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MiR-1294 acts as a tumor suppressor in clear cell renal cell carcinoma through targeting HOXA6

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Abstract. – OBJECTIVE: Renal cancer represents about 3% of all human cancers. Clear cell renal cell carcinoma (ccRCC) is the main type of renal cancer. MicroRNAs (miRNAs) have been reported to play crucial roles in the carcinogenesis of human cancers. This study was aimed to investigate the expression of miR-1294 and the mechanisms underlying miR-1294-mediated ccRCC progression.

MATERIALS AND METHODS: The miR-1294 expression levels in ccRCC cell lines were analyzed by quantified real time-PCR (qRT-PCR). The effect of the miR-1294 expression on the overall survival of ccRCC patients was analyzed by the Kaplan-Meier Plotter. Cell proliferation, colony growth, and cell invasion were examined by cell counting kit-8 assay, colony formation assay, and transwell invasion assay, respectively. The luciferase activity reporter assay and Western blot assay were conducted to validate the connection between miR-1294 and homeobox A6 (HOXA6).

RESULTS: MiR-1294 was downregulated in ccRCC cell lines and correlated with the poor overall survival of ccRCC patients. The overexpression of miR-1294 inhibits ccRCC cell proliferation, colony growth, and cell invasion. HOXA6 was validated as a target of miR-1294 and negatively regulated by miR-1294. The overexpression of HOXA6 attenuated the miR-1294-mediated effects on ccRCC cellular functions.

CONCLUSIONS: Our results indicated that miR-1294 functions as a tumor suppressor in ccRCC. MiR-1294 suppressed cell proliferation, colony formation, and invasion in ccRCC partially via targeting HOXA6.

Key Words

MiR-1294, HOXA6, Clear cell renal cell carcinoma, Proliferation, Prognosis.

Introduction

Clear cell renal cell carcinoma (ccRCC), represents 70-80% of all renal cell carcinoma cases and is characterized by high morbidity and poor prognosis[1]. The conduction of surgical resection has a good treatment effect for local ccRCC[2]. However, the treatment measures for advanced ccRCC remain limited, due to the poor understanding of the detailed mechanisms behind the carcinogenesis of ccRCC[3].

MicroRNAs (miRNAs) were non-coding RNAs and firstly identified in *Caenorhabditis elegans* in 1993 at the length of 19 to 24 nucleotides[4]. MiRNAs were wildly recognized as crucial modulators to regulate multiple cellular bioprocesses mainly through complementary binding with the 3'-untranslated region (3'-UTR) of targeted genes[5,6]. In addition, miRNAs were shown to have dual roles in the carcinogenesis of human cancers to function as tumor suppressive or oncogenic miRNA at a cancer-specific manner[7,8].

Abnormal expression of miR-1294 has been found in various human cancers including glioma, oral squamous cell carcinoma, ovarian cancer, gastric cancer, and osteosarcoma[9-13]. MiR-1294 was found to reduce the expression in glioma and promote the chemosensitivity of glioma cells to temozolomide by regulating the expression targeting protein for Xenopus kinesin-like protein 2[9]. Besides that, the growth of oral squamous cell carcinoma cell could be inhibited by miR-1294[10]. In addition, miR-1294 was shown to regulate the response of ovarian cancer cell to cisplatin via regulating IGF1R[11]. Moreover, it was found that miR-1294 was associated with the prognosis of patients with gastric cancer and osteosarcoma, which highlighted the importance of miR-1294 in cancers[12,13]. However, we still did not understand the role of miR-1294 in ccRCC until now.

A total of 39 Homeobox (HOX) genes located at chromosomes 7p15, 17q21.2, 12q13, and 2q31 have been identified in human to date[14]. These genes were further classified into four sub-families, named HOXA, HOXB, HOXC, and HOXD[14]. Of note, these genes were found to be involved with multiple cell behaviors and have a role in tumor pathogenesis and progression[14,15]. Recently,

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HOXA9, a member of the HOX family, was identified as a direct target of miR-1294[13]. Therefore, it is reasonable to suspect that miR-1294 may have a connection with HOXA6.

In this work, we analyzed the expression level of miR-1294 in ccRCC cell lines using quantified real time-polymerase chain reaction (qRT-PCR). The effect of the miR-1294 expression on the overall survival of ccRCC patients was analyzed by the Kaplan-Meier Plotter website. The association of miR-1294 and HOXA6 was validated by luciferase activity reporter assay and Western blot assay. The effects of miR-1294 or HOXA6 expression on cell proliferation, colony growth, and cell invasion were examined by cell counting kit-8 assay, colony formation assay, and transwell invasion assay, respectively.

Materials and Methods

Cell Lines and Cell Culture

Human ccRCC cell lines (Caki-1 and Caki-2) and normal human renal tubular epithelial cell (HK-2) purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin at a humidified 37°C incubator 5% of CO_2.

Overexpression of MiR-1294 and HOXA6 in ccRCC Cell Lines

To manipulate the expression of miR-1294, the selected ccRCC cell lines were transfected with miR-1294 mimic and negative control (miR-NC) purchased from Sangon Biotechnology Co., Ltd (Shanghai, China). In addition, the pcDNA3.1 expression vector containing the open reading frame of HOXA6 (pcHOXA6) obtained from GenScript (Nanjing, China) was employed to regulate the expression of HOXA6. The lipofectamine 2000 (Invitrogen) was used for the synthetic miRNAs or expression vectors transfection according to the manufacturer's instructions.

Isolation of Total RNA and qRT-PCR

TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to isolate total RNA from cultured cells according to the supplier's protocol. First-strand complementary DNA was synthesized from the extracted RNA sample using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). The qRT-PCR was conducted using SYBR Green Mix (TaKaRa, Dalian, China) at ABI7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following procedure: 1 cycle of 94°C for 5 min; 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, and 1 cycle of 72°C for 10 min. U6 small nuclear RNA (U6 snRNA) was used as an endogenous control to normalize the expression level of miR-1294. The primer sequences for miR-1294 were forward: 5'-TATGATCTCACCGAGTCCT-3', reverse: 5'-CACCTTCCTAATCCTCAGTT-3', U6 snRNA were forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Isolation of Total Protein and Western Blot

The cells were collected and lysed in Radioimmunoprecipitation Assay buffer plus protease inhibitors and phosphatase inhibitors (Beyotime, Haimen, Jiangsu, China). After quantified by bicinchoninic acid (BCA) kit (Beyotime, Haimen, Jiangsu, China), an equal amount of protein sample was separated at 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with the corresponding primary antibodies (anti-HOXA6: ab74064, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH): ab181602; Abcam, Cambridge, MA, USA) at 4°C for overnight following fat-free milk incubation. After that, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (ab6721; Abcam, Cambridge, MA, USA) at 37°C for 4 h. The protein bands were developed by BeyoECL Star (Beyotime, Haimen, Jiangsu, China) according to the manufacturer's protocol.

Cell Counting Kit-8 (CCK-8) Assay

The cell proliferation was measured with CCK-8 assay. The cells were seeded into 96-well plate at the density of 3×10^3 cells/well. At indicated time points, CCK-8 reagent (Beyotime, Haimen, Jiangsu, China) was added to the plate and incubated for another 4 h. Then, the optical density at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Colony Formation Assay

Approximately 5,000 cells were seeded in 6-well plates filled with serum-free medium. After incubation for 2 weeks, the cells were washed with phosphate buffered solution, fixed with 4% paraformaldehyde, and then stained with 0.1% crystal violet. The number of colonies generated was counted using Image J 1.42 software (National Institutes of Health, Bethesda, MD, USA).

Transwell Invasion Assay

Cell invasive capability was measured using the transwell invasion assay. The insert was coated with the matrigel (BD Biosciences, San Jose, CA, USA). The cells were seeded in the upper chamber filled with serum-free DMEM, while the bottom chamber was filled with DMEM containing 10% FBS. After incubation for 24 h, the invasive cells were fixed by 4% paraformaldehyde and stained with 0.1% crystal violet.

Prediction of MiR-1294 Targets

The potential targets of miR-1294 were predicted and analyzed using the online bioinformatics algorithms including TargetScan and miRDB. The results indicated that HOXA6 contains a binding site for miR-1294 in its 3'-UTR.

Dual-Luciferase Activity Reporter Assay

The wild-type HOXA6 3'-UTR or the mutant HOXA6 3'-UTR containing predicted binding sites of miR-1294 were cloned into pGL3 vector (Promega, Madison, WI, USA) and named as wt-HOXA6 and mt-HOXA6. The cells were co-transfected with wt-HOXA6 or mt-HOXA6, and miR-1294 mimic or NC-miR using Lipofectamine 2000. After 48 h of transfection, the luciferase activity was measured using a Dual-luciferase assay system (Promega) according to the manufacturer's instructions.

Kaplan-Meier Survival Analysis

The Kaplan-Meier plotter (www.kmplot.com) was used to assess the effect of the miR-1294 expression on the overall survival of ccRCC patients. The cut-off value was auto-selected in the algorithm.

Statistical Analysis

The data were presented as mean ± Standard Deviation and analyzed at SPSS 17 software (SPSS Inc., Chicago, IL, USA). The one-way ANOVA followed by Tukey's post-hoc test was used to measure the difference among multiple groups, while the Student's *t*-test was used to assess the difference between the two groups. A log-rank test was used to calculate the survival difference between low or high expression groups. $p<0.05$ was considered statistically significant.

Results

Low Expression of MiR-1294 in ccRCC

First, we detected the expression of miR-1294 in ccRCC cell lines using RT-qPCR. We found that miR-1294 expression was markedly downregulated in ccRCC cell lines (Caki-1 and Caki-2) compared with normal human renal tubular epithelial cell (HK-2) (Figure 1A). In

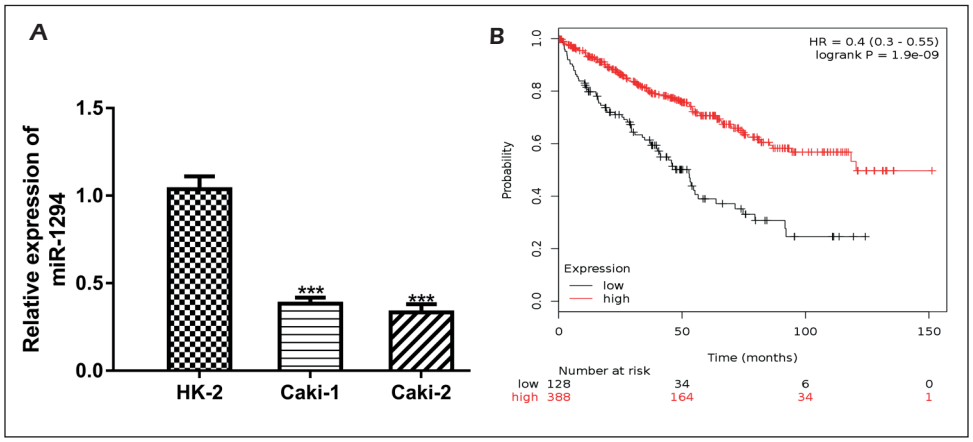

Figure 1. Downregulation of miR-1294 in ccRCC. **A**, miR-1294 expression level in ccRCC cell lines (Caki-1 and Caki-2) and normal human renal tubular epithelial cell (HK-2). **B**, Low miR-1294 expression level predicts poor overall survival of ccRCC patients. ***$p<0.001$. miR-1294: microRNA-1294; ccRCC: clear cell renal cell carcinoma.

addition, the Kaplan-Meier survival curve analyzed through the KM Plotter website indicated that patients with low miR-1294 expression tend to have a poorer overall survival compared to those with high miR-1294 expression (Figure 1B; p = 1.9e-09). Together, our findings revealed that miR-1294 expression was downregulated in ccRCC.

Overexpression of MiR-1294 Inhibits ccRCC Cell Proliferation, Colony Formation, and Invasion

Then, we investigated the effects of miR-1294 expression on ccRCC cell behaviors. We introduced synthetic miRNAs into ccRCC cell lines, and we found that miR-1294 level was significantly elevated by miR-1294 mimic compared with NC-miR (Figure 2A). To examine the effect of miR-1294 on cell proliferation, CCK-8 assay and colony formation assay were conducted. CCK-8 assay revealed that the introduction of miR-1294 mimic impaired the proliferation rate when compared with NC-miR (Figure 2B). The inhibitory effect of miR-1294 on proliferation was further confirmed by the colony formation assay. The results indicated that the ectopic expression of miR-1294 impaired colony growth (Figure 2C). Transwell invasion assay showed that the overexpression of miR-1294 inhibited cellular invasion (Figure 2D). Collectively, these results demonstrated the inhibitory effects of miR-1294 on ccRCC cell behaviors.

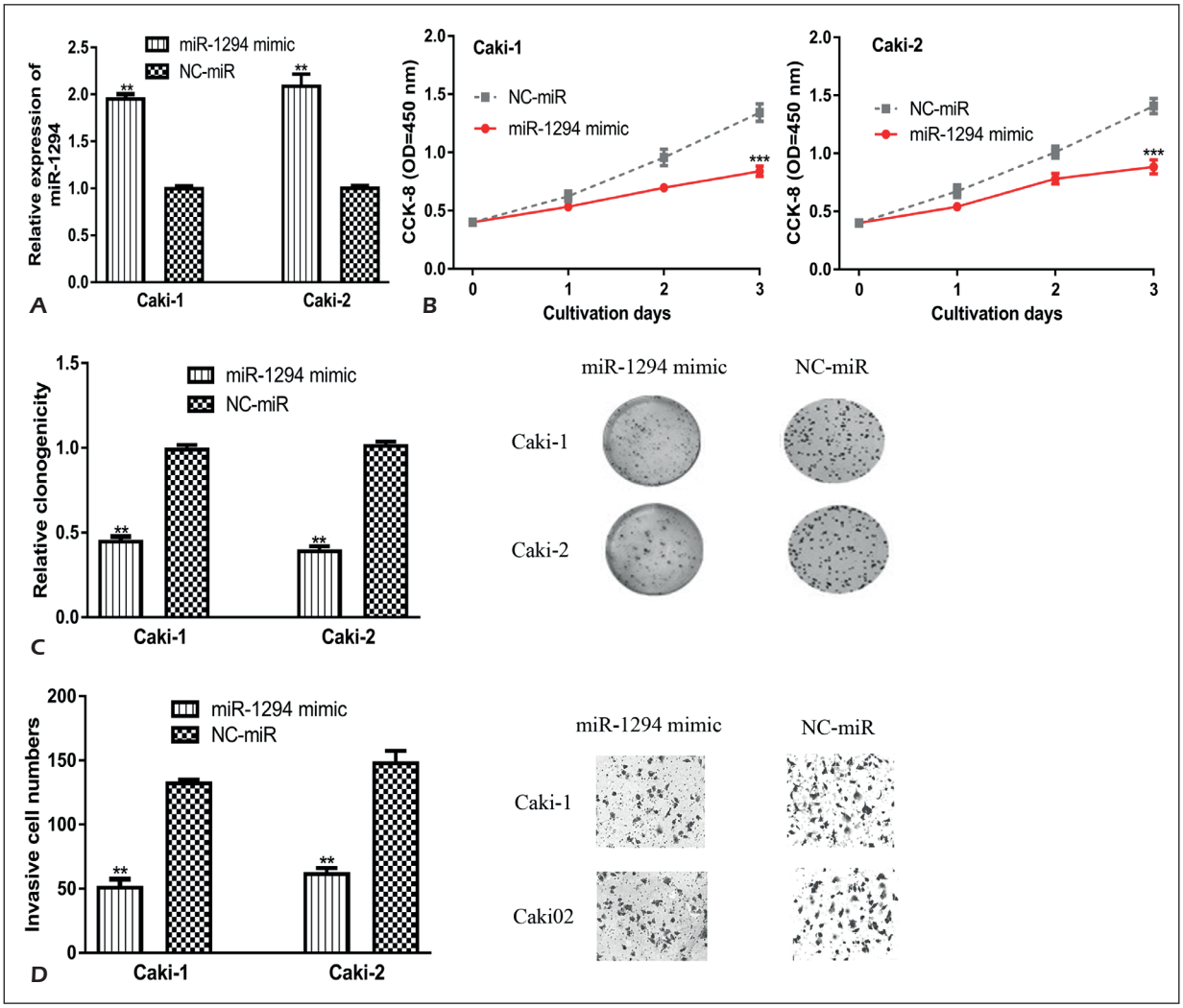

Figure 2. Overexpression of miR-1294 inhibits ccRCC cell proliferation, colony formation, and cell invasion. **A**, miR-1294 expression; **B**, cell proliferation; **C**, colony formation, and (**D**) cell invasion in ccRCC cells transfected with miR-1294 mimic or NC-miR. Magnification 200x. **p<0.01, ***p<0.001. miR-1294: microRNA-1294; ccRCC: clear cell renal cell carcinoma; NC-miR: negative control miRNA.

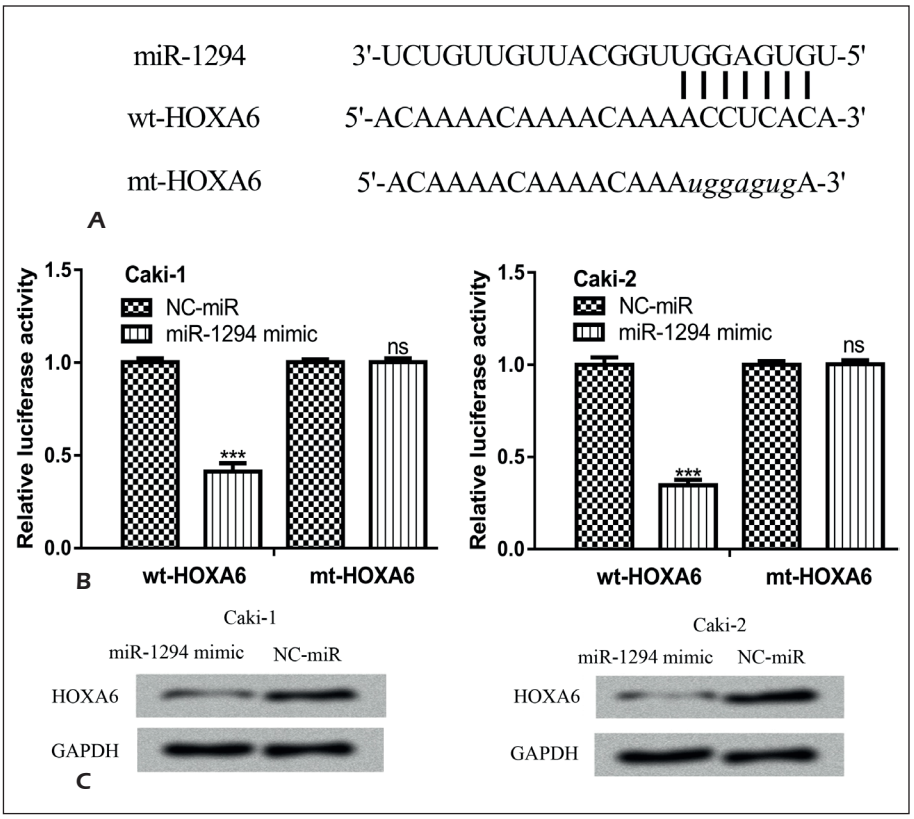

Figure 3. HOXA6 was a direct target of miR-1294. **A**, Binding site between miR-1294 and the 3'-UTR of HOXA6. **B**, Relative luciferase activity of cells transfected with luciferase activity reporter vector and synthetic miRNAs. **C**, HOXA6 expression in cells transfected with synthetic miRNAs. ns not significant, ***$p<0.001$. miR-1294: microRNA-1294; UTR: untranslated region; wt: wild-type; mt: mutant; NC-miR: negative control miRNA; HOXA6: homeobox A6.

HOXA6 Was a Direct Target of MiR-1294

The bioinformatic algorithms suggested that HOXA6 could bind to miR-1294 (Figure 3A). This prediction was validated by the Dual-Luciferase Activity Reporter Assay. As shown in Figure 3B, the transfection of miR-1294 mimic inhibited the luciferase activity of cells transfected with wt-HOXA6. However, miR-1294 mimic did not affect the luciferase activity of cells transfected with mt-HOXA6 (Figure 3B). The Western blot assay showed that miR-1294 mimic transfection decreased HOXA6 expression compared with NC-miR (Figure 3C). These results suggested that HOXA6 was a direct target of miR-1294.

HOXA6 Was a Functional Downstream Target in the MiR-1294-Mediated ccRCC Cell Behaviors Inhibition

To elucidate whether miR-1294 regulates ccRCC cell behaviors by targeting HOXA6, a rescue experiment was performed. Western blot showed that HOXA6 expression was significantly upregulated by pcHOXA6 transfection (Figure 4A). The overexpression of HOXA6 increased cell proliferation, colony formation, and cell invasion (Figure 4B-4D). HOXA6 overexpression partially reversed the effects of miR-1294 on these cell behaviors (Figure 4B-4D). These results suggested that HOXA6 was a functional target of miR-1294.

Discussion

The dysregulation of miRNAs and mRNAs can trigger the abnormal status of multiple biological processes to contribute to the carcinogenesis of human cancers[16]. Since miRNAs exert their effects by regulating the expression of mRNAs, it is reasonable to deduct that miRNAs has a crucial role in the tumorigenesis of human cancers[17].

In the current study, miR-1294 expression was revealed to be downregulated in ccRCC cell lines compared with the normal cell line. In addition, low miR-1294 expression was found as a predictor for the poor prognosis of ccRCC patients. These results indicated that miR-1294 functions as a tumor suppressive miRNA in the progression of ccRCC, which is the same as its role in glioma, oral squamous cell carcinoma, ovarian cancer,

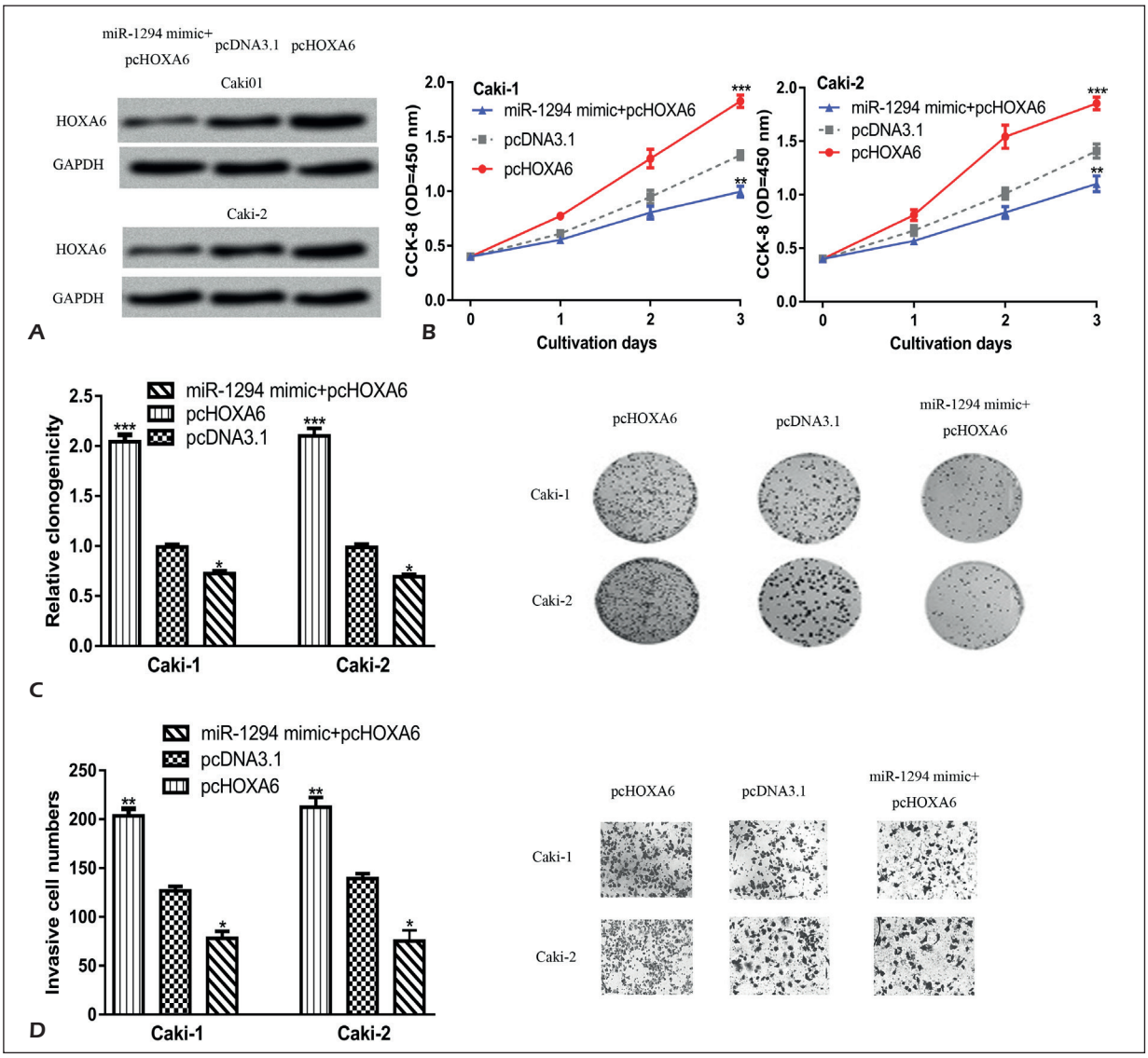

Figure 4. miR-1294 regulates ccRCC cell behaviors by targeting HOXA6. **A**, HOXA6 expression; **B**, cell proliferation; **C**, colony formation, and (**D**) cell invasion in ccRCC cells transfected with pcHOXA6, pcDNA3.1, or pcHOXA6 and miR-1294 mimic. Magnification 200x. *p<0.05, **p<0.01, ***p<0.001. miR-1294: microRNA-1294; HOXA6: homeobox A6; ccRCC: clear cell renal cell carcinoma.

gastric cancer, and osteosarcoma[9-13]. Then, CCK-8 assay, colony formation assay, and transwell invasion assay were conducted to investigate the biological role of miR-1294 in ccRCC. CCK-8 assay indicated the introduction of miR-1294 mimic impaired cell proliferation rate. The colony formation assay confirmed the results of the CCK-8 assay. The transwell invasion assay revealed that miR-1294 overexpression could inhibit cell invasion. Collectively, these results indicated that the inhibitory effect of miR-1294 on ccRCC cell behaviors *in vitro* and overexpression of miR-1294 hindered cancer progression.

Multiple targets of miR-1294 have been identified in human cancers, including targeting protein for Xenopus kinesin-like protein 2, c-Myc, IGF1R, and HOXA9[9-11,13]. Here, the bioinformatics algorithms showed that HOXA6 was also a putative target of miR-1294. HOXA6 was reported to be upregulated in cancers including cervical cancer, colorectal cancer, and non-small cell lung cancer[18-20]. The overexpression of HOXA6 was shown to promote malignancy behaviors of cancer cell and to be correlated with the poor survival outcome of cancer patients[18-20]. The luciferase activity reporter assay and Western blot

assay together confirmed this prediction. To directly address whether the effects of miR-1294 in regulating ccRCC cell behaviors can be attributed to its regulation of HOXA6, rescue experiments were performed. It was found that the overexpression of HOXA6 resulted in increased proliferation rate and invasive ability compared with the pcDNA3.1 group. HOXA6 overexpression decreased the miR-1294-mediated suppression on cell proliferation and invasion.

Conclusions

The current study provided evidence that miR-1294 functions as a tumor suppressor by negatively regulating HOXA6 to suppress ccRCC cell proliferation, colony formation, and cell invasion, and thereby may be developed as a novel therapeutic target for ccRCC treatment.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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