down-regulated after treatment with 10nM of DHT in the LNCaP cells. Furthermore, we found that catalase mRNA levels (Fig. 3B) and protein levels (Fig. 3C and Fig. 3D) were also significantly reduced in the LNCaP cells after DHT treatment.

The effect of over-expression of FOXO3a and knockdown of FOXO3a on the gene expression in LNCaP cells

To explore whether FOXO3a affected the expression of catalase in LNCaP cells, the recombinant plasmid vectors containing FOXO3a over-expressing fragment were transfected into the LNCaP cells. We found that the expression of FOXO3a was increased significantly (p 0.05) (Fig. 4A). Although DHT treatment induced a decrease in the mRNA levels of catalase in LNCaP cells, over-expression of FOXO3a can restore the expression of catalase (Fig. 4B).

To further investigate whether FOXO3a knockdown can suppress the expression of catalase, two FOXO3a siRNAs were transfected into LNCaP cells. We found that both of FOXO3a siRNAs reduced the elevated gene expression of FOXO3a by over-expression vector (Fig. 4C). In addition, FOXO3a siRNAs also decreased the elevated gene expression of catalase (Fig. 4D). These results indicate that FOXO3a plays a regulated role on the upstream of catalase.

DHT treatment suppressed the catalase activity in LNCaP cells

To study the effect of DHT on the catalase activity, we tested the catalase activity after DHT(1nM,10nM,100nM) treatment by 48h. Compared with the control group, the catalase activity of DHT group (10nM, 100nM) was significantly reduced (Fig. 5A). Then we investigated the involvement of ROS by adding DCFH-DA to detect ROS production. We found that the ROS of DHT group was increased by 80% compared with the control group (p 0.001) (Fig. 5B).

The effect of the ROS scavenger Tiron on the cell proliferation in LNCaP cells

DHT-induced proliferation involved the production of ROS in LNCaP cells. Therefore, we studied the involvement of ROS in DHT-induced by adding the ROS scavenger Tiron (1mM) to the cell cultures. We found that Tiron treatment reduced DHT-induced proliferation (Fig. 6A) and ROS production (p 0.05) (Fig. 6B). This data further proves that DHT promotes cell proliferation by reducing the expression of catalase and then increasing ROS. At the same time, the level of ROS in the Tiron treatment group also decreased, indicating that Tiron eliminates intracellular ROS, thereby reducing the proliferation effect of DHT.

Discussion

FOXO3a is a member of the FOXO subfamily of forkhead transcription factors.It can induce most of the cellular processes,such as cell apoptosis, cell proliferation, cell cycle progression, DNA damage, and tumorigenesis. The post-transcriptional inhibition of microRNAs (miRNAs), PTMs and protein-protein interaction can regulate the function of FOXO3a [4]. In this study, we have identified a novel mechanism through which decreased the expression of FOXO3a promoted cell proliferation by activated ROS signaling. Our observations found that DHT treatment promoted cell proliferation via inhibiting the expression of FOXO3a and then we demonstrated that FOXO3a could inhibit the catalase activity, which in turn, increased ROS levels of PCa cell.

Several studies have already reported that FOXO3a was decreased in human prostate cancer and and they found FOXO3a could promote prostate cancer progression[14, 15]. For example, decreased FOXO3a expression and reduced p27/Kip1 promoter transactivation appeared in prostate cancer progression from androgen dependence to androgen independence [16]. Consistent with these published data, we also demonstrated that DHT treatment promoted the proliferation of LNCaP cells in a dose-dependent manner, and down-regulated the expression of FOXO3a. The expression of FOXO3a was also decreased when LNCaP cells from androgen dependence to androgen independence (the data was not shown). These results indicated that FOXO3a played a major role in the PCa cells. It also showed that catalase expression was reduced in PCa by UALCAN database. Through the GEPIA database, it can be found that

the expression of FOXO3a was positively correlated with the expression of catalase. The PPI net network constructed using the STRING database also found that FOXO3a can interact with catalase. Indeed, experimental evidence showed that FOXO3a regulated the expression of catalase had been mainly observed in rat cells. In rats, FOXO3a can bind to the ATAAATA sequence in catalase promoter and then positively regulated catalase expression[4, 17, 18]. Consequently, silencing FOXO3a induced a reduction in the catalase mRNA and protein in isolated rat cardiomyocytes[17, 18]. In addition, our data showed that the mRNA and protein levels of FOXO3a and catalase were significantly decreased in the LNCaP cells after DHT treatment. Recombinant plasmid vector and siRNA transfection identified that FOXO3a played a regulatory role on the upstream of catalase.

The expression of catalase is altered significantly in human tumors. It was coded by *CAT* gene, and is a key enzyme in the metabolism of H_2O_2 and ROS [19]. As compared to adjacent normal tissues of the sample, some authors found that catalase expression was increased [19, 20], whereas other researchers showed the opposite result[19, 21]. These suggested that cancer cells are sensitive to oxidative stress. We investigated the catalase activity after DHT treatment in PCa cell. Compared with the control group, the catalase activity of DHT group was significantly reduced. Furthermore, we found that the ROS level of DHT group was increased compared with control group. Increased ROS production has been demonstrated in various cancers and shown to have several roles, for example, they can activate pro-tumor signals, and then enhance the survival and proliferation ability of cell [22, 23]. Our observations indicated that the increased level of ROS by DHT treatment induced the proliferation of PCa cells. Some researchers also reported that ROS can promote anti-tumor signals and initiate oxidative stress-induced cancer cells death[22–24]. Therefore, it is critical for cancer cells to maintain an optimal ROS level, which identifies ROS manipulation as a potential target for cancer therapies.

Antioxidants have a certain effect in suppressing the activities of ROS[25]. Catalase is one of the endogenous antioxidant system[26]. It must add exogenous antioxidants to reduce the excessive production of ROS when the endogenous antioxidant system is un-active. Tiron is one of the ROS scavenger, and it can inhibit the release of cytochrome c, caspase-3 activity, and then increase the cell survival rate from suppressing cell apoptosis[27]. In our experiments, Tiron reduced the DHT-induced proliferation and ROS production. All these data suggested that DHT promoted cell proliferation by reducing the expression of catalase and then increasing ROS. Tiron can reduce the intracellular ROS and then inhibit the proliferation of PCa. It was further demonstrated that antioxidant played an important role in cancer chemoprevention by suppressing oxidative stress-induced cell survival.

Conclusions

Above all, results shown here revealed the mechanisms by which DHT promoted PCa cell proliferation was that FOXO3a suppressed catalase expression and activated ROS signaling.

Abbreviations

PCa
Prostate cancer; ROS:Reactive oxygen species; FOXO:Forkhead box O transcription factors; GEPIA:Gene expression profiling interactive analysis; TCGA:The Cancer Genome Atlas; PPI:protein-protein interaction; DHT:Dihydrotestosterone; DCFH-DA:Dichloro-dihydro-fluorescein diacetate; CRPC:Castration-resistant prostate cancer; qRT-PCR:Quantitative real-time-PCR; PTMs:Post-translational modifications.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

All authors have read and approved the final manuscript. HM and WZ conceived the research. TY, LJ, SF, and ZJ performed the experiments. LL and LS analyzed the data.TY wrote the original draft of the manuscript. HM reviewed and edited the manuscript. WZ supervised the study.

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Availability of data and materials

All data analysed during this study are included in this published article. Generated data from each experimentalrepeat are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

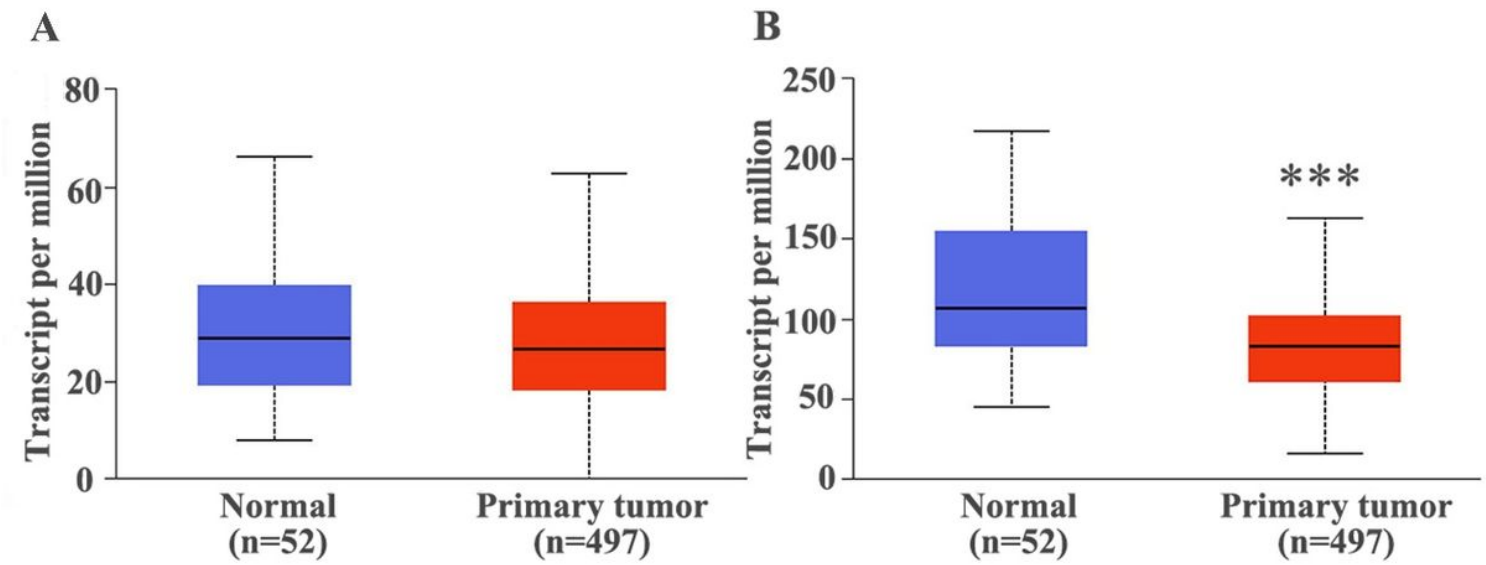

Figure 1

The mRNA expression levels of FOXO3a (A) and catalase (B) in prostate cancer. (*** p□0.001)

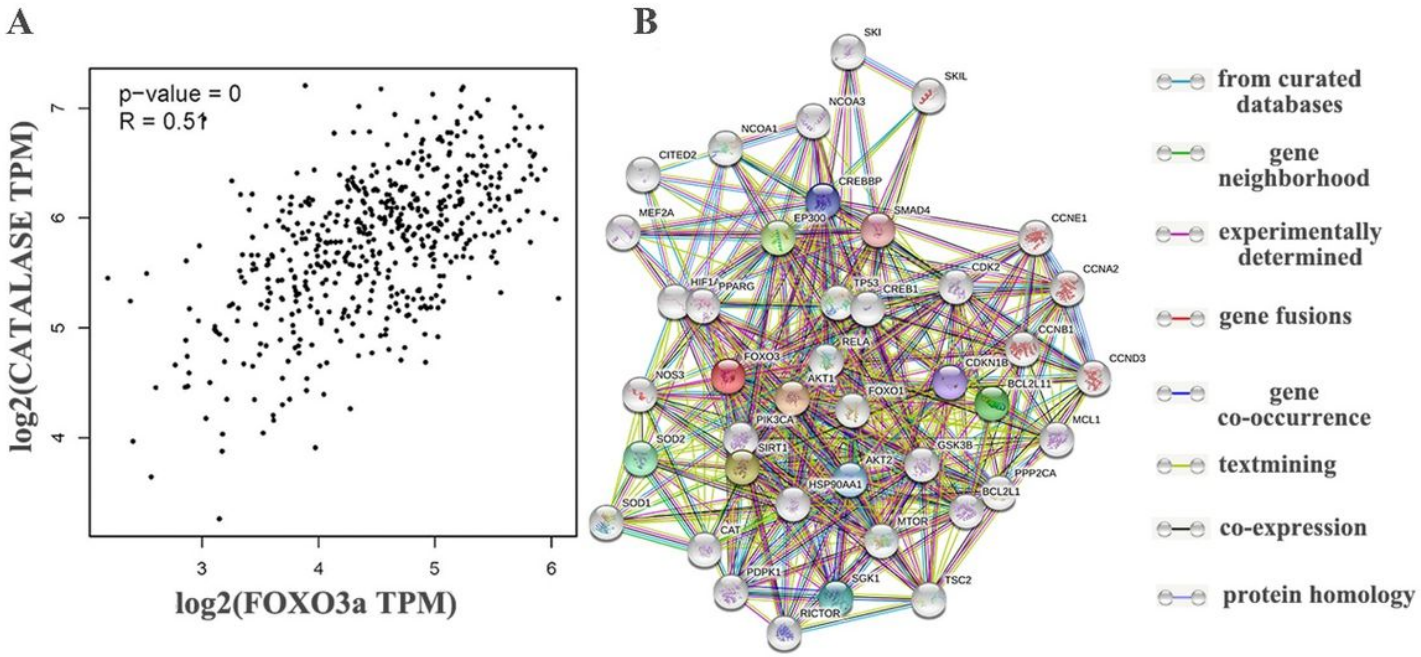

Figure 2

The correlation between FOXO3a and catalase at the gene and protein levels. (A)Scatterplots of correlations between FOXO3a expression and catalase in PCa. (B) The PPI network, which contained 41 nodes, was constructed using the STRING database.

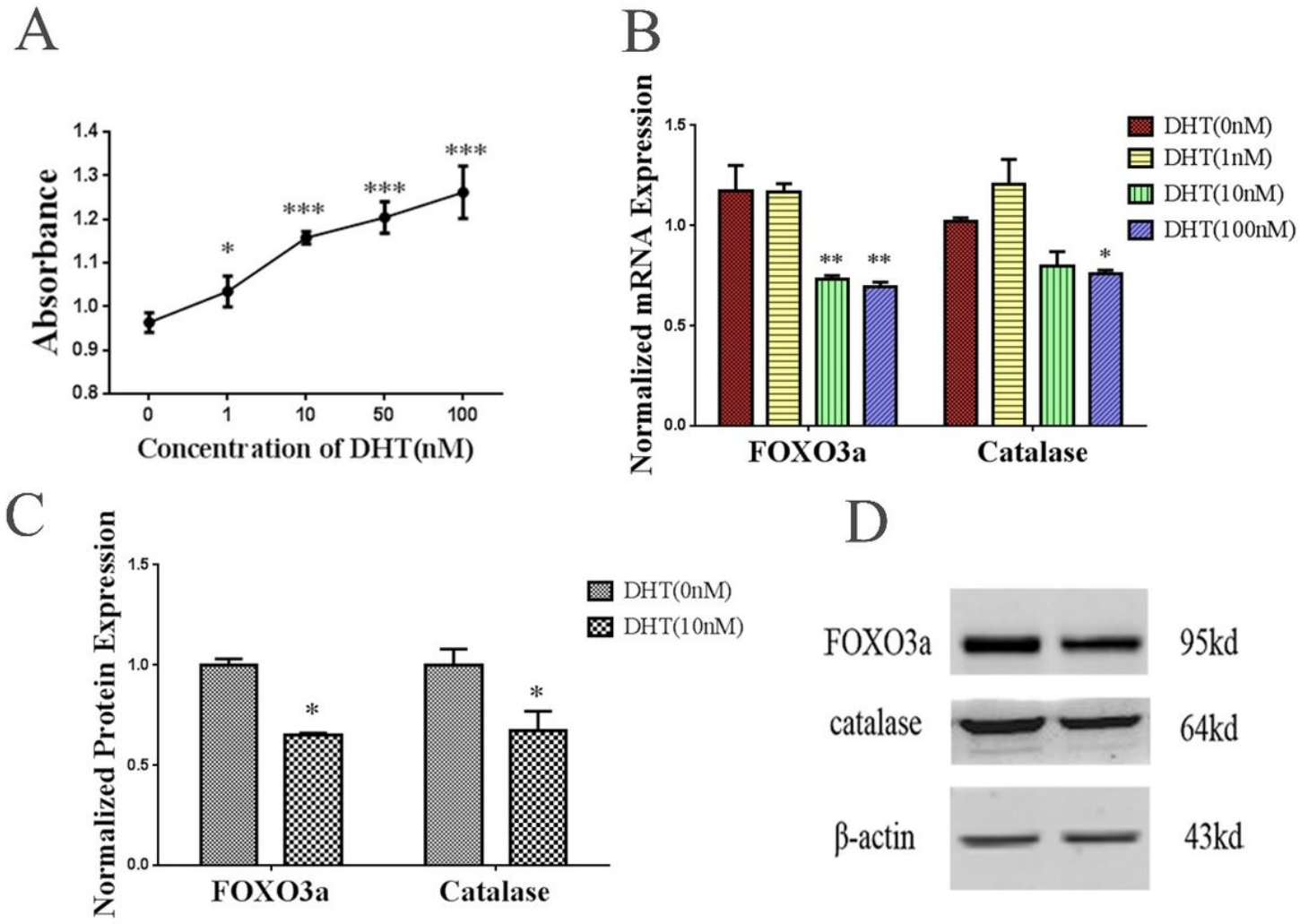

Figure 3

The effect of DHT in the LNCaP cells. (A) DHT treatment promoted proliferation in LNCaP cells. (B) DHT treatment down-regulated the mRNA expression of FOXO3a and catalase in the LNCaP cells. (C) and (D) DHT treatment downregulated the protein expression of FOXO3a and catalase in the LNCaP cells. (* p 0.05, ** p0.01, *** p0.001)

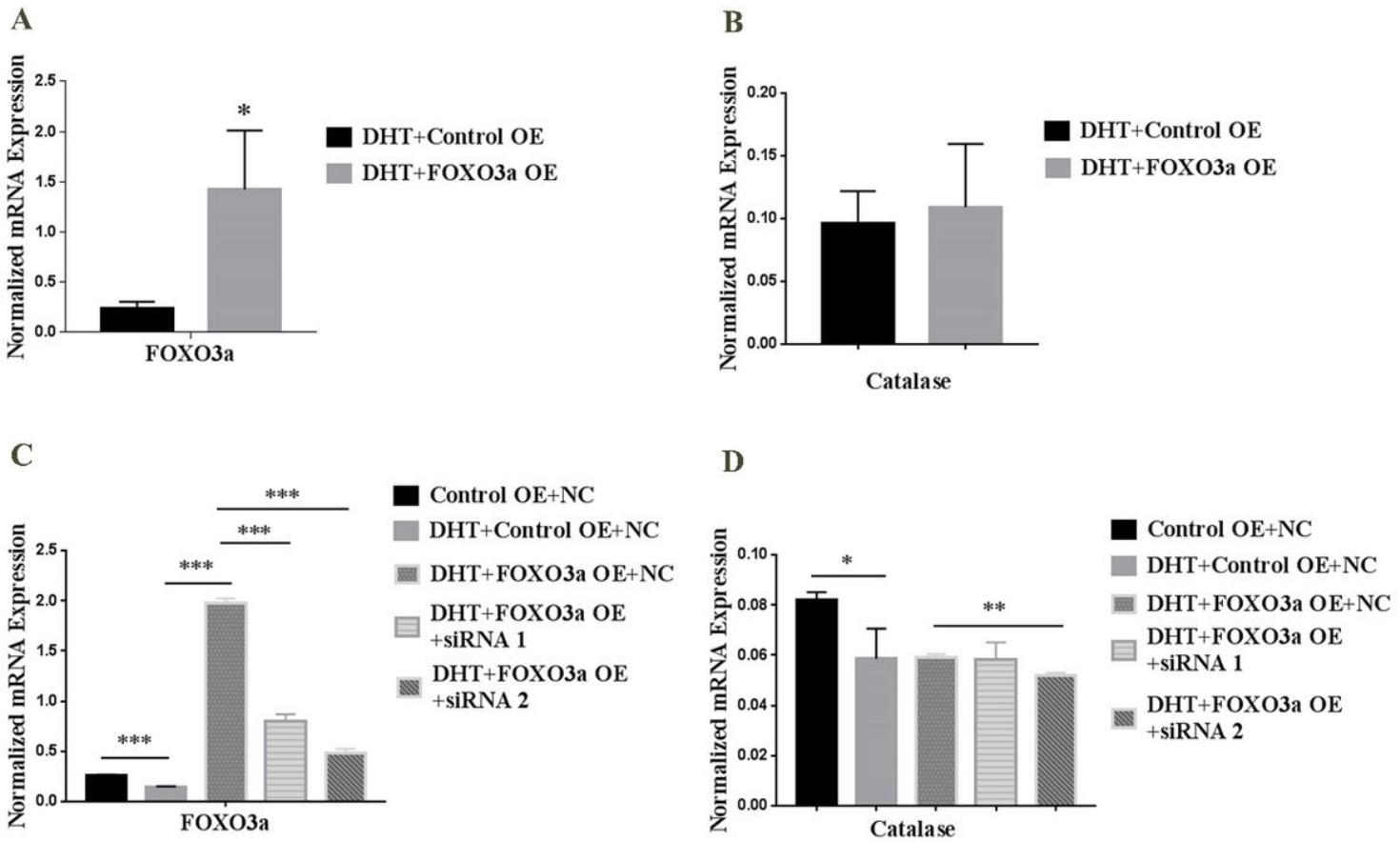

Figure 4

The effect of over-expression of FOXO3a and knockdown of FOXO3a on the gene expression in LNCaP cells. (A) Over-expression of FOXO3a increased the expression of FOXO3a and catalase after DHT treatment in LNCaP cells. (B) FOXO3a siRNAs can decrease the elevated gene expression of FOXO3a and catalase after DHT treatment and over-expression of FOXO3a. Control OE, over-expressing control vector; FOXO3a OE, FOXO3a over-expressing vector; NC, negative control siRNA; siRNA1, first FOXO3a-specific siRNA; siRNA2, second FOXO3a-specific siRNA. (* p□0.05, ** p□0.01, *** p□0.001)

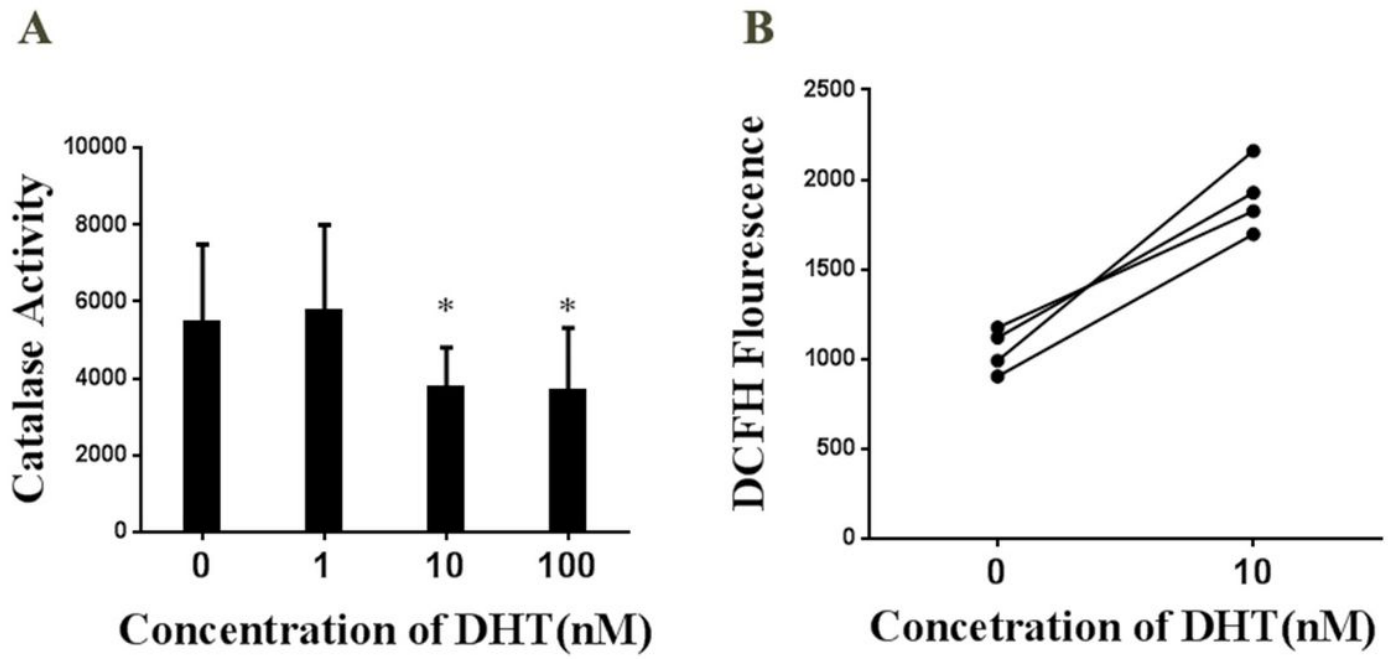

Figure 5

DHT treatment suppressed the catalase activity in LNCaP cells. (A) The catalase activity was decreased after DHT treatment. (B) The level of ROS was increased after DHT treatment. (* p□0.05)

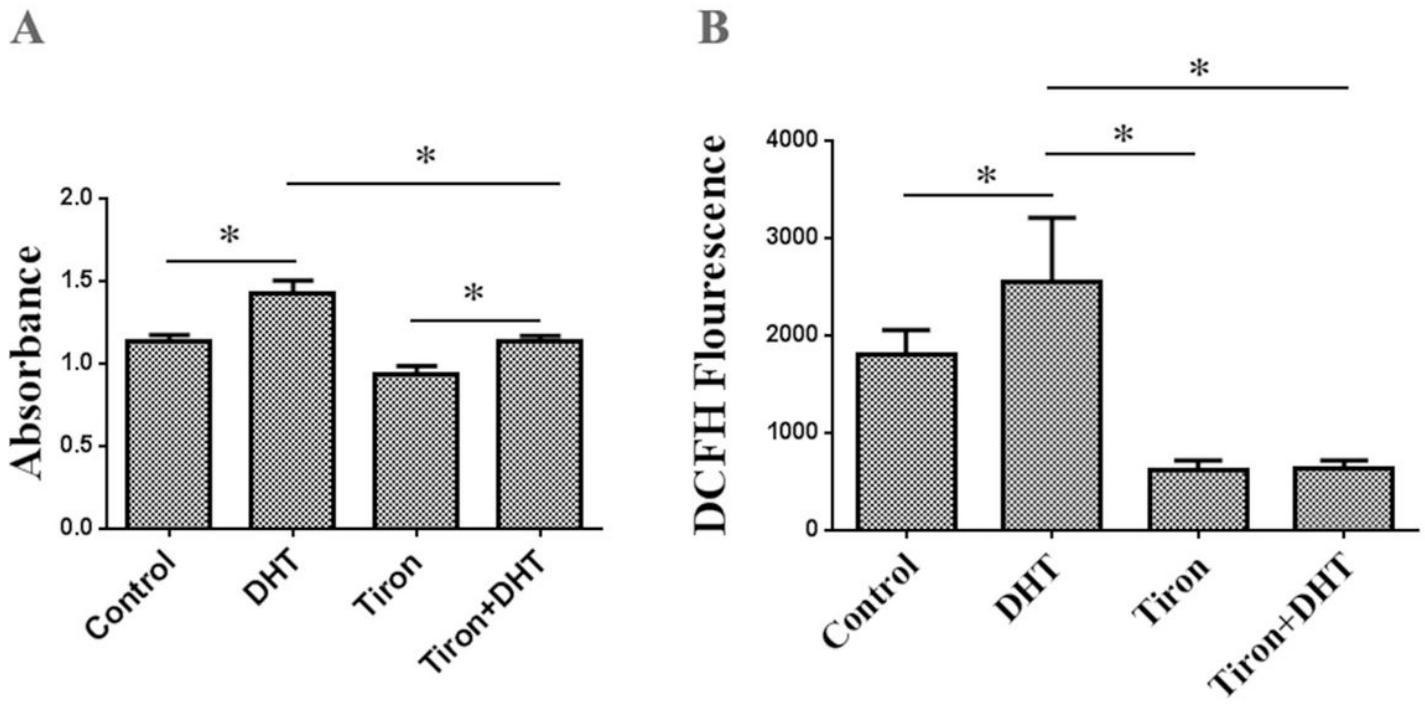

Figure 6

The effect of the superoxide anion scavenger Tiron on the cell proliferation in LNCaP cells. (A) Tiron treatment reduced DHT-induced proliferation. (B) Tiron treatment reduced DHT-induced ROS production. (* p□0.05)