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Regulatory roles of miR-593 in the proliferation and invasion of human hepatic carcinoma

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Abstract. – OBJECTIVE: The aim of this study was to explore the expression manuals of microRNA-593 (miR-593) in hepatocellular carcinoma (HCC), and to investigate its role in mediating the proliferation, migration and invasion of HCC cells.

PATIENTS AND METHODS: Real-time quantitative polymerase chain reaction (RT-qPCR) was used to detect miR-593 expression in HCC tissues and cells. HCC cells were, then, transfected with miR-593 mimic to exogenously overexpress miR-593. Cell counting kit 8 (CCK8) assay was exploited to detect the proliferation ability of HCC cells. In addition, wound healing assay and transwell assay were adopted to determine the migration and invasion abilities of HCC cells, respectively.

RESULTS: MiR-593 was abnormally down-expressed in HCC tissues and cells. Up-regulation of miR-593 significantly inhibited the proliferation, migration and invasion of HCC cells.

CONCLUSIONS: MiR-593 acted as a tumor suppressor gene in the development of HCC. Hence, we proposed that miR-593 might be a new potential therapeutic target marker for HCC treatment.

Key Words:
Hepatocellular carcinoma (HCC), MicroRNA-593, Treatment, Suppressor.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. It ranks fifth in terms of incidence rate and second in mortality rate. HCC is characterized by latent development, high malignancy and poor prognosis, severely endangering the life and health of people[1]. As to its treatment, surgical resection, transcatheter arterial chemoembolization, and targeted drug therapy are mainly adopted[2-4]. All these treatment measures aim to prolong the survival time, relieve pain and improve the life quality of patients. In recent years, great progress and improvement have been made in the diagnosis and treatment techniques for HCC[5,6]. However, there is still no amelioration in the outcome that HCC patients are prone to die from recurrence and metastasis after surgery since the blood supply to the liver is abundant. Meanwhile, most patients tend to be at the middle or advanced stage when first diagnosed[7]. Therefore, searching for tumor markers with strong specificity and high sensitivity in the development of HCC, and uncovering their regulatory mechanisms are of great significance for improving the survival and prognosis of HCC patients.

Micro ribonucleic acids (miRNAs) are widely detected in eukaryotes. They participate in a series of important life processes, including early embryonic development, proliferation, differentiation, apoptosis and death of cells, and metabolism of many substances[8-10]. MiRNAs may bind to the complementary sequence in the 3'-untranslated region (3'-UTR) of mature messenger RNAs (mRNAs) to repress gene transcription or force mRNAs to degrade to inhibit its expression, thus exerting their important regulatory roles in the body[11,12]. Besides, miRNAs have diverse sequences, structures, abundances and expressions, greatly affecting the development of individuals as regulators of mRNA-encoded proteins *in vivo*. About 50% of known miRNAs are associated with the development of human malignant tumors[13-15]. Some miRNAs are abnormally highly or lowly expressed in tumor tissues and cells; therefore, these miRNAs may become new biomarkers for the diagnosis and prognosis of malignant tumors.

Recently, miR-593 has been observed closely involved in several kinds of human tumors, such as colon cancer[16], non-small cell lung cancer[17], esophageal cancer[18] and breast cancer[19]. However, the exact role of miR-593 in HCC development still remains unclear. In this study, we mainly explored the effects of miR-593 on the proliferation

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migration and invasion of HCC cells, so as to provide theoretical support for HCC treatment.

Patients and Methods

HCC Tissues Collection

In this study, 59 pairs of HCC tissues and adjacent normal tissues (2 cm away from the tumor edge) surgically resected from patients who received treatment in our University's Cancer Center from December 2017 to June 2019 were collected. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). All enrolled patients were diagnosed with primary HCC *via* postoperative pathology. No patient received any chemotherapy, radiotherapy, local ablation therapy, and targeted drug therapy before operation. This investigation was approved by the Ethics Committee of Chongqing Medical University. Informed consent was obtained from each subject before the study.

Cell Culture

Human HCC cell lines (including HCCLM3, Hep-3B, SMMC-7721 and Huh7), and human normal liver L02 cell line were cultured with complete medium in a 5% CO_2 cell thermostatic incubator at 37°C. Cell growth status was observed using a microscope. When the cells covered 80-90% of the bottom of the culture flask, 1 mL of trypsin digestion solution was added. After shaking evenly to make trypsin cover the whole surface, the cells were placed in an incubator for 1-2 min. Next, the cells were observed again under a microscope. An appropriate volume of complete medium was then added to terminate digestion when retracted cytoplasm, round cells, increased space, and suspension of cells were observed. Then, the cells were pipetted evenly and centrifuged. Thereafter, the resulting single cell suspension was dispensed into new culture dishes at 1:3 and transferred to the cell incubator.

Cell Transfection

The cells were first digested, collected and planted into 6-well plates, followed by incubation in a 37°C and 5% CO_2 incubator overnight. MiR-593 mimic RNA and scramble RNA with concentration of 200 nM was used for cell transfection. In brief, miR-593 and lipofectamine 2000 solution (Invitrogen, Carlsbad, CA, USA) were mixed and incubated at room temperature for 10 min. The mixture was then added into cells, followed by culture for 6 h. Thereafter, the cells were replaced with fresh medium containing fetal bovine serum (FBS), and cultured at constant temperature for 24 h. Accordingly, the cells were divided into two groups, including NC group (transfection of miR-593 scramble RNA) and miR-593 mimic group (transfection of miR-593 mimic RNA). Transfected cells were collected for subsequent experiments.

Cell Counting Kit 8 (CCK8) Assay

Transfected cells were first inoculated into 96-well cell culture plates at a density of 2000 cells/well, and cultivated at 37°C and 5% CO_2 for 4 d. At the same time point every day, the plate was taken out and added with CCK8 solution (Dojindo Molecular Technologies, Kumamoto, Japan), followed by incubation for another 4 h in the dark. After discarding the medium, each well was added with 150 μL of dimethyl sulfoxide (DMSO) solution (Sigma-Aldrich, St. Louis, MO, USA), and cultured in dark for 10 min. Optical density (OD) value was finally determined by a micro-plate reader (wavelength: 450 nm).

Transwell Assay

Transfected cells were first re-suspended with serum-free Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA). Transwell chambers were placed in a 24-well plate with complete medium. 100 μL of cell suspension (3×10^4 cells) was transferred into the upper chamber of transwell chamber using a pipette. Meanwhile, complete medium was added to the lower chamber. Subsequently, the plate was gently shaken to mix the cells, followed by culture in an incubator for 24 h. Next, the cells were fixed with 600 μL of 4% paraformaldehyde for 30 min and stained with 600 μL of Giemsa staining for 45 min at room temperature. Cells not passing through the membrane were wiped off with cotton swabs. After drying, migrating cells were observed in 5 randomly selected fields under a microscope, and the number of migrating cells was counted.

Wound Healing Assay

After transfection, the cells were digested, seeded into 6-well plates, and cultured in a humidified incubator with 5% CO_2 at 37°C. After 24 h of incubation, a 10 μl tip was used for scratching in the middle of the plate. Then, the cells were washed 3 times with phosphate-buffered saline

(PBS) and added with complete fresh medium for another 48 h of incubation. Finally, the cells were observed and photographed under an inverted microscope (Olympus, Tokyo, Japan).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was first added to extract total RNA in tissues or cells. The concentration of extracted RNA was detected by Nanodrop. For RT-qPCR analysis, three replicate wells were designed for each sample. The prepared system was then performed on an Applied Bio Step One Plus systems (Applied Biosystems, Foster City, CA, USA) with the following conditions: pre-denaturation at 95°C for 30 s; reaction at 95°C for 5 s, and at 60°C for 30 s (for a total of 40 cycles). U6 was used as an internal reference. Gene expression was quantified by the $2^{-\Delta\Delta CT}$ method. Primers used in this study were as follows: miR-593 forward 5'-CACCAGCCAGGCATTGCTC-3', miR-593 reverse 5'-CTCAACTGGTGTCGTGGA-3'. U6 forward 5'-TTATGGGTCCTAGCCTGAC-3', U6 reverse 5'-CACTATTGCGGGTCTGC-3'.

Statistical Analysis

GraphPad Prism 7.01 statistical software (La Jolla, CA, USA) was used for all statistical analysis. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

MiR-593 was Lowly Expressed in HCC Tissues

To explore the role of miR-593 in HCC, we first examined its expression in HCC tissues and adjacent normal tissues. QRT-PCR results showed that miR-593 expression decreased significantly in HCC tissues compared with corresponding adjacent normal tissues ($p<0.05$; Figure 1A). In addition, we detected the expression levels of miR-593 in four different HCC cell lines (HCCLM3, Hep-3B, SMMC-7721, Huh7) and L02 cell lines. The results found that the expression of miR-593 in HCC cell lines was significantly lower than L02 cells ($p<0.05$; Figure 1B). These findings indicated that miR-593 was lowly expressed in HCC and was correlated with the development of HCC.

MiR-593 was Highly Expressed in HCC Cells after MiR-593 Mimic Transfection

To better illustrate the effect of miR-593 on HCC cells, Hep-3B cells and Huh7 cells were selected as research objects based on qRT-PCR results. Subsequently, the cells were transfected with miR-593 mimic. As shown in Figure

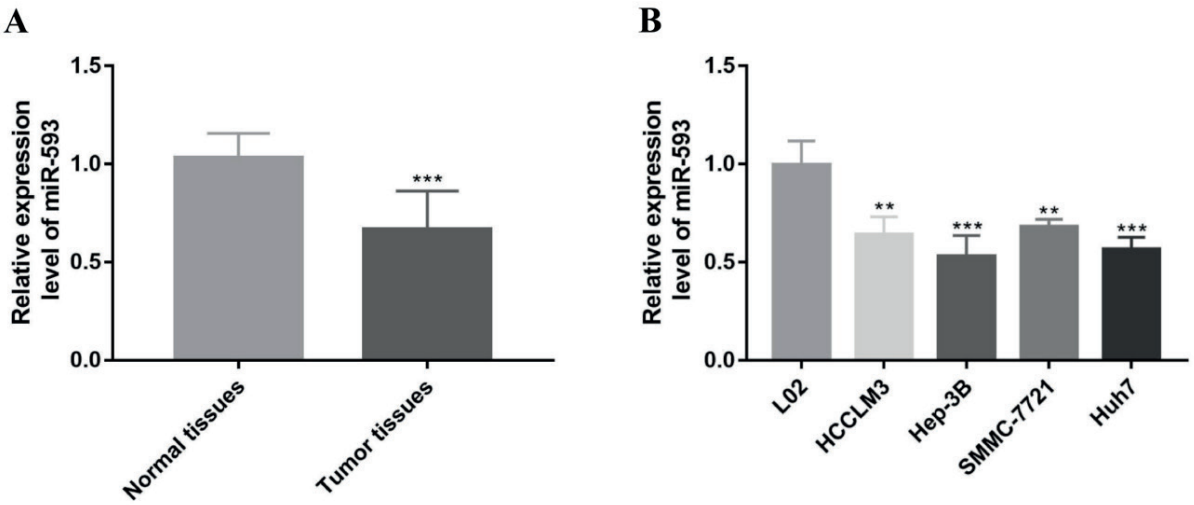

Figure 1. Expression level of miR-593 in HCC tissues and cells. **A,** MiR-593 was down-regulated in HCC tissues compared with adjacent normal tissues. **B,** MiR-593 was down-expressed in HCC cell lines compared with normal cells. (**$p<0.01$, ***$p<0.001$).

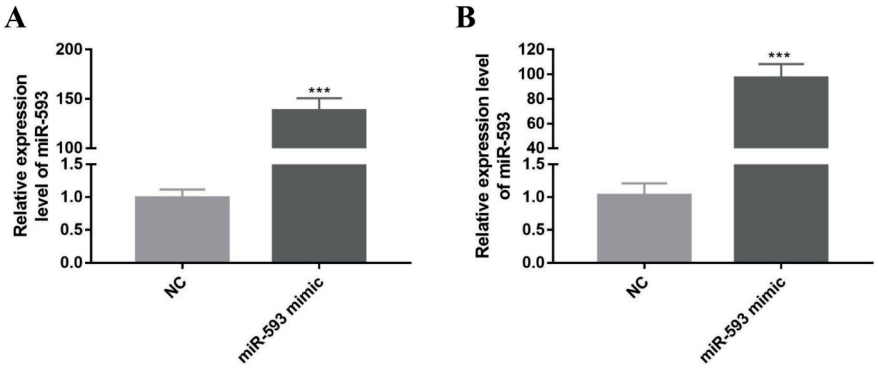

Figure 2. Transfection of miR-593 mimic up-regulated miR-593 expression in HCC cells. The expression of miR-593 was higher in Hep-3B cells (**A**) and Huh7 cells (**B**) after transfection with mimic compared with HCC cells without transfection. (**p<0.01).

2, compared with NC group, both Hep-3B cells and Huh7 cells transfected with miR-593 mimic showed significantly up-regulated miR-593 expression (p<0.05).

Regulatory Roles of MiR-593 on the Proliferation Ability of HCC Cells

CCK-8 assay was then performed to verify the effect of miR-593 on the proliferation of HCC cells. The results in Figure 3A suggested that Hep-3B cells with high expression of miR-593 exhibited significantly inhibited proliferation ability compared with normal Hep-3B cells (p<0.05). Similarly, the same results were observed in Huh7 cells (Figure 3B). These results suggested that overexpression of miR-593 inhibited the proliferation ability of HCC cells.

MiR-593 Inhibited the Migration Ability of HCC Cells

Wound healing assay was applied to detect the migration ability of HCC cells. The results revealed that, compared with NC group, the percentage of relative wound healing rate in miR-593 mimic group decreased remarkably, and the difference was statistically significant (p<0.05; Figure 4). These results indicated that miR-593 overexpression significantly impeded the migration ability of HCC cells.

MiR-593 Repressed the Invasion Ability of HCC Cells

We performed transwell assay to examine the impact of miR-593 on the invasion ability of HCC cells. The results showed the number of invasive

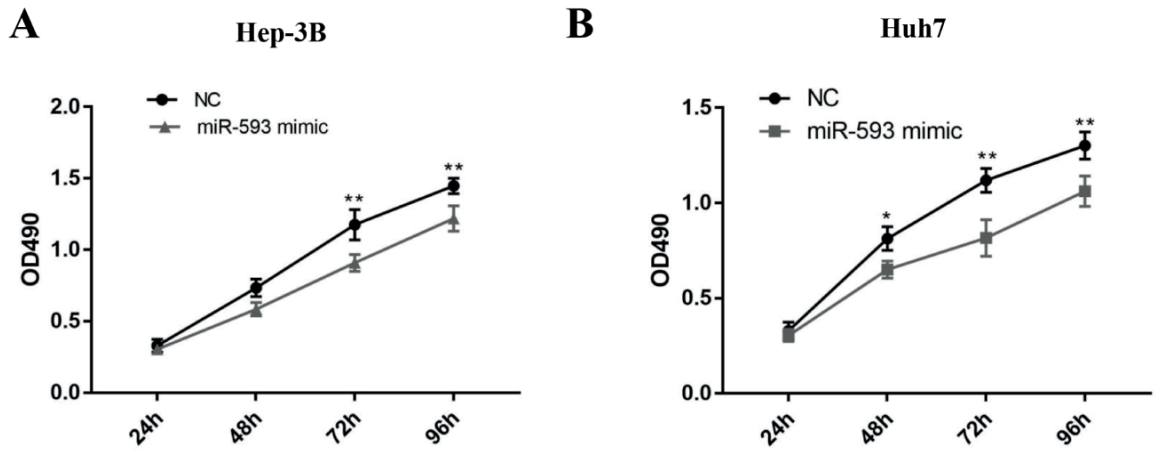

Figure 3. Regulatory roles of miR-593 on the proliferation ability of HCC cells. The proliferation ability of Hep-3B cells (**A**) and Huh7 cells (**B**) in miR-593 mimic group was reduced than NC group. (*p<0.05, **p<0.01).

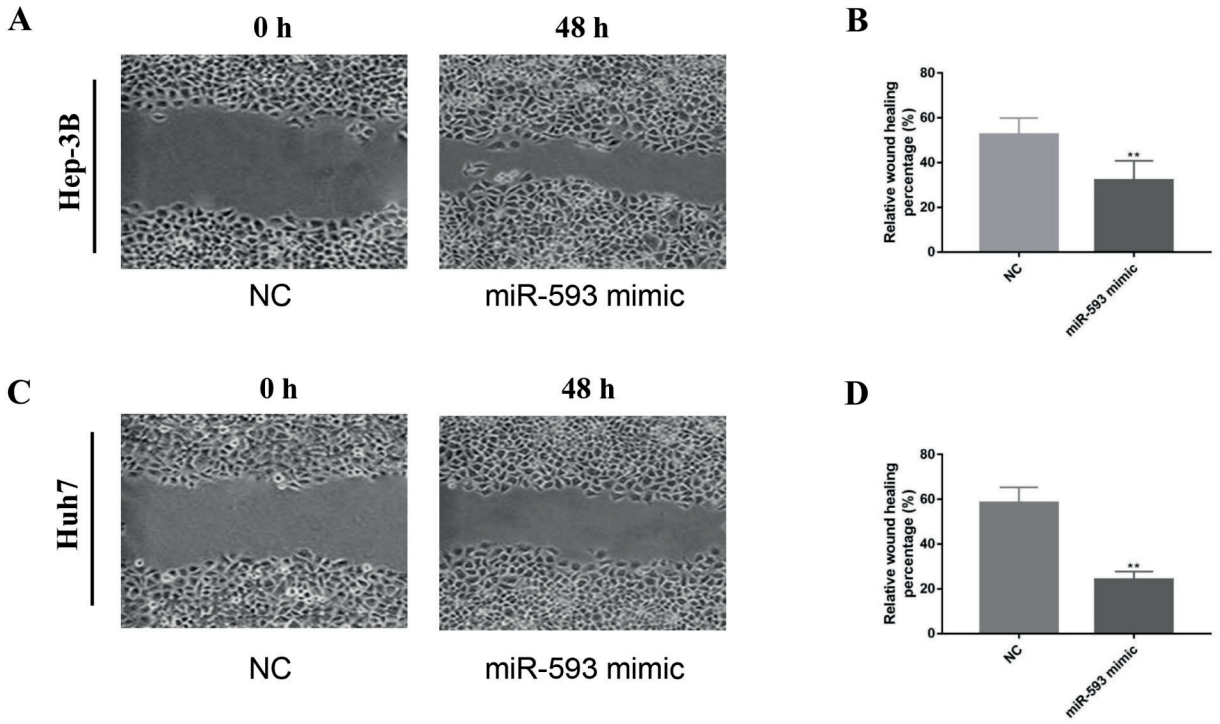

Figure 4. MiR-593 inhibited the migration ability of HCC cells. The migration ability of Hep-3B cells (**A-B**) and Huh7 cells (**C-D**) in miR-593 mimic group decreased significantly in comparison with NC group (magnification: 40×). (**p<0.01).

cells in miR-593 mimic group decreased notably compared with NC group (p<0.05; Figure 5). These findings indicated that overexpression of miR-593 could significantly impair the invasion ability of HCC cells.

Discussion

Currently, surgical treatment is still the major treatment method for HCC. After surgical resection, the five-year survival rate of early HCC can reach 50-70%. Due to the reason that HCC has no typical clinical symptoms in the early stage and is easy to be treated as other non-specific digestive diseases the diagnosis rate of HCC patients is relatively low and the postoperative recurrence rate is high[20]. Moreover, patients are prone to metastasis and recurrence after surgery due to the metastasis of residual cancer cells[21,22]. The reason is that without effectual clinical adjuvant treatments, it is hard to remove all original lesions and cancer cells in patients *via* surgery. As a result, early diagnosis and treatment are particularly important for HCC[23,24]. However, highly-specific and -sensitive tumor markers for screening of early HCC are absent at present. Therefore, searching for available tumor markers for early HCC is of great significance.

MiRNAs are a class of small non-coding single-stranded RNAs with about 20-25 nucleotides in length, which are encoded by endogenous genes. The main function of miRNAs is to induce mRNA degradation or suppress mRNA translation by specifically binding to mRNA 3-'UTR[25,26]. At present, miRNAs have been confirmed as important players in modulating many physiological processes of human tumors, such as early development, differentiation, proliferation and apoptosis[27,28].

As a member of miRNAs, the relationship between miR-593 and cancer was first reported by Ito et al[17]. They found that miR-593 suppressed the proliferation and increased cell proportion of G2/M phase in esophageal cancer. However, miR-593 inhibitor exhibited the opposite effect. Dong et al[28] have reported that miR-593 is involved in the proliferation and EMT phenotype of gastric cancer cells. Meanwhile, Song et al[18] have indicated that miR-593 participates in the

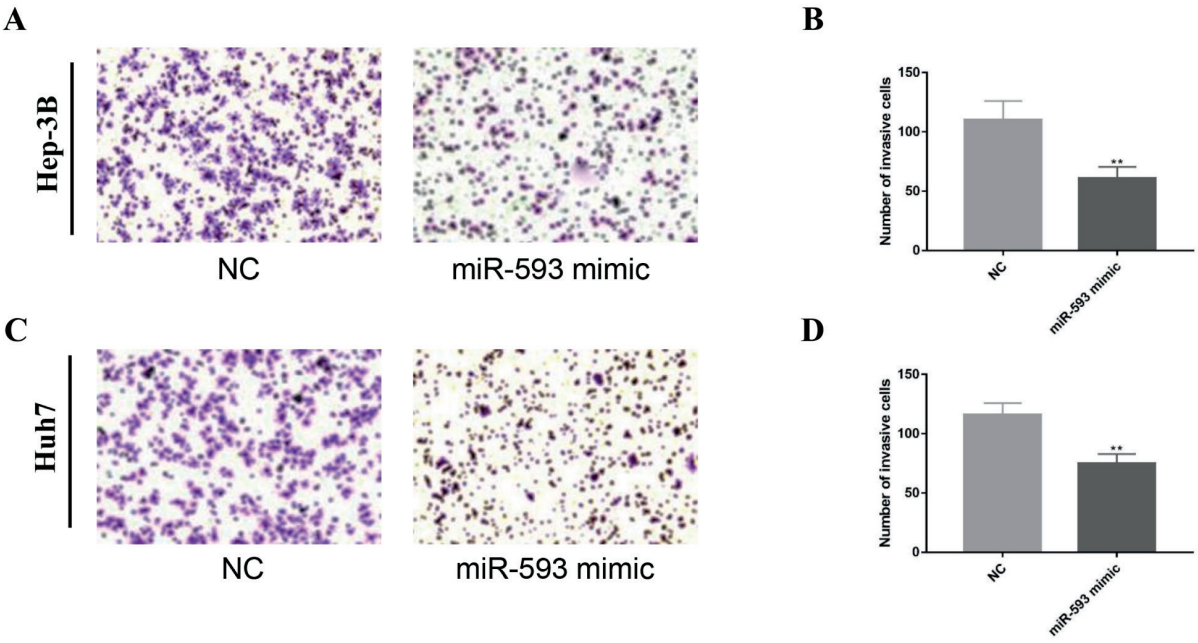

Figure 5. MiR-593 repressed the invasion ability of HCC cells. The invasion ability of Hep-3B cells (**A-B**) and Huh7 cells (**C-D**) in miR-593 mimic group was significantly inhibited compared with cells in NC group (magnification: 40×). (**p<0.01).

proliferation and invasion of breast cancer cells. Besides, Yu et al[29] have shown that miR-593-5p is abnormally down-expressed in gastric cancer tissues and cell lines. Meanwhile, it inhibits cell proliferation, migration, and invasion as well as arrested cell cycle at the G0/G1in gastric cancer cell lines.

To date, there is still no report on the relationship between miR-593 and HCC. Therefore, the main purpose of this study was to verify the effect of miR-593 on the biological function of HCC cells. Paired HCC tissues and corresponding normal tissues were first collected from patients diagnosed with HCC. By performing RT-qPCR, we found that miR-593 expression in HCC tissues was markedly lower than that of adjacent normal tissues. *In vitro* experiments, including CCK8 assay, wound healing assay and transwell assay, were applied to further verify the molecular role of miR-593 in HCC. Unsurprisingly, we found that exogenous overexpression of miR-593 not only inhibited the proliferation of HCC cells, but also impaired their ability to invade and migrate. These results are consistent with previous reports[17,29]. All our findings suggest that miR-593 is very likely to become a new molecular target for the detection and treatment of HCC.

Conclusions

MiR-593 was lowly expressed in both HCC tissues and cell lines. Over-expression of miR-593 inhibited the proliferation, migration and invasion of HCC cells. The novelty of this study was that miR-593 might serve as a new potential diagnostic and therapeutic molecular marker for HCC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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