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Increased expression of microRNA-31-5p inhibits cell proliferation, migration, and invasion via regulating Sp1 transcription factor in HepG2 hepatocellular carcinoma cell line

Guoliang Zhao [a], Chuangye Han [a], Zhi Zhang [b], Lei Wang [c], Jing Xu [a, *]

[a] Department of Hepatobiliary Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Province, China
[b] Department of Hepatobiliary Surgery, The First People's Hospital of Nanning, Nanning 530022, Guangxi Province, China
[c] Department of Ultrasound, Shandong Provincial Hospital, Jinan 250021, Shandong Province, China

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ABSTRACT

Accumulating evidence has suggested that microRNA-31-5p (miR-31-5p) is dysfunctional in hepatocellular carcinoma (HCC). However, the molecular mechanism of HCC remains unclear. In this study, we investigated the role of miR-31-5p in tumor formation and development of HCC. The expression of miR-31-5p was detected in HCC tissues, corresponding adjacent tissues, normal liver tissues, and HCC cell lines. miR-31-5p mimics and an inhibitor were transfected into HepG2 cells to assess the effects of miR-31-5p on cell proliferation, apoptosis, cell cycle, migration, and invasion assays. Western blotting was used to detect the expression of Sp1 transcription factor (SP1), cyclin D1, and survivin in transfected HCC cells and control cells. The expression of miR-31-5p was significantly decreased in HCC cells and HCC tissues. Overexpression of miR-31-5p inhibited HCC cell growth, migration, and invasion. Overexpression of miR-31-5p reduced the expression of SP1 and cyclin D1, and knockdown of SP1 decreased cyclin D1 expression. The dual luciferase assay showed that miR-31-5p directly targeted SP1 in HepG2. Together, the results suggested that miR-31-5p acted as a tumor suppressor to regulate SP1, and that miR-31-5p could be used as a therapeutic target for the treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the main type of primary liver cancer that is nearly equal in morbidity and mortality. It is the third leading cause of cancer deaths worldwide. Approximately 745,500 people die of liver cancer each year [1,2], and there is currently no consistently effective treatment for HCC [3]. Current treatments for HCC that include surgery, local ablation, chemotherapy, and radiotherapy are not satisfactory [4,5]. Hence, the prognosis and 5 year survival of liver cancer are very poor. Previous studies reported that tumor suppressor inactivation and abnormal regulation of signaling pathways were closely related to the recurrence of HCC [6]. Although the mechanism of HCC pathogenesis is unclear, knowledge of the biological mechanisms of liver cancer cells could help develop effective treatment strategies.

Some studies reported that miRNAs can induce gene degradation or overexpression and may play a key role in physiological processes of different cancers [7]. We have also reported that miRNAs play an important role in tumorigenesis, proliferation, and apoptosis in different cancers. Multiple miRNAs have been reported to promote or inhibit metastasis [8]. Increasing evidence has suggested that miRNAs may be used as tissue-specific biomarkers for HCC [9,10], and the miRNAs may be an effective treatment for liver cancer. It is therefore necessary to first characterize the endogenous regulation of miRNAs in HCC.

Many epigenetic events during tumor development have been reported [11]. The microRNAs (miRNAs) are a group of small non-coding RNAs that are 20–24 nucleotides in length that have different modes of expression in different tumors [12,13]. They can combine with the complementary sequence of target genes to regulate the expression of genes. Previous studies [5], including studies of miR-744 [14], miR-590-5p [15], and miR-31 [16], have reported that miRNAs play important roles in tumorigenesis and tumor suppression. Among these miRNAs, miR-31-5p is expressed in various tissues and cell types in humans. Kim et al. reported that miR-31 acted as a tumor suppressor in HCC by regulating the cell cycle and epithelial mesenchymal transition [16]. The miR-31 also plays an important role in other tumors such as cervical cancer [17],

* Corresponding author.
E-mail address: dr_xujing@163.com (J. Xu).

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gastric cancer [18], colorectal cancer [19], esophageal squamous cell carcinoma [20], and breast cancer [21].

It is possible to predict the targets of a specific miRNA by using a bioinformatics database. Many targets of miR-31 have been reported, including GNA13 [21], HDAC2 [16], CDK2 [16], Smad4 [18], E2F2 [19], LATS2 [22], PPP2R2A [22], and WAVE3 [23]. Using bioinformatics, we found that SP1 was a potential target of miR-31-5p. Although miR-31-5p has been associated with the occurrence and development of HCC, the specific molecular mechanisms of these processes remain unknown.

In this study, we investigated the roles of miR-31-5p in HCC that may provide a candidate molecule and model for therapeutic intervention of HCC.

2. Materials and methods

2.1. Cell culture conditions

HCC cell lines (SMMC-7721, MHCC97H, MHCC97L, and HepG2) and immortal normal liver cell line HL-7702 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and were maintained at 37 °C with an atmosphere of 5% CO_2.

2.2. Tissue samples

Sixteen normal liver tissues, and 30 paired HCC tissues and adjacent non-tumor liver tissue were obtained from the First Affiliated Hospital of Guangxi Medical University. All samples were frozen using liquid nitrogen, then stored at −80 °C. The patients did not have any other treatment before surgery. All liver cancer patients were diagnosed by pathological diagnoses. All samples were obtained after informed consent, and the study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University.

2.3. Transfection and assays

All mimics and inhibitors were chemically synthesized and purified by Thermo Scientific (Waltham, MA, USA). When the density of HepG2 cells reached approximately 80% in six-well culture plates, 50 nM of miR-31-5p mimics or negative control mimics, with either 100 nM of miR-31-5p inhibitors or negative control inhibitors was transfected into the HepG2 cells using the Lipo6000™ transfection reagent (Beyotime Biotechnology, Shanghai, China). Gene expression was detected after 24 h of transfection, and protein expression was detected after 72 h of transfection. To knockdown human SP1, siRNA targeting was performed using the Lipo6000™ transfection reagent, and the protein expression was measured after 72 h of transfection.

2.4. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was isolated from HCC cell lines and HCC tissues using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, then reverse transcribed into cDNA using the miRcute Plus miRNA First-Strand cDNA Synthesis Kit (Tiangen Biotech, Beijing, China). U6 small RNA was used as an internal control for normalization and quantification of miR-31-5p(miRBase Accession number: MIMAT0000089) expression. Real-time PCR was performed using the miRcute Plus miRNA qPCR Detection Kit (SYBR Green) (Tiangen Biotech, Beijing, China) and the ABI7500 real-time PCR system, including a reverse primer according to the manufacturer's instructions. All experiments were done in triplicate. The $2^{-\Delta\Delta Ct}$ method for relative quantification of gene expression was used to determine miRNAs expression levels. All primers (U6 forward primer, catalogue number, CD201-0145; miR-31-5p forward primer, catalogue number, CD201-0033) were synthesized by Tiangen Biotech.

2.5. Western blot analysis

After extracting protein from the cells, the lysates containing equal amounts of protein were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for electrophoresis and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk for 2 h, and then incubated with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 h at 4 °C, followed by 2 h of incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Finally, the proteins were detected by enhanced chemiluminescence using the ECL Plus Kit (Amersham, Buckinghamshire, UK). The western blot bands were quantified using the LAS 3000 (Fuji Film, Tokyo, Japan). The antibodies used were anti-SP1(sc-420, 1:500), anti-cyclin D1 (sc-8396, 1:500), anti-GAPDH(sc-32233, 1:1000), anti-survivin(sc-17779, 1:250), which were purchased from Santa Cruz Biotechnology.

2.6. Cell proliferation assay

Cells (1.0×10^4 cells/mL) were plated onto 96-well plates in complete medium and exposed to fresh media every other day. The cells were treated with 50 μg/well of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (Sigma-Aldrich, St. Louis, MO, USA) every 24 h and incubated for 4 h. The resulting precipitate was dissolved in dimethylsulfoxide, and the absorbance was measured at 570 nm using a multilabel plate reader.

2.7. Cell migration and invasion assays

A wound healing assay was used to determine the cell migration ability. When the cell density reached approximately 90%

Table 1
Correlation between the clinicopathologic characteristics of 30 HCC patients and the expression of miR-31-5p in HCC tissues.

Characteristics	n = 30	miR-31-5p		
		Higher	Lower	*P*-value
Gender				
Male	26	14	12	0.598
Female	4	1	3	
Age (years)				
<60	22	10	12	0.682
≥60	8	5	3	
Differentiation				
Poorly	5	2	3	1.000
Moderate/well	25	13	12	
BCLC stage				
0/A	19	13	6	**0.021**
B	11	2	9	
Hepatitis B				
Negative	3	1	2	1.000
Positive	27	14	13	
Tumor size				
<5 cm	9	3	6	0.472
≥5 cm	21	12	9	

Note: The median of mRNA expression in HCC patients was used as the cut-off point to define lower and higher expression groups. *P*-value < 0.05 was considered statistically significant.

confluency, a 100-μL sterile pipette tip was used to scrape a wound, and the cell migration distance to the scraped area was recorded at 0 and 24 h. A Transwell® invasion assay was used to assess the cell invasion ability. A total of 2.5×10^4 cells suspended with serum-free DMEM were plated onto ECMatrix™-coated inserts (Millipore), and 24-well companion plates were maintained using 10% fetal bovine serum (FBS) in DMEM. After incubation for 24 h, the inserts were washed three times with phosphate-buffered saline (PBS), fixed for 20 min with methanol, and stained with 0.5% Crystal Violet for 30 min. The cells in the upper chamber were carefully removed using a cotton tip, and the invaded cells were photographed using a bright field microscope. Each insert was counted using five fields at a magnification of 20×.

2.8. Flow cytometry analysis

The HepG2 cells were digested using trypsin, washed with ice-cold PBS, and centrifuged at 1000 rpm for 5 min. The cells (1×10^6) were fixed in pre-chilled 70% ethanol at −20 °C for 1 h and incubated with 0.25 mg/mL RNase A at 37 °C for 30 min. Propidium iodide (PI; KeyGen, Nanjing, China) was incubated with the cells at 37 °C in the dark for 30 min. A FACS Calibur Flow Cytometer (BD

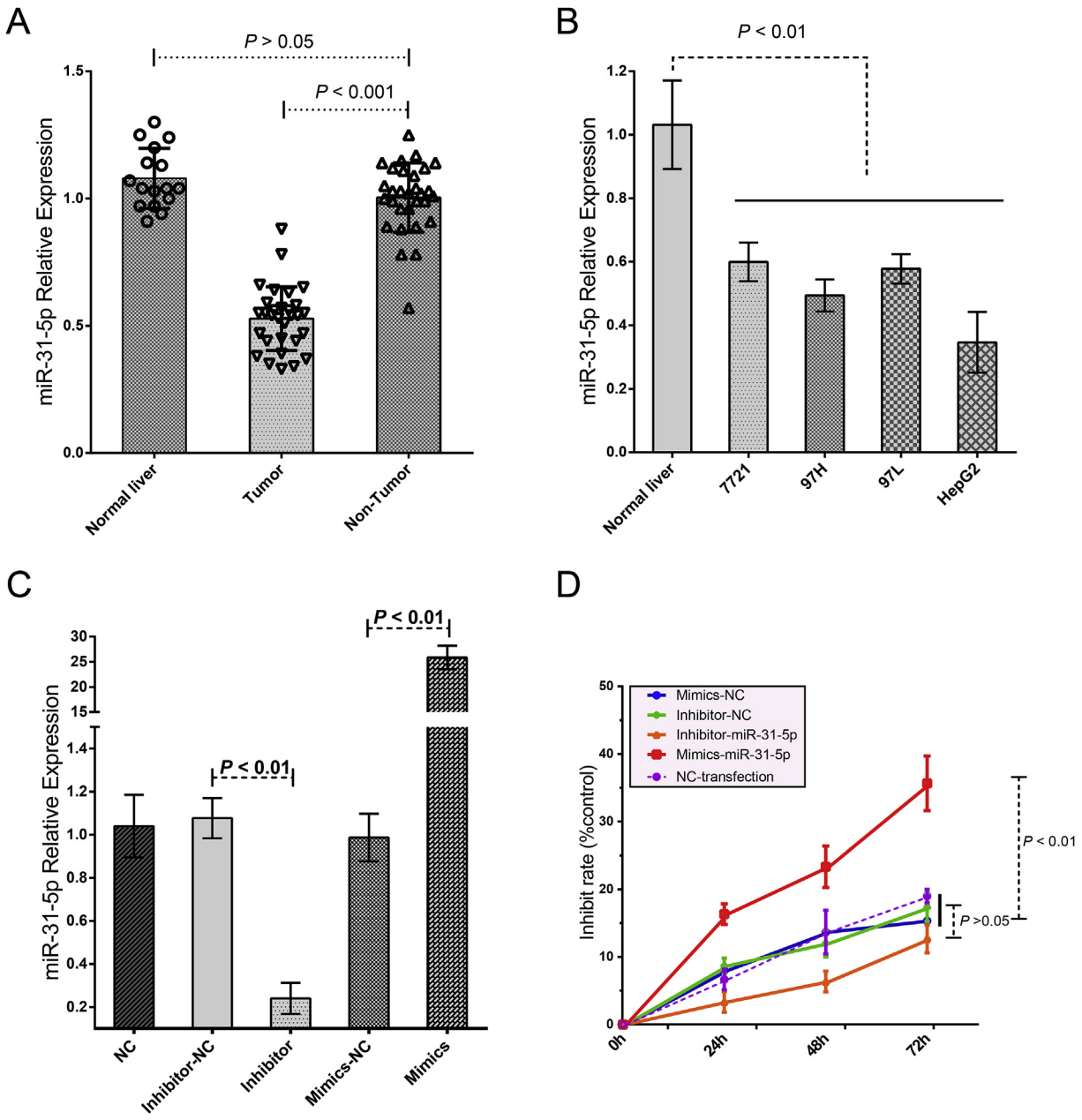

Fig. 1. The expression of miR-31-5p was significantly decreased in hepatocellular carcinoma (HCC) tissues and HCC cell lines, and overexpression of miR-31-5p inhibited HepG2 growth. (A) The expression of miRNA-31-5p in 16 human normal liver and 30 pairs of HCC and adjacent tissues. The expression of miR-31-5p was significantly decreased in HCC tissues compared with the adjacent tissues ($P < 0.001$). (B) The expression of miR-24-3p in HCC cell lines (SMMC-7721, MHCC-97H, MHCC-97L, and HepG2) and immortal cancer cell line HL-7702. The expression was significantly decreased in HCC cell lines compared with immortal cancer cell line HL-7702 ($P < 0.001$). (C) The transfection of miR-31-5p mimics and inhibitors increased or decreased the expression of miR-24-3p in HepG2 cells. (D) The transfection of miR-31-5p mimics inhibited HepG2 cells growth compared with the other transfection groups ($P < 0.01$), detected by MTT method. NC = normal control (50 nM of miR-31-5p mimics or negative control mimics, with either 100 nM of miR-31-5p inhibitors or negative control inhibitors was transfected into the HepG2 cells).

Biosciences, Franklin Lakes, NJ, USA) was then used for analyses of the cell cycle. An annexin V-FITC/PI Apoptosis Kit (BD Biosciences) was used to detect apoptosis according to the manufacturer's instructions. The annexin fluorescence intensity was measured using FCM flow cytometry.

2.9. Luciferase reporter assay

TargetScan (www.targetScan.org) was used for the prediction of miRNA target genes. The predicted results show that SP1 is one of the target genes for miR-31-5p. To confirm the regulation between miR-31-5p and SP1, a luciferase reporter assay was performed in HEK293T cells. The cells were grown to 60–70% confluency in 24-well plates with luciferase reporter plasmids carrying wild-type SP1 3′-UTR, mutant SP1 3′-UTR, and a 3′-UTR negative control and hsa-miR-31-5p or hsa-miR negative control plasmid and renilla plasmid cotransfected into HepG2 cells. Cells were collected 48 h after transfection and analyzed using the Luciferase Assay System, according to the manufacturer's instructions (Promega Corporation, Madison, Wisconsin, USA).

2.10. Statistical analysis

All experiments were repeated at least three times. The data were evaluated using the Student's *t*-test for unpaired data using SPSS statistical software for Windows, version 17.0 (SPSS, Chicago, IL, USA), with error bars representing the mean ± SD. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-31-5p was downregulated in human HCC tissues and cell lines

Correlation between the clinicopathologic characteristics of 30 HCC patients and the expression of miR-31-5p in HCC tissues were showed in Table 1, We found that the expression of miR-31-5p was significantly associated with BCLC stage ($P = 0.021$). We determined the expression levels of miR-31-5p in several HCC cell lines, paired HCC tissues and adjacent non-tumor liver tissue, and normal liver tissues, and found that miR-31-5p levels were significantly

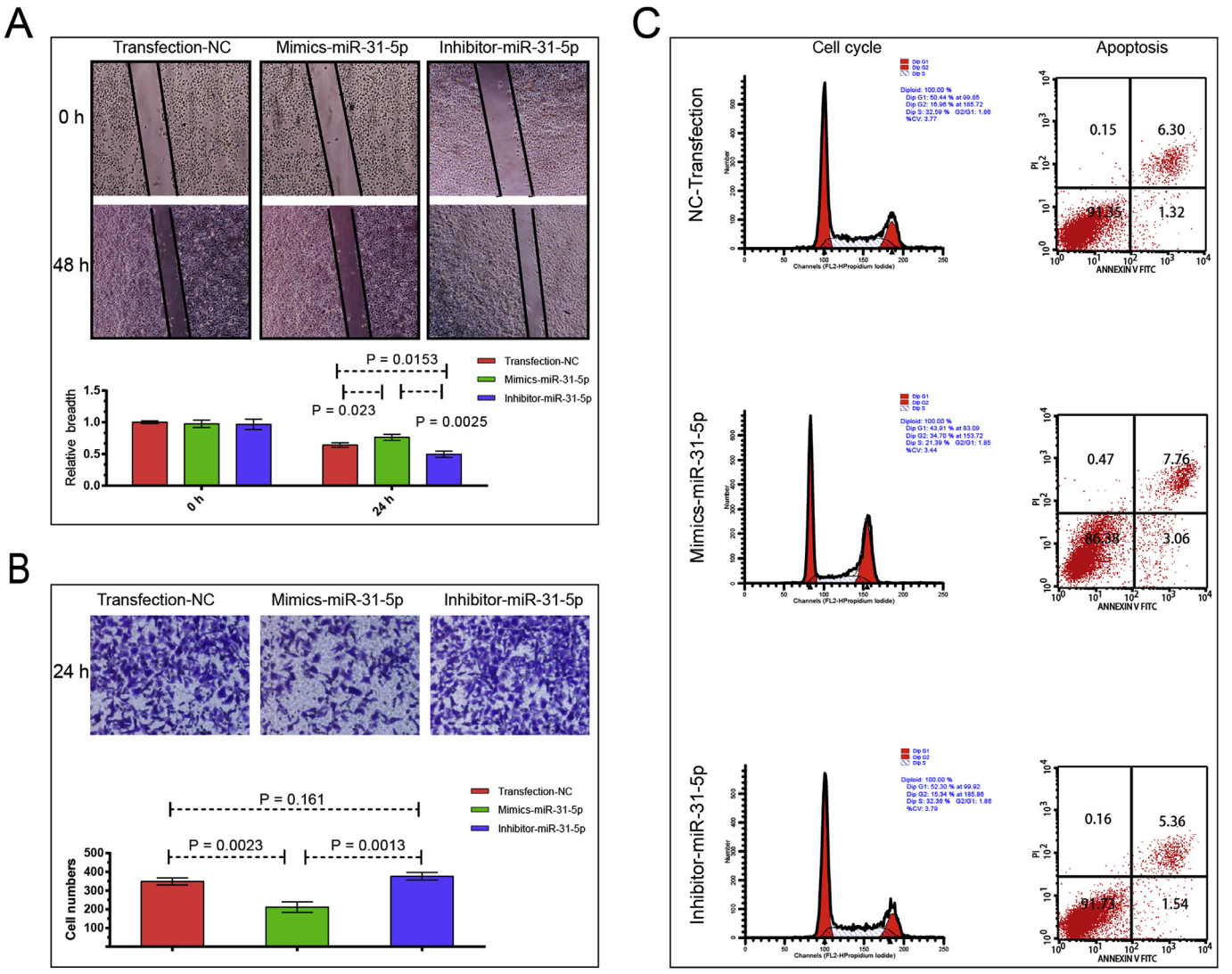

Fig. 2. The overexpression of miR-31-5p in HepG2 cells induced inhibition of invasion, metastasis, and cycle arrest(50 nM of miR-31-5p mimics or negative control mimics, with either 100 nM of miR-31-5p inhibitors or negative control inhibitors was transfected into the HepG2 cells). (A) Transfection of miR-31-5p mimics and inhibitors increased or decreased the invasion ability in HepG2 cells. (B) The transfection of miR-31-5p mimics and inhibitors increased or decreased the metastasis in HepG2 cells. (C) The overexpression of miR-31-5p by transfection of mimics in HepG2 cells induced cycle arrest, but did not promote apoptosis.

decreased in HCC tissues versus adjacent non-tumor liver tissue and normal liver tissues ($P < 0.001$; Fig. 1A). Furthermore, the levels of miR-31 in the HCC cell lines were lower than that in immortal normal liver cell line HL-7702 ($P < 0.01$; Fig. 1B). Together, these results showed that miR-31-5p was significantly downregulated both in HCC tissues and cell lines, and suggested that miR-31-5p may play a role in the development of HCC.

3.2. The overexpression of miR-31-5p inhibited HCC cell growth, migration, and invasion

To evaluate the effects of miR-31-5p on proliferation of HepG2 cells, we transfected miR-31-5p mimics, inhibitors, and negative control oligonucleotides into HepG2 cells. Fig. 1C shows that transfection of miR-31-5p mimics resulted in overexpression, and transfection of miR-31-5p inhibitors silenced its expression. At 72 h after transfection, overexpression of miR-31-5p inhibited the proliferation of HepG2 cells (Fig. 1D).

The wound healing assay and the Transwell® invasion assay were then used to determine the metastatic potential of HCC cells. The results showed that overexpression of miR-31-5p inhibited cell migration and invasion compared with the control groups (both, $P < 0.05$; Fig. 2A and B).

3.3. The overexpression of miR-31-5p promoted HCC cell cycle arrest

To assess the effects of miR-31-5p on cell proliferation, we analyzed the cell cycle using flow cytometry after transfection of miR-31-5p mimics and inhibitors into HepG2 cells. The cell cycle was arrested in the G2 phase, with 34.70% of the miR-31-5p-transfected group in the G2/M interface versus 16.96% of the negative control oligonucleotide-transfected group in the G2/M interface (Fig. 2C). In addition, overexpression of mir-31-5p did not significantly induce apoptosis of HepG2 cells (Fig. 2C). Together, the results showed that miR-31-5p promoted cell cycle arrest, but did not promote apoptosis in human HepG2 cells.

3.4. miR-31-5p negatively regulated SP1, resulting in an altered expression of cyclin D1

SP1 is an important gene involved in the regulation of the cell cycle, apoptosis, cell proliferation, and cell invasion. Previous studies reported that SP1 miR-31-5p targeted SP1 in gastric

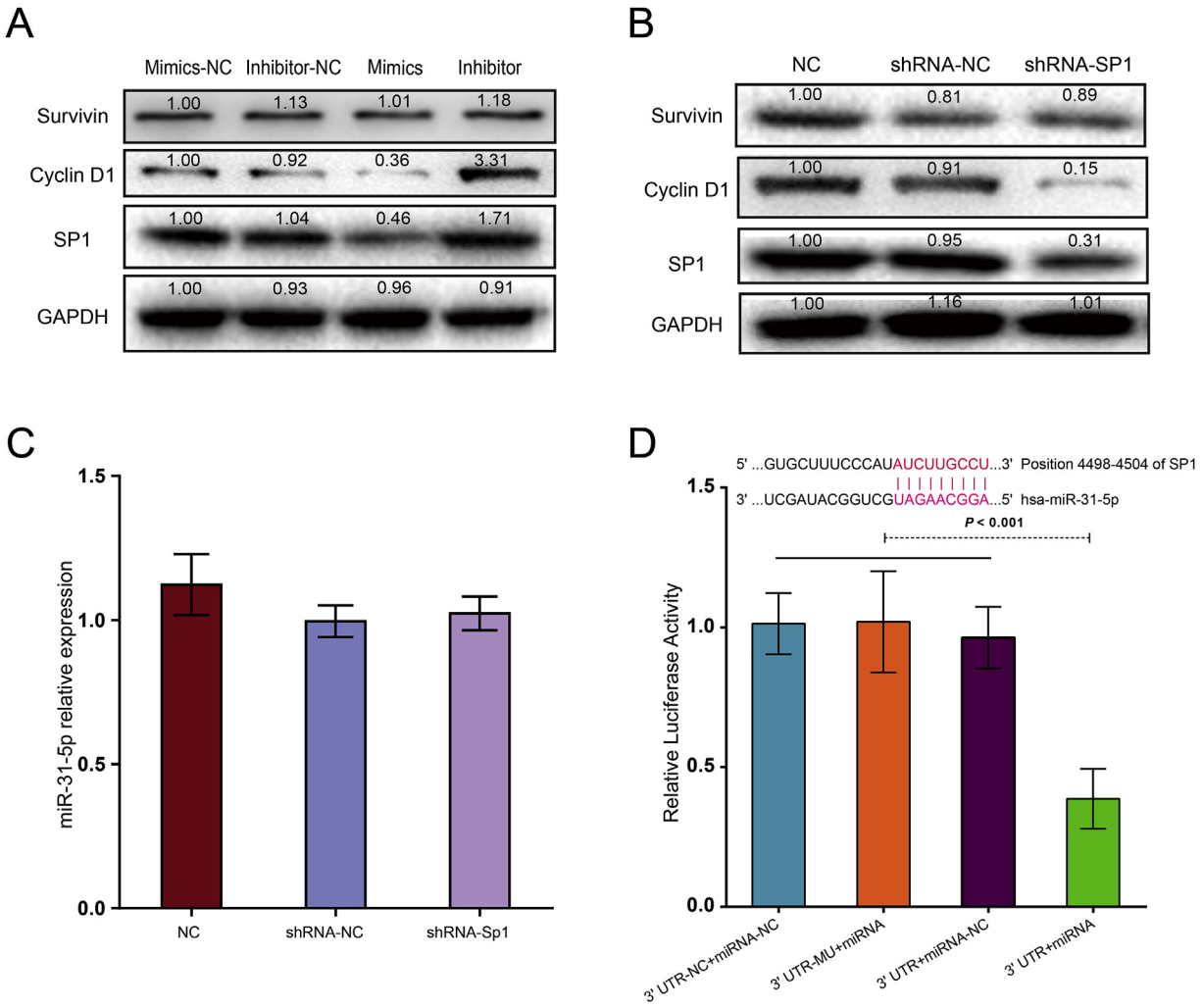

Fig. 3. The miRNA-31-5p directly targeted SP1, and SP1 was involved in regulating cyclin D1 expression. (A) The miR-31-5p mimic and inhibitor regulated SP1, cyclin D1, and survivin protein expression, as detected by western blots. (B) The protein expression of SP1, cyclin D1, and survivin in HepG2 cells was detected by western blotting after silencing SP1 for 48 h. (C) Silencing of SP1 did not significantly affect the expression of miR-31-5p in HepG2 cells. (D) The dual luciferase reporter assay showed that miR-31-5p binds to the 3′-UTR of SP1, which inhibited the expression of SP1 in HepG2 cells ($P < 0.001$).

cancers. To determine if miR-31-5p can regulate SP1 in HCC cells, we performed western blot analyses in different groups. Fig. 3A shows that SP1 significantly downregulated expression of the miR-31-5p group and the negative control group, and upregulated expression of the inhibitor group compared with the control group. We also found a decreased expression of a cell cycle-related protein gene cyclin D1 in the miR-31-5p group and the increased inhibitor group (Fig. 3A). The results showed that miR-31-5p negatively regulated SP1 and cyclin D1 expression. In addition, the luciferase assay showed that miR-31-5p can directly target SP1 in HepG2 cells (Fig. 3D). miR-31-5p may therefore inhibit cell proliferation by upregulating SP1 and cell cycle-related genes.

3.5. The knockdown of SP1 reduced cyclin D1 expression

We then transfected SP1 siRNA or the negative control into HepG2 cells. The results showed that expression was inhibited at the mRNA and protein expression levels in HCC cells (Fig. 3B). Furthermore, western blots showed that SP1 siRNA inhibited the protein expression of SP1, but did not affect expression of miR-31-5p (Fig. 3B and C).

Survivin is an important apoptosis inhibition factor. We silenced the expression of SP1 using siRNA. Then, we detected the expression of survivin and found that silencing of SP1 did not significantly reduce the expression of survivin. We also confirmed that the effects of SP1 in HCC cell lines were through the regulation of survivin. Together, the results suggested that SP1 regulated cyclin D1 expression to regulate cell proliferation and cell cycle arrest in HCC cells, which was regulated by miR-31-5p and SP1.

4. Discussion

In the present study, we characterized the expression of miR-31-5p in four hepatocellular carcinoma cell lines, 16 normal liver tissues, and 30 paired HCC tissues and adjacent normal tissues. miR-31-5p was significantly downregulated in HCC cell lines and HCC tissues, and overexpression of miR-31-5p contributed to the inhibition of HCC cell proliferation, migration, and invasion. Moreover, our results indicated that SP1 was a direct target of miR-31-5p. Taken together, our studies showed that miR-31-5p inhibited cell proliferation, migration, and invasion by the targeting of SP1. The results increased our understanding of the function of miR-31-5p and its possible roles in the formation and development of HCC.

miR-31-5p is located on the 9p21.3 chromosome. Previous studies have reported that miR-31-5p is differentially expressed in various kinds of cancers, such as upregulated expression in head-and-neck squamous cell carcinoma [24] and lung cancer [22], and downregulated expression in colorectal cancer [25], certain T-cell leukemias [26], and gastric cancer [18]. A previous study also reported that miR-31 is associated with the clinical pathological characteristics of cancer patients [27]. Whether miR-31-5p acts as a inducer or suppressor of tumors may depend on the type of tumor. Although previous studies found that miR-31 regulated cell cycle protein and the EMT in HCC [16], the specific mechanism of miR-31 in hepatocarcinogenesis is still unclear.

Based on our study, the expression of miR-31-5p was significantly lower in four HCC cells lines, indicating that miR-31-5p may act as a tumor suppressor in HCC. These results are consistent with previous reports [16], showing that upregulation of miR-31-5p inhibits HCC cell proliferation and cell cycle arrest.

Analysis of miRWalk (a sequence database) suggested a potential regulation between transcription factors and miRNAs, further indicating a possible connection between the well-known SP1 and miR-31 expression. SP1 is a sequence-specific DNA binding protein that is highly expressed in a variety of cancers. SP1 participates in the transcription of many important regulatory genes that have been correlated with cancer development [28,29]. In addition, it has been shown to be involved in many physiological processes, including angiogenesis and cell cycle progression [30]. Yao et al. reported that high expression of miR-200b inhibited breast cancer cell proliferation, induced apoptosis, and acted on the cell cycle by targeting SP1 [31]. The expression of SP1 in HCC tissues was significantly higher than in the corresponding adjacent tissues, and overexpression of SP1 may functionally participate in HCC occurrence and development [32].

Cyclin D1 is overexpressed in most human tumors, and can play an important role in cell proliferation and cycle. The results of our study showed that SP1 promoted HCC cell proliferation and that knockdown of Sp1 reduced cyclin D1 expression. In addition, miR-31 decreased SP1 expression in HCC cells, and resulted in the downregulation of cyclin D1 expression. These results showed that SP1 was a direct and functional target of miR-31 in HCC HepG2 cells.

In conclusion, miR-31-5p was downregulated in HCC cell lines when compared with normal liver cell lines. Upregulated miR-31-5p inhibited HCC cell proliferation, migration, and invasion, and induced cell cycle arrest by targeting SP1. These findings suggested that miR-31-5p can be used as a target for HCC therapy.

Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Declaration of interest

The authors report no conflicts of interest.

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