LncRNA HOXA11-AS modulates the miR-148b-3p/MLPH axis to promote prostate cancer cell proliferation

Shuai Yang, Han Guan, Zhijun Chen, Sheng Wang, Hongliang Wu, Qingwen Li*

Department of Urology, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233000, Anhui Province, China

ARTICLE INFO

Original paper

Article history:
Received: January 10, 2023
Accepted: March 22, 2023
Published: March 31, 2023

Keywords:
Prostate cancer; HOXA11-AS; miR-148b-3p; MLPH

ABSTRACT

Prostate cancer refers to the epithelial malignant tumor of the prostate. It has a high incidence and mortality rate, seriously endangering the lives of men. In recent years, lncRNAs have become a hot topic for lots of scholars for their regulation functions on assorted cancers. Several lncRNAs have been proven they can take part in the regulation of prostate cancer development. Nevertheless, how HOXA11-AS (homeobox A11 antisense RNA) functioned in prostate cancer is not explained. In our research, the expression of HOXA11-AS in prostate cells was evaluated through qRT-PCR. Colony formation experiments, EdU experiments, Tanswell and TUNEL experiments, as well as caspase-3 detection, were designed to test cell proliferation, migration, invasion and apoptosis. RIP, pull down and luciferase reporter experiments examined the correlations of HOXA11-AS, miR-148b-3p and MLPH. We discovered a high level of HOXA11-AS in prostate cancer cells. HOXA11-AS silence could restrain the mentioned cell malignant behavior. Mechanically, HOXA11-AS could sponge miR-148b-3p to target MLPH. MLPH was positively associated with HOXA11-AS and overexpressed it accelerated the progression of prostate cancer. Taken together, HOXA11-AS elevated MLPH expression by sponging miR-148b-3p and accelerated prostate cancer cell proliferation.

Introduction

The epithelial malignant tumor of the prostate is known as prostate cancer. The incidence of prostate cancer keeps increasing over the past years. With the growth of males age, the incidence of the disease is higher. The high incidence age of prostate cancer is 70 years old. With the development of medicine, radical treatment can be adopted for early prostate cancer, such as radical prostatectomy and radical external radiotherapy. Since the early symptoms of this cancer are not obvious, prostate cancer has developed into an advanced stage when patients are diagnosed. The overall survival rate is not satisfactory. Thus, it is urgent to find effective biomarkers (1).

Long non-coding RNAs (lncRNAs) refer to RNAs with a length of t more than 200 nucleotides. In addition, they have limited power to encode proteins (1). Evidences have proved dissimilar functions were exerted by lncRNA in cancer. The progression of cancer has been proven to be correlated with maladjusted lncRNAs. For instance, dysregulated FTX was blamed for the abnormally activated malignant phenotype of renal cell carcinoma cells (2). Up-regulation of H19 betokens a bad prognosis of cholangiocarcinoma and accelerates cell growth (3). What’s more, disordered CASC9.5 expedites the cell proliferative capability of lung adenocarcinoma (4). At the same time, lots of lncRNAs have been indicated that they can regulate the development of prostate cancer, like HOXD-AS1 and LINC00844 (5, 6). They served as the tumor promoter in prostate cancer and accelerate its progression by regulating genes expressions. Increasing number of researches demonstrated that the progression of cancer could be control by lncRNAs via serving as ceRNAs. The ceRNA refers to competing endogenous RNAs which can release mRNAs through completely sponging miRNAs (7). For example, SNHG5 competitively binds to miR-32 against KLF4 to regulate gastric cancer cell growth (8). And TUG1 accelerates papillary thyroid cancer cell growth through targeting miR-145 (9). As a HOXA11 antisense lncRNA, HOXA11-AS (homeobox A11 antisense RNA) has been reported to exert the carcinogenic effect on gastric cancer and melanoma and it accelerated the progressions of them (10, 11). Nevertheless, the specific biological functions of HOXA11-AS in prostate cancer are still unclear.

Hence, we mainly intend to uncover the latent molecular mechanisms and functions of HOXA11-AS in prostate cancer. This object may provide new insights into curing prostate cancer.

Materials and Methods

Cell culture

Human prostate cancer cell lines (DU145, PC3, LNCAP, 22RV1, VCaP) and human normal prostate epithelial cell line (RWPE-1), from the ATCC cell bank (Rockville, MD), were maintained under 5% CO2, and 37°C. RPMI-1640 (HyClone, Logan, UT), with 10% FBS and 1% antibiotic, was acquired for cell culture.

Quantitative real-time PCR (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA) was applied
for the extraction of RNAs from cell samples. Then, total RNA was converted into cDNA using Reverse Transcription Kit (Takara, Tokyo, Japan). RT-qPCR was progressed using SYBR-Green Real-Time PCR Kit (Takara). Fold expression changes were determined with the utilization of the 2^(-ΔΔCt) method, with GAPDH/U6 utilized as the reference gene.

**Plasmid transfection**

GenePharma (Shanghai, China) helps to provide all plasmids for gene overexpression and inhibition. shRNAs were synthesized to silence HOXA11-AS and MLPH in cells of DU145 and LNCAP using Lipofectamine 2000 (Invitrogen), and the control plasmids sh-NC were provided at the same time. Moreover, the miR-148b-3p mimics/inhibitor and NC mimics/inhibitor, as well as pcDNA3.1/MLPH and NC-pcDNA3.1, were also purchased to conduct 48 h of plasmid transfection.

**Colonies formation**

After 2-week culture, DU145 and LNCAP cells in 6-well plates (500 cells / well) were stained with crystal violet. Colonies were counted manually.

**EdU staining**

EdU staining assay of DU145 and LNCAP cells was completed in line with the manufacturer’s protocol forth the EdU kit (Ribobio, Guangzhou, China). Cells in 96-well plates were stained by EdU. Hoechst 33342 solution was added for 30-min staining of cell nuclei in the dark. A fluorescence microscope (Olympus, Tokyo, Japan) was procured for sample observation.

**TUNEL staining**

The apoptosis cells were evaluated by using TUNEL Apoptosis Kit (Beyotime, Shanghai, China) based on the user’s guide. TUNEL reaction mixture was added to incubate the dehydrated samples. After DAPI counterstaining, the TUNEL-positive cells were counted under a fluorescence microscope.

**Caspase activity detection**

The caspase-3/8/9 activity kits were available to detect the caspase-3/8/9 activities as per manual Solarbio (Beijing, China). Cell proteins were added to a 96-well plate containing the caspase substrate and provided a reaction buffer. Absorbance at 405 nm was examined by a microplate reader.

**Transwell assay**

Cell migration was monitored by Transwell chambers (Corning, Corning, NY). The basal chamber was filled with a culture medium containing 10% FBS, while the apical chamber was filled with cells in a medium without serum. Invasion analysis was the same except that the upper chamber was coated with matrigel. Migrating or invading cells after were determined with the help of 0.1% crystal violet staining using microscope h.

**Subcellular fraction**

Cytoplasmic and Nuclear RNA Purification kits (Norgen, Ontario, Canada) were applied for the isolation and purification of cytoplasmic and nuclear RNA of DU145 and LNCAP cells based on the specification. HOXA11-AS abundance was assessed by qRT-PCR. GAPDH and U6 were considered cytoplasm and nuclear control.

**FISH**

After fixing by 4% formaldehyde, cells were dehydrated and then cultured in a hybridization buffer with the HOXA11-AS-FISH probe (Ribobio). DAPI staining was performed prior to observation with a fluorescence microscope.

**RNA immunoprecipitation (RIP)**

Magna RNA-binding protein immunoprecipitation kit was employed for RIP assay in accordance with the direction. DU145 and LNCAP cells in RIP buffer were immunoprecipitated with antibodies against Ago2 or normal IgG, followed by qRT-PCR analysis. The kit and antibodies used were provided by Millipore (Bedford, MA).

**RNA pull-down**

The miR-148b-3p fragments carrying wild-type and mutated HOXA11-AS or MLPH binding sites were biotinylated to build Bio-miR-148b-3p-WT/Mut. Cell protein extracts were harvested for co-culturing with the biotin probes and the streptavidin-coupled agarose beads were added to collect the pull-downs.

**Luciferase reporter assay**

Binding sequences of miR-148b-3p in HOXA11-AS or MLPH fragments (wild or mutant) were inserted into the pmirGLO luciferase reporter vector (Promega, Madison, WI). After the constructs and miR-148b-3p mimics/NC mimics were co-transfected for 48 h, the luciferase activity was achieved by applying a dual luciferase system (Promega).

**Statistical analyses**

All experiments were conducted thrice independently. Results after data analysis were shown as means ± SD. Statistical analysis was completed depending on the processing of GraphPad Prism 5 software (GraphPad Software, San Diego, CA). The significance of the statistical difference between two or multiple groups was analyzed by Student’s t-test or ANOVA. P<0.05 denoted the threshold for statistical significance.

**Results**

**HOXA11-AS deficiency represses prostate cancer cell malignant phenotype**

To investigate HOXA11-AS function in prostate cancer, we measured its expression in prostate cancer cells at the very beginning. According to the outcomes of qRT-PCR, HOXA11-AS demonstrated a high level in prostate cancer cells, especially in DU145 and LNCAP cells (Fig. 1A). Thus, we detected the efficacy of HOXA11-AS silencing in the two cell lines (Fig. 1B), observing visible decline in HOXA11-AS expression upon transfection. Subsequently, we corroborated that the proliferative ability of DU145 and LNCAP cells was suppressed responding to HOXA11-AS silencing since the colonies number, as well as EdU positive cells rate, were decreased (Fig. 1C-D). Furthermore, the effect of HOXA11-AS depletion on cell apoptosis was also assessed through TUNEL experiments and spectrophotometer detection of caspase-3 acti-
HOXA11-AS sponges miR-148b-3p in prostate cancer cells

For probing the latent molecular mechanisms of HOXA11-AS in prostate cancer, the subcellular distribution of HOXA11-AS was determined first. Experimental results revealed the primarily cytoplasmic accumulation of HOXA11-AS in prostate cancer cells (Fig. 2A-B). Then, RIP assays uncovered that HOXA11-AS was enriched in Ago2, indicating that HOXA11-AS could exert the sponge effects (Fig. 2C). Accordingly, starBase (http://starbase.sysu.edu.cn/) was involved in the prediction of the potential miRNAs, and ultimately, miR-148b-3p was found since it met our screening criteria (pan-Cancer >= 10). MiR-148b-3p was found to possess a low expression in prostate cancer cells, especially in DU145 and LNCAP cells (Fig. 2D). As HOXA11-AS was seen to be largely pulled down by biotin-labeled miR-148b-3p-WT, the combination relationship between HOXA11-AS and miR-148b-3p was validated (Fig. 2E). The binding sites were presented in Fig. 2F. Following the verification of miR-148b-3p overexpression efficacy (Fig. 2G), the outcomes of luciferase reporter experiments illustrated that the luciferase activity of HOXA11-AS-WT was visibly diminished in response to miR-148b-3p augmentation, which represented that miR-148b-3p had a strong affinity to HOXA11-AS-WT (Fig. 2H). Overall, miR-148b-3p was sequestered by HOXA11-AS in prostate cancer cells.

MLPH is miR-148b-3p target

On the basis of the prediction of RAN22, miRmap and miRanda databases, 14 mRNAs were selected as the possible target of miR-148b-3p (Fig. 3A). In order to select the most suitable mRNA, we implemented the qRT-PCR and the outcomes indicated that 4 mRNAs (VPS45, UBA6, MLPH and ROBO1) could be reduced under miR-148b-3p overexpression (Fig. 3B). Moreover, the levels of VPS45 and MLPH were decreased evidently, while that of UBA6 and ROBO1 was barely affected by HOXA11-AS silence (Fig. 3C). We also detected a high expression of MLPH in prostate cancer cells (Fig. 3D), whereas VPS45 did not present the notably high expression profile. Thus, MLPH was selected to take further experiments. RIP experiments

vity (Fig. 1E-F). The outcomes depicted that the TUNEL positive cells ratio ascended. The activity of caspase-3/8/9 was also observed to be enhanced when HOXA11-AS was subjected to down-regulation. It meant that cell apoptotic ability could accelerate by the lack of HOXA11-AS. Additionally, Transwell experiments uncovered that the migratory and invasive capabilities of DU145 and LNCAP cells were weakened after HOXA11-AS knockdown (Fig. 1G). Overall, HOXA11-AS depletion restrained proliferation, migration and invasion of prostate cancer cells but accelerated cell apoptosis.

HOXA11-AS sponges miR-148b-3p in prostate cancer cells

For probing the latent molecular mechanisms of HOXA11-AS in prostate cancer, the subcellular distribution of HOXA11-AS was determined at first. Experimental results revealed the primarily cytoplasmic accumulation of HOXA11-AS in prostate cancer cells (Fig. 2A-B). Then, RIP assays uncovered that HOXA11-AS was enriched in Ago2, indicating that HOXA11-AS could exert the sponge effects (Fig. 2C). Accordingly, starBase (http://starbase.sysu.edu.cn/) was involved in the prediction of the potential miRNAs, and ultimately, miR-148b-3p was found since it met our screening criteria (pan-Cancer >= 10). MiR-148b-3p was found to possess a low expression in prostate cancer cells, especially in DU145 and LNCAP cells (Fig. 2D). As HOXA11-AS was seen to be largely pulled down by biotin-labeled miR-148b-3p-WT, the combination relationship between HOXA11-AS and miR-148b-3p was validated (Fig. 2E). The binding sites were presented in Fig. 2F. Following the verification of miR-148b-3p overexpression efficacy (Fig. 2G), the outcomes of luciferase reporter experiments illustrated that the luciferase activity of HOXA11-AS-WT was visibly diminished in response to miR-148b-3p augmentation, which represented that miR-148b-3p had a strong affinity to HOXA11-AS-WT (Fig. 2H). Overall, miR-148b-3p was sequestered by HOXA11-AS in prostate cancer cells.

MLPH is miR-148b-3p target

On the basis of the prediction of RAN22, miRmap and miRanda databases, 14 mRNAs were selected as the possible target of miR-148b-3p (Fig. 3A). In order to select the most suitable mRNA, we implemented the qRT-PCR and the outcomes indicated that 4 mRNAs (VPS45, UBA6, MLPH and ROBO1) could be reduced under miR-148b-3p overexpression (Fig. 3B). Moreover, the levels of VPS45 and MLPH were decreased evidently, while that of UBA6 and ROBO1 was barely affected by HOXA11-AS silence (Fig. 3C). We also detected a high expression of MLPH in prostate cancer cells (Fig. 3D), whereas VPS45 did not present the notably high expression profile. Thus, MLPH was selected to take further experiments. RIP experiments
unveiled that MLPH, miR-148b-3p, as well as HOXA11-AS, were all enriched in the Anti-Ago2-precipitated complex (Fig. 3E). MLPH was also detected in the pull-down products of Bio-miR-148b-3p-WT (Fig. 3F). Based on the binding sites of MLPH and miR-148b-3p (Fig. 3G), luciferase reporter experiments appraise the potential of the combination. (I) qRT-PCR assessed the efficacy of miR-148b-3p inhibition. (J) MLPH expression was tested when HOXA11-AS and miR-148b-3p were suppressed.*P <0.05, **P < 0.01.

**Figure 3.** MLPH is a target of miR-148b-3p in prostate cancer cells. (A) The potential targets of miR-148b-3p were projected on the bioinformatics database. (B) qRT-PCR detected candidate mRNAs expression in response to miR-148b-3p augmentation. (C) qRT-PCR tested potential mRNAs expression after HOXA11-AS silencing. (D) qRT-PCR monitored mRNAs expression in prostate cancer cells. (E-F) RIP and pull-down experiments examined the combination of HOXA11-AS, miR-148b-3p and MLPH. (G) The binding sequences of miR-148b-3p and MLPH were presented. (H) Luciferase reporter experiments appraise the potential of the combination. (I) qRT-PCR assessed the efficacy of miR-148b-3p inhibition. (J) MLPH expression was tested when HOXA11-AS and miR-148b-3p were suppressed.*P <0.05, **P < 0.01.

**Figure 4.** HOXA11-AS conduces to prostate cancer cell growth via miR-148b-3p/MLPH axis. (A) qRT-PCR evaluated the efficacy of MLPH augmentation. (B-C) Colony formation and EdU assays estimated the rescue effect on cell proliferation upon indicated transfections. (D-E) TUNEL and caspase-3 activity detection monitored the variations of cell apoptosis under different conditions. (F) Transwell experiments examined the migration and invasion of prostate cancer cells with indicated transfections. **P < 0.01.

**HOXA11-AS accelerates prostate cancer cell growth by regulating miR-148b-3p/MLPH axis**

To figure out whether HOXA11-AS/miR-148b-3p/MLPH axis was involved in regulating prostate cancer cell growth, rescue experiments were implemented. Above all, the efficiency of MLPH overexpression in DU145 and LNCAP cells was examined (Fig. 4A). Then, we noticed that the suppressive impact of HOXA11-AS silencing on prostate cancer cell proliferation was abrogated by miR-148b-3p inhibition or MLPH up-regulation (Fig. 4B-C). On the other side, the facilitated cell apoptosis caused by HOXA11-AS depletion could be restored by miR-148b-3p repression or MLPH augmentation (Fig. 4D-E). In the end, Transwell experiments demonstrated that HOXA11-AS silencing impaired the migratory and invasive ability of prostate cancer cells, and such effect was abolished by miR-148b-3p inhibition or MLPH overexpression (Fig. 4F).

**MLPH silencing results in the suppression of prostate cancer cell growth**

We further proved how MLPH functioned in prostate cancer cells. After the effective MLPH knockdown was confirmed (Fig. S1A), EdU and colony formation experiments were conducted. As presented in Fig. S1B-C, cell proliferation was hindered on account of MLPH down-regulation. Additionally, cell apoptosis was expedited upon MLPH inhibition based on TUNEL assay and caspase-3 activity detection (Fig. S1D-E). In the end, cell migrated and invaded capabilities were estimated through Transwell experiments and the outcomes demonstrated that both of the two abilities could be suppressed after silencing MLPH (Fig. S1F). Taken together, MLPH silencing conduces to the impeded proliferation, migration and invasion of prostate cancer cells.
Discussion

Current evidence elaborates that lncRNAs display anomalous expressions in varied cancers, and have been recognized as vital regulatory factors involved in diverse biological processes (12). For example, IncRNA AK027294 engages in affecting the proliferation, migration, and apoptosis of colorectal cancer cells (13). Moreover, IncRNA PVT1 modulates the miR-143/HK2 axis to facilitate gallbladder cancer development (15). In the same way, IncRNAs have been proven that they exert their regulatory functions in prostate cancer. For instance, SOCS2-AS1 conducted to prostate cancer cell growth, while impeding cell apoptosis (16). HOXA11-AS has also been unveiled to work as a carcinogenic gene in assorted cancers and accelerate their progressions. Specifically, HOXA11-AS works as a miR-140-5p sponge contributing to glioma tumorigenesis (18). HOXA11-AS also has a regulatory effect on the cisplatin resistance of lung adenocarcinomas (17). Herein, we uncovered the intense and anomalous expression of HOXA11-AS and corroborated that HOXA11-AS silencing resulted in the restrained malignant behavior of prostate cancer cells. Thus, HOXA11-AS acted as the oncogene in prostate cancer cells.

LncRNAs can sequester microRNAs (miRNAs) to impact the downstream target gene expression in assorted cancers (19). MiRNAs are small RNAs consisting of 20-22 nucleotides. They served as crucial regulators in cancers (20). A large number of reports have unmasked that the expressions of miRNAs are maladjusted in prostate cancer. Referring to existing evidence, miR-148b-3p has been identified as the tumor inhibitor in hepatocellular carcinoma (21), gastric cancer as well as osteosarcoma (26). In our research, miR-148b-3p was revealed to be sequestered by HOXA11-AS and display a low expression in prostate cancer cells. In addition, miRNAs influence gene expressions by binding to the 3’ UTRs of mRNAs (27). For example, lncRNA TUG1-induced miR-142-3p inhibition facilitates metastasis and the EMT of hepatocellular carcinoma by targeting ZEB1 (28). The present work uncovered that MLPH targeted by miR-148b-3p, exhibited a high level in prostate cancer cells. Moreover, overexpressed MLPH abrogated the inhibitory impact of HOXA11-AS silencing on prostate cancer cell growth.

To sum up, our research proved that HOXA11-AS contributed to prostate cancer cell proliferation by modulation on miR-148b-3p/MLPH. This discovery might offer new insight for searching the novel therapeutic target for curing prostate cancer.

Acknowledgement

All supports from our experimenters are appreciated.

Conflicts of interest

None.

Fundings

The research is supported by: Key Projects of Natural Science Foundation of Colleges and Universities in Anhui Province (No. KJ2021A0777) Key Projects of Natural Science Foundation of Colleges and Universities in Anhui Province (No. KJ2019A0355); Science Foundation of Bengbu Medical College (No. BYKY1840ZD).

References

9. LeiH, Gao Y, Xu X. LncRNA TUG1 influences papillary thyroid cancer cell proliferation, migration and EMT formation through targeting miR-145. Acta Biochim Biophys Sin (Shanghai)2017; 49(7): 588-597.
13(9): 1347-57.


