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MiRNA-1246 suppresses the proliferation and migration of renal cell carcinoma through targeting CXCR4

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Abstract. – OBJECTIVE: To uncover the role of miRNA-1246 in influencing the proliferative and migratory capacities of RCC by binding CXCR4 and downregulating its level.

PATIENTS AND METHODS: Relative levels of miRNA-1246 and CXCR4 in 40 paired RCC tissues and adjacent normal tissues were determined. The binding relationship between miRNA-1246 and CXCR4 was confirmed by Dual-Luciferase reporter assay. Proliferative and migratory abilities of RCC regulated by miRNA-1246 and CXCR4 were assessed.

RESULTS: MiRNA-1246 was downregulated in RCC tissues and cell lines. Overexpression of miRNA-1246 attenuated proliferative and migratory capacities of 786-O and 769-P cells. CXCR4 was the direct target of miRNA-1246 and its level was negatively regulated by miRNA-1246. Silence of CXCR4 inhibited RCC to proliferate and migrate.

CONCLUSIONS: MiRNA-1246 attenuates proliferative and migratory abilities of RCC by downregulating CXCR4. MiRNA-1246/CXCR4 axis could be potential therapeutic target for RCC.

Key Words:
MiRNA-1246, CXCR4, Proliferation, Migration.

Introduction

Renal cell carcinoma (RCC) cases account for 4% of adult malignant tumors. The main histological subtypes of RCC include renal clear cell carcinoma (70%-80%), papillary carcinoma (10%-15%), chromophobe cell carcinoma, and collecting duct carcinoma[1]. RCC is prone to recurrent and exerts a high mortality over 40%[2]. Currently, the incidence of RCC is on the rise. It has become the seventh and the eighth most-common cancer in American males and females, respectively[3,4]. Generally, early-stage RCC can be diagnosed by B-ultrasound, CT, and MRI. Symptoms of hematuria, low back pain, and lumbar mass predict the metastasis of RCC. It is reported that about 30% RCC patients suffer from metastatic loci at the time of diagnosis, who have extremely poor prognosis and overall survival[5,6]. Radical nephrectomy is only suitable for early stage RCC patients with clinical indications[7,8]. For advanced RCC, molecular targeted drugs are generally applied, including sorafenib and sunitinib. However, innate or secondary drug resistance greatly restricts the therapeutic efficacy[9,10]. Searching for novel targets for RCC treatment is of great significance.

MiRNAs negatively regulate target gene expressions at the post-transcriptional level by complementary base pairing to the mRNA 3'UTR[11]. In 2558 discovered miRNAs, the vast majority are functional non-coding RNAs with 20-23 nucleotides long[12]. Abnormally expressed miRNAs can activate or inactivate intracellular signaling pathways and cell cycle-associated genes, thus leading to diseases[13]. Many dysfunctional miRNAs are associated with tumorigenesis[13]. In addition, abnormally expressed miRNAs may result in proto-oncogene dysregulation and abnormalities of cancer-related pathways[14]. During the malignant progression of tumors, mechanisms underlying the dysfunctional miRNAs are diverse. Firstly, miRNA expression changes during gene amplification and product modification. Secondly, miRNAs are abnormally expressed as transcriptional regulators of proto-oncogenes (such as c-myc). Thirdly, genes related to miRNA mutations are mutant (such

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as DICER)[15]. Duensing and Hohenfellner[16] have found that miRNAs exert a carcinogenic or tumor-suppressor effect on the progression of RCC.

In 2008, miRNA-1246 was initially discovered in sequencing analysis on the human embryonic stem cells[17]. MiRNA-1246 can stimulate the activation of angiogenesis-related pathways by activating P53 and DYRK1A, thus stimulating tumor angiogenesis and tumor cell metastasis[18]. Serum level of miRNA-1246 is elevated in patients with esophageal squamous cell carcinoma, indicating that miR-1246 may serve as a serological molecular marker[19]. In addition to the involvement in tumor diseases, miRNA-1246 also participates in inflammation and immunity. In mesenchymal stem cells/stromal cells, miRNA-1246 stimulates the inflammatory response by targeting PKA and PP2A[20]. MiRNA-1246 is involved in the activation of B cells and the malignant transformation of induced bronchial epithelial cells[21]. However, the biological function of miRNA-1246 in RCC is unclear. Our study previously found that miRNA-1246 was downregulated in RCC. This paper aims to clarify the molecular mechanism of miRNA-1246 on influencing the progression of RCC.

Patients and Methods

Sample Collection

According to international guidelines on RCC[22], a total of 40 paired RCC tissues and adjacent normal tissues were surgically resected, immediately placed in liquid nitrogen, and preserved at -80°C. None of enrolled RCC patients received preoperative anti-tumor therapies. Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of Daqing Oilfield General Hospital and in accordance with the Declaration of Helsinki. All recruited patients provided written informed consent before the study.

Cell Culture

Renal cortical proximal tubular epithelial cells (HK2) and RCC cell lines (A498, Caki-1, 786-O and 769-P) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine. Cells were maintained in a 37°C, 5% CO_2 incubator. Medium was replaced every 2-3 days.

Transfection

Transfection plasmids were provided by GenePharma (Shanghai, China). Cells pre-seeded in the 6-well plates were cultured to 70% confluence and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 24-48 h, cells were harvested for subsequent experiments.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The mixture was placed on ice for 5-10 min, followed by incubation with 4 μL reverse transcriptase buffer, 2 μL DTT, and 1 μL dNTPs. The mixture was then incubated at 42°C for 2 min. 1 μL of reverse transcriptase was then added for 1 h incubation at 42°C. Reverse transcriptase activity was quenched at 65°C. RNA was purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Primer sequences were shown as follows: MiRNA-1246, F: 5'-TGCGGAATGGATTTTTGG-3'; R: 5'-CCAGTGCAGGGTCCGAGGT-3'; CXCR4, F: 5'-TTTGCTTTCAGAGACGTTGG-3'; R: 5'-GCGTCAGTGCTCTGCAAC-3'; GAPDH, F: 5'-GGTGGTCTCCTCTGACTTCAA-3'; R: 5'-GTTGCTGTAGCCAAATTCGTTGT-3'; U6, F: 5'-AGAGAAGATTAGCATGGCCCCTG-3'; R: 5'ATCCAGTGCGGGTCCGAGG-3'.

Cell Counting Kit-8 (CCK-8)

Cells were seeded in the 96-well plate with 5×10^3 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

Colony Formation Assay

Cells were inoculated in a 6-well plate with 500 cells per well and incubated for 2 weeks. Subsequently, cells were fixed in 100% methanol and dyed with 0.5% crystal violet for 20 min. Colonies were finally captured and calculated.

Transwell Migration Assay

1.0×10^4 cells were inoculated in the upper side of transwell chamber (Corning, Corning, NY, USA) inserted in a 24-well plate. In the bottom side, 700 μL of medium containing 10% FBS was applied. After 48 h of incubation, cells migrated to the bottom side were subjected to fixation in methanol for 15 min, crystal violet staining for 20 min, and cell counting using a microscope. The number of migratory cells was counted in 5 randomly selected fields per sample (magnification 200×).

Western Blot

Extraction of protein samples was performed using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China). Protein concentration was detected by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein samples were applied for electrophoresis and transferred on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membrane was blocked in 5% skim milk for 2 hours, incubated with primary and secondary antibodies. Band exposure was conducted by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA) and was further analyzed by Image Software.

Dual-Luciferase Reporter Assay

Potential binding sequences in the 3'UTR of miRNA-1246 and CXCR4 were predicted by TargetScan. Cells were co-transfected with miRNA-1246 mimics/NC and wt CXCR4 3'UTR/mut CXCR4 3'UTR using Lipofectamine 2000. After 24 hours, co-transfected cells were collected for determining Luciferase activity using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 7 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the *t*-test. Spearman regression test was performed to evaluate the relationship between two genes. $p<0.05$ was considered as statistically significant.

Results

MiR-1246 Was Downregulated in RCC

Compared with matched normal tissues, miRNA-1246 was downregulated in RCC tissues (Figure 1A). Identically, miRNA-1246 was lowly expressed in RCC cell lines (Figure 1B). In the subsequently experiments, 786-O and 769-P cells were selected for establishing miRNA-1246 overexpression model. Transfection of miRNA-1246 mimics markedly upregulated miRNA-1246 level in 786-O and 769-P cells (Figure 1C, 1D).

MiRNA-1246 Attenuated RCC Cells to Proliferate and Migrate

In 786-O and 769-P cells overexpressing miRNA-1246, their viabilities were markedly reduced relative to controls (Figure 2A, 2B). Colony formation assay revealed the decreased colony number in RCC cells after transfection of miRNA-1246 mimics, identically suggesting the inhibited proliferative ability (Figure 2C, 2D). Moreover, transfection of miRNA-1246 mimics reduced the number of migratory cells, indicating the attenuated migratory ability (Figure 2E). Collectively, proliferative and migratory abilities of RCC were suppressed by miRNA-1246 overexpression.

MiRNA-1246 Bound to CXCR4

Through online prediction, potential binding sequences in the 3'UTR of miRNA-1246 and CXCR4 were identified (Figure 3A). A remarkably decline in Luciferase activity was observed in 786-O and 769-P cells co-transfected with miRNA-1246 mimics and wt CXCR4 3'UTR. However, overexpression of miRNA-1246 failed to regulate Luciferase activity in mut CXCR4 3'UTR, verifying the binding between miRNA-1246 and CXCR4 (Figure 3B, 3C). Relative level of CXCR4 was markedly downregulated by transfection of miRNA-1246 mimics in 786-O and 769-P cells (Figure 3D, 3E). To further explore the potential role of CXCR4 in RCC, we constructed si-CXCR4 and tested its transfection

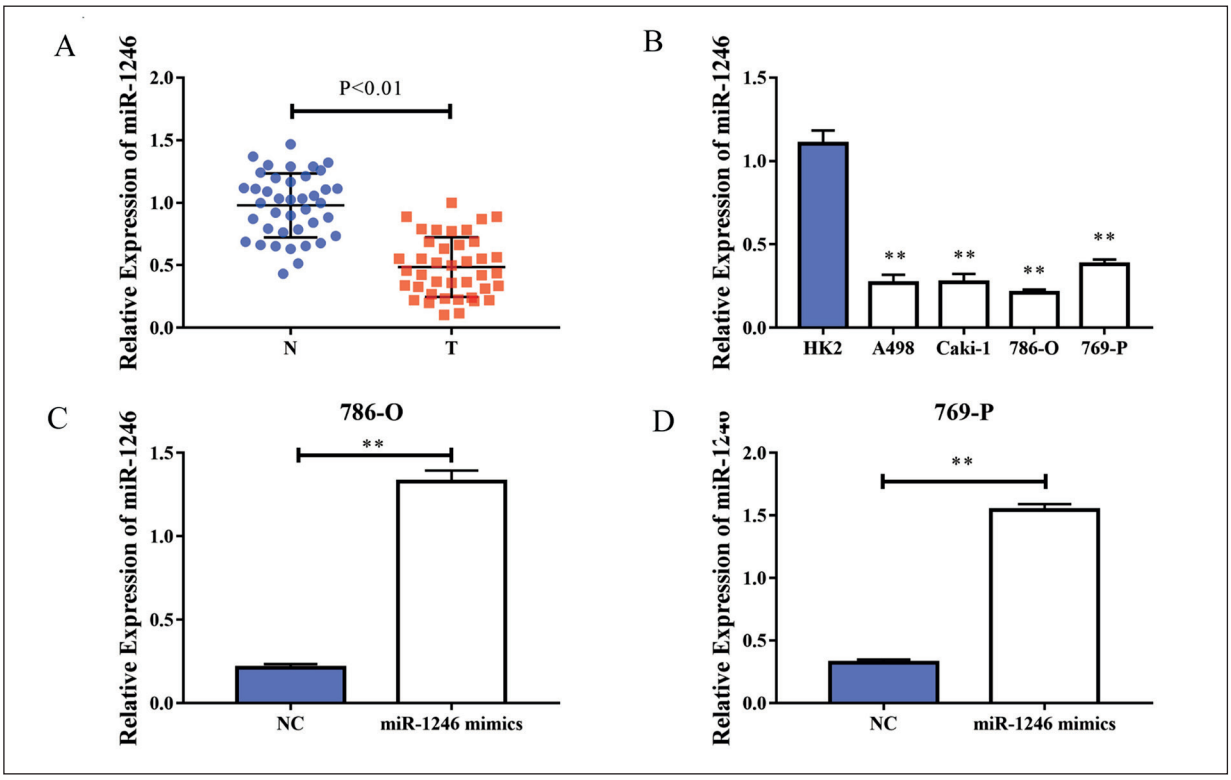

Figure 1. MiR-1246 was downregulated in RCC. **A,** Relative level of miRNA-1246 in RCC tissues and adjacent normal tissues. **B,** Relative level of miRNA-1246 in renal cortical proximal tubular epithelial cells (HK2) and RCC cell lines (A498, Caki-1, 786-O and 769-P). **C,** Transfection efficacy of miRNA-1246 mimics in 786-O cells. **D,** Transfection efficacy of miRNA-1246 mimics in 769-P cells.

efficacy (Figure 3F). After transfection of si-CXCR4, relative level of miRNA-1246 was markedly elevated (Figure 3G). The above data indicated that CXCR4 was bound to miRNA-1246 and negatively regulated by it.

CXCR4 Was Upregulated in RCC

Compared with matched normal tissues, CXCR4 was highly expressed in RCC tissues (Figure 4A). A negative correlation was identified in RCC tissues between expression levels of miRNA-1246 and CXCR4 (Figure 4B). CCK-8 assay showed an elevation in the viability after knockdown of CXCR4 in 786-O and 769-P cells (Figure 4C, 4D). Silence of CXCR4 decreased the number of colonies formed in 786-O and 769-P cells, indicating the attenuated proliferation (Figure 4E, 4F). Additionally, transfection of si-CXCR4 decreased the number of migratory cells (Figure 4G). It is concluded that silence of CXCR4 attenuated the proliferative and migratory abilities of RCC.

Discussion

RCC cases account for approximately 39% of all adult tumors and 90% of all kidney cancers. Renal clear cell carcinoma is the most common subtype of RCC. Patients with renal clear cell carcinoma are mostly prone to develop metastases with an extreme poor prognosis[23]. RCC is characterized by lacked early-stage symptoms, atypical clinical manifestations, and radiotherapy/chemotherapy resistance[24]. The latest research found that systemic treatment has greatly improved the prognosis of affected patients[25]. Therefore, searching effective and sensitive tumor markers or therapeutic targets for RCC is of great significance. MiRNAs are a class of non-coding RNAs that participate in the development and progression of RCC by targeting key genes (i.e. HIF, mTOR, VEGF and VHL)[26,27]. Many studies have shown that tumor metastasis and inflammatory cell infiltration exhibit the similar formation process, involving directed migration, transendothelial migration and adhesion of cells[28].

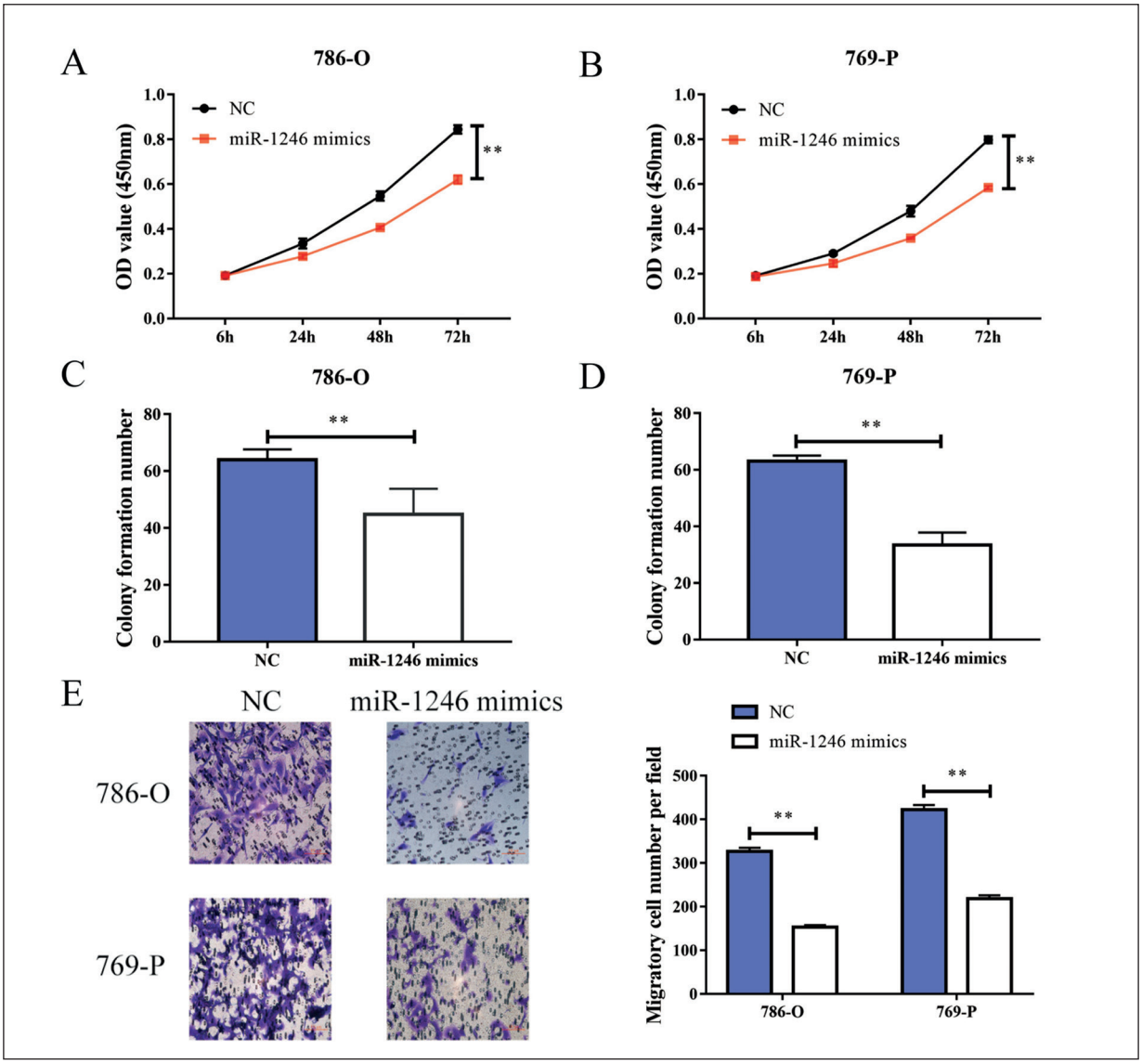

Figure 2. MiRNA-1246 attenuated RCC cells to proliferate and migrate. **A,** CCK-8 assay showed the viability in 786-O cells transfected with NC or miRNA-1246 mimics. **B,** CCK-8 assay showed the viability in 769-P cells transfected with NC or miRNA-1246 mimics. **C,** Colony formation assay showed the colony number in 786-O cells transfected with NC or miRNA-1246 mimics. **D,** Colony formation assay showed the colony number in 769-P cells transfected with NC or miRNA-1246 mimics. **E,** Transwell assay showed the migratory cell number in 786-O and 769-P cells transfected with NC or miRNA-1246 mimics (magnification 200×).

Chemokines provide the signal to stimulate the activation of inflammatory cells, and thereby initiate their targeted movement and accurate localization[29]. Many tumor cells can express certain chemokines or chemokine receptors in a limited manner. Some chemokine signaling pathways are dysregulated in tumors[30]. CXCR4 (C-X-C motif chemokinereceptor 4) is a member of the G protein-coupled receptor family containing seven transmembrane regions. It is extensively expressed in a variety of tissues and organs[31]. CXCR4 is involved in multiple physiological processes, including HIV-1 infection, hematopoietic function, embryonic development and tumorigenesis[32]. Stromal cell-derived factor 1 (SDF-1) is the only ligand for CXCR4. SDF-1/CXCR4 pathway is activated in a variety of malignancies[33,34]. CXCR4 and its ligand SDF jointly determine the metastasis of breast cancer. It can stimulate the chemotaxis of breast cancer cells. CXCR4 antibody could abolish the

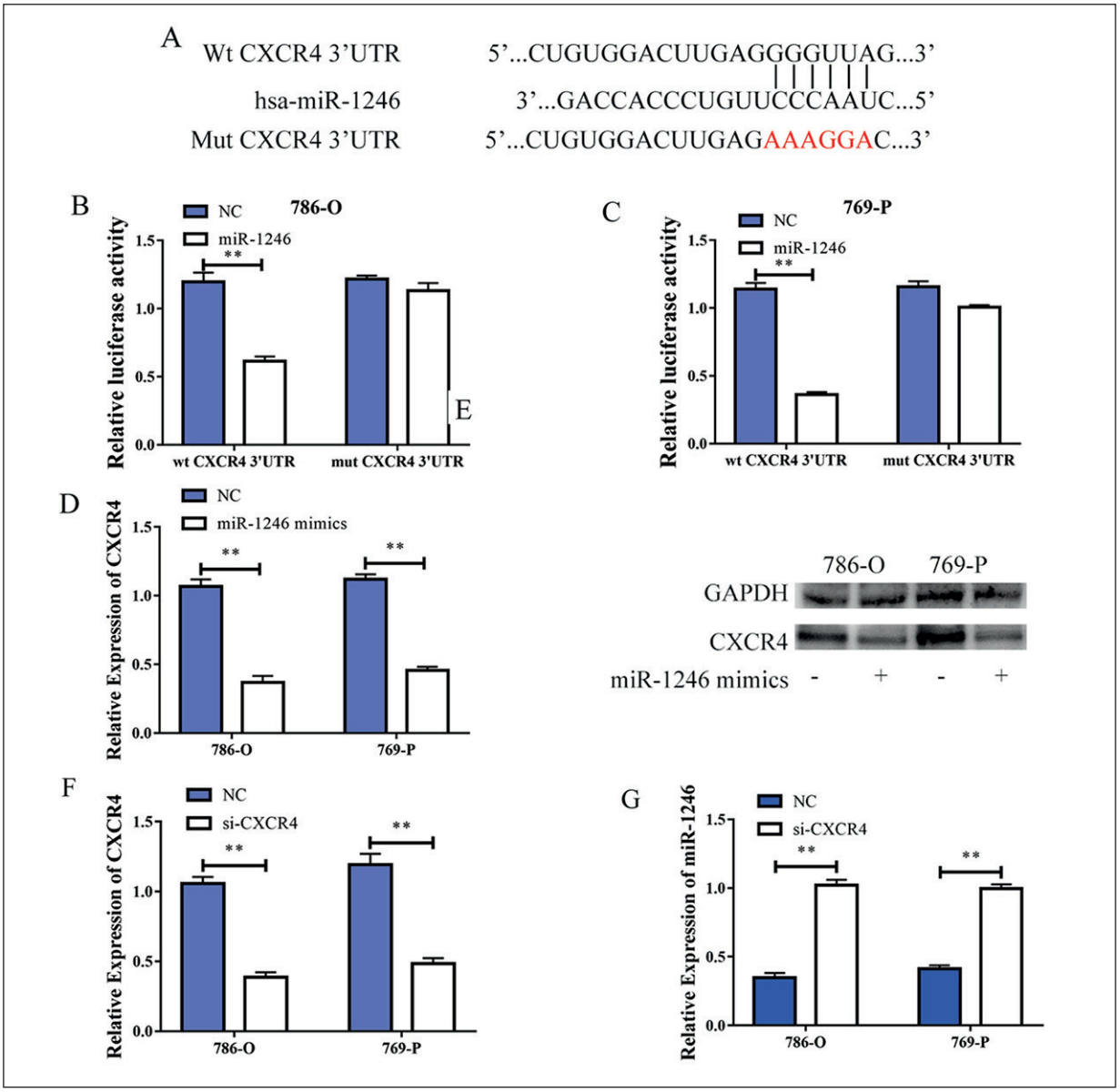

Figure 3. MiRNA-1246 bound to CXCR4. **A,** Potential binding sequences between miRNA-1246 and CXCR4. **B,** Luciferase activity in 786-O cells co-transfected with miRNA-1246 mimics/NC and wt CXCR4 3'UTR/mut CXCR4 3'UTR. **C,** Luciferase activity in 769-P cells co-transfected with miRNA-1246 mimics/NC and wt CXCR4 3'UTR/mut CXCR4 3'UTR. **D,** mRNA level of CXCR4 in 786-O and 769-P cells transfected with NC or miRNA-1246 mimics. **E,** Transfection efficacy of si-CXCR4 in 786-O and 769-P cells. **F,** Protein level of CXCR4 in 786-O and 769-P cells transfected with NC or miRNA-1246 mimics. **G,** Relative level of miRNA-1246 in 786-O and 769-P cells transfected with NC or si-CXCR4.

chemotactic ability of breast cancer to metastasize to target organs, which explains for organ-specific metastasis[34].

In this paper, miRNA-1246 was downregulated in RCC tissues and cell lines. Overexpression of miRNA-1246 attenuated proliferative and migratory capacities of 786-O and 769-P cells. CXCR4 was the direct target of miRNA-1246 and its level was negatively regulated by miRNA-1246. Silence of CXCR4 also inhibited RCC to proliferate and migrate. It is believed that miRNA-1246 could alleviate the proliferative and migratory abilities of RCC by downregulating CXCR4.

In the future, we will expand the simple size and collect the follow-up data to perfect our re-

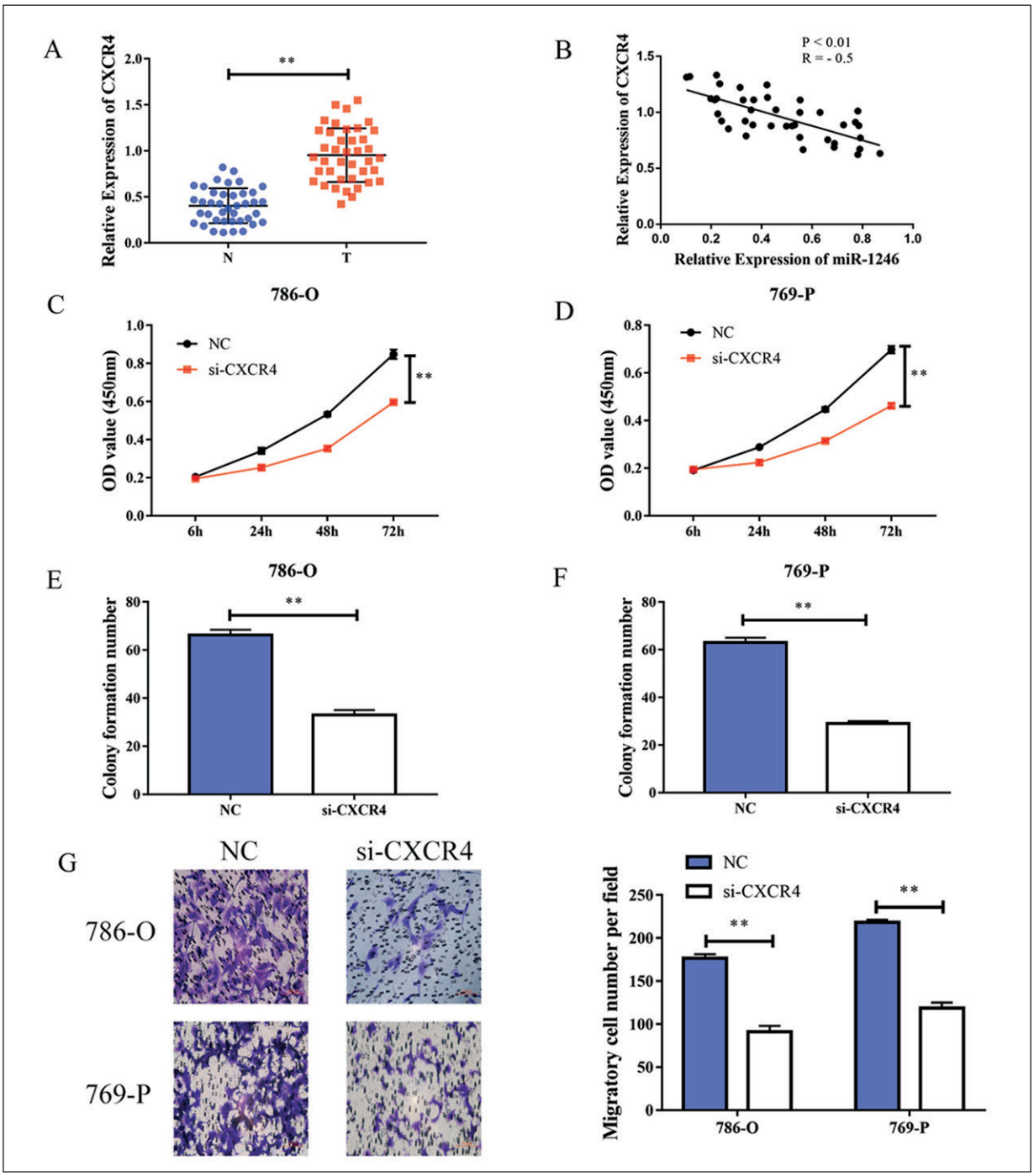

Figure 4. CXCR4 was upregulated in RCC. **A,** Relative level of CXCR4 in RCC tissues and adjacent normal tissues. **B,** A negative correlation in RCC tissues between expression levels of miRNA-1246 and CXCR4. **C,** CCK-8 assay showed the viability in 786-O cells transfected with NC or si-CXCR4. **D,** CCK-8 assay showed the viability in 769-P cells transfected with NC or si-CXCR4. **E,** Colony formation assay showed the colony number in 786-O cells transfected with NC or si-CXCR4. **F,** Colony formation assay showed the colony number in 769-P cells transfected with NC or si-CXCR4. **G,** Transwell assay showed the migratory cell number in 786-O and 769-P cells transfected with NC or si-CXCR4 (magnification 200×).

search. The relationship between the expression level of miR-1246 and the prognosis of RCC patients will be analyzed. Moreover, we will uncover the diagnostic potential in RCC.

Conclusions

The novelty of this study was that miRNA-1246 attenuates proliferative and migratory capacities of RCC by downregulating CXCR4. MiRNA-1246/CXCR4 could be potential therapeutic targets for RCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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