Investigation of lncRNA H19 in prostate cancer cells and secreted exosomes upon androgen stimulation or androgen receptor blockage

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ABSTRACT

OBJECTIVES: In the present study, cellular or exosomal expression of H19, an oncofetal lncRNA gene, was evaluated during androgen stimulation via dihydrotestosterone (DHT) or AR blockage via enzalutamide in cultured hormone-sensitive Pca cells which overexpress AR (LNCaP-AR⁺).

BACKGROUND: Prostate cancer (PCa) is an androgen-dependent disease. Androgen receptor (AR) antagonists (i.e. enzalutamide) have been used for the treatment of patients with metastatic castration-resistant prostate cancer (CRPC). Exosomes and their contents (non-coding RNA) play an important role in tumor development and progression.

METHODS: Cells were treated with DHT (10 nM) and/or enzalutamide (10 μM) for 24 h. Cellular and exosomal expression of H19 was investigated using a quantitative polymerase chain reaction assay.

RESULTS: Our findings reveal that cellular H19 expression decreased approximately 2.3-fold in mean upon androgen stimulation of Pca cells. AR blockage using enzalutamide restored DHT effect and we found increased H19 expression (≤ 2.5-fold, p < 0.05) upon the combined use of DHT and enzalutamide compared to control cells. Similar to its cellular effect, DHT treatment also led to declined exosomal expression of H19 (≤ 3-fold, p < 0.0001). Restorative effect of enzalutamide on decreased H19 expression induced by androgen stimulation was not observed 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 intercellular communication via exosomes (Fig. 2, Ref. 32). Text in PDF www.elis.sk.

KEY WORDS: prostate cancer, exosomes, H19, androgen, enzalutamide.
in exosomes released from cultured cells relative to their cellular levels (16).

LncRNAs are longer than 200 nucleotides with restricted protein-encoding ability (17, 18). Aberrant expression of many lncRNAs were detected in multiple human cancers, such as colorectal, lung, breast, liver, bladder cancer, and PCa (19, 22). LncRNAs play critical roles in all stages of PCa development and progression (23). H19, an oncofetal RNA, is the first discovered lncRNA molecule and has been shown to possess regulatory functions in cancer development (24). 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.

Materials and methods

Cell culture

We used LNCaP-AR+ cells, kindly gifted by Charles Sawyers Lab (Memorial Sloan Kettering Cancer Center, New York, NY, USA) which are androgen sensitive and 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 (37 °C and 5 % CO2 humidity).

Androgen stimulation and androgen receptor blockade

Cells were seeded at a density of 2×10^5 cells in culture plates and grown for 72 hours in the growth medium supplemented with exosome-depleted charcoal/dextran stripped FCS. The medium was then replaced by a fresh one containing dihydrotestosterone (DHT) (Sigma-Aldrich, USA), enzalutamide (kindly donated by Astellas Inc., Northbrook, IL, USA) or the control (dimethyl sulfoxide, DMSO). DHT is active metabolite of androgen. Cells were then replaced by a fresh one containing dihydrotestosterone (DHT) (Sigma-Aldrich, USA) which are androgen sensitive and modiﬁed to overexpress AR. Cells were cultured in the RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Biochrom) and antibiotics under standard conditions (37 °C and 5 % CO2 humidity).

Exosome extraction from the culture medium was performed using the Total Exosome Isolation reagent (Thermo Fisher Scientiﬁc, Inc., Waltham, MA, USA) according to the manufacturer’s instructions. 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) according to the manufacturer’s instructions.

Quantitative PCR

Total RNA and exosomal RNA were used for 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. Changes in gene expression levels relative to control cells were expressed as ‘fold changes’ and mean values were statistically compared using the ANOVA Tukey multiple comparison analysis method. P < 0.05 was considered as a level of signiﬁcance. Statistical analyses were conducted using the GraphPad Prism 5 software.

Results

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 control cells. The extent of the decline of H19 level was not statistically signiﬁcant. Treatment of cells with enzalutamide (10 uM) alone had no effect on the H19 expression. Interestingly, upon the combined use of DHT and enzalutamide on LNCaP-AR+ cells we noticed restoration of the DHT effect by enzalutamide as H19 expression in cells treated with DHT and enzalutamide was 2.5-fold higher than that in control cells and 5-fold higher than in cells stimulated with DHT (p < 0.05) (Fig. 1).

Next, we examined the expression of H19 in exosomes extracted from culture media secreted by LNCaP-AR+ cells. Interestingly, we noticed that H19 was highly enriched in exosomes compared with its cellular level. Similar to its cellular expression, exosomal level of H19 was approx. 3-fold declined in DHT-treated cells.

Fig. 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.
studies lead to contradictory findings on whether H19 RNA is an oncogenic or tumor suppressor.

We revealed that hormone treatment of PCa cells leads to decreased expression of cellular H19. Similar finding has been reported by Berteaux et al (29). We also found that blocking the androgen receptor by enzalutamide restores the testosterone effect suggesting that H19 might have tumor suppressor activity in prostate cancer.

We showed that H19 was enriched in secreted exosomes. This is consistent with our previous report including some other lncRNAs that have been found to accumulate in secreted exosomes (16). This may be associated with any role of H19 in cellular communication as reported in liver cancer cells in which exosomal H19 has been shown to be transferred to endothelial cells and regulate various processes via VEGF (30). Decreased expression of H19 in LNCaP-AR- cells upon DHT treatment was also reflected by exosomal level of H19. Even if enzalutamide treatment alone had no significant effect on cellular expression of H19, exosomal level of H19 was decline upon enzalutamide. This effect was increased with combined use of DHT and enzalutamide. The basis of this observation is clear at the moment but might be associated with the role of H19 in intercellular communication (31, 32).

In conclusion, our experimental study provides evidence that H19 might be involved in androgen/receptor pathway as testosterone treatment of hormone-dependent receptor-overexpressing cells suppresses H19. Accordingly, exosomal levels of H19 also decline in parallel to its cellular levels. Blockage of AR by enzalutamide restores the testosterone effect on H19 expression in cells but not in exosomes. Further research is needed to explore the role of H19 in PCa and intercellular communication via exosomes.

Discussion

In this study, we aimed to investigate the effect of androgen pathway on the exosomal expression of H19 gene in AR-overexpressing prostate cancer cells (LNCaP-AR+). The H19 is a typical molecule for lncRNA genes that are maternally expressed and paternally suppressed. It has been reported to exert oncogenic functions in multiple cancers whereas some studies have also described a tumor suppressor role, depending on the type of cancer and cellular content (25, 26). H19 negatively regulates the p53 protein and cell cycle progression (26) or acts as a molecular sponge to regulate the let-7 family of microRNAs (miRNAs) (27).

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