



Original Research

Down-regulation of miRNA-196b expression inhibits the proliferation, migration and invasiveness of HepG2 cells while promoting their apoptosis via the PI3K/Akt signaling pathway

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Abstract: The purpose of this study was to investigate the effects of microRNA-196b (miRNA-196b) on proliferation, migration, invasiveness and apoptosis of hepatocellular carcinoma cell line (HepG2), and the mechanism involved. MiRNA-196b inhibitor or negative control were transfected into HepG2 cells, while empty liposome vector was used as normal control. The results of transfection were assessed using real-time quantitative polymerase chain reaction (qRT-PCR). Cell proliferation, migration, invasiveness and apoptosis were determined using cell counting kit 8 (CCK-8), scratch test, Transwell invasion assay, and flow cytometric analysis, respectively. The expressions of PIK3, Akt and p-Akt proteins were determined using Western blotting. The HepG2 cells were also treated with PI3K/Akt signaling pathway inhibitor LY294002, and its effect on cell proliferation, migration, invasion, and apoptosis, and expressions of PIK3, Akt, and p-Akt proteins were determined. The results of RT-PCR showed that the relative expression of miRNA-196b in the inhibitor group (0.42 ± 0.13) was significantly lower than that in the blank control group (0.96 ± 0.10) and the negative control group (1.01 ± 0.32) ($p < 0.05$). The miRNA-196b inhibitor significantly and time-dependently reduced the invasiveness, proliferation migration abilities of HepG2 cells, while promoting their apoptosis ($p < 0.05$). The expressions of PIK3 and p-Akt proteins were significantly down-regulated in the inhibitor group, when compared with normal and negative control groups ($p < 0.05$). However, there were no significant differences in the expression of Akt protein among the groups ($p > 0.05$). After treatment of HepG2 cells with PI3K/Akt signaling pathway inhibitor LY294002, the proliferative, migratory and invasive abilities of cells in the treatment group were significantly enhanced, while cell apoptosis was significantly reduced ($p < 0.05$). Similarly, the protein expressions of PIK3 and p-Akt in the non-treatment group was significantly upregulated, relative to the treatment group ($p < 0.05$), but there was no significant difference in the expression of Akt protein between the two groups ($p > 0.05$). Downregulation of miRNA-196b expression inhibits the proliferation, migration and invasiveness of HepG2 cells, while promoting their apoptosis via a mechanism involving the PI3K/Akt signaling pathway.

Key words: Liver cancer; MiRNA-196b; PI3K/Akt signaling pathway; Proliferation; Apoptosis.

Introduction

Liver cancer is a common malignant tumor characterized by high incidence of mortality. Its pathogenesis remains unclear. However, it is speculated to be caused by gene mutation, infection, immune injury and other factors (1). MicroRNA (miRNA) is a small non-coding RNA molecule found in plants, animals and some viruses, and functions in RNA silencing and post-transcriptional regulation of gene expression. The expression and processing patterns of miRNA vary depending on the type of tumor. By targeting dozens-to-hundreds of genes, the basic biological functions and pathways involved in tumorigenesis can be unraveled (2). MicroRNAs have been shown to play a role in the pathogenesis and progression of liver cancer. MiRNA-196b is highly expressed in gastric, colorectal, breast and pancreatic cancers (3-6). Studies on the role of miRNA-196b in liver cancer are scanty. It has been reported that miRNA-196b may promote the proliferation and invasion

of hepatocellular carcinoma (HCC) cells by targeting forkhead box p2 gene (FOXP2) (7).

Phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) signaling pathway is an important pathway in the pathogenesis and progression of tumor and inflammation. The binding of PI3K to its upstream molecule induces a conformational change in the protein, and results in the phosphorylation of Akt, thereby activating it. Activated Akt regulates downstream molecules, thereby participating in the regulation of tumors. The PI3K/Akt signaling pathway is overexpressed and activated to varying degrees in most malignant tumors such as gastric, breast, and liver cancers (8-10). Little or nothing is known about the involvement of PI3K/Akt signaling pathway in the regulation of biological behavior of cancer cells by miRNA-196b. In a previous study, it was reported that the antiproliferative and anti-invasive effects of miRNA-196b in lung cancer cells were exerted via activation of PI3K/Akt signaling pathway (11). The aim of this study was to investigate the effect of miR-

NA-196b on proliferation, migration, invasiveness and apoptosis of HepG2 cells, and the mechanism involved.

Materials and Methods

Materials and reagents

Human liver cancer cell line (HepG2) was obtained from Shanghai Huiying Biotechnology Co. Ltd. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Shanghai United Cell Biotechnology Co. Ltd. Lipofectamine 2000 transfection reagent was a product of Thermo Fisher Scientific Co. Ltd. (USA); PI3K/Akt signaling pathway inhibitor (LY294002) was obtained from Beijing Biolab Technology Co. Ltd. The MiRNA-196b inhibitor and negative control were purchased from Shanghai Jima Pharmaceutical Technology Co. Ltd. Cell counting kit 8 (CCK8) was obtained from Shanghai Yuduo Biotechnology Co. Ltd; Transwell chamber was purchased from Shanghai Yanhui Biotechnology Co. Ltd. RT-PCR detection kit was a product of Biomics Biotechnology Co., Ltd. Annexin V-FITC/PI apoptosis assay kit was product of Amyjet Scientific Co. Ltd. Rabbit anti-human PIK3, Akt and p-Akt monoclonal antibodies, and sheep anti-rabbit IgG were purchased from Sigma-Aldrich (USA). The miRNA-196b primer sequences were designed by Shanghai Sangon Co. Ltd. Microplate reader was purchased from Analytik Jena AG (Germany). Flow cytometer was product of BD Biosciences (USA).

Cell culture, transfection and grouping

The HepG2 cells were cultured in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO₂ and 95 % air. The culture medium was changed once every 2 days, and sub-cultured with 0.25 % trypsin for 3 to 5 days. After digestion, the cells were inoculated in 6-well plates until they attained 60 to 80 % confluency. Then, the medium was replaced with serum-free medium, and incubated for 12 h, and thereafter transfected using lipofectamine 2000 transfection reagent. The density of HepG2 cells was adjusted to 1 × 10⁶ cells/mL, and 2 mL aliquot was seeded into 6-well plates. MiRNA-196b inhibitor or negative control, each at a concentration of 10 nmol/L, were transfected into the cells using Lipofectamine 2000 transfection reagent, and they served as inhibitor group and negative control group, respectively. The normal control cells (empty liposome) were not transfected.

qRT-PCR

The level of expression of miRNA-196b was determined using qRT-PCR. Trizol RNA extraction reagent was used to extract total RNA from cells in each group, while cDNA synthesis kit was used to perform cDNA synthesis reaction according to the instructions of the manufacturer. Light Cycler 1536 RT-PCR detection system was used for the estimation of the mRNA expression of the gene. Variation in the cDNA content was normalized using GAPDH. The miRNA-196b primer sequences were:

forward: 5'-CGGTAAACCCCTTCCTTGACGCATTTG-3'; and reverse: 5'-CGACTCGAGAACCCTAACCCCTACCTGCTGTGA-3'.

The reaction conditions of qRT-PCR were: pre-denaturation at 95°C for 30 sec; PCR reaction at 95°C for 5 sec, 60°C for 30 sec, with a total of 40 cycles. The PCR reaction mixture (20 μL) consisted of 6.4 μL of dH₂O, 1.6 μL of gene-specific primer (10 μM), 2 μL of synthesized cDNA and 10 μL of SYBR Premix Ex Taq™ II. The Ct value of U6 was taken as the internal parameter, and 2^{-ΔΔCt} was used to calculate the relative expression level of the protein.

Determination of cell proliferation

Cell proliferation was determined using CCK-8 assay kit. The

cells were seeded in 96-well plates at a density of 1 × 10⁴ cells/well, and cultured for 24 h at 37 °C in a humidified atmosphere of 5 % CO₂ and 95 % air. Then, 10 μL of CCK-8 was added to each well, and cultured at different time points (24, 48, and 72 h). The absorbance of the samples was read in a microplate reader at 450 nm. The assay was performed in triplicate. Cell proliferation was calculated as shown in Equation 1:

$$\text{Cell proliferation (\%)} = \frac{\text{Absorbance of the experimental group} \times 100}{\text{Absorbance of the control group}} \quad (1)$$

Determination of cell migration

The migratory ability of HepG2 cells was determined using scratch test. Cells in logarithmic growth phase were seeded in 6-well plates until they attained 90 - 100 % confluency, and scratches were made on cell monolayers. After washing thrice with serum-free medium, the cells were further cultured for 24 and 48 h, and then observed and analyzed using Image Pro Plus (6.0). Cell motility was calculated as shown in Equation 2.

$$\text{Cell mobility} = \frac{1 - \text{scratch width during measurement} \times 100\%}{\text{Initial scratch width}} \quad (2)$$

Cell invasion assay

The degree of invasiveness of HepG2 cells was assessed using Transwell invasion assay. The cells (2 × 10⁵ cells/mL) were placed in Transwell chamber coated with substrate and cultured in serum-free medium. Then, medium containing 10 % FBS was added to the lower chamber. After 24 h, the cells that passed through the matrix gel membrane were stained with crystal violet after fixation, photographed and counted using an inverted microscope. The invasive ability of the cells was calculated as shown in Equation 3.

$$\text{Invasive ability (\%)} = \frac{\text{Number of cells penetrating the filter membrane} \times 100\%}{\text{Number of cells in the corresponding wells}} \quad (3)$$

Apoptosis assay

The cells were seeded at a density of 2.5 × 10⁶ cells/well in 6-well plates and cultured at 37°C for 72 h. Thereafter, they were washed with phosphate-buffered saline (PBS), and thoroughly mixed with 500 μL binding buffer. The cells were then stained with Annexin V-fluorescein isothiocyanate and propidium iodide (10 μL each) within 10 min at room temperature in the dark. Cell apoptosis was assessed using a flow cytometer fitted with argon laser operated at 485 nm.

Western blotting

The cells were washed with PBS, and were thereafter lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitor. The resultant lysate was centrifuged at 15,000 rpm for 15 min at 4°C, and the protein concentration of the supernatant was determined using bicinchoninic acid (BCA) protein kit. A portion of total cell protein (30 μg) from each sample was separated on 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90°C for 120 min. Subsequently, non-fat milk powder (3 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37°C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4°C with primary antibodies of PIK3, p-Akt, Akt and GAPDH, each at a dilution of 1: 1000. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1.5 h at room temperature. The blot was developed using an X-

ray film. Grayscale analysis of the bands was performed using Bio-rad gel imaging system. Respective protein expression levels were normalized to that of GAPDH which was used as a standard.

Determination of the effect of PI3K/Akt signaling pathway inhibitor LY294002 on the biological behavior of HepG2 cells

The inhibitor of PI3K/Akt signaling pathway (LY294002, 15 $\mu\text{mol/L}$) was used to treat HepG2 cells. This served as treatment group, while cells without inhibitor served as non-treatment group. Changes in cell proliferation, migration, invasiveness and apoptosis; and expressions of PIK3, p-Akt, and Akt were determined after 48 h.

Statistical analysis

Data are expressed as mean \pm SEM. Statistical analysis was performed using SPSS (19.0). Groups were compared using Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Level of expression of miRNA-196b in HepG2 cells after transfection

As shown in Figure 1, miRNA-196b inhibitor significantly down-regulated the expression of miRNA-196b. ($p < 0.05$).

Effect of miRNA-196b inhibitor transfection on cell proliferation

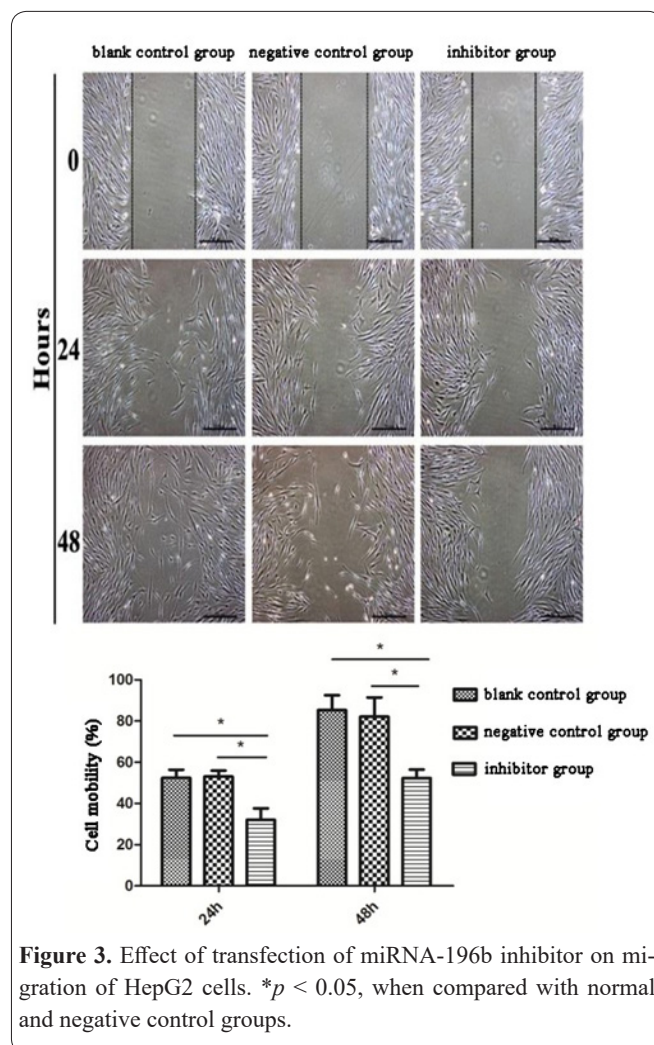
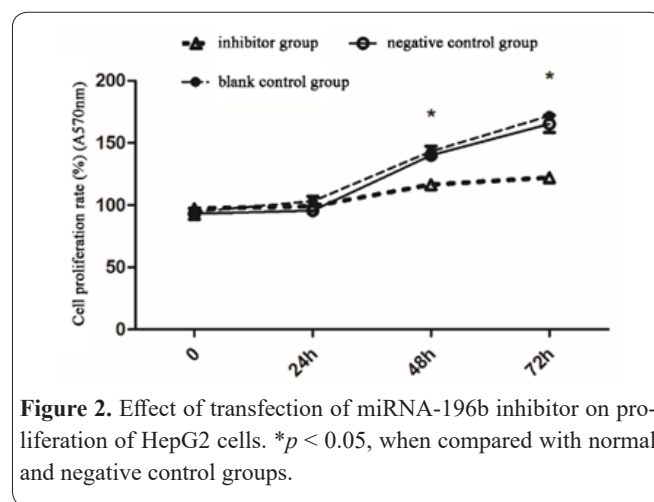
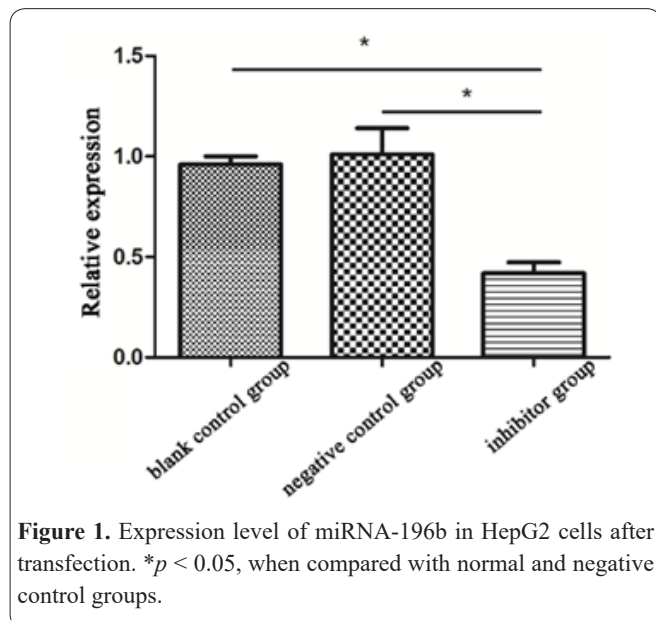
There were no significant differences in cell proliferation among the groups at 24 h ($p > 0.05$). However, at 48 and 72 h, cell proliferation was significantly lower in the inhibitor group than in normal and negative control groups ($p < 0.05$). These results are shown in Figure 2.

Effect of transfection of miRNA-196b inhibitor on migratory ability of HepG2 cells

As shown in Figure 3, miRNA-196b inhibitor significantly and time-dependently reduced the migratory ability of HepG2 cells ($p < 0.05$).

Effect of transfection of miRNA-196b inhibitor on the invasive ability of HepG2 cells

MicroRNA-196b inhibitor significantly reduced the invasive ability of HepG2 cells ($p < 0.05$; Figure 4).



Effect of transfection of miRNA-196b inhibitor on apoptosis of HepG2 cells

As shown in Figure 5, miRNA-196b inhibitor significantly promoted apoptosis of HepG2 cells ($p < 0.05$).

Effects of transfection of miRNA-196b inhibitor on the protein expressions of PI3K, Akt and p-Akt in HepG2 cells

As shown in Figure 6, the expressions of PIK3 and p-Akt proteins were significantly down-regulated in inhibitor group, when compared with normal and negative control groups ($p < 0.05$), but there were no significant differences in the expression of Akt protein among the groups ($p > 0.05$).

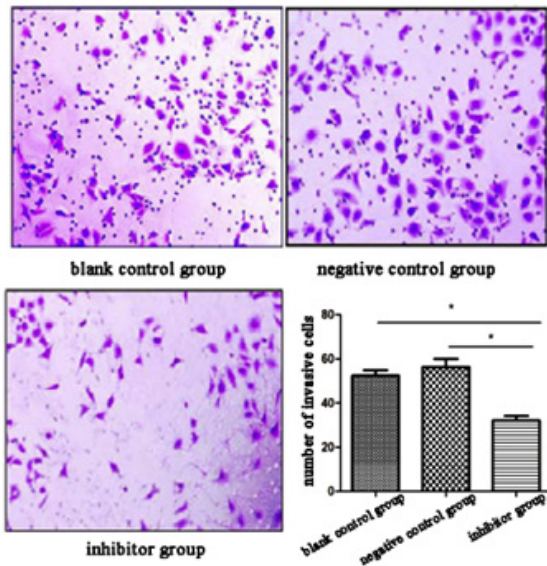


Figure 4. Effect of miRNA-196b inhibitor transfection on the invasive ability of HepG2 cells. * $p < 0.05$, when compared with normal and negative control groups.

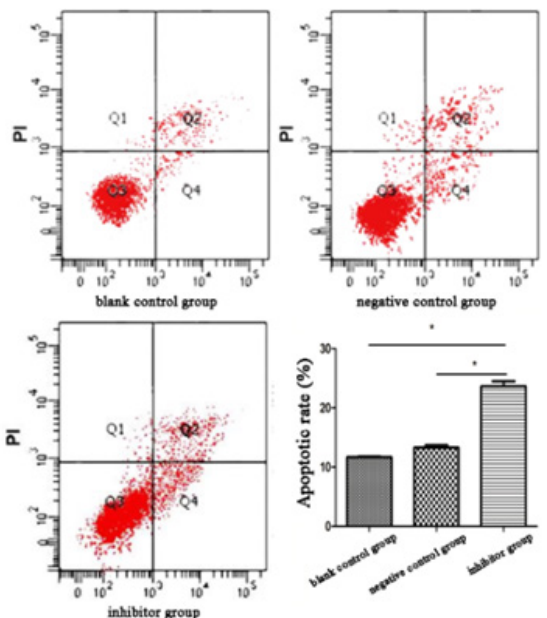


Figure 5. Effect of miRNA-196b inhibitor transfection on HepG2 cell apoptosis. * $p < 0.05$, when compared with normal and negative control groups.

Effect of PI3K/Akt signaling pathway inhibitor LY294002 on HepG2 cells

After treatment of HepG2 cells with PI3K/Akt signaling pathway inhibitor LY294002, the proliferative, migratory and invasive abilities of HepG2 cells in the treatment group were significantly promoted, but cell apoptosis was significantly reduced ($p < 0.05$). The expressions of PIK3 and p-Akt proteins in the non-treatment group were significantly upregulated, relative to the treatment group ($p < 0.05$), but there was no significant difference in the expression of Akt protein between the two groups ($p > 0.05$). These results are shown in Figure 7.

Discussion

Liver cancer, one of the most common malignant

tumors in the world, is characterized by high invasiveness and recurrence (12). Studies have shown that miRNA is a potential therapeutic target that can be used for the diagnosis and prognosis of hepatocellular cancer (13, 14). It is an endogenous non-coding RNA that regulates protein translation, cell development, apoptosis, and metabolism. MicroRNA-196 has been reported to play a role in the pathogenesis and progression of liver cancer. It comprises miRNA-196a and miRNA-196b. MicroRNA-196a is associated with liver cancer and its recurrence after liver transplantation (15, 16). In contrast, miRNA-196b is involved in the proliferation and invasiveness of hepatocellular carcinoma cells (17). Reports on the effect of miRNA-196b on the biological behavior of liver cancer cells are scanty. Studies have shown that miRNA-196b promotes the proliferation and invasiveness of hepatocellular carcinoma (HCC) cells. In one of such studies, it was reported that knockout of miRNA-196b gene significantly reduced the metastasis of HCC in mice. It has also been reported that miRNA-196b promotes etoposide-induced apoptosis in HepG2 cell by targeting insulin-like growth factor-2 mRNA-binding protein 1 (IGF2BP1) (18). In the pres-

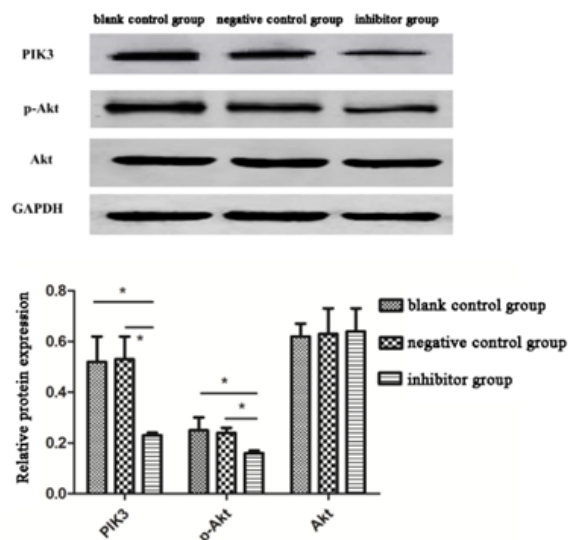


Figure 6. Effect of transfection of miRNA-196b inhibitor on PI3K, Akt and p-Akt protein expressions in HepG2 cells. * $p < 0.05$, when compared with normal and negative control groups.

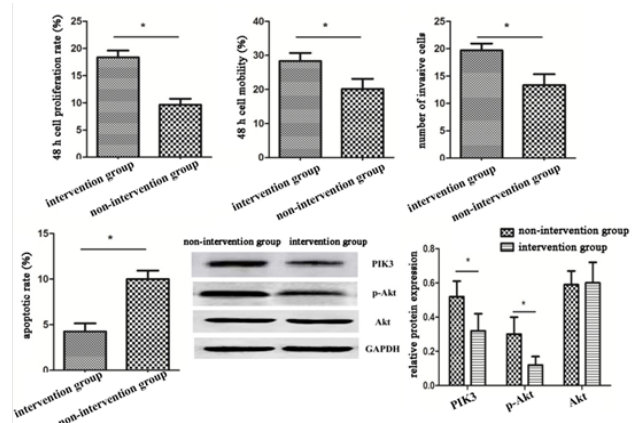


Figure 7. Effect of PI3K/Akt signaling pathway inhibitor LY294002 on proliferation, migration, invasion, apoptosis, and expressions of PI3K, Akt and p-Akt proteins in HepG2 cells. * $p < 0.05$, when compared with nontreatment group.

ent study, HepG2 cells were used. The results showed that transfection of miRNA-196b inhibitor significantly and time-dependently reduced the proliferative and migratory abilities of HepG2 cells. It also significantly reduced the invasiveness of the cells, while promoting their apoptosis. These results suggest that the down-regulation of miRNA-196b expression may inhibit the proliferation, migration and invasiveness of HepG2 cells, while enhancing their apoptosis. It is likely that miRNA-196b plays a key role in the initiation and progression of liver cancer.

The PI3K/Akt signaling pathway plays a role in the pathogenesis of tumors. Dysregulation of this pathway due to abnormal expressions of related proteins leads to abnormal proliferation, invasion and apoptosis of cancer cells (19). Phosphatidylinositol-3-kinase (PI3K) is activated on binding to upstream tyrosine receptor kinase via the SH2 region. This interaction induces a conformational change in the protein. It is also activated by the binding of p110 β to G protein-coupled receptors (GPCRs). After activation, its substrate phosphatidylinositol 4, 5-bisphosphate (PIP2), and product phosphatidylinositol 3, 4, 5-triphosphate (PIP3) serve as second messengers which activate different target proteins and form signal cascade complexes. The activation of Akt via phosphorylation regulates biological behaviors such as cell proliferation, invasiveness, and differentiation (20). Studies have shown that miRNA-196b regulates the proliferation and invasive abilities of gastric cancer cells by targeting the PI3K/Akt/mTOR signaling pathway (21). The results of this study showed that the expressions of PI3K and p-Akt proteins were significantly down-regulated after inhibition of miRNA-196b, an indication that miRNA-196b may regulate PI3K/Akt signaling pathway in HepG2 cells. After treatment of HepG2 cells with PI3K/Akt signaling pathway inhibitor LY294002, the proliferation, migration and invasive ability of cells in the treatment group were significantly promoted, but cell apoptosis was significantly reduced. In addition, the expressions of PI3K and p-Akt proteins in the nontreatment group was significantly upregulated relative to the treatment group. These results suggest that miRNA-196b may regulate the biological activity of HCC cells via the PI3K/Akt signaling pathway.

The limitations of this study were: (a) The non-inclusion of other biological behaviors outside proliferation, apoptosis and invasion; (b) The targeted regulation of PI3K/Akt signal pathway by miRNA-196b was not directly studied; (c) This study was conducted *in vitro*, and did not investigate the relationship between miRNA-196b and liver cancer metastasis and prognosis.

The down-regulation of miRNA-196b expression inhibits the proliferation, migration and invasiveness of HepG2 cells, while promoting their apoptosis via a mechanism involving the PI3K/Akt signaling pathway.

Acknowledgements

None.

Conflict of Interest

There are no conflicts of interest in this study.

Author's contribution

All work was done by the author s named in this article

and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Chunzhao Yu; Fanggui Xu, Feng Zhu, Wulin Wang, Wenjie Gao, Xin Chen, Chunzhao Yu collected and analysed the data; Fanggui Xu wrote the text and all authors have read and approved the text prior to publication.

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