

MiR-5692a promotes the invasion and metastasis of hepatocellular carcinoma via MMP9

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Abstract. – OBJECTIVE: To investigate the role of miR-5692a in hepatocellular carcinoma (HCC), and to further study the relationship between miR-5692a expression and clinical pathology as well as the prognosis of HCC.

PATIENTS AND METHODS: The expression level of miR-5692a in 96 pairs of HCC tissues and para-cancerous tissues were detected by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The relationship between miR-5692a and pathological indicators as well as the prognosis of HCC was analyzed by Kaplan-Meier curves. For *in vitro* experiments, qRT-PCR was used to detect the expression of miR-5692a in HCC cell lines. Furthermore, small interference sequence of miR-5692a was constructed. Cellular functions of HCC cells after miR-5692a knockdown were detected by cell counting kit-8 (CCK-8), colony formation and transwell assay, respectively. The underlying mechanism of miR-5692a in regulating the development of HCC was detected by Western blot.

RESULTS: MiR-5692a was overexpressed in HCC tissues than that of para-cancerous tissues. HCC patients with higher miR-5692a expression exhibited a higher prevalence of lymph node metastasis and distant metastasis, as well as lower overall survival than those patients with lower level of miR-5692a expression. *In vitro* experiments demonstrated that miR-5692a knockdown resulted in decreased proliferation and invasion, and increased apoptosis of HCC cells. Western blot results revealed that EMT-related (epithelial-mesenchymal transition) genes, including N-cadherin, Vimentin, β -catenin and MMP9, were downregulated after miR-5692a knockdown. Rescue experiments indicated that miR-5692a promoted malignant progression of HCC via regulating MMP9.

CONCLUSIONS: MiR-5692a was overexpressed in HCC patients, which was remarkably correlated with HCC stage, distant metastasis

and poor prognosis. In addition, miR-5692a promoted the malignant progression of HCC via regulating MMP9.

Key Words:

MiR-5692a, MMP9, Hepatocellular carcinoma (HCC), Malignant progression.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. In United States, there are about 42,220 new cases and 30,200 deaths of HCC every year, ranking the 10th of cancer incidence and the 5th of cancer death, respectively^{1,2}. In China, there are around 390,000 new HCC cases every year. The incidence of HCC ranks the 5th, and death rate of HCC ranks the 2nd^{3,4}. Epidemiological investigations^{5,6} have shown that about 80% HCC cases are associated with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infection. In addition to viral infection, alcoholic liver disease, smoking and obesity are also related to HCC incidence^{7,8}. Seriously, the incidence of HCC in China is increasing year by year, which seriously affects the life and health of the infected population. The high hepatitis infection rate in China also leads to a serious burden on HCC patients^{4,7}. Clinically, over 50% HCC patients experienced micro-metastases before radical surgery. Some factors, including genetics, diet, unhealthy lifestyles and precancerous lesions, are all closely related to the incidence of HCC⁹. Unfortunately, the unclear pathogenesis of HCC brings huge difficulty in precise diagnosis and treatment^{9,10}. Therefore, it

is of great clinical significance to clarify the molecular mechanism of HCC metastasis. With the rapid development of molecular biology and genetic diagnosis technology, HCC is considered as an outcome of the long-term interaction of genetic and environmental factors. Malignant transformation and irreversible genetic changes eventually lead to cell-specific physiological dysfunctions, including proliferation, apoptosis and differentiation^{11,12}. Despite that great progresses have been made so far, other mechanisms involved in the incidence and development of HCC still need to be further explored.

MicroRNA (miRNA) is a type of eukaryotic endogenous small-molecule RNA with 18-25 nt in length. MiRNA and mRNA are not completely complementary paired, thereby exerting a targeted regulatory function on the post-transcriptional level^{13,14}. Therefore, abnormal miRNA expression can lead to the dysfunction of relevant protein¹⁴. Relative experiments¹⁵⁻¹⁷ have shown that differentially expressed miRNAs are closely related to malignancies and can be served as biomarkers in tumors. Recent works¹⁶ have found that miRNAs are tissue-specific, which can promote the proliferation, invasion and metastasis of tumor cells through various mechanisms, eventually participating in the incidence and development of tumors¹⁸. MiRNAs have been found to be involved in multiple tumors, such as colorectal cancer, breast cancer, osteosarcoma and non-small cell lung cancer¹⁹⁻²¹. The specific role of miRNAs in regulating tumor development, however, is not clearly elucidated. It is currently believed that miRNAs play an important role in regulating the proliferation, cell cycle, apoptosis, differentiation and migration of tumor cells *via* different aspects^{17,22}. MiR-876-5p, miR-520f and other miRNAs have been found to participate in the development of HCC²³⁻²⁵. Relevant researches²⁵ have demonstrated that the Wnt pathway, the epidermal growth factor receptor (EGFR) pathway, transforming growth factor-Beta (TGF- β) inhibition, p53 mutation and epithelial-mesenchymal transition (EMT), are the potential mechanisms of HCC development.

In the present work, we detected the expression of miR-5692a in HCC tissues and paired para-cancerous tissues. Further analysis detected the role of miR-5692a in regulating cellular functions of HCC cells. The aim of this study was to explore the relationship between miR-5692a expression and pathological indicators as well as the prognosis of HCC.

Patients and Methods

HCC Samples

A total of 96 pairs of surgically resected HCC tissues and para-cancerous tissues were collected. All patients were pathologically diagnosed as HCC according to the 8th edition of UICC/AJCC. HCC patients did not receive preoperative anti-tumor therapies. This study was approved by the Ethics Committee of Yantai Yuhuangding Hospital. Informed consent was obtained from all the patients.

Cell Culture

Six human HCC cell lines (Bel-7402, HepG2, MHCC96H, SMMC-7221, Huh7 and Hep3B) and one normal liver cell line (LO2) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). All cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (FBS), and were maintained in a 37°C, 5% CO₂ incubator (Gibco, Rockville, MD, USA).

Cell Transfection

Negative control (si-RNA-NC) and small interference sequence of miR-5692a (si-miR-5692a) were obtained from GenePharma (Shanghai, China). Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) when the confluence reached 70%. After transfection for 48 h, cells were collected for the following experiments.

Cell Counting Kit-8 (CCK-8) Assay

The cells were seeded in 96-well plates at a density of 2×10^3 /well with 6 replicates in each group. 10 μ L CCK-8 (Dojindo, Kumamoto, Japan) solution was added to each well, followed by incubation for another 2 h. The absorbance at a wavelength of 490 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Transfected cells were seeded into 6-well plates at a density of 200 cells per well. After cell culture for 2 weeks, cells were washed with phosphate-buffered saline (PBS) and fixed with 2 ml methanol for 20 min. After washing with PBS, the colonies were stained with 0.1% crystal violet for 20 min, followed by image capture by using an inverted microscope.

Cell Apoptosis

HepG2 cells in the logarithmic growth phase were seeded into 6-well plates. After specific treatment for 24 h, cells were digested, washed twice with 4°C pre-cooled PBS and centrifuged at 1000 rpm for 5 min. The cells were then resuspended in 100 µL Annexin V Binding Buffer mixed with 5 µL Annexin V-fluorescein isothiocyanate (FITC) and 5 µL PI (propidium iodide) (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the cells were incubated at 4°C for 15 min in the dark, followed by flow cytometric analysis (Partec AG, Arlesheim, Switzerland).

Transwell Assay

50 µL FN and 100 µL matrigel were added into the lower and the upper chamber, respectively. Cells were seeded in the upper chamber at a density of 2×10^5 cells per well. After 24 h, the transwell chamber was removed from the incubator and placed in a 24-well plate with 500 µL methanol for fixation at 4°C overnight. After washing with PBS for three times, 5 randomly selected images were captured by using an inverted microscope (Nikon, Tokyo, Japan).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the tissues and cells according to the instructions of TRIzol kit (Invitrogen, Carlsbad, CA, USA). The concentration of extracted RNA was detected by an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary deoxyribose nucleic acid (cDNA) was then synthesized according to the instructions of the Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The relative expression level of target genes was calculated by $2^{-\Delta\Delta C_t}$. Primers used in this study were as follows: MiR-5692a: 5'-AGAGGGGTGGAGGGGAGACTAG-3'; U6: 5'-TGCGGGTGCTCGCTTCGGCAGC-3'; MMP9: forward, 5'-TTCCAAACCTTTGAGGGCGA-3', reverse, 5'-CAAAGGCGTCGTCAATCACC-3'; β -actin: forward, 5'-CCTGGCACCCAGCACAAT-3', reverse, 5'-TGCCGTAGGTGTCCCTTTG-3'.

Western Blot

The radio-immunoprecipitation assay (RIPA) protein lysate (Beyotime, Shanghai, China) was used to extract total protein of cells and tissues in each group. The BCA (bicinchoninic acid) method (Pierce, Rockford, IL, USA)

was performed to quantitate protein concentrations. Subsequently, protein samples were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After washing with Tris-buffered saline and Tween (TBST), the membranes were then incubated with secondary antibody. Chemiluminescence was used to expose the protein bands.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 Software Package (IBM, Armonk, NY, USA) was used for all statistical analysis. The Kaplan-Meier curve was performed for analyzing the prognosis of HCC patients. Difference in curves was compared by using the Log-rank test. The *t*-test was used to analyze the difference between two groups, while the χ^2 -test was used for the analysis of classification data. $p < 0.05$ was considered statistically significant.

Results

MiR-5692a was Overexpressed in HCC Tissues and Cell Lines

QRT-PCR results indicated that the expression of miR-5692a in HCC tissues was higher than that of para-cancerous tissues (Figure 1A and 1B). Besides, miR-5692a was overexpressed in HCC cell lines than that of LO2 cells (Figure 1C). Meanwhile, the Bel-7402 and HepG2 cells exhibited highest expression of miR-5692a, which were then selected for the following experiments.

MiR-5692a Expression was Correlated with Clinical Stage, Lymph Node and Distance Metastasis and Overall Survival in HCC Patients

Based on the expression of miR-5692a, all HCC patients were assigned into the high expression group and the low expression group. The information of age, gender, clinical stage, and lymph node and distant metastasis of each HCC patient were recorded. We found that miR-5692a expression was positively correlated with clinical stage, lymph node and distant metastasis, whereas was not correlated with age and gender of HCC patients (Table I). Follow-up data of these HCC

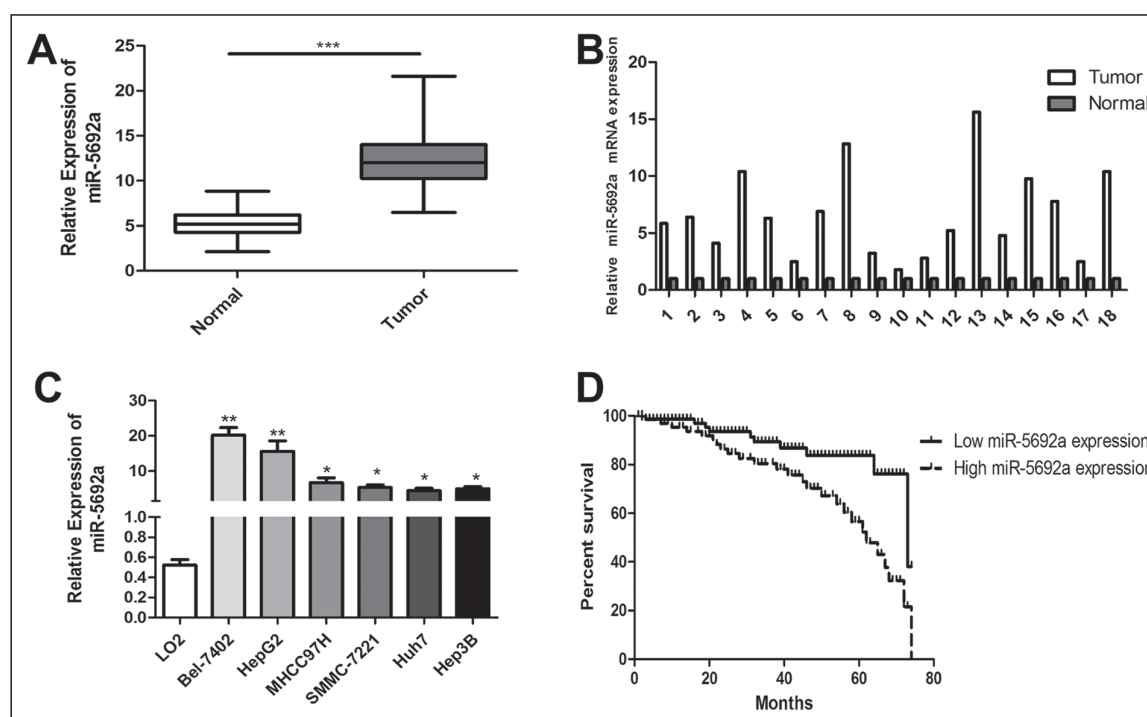


Figure 1. MiR-5692a was overexpressed in HCC tissues and cell lines. **A, B,** The expression of miR-5692a in HCC tissues was significantly increased. **C,** The expression level of miR-5692a in 6 HCC cell lines (Bel-7402, HepG2, MHCC96H, SMMC-7221, Huh7 and Hep3B) and 1 normal hepatic cell line (LO2). **D,** Kaplan-Meier survival curves of HCC patients based on miR-5692a expression. The prognosis of patients in the high expression group was poorer than those in low expression group.

patients were collected for analyzing the overall survival. The Kaplan-Meier curve showed that miR-5692a expression was negatively correlated with the prognosis of HCC patients ($p < 0.05$, Figure 1D).

Knockdown of miR-5692a Inhibited Cell Proliferation and Colony Formation Ability

We firstly constructed si-NC and si-miR-5692a. Cell transfection was then performed, and the transfection efficacy was verified by

Table I. Association of miR-5692a expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	Number of cases	miR-5692a expression		p-value
		Low (%)	High (%)	
Age (years)				0.569
< 60	42	25	17	
≥ 60	54	29	25	
Gender				0.143
Male	47	30	17	
Female	49	24	25	
T stage				0.032
T1-T2	53	35	18	
T3-T4	43	19	24	
Lymph node metastasis				0.035
No	55	36	19	
Yes	41	18	23	
Distance metastasis				0.023
No	70	45	25	
Yes	26	10	16	

qRT-PCR (Figure 2A and 2B). CCK-8 assay indicated that the knockdown of miR-5692a inhibited the proliferative ability of HCC cells

(Figure 2C and 2D). Similar results were obtained from the colony formation assay (Figure 2E).

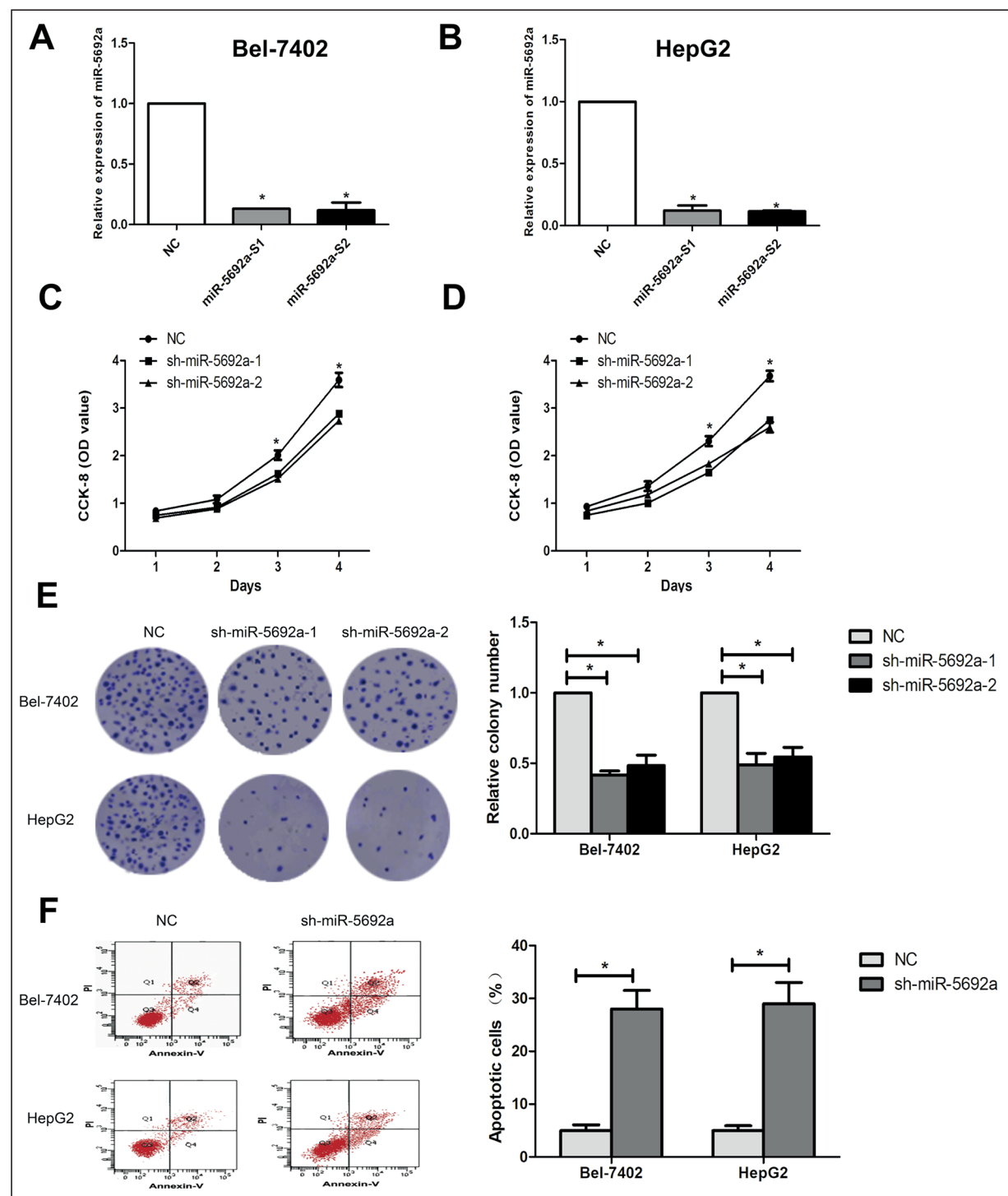


Figure 2. The effect of miR-5692a on cellular functions of HCC cells. **A, B**, QRT-PCR was used to verify the transfection efficiency of miR-5692a knockdown in Bel-7402 and HepG2 cells. **C, D**, Growth curve analysis showed the cell growth of Bel-7402 and HepG2 cells after miR-5692a knockdown. **E**, The efficiencies of colony formation in Bel-7402 and HepG2 cells after miR-5692a knockdown. **F**, The efficiencies of cell apoptosis in Bel-7402 and HepG2 cells after miR-5692a knockdown.

Knockdown of miR-5692a Induced Apoptosis in HCC Cells

To explore the effect of miR-5692a on the apoptosis of HCC cells, we conducted flow cytometry and found that miR-5692a knockdown remarkably increased the apoptotic rate of HCC cells (Figure 2F).

Knockdown of miR-5692a Inhibited Cell Migration and Invasion

Transwell assay was performed to detect the role of miR-5692a in the migration and invasion ability of HCC cells. Experimental data showed that the ratio of penetrating cells was remarkably lower after transfection with si-miR-5692a when compared with those transfected with si-NC (Figure 3C). Invasion assay demonstrated the same results (Figure 3D), indicating that miR-5692a knockdown inhibited cell migration and invasion.

Knockdown of miR-5692a Downregulated EMT-Related Genes

To further explore the potential mechanism of miR-5692a in regulating HCC cells, we detected the protein expression of EMT-related genes. Knockdown of miR-5692a led to the downreg-

ulated expression of EMT-related genes, including N-cadherin, Vimentin, β -catenin and MMP9 (Figure 4A).

MMP9 Modulated the Expression of miR-5692a in Human HCC Cells

Bioinformatics methods have reported that MMP9 can interact with miR-5692a. In the present study, we found that MMP9 was downregulated in HCC tissues than that of para-cancerous tissues (Figure 4B). Moreover, the expression of MMP9 in HCC cell lines was lower than that of normal liver cell line (Figure 4C).

Furthermore, miR-5692a was found upregulated after MMP9 knockdown in HCC cells. We speculated that whether miR-5692a could regulate MMP9. Hence, we detected the expression of miR-5692a and MMP9 in 16 pairs of HCC tissues and para-cancerous tissues. Both protein and mRNA levels of miR-5692a were negatively correlated with MMP9 expression (Figure 4D). For *in vitro* experiments, MMP9 was also downregulated in HCC cells when compared with that of LO2 cells (Figure 5A). Subsequently, we constructed si-MMP9 for cell transfection, and the transfection efficacy was verified by Western blot (Figure

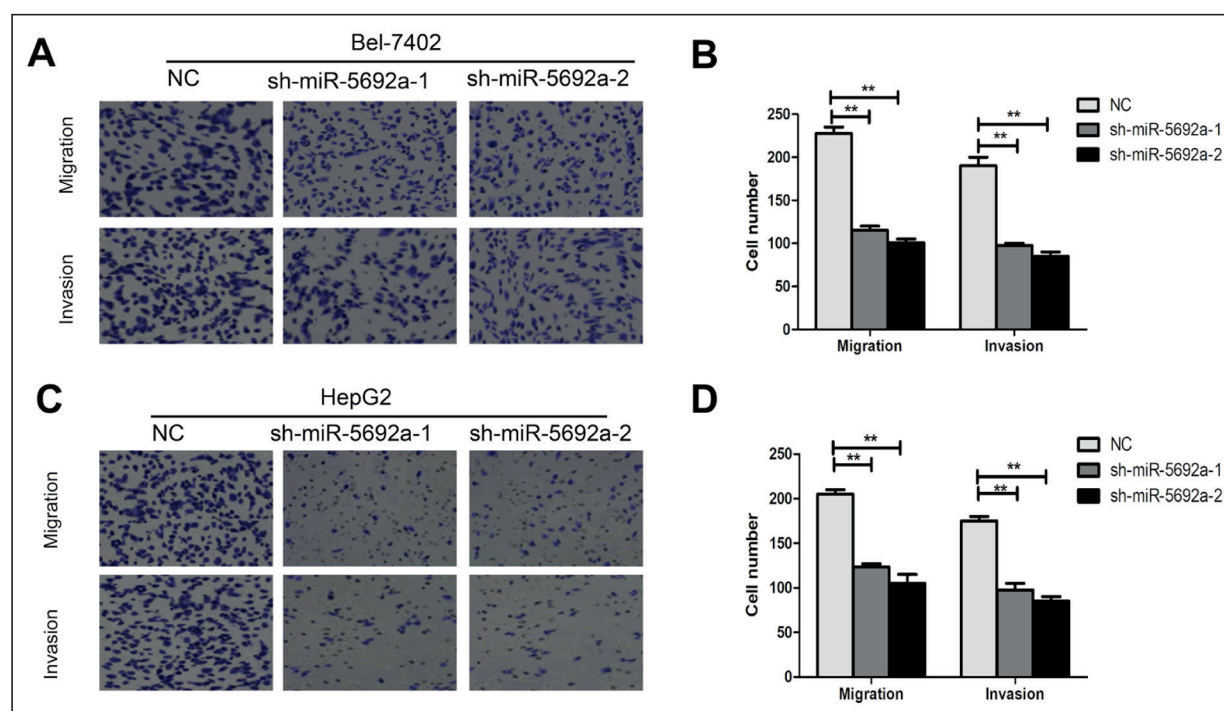


Figure 3. Knockdown of miR-5692a inhibited the migration and invasion of HCC cells. **A, B**, Bel-7402 and HepG2 cells transfected with si-miR-5692a displayed significantly lower migration capacity. **C, D**, Bel-7402 and HepG2 cells transfected with si-miR-5692a showed significantly lower invasion capacity.

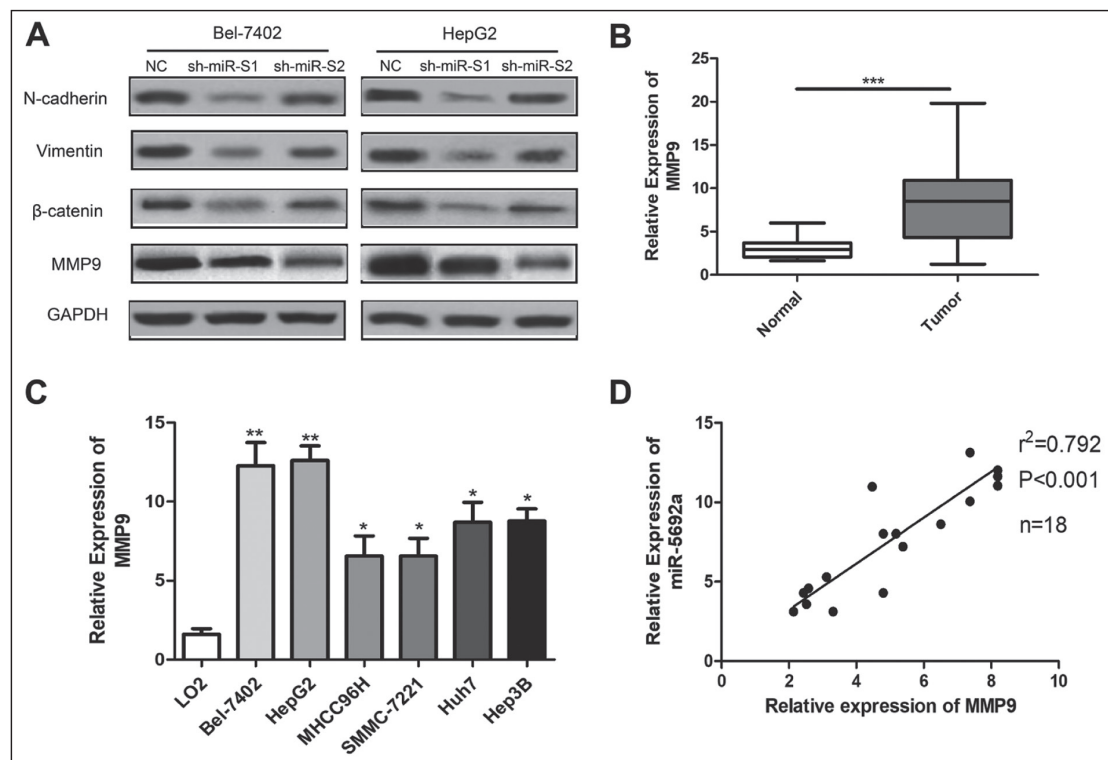


Figure 4. MiR-5692a regulated HCC development via regulating the EMT pathway. **A**, Knockdown of miR-5692a significantly decreased the expression of EMT-related genes, including N-cadherin, Vimentin, β-catenin and MMP9. **B**, **C**, The mRNA expression level of MMP9 relative to GAPDH in human HCC tissues and corresponding adjacent tissues, and cell lines were detected by qRT-PCR. **D**, A negative correlation was found between miR-5692a and MMP9 in tumor samples.

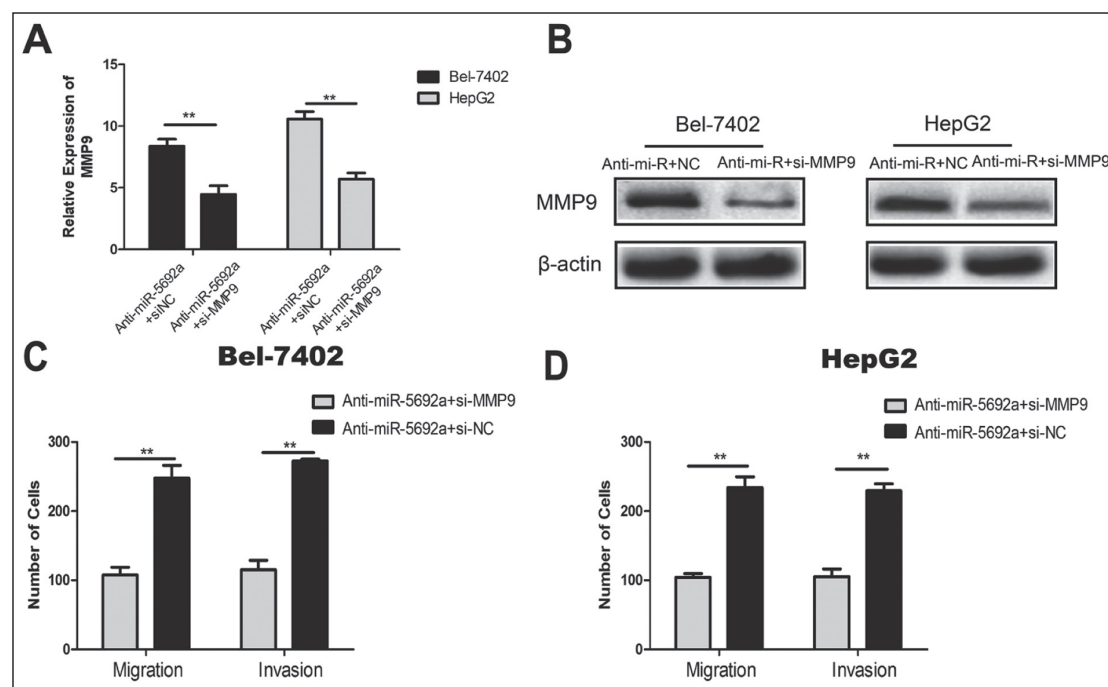


Figure 5. MiR-5692a regulated HCC cells via regulating MMP9. **A**, The mRNA expression of MMP9 was verified by qRT-PCR in co-transfected cell lines. **B**, Western blot was used to verify the protein expression of MMP9. **C**, **D**, The role of miR-5692a and MMP9 in the regulation of cell migration and invasion was examined by transwell assay. A representative data set was displayed as mean ± SD values (* $p < 0.05$, ** $p < 0.01$).

5B). The inhibited proliferative and invasive abilities induced by miR-5692a knockdown in Bel-7402 cells were reversed by si-MMP9 transfection, indicating that miR-5692a could negatively regulate MMP9.

Discussion

HCC poses a great burden to the affected population worldwide^{1,2}. In recent years, the morbidity and mortality of HCC in China have gradually increased. More seriously, the early diagnosis rate of HCC patients in our country is extremely low. Most of the patients are already in the advanced or late stage when they are diagnosed. Therefore, advanced HCC cases account for the majority⁷. HCC is manifested as high malignancy, easy relapse and metastasis, and poor prognosis. Early diagnosis and accurate treatment contribute to the improvement of clinical outcomes. It is of great significance to explore effective biomarkers for better treatment of HCC^{4,9}. Genetics, diet, unhealthy lifestyles, and precancerous lesions are closely related to the incidence of HCC. Clinically, over 50% HCC patients have experienced micro-metastases before undergoing radical surgery, which is the direct cause of metastasis and postoperative recurrence of HCC⁹. Researches on exploring postoperative metastasis, recurrence and adjuvant therapy of HCC have been well recognized^{9,12}. Molecular changes in HCC cells, such as changes in gene copy number and the disruption of coding sequences, exert important effects on tumor phenotype^{11,12,25}. Multiple differentially expressed miRNAs have been found in HCC, which may play a crucial role in the diagnosis, treatment and prognosis of HCC²⁵. Hence, the relationship between differentially expressed miRNAs in HCC and clinical prognosis needs to be well investigated, so as to improve the outcomes of HCC patients.

Accumulating evidence has shown that miRNAs participate in the biological behavior of HCC, which may provide a new direction for the diagnosis and treatment of HCC in the future²⁵. MiRNA is a single-stranded non-coding RNA with 22 nucleotides in length. Functionally, miRNA degrades target mRNAs or inhibits its translation, thereby regulating the expression of downstream genes^{13,14}. MiRNAs can affect cell proliferation, apoptosis, sensitivity to chemotherapy and radiotherapy, tumor metastasis, and may

even define the phenotype of cancer stem cells¹⁸. It has been believed that miRNAs may serve as targets for the development of novel anti-tumor drugs²⁵. In the present study, we explored the role of miR-5692a in HCC and investigated the potential mechanism. We found that miR-5692a was overexpressed in HCC tissues than that of para-cancerous tissues. The expression of miR-5692a was positively correlated with the clinical stage of HCC, lymph node metastasis, distant metastasis and poor prognosis. To further explore the effects of miR-5692a on the biological functions of HCC cells *in vitro*, we constructed small interfering sequence of miR-5692a. Subsequent cellular experiments demonstrated that miR-5692a knockdown remarkably inhibited the development of HCC.

The EMT pathway is an important signaling pathway related to tumorigenesis^{26,27}. EMT has been greatly focused on because of its role in the occurrence and development of epithelial cell malignancy²⁸. Multiple studies have suggested that EMT plays a pivotal role in the primary and secondary metastases of breast cancer, colon cancer, lung cancer, prostate cancer, liver cancer, and pancreatic cancer^{28,29}. Therefore, studying the occurrence and regulatory mechanism of EMT is of great significance for finding novel targets for the treatment of malignant tumors, especially the metastasis of tumor cells³⁰. In this experiment, the knockdown of miR-5692a significantly decreased the expression of EMT-related genes, such as N-cadherin, Vimentin, β -catenin, and MMP9, indicating that miR-5692a might promote HCC via the EMT pathway.

Matrix metalloproteinases (MMPs) are a group of metal ion-dependent proteases that can degrade various proteins in the extracellular matrix. MMP activity is closely related to the invasion and metastasis of tumors³¹. MMP9 is a member of the MMPs family, which can degrade almost all the components of the extracellular matrix³². Recent studies^{33,34} have shown that the upregulation of HIF-1 and MMPs mediated by hypoxia may be correlated with tumor angiogenesis. MMP9 amplification is associated with increased cell differentiation, local and distant metastasis, migration, decreased apoptosis, accelerated angiogenesis, and tumor invasion³⁵. Under physiological conditions, MMP9 participates in embryonic development and wound repair. However, it can promote tumor growth, invasion and metastasis by destroying the extracellular matrix and basement membrane barrier of local tissues

because of external stimuli^{32,35}. Our study found that higher positive-expressed MMP9 in HCC tissues was positively related to poor differentiation, high lymph node metastasis and high clinical staging, which were consistent with previous studies. Overexpressed MMP9 could promote the metastasis of tumor cells via regulating adhesion factors³⁶. Our data demonstrated that miR-5692a regulated HCC development via MMP9.

To prove whether miR-5692a promoted HCC development through regulating the EMT signaling pathway, we detected the expression of EMT-related genes, including N-cadherin, Vimentin, β -catenin, and MMP9 after miR-5692a knockdown. Western blot results elucidated that the protein expression of EMT-related genes was remarkably downregulated, indicating miR-5692a promoted HCC development via regulating the EMT pathway. Moreover, MMP9 expression was decreased after the knockdown of miR-5692a, further suggesting that miR-5692a participated in the development of HCC via regulating MMP9.

Conclusions

We showed that miRNA-5692a was over-expressed in HCC, which was remarkably correlated with clinical stage, distant metastasis and poor prognosis of HCC. In addition, miR-5692a promoted malignant progression of HCC via regulating MMP9.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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