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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 [illegible] 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 [illegible] para-cancerous tissues. HCC patients with higher miR-5692a expression exhibited a higher incidence 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 HCC[9]. Unfortunately, the unclear pathogenesis of HCC brings huge difficulty in precise diagnosis and treatment[9,10]. Therefore, it

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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 protein [14]. Relative experiments[15-17] have shown that differentially expressed miRNAs are closely related to malignancies and can be served as biomarkers in tumors. Recent works[16] have found that miRNAs are tissue-specific, which can promote [illegible] liferation, invasion and metastasis of [illegible] through various mechanisms, eventually [illegible] ipating in the incidence and development [illegible] mors[18]. MiRNAs have been found to be [illegible] in multiple tumors, such as [illegible] breast cancer, osteosarcoma [illegible] cell lung cancer[19-21]. The [illegible] of [illegible] in regulating tumor [illegible] how[illegible] not clearly elucidated. It is [illegible] miRNAs play an [illegible] regulating the proliferation, [illegible] cycle, [illegible] differentiation and [illegible] tumor [illegible] different aspects[17,22] [illegible], miR-520f and other miRNAs [illegible] been [illegible] participate in the [illegible] of HCC[23-25]. [illegible] researches[25] have [illegible] that the [illegible] pathway, the epidermal [illegible] receptor (EGFR) pathway, [illegible] factor-Beta (TGF-β) inhibition, [illegible] and [illegible] epithelial-mesenchymal [illegible] T), [illegible] potential mechanisms [illegible] C [illegible]

[illegible] present [illegible] work, we detected the expression [illegible] miR-5692a in HCC tissues and paired [illegible] tissues. Further analysis detected [illegible] role of miR-5692a in regulating cellular functions of HCC cells. The aim of this study was [illegible] 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 [illegible] tissues and para-cancerous [illegible] collected. All patients were [illegible] diagnosed as HCC according to the 8th [illegible] UICC/AJCC. HCC patients did [illegible] ative anti-tumor therapies. [illegible] was [illegible] by the Ethics [illegible] Hospital. Informed [illegible] from all the patients.

Cell Culture

Six [illegible] cell lines ([illegible], HepG2, MHCC9[illegible]7221, Huh7 [illegible] Hep3B) and one [illegible] liver [illegible] (LO2) were obtained from [illegible] Collection (ATCC) ([illegible] VA, USA). [illegible] were cultured in [illegible] (Dulbecco's Modified Eagle Medium) [illegible] 10% [illegible] bovine serum (FBS), and [illegible] maintained [illegible] a 37°C, 5% CO_2 incubator ([illegible] Rockvi[illegible] MD, USA).

Cell [illegible]

Negative control (si-RNA-NC) and small [illegible] sequence of miR-5692a (si-miR-[illegible]) 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 μL 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, [illegible], USA). The concentration of extracted RNA was [illegible] by an ultraviolet spectrophotometer ([illegible]kyo, Japan). The complementary deoxyribose nucleic acid (cDNA) was then synthesized according to the instructions of the [illegible] RT Reagent ([illegible], Shiga, Japan). The relative expression level of target genes was calculated by [illegible]$^{\Delta\Delta Ct}$. Primers used in this study were as follows: MiR-5692a: [illegible]-AGAGGGGT-GGA[illegible]GGAG[illegible]TAG-3’; U6: 5’-TGCGGGT-GC[illegible]CTTC[illegible]GCAG[illegible]3’; MMP9: forward, 5’-TT[illegible]CTTTT[illegible]GGGCGA-3’, reverse, [illegible]CAAA[illegible]GTC[illegible]AATCACC-3’; β-actin: forward, 5’[illegible]ACCCAGCACAAT-3’, reverse, [illegible]’-TGCC[illegible]AGGTGTCCCTTTG-3’.

[illegible]

The radio-immunoprecipitation assay (RI[illegible]) 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 [illegible] transferred to polyvinylidene difluor[illegible] membranes (Merck Millipore, [illegible], MA, USA). After blocking with 5% [illegible]med milk, the membranes were incubated [illegible] primary antibody (Cell Signaling Technology, [illegible], MA, USA) at 4°C overnight. After washing [illegible] Tris-buffered saline [illegible] Tween (TBST), [illegible] membranes were then incubated with secondary antibody. Chemilum[illegible] was used [illegible] expose the protein bands.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22[illegible] Software Package (IBM, Armonk, NY, USA) was used [illegible] all statistical analysis. The Kaplan-Meier curve was performed for analyzing the prognosis of HCC patients. Difference [illegible] curves was compared by using the Log-rank [illegible]. The *t*-test was used to analyze the difference between two groups, while the x^2-test was used for [illegible] 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

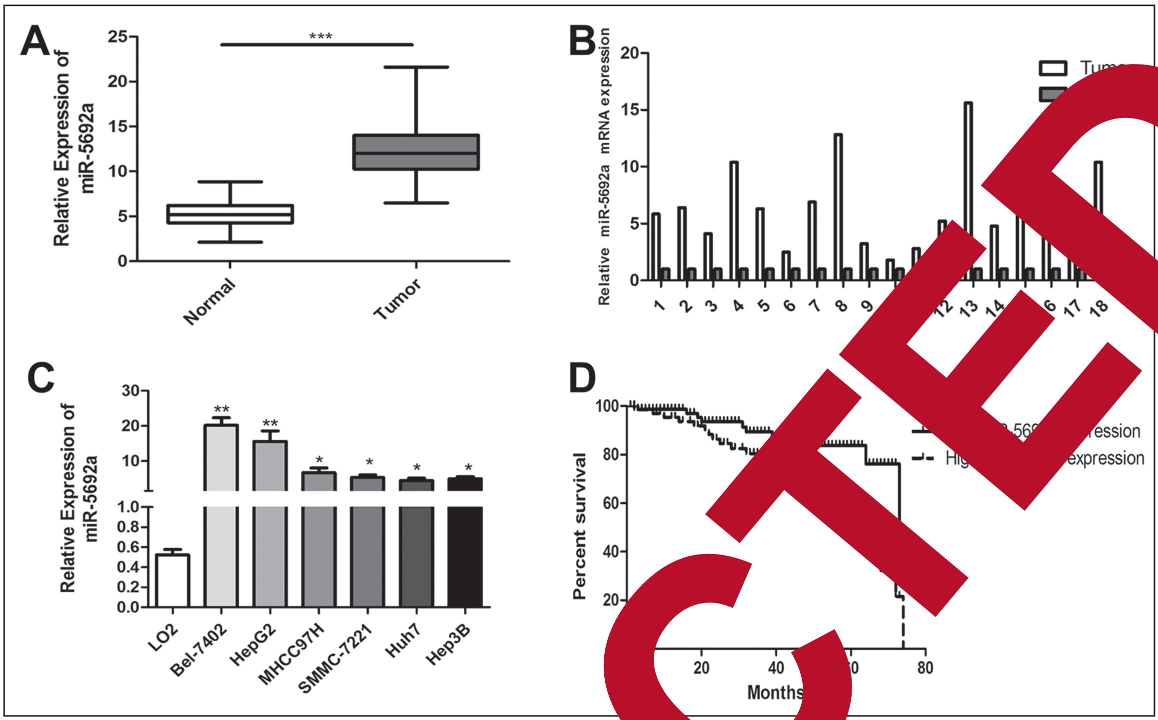


Figure 1. MiR-5692a was overexpressed in HCC tissues and cell li[illegible] **B,** Th[illegible]ession of miR-5692a in HCC tissues was significantly increased. **C,** The expression level of miR-5692a in 6 HC[illegible] (Bel-7402, HepG2, MHCC96H, SMMC-7221, Huh7 and Hep3B) and 1 normal hepatic cell [illegible]02). **D,** Kaplan-Meier survival curves of HCC patients based on miR-5692a expression. The prognosis of patients i[illegible] [illegible]on group was poorer than those in low expression group.

patients were collected for analy[illegible] the overa[illegible] survival. The Kaplan-Meier [illegible]ved that miR-5692a expression was [illegible]atively [illegible]rrelated with the prognosis of H[illegible] patients [illegible] 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. Associatio[illegible] 5692aexpress[illegible] clinicopathologic characteristics of hepatocellular carcinoma.

Para[illegible]rs	[illegible]er of cases	miR-5692a expression Low (%)	miR-5692a expression High (%)	*p*-value
Age [illegible]s)				0.569
[illegible]	42	25	17	
≥[illegible]	54	29	25	
Gende[illegible]				0.143
[illegible]ale	47	30	17	
[illegible]ale	49	24	25	
T[illegible]				0.032
[illegible]2	53	35	18	
[illegible]	43	19	24	
[illegible]mph no[illegible]etastasis				0.035
No	55	36	19	
[illegible]es	41	18	23	
[illegible]e metastasis				0.023
[illegible]	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).

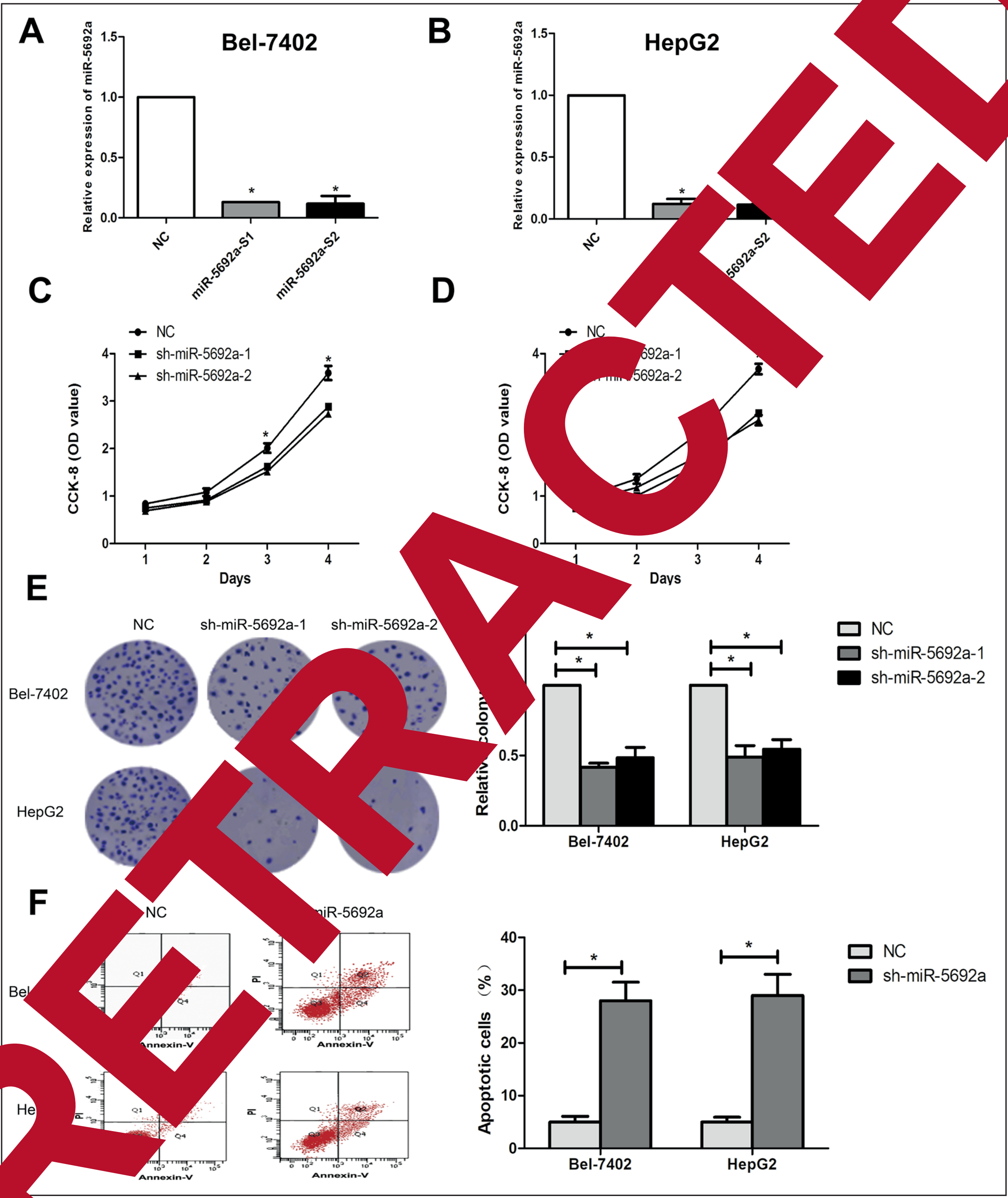


Figure 2. The effect of miR-5692a on cellular functions of HCC cells. **A, B,** QRT-PCR was used to verify the transfection efficiency of miR-5692a knockdown in Bel-7402 and HepG2 cells. **C, D,** Growth curve analysis showed the cell growth of Bel-7402 and HepG2 cells after miR-5692a knockdown. **E,**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

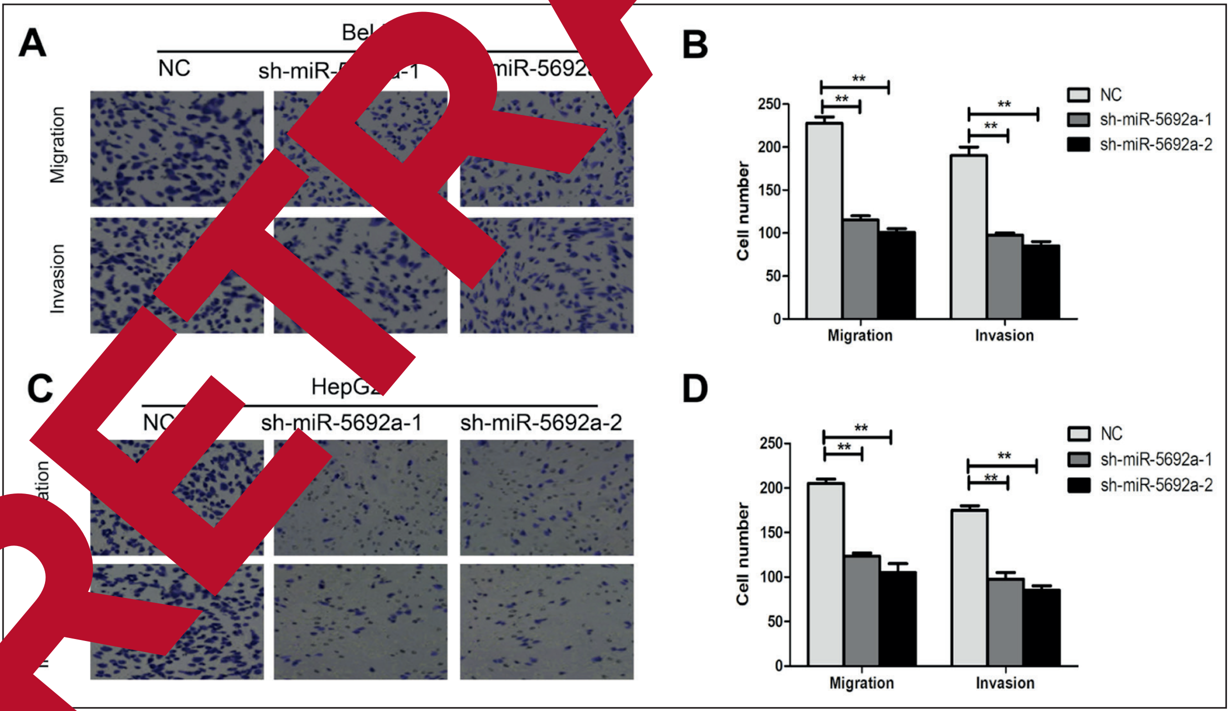
Knockdown of miR-5692a Downregulated EMT-Related Genes

To further explore the potential mechanism of miR-5692a in regulating HCC cells, we det[illegible]d the protein expression of EMT-related [illegible] Knockdown of miR-5692a led to the dow[illegible]gulated expression of EMT-related genes, including N-cadherin, Vimentin, β-catenin and MMP9 (Figure 4A).

MMP9 Modulated the Expres[illegible] of miR-5692a in Human HCC [illegible]

Bioinformatics methods have [illegible]ted that MMP9 can interact with mi[illegible]5692a. [illegible] present study, we found that [illegible]P9 was do[illegible]lated in HCC tissues th[illegible]hat of [illegible]ra-canc[illegible] tissues (Figure 4B). [illegible]eover, [illegible] expression of MMP9 in HCC cell [illegible] wa[illegible]ver tha[illegible]at of normal liver cell[illegible] (Fi[illegible]C).

Furthermor[illegible]miR-5692[illegible] f[illegible]d upregulated afte[illegible]9 knockdow[illegible]HCC cells. We specu[illegible]d [illegible] whether mi[illegible]5692a could regulate [illegible]MP9. [illegible] we detected the expressi[illegible]f miR-5692[illegible] MMP9 in 16 pairs of [illegible]issues and pa[illegible]-cancerous tissues. [illegible] protein and mRNA levels of miR-5692a [illegible]e negativel[illegible]orrelated with MMP9 ex[illegible]sion (Figure [illegible]). For *in vitro* experiments, M[illegible]9 was als[illegible]ownregulated in HCC cells whe[illegible]with that of LO2 cells (Figure 5A). Su[illegible]quently, we constructed si-MMP9 for cell transfection, and the transfection ef[illegible]as verified by Western blot (Figure



Fig[illegible]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.

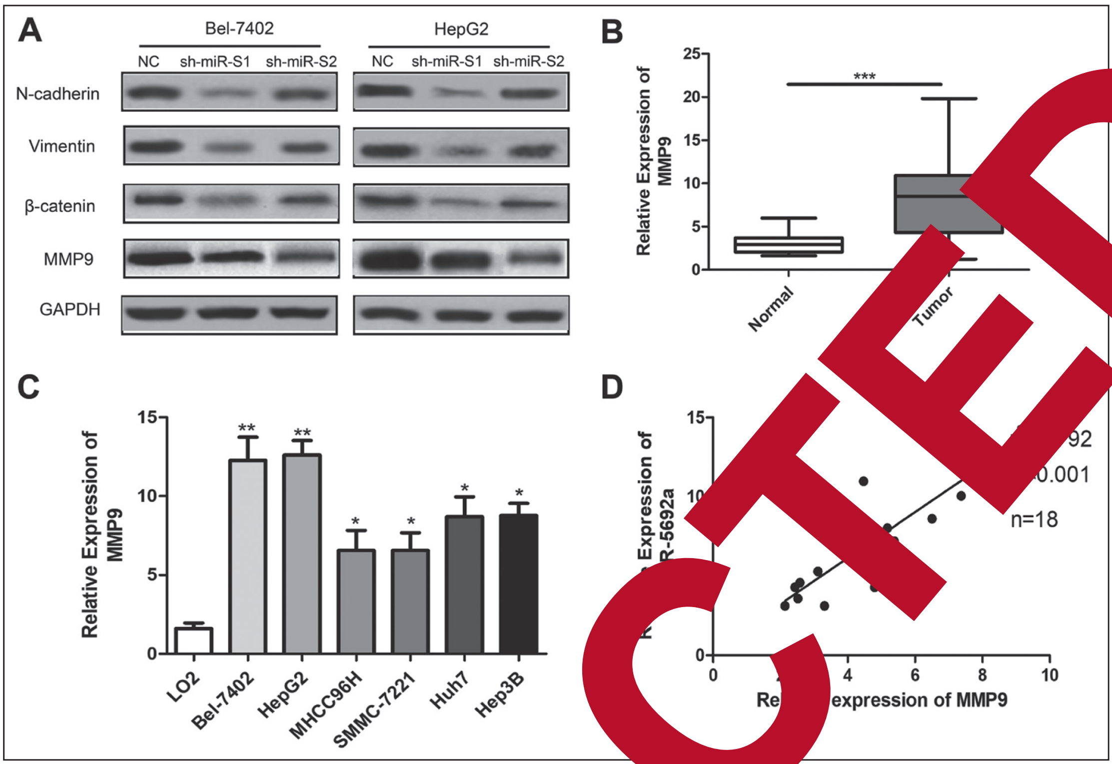
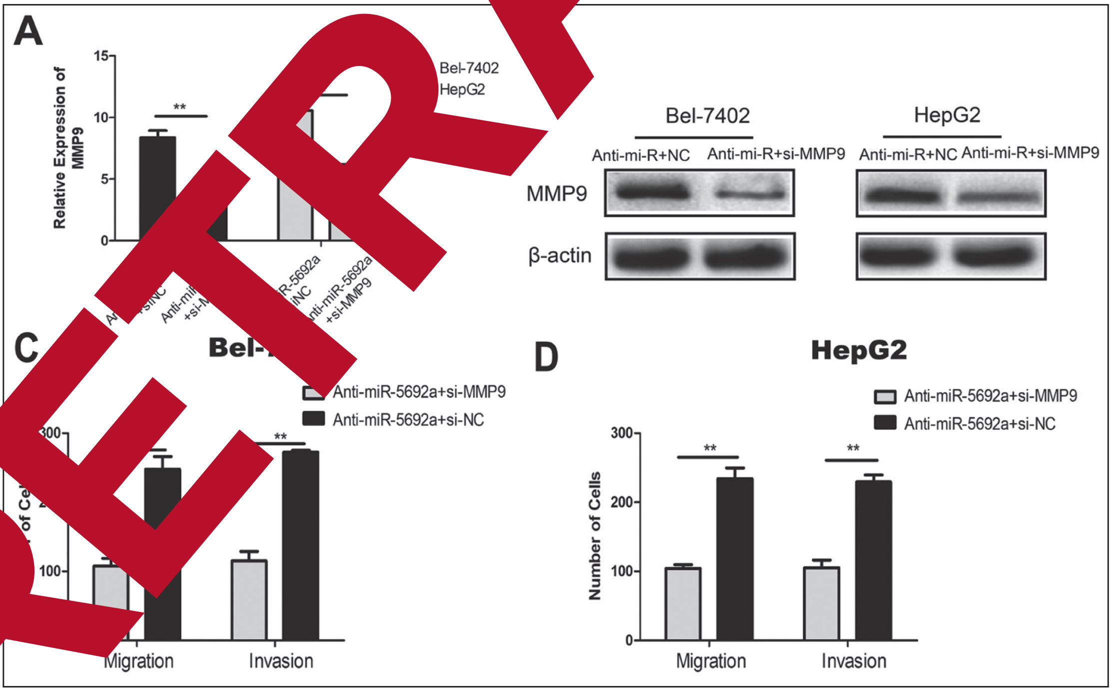


Figure 4. MiR-5692a regulated HCC development vi[illegible]lating the EMT pa[illegible]. **A,** Knockdown of miR-5692a significantly decreased the expression of EMT-related genes, i[illegible]herin, Vimentin, β-catenin and MMP9. **B, C,** The mRNA expression level of MMP9 relative to GAPDH in h[illegible] HC[illegible] corresponding adjacent tissues, and cell lines were detected by qRT-PCR. **D,** A negative correlation was [illegible]d betw[illegible]2a and MMP9 in tumor samples.



[illegible] **5.** MiR-5692a regulated HCC cells via regulating MMP9. **A,** The mRNA expression of MMP9 was verified by qRT-PC[illegible]6-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 majority[7]. 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, ov[illegible] HCC patients have experienced micro-metastases before undergoing radical surgery, whi[illegible] the direct cause of metastasis and postoperative recurrence of HCC[9]. Research[illegible] exploring postoperative metastasis, rec[illegible] and adjuvant therapy of HCC have been w[illegible] recognized[9,12]. Molecular chan[illegible] in HCC [illegible] such as changes in gene copy n[illegible] a[illegible]tion of coding seque[illegible], exe[illegible]rtant effects on tumor phenoty[illegible],25. Multip[illegible] differentially expressed miR[illegible] have been fou[illegible] HCC, which may pl[illegible] cr[illegible] role in the diagnosis, treatment and prognos[illegible] HCC[25]. Hence, the relationship between differentially expressed miRNA[illegible] HCC and clinical prognosis needs to be well investigated, so as to improve the ou[illegible] of [illegible] patients.

Acc[illegible]g evid[illegible] has shown that miRNAs pa[illegible] in[illegible] biological behavior of [illegible] which [illegible]ovide a new direction for the [illegible]gnosis a[illegible] treatment of HCC in the future. MiRNA is a single-stranded non-coding R[illegible] nucleotides in length. Functionally, [illegible]RNA degrades target mRNAs or inhibits its [illegible]lation, thereby regulating the expression of d[illegible]eam 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 cells[18]. It has been believed that miRNAs may serve as targets for the development of novel a[illegible] drugs[25]. In the present study, we [illegible] role of miR-5692a in HCC and investigated the potential mechanism. We found [illegible] miR-5692a was overexpressed in HCC tissues [illegible] that of para-cancerous tissues. The expression [illegible] miR-5692a was positively correlated with the [illegible] stage of HCC, lymph[illegible] metastasis, d[illegible] metastasis and poor prognosis. [illegible] further explore the effects of [illegible] the biological functions of HC[illegible] cells [illegible], we constructed small interfering sequence[illegible] miR-5692a. Subsequent cell experiments [illegible]strated that miR-5692[illegible] remarkably inhibited the development of HC[illegible]

The EMT pathway [illegible] important signaling pa[illegible] related to tumor[illegible]enesis[26,27]. EMT has [illegible] greatly focused on because of its role in the [illegible]urrence and [illegible]velopment of epithelial cell [illegible]gnancy[28]. M[illegible]ple studies have suggested th[illegible]MT plays [illegible]votal role in the primary and sec[illegible]ases of breast cancer, colon cancer, [illegible] cancer, prostate cancer, liver cancer, and pancreatic cancer[28,29]. Therefore, studying the [illegible]ce and regulatory mechanism of EMT [illegible] of g[illegible]at significance for finding novel targets for the treatment of malignant tumors, especially the metastasis of tumor cells[30]. 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 tumors[31]. MMP9 is a member of the MMPs family, which can degrade almost all the components of the extracellular matrix[32]. 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 invasion[35]. 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 factors[36]. 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 [illegible]r-expressed in HCC, which was remark[illegible] correlated with clinical stage, distant met[illegible]tasis and poor prognosis of H[illegible] addition[illegible] miR-5692a promoted malig[illegible]ssion o[illegible] HCC via regulating MMP[illegible]

Acknowledgement[illegible]
This study was suppo[illegible]y Shandon[illegible]ncial Natural Science Foundation [illegible]6HL33).

Conflict o[illegible]terest
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Refe[illegible]ces

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