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Research Article

Propranolol Inhibits Androgen Deprivation-induced Neuroendocrine Differentiation of Prostate Cancer

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Abstract

Background and Objective: Clinical studies have shown that castration-resistant prostate cancer (CRPC) is the ultimate cause of death in men with advanced prostate cancer. Among them long-term androgen deprivation therapy (ADT)-induced neuroendocrine differentiation (NED) and neuroendocrine prostate cancer (NEPC) are most important causes to CRPC. NED is a kind of undifferentiated small cell carcinoma with high-invasiveness which could be induced by long time ADT, leading to a highly invasive and metastatic clinical process. Therefore, it is a current research hotspot to develop targeted therapeutic drug for NED. This study aimed to investigate the role of propranolol in the progression of NED induced by ADT and the related mechanisms. **Materials and Methods:** Androgen receptor (AR) antagonist enzalutamide (MDV3100) was used to induce androgen deprivation. MTT and cell migration assays were performed to assess the effect of propranolol on cell viability and migration. Western Blot was used to measure the expression of NED/NEPC biomarkers such as CHGA, ENO2 as well as CREB (cAMP response element binding protein) and phosphorylation level of CREB (p-CREB). A qRT-PCR was performed to test the mRNA level of CHGA, ENO2, KLK3. **Results:** ADT promoted prostate cancer cell growth and migration, up-regulated the CHGA expression and down-regulated AR expression. Propranolol could reverse the NED progression through inhibiting CREB phosphorylation and finally down-regulating CHGA expression. **Conclusion:** Propranolol might suppress the progression of NED via inhibiting CREB phosphorylation possibly.

Key words: Propranolol, prostate cancer, neuroendocrine differentiation, androgen deprivation, *in vitro* assay, off-label use, gene expression, adenocarcinoma, androgen receptors

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Prostate cancer is a common malignant tumor in the male genitourinary system. The latest global cancer statistics indicate that prostate cancer has become the second most common malignant tumor after lung cancer[1]. In the United States, the incidence of prostate cancer is even higher than that of lung cancer with ranking first[2]. In recent years, the incidence of prostate cancer in China is gradually increasing. Age effect on incidence and mortality presented an increasing trend in older people and period effect showed increasing trends. The incidence rate of prostate cancer is increasing at an alarming rate in all age groups, which may adversely impact the mortality rates. Mortality began to increase since 2005; thus, timely intervention should be conducted, especially for earlier birth cohorts at high risk[3]. However, because of the hidden symptoms of prostate cancer, about 50% of patients have progressed to locally advanced tumors at the time of diagnosis. Metastasis and recurrence are the main causes of death. Therefore treatment and prevention of metastasis are particularly important to improve survival rate.

Neuroendocrine cells have been recognized as a component of normal prostate ducts and acini. Nearly 50% of prostate cancer emerged neuroendocrine cell mutation. Neuroendocrine differentiation (NED) of prostate cancer also known as undifferentiated small cell carcinoma is a highly aggressive subtype of prostate cancer[4]. It can be primary, but most of it comes from advanced prostate adenocarcinoma after hormone therapy. Clinical studies have shown that long-term androgen deprivation therapy (ADT) can induce neuroendocrine transformation of cells[5]. Therefore, the incidence of castration-resistant prostate cancer (CRPC) may increase with the introduction of ADT, leading to a highly invasive and metastatic clinical process[6].

The poor prognosis of neuroendocrine prostate cancer (NEPC) is associated with neuroendocrine differentiation (NED) of prostate cancer cells. Up to date the treatment outcome of chemo- and/or radio-therapy for NEPC is far from satisfactory, with a short survival time. The progress on molecular mechanism studies involved in NEPC is also limited. Therefore, elucidating the molecular mechanism of NED and developing targeted therapeutic drugs for NED are currently a research hotspot in the field of prostate cancer.

During NED progress, cells usually highly expressed neuroendocrine biomarker such as chromogranin A (CHGA) and specific enolase 2 (ENO2)[7]. cAMP responsive element binding protein(CREB) is a transcription factor in the leucine zipper family. It is a proto-oncogene and its expression in tumor tissues is significantly higher than that in paracancerous tissues. The expression and activity of CREB in various tumors, such as leukemia[8], melanoma[9] and breast cancer[10], are significantly increased. The increase in intracellular phosphorylated CREB (pCREB) levels induces the expression of the downstream gene cAMP response element (CRE), thereby regulating cell survival, proliferation and differentiation. In androgen receptor positive (AR^+) prostate cancer cells, CREB has been shown to act as an AR coactivator in transcriptional activation of AR target genes[11]. However, it is still largely unclear how CREB activation promotes AR in different NED.

Propranolol, a widely used non-selective β-adrenergic receptor blocker, was recently shown to display anticancer properties. Its potential to synergize with certain drugs has been also outlined[12-14]. A new prospective clinical cohort study demonstrated that propranolol adjuvant therapy can reduce 80% recurrence in melanoma patients[15]. Propranolol could also block CREB-mediated intracellular signal transduction to prevent the development of pancreatic ductal adenocarcinoma[16]. Latest research report that treatment of cancer cells with propranolol in combination with the glycolysis inhibitor induced a massive accumulation of autophagosome due to autophagy blockade. The propranolol+glycolysis inhibitor treatment efficiently prevents prostate cancer cell proliferation, induces cell apoptosis, alters mitochondrial morphology, inhibits mitochondrial bioenergetics and aggravates ER stress *in vitro* and also suppresses tumor growth *in vivo*[17]. This study hypothesized for the first time that propranolol might inhibit the progression of NEPC by inhibiting the phosphorylation of CREB and thus decreasing pCREB levels, underlining the interest to take advantage of the ability of propranolol to broaden new anti-cancer therapies.

MATERIALS AND METHODS

Cell culture: The human prostate cancer cell lines LNCaP, PC3 and endothelial cell line SVEC4-10 were purchased from Shanghai Cell Bank, Academy of Sciences and stored in a laboratory at Fengxian Hospital, Shanghai. Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlbad, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), 100 IU mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin at 37°C and 5% CO_2 in a humidified atmosphere. Cells were passaged once every 4 days. Cells in the logarithmic growth phase were selected for experiments. Microscope was used to observe morphological change under different treatment.

Reagents: Propranolol was purchased from Sigma-Aldrich (St. Louis, USA). Enzalutamide (MDV3100), androgen receptor antagonist, was purchased from Adooq Bioscience (Irvine, USA). Propranolol and enzalutamide stock solution was prepared at a concentration of 10 and 80 μM, respectively and filtered through a 0.22 μm membrane, dispensed in a 1.5 mL centrifuge tube and stored in the dark at -20°C. DMEM was used to dilute and prepare series working concentration solutions. The following supplies and reagents were used: CHGA rabbit polyclonal antibody (Abcam, 1:100), β-actin mouse monoclonal antibody (Abcam), rabbit anti-mouse IgG (Merck) and goat anti-rabbit IgG (Sigma). SYBR Green fluorescence-based quantitative real-time-polymerase chain reaction (qRT-PCR) reagent kit (TaKaRa), 0.25% trypsin (Gibco, USA), fetal bovine serum (FBS) (Hangzhou Sijiqing Co., Ltd.), primer synthesis (Invitrogen, USA), 0.22 μm polyvinylidene fluoride (PVDF) membrane (Millipore) and Annexin V-FITC/PI apoptosis detection kit (Sigma, USA). Other biochemical reagents including dihydrotestosterone (DHT) were purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China).

Effect of androgen deprivation treatment on neuroendocrine differentiation: LNCaP cells were incubated with or without enzalutamide (MDV3100) 10 μM for 48 h. Cell morphology was observed under microscope to compare the difference. Cells treated with enzalutamide were further divided into 2 groups, one continued with another 24 h of enzalutamide treatment and the other followed by 24 h of enzalutamide and 5 nM dihydrotestosterone (DHT) co-treatment. The 3 groups of cells were collected for mRNA and protein expression determination.

Western blot analysis: The cells were lysed for 1 h at 4°C in a lysis buffer (30 mM Tris, 200 nM NaCl, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 20% Glycerol, 1% NP-40, 1 mM DTT) containing a protease inhibitor cocktail. Protein concentrations were determined using Pierce BCA protein assay kit (Thermo Scientific). The samples were then separated by 10% SDA-PAGE and transferred to PVDF membranes (Bio-Rad). The membrane was then blocked with 5% milk in TBST for 1 h at room temperature, followed by incubation of a primary antibody overnight at 4°C. Primary antibody used were as follows: the anti-β-actin and anti-CHGA antibodies (1:1000, Santa Cruz), anti-pCREB, anti-CREB, anti-AR antibodies (Cell Signaling Technology, 1:1000). After three times of washing with PBS, the HRP-conjugated secondary antibodies were added and incubated for 2 h at room temperature. The lots were then detected by Pierce ECL Western Blot Substrate (Thermo Scientific) on Blue Basic Autoradiography Films (Bioexpress).

Table 1: Primers used in real-time PCR

Genes	Direction	Sequences (5′-3′)
CHGA	Upward	TACAAGGAGATCCGGAAAGG
CHGA	Reverse	CCATCTCCTCCTCCTCCTCT
ENO2	Upward	CTGTGGTGGAGCAAGAGAAA
ENO2	Reverse	ACACCCAGGATGGCATTG
β-actin	Upward	CCAACCGCGAGAAGATGA
β-actin	Reverse	CCAGAGGCGTACAGGGATAG
KLK3	Upward	CGTGACGTGGATTGGTGC
KLK3	Reverse	GCCGCAGACTGCCCT G

qRT-PCR: Treated LNCaP cells were collected. A total RNA Isolation Kit (Qiagen) was used to extract total RNA and measure RNA concentration and integrity. Reverse transcription of RNA into complementary DNA (cDNA) was conducted using a Prime Script Reverse Transcription Kit (Takara) according to the information provided in the kit. The reaction conditions were as follows: 37°C for 15 min and 85°C for 5 sec. Real-time PCR was performed using a SYBR Prime Script Real Time-PCR Kit (Takara) and the reaction system was configured based on the information provided by the kit. The reaction system was as follows: 95°C for 5 min; 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. β-actin was used as an internal reference. The primer sequences were shown in Table 1.

MTT assay for cell viability evaluation: LNCaP cells were treated with various concentrations of enzalutamide (0, 2.5, 5, 10, 20, 40 and 80 μM) for 48 h. Cells in the logarithmic growth phase were collected, adjusted to a cell density of 1×10^4 mL^{-1}, seeded with 100 μL/well in96-well plate and divided into the propranolol (10 μM) or non-propranolol treatment group. The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay was performed at 72 h. About 10 μL (5%) of MTT solution was added to each well and the cells were cultured in an incubator at 37°C with 5% CO_2. After culturing for 3 h, the supernatant was removed and 150 μL of DMSO was added to each well, the plate was then placed on a shaker for 30 min. The absorbance at 490 nm was measured with a microplate reader. Each group consisted of 4 parallel wells and the experiment was repeated 3 times.

Cell migration assay: LNCaP cells were divided into the blank control group, the M group (enzalutamide/MDV3100 treatment for 48 h) group and the MP group (enzalutamide/

MDV3100 treatment for 48 h followed by propranolol treatment for 24 h). These 3 groups of LNCaP cells above and other SVEC-10 endothelial cells were grown to 70% confluence and then continued to starvation culture in serum-free medium overnight. The SVEC4-10 starvation cells were harvested with trypsin and re-suspended in serum-free medium. A total of 5×10^4 starvation cells were seeded into the upper chamber of Boyden chamber (8 μm, BD Biosciences), meanwhile conditioned medium obtained from above mentioned 3 groups of starvation LNCaP cells was added to the lower chamber as the inducer. SVEC4-10 endothelial cells were then allowed to migrate for 4 h. The upper chamber was fixed, stained with crystal violet dye and photographed under a microscope to observe migrated cells. The migration of cells in each group was qualitatively analyzed by calculating the number of migrated cells in each high-power field.

Cells transfection: LNCaP and PC3 cells were transfected with CREB short hairpin RNA (shRNA) lenti viruses. The cell density for each well in 6-well plates was adjusted to 4×10^5 mL^{-1} 24 h before transfection. The virus was infected with Polybrene virus infection solution. The CREB shRNA target sequence used in this study was 5'-GAGAGGTCCGTCTAATG-3'. The CREB specific shRNA coding plasmid (SABioscience) or empty vector (used as a control) was transferred to each well. The cells were incubated overnight at 37°C in an incubator. Puromycin (2 μg mL^{-1}) was added to each well and the cells were cultured for approximately 3 weeks to select purine-resistant colonies.

Statistical analysis: Data are expressed as Mean±standard error. Statistical analysis was performed using GraphPad Prism software. Data were first tested for homogeneity of variance and then one-way analysis of variance (ANOVA) was performed. The Dunnett method was used for intergroup comparisons. The $p<0.05$ was considered statistically significant.

ADT promote the neuroendocrine differentiation: LNCaP cells were incubated with adrenergic receptor (AR) antagonist enzalutamide (MDV3100) 10 μM for 48 h. Under microscope cell morphology was witnessed as spindle shaped cells with more tentacle compared with control (Fig. 1a). The western

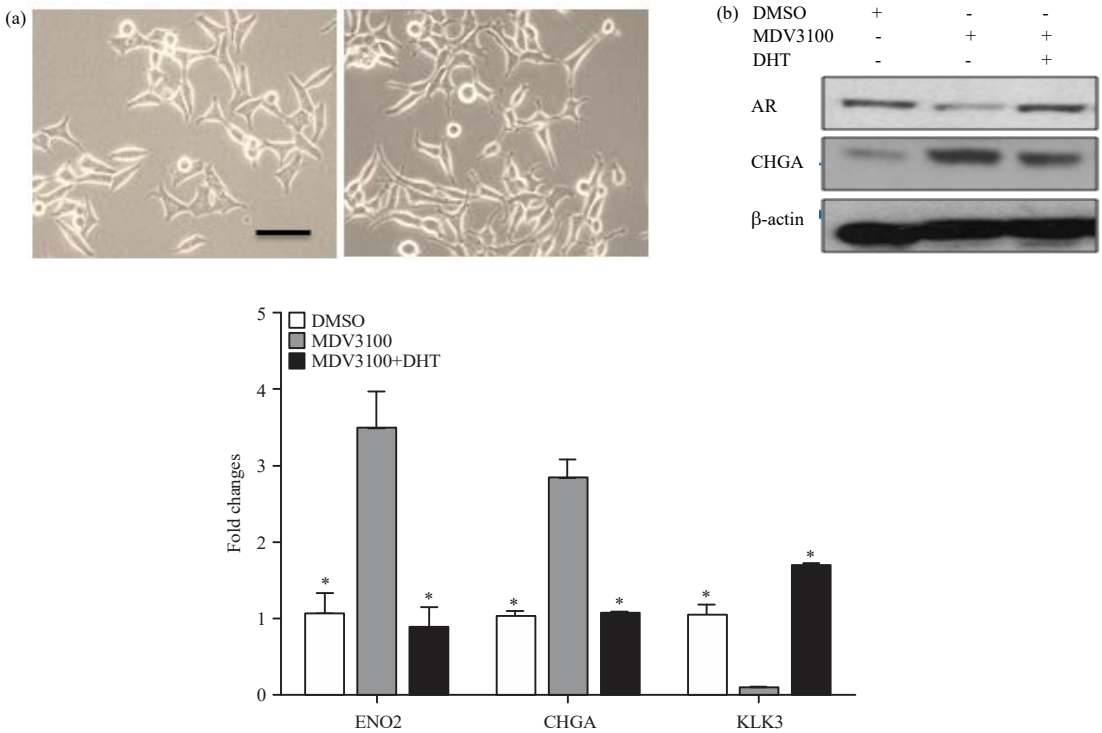

Fig. 1(a-c): (a) Control cell morphology and enzalutamide (MDV3100) treated cells morphology, (b) Protein expression of AR and CHGA in LNCaP cells incubated with enzalutamide (MDV3100) (10 μM, 72 h) and DHT (5 nM, 24 h) and (c) mRNA expression of ENO2, CHGA and KLK3 in different treatment
*$p<0.05$ compared with the MDV3100 group, Scale bar: 50 μm

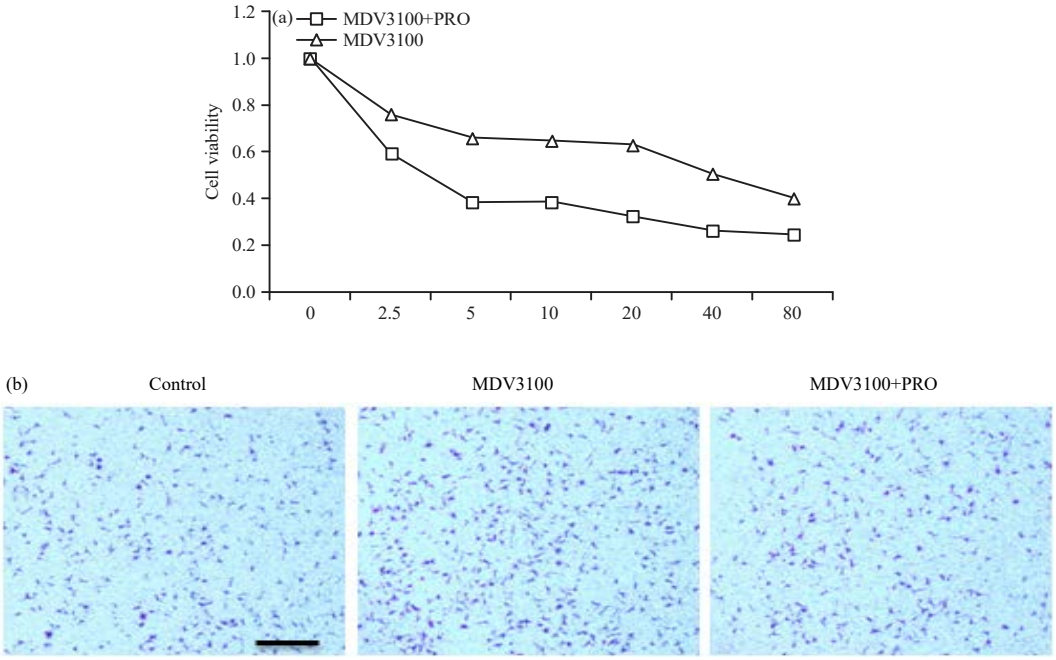

Fig. 2(a-b): (a) LNCaP cell viability treated by enzalutamide alone and with propranolol (10 μM L^{-1}, 72 h) and (b) Cell migration with different treatment, from left to right: Control: Enzalutamide treatment (MDV3100, 10 μM, 72 h), Enzalutamide+propranolol co-treatment (MDV310010 μM, 72 h+propranolol 10 μM, 24 h)
Scale bar = 50 μm

blot results showed that after treatment with the enzalutamide(MDV3100,10 μM, 72 h), the expression of AR was down-regulated meanwhile the expression of CHGA was up-regulated, which meant LNCaP underwent neuroendocrine differentiation (NED). However, if the cells were co-treated by dihydrotestosterone (DHT, 5 nM, 24 h), the regulation effects of enzalutamide were partly reversed (Fig. 1b). qRT-PCR results also confirmed that enzalutamide treatment down-regulated the mRNA expression of the prostate-specific antigen KLK3 but up-regulated the NED biomarkers ENO2 and CHGA, which was also partly reversed by androgen DHT (Fig. 1c).

Propranolol inhibits cell viability and migration of prostate cancer treated by ADT: After 72 h the cell viability assay was conducted and data was shown in Fig. 2a. Boyden chamber cell migration assay results were shown in Fig. 2b. The figure demonstrated that compared with enzalutamide (MDV3100) group, propranolol co-treatment inhibited cell viability and decreased cell migration.

Propranolol inhibits CREB phosphorylation: As cAMP responsive element binding protein(CREB) is a transcription factor in the leucine zipper family and highly expressed in tumor tissues, we compared the CREB and CHGA expressions both in normal LNCaP and in normal PC3 cells. Figure 3a displayed that CREB and CHGA expression in PC3 cells were less than those in LNCaP cells since LNCaP cells have neuroendocrine character. When CREB was knockdown with transfecting shRNA lenti viruses in LNCaP cells, the CREB expression was significantly reduced compared to control (Fig. 3b). Furthermore with CREB knockdown, CREB, as well as pCREB and NED biomarker CHGA expression were down-regulated. Co-treatment with enzalutamide (MDV3100) and shCREB could up-regulated CREB, pCREB and CHGA expression (Fig. 3c). Co-treatment with propranolol could inhibit pCREB and CHGA expression rather than CREB expression (Fig. 3d). The underlying mechanism is not clear.

DISCUSSION

The incidence of treatment-related neuroendocrine prostate cancer is rising as more potent drugs targeting the androgen signaling axis are clinically implemented. Neuroendocrine differentiation is induced by androgen-deprivation therapy (ADT) or anti-androgens and by activation of the β2-adrenergic receptor in prostate cancer cell lines[18]. Thus, understanding whether β2-adrenergic receptor and its blockers are involved in ADT-initiated neuroendocrine

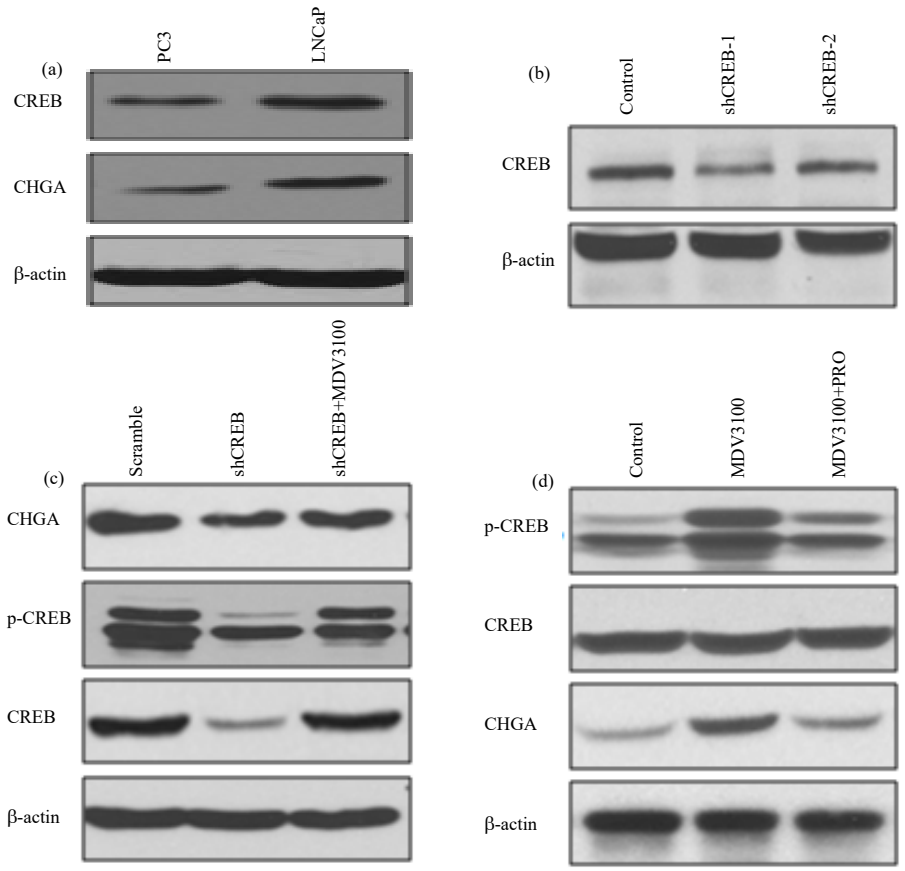

Fig. 3(a-d): (a) CREB and CHGA expression in PC3 and LNCaP cells, (b) CREB knockdown (shRNA) reduce CREB expression in LNCaP cells, (c) p-CREB and CHGA in LNCaP-shCREB-1 cells reduction with CREB knockdown and (d) Propranolol inhibit p-CREB and CHGA expression rather than CREB expression

differentiation may assist in developing treatment strategies that can prevent or reverse neuroendocrine differentiation emergence, thereby prolonging therapeutic responses. A potential application of β-blockers is suggested to prevent cancer cells committed to a neuroendocrine lineage from evolving into neuroendocrine differentiation. Based on the hypothesis, we investigated the effect of propranolol, anon-selective β-adrenergic receptor blocker, on ADT-induced neuroendocrine differentiation. We found that ADT promoted prostate cancer cell growth and migration, up-regulated the CHGA expression and down-regulated AR expression. For the first time we found that propranolol reversed the NED progression through inhibiting CREB phosphorylation and finally down-regulating CHGA expression. Thus, propranolol might suppress the progression of NED via inhibiting CREB phosphorylation possibly.

Androgen deprivation therapy (ADT) is one of important treatment tool in prostate cancer, however, long time ADT might develop NED, NEPC and finally lead to fatal castration-resistant prostate cancer (CRPC). The progression from CRPC to is a major negative consequence of prostate cancer therapy[19]. It is generally believed that in NEPC, prostate-specific antigen (PSA) expression is decreased and NE biomarker CHGA and ENO2 expression is up-regulated. Yuan *et al.*[6] found that ADT can induce the development of NED and androgen-free medium can promote NEPC biomarkers ENO2 and CHGA expression in LNCaP cells, with cell morphology similar to that of NE cells, which is consistent with our data in this study.

CREB is a transcription factor that is highly expressed in a variety of tumors. In non-small cell lung cancer patients, CREB expression and its phosphorylation level are higher than those in the normal population[20]. CREB also plays a role as a proto-oncogene in leukemia[21] and a reduction inpCREB levels significantly reduces the growth and migration of human melanoma[6]. The molecular mechanisms may be related to the regulation of Bcl-2 expression and blocking the binding sites of cyclin A and cyclin D1[22]. CREB has been shown to promote

the progression of prostate cancer[23]. In this study, we found that the CREB expression might be accompanied by NE biomarkers expression in LNCaP cells with neuroendocrine character. However, in spite of CREB knockout and rogen receptor antagonist enzalutamide (MDV3100) could induce CREB and p-CREB expression. Our preliminary study found that the p-CREB and NE biomarker CHGA expression could be inhibited by propranolol. Propranolol can inhibit tumor progression through a variety of mechanisms. It can inhibit the activation of norepinephrine-mediated SCR to delay the progression of ovarian cancer and inhibit stress-induced macrophage infiltration and inhibit tumor metastasis to distant tissues[24]. Because propranolol has been used clinically for hypertension and cardiovascular disease for decades, its safety is not in question. The mechanism is worth studied further.

CONCLUSION

This study found that propranolol might inhibit the neuroendocrine differentiation of prostate cancer cells induced by androgen deprivation therapy.

SIGNIFICANCE STATEMENTS

This study discovers that the propranolol might suppress the progression of neuroendocrine differentiation of prostate cancer cells induced by androgen deprivation that can be beneficial for combination chemotherapy. This study will help the researcher to uncover the possible mechanisms of propranolol to inhibit differentiation.

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