Long non-coding RNA RNCR3 promotes prostate cancer progression through targeting miR-185-5p

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Abstract: Long non-coding RNAs (lncRNAs) have been suggested to play important roles in the development of numerous kinds of human cancers. Increasing data has indicated that lncRNA RNCR3 has been involved in some human diseases. However, the exactly biological function and potential mechanisms of RNCR3 in the development of prostate cancer is still unclear. Here, our results confirmed that the RNCR3 expression was increased in prostate cancer compared to the corresponding adjacent normal prostate tissues. Moreover, our data showed that the increased expression of RNCR3 is significantly associated with tumor progression and poor survival of prostate cancer patients. Additionally, we found that RNCR3 knockdown could suppress the ability of proliferation, colony formation, and invasion of prostate cancer cells. Finally, we further confirmed that RNCR3 binds to miR-185-5p, which has been identified as a tumor suppressor in some human cancers, including prostate cancer. We also confirmed that the oncogenic function of RNCR3 in prostate cancer are partly mediated by negative regulation of miR-185-5p targeted BRD8 ISO2. Our data revealed that RNCR3 functions as an tumor-promoting lncRNA in prostate cancer and may serve as a novel important biomarker for the diagnosis and treatment of prostate cancer.

Keywords: RNCR3, prostate cancer, miR-185-5p, prognosis

Introduction

Prostate cancer has been proved to be one of the most common malignant tumor in both the developed and developing countries, which become the second leading cause of cancer-related death among American men [1]. Although the prognosis of early stage prostate cancer patients is good, the 5-year survival rate of advanced metastatic prostate cancer patients is only 28%. Moreover, prostate cancer patients have been diagnosed at younger ages in recent years. It is widely accepted that prostate cancer has become a great threat to the human health. Therefore, identification of novel biomarker and a better understanding of the mechanism involved in the tumorigenesis and development of prostate cancer are instantly required.

Retinal non-coding RNA3 (RNCR3 also known as LINC00599) is an lncRNA transcribed from the intergenic regions of the genome and conserved in mammals [2].

It is reported that RNCR3 can serve as an important regulator in cell proliferation, differentiation and atherosclerosis [3, 4]. Moreover, knockdown of RNCR3 can also result to higher expression of IL-6, CCL2 and TNF-α in blood plasma, indicating that RNCR3 may have a biological function in immune system [5]. However, the expression, biological role and potential mechanism of RNCR3 in human cancer, including prostate cancer remains unclear.

In this study, we examined the expression of RNCR3 in prostate cancer tissues and in paired adjacent normal prostate tissues, and we further explored the biological function of RNCR3 in prostate cancer cell lines. We also assessed the interaction between RNCR3 and miR-185-5p to reveal the underlying mechanism of RNCR3 in prostate cancer. To the best of our
knowledge, our study is the first to show that RNCR3 functions as an oncogene in the development of prostate cancer.

Materials and methods

Human tissue samples

Totally, 52 pairs of prostate cancer tissue and adjacent normal prostate tissue were collected from patients undergoing resection surgery at the Department of Urology, Peking University Third Hospital, and Department of Urology, Fenjinting Hospital from 2006-2011. All of the tissue were frozen and stored in liquid nitrogen until further research. None of the patients received preoperative chemotherapy or radiation. This study was performed with approval from the Ethics and Research Committees of Fenjinting Hospital and was performed in accordance with the Declaration of Helsinki Principles. All of the subjects provided written informed consent. The clinical characteristics of all the patients are summarized in Table 2.

Table 1. Expression of RNCR3 in relation to pathologic and clinical variables

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>N</th>
<th>RNCR3 expression</th>
<th>χ²</th>
<th>P value</th>
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<tbody>
<tr>
<td>All</td>
<td>52</td>
<td>33 19</td>
<td>0.224</td>
<td>0.374</td>
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<tr>
<td>Age (years)</td>
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<td>5.773</td>
<td>0.022</td>
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<tr>
<td>&lt; 65</td>
<td>31</td>
<td>20 11</td>
<td>0.224</td>
<td>0.374</td>
</tr>
<tr>
<td>≥ 65</td>
<td>21</td>
<td>14 4</td>
<td>5.773</td>
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<td>Tumor size</td>
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<tr>
<td>&lt; 2.5 cm</td>
<td>22</td>
<td>12 10</td>
<td>0.136</td>
<td>0.428</td>
</tr>
<tr>
<td>≥ 2.5 cm</td>
<td>30</td>
<td>24 6</td>
<td>0.136</td>
<td>0.428</td>
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<tr>
<td>Gleason score</td>
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<td></td>
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<td>0.221</td>
</tr>
<tr>
<td>&lt; 7</td>
<td>23</td>
<td>14 9</td>
<td>1.294</td>
<td>0.221</td>
</tr>
<tr>
<td>≥ 7</td>
<td>19</td>
<td>15 4</td>
<td>1.294</td>
<td>0.221</td>
</tr>
<tr>
<td>Preoperative PSA level (ng/mL)</td>
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<td></td>
<td>3.935</td>
<td>0.041</td>
</tr>
<tr>
<td>&lt; 10</td>
<td>14</td>
<td>8 6</td>
<td>3.935</td>
<td>0.041</td>
</tr>
<tr>
<td>≥ 10</td>
<td>38</td>
<td>25 13</td>
<td>3.935</td>
<td>0.041</td>
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<tr>
<td>Lymph node metastasis</td>
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<td>3.935</td>
<td>0.041</td>
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<tr>
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<td>38</td>
<td>22 16</td>
<td>3.935</td>
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<tr>
<td>Positive</td>
<td>14</td>
<td>9 5</td>
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<td>Clinical stage</td>
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<td>0.041</td>
</tr>
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<td>23 18</td>
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<td>0.041</td>
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<tr>
<td>T3-T4</td>
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<td>7 4</td>
<td>3.935</td>
<td>0.041</td>
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Table 2. Primer list

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<th>Reverse primer</th>
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<td>GCTGGCTCCTTCTTGTCCACATA</td>
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<tr>
<td>GAPDH</td>
<td>CGCTCTCTGCTCCTCCTGTCACATTACACCGACTCTTCAC</td>
<td>ATCCGTTGACCTCGACCTTCAC</td>
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</tbody>
</table>

Cell culture

The prostate epithelial cell line RWPE-1 and human prostate cancer cell lines LNCaP, DU145 and PC-3 were purchased from American Type Culture Collection (ATCC).

RWPE-1 cell line was cultured in keratinocyte serum free media (Gibco). LNCaP, DU145 and PC-3 were maintained in RPMI 1640 (Life Technologies, CA) with 10% fetal bovine serum (Invitrogen), 50 U/ml penicillin, and 0.1 mg/ml streptomycin. All the cells were placed in a 5% CO₂ cell culture incubator.

RNCR3 short hairpin RNA and small interfering RNA

Lentivirus-encoding short hairpin RNA (shRNA) targeting RNCR3, sh-RNCR3, was purchased from Shanghai GenePharma Co. Ltd (Shanghai, China). AntimiR-185, miR-185 mimic, and scrambled siRNA (si-NC) were ALSO purchased from Shanghai Gene Pharma Co. Ltd (Shanghai, China). Transfections were performed using the Lipofectamine 2000 kit (Invitrogen) according to the manufacturer’s protocol. The knockdown efficiency was examined by qRT-PCR.

RNA extraction and real-time PCR

The total RNA of tissues and cells was isolated using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. The isolated RNA was reverse transcribed with the PrimeScript RT Reagent Kit (Invitrogen, USA) and qRT-PCR was conducted using SYBR Premix Ex Taq (TaKaRa, China) according to the manufacturer’s protocol. GAPDH was used as an internal control. The primer sequences for RNCR3 and GAPDH are listed in Table 1. qRT-PCR was conducted using the ABI PRISM 7500

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PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol.

**Cell counting kit-8 assay**

Cell proliferation was assessed by the Cell Counting Kit-8 (CCK8, Dojindo Molecular technologies, Inc., Kyushu, Japan) assay every 24 h following the manufacturer’s instructions. In brief, 3000 cells were plated into the wells of 96-well plates, and then 10 μl of CCK8 solution was added and incubated for 2 h. The concentration of formazan present was evaluated by determining the absorbance at 450 nm according to the manufacturer’s protocol.

**Colony formation assay**

Cells were seeded in a 6-well plate (500 cells per well) and then cultured in RPMI-1640 medium containing 10% FBS. After 10 days, colonies were then fixed with methanol for 10 min and stained with 0.5% crystal violet for 5 min. Then the number of visible colonies was counted under the fluorescent microscope (Olympus, Tokyo, Japan).

**Cell invasion assays**

BD 24-well transwell chambers (Costar, Massachusetts, USA) with a matrigel coating were used to assess cell invasion according to the manufacturer’s protocol. 1×10⁵ cells suspended in 300 μl of serum-free medium were added to the upper compartments of 24-well plates and 800 μl of DMEM supplemented with 10% FBS were added to the lower compartments. After 24 h, the membranes in the lower chambers were fixed with 4% formaldehyde and stained with 1% crystal violet. The number of cells that invaded through the membrane was determined by microscopy using at least five fields. These experiments were performed in triplicate.

**Dual-luciferase reporter assay**

A reporter plasmid containing RNCR3 wild type (wt) or RNCR3 mutant type (mut) was co-transfected with the miR-185 mimic or the si-NC RNA into PC-3 cells. And then, the reporter plasmid and the internal control plasmid containing renilla luciferase were transfected using Lipofectamine 3000 (Invitrogen) and 48 h after transfection, luciferase activity was examined using the dual-luciferase reporter gene assay system (Promega, Madison, USA). Renilla luciferase activity was normalized to firefly luciferase activity.

**Western blot analysis**

Total protein was obtained from cells with RIPA buffer (Beyotime, Shanghai, China) and then centrifuged at 14,000 g and 4℃ for 15 min. Cell protein lysates were 10% sodium dodecyl sulfate-polyacrylamide gel el ectrophoresis (SDS-PAGE), and then transferred to PVDF membranes (Bio-Rad). After blocking in 5% nonfat milk for 1.5 h at room temperature, membranes were probed with primary antibodies at 4℃ overnight. β-actin was used as control. The immunoblots were detected using an electrochemiluminescence kit (Santa Cruz, Dallas, TX, USA) and visualized after X-ray film exposure.

**Statistical analysis**

All statistical analyses were conducted using the SPSS 20.0 software (IBM, SPSS, Chicago, IL, USA), and were from at least three independent experiments. Chi-square tests were conducted to analyze associations between RNCR3 levels and clinicopathological factors. The Kaplan-Meier method was used to create survival curves and the log-rank test was used to determine statistical significance. Differences between groups were analyzed by the Student’s t test for continuous variables and χ² test for categorical variables. Pearson correlation analysis was conducted to evaluate the relationship between RNCR3 expression and miR-185 expression. Data were considered statistically significant when P < 0.05.

**Results**

**Expression of RNCR3 was upregulated in prostate cancer tissues and cell lines**

To explore the biological function of RNCR3 in prostate cancer, we measured relative expression of RNCR3 in 52 pairs of prostate cancer tissue and adjacent non-tumor prostate tissue by qRT-PCR analysis. The results showed that RNCR3 expression was higher in prostate cancer tissues than in the corresponding adjacent non-tumor tissues (Figure 1A). Then, we
examined RNCR3 expression in 5 human prostate cancer cell lines and in the normal prostate epithelial cell line RWPE-1. We found higher RNCR3 expression levels in the prostate cancer cell lines than in the normal prostate epithelial cell line (Figure 1B). These data indicated that there is an increase in RNCR3 expression in human prostate cancer tissues and in prostate cancer cell lines.

*Increased expression of RNCR3 was associated with progression and poor prognosis of prostate cancer patients*

Next, we further investigated associations between RNCR3 expression levels and clinicopathological factors in 52 prostate cancer patients (Table 2). The results showed that increased RNCR3 expression correlated with tumor size (P = 0.022), Gleason score (P = 0.032), and clinical stage (P = 0.041), but there were no significant associations between RNCR3 expression and age, preoperative PSA level, and lymph node metastasis. Finally, Kaplan-Meier survival curves showed that prostate cancer patients who had lower expression levels of RNCR3 in prostate cancer tissues had significantly better survival rates than prostate cancer patients who had higher expression levels of RNCR3 in their prostate cancer tissues (Figure 1C).

**miR-185-5p expression was down-regulated in prostate cancer cell lines and tissues**

The miR-185-5p expression was higher in 5 prostate cancer cell lines (22Rv1, C4-2B, VCaP, LNCaP, and PC-3) than in normal prostate epithelial cell line RWPE-1 (Figure 2A). The expression level of miR-185-5p in 52 pairs of prostate cancer tissues and adjacent non-tumorous prostate tissues was evaluated by qRT-PCR, the results showed that the expression level of miR-185 was significantly lower in prostate cancer tissues compared with that in the adjacent normal prostate cancer tissues (Figure 2B). Moreover, the expression level of miR-185-5p was negatively correlated with the expression of RNCR3 in prostate cancer tissues (Figure 2C).
RNCR3 in prostate cancer

Figure 2. miR-185-5p expression was down-regulated in prostate cancer tissues and cell lines. A. Down-gulation of miR-185-5p in prostate cancer cells compared with normal prostate epithelial cells RWPE-1. *P < 0.05. B. Relative expression of miR-185-5p in 52 pairs of prostate cancer tissue and adjacent non-tumor tissues by qRT-PCR analysis. C. The expression of miR-185-5p was negatively related with the expression of RNCR3 in the prostate cancer tissues.

Figure 3. Knockdown of RNCR3 suppressed the miR-185-5p expression. A. The RNCR3 expression in the LNCaP and PC-3 cell was analyzed by qRT-PCR analysis. **P < 0.01. B. Knockdown of RNCR3 suppressed the expression of miR-185-5p. **P < 0.01. C. The mRNA expression of BRD8 ISO2 was analyzed by qRT-PCR analysis. **P < 0.01. D. The protein expression of BRD8 ISO2 was analyzed by western blot.
Knockdown of RNCR3 increased the expression of miR-185-5p

qRT-PCR assay showed that LNCaP, and PC-3 cells transfected with RNCR3 shRNA expressed lower levels of RNCR3 than control untransfected LNCaP, and PC-3 cells (Figure 3A). Knockdown of RNCR3 significantly increased the expression of miR-185-5p (Figure 3B). In addition, Knockdown of RNCR3 suppressed the BRD8 ISO2 mRNA and protein expression (Figure 3C and 3D).

BRD8 ISO2 was a direct target gene of miR-185-5p

Open-source software Targetscan and miRanda were used to find the potential target gene of miR-185-5p and the potential 3’UTR binding site of BRD8 ISO2 of miR-185-5p was shown in Figure 4A. The results of qRT-PCR assay showed that the miR-185-5p expression was remarkably increased in the PC-3 cell that was treated with miR-185-5p mimic (Figure 4B). Overexpression of miR-185-5p significantly decreased luciferase activity of the WT BRD8 ISO2 3’UTR but not the mutant vector (Mut BRD8 ISO2 3’UTR) (**P < 0.01. D. Overexpression of miR-185-5p suppressed the BRD8 ISO2 expression.

Knockdown of RNCR3 suppressed the cell proliferation, colony formation, and invasion in prostate cancer line

Knockdown of RNCR3 inhibited cell proliferation in prostate cancer line LNCaP (Figure 5A) and PC-3 (Figure 5B). Knockdown of RNCR3 reduced cell colony formation in LNCaP and PC-3 (Figure 5C). Furthermore, Knockdown of RNCR3 inhibited cell invasion in LNCaP and PC-3 (Figure 5D).

The oncogenic function of RNCR3 in prostate cancer cell is dependent on miR-185-5p

Rescue experiments were performed to explore whether knockdown of RNCR3 suppressed prostate cancer cell proliferation, colony formation, and invasion in a miR-185-5p-dependent
manner. As shown in Figure 6A, anti-miRNA-NC or miR-185-5p inhibitor was transfected stably into PC-3 cells already transfected with sh-RNCR3. The results of CCK-8 assays demonstrated that the inhibition of proliferation of PC-3 cells that was induced by knockdown of RNCR3 was partially abolished in the presence of the miR-320a inhibitor (Figure 6B). Moreover, the presence of the miR-185-5p inhibitor rescued the suppression of colony formation and invasion of PC-3 cells that was induced by knockdown of RNCR3 (Figure 6C and 6D). Collectively, these results revealed that the oncogenic function of RNCR3 in prostate cancer cells involved, at least partially, negatively regulation of miR-185-5p.

Discussion

LncRNAs) are non-coding RNA transcripts that are longer than 200 nucleotides [6]. For a long time, LncRNAs were considered as transcriptional “noise” without biological role because they do not code for proteins [7]. In recent years, mounting evidence has accumulated indicating that LncRNAs play important roles in various physiological and pathological processes, such as cell proliferation, apoptosis, differentiation, and the development of cancers [8, 9]. RNCR3, also known as LINC00599, is a long intergenic non-coding RNA. RNCR3 was previously reported to be expressed during mouse retinal development [10]. Moreover, RNCR3 was confirmed as an important regulator of neurons and oligodendrocyte differentiation and the maintenance of ocular microvascular function [2, 3, 11, 12].

In this study, we found that the expression of RNCR3 was elevated in prostate cancer tissues and cell lines. And the increased RNCR3 expression correlated with tumor size, Gleason score, and clinical stage. Moreover, prostate cancer patients who had lower expression levels of RNCR3 in prostate cancer tissues had significantly better survival rates than prostate cancer patients who had higher expression levels of RNCR3 in their prostate cancer tissues. Knockdown of RNCR3 promoted miR-185-5p expression in prostate cancer cell. Besides, we further showed that the expression of miR-185-5p was down-regulated in prostate cancer tissues and cell lines. Further, the expression of miR-185-5p was negatively correlated with the expression of RNCR3 in prostate cancer tissues. Moreover, we identified that BRD8 ISO2 was a direct target gene of miR-185-5p. Overexpression of miR-185-5p significantly suppressed the BRD8 ISO2 expression. Knockdown of RNCR3 also inhibited the expression of BRD8 ISO2. In addition, knockdown of RNCR3c suppressed prostate cancer cell proliferation, colony formation and invasion through targeting miR-185-5p. miR-185-5p was confirmed as an important tumor-suppressor in prostate cancer [13-15]. Collectively, these data indicated that RNCR3 might act as an oncogene in the development of prostate can-
ceRNAs or as molecular sponges [17]. For instance, it has been reported IncRNA Unigene56159 promoted the epithelial mesenchymal transition in hepatocellular carcinoma by acting as a ceRNA of miR-140-5p [18]. Zhang et al. reported that IncRNA UCA1 promoted prostate cancer progression by acting as a ceRNA of ATF2 [19]. Our data further supported this novel regulatory mechanisms, RNCR3 promoted the progression of prostate cancer by acting as a ceRNA of miR-185-5p.

In conclusion, our study, for the first time, showed an increase in RNCR3 expression in prostate cancer tissues and cell lines. Furthermore, RNCR3 promote cell proliferation, colony formation, and invasion of prostate cancer cells by binding to and suppressing miR-185-5p. These results demonstrated that RNCR3 may serve as an important prognostic indicator for prostate cancer patients and may be a potential biological target for future prostate cancer therapeutics.

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Disclosure of conflict of interest

None.

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References

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