RESEARCH COMMUNICATION

Changes of Androgen Receptor and Insulin-Like Growth Factor-1 in LNCaP Prostate Cancer Cells Treated with Sex Hormones and Flutamide

Da Yue Tong[1], Xing Qiao Wen[2], Yi Jin[2], Zhuo Wei Liu[3], Hong Yu Sun[1], Fang Jian Zhou[3], Xin Yao Wu[1*]

Abstract

Changes of androgen receptor (AR) and insulin-like growth factor-1 (IGF-1) were investigated in LNCaP cells treated with 5α-dihydrotestosterone (DHT), estrone and flutamide. Real-time PCR, immunocytochemistry and western blotting were used to detect the expression of AR and IGF-1 in the presence or absence of various kinase inhibitors. Low concentrations of DHT, estrone and flutamide increased the expression of AR and IGF-1, especially estrone, with concentration and time dependence. With DHT and flutamide, there was a significant alteration in AR expression ($p<0.001$). The results indicated expression of AR and IGF-1 genes to be influenced by DHT, estrone and flutamide in LNCaP cells, regulated by multiple signal pathways.

Keywords: Androgen receptor - DHT - insulin-like growth factor-1 - LNCaP cells - real-time PCR

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Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer-related deaths among North American men (Jemal et al., 2003; Greenlee et al., 2006). The patients are sensitive to initial anti-androgen therapies. After a medium time of 18 months to 3 years, they will switch to the stage of an androgen-independent prostate cancer (AI-PCa), which is rapidly fatal. Currently, the molecular mechanism of AI-PCa is not completely understood (Naiki et al, 2009; Galbraith and Duchesne, 1997). Recent evidence suggests that both estrone and IGF-1 may regulate the growth, apoptosis and invasion of prostate cancer (LeRoith and Roberts, 2003; Tong and Wu, 2006; Mikami et al., 2009). Clinical and epidemiologic studies indicate that increased serum IGF-I levels are associated with an increased risk of prostate cancer (Djavan et al., 2001). There is consistent evidence, both in vitro and in vivo, that IGF-1 can have an effect on PCa.

To understand fully the role of AR and IGF-I, in prostate cancer, we investigated the expression change of these two genes in LNCaP cells treated with 5α-DHT, estrone and flutamide. The roles of signal pathways such as protein kinase C (PKC); mitogen-activated protein kinase (p38MAPK); extracellular regulated protein kinase (ERK)1/2; and c-Jun NH2-terminal kinase (JNK) were also examined.

Materials and Methods

Cell Culture

LNCaP cells were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai) and maintained in Ham's F12-media (Invitrogen Life Technologies, Inc., Carlsbad, CA) containing 10% fetal bovine serum (FBS); (Invitrogen Life Technologies, Inc) (Thalmann et al., 1994). The cells were grown in Ham's F12 medium, supplemented with 10% fetal calf serum (FCS), 20 mM HEPES buffer, 50 μg/ml gentamicin (growth medium), 100 U/ml penicillin and 100 μg/ml streptomycin sulphate, at 37 °C, in a humidified atmosphere of 5% CO_2. LNCaP cells were seeded on poly-l-lysine coated flasks and wells (Corning incorporated, NY).

Androgen, Estrone, Anti-androgen and Kinase inhibitors Treatments

Cells were seeded in 6-well plates (2.5×10^5 cells/well) and grown for 48 h in growth medium to allow adhesion. They were then washed and cultured in Ham's F12 medium containing 10% FBS for 24 hr. They were then washed again and taken count of cell at this point, considered as time-0, were exposed to DHT, estrone and flutamide complete culture medium to which concentrated of DHT, estrone and solutions were added, for time and dose response studies. F12 is a serum-free medium based on phenol red-free RPMI-1640, supplemented with

*[1]Department of Forensic Medicine, Zhongshan School of Medicine, Sun Yat-sen University, [2]The Third Affiliated Hospital of Sun Yat-sen University, [3]Sun Yat-sen University Cancer Centers, China *For correspondence : wuxinyao@mail.sysu.edu.cn*

DHT, estrone and flutamide, including (2, 5, 10, 20, 50, 100 nmol/L), Control cultures without hormones were supplemented with 0.01% v/v ethanol (0 nmol/L) and 1, 3, 7 days. To evaluate the involvement of the PKC, p38MAKP, ERK and JNK pathway in PCa progress, These cells were incubated with DHT or IGF-1 in the presence or the absence of various kinase inhibitors, including SB202190 (a p38MAPK inhibitor), LY294002 (a PI3 kinase inhibitor), PD98059 (a MEK1/2 inhibitor), and SP600125 (a JNK inhibitor). IGF-1 and all inhibitors used in this study were purchased from Sigma (ST.Louis).

Immunocytochemistry Studies

Cells that were exposed to DHT, estrone and flutamide were collected and fixed with 4% poly-formaldehyde. Immunocytochemistry staining was performed by avidin-biotin immuneperoxidase system (Saichi Biotechnology Ltd, Beijing). The endogenous peroxidase blocked with 3% H_2O_2 in 70% methanol. The nonspecific protein-binding sites were blocked with 3% normal goat serum (Ruite Biological company, Guangzhou) to reduce background staining. A commercially available monoclonal antibody against IGF-1 (1:50 dilution; Labvision Corporation, Suffolk) and a monoclonal antibody against AR (1:100 dilution; Labvision Corporation, Suffolk) were used as the primary antibodies. The optimum working dilutions were determined by serial titration. The sections were visualized using 0.05% (w/v) 3, 3' -diaminobenzidine and 0.010% (v/v) hydrogen peroxide in PBS (10 mM, pH 7.4). These sections were counter stained with hematoxylin and mounted with entellan. Control procedure were undertaken to insure the specificity of immunoreaction. Negative controls were carried out by replacing the primary antibodies with PBS. Screen shots were taken with Olympus Digital camera (DP 20) attached at Olympus BX51 microscope. The intensity of immunocytochemistry staining was graded semi-quantitatively as follows: (–) no immunostaining; (+) weak staining; (++) moderate staining; (+++) strong staining.

Western Blot Analysis

Whole cell lysates were lysed in buffer A (PBS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, a 1× protease inhibitor mixture) (Complete™ Nissui, Tokyo), and 1 mM dithiothreitol, and sonicated, and 50 μg of each sample were processed for SDS-PAGE and electrophoretic transfer to nitrocellulose. IGF-I was determined by immunoblotting with the antibody of IGF-1 and AR (Labvision Corporation, Suffolk). The second antibody with HRP (horseradish peroxidase) was Rabbit anti-mouse polyclonal antibody (Saichi Biotechnology Ltd, Beijing) and visualized by chemiluminescence (ECL; Amersham Pharmacia, Buckinghamshire).

Real-Time Polymerase Chain Reaction

Single-stranded cDNAs were generated from DNase I-treated total RNA isolated using TRIzol reagent (Invitrogen Life Technologies Inc, Austin) and using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies Inc, Austin) according to the manufacturer's instructions. RNA (2 μg) was primed for cDNA synthesis using a random hexamer primer (0.5 μg/μL). The comparative threshold cycle method was used to measure IGF-I and AR mRNA levels under different treatment conditions using the GeneAmp 7300 Sequence Detection System and GeneAmp 7300 system SDS software (Applied Biosystems, Foster City, CA). The probe was labelled with a FAM/TAMRA quencher/ reporter. The β-actin mRNA was a suitable control for normalizing the results to total mRNA levels. The real-time polymerase chain reactions (PCRs) were performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City,CA). The primer sequence of AR: Forward: 5'-AAGCCATTGAGCCAGGTGTAG-3', Tm: 58.6, anneal temperature: 52.4 and numbers of bp: 21. Reverse: 5'-CAAGCTGTCTCTCTCCCAGTTC-3', Tm: 58.9, anneal temperature: 54.5 and numbers of bp: 22. Human AR TaqMan probe: FAM-ACACGACAACAACCAGCCCGACTCCT-TAMRA, Tm: 69.2, anneal temperature: 57.7, numbers of bp: 26, Product size: 105 bp. The primer sequence of IGF-1: Forward: 5'-GCTATGGCTCCAGCATTCG-3', Tm: 58.3, anneal temperature: 52.1 and numbers of bp: 20. Reverse: 5'-GCTCCGGAAGCAACACTCA-3', Tm: 57.9, anneal temperature: 51.9 and numbers of bp: 19. Human IGF-1 TaqMan probe FAM-AGGGCACCTCAGACAGGCATTGTGG-TAMRA. Tm: 67.4, anneal temperature: 56.1 and numbers of bp: 25, product size: 134 bp.

Data Statistical Analysis

Excluding the immunohistochemistry, all results are mean ± SD. Differences in measured variables between the experimental and control groups were assessed by Student's t test. Statistical calculations were performed on a Windows personal computer with the software package SPSS 13.0 (SPSS Incorporated, Chicago, USA). $P < 0.05$ were considered as statistically significant.

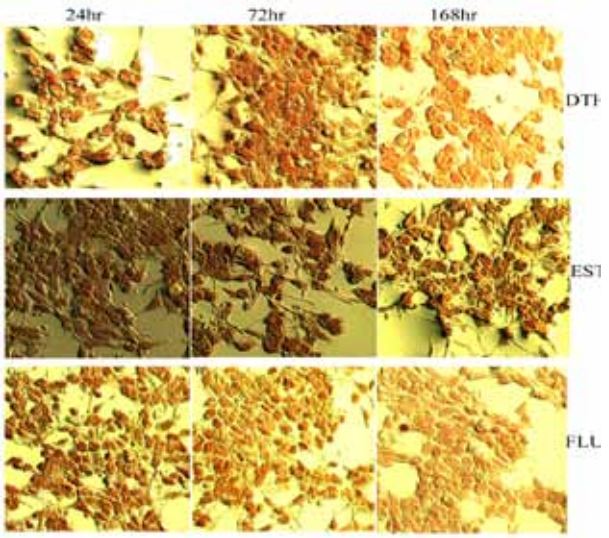

Figure 1. Immunocytochemistry Staining of AR Expression in LNCaP Cells Treated with DHT, Estrone (EST) and Flutamide(FLU). A: DHT ++. B: DHT +++. C: DHT +. D: estrone +. E: estrone ++. F: estrone, +++. G: flutamide ++. H: flutamide +. I: flutamide ± (Original magnifications: A-I ×200)

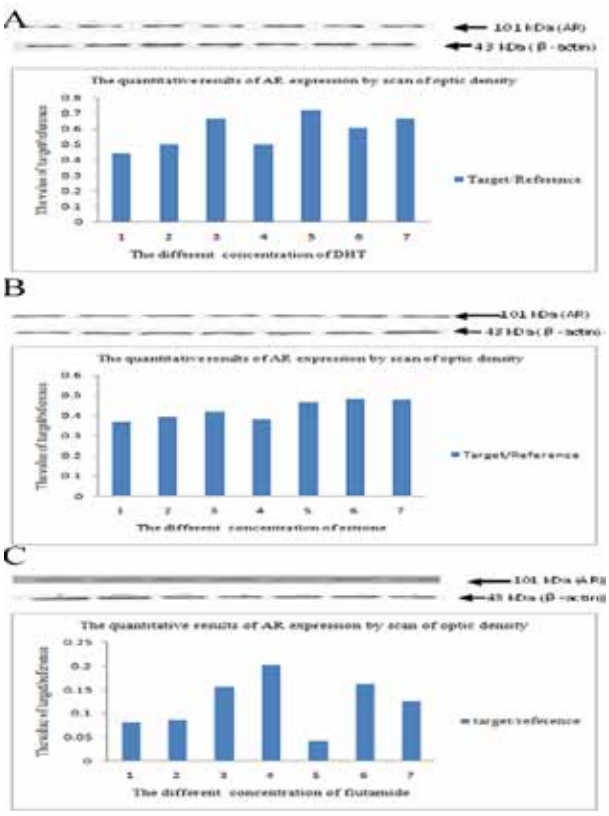

Figure 2. AR Expression in LNCaP Cells Treated with DHT, Estrone or Flutamide. A: Western blot and optic density scan with DHT (upper), B:estrone(middle) or C:flutamide(lower). No:1-7 concentrations of 0, 2, 5, 10, 20, 50 ,100 nmol/L respectively

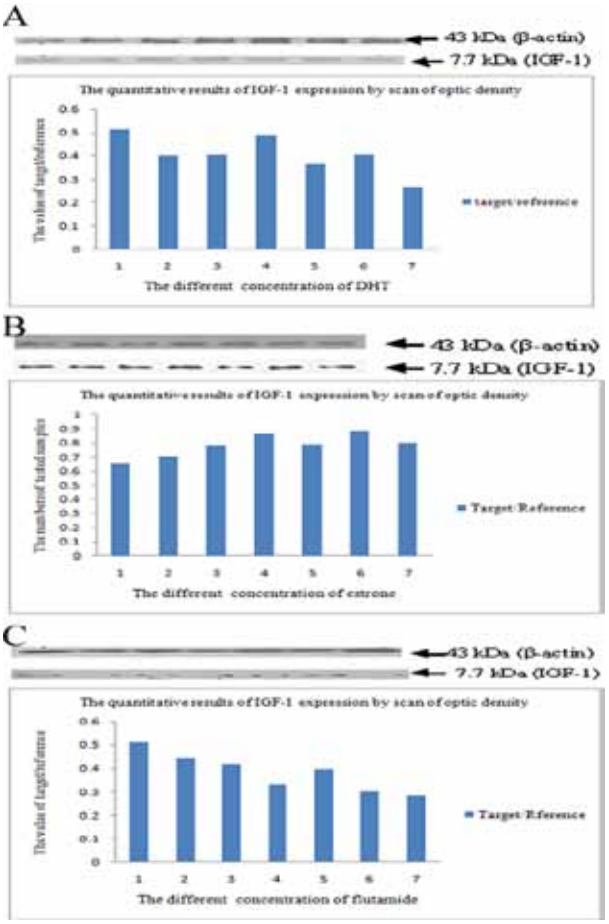

Figure 3. IGF-1 Expression in LNCaP Treated with DHT, Estrone and Flutamide. A: Western blot and optic density scan with DHT (upper), B:estrone(middle) or C:flutamide(lower). No:1-7 concentrations of 0, 2, 5, 10, 20, 50 ,100 nmol/L respectively

Results

Immunocytochemistry Staining

The expression of AR showed a dose- and time-dependent relationship in the concentration range of DHT <20 nmol / L as well as the reaction time <3 days (Figure 1). It was reverse time-dependent on when cells were treated with flutamide. Dose-dependence was also noted with estrone in all studied concentrations. The expression of IGF-1 was not dose-dependent when LNCaP cells were treated with DHT, estrone and flutamide (data not shown).

Quantitative Results for AR and IGF-1 Expression

The quantity and ratio of the AR and IGF-1 expression in target and reference cells with western-blot and optic density scans were shown Figures 2 and 3 (the value of scan and ratio see supplement).

Expression of mRNA of AR and IGF-1 gene in LNCaP cells

The results of AR-mRNA expression in LNCaP cells treated with DHT were significantly difference from those treated with flutamide 2.5 times (p<0.001). The results of AR-mRNA expression in LNCaP cells treated with estrone were not significantly difference from those treated with flutamide (0.61 time, p>0.05). The results of IGF-1-mRAN expression have not been found to be significantly difference, while LNCaP cells treated with DHT and estrone (0.89 times, p>0.05). However, asignificantly difference were found between treated with DHT, estrone and flutamide (8.5 or 9.0 times, p<0.001), Figure 4A-F.

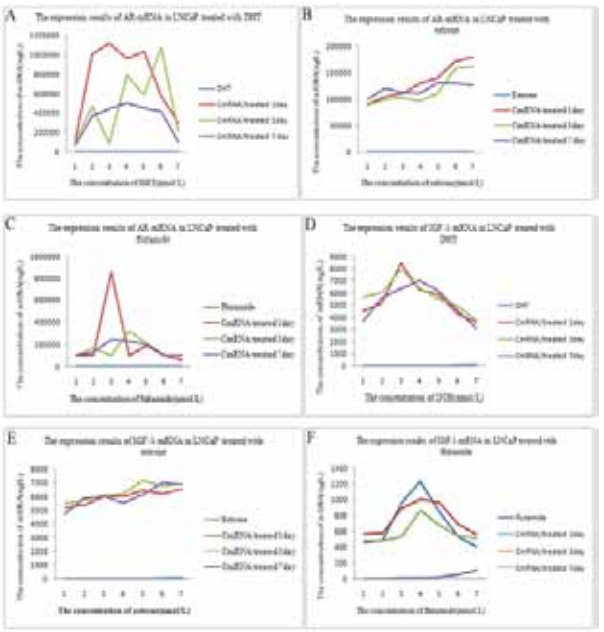

Figure 4. mRNA Expression in LNCaP Cells Treated with DHT, Estrone or Flutamide A, C, E: AR-mRNA expression after 1, 3,7 days with DHT, estrone and flutamide, respectively. B, D, F: IGF-1-mRNA expression after 1, 3,7 days with DHT, estrone and flutamide, respectively

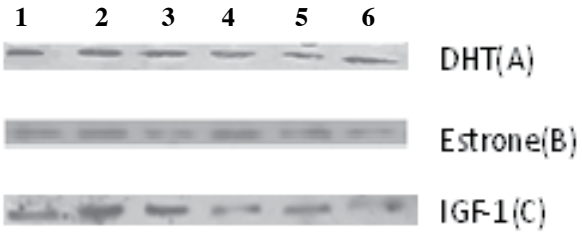

Figure 5. AR Expression when LNCaP Cells were Treated with DHT, Estrone and IGF-1 in Absence or Presence Signal Pathways Inhibitors. A: 1: Control cells. 2: Treated with DHT absence inhibitors; 3: The cells treated with DHT presence SB202190; 4: The cells treated with DHT presence LY294002; 5: The cells treated with DHT presence PD98059, 6: The cells treated with DHT presence SP600125, respectively. B: 1: The control cells. 2: The cells treated with estrone absence inhibitors; 3: The cells treated with estrone presence SB202190; 4: The cells treated with estron presence LY294002; 5: The cells treated with estrone presence PD98059; 6: The cells treated with estrone presence SP600125, respectively.C: 1: The control cells, 2:The cells treated with IGF-1 absence inhibitors; 3: The cells treated with IGF-1 presence SB202190, 4:The cells treated with IGF-1 presence LY294002, 5: The cells treated with IGF-1 presence PD98059, 6: The cells treated with IGF-1 presence SP600125, respectively

AR expression in the presence or the absence of various kinase inhibitors

The inhibitors of PKC, p38MAPK, ERK and JNK suppressed the expression of AR in LNCaP cells when DHT (Figure 5A) and estrone (Figure 5B) presence. It was observed that the inhibitors of PKC, p38MAPK, ERK and JNK suppress the expression of AR in LNCaP cells while IGF-1 presence (Figure 5C). The effects of inhibitors were more significant that PI3, ERK and JNK pathway inhibitors than p38MAPK inhibitor.

Discussion

This study investigated the change of the expression of androgen receptor (AR) and insulin like growth factor 1 (IGF-1) on cellular and molecular levels and analysed the effect of signaling pathway that may be involved in regulation of the expression of AR and IGF-1 thus providing new experimental evidence for clinical practice concerning the influence of hormone on expression and regulation of PCa cells.

This study found that after processed by DHT, the LNCaP cell showed a dose- and time- dependent relationship betewwn expression of AR and concentration range of DHT (<20 nmol / L) as well as the reaction time (<3 days). The mRNA expression level detected by RT-PCR was similar with the protein expression level by immunocytochemistry and Western Blot. LNCaP cells showed tolerance to high concentration of DHT, and decreased expression of AR was a typical performance of androgen-independent cells (Wang et al., 2005). These results are partially consistent with those report by (Lv et al., 2007; Narimoto et al., 2010). The expression of AR was proportional to LNCaP cells processed by estrone in the range of experimental time and concentration. There was no tolerance phenomenon showed between AR-mRNA expression and function of estrone of the experimental concentration and time. Similar results or mechanisms have not been reported yet. As an "exception" phenomenon, flutamide of a low concentration could stimulate the expression of AR in LNCaP cells has been reported (So et al., 2005; Takahara et al., 2009). The above "adaptation" and "tolerance" phenomenon of cancer cells indicates that prostate cancer cells are sensitive to non-specific ligands other than specific ligands.

DHT and estrone could also stimulate the expression of IGF-1 in LNCaP cells, while flutamide could inhibit the expression. The effect of flutamide on the expression of IGF-1 was different from that on AR due to the different cell conduction systems and cytokines participated in the IGF expression and regulation (Fuse et al., 2007). After treated by DHT, estrone and flutamide, IGF-1 expression level of LNCaP cells showed no obvious change compared with that of AR. The promoting effect of DHT on IGF-1-mRNA was consistent with the effect reported by Genua et al., (2009). In addition, there was an interactive reaction between expression of AR and IGF-1 with a synergistic effect.

After detection of cells affected by DHT, estrone, and IGF-1 with signaling pathway inhibitor, the different impact degrees of PKC, MAPK, ERK and JN inhibitors on the expression of AR were found (Genua et al., 2009), in particular JNK, MEK1/2 pathway inhibitors had more obvious effect on the expression. Findings of the study have instruction significance for the clinical treatment and medication of prostate cancer, which would help the diagnosis and treatment of androgen dependent prostate cancer, and also provide experimental evidence for the pathogenesis of androgen-independent prostate cancer.

This study also has some defects. We found that AR expression induced by GF-1 is influenced by p38MAPK, MEK1/2 and JNK signaling pathway inhibitor, and this mechanism needs further research. And no research has been done concerning the influence concentration and time of AR and IGF-1 expression by estrone .

In conclusion, this study found that: 1. DHT and estrone could induce the expression of AR and IGF-1, while AR and IGF-1 have certain synergy effect. 2. Flutamide in a low concentration could induce an increased expression of AR, while high concentrations of Flutamide could inhibit the "tolerance" performance of the cancer cells. 3. IGF-1 could induce the expression of AR, which is consistent with the characteristics of androgen-independent cancer cells reported previously (Nickerson et al, 2001; Yanase et al,. 2009). The emergence and development of androgen-independent cancer cells is related with its corresponding signaling pathway, which further explains the autocrine role of IGF-1 in the development of AI (Schaffer et al., 2003).

This study also proved that LNCaP cell line is ideal for investigation of the expression of AR and IGF-1 and the regulation of signaling pathway of prostate cancer in vitro, which could also be a transformation model from AD to AI to elucidate the pathogenesis of AI, which explains the phenomenon of "tolerance" and "exception" found in this study.

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