ER α -NRF2 signaling axis promotes bicalutamide resistance in PCa

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Method Article

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Abstract

Background

Bicalutamide is a potent and specific non-steroidal anti-androgen widely used as a first-line clinical treatment for prostate cancer (PCa). Although patients initially show an effective response to bicalutamide treatment, resistance to bicalutamide frequently occurs and leads to the development of castration-resistant PCa (CRPC). Estrogen receptor alpha (ER α) is significantly increased in bicalutamide-resistant PCa cell lines. Here, we investigated the roles of the estrogen-ER α signaling pathway in bicalutamide-resistant PCa cells.

Methods

Bioinformatics analyzed the expression and the correlation of ER α and nuclear factor E2-related factor 2 (NRF2) in human clinical datasets and tissue samples. RT-PCR, Western blotting, and immunofluorescence staining were used to evaluate the expression of ER α , NRF2 and resistance-related genes in cells treated with bicalutamide. The estrogen response element presents on the NRF2 promoter and facilitation of ER α binding to the NRF2 promoter were validated by the chromatin immunoprecipitation assay. The sensitivity of PCa cells to bicalutamide was evaluated by the MTT assay.

Results

ER α and NRF2 expression was significantly increased and positively correlated in CRPC cells. E2 promoted the expression of ER α and NRF2. ER α antagonist tamoxifen inhibited the upregulation of NRF2 induced by bicalutamide treatment. ER α -induced estrogen effects promote the activation of NRF2 signaling pathway via binding to NRF2 promoter. Moreover, ER α -NRF2 signaling axis resensitized PCa cells to bicalutamide and enhanced cell invasion and migration.

Conclusion

ER α -mediated estrogen effects promote bicalutamide resistance in CRPC cells by directly regulating NRF2 expression. This study suggests the molecular mechanism of PCa resistance to bicalutamide.

Background

Among men worldwide, prostate cancer (PCa) is the second most common cancer and the fifth leading cause of cancer-related deaths^[1]. Since Huggins and Hodges first proposed the concept of androgen dependence in 1972, androgen deprivation therapy (ADT), has been the standard initial treatment for

PCa^[2]. Initial ADT involved surgical castration (for example bilateral orchiectomy) and chemical castration to remove circulating serum testosterone produced by the testes^[3, 4]. Persistence of androgen receptor (AR) signaling axis during systemic castration via the production of androgens in tumors undermines therapeutic efficacy. Some second-generation androgen receptor antagonists including the steroidal antiandrogen cyproterone acetate and megestrol acetate^[5–6] as well as nonsteroidal antiandrogens including flutamide, nilutamide, and bicalutamide^[7–9] are often used in combination with ADT to prevent androgens from binding to the AR. Although bicalutamide treatment initially exhibits favorable responses for prostate cancers, they eventually becomes refractory and acquires resistance to bicalutamide, resulting in progressing to castration-resistant PCa (CRPC), which remains incurable^[10, 11]. The molecular mechanisms related to bicalutamide resistance in CRPC are incompletely understood.

Regarding to the mechanisms of CRPC resistance transformation, many findings support the viewpoint that the androgen signaling axis continues to play an important role^[12–14]. During the process of PCa entering CRPC, certain PCa cells can offset its sensitivity to androgens, so androgens and AR signaling axis are no longer necessary for tumors cell survival^[11]. The continued survival and proliferation of these androgen-independent PCa cells eventually prove fatal to the prostate cancers^[15].

Bonkhoff *et al.* first described the presence of the classic estrogen receptor α (ER α) in premalignant and advanced PCa in 1999^[16]. Estrogen effects appear to be related to the progression of CRPC. The most significant ER α mRNA and protein expression has been observed in metastatic lesions and CRPC tissues, and there is a significant amount of estrogen-responsive cells in these tumors^[17]. Previous reports have shown that advanced PCa cells can switch from AR signaling to ER α signaling in response to ADT to maintain tumor cell proliferation in an androgen deprived condition^[18]. Our laboratory found that 17 β -estradiol (E2) promoted tumor formation and metastasis in castrated PC3 xenograft mice, and the ER α –NOTCH1 signaling axis enhanced the basal stem-like phenotype and epithelial-mesenchymal transition in PCa cells^[19]. Furthermore, we found that aromatase expression was significantly increased in CRPC cell lines and tissues, whereas highly expressed aromatase increased the concentration of endogenous estrogen metabolized by testosterone and ER α transcriptional activity was enhanced. Treatment with bicalutamide can upregulate the expression of aromatase leading to CRPC progression^[20]. However, the effects of estrogen on the development of resistance to bicalutamide treatment, which contributes to the resistance transformation of CRPC, is still urgently needed. It has been shown that activation of Nuclear factor E2-related factor 2 (NRF2) is activated to protect against oxidative stress in cancer associated with ROS production induced by drugs^[21, 22]. We used mRNA sequencing (mRNA-seq) to compare bicalutamide-resistant cell lines to their parental cell lines in order to identify the potential estrogen pathways that contribute to resistance. We found that the NRF2 pathway is linked to bicalutamide resistance in PCa. Moreover, the correlation of NRF2 and ER α from The Cancer Genome Atlas (TCGA) and Genotype-Tumor Expression (GTEx) project was higher in PCa than in normal healthy prostate tissue.

In this study, we investigated the role and molecular mechanisms of estrogen effect in promoting the resistance of CRPC to bicalutamide via regulating NRF2. These findings revealed a molecular link

between ER α -induced estrogen effects, NRF2 and bicalutamide-resistant in PCa.

Materials And Methods

Cell culture and bicalutamide treatment

Androgen-independent PCa cells LNCaP abl (a kind gift from Helmut Klocker; (Department of Urology, Medical University of Innsbruck, Innsbruck, Austria) were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 1% penicillin/streptomycin (P/S; HyClone, Logan, UT, USA) and 10% charcoal dextran stripped fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). LNCaP and PC3 PCa cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 1% P/S and 10% FBS (Gibco). LNCaP and LNCaP abl cells were treated with 30 μ M bicalutamide (Selleck Chemicals, Houston, TX, USA) for 7 days. LNCaP, LNCaP abl, PC3 were treated with 17 β -estradiol (Sigma) at a concentration of 10 nM. Bicalutamide and 17 β -estradiol were dissolved in dimethyl sulfoxide (DMSO).

Bioinformatics analysis

Best.C (GSE2443)^[23], Goodison.S (GSE25136)^[24], Monzon (GSE6919)^[25, 26], and Nelson (GSE74367)^[27] Gene Expression Omnibus (GEO) databases (<http://www.ncbi.nlm.nih.gov/geo/>) from PubMed were used in our study. For the correlation of ER α and NRF2, we used mRNA expression data from human prostate cancer databases of the TCGA including 498 prostate tumor tissues and 52 adjacent normal tissues and Genotype-Tissue Expression (GTEx), including 100 normal prostate tissues. Gene set enrichment analysis (GSEA) analyses were performed using the GSEA software package (GSEA-3.0.jar)^[28]. Clinical outcome analyses were performed using GraphPad Prism 5.

Generation of PCa cell lines stably expressing NRF2 short hairpin RNA

The lentiviral shuttle plasmid and its original packaging vector plasmids were prepared (pspax2, pMD2G, pHBLV). An endotoxin-free plasmid extraction kit was used for plasmid extraction, followed by transfection of 293T cells with 5 μ g NRF2 short hairpin RNA (shNRF2; sequence is shown in Additional file 1: Table S1), pspax2 plasmid, and pMD2G plasmid; 5 μ g pHBLV plasmid was used as a control. Lentiviral particles containing supernatant were collected at 48 and 72 h. After the control cells were killed by puromycin, half of the final concentration of puromycin was used to culture cells of the virus-infected group.

Small interfering RNA and transient transfection

Control small interfering RNA (siRNA) and siRNAs targeting ER α , ER β , G protein-coupled receptor 30 (GPR30), AR, and NRF2 were purchased from GenePharma (Shanghai, China). The siRNA sequences are listed in Additional file 1: Table S2. All transfections with siRNA and plasmids were conducted using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions.

Chromatin immunoprecipitation

PC3 cells were treated with DMSO or 10 nM 17 β -estradiol for 30 min before harvesting. Chromatin immunoprecipitation (ChIP) assays were performed as previously described^[32]. The DNA was sonicated and purified, extracted, and analyzed by PCR. The primers used in the ChIP assays are shown in Additional file 1: Table S2, and an ER α antibody (ab32063; Abcam, Cambridge, MA, USA) was used.

Western blot analysis and quantitative PCR

Total RNA was extracted from PCa cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions, followed by reverse transcription into cDNA. Quantitative PCR (qPCR) was performed as previously described^[29]. The housekeeping gene of PCa cells is hypoxanthine phosphoribosyl transferase 1. The primer sequences are shown in Table S2. Protein lysis buffer (RIPA; Thermo Fisher, Waltham, MA, USA) was used to extract total protein from the cells, and Western blot analysis was performed as previously described^[30]. The primary antibody dilutions used are provided in Additional file 1: Table S3.

Immunohistochemistry and immunofluorescence analyses

Prostate tissue samples from CRPC patients (age 63–78 years, n = 28) who underwent surgical castration and PCa tissues from patients who underwent radical prostatectomy (age 63–78 n = 34) were obtained from the Department of Pathology and the Department of Urology, the Second Affiliated Hospital of Tianjin Medical University (Tianjin, China). Normal human prostate specimens from patients undergoing radical cystectomy for bladder cancer (age 53–67, n = 21) were obtained from the Department of Urology, Shanghai First People's Hospital (Shanghai, China). All samples were obtained with the informed consent of patients, and approval of the study was obtained from the Ethics Committee of Nankai University (Tianjin, China). Immunohistochemistry (IHC) and immunofluorescence (IF) were performed on prostate tissues as previously described^[31]. The primary antibody dilutions are provided in Additional file 1: Table S3. For IF, LNCaP and LNCaP abl cells were treated with bicalutamide or 10 nM E2 for 72 h, and IF images were taken using a fluorescence microscope (Leica, Wetzlar, Germany). IHC images were taken using an Olympus microscope (BX43; Olympus, Tokyo, Japan).

Luciferase reporter activity assay

The promoter fragments with ER α binding to normal sites and mutation sites used in the experiment were all obtained by Shanghai General Biology (Anhui) through gene synthesis, and the pGL3-basic vector to obtain Normal-luc and Mutation-luc plasmids were constructed. Plasmids were transfected into PCa cells after the indicated treatment for 24 and 48 h. The sequence used for the ERE-luc assays are shown in Additional file 1: Table S4. The luciferase activity was detected using a dual-luciferase assay kit (Promega, Madison, WI, USA) as previously described^[33].

Cell proliferation, scratch migration, and Matrigel invasion assays

To monitor proliferation, the cells were pretreated with shRNAs or ER α and NRF2 overexpression plasmids for 24 h, followed by bicalutamide treatment (0, 10, 20, 30, 40, 50 μ M). After 48 h, the sensitivity of the

different treatment groups to bicalutamide was determined by MTT assays (at 570 nm). The scratch test was used to detect the migration of PC3 cells, which were divided into different treatment groups. The cells were cultured in a 6-well plate and scratched with the fine end of a 1 mL pipette tip. Pictures were taken at 0 and 24 h. Transwell assays were used to evaluate the invasive capability of the PC3 cells. Briefly, RPMI 1640 was added to the transwell migration chambers (Transparent PET Membrane 24-well, 8.0 μ m pore size; Corning Inc., Corning, NY, USA), and RPMI 1640 supplemented with 10% FBS was added to the lower wells. The cells that migrated through the filter to the lower surface were fixed in 4% paraformaldehyde and stained with a 0.5% crystal violet solution (BBI Life Sciences, Shanghai, China) after an overnight incubation. Finally, the cells were counted in five randomly selected areas in each chamber.

Statistical analyses

Data are shown as the mean $\pm$ standard error of the mean (SEM). Student's t-test was used for analysing differences between two groups, and more than two groups were compared with one-way ANOVA. $P < 0.05$ was considered significant.

Results

High expression and positive correlation of NRF2 with ER α in human CRPC tissues and cell lines

We examined the expression of NRF2 and ER α in different PCa tissues from three publicly available datasets (Best.C, 10 androgen-dependent PCa and 10 androgen-independent PCa samples, Goodison S, 40 non-recurrent primary PCa and 39 recurrent primary PCa samples, Monzon, 64 primary PCa and 25 metastatic PCa samples). NRF2 expression was significantly higher in CRPC than primary PCa (Fig. 1a). Then we selected ER α^{High} and ER α^{Low} PCa tumor samples individually from GEO datasets (Nelson, 11 primary PCa tumor and 45 CRPC tumor samples). The mean NRF2 gene expression was significantly higher in ER α^{High} prostate adenocarcinoma samples than in ER α^{Low} prostate adenocarcinoma samples (Fig. 1b). Thus, we focused on the relationship between ER α and NRF2. We analyzed the correlation of ER α and NRF2 from TCGA and GTEx project. The results showed that the correlation of ER α and NRF2 was more significant in PCa than in normal prostate tissue (Fig. 1c). Next, we performed qPCR and Western blotting to assess the mRNA and protein expression of ER α and NRF2, respectively, in PCa cell lines. ER α and NRF2 expression was significantly upregulated in androgen-independent PCa cell lines (LNCaP abl and PC3) compared with the androgen-dependent PCa cell line (LNCaP). AR had the opposite expression pattern of ER α and NRF2 (Fig. 1d). In addition, IHC was performed to evaluate the protein expression of ER α and NRF2 in normal prostate (n = 21), primary PCa (n = 34), and CRPC (n = 28) samples. ER α and NRF2 were both significantly upregulated in prostate tumors compared with normal tissues. In the primary PCa samples, high Gleason score (Gleason 8–10) tumors had stronger ER α and NRF2 staining than low Gleason score (Gleason $\leq$ 6) tumors. The CRPC samples displayed higher ER α and NRF2 expression than the primary PCa samples (Fig. 1e).

NRF2 expression was enhanced in association with ER α upregulation upon bicalutamide treatment

To identify the role of NRF2 pathway activation in the progression of bicalutamide-resistant PCa, we cultured LNCaP and LNCaP abl cell lines and treated them with an AR blocker, bicalutamide. After 7 days of exposure to 30 μ M bicalutamide, we conducted GSEA from the mRNA-seq dataset. The reactive oxygen species (ROS) pathway was enriched after treatment of LNCaP and LNCaP abl cell lines with bicalutamide (Fig. 2a). At the cellular level, we detected a significant increase in ER α and NRF2 protein expression in LNCaP and LNCaP abl cells (Fig. 2b). At the mRNA level, increased expression of ER α and NRF2, along with higher target genes of NRF2 and lower AR after treatment of LNCaP and LNCaP abl cell lines with bicalutamide were observed (Fig. 2c-2d). LNCaP abl cells are sensitive to bicalutamide although they are an androgen-insensitive PCa cell line. Because LNCaP abl cells are already adapted to growth in steroid-ablated conditions, we treated these cells with 10 nM 17 β -estradiol to investigate the expression of ER α and NRF2 after bicalutamide treatment. E2 treatment significantly upregulated the expression of NRF2 compared to bicalutamide alone. Moreover, IF was conducted to further investigate the relationship between ER α and NRF2 expression in LNCaP cells after bicalutamide treatment. Increased expression and good co-expression of ER α and NRF2 were observed (Fig. 2e). Thus, we hypothesized that estrogen effects promote NRF2 signaling in PCa. We detected a significant reduction of ER α and NRF2 expression following bicalutamide treatment with tamoxifen (TAM) in AR-blocking conditions (Fig. 2f). This indicated that NRF2 may be regulated by ER α -induced estrogen effects. To determine whether E2 regulates NRF2, we detected the expression of NRF2 in LNCaP, LNCaP abl, and PC3 cells treated with E2. The results showed that E2 promoted the expression of NRF2 and its target genes (Fig. 2g). IF confirmed the above conclusion in LNCaP abl cells (Additional file 2: Fig. S1A). We established PC3 and LNCaP abl cell lines with stable knockdown of NRF2, and Western blotting was used to determine the knockdown efficiency (Additional file 2: Fig. S1B). In PC3 and LNCaP abl cells treated with E2, ROS production was increased. Interestingly, the changes in gene expression induced by E2 were inhibited by NRF2 knockdown. These results illustrate that ROS-producing pathways are responsive to the effects of estrogen (Additional file 2: Fig. S1C).

ER α binds directly to the ERE site of the NRF2 promoter.

To explore the molecular mechanisms underlying E2 regulation of NRF2, ER α was knocked down in LNCaP abl and PC3 cells and overexpressed in LNCaP cells. The results showed that the expression of NRF2 was downregulated in LNCaP abl and PC3 cells with ER α knockdown, and overexpression of ER α in LNCaP cells significantly increased NRF2 at the protein level (Fig. 3a). Furthermore, induction of the NRF2 pathway by E2 was verified at the mRNA level (Fig. 3b). Next, we knocked down ER α in LNCaP abl and PC3 cells treated with or without E2. ER α knockdown notably reduced the effects of E2 on LNCaP abl and PC3 cells (Fig. 3c). Similarly, the ER α inhibitors TAM inhibited the expression of NRF2 induced by E2 (Fig. 3d). The same results were observed for another estrogen receptor antagonist, ICI 182780 (Additional file 2: Fig. S2A). Next, we assessed the NRF2 promoter for the presence of ERE, which facilitates the direct binding of ER α to the promoter sequence. Analyses revealed the presence of putative ERE, and the ChIP assay verified these findings. Our findings indicated that treatment with E2 stimulated NRF2 transcription by inducing ER α binding to a specific ERE site in the NRF2 promoter region (Fig. 3e). The Dual-Luciferase® Reporter Assay System was used to evaluate the activity of the normal ERE (Normal) and mutated ERE

(Mutation) in LNCaP and LNCaP abl cells treated with or without E2. In LNCaP and LNCaP abl cells, the normal ERE-luc significantly increased the activity of the NRF2 promoter after treatment with E2 for 24 and 48 h. On the other hand, the mutated ERE-luc did not change the activity of the NRF2 promoter (Fig. 3f). Finally, to determine whether AR regulates the expression of NRF2, the results showed that knocking down the AR in LNCaP, LNCaP abl, and PC3 cells had no effect on the expression of NRF2 (Additional file 2: Fig. S2B). To verify which estrogen receptor regulates NRF2, ER α , ER β and GPR30 were knocked down in LNCaP abl and PC3 cells. Only knockdown of ER α decreased NRF2 expression, suggesting that ER α regulates NRF2 (Additional file 2: Fig. S2C).

Inhibition of the ER α -induced NRF2 signaling pathway resensitizes bicalutamide-resistant cells to bicalutamide

To determine the mechanism by which the ER α –NRF2 signaling axis sensitizes CRPC cells to bicalutamide, we found that expression of the antiapoptosis gene Bcl-2, ATP-binding cassette transporters such as ABCG2 and ABCB1, and stemness genes CD44 and CD49f were upregulated after treatment of LNCaP and LNCaP abl cells with bicalutamide, as determined by the mRNA-seq assay (Fig. 4a). We defined the above genes as bicalutamide resistance-related genes. Then qPCR and Western blot analysis verified that the expression of resistance-related genes in LNCaP and LNCaP abl cells was higher after treatment with bicalutamide (Fig. 4b–4c). IF analysis was performed to analyze the increased expression and co-expression of NRF2 and CD44/ABCG2/CD49f in LNCaP cells after bicalutamide treatment (Fig. 4d–4f). Furthermore, qPCR analysis confirmed that the ER α –NRF2 signaling axis can increase the expression of resistance-related genes in PCa. ER α could not regulate the expression of resistance-related genes in LNCaP and PC3 cells under conditions of NRF2 silencing, but NRF2 could rescue the expression of resistance-related genes in LNCaP and PC3 cells in the condition of ER α silencing (Fig. 4g). Then to determine whether ER α –NRF2 signaling regulates bicalutamide resistance in PCa cells, shRNAs of NRF2 or ER α were used to silence the expression of NRF2 or ER α . The MTT assay showed that ER α did not regulate the sensitivity of LNCaP abl cells to bicalutamide in the condition of NRF2 silencing. However, overexpressed NRF2 rescued the viability of LNCaP cells upon ER α knockdown (Fig. 4h). In addition, knockdown of ER α and NRF2 significantly decreased PC3 cell invasiveness. Overexpressed NRF2 rescued the invasion of PC3 cells in conditions of ER α inhibition, but overexpressed ER α in conditions of NRF2 inhibition had no effect (Fig. 4i). The cell scratch test showed the same results (Additional file 2: Fig. S3A). These results indicate that the ER α –NRF2 signaling axis can promote CRPC progression by bicalutamide resistance.

Discussion

Drug resistance remains a major obstacle for the treatment of PCa. Generally, several factors have been shown to be involved in the mechanisms of bicalutamide resistance in PCa^[34–36]. Our laboratory proposed that the expression of aromatase is increased upon treatment bicalutamide, and endogenous estrogen catalyzed by testosterone is increased. Mechanistically, aromatase-induced estrogen promotes ER α binding to the matrix metalloproteinase 12 promoter ERE site upon bicalutamide treatment in PCa

cells, leading to CRPC progression^[20]. This article further clarified the molecular mechanisms underlying the effects of estrogen on promoting resistance transformation to bicalutamide in PCa cells.

As previously described, estrogen mediates the biological effects of target tissues mainly by binding to specific intracellular receptors, ER α and ER β ^[37–39]. However, recent studies have provided evidence that luminal cells with high expression of ER β are particularly fragile and undergo programmed cell death after ADT, radiation, and chemotherapy. Basal cells that express ER α are multidrug-resistant and can survive in cytotoxic conditions^[19]. Previous studies indicated that NRF2 plays a role in the drug resistance of a variety of cancers such as breast cancer, hepatocellular carcinoma, bladder cancer, and pancreatic adenocarcinoma^[40]. Oxidative stress promotes the initiation, progression, and transition of PCa to CRPC^[41–43], and NRF2 significantly decreases transactivation of the AR and suppresses the dihydrotestosterone-induced activity of the AR^[44, 45]. However, the regulation of estrogen effects on NRF2 is poorly understood in PCa cells resistant to bicalutamide. In this study, by analyzing the PCa sample data obtained from the GEO and TCGA database, we found that the expression of NRF2 was significantly upregulated in androgen-independent PCa cells and CRPC tissues, and was significantly positively correlated with ER α . The expression of ER α and NRF2 was significantly increased and showed a good co-expression and co-localization relationship after treated with bicalutamide. Additionally, inhibiting the expression of ER α not only reduces NRF2 expression but also reduces the upregulation of NRF2 induced by E2. Based on these findings, we hypothesized that ER α -NRF2 signaling axis is potential pathway to contribute to bicalutamide resistance of PCa cells. As expected, overexpression of NRF2 resensitized PCa cells to bicalutamide and rescued the ability of cell invasion and migration inhibited by ER α knockdown. Moreover, NRF2 knockdown reduced the activity of detoxifying enzymes induced by E2 indicating that estrogen effect is involved in bicalutamide resistance through regulating reactive oxygen balance, suggesting a critical role of ER α -NRF2 signaling axis in regulating bicalutamide resistant in PCa cell lines and providing a potential diagnostic and predictive biomarker for PCa.

We further investigated the regulation mechanism of ER α and NRF2. The ER α antagonist MPP inhibits NRF2 activity in MCF-7 breast cancer cells, and estrogen increases NRF2 activity in MCF7 breast cancer cells by activating the phosphoinositide 3-kinase/glycogen synthase kinase 3 beta (PI3K/GSK3 β) pathway^[46]. Our results showed that E2 regulates NRF2 via ER α binding to the ERE site on the promoter of NRF2 upon bicalutamide treatment in PCa cells, indicating that NRF2 is a direct target gene of ER α and participates in the bicalutamide resistance of CRPC as an ER α -mediated estrogen downstream effect. However, estrogen has been shown to recruit ER α at the NQO1 promoter resulting in inhibition of NQO1 transcription, which is NRF2-dependent detoxifying enzymes, contributing to breast cancer prevention^[47]. Further study is needed to understand regulatory relationship of ER α and NRF2 in different cancer types.

CD44 was consequently been recognized as a cancer stem cell marker in several types of cancers that has been proven to participate in tumor progression, metastasis and drug resistance^[48–50]. Previous studies suggested that PCa stem-like cells are a key contributor to CRPC. CD44⁺ stem-like cells are increased with PCa progression after ADT and aromatase is mostly expressed in CD44⁺ PCa cells^[24]. Our

data showed that ER α -induced estrogen effects increase the expression of CD44 by regulating NRF2. While NRF2 knockdown reduced CD44 expression, increased the sensitivity to bicalutamide. Moreover, CD44 and NRF2 showed good co-expression in CRPC tissue after bicalutamide treatment. Therefore, NRF2 might promote the bicalutamide resistance of CRPC cells by regulating the expression of CD44. However, Ryoo *et al.*^[51] reported that the molecular mechanisms of CD44-mediated NRF2 activation involve high p62 expression, and high levels of CD44 lead to p62-associated NRF2 activation in CD44^{high} breast cancer stem cell (CSC)-like cells. Therefore, future studies are needed to determine the role of the interaction between CD44 and NRF2 in CRPC.

Animal experiments to verify the mechanism of ER α -mediated NRF2 in bicalutamide resistance remain to be explored *in vivo*. Future studies are also needed to determine if there is a similar molecular mechanism as bicalutamide in PCa for resistance to other androgen receptor antagonists.

Conclusions

Our results identify bicalutamide-resistance prostate cancer cells with high ER α and NRF2 expression, ER α -induced estrogen effects promote the expression of NRF2 via binding ERE site of the NRF2 promoter and enhances NRF2 expression. ER α -NRF2 signaling axis enhances the resistance to bicalutamide of CRPC cells by up-regulating bicalutamide resistance-related genes, promoting the progress of CRPC. We revealed high estrogen effect resulting enhanced NRF2 expression might be one of the mechanisms underlying bicalutamide resistance transformation in CRPC. This enriches the study of bicalutamide resistance of PCa and provides a theoretical basis for the clinical development of NRF2 as a new molecular target.

Abbreviations

ADT Androgen deprivation therapy

AR Androgen receptor

Bic Bicalutamide

ChIP Chromatin immunoprecipitation

CRPC Castration-resistant prostate cancer

E2 17 β -estradiol

ER α Estrogen receptor α

ERE Estrogen response element

ICI ICI 182780

IF Immunofluorescence

IHC Immunohistochemistry

NRF2 Nuclear factor E2-related factor 2

PCa Prostate cancer

TAM Tamoxifen

Declarations

Ethics approval and consent to participate

Human tissue were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Nankai University and the Department of Pathology and the Department of Urology, the Second Affiliated Hospital of Tianjin Medical University.

Consent for publication

All authors have approved the manuscript for submission.

Availability of supporting data

The datasets supporting the conclusions of this article are included within the article and the additional file. More supporting data is available under reasonable request.

Authors' contributions

L.T designed and conducted the study, analyzed the data, and drafted the manuscript. K.Y provided prostate cancer tissue paraffin sections for IHC. ZX.L conducted the study. XL.D. provided the study materials. J.Z. and JD.S. provided the study materials and helped to draft the manuscript. All authors read and approved the final manuscript.

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

Data Availability Statement

The human clinical datasets utilized in the study are included in the Methods sections.

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Figures

Figure 1

Expression of ER α and NRF2 in PCa. **a**, Box & whisker plots present ER α and NRF2 mRNA expression in different PCa tissues from three publicly available datasets (t-test). **b**, Mean mRNA analysis of NRF2 for ER α ^{High} samples vs. ER α ^{Low} samples in prostate adenocarcinoma (PRAD) from Nelson dataset (t-test). **c**, The correlation of ER α and NRF2 in normal prostate and PRAD patients from TCGA (num (Normal) = 52, num (PRAD) = 498) and GTEx (num (Normal) = 100) datasets. **d**, The mRNA levels of ER α , NRF2 and AR in LNCaP, LNCaP abl, and PC3 cells are shown (one-way ANOVA). **e**, Immunohistochemical analyses of ER α and NRF2 in normal prostate (n = 21), low Gleason score (Gleason $\leq$ 6) PCa tumor (n = 17), high Gleason score (Gleason 8–10) PCa tumor (n = 17) and CRPC tissues (n = 28); representative microphotographs are shown; scale bar, 100 μ m. The right panel shows the average optical density of ER α and NRF2 for normal prostate vs. low Gleason score vs. high Gleason score tissues and primary tumor vs. CRPC tissues (t-test). The average optical density was measured by Image-Pro Plus 6.0 software; the values are the mean $\pm$ SEM; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2

NRF2 was enhanced in association with ER α upregulation upon bicalutamide treatment in PCa. LNCaP and LNCaP abl cells were treated with 30 μ M bicalutamide for 7 days, and LNCaP abl cells were exposed to 10 nM 17 β -estradiol (E2). **a**, GSEA plot of the ROS pathway gene set activity in PCa from mRNA-seq. **b**, The protein levels of ER α and NRF2 were analyzed. **c-d**, The mRNA levels of ER α , NRF2, AR, and NRF2 target genes were analyzed in LNCaP (t-test) and LNCaP abl (one-way ANOVA). **e**, Representative immunofluorescence graphs of ER α and NRF2 in LNCaP cells before and after bicalutamide treatment; scale bar, 50 μ m. **f**, The mRNA levels of ER α , NRF2, and its target genes in LNCaP cells and LNCaP abl cells exposed to bicalutamide and tamoxifen (one-way ANOVA). **g**, ER α , NRF2 and its target genes mRNA and protein levels in LNCaP and LNCaP abl cells treated with DMSO or 10 nM E2 treatments (t-test). All studies were repeated at least three times; the values are the mean $\pm$ SEM; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3

The mechanisms of NRF2 regulation by E2. **a**, Western blot analysis of the expression of NRF2 in LNCaP abl and PC3 cells with ER α knockdown, and LNCaP with ER α overexpression. **b**, qPCR analysis of the expression of NRF2 and its target genes (t-test). **c**, qPCR and Western blot analysis of the indicated in LNCaP abl or PC3 cells with or without ER α knockdown after treatment with either DMSO or 10 nM E2 (one-way ANOVA). **d**, qPCR analysis of the indicated in LNCaP abl or PC3 cells with or without TAM after treatment with either DMSO or 10 nM E2 (one-way ANOVA). **e**, ChIP assay showing that ER α binds directly to the ERE site of the NRF2 promoter region. **f**, ER α transcriptional activity in LNCaP and LNCaP abl cells after different treatment (one-way ANOVA). All studies were repeated at least three times; the values are the mean $\pm$ SEM; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4

ER α -NRF2 signaling axis enhance bicalutamide resistance. **a**, mRNA-seq of LNCaP and LNCaP abl cells treated with DMSO or 30 μ M bicalutamide (t-test). **b-c**, The mRNA and protein levels of bicalutamide resistance-related genes were analyzed in LNCaP cells (t-test) and LNCaP abl cells (one-way ANOVA). **d-f**, Representative IF graphs between NRF2 with CD44 and ABCG2 in LNCaP cells. scale bar, 50 μ m. **g**, LNCaP abl with ER α knockdown and LNCaP with ER α overexpression. qRT-PCR analysis showing expression changes of resistance-related genes in different treatment (one-way ANOVA). **h**, MTT assay of LNCaP and LNCaP abl cells (t-test) after treatment with 30 μ M bicalutamide. **i**, Invasion capability was assessed in PC3 cells with different treatments (one-way ANOVA). scale bar, 500 μ m. All studies were repeated at least three times; the values are the mean $\pm$ SEM; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5

The total signaling pathway. ER α -induced estrogen effects promote the expression of NRF2 by binding NRF2 promoter at -1434 and -1417 bp. The ER α -NRF2 signaling axis enhances the sensitivity of prostate cancer cells to bicalutamide by promoting the expression of resistance-related genes.

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