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MicroRNA-328-3p inhibits malignant progression of hepatocellular carcinoma by regulating MMP-9 level

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Abstract. – OBJECTIVE: This study aims to explore the influence of microRNA-328-3p on proliferative and apoptotic abilities of hepatocellular carcinoma (HCC) cells, and the potential regulatory mechanisms.

PATIENTS AND METHODS: Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was performed to detect the expression of microRNA-328-3p in 52 tumor tissue samples and paracancerous ones of HCC patients. The potential interaction between microRNA-328-3p level and clinical indicators of HCC patients was analyzed. Subsequently, the microRNA-328-3p overexpression model was established. In addition, the influence of microRNA-328-3p on the biological functions of the HCC cells was analyzed by the cell counting kit-8 (CCK-8), colony formation assay, and flow cytometry. Finally, the potential downstream gene of microRNA-328-3p was explored by bioinformatics analysis. Recovery experiments were performed to explore the regulation mechanism.

RESULTS: QRT-PCR results revealed that microRNA-328-3p level in tumor tissue specimens of HCC patients was remarkably lower than that in adjacent ones, and the difference was statistically significant. Compared with patients with high expression of microRNA-328-3p, those with low expression of miR-328-3p had more advanced pathological staging and lower overall survival. The overexpression of microRNA-328-3p decreased the proliferative capacity and increased apoptotic rate in HCC cells. Subsequently, MMP-9 expression was found to be highly expressed in HCC tissues and cells, and negatively correlated with microRNA-328-3p level. In addition, microRNA-328-3p overexpression significantly down-regulated the protein expressions of CD31, Ki-67, c-Myc, MMP-2, and MMP-9. In the cell reverse experiment, the overexpression of MMP-9 could counteract the influence of the overexpressed microRNA-328-3p on proliferation and apoptosis in HCC cells, so as to regulate the malignant progression of HCC.

CONCLUSIONS: MicroRNA-328-3p could inhibit the malignant progression of HCC. Its level is remarkably associated with the pathological staging and prognosis of HCC patients. In addition, it is found that microRNA-328-3p might suppress the proliferative ability and promote apoptosis of HCC cells *via* modulating MMP-9.

Key Words:
MicroRNA-328-3p, MMP-9, Hepatocellular carcinoma, Proliferation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. Similar to other malignancies, the molecular mechanisms of HCC include the inactivation of tumor-suppressor genes and/or the activation of proto-oncogenes in a multi-step and multi-stage process[1-3]. The etiology of HCC has been basically clarified. Currently, viral hepatitis, alcoholism, and non-alcoholic fatty liver lesions are considered as the main causes of HCC[4,5]. The mortality rate of HCC is extremely high, ranking the third among malignant tumors worldwide[5,6]. In China, the mortality rate of HCC is second only to gastric cancer. About 110,000 people die of liver cancer every year, so it has become a serious threat to the

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health and lives of Chinese people[7,8]. Symptoms and signs of HCC in the early stage are atypical, and most patients are already in the middle and late stages when they are diagnosed. The commonly used imaging examination and detection of serum AFP have limited abilities for the diagnosis of early-stage HCC. Clinically, the overall prognosis of HCC patients is extremely poor, and the 5-year survival rate is low. Therefore, it is especially important to actively seek more effective diagnosis and treatment for HCC[9,10]. In recent years, microRNA (miRNA) has become a research hotspot in the field of molecular biology, and its abnormal expression has a good application prospect in the diagnosis, treatment, prognosis, and other aspects of tumor diseases[11,12].

MiRNAs are a class of highly conserved, single-strand, non-coding RNAs with a length of about 18-25 nucleotides, which are found not only in tissues and cells, but also in the circulatory system[13,14]. MiRNA can negatively regulate the expression of the target gene and eventually leads to tumor formation through complete or/and incomplete pairing with the 3' terminal non-translation region (3'UTR) of the target gene[15]. Each miRNA can regulate the expressions of multiple target genes, and multiple miRNAs can also regulate the same target gene at the same time, thus forming a complex regulatory network[16,17]. Mature miRNAs can participate in a variety of biological activities, including cell proliferation, apoptosis, cell division, cell migration, hormone secretion, and cell infiltration[18,19]. Previous studies[20,21] have revealed that some miRNAs are up-regulated in HCC as oncogenes, which are called "tumor miRNAs", while some miRNAs are down-regulated in HCC as tumor-suppressor genes. MicroRNA-328-3p is a newly discovered cancer-related miRNA, which has been confirmed to be down-regulated in osteosarcoma, ovarian cancer, and other malignant tumors, playing the role of a tumor-suppressor gene[22,23]. However, its expression and function in HCC are rarely reported. Through bioinformatics analysis, it was speculated that microRNA-328-3p might target MMP-9 and inhibit the malignant progression of HCC, thus becoming a new direction of tumor-targeted therapy.

Based on the above researches, in this study, we elaborated the possible roles of microRNA-328-3p and MMP-9 in the occurrence and development of HCC, and the underlying mechanism, so as to bring new ideas for the diagnosis and treatment of HCC.

Patients and Methods

Patients and HCC Samples

The tumor tissue samples and paracancerous ones were collected from 52 HCC patients aged 32-89 years who underwent radical resection. None of the patients received any radiotherapy or chemotherapy before surgery. The pathological subtype and staging criteria of HCC were performed according to the guidelines proposed by the Union for International Cancer Control (UICC). Patients and their families in this study had been fully informed. This study was approved by the Ethics Committee of Jinan Infectious Disease Hospital Affiliated to Shandong University. The written signed informed consents were obtained from all participants before the study.

Cell Lines and Reagents

Six human HCC cell lines (Bel-7402, HepG2, MHCC88H, SMMC-7221, Huh7, Hep3B) and one human normal liver cell line (LO2) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and the Dulbecco's Modified Eagle's Medium (DMEM) medium and the fetal bovine serum (FBS) were obtained from Gibco (Rockville, MD, USA). The cells were cultured in high-glucose DMEM containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C with 5% CO_2. Until 80%-90% confluence, the cells were digested with 1×trypsin+EDTA (ethylenediaminetetraacetic acid).

Transfection

The negative control (NC) and the microRNA-328-3p mimics were purchased from GenePharma (Shanghai, China). After, the cells were plated in 6-well plates and grown to a cell density of 70%, transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 hours, the cells were collected for qRT-PCR analysis and functional experiments.

Cell Counting Kit-8 (CCK-8) Assay

The cells were harvested and inoculated into 96-well plates with 2000 cells per well. After cell culture for 24 h, 48 h, 72 h, and 96 h, CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was applied. After incubation for 2 hours, the optical density (OD) value of each well was measured in the microplate reader.

Colony Formation Assay

After 48 h of transfection, the cells were collected, and 200 cells were seeded in each well of a 6-well plate and cultured in complete medium for 2 weeks. The medium was changed after one week, and then twice a week. The medium should not be replaced as much as possible in the first week to avoid cell adhesion. After 2 weeks, the cells were washed twice with PBS. The cells were fixed in 2 ml of methanol for 20 minutes. After the methanol was aspirated, the cells were stained with 0.1% crystal violet staining solution for 20 minutes, washed 3 times with PBS, and photographed and counted under a light-selective environment.

Flow Cytometry

The cells in the logarithmic growth phase were seeded into 6-well plates. After 24 hours of drug treatment, the cells were collected, washed twice with PBS, and resuspended in the binding solution. After incubation at room temperature for 15 min in the dark, the cells were gently mixed with 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and Propidium Iodide (PI). The apoptotic rate was measured using flow cytometry.

QRT-PCR

After the cells were accordingly treated, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and the total RNA was extracted. The initially extracted RNA was treated with DNase I to remove the genomic DNA and to purify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, the Real Time-PCR was performed according to the SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) kit instructions, and the PCR reaction was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Three replicate wells were set for each sample and the assay was repeated twice. Bio-Rad (Hercules, CA, USA) PCR instrument was used to analyze and process the data with software iQ5 2.0. β-actin and U6 genes were used as internal controls, and the gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used were as follows: MMP-9, F: 5'-GCGAGGTGCGCCTTAGTC-3', R: 5'-CGGTGCCGGATAGTCCACAGGA-3'; microRNA-328-3p, F: 5'-GCTGTACAATATCCTACGACTAG-3', R: 5'-AGTTGCAGTCGAGGACGTCAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GCTGATCCACATCTGCTGGAA-3'.

Western Blot Assay

The cells or tissue specimens were lysed using cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 × g for 15 minutes at 4°C. The total protein concentration was calculated by the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, Il, USA). Anti-CD31, Ki-67, c-Myc, MMP-2, and the MMP-9 monoclonal antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA), while horseradish peroxidase-labeled goat anti-rabbit secondary antibody was purchased from Genscript (Nanjing, China). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference control. The protein samples were separated using the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked overnight, and then the primary antibodies and secondary antibodies were in turn added for enhanced chemiluminescence (ECL) coloration. The images were semi-quantitatively analyzed by the Alpha SP image analysis software.

Statistical Analysis

The statistical analysis was performed using the Statistical Product and Service Solutions (SPSS) 22. 0 software (IBM Corp., Armonk, NY, USA). The univariate analysis was performed using the χ^2-test and the Fisher test. The multivariate analysis was performed using the COX regression analysis. The Kaplan-Meier method was introduced for survival analysis, followed by the Log-rank test to compare the intergroup curves. The data were expressed as mean ± standard deviation, and $p<0.05$ was considered to be statistically significant.

Results

MicroRNA-328-3p Was Downregulated in HCC Tissues, and Its Level Was Correlated with Advanced Clinical Staging and Poor Prognosis

In order to clarify the role of microRNA-328-3p in HCC, we collected 52 pairs of HCC tissue samples and adjacent ones, and detected the expression of microRNA-328-3p using qRT-PCR.

The results showed that microRNA-328-3p level was remarkably lower in HCC tissues than in the adjacent ones (Figure 1A). In addition, microRNA-328-3p was remarkably down-regulated in HCC cells compared with normal hepatic cell line LO2, especially in the Huh7 and Bel-7402 cell lines. As a result, we chose these two cell lines for follow-up experiments (Figure 1B). According to the expression of microRNA-328-3p in tumor tissue samples and paracancerous ones of 52 pairs of HCC, the tissue specimens were divided into high microRNA-328-3p and low microRNA-328-3p expression group. Subsequently, we analyzed the relationship between microRNA-328-3p expression and age, gender, pathological staging, lymph node, or distant metastasis of HCC patients. As shown in Table I, the low expression of microRNA-328-3p was positively correlated with the pathological staging of HCC, but not with other indicators. In addition, the Kaplan-Meier survival curves demonstrated that the low expression of microRNA-328-3p was remarkably relevant to the poor prognosis of HCC (p<0.05; Figure 1C).

Therefore, the above results suggested that microRNA-328-3p might be a new biological indicator for predicting the malignant progression of HCC.

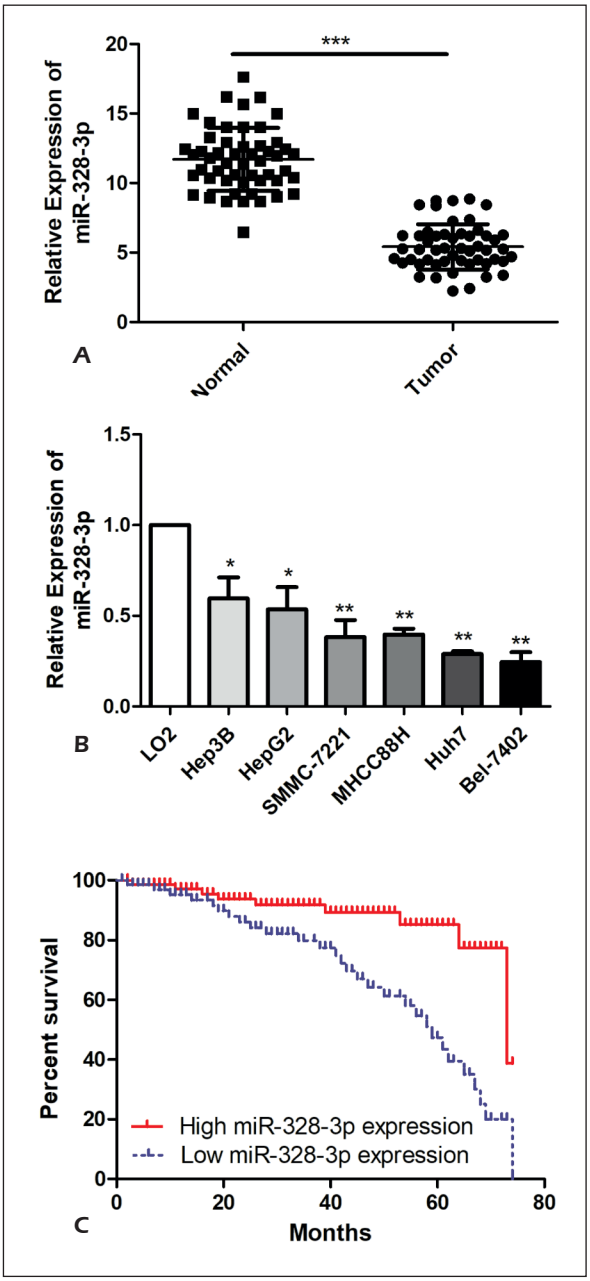

Figure 1. MiR-328-3p is down-regulated in hepatocellular carcinoma tissues and cell lines. **A**, qRT-PCR was used to detect the expression level of miR-328-3p in tumor tissues and adjacent tissues of hepatocellular carcinoma. **B**, qRT-PCR was used to detect the expression level of miR-328-3p in the hepatocellular carcinoma cell lines. **C**, The Kaplan-Meier survival curves of the hepatocellular carcinoma patients based on miR-328-3p expression. The data are expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Upregulation of MicroRNA-328-3p Inhibited Cell Proliferation and Promoted Cell Apoptosis

To explore the effects of microRNA-328-3p on the function of HCC cells, microRNA-328-3p overexpression model was first successfully constructed (Figure 2A). The cell proliferation and flow cytometry assays were subsequently performed in the Huh7 and Bel-7402 cell lines. CCK-8 assay showed that the cell proliferative rate remarkably decreased after the overexpression of microRNA-328-3p (Figure 2B). A similar result was observed in the colony formation assay (Figure 2C). On the contrary, the flow cytometry analysis revealed that the apoptosis of HCC cells remarkably increased after the overexpression of microRNA-328-3p compared with the NC group (Figure 2D). These results suggested that the up-regulation of microRNA-328-3p inhibited cell proliferation and promoted cell apoptosis in HCC.

MMP-9 Was Highly Expressed in HCC Tissues and Cell Lines

QRT-PCR was performed to verify the expression of MMP-9 in HCC tissues and cell lines, and the results showed that MMP-9 level was remarkably up-regulated in HCC tumor tissues compared with adjacent tissues (Figure 3A). In addition, MMP-9 was also found to be highly expressed in HCC cells than in LO2 cells (Figure 3B).

Table I. Association of miR-328-3p expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	Number of cases	miR-328-3p expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.812
<60	21	11	8	
≥60	31	19	12	
Gender				0.357
Male	25	17	8	
Female	27	15	12	
T stage				0.004
T1-T2	31	24	7	
T3-T4	21	8	13	
Lymph node metastasis				0.111
No	33	23	10	
Yes	19	9	10	
Distance metastasis				0.093
No	38	26	12	
Yes	14	6	8	

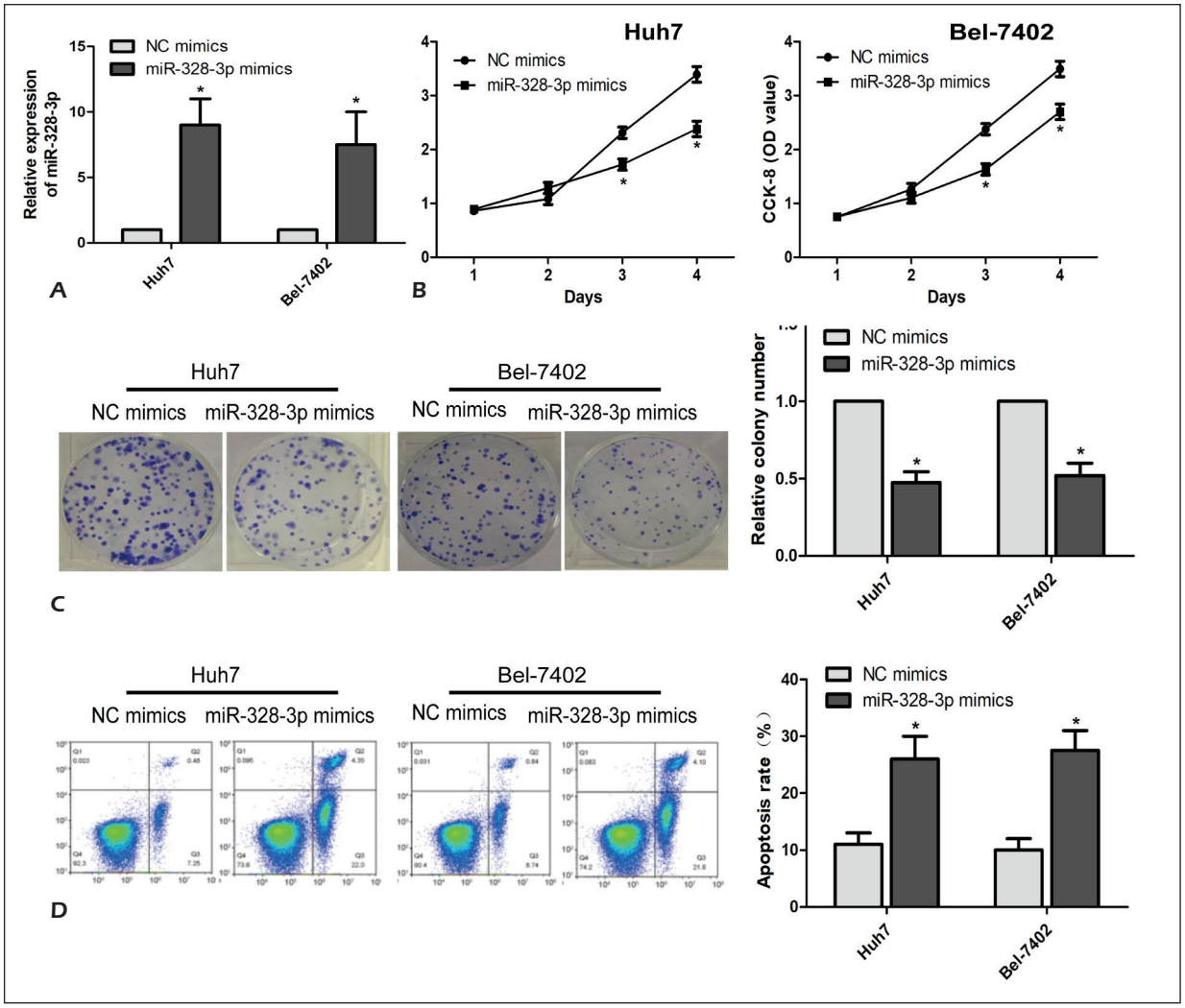

Figure 2. MiR-328-3p overexpression inhibits the proliferation of the hepatocellular carcinoma cells and promotes apoptosis. **A**, qRT-PCR verified the transfection efficiency of miR-328-3p mimics in Huh7 and Bel-7402 cells. **B**, CCK-8 assay detects the effect of miR-328-3p on the proliferation in Huh7 and Bel-7402 cells. **C**, The colony formation assay detects the effect of miR-328-3p on the clonality in Huh7 and Bel-7402 cells (magnification × 40). **D**, The flow cytometry assay was used to detect the effect of miR-328-3p on apoptosis in Huh7 and Bel-7402 cells. The data are expressed as mean ± SD, **p*<0.05.

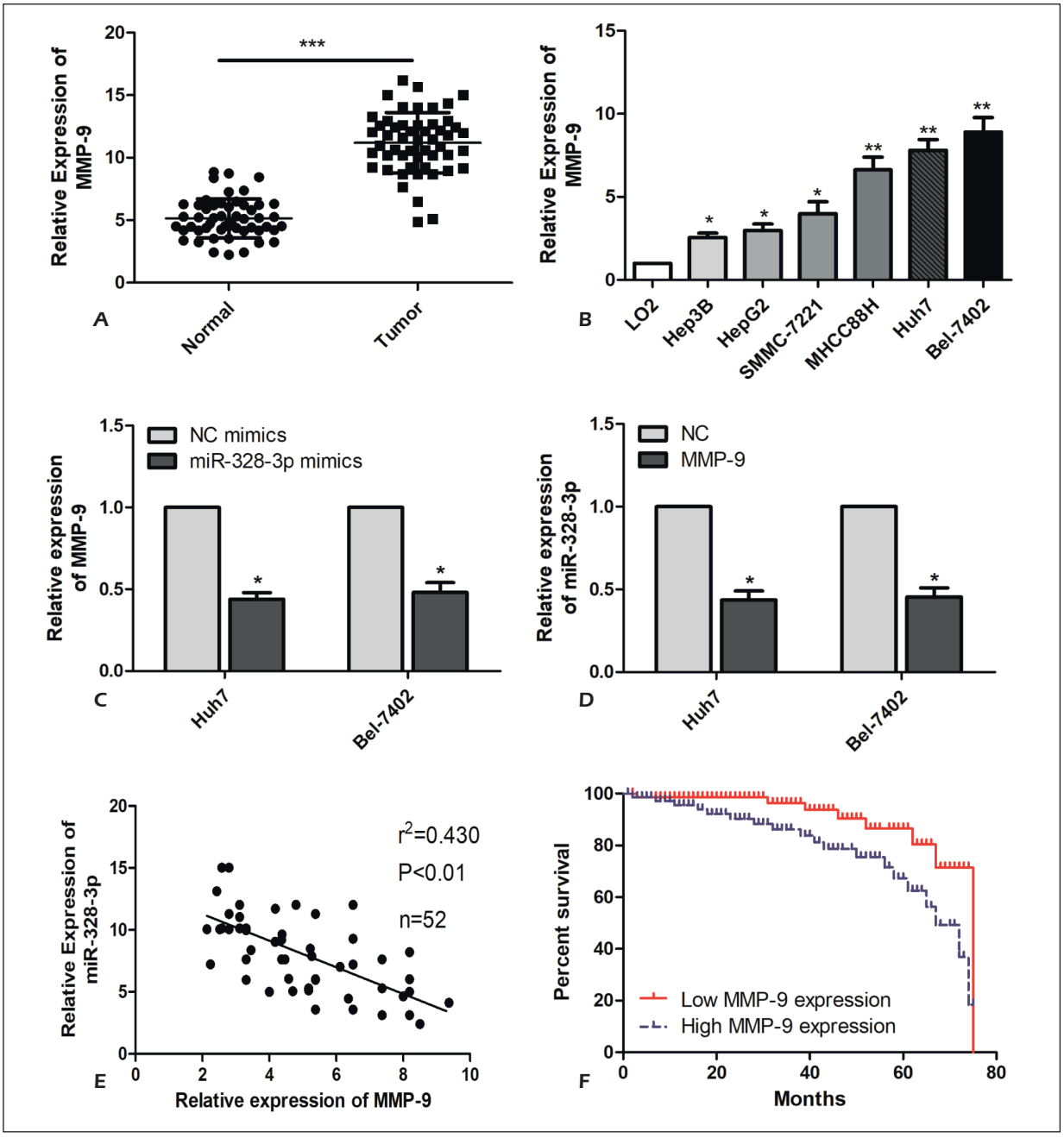

Figure 3. MMP-9 is highly expressed in hepatocellular carcinoma tissues and cell lines. **A**, qRT-PCR was used to detect the expression level of MMP-9 in hepatocellular carcinoma tumor tissues and adjacent tissues. **B**, qRT-PCR was used to detect the expression level of MMP-9 in hepatocellular carcinoma cell lines. **C**, qRT-PCR verified the expression level of MMP-9 after transfection of miR-328-3p mimics in Huh7 and Bel-7402 cells. **D**, qRT-PCR verified the expression level of miR-328-3p after the overexpression of MMP-9 in Huh7 and Bel-7402 cells. **E**, A significant negative correlation between miR-328-3p and MMP-9 expression levels in the hepatocellular carcinoma tissues. F, The Kaplan-Meier survival curves of the hepatocellular carcinoma patients based on the MMP-9 expression. The data are expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Besides, MMP-9 expression significantly decreased in Huh7 and Bel-7402 cells after the overexpression of microRNA-328-3p (Figure 3C). Likewise, microRNA-328-3p level remarkably decreased after the overexpression of MMP-9 (Figure 3D). Additionally, the expression levels of microRNA-328-3p and MMP-9 presented a significant negative correlation in tumor tissues of HCC patients (Figure 3E). According to the MMP-9 level in 52 HCC tissue samples and

paracancerous ones, the relationship between the expression of MMP-9 and the prognosis of HCC patients was explored. The Kaplan-Meier survival curves revealed that the high expression of MMP-9 was remarkably associated with poor prognosis of HCC (p<0.05; Figure 3F).

Up-Regulation of MicroRNA-328-3p Decreased the Expressions of MMP-9-Related Signaling Pathway Genes

In order to explore how microRNA-328-3p promoted the malignant progression of HCC, the expressions of the key proteins in MMP-9-related pathways after overexpression of microRNA-328-3p were detected by the Western Blot. The results showed that the expressions of the key proteins, including CD31, Ki-67, c-Myc, MMP-2, and MMP-9 in the signaling pathway significantly decreased (Figure 4).

MicroRNA-328-3p Directly Inhibited MMP-9 Expression

It is speculated that MMP-9 was responsible for the malignant progression of HCC influenced by microRNA-328-3p. QRT-PCR assay examined the microRNA-328-3p level (Figure 5A) and the Western Blot examined MMP-9 level (Figure 5B) in the co-transfected cells. Subsequently, the overexpression of MMP-9 was demonstrated to counteract the effects of the overexpressed microRNA-328-3p on the proliferation and apoptosis of HCC cells (Figures 5C and 5D). Therefore, these results revealed that microRNA-328-3p regulated the HCC progression by modulating the MMP-9 level.

Discussion

HCC accounts for more than 90% of primary HCC cases, and more than 520,000 people die of HCC every year. China is a typical high-risk region, accounting for more than 50% of HCC cases in the world[4-6]. The insidious onset and high degree of malignancy of HCC lead to the loss of effective treatment opportunities for HCC patients at the time of diagnosis. Early detection and prompt treatment for HCC contribute to prolong the survival period of patients[7,8]. The diagnosis and screening of HCC today are mainly based on imaging examination and detection of tumor markers, which play a very important role in early diagnosis, prognosis judgment, recurrence monitoring, and other aspects of HCC[9,10]. However, the tumor markers have certain false positive rates, low sensitivity, and specificity[8]. Therefore, new tumor markers related to HCC are urgent to be discovered, so as to improve the early diagnosis rate of HCC[6-9].

MiRNA is a large family of endogenous, non-coding RNA with a length of about 18-25 nucleotides. The most primitive pri-miRNA is generated in the nucleus and then transported to the cytoplasm after a series of complex processing, eventually becoming mature miRNA[13,14]. It mainly plays an important regulatory role in the occurrence and development of the diseases by binding specific target genes or target proteins[15]. In recent years, the abnormally expressed miRNAs have been proved to regulate a series of biological processes, including

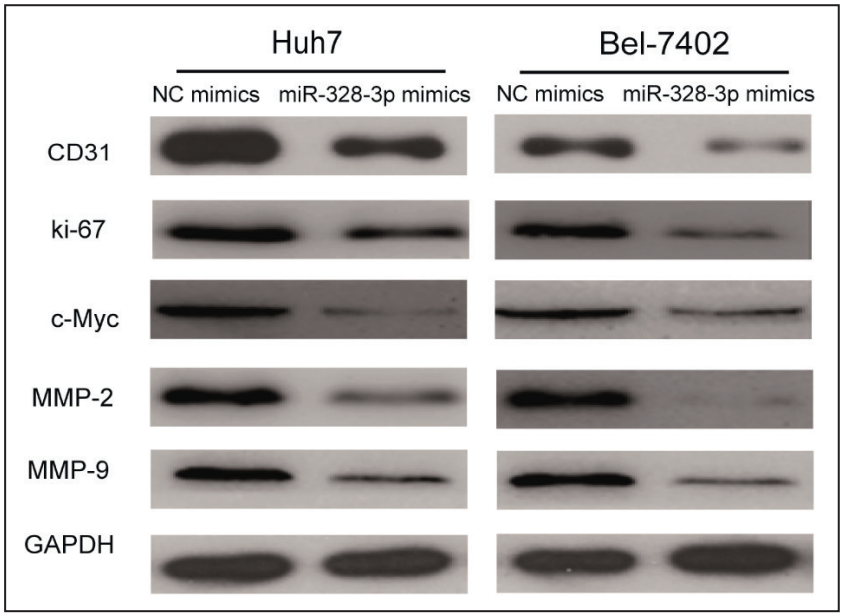

Figure 4. The Western blotting assays of the protein levels of the key proteins in the MMP-9 pathway, including CD31, Ki-67, c-Myc, MMP-2, and MMP-9, in Huh7, and Bel-7402 cells transfected with miR-328-3p mimics.

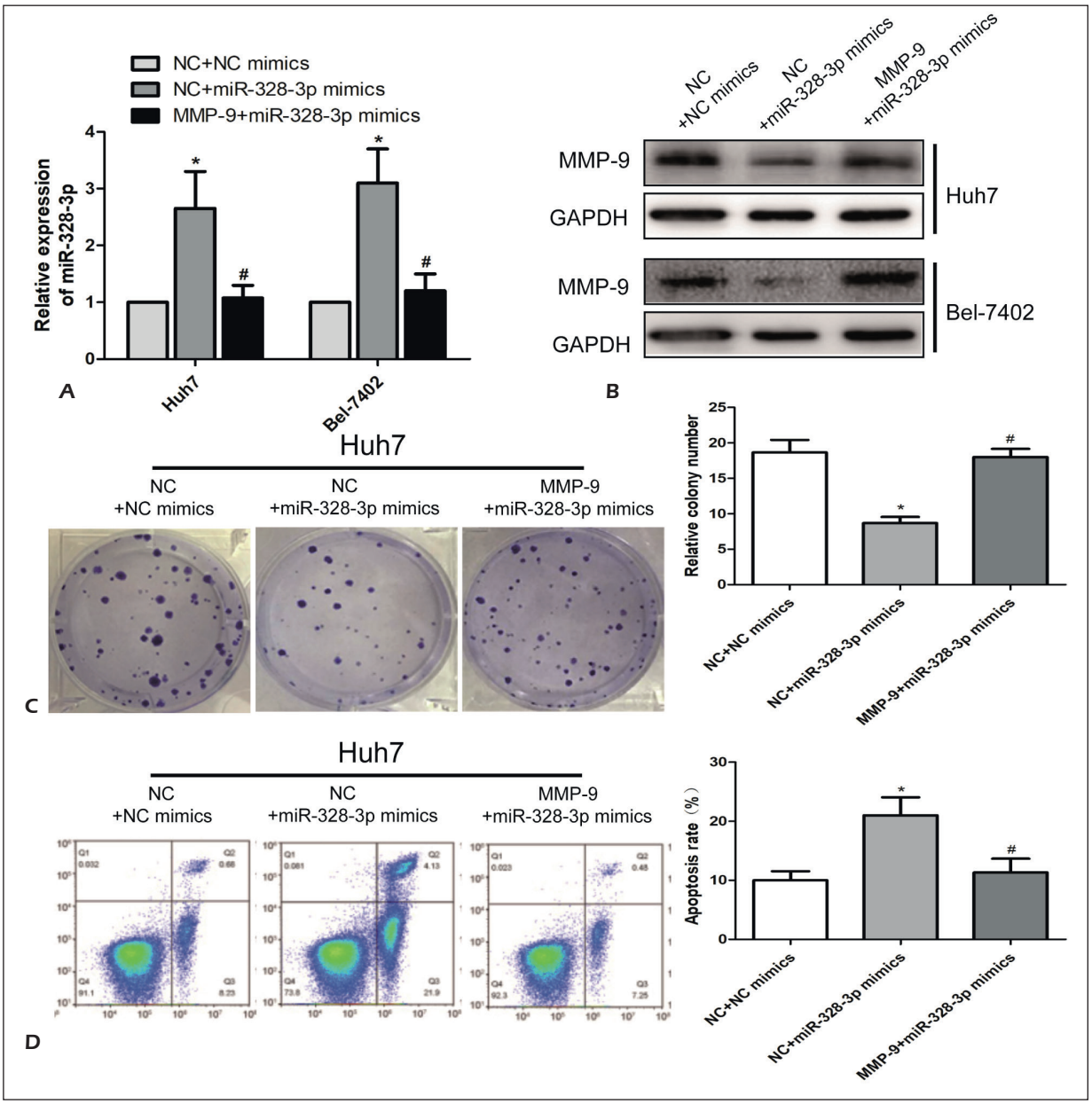

Figure 5. MiR-328-3p regulates the expression of MMP-9 in hepatocellular carcinoma cell lines. **A**, The expression level of miR-328-3p in the co-transfected cells detected by qRT-PCR. **B**, MMP-9 expression in the co-transfected cells detected by the Western Blot. **C**, The colony formation assay detects clonality in the co-transfected cells (magnification × 40). **D**, The flow cytometry assay detects the apoptosis in the co-transfected cells. The data are expressed as mean ± SD, *#p<0.05.

cancer cell proliferation, differentiation, apoptosis, invasion, and metastasis. Besides, they are involved in the changes of the tumor microenvironment, domestication, and stemness of tumor cells, suggesting their potential for diagnosis, prognosis, and therapeutic intervention in tumor patients[16-19]. MicroRNA-328-3p is a newly discovered miRNA associated with HCC analyzed through miRNA microarray technology, which is located on human chromosome 16q22.1[22,23]. In order to explore the role of microRNA-328-3p in the occurrence and development of HCC, this study firstly detected the expression of microRNA-328-3p in 52 cases of HCC tissues and its paracarcinoma paired tissues. It was found that the expression of microRNA-328-3p in HCC tissues was remarkably down-regulated, and the expression in HCC tumor tissues was remarkably lower than that in paracarcinoma tissues. Therefore, we believed that microRNA-328-3p may play a role in tumor inhibition in HCC.

Besides, the expression level of microRNA-328-3p was the lowest between Huh7 and bel-7402 cell lines, which laid a foundation for future experiments. To further explore the effect of microRNA-328-3p on the biological function of HCC, the overexpression model of microRNA-328-3p was constructed in this study. MicroRNA-328-3p was able to inhibit the proliferation and promote the apoptosis in HCC. In addition, after the overexpression of microRNA-328-3p, the expression levels of CD31, ki-67, c-myc, MMP-2, and MMP-9 were remarkably down-regulated.

At the same time, a further understanding of the molecular biological mechanism of HCC formation and progression can provide new specific diagnostic and therapeutic targets for human HCC[10-12]. Authors[14,15] have found that the same miRNA can regulate multiple target genes. Similarly, we used bioinformatics software to predict the downstream target gene of microRNA-328-3p and MMP-9 was discovered. MMP-9 was found to promote the malignant progression of the tumor cells in HCC[24]. The expression level of MMP-9 was remarkably down-regulated after the transfection of microRNA-328-3p mimics. Subsequently, the overexpression of MMP-9 was found to reverse the biological effect of microRNA-328-3p on HCC cells, thus affecting the malignant progression of HCC.

Conclusions

We demonstrated that microRNA-328-3p was remarkably associated with pathological staging and poor prognosis in HCC. MicroRNA-328-3p might inhibit the malignant progression of HCC *via* regulating MMP-9.

Conflict of Interests

The authors declared that they have no conflict of interests.

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