

ORIGINAL ARTICLE

Expression of miR-410 in peripheral blood of patients with clear cell renal cell carcinoma and its effect on proliferation and invasion of Caki-2 cells

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Summary

Purpose: To explore the significance of miR-410 expression in clear cell renal cell carcinoma (CCRCC) and its biological function in CCRCC.

Methods: A total of 113 patients with CCRCC admitted to our hospital and 113 healthy individuals over the same period were enrolled. MiR-410 in the tissues and serum of patients with CCRCC was quantified, and the diagnostic value of miR-410 in CCRCC and the relationship between miR-410 and prognosis of patients with CCRCC were analyzed. In addition, miR-410 mimic and miR-410 inhibitor were adopted to regulate miR-410 in CCRCC cells (Caki-2), and then the changes in the proliferation, migration, invasion, and cell cycle of Caki-2 cells were determined. Moreover, tumorigenicity in nude mice was carried out to determine the effect of miR-410 on the tumor growth of CCRCC.

Results: MiR-410 was expressed at a high level in CCRCC patients, and had a high diagnostic accuracy [area under the curve (AUC) = 0.916]. In addition, miR-410 was an independent risk factor for the survival prognosis of patients with CCRCC, and its high expression indicated poor prognosis of the patients. Inhibiting miR-410 suppressed cell proliferation, cycle progression, migration, invasion and tumor growth in vivo and promoted cell apoptosis.

Conclusion: MiR-410 is a possible biological indicator for the diagnosis and prognosis of CCRCC, and is also an independent risk factor for the survival prognosis of CCRCC patients. In addition, miR-410 plays a role as an oncogene in CCRCC and promotes the malignant progression of CCRCC.

Key words: clear cell renal cell carcinoma, diagnosis, miR-410, prognosis, proliferation

Introduction

Clear cell renal cell carcinoma (CCRCC) is an aggressive histological subtype of renal cell carcinoma (RCC), accounting for approximate 3% of human tumors and 75% of renal tumors. There are more than 270,000 new CCRCC cases worldwide every year. About one third of patients with RCC have already metastases at the time of initial diagnosis, and the mortality of patients at stage IV is

close to 60% [1-5]. Currently, little is known about the pathogenesis of CCRCC and no sensitive tumor markers have been found. Therefore, a clearer explanation of the pathogenesis of CCRCC is of great value for pursuing new therapeutic targets to improve the prognosis of CCRCC patients.

MicroRNAs (miRs) play a crucial role in epigenetic regulation. They take part in the regulation of

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cell differentiation, proliferation, stress response, and other basic biological processes and signaling pathways, and they are also involved in the development of more than 300 diseases including tumors [6-8]. In recent years, it has been found that miR-410 is involved in the development of tumors. One study by Abedi et al [9] has revealed miR-410, was highly expressed in patients with colon cancer and correlated with the TNM staging of colon cancer, and it was an independent risk factor for the prognosis of patients with this disease. Additionally, studies have verified that miR-410 activates the wnt/ β -catenin signaling pathway and induces proliferation, migration, as well as invasion of colon cancer cells by targeting Dickkopf-related protein 1 [10,11]. MiR-410 is related to the chemotherapeutic efficacy in RCC according to recent studies. Gámez-Pozo et al [12] have found that miR-410 is related to prolonged response to sunitinib therapy, and Kovacova et al [13] have also found that miR-410 is related to the efficacy of sunitinib.

At present, there is no research report on the significance of miR-410 expression in CCRCC and its effect on the biological behaviors of CCRCC cells. Therefore, this study performed an analysis to seek for more molecular mechanisms linked to the development and progression of CCRCC.

Methods

Study participants

Altogether 113 patients with CCRCC treated in our hospital from December 2011 to December 2014 were enrolled, including 48 males and 65 females, with an average age of 57.2 years (range 35-78), and cancer tissues, paracancer tissues, and peripheral blood were sampled from each patient. All patients were diagnosed as CCRCC by postoperative pathology. In addition, 113 healthy individuals during the same period were enrolled, including 55 males and 58 females, with an average age of 53.6 years (range 35-78), and peripheral blood was sampled from each individual. The study was carried out with permission from the Ethics Committee of our hospital, and all enrolled participants signed informed consent forms.

Collection of general data about the patients

The data about sex, age, TNM staging, Fuhrman pathological grading, and 5-year survival prognosis of the patients were collected from their medical records, and analyzed using Excel (Table 1).

Collection of peripheral blood

Fasting peripheral blood was sampled from patients with CCRCC and healthy individuals in our hospital in the morning, and transferred for detection within 1 h after being collected. The sampled blood was centrifuged at 1000×g and 4°C for 30 min to take the serum, and the expression of miR-410 in the serum was quantified.

Extraction of total RNA in tissue/serum specimens and quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was obtained from tissues and serum with Trizol reagent (Invitrogen, USA), and miR was reversely transcribed with a miRcute miR first-strand cDNA kit (Tiangen, China). In addition, cDNA amplification was carried out using a one-step RT-PCR kit (TransGen, China). With U6 as an internal reference, the relative expression of miR-410 was calculated using the $2^{-\Delta Ct}$ method. All assays were conducted in triplicate, and each sample was determined three times.

Cell culturing

Human CCRCC cells (Caki-2) from American Type Culture Collection (ATCC) (ATCC®HTB-47™, USA) were incubated in McCoy's 5a Medium Modified (30-2007, USA) supplemented with 10% fetal bovine serum (FBS) (30-2020, USA) under 95% air and 5%CO₂ at 37°C.

Vector construction and transfection

MiR-410 mimic, mimic-NC, miR-410 inhibitor, and inhibitor-NC were designed and constructed by Thermo Fisher Scientific (USA), and transfected into cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according

Table 1. General patient data

| Patients with CCRCC | Clinical features n |
|------------------------------|------------------------|
| Sex | |
| Male | 48 |
| Female | 65 |
| Age (years) | 57.2±6.3 |
| Tumor size (cm) | 6.1±2.2 |
| T stage | |
| T1+T2 | 84 |
| T3+T4 | 29 |
| N stage | |
| N0+NX | 104 |
| N1 | 9 |
| M stage | |
| M0+MX | 89 |
| M1 | 24 |
| TNM staging | |
| I+II | 81 |
| III+IV | 32 |
| Fuhrman pathological grading | |
| G1-G2 | 58 |
| G3-G4 | 55 |
| Recurrence (within 5 years) | |
| Yes | 23 |
| No | 90 |
| 5-year survival rate | |
| Surviving | 56 |
| Dead | 57 |

to kit instructions. After 48 h of transfection, the transfection results were quantified using qRT-PCR assay.

Cell proliferation assay

The proliferation ability of cells was detected through the MTT method using a MTT assay kit (Sigma-Aldrich, Germany) as follows: 1000 cells were transferred to 96-well plates, and added with 100 μ L MTT solution (0.5 mg/ml) at different time points (0, 12, 24, 36, 48, and 72 h), followed by 4-h incubation at 37°C. Afterwards, the cells were centrifuged at 1000 \times g in room temperature for 10 min, and added with 100 μ l 0.04 mol/l HCl-isopropanol. Finally, the optical density of cells at 490 nm was detected by a microplate reader (BioTek Instruments, Beijing, China). The assay was repeated three times.

Wound healing assay

Cells were transferred to a 10% fetal bovine serum (FBS) solution. After monolayer cells were fused and formed, a 200 μ L sterile pipette was used to scratch the cell layer. Subsequently, the culture medium was replaced. The wound healing was analyzed with a light microscope (\times 100) at 0 and 12 h after the scratching, and wound photos were taken. Migration index=scratch width/the initial width of the same scratch. The experiment was conducted in triplicate.

Cell invasion assay

The invasion ability of cells was determined using the Transwell method. Matrigel (30 μ L, BD Biosciences, USA) was fully mixed into 150 μ L RPMI-1640 serum-free medium at 37°C. Altogether, 1×10^5 cells were resuspended in 100 μ L serum-free medium, and then transferred to the upper compartment of the Transwell chamber (8 μ m; Corning Incorporated, USA) and 500 μ L RPMI-1640 supplemented with 10% FBS were placed into the lower compartment. The chamber was cultured at 37°C, and 24 h later the cells were immobilized, and dyed with methanol and crystal violet. The assay was repeated in triplicate.

Cell cycle and apoptosis assays

Cells were immobilized in 70% ethanol overnight, and then cultured with RNase (50 μ g/mL) and propidium iodide (PI) (50 μ g/mL) (BD, San Jose, CA, USA) for 30 min. The cell cycle was analyzed using a FACScan flow cytometer (BD, USA) and CELL Quest 3.0 software (BD) data analysis system. In addition, the cell apoptosis was determined using Annexin V-FITC apoptosis assay kit (Beyotime, China) as follows: Cells were resuspended with PBS, and then cultured with Annexin V binding buffer, followed by double staining through Annexin-V/FITC (5 μ L) and PI (10 μ L). Subsequently, the cells were cultured with dark surroundings for 15 min, and the cell apoptosis was determined by a FACSCalibur cytometer.

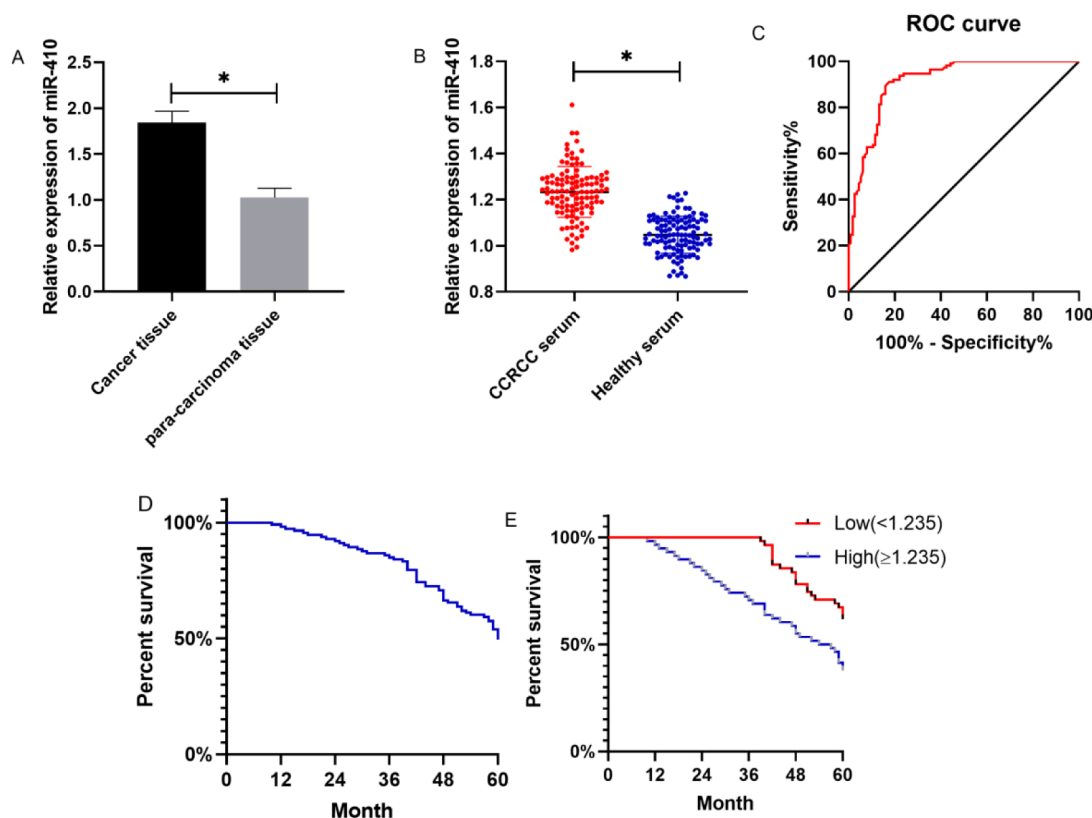


Figure 1. Expression of miR-410 in patients with CCRCC. **A:** Expression of miR-410 in tissues. **B:** Expression of miR-410 in serum. **C:** Diagnostic value of miR-410 for CCRCC. **D:** Overall 5-year survival rate of patients with CCRCC. **E:** Relationship between miR-410 and 5-year survival rate of patients with CCRCC. The 5-year survival rate of patients with high miR-410 expression was lower than that of patients with low miR-410 expression (*p<0.05).

Tumorigenicity in nude mice

A total of 5×10^6 Caki-2 cells were subcutaneously seeded into the left armpit of male thymus-free BALB/c nude mice (Beijing Hfk Bioscience, Beijing, China), and normal saline was used as control. Tumor size of each mouse was measured every 3 days, and all nude mice were euthanized after 4 weeks, and their tumors were removed. Tumor volume (mm^3) = long diameter (mm) \times short diameter (mm)²/2; tumor growth rate = tumor volume (mm^3)/ tumor growth time (s).

Statistics

SPSS 17.0 software (SPSS, Inc., USA) was adopted for statistical analyses. Data were presented as mean \pm standard deviation (SD). Enumeration data were compared using χ^2 test, and measurement data were compared between groups using t-test, and compared among multiple groups using one-way ANOVA. *Post hoc* test was carried out using the least significant difference (LSD) test. In addition, receiver operating characteristic (ROC) curves were used for analysis of diagnostic value, and Cox proportional hazards model was used for analy-

sis of risk factors affecting the prognosis of patients with CCRCC. $P < 0.05$ suggested a remarkable difference.

Results

Expression of miR-410 in CCRCC patients

The qRT-PCR assay revealed that the expression of miR-410 in CCRCC cancer tissues was higher than that in corresponding paracancer tissues, and the expression of serum miR-410 in CCRCC patients was also higher than that in healthy individuals. According to the ROC analysis, the area under the curve (AUC), sensitivity, specificity, 95%CI, and diagnostic level of serum miR-410 for diagnosing CCRCC were 0.916%, 90.27%, 83.19%, 0.880-0.952, and 1.142, respectively. With the median value of miR-410 expression (1.235) as the critical point, CCRCC patients were divided into high and low expression groups. According to the Kaplan-Meier curve and log-rank test, patients with high miR-410 expression showed

Table 2. Correlation between miR-410 and clinical features of CCRCC

| Patients with CCRCC | Clinical features | Low (<1.235) n (%) | High (\geq 1.235) n (%) | χ^2 | p value |
|------------------------------|-------------------|-----------------------|-------------------------------|----------|---------|
| Sex | | | | 2.571 | 0.109 |
| Male | 48 | 28 (50.00) | 20 (35.09) | | |
| Female | 65 | 28 (50.00) | 37 (64.91) | | |
| Age (years) | | | | 1.505 | 0.220 |
| <55 | 46 | 26 (46.43) | 20 (35.09) | | |
| \geq 55 | 67 | 30 (53.57) | 37 (64.91) | | |
| Tumor size (cm) | | | | 7.631 | 0.006 |
| <5 | 77 | 45 (80.36) | 32 (56.14) | | |
| \geq 5 | 36 | 11 (19.64) | 25 (43.86) | | |
| T stage | | | | 7.534 | 0.006 |
| T1+T2 | 84 | 48 (85.71) | 36 (63.16) | | |
| T3+T4 | 29 | 8 (14.29) | 21 (36.84) | | |
| N stage | | | | 2.057 | 0.040 |
| N0+NX | 104 | 55 (98.21) | 49 (85.96) | | |
| N1 | 9 | 1 (1.79) | 8 (14.04) | | |
| M stage | | | | 2.251 | 0.024 |
| M0+MX | 89 | 49 (87.50) | 40 (70.18) | | |
| M1 | 24 | 7 (12.50) | 17 (29.82) | | |
| TNM staging | | | | 13.685 | <0.001 |
| I+II | 81 | 49 (87.50) | 32 (56.14) | | |
| III+IV | 32 | 7 (12.50) | 25 (43.86) | | |
| Fuhrman pathological grading | | | | 37.449 | <0.001 |
| G1+G2 | 58 | 45 (80.36) | 13 (22.81) | | |
| G3+G4 | 55 | 11 (19.64) | 44 (77.19) | | |
| Recurrence (within 5 years) | | | | 15.402 | <0.001 |
| Yes | 90 | 53 (94.64) | 37 (64.91) | | |
| No | 23 | 3 (5.36) | 20 (35.09) | | |

a higher 5-year survival rate than patients with low miR-410 expression (Figure 1).

Correlation between miR-410 and clinical features of CCRCC

For the purpose of analyzing the relationship between miR-410 and clinical features of CCRCC,

we assigned the patients into high and low expression groups with the median expression of miR-410 as the critical value. It came out that miR-410 was related to tumor size, T, N, and M stages, TNM staging, Fuhrman pathological grading, and recurrence (within 5 years) of patients with CCRCC (Table 2).

Table 3. Univariate and multivariate analyses of patients with CCRCC

| | Univariate | | Multivariate | |
|--|----------------|-----------------------|----------------|----------------------|
| | <i>p</i> value | HR (95% CI) | <i>p</i> value | HR (95% CI) |
| Sex (male/female) | 0.953 | 0.958 (0.247-3.581) | | |
| Age (<55/≥55) | 0.864 | 1.115 (0.314-0.796) | | |
| Tumor size (cm <5/≥5) | 0.033 | 0.513 (0.282-0.942) | 0.214 | 0.388 (0.068-1.816) |
| T stage (T1+T2/T3+T4) | <0.001 | 10.535 (7.349-14.847) | 0.003 | 1.758 (1.268-2.036) |
| N stage (N0+NX/N1) | <0.001 | 12.035 (8.002-18.098) | 0.002 | 3.124 (1.009-5.251) |
| M stage (M0+MX/M1) | <0.001 | 12.737 (8.552-18.988) | 0.006 | 4.381 (2.146-6.971) |
| TNM staging (I+II/III+IV) | <0.001 | 3.028 (2.216-4.087) | 0.014 | 4.522 (2.771-6.916) |
| Fuhrman pathological grading (G1+G2/G3+G4) | 0.017 | 4.138 (1.295-13.830) | 0.028 | 3.414 (1.072-11.582) |
| Recurrence (Yes/No) | <0.001 | 5.017 (3.622-6.855) | <0.001 | 3.173 (2.165-4.544) |
| MiR-410 (low/high) | <0.001 | 3.085 (2.184-4.355) | <0.001 | 2.647 (1.854-3.776) |

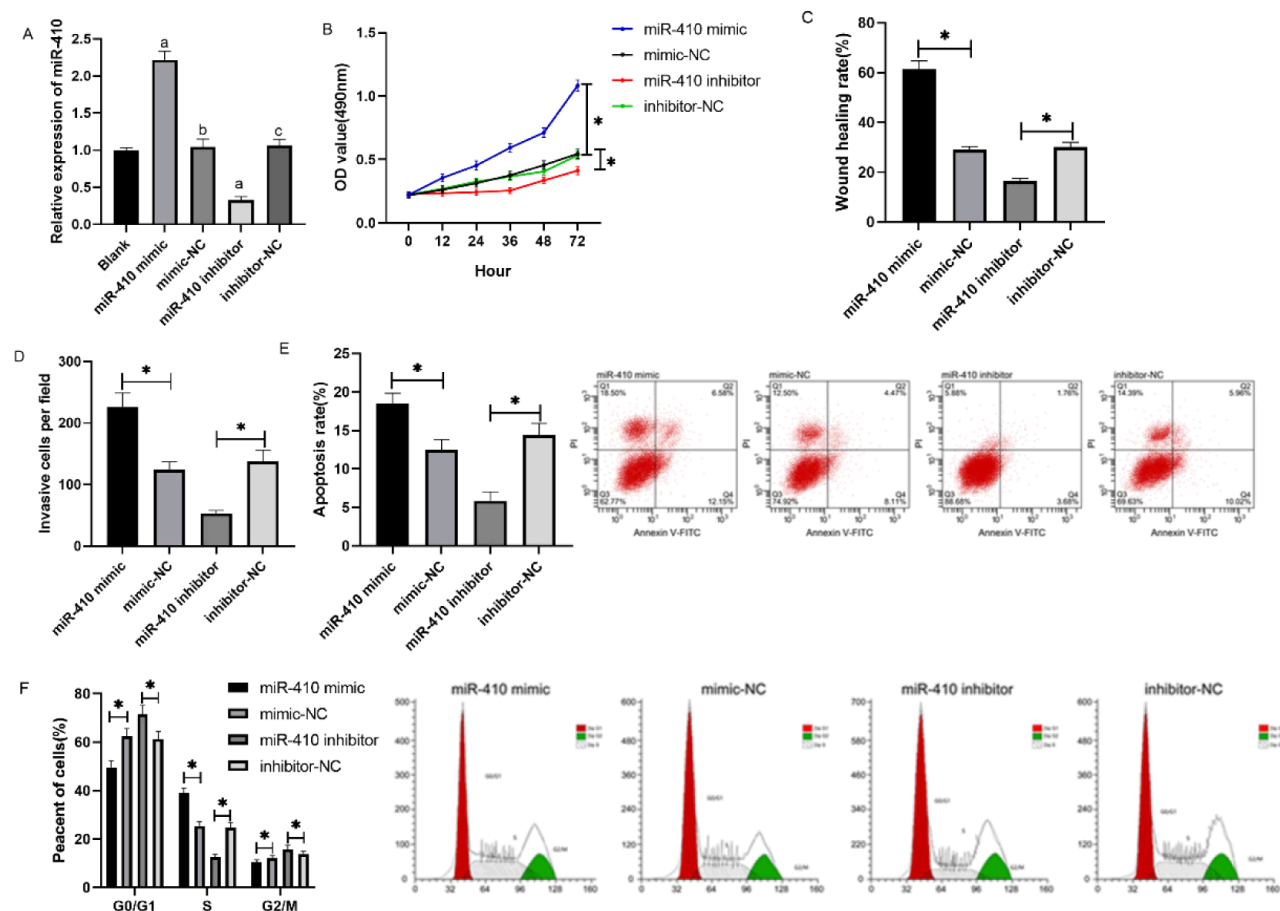


Figure 2. Influence of miR-410 on the biological behaviors of CCRCC cells. **A:** Effect of miR-410 mimic and miR-410 inhibitor on the expression of miR-410 in Caki-2 cells. **B:** Effect of miR-410 on the proliferation of Caki-2 cells. **C:** Effect of miR-410 on the migration of Caki-2 cells. **D:** Effect of miR-410 on the invasion of Caki-2 cells. **E:** Effect of miR-410 on the apoptosis of Caki-2 cells. **F:** Effect of miR-410 on the cycle of Caki-2 cells (**p*<0.05).

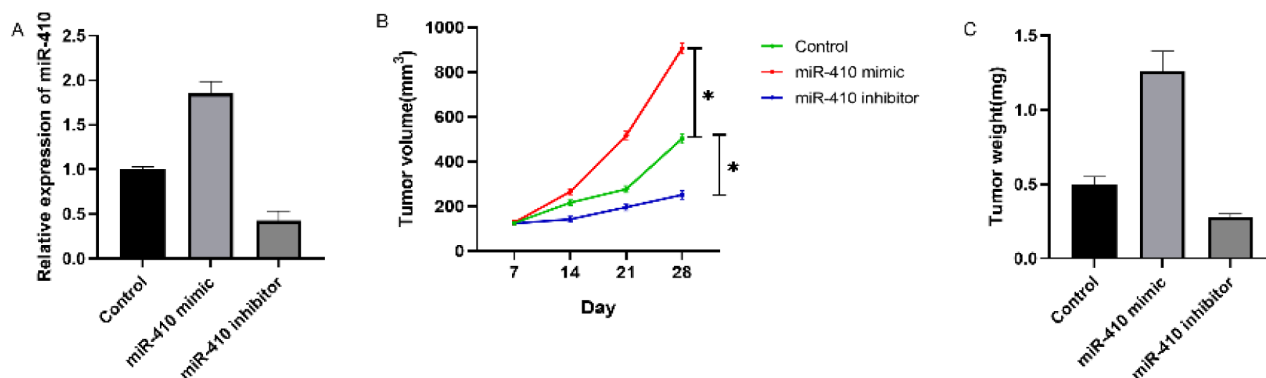


Figure 3. Effect of miR-410 on the tumorigenesis of CCRCC. **A:** Expression of miR-410 in tumor tissues. **B:** Effect of miR-410 on the tumor volume of CCRCC. **C:** Effect of miR-410 on the tumor mass of CCRCC (* $p < 0.05$).

Prognostic risk factors of CCRCC

Cox proportional hazards model was adopted for analysis of risk factors affecting the survival prognosis of CCRCC. Univariate analysis showed that tumor size, TNM staging, Fuhrman pathological grading, recurrence and miR-410 expression were related to the survival prognosis of patients, and multivariate analysis showed that formal TNM staging, Fuhrman pathological grading, recurrence and miR-410 expression were independent risk factors for the survival prognosis of patients with CCRCC (Table 3).

Influence of miR-410 on the biological behaviors of CCRCC cells

In order to explore the biological role of miR-410 in CCRCC, we regulated miR-410 in CCRCC cells (Caki-2) with miR-410 mimic and miR-410 inhibitor, and verified the transfection results through qRT-PCR assay. It was found that miR-410 mimic significantly promoted the proliferation, migration, invasion and cell cycle of Caki-2 cells, and inhibited their apoptosis. On the contrary, miR-410 inhibitor exerted significant inhibitory effect on the growth of Caki-2 cells (Figure 2).

Effect of miR-410 on the tumorigenesis of CCRCC

In order to verify whether miR-410 affected the tumorigenesis of CCRCC *in vivo*, we injected nude mice with Caki-2 transfected with miR-410 mimic or miR-410 inhibitor. Four weeks later, it was found that miR-410 mimic significantly promoted tumor growth, while miR-410 inhibitor inhibited tumor growth (Figure 3).

Discussion

In recent years, growing evidence has shown that cancer is often accompanied by dysregulation

of miRs, and regulation of miRs on the expression of target genes changes the risk and treatment results of cancer [14-16]. Genome-wide microarray-based analysis of miRs expression in cancer and paracancer tissues of patients with CCRCC revealed that dozens of miRs are up-regulated or down-regulated in cancer tissues of patients with CCRCC [17,18].

MiRs are small non-coding endogenous single-stranded RNAs, which have been adopted as diagnostic markers and potential therapeutic targets in many studies [19-21]. This study explored the clinical value of miR-410 expression in CCRCC patients and its biological role in CCRCC. In this study it was found that miR-410 was expressed at a high level in patients with CCRCC, and had a high diagnostic accuracy in CCRCC (AUC=0.916). Treatment of patients with CCRCC is limited by difficulties in early diagnosis and lack of reliable specific biomarkers [22]. One previous study has reported that miR-410 can be used as a diagnostic serum marker for prostate cancer, with AUC of 0.810 [23]. Many miRs have been found to be potential diagnostic markers for CCRCC [24]. Lou et al [25] have revealed that the AUC of miR-144-3p in distinguishing patients with CCRCC from healthy individuals was 0.91, and the sensitivity and specificity were 87.10% and 83.02%, respectively. Liu et al [26] have screened candidate miRs based on miR microarray analysis, and have found that the AUCs of serum miR-508-3p and miR-885-5p were 0.80 and 0.87, respectively. In this study, we also found that miR-410 was an independent risk factor for survival prognosis of CCRCC patients, and its high expression indicated poor prognosis of the patients. Currently, there are rare reports on circulating miR-410 as a diagnostic biomarker for CCRCC. As far as we know, this is the first time to study the significance of miR-410 expression in CCRCC in clinical practice.

We have also analyzed the biological effects of miR-410 on CCRCC for the first time. In this study, inhibiting miR-410 suppressed cell proliferation, cycle progression, migration, invasion, and tumor growth *in vivo*, and promoted cell apoptosis. Up-regulation of miR-410 is common in many cancers. Earlier studies have revealed that miR-410 is up-regulated in cancers including pituitary adenoma [27], prostate cancer [28], colon cancer [29], as well as lung cancer [30], and promotes the malignant development of tumors. In terms of renal diseases, miR-410 inhibits the expression of interleukin 6 and participates in renal fibrosis of lupus nephritis [31], while it is also involved in renal-related repair of mesenchymal stem cells [32]. The mechanism of miR-410 in tumors has also been found in many studies. MiR-410 induces apoptosis of lung cancer cells by down regulating the JAK/STAT/suppressor of cytokine signaling-3 (SOCS-3) intracellular signaling pathway [33]. In fact, miR-410 plays a double-edged sword in tumors [34]. This MiR inhibits the proliferation, migration, invasion, and epithelial-mesenchymal transformation of osteosarcoma cells by targeting TRIM 44 [35]. In addition, miR-410 is also regulated by long non-coding RNAs (lncRNAs). LncRNA OPA-interacting pro-

tein 5 antisense transcript 1 (OIP5-AS1) specifically blocks the Wnt-7b/catenin pathway through targeted up-regulation of miR-410, thus inhibiting the growth, invasion, as well as migration of glioma cells and promoting their apoptosis [36]. The ability of miR-410 in regulating the expression of multiple targets demonstrates that miR-410 plays a crucial role in the regulatory network of tumor development, but the mechanism of miR-410 in CCRCC is still unclear, and the comprehensive regulatory mechanism of miR-410 on biological behaviors such as cell proliferation and invasion of CCRCC and its related signal pathways need to be further explored.

To sum up, miR-410 was up-regulated in patients with CCRCC, had a good diagnostic value for CCRCC and was an independent risk factor affecting the prognosis of survival of CCRCC patients. In addition, miR-410 played a role as an oncogene in CCRCC and promoted the malignant progression of CCRCC.

Conflict of interests

The authors declare no conflict of interests.

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