LncRNA HEIRCC promotes prostate cancer growth through activating Wnt/β-catenin pathway

Ren Wang1*, Xia Hong2*, Rui-Liang Wang3, Bing Hu1

1Department of Ultrasound in Medicine, Shanghai Jiaotong University Affiliated Sixth People’s Hospital, Shanghai Institute of Ultrasound in Medicine, Shanghai, China; 2Child Health Care Center, Shanghai Children’s Hospital, Affiliated to Shanghai, Shanghai, China; 3Department of Geriatrics, Shanghai Jiaotong University Affiliated Xinhua Hospital, Shanghai, China. *Equal contributors.

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Abstract: Recent studies showed that long non-coding RNAs (lncRNAs) were associated with tumor progression. However, the contributions of lncRNAs to prostate cancer (PCa) were still unclear. Thus, we aimed to explore the role of lncRNA HEIRCC (high-expressed in renal cell carcinoma) and its underlying mechanism in PCa progression. In the current study, we firstly reported that HEIRCC expression was overexpressed in PCa tissues and cell lines, increased expression of HEIRCC was associated with advanced histological grade, distant metastasis and poor overall survival of patients with PCa. Function assays showed that HEIRCC silencing suppressed PCa cell proliferation, arrested cell cycle and induced cell apoptosis in vitro, and inhibited tumorigenicity of PCa cells in vivo. In addition, we showed that HEIRCC inhibition suppressed Wnt/β-catenin signaling activity in PCa cells. Therefore, we concluded that lncRNA HEIRCC could act as a tumor oncogene in PCa by promoting cell proliferation through Wnt/β-catenin signaling. Thereby lncRNA HEIRCC may function as a potential therapeutic target for the treatment of PCa.

Keywords: Long non-coding RNAs, HEIRCC, prostate cancer, Wnt/β-catenin signaling

Introduction

Prostate cancer (PCa) is one of the most prevalent solid tumors and the second leading cause of cancer related death in men worldwide [1]. Androgen receptor plays critical roles in the progression of PCa, and androgen deprivation therapy is the first line therapy for most first time diagnostic PCa patients [2]. However, despite initial response rates of 80-90%, patients will progress to castration-resistant prostate cancer (CRPC) and even metastatic PCa [3]. Therefore, understanding the carcinogenesis mechanism of PCa is important for developing new therapies, especially for patients with CRPC and metastasis.

Long non-coding RNAs (lncRNAs) are a kind of non-coding RNA transcript with more than 200 nucleotides without protein coding function [4]. Recent studies showed that lncRNAs were involved in multiple cellular biological processes, ranging from transcriptional and post-transcriptional regulation to cell cycle distribution, cell differentiation and epigenetic modifications [5-7]. Furthermore, lots of lncRNAs were also important factors involving in cancer development and progression [8]. For example, Yin et al revealed that lncRNA GAS5 affected cell proliferation and predicts a poor prognosis in patients with colorectal cancer [9]. Zhang et al found that lncRNA ANRIL indicated a poor prognosis of cervical cancer and promotes carcinogenesis via PI3K/Akt pathways [10]. Wang et al showed that lncRNA Malat1 promoted gallbladder cancer development by acting as a molecular sponge to regulate miR-206 [11]. However, the biological role of HEIRCC and its mechanism in PCa is still unclear.

In the present study, we found that the expression of lncRNA HEIRCC was upregulated in PCa tissues and cell lines. Increased expression of HEIRCC was associated with advanced clinical features and poor prognosis of PCa patients. Function assays revealed that HEIRCC silencing suppressed PCa cell proliferation, arrested cell cycle, induced cell apoptosis in vitro, and inhib-
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Materials and methods

Human tissue samples

PCa tissues and matched adjacent non-tumor tissues were obtained from 68 patients who underwent radical prostatectomy in the period from 2011 to 2012 at Shanghai Jiaotong University Affiliated Sixth People’s Hospital. None of the patients has received any previous chemotherapy or radiotherapy before surgery. All of the tissue samples were immediately snap frozen in liquid nitrogen after surgical excision and stored at -80°C until total RNA was extracted for experiments. Informed consents were obtained from all patients, and this study was approved by the Clinical Research Ethics Committee at the Shanghai Jiaotong University.

Cell culture and transfection

Human PCa cell lines (PC3, DU145, and LNCaP) were all acquired from the American Type Culture Collection. Normal human prostate epithelial cell line RWPE-1 was purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) which was contained in a humidified atmosphere with 5% CO₂ at 37°C.

Transfections were performed using the Lipo-fectamine 2000 kit (Invitrogen) according to the manufacturer’s instructions. The siRNAs to HEIRCC (si-HEIRCC) and scrambled siRNA were purchased from Genepharma Co., Ltd. (Shanghai, China). The siRNA sequences for HEIRCC were as followed: si-HEIRCC-1, 5’-GGAAGAGUCUCUCCCUUUTT-3’; si-HEIRCC-2, 5’-UCACCU-GUUUAUCGGAGUUTT-3’ [12]. The scrambled siRNA was used as the negative control (si-NC).

Cell proliferation assay

The cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo) according to the manufacturer’s protocol. Briefly, a total of approximately 5 × 10⁴ PCa cells were plated in 96-well plates, treated with 20 µl/well of CCK-8 solution during the last 4 h of culture, and the cell proliferation curves were plotted using the 450 nm absorbance at each time point. All experiments were performed in triplicate.

Cell cycle assay

After 48 h transfection, the cells were collected and washed as well as re-suspended with PBS and fixed with 75% ethanol overnight at 4°C. On second day, the cells were incubated with RNase at 37°C for 30 min. Then the cells were stained with propidium iodide for 30 min. Culture were collected and analyzed for cell cycle using a Flow Cytometer. All experiments were performed in triplicate.

Cell apoptosis assay

After 48 h transfection, the cells were collected and washed twice with cold PBS and re-suspended in binding buffer. Then, Apoptosis was evaluated using an Annexin-Fluos and Propidium Iodide (PI) Apoptosis Detection Kit (Sigma) by fluorescence activated cell sorter (FACS, BD Bioscience) according to the manufacturer’s protocol. All experiments were performed in triplicate.

Tumor xenograft model in nude mice

PC3 cells were transfected with si-HEIRCC and the scrambled siRNA (si-NC). A total of 3 × 10⁶ transfected cells were suspended in 100 µl of serum-free DMEM culture medium and subcutaneously injected into 6 week-old female nude mice in the oxter. Mouse weights and tumor sizes were measured every 3 days after 7 days of injection. The tumor volume was calculated as follows: length × width² × 1/2. All mice were sacrificed 21 days after injection. The tumors were isolated from the mice and stored at -80°C.

RNA extraction and quantitative real-time PCR

Total RNAs were extracted using TRIzol reagent (Sigma) following the manufacturer’s instructions. Reverse transcript PCR was carried out using PrimeScript RT Master Mix (Takara) according to the manufacturer’s instructions. Real-time qPCR was performed using AceQ
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Results

LncRNA HEIRCC expression were upregulated in PCa tissues and cell lines

To explore the function of IncRNA HEIRCC in the progression of PCa, we first determined the expression of HEIRCC in 68 pairs of PCa tissues and adjacent non-tumor tissues by qRT-PCR and results were expressed as mean ± SD (standard deviation, SD). Statistical analyses were performed using Student’s t test, chi-square tests or One-way ANOVA as appropriate. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to compare the differences between patient groups. P<0.05 was considered statistically significant.

Luciferase reporter assay

Indicated cells were seeded in 96-well plates and transfected with TCF/β-catenin reporter plasmid (Wnt/β-catenin signaling) and 10 ng Renilla following the recommended protocol for the Lipofectamine 2000 transfection system. After 48 h incubation, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega) from the cell lysates.

Western blot analysis

Cells were washed once with cold PBS and lysed in RIPA buffer containing protease inhibitors. Approximate 30 μg of protein was separated with 8-12% SDS-PAGE gel and blotted onto nitrocellulose membranes. Then membranes were blocked with 5% milk at room temperature for 1 h and then incubated with primary antibodies at 4°C overnight, followed by TBST wash and 1 h incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature. Protein bands were visualized by a Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories).

Statistical analysis

All statistical analyses were performed using SPSS 17.0 and results were expressed as mean ± SD (standard deviation, SD). Statistical analyses were performed using Student’s t test, chi-square tests or One-way ANOVA as appropriate. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to compare the differences between patient groups. P<0.05 was considered statistically significant.

Figure 1. Relative expression of IncRNA HEIRCC in PCa tissues and adjacent non-tumor tissues. IncRNA HEIRCC expression was examined by qRT-PCR SYBR Green Master Mix (Vazyme Biotech co.,ltd) on Roche LightCycler 480. The PCR primers for HEIRCC or GAPDH were as follows: HEIRCC forward, 5’-GCTGCTATTCTGGTGCCC-3’ and reverse, 5’-TCAACTCCGATAAACAGGTGA-3’; GAPDH forward, 5’-CGCTCTCTGCTCCTCCTGTTC-3’ and reverse, 5’-ATCGGTGACTCCGACCTTCAC-3’. The Ct values were normalized using GAPDH as internal control to estimate the different expression of genes. Each sample was run in triplicate to ensure quantitative accuracy.
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Table 1. The relationship between IncRNA HEIRCC expression and clinicopathological features in PCa

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number</th>
<th>Low expression</th>
<th>High expression</th>
<th>P value</th>
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<tr>
<td>Age (years)</td>
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<tr>
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<tr>
<td>≥65</td>
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<td>24</td>
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<td></td>
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<tr>
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<td>20</td>
<td>17</td>
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<tr>
<td>≥2.5</td>
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<td>14</td>
<td>17</td>
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<td>14</td>
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</table>

As shown in Figure 1A, increased expression of HEIRCC was observed in PCa tissues compared with adjacent non-tumor tissues (P<0.05). We then detected HEIRCC expression levels in three PCa cell lines and cultured normal human prostate epithelial cell line RWPE-1. As shown in Figure 1B, HEIRCC expression was higher in PCa cell lines (PC3, DU145, and LNCaP) compared to RWPE-1 cells (P<0.05). These results indicated that IncRNA HEIRCC play important roles in PCa pathogenesis.

High IncRNA HEIRCC expression correlated with poor overall survival of PCa patients

According to the median value of relative HEIRCC expression in PCa tissues, PCa patients were divided into two groups: high HEIRCC group and low HEIRCC group. The correlation between expression of HEIRCC and clinicopathological features were assessed in Table 1. HEIRCC expression levels were associated with advanced histological grade and distant metastasis (P<0.05). However, there was no significant difference between HEIRCC expression and other clinicopathological features, such as age, tumor diameter, Gleason score, lymph node metastasis, tumor stage and Capsule invasion (P>0.05). Kaplan-Meier survival curve analysis with a log-rank comparison showed that high HEIRCC expression group had a poor overall survival than the low HEIRCC expression group (Figure 1C, P<0.05). These findings indicated that IncRNA HEIRCC could act as a prognosis biomarker of PCa.

Knockdown of IncRNA HEIRCC suppressed PCa cell proliferation in vitro

To investigate the biological function of IncRNA HEIRCC in PCa tumorigenesis, we detected the effects of HEIRCC on the cell proliferation, cell cycle and cell apoptosis. The knockdown efficiency of HEIRCC in PC3 cells were shown in Figure 2A. CCK-8 assay showed that the cell proliferation ability was significantly decreased when HEIRCC was inhibited in PC3 cell lines (Figure 2B, P<0.05). Next, we examined the impact of HEIRCC on cell cycle and apoptosis in PC3 cells. Flow cytometric analysis showed that reduced expression of HEIRCC increased the number of PC3 cells in G0/G1 phase and decreased the number of PC3 cells in S phase (Figure 2C, P<0.05). Furthermore, compared with si-NC group, HEIRCC inhibition markedly increased PC3 cells apoptosis (Figure 2D, P<0.05). These data indicated that HEIRCC promoted PCa cells proliferation by promoting cell cycle progression and inhibiting cell apoptosis.

Knockdown of IncRNA HEIRCC inhibited PCa cell growth in vivo

To further confirm the effects of HEIRCC on PCa tumorigenesis, si-HEIRCC or si-NC transfected PC3 cells were inoculated into nude mice. All mice developed xenograft tumors at the injection site. As shown in Figure 3A and 3B, tumor
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Subsequently modulates cell proliferation. As shown in Figure 4A, luciferase reporter assays revealed that silencing HEIRCC impaired the activation of Wnt/β-catenin signaling in PC3 cells (P<0.05). We then examined the expression level of β-catenin, cyclin D1 and c-myc (classic downstream genes of the Wnt/β-catenin signaling pathway) in HEIRCC inhibited PC3 cells. Western blot showed that reduced expression of HEIRCC suppressed the protein expression of β-catenin, cyclin D1 and c-myc expression compared to control groups (Figure 4B). These data suggested that upregulation of HEIRCC expression contributes to the tumorigenesis might by regulating Wnt/β-catenin signaling in PCa.

Figure 2. Effects of lncRNA HEIRCC on PCa cell proliferation, cell cycle and apoptosis in vitro. A. Relative expression of lncRNA HEIRCC after PCa cells transfected with si-HEIRCC and si-NC. B. CCK-8 assay showed knockdown of lncRNA HEIRCC suppressed cell proliferation of PC3 cells. C. Flow cytometric analysis showed knockdown of lncRNA HEIRCC increased PC3 cells in G0/G1 phase and decreased cells in S phase. D. Flow cytometric analysis showed knockdown of lncRNA HEIRCC increased apoptotic cells in PC3 cells. *P<0.05.

Growth in si-HEIRCC group was significantly slower than that in si-NC group (P<0.05). Furthermore, the average tumor weight in si-HEIRCC group was obviously lower than in si-NC group (Figure 3C; P<0.05). The expression level of lncRNA HEIRCC was also detected, and the result showed that HEIRCC was knocked down effectively (Figure 3D; P<0.05). These data indicated that lncRNA HEIRCC inhibition could suppress PCa cell proliferation in vivo.

LncRNA HEIRCC regulated the activation of Wnt/β-catenin signaling in PCa

As Wnt/β-catenin signaling could regulate these cyclins [13], we then asked whether lncRNA HEIRCC regulates Wnt/β-catenin signaling then subsequently modulates cell proliferation. As shown in Figure 4A, luciferase reporter assays revealed that silencing HEIRCC impaired the activation of Wnt/β-catenin signaling in PC3 cells (P<0.05). We then examined the expression level of β-catenin, cyclin D1 and c-myc (classic downstream genes of the Wnt/β-catenin signaling pathway) in HEIRCC inhibited PC3 cells. Western blot showed that reduced expression of HEIRCC suppressed the protein expression of β-catenin, cyclin D1 and c-myc expression compared to control groups (Figure 4B). These data suggested that upregulation of HEIRCC expression contributes to the tumorigenesis might by regulating Wnt/β-catenin signaling in PCa.
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Discussion

Increasing studies suggested that dysregulation of IncRNAs involved in tumor occurrence and development in multiple cancers, including PCa. For example, Wang et al suggested that overexpression of IncRNA LOC400891 promoted tumor progression and poor prognosis in prostate cancer [14]. Zheng et al suggested that the up-regulation of IncRNA CCAT2 indicated a poor prognosis for prostate cancer and promoted metastasis by affecting epithelial-mesenchymal transition [15]. Xue et al showed that IncRNA GAS5 inhibited proliferation and progression of prostate cancer by targeting miR-103 through AKT/mTOR signaling pathway [16]. These studies suggested that IncRNAs could serve as a therapeutic target in the treatment of PCa. However, the overall pathophysiological roles of IncRNAs to PCa remain largely unknown.

HEIRCC (high-expressed in renal cell carcinoma) was found to be significantly upregulated in renal cell carcinoma tissues and overexpression of HEIRCC was associated with larger tumor size, poor differentiation and lymphatic metastasis of patients. Furthermore, they found that HEIRCC knockdown could inhibit cell proliferation, migration, invasion and the epithelial to mesenchymal transition (EMT) program [12]. However, to our knowledge, the clinical significance and biological function of HEIRCC in prostate cancer remains unclear.

In our study, we found that IncRNA HEIRCC expression was increased in PCa tissues and cell lines, and its increased expression of HEIRCC was associated with advanced clinicopathological features and poor overall survival of PCa patients. Function assays showed that HEIRCC silencing suppressed PCa cell proliferation, arrested cell cycle and induced cell apoptosis in vitro. In addition, our data showed that HEIRCC suppression inhibited tumorigenicity of PCa cells in vivo. These findings suggested that high HEIRCC expression may represent a novel biomarker of poor prognosis and a potential therapeutic target for the treatment of PCa.

Wnt/β-catenin signaling contributes to tumour progression characteristics (invasion, EMT, metastasis, and angiogenesis) [17, 18]. Recent
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studies showed that IncRNAs could regulate tumor progression via Wnt/β-catenin signaling pathway. For example, Li et al found that IncRNA HOTAIR led to chemoresistance by activating the Wnt/β-catenin pathway in human ovarian cancer [19]. Shao et al suggested that highly expressed IncRNA CRNDE promoted cell proliferation through Wnt/β-catenin signaling in renal cell carcinoma [20]. Zhao et al revealed that up-regulated expression of IncRNA NEAT1 promoted progression of osteosarcoma by regulating the activity of Wnt/β-catenin pathway [21]. In the current study, luciferase reporter assays revealed that silencing HEIRCC impaired the activation of Wnt/β-catenin signaling in PC3 cells. In addition, we found that reduced HEIRCC expression could suppress the protein expression of β-catenin, cyclin D1 and c-myc expression in PCa cells. These data suggested that upregulation of HEIRCC contributes to the tumorigenesis might by regulating Wnt/β-catenin signaling. However, the detailed mechanisms of how HEIRCC regulated Wnt/β-catenin signaling remains to be elucidated in further study.

Taken together, our data showed that IncRNA HEIRCC, which is increased in PCa, plays critical roles in PCa cell proliferation via the Wnt/β-catenin signaling pathway. Thus, these data indicated that IncRNA HEIRCC may act as a potential therapeutic target and prognostic factor for the treatment of PCa.

Disclosure of conflict of interest

None.

Address correspondence to: Ren Wang, Department of Ultrasound in Medicine, Shanghai Jiaotong University Affiliated Sixth People’s Hospital, Shanghai Institute of Ultrasound in Medicine, Shanghai 200233, China. E-mail: jinjinchen76@163.com

References

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