LncRNA HCG18 promotes Prostate Cancer progression by regulating miR-512-3p/HK-2 axis

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Abstract

Long non-coding RNA (lncRNA) plays an important role in tumor progression. Numerous studies show that lncRNA is strongly associated with prostate cancer progression. Our study confirmed that lncRNA HCG18 was highly expressed in prostate cancer (PC) and correlated with tumor progression in databases and cell lines. Western blot, RNA Pull-down, dual luciferase assay and rescue assays verified the correlation among lncRNA HCG18, miR-512-3p and hexokinase-2 (HK-2). In general, the results showed that lncRNA HCG18 accelerated cell proliferation, migration, and invasion of PC via up-regulating HK-2 through sponging miR-512-3p, which provided a new direction for the diagnosis and treatment of PC.

Introduction

Prostate cancer (PC) is the second most common cancer in men, with more than 1.2 million new cases diagnosed annually[1]. The prognosis of PC is depending on the grade of tumor and stage at primary diagnosis[1]. Once distant metastases occur, the 5-year survival rate for prostate cancer is only 30%[2]. Therefore, it is critical to explore the pathogenesis of prostate cancer and identifying novel therapeutic targets is critical for the development of future effective therapies for PC.

Long non-coding RNAs (lncRNAs) are a class of RNA transcripts with a length larger than 200 nucleotides which can’t code for proteins[3]. However, lncRNAs have been confirmed to be involved in a variety of biological processes[4]. Especially, lncRNAs have been proven to regulate tumor progression[5]. The mechanism of lncRNAs in PC has been extensively studied. Shang et al. showed that lncRNA PCAT1 is important in the progression of castration-resistant prostate cancer[6]. Lang et al. demonstrated the involvement of lncRNA PCAT6 in the bone metastasis process of PC[7]. LncRNA HCG18 has been confirmed to be involved in the occurrence and development of a variety of tumors like osteosarcoma, hepatocellular carcinoma and clear cell renal cell carcinoma through its spongy effect on miRNA[8–10]. Bioinformatics studies have shown that lncRNA HCG18 may be associated with PC, but the specific mechanism remains unclear[11].

MicroRNAs (miRNAs) are evolutionarily conserved short noncoding single-stranded RNA molecules, with a length of 18–22 nucleotides[12]. A large number of miRNAs have been demonstrated to be involved in the progression of PC[12]. For example, Wang et al. showed that long non-coding RNA AFAP1-AS1 promoted proliferation and invasion in prostate cancer via targeting miR-512-3p[13]. Playing the role of cancer inhibitor, miR-512-3p has been found to be down-regulated in the majority of cancer cells. For example, miR-512-3p was confirmed to enhance chemosensitivity and decrease metastatic potential in breast cancer[14]. Meanwhile, miR-512-3p has also been shown to be regulated by lncRNA PART1 in ovarian cancer to enhance drug resistance[15]. However, Rao et al. showed that miR-512-3p can promote cell proliferation in prostate cancer[16]. This indicates that the role of miR-512-3p in prostate cancer may still be controversial, suggesting the important significance of exploring it.
Metabolic reprogramming is an important feature of tumor cell development[17]. In contrast to normal cells, tumor cells can obtain energy through anaerobic glycolysis even under oxygen-enriched conditions, a change known as the Warburg effect[18, 19]. Glycolysis is a tightly regulated process in which hexokinases (HKs), phosphofructokinases (PFK) and pyruvate kinases (PK) play a crucial role, and overexpression of HKs (mainly HK-2), as well as enhanced activity, has been found in many types of cancer[20]. Hexokinases are involved in the glycolytic process of tumor cells on the one hand and act on voltage-dependent ion channels to inhibit apoptosis on the other hand[21]. In addition, hexokinase has also been shown to be involved in tumor progression by affecting tumor vascular function, and Zhang et al. showed that in colorectal cancer, HK-2 affects cellular sensitivity to drugs[17, 22]. Therefore, HK-2 is now considered to be an important target for anti-cancer drugs[19]. It has been shown that HK-2 is highly expressed in prostate cancer cells and is a good marker for high-risk prostate cancer, and HK-2 is closely associated with prostate cancer progression, but its mechanism has not been fully elucidated[23]. Meanwhile, in a variety of tumors, lncRNAs have been shown to regulate HK-2 by targeting miRNAs, thereby affecting tumor progression[24–26].

In this study, we explored the role of lncRNA HCG18/miR-512-3p/HK-2 axis in the development of PC. and provide a theoretical basis for the diagnosis and treatment of PC.

**Materials And Methods**

**Bioinformatics analysis**

The data of lncRNA HCG18 expression were obtained from the Cancer Genome Atlas (TCGA) database. The Kaplan-Meier curve was used to test lncRNA association with time to progression. MiRNA was predicted through the starBase, MicroRNA Target Prediction Database(miRDB), miRwalk and IncRNASNP2.

**Cell Culture**

Five PC cell lines PC-3, C4-2, DU145, LNCAP;22RV1 and prostate epithelial cells RWPE1 were all obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI-1640(Sigma, USA) containing 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 1% penicillin-streptomycin (HyClone, Logan, UT, USA) and then preserved in the atmosphere with 5% carbon dioxide (CO2) at 37°C.

**Cell Transfection**

Plasmids, miRNA inhibitor and mimic were commercially purchased from Youze Biological Corporation. As per the manufacturer, cell transfection was performed using Lipofectamine 2000 (Invitrogen, Inc., CA, USA).
Quantitative Real-time Pcr (Qrt-pcr) Analysis

The total RNA was extracted using TRIzol reagent. An equivalent of 1 µg of total RNA was subjected to reversed transcription into cDNA using PrimeScript™ RT reagent Kit (Takara, Kyoto, Japan) and Mir-X™ miRNA qRT-PCR SYBR® Kit (Takara, Kyoto, Japan). The mRNA and miRNA expression of the target gene was determined by qRT-PCR conducted on ABI-7900 system with SYBR Green (Takara, Kyoto, Japan). The expression of a target gene was normalized to that of GAPDH or U6. HCG18: Forward: GCTAGGTCCCTACTTTT, Reverse: CAGAAAGTAGGACCTAGC.


Western Blot Analysis

Cells were harvested and lysed in RIPA buffer with 1% phosphatase and protease inhibitors. Protein content was quantified by BCA protein quantification kits. 20 µg of proteins were separated and transferred onto NC membranes (Millipore). After blocking membranes for 1 h at room temperature, the NC membranes were incubated with dilutions containing specific primary antibodies (HK-2, abcam, ab209847. GAPDH, abcam, ab8245) overnight at 4°C. The next day, the membrane was washed three times with 1 × TBST and incubated with HRP-conjugated secondary antibodies (Beyotime, China, A0208) for 1 h at room temperature. Finally, the membrane was washed three times again and visualized using the ECL exposure system (Thermo Fisher Scientific).

Transwell Assay

Transwell chamber (24-well) with Matrigel was applied to determine cell invasion following the manufacturer’s instructions. 200 µl transfected PC3 or LNCaP cells (1×10^5) were added in the upper chamber with RPMI1640 medium without FBS. 500 µl RPMI-1640 medium containing 10% FBS was added to the lower chamber. PC3 cells were cultured for 12 h and LNCAP for 36h at 37°C with 5% CO2. The cells were fixed with formaldehyde for 15 min and then stained with 0.1% crystal violet for 15 min. The invasive cell number was counted using a Zeiss Microscope (Nikon Corporation, Japan).

Cell Counting Kit

Cells were selected in the logarithmic growth phase and seeded in 96-well plates at 2 × 10^3 cells/well for 0h,24h,48h and 72h. 10µL CCK8 solution (Dojindo, Japan) was added and the plate was incubated at 37°C for 2h in the dark. The absorbance was detected at 450 nm using a microplate reader (Thermo Fisher Scientific).

Wound Healing Assay
Cells were plated in 6-well plates and scratched vertical wounds with 10 µl tips after completely adherent. Cells were cultured in serum-free RPMI-1640 and photographed at 0h and 48h.

**Rna Pull-down Assay**

Biotin was attached to miR-512-3p (Bio-miR-512-3p) or miR-negative control (Bio-miR-negative control). Bio-miR-512-3p or Bio-miR-negative control was transfected into LNCAP cells. Cells were collected and lysed after transfection for 48 h. RNA pull-down assay was conducted according to the protocol. QRT-PCR was conducted to examine the level of HCG18 in LNCAP cells.

**Luciferase Reporter Assay**

Relative luciferase activity was determined with the Dual-Luciferase® Reporter Assay System based on supplier’s instructions (Promega).

**Subcutaneous Tumor Formation Assay**

BALB/c nude mice (male; 4–6 w; 18–22 g) were selected from Shanghai Slake Experimental Animal Co., Ltd. The animal experiment was approved by the Ethics Committee of the Tongji Hospital, Tongji University of Medicine. 1 × 10⁶ cells were injected subcutaneously into the left shoulder of each nude mouse, and the nude mouse was executed 45 days after the injection and the tumor size and weight were measured. The treatment of animals in all experiments conforms to the ethical standards of experimental animals. In this animal experiment, the largest tumor diameter was 1.4 cm, and the largest tumor volume was 0.903 cm³, both of which did not exceed the range of ethical requirements.

**Statistical analysis**

Data were from three independent experiments and presented as the mean ± standard deviation (SD) and analyzed using SPSS 19.0 software (SPSS Inc., USA). The statistical methods include ANOVA, Student’s t-test, Pearson's and Spearman's correlation analysis for the relationship between variables. p < 0.05 shows statistical significance.

**Results**

**LncRNA HCG18 is markedly increased in PC and related to cells proliferation, migration, and invasion**

To explore the association between HCG18 and prostate cancer, the data from TCGA database and STARBASE database were analyzed. The data showed that HCG18 expression levels were higher in prostate cancer samples than in normal samples (Fig. 1A). In addition, HCG18 was negatively correlated
with the survival of patients, the higher the expression of HCG18, the worse the survival of patients (Fig. 1.B). Then, we compared the expression level of HCG18 in several commonly used prostate cancer cell lines (PC-3, C4-2, DU145, LNCAP, 22RV1) and prostate epithelial cells (RWPE1) by qPCR, and found that HCG18 in prostate cancer cell lines was higher than that in prostate cancer epithelial cells (Fig. 1.C). To test the role of HCG18 in prostate cancer, shRNA was transfected into PC3 cells and LNCAP cells to knock down HCG18. Compared with control groups, the proliferation ability of cells decreased significantly in sh-HCG18 transfected cells (Fig. 1.D). Transwell and wound healing assay showed that HCG18 inhibition remarkably decreased invasion and migration ability in cells (Fig. 1.E and F).

**Hk-2 Promotes Pc Progression And Can Be Regulated By Lncrna Hcg 18**

A large number of studies have reported that IncRNA regulates HK-2 expression through miRNA to affect tumor progression [25, 27, 28]. We hypothesized that IncRNA HCG 18 might also be an upstream molecule regulating HK-2 expression. To test this hypothesis, HCG18 was knocked down in PC3 and LNCAP cells and Western blotting was performed to compare HK-2 expression. Inhibition of HCG18 significantly reduced HK-2 expression compared with the control group (Fig. 2.A). After HK-2 knockdown in PC3 and LNCAP cells, CCK-8 assay, Wound Healing assay and Transwell assay were performed. The results showed that HK-2 knockdown significantly reduced the proliferation, migration and invasion of PC3 and LNCAP cells compared with the control group (Fig. 2.B, C and D). These results suggest that HK-2 is indeed involved in the progression of prostate cancer and can be regulated by HCG18.

**Mir-512-3p Directly Interacts With Hcg18 And Hk2 And Inhibits The Progression Of Prostate Cancer Cells**

To explore the intermediate pathway between HCG18 and HK-2, we searched the database and screened out two target molecules, Mir-512-3p and Mir-676-3p (Fig. 3.A). By reviewing the literature, we found that the role of Mir-512-3p in PC has been reported, however, the results obtained from different studies are inconsistent, so we think it is more valuable to investigate Mir-512-3p. We employed biotinylated miR-512-3p probe to pull down the IncRNA HCG18. Data indicated endogenous IncRNA HCG18 was enriched specifically in miR-512-3p probe detection compared with the control group, suggesting that miR-512-3p is a direct inhibitory target of HCG18 (Fig. 3.B). Dual luciferase reporter assay showed that luciferase activity was remarkably decreased in cells co-transfected with WT-HK-2 and miR-512-3p mimic (Fig. 3.C and D). These indicated that miR-512-3p could directly bind to 3'-UTR of HK-2. MiR-512-3p significantly impaired cell migratory and invasive capacity in PC-3 and LNCAP cells (Fig. 4. A, B, C and D). Meanwhile, CCK-8 assay showed a significant decrease in cell proliferation after transfection with Mir-512-3p mimic. (Fig. 4.E)
Lncrna Hcg18 Promotes Pc Progression Via The Lncrna-hcg18/mir-512-3p/hk-2 Axis

Rescue assays were conducted to test the effect of lncRNA-HCG18/miR-512-3p/HK-2 axis on the progression of prostate cancer. It was indicated from CCK-8 assay that the falling tendency of cell proliferation caused by HCG18 silencing was recovered by miR-512-3p depletion or the overexpression of HK-2 (Fig. 5.B). It was discovered through Wound healing assay and Transwell assay that HCG18 knockdown suppressed PC cell migration and invasion, the descending trend was reversed after miR-512-3p inhibitor or HK-2 overexpression plasmid were transfected into cells (Fig. 5.A and C). In conclusion, HCG18 expedited the progression of PC through sponging miR-512-3p to up-regulate HK-2 expression.

Lncrna-hcg18/mir-512-3p/hk-2 Axis Modulated Pc Tumor Growth In Vivo

To verify the role of lncRNA-HCG18 / Mir-512-3p /HK-2 axis in vivo, we conducted experiments in nude mice. The results indicated knockdown of HCG18 or miR-512-3p mimic could slowdown mice tumor growth, and miR-512-3p inhibitor accelerated tumor growth (Fig. 6.A). Meanwhile, the tumor weight of mice in sh-HCG18 + miR-512-3p inhibitor group was smaller than that in control group, while larger than that in sh HCG18 group and miR-512-3p mimic group (Fig. 6.B). The vivo experiments confirmed the oncogenic effect of miR-512-3p and the role of this signaling axis in PC.

Discussion

Prostate cancer (PC) is the second most common cancer in men. According to the competing endogenous RNA hypothesis, lncRNAs are capable of regulating gene expression through titrating miRNAs to participate in tumor progression. We focused on the role of LncRNA HCG18/ miR-512-3p /HK2 axis in prostate cancer progression, where HCG18 regulates HK-2 expression by titrating miR-512-3p thereby promoting prostate cancer progression.

By bioinformatics analysis and qRT-PCR, we confirmed the high expression of HCG18 in PC. By transwell assay, cell counting kit and wound healing assay, we confirmed that knockdown of HCG18 could reduce the proliferation, migration and invasion ability of PC cells, suggesting its carcinogenic role. Similarly, Chen et al. speculated that HCG18 was associated with bone metastasis and poor prognosis of prostate cancer through bioinformatics analysis, which was consistent with our experimental results[11]. Subsequently, to test the hypothesis that HCG18 is an upstream gene of HK-2, we knocked down HCG18 in PC cells and then compared the changes of HK-2 by western blot analysis. The results showed that the expression of HK-2 decreased with less HCG18. Then, to probe the intermediate factors of HCG18 and HK-2, we targeted miR-512-3p by bioinformatics analysis and reading the literature. By luciferase reporter assay and RNA pull-down assay, we confirmed that miR-512-3p has binding sites to both HCG18 and HK-2 and may be an intermediate factor in the regulation of HK-2 by HCG18. However, although a large body
of literature has consistently shown that miR-512-3p has a tumor suppressor effect in a variety of tumors, two studies have yielded very different results regarding the role of miR-512-3p in prostate cancer. Wang et al. showed that in PC, miR-512-3p is tumor suppressive and can be titrated by the tumor-promoting LncRNA AFAP1-AS1, however, Rao et al. concluded that miR-512-3p may have a pro-proliferative function in PC cells using raw bioinformatics Analysis analysis[13, 16]. In our experiments, in vitro experiments showed that miR-512-3p mimic could reduce the proliferation, invasion, and migration ability of PC cells, while inhibitors of miR-512-3p had the opposite effect; in vivo experiments also showed that analogs of miR-512-3p could inhibit tumor growth, while inhibitors could promote tumor growth. Our experimental results are consistent with those of Wang et al. but contrary to those of Rao et al. We believe that this inconsistency may be related to the insufficient number of specimen cases included in the bioinformatics database or the selection of different databases.

Our results combined LncRNA with Warburg effect and proposed that HCG18 could regulate HK-2 via miR-512-3p and thus participate in the mechanism of Warburg effect affecting tumor progression. In addition, our study validated the role of miR-512-3p with more experiments, providing a basis for the oncogenic effect of miR-512-3p in PC. Our study also has limitations; this study has not yet included a clinical sample for analysis, and we will further validate the proposed pathway in this study by collecting a large amount of clinical data and long-term follow-up in a follow-up study. Overall, our study suggests that the LncRNA-HCG18 /miR-512-3p/HK-2 axis is involved in PC progression and this study may contribute to the development of diagnostic and therapeutic tools for PC.

**Declarations**

**Conflict of Interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Author Contributions**

All authors contributed to the conceptualization and design of the study. YRZ, ZJW, HPL, and ZR made substantial contributions to the acquisition of data, analysis, and interpretation of data and drafted the manuscript. TZ and XQ revised the manuscript critically for important intellectual content. WHZS, XC, and GW are respond for statistic and quality review. All authors read and approved the final manuscript.

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**Ethics statement**
This study was approved and supervised by the animal ethics committee of the Ethics Committee of the Tongji Hospital, Tongji University of Medicine (2021-DW-(001)). The treatment of animals in all experiments conforms to the ethical standards of experimental animals. In this animal experiment, the largest tumor diameter was 1.4 cm, and the largest tumor volume was 0.903 cm$^3$, both of which did not exceed the range of ethical requirements.

**Research Data Policy and Data Availability Statements**

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**References**


LncRNA HCG18 is markedly increased in PC and related to cells proliferation, migration, and invasion

**Figure 2**

HK-2 promotes PC progression and can be regulated by LncRNA HCG 18

Figure 3

MiR-512-3p correlates with HCG18 and HK-2

A) Bioinformatic analysis for screening miRNAs. B) HCG18 interacts directly with miR-512-3p. C) Binding sites of miR-512-3p and HK-2. D) MiR-512-3p can combine with HK-2
Figure 4

Mir-512-3p directly interacts with HCG18 and HK2 and inhibits the progression of prostate cancer cells

Figure 5

LncRNA HCG18 promotes PC progression via the LncRNA-HCG18/miR-512-3p/HK-2 axis

Figure 6

Animal experiments

A) Tumor visualization. B) Tumor weight difference statistics chart.