miRNA-370 acts as a tumor suppressor via the downregulation of PIM1 in hepatocellular carcinoma

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Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC) is a primary malignancy of liver. Nowadays HCC is one of the most common and aggressive malignancies worldwide. This study shows the aberrant up-regulation or down-regulation of miR-370 correlates with the development and prognosis of different human malignancies including HCC.

MATERIALS AND METHODS: The HCC cell lines were used as model cell lines and the anti-tumor effect of miR-370 in vitro were examined. The level of miR-370 in HCC cells was determined by qRT-PCR and restored in GC cells by using miR-370 mimic. Moreover, the target gene of miR-370 was then identified.

RESULTS: The expression of miR-370 in HCC cell lines was significantly lower than that in the other human liver cells. The miR-370 acted as a tumor suppressor in HCC. Moreover, it showed that miR-370 exerted anti-tumor effect by targeting PIM1 directly, a serine/threonine-specific kinase involved in the development and progression of HCC.

CONCLUSIONS: miR-370 acted as the tumor suppressor in HCC and was a potential therapeutic target for HCC treatment.

Key Words: Hepatocellular carcinoma, miR-370, PIM1.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignancies worldwide, and causes more than 1 million cases of death every year¹. HCC is much more prevalent in developing countries, such as China and many African countries, than that in developed countries². Therefore, the discovery of new potential therapeutic targets has been a research hotspot of HCC³.

MicroRNAs (miRNAs), account for a class of small non-coding RNAs with a length of 21-25 nucleotides, play an important role in a variety of cellular physiological activities and processes, such as proliferation, development, apoptosis and differentiation through post-transcriptionally regulating expression of the target genes, namely mRNA⁴,⁵. A large number of evidences have shown that the aberrant up-regulation or down-regulation of miRNAs correlates with the development and prognosis of different human malignancies including HCC⁶. For instance, down-regulation of miR-148b has been found of correlating with poor outcome of HCC⁷. Shi et al⁸ reported that the elevated level of miR-522 predicted poor prognosis in patients with HCC. In hepatocellular carcinoma cells, miR-370 showed the potential of suppressing metastasis by inhibiting migration and invasion⁹. Sun et al¹⁰ also showed that the increasing miR-370 expression promoted the cell death of liver cancer cells in vitro. However, the mechanism by which miR-370 affects the biological behavior of HCC cells was still unclear.

PIM (Proto-oncogene serine/threonine-protein) family kinases, a family of small, constitutively active and highly evolutionarily conserved serine/threonine-specific kinases, include three members, PIM1, PIM2 and PIM3. As oncogene, PIM is found of being aberrantly highly expressed in various human malignancies¹¹. Particularly, PIM1 can regulate a variety of cellular activities of tumor cells, including the cellular proliferation, the cell cycle, the apoptosis and the metabolism¹¹. Currently, we addressed that the crucial facts on the regulatory mechanism between PIM1 and miRNAs. PIM1 was found of being directly targeted by miR-370 and miR-370 suppressed cell growth and invasiveness, and enhancing apoptosis in HCC cell lines; moreover, PIM1 was found of mediating the tumor suppressor effect of miR-370.
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**Materials and Methods**

**Cell Lines and Cultures**

Human HCC cells SMMC7721 and HepG2 were purchased from ATTC: American Type Culture Collection (Manassas, VA, USA). The immortalized liver cell line L02 was purchased from the Cell Resource Center of Chinese Academy of Sciences (Shanghai, China). All cells were maintained at RPMI (Roswell Park Memorial Institute)-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 4 m/L glutamine, 3.7 g/L sodium bicarbonate, 4.5 g/L glucose and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). The cells were maintained in a 5% CO₂ humidified incubator at 37°C.

**Quantitative Real-Time PCR (qRT-PCR)**

TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to extract total RNA from cultured cells. MiR-370 expression was quantified by real time PCR with a TaqMan Probe (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Briefly, cDNA was obtained by High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). A TaqMan PCR kit and the ABI 7500 System (Thermo Fisher Scientific, Waltham, MA, USA) were used to conduct the qRT-PCR analysis. U6 was taken as the internal control to normalize the relative expression of miR-370. For PIM1 mRNA expression, the primer was synthesized based on published sequence. The first-strand cDNA was reversely transcribed from 1 μg RNA in total by using the Super M-MLV Reverse transcriptase (BioTeke Co., Beijing, China). PCR reaction solution included a master mix including SYBR GREEN mastermix (Solarbio Co., Beijing, China), forward primer, reverse primer and 10 ng template cDNA. GADPH (glyceraldehyde-3-phosphatedehydrogenase) was used as internal control to normalize PCR results. PCR results were analyzed by using the comparative ΔCt method (ABPrism software, Applied Biosystems, Foster City, CA, USA).

**Western Blot**

Proteins were isolated from tissues by lysing frozen tissues in RIPA (radioimmuno-precipitation assay) buffer (Sigma-Aldrich, St. Louis, MO, USA). Proteins were extracted from cells after cells had been lysed by lysis buffer (Beyotime, Shanghai, China) containing protease inhibitors (Sigma Aldrich, St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) was utilized to analyze the protein concentration. Extracted proteins were separated on SDS-PAGE (polyacrylamide gel electrophoresis) and then transferred electrophoretically onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Proteins were probed into with specific antibodies following the standard protocol. Specific primary antibodies against PIM1 and β-actin were purchased from Abcam (Shanghai, China). The second antibodies used in this study included the goat anti-rabbit IgG-HRP, the goat anti-mouse IgG-HRP and the donkey anti-goat IgG-HRP (Beyotime Institute of Biotechnology, Shanghai, China). Signals were detected by using the chemiluminescent substrate (KPL, Guildford, UK) and the blot intensity were quantified by using BandScan software (Glyko, Novato, CA, USA).

**Construction of Reporter Plasmids and Luciferase Assay**

The reporter plasmid was constructed as previously described. A fragment containing PIM1 3'UTR were amplified by PCR from human genomic DNA by utilizing specific primers and inserted into a pGL3 vector (Promega, Madison, WI, USA) downstream the stop codon of firefly-luciferase reporter gene, thus resulting in the pGL3-3'UTR/PIM1 construct. For luciferase assay, 293T recipient cells were transiently co-transfected with 0.2 μg of pGL3-3'UTR/PIM1 constructs, 0.02 μg of pRL-TK-Renilla luciferase reporter plasmids (Promega, Madison, WI, USA) containing the Renilla-luciferase for normalization and with 5 pmol of miR-370 overexpression construct or control. 24 hours after transfection, the cells were lysed and the luciferase activity was measured with a luminometer by using the dual-luciferase reporter assay system according to the manufacturer’s instructions.

**MiR-370 Knockdown or Overexpression**

The lentiviral miR-370 mimic and miR-370, and miR-370 inhibitor were obtained from Qiagen (Hilden, Germany). Cells were transfected with miR-370 mimic and miR-370, or miR-370 inhibitor in accordance with the manufacturer’s protocols. The expression of miR-370 in transfected cells was examined by qRT-PCR.
PIM1 Knockdown
The shRNA (short hairpin RNA) oligos targeting PIM1 genes were obtained from GenePharma (Shanghai, China). PIM1 targeting shRNA sequence and one scramble sequence as control were inserted into plasmid vector pGCsi-H1. The resulting plasmids were named pGCsi-H1-PIM1 and pGCsi-H1-control, respectively. For transfection, the cells in logarithmic growth phase were seeded in a 6-well plate and transfected with constructed plasmids in accordance with the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). The transfected cells were incubated for another 48 hours and the knockdown was verified by the Western blot analysis.

PIM1 Overexpression
PIM1 were overexpressed through transfection with the expressing construct for using Lipofectamine 3000 reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. PIM1 overexpressing vector was constructed as previously described by using a plasmid vector pGCsi-H113. Cells transfected with empty vector were used as controls. 48 hours after transfection, the cells were rinsed before re-suspended in a fresh culture media and the overexpression was verified by the Western blot analysis.

Cell Viability Assay
The cell viability was determined by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Sigma-Aldrich, MO, USA). Briefly, the cells were plated at a density of 5x10⁴ cells/well in 96-well culture plates. After treatment, 20 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated for 2 hours. MTT formazan was dissolved in 150 µl of isopropanol and the absorbance was measured at 595 nm with an ELISA (enzyme linked immunosorbent assay) reader (Tecan Group Ltd, Männedorf, Switzerland).

Cell Apoptosis Analysis
The cell apoptosis was evaluated by flow cytometry by using an FITC (fluorescein isothiocyanate) Annexin V apoptosis kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions. In brief, cells at a concentration of 1x10⁶ cells/ml were stained with annexin V-FITC and propidium (PI) for 15 minutes in dark before analysis with flow cytometer (Beckman Coulter Inc., Miami, FL, USA).

Cell Invasion Assay
The 24-well Transwells coated with Matrigel (8-µm pore size, BD Biosciences, San Jose, CA, USA) were used for cell invasion assays. 1x10⁴ cells were maintained in the serum-free medium for overnight. Then the cells were trypsinized and adjusted to 2x10⁵ cells/ml in a DMEM (Dulbecco’s modified Eagle medium) medium supplemented with 1% fetal bovine serum (FBS) before it was added to the upper chamber. DMEM supplemented with 10% FBS was added in the lower chamber as chemoattractant. After 48 hours, the Matrigel and the cells left in the upper chamber were cleaned. After fixation, the hematoxylin staining was performed to count the cells on the lower surface of the membrane were stained with solution after the cells were fixed with formaldehyde solution.

Statistical Analysis
All statistical analysis was performed by using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Values were presented as the mean ± SD. Statistical comparisons were performed by one-way ANOVA followed by Dunnett’s t-test. The difference with a p-value less than 0.05 was defined as statistically significant.

Results
miR-370 Functioned as a Tumor Suppressor in HCC Cells
The role of miR-370 in HCC was studied in two HCC cell lines SMMC-7721 and HepG2. As shown in Figure 1A, the expression of miR-370 in both HCC cells was significantly lower when compared with the human liver cells L02. Then miR-370 mimic and inhibitor as well as negative controls (NC) were transfected into HCC cells to examine the role of miR-370 in cellular activities. As shown in Figure 1B, miR-370 mimic was able of significantly increasing the expression of miR-370 while miR-370 inhibitor transfection was associated with the markedly decreased level of miR-370.

Methylthiazolyl Tetrazolium (MTT)
(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay revealed that miR-370 overexpression significantly inhibited the growth of HCC cells when compared with the control cells while miR-370 knockdow enhan-
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miRNA-370 acts as a tumor suppressor via the downregulation of PIM1 in HCC under test. Collectively, these results provided experimental evidences that miR-370 functioned as the tumor suppressor in HCC cells.

**PIM1 is Directly Targeted by miR-370**

MiRNAs regulated the target gene expression through the post-transcriptional mechanisms; therefore, two computational algorithms, TargetScan14 and online tool (microRNA.org)15, were utilized together to dig the possible target that mediated the anti-tumor effect of miR-370 in HCC. Both methods predicted an interaction between miR-370 and the target sites in the PIM1 3’-UTR, as shown in Figure 3A. To further examine the regulating mechanism of miR-370 on PIM1, the mRNA and protein expression of PIM1 were measured in cells transfected with miR-370 mimic and miR-370 mimic. As shown Figure 3B, miR-370 overexpression in HCC cells resulted in significant decrease of PIM1 protein expression. In contrast, the knockdown miR-370 was associated with the significantly higher expression of PIM1 protein (Figure 3B). Next, we adopted the luciferase assay to determine whether miR-370 regulated the expression of PIM1 by directly targeting the 3’-UTR of PIM1. As shown in Figure 3C, miR-370 mimic significantly decreased the luciferase reporter activity while miR-370 inhibitor significantly increased the reporter activity, compared with the controls, indicating that PIM1 was a direct target of miR-370. Taken together, our findings showed that miR-370 regulated the expression of PIM1 at the protein levels.

**The Anti-Tumor Effect of miR-370 was Mediated by PIM1**

In order to verify the role of PIM1 in the anti-tumor effect of miR-370, the expression of PIM1 was manipulated in both HepG2 and SMMC7721 cells by using shRNAs or overexpression plasmid. The effect of PIM1 knockdown on cell proliferation, apoptosis and invasion was examined in cells when transfected with miR-370 inhibitor. As shown in Figure 4A, 4B and 4C, the promoting effect of miR-370 inhibitor on cell proliferation and invasion as well as the suppressing effect of miR-370 inhibitor on apoptosis were almost completely abrogated by PIM1 shRNAs; on the other hand, the ectopic overexpression of PIM1 significantly attenuated the suppression of proliferation and invasion, and apoptosis induced by miR-370 mimic (Figure 5A,B,C). Our results provided strong evidences that the anti-tumor effect of miR-370 was mediated by PIM1.
Dysregulation of miR-370 has been described in a variety of human malignancies, but the functional role of miR-370 in cancer has been still controversial. MiR-370 may act as a key regulator in tumorigenesis and progression by functioning as either an oncogene or a tumor suppressor gene. For instance, miR-370 was reported down-regulated and to function as a tumor suppressor gene in the laryngeal squamous cell carcinoma, *Helicobacter pylori*-induced gastric carcinoma and acute myeloid leukemia\textsuperscript{16-18}. In cholangiocarcinoma, ectopic overexpression of miR-370 was reported to exert anti-proliferative effect on cholangiocarcinoma cells\textsuperscript{19}. In hepatocellular carcinoma cells, miR-370 showed potential to suppress metastasis by inhibiting migration and invasion\textsuperscript{9}; moreover, the restoration of miR-370 was found to resensitize the endometrioid ovarian cancer cell to cisplatin\textsuperscript{20}. On the contrary, a number of studies have provided experimental results to demonstrate the role of miR-370 as oncogenic miRNAs\textsuperscript{21}. Cao et al\textsuperscript{22} have found that miR-370 overexpression may...
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result in enhanced cell growth, enhanced expression of cyclin-dependent kinase inhibitors, and down-regulation of cyclin D1 in the Wilms tumor G401 cell line, suggesting the pro-proliferative role of a role of miR-370. In human prostate and gastric cancers, miR-370 was abnormally highly expressed and acted as an oncogene by directly regulating FOXO123,24. Sim et al25 also identified miR-370 as oncogenic miRNAs in breast cancer. All these studies suggested that whether miR-370 can function as an oncogene or tumor suppressor may depend on the key target genes regulated by miR-370, which may be cell specific. As far as we know, the clinical significance of miR-370 and the functional role of miR-370 in HCC are not fully understood. In present work, our findings have revealed that miR-370 is significantly down-regulated in HCC cell lines when compared with the normal liver cells. Next, the effects of ectopic overexpression of miR-370 or inhibition on cell proliferation and apoptosis were assessed. Our results showed that ectopic overexpression of miR-370 inhibited proliferation and induced apoptosis while miR-370 inhibitor promoted cell proliferation and suppressed apoptosis in HCC cells in vitro; moreover, the overexpression of miR-370 in HCC cells can significantly suppress invasion. Collectively, our data suggested that miR-370 function as tumor suppressor in HCC.

Given the regulatory role of PIM1 in a variety of biological activities of cancer cells, PIM1 has been considered as a promising target for anticancer drug discovery11. In the context of HCC, PIM1 is overexpressed in the primary and metastatic HCC tissues13. In present work, our results suggested that that knockdown of PIM1 by using
In hepatocellular carcinoma, PIM1 is reported to be involved in cell metabolism and able to promote tumor progression by enhancing glycolysis. Therefore, by targeting PIM1, miR-370 might also play a key role in cell metabolism, although further data are needed to be collected.

shRNAs suppressed cell growth and induced apoptosis in HCC cells, whereas overexpressing PIM1 had opposite effects, thus validating the role of PIM1 as an oncogene in HCC. In addition to proliferation and apoptosis, PIM1 is also involved in other physiological behaviors of cancer cells.

**Figure 4.** The promoting effect of miR-370 inhibitor on cell proliferation and invasion as well as the suppressing effect of miR-370 inhibitor on apoptosis were almost completely abrogated by PIM1 shRNA. **p < 0.01.
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Conclusions

We observed that miR-370 functioned as a tumor suppressor gene in HCC; furthermore, we identified PIM1 as a direct target of miR-370. Our results suggested the potential value of miR-370 and PIM1 as new therapeutic targets for HCC.

Figure 5. The ectopic overexpression of PIM1 significantly attenuated the suppression of proliferation and invasion, and apoptosis induced by miR-370 mimic. **p<0.01.


miR-370 suppresses proliferation and promotes endometrial ovarian cancer chemosensitivity to cDDP by negatively regulating ENG. Cancer Lett 2014; 353: 201-210.

