

miR-122 Inhibits Hepatocarcinoma Cell Progression by Targeting LMNB2

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In the present study, we investigated the role of miR-122 in hepatocarcinoma progression and explored the mechanism. In hepatocarcinoma tissues and cells, we used qRT-PCR to validate the miR-122 expression level. Next, we used colony formation by crystal violet staining assay to compare cell proliferation ability, and we used scratch test or Transwell assay to compare cell migration or invasion ability. We then conducted bioinformatics or luciferase reporter gene assay to prove the regulation effect of miR-122 on lamin B2 (LMNB2), and the biological function of LMNB2 was analyzed. We used nude mouse tumorigenicity assay to test the inhibition effect of miR-122 ASO therapy against hepatocarcinoma. miR-122 was reduced in hepatocarcinoma tissues compared to the paracarcinoma tissues, which was relatively low or high in hepatocarcinoma cell line SMMC7721 or Hep3B, and overexpressed miR-122 inhibited proliferation, migration, and invasion in hepatocarcinoma cells. Additionally, some reports showed that LMNB2 was regulated by miR-122, which inhibited the expression of LMNB2. Moreover, LMNB2 functioned to promote cell proliferation, migration, and invasion. We could achieve the inhibition of hepatocarcinoma using miR-122 therapy through decreasing LMNB2 expression *in vivo*. Our data indicated that miR-122 could inhibit hepatocellular carcinoma cell progression by targeting LMNB2 and as a therapeutic target for hepatocarcinoma treatment.

Key words: miR-122; Hepatocarcinoma; Progression; Lamin B2 (LMNB2); Target

INTRODUCTION

Hepatocarcinoma is a neoplasm ranking third in mortality in human cancers and the fifth most common incidence, which is rising; in 2017 it was estimated that there would be 40,710 Americans who would be diagnosed with hepatocarcinoma and 28,920 of them would die from the disease¹. Presently, surgery with liver transplantation is the only treatment option for hepatocarcinoma in many cases, and patient resistance to standard chemo- and radiotherapy is becoming challengeable for the therapy²⁻⁵. In spite of the advances in functional genomics or targeted therapy, there are still some limitations including chemoresistance²⁻⁵.

MicroRNAs (miRNAs) as an abundant group of small endogenous noncoding RNA molecules (about 22 nucleotides) are single-stranded and bind to target mRNAs mainly at their 3'-UTR²⁻⁹. Recent progress in cancer biology has shown that miRNAs are relevant to cellular processes in various types of human cancer including hepatocarcinoma²⁻¹¹. To date, several studies

showed that changes in expression level in miRNAs were linked to the development of hepatocarcinoma, and therapies combined with miRNA-based anticancer treatment are being developed to improve disease response for increasing patient survival⁹⁻¹³. Numerous studies have shown that miR-122 has a tumor suppressor function: the downregulated miR-122 expression is found in hepatocarcinoma tissue, and miR-122 has some downstream targets, such as serine/threonine kinase (Akt), cyclin G1, Bcl2-like protein 2, CCAAT-displacement protein, and paternally expressed gene 10 (PEG10), all of which are overexpressed in hepatocarcinoma patients^{4,14-37}. More recently, it has been reported that lncRNA ANRIL promotes cell proliferation and invasion by regulating miR-122-5p in hepatocarcinoma⁵. However, it is not enough for the many important roles of miR-122; the functional study of miR-122 in hepatocarcinoma has been limited.

In the present study, we first examined the level of miR-122 in hepatocarcinoma tissues and cell lines, and we explored its function on proliferation, migration, and

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invasion. Moreover, our data demonstrated that lamin B2 (LMNB2), a biomarker for the carcinogenesis in hepatocarcinoma, was a direct target of miR-122. Finally, our results indicated that miR-122 could inhibit hepatocarcinoma progression by inhibiting the expression level of LMNB2. Therefore, miR-122 has the potential to become a novel target for hepatocarcinoma.

MATERIALS AND METHODS

Ethics

After obtaining ethics committee approval in the First Hospital of Shijiazhuang, 15 paired tissue sections were obtained from 15 patients undergoing surgical resection from July 2013 to May 2015. The human samples used in this study were approved by the ethics committee of the First Hospital of Shijiazhuang. Written informed consent was provided by all the participants in this study.

Cell Culture and Reagents

Human hepatocarcinoma cell lines SMMC7721 and Hep3B were obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, P.R. China). The cells were maintained in DMEM medium with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. After obtaining ethics committee approval, 15 paired tissue sections were obtained from 15 hepatocarcinoma patients undergoing surgical resection. The expression levels of miR-122 in each paired tissue sections were compared, and the qRT-PCR (below) experiment was repeated three times for statistical analysis. The expression level of miR-122 in the adjacent tissues was taken as the relative value base 1, and the corresponding level in the tumor tissues was calculated.

RNA Analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then cDNA was generated using a PrimeScript RT reagent kit (Invitrogen) for further analysis. Duplex-qRT-PCR analysis was performed by the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA) according to the following conditions: 95°C for 5 min followed by 35 cycles of amplification at 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. The expression level of genes was normalized with β -actin. The different expression levels in the groups were compared with the 2^{-Ct} method. The expression level of miR-122 was detected using TaqMan microRNA assays, and RNU66 was evaluated using Applied Biosystems. The primers used for the qRT-PCR analysis were as follows²³: LMNB2 5'-TAGACACTGTTGTTGCTCAGCC-3' (forward) and 5'-TTTGACCAAATGGTGAGATGAG-3' (reverse). Each qRT-PCR analysis was carried out independently three times.

Transfection Assays

The miR-122 mimics and miR-122 antisense oligodeoxynucleotide (ASO) were purchased from Guangzhou RiboBio (Guangzhou, Guangdong Province, P.R. China). The final concentration of miR-122 ASO was 100 nM. LMNB2 plasmid was supplied by Origene (Rockville, MD, USA). The empty vector plasmid (pCMV6) was performed as negative control. LMNB2 small interfering RNA (siRNA; Cat. No. sc-61885) and the control plasmid (Cat. No. sc-37007) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The transfection method was as described previously²⁴.

Western Blot Assay

Cells were lysed in buffer containing 60 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 0.25% bromophenol blue, 1.25% 2-mercaptoethanol. Prior to use, the protease inhibitor was added. The BCA protein assay (Pierce/Thermo Fisher Scientific, Carlsbad, CA, USA) was used to test the protein concentration following the manufacturer's instructions. Then the protein (30–50 mg) was separated by SDS-PAGE (10%) and transferred onto nitrocellulose membrane, and it was detected by LMNB2 antibody (ab8983; dilution 1:1,000; Abcam, Cambridge, MA, USA) and β -actin antibody (sc-8432; 1:5,000) overnight at 4°C. Then the secondary antibodies, anti-rabbit (1:2,500) or anti-mouse (1:2,500), were incubated at room temperature for 1 h.

Cell Proliferation Assay

Complete medium (2 ml) with 500 cells was added into wells in six-well plates and incubated for 2 weeks. Cells were fixed by 100% methyl hydrate for 10 min and stained. The number of colonies was identified by manual count under microscope.

Scratch Assays

The cells were seeded in six-well plates overnight. A sterile pipette tip was used to introduce a scratch in the middle of the well. The migration of cells toward the center of the wound was measured at indicated time points.

Transwell Assay

For Transwell assay, 2 × 10⁵ cells with Matrigel (BD Bioscience, Mountain View, CA, USA) were added into the upper chambers (8.0 μ m; Corning Costar, Corning, NY, USA), and completed medium with 10% FBS was added into the lower chambers. After 1 day, cells in the bottom filter were stained.

Bioinformatic Analysis of miR-122 Target Genes

The putative miR-122 targets were predicted using several different algorithms, including microRNA.org (<http://www.microRNA.org>), TargetScan (<http://www.targetscan.org/>), and miRanda (<http://microrna.sanger.ac.uk/>).

*Dual-Luciferase Reporter Assay*²⁵

For detection of luciferase activity, the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) was used as described by the manufacturer. Cells were transfected (LMNB2 3'-UTR or mutant 3'-UTR) using Lipofectamine 2000. After 2 days, PBS was used to wash cells in each well, cells were subjected to lysis, and the luciferase activities were detected by dual-luciferase assay kit (Promega). *Renilla* luciferase activities were used to normalize the results. The wild-type and mutant miR-122 target sites in LMNB2 3'-UTR were supplied by Shanghai Genechem Co. Ltd. (Shanghai, P.R. China).

Xenografts Assays

The Animal Experimentation Ethics Committee in the First Hospital of Shijiazhuang approved the following animal study. All efforts were made to minimize the pain. Nude mice (aged 5–6 weeks) were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, P.R. China), and all mice were housed in a pathogen-free animal facility and randomly allocated to the control group or experimental group (four mice per group)²⁶. SMMC7721 cells (3×10^6) resuspended in 0.1 ml of DMEM were subcutaneously injected into the right flank of each nude mouse²⁶, and five groups that were treated dissimilarly on day 14 (the tumor volume reached about 100 mm³) were as follows: the mice were injected with miR-122 control and miR-122 once a day (15 times)^{5,22}. miR-122 control or miR-122 was injected into tumors, and the final concentration was 100 nM. The tumors were measured every 3 days, and the volume of tumors was calculated as $V=0.5 \times L \times W^2$ (V =volume, L =length, W =width). After 29 days, all mice were killed, and tumors were removed.

Statistical Analysis

All results were demonstrated as mean \pm SD. Statistical comparisons were conducted using one-way analysis of variance, which revealed significant differences between groups, and the Student's *t*-test, which revealed significant differences between two sample means. The expression of miR-122 in tissue was analyzed using the Mann–Whitney test. SPSS 22.0 was used to perform all the calculations, and the level of significance was set to $p < 0.05$.

RESULTS

miR-122 Was Decreased in Hepatocarcinoma Tissues Compared to the Paracarcinoma Tissues, and Overexpressed miR-122 Could Inhibit Cell Proliferation, Migration, or Invasion In Vitro

First of all, the expression level of miR-122 was detected in 15 tissues derived from paired tumors and

adjacent tissues. We observed that the expression level of miR-122 decreased in hepatocarcinoma cancer compared with adjacent normal tissues ($p < 0.05$). To test the effect of miR-122 in hepatocarcinoma cells, we examined the level of miR-122 in the hepatocarcinoma cell lines (data not shown), and the results were similar with a previous article³⁸. Therefore, we could use the similar method to explore the function and the mechanism of miR-122 in our study³⁹: we overexpressed miR-122 in the cell line SMMC7721 with miR-122 mimics and suppressed it with miR-122 ASO in the cell line Hep3B (Fig. 1B and C). The results of cell function experiments revealed that increased or decreased miR-122 expression could inhibit or promote proliferation, migration, or invasion in vitro, respectively ($p < 0.05$) (Fig. 2A–C). Taken together, these results indicated a decreased miR-122 expression in hepatocarcinoma tissues, and miR-122 could inhibit cell proliferation, migration, and invasion in hepatocarcinoma cells.

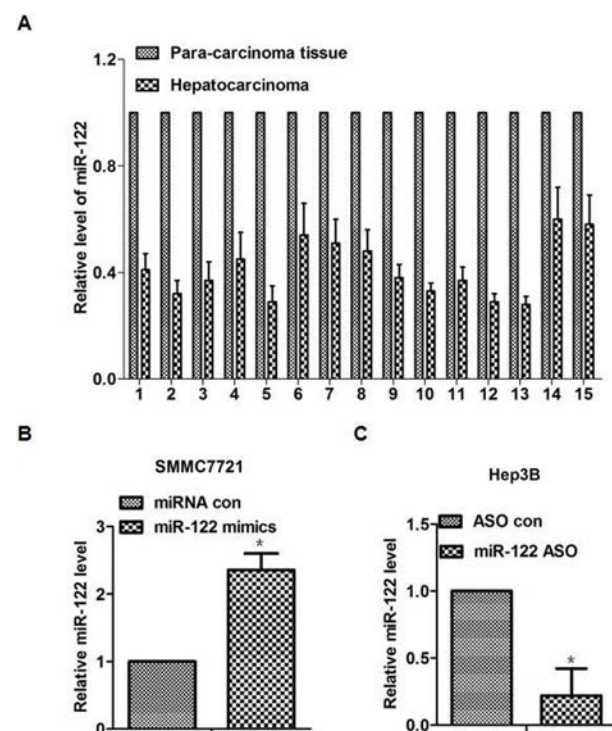


Figure 1. The expression levels of miR-122 in 15 pairs of human tissues (hepatocarcinoma and paracarcinoma tissues). (A) The miR-122 expression level had an average of 44% reduction in hepatocarcinoma tissues compared with paired paracarcinoma tissues. (B) The expression level of miR-122 transfected with miR-122 mimics or negative control in the SMMC7721 cells ($p < 0.05$). (C) The different miR-122 expression level of miR-122 antisense oligodeoxynucleotide (ASO) transfection and control group in the Hep3B cells ($p < 0.05$). * $p < 0.05$.

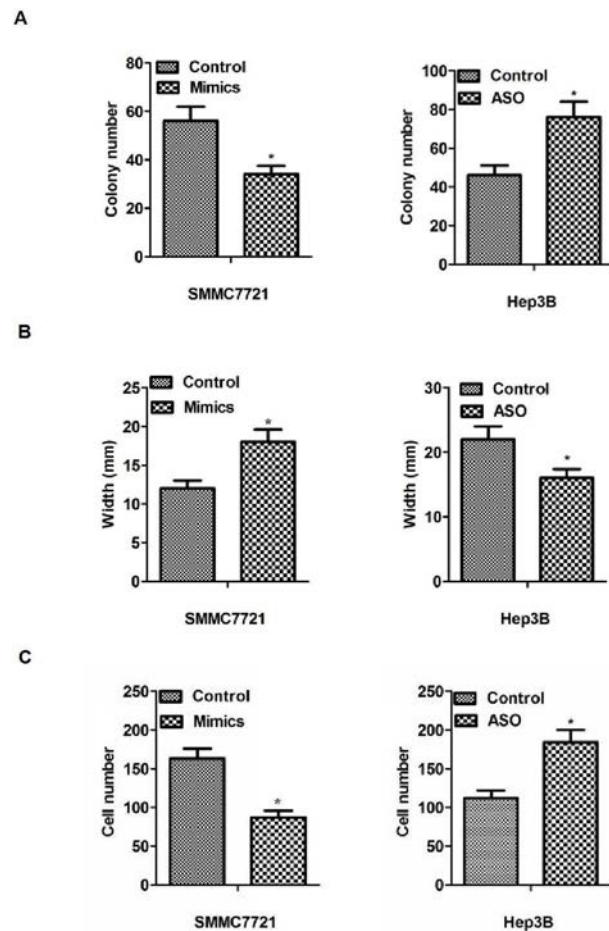


Figure 2. The function of miR-122 on the proliferation, migration, and invasion of hepatocarcinoma cells in vitro. (A) The results of colony formation. Cell proliferation was inhibited or promoted by transfecting miR-122 mimics or ASO. (B) The results of migration detected by scratch assays. The results showed a significantly larger or smaller width in the transfecting miR-122 mimics or ASO group compared with control in vitro ($p < 0.05$). (C) The results of invasion detected by Transwell assays. The results showed significantly less or more cells in the treated group compared with control in vitro ($p < 0.05$). * $p < 0.05$.

LMNB2 Was a Downstream Target of miR-122, and miR-122 Reduced lmn2 Expression in Hepatocarcinoma Cells

We found that LMNB2 was on the list of miR-122 targets suggested by a bioinformatic software (<http://www.microrna.org>) and that LMNB2 was the target gene of miR-122 by bioinformatic analysis (<http://www.microrna.org>). As shown in Figure 3A, to test the possible link between miR-122 and LMNB2, the wild type or mutant of LMNB2 UTR was inserted into firefly luciferase downstream to create the pGL3-LMNB2 UTR WT or the pGL3-LMNB2 UTR Mut construct, respectively. Then those plasmids were transfected into SMMC7721 cells.

Dual-luciferase reporter assay proved that wild-type 3'-UTR inhibited luciferase activity compared with mutant 3'-UTR, showing that miR-122 significantly decreased the relative (A1/A2: pGL3-LMNB2 UTR WT or pGL3-LMNB2 UTR Mut construct group/pGL3 construct group) luciferase activity of the wild-type miR-122 3'-UTR compared to the mutant miR-122 3'-UTR (Fig. 3B), suggesting that LMNB2 was regulated by miR-122 through binding to 3'-UTR. To investigate the mechanism of miR-122 in the progression of hepatocarcinoma cells via its own ability to repress its downstream gene LMNB2 expression, the expression level of LMNB2 in a different treatment with miR-122 was investigated by Western blot. The results showed that the expression level of LMNB2 was low with miR-122 mimics compared with control in SMMC7721 cells (Fig. 3C). In addition, the expression level of LMNB2 was high when treated with miR-122 ASO compared with ASO control in Hep3B cells (Fig. 3D). To sum up, miR-122 could downregulate the LMNB2 expression in hepatocarcinoma cells.

LMNB2 Could Inhibit Cell Proliferation, Migration, or Invasion in Hepatocarcinoma Cells

To demonstrate the importance of LMNB2 on the biological function of hepatocarcinoma cells, we examined the level of LMNB2 in the hepatocarcinoma cell lines (data not shown). The results were similar with a previous study³⁸, so we could use the similar method to explore the function and the mechanism of LMNB2³⁹: LMNB2 siRNA or plasmid was used to decrease or increase the expression of LMNB2 in SMMC7721 or Hep3B cell lines. As depicted in Figure 4A, the expression level of LMNB2 was decreased or increased in SMMC7721 or Hep3B cells, respectively ($p < 0.05$). The colony formation assay demonstrated that capacity for proliferation caused by the increased or decreased LMNB2 was significantly enhanced or weakened compared to the control in Hep3B or SMMC7721 cells, respectively ($p < 0.05$) (Fig. 4B). The results of scratch assay indicated significantly smaller or larger width caused by increased or decreased LMNB2 compared with the control in Hep3B or SMMC7721 cells, respectively ($p < 0.05$) (Fig. 4C). The results of Transwell assay demonstrated significantly more or less cells caused by the increased or decreased expression of LMNB2 than that in the control in Hep3B or SMMC7721 cells, respectively ($p < 0.05$) (Fig. 4D). The results indicated that overexpression or loss of LMNB2 level promoted or inhibited cell proliferation, migration, and invasion in hepatocarcinoma cells.

Inhibiting Effect of miR-122 ASO by Increasing LMNB2 In Vivo

To determine the inhibition effect of miR-122 and to investigate whether LMNB2 expression was regulated by

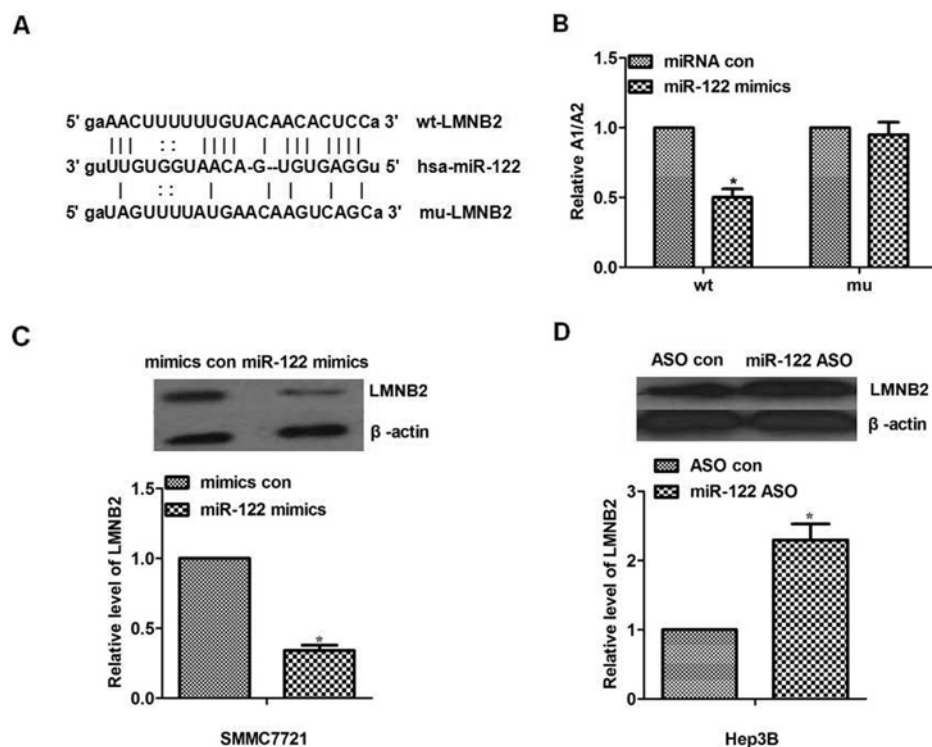


Figure 3. miR-122 downregulated lamin B2 (LMNB2) by targeting 3'-UTR. (A) Bioinformatics results. The 3'-UTR of LMNB2 contains a potential miRNA-binding site for miR-122. We inserted the LMNB2 UTR with either a wild-type or mutant miR-122 target sequence downstream of the firefly luciferase gene into the pGL3-control vector (Promega) to create the pGL3-LMNB2 UTR WT or the pGL3-LMNB2 UTR Mut construct, respectively. Bioinformatics predicts binding sites between miR-122 and 3'-UTR of LMNB2. (B) The result of luciferase reporter assay. In the wild-type group, SMMC7721 cells were transfected with pGL3-LMNB2 UTR WT, pGL3 control plasmid. In the mutant group, SMMC7721 cells were transfected with pGL3-LMNB2 UTR WT, pGL3 control plasmid. miR-122 significantly decreased the relative (A1/A2: pGL3-LMNB2 UTR WT or pGL3-LMNB2 UTR Mut construct group/pGL3 construct group) luciferase activity of the wild-type miR-122 3'-UTR compared to the mutant miR-122 3'-UTR ($p < 0.05$). (C) The expression level of LMNB2 was detected using Western blot. The LMNB2 expression treated with miR-122 mimics was decreased in SMMC7721 cells ($p < 0.05$). (D) The expression level of LMNB2 was detected using Western blot. The LMNB2 expression treated with miR-122 ASO was increased in Hep3B cells ($p < 0.05$). * $p < 0.05$.

miR-122 in vivo, SMMC7721 cells were injected subcutaneously into the right flank of nude mice. The result showed that the volume of tumors in miR-122 groups was decreased compared with the control group (Fig. 5A). We also investigated LMNB2 expression in the tumors followed by Western blot assay, and the results demonstrated that LMNB2 protein expression was reduced by miR-122 ($p < 0.05$) (Fig. 5B). These results suggested that miR-122 has an antitumor effect and was safe to use in vivo. In summary, our data suggested that miR-122 might target LMNB2 in vivo to inhibit the progression of hepatocarcinoma.

DISCUSSION

miRNAs act with diverse function (promote or suppress development) in the cellular process of various kinds of cancer, including hepatocarcinoma^{2,6-13}. miR-122 has reduced expression, and it is an important tumor suppressor in patients with hepatocarcinoma^{2-4,27}. Studies

have shown the potential role of miR-122 as a tumor suppressor, and the evidence was from clinical tissue specimens, animals in vivo, and the cell lines in vitro^{2-4,27}. Furthermore, it was reported that several liver-enriched transcription factors, such as C/EBP, HNF1, HNF3, HNF4, and HNF6, were shown to activate miR-122 gene expression in hepatic cell lines²⁷⁻³⁰. It was speculated that diminished level of miR-122 during hepatocarcinoma might be one of the reasons for tumor progression by altering the expression of its target genes²⁷⁻³¹. Up to now, miR-122 has been verified to be associated with chemoresistance in types of chemotherapy drugs including doxorubicin, sorafenib, adriamycin, and vincristine in cancer³²⁻³⁵. Another two studies reported that the multifunctional miR-122 loaded graphene-gold composites on drug-resistant liver cancer^{36,37}. However, different from these studies, a recent study demonstrated that miR-122 could promote 5-FU resistance by targeting PCDH20 gene in hepatocarcinoma, and in this study miR-122 could

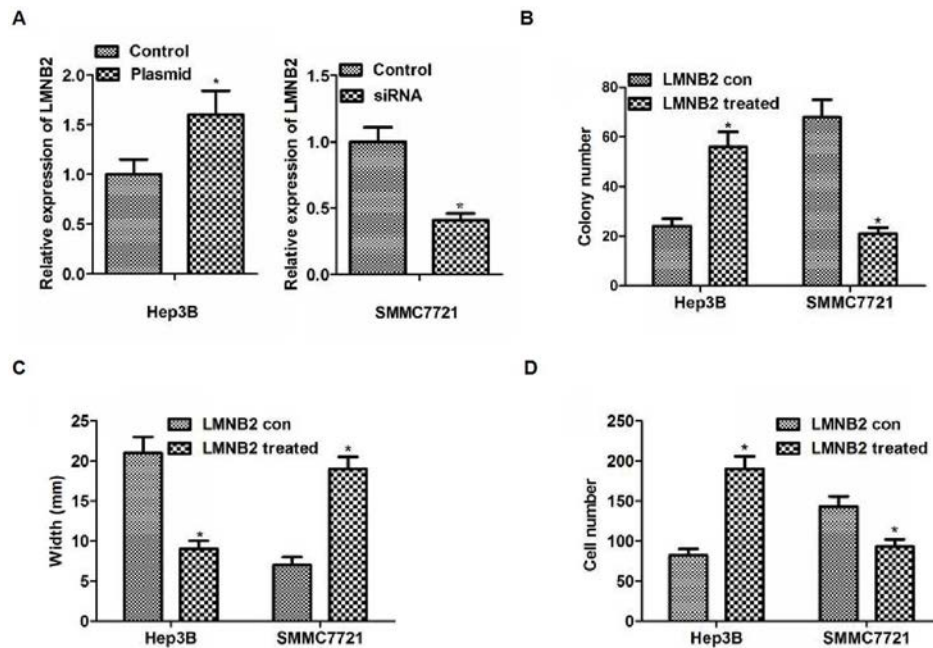


Figure 4. Influence of LMNB2 on the biological behaviors of hepatocarcinoma cell lines. (A) Hep3b cells or SMMC7721 transfected with LMNB2 overexpression plasmid or siRNA, respectively. Western blot was used to detect protein level of LMNB2. The LMNB2 expression level was increased in Hep3B cells with overexpression LMNB2, and it was decreased in SMMC7721 cells transfected with siRNA ($p < 0.05$). (B) The images of colony formation assays (up) and quantification (bottom). The colony number was increased in Hep3B cells with overexpression LMNB2, and colony numbers was decreased in SMMC7721 cells transfected with siRNA ($p < 0.05$, respectively). (C) The results of migration detected by scratch assays. The results showed a significantly smaller or bigger width in the plasmid or siRNA of LMNB2 group compared with control in vitro ($p < 0.05$). (D) The results of invasion detected by Transwell assays. The results showed significantly more or less cells in the plasmid or siRNA of LMNB2 group compared with control in vitro ($p < 0.05$). * $p < 0.05$.

promote cell proliferation and inhibit cell apoptosis in hepatocarcinoma cells treated with 5-FU⁵. This provides a new insight on the function of miR-122 in hepatocarcinoma, which suggests that miR-122 has different effects in different stages of hepatocarcinoma development.

The aim of the present study was to demonstrate the biological function of miR-122 in the development of hepatocarcinoma and to investigate its mechanism. First of all, we found that miR-122 was decreased in hepatocarcinoma tissues compared to the paracarcinoma tissues,

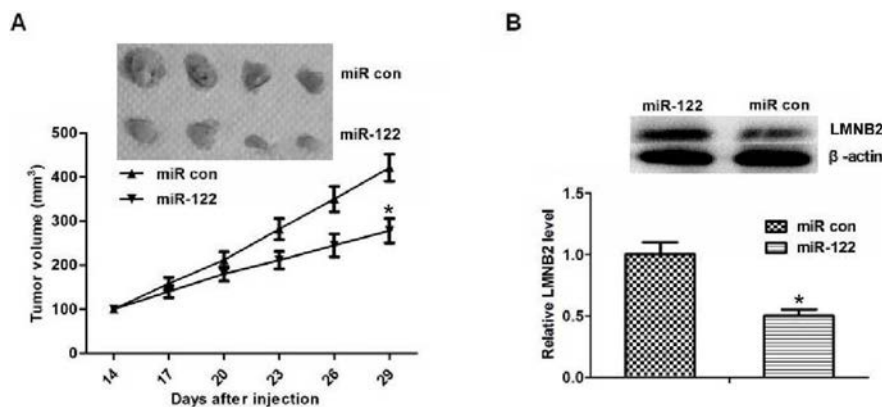


Figure 5. Inhibiting effect of miR-122 by reducing LMNB2 in vivo. (A) The mice tumor volume in different groups. After 29 days of treatment, the miR-122 therapy decreased the tumor volume compared to the control group, and the average tumor volume in miR-122 group was decreased significantly ($p < 0.05$). (B) Western blot results of LMNB2 expression in different groups. LMNB2 expression was decreased in the miR-122-treated group in hepatocarcinoma cells injected with tumor xenografts in nude mice, and its expression was lower in the miR-122-treated group than in the control group ($p < 0.05$). * $p < 0.05$.

and overexpressed miR-122 could inhibit cell proliferation, migration, or invasion *in vitro*, which was consistent with previous studies^{38,40}. Moreover, we confirmed that LMNB2 was a direct target of miR-122 in hepatocarcinoma cell lines. Thus, we demonstrated that the aberrant alteration between miR-122 and LMNB2 might be associated with the progression in hepatocarcinoma. Then we found that LMNB2 was a target gene of miR-122 by software such as TargetScan. The direct relationship was further supported by luciferase reporter assay. Finally, we proved the relationship between miR-122 and LMNB2 through the restoration experiment *in vitro* and the xenografts assay *in vivo*.

LMNB2, an intermediate filament protein that belongs to the lamins family known as the nuclear lamina, has been reported to be overexpressed in non-small cell lung cancer^{41–44}. There are LMNA, LMNB1, and LMNB2 genes in the lamins family, and many studies have reported the link between lamins and cancer. Low LMNA expression is associated with colon cancer and lung adenocarcinoma^{41,45}. LaminA/C is overexpressed in neuroblastoma, prostate cancer, hepatocellular carcinoma, breast cancer, and low-grade endometrial cancer^{46–51}. LaminB1 expression correlates with poor prognosis in hepatocellular carcinoma, prostate cancer, and pancreatic cancer^{52,53}. Recently, different from the low expression of LMNB1 in lung adenocarcinoma, LMNB2 was reported to be overexpressed in patients with non-small cell lung cancer and to be a prognostic marker for proliferation and metastasis^{41,42}. In accord with these results, we showed that LMNB2, as a tumor oncogene, promoted cell proliferation, migration, and invasion in hepatocarcinoma. Moreover, we found that the expression level of LMNB2 was high or low in SMMC7721 or Hep3B cell line in the literature⁴¹, which might provide a clue to the relationship with miR-122. To sum up, the above provided the indirect evidence for the hypothesis that miR-122 could promote hepatocarcinoma development via targeting LMNB2. Furthermore, our data showed that LMNB2 was regulated by miR-122, and we could also perform in a similar method to prove their correlation from previous studies^{5,32–35}. Therefore, we used a similar method to further explore the mechanism. The bioinformatic analysis, dual-luciferase reporter assay, and the regulation results (with the same tendency of the relations between the upstream and downstream genes) demonstrated that LMNB2 as an miR-122 target gene functions on cell proliferation, migration, and invasion.

As shown in our study, we could observe the inhibiting effect of miR-122 by reducing the LMNB2 expression against hepatocarcinoma *in vitro* and *in vivo*. Nevertheless, future studies should also determine the above mechanism in various kinds of hepatocarcinoma cells.

To sum up, our data demonstrated that miR-122 might promote hepatocarcinoma cell progression by targeting

LMNB2, and miR-122-LMNB2 might serve as a therapeutic target for hepatocarcinoma.

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