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ORIGINAL ARTICLE

MiR-384 represses tumorigenesis by regulating CDK6 and predicts prognosis of clear cell renal cell carcinoma

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Summary

Purpose: *Clear cell renal cell carcinoma (ccRCC) accounts for 70-80% of renal cell carcinomas. Various microRNAs (miRs) have been reported to affect the tumorigenesis of ccRCC. However, the role of miR-384 in ccRCC is still unknown. Thus, the purpose of this study was to investigate the function of miR-384 in ccRCC.*

Methods: *qRT-PCR or Western blot were employed to examine the expressions of miR-384 and CDK6 in ccRCC tissues or cell lines. MTT and transwell assays were applied to measure cell proliferation and motility. The survival rate was analyzed by Kaplan–Meier method accompanied with log-rank test. Dual luciferase assay was used to verify the relationship among miR-384 and CDK6 in ccRCC cells.*

Results: *MiR-384 was downregulated in ccRCC tissues and cell lines, and its overexpression inhibited cell proliferation and motility in ccRCC. In addition, miR¬384 was found to be a marker for good prognosis in ccRCC. Furthermore, CDK6 was confirmed to be directly targeted by miR-384, and upregulation of CDK6 restored the inhibitory effects of miR-384 in ccRCC.*

Conclusion: *MiR-384 repressed tumorigenesis by regulating CDK6 in ccRCC and predicted good prognosis for ccRCC.*

Key words: *CDK6, clear cell renal cell carcinoma, miR-384, prognosis, proliferation*

Introduction

Renal cell carcinoma (RCC) is a malignant tumor originating from the renal parenchymal epithelial system, accounting for about 2-3% of adult malignant tumors and 80-90% of renal malignancies in adults [1]. Most of them are ccRCC, accounting for 70-80% of renal cancers [2]. Early ccRCC is asymptomatic, or has only general systemic symptoms such as fever and fatigue which can not be attributed to the disease until the tumor volume increases [3]. Nowadays, surgical treatment of RCC is still the preferred treatment method which can be curative [4]. The 5-year survival rates of patients with RCC were 92, 86, 64 and 23%, at I, II, III and IV stages, respectively. Immunotherapy and chemotherapy can prolong the life of patients with RCC [5], however, its prognosis still remains unknown.

In recent years, an increasing number of miRs has been observed to participate in the occurrence and formation of human tumors [6]. For instance, miR-17-5p functions as a tumor suppressor in breast cancer [7]. MiR-182 was associated with growth, migration and invasion in prostate cancer via suppression of FOXO1 [8]. In RCC, miR-429 and miR-630 were found to be associated with metastasis and prognosis [9,10]. As for ccRCC, miR-30e and miR-514a were found to be downregulated [11,12] and upregulation of miR-93 and miR-144 was identified in ccRCC [13,14]. Among them, miR-384 has been identified as a tumor suppressor in

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non-small-cell lung cancer [15], colorectal cancer [16], and hepatocellular carcinoma [17]. However, almost no research of miR-384 was reported in ccRCC as far as we know.

Cyclin-dependent kinase 6 (CDK6), belonging to the family of serine-threonine kinases, is involved in the G1/S cell cycle transition [18] and abnormal expression of CDK6 has been found in osteosarcoma [19], colorectal adenoma [20] and ovarian cancer [21], indicating that CDK6 also regulated the development of human cancers like miRNAs. Moreover, upregulation of CDK6 was confirmed to be related with poor prognosis of bladder cancer [22]. However, the function of CDK6 in ccRCC is still fuzzy.

In this study, miR-384 level was detected and the relationship among miR-384 and prognosis was also investigated in ccRCC. Moreover, the function of miR-384 regulated by CDK6 was explored. We maintain that the findings of our study would lead to the development of novel pathways for the diagnosis and treatment of ccRCC.

Methods

Ethics approval and consent to participate

This study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University. Signed informed consents were obtained from the patients and/or their guardians.

Cancer specimens

Fifty-three paired surgical ccRCC specimens and adjacent tissue samples were obtained from the Department of Pathology, China-Japan Union Hospital of Jilin University after receiving signed informed consent from the ccRCC patients. No patient with ccRCC received treatment prior to the operation. Then, these tissues were frozen in liquid nitrogen and stored in -80°C until assessed. Tumor specimens were divided based on the 2002 TMN System and the 2004 WHO classification.

Cell culture and transfection

The 786O, A498, HK-2 and HEK293T cell lines were used for this study. The 786O, A498 and HEK293T cell lines were procured from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Human normal kidney proximal tubule epithelial cell line HK-2 was purchased from the American Type Culture Collection (ATCC, USA). All cells were seeded in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Shanghai, China) with 10% fetal bovine serum (FBS) and cultured at 37°C with 5% CO_2.

The miR-384 mimic and inhibitor, CDK6 siRNA (si-CDK6) were purchased from Genepharma (Shanghai, China) and then they were transferred into 786O ccRCC cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

Quantitative RT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for extracting total RNA containing miR to quantitate miR-384 expression in ccRCC tissues and cell lines. Quantitative RT-PCR was carried out through the SYBR Green PCR Master Mix (Applied Biosystems) on ABI 7500 Fast Real-Time PCR system (Applied Biosystems). U6 and GAPDH were used as control for miR-384 and CDK6, and the expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Dual luciferase assay

The wild or mutant type of 3'-UTR of CDK6 was inserted into the pmiRGLO luciferase vector (Promega, Madison, WI, USA) for luciferase reporter experiments. Then, wild or mutant type of 3'-UTR of CDK6 and miR-384 mimic were transfected into HEK293T cells. Subsequently, the Dual Luciferase Assay System (Promega, Beijing, China) was applied to analyze luciferase activity.

MTT assay for cell proliferation

The MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) assay was applied to measure cell proliferation. Cells (4×10^3/well) were seeded onto 96-well plates in RPMI 1640 medium. The cells containing miR-384 mimic or inhibitor were incubated for 1 24, 48, 72 and 96 hrs. After incubation, MMT was added to the cells (Sigma, Shanghai, China) which were incubated for 4 hrs at 37°C. The absorbance at 490 nm (optical density/OD = 490 nm) was detected with a spectrophotometer.

Transwell assays for cell migration and invasion

Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA) were used to evaluate the migratory and invasive ability of ccRCC cells in 24-well plates. 5×10^4 ccRCC cells without serum were put in the upper chamber on the non-coated membrane, and the lower chamber was filled with 10% FBS to induce ccRCC cells to migrate or invade through the membrane. The cells were put also in the upper chamber with the coated membrane for invasion assay. Then, these cells were incubated for 48 hrs for migration and invasion evaluation and stained with 0.1% crystal violet. A microscope was used for counting migrated and invaded cells.

Western blot analysis

The protein samples were obtained using RIPA lysis buffer. Proteins were separated through a 10% SDS-PAGE and incubated with 5% non-fat milk in PVDF membranes at room temperature. Next, we incubated the membranes overnight at 4°C with rabbit monoclonal anti-CDK6 (ab151247; 1:2000; Abcam, Cambridge, MA, USA), rabbit monoclonal anti-GAPDH antibody (EPR16891; 1; 2000; Epitomics, Burlingame, CA, USA) and subsequently incubated with goat polyclonal anti-Rabbit IgG H&L secondary antibody (ab150077; 1:2000; Abcam, Cambridge, MA, USA). Then, protein expression levels were measured by ECL (ECL, Pierce, Shanghai, China).

Statistics

The data were analyzed by SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) and Graphpad Prism 6 (Graphpad Software Inc., San Diego, CA, USA). The associations among miR-384 level and clinical variables were analyzed by Pearson's x^2 test. The relationship between miR-384 level and the survival rate was performed by Kaplan-Meier method to figure out the percent probability of patients' disease-free survival (DFS) and overall survival (OS). Log-rank test was employed to evaluate the differences of between groups. Significant difference was defined as $p<0.05$.

Results

MiR-384 levels in ccRCC tissues and cell lines

Firstly, we detected the miR-384 levels in ccRCC and normal tissues via qRT-PCR. Lower expression of miR-384 was found in ccRCC tissues compared to normal tissues (Figure 1A). In addition, the association among miR-384 level and clinicopathological characteristics of patients was studied and showed significant correlation between miR-384 level and tumor stage (p=0.002), whereas no association was found between miR-384 level and other clinicopathological parameters (Table 1). Furthermore, miR-384 levels in 786O, A498 and HK-2 cell lines were evaluated through qRT-RCR. Similarly, decreased expression of miR-384 was found in 786O and A498 cell lines compared to HK-2 cells (Figure 1B). All the results suggested that miR-384 would be involved in the tumorigenesis of ccRCC.

MiR-384 overexpression inhibited cell proliferation and metastasis in ccRCC

Next, we transfected miR-384 mimic or inhibitor into 786O cells to investigate its function in ccRCC and the miR-384 level in these transfected cells was observed through qRT-PCR (Figure 2A). The MTT assay indicated that the cell proliferation was enhanced in cells with miR-384 mimic, while it was repressed in cells with miR-384 inhibitor (Figure 2B). Besides, the effect of miR-384 on cell metastasis in ccRCC was evaluated as well. The Transwell assay suggested that high miR-384 expression inhibited cell migration and the opposite function was observed with low miR-384 expression in ccRCC (Figure 2C). Concurrently, similar function of miR-384 was also found for cell invasion in ccRCC (Figure 2D). In brief, the capacities of cell proliferation and metastasis were impaired by miR-384.

MiR-384 was a marker for good prognosis in ccRCC

Kaplan-Meier survival showed longer DFS (p=0.0352) in patients with high miR-384 level compared to patients with low miR-384 level (Figure 3A). Similar tendency of miR-384 for OS

Table 1. Relationship between miR-384 levels and clinicopathological characteristics in ccRCC

Characteristics	*Number of cases (n=41)*	*miR-384*		*p value*
		High	*Low*	
Age (years)				0.491
≥ 61	22	7	15	
<61	19	3	16	
Gender				0.09
Male	24	10	14	
Female	17	8	9	
Tumor size (cm)				0.239
≥ 4	23	10	13	
<4	18	8	10	
Laterality				0.09
Left	24	10	14	
Right	17	7	10	
Tumor stage				0.002
I + II	27	7	20	
III + IV	15	6	9	
TNM stage				
I + II	22	10	12	0.491
III + IV	19	8	11	

Statistical analyses were performed by the x^2 test

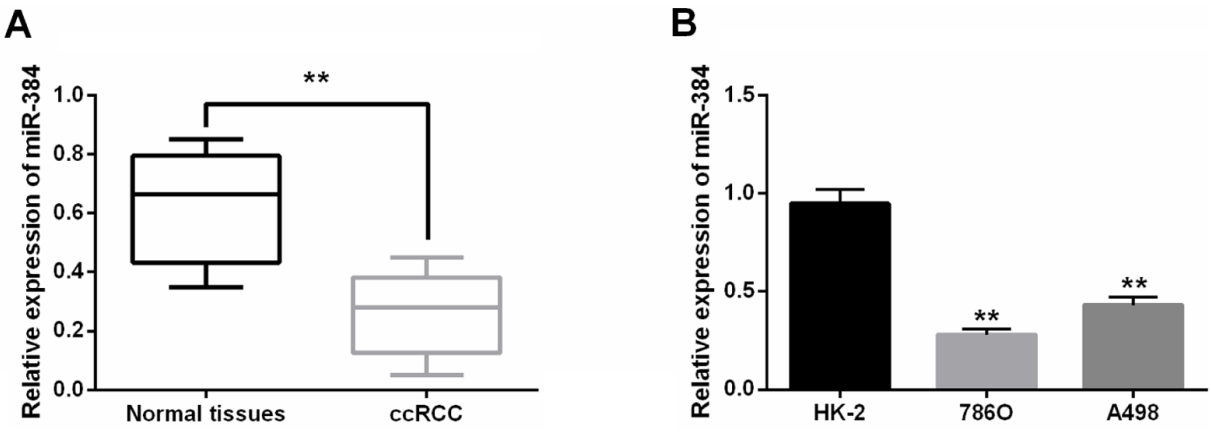

Figure 1. MiR-384 levels were examined in ccRCC tissues and cell lines. **A:** The expressions of miR-384 in ccRCC tissues and normal tissues as detected via qRT-PCR. **B:** The miR-384 expression in 786O, A498 and HK-2 cells. **p<0.01.

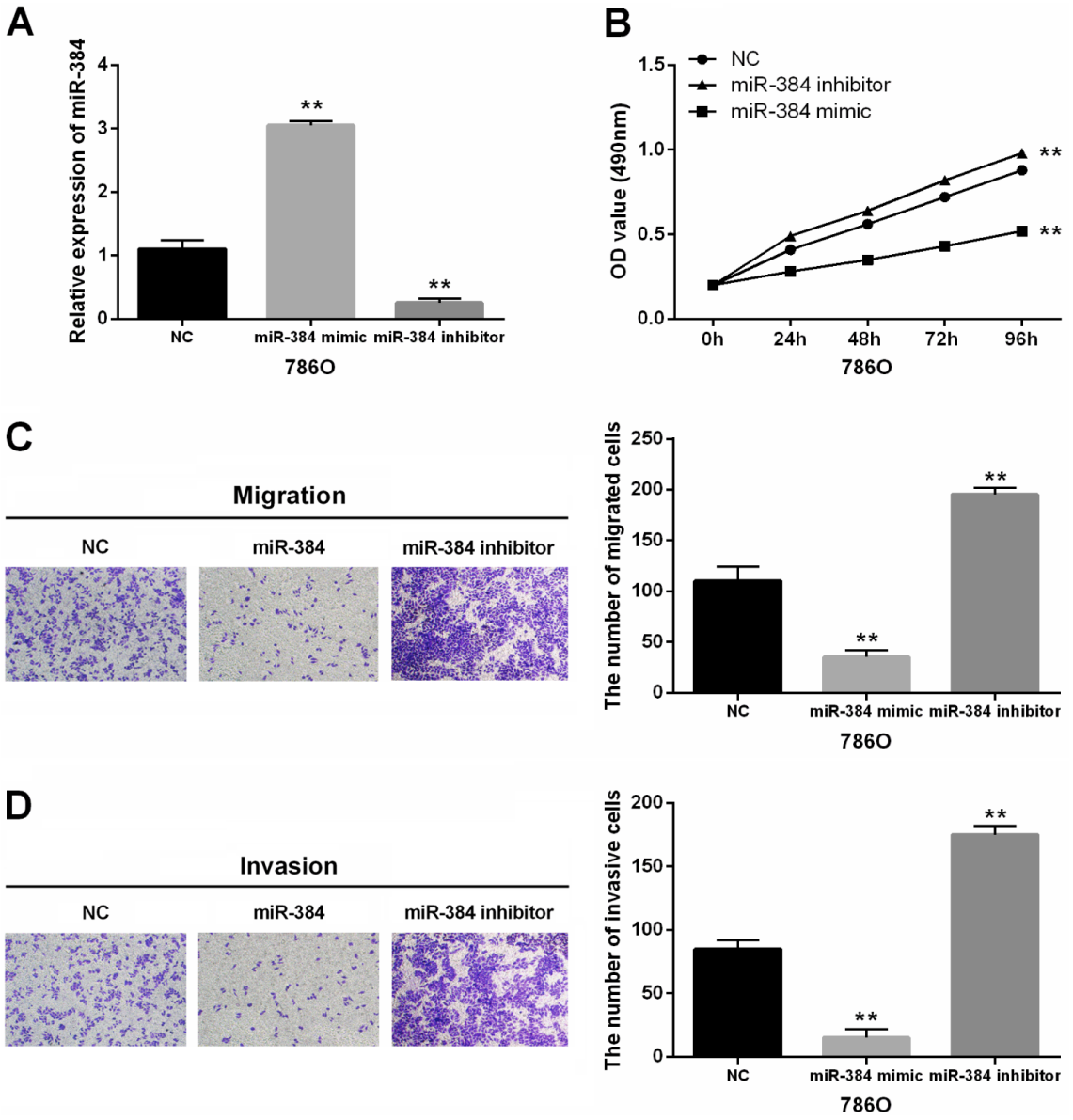

Figure 2. MiR-384 overexpression inhibited cell proliferation and metastasis in ccRCC. **A:** The expression of miR-384 was examined in 786O cells containing miR-384 mimics or inhibitor via qRT-PCR. **B:** The cell proliferation was measured in cells containing miR-384 mimics or inhibitor via MTT. **C,D:** The cell migration and invasion was measured in cells with miR-384 mimics or inhibitor via Transwell analysis. **p<0.01.

(p=0.0198) was also found in ccRCC (Figure 3B). Moreover, the survival rate was also studied in patient subgroups classified according to tumor size and stage. In patients with tumors >4 cm, longer DFS (p=0.0156) and OS (p=0.0248) were identified in patients with high miR-384 level than in patients with low miR-384 level (Figure 3C,3D). In general, miR-384 was a marker for good prognosis in ccRCC.

CDK6 was directly targeted by miR-384

Furthermore, CDK6 was found to be targeted by miR-384, predicted by TargetScanHuman (http://www.targetscan.org/vert_71/) and the binding site between miR-384 and wild type of CDK6 is shown in Figure 4A. We performed dual luciferase reporter assay to confirm the above prediction. The result indicated that wild type of CDK6 significantly reduced the luciferase activities in HEK-293T cells with miR-384. As expected, the mutant type of CDK6 did not impact the luciferase activities (Figure 4B). In addition, CDK6 levels in 786O, A498 and HK-2 cell lines were examined through qRT-RCR and increased expression of CDK6 was found in 786O and A498 cell lines than in HK-2 cells (Figure 4C). Moreover, we also observed the CDK6 level in 786O cells with miR-451 mimic or inhibitor to further verify their relationship. The qRT-PCR suggested that miR-384 mimic apparently blocked the expression of CDK6 and miR-384 inhibitor enhanced CDK6 level in 786O cells (Figure 4D). Western blot assay also showed the same trend which confirmed these results again (Figure 4E). Altogether, CDK6 was directly targeted by miR-384 and negatively correlated with miR-384.

Upregulation of CDK6 restored the inhibitory effects of miR-384 in ccRCC

Conclusively, to investigate how CDK6 regulated miR-384 in ccRCC, CDK6 vector and miR-384 were co-transfected into 786O cells. The miR-384 levels were examined by qRT-PCR and Western

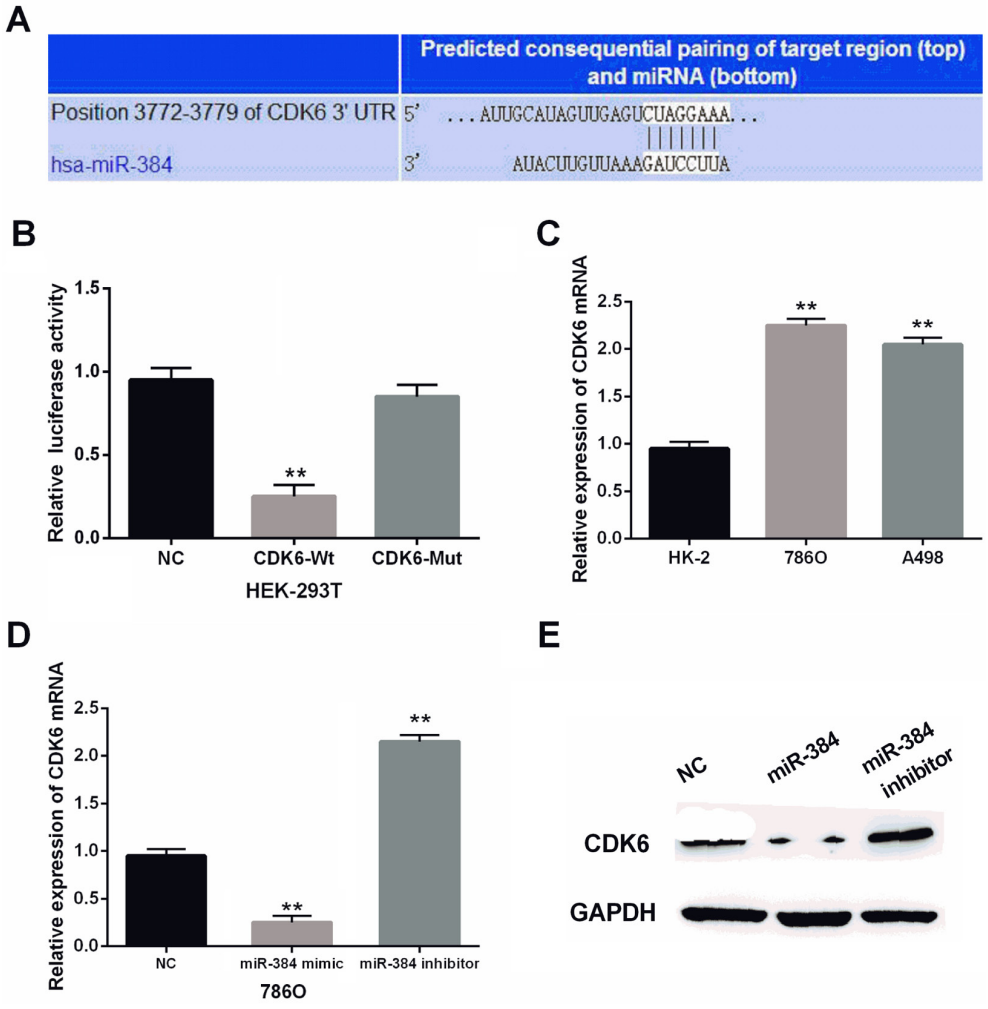

Figure 3. CDK6 was directly targeted by miR-384. **A:** The binding sites of miR-384 on the 3'-UTR of CDK6. **B:** Luciferase reporter assay. **C:** The CDK6 expression in 786O, A498 and HK-2 cells. **D,E:** The mRNA and protein expressions of CDK6 were analyzed in cells containing miR-384 mimics or inhibitor. **p<0.01.

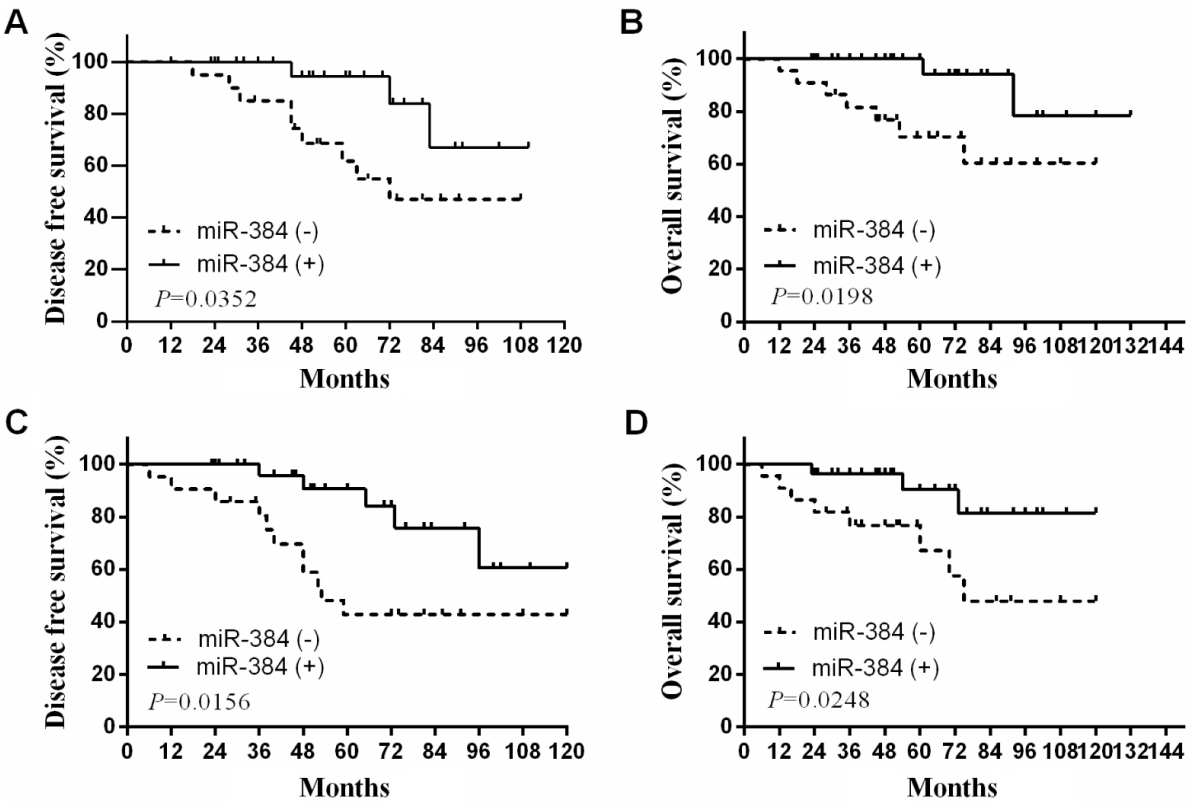

Figure 4. MiR-384 was a marker for good prognosis in ccRCC. **A,B:** miR-384 overexpression patients showed longer DFS and OS. **C,D:** In the subgroup of patients with tumor size >4 cm, patients with miR-384 overexpression showed longer DFS and OS.

blot (Figure 5A,5B). Functionally, the abilities of cell proliferation in cells containing miR-384 mimic and CDK6 vector were regained in comparison with the cells with only miR-384 mimic (Figure 5C). Similar tendency was also found for cell migration and invasion (Figure 5D,5E). In brief, upregulation of CDK6 restored the inhibitory effects of miR-384 in ccRCC.

Discussion

In current research, miR-384 was downregulated in ccRCC tissues and cell lines and miR-384 overexpression inhibited cell proliferation, migration and invasion in ccRCC. In addition, miR-384 was found to be a marker for good prognosis in ccRCC. Furthermore, CDK6 was confirmed to be directly targeted and negatively regulated by miR-384 and upregulation of CDK6 restored the inhibitory effects of miR-384 in ccRCC. Thus, miR-384 would function as a tumor suppressive miR through regulating CDK6 expression in ccRCC.

In the last few years, dysregulated expressions of many miRs have been observed to affect various bioprocesses included in the occurrence and formation of ccRCC tumors. For instance, downregulation of miR-1274a induced cell apoptosis of ccRCC by regulating BMPR1B [23]. MiR-182 suppressed ccRCC migration and invasion via regulating IGF1R [24]. And miR-138 attenuated EMT through suppressing SOX4 in ccRCC [25]. Moreover, miR-19a had been reported to correlate with poor prognosis of patients with ccRCC via promoting cell proliferation [26]. These studies indicated that miRs play a significant role in the development of ccRCC. Dysregulated expression of miR-384 had been identified to participate in tumorigenesis of many human cancers and diseases. For example, miR-384 overexpression repressed cell proliferation, migration and invasion in hepatocellular carcinoma [27] and was found to be related to lymph node metastasis, distant metastasis, and invasive depth of colorectal cancer [16]. Moreover, downregulation of miR-384 and its inhibitory effect on cell growth and invasion were identified in RCC through suppressing AEG-1 [28]. The same results were also found in ccRCC in our study. Besides that, we also examined the function of miR-384 for cell proliferation in ccRCC and found that it could predict good prognosis of ccRCC patients, which has not been reported in previous studies. In addition, the relationship between miR-384 and CDK6 in ccRCC has not been investigated in previous studies.

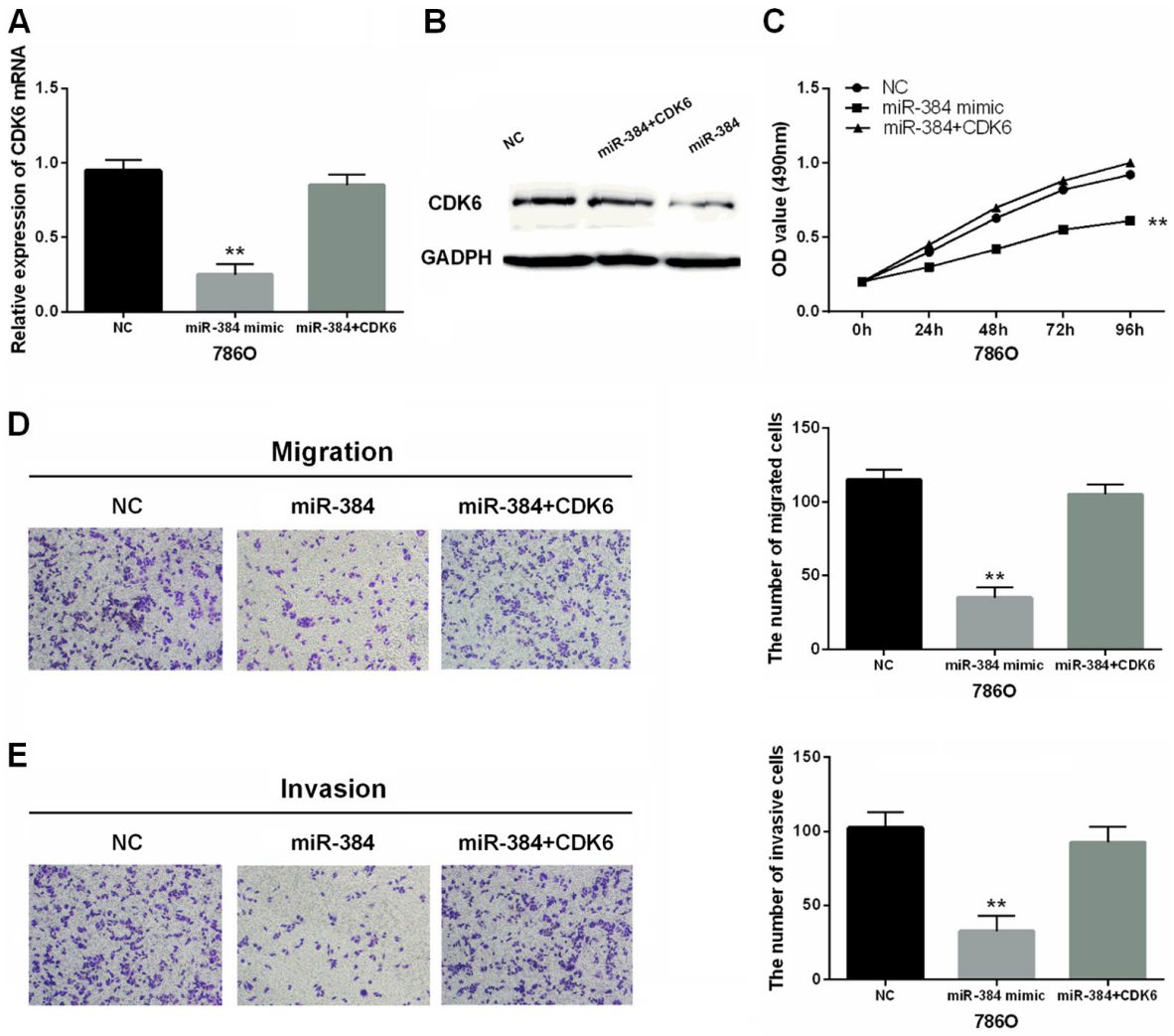

Figure 5. Upregulation of CDK6 restored the inhibitory effects of miR-384 in ccRCC. **A,B:** The mRNA and protein expressions of CDK6 were measured in cells containing CDK6 vector and miR-384. **C:** The cell proliferation in cells containing CDK6 vector and miR-384. **D,E:** The cell migration and invasion in cells containing CDK6 vector and miR-384. **p<0.01.

In this study, CDK6 was verified as a target gene of miR-384 which has an ability to affect cell cycle [29]. Moreover, the oncogenic effect of CDK6 had been detected in many human cancers. For instance, upregulation of CDK6 was found in non small cell lung cancer (NSCLC) which was similar to our result of CDK6 in ccRCC [30]. Also, Zhu et al. reported that CDK6 promoted cell proliferation of hepatocellular carcinoma, a result similar to the findings of this research [31]. Besides that, miR-186 was reported to inhibit cell proliferation and tumor growth via suppression of CDK6 [32]. Herein, we found that upregulation of CDK6 restored the inhibitory effects of miR-384 in ccRCC. As far as we know, this is the first time that miR-384 repressed tumorigenesis of ccRCC by regulating CDK6.

In conclusion, in the present study, downregulation of miR-384 was examined in ccRCC which was correlated with tumor stage. Additionally, high expression of miR-384 was related to longer DFS and OS. Moreover, miR-384 overexpression inhibited the cell proliferation and motility through suppressing CDK6 expression in ccRCC. In total, miR-384 repressed tumorigenesis of ccRCC by regulating CDK6 and predicted its prognosis.

Authors' contributions

GL contributed significantly to analysis and wrote the manuscript. WD performed the data analyses. ZZ, as the corresponding author, contributed to the conception of the study. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflict of interests.

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