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Original Article

Enzalutamide restores the testosterone effect on H19 expression in prostate cancer cells but not in exosomes

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Abstract

Aim: Cellular or exosomal expression of an oncofetal lncRNA gene H19, was evaluated during androgen stimulation via dihydrotestosterone (DHT) or androgen receptor (AR) blockage via enzalutamide in cultured hormone-sensitive prostate cancer (PCa) cells which overexpress the androgen receptor (LNCaP-AR+) in the present study.

Material and Methods: Cells were treated using DHT (10 nM) and/or enzalutamide (10 uM) for 24 hours. Cellular, and exosomal expression of H19 was investigated using a quantitative polymerase chain reaction assay.

Results: Our findings revealed that the mean cellular H19 expression decreased approximately 2.3 fold after androgen stimulation of PCa cells. Enzalutamide restored DHT effect with AR blockage, and we found increased H19 expression with the combined use of DHT, and enzalutamide compared with the levels in the control cells ($p<0.05$). Similar to its cellular effect, DHT treatment also led to declined exosomal expression of H19 ($p<0.0001$). No restorative effect of enzalutamide was observed on decreased H19 expression induced androgen stimulation in exosomes. Stimulation of cells with enzalutamide caused a significant reduction of H19 in exosomes.

Conclusion: This experimental study provides evidence that H19 might be involved in androgen receptor pathway. Further research is needed to explore the role of H19 in PCa, and the intercellular communication via exosomes.

Keywords: Prostate cancer; exosomes; H19; androgen; enzalutamide .

INTRODUCTION

Prostate cancer (PCa) is the second most common cancer in men worldwide (1). PCa is associated with androgen receptor (AR) pathway as its development is androgen-dependent. Therefore, the suppression of androgen pathway by surgical or medical castration is the approach of treatment of this disease. Although the response rate is higher with androgen deprivation, many patients with metastatic disease progress to castration-resistant prostate cancer (CRPC) as a consequence of continuous AR activation (2). In recent years, the second generation AR inhibitor enzalutamide (3), and androgen biosynthesis inhibitor abiraterone acetate (4) were developed as a treatment option in patients with metastatic CRPC. Enzalutamide acts in multiple ways that it blocks the binding of androgens to the AR, and with the transition of active AR into the nucleus, and prevents the binding of the DNA to the AR (5).

Prostate specific antigen (PSA) is the most widely used biomarker in the management of PCa. The serum PSA levels also increase in prostatitis or benign prostatic hyperplasia (BPH), therefore its diagnostic value is limited (6). Thus, identifying more specific biomarkers will improve the diagnostic accuracy of PCa.

Recently, exosomes which are membrane-bound secreted vesicles (30-150 nm), constituted a valuable source for biological marker discovery (7, 8). Exosomes are involved in the regulation of many physiological or pathological processes including tumor development, and progression. Exosomes were shown to include most types of macromolecules including lipids, proteins, DNA, messenger RNA, microRNA, and long noncoding RNAs (lncRNAs). They mediate intercellular communication by delivering their loads to target cells. We previously demonstrated that some lncRNAs were enriched in exosomes released from cultured cells relative to their

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cellular levels (9).

LncRNAs are longer than 200 nucleotides with restricted protein-encoding ability (10,11). Aberrant expression of many lncRNAs were detected in multiple human cancers, such as colorectal, lung, breast, liver, bladder cancer, and PCa (12-15). LncRNAs play critical roles in all stages of PCa development, and progression (16). An oncofetal RNA H19, is the first discovered lncRNA molecule and has been shown to possess regulatory functions in cancer development (17). In this experimental study, we aimed to study whether cellular or exosomal levels of H19 are adjusted during androgen stimulation or AR inhibition in cultured PCa cells using a cell line overexpressing AR as the cellular model.

MATERIAL and METHODS

Cell culture

We used the androgen sensitive LNCaP-AR+ cells, kindly gifted by Charles Sawyers Lab (Memorial Sloan Kettering Cancer Center, New York, NY, USA) that were modified to overexpress AR. Cells were cultured in the RPMI-1640 medium containing NaHCO3 (3.7 g/L), glucose (1 g/L) and stable glutamine (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom) and antibiotics under standard conditions (at 37˚C and 5% CO2 humidity).

Androgen stimulation and androgen receptor blockade

Cells were seeded at a density of 2×105 cells in culture plates, and were grown for 72 hours in the growth medium supplemented with exosome-depleted charcoal/dextran stripped FCS. The medium was then replaced by a another medium which contained dihydrotestosterone (DHT) (Sigma-Aldrich, USA), enzalutamide (kindly donated by Astellas Inc., Northbrook, IL, USA) or the control (dimethyl sulfoxide, DMSO). DHT is an active metabolite of androgen. Cells were grown for further 24 h, and harvested along with culture medium and stored for subsequent analysis.

Isolation of exosomes from culture medium, and of exosomal RNA

Exosome extraction from the culture medium was performed using the Total Exosome Isolation reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in accordance with the manufacturer's instructions. Briefly, the culture medium was centrifuged at 2,000 x g for 30 min to remove and cells and debris. Cell medium, and the exosome isolation reagent were mixed using the brief vortexing and incubated at 4°C overnight before centrifuged at 4 °C at 10,000 x g for 1 h. The pellet containing exosomes were resuspended in PBS. RNA was isolated from the extracted exosomes using the Total Exosome RNA & Protein Isolation kit (Thermo Fisher Scientific, Inc.) as per the manufacturer's instructions. Total cellular RNA was extracted using the RNA isolation solution (Roche Diagnostics GmbH) in accordance with the manufacturer's instructions.

Quantitative PCR

Total RNA and exosomal RNA were used for the complementary DNA (cDNA) synthesis using a First-Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA, USA), in accordance with the manufacturers' instructions. Expression analysis was performed using the LightCycler 480 instrument (Roche), and SYBR Green (Roche) as the fluorescent molecule. H19 expression levels were normalized with the internal control 18S rRNA. The PCR program included an initial hot start of 10 min, followed by 45 cycles of amplification. Each cycle consisted of a denaturation step at 95˚C for 10 s, annealing starting at 60˚C for 20 s and decreasing 2˚C every 2 cycles until 55˚C, as well as amplification at 72˚C for 30 s. The results of three different experiments were used for the calculation of expression level of H19.

Statistical analysis

We assessed the results of three independent cell culture experiments. The changes in gene expression levels relative to control cells were expressed as 'fold changes' and mean values were statistically compared using the one-way ANOVA analysis method. $P < 0.05$ was considered as the level of significance. Statistical analyses were conducted using the GraphPad Prism 5 software.

Results

Enzalutamide removes the effect of DHT on the H19 gene

We studied the effect of androgen stimulation of LNCaP-AR+ cells and AR suppression via enzalutamide on cellular and exosomal expression of H19. DHT stimulation (10 nM) of LNCaP-AR+ cells for 24 h resulted in a 2.3-fold decrease of cellular expression of H19 compared with the control cells. The extent of the decline of H19 level was statistically insignificant. Treatment of cells with enzalutamide (10 uM) alone had no effect on the H19 expression. Interestingly, the combined use of DHT and enzalutamide on LNCaP-AR+ cells showed that the restoration of the DHT effect by enzalutamide as H19 expression in cells treated with DHT, and with enzalutamide was 2.5-fold higher than that in the control cells and 5- fold higher than in the cells stimulated with DHT ($p<0.05$) (Figure 1).

Decreased expression of H19 in exosomes

We examined the expression of H19 in exosomes extracted from culture media secreted by LNCaP-AR+ cells. Interestingly, H19 was highly enriched in exosomes compared with its cellular level. Similar to its cellular expression, exosomal levels of H19 declined approximately 3-fold in DHT-treated cells ($p<0.0001$). Intriguingly, the effect of enzalutamide was more significant on exosomal expression of H19 as we detected approximately 19-fold decrease of H19 level in enzalutamide-exposed cells ($p<0.0001$). The combination of DHT, and enzalutamide resulted in a highly decreased expression of H19 in exosomes (approx. 22-fold, $p<0.0001$) (Figure 2).

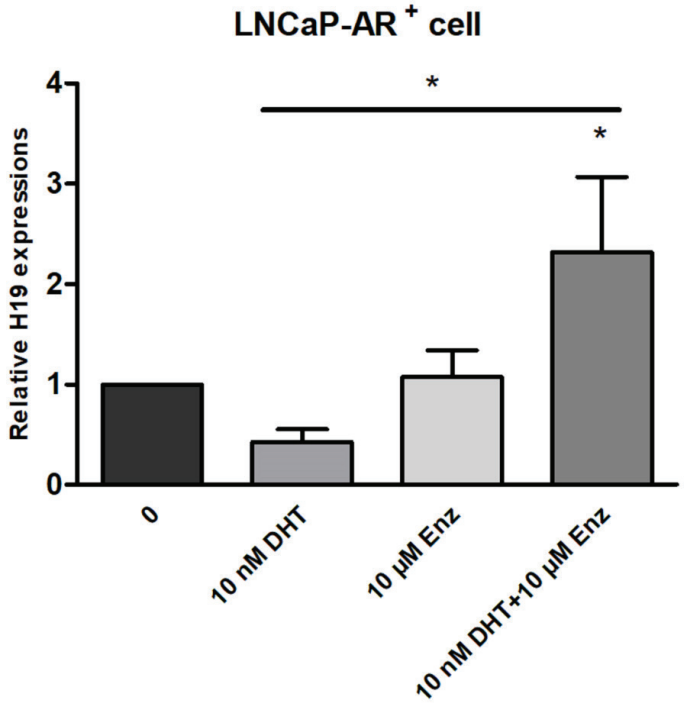

Figure 1. Expression of lncRNA H19 in DHT- or enzalutamide- treated LNCaP-AR+ cells. Results of three independent cell culture experiments were used to calculate the average value of relative gene expression. Each column represents mean + SE and * p< 0.05 indicates statistically significant differences compared to untreated cells and DHT-treated cells.

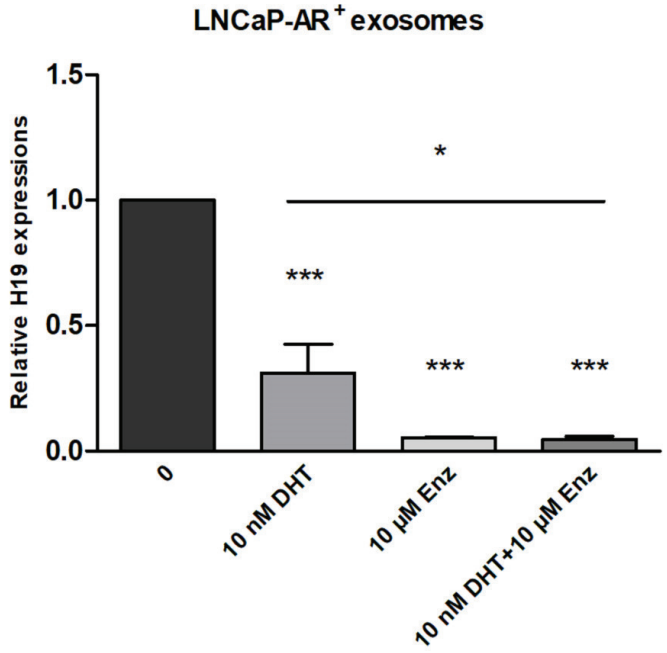

Figure 2. Exosomal expression of lncRNA H19 in DHT- or enzalutamide-treated LNCaP-AR+ cells. Results of three independent cell culture experiments were used to calculate the average value of relative gene expression. Each column represents mean + SE and *** p < 0.0001 indicates statistically significant differences compared to untreated cells. * p < 0.05 indicates significant change compared with DHT-treated cells.

DISCUSSION

We aimed to investigate the effect of androgen pathway on the exosomal expression of H19 gene in AR-overexpressing prostate cancer cells (LNCaP-AR +) in the present study. The H19 is a typical molecule for lncRNA genes which are maternally expressed, and are paternally suppressed. H19 was reported to exert oncogenic functions in multiple cancers however, some studies have also described its tumor suppressor role depending on the type of cancer, and cellular content (18,19). H19 negatively regulates the p53 protein, and cell cycle progression (19) or acts as a molecular sponge to regulate the let-7 family of microRNAs (miRNAs) (20). However, H19 and H19-produced miR-675 were both significantly downregulated in metastatic prostate cancer cells compared to non-metastatic prostate cancer cells in a recent study (21). These studies reported controversial results whether H19 RNA was an oncogenic or a tumor suppressor.

We showed that the hormone treatment in PCa cells leads to decreased expression of cellular H19. Berteaux et al. reported similar findings (22). We also found that the blocking of the androgen receptor using enzalutamide restored the testosterone effect which suggesting that H19 might have a tumor suppressor activity in prostate cancer.

We showed that H19 was enriched in the secreted exosomes. This is consistent with our previous report which included some lncRNAs that were found to accumulate in secreted exosomes (9). This may be associated with role of H19 in cellular communication as reported in liver cancer cells in which exosomal H19 was shown to be transferred to endothelial cells, and regulate various processes through VEGF (23). The decreased expression of H19 in LNCaP-AR + upon DHT treatment was also reflected by exosomal level of H19. Although enzalutamide treatment alone had no significant effect on cellular expression of H19, exosomal level of H19 declined after enzalutamide. This effect was increased on combined use of DHT, and enzalutamide. The basis of this observation is yet unclear, however might be associated with any role of H19 in intercellular communication (24, 25).

Exosomes can shield tumor cells from the attack of therapeutic agents and this leads to failure of treatment (26). In prostate cancer, exosomal ingredients could play a role in drug resistance. As androgen-receptor splice variant 7 (AR-V7), an isoform of AR, lacks the ligand binding site and is associated with resistance to enzalutamide and abiraterone therapies (27), exosomal ingredients including H19 and AR-V7 can be utilized to monitor resistance to hormonal treatment in patients with prostate cancer.

CONCLUSION

In conclusion, our experimental study provides evidence that H19 might be involved in androgen/receptor pathway as the testosterone treatment of hormone dependent receptor-overexpressing cells suppress H19. Accordingly, exosomal levels of H19 also decline parallel to its cellular levels. Blockage of AR by enzalutamide restores the testosterone effect on H19 expression in the cells however

does not restore the testosterone effect on H19 expression in the exosomes. Further research is needed to explore the role of H19 in PCa and the intercellular communication through exosomes.

Competing interests: The authors declare that they have no competing interest.

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Ethical approval: Permission was obtained from the local ethics committee for the study.

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