Involvement of estrogen receptor β in androgen receptor-induced growth inhibition in prostate cancer PC-3 cells

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Abstract. Previous studies have suggested that changes in sex hormone receptor expression may be associated with the initiation and progression of prostate cancer (PCa). Therefore, the present study aimed to investigate the association and possible pathways between two sex hormone receptors and PCa by measuring the expression levels of the androgen receptor (AR) and the estrogen receptor subtypes alpha (ERα) and beta (ERβ) in prostatic cancer PC-3 cell lines. The pcDNA3.1-hERβ plasmid was transfected into PC-3 cell lines. The expression levels of AR, ERα and ERβ were detected at the mRNA level by reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR). The results demonstrated that the expression levels of AR, ERβ and ERα were downregulated to different degrees: ERβ test group vs. PC-3 cell group (P=0.000; 95% confidence interval: 0.9803-1.6331). ERβ and AR expression was detected continuously in the PC-3 cells, but the expression of ERα was not. AR expression levels exhibited an upward trend whilst the expression of ERβ demonstrated a marked downward trend. There is a correlation between the expression levels of ERβ and the incidence of PCa, and ERβ may inhibit the growth of PC-3 cell lines by regulating the expression levels of AR. ERβ may provide a novel target for PCa therapies.

Introduction

Prostate cancer (PCa) is a common, hormone-dependent, type of malignant tumor regularly observed in men >60 years in the united states and european countries (1,2). However, the pathogenesis and mechanisms of progression remain unclear.

Materials and methods

Cell culture. The human PCa PC-3 cell line was obtained from the Pathology department of the West China Hospital of Sichuan University (Chengdu, China). The PC-3 cells were grown and maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 units/ml penicillin, and 100 µg/ml streptomycin. The cell lines were maintained in humidified incubators with 5% CO₂ at 37°C. Subsequent to cell adhesion to the base of cell culture dish, cells were subcultured into three equal dishes.

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Cell counting and seeding. The PC-3 cells were seeded in 6-well tissue culture dishes at a density of 175,000 cells/well in high glucose DMEM supplemented with 10% FBS, and were maintained in humidified incubators with 5% CO$_2$ at 37°C for 48 h. Once the cells covered between 80-90% of the base of the dish, the media were discarded and the cells were washed twice with phosphate buffered saline (PBS). A total of 2 ml/well medium, without antibiotics and without FBS was then added to each well. All the assays were carried out in triplicate.

Cell grouping and treatment. According to the different reagents added, the cells were divided into pcDNA3.1-hERβ plasmid, the plasmid, Lipofectamine® 2000 and PC-3 cells control groups. The pcDNA3.1-hERβ plasmid, the blank plasmid and Lipofectamine® 2000 were purchased from the Shanghai GenePharma Company (Shanghai GenePharma Co.Ltd., Shanghai, China). A total of 115 µl/well of the configured mixture of pcDNA3.1-hERβ plasmid-Lipofectamine® 2000 was added into the treated cell groups to induce transfection, following the protocol of the manufacturer. Lipofectamine® 2000, blank plasmids and DMEM were added to the remaining 3 groups. All of the groups were maintained in humidified incubators with 5% CO$_2$ at 37°C for 4 h. The media were removed and the cells were washed twice with PBS, then 2 ml/well high glucose DMEM supplemented with 10% FBS was added. The cells were cultured for an additional 48 h and harvested. All cell groups were prepared in triplicate, and images were captured using an inverted phase contrast microscope (TS100; Nikon Corporation, Tokyo, Japan).

RNA isolation. The total RNA from all groups were isolated using TRizol reagent and (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the protocol of the manufacturer. RNA samples were treated with DNase I (50 U/ml) (Thermo Fisher Scientific, Inc.) prior to analysis. The content and purity of the RNA was assayed with the DU 730 nucleic acid protein analyzer (Beckman Coulter, Inc., Brea, CA, USA), and measured between 1.6 and 1.9 at A260 nm. The amount of RNA was estimated from the optical density at 260 nm. The total RNA was then isolated and was used for qPCR analysis. The total RNA was isolated from the PC-3 cells treated with the pcDNA3.1-hERβ plasmid, the plasmid, Lipofectamine® 2000 and medium for 4 h, and reverse-transcribed into complementary (c)DNA. The cDNA was used for TaqMan analysis according to the protocol of the manufacturer. The PCR primers and TaqMan probes for AR, ERα and ERβ were purchased from the Shanghai GenePharma Co., Ltd. GAPDH was used as the internal control for data normalization. The obtained quantification cycle values of the interest gene were indicated with a standardized ΔΔCq value (17).

Fluorescence qPCR. The cDNA was quantified by fluorescence qPCR using the ABI 7300 real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR primers and fluorescence probes for AR (Gene ID: 367), ERα and ERβ (assay ID: Hs00174860_ml and Hs00230957_ml, respectively) were purchased from Shanghai GenePharma Co., Ltd. All sequences are summarized in Table I. The GAPDH (assay ID: No. 4352934E; Shanghai GenePharma Co., Ltd.) was used as the internal control for data normalization. The fluorescence qPCR reaction system was configured according to the protocol of the manufacturer, as summarized in Table II. Table III demonstrates the sequence information of the oligonucleotide primers and probes used. The PCR reaction was carried out according to the protocol of the manufacturer. The cycles were as follows: 95°C for 1 min for prior degeneration; 95°C for 12 sec and 62°C for 40 sec to measure fluorescence, for 40 cycles. Each incident of mRNA expression of the target genes was indicated with a standardized ΔΔCq value (17). The obtained quantification cycle values of the interest gene were evaluated by the relative standard curve method and

| Reaction reagent | Application amount (µl) |
|------------------|------------------------|
| Total RNA        | 1                      |
| 5x reverse transcription buffer | 2                  |
| Random hexamers (50 µm)    | 0.5                   |
| Oligo d'T Primer (100 µm)   | 0.5                   |
| PrimeScript™ RT Enzyme Mix I | 0.5             |
| DEPC water       | 5.5                    |
| Total volume     | 10                     |

DEPC, diethylpyrocarbonate.

| Reaction reagent            | Application amount (µl) |
|-----------------------------|------------------------|
| Primers                     | 0.8                    |
| 2x quantitative PCR Master Mix | 10                   |
| Fluorescent probe (10 µm)   | 0.4                    |
| cDNA template               | 2                      |
| Taq DNA polymerase (5 u/µl) | 0.4                    |
| Double distilled water      | 6.4                    |
| Total volume                | 20                     |

PCR, polymerase chain reaction; cDNA, complementary DNA.
normalized using the respective values of the internal control GAPDH.

**Statistical analysis.** IBM SPSS 19.0 (Armonk, NY, USA) for Windows was used to establish the database and to conduct the statistical analysis. All results are reported as the mean ± standard deviation, using the independent samples t-test to detect the differences in each test group and the control group. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Cell culture and plasmid transfection.** The PC-3 PCa cells cultured in high glucose DMEM supplemented with 10% FBS are illustrated in Fig. 1A. Once the pcDNA3.1-hERβ-plasmid had been transfected into the PC-3 cells, the cell-growth exhibited a very poor status, namely a high level of apoptosis, small nuclei, a reduced level of cytoplasm and few synapses were all observed (Fig. 1B).

**Expression of AR, ERβ and ERα.** Through the fluorescent qPCR, the patterns of expression levels of the mRNA of ERβ, ERα and AR were assayed in the recombinant plasmid, the empty plasmid, lipofectamine-2000 and the control groups. As demonstrated in Fig. 2, the results suggest that AR mRNA was expressed in all cell samples and expression of AR could be detected in all samples when compared with the blank plasmid group and blank cells. No difference was observed in the levels of expression of AR (pcDNA3.1-hERβ-plasmid transfection vs. PC-3 cell group, P=0.889) between all groups.

As illustrated in Fig. 3, the results also suggest that ERβ was expressed in all cells, and the expression of ERβ positive rate was 100%. The difference in the levels of ERβ expression was statistically significant in the pcDNA3.1-hERβ-plasmid transfection group compared with the PC-3 cell group (P<0.0001). A statistically significant difference was identified between all groups (P<0.0001), as illustrated in Fig. 3.

A statistically significant difference was observed between the levels of expression of ERβ, as measured by
Table IV. ΔΔCq value of AR, ERβ, ERα.

|        | AR      | ERβ     | ERα     |
|--------|---------|---------|---------|
| Test   | 1.179±1.277 | 0.824±0.186 | 1.055±0.964 |
| Blank plasmid | 0.670±0.328 | 1.406±0.218 | 0.807±0.758 |
| Lipofect-2000 | 0.722±0.393 | 1.771±0.071 | 1.643±0.811 |
| Control cell | 1.263±0.209 | 1.790±0.032 | 1.202±1.119 |

AR, test group vs. control cell group, P=0.889; ERβ, test group vs. control cell group, P<0.0001; ERα, test group vs. control cell group, P=0.79; ERβ, blank plasmid group vs. control cell group, P<0.0001; ERα, Lipofectamine® 2000 vs. control cell group, P<0.0001.

ΔΔCq value, in the pcDNA3.1-hERβ + plasmid transfection group compared with the control blank PC-3 cell group, 0.824±0.186 vs. 1.790±0.032, (P=0.000, 95% confidence interval (CI), 0.9803-1.6331), as demonstrated in Table IV. A statistically significant difference was also observed in the pcDNA3.1-hERβ + plasmid transfection group compared with the blank plasmid control group and lipofect2000 control group, (P<0.0001; 95% CI, -1.200-0.7066) and (P<0.0001; 95% CI, -0.8545-0.3093), respectively, suggesting that the pcDNA3.1-hERβ + plasmid, blank plasmid and Lipofectamine® 2000 treated PC-3 cell lines exhibited alterations in the expression levels of ERβ. The most marked effect was observed in the pcDNA3.1-hERβ + plasmid transfected cell lines.

When considering ERα, the results suggest that the expression level of ERα was lower compared with the expression level of AR and ERβ in the PC-3 cells, and that the positive rate of ERα expression was only 56%. There was no statistically significant difference observed in the levels of ERα expression in the pcDNA3.1-hERβ+plasmid group compared with blank cells group (P=0.79). When the rates of all groups were compared, there was no significant difference observed (P>0.05), as demonstrated in Fig. 4.

Expression levels of AR, ERα and ERβ in human PCa PC-3 cell lines transfected with the pcDNA3.1-hERβ plasmid. In the present study, qPCR detected the patterns of ERβ, ERα and AR mRNA expression levels in the recombinant plasmid, the empty plasmid, Lipofectamine® 2000 and normal PC-3 cells groups. As illustrated in Fig. 5, the expression of AR and ERβ was detected in all cells, with the AR and ER positive expression rates at 100%. However, the expression of ERα was only observed in 61% of the samples.

Subsequent to the transfection of the PC-3 cells with the pcDNA3.1-hERβ+plasmids, the expression levels of the 3 receptors were lower compared with the blank/control cell group: AR1.179±1.277 vs. 1.263±0.209; ERα: 1.055±0.964 vs. 1.202±1.119 and ERβ: 0.824±0.186 vs. 1.790±0.032, as demonstrated in Table IV and Fig. 6. Of these, the decrease was most marked in the expression levels of ERβ. There was also a marked increase in the level of apoptosis in the transfected cells, and the ratio of ERβ/AR was <1 (0.824±0.186/1.179±1.277 <1) compared with the control group in which the ERβ/AR ratio was >1 (1.790±0.032/1.263±0.209 >1). This demonstrates that ERβ exhibited a downward trend in expression levels, and AR expression levels exhibited an upward trend in the PCa PC-3 cell lines. When the ΔΔCq value of the expression level of ERβ between the pcDNA3.1-hERβ+plasmid transfection, 1.055±0.964, and the control blank PC-3 cells groups, 1.202±1.119, was compared, no statistical significance was observed (P=0.079, 95% CI, 0.079-1.0166). This suggests that...
In present study, the pcDNA3.1-hERα-plasmid was successfully transfected into the PC-3 cell lines using the eukaryotic cell transfection technique. The levels of expression of AR, ERβ and ERα were measured using RT-PCR and fluorescence qPCR, which demonstrated that AR and ERβ are constantly expressed in PC-3 cell lines. However, the ERα were only expressed in ~50% of the PC-3 cell lines. These results suggest that the PC-3 cell line was an ideal cell model and may indicate the interaction between sex hormones and corresponding receptors. Therefore, PC-3 cell lines may be used to investigate the interaction between the hormone and its receptors in vitro. DHT serves an important role in the AR-mediated regulation of the development of the prostate (21). Mutations in the AR receptor may lead to an attenuated ligand-binding ability, which may cause complete or partial androgen resistance. Partially mutated AR may be activated by antagonists, which may lead to the development of hormone refractory PCa (21).

It has been hypothesized that the expression of AR is exhibited throughout PCa tissue: In castration-resistant PCa, AR is still expressed (22). In contrast, Suryavanshi et al (23) demonstrated that there was a significant loss in AR expression in certain cases of late hormone refractory PCa. As the level of expression of ER remains the same, Kleb et al (24) suggested that small cell PCa does not express AR or respond to hormonal therapies, as the expression of AR does not demonstrate the corresponding decline or deficiency. The current study indicates that there is an association between expression levels of AR and the progress of PCa, and demonstrated that the expression level of AR was constant in all groups, although no significant difference between the pre- and post-transfection cells was observed. However, as levels of ERβ expression exhibited a downward trend, the level AR expression exhibited an upward trend in the PC-3 cell lines.

The prostate gland is not a classical target organ of estrogen and exhibits low or undetectable expression of ERα. Simultaneously, the prostate gland demonstrates a significant expression of ERβ (14). Weihua et al (25) and McPherson et al (26) reported the ERα was mainly expressed in the stromal cells of the prostate, whereas ERβ expression levels were marked in the luminal epithelial cells. However, Lau et al (27) detected the expression of ERβ and ERα in the androgen-independent PC-3 cell lines, and revealed that estrogen and anti-estrogen negatively regulate PC-3 cell growth. The present study demonstrated that unlike the significant levels of expression of AR and ERβ, ERα exhibited no constant expression levels.

The loss of ERα expression at the mRNA level was identified in ~50% of the cell groups, comparing the transfection and control groups. The expression of ERα also exhibited no significant change, which suggests that ERα may not serve a major role in PC-3 cell growth, and demonstrates that ERα...
was not the predominant ER subtype in the PC-3 cell line. It also suggests that the prostate gland is not a classical estrogen target organ.

Cell proliferation in early PCa is attributed to the inhibition of apoptosis of cells (28). Previous studies report that ERβ may promote apoptosis via the downregulation of the protein kinase B signaling pathway (7). This is an important signaling pathway that may promote the growth of tumor cells and blood vessels and enhance the metastatic efficacy of the tumor cells. Concurrently, ERβ may promote the expression levels of B-cell lymphoma-2-like protein 4 and the apoptosis promoter protein-caspase-3 (28). A previous study reported that ERβ may serve an anti-proliferative role via downregulating the expression levels of androgen receptors (25). In the present study, it was demonstrated that PC-3 cells may consistently express ERβ. Furthermore, when the PC-3 cells were transfected the pcDNA3.1-hERβ-plasmid, the expression of ERβ exhibited a marked downward trend, which was evidently different compared with the control groups. The PC-3 cells illustrated a clear inhibition of proliferation, which indirectly suggests an association between the lack of ERβ with the levels of apoptosis of the PC-3 cells, and also indirectly confirms that the ERβ recombinant plasmid was successfully transfected into the PC-3 cell lines.

With a marked downward regulation of the expression levels of ERβ, the expression levels of AR and ERα were compared, and it was demonstrated that although the expression levels of AR were also downregulated, the degree was less compared with ERβ. Concurrently, the respective proportional upward and downward trends of expression levels of AR and ER-β exhibited when the transfection and blank control groups were compared provides data to suggest that ERβ may regulate PCa cell growth via the expression of AR.

To the best of our knowledge, the present study is the first to demonstrate the hypothesis that ERβ may regulate the expression of AR to inhibit the growth of PCa PC-3 cells. It was difficult to determine the association between ERα and AR, as the expression levels of ERα were not consistent between the PC-3 cell lines.

In the present study, subsequent to pcDNA3.1-hER-plasmid transfection, it was demonstrated that the expression levels of the three receptors AR, ERβ and ERα were lower in the transfection group compared with the control group. There were different degrees of downregulation, in particular the expression of ERβ was markedly decreased, which suppressed PCa PC-3 cell lines growth in vitro. These data support the hypothesis that ERβ performs the opposite regulatory action to cell growth: ERβ may serve a role in the direct suppression of PC-3 cell proliferation. However, the data do not fully describe and analyze the interaction between ERs and ARs due to the low number of cell samples, use of a singular cell line and the lack of confirmation of results through crosschecking analysis between other cell lines, such as Du145 and LNCaP. Therefore, additional studies are required to investigate the molecular mechanisms underpinning the suppression of the growth of prostate cancer cells by hormone receptors.

In conclusion, in PCa PC-3 tissues, the expression of AR demonstrated an upward or variant trend, and ERβ expression was downregulated. Therefore, it is hypothesized that the variation in expression levels of ER and AR may serve an important role in the pathogenesis of PCa. At an mRNA level, the PCa PC-3 cell line constantly expressed AR and ERβ, whereas the level of ERα expression was inconsistent. These results support the hypothesis that ERβ is a candidate gene: Increasing the ERβ expression level in PCa cells may be an effective therapeutic strategy to treat PCa.

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