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Analysis of the relationship between microRNA-31 and interferon regulatory factor-1 in hepatocellular carcinoma cells

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) play a role in the pathogenesis of hepatocellular carcinoma (HCC). This study was designed to elucidate the role of microRNA-31 (miR-31) in HCC.

MATERIALS AND METHODS: HuH7 cell lines were transfected with miR-31 mimic or miR-31 inhibitor to investigate the role of miR-31 in regulating interferon regulatory factor-1 (IRF-1). The mRNA and protein expression levels of IRF-1 were quantitatively detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot, respectively. Subsequently, Dual-Luciferase reporter assay was also performed.

RESULTS: The expression level of miR-31 was significantly up-regulated in HuH7 cells when compared with that in primary human hepatocytes (hHC). Dual-Luciferase reporter assay indicated that IRF-1 was the direct target of miR-31. The expression levels of IRF-1 were decreased in HuH7 and HepG2 cell lines. IRF-1 was negatively correlated with miR-31 in HCC tissues and paired adjacent tissues. The expression level of miR-31 was inversely correlated with IRF-1. MiR-31 inhibitor up-regulated the expression levels of IRF-1 in HuH7 cells, whereas miR-31 mimic down-regulated the expression levels of IRF-1. Furthermore, the miR-31 mimic repressed IRF-1-3'UTR reporter activity, whereas the miR-31 inhibitor enhanced IRF-1-3'UTR reporter activity depending on the concentration of miR-31 mimic and miR-31 inhibitor.

CONCLUSIONS: These results indicated that miR-31 can regulate the expression level of IRF-1 in HCC, which probably provided novel theoretical evidence for the application of target miR-31 treatment of HCC.

Key Words:
GMiR-31, IRF-1, Hepatocellular carcinoma, Relationship.

Introduction

Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer-related mortality[1]. Despite advancement has been obtained in the treatment of liver cancer, including liver transplantation, immunotherapy, and chemotherapy, the clinical prognosis of most patients with HCC is still unsatisfactory. The overall survival of patients diagnosed with advanced HCC is estimated to be 6 to 20 months. Aberrant expression of miR-31 has been proven to be associated with the pathogenesis of HCC[2].

MicroRNAs (miRNAs) are a group of small non-coding RNAs (ncRNAs), which can repress the translation target mRNAs by base pairing to partially complementary sequences in their 3'-untranslated region (UTR), eventually lead-

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ing to suppressing the translation to protein or mRNA degradation[1]. MiRNAs are capable of regulating multiple functions, such as cell differentiation, cell proliferation, and cell apoptosis, and regulating the expression profile of many target genes[2].

Abnormal expression of miRNAs has been proven to be associated with various categories of cancers. Aberrant expression of miR-31 has been identified in multiple types of malignant tumors. In fact, the expression of miR-31 is down-regulated in patients with bladder cancer[3] and gastric cancer[4], whereas significantly up-regulated in patients with colon cancer[5] and ovarian cancer[6]. In addition, miR-31 has been found to be highly over-expressed in patients with pancreatic cancer[7]. Previously Kim et al[8] have demonstrated that miR-31 is a tumor repressor, which can directly or indirectly regulate the expression levels of specific proteins rather than interferon regulatory factor-1 (IRF-1). In addition[9], miR-31 has been found to play a role in promoting oncogenesis in intrahepatic cholangiocarcinoma cells via the direct suppression of Ras p21 protein activator (RASA1). MiR-31 can possess complex functions by binding to different targets.

IRF-1 plays a certain role in cell cycle, cell apoptosis, immune response, tumor suppression, and so on[10-13]. In the present study, the expression profile of miR-31 was quantitatively measured and compared between primary HCC tissues and paired adjacent normal tissues to identify whether miR-31 can modulate the expression of IRF-1 in HCC tissues. In addition, we explored the potential association between miR-31 and IRF-1 in HCC and unraveled the possible role and underlying mechanism of miR-31 in the pathogenesis of HCC.

Materials and Methods

Cell Line Preparation and Culture

HuH7 and HepG2 cell lines (hepatocellular carcinoma) and HCT116 (colon cancer cell) were obtained from the China Center for Type Culture Collection of Wuhan University (Wuhan, China). HuH7 cells and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza Investments Co., Basel Switzerland), and HCT116 cells were cultured in the McCoy's 5A medium (Gibco, Grand Island, NY, USA). All cell lines were incubated at 37°C in a humid atmosphere consisting of 5% CO_2.

Clinical Specimen Preparation

Thirty paired human HCC tissues and adjacent liver tissues were collected at the Liver Cancer Center of the University of Pittsburgh School of Medicine (Pittsburgh, PA, USA). Surgically removed tissues were frozen in liquid nitrogen and stored at -80°C. This research was approved by the Ethical Institutions of the University of Pittsburgh Institutional Review Board (IRB).

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

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufactures' protocol. 2 μg of total RNA from each sample was reversely transcribed to single-stranded cDNA by using RNA to cDNA EcoDry™ Premix (Clontech, San Francisco, CA, USA). 1 μg of cDNA was diluted by five folds with nuclease-free water and used as a template for the following qRT-PCR. The mRNA expression of IRF-1 was quantified using the IRF-1 primer by One Plus Real-Time PCR system as previously described[14]. IRF-1: forward primer: 5'-ACCCTGGCTAG AGATGCAGA-3', reverse primer: 5'-GCTTTGTATCGGCCTGTGTG-3'. GAPDH: forward primer: 5'-GGGAAGCTTGTCATCA ATGG-3', reverse primer: 5'-CATCGCCCCACTT GATTTTG-3'. The qRT-PCR cycling conditions were performed as follows: 95°C for 10 min, 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 1 min. Exponential amplification was conducted for 40 cycles. The relative expression of IRF-1 was calculated using the $2^{-\Delta\Delta Ct}$ methods.

The expression of miR-31 and U6 snRNA was measured by using qRT-PCR with TaqMan miRNA assay according to the manufacturers' protocol. Each RT reaction 15 μL multiplex reaction contained 150 ng total RNA. Before performed qRT-PCR, the multiplex RT-reactions were diluted with 25 μl nuclease-free water. The diluted RT-products were mixed with TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), without UNG (Applied Biosystems, Foster City, CA, USA). U6 snRNA was used for normalization. MiR-31 and U6 snRNA primers were purchased from Applied Biosystems (Waltham, MA, USA). The qRT-PCR cycling conditions were performed as follows: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s and 60°C for 1 min. The relative expression levels of miR-31 and IRF-1 mRNA were calculated by using the $2^{-\Delta\Delta CT}$ method.

Western Blot

The whole protein was extracted and treated with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA). Nuclear protein was extracted as previously described[15]. According to the previous approach[14], 20 μg of nuclear protein was electrophoresed on the 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane. After 5% non-fat milk blocked at room temperature for 1 h, the membranes were incubated with 1:1000 diluted anti-IRF-1 (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. Lamin A/C (1:2000, Cell Signaling Technology, Danvers, MA, USA) was used as the control. The membranes were washed for three times with Tris-Buffered Saline and Tween-20 (TBST) and incubated with 1:10000 diluted goat anti-rabbit secondary antibody (ZSGB Biotech Co., Ltd., Beijing, China) for 1 h, and developed onto X-ray film using Novex™ ECL Chemiluminescent Substrate Reagent Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Luciferase Reporter Assay

HCT116 cells and HuH7 cells were cultured in a 12-well plate and co-transfected with the pMIR-IRF-1-3'UTR plasmid and the miR-31 mimic (60 pmol/well, Baomaco Biotechnological Technology Co., Ltd., Nantong, China) or miR-31 inhibitor (30 pmol/well, Baomaco Biotechnological Technology CO., LTD., Nantong, China). The relative luciferase unit (RLU) was measured by using the Dual-Luciferase report assay (BioTek, Winooski, VT, USA).

Cell Transfection

HuH7 cells were seeded in a 6-cm dish, and then transfected with Lipofectamine 2000 (Invitrogen, MA, USA), hsa-miR-31 mimic (Baomaco Biotechnological Technology Co., Ltd., Nantong, China) or hsa-miR-31 inhibitor (Baomaco Biotechnological Technology Co., Ltd., Nantong, China) for 6 h, and subsequently replaced by DMEM. After infection for 24 h, HuH7 cells were harvested. The total RNA was extracted to quantitatively detect the expression levels of IRF-1.

Immunofluorescent Staining

Immunofluorescent staining was performed according to the procedures described in our previous study[16]. HuH7 cells were placed on the coverslips and co-cultured with the primary IRF-1 antibody (1:150; Cell Signaling Technology, Danvers, MA, USA) for 2 h and then incubated with Alexa Fluor 488 anti-rabbit IgG antibody (1:250; Invitrogen, Carlsbad, MA, USA) for 90 min at room temperature. After rinsed with phosphate-buffered saline (PBS) for 10 min, the slides were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) for 2 min and mounted overnight and subsequently observed under a Olympus Fluoview FV1000 II microscope (Olympus, Tokyo, Japan).

MTT Assay

Cell proliferation was measured using the 3-[4,5-di-methyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay. Cells were seeded into 96-well plates at a density of 2 × 103 cells/well, and cultured for 24 h, 48 h, and 72 h. Then, 10 μL of MTT (5 mg/ml) was added into each well for 4-h incubation at 37°C. The culture medium was discarded, dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added (100 μL/well) to dissolve the precipitate overnight. The absorbance value was measured by using a plate reader at a wavelength of 570 nm.

Flow Cytometry

Cell apoptosis was detected by using the Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturers' instructions. Cells were collected, washed, and stained with Annexin V-FITC and propidium iodide (PI). After incubation for 15 min in darkness, the cells were examined by flow cytometry (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Statistical analysis was performed using SPSS statistical software (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± standard deviation (SD). Raw data analysis was carried out by using the Student's *t*-test. A *p*-value less than 0.05 was considered as statistical significance.

Results

Aberrant Expression of MiR-31 and IRF-1 in HCC Tissues and Cell Lines

To investigate the relationship between miR-31 and IRF-1 in HCC, the expression profile of

miR-31 and IRF-1 in HCC tissues and HuH7 cells was detected. As shown in Figure 1A, the expression level of miR-31 was negatively correlated with that of IRF-1 in HCC tissues when compared to that in the paired normal adjacent tissues (both $p<0.01$). In addition, the expression levels of miR-31 and IRF-1 in the HuH7 and HepG2 cell lines were also detected. As illustrated in Figure 1B and C, the expression levels of miR-31 in the HCC cells were significantly up-regulated in comparison with those in hHC cells ($p<0.01$). These results indicated that miR-31 was probably associated with the pathogenesis of HCC.

IRF-1 Was a Target of MiR-31

To investigate the tumorigenesis mechanism of miR-31, the microrna.org on the results of the bioinformatics analysis was employed. Then, Dual-Luciferase reporter assay was carried out to investigate whether IRF-1 was a direct target of miR-31 (Figure 2A). The results showed that the relative luciferase value was notably decreased when miR-31-3p mimic and IRF-1-3'-UTR were co-transfected to HCT116 and HuH7 cells ($p<0.01$, Figure 2B). However, the relative luciferase value was significantly increased in the two cell lines co-transfected with miR-31-inhibitor and RF-1-3'-UTR ($p<0.01$, Figure 2B).

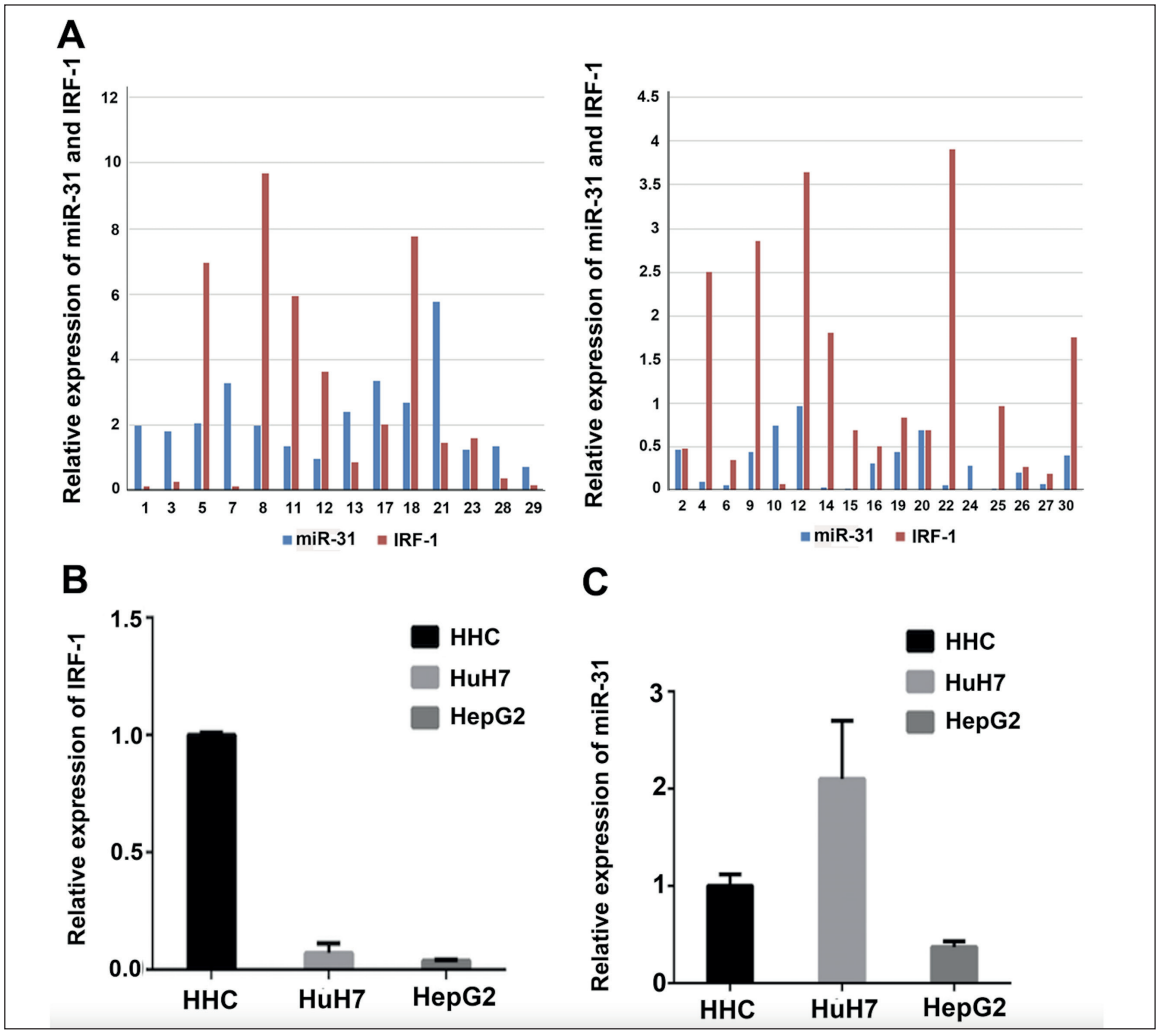

Figure 1. Aberrant expression of miR-31 and IRF-1 in HCC tissues and cell lines. **A,** QRT-PCR of IRF-1 in HCC tissues and paired normal adjacent tissues. Spearman correlation analysis showed a negative relationship between the expression level of miR-31 and the mRNA expression level of IRF-1in 30 HCC tissue samples and adjacent normal tissues. **B,** Expression levels of miR-31 in HCC cell lines (HuH7cells and HepG2 cells) and hHC cells ($p<0.05$). **C,** Relative expression of IRF-1 in HuH7 cells and HepG2 cells when compared with that in hHC cells ($p<0.001$).

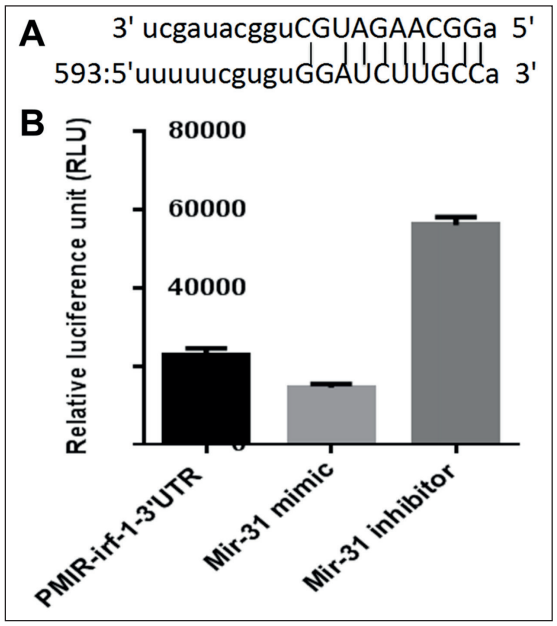

Figure 2. A, MiR-31 regulated mRNA expression of IRF-1 by binding to the IRF-1 3'-untranslated region (3'UTR Bioinformatics analysis indicated a putative miR-31-3p specific binding site in the IRF-1 3'UTR). **B,** Luciferase assay was used to assess whether IRF-1 was the direct target of miR-31. The relative luciferase value was decreased in miR-31-3p mimic, whereas the value was significantly increased in miR-31-3p inhibitor.

IRF-1 Was Negatively Regulated by MiR-31

The expression level of IRF-1 was significantly up-regulated in the HuH7 cells transfected with miR-31 inhibitor ($p<0.01$, Figure 3A). Nevertheless, the expression level of miR-31 was remarkably down-regulated after the HuH7 cells were transfected with miR-31 inhibitor ($p<0.01$, Figure 3B). The expression level of nuclear protein IRF-1 was up-regulated after the cells were transfected with miR-31 inhibitor (Figure 3C).

Hsa-miR-31 Regulated the Expression of IRF-1

To investigate the relationship between miR-31 and IRF-1, miR-31 mimics or miR-31 inhibitors were transfected with the cells to up-regulate or down-regulate the expression level of miR-31. At 24 h after cell transfection, immunofluorescence staining was employed to evaluate the effect of miR-31 upon the expression of nuclear protein IRF-1. The expression level of IRF-1 was decreased in the hUH7 cells (Figure 4A), whereas it was significantly up-regulated in the hUH7 cells transfected with the hsa-miR-31-inhibitor (Figure

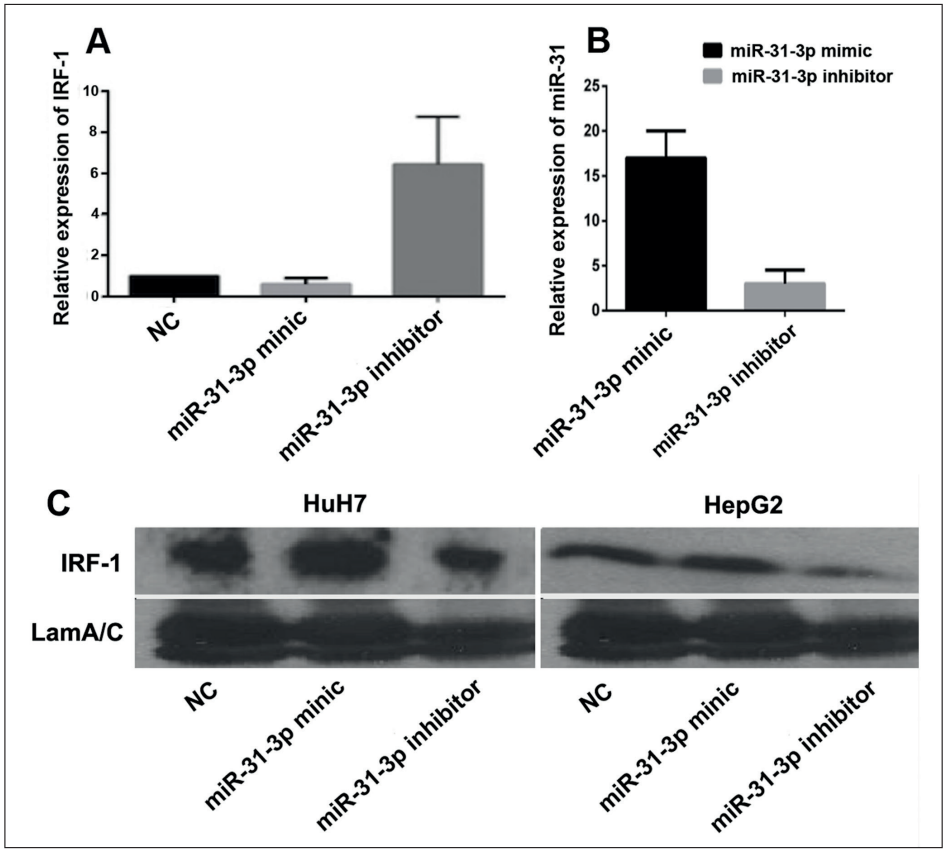

Figure 3. IRF-1 was negatively regulated by miR-31. **A,** QRT-PCR was used to detect the expression of IRF-1 in HuH7 cells transfected with miR-31 inhibitor. $^{**}p<0.01$ **B,** QRT-PCR was used to detect the expression of miR-31 in HuH7 cells transfected with miR-31 inhibitor. $^{**}p<0.01$ **C,** Western blot was performed to examine the expression of IRF-1 in HuH7 cells transfected with miR-31 inhibitor. $^{**}p<0.05$.

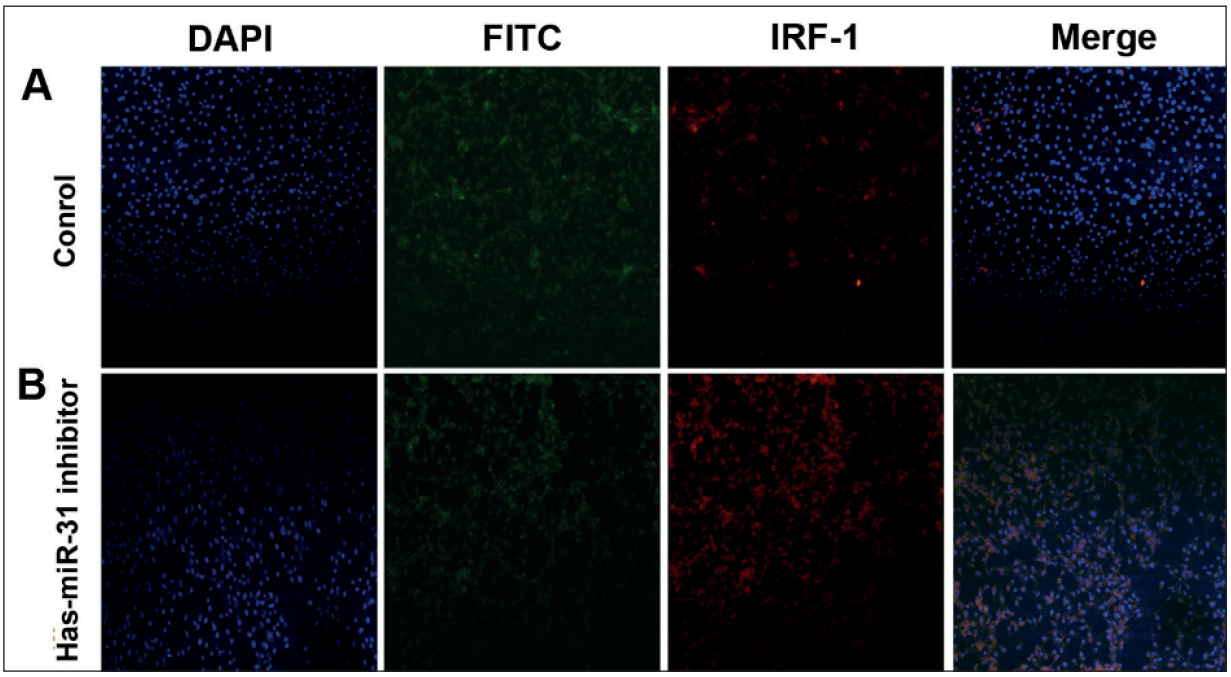

Figure 4. The expression of IRF-1 nuclear protein in HuH7 cells transfected with the hsa-miR-31 inhibitor. Images were representative images of 3 independent samples. **A,** Immunofluorescence staining showed low expression. **B,** Nuclear protein expression of IRF-1 was up-regulated in HuH7 cells transfected with the hsa-miR-31 inhibitor (200 × magnification).

4B). These findings indicated that endogenous miR-31 mimic suppressed the mRNA expression level of IRF-1in HuH7 cells.

MTT Assay and Flow Cytometry

MTT assay and flow cytometry demonstrated that up-regulating or down-regulating the expression level of miR-31 could exert effect upon the proliferation and apoptosis of HuH7 cells. MiR-31 inhibitor up-regulated the expression level of IRF-1, which inhibited the cell proliferation and promoted cell apoptosis of HuH7 cells, whereas miR-31 mimic down-regulated the expression level of IRF-1, which could promote cell proliferation and decrease cell apoptosis (Figure 5).

Discussion

Hepatocellular carcinoma (HCC) refers to a heterogeneous tumor with multiple factors implicated in the incidence and progression. Chronic infection and cirrhosis by hepatitis B virus (HBV) is the most prevalent cause. Liver cirrhosis induced by alcohol consumption, metabolic syndrome, and viral infection with the hepatitis C virus (HCV) is equally associated with the progression of HCC. Nevertheless, clinical treatment of HCC is still unsatisfactory. Therapeutic management of HCC consists of liver resection, ablation, chemoembolization, and liver transplantation depending upon the liver function, tumor staging, patient performance status, and so on. The participation of different signaling pathways in the incidence and progression of HCC based upon clinical trial data provides a convincing rationale for exploring the anti-cancer agents targeting key components of those signaling pathways.

Previous studies[15,16] have demonstrated that kinds of miRNAs are involved in the pathogenesis of HCC, including miR-21, miR-122, miR-145, miR-146a, and miR-204. MiRNAs can regulate the expression levels of their targets at a post-transcriptional level that are associated with the pathogenesis of malignant tumors. MiR-31 has been shown to be aberrantly expressed in various gastroenterological carcinoma, including colon cancer[17], esophageal adenocarcinoma[18], and gastric cancer[19]. MiR-31 has been served as both an oncogene and a tumour suppressor gene[20,21]. However, the role of miR-31

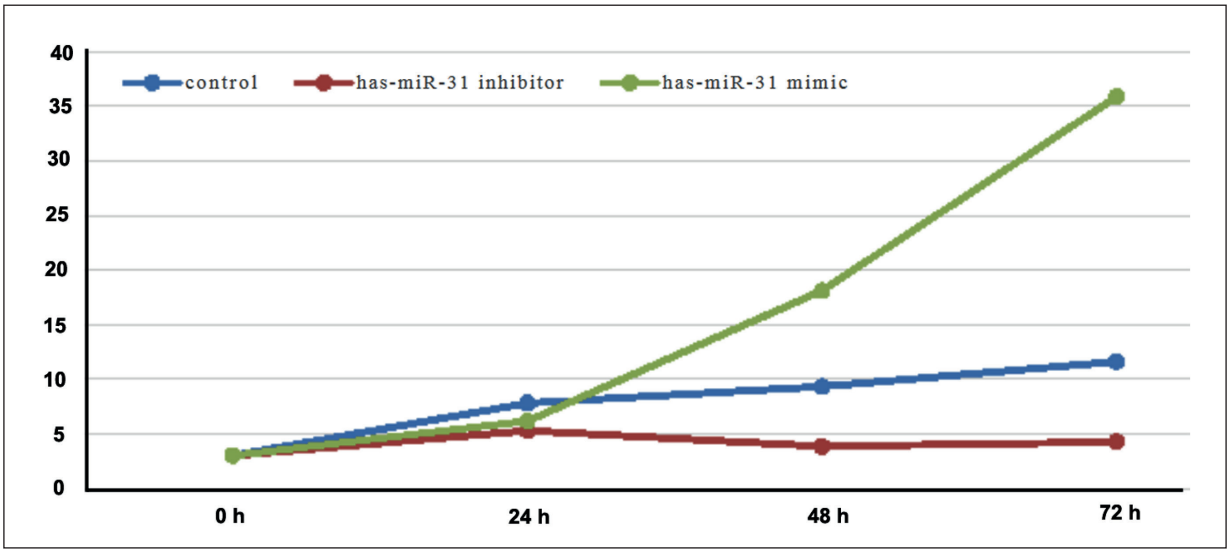

Figure 5. Cell proliferation was reduced in HuH7 cells transfected with hsa-miR-31 inhibitor when compared to the control HuH7 cells, whereas cell proliferation was significantly increased in HuH7 cells transfected with has-miR-31 mimic.

in the HCC sample remains to be elucidated and the underlying mechanism in patients with HCC has not been reported. In addition, the relationship between miR-31 and IRF-1 has been largely unknown.

In the present study, the expression level of miR-31 was inversely correlated with that of IRF-1 in the obtained HCC tissues when compared to that in the paired adjacent tissues. In addition, bioinformatics methods were employed to predict the potential targets of miR-31. Our results indicated IRF-1 was a direct target of miR-31, the protein expression level of IRF-1 was significantly down-regulated in the HCC tissues when compared with that in the paired adjacent tissues. Based on the contrasting expression patterns of miR-31 and IRF-1, we hypothesized that miR-31 was involved in the pathogenesis of HCC by directly down-regulating the expression profile of IRF-1. Further investigation was performed to sustain this opinion. Experimental results demonstrated that the down-regulation of miR-31 could up-regulate the expression level of IRF-1 and subsequently suppress the proliferation and enhance the cell apoptosis of HCC cells. Nevertheless, down-regulation of miR-31 could up-regulate the expression level of IRF-1 and increase cell apoptosis, indicating that IRF-1 acted as a downstream target gene of miR-31 in HCC cells. Our previous studies[16] have indicated that IRF-1 is a tumor-suppressor gene induced by interferon-γ (IFNγ). IRF-1 plays an important role in the cell growth and apoptosis of HCC, but the molecular mechanisms of IRF-1 suppression have not been clarified. The findings obtained from the present study could probably answer this question.

Conclusions

These results indicated that miR-31 played a significant role in regulating the expression profile of IRF-1 in the HCC specimens, which probably served as a novel target for the clinical management of patients with HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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