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Effect of MicroRNA-218 on the viability, apoptosis and invasion of renal cell carcinoma cells under hypoxia by targeted downregulation of CXCR7 expression

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ABSTRACT

Objective: To investigate the effect of microRNA-218 on the viability, apoptosis and invasion of renal cell carcinoma cells under hypoxia by targeted regulation of expression of chemokine receptor 7 (CXCR7).
Methods: The expression of miR-218 in renal cell carcinoma cell lines under normal and hypoxia conditions, as well in normal renal tubular epithelial cells (HK2) was measured using RT-PCR. MiR-218 mimic and NC were transfected into renal cell carcinoma cell line ACHN using Lipofectamine™ 2000. The expression of miR-218 was analyzed using RT-PCR. The viability, apoptosis, migration and invasion of the transfected cells were assayed using the MTT assay, flow cytometry and transwell assays. The expression of CXCR7 was assayed using RT-PCR and Western blot. Luciferase reporter was used to verify the downstream target of miR-218.
Results: The expression of miR-218 was lower than in renal cell carcinoma cell lines ACHN, 769-p and Caki-1 that in HK-2. The expression of miR-218 in the renal carcinoma cell lines was lower under hypoxia than under normal oxygen conditions. The expression of miR-218 in ACHN cells under normal and hypoxic conditions was significantly increased after transfection with miR-218 mimic. Compared with NC transfected cells under normal oxygen condition, the mimic-transfected cells had reduced viability, migration ability and invasion ability, and increased apoptosis, and mimic transfected-cells under hypoxia had significantly reduced viability, migration ability and invasion ability, and increased apoptosis. Overexpression of miR-218 mimic resulted in significant reduction in the expression of CXCR7 at protein and mRNA levels under normal and hypoxic conditions. Luciferase reporter assay confirmed that CXCR7 is the target protein of miR-218.
Conclusion: Up-regulation of miR-218 expression in renal cell carcinoma under hypoxia can result in significant and targeted down-regulation of CXCR7 expression, which could reduce cell viability, migration and invasion ability and induce apoptosis in the cancer cells.
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1. Introduction

Renal cell carcinoma (RCC) is a malignant tumor originated from renal tubular epithelial cell, and its incidence ranks the second among the urinary system tumors [1,2]. In recent years, the incidence and mortality of RCC have been increased remarkably. At present, surgical treatment remains the most effective management option for the cancer. However, there are still about 30% of metastasis of locally progressed RCC and the five-year survival rate is less than 10%. Furthermore, the cancer is not very responsive to conventional chemotherapy and radiotherapy [2]. Therefore, it is an important to have a better understanding of the mechanisms underlying the disease and to develop new and effective therapeutic methods. Hypoxia is considered to be an important feature in tumor growth microenvironment, and is also one of the reasons for the failure and poor prognosis in clinical tumor treatment [3]. Hypoxia inducible factor-α (HIF-α) is a key transcriptional factor in hypoxic environment that regulates the

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expression of a variety of protein-coding and non-coding genes [4,5]. It is also shown that miRNA expression is not only closely related to the development of tumor, but also to the hypoxic environment. miRNA expression in tumor cells varies significantly between in hypoxic and normal cells [6]. For example, miR-218 expression is found significantly lower in clear cell RCC than in the adjacent normal renal tissues, and increasing its expression can significantly inhibit cell proliferation, suggesting that miR-218 is closely related to the development of RCC and might be a tumor repressor in clear cell RCC [7,8]. Hwang et al. [9] confirmed that HIF- α is highly expressed in glioma cells, which affects the biological behavior of the cancer cells by targeted down-regulation of miR-218 expression. Thus, we speculated that miR-218 may be also closely related to hypoxic environment and abnormally expressed in RCC under hypoxic environment, and subsequently involved in tumor development, apoptosis, and invasion. In this study, we analyzed the expression of miR-218 in normal renal tissues and RCC cells under normal and hypoxic conditions and investigated the effect of miR-218 on cell viability, apoptosis, migration and invasion ability and expression of downstream target. The findings would provide new information regarding the role of miR-218 in RCC development and progression and clues for new therapeutic strategies.

2. Materials and methods

2.1. Cell lines and culture

Human RCC cell lines ACHN, 769-p and Caki-1 were purchased from Cell Bank of Chinese Academy (Beijing, China). Renal tubular epithelial cell line HK-2 was purchased from American Type Culture Collection. The cells were maintained in DMEM medium containing 10% fetal bovine serum before being used for experiments. For analysis of oxygen effects, the cells were cultured at 37 °C in a 5% CO_2 incubator in normal oxygen condition or hypoxic condition containing 2% O_2 93% N_2 and 5% CO_2.

2.2. miRNA transfection

2 mL ACHN cells ($4-5\times10^4$ cells) were seeded in 6-well plates and cultured to 50–70% confluence before being used for transfection. 250 μL miRNA (miR-218 mimic and scramble control miR-218 NC, Shanghai GenePharma Co., Ltd., Shanghai, China) was diluted in serum-free Opti-MEM® I (Invitrogen USA) medium and mixed with Lipofectamine 2000 (Invitrogen) for transfection following the manufacturer's instruction. The transfected cells were seeded into 6-well plates and cultured in DMEM medium (Hyclone, USA) for 48 h at 37 °C in a CO_2 incubator, and then in normal oxygen condition or hypoxic conditions described above. The cells were grouped as normal oxygen (transfected with miR-218 NC and cultured in normal oxygen condition), normal oxygen with overexpressed miR-218 mimic, hypoxia (transfected with miR-218 NC and cultured in hypoxic condition) and hypoxia with overexpressed miR-218 mimic.

2.3. qRT-PCR

Total RNA was extracted using the Trizol kit (Invitrogen, USA) according to manufacturer's instructions. Primers used for PCR are shown in Table 1. Reverse transcription was performed with 200 ng of RNA in a total volume of 10 μL using the High Capacity cDNA Transcriptase Reverse kit (Applied Biosystems by Life Technologies, Carlsbad, California, USA) according to manufacturer's recommendations. Reactions were performed on a 96 thermal cycler with the following conditions: 10 min at 25 °C, 2 h at 37 °C and 5 min at 85 °C. A total of 2.5 μL of the resulting cDNA was subjected to pre-amplification using the TaqMan Pre-Amp Master Mix (Applied Biosystems) in a total volume of 12 μL. Pre-amplification cycling conditions were 10 min at 95 °C followed by 35 cycles, each consisting of denaturing at 94 °C for 45 s, annealing at 59 °C for 45 s, and elongation at 72 °C for 60 s. RT-PCR was performed on 7900HT Fast Real-Time PCR system using TaqMan gene expression assays (Applied Biosystems). 5 μL amplified product was separated on 2% agarose gel after electrophoresis and photographed for further analysis. The PCR reactions were run in 25 μL volume, with 35 cycles of denaturing at 94 °C for 45s, annealing at 59 °C for 45s, and elongation at 72 °C for 60 s. Human GADPH (Hs03929097_g1) was used as an internal control.

Table 1
RT-PCR primers.

Gene	Primer (5′-3′)
miR-218	For: GATCGCTCGAGCTAGGTGGAGGCAAGTGGAA
	Rev: GGCCGCTCTAGGTTTAAACGACTGGCTTGCCTTATCTGG
U6	For: GCTTCGGCAGCACATATACTAAAAT
	Rev: CGCTTCACGAATTTGCGTGTCAT
CXCR7	For: AGAGGCTCCTTTCTGCAGTG
	Rev: AGTCGAAGAGATGCAGATCC
GADPH	For: AGCCACATCGCTCAGACA
	Rev: TGGACTCCACGACGTACT

The data were managed using the Applied Biosystems software RQ Manager v1.2.1. Relative expression was calculated using the comparative Ct method and the fold change value ($2-D\Delta Ct$) was obtained according to previously described protocol [10].

2.4. Cell viability assay

The viability of infected cells was assayed as reported [11]. Briefly, 20 μL MTT (5 mg/mL, Gibco, USA) was added to the transfected cells and the cells were cultured for another 4 h. After aspiration of culture medium, 150 μL DMSO was added to each well, and the plates were shaken to dissolve the crystals and measured at 560 nm using a plate reader for OD as relative cell viability.

2.5. Cytometry

Apoptosis was assayed as reported [11]. Briefly, the cells were harvested, strained with AnnexinV/PI (Keygen Biotech, Nanjing) and analyzed according to the manufacturer's protocol.

2.6. Cell migration and invasion assays

Migration ability was measured using the transwell assay [12]. Briefly, the transfected cells were trypsinized and added to the upper chambers in transwells (Corning, USA) and the bottom chambers contained DMEM medium with 5% fetal bovine serum (Hyclone, USA). For the assessment of invasion, transfected cells were added into the upper chambers of inserts coated with Matrigel (BD Bioscience, USA). The cells were cultured for 24 h and the inserts were removed from the chambers, washed with PBS, fixed by submerging the inserts in 10% formalin for 10 min, stained with hematoxylin. The migrated cells were counted in five fields under an inverted light microscope.

2.7. Western blot

Western blot was preformed as reported [11]. Briefly, the transfected cells were harvested, lysed in RIPA buffer and vortexed for 30s every 10 min for 40 min. The lysate was centrifuged at

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