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MIIP inhibits malignant progression of hepatocellular carcinoma through regulating AKT

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Abstract. – OBJECTIVE: This study was aimed to investigate the expression characteristics of MIIP in hepatocellular carcinoma (HCC), and to further explore whether it can inhibit the malignant progression of this disease via regulating AKT expression.

PATIENTS AND METHODS: Real-time quantitative PCR (qRT-PCR) was performed to examine the expression of MIIP in tumor and paracancerous tissue specimens of 39 patients with HCC, and to analyze the interplay between MIIP expression and clinical indicators and prognosis of HCC patients. At the same time, in HCC cell lines, the expression of MIIP was further verified using qRT-PCR. In addition, MIIP overexpression and knockdown models were constructed using lentivirus in HCC cell lines (Bel-7402 and Hep3B), and the influence of MIIP on the biological function of HCC cells was analyzed through CCK-8 and transwell migration assays. Finally, luciferase reporting assay and cell reverse experiments were applied to further explore the potential molecular mechanism and the interaction between MIIP and AKT.

RESULTS: The results of qRT-PCR showed that the expression level of MIIP in HCC tissue samples was remarkably lower than that in adjacent ones, with a statistically significant difference. Compared with patients with high expression of MIIP, patients with low MIIP expression had a higher occurrence of distant metastasis and a lower overall survival rate. Similarly, compared with control group, the proliferation and migration ability of HCC cells in MIIP knockdown group (sh-MIIP) was remarkably enhanced, while the opposite result was observed in MIIP overexpression group. In addition, qRT-PCR results also revealed that AKT and MIIP were negatively correlated in HCC tissues. At the same time, the results of luciferase reporter gene assay demonstrated that MIIP can be targeted by AKT through certain binding site. Additionally, cell reverse experiment found that there might exist a mutual regulation between MIIP and AKT, thereby jointly regulating the malignant progression of HCC.

CONCLUSIONS: MIIP expression is remarkably decreased both in HCC tissues and cell lines; meanwhile, the low expression of MIIP is positively correlated with the occurrence of distant metastasis and poor prognosis of patients with HCC. In addition, MIIP may be able to inhibit the malignant progression of HCC by modulating AKT expression.

Key Words:
MIIP, AKT, Hepatocellular carcinoma, Malignant progression.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid malignancies in the world, ranking third in the world and second in China in the causes of death of malignancies, and its incidence has been increasing in recent years[1-3]. Surgical resection and liver transplantation are still the most effective treatments at present, but the curative effect is not good, due to the high postoperative recurrence and metastasis rates of this cancer[4-6]. With the improvement of liver surgical technique and perioperative treatment level, the surgical resection rate of HCC is continuously improved, and the hospital mortality

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rate of HCC is close to zero[6]. Despite this, the mortality rate of patients with HCC remains high, with a 5-year survival rate of only 40%-50%[7,8]. Recurrence and metastasis of HCC is the most important cause of high mortality in HCC patients, which apparently has become a bottleneck that severely restricts the long-term survival rate of HCC patients[9,10]. Therefore, in-depth study of the mechanism and active exploration of effective anti-relapse treatments are the focus and difficulty in the current diagnosis and treatment of HCC, and it is of great significance to further improve the long-term survival rate of HCC patients[10]. Migration invasion suppressor protein (MIIP), also known as invasion suppressor protein 45 (IIP45), was first identified in 2003 in the study of glioblastoma multiforme[11-13]. The MIIP gene is located in the short arm 3 region 6 band (1p36) of chromosome 1, with a total length of 12.6 kb and a molecular weight of 45 kD. There are 10 exons and 6 single nucleotide polymorphisms in this region, which are absent in various tumor tissues[13,14]. The MIIP-encoded products are all hydrophilic proteins with a particularly strong affinity, consisting of up to 388 amino acids[14]. Therefore, MIIP can play a part in tumor suppression and plays a complex and important role in a variety of cellular signaling. The change of the gene's tumor-inhibiting ability has nothing to do with gene mutation, but is closely related to the gene haploid dose deficiency and allele deletion[15]. However, the role of MIIP in HCC still remains unclear; therefore, this study comprehensively analyzed the expression and biological effects of MIIP in HCC, and initially explored the molecular mechanism of its tumor regulation.

Although previous studies have shown that the up-regulation of MIIP expression is closely related to tumor invasion and metastasis and MIIP can inhibit tumor intraperitoneal implantation, there is no research to elucidate the specific mechanism of MIIP regulating invasion and metastasis of liver cancer[13,16,17]. Therefore, MIIP overexpression and knockdown models were constructed in this study using lentivirus to explore the molecular mechanism by which MIIP regulates the malignant progression of HCC.

Patients and Methods

Patients and Hepatocellular Carcinoma Samples

In this study, 39 pairs of specimens were selected from surgically treated HCC cases, and HCC tissues and their corresponding adjacent tissues were collected and then stored at -80°C. The collection of clinical specimens was approved by the Ethics Monitoring Committee, and patients and their families had been fully informed that their specimens would be used for scientific research, and all participating patients had signed informed consent.

Cell Lines and Reagents

Six human HCC cells (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), while Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from American Life Technologies (Gaithersburg, MD, USA). The HCC cells were cultured in DMEM high glucose medium containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were cultured in a 37°C, 5% CO_2 incubator, and were passaged with 1% trypsin + ethylene diamine tetraacetic acid (EDTA) for digestion when grown to 80%-90% confluence.

Transfection

Control group (sh-NC or NC) and the lentivirus (sh-MIIP or MIIP) containing the MIIP overexpression and knockdown sequences were purchased from Shanghai Jima Company (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 40%, followed by transfection according to the manufacturer's instructions. After 48 h, cells were collected for qRT-PCR analysis and cell function experiments.

Cell Counting Kit (CCK-8) Assay

After 48 h of transfection, cells were collected and plated into 96-well plates at 2000 cells per well. The cells were cultured for 24 h, 48 h, 72 h, and 96 h, respectively, and then added with CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent. After incubation for 2 hours, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

Transwell Assay

The cells after transfection for 48 hours were digested, centrifuged, and resuspended in serum-free medium to adjust the density to 5 x 10^5 cells/mL. A cell suspension of 200 uL (1×10^5 cells) was added to the upper chamber, while 700 uL of a medium containing 20% FBS was added to the lower chamber. According to the different

migration abilities of each cell line, they were put back into the incubator and continued to be cultured for a specific time. Afterwards, the transwell chamber was clipped, washed 3 times with 1 x phosphate-buffered saline (PBS), and placed in methanol for 15 min cell fixation. After the chamber was stained in 0.2% crystal violet for 20 min, the cells on the upper surface of the chamber were carefully wiped off with a cotton swab. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed in 5 randomly selected fields of view under the microscope.

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

After the cells were treated accordingly, 1 ml of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and repurify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, 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 repeated for each sample and the assay was repeated twice. The Bio-Rad PCR instrument was used to analyze and process the data with the software iQ5 2.0 (Bio-Rad, Hercules, CA, USA). The GAPDH and U6 genes were used as internal parameters, and the gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers were as follows: MIIP, forward: 5'-ATACCTGGGCTATGACTGGATT-3', reverse: 5'-AGTACACGCATTCATGGTCTTC-3'; AKT, forward: 5'-AGCGACGTGGCTATTGTGAAG-3', reverse: 5'-GCCATCATTCTTGAGGAGGAAGT-3'; GAPDH, forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Western Blot

The transfected cells were lysed using cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 x g for 15 min at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The extracted proteins were separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and later transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. The primary antibodies against MIIP, AKT, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as well as the secondary antibodies against mouse and rabbit, were all purchased from Cell Signaling Technology (Danvers, MA, USA).

Dual-Luciferase Reporter Assay

A reporter plasmid was constructed in which a specific fragment of the target promoter was inserted in front of the luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected into Bel7402 and Hep3B cells or other related cell lines with the reporter plasmid. If the transcription factor could activate the target promoter, the luciferase gene would be expressed, and the amount of luciferase expression was directly proportional to the intensity of the transcription factor. A specific luciferase substrate was added, luciferase reacted with the substrate to generate fluorescence, and the activity of the luciferase was determined 48 hours later using the Promega luciferase kit (Madison, WI, USA) to detect the intensity of the fluorescence.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (Version X; La Jolla, CA, USA). 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). Independent experiments were repeated at least three times for each experiment, and the data were expressed as average ± standard deviation ($\bar{x}\pm s$). There were three levels of $p<0.05$, $p<0.01$, and $p<0.001$ at the significance level, and $p<0.05$ was considered statistically significant.

Results

MIIP Was Lowly Expressed in Hepatocellular Carcinoma Tissues and Cell Lines

To determine the role of MIIP in HCC, a total of 39 pairs of tumor tissues and paracancerous tissues of HCC patients were collected, and the expression of MIIP was detected by qRT-PCR. The

results showed that the expression of MIIP in tumor tissues of patients with HCC was remarkably lower than that of adjacent tissues (Figure 1A, 1B). In addition, MIIP was remarkably lower in HCC cell lines than that in human normal liver cell lines (LO2), and the difference was statistically significant (Figure 1C), suggesting that MIIP may play a role as a tumor suppressor gene in HCC

MIIP Expression Was Correlated With Distant Metastasis and Overall Survival in Hepatocellular Carcinoma Patients

According to the mRNA results of MIIP in 39 tumor tissues and paracancerous tissues of patients with HCC, MIIP expression was divided into high expression group and low expression group. The relationship between MIIP expression and age, sex, pathological stage, lymph node metastasis of HCC patients was analyzed. As shown in Table I, the low expression of MIIP was positively correlated with the incidence of distant metastasis of HCC, but not with age, gender, pathological stage, and lymph node metastasis. Then, based on 39 mRNA results of MIIP in HCC tissues and paracancerous tissues, MIIP expression was divided into high expression group and low expression group, and the relationship between MIIP expression and poor prognosis of HCC patients was analyzed. As shown in Figure 1D, low expression of MIIP was closely related to poor prognosis of HCC. These results suggested that MIIP expression is correlated with distant metastasis and overall survival in hepatocellular carcinoma patients.

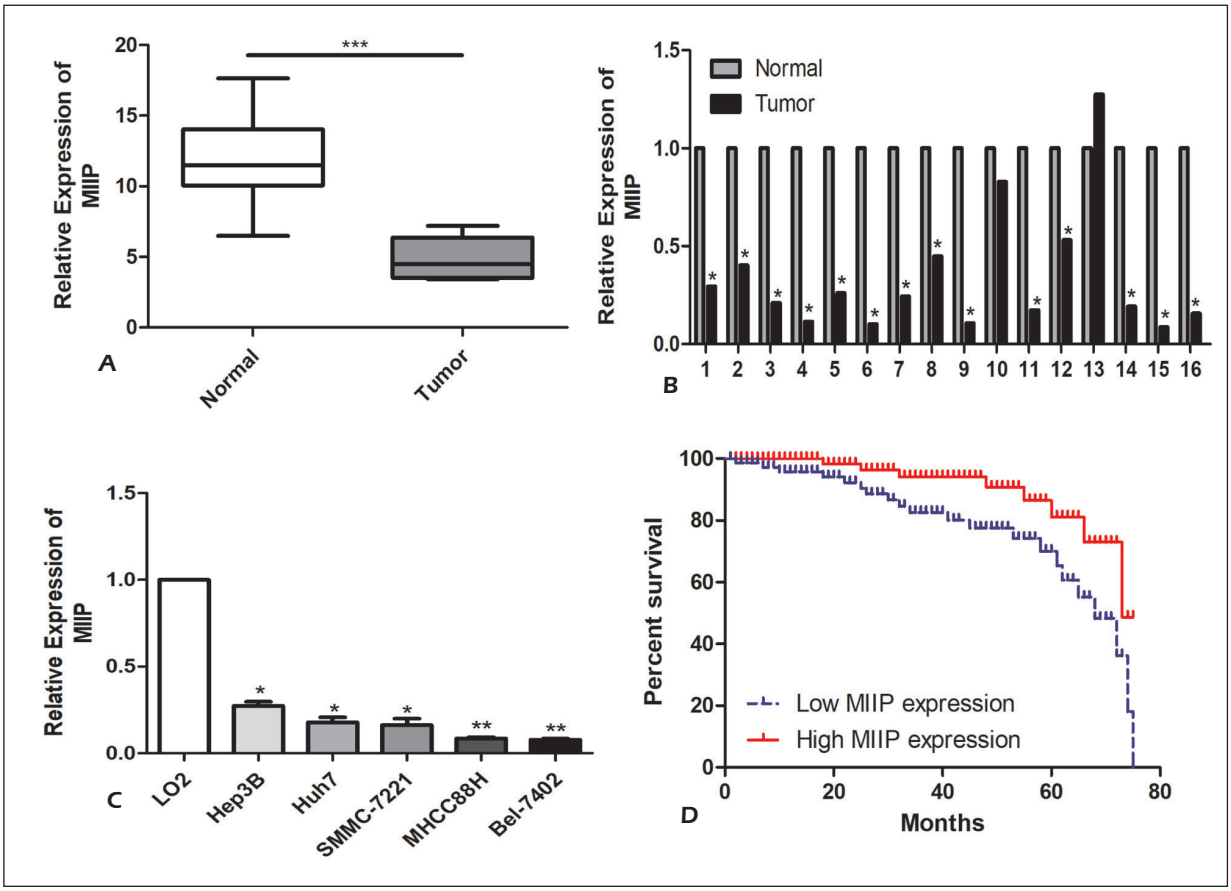

Figure 1. MIIP is underexpressed in hepatocellular carcinoma tissues and cell lines. **A**-**B**, QRT-PCR was used to detect the differential expression of MIIP in hepatocellular carcinoma tumor tissues and adjacent tissues; **C**, QRT-PCR was used to detect the expression level of MIIP in hepatocellular carcinoma cell lines; **D**, Kaplan Meier survival curve of patients with hepatocellular carcinoma based on MIIP expression was shown; the prognosis of patients with low expression was found significantly worse than that of high expression group. Data are mean ± SD, *p<0.05, **p<0.01, ***p< 0.001.

Table I. Association of MIIP and AKT expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	No. of cases	MIIP expression		*p*-value	AKT expression		*p*-value
		High (%)	Low (%)		High (%)	Low (%)	
Age (years)				0.102			0.231
<60	15	10	5		4	11	
≥60	24	10	15		11	13	
Gender				0.264			0.265
Male	20	12	8		6	14	
Female	19	8	11		9	10	
T stage				0.431			0.073
T1-T2	25	14	11		7	18	
T3-T4	14	6	8		8	6	
Lymph node metastasis				0.648			0.196
No	28	15	13		9	19	
Yes	11	5	6		6	5	
Distance metastasis	0.034			0.013			
No	25	16	9		6	19	
Yes	14	4	10		9	5	

Knockdown/Overexpression of MIIP Promoted/Inhibited Cell Proliferation and Migration

To investigate the cytological function of MIIP in HCC, overexpression and knockdown of MIIP lentiviral vectors were constructed. After transfection of the MIIP lentiviral vector in the Bel-7402 and Hep3B cell lines, Western Blotting and qRT-PCR experiments were performed to verify the interference efficiency, and it was found that the difference was statistically significant (Figure 2A). The proliferation and migration ability of HCC cells was detected by CCK-8 and transwell migration assay after knocking down MIIP in the Bel-7402 cell line and overexpressing MIIP in Hep3B cell line. The results showed that compared with the sh-NC group, the proliferation and migration ability of hepatocyte cancer cells in the MIIP expression knockdown group was remarkably increased. Compared with the NC group, the proliferation and migration ability of hepatocyte cancer cells in the MIIP overexpression group was significantly lower (Figure 2B, 2C). These results suggested that MIIP can regulate the cell proliferation and migration in HCC.

MIIP Bounded to AKT

As shown in Figure 3A, to further verify the targeting of AKT to MIIP, a luciferase reporter assay was performed. The results showed that overexpression of AKT remarkably attenuated the luciferase activity of the wild-type MIIP vector ($p<0.05$) without attenuating the luciferase activity containing the mutant vector ($p>0.05$) or the empty vector. It was further demonstrated that MIIP can be targeted by AKT through this binding site. After constructing MIIP overexpression and knockdown lentiviral vector in Bel-7402 and Hep3B cell lines, Western blotting results showed that the expression level of AKT in MIIP knockdown group was remarkably increased, while that of AKT in MIIP overexpression group was remarkably decreased (Figure 3B). The qRT-PCR experiment also reflected the same trend (Figure 3C). These results recommended that MIIP can bind to AKT and regulate the expression levels of AKT.

AKT Was Highly Expressed in Hepatocellular Carcinoma Tissues and Cell Lines

The expression of MIIP and AKT in HCC tissues was detected by qRT-PCR. The results showed that MIIP and AKT were negatively correlated in HCC tissues (Figure 3D). Besides, qRT-PCR experiment showed that the expression level of AKT in HCC tissues was remarkably increased compared with paracancerous tissues, and the difference was statistically significant (Figure 3E), followed by simultaneous analysis of clinical indicators and prognosis of AKT in HCC. As shown in Table I, high expression of AKT was positively correlated with the occurrence of distant metastasis of HCC, but not with age, gender, pathological stage, and lymph node metastasis. Furthermore, as shown in Figure 3F, high expression of AKT was closely related to poor prognosis of HCC.

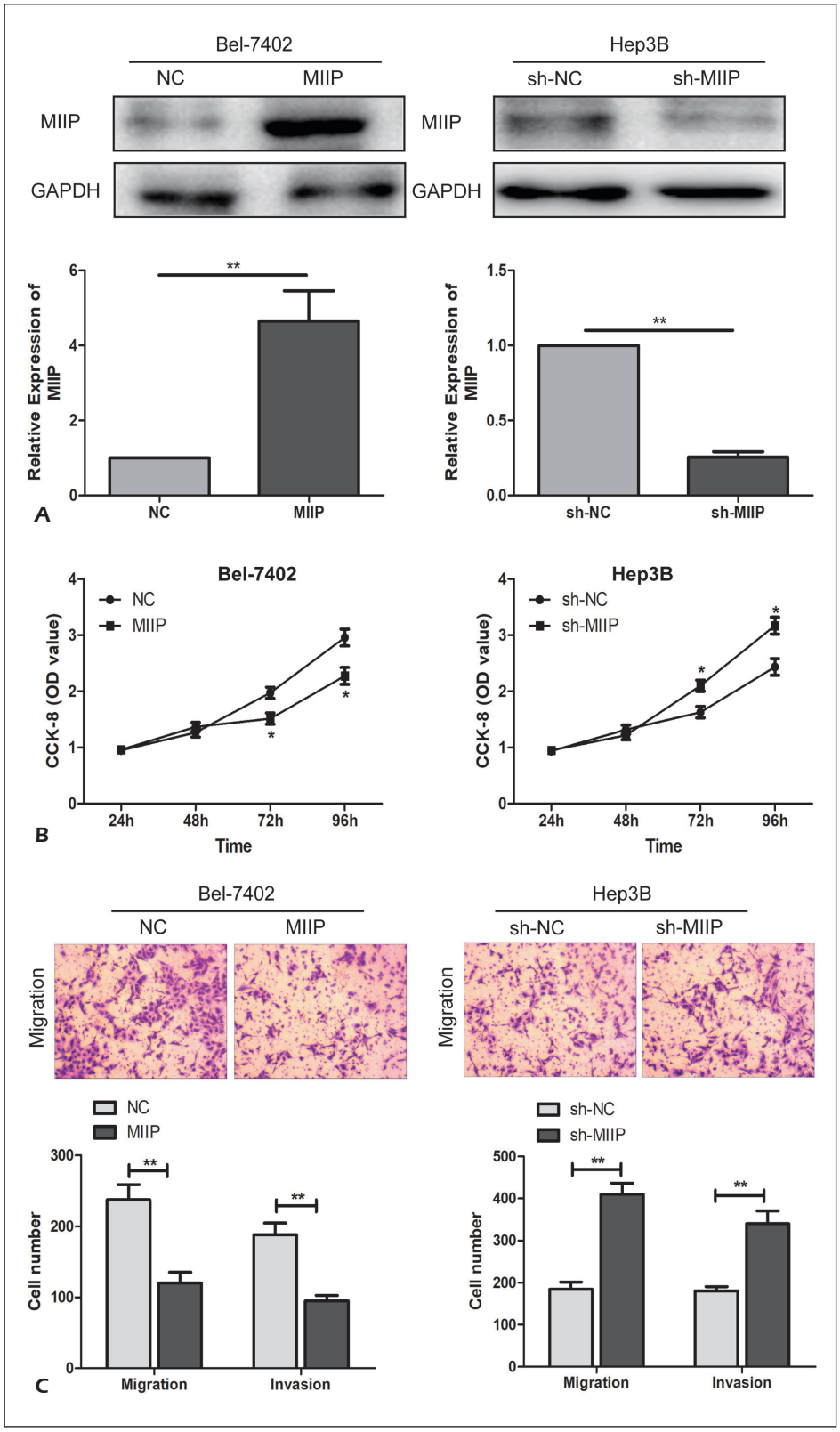

Figure 2. Overexpression/silencing of MIIP inhibits/promotes hepatocyte cancer cell proliferation. **A**, Western Blotting and qRT-PCR verified the interference efficiency of MIIP after transfection of MIIP lentiviral vector in Bel-7402 and Hep3B cell lines; **B**, CCK-8 assay detected the effect of transfection of MIIP on proliferation of hepatocellular carcinoma cells in Bel-7402 and Hep3B cell lines; **C**, Transwell assay detected the effect of transfection of MIIP on the metastasis of hepatocellular carcinoma cells in the Bel-7402 and Hep3B cell lines (magnification: 20X). Data are mean ± SD, *$p<0.05$.

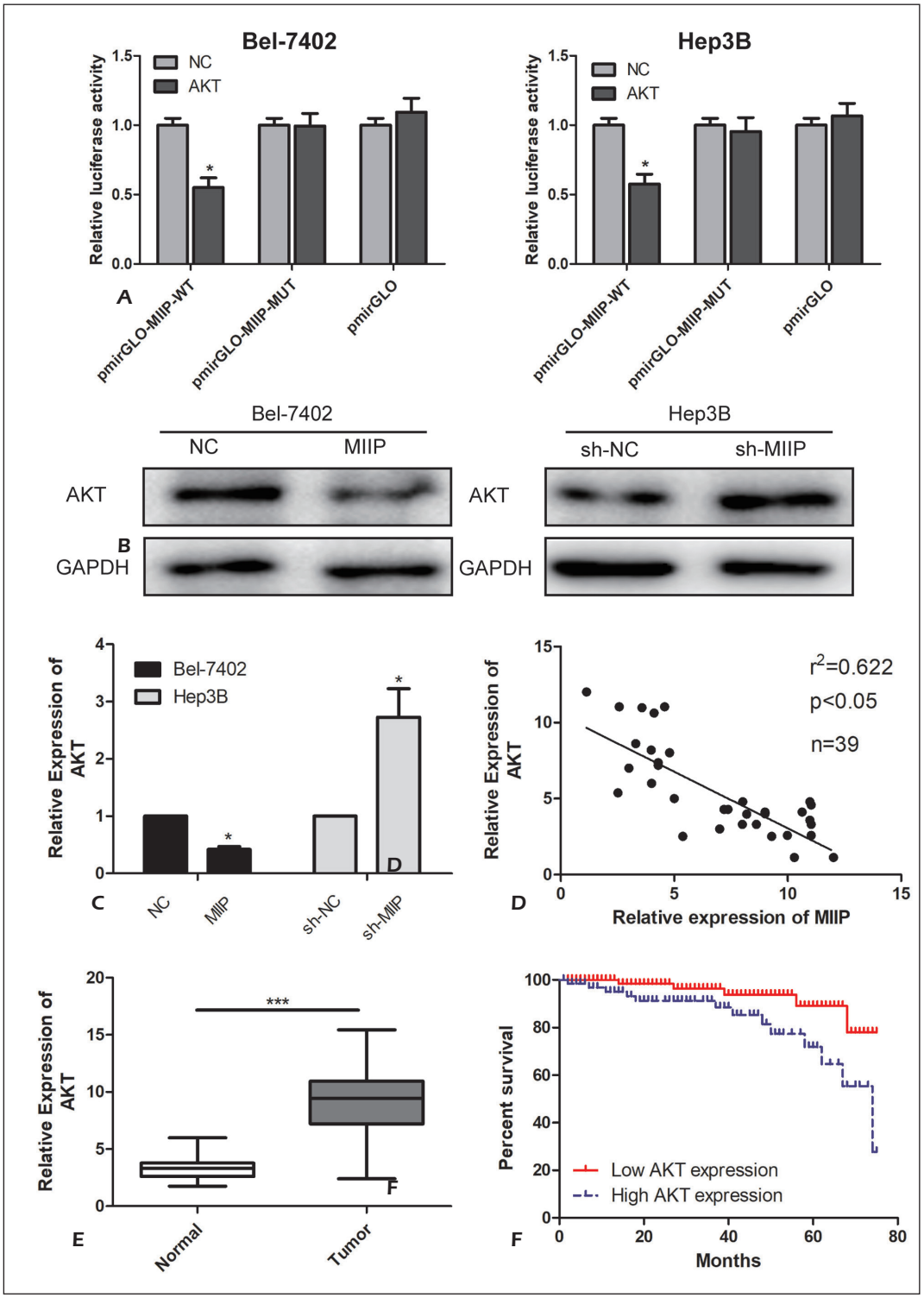

Figure 3. Direct targeting of AKT by MIIP was indicated. **A**, Dual-Luciferase reporter assays validated the direct targeting of MIIP and AKT; **B**, Western Blotting verified the interference efficiency of AKT after transfection of MIIP lentiviral vector in Bel-7402 and Hep3B cell lines; **C**, qRT-PCR verified the interference efficiency of AKT after transfection of MIIP lentiviral vector in Bel-7402 and Hep3B cell lines; **D**, There was a significant negative correlation between the expression levels of MIIP and AKT in hepatocellular carcinoma tissues; **E**, QRT-PCR was used to detect the difference in expression of AKT in hepatocellular carcinoma tumor tissues and adjacent tissues; **F**, Kaplan Meier survival curve of patients with hepatocellular carcinoma based on AKT expression was shown; the prognosis of patients with high expression was found significantly worse than that of low expression group. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Overexpression/Knockdown of AKT Promoted/Inhibited Cell Proliferation and Migration

To investigate the cytological function of AKT in HCC, overexpression and knockdown of AKT vectors were constructed. After transfection of the AKT vector in the Bel-7402 and Hep3B cell lines, qRT-PCR experiments were performed to verify the interference efficiency, and the difference was statistically significant (Figure 4A). Next, cell proliferation and migration ability were measured by CCK-8 and transwell migration assays. The results showed that compared with the sh-NC group, the proliferation and migration ability of hepatocyte cancer cells in the AKT expression knockdown group was remarkably decreased. Compared with NC group, the proliferation and migration ability of hepatocyte cancer cells in AKT overexpression group was significantly increased (Figure 4B, 4C), suggesting that AKT can regulate the HCC cell proliferation and migration.

MIIP Modulated AKT Expression in Hepatocellular Carcinoma Cell Lines

To further explore the role of MIIP in malignant progression of hepatocellular carcinoma, a possible relationship between MIIP and AKT was found through relevant bioinformatics analysis. In addition, to explore the interaction between MIIP and AKT in HCC cells, AKT was silenced in the Hep3B cell line that had silenced MIIP, and AKT was overexpressed in the Bel-7402 cell line that had overexpressed MIIP to explore their mutual relationship in the liver. Transfection efficiency was examined by Western Blotting and qRT-PCR experiments (Figure 5A, 5B). Next, it was found by CCK-8 and transwell migration experiments that AKT could counteract the effect of MIIP on proliferation and migration of HCC cells (Figure 5C, 5D). In sum, we concluded that MIIP modulated AKT expression in hepatocellular carcinoma cell lines.

Discussion

As a common malignant tumor, hepatocellular carcinoma (HCC) is a serious threat to human health. In all new cases of malignant tumors each year, HCC accounts for 5.6%, ranking fifth. Currently, surgical treatment is still the main method, but most patients have lost the chance of surgery at the time of diagnosis, so the prognosis of the disease is extremely poor, and the recurrence rate after liver cancer resection remains high, with a dissatisfactory treatment effect[1-5]. The development of HCC involves multiple steps, including the regulation of genes by epigenetic changes, which in turn activates proto-oncogene activation and tumor suppressor genes[6,8,9].

The MIIP gene is located on chromosome lp36.22 with 10 exons, and its expression product contains 388 amino acids, one RGD sequence, and three structural fragments[11-13]. MIIP is widely distributed in tumors such as colon cancer, lung cancer, and prostate cancer, whose discovery is a milestone in tumor research. When the MIIP gene is deleted, the cell mitosis is prone to be out of control, the tumor cells are easy to form, and tumors easily progress with migration and invasion, so MIIP gene acts as a tumor suppressor gene that can inhibit tumor cell proliferation, invasion, and metastasis[14,15]. In this study, MIIP overexpressing and knocking down models were established by lentivirus to study the role of MIIP in the malignant progression of HCC, and finally determine the relationship between MIIP and the development of HCC. Through tissue validation, it was found that the expression of MIIP in HCC was remarkably lower in HCC tissues than that in adjacent tissues, and it was positively correlated with the occurrence of distant metastasis and poor prognosis in patients with HCC, suggesting that MIIP may play a role in HCC. To further study the molecular mechanism of MIIP in the development of liver cancer, it was proved on *ex vivo* cells that MIIP was a disease-related gene. Later, CCK-8 and transwell experiments were performed. The results showed that compared with that in sh-NC group, the proliferation and migration ability of hepatocyte cancer cells in the MIIP expression knockdown group was remarkably increased; compared with NC group, the ability of cancer cells to proliferate and migrate in MIIP overexpression group was remarkably reduced.

At present, it is believed that the gene can competitively bind to the mRNA of target gene, thereby affecting the regulation of its target mRNA[18,19]. Previous studies have predicted through bioinformatics analysis that AKT may interact with MIIP. The results of this study showed that AKT was highly expressed in HCC tissues compared with adjacent tissues, and AKT can promote proliferation and migration and metastasis in HCC cells. In addition, the bioinformatics method was used to analyze the MIIP sequence containing an AKT binding site, and the direct binding of MIIP to the downstream AKT was detected by molecular biological experiments such as the Dual-Luciferase reporter gene assay. As

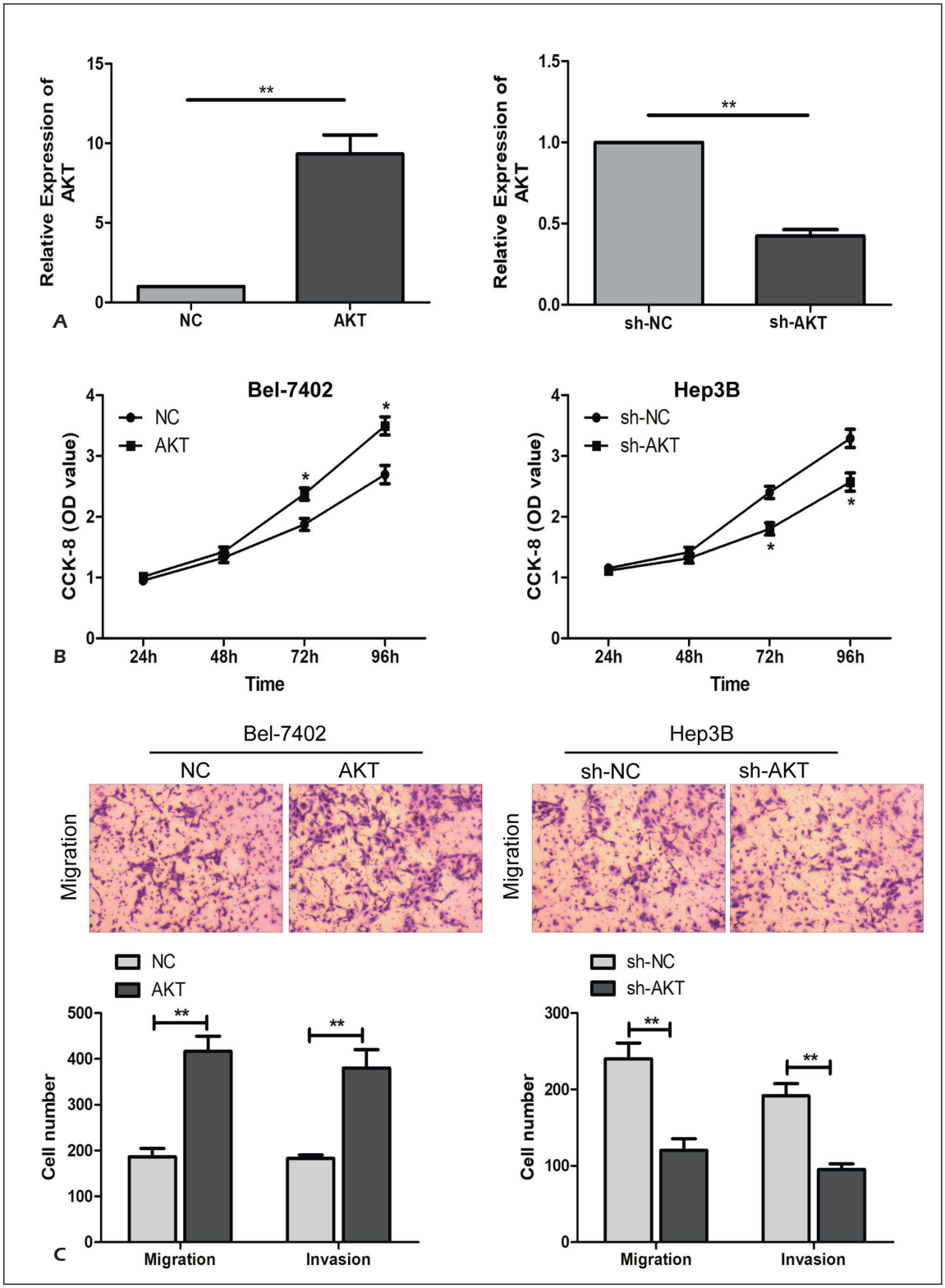

Figure 4. Silencing/overexpression of AKT inhibits/promotes hepatocyte cancer cell proliferation. **A**, QRT-PCR verified the interference efficiency of MIIP after transfection of AKT vectors in Bel-7402 and Hep3B cell lines; **B**, CCK-8 assay detected the effect of transfection of AKT on proliferation of hepatocellular carcinoma cells in Bel-7402 and Hep3B cell lines; **C**, Transwell assay detected the effect of transfection of AKT on hepatocyte cancer cells in the Bel-7402 and Hep3B cell lines (magnification: 20X). Data are mean ± SD, *$p<0.05$.

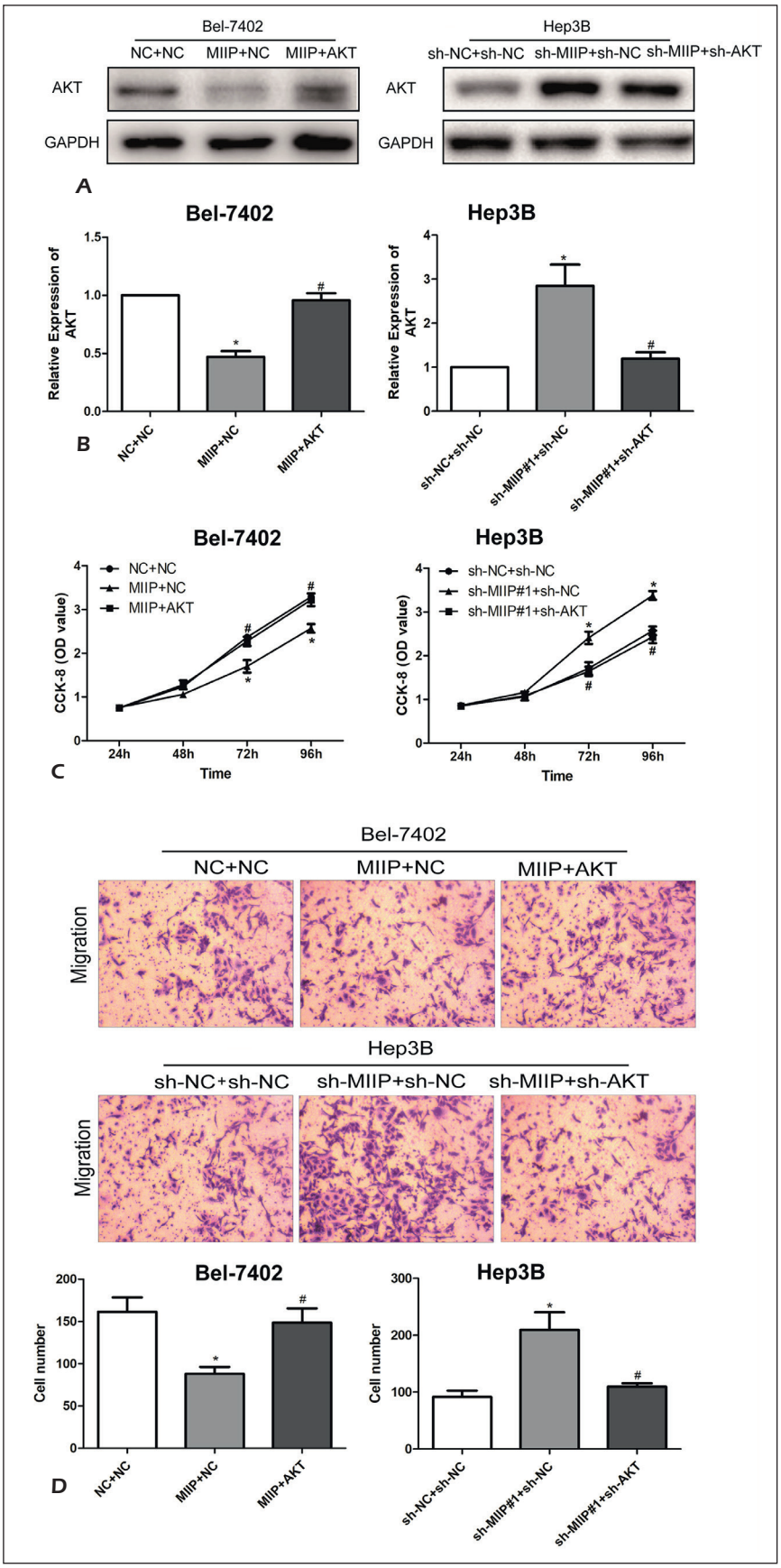

Figure 5. MIIP regulates the expression of AKT in hepatocellular carcinoma tissues and cell lines. **A**, MIIP expression levels in MIIP-AKT co-transfected hepatocellular carcinoma cell lines were detected by Western Blotting; **B**, Level of MIIP expression in the co-transfected hepatocellular carcinoma cell line between MIIP and AKT was detected by qRT-PCR; **C**, CCK-8 assay detected the effect of MIIP and AKT co-transfection on the proliferation of hepatocellular carcinoma cells; **D**, Transwell assay detected the migration of hepatocellular carcinoma cells after co-transfection of MIIP and AKT (magnification: 20X). Data are mean ± SD, *$p<0.05$.

shown in the results, the MIIP vector lacking the AKT binding site failed to enrich AKT, further confirming that MIIP can directly target AKT. To further explore the expression and regulation of MIIP and AKT in HCC cell lines, the expression levels of MIIP and AKT were detected in HCC tissues and cell lines, which were found to be just negatively correlated. In addition, reverse experiments showed that AKT can counteract the effect of MIIP on cell biology in HCC cells. In summary, the study found that MIIP can inhibit the expression of AKT, thereby inhibiting the malignant progression of HCC.

Conclusions

MIIP expression was found remarkably decreased both in HCC tissues and cell lines. The low expression of MIIP was positively correlated with the occurrence of distant metastasis and poor prognosis of patients with HCC. In addition, MIIP might be able to inhibit the malignant progression of HCC by modulating AKT expression.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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