miR-183-5p promotes proliferation and migration in hepatocellular carcinoma by targeting IRS1 and its association with patient survival

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Abstract
Background: MiR-183-5p plays an important role in the pathophysiology of many tumors, while the role of MiR-183-5p in liver cancer is unclear.
Methods: In this study, quantitative reverse transcription-polymerase chain reaction and Western blotting were used to detect the expression of miR-183-5p in liver cancer cell lines, liver cancer tissues, and normal tissues adjacent to the cancer, and to explore the mechanism of miR-183-5p regulating liver cancer progression. The in vitro effects of miR-183-5p were evaluated by CCK-8, colony formation test, and wound healing test. Various databases were used to predict the target mRNA of miR-183-5p and verified by luciferase report analysis. In addition, the effects of miR-183-5p and its target gene on the survival of patients with liver cancer were also analyzed.
Results: miR-183-5p was highly expressed in hepatocellular carcinoma cells and tissues, and was related to some clinicopathological features. MiR-183-5p can promote the proliferation and migration of liver cancer cells. Using the bioinformatics database, we proved that miR-183-5p is related to the survival of liver cancer patients. Insulin receptor substrate 1 (IRS1) is a target of miR-183-5p, and luciferase analysis confirmed that miR-183-5p combines with the 3′-untranslated region (3′-UTR) of IRS1.
Conclusion: The miR-183-5p/IRS1 axis may be a new target for liver cancer research.

Keywords
miR-183-5p, IRS1, hepatocellular carcinoma, cell proliferation, prognosis

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Introduction
Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide—and especially in Asia. MicroRNAs (miRNAs) play an important role in the occurrence and development of various cancers. Many studies have confirmed that miRNAs are involved in the proliferation, differentiation, migration, and apoptosis of liver cancer cells. It is well known that miR-183 is upregulated in HCC and is considered to be an oncomiR. Functionally, the miR-183 family (miR-183, -96 and -182) promotes the cellular invasion and migration of HCC cells. Some researchers have confirmed that miR-183-5p acts as a tumor promoter in some cancers, and some researchers have confirmed that it can promote cell proliferation of cancer cell lines. The latest research shows that miR-183-5p can promote the proliferation of HCC cells by inhibiting the expression of tumor suppressors (including AKAP12, DYRK2, FOXN3, FOXO1, and LATS2).

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However, the role of miR-183-5p in hepatocellular carcinoma is still not fully understood.

Insulin receptor substrate-1 (IRS1) is a member of the insulin receptor substrate family. In the insulin receptor (IR) and insulin-like growth factor I receptor (IGF1R) signaling transduction, IRS1 was first considered to be a typical cytoplasm adaptor protein. Recent studies have confirmed that IRS1 also plays a role in promoting mitotic and apoptotic resistance, malignant transformation, and proliferation.

Although the direct interaction between nuclear IRS1 and double-stranded DNA has not been reported, recent studies have suggested that nuclear IRS1 may be involved in regulating the transcriptional activities of genes related to cell growth and cell proliferation. In addition, researchers point out that nuclear IRS1 can act as an AR and ER transcriptional regulator, suggesting that it is involved in the development and progression of breast and prostate cancer. At present, there is no research report regarding the interaction between miR-183-5p and IRS1.

The purpose of this study was to investigate the role of miR-183-5p in the pathogenesis of liver cancer and its molecular mechanism, and to provide new ideas for its treatment.

**Materials and methods**

**Cell culture and gene transfection**

Human liver cancer cell lines HUH-7, HEPG2, and HB611 were purchased from the Shanghai Chinese Academy of Sciences. L02 is a human normal liver cell line, purchased from Zhongqiao Xinzhou Biotechnology (Shanghai, China). L02 cells were cultured in BEGM (Lonza; Walkersville, MD, USA), and the flasks were pre-coated with fibronectin from bovine plasma (Sigma Aldrich, St. Louis, MO, USA). Others add 10% fetal bovine serum ( Gibco) to Durbeco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) in a 37°C, 5% carbon dioxide cell incubator. HUH-7, HEPG2, and HB611 cells were passaged every 2–3 days, and L02 cells were passaged every 4–5 days.

MiR-183-5p mimic (MiR-183-5p), inhibitor and negative control (NC) were chemically synthesized by Guangzhou Ribose Biological Co., Ltd (Guangzhou, China). Full-length IRS1 cDNA was cloned into the expression vector as an overexpression plasmid (Guangzhou Ribose Biological Co.); MiR-183-5p mimics/inhibitor/NC or plasmid duplexes (20 nM) were transfected to 6×10^5 cells in 6-well plates using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) for 48 hours at 37°C in a CO2 incubator according to the manufacturer’s instructions.

**Western blotting analysis**

Cell pellets were resuspended in a lysis buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 2 mM Na2EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS)) to which a mixture of protease inhibitor and phosphatase inhibitor was added, and a cell lysate was prepared. After 20 minutes of incubation on ice, the supernatant was separated for 20 minutes at 14,000 rpm. The protein concentration was determined using a BCA protein detection kit (Thermo Scientific Pierce, Waltham, MA, USA). SDS-PAGE gel electrophoresis was performed using standard techniques and transferred to a polyvinylidene fluoride membrane (Millipore, Temecula, CA, USA). The blots were probed with antibodies Anti-IRS1 (ab131487, 1:500 dilution; Abcam, Cambridge, UK), GAPDH (sc-25778, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody was used to a control for loading. Proteins were detected using an enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech, Piscataway, New Jersey, USA).

**Cell viability assessment**

The test was performed in accordance with the manufacturer’s instructions in triplicate. One day before treatment, cells were seeded in 96-well plates at a density of 3000 cells per well. Then, viability tests were performed before and 24 or 48 hours after treatment, respectively, for comparison. Detailed operation instructions are as follows: 20 μL of CCK-8 (10% in culture medium) was added to the cells at each time point. The cells were incubated at 37°C for 2 hours. After stirring for 10 minutes on a shaker, the absorbance at 562 nm was read using a scanning microtiter (Perkin Elmer, Waltham, MA, USA).

**Colony formation assay**

Forty-eight hours after transfection, the transfected cells were plated into six-well plates at a rate of 500 cells per well. These cells were then cultured for 7–10 days, with medium changed every 3–4 days. After 7–10 days, the cells were washed with phosphate buffered saline (PBS), dried and fixed with 95% ethanol, and stained with 0.1% crystal violet solution. Then, the cells were washed with water. Finally, photos were taken as soon as the plate was dry.

**Wound-healing assay**

After transfection of HUH-7 cells in a six-well plate, when the cell confluence reached about 90%, scratches were generated using 200 μL pipette tips in each well. The tip must be perpendicular to the board. It was then washed three times with PBS and cultured in DMEM medium without FBS and antibiotics. The wound healing was observed under a light microscope, and cell movement was observed at the same location at 0 and 24 hours.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction**

Total RNA of the cells was extracted by using TRIzol (Invitrogen, USA) according to the protocol. The primer
sequences of miR-183-5p and U6 were chemical synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). cDNA was synthesized by reverse transcription using the PrimeScript™ RT-PCR kit according to the manufacturer’s protocol (Takara, Tokyo, Japan). Real-time PCR was performed on a 7900HT fast RT-PCR instrument (Applied Biosystems, Singapore). The amplification procedure was as follows: 3 minutes at 95°C followed by 40 cycles at 95°C for 3 seconds and 65°C for 30 seconds. The qRT-PCR results were analyzed with the method \(2^{-\Delta\Delta C_T}\).

Dual-luciferase reporter assay

A total of 293T cells were seeded in 48-well plates. When the cell fusion rate reached 80%, replace the medium with FBS, penicillin, and streptomycin (250 μL/well). Psicheck-2/IRS1 3′-UTR and Psicheck-2/IRS1 3′-UTR mutation reporting plasmids were purchased from Comprehensive Biotechnology Solutions (Shanghai, China). 293T cells were co-transfected with Psicheck-2/IRS1 3′-UTR and Psicheck-2/IRS1 3′-UTR mutation reporter plasmids, miR-183-5p or NC and Lipofectamine 2000. After 24 hours, the firefly and renin luciferase activities were measured by the double luciferase method (Promega, Madison, WI, USA). Finally, the firefly luciferase activity was normalized to renin, and the ratio of firefly to renin was recorded.

Statistical Analysis

Graphpad Prism version 7.0 was used. Data were dealt with the mean ± standard for three separate experiments. \(P<0.05\) was considered statistically significant.

Results

**MiR-183-5p is highly expressed in HCC cells and tissues**

qRT-PCR was used to detect the mRNA expression of miR-183-5p in three liver cancer cell lines HUH-7, HB611, HEPG2, and normal liver cell line, LO2. We found that miR-183-5p was more highly expressed in HCC cells, especially in the HUH-7 cell line compared to normal hepatocytes (Figure 1(a), two-way ANOVA, \(P<0.001\)).
MiR-183-5p expression was also measured and compared in surgically resected liver cancer tissues (and adjacent normal tissues). As shown in Figure 1(b) in 41 pairs of matched tissue samples, miR-183-5p was also highly expressed in liver cancer. The relative expression levels of miR-183-5p are shown in Table 1. There were no significant differences in miR-183-5p levels with gender, age, and histological grade. There were significant differences in tumor stage and lymph node metastasis when miR-183-5p were shown. These results suggest that miR-183-5p may be a potential independent prognostic marker for liver cancer.

The survival curve of miR-183-5p in HCC was then drawn in the kmplot website (http://kmplot.com/analysis/index.php?p=service&cancer=liver_mirna). The selected platform was the RNA-seq (n=372) from the TCGA database (Figure 1(c)). As the figure illustrates, high expression of miR-183-5p had a lower survival rate than those that had low expression. Similar results were obtained when we restricted the race in the study to Asians (Figure 1(d)). The above results indicated that miR-183-5p could be a tumor promoter in HCC.

**MiR-183-5p promotes HCC cell migration**

Next, we investigated whether miR-183-5p can promote the migration process of liver cancer cells. Through the wound healing test, Figure 2(d) shows that compared with the 72-hour image and the 0-hour image of the HUH-7 and HEPG2 cell lines, the miR-183-5p mimic group migrated significantly faster than the inhibitor group. In conclusion, miR-183-5p can promote the migration of liver cancer cells.

**MiR-183-5p targets 3'-UTR of IRS1 and inhibits its expression**

Through the Starbase v3.0 database (http://starbase.sysu.edu.cn), the TargetScan database (http://www.targetscan.org/vert_72/) and the LinkedOmics database (http://www.linkedomics.org/), we predict that IRS1 is a potential target for miR-183-5p. We found a strong correlation between IRS1 and miR-183-5p on all three platforms (target prediction data are shown in Suppl. Table 1 to Table 3), and this relationship is particularly evident in liver cancer tissues (Figure 3(a)). Our Western blot experiments also demonstrated that the transfection of miR-183-5p mimics resulted in the reduced expression of IRS1 protein in HCC cells (image J software converted to grayscale) (Figure 3(b)). In order to confirm that IRS1 is a target gene of miR-183-5p, we also constructed IRS1 overexpression plasmid to transfected 293T cells, and then transfected miR-183-5p mimic or NC into different groups. As a result, the IRS1 protein overexpressed only in the miR-183-5p NC group (Figure 3(b)). This verifies that miR-183-5p can down-regulate IRS protein expression in different cells. Then, we performed a dual-luciferase reporter assay analysis. Wild-type and mutant-type binding sites of miR-183-5p and IRS1 were constructed. When the IRS1 conserved target region of miR-183-5p was mutated, the reporter gene's luciferase activity was restored. As shown in Figure 3(c), miR-183-5p reduced luciferase activity in the wild-type group, but did not change significantly in the mutant group. These results suggest that IRS1 is a direct target of liver cancer miR-183-5p.

**Bioinformatics and survival analysis of miR-183-5p and IRS1 in HCC**

To explore the possible functions of miR-183-5p, GO enrichment and KEGG pathway analyses were also performed as described in the supplementary section. According to the results of the GO analysis, the main functions and biological processes related to miR-183-5p were biological regulation,
metabolic process, and protein/iron/nucleic acid binding (Suppl. Figure 1(a)). The miR-183-5p/IRS1 axis was probably associated with semaphoring-plexin signaling pathway and oxidoreductase activities (Suppl. Figure 1(b) as a function term and Suppl. Figure 1(c) as a pathway term).

According to the Kaplan–Meier analysis, IRS1 was also significantly ($P = 0.002$) associated with HCC patient overall survival (OS) (Figure 3(d)). We also performed receiver operating characteristic curve to assess the sensitivity and specificity of survival predictions (data from the LinkedOmics database). Here, the 5-year survival area under curve (AUC) value of miR-183-5p is 0.576, and the AUC value of IRS1 is 0.578 (Figure 3(e)). It is suggested that miR-183-5p/IRS1 signal has certain accuracy in predicting the prognosis survival rate of liver cancer patients.

**Discussion**

This study aimed to investigate the role of miR-183-5p and its possible target genes in the pathogenesis of liver cancer cells. In functional studies, we found that overexpression of miR-183-5p promotes the proliferation and migration of liver cancer cells. At the same time, miR-183-5p decreased the expression of IRS1 protein in cells. On the contrary, inhibit miR-183-5p constrained proliferation and migration processes of HCC cells and increase the expression of IRS1 protein. By constructing a luciferase reporter gene, we determined that IRS1 is a functional target of miR-183-5p.

MiR-183-5p is a member of the MiR-182-183 miRNA cluster and is located at positions 7q31-34.15 This miRNA cluster includes three miRNAs, namely miR-96, miR-182, and miR-183-5p, which have highly homologous 5′-seed sequences. Motoyama et al.16 found that miR-183-5p is overexpressed in human colorectal cancer, and Lin et al.17 have reported that miR-183-96-182 clusters are frequently amplified in melanoma. At the same time, miR-183-5p may play an oncogene role in many tumor types. For example, miR-183-5p is considered a potential metastasis inhibitor in lung cancer cells.18 These data suggest that the role of miR-183-5p as an oncogene is cell-type dependent.
IRS1 is a docking protein that is highly expressed in a variety of cancers. Transgenic mice that overexpress IRS1 or IRS-2 in the mammary glands show progressive breast hyperplasia, tumorigenesis, and metastasis. In addition, IRS1 overexpression can target upstream binding factor-1 (UBF-1), which affects cell size. These findings suggest that IRS1 may play a major role in cell growth, proliferation, and differentiation. IRS1 has been shown to introduce extracellular signals into colorectal cancer cells via miR-145 and via the AKT and ERK1/2 signal transduction pathways. Li et al. also found that the effects of miR-183 on cell viability and apoptosis are mediated by affecting the activation of cell autophagy, and down-regulating miR-183 activates the PI3K/AKT/mTOR pathway. Here we found that endogenous miR-183-5p and exogenous miR-183-5p mimics can inhibit IRS1 pathway. Our results and previous studies have further confirmed that miRNAs and their target genes do not have a one-to-one correspondence, but instead, one miRNA can regulate several pathways by targeting different mRNAs, and one
target mRNA can be regulated by several miRNAs and pathways in the same human tumor.

In short, miR-183-5p promotes the proliferation and migration of liver cancer cells and affect the OS of liver cancer patients. MiR-183-5p targeting IRS1 may be a new potential biomarker for liver cancer. However, the role of miR-183-5p in liver cancer needs to be further studied in vivo.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical standards
This work has been carried out in accordance with the Declaration of Helsinki (2010) of the World Medical Association. This study was approved ethically by First Affiliated Hospital of Xi’an Jiaotong University of College of Medicine, Shaanxi, China. All patients provided informed written consent.

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Supplemental material
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