Original Article

Hypoxia promoted renal cell carcinoma cell migration through regulating IncRNA-ENST00000574654.1

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Received October 31, 2019; Accepted April 28, 2020; Epub July 15, 2020; Published July 30, 2020

Abstract: Background: Hypoxia is common in solid tumor masses that has functional consequences for tumor progression. Previous studies demonstrated that nearly 80% renal cell carcinoma (RCC) are under hypoxia. However, effect and its mechanism of hypoxia on RCC cell invasion remains to be defined. Methods: The shRNA expression vectors, which were constructed to express a short hairpin RNA against IncRNA and overexpression of IncRNA, were transfected into the RCC cell lines (SW839 and OSRC-2). Levels of lncRNA-ENST00000574654.1, VEGF-A and VEGF-C mRNA and protein were examined by real-time quantitative-fluorescent PCR and Western blot analysis respectively. The effects of lncRNA silencing and overexpression on cell invasion of SW839 and OSRC-2 cells were evaluated with cell migration assay. Results: Hypoxia significantly stimulated cell invasion in both RCC cell lines (SW839: 2.38 ± 0.19 of normoxia vs 7.83 ± 0.38 of hypoxia, \( P < 0.05 \); and OSRC-2: 1.00 ± 0.08 of normoxia vs 5.88 ± 0.32 of hypoxia, \( P < 0.05 \)). LncRNA microarray analysis found that IncRNA-ENST00000574654.1 was down-regulated under hypoxia. Consistently, over-expression of IncRNA-ENST00000574654.1 resulted in significant blockade of hypoxia-induced RCC migration. Furthermore, expression of IncRNA-ENST00000574654.1 was regulated by HIF-1α and VEGF-A through interacting with hnRNP, which in turn regulated the RCC cell invasion. Conclusions: These findings suggested that hypoxia promoted RCC cell invasion through HIF-1α/IncRNA (ENST00000574654.1)/hnRNP/VEGF-A pathway. Targeting this pathway could potentially improve therapeutic outcomes of renal cell carcinoma.

Keywords: Renal cell carcinoma (RCC), long non-coding RNA (lncRNA), invasion, hypoxia, vascular endothelial growth factor (VEGF)

Introduction

Renal cell carcinoma (RCC) is the ninth most common malignant tumor representing approximately 2-3% of all adult malignancies [1]. In the U.S., renal cell carcinoma (RCC) is the 6th leading cause of cancer deaths in men and the 8th leading cause in women, and the incidence of RCC continues to rise [2]. Until recently, there was a worldwide annual increase in the incidence of nearly 2%. Approximately 84,400 new RCC cases were diagnosed resulting in more than 34,700 kidney cancer-related deaths within the European Union in 2012 [3]. Presently, surgery is still the standard treatment for primary RCC, while seven targeted therapies have been FDA-approved for metastatic RCC [4]. Although these therapies have extended survival of patients with advanced RCC, the response rate is low and the 5-year survival rate of patients with metastatic RCC remains less than 10%. Therefore, more efficacious approaches are needed for treatment of metastatic disease as well as for neoadjuvant and adjuvant therapy of localized RCC.

In order for tumors to metastasize and grow, neoplastic and endothelial cells must invade and migrate into surrounding tissues [5]. Hypoxia is a frequent occurrence in solid tumor masses, which has functional consequences for cells to invade and migrate [6]. In addition, previous study demonstrated that nearly 80% RCC are under hypoxia [7]. Furthermore, the expression levels of a number of lncRNAs are associated with hypoxia including NEAT1, H19,
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HOTAIR, MALAT1, and UCA1 [8]. The current study was, therefore, designed to examine the role of IncRNAs under hypoxia during RCC progression. This is particularly relevant since a majority of RCC carry a mutation in Von Hippel-Lindau (VHL), an E3 ubiquitin ligase that targets hypoxia inducible factor (HIF), thus implicating oxygen sensing as a critical factor for RCC development and progression [9].

Cancer metastasis is a complex process, which involves several signal-transduction pathways that allow cancer cells to proliferate, remodel their surrounding environment, and invade to new tissues. In this process, cell invasion is the key steps, which plays an important role in cancer metastasis. Cellular invasion is governed at both the extracellular and intracellular levels by several factors, and depends on the cell’s carefully balanced dynamic interaction with the extracellular matrix (ECM). Increasing evidence suggests that hypoxia play an important role in cell invasion [10].

In this study, using IncRNA microarray analysis, we first searched the IncRNA, which were regulated by hypoxia, and further demonstrated that hypoxia could promote RCC cell invasion through decreasing the IncRNA-ENST00000574654.1. A novel pathway of hypoxia modulation on cell invasion as well as development and progression of RCC was also investigated.

Methods

Cell culture

Human RCC cell lines, SW839 and OSRC-2, were obtained from Chinese Academy of Science (Chinese Academy of Science, China). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO₂ incubator (normoxia) or 1% oxygen (1% O₂, 5% CO₂, 94% N₂; hypoxia) and were routinely passaged at 3- or 4-day intervals.

Plasmids containing shRNA targeting IncRNA-ENST00000574654.1 and its transfection

Short hairpin RNAs (shRNAs) were designed and their expression vectors were constructed by Sangon Biotech company (Sangon Biotech, China). Interference plasmid and the negative control plasmid (empty plasmid, pLV-THM) were transfected to RCC cells with Lipofectamine 2000 transfection reagent (Invitrogen). After 48 h transfection, total RNA and protein were extracted from the cells, and the expression levels of IncRNA, VEGF-A and VEGF-C mRNA as well as protein were examined by RT-PCR and immunoblotting, respectively.

RNA isolation and real time PCR

Total RNA of SW839 and OSRC-2 cells was extracted by Trizol reagent (Life Technologies) following the manufacturer’s instructions. Concentration and quality of the extracted total RNA were determined by measuring OD260 and the OD260/OD280 ratio. Total RNA was treated with DNase before reverse transcription. The reverse transcription of 1 µg RNA into cDNA was carried out using Superscript II reverse transcriptase (TakaRa, Japan) and stored at -80°C until use.

Primers for human IncRNA, VEGF-A, VEGF-C and GAPDH were designed with Primer Express 2.0 software (Applied Biosystems) and synthesized by Sangon. The basic information on primers, including gene name, forward primer, reverse primer and temperature was presented in Table 1. Real-time PCR was done in triplicated wells for each sample in a 20 µL reaction mixture, which consisted of template DNA (2 µL), primers (1 µL), SYRB premix (10 µL), ddH2O (7 µL) (ExScript real-time PCR Kit, TaKaRa). PCR was done with a 7900HT Fast real-time PCR instrument using the following thermal cycles: The cycling variables were 95°C (30 s), 40 cycles of 95°C (5 s), 60°C (30 s). According to the method tested by Pfaffl, the relative expression ratio of a targeted gene was calculated based on efficiency and the Ct compared with a reference gene (GAPDH).

Immunoblotting analysis

Cells were lysed with RIPA buffer and proteins (30 µg) were separated by 8-10% SDS/PAGE gel and then transferred onto PVDF membranes (Millipore, Billerica, MA). After blocking, the membranes were incubated with 1:200 dilutions of specific primary antibodies, mouse anti-human VEGF-A and anti-human VEGF-C (Abcam China, Shanghai, China). GAPDH was
used as loading control. The blots were then incubated with HRP-conjugated secondary antibodies and visualized using ECL system.

**Cell invasion assay**

The invasion capability of RCC cells was determined by the transwell assay. The upper transwell chambers (Corning Inc., Corning, NY, USA) were pre-coated with diluted growth factor-reduced matrigel (1:5 serum free RPMI) and put into the incubator for 5 h. RCC cells were harvested and seeded at 1 × 10^5 cells/well with serum-free DMEM into the upper chamber and the lower chamber contained DMEM with 10% FBS. After 24 h incubation at 37°C, the invaded cells attached to the lower surface of the membrane were fixed with paraformaldehyde and stained with crystal violet. Cell numbers were counted in five randomly chosen microscopic fields.

**RNA immunoprecipitation (RIP)**

The 293T cells were lysed in RIPA lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2 EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na2VO4, 1 µg/ml leupeptin) supplemented with anti-RNase, protease inhibitor cocktail. RNase-free DNase (NEB) (400 U) was then added to the lysates and incubated on-ice for 30 min. The cell lysates were diluted in the RIPA buffer and 50 µl of the supernatant was added as input for PCR analysis. Five-hundreds microliter of the supernatant was incubated with 4 µg of anti-heterogeneous nuclear ribonucleoproteins (anti-hnRNP C1/C2, Santa Cruz, cat#: sc-515-938) or IgG antibody. Protein A/G beads were pre-blocked by 15 mg/ml BSA in PBS. Then pre-blocked beads were added to the antibody-lysate mixture and incubated for another 2 hours. The RNA/antibody complex was washed four times by RIPA buffer supplemented with anti-RNase, protease inhibitor cocktail. The RNA was extracted using Trizol (Invitrogen) following the manufacturer’s protocol and subjected to real time RT-PCR analysis. For UV cross-linking and RIP, cells were first subjected to UV cross-linking (200 mJ/cm²) and then conducted as native RIP protocol.

**Statistical analysis**

All statistical analyses were carried out with SPSS 19.0 (SPSS Inc, Chicago, IL). The data values were presented as the mean ± SD. Differences in mean values between two groups were analyzed by two-tailed Student’s t test and the mean values of more than two groups were compared with one-way ANOVA followed by Bonferroni post-correction. P ≤ 0.05 was considered as statistically significant.

**Results**

**LncRNA-ENST00000574654.1 was significantly down regulated in RCC under hypoxia**

The effect of hypoxia on RCC cell migration was first examined and found that hypoxia could promote cell migration in both RCC OSRC-2 (1.00 ± 0.08 of normoxia vs 5.88 ± 0.32 of hypoxia, P < 0.05) and SW839 cells (2.38 ± 0.19 of normoxia vs 7.83 ± 0.38 of hypoxia, P < 0.05, Figure 1A). Using lncRNA microarray analysis [11], expression of lncRNAs in SW839 cell line in response to hypoxia (1% oxygen level for 24 h) was assessed by a comparative analysis on the expression of 40,000 lncRNAs, and it was found that nearly 7,000 lncRNAs were increased while nearly 13,000 lncRNAs were decreased in response to hypoxia. Total of 20
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IncRNAs with significant down-regulation were further investigated (Figure 1B). Specifically, to link those 20 IncRNAs to the cell invasion under hypoxia condition, the GEO database (GSE96574) were analyzed and found that expression of IncRNA-ENST00000574654.1 was significantly reduced in the RCC tumor tissue (Figure 1C).

**LncRNA-ENST00000574654.1 modulated RCC cell migration in response to hypoxia**

To examine biological function of IncRNA-ENST00000574654.1, two shRNAs that specifically suppressing IncRNA-ENST00000574654.1 was synthesized and a plasmid that overexpressing IncRNA-ENST00000574654.1 was constructed. As shown in Figure 2A, IncRNA-ENST00000574654.1 was significantly inhibited by shRNAs, especially by shRNA1. In contrast, expression of IncRNA-ENST00000574654.1 was significantly increased in the cells transfected with the overexpressing plasmid.

After transfection of the plasmids containing shRNA or sequence of overexpressing IncRNA-ENST00000574654.1, cell migration under normoxia or hypoxia condition was assessed. It was fund that, under normoxia or hypoxia condition, suppression of IncRNA-ENST00000574654.1 by shRNA resulted in increased RCC cell invasion, while overexpression of IncRNA (ENST00000574654.1) resulted in significant reduction in cell invasion in both SW839 and OSRC-2 cell lines (Figure 2B).
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LncRNA-ENST00000574654.1 expression was regulated by HIF-1α

To explore the mechanism of hypoxia regulation on the expression of LncRNA-ENST00000574654.1, hypoxia-inducible factor (HIF), a transcriptional complex that plays a central role in the regulation of gene expression by oxygen, was investigated in the SW839 and OSRC-2 cell lines by RNA interfering HIF-1α and HIF-2α. To accomplish this, cells were transfected with plasmid containing shRNA specifically targeting HIF-1α or HIF-2α as we have previously published [12].

It was found that suppression of HIF-1α by shRNA significantly blocked the reduction of LncRNA-ENST00000574654.1 expression in...
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Mechanism of IncRNA-ENST00000574654.1 regulation on RCC cell invasion in response to hypoxia

Previous studies suggested that VEGF-A and VEGF-C were associated with cancer metastasis [13]. Gene expression as well as protein levels of the VEGF-A and VEGF-C in the RCC cell lines were, therefore, assessed by real time PCR and immunoblotting analyses, respectively. As shown in Figure 4A, VEGF-A mRNA expression was significantly up regulated in both SW839 and OSRC-2 cells lacking IncRNA-ENST00000574654.1 (3.05 ± 0.12 of control shRNA vs 1.08 ± 0.16 of sh-IncRNA, P < 0.05), while it was not significantly altered in the cells over-expressing IncRNA-ENST00000574654.1. Consistently, VEGF-A protein was significantly increased in the cells transfected with shRNA-IncRNA compared to the cells transfected with scramble shRNA (3.53 ± 0.09 of control shRNA vs 1.02 ± 0.07 of shRNA-IncRNA, P < 0.05), while it was not significantly altered in the cells over-expressing the IncRNA (Figure 4B). Interestingly, however, VEGF-C mRNA and protein were not significantly altered in the cells regardless of suppressing or over-expressing the IncRNA (Figure 4A).

To explore which motif of IncRNA-ENST00000574654.1 has a function of regulating renal cell carcinoma cell invasion, a bioinformatic analysis of likely protein-RNA interactions (RBP map) was analyzed and it was found that ENST00000574654.1 might be able to interact with hnRNP, a protein reported to be a regulator of VEGF [14, 15] and a transcriptional factor critical for promoting cell invasion. Results from RIP assays revealed that IncRNA-ENST00000574654.1 indeed interacted with hnRNP compared to IgG (Figure 4C).
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Discussion

Although detection of small renal masses has been improved through recently advanced diagnosis techniques, approximately one-third of patients still miss the early diagnosis and develop metastatic lesions during the course of the renal cell cancer development [16]. In addition, up to 40% of patients with loco regional renal cell carcinoma have a relapse with metastasis after nephrectomy given nephrectomy (radial or partial) is still the major treatment for localized RCC [17]. Recently, strategies targeting critical biological pathways, including vascular endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR), have produced robust clinical effects and revolutionized the treatment of metastatic RCC [18]. However, some patients are inherently resistant to these approaches and most, if not all, patients acquire resistance over time [19]. Therefore, there is still a challenge to urologist to treat RCC. The current study found that hypoxia-induced RCC migration was significantly suppressed by over expression of IncRNA-ENST00000574654.1, suggesting ENST00000574654.1 might be a tumor suppressor in RCC.

The von Hippel-Lindau tumor suppressor, pVHL, is a key player in one of the best characterized hypoxia signaling pathways, the VHL-hypoxia-inducible factor (VHL-HIF) pathway. In RCC, 60-80% of clear cell RCC cases display loss-of-function coding mutations in the VHL gene, chromosomal aberrations on chromo-
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Figure 5. Schematic illustration on the role of HIF1α and IncRNA-ENST00000574654.1 in regulating VEGF-A and cell migration in RCC cells.

Increasing evidence has suggested that VEGF-A is upregulated in numerous cancer cell types, including RCC [26]. VEGF-A has been revealed to be involved in angiogenesis, cell proliferation, migration, invasion and tumor angiogenesis [27, 28]. Shan et al reported that polypyrimidine tract-binding protein 1 (PTBP1) regulated HIF-1α and VEGF expression. Specifically, they found that PTBP1 silencing resulted in downregulation of HIF-1α and VEGF, and overexpression of HIF-1α could rescue decreased VEGF expression following PTBP1 suppression by siRNA [29]. Consistently, the current study found that VEGF-A was up regulated in the cells lacking IncRNA-ENST00000574654.1, but dramatically suppressed in the cells over-expressing IncRNA-ENST00000574654.1. Furthermore, the current study found that VEGF-A was down regulated in the cells over-expressing IncRNA-ENST00000574654.1 under normoxia, but it was up regulated in the cells lack of IncRNA-ENST00000574654.1 by shRNA-lncRNA. However, while we found that IncRNA-ENST00000574654.1 might interact with hnRNP, motif of IncRNA-ENST00000574654.1 that was responsible for regulating VEGF through interacting with hnRNP remains to be identified in the future study. These findings established the foundation to enhance suppression of RCC invasion through regulating IncRNA expression and targeting VEGF pathway in RCC treatment.

Transcription factor HIF is a heterodimer consisted of one of three alpha (α) subunits and a beta (β) subunit. While HIF-6 is constitutively expressed, HIF-α is induced by hypoxia. HIF-α family includes HIF-1α, -2α, and -3α [30-33]. While HIF-1α is the most well-established and...
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Extensively investigated in various kinds of cell types, recently, HIF-2α has also been an attractive therapeutic target in renal cell cancer [34], and several reports described the antitumor effects of a small-molecule HIF-2α antagonist that binds to a hydrophobic binding pocket discovered in HIF-2α PAS-B domain [35-37] as well as siRNA targeting HIF-2α in the renal cell cancer [38]. In the current study, however, neither shRNA-HIF-2α nor the HIF inhibitor (FG-4592) could significantly alter expression of lncRNA-ENST00000574654.1 in RCC cells, suggesting HIF-2α may not be involved in regulating lncRNA-ENST00000574654.1 in RCC cells.

Conclusions

Φ-1α was up-regulated under hypoxia in RCC cells, which mediated up-regulation of VEGF and promoted RCC cell migration through down-regulation of lncRNA-ENST00000574654.1. Findings of the current study suggested that hypoxia-induced RCC cell invasion may be modulated by HIF-1α/lncRNA (ENST00000574654.1)/hnRNP/VEGF-A pathway. Targeting this pathway could potentially improve RCC therapeutic outcomes.

Acknowledgements

This work was supported in part or in whole by the National Natural Science Foundation of China (No. 81802518). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding received for this study.

Disclosure of conflict of interest

None.

Abbreviations

ECM, extracellular matrix; ESC, embryonic stem cell; HIF, hypoxia inducible factor; LncRNA, long non-coding RNA; RCC, renal cell carcinoma; shRNAs, short hairpin RNAs; VEGF, vascular endothelial growth factor; VHL, Von Hippel-Lindau; VHL-HIF, VHL-hypoxia-inducible factor.

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