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Original Article

miRNA-221 promotes proliferation, migration and invasion by targeting TIMP2 in renal cell carcinoma

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Abstract: Introduction: MicroRNAs (miRNAs) play important roles in tumorigenesis. In this study, we investigated the role of miR-221 in the development and progression of clear cell renal cell carcinoma (ccRCC). Methods: Quantitative real-time PCR (qRT-PCR) was used to measure the expression level of miR-221 in ccRCC tissues and cell lines. Then, we investigated the role of miR-221 to determine its potential roles on renal cancer cell proliferation, migration and invasion in vitro. A luciferase reporter assay was conducted to confirm the target gene of miR-221 and the results were validated in renal cancer cells. Results: In the present study, we found that miR-221 was significantly increased in ccRCC tissues and cell lines. Knocked-down expression of miR-221 remarkably inhibited cell proliferation, migration and invasion of renal cancer cells. Moreover, at the molecular level, our results suggested that TIMP2 as a direct target of miR-221 through which miR-221 promoted tumor cell proliferation, migration and invasion. Conclusions: These findings suggested that miR-221 play an oncogenic role in the renal cancer cell proliferation, migration and invasion by directly inhibiting the tumor suppressor TIMP2, indicating miR-221 act as a potential new therapeutic target for the treatment of ccRCC.

Keywords: Clear cell renal cell carcinoma, miR-221, TIMP2

Introduction

Renal cell carcinoma (RCC) is the most common urologic malignancy, accounting for approximately 3% of adult malignancies and causes about 90,000 deaths worldwide annually [1]. Clear cell renal cell carcinoma (ccRCC) is the largest subtype of RCC and accounts for approximately 70% of these tumors [2]. RCC respond poorly to chemotherapy and radiotherapy and surgery remains the only curative treatment [3]. Early detection is of great importance for patient outcome, the 5-year survival for patients diagnosed with organ-confined disease is approximately 90%, whereas the prognosis of patients with distant metastasis remains poor with a 5-year survival of less than 10% [4, 5]. Therefore, increased understanding of the molecular mechanisms of RCC progression and metastasis is urgently needed.

MicroRNAs (miRNAs) are non-coding small RNAs, usually 18-25 nucleotides in length, which repress translation and cleave mRNA by base pairing to the 3' untranslated region (UTR) of the target genes [6]. miRNAs are implicated in the regulation of various cellular processes, including proliferation, differentiation, cell death and cell mobility [7]. In addition, miRNA profiles also indicate that miRNAs can function either as oncogenes or tumor suppressors in tumor progression [8]. To date, several miRNAs have been implicated in the development and progression of ccRCC, such as miR-708, miR-21, miR-205 and miR-646 [9-12].

Previous studies have shown that miR-221 was significantly up-regulated in several types of human cancers. For example, Fornari et al showed that miR-221 is up-regulated in of human hepatocellular carcinoma (HCC) and has an oncogenic function through the inhibition of

CDKN1C/p57 and CDKN1B/p27 protein expression [13]. Zhang et al found that miR-221 is increased gastric carcinoma and regulate radiosensitivity and cell growth and invasion of gastric cancer cells via direct modulation of PTEN expression [14]. Yang et al demonstrated that down-regulation of miR-221 restrain prostate cancer cell proliferation and migration that is partly mediated by activation of SIRT1 [15]. Yao et al suggested that miR-221 is significantly increased in colorectal carcinoma and miR-221 can be used as a non-invasive biomarker for the detection of colorectal carcinoma [16]. However, no studies apparently have been conducted to investigate the role of miR-221 in ccRCC.

In this study, our data showed that miR-221 was up-regulated in ccRCC tissues and cell lines. Down-regulated expression of miR-221 could inhibit cell proliferation, migration and invasion in vitro assays. We identified TIMP2 was a direct target of miR-221 and showed that miR-221 functions as a tumor oncogenes by down-regulating TIMP2 expression, providing a potential diagnostic and therapeutic target for the treatment of ccRCC.

Materials and methods

Patients and specimens

A total of 28 ccRCC tissues and adjacent non-tumor tissues were obtained from patients who underwent radical nephrectomy surgery at the First Affiliated Hospital of Xinxiang Medical University. Fresh specimens were collected, snap frozen in liquid nitrogen immediately after resection, and directly stored at -80°C until RNA extraction was performed. Samples from cancerous areas were isolated from non-necrotic parts of the tumor tissue. Tumor classification and staging were performed according to the 2004 World Health classification and the 2002 TNM System. The study was approved by the Ethical Review Board of the Xinxiang Medical University and written informed consent was obtained from all patients.

Cell culture and transfection

Renal cell lines (786-0, ACHN, Caki-1, Caki-2) and Immortalized normal human proximal tubule epithelial cell line HK-2 were obtained from American Type Culture Collection (ATCC, USA). HK-2 cells were cultured in KSFM medium (Gibco), and other cells were cultured in RPMI-1640 medium (HyClone) with 10% fetal bovine serum (Gibco). The culture media were all supplemented with 10% fetal bovine serum, 50 U/ml of penicillin and 50 μg/ml of streptomycin. All cells were maintained at 37°C in a humidified incubator with 5% CO_2.

miR-221 inhibitor and miR-221 mimics were synthesized by RiboBio (China). Small interfering RNA against TIMP2 (si-TIMP2) and negative control (si-NC) were designed by Genepharma (China). Transfection was carried out using Lipofectamine 2000 (Invitrogen) method.

MTT assay

Cell proliferation was measured by 3-[4,5-dimethylthi-azol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were grown in a 96-well plate for 24 h and cultured in normal medium. Cells were then incubated in 0.1 mg/ml MTT at 37°C for 5 h and lysed in DMSO at room temperature for 10 min at 0, 24, 48, 72, and 96 h after transfection. The absorbance in each well was then measured at 570 nm, with a SpectraMax M5 Microplate Reader (Molecular Deviced, Sunnyvale). Each experiment was performed at least three times.

Transwell migration and invasion assays

Cell migration and invasion were assessed using transwell assay. For migration, 2×10^4 cells in serum-free medium were seeded into the top chambers of an insert (8 μm pore size, Merck Millipore), which were soaked into the bottom chambers filled with complete medium. For invasion, the same density of cells was placed into the upper chambers, which were pre-coated with Matrigel (BD Biosciences). After 24 h of incubation, the chambers were fixed with 4% para-formaldehyde and then stained with 0.1% crystal violet. Numbers of invaded cells were counted in five randomly selected fields under a microscope (Nikon).

Luciferase reporter assay

The 3' untranslated region (UTR) of human TIMP2 gene that was predicted to interact with miR-221 was synthesized and inserted into pMIR-REPORT (Ambion), yielding pMIR-REPORT TIMP2. Mutations within potential miR-221 binding sites were generated by nucleotide replacement of wild-type sequence to inhibit

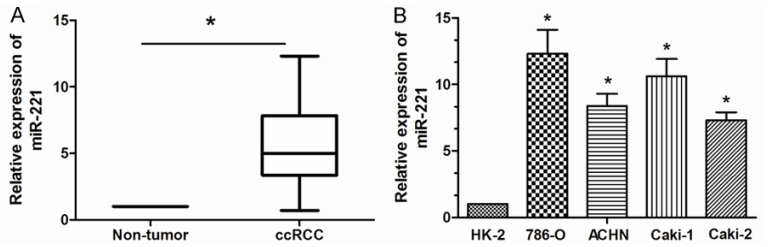

Figure 1. miR-221 was increased in human ccRCC tissues and cell lines. A. The expression of miR-221 in 28 paired ccRCC tissues and their corresponding non-tumor tissues (Non-tumor) was measured by qRT-PCR. GAPDH was used as an internal control. B. The expression levels of miR-221 in the human normal proximal tubule epithelial cell line HK-2 and four renal cancer cell lines. *P<0.05.

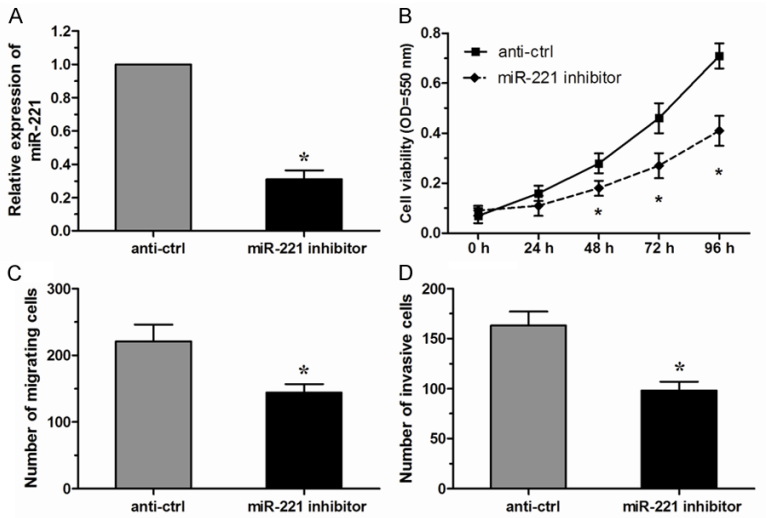

Figure 2. Down-regulated expression of miR-221 suppressed renal cancer cell proliferation, migration and invasion. (A) The relative expression level of miR-221 in 786-0 cells transfected with miR-221 inhibitor or inhibitor control (anti-ctrl) was determined by qRT-PCR. (B) MTT assay was performed to analyze the effect of miR-221 on cell proliferation of 786-0 cells. (C), (D) Transwell assays were used to analyze the effect of miR-221 on cell migration (C) and invasion (D) of 786-0 cells. *P<0.05.

miR-221 binding. Cells were transfected with the pMIR-REPORT vectors containing the 3′UTR variants and miR-221 mimics for 24 h. The pRL-SV40 Vector (Promega) carrying the Renilla luciferase gene was used as an internal control to normalize the transfection efficiency. Luciferase values were determined using the Dual-Luciferase Reporter Assay System (Promega).

RNA isolation and quantitative real-time PCR

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacture's protocol. The cDNA strand was synthesized from total RNA with M-MLV Reverse Transcriptase (Promega) in a 25 μl volume. Real-time PCR was carried out with the reagents of a Sybr green I mix (Takara) in a 20 μl reaction volume on an ABI PRISM 7000 Fluorescent Quantitative PCR System (Applied Biosystems). The PCR cycling parameters were: 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 30 s. Relative expression fold c hanges were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Proteins were separated on 10% SDS-PAGE gel and then transferred to nitrocellulose membrane (Bio-Rad). After blocked with 5% nonfat milk, the membrane was incubated with TIMP2 antibody (1:1000, Cell Signaling Technology), β-Actin antibody (1:2000, Cell Signaling Technology). The proteins were visualized using ECL reagents (Beyotime).

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software (IBM). Data are expressed as the mean ± SD from at least three separate experiments. Data were analyzed using Student's t test or one-way ANOVA analysis. Values of $P<0.05$ were considered statistically significant.

Results

miR-221 levels were increased in ccRCC tissues and cell lines

The expression of miR-221 in 28 pairs of ccRCC tissues and their adjacent non-tumor tissues was measured using qRT-PCR. Our results revealed that miR-221 expression was significantly increased in ccRCC tissues compared with adjacent non-tumor tissues (**Figure 1A**). In

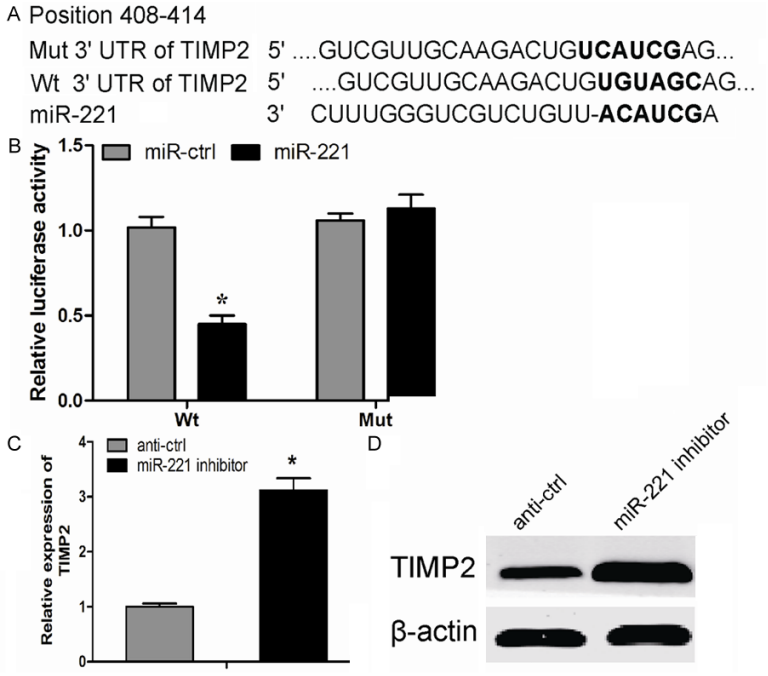

Figure 3. miR-221 negatively regulated TIMP2 by binding to the TIMP2 3'-UTR. A. The wild type or the mutated sequences of the TIMP2 mRNA 3'-UTR. B. The luciferase activity of the wild type TIMP2 3'-UTR (Wt) and mutant TIMP2 3'-UTR (Mut) co-transfected with miR-221 mimics (miR-221) or a miRNA negative control (miR-Ctrl) was measured. C. The mRNA level of TIMP2 in 786-O cells transfected with miR-221 inhibitor or inhibitor control (anti-ctrl) was determined by qRT-PCR. D. The protein level of TIMP2 in 786-O cells transfected with miR-221 inhibitor or anti-ctrl was determined by Western blot. *P<0.05.

addition, the expression of miR-221 in four renal cancer cell lines was determined. We found that the relative expression of miR-221 in these renal cancer cells was strikingly elevated compared with that of the human normal proximal tubule epithelial cell line HK-2 (**Figure 1B**). These data suggested that miR-221 may contribute to the progression of ccRCC.

Inhibition of miR-221 suppressed proliferation, migration and invasion of renal cancer cells

To evaluate the effects of miR-221 in the progression of ccRCC, 786-O cells were transfected with miR-221 inhibitor. Decreased expression of miR-221 was confirmed by qRT-PCR (**Figure 2A**). We found that down-regulated expression of miR-221 significantly inhibited the proliferation of 786-O cells compared to their corresponding controls (**Figure 2B**). Furthermore, transwell assays showed that decreased expression of miR-221 could inhibit the migratory and invasive abilities of 786-O cells (**Figure 2C** and **2D**). Taken together, these results suggested that decreased expression miR-221 could inhibit renal cancer cell proliferation, migration and invasion in vitro.

TIMP2 was a direct target of miR-221

To elucidate the underlying mechanisms by which miR-221 exerts its function, we used TargetScan bioinformatics algorithm. TIMP2 was found to be a potential target (**Figure 3A**). Luciferase reporter assay was commonly used to validate the suppressive effects of miRNAs on their target mRNAs. Here, we found that miR-221 significantly decreased the luciferase activity of the wild-type (Wt) but not the Mut 3'-UTR of TIMP2 in HEK293 cells (**Figure 3B**). Moreover, inhibition of miR-221 significantly increased expression levels of both TIMP2 mRNA and protein (**Figure 3C** and **3D**). Taken together, these data strongly suggested that TIMP2 is a direct target of miR-221 in ccRCC.

TIMP2 was involved in miR-221 induced proliferation, migration and invasion in renal cancer cells

To test whether down-regulation of TIMP2 might function in miR-221 induced 786-O cell proliferation, migration and invasion, we inhibited TIMP2 expression with siRNA in 786-O cells (**Figure 4A**). The MTT assay revealed that both TIMP2 and miR-221 depleted cells showed increased proliferation compared with those cells with only depleted miR-221 (**Figure 4B**). Moreover, transwell assays showed that TIMP2 silencing could significantly enhance migration and invasion ability in miR-221 depleted 786-O cells (**Figure 4C**, **4D**). Collectively, these results demonstrated that miR-221 could promote the proliferation, migration and invasion of renal cancer cells via directly down-regulating TIMP2 expression.

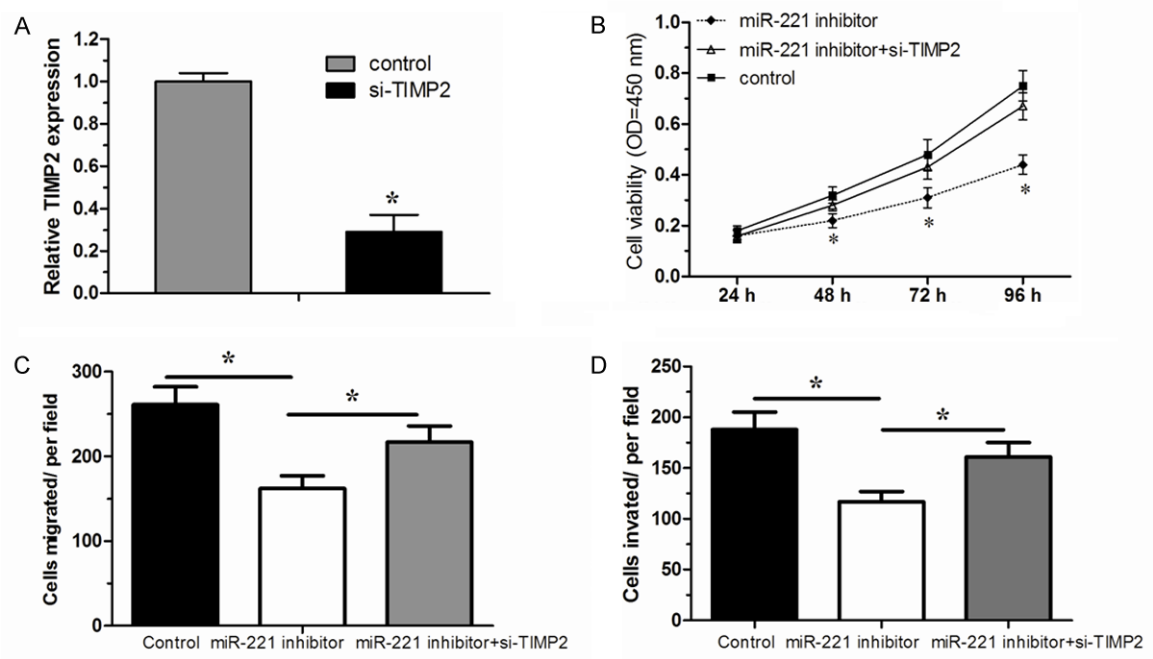

Figure 4. Decreased expression of TIMP2 partially attenuated the tumor oncogenic effects of miR-221. (A) Knockdown of TIMP2 by siRNA (si-TIMP2) was confirmed by qRT-PCR in 786-0 cells. (B) MTT assay was used to detect the effects on cell proliferation of 786-0 cells transfected with miR-221 inhibitor with or without si-TIMP2. (C) Transwell migration assay was performed to detect the effects on cell migration of 786-0 cells treated as described in (B). (D) Transwell invasion assay was utilized to detect the effects on cell invasion of 786-0 cells treated as described in B. *P<0.05.

Discussion

In this study, we investigated the roles of miR-221 in cell proliferation, migration and invasion of ccRCC. Our results showed that miR-221 was increased in ccRCC tissues and cell lines. Further studies suggested that down-regulated expression of miR-221 inhibited renal cancer cell proliferation, migration and invasion. These findings indicated that miR-221 could act as onco-MicroRNA in the progression of ccRCC. However, further studies are still needed to investigate its underlying mechanism.

At the molecular level, our results indicated that TIMP2 is a direct target of miR-221 in renal cancer cells and showed miR-221 down-regulation is correlated with TIMP2 over-expression leading to the inhibition of cell proliferation, migration and invasion. Our data revealed that the tumor oncogene role of miR-221 is mediated by the regulation of TIMP2 expression. TIMP2 which is a member of tissue inhibitor of metallopeptidases (TIMPs) family which can degrade the extracellular matrix (ECM) and basement membrane (BM), the degradation of which is a prerequisite for tumor metastasis [17]. It has been reported that TIMP2 combines with activated MMP2 through 1 to 1 non-covalent forms and abrogates its collagenases and gelatinases activity, reduces the degradation of ECM, and maintains the integrity of BM [18]. In addition to an inhibitory role against metalloproteinases, TIMP2 has a unique role among TIMP family members in its ability to directly suppress the proliferation of endothelial cells with MMP-independent mode [19]. Recently, Zhu et al showed that up-regulation of miR-106a may exert oncogenic role with promoting effects on cell proliferation and especially metastasis by targeting TIMP2, and conversely, knockdown of TIMP2 could mimic the miR-106a induced cancer development benefits [20]. Wu et al found that miR-19a is correlated with prognosis and apoptosis of laryngeal squamous cell carcinoma by regulating TIMP-2 expression [21]. Therefore, our results added a novel mechanism for the dysregulated TIMP2 expression in tumors.

In summary, the present study showed that miR-221 was increased in ccRCC tissues and

cell lines, and decreased expression of miR-221 could inhibit renal cancer cell proliferation, migration, and invasion. The tumor oncogenes function of miR-221 was mediated by the down-regulation of its downstream target gene TIMP2. These findings demonstrated that miR-221 might be a helpful therapeutic strategy for renal cancer treatment in the future.

Disclosure of conflict of interest

None.

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