Short Communication

Androgen Receptor Blockade Using Enzalutamide Suppresses Long Non-Coding RNA *ARLNC1* in Prostate Cancer Cells

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Abstract

Prostate cancer (PCa) is a common malignant disease with high mortality rates that develops and progresses in an androgen-dependent way. In recent years, RNA sequencing enabled identification of many PCa-related long non-coding RNAs including androgen receptor-regulated long non-coding RNA 1 (*ARLNC1*) and prostate cancer-associated transcript 1 (*PCAT1*). In the present study, our goal was to illuminate expression changes of *ARLNC1* and *PCAT1* in the context of androgen stimulation or androgen receptor (AR) blockade with respect to AR expression status. In this experimental study, LNCaP cells and higher AR-expressing LNCaP-AR++ cells were used as cell models. Cells were treated with dihydrotestosterone (DHT) as an androgen stimulator and/or enzalutamide as an AR inhibitor. Cell viability was assessed using annexin V and propidium iodide (PI) staining in flow cytometry. Androgen stimulation prompted baseline *ARLNC1* levels by 53.5-fold in the LNCaP cells (P=0.01) and by 25-fold in the LNCAP-AR+ cells (P=0.18). AR inhibition by enzalutamide reduced baseline *ARLNC1* in LNCaP-AR++ cells by 2-fold (P=0.01), but to a lesser extent in LNCaP cells. Co-treatment of cells with DHT and enzalutamide led to a remarkable decrease in the DHT effect on *ARLNC1* expression. No specific effect of androgen stimulation or AR blockade on *PCAT1* expression was detected. Our results revealed that the extent of induction of *ARLNC1* by androgen is modulated by receptor expression status. In addition, we determined that AR blockade, via enzalutamide, effectively suppresses *ARLNC1* both at baseline and after induction by DHT.

Keywords: Androgen, Androgen Receptor, Enzalutamide, Long Non-Coding RNAs, Prostate Cancer

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Prostate cancer (PCa) is the second most common malignancy in men with an incidence of approximately 36 cases per 100,000 worldwide and in Turkey (1). In most of patients diagnosed with PCa, the disease is locally confined to the prostate and does not affect their natural life expectancy following the appropriate treatment options. However, in some patients, the disease may progress and metastasize to other sites outside the prostate, significantly shortening life expectancy. Indeed, 5-year survival in patients with metastatic PCa is only around 30% (2). Although many molecular/epigenetic alterations occur in the development and progression of PCa, the most fundamental component of prostate carcinogenesis is the androgen receptor (AR) pathway, as development and progression of PCa is androgen-dependent (3). Thus, suppressing androgen pathway by surgical or medical castration is the path to treatment of the disease. A higher response rate is achieved with androgen deprivation in the majority of patients. However, due to the increased receptor expression or emergence of mutations leading to resistance of the receptor to inhibitors, many patients with metastatic disease progress to castration-resistant PCa (CRPCa) (4). Recently, the second-generation of AR inhibitor, enzalutamide, has been established as a treatment option in patients with metastatic CRPCa (5).

Identification and characterization of non-coding RNAs have been some of the major advancements in molecular cell biology in the last two decades (6). Non-coding RNAs include a wide range of RNA types, among which long non-coding RNAs (lncRNAs), with a diverse variety and uncharacterized biologic functions, constitute a significant sub-group. So far characterized lncRNAs have been shown to be implicated in the regulation of many molecular and cellular events such as gene expression and chromatin regulation, epigenetic regulation, genomic imprinting, differentiation, cell growth and death (7). High-throughput RNA sequencing (RNA-seq) studies have enabled identification of oncogenic and tumor-suppressing lncRNAs that contribute to the pathogenesis of PCa, as with other cancers.

Androgen receptor-regulated long non-coding RNA 1 (*ARLNC1*) is a recently identified cancer-related lncRNA and it has been shown to be associated with AR signaling in PCa. *ARLNC1* and AR protein mutually regulate each other, because *ARLNC1* is induced by AR, whereas *ARLNC1* stabilizes AR transcript via RNA-RNA interaction (8). Prostate cancer-associated transcript (*PCAT1*) is a PCa-overexpressed lncRNA and it has been shown to induce proliferation of PCa cells in a c-Myc-dependent way (9, 10). Herein, we intended to elucidate *ARLNC1* and *PCAT1* expression changes in the context of androgen stimulation or AR blockade with respect to AR expression status, where two cell lines (LNCaP and LNCaP-AR^{++}) were employed as cellular models with differential AR expression.

In the present experimental study, we used the LNCaP and LNCaP-AR^{++} PCa cell lines. LNCaP cells were obtained from a lymph node metastasis of the patient with prostate adenocarcinoma in 1977 that expressed AR and thus was androgen-sensitive (11). The LNCaP-AR^{++} cell line is genetically engineered from ancestral LNCaP cells and expresses more AR (5). The LNCaP cell line (ATCC# CRL-1740) was previously acquired from the American Type Culture Collection. The AR^{++} cell line was attentively provided by Memorial Sloan Kettering Cancer Center (New York, USA). Standard cell culture protocols were followed to handle the cells. LNCaP and LNCaP-AR^{++} cells were grown in standard growth conditions (5% CO_2, 95% humidity at 37˚C) with RPMI, as the growth medium with added fetal bovine serum (FBS, 10%, Biochrom, Germany) and antibiotics (1% penicillin and streptomycin).

For androgen stimulation in PCa cells, we used dihydrotestosterone (DHT), as a potent agonist of ARs that binds to tissue ARs more strongly than its precursor testosterone, thus harboring a more pronounced androgenic effect at lower concentrations (12). A stock solution (100 mM) was prepared by dissolving DHT in ethanol and stored at -20˚C in aliquots. Enzalutamide, a non-steroidal anti-androgen currently used in the treatment of metastatic CRPCa, was used as an AR inhibitor and it was kindly provided by Aztellas. Enzalutamide was dissolved in dimethyl sulfoxide (DMSO) and stored at -20˚C as 1 mM stocks. Enzalutamide acts in multiple ways either by blocking the binding of androgens to the AR and transition of active AR into the nucleus or by preventing AR binding to its binding sites in DNA (5).

Before treating cells with DHT and/or enzalutamide, serum growth hormones were removed from FBS using charcoal treatment, as described previously (13). For DHT and/or enzalutamide administration, approximately 200000 cells were plated into each well of six-well Petri dishes and they were kept in hormone-free medium conditions for 24 hours. The cells were then further cultured for 24 hours in a complete medium containing 10 nM DHT (14) and/or 10 µM enzalutamide (15). We considered induction and suppression of prostate-specific antigen (PSA) expression, as proof of effective androgen stimulation by DHT or AR inhibition by enzalutamide, respectively (data not shown).

For assessing effect of AR blockade on the viability of cells, we utilized annexin V and propidium iodide (PI) staining assay using the Annexin V Apoptosis Detection Kit (Thermo Fisher Scientific, USA) in the flow cytofluorimetric analysis. Annexin V staining is aimed to detect cellular apoptosis, whereas PI detects necrotic or late apoptotic cells that lose membrane integrity. In brief, the cells were harvested and adjusted to 1×10^6 cells/100 µl in appropriate tubes. After several wash steps, the cells were resuspended in 100 µl of 1x annexin V binding buffer, followed by adding 5 µl annexin V-FITC and 1 µl PI from 100 µg/ml stock. Subsequently, they were incubated in dark (15 minutes). Fluorescence measurement was performed with a FACSCalibur® flow cytometer (BD Biosciences, USA) and analyzed using the CellQuest software. The generated data were illustrated in two-dimensional dot plots in which PI was displayed versus annexin V-FICT. The Q2-3 in the plots represented (Fig.1) viable cells that were not stained with either probe; Q2-4 included early apoptotic cells that were only positive for annexin V; in Q2-2, late apoptotic cells were compromised and they were positive for both dyes, while the cells in Q2-1 were necrotic/dead cells and only stained with PI.

Expression of the target molecules was determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For this, total RNA was isolated from the cells using a commercially available RNA isolation solution (Life Technologies, USA) according to the instructions. Integrity and purity of the RNA were checked using electrophoresis and spectrophotometrically. RNA was used as a template to synthesize complementary DNA (cDNA) according to the instructions of the kit (Life Technologies, USA), which was then used as a template in qRT-PCR. The assay included the primer sets displayed in Table 1 and the SYBR Green dye (Life Technologies, USA) for fluorescence detection. The PCR protocol included initial denaturation of 10 minutes and 40 cycles of amplification according to the instructions of the SYBR Green assay kit and run in a LightCycler 480 PCR device (Roche Diagnostics, Germany). Expressions of *ARLNC1* and *PCAT1* in the PCa cells were determined semi-quantitatively using the housekeeping gene *GAPDH*, as the internal control, and their relative expression was calculated using the $2^{-\Delta\Delta cCt}$ method.

Outcomes of the three independent qRT-PCR measurements were used for inter-group comparisons. Changes relative to baseline levels in the control cells were expressed as 'fold changes,' and mean values were statistically compared using an independent sample t test between test groups.

Table 1: The utilized primer sequences

Gene	Primer sequences (5′-3′)	Reference
ARLNC1	F: CCTTGTCCACTGGAACTCGT R: TATAACCTTGGGGGCCATGA	(8)
PCAT1	F: TGAGAAGAGAAATCTATTGGAACC R: GGTTTGTCTCCGCTGCTTTA	(9)
PSA	F: TGAACCAGAGGAGTTCTTGAC R: CCCAGAATCACCCGAGCAG	(16)
AR	F: TATCCCAGTCCCACTTGTGTC R: CTTGTGCATGCGGTACTCATTG	(17)
GAPDH	F: AGCCACATCGCTCAGACAC R: GCCCAATACGACCAAATCC	(18)

Outcomes of the three independent qRT-PCR measurements were used for inter-group comparisons. Changes relative to baseline levels in the control cells were expressed as 'fold changes,' and mean values were statistically compared using an independent sample t test between test groups.

Viability of the PCa cells treated with DHT or enzalutamide was investigated using annexin V/PI counterstaining (Fig.1). DHT strongly induced cell proliferation in the both LNCaP (92%) and LNCaP-AR^{++} cells (95.4%) (P=0.01). In the enzalutamide-treated LNCaP cells, proportion of the late apoptotic cells was increased from 6.4% to 9.4%, while in the LNCaP-AR^{++} cells it was increased from 10.8% to 13%. In the cells co-treated with DHT and enzalutamide, DHT effect was dominant in the viability of the cells.

We validated the elevated AR expression in LNCaP-AR^{++} cells compared to the parental LNCaP cells, because these cells have been artificially engineered to express higher AR (5). Figure 2A depicts 4.6-fold higher AR expression in the LNCaP-AR^{++} than the LNCaP cells (P=0.02), providing further evidence of the AR-regulation of *ARLNC1*. Similar to AR, baseline *ARLNC1* expression was 1.7-fold higher in the LNCaP-AR^{++}, rather than the LNCaP cells, whereas *PCAT1* molecules had the opposite expression pattern with 3.8-fold lower expression in the LNCaP-AR^{++} cells than the LNCaP cells.

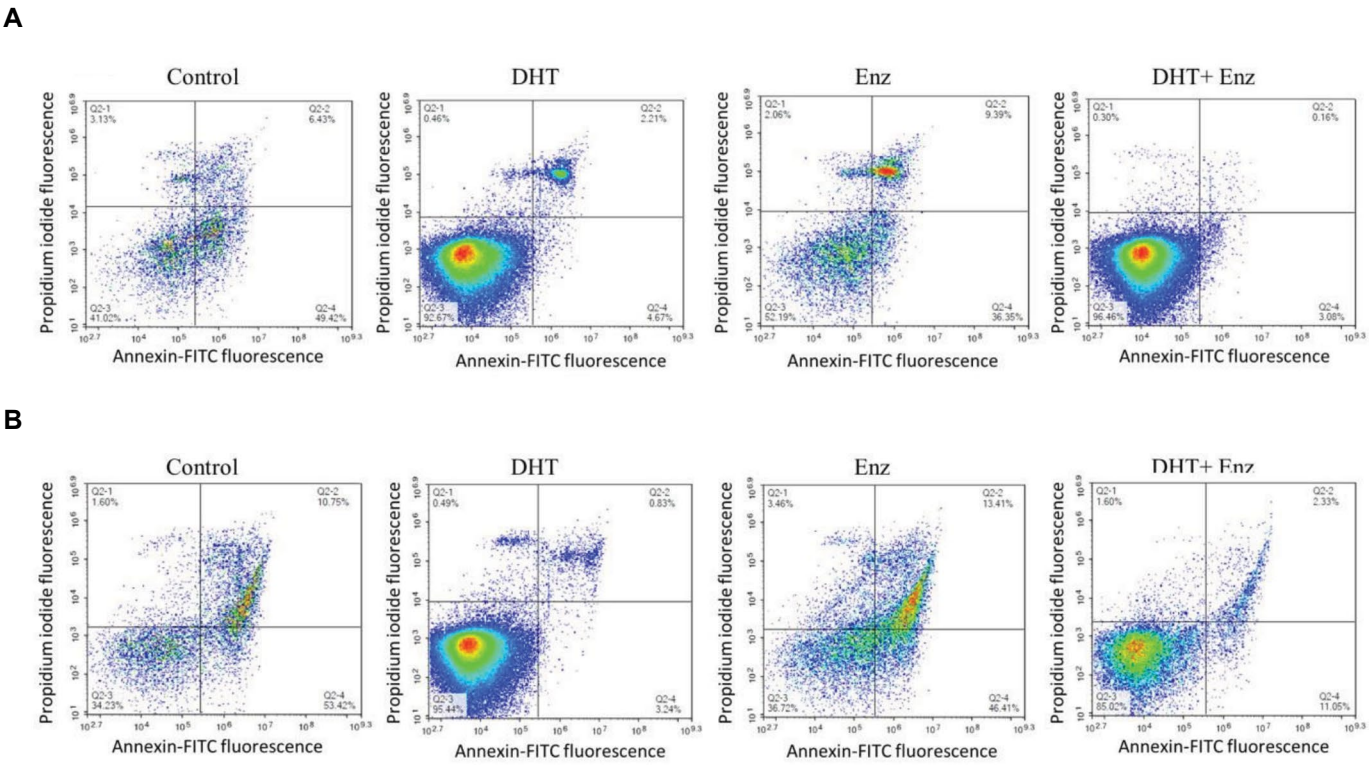

Fig.1: Cell viability assessment in PCa cells by flow cytometry. Approximately 200,000 cells were plated and kept in hormone-free medium conditions for 24 hours. The cells were then further cultured for 24 hours in a complete medium containing 10 nM dihydrotestosterone and/or 10 µM enzalutamide. After treatment, the cells were collected and washed, followed by staining with annexin V-FITC and propidium iodide. Following the measurement of fluorescence from cells, propidium iodide (y-axis) was plotted against annexin V-FITC (x-axis). The cells in Q2-3 plots included viable cells stained with neither PI nor FITC; the cells in Q2-4 were apoptotic cells and annexin-positive; Q2-2 illustrates late apoptotic cells that were stained with both dyes, and the cells in Q2-1 were dead cells, which were positive for propidium iodide only. **A**. LNCaP cells and **B**. LNCaP-AR^{++} cells. DHT; dihydrotestosterone and Enz; Enzalutamide.

Neither androgen stimulation nor AR blockade increased *PCAT1* expression. However, *PCAT1* expression levels were lower in the all experimental conditions compared to the levels in the control cells (Fig.2C). Thus, no specific conclusions could be drawn on the effect of androgen stimulation or AR inhibition on *PCAT1* expression.

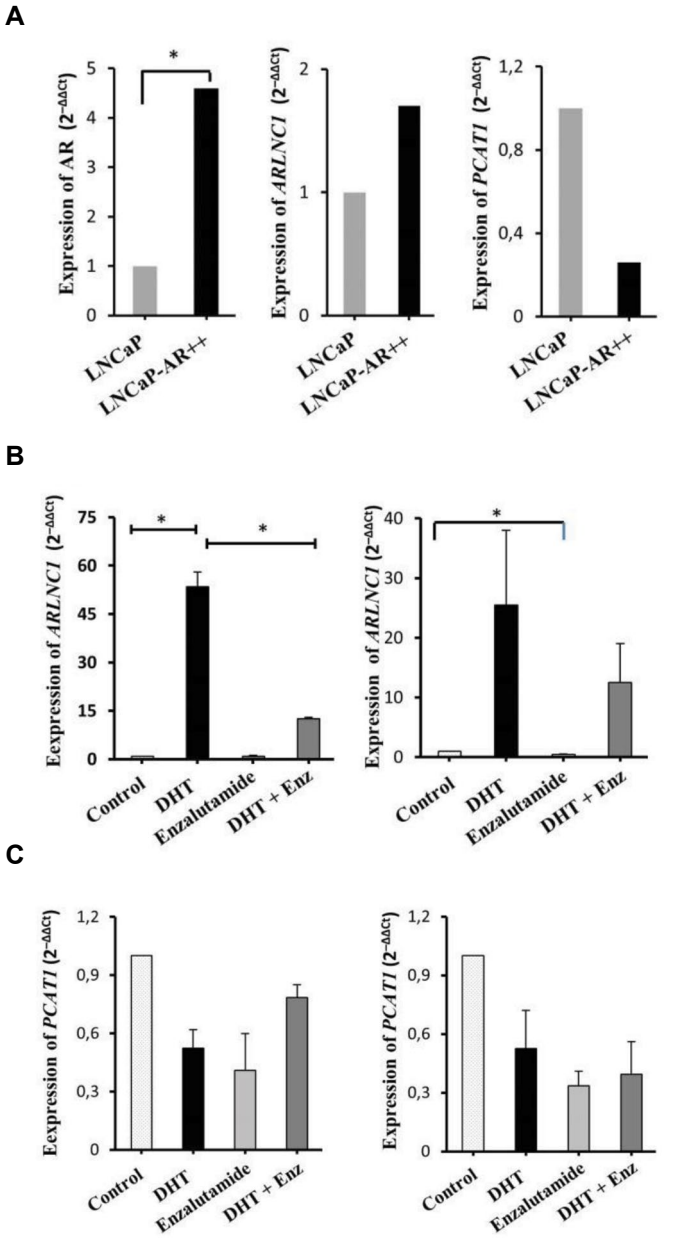

Fig.2: Expression analyses of androgen receptor, *ARLNC1* and *PCAT1* in prostate cancer cells. Total RNA was isolated from the cells treated with dihydrotestosterone and/or enzalutamide and it was used for complementary DNA (cDNA) synthesis. cDNA was then employed as a template in reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Expression levels of *ARLNC1* and *PCAT1* was determined semi-quantitatively with *GAPDH*, as the internal control. Their relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. **A**. Baseline relative expression of androgen receptor, *ARLNC1* and *PCAT1* in the LNCaP cells and LNCaP-AR^{++} cells. **B**. Relative *ARNC1* expression in the control cells, dihydrotestosterone- and /or enzalutamide-treated cells (left: the LNCAP cells, right: the LNCaP-AR^{++} cells). **C**. *PCAT1* expression in the control cells, dihydrotestosterone- and/or enzalutamide-treated cells (left: the LNCAP cells, right: the LNCaP-AR^{++} cells). Bar graphs show mean values and standard deviations. * indicates statistical significance. The independent sample t-test was used to investigate statistical differences between the test groups.

Present study aimed to interrogate *ARLNC1* and *PCAT1* expression changes in the context of androgen stimulation or AR blockade with respect to AR expression status. Differential AR expression status was accomplished by the utilizing two cell lines (LNCaP and LNCaP-AR^{++}) which express AR in various extent (5). We validated higher AR expression in LNCaP-AR^{++} cells which were also shown to expresses higher *ARLNC1* at baseline than parental LNCaP cells. Interestingly, the extent of *ARLNC1* induction by androgen stimulation was lower in the LNCaP-AR^{+} cells than in LNCaP cells (25-fold vs. 53.5-fold, respectively). In contrast to this, AR blockade reduced basal *ARLNC1* expression stronger in the LNCaP-AR^{++} cells, and attenuated the DHT effect on *ARLNC1* expression in both cell types remarkably. Our findings showed that AR inhibition had no suppressive effect on the DHT-stimulated cell proliferation in cells co-treated with DHT and enzalutamide, whereas DHT-stimulated *ARLNC1* was effectively suppressed by enzalutamide. This indicated the prompt responsiveness of *ARLNC1* to AR blockade. Thus, enzalutamide, which is currently used in CRPCa (19), may be beneficial in targeting *ARLNC1,* a potential therapeutic target in the progression of AR-positive castration-sensitive PCa (8).

In contrast to *ARLNC1, PCAT1* expression was affected neither by androgen stimulation nor AR blockade in a specific way, and lower in the all experimental conditions compared to the levels in the control cells. Although *PCAT1* has been identified as a PCa-related lncRNA (9), recent data indicated that it was not specific to PCa because *PCAT1* has been found to be overexpressed in different types of cancer. It was also implicated in several processes such as cell proliferation, invasion, metastasis, apoptosis, cell cycle and chemoresistance (20). Our finding that *PCAT1* was not up-regulated by androgen stimulation supported the notion that *PCAT1* acted independently on the AR pathway in the carcinogenesis of PCa.

Our findings revealed that the extent of androgen stimulation of *ARLNC1* varies according to its baseline cellular levels and it may be modulated by AR expression status in PCa cells. *PCAT1*, de-regulated in many cancer types, seems not to be regulated by androgens. We also demonstrated that AR blockade via enzalutamide effectively suppressed baseline *ARLNC1* expression in LNCaP-AR^{++} cells and remarkably reduced the DHT-stimulated *ARLNC1* expression in the both cell types. Our study possesses some limitations including lack of mechanistic work for elucidating the mechanism of *ARLNC1* suppression by enzalutamide and clinical implications of this in the management of patients.

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Authors' Contributions

U.G.; Were responsible for overall supervision. G.H., E.E.Y., U.G.; Contributed to conception and design. G.H., E.Ö., S.B.G.; Contributed to the all experimental works, data and statistical analysis, as well as interpretation of data. G.H.; Drafted the manuscript, which was revised by U.G. All authors read and approved the final manuscript.

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