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MiR-370 promotes cell death of liver cancer cells by Akt/FoxO3a signalling pathway

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Abstract. – OBJECTIVE: MicroRNAs have emerged as key regulators in cancer cell biology. In the present study, we investigate the role and the involving mechanism of miR-370 in the progression of liver cancers.

MATERIALS AND METHODS: MiR-370 levels were detected by real-time PCR assay. Cell proliferation of HepG2, MHCC-97H and SMMC-7721 was determined by MTT assay. PI staining was detected by FACS analysis. Colony formation was used to test liver cancer cell growth. FoxO3a and Akt expression was determined by western blotting analysis.

RESULTS: MiR-370 level was significantly down-regulated in liver cancer cells. Functional analysis revealed that miR-370 mimics suppressed cell proliferation of liver cancer cells, while transfection with miR-370 inhibitor increased cell proliferation of liver cancer cells. Moreover, miR-370 mimics induced cell death of HepG2. Furthermore, Western blotting analysis results demonstrated that miR-370 inhibited the proliferation of liver cancer cells by activating FoxO3a.

CONCLUSIONS: MiR-370 inhibited cell proliferation of liver cancer cells by PI3K/Akt signaling pathway. It worked as a tumor suppressor to suppress the progression of human liver cancers.

Key Words:
MiR-370, Liver cancer, FoxO3a, Akt.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide[1,2]. The incidence and mortality rates are rapidly rising in recent years[3]. Compared with developed countries, liver cancer is much more commonly seen in developing countries, such as China and Africa[4,5]. The pathogenic factors for HCC are diverse and complex. Chronic HCV and chronic hepatitis B virus (HBV) infection are the most common reasons in patients with liver cancer[6]. The therapeutic options are mainly including liver resection, transplantation, systemic and local therapy, which should be tailored to each patient with HCC. Although emerging papers about the mechanism and progression of liver carcinoma have been published, the mechanism for hepatocarcinogenesis and development is not clearly clarified[7,8]. Looking for the new diagnostic markers and therapeutic targets for liver cancers is the hotspot in the research area.

MicroRNAs (miRNAs) are a class of small, evolutionary conserved, endogenous, single-stranded, non-coding RNAs of approximately 19-22 nucleotides in length, which regulate gene expression in a sequence-specific manner[9,10]. MicroRNAs are transcribed by RNA polymerases II and III, and the generating precursors are cleaved to form mature microRNA[11]. The mature miRNA binds to the target mRNA and typically in the 3'-untranslated region (3'-UTR), leading to the targeted mRNA degradation or translational repression[12]. Individual miRNAs can target multiple mRNAs and a single mRNA can bind and is regulated by multiple miRNAs[13].

Recently, miR-370 was reported to regulate the proliferation and progression of a variety of tumors[14-16]. A preliminary screening study suggested that down-regulation of miR-370 occurred in oral squamous cell carcinoma (OSCC) tissue and targeted to insulin receptor substratre-1 (IRS-1) to inhibit the tumor progression[16]. In laryngeal squamous cell carcinoma (LSCC), miR-370 targeted Forkhead Box ml (FoxM1) and functioned as a tumor suppressor[17]. Moreover, H.

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pylori infection and CagA upregulated FoxM1 expression, which is negatively regulated by miR-370, altered the expression of p27 (Kip1), and promoted proliferation of gastric cells[18]. However, there were also several papers holding the opposite point of view, e.g. Garcia-Orti[19] had reported that miR-370 was upregulated and targeted NF1 in acute myeloid leukemia. However, it is not clarified whether miR-370 affected the progression of human liver cancers. In the present study, we investigated the role of miR-370 and explored the involving mechanism of miR-370 in liver cancer cells. It is helpful to provide some new clues to find new targets for the therapy of human hepatocellular carcinoma.

Materials and Methods

Cell Line and Agent

Human hepatocellular carcinoma cell lines, MHCC97H, HepG2 and SMMC-7721 and the immortalized liver cell line $L0_2$ were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cell lines were cultured in DMEM medium with 10% fetal bovine serum. Moreover, 1% penicillin and 1% streptomycin was added into the DMEM medium. Dulbecco's Modified Eagles Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Hyclone Co. (Logan, UT, USA). MTT agent was purchased from Sigma-Aldrich Inc. (Saint Louis, MO, USA). LY294002 (Cat. No. S1737), PI3K inhibitor, was obtained from Beyotime Co. (Shanghai, China). The miR-370 mimics and miR-370 inhibitor with its corresponding negative control were obtained from Ribobio Co. (Guangzhou, China), and the information was listed as follows:

- micrOFF™ hsa-miR-370 inhibitor (Cat. No. miR20000722-1-2);
- micrOFF™ inhibitor Negative Control #22 (Cat. No.miR02101-1-2);
- micrON™ hsa-miR-370 mimic (Cat. No. miR10000722-1-5);
- micrON™ agomir Negative Control #24(Cat. No. miR04201-1-2).

MTT assay

The cell viability in the different groups was detected by methyl thiazolyl tetrazolium (MTT) assay as described[20,21]. Briefly, cells were transfected with miR-370 mimics and negative control #24, miR-370 inhibitor and negative control #22. The cells were cultured for 1 d, 2 d, 3 d, 4 d and 5 d, respectively. Four hours before testing, 5 mg/mL of MTT agent was added into each well. Before the test, the purple crystals were dissolved in dimethyl sulfoxide (DMSO) and the data were tested at a test wavelength of 490 nm.

Real-time PCR assay

The liver cancer cell lines, MHCC97H, HepG2 and SMMC-7721, and normal control cell line $L0_2$ were cultured for 8 hours. Total RNA in each sample was extracted with an RNApure kit (Bioteke, Beijing, China) according to the protocols. The samples of cDNA were transcribed using the PrimeScript-RT reagent kit (Takara Co., Ltd., Otsu, Shiga, Japan) in a 20 mL final reaction volume according to the protocols. Real-time quantitative PCR assay was performed to evaluate miR-370 levels using the SYBR Premix ExTaqTM II (Takara Co., Ltd., Otsu, Shiga, Japan) on ABI 7500 system. U6 snRNA was used for normalization and each sample was analyzed in triplicate. No template and no reverse transcription were included as negative controls. The sequence of primers was as follows.

For real-time PCR reaction

- hsa-mir-370 sense: GCCUGCUGGGGUGGAACCUGGU
- anti-sense: CAGGUUCCACCCCAGCAGGCUU
- U6 sense: 5′-CTCGCTTCGGCAGCACA-3′
- Anti-sense: 5′-AACGCTTCACGAATTTGCGT-3′

Clone formation assay

The liver cancer cells were transfected with miR-370 mimics, miR-370 inhibitor and negative controls for 48 hours. Then, the cells were planted into 6-well plates (5×10^3 cells/well) and cultured for at least 2 weeks. The cultured medium was refreshed every 4 days. At the end of the experiment, methanol was used to fix the surviving colonies and the colonies were stained with 1.25% crystal violet. The colonies were counted under a light microscope.

Western blotting analysis

HepG2 cells were treated with 20 μM and 40 μM for 24 hours. The other group of HepG2 was transfected with miR-370 mimic or miR-370 inhibitor for 48 hours. The expression levels of p-Akt, Akt and FoxO3a were detected by Western blotting analysis. Briefly, the cells were lysed by

RIPA-buffer (Roche Diagnostics, Indianapolis, IN, USA) and the proteins in cell lysates were separated by PAGE. Then, the proteins were transferred onto PVDF membranes (Bio-Rad, Richmond, VA, USA) at 400 mA for 1 hour. The membranes were blocked in 5% BSA/PBS for 30 min, followed by incubating with related primary at 4°C overnight. The membranes were washed with PBST for three times and were incubated with secondary antibodies at the room temperature for 30 min. The membrane was washed for three times. The bands were detected in a dark room using chemiluminescence techniques. Here, β-actin was used as an internal reference. The antibodies used here were listed as following. FoxO3a (75D8) (Rabbit mAb #2497) is a monoclonal antibody for detecting exogenous and endogenous levels of total FoxO3a protein, which is purchased from Cell Signaling Technology Co. Both of the p-Akt antibody (Cat. No. sc-7985-R) and AKT antibody (BDI111, Cat. No. sc-56878) was obtained from Santa Cruz Biotechnology. p-Akt1/2/3 (Ser 473)-R Antibody is a rabbit polyclonal IgG provided at 200 μg/ml and AKT Antibody is a mouse monoclonal IgG1 provided at 50 μg/ 0.5 ml. Mouse monoclonal anti- -actin antibody (Cat. No. sc-47778), goat anti-rabbit IgG-HRP (Cat. No. sc-2004) and goat anti-mouse IgG-HRP (Cat. No. sc-2005) were obtained from Santa Cruz Co. (Santa Cruz, CA, USA).

FACS assay

The liver cancer cells were transfected with miR-370 mimic and negative control, miR-370 inhibitor and negative control. The cells were cultured for 48 hours. Then, they were collected and resuspended with phosphate buffered saline (PBS) buffer. Finally, 0.05 μg/μl of propidium iodide (PI) was added, and the samples were detected by flow cytometry.

Statistical Analysis

The data in the present study were analyzed by SPSS software (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to analyze the statistical significance. Data were shown as means ± SD. $p < 0.05$ was considered statistically significant.

Results

MiR-370 Level is Significantly Downregulated in Liver Cancer Cell Lines

It has been reported that altered miRNA expression likely contributes to human disease, including cancer. In order to investigate the role of miR-370 in the progression of human liver cancer carcinomas, we extracted total RNA from three liver cancer cell lines, including HepG2, MHCC-97H and SMMC-7721 and detected the expression levels of miR-370 by real-time PCR assay. The results demonstrated that the expression levels of miR-370 were significantly downregulated in liver cancer cell lines (Figure 1), compared with that of the immortalized liver cell line $L0_2$.

The miR-370 mimics inhibit the proliferation of liver cancer cells in a time-dependent manner

To investigate the role of miR-370 in the proliferation of liver cancer cells, miR-370 mimics were transfected into liver cancer cell HepG2 for 1 d to 5 d. Cell viability was determined by MTT assay. As shown in Figure 2, the OD490 values in miR-370 mimics-transfected HepG2 cells were obviously decreased than that of negative control cells since the 3rd day. This was consistent with that of the results in MHCC-97H cells and SMMC-7721 cells ($*p < 0.05$, $**p < 0.01$ compared with negative ctrl.). All of the data demonstrated that miR-370 expression in HepG2, MHCC-97H and SMMC-7721 cells dramatically decreased the proliferation of liver cancer cells.

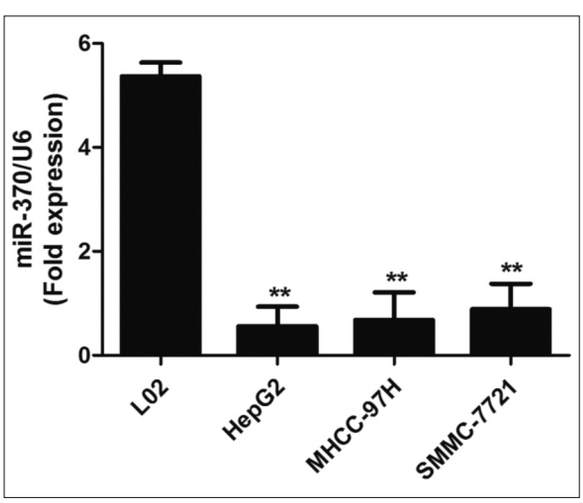

Figure 1. MiR-370 level is significantly downregulated in liver cancer cell lines. Total RNA was extracted and reverse transcription was performed to get the cDNA from liver cancer cell lines HepG2, MHCC-97H and SMMC-7721 as described in Materials and Methods. The expression levels of miR-370 were detected by real-time PCR assay. $**p < 0.01$, compared with that in $L0_2$ cells.

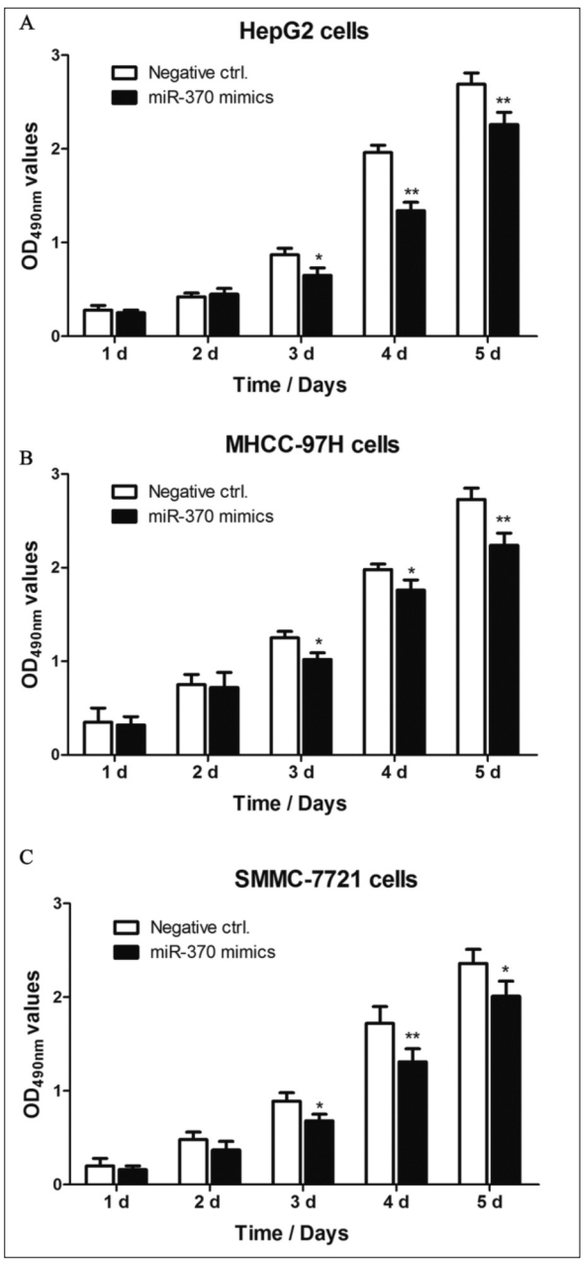

Figure 2. The miR-370 mimics inhibit the proliferation of liver cancer cells in a time dependent manner. The liver cancer cells HepG2 ***(A)***, MHCC-97H ***(B)*** and SMMC-7721 ***(C)*** were plated into 48-well plate. After cultured for 8 hours, the cells were transfected with miR-370 mimics and negative ctrl for 1 d, 2 d, 3 d, 4 d and 5 d, respectively. The OD 490 nm values were tested by MTT assay. *$p < 0.05$, **$p < 0.01$, compared with negative control group.

MiR-370 regulates the proliferation of liver cancer cells

In order to further confirm the role of miR-370 in the progression of liver cancer cells, the miR-370 mimics and inhibitors were transfected to increase or decrease the levels of miR-370 in liver cancer cells. Briefly, the liver cancer cells were transfected with miR-370 mimics and miR-370 inhibitor for 1 d to 5 days, and the cell viability was determined by MTT assay. As shown in Figure 3, the OD490 nm values were significantly decreased in miR-370 transfected cells, while transfection with miR-370 inhibitor could significantly increase the proliferation of HepG2 and SMMC-7721 cells (**$p < 0.01$, *$p < 0.05$, compared with negative ctrl. group).

The effect of miR-370 on liver cancer cell growth is further confirmed by colony formation assay

The colony formation assay is a widely used method for measuring the effects of miRNAs on the proliferation of cancer cells *in vitro*. As shown in Figure 4, compared to cells transfected

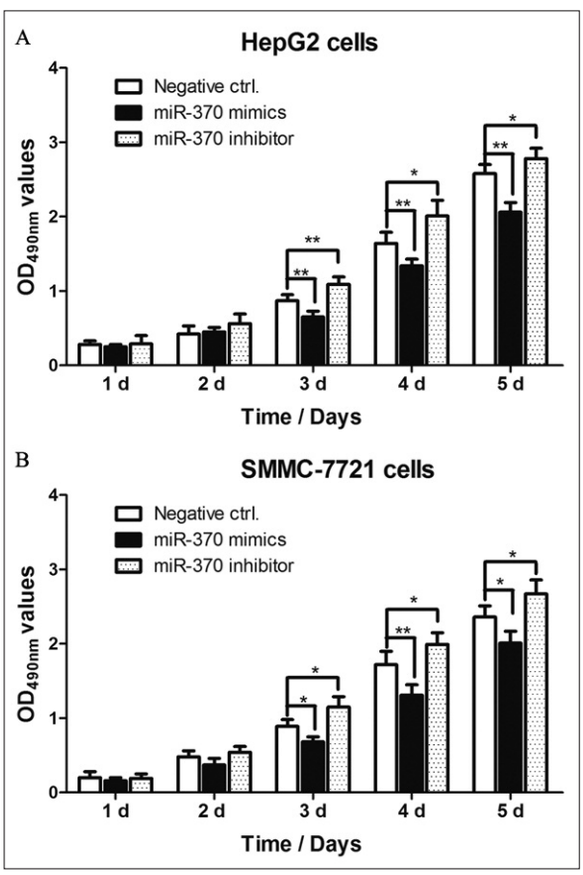

Figure 3. MiR-370 regulates the proliferation of liver cancer cells. The liver cancer cells HepG2 and SMMC-7721 cells were transfected with miR-370 mimics and inhibitor for 1 d, 2 d, 3 d, 4 d and 5 d, respectively. The cell viability was determined and OD490 nm values were tested by MTT assay. *$p < 0.05$, **$p < 0.01$, compared with negative control group.

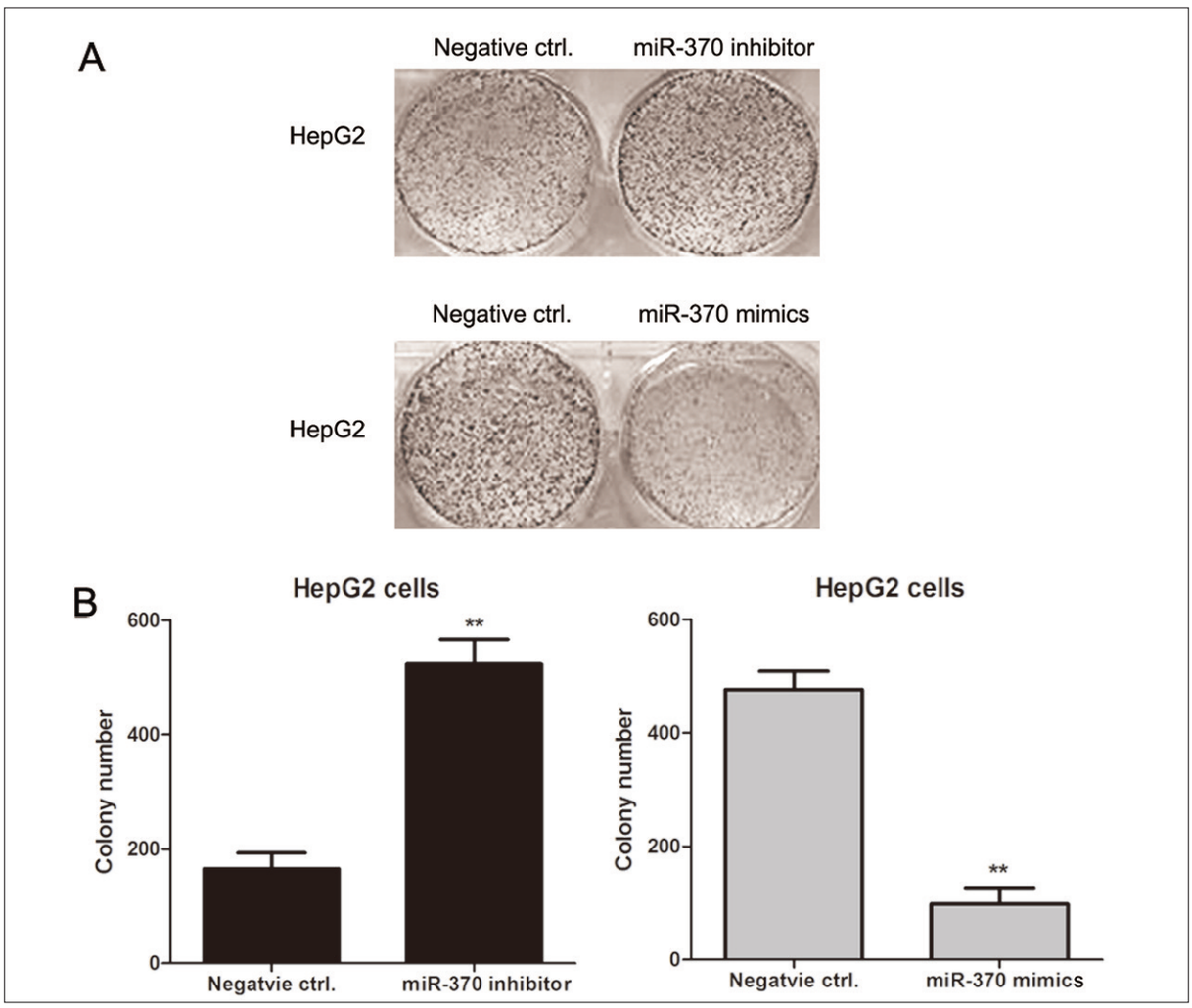

Figure 4. The effect of miR-370 on liver cancer cell growth is further confirmed by colony formation assay. ***A,*** The HepG2 cells were transfected with miR-370 mimics or inhibitor for 3 days, and the colony number were shown in every group. ***B,*** Colony number of miR-370 mimics or miR-370 inhibitor transfected HepG2 cells was shown in histogram. *$p < 0.05$, **$p < 0.01$, compared with negative control group.

with negative control, the number of colonies was significantly increased in cells transfected with miR-370 inhibitor. However, the number of colonies was significantly decreased in miR-370 mimics- transfected cells than that of negative control transfected HepG2. Taken together, higher levels of miR-370 significantly led to the lower proliferation of liver cancer cells and miR-370 acted as a potential tumor suppressor.

Transfection with miR-370 mimics increases the cell death of HepG2 by PI-staining analysis

In order to further to detect the role of miR-370 in the proliferation of liver cancer cells, propidium iodide was used for identifying dead cells in miR-370 mimics or miR-370 inhibitor transfected HepG2 cells. As shown in Figure 5, FACS assay results demonstrated that the HepG2 cells were transfected with miR-370 mimics for 3 days, died cells were more than that of the negative control group. However, transfection with miR-370 inhibitor in HepG2 cells had not affected the cell death rate of HepG2, compared with that of negative control cells.

Activation of FoxO3a suppresses the proliferation of liver cancer cells

Next, we wanted to know whether the PI3K/Akt signaling pathway was involving in miR-370 in HepG2 cells, Western blotting analysis was performed to detect the levels of p-Akt and

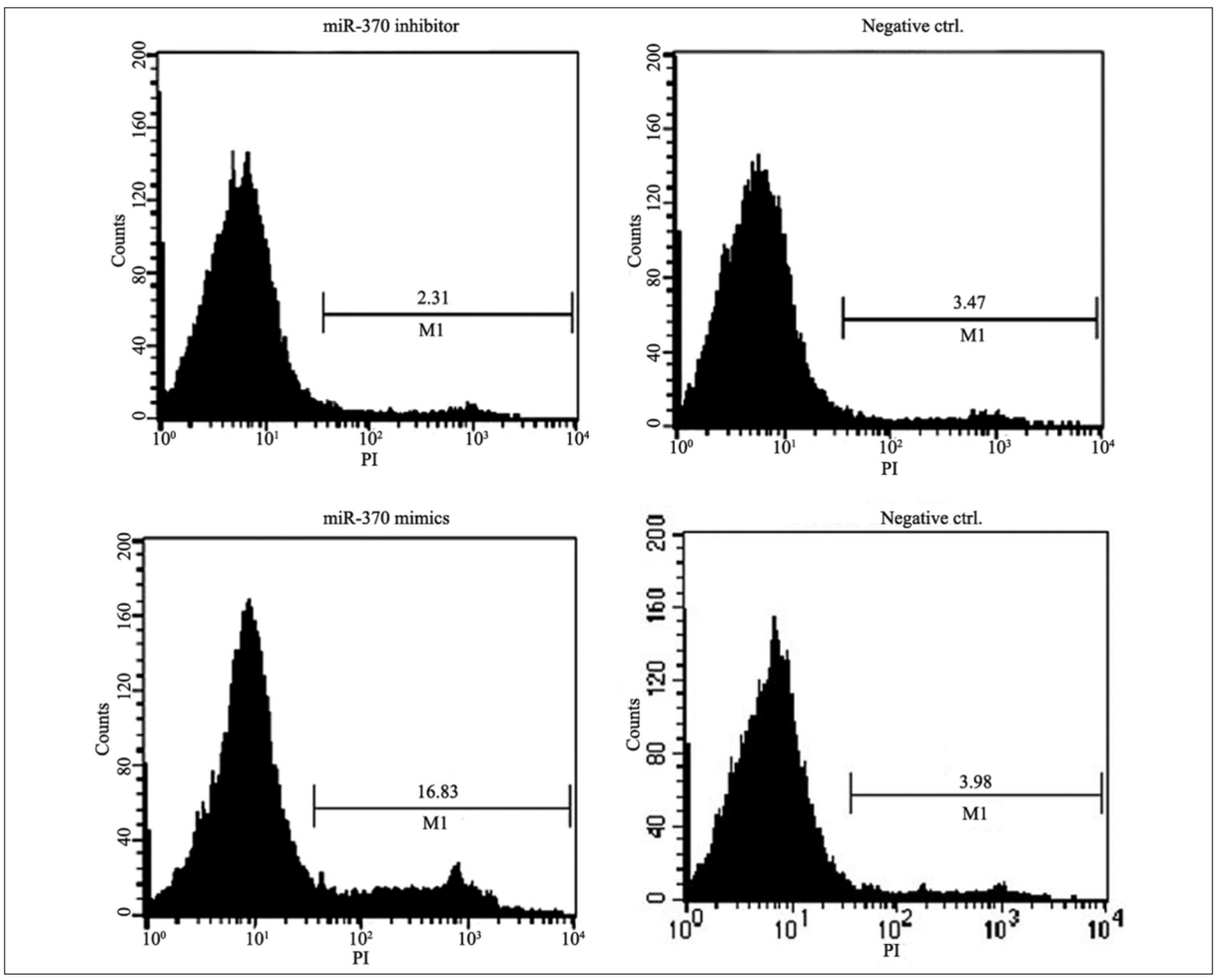

Figure 5. Transfection with miR-370 mimics increases the cell death of HepG2 by PI-staining analysis. The cell death was analyzed by FACS as described in Materials and Methods. The values represent the ratio of the mean fluorescence intensity (MFI) values of dead cells in each group.

FoxO3a in miR-370 mimics or inhibitor-treated cells. LY294002 was a highly selective inhibitor of phosphatidylinositol-3 (PI-3) kinase. We used LY294002 (20 μM and 40 μM) to treat HepG2 cells for 24 hours. As shown in Figure 6a, the phospho-Akt level was decreased and the level of FoxO3a was significantly increased as the increasing concentration of LY294002. Meanwhile, we also found that in miR-370 mimics-transfected HepG2 cells, lower level of p-Akt was accompanied by a higher level of FoxO3a, compared with negative control cells. Additionally, the miR-370 inhibitor-transfected cells had a higher level of p-Akt and lower level of FoxO3a compared with negative control cells. All of the data suggested that miR-370 inhibited the proliferation of liver cancer cells by activating FoxO3a, which was mediated by PI3K/Akt signaling pathway.

Discussion

Till now, surgery is still the preferred method in the therapy for liver cancer[22]. But in most cases, the patients with hepatocellular carcinoma were diagnosed in an advanced stage, which had a poor effect of surgery or lost the opportunity of surgery[23]. Moreover, postoperative complications and high of recurrence and metastasis after surgery severely limited the application and effect of liver cancer surgery. Liver transplantation is by far the most effective way to cure liver cancer[24]. As for the shortage of transplanted liver, the limited surgery conditions and the postoperative complications, the overall mortality rates of liver cancer have not been greatly improved than 10 years ago. Additionally, the chemotherapeutic resistance of chemotherapy drugs for liver cancer

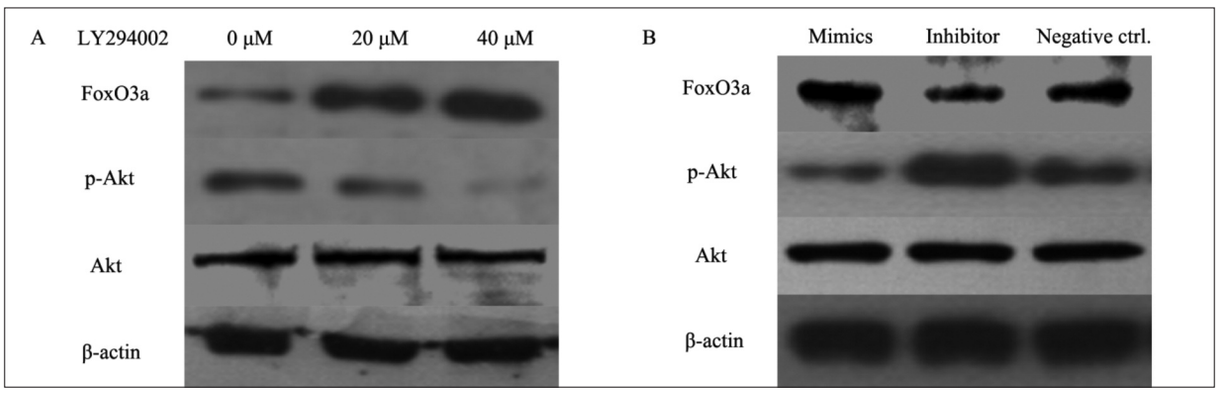

Figure 6. Activation of FoxO3a suppresses the proliferation of liver cancer cells. ***A,*** HepG2 cells were treated with LY294002, the PI3K inhibitor, at the concentration of 20 μM and 40 μM for 24 hours. The expression levels of p-Akt, Akt and FoxO3a were detected by Western blotting analysis. ***B,*** HepG2 cells were transfected with miR-370 mimics and miR-370 inhibitor for 3 days. Western blotting analysis was used to detect the expression levels of p-Akt, Akt and FoxO3a. Here, β-actin was used as the internal reference gene.

is a big obstacle to cure liver cancer. Thus, it is urgent to clarify and understand the mechanism of occurrence, development and transfer of liver cancers.

The mature miRNA recognized the target mRNAs by incorporating into a RNA-induced silencing complex (RISC) resulting in translational inhibition or destabilization of the target mRNA[25-27]. In the present study, the MTT assay and clone formation assay was used to determine the influence of miR-370 on cell proliferation of hepatocellular carcinoma. We first selected three liver cancer cell lines, such as HepG2, MHCC-97H and SMMC-7721 and an immortalized liver cancer cell line $L0_2$ as cell models. The total RNA was extracted and the miR-370 was tested in every liver cancer cell line and we found that the levels of miR-370 were significantly down-regulated in liver cancer cells than that of $L0_2$. The results suggested that miR-370 worked as a tumor suppressor and this was consistent with the results in laryngeal squamous cell carcinoma[17]. However, the results were inconsistent with the founding in gastric cancers[18,28], which was partly due to single miRNA targeting different m RNA in various tumor tissues.

Next, MTT assay and colony formation assay was also performed to test the effects of miR-370 mimics and miR-370 inhibitor in the proliferation of liver cancer cells. The functional analysis demonstrated that miR-370 mimics significantly inhibited cell proliferation of liver cancer cells, and miR-370 inhibitor increased cell proliferation of HepG2, MHCC-97H and SMMC-7721. Additionally, miR-370 mimics or miR-370 inhibitor were used to treat liver cancer cells and the death rates were tested by PI staining which was detected by FACS analysis. All the results obviously revealed that overexpression of miR-370 inhibited the proliferation and promoted cell death of HepG2 cells.

It has been reported that miR-125a inhibited the invasive ability of hepatocellular carcinoma cells via regulation of the PI3K/AKT/mTOR pathway[29] and PI3K/AKT signaling pathway was involved in the regulating the progression of liver cancers[30-32]. Furthermore, we also detected the underlying mechanism by western blotting analysis. The data suggested that miR-370 suppressed the proliferation of liver cancer cells by activating FoxO3a, which was also involved in and mediated by PI3K/Akt signaling pathway. In conclusion, higher levels of miR-370 promoted cell death and inhibited cell proliferation of liver cancer cells by regulating the Akt/FoxO3a signaling pathway. The research would give some new clues and provide a new target for the therapy of human liver cancers.

Conclusions

MiR-370 inhibited cell proliferation of liver cancer cells by PI3K/Akt signaling pathway. It worked as a tumor suppressor to suppress the progression of human liver cancers.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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