MiR-331-3p is down-regulated in renal cell carcinoma and inhibits cell proliferation and invasion by targeting HER2 and NRP2

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Abstract: Recently, microRNA (miR) has been revealed in many tumors in the expression and role. However, the relationship between miR-331-3p and renal cell carcinoma is unclear. This study investigates the expression and role of miR-331-3p in renal cell carcinoma. The expression of miR-331-3p in renal cell carcinoma was significantly lower than that in adjacent normal tissues. The upregulation of miR-331-3p significantly inhibited the proliferation, migration and invasion of renal cell carcinoma cells. Compared with the control group, miR-331-3p up-regulation of HER and NRP2 expression was inhibited, suggesting that miR-331-3p inhibits renal cell carcinoma progression by down-regulating HER and NRP2 expression. Therefore, our data reveal the down-regulation of miR-331-3p in RCC and its tumor suppressor, and initially found the mechanism of miR-331-3p in promoting RCC progression.

Keywords: miR-331-3p, ccRCC, HER, NRP2, tumor progression

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer, accounting for 2.4% of all adult malignancies [1]. Transparent cell renal cell carcinoma (ccRCC) accounts for the majority (about 80-90%) of the RCC histological subtype [2]. Due to the limited early warning, most RCC patients are diagnosed at a later stage. Because of the ease of recurrence after surgery and the resistance to chemotherapy and radiotherapy, RCC patients’ treatment strategies are a serious problem that needs to be addressed. Therefore, the early detection of RCC and the disclosure of molecular mechanisms are important. MicroRNA (miR) is a group of 20-25 nucleotides of RNA that regulates post-transcriptional gene expression by targeting specific mRNA [3, 4]. Recently, some studies have demonstrated that miRNAs expression are abnormal in a variety of human cancers and involve epigenetic processes of cancer cells [5]. Studies have shown that miR-331-3p is involved in a variety of cancers, including cervical cancer, gastric cancer, prostate cancer, colorectal cancer, hepatocellular carcinoma and glioblastoma [3, 6-11]. It has been reported that miR-331-3p is downregulated in cervical cancer and colorectal cancer tissues and is involved in cancer cell proliferation, migration and invasion [10, 12]. However, the role of miR-331-3p in RCC and its potential function is unclear.

In this study, miR-331-3p was down-regulated in normal tissues and normal cell lines in RCC tissues and cell lines, and miR-331-3p may inhibit the proliferation, migration and invasion of RCC cells and regulate NRP2 expression by modulating HER2.

Materials and methods

Patient samples

In this study, 60 patients with RCC (January 2013 to April 2014) were enrolled in the first
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hospital in Fangshan District, Beijing, China. Collect pairs of tumors and adjacent normal tissues and quickly freeze in liquid nitrogen until RNA is extracted. All participants received informed consent under the Helsinki Declaration, and the ethical approval of this study was provided by the Founder Mountain First Hospital Urology Ethics Committee (Beijing, China).

Cell lines and cell culture

Renal cell carcinoma cell lines (ACHN, 786-0, 769-P) and normal kidney cell line (proximal tubular cell line (HK-2)) from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 (Hyclone, Logan, Utah, USA) containing 10% FBS (Gibco, Austria) supplemented with 1% penicillin/streptomycin respectively, and incubated at 37°C in a humidified atmosphere with 5% CO₂.

Cell transfections

MiR-331-3p mimics (ID: PM10881) and negative control miR-RNA (ID: AM17110) were synthesized by GenePharma (Suzhou, China). The mimics and controls were transfected into ACHN and 786-0 cells using Lipofectamine 3000 Transfection Kit (invitrogen, USA) according to the manufacturer’s instructions. Further experiments were performed after transfection for 24 h.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from tissues and cell lines using RNAsimple Total RNA kit (TIANGEN, Beijing, China) following the manufacturer’s instructions. Then, the complementary DNA (cDNA) was synthesized using FastQuant RT Kit (TIANGEN, Beijing, China), and stored at -20°C. Quantitative real-time PCR polymerase chain reaction was carried out using KAPA SYBR FAST Universal qPCR Kit (KAPA) on an iQ5 real-time detection system (Bio-Rad Laboratories, Hercules, CA, USA). U6 small nuclear RNA was used as control.

Western blot analysis

The cells were lysed on ice in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA), which was supplemented with a 1% protease inhibitor cocktail. The protein concentration was assessed using BCA protein assays (Solarbio, Beijing, China). Equal amounts of protein were separated with 10% or 12.5% SDS-PAGE gels, and then transferred onto nitrocellulose membranes (Millipore, Bedford, Massachusetts, USA). The membranes were blocked in 5% skimmed milk for 1 h and incubated with primary antibodies at 4°C over night. After incubating with secondary antibody for 1 h at room temperature, the immunoreactive bands were detected with Super Signal West Pico Chemiluminescent Substrate (Thermo, USA). Densitometric analysis was performed with Image J software. The primary antibodies were HER rabbit monoclonal antibody (1:1000; Abcam, USA), GAPDH rabbit monoclonal antibody (1:3000; CST, USA), NRP2 rabbit monoclonal antibody (1:1000; CST, USA). The secondary antibodies were goat anti-rabbit antibodies. GAPDH was used as loading control and the intensity of target proteins was normalized to the intensity of GAPDH.

Cell proliferation assay

Cell proliferation was evaluated using the CCK8 Cell Proliferation Assay Kit (Invitrogen, Carlsbad,
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California, USA). 2x10^3 ACHN and 786-0 cells were plated in 96-well plates and transfected with the miR-195 or NC. At the indicated time-point, 10 μl CCK8 solutions were added into each well and incubated for 2 h. The absorbance at 450 nm was measured to calculate the number of viable cells. Cell proliferation was measured on days 0, 1, 2, 3, 4 and 5. Each condition was assessed in triplicate, and the assay was repeated three times.

Cell migration assay

Twenty-four hours after transfection with miR-331-3p or NC, the ACHN and 786-0 cells were starved for 6 h. Then, 4x10^4 cells in 250 μL serum-free media of each group were added into the upper chamber of a 24-well invasion (Corning, New York, USA) with a polycarbonate filter (8 μm pore size). The bottom chamber contained conditioned medium with 10% FBS. After 24 h incubation, the non-migrated cells in the upper chamber were scraped off with a cotton swab, and the migrated cells on the bottom were fixed with methanol and stained with haematoxylin. The number of cells was counted in 6 randomly chosen fields (magnification, ×100). Each experiment was performed in triplicate, and the assay was repeated three times.

Statistical analysis

Expression of miR-331-3p in ccRCC tissues was analyzed using Mann-Whitney U test. Other data were expressed as mean ± SEM, and comparisons between two groups were carried out by Student’s t-test. Value of P<0.05 was considered as statistical significance.

Results

miR-331-3p was downregulated in RCC tissues and cell lines compared with normal tissues and cell line

In order to study the expression of miR-331-3p in RCC and adjacent non-tumorous tissues, q-PCR was performed. MiR-331-3p was significantly downregulated in RCC tissue compared to adjacent non-tumor tissue (Figure 1A). In addition, the expression of miR-331-3p in RCC cell lines was lower than that in normal cell lines (Figure 1B).

miR-331-3p inhibited the proliferation of RCC cells

To investigate the potential role of miR-331-3p in RCC cell proliferation, CCK-8 was performed

Figure 2. miR-331-3p inhibited the proliferation of RCC cell lines. A. After transfection, the expression of miR-331-3p in ACHN and 786-0 cells was increased significantly in miR-331-3p groups. The results were acquired from q-PCR. B. CCK-8 assays showed that the proliferation of ACHN and 786-0 cells transfected with miR-331-3p mimics were inhibited compared with the Controls.
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![Image of cell cultures and graphs]

**Figure 3.** miR-331-3p inhibited the migration of RCC cells. After transfected with miR-331-3p mimics, the number of migrated ACHN and 786-0 cells were decreased.

- **Figure 4.** miR-331-3p suppressed the invasion of ccRCC cells. Compared with Control, the number of invaded miR-331-3p-transfected ACHN and 786-0 cells was decreased.

Using ACHN and 786-0 cells. As a result, proliferation of ACHN and 786-0 cells transfected with miR-331-3p was significantly inhibited compared to NC cells (**Figure 2B**). Our results suggest that miR-331-3p may be the cause of RCC cell proliferation.

**miR-331-3p significantly suppressed the migration of RCC cells**

To investigate the impact of miR-331-3p on RCC cell migration, transwell assays were performed. Compared with controls, upregulation of miR-331-3p led to a decrease in the number of cells that crossed over the filter (**Figure 3**).

**miR-331-3p reduced the invasion ability of RCC cells**

After finding the role of miR-331-3p in migration of cells, we aimed to explore whether miR-331-3p was involved in the invasion of RCC cells. Therefore, we performed invasion assays using the same number of ACHN and 786-0 cells.
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transfected with miR-331-3p or NC. As a result, miR-331-3p-expressing significantly decreased the number of invading cells comparing with controls (Figure 4).

miR-331-3p repressed the expression of HER2 and NRP2 in RCC cells

To explore the mechanisms of miR-331-3p in repressing the progression of RCC, we detected some of human genes that were related with cell proliferation and cell migration. Compared with NC, the expression of HER and NRP2 in miR-331-3p groups were significantly decreased at both mRNA and protein levels (Figure 5A and 5B). Our data indicated that miR-331-3p repressed the expression of HER and NRP2 in RCC cells.

Discussion

In this study, the expression and potential function of miR-331-3p was studied in RCC. Our data show that miR-331-3p is downregulated in RCC tissues and cell lines compared to adjacent normal tissues and cell lines. In addition, the upregulation of miR-331-3p affected the proliferation, migration and invasion of RCC cells. Enhanced cell proliferation is associated with tumor growth, whereas cell migration and invasion contribute to metastasis [13, 14]. Thus, our data suggest that miR-331-3p may be involved in RCC tumor growth and metastasis processes.

Various studies have shown that miR-331-3p is upregulated in various tumors, including hepatocellular carcinoma, and plays a role in tumor promoters [4, 9]. However, our study shows that miR-331-3p downregulates in RCC and plays a role in tumor suppressor, consistent with studies of miR-331-3p in prostate cancer, gastric cancer and cervical cancer. Our data and previous studies have shown that miR-331-3p may play a different role in different types of tumors, as well as other members of miRNAs [15]. It has been reported that miR-143 downregulates colon cancer and inhibits colon cancer cell migration and invasion [16], and inhibit the migration and invasion of glioma cells by targeting N-RAS [17]. In RCC, the accumulation of studies reported the different effects of different miRNAs. For example, miR-144-3p, miR-28-5p miR-144 and miR-429, by modulating different pathways as tumor suppressors [18-20], however, miR-19a, miR-630 and miR-184 play a role in tumor-promoting factors in the development of RCC [21-23]. Thus, different miRNAs played complicated functions in the same human cancers, and the same miRNA in different tumors also played complicated roles in the tumorigenesis and progression of tumors. Our data demonstrated that miR-331-3p played as a tumor suppressor in RCC.

HER2 (also known as Erb-B2 receptor tyrosine kinase 2) is a member of the HER gene family consisting of HER1, HER2, HER3 and HER4 [24].

Previous studies have reported that HER2 is commonly involved in various types of tumors, including breast, lung and renal cell carcinomas, and may be involved in the proliferation, migration and invasion of cancer cells [25, 26]. In renal cell carcinoma, HER2 is overexpressed as a tumor promoting factor [27, 28].
In our study, the expression of HER2 was inhibited after miR-331-3p upregulation, suggesting that HER2 may be a target for miR-331-3p. MiR-331-3p may inhibit the proliferation, migration and invasion of RCC cells by targeting and inhibiting the expression of HER2. NRP-2 is reported to involve advances in many types of tumors and promising therapeutic targets [29]. In many tumors, NRP-2 acts as a tumor promoting factor. For example, NRP-2 is essential for the initiation of breast cancer and is reported to promote migration and invasion of thyroid and prostate cancer cells [30-32].

In our study, the expression of NRP-2 was upregulated in miR-331-3p in RCC cell lines, which was consistent with previous studies. [6] In addition, previous studies have shown that NRP-2 is overexpressed in RCC tissues and accelerates RCC progression [33]. Our data suggest that miR-331-3p may inhibit RCC progression and inhibit NRP-2 expression by targeting NRP-2.

In conclusion, miR-331-3p downregulates in renal cell carcinoma and inhibits the proliferation, migration and invasion of RCC cells in vitro. These data suggest that miR-331-3p plays an inhibitory role in RCC and may be a potential therapeutic target for RCC.

Disclosure of conflict of interest

None.

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