

# MiR-27a Promotes Hepatocellular Carcinoma Cell Proliferation Through Suppression of its Target Gene Peroxisome Proliferator-activated Receptor $\gamma$

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## Abstract

**Background:** MicroRNAs (miRNAs) function as essential posttranscriptional modulators of gene expression, and are involved in a wide range of physiologic and pathologic states, including cancer. Numerous miRNAs are deregulated in hepatocellular carcinoma (HCC). This study aimed to investigate the role of miR-27a in the development of HCC.

**Methods:** The expression of MiR-27a was measured by quantitative real-time polymerase chain reaction (qRT-PCR). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to examine changes in the viability of HepG2, Bel-7402, Bel-7404 hepatoma cell lines associated with up-regulation or down-regulation of miR-27a. A dual-luciferase activity assay was used to verify a target gene of miR-27a. Immunohistochemistry, qRT-PCR, Western blotting analysis, and cell cycle and apoptosis flow cytometric assays were used to elucidate the mechanism by which miR-27a modulates liver cancer cell proliferation.

**Results:** The expression of miR-27a was significantly increased in HCC tissues and HepG2, Bel-7402, Bel-7404 hepatoma cell lines ( $P < 0.05$ ). We also found that the down-regulation of miR-27a in HepG2 cells dramatically inhibited proliferation, blocked the G1 to S cell cycle transition and induced apoptosis ( $P < 0.05$ ). In addition, miR-27a directly targeted the 3'-untranslated region of peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ), and ectopic miR-27a expression suppressed PPAR- $\gamma$  expression on the mRNA and protein levels. The rosiglitazone-induced overexpression of PPAR- $\gamma$  attenuated the effect of miR-27a in HCC cells.

**Conclusions:** Our findings suggested that miRNA-27a promoted HCC cell proliferation by regulating PPAR- $\gamma$  expression. MiR-27a may provide a potential therapeutic strategy for HCC treatment.

**Key words:** Cell Proliferation; Hepatocellular Carcinoma; MiR-27a; Peroxisome Proliferator-activated Receptor  $\gamma$

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human malignancies, which shows exceptional heterogeneity in cause and outcome and is especially prevalent among Chinese populations.<sup>[1]</sup> It ranks as the second leading cause of cancer-related death in males and the third leading cause of cancer-related death in females, with a total mortality rate of 26.1 per 100,000 in China.<sup>[2]</sup> Despite successful local therapies such as surgery or transcatheter arterial chemoembolization, patients with HCC have a high rate of recurrence due to local invasion and intrahepatic

metastasis.<sup>[3]</sup> Given this context, there is an urgent need to discover novel early diagnostic biomarkers and new therapeutic strategies for HCC.

Although various genetic and epigenetic changes that lead to HCC have been revealed, the underlying molecular mechanisms for liver cancer are not fully elucidated. Recent research highlighted the role of dysregulated microRNAs (miRNAs) in HCC.<sup>[4]</sup> MiRNAs are small noncoding RNAs that bind to the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs) and inhibit gene expression by cleaving the target mRNA or repressing mRNA translation.<sup>[5]</sup> Through their influence on target genes, miRNAs with altered expression patterns are capable of regulating tumor cell growth, apoptosis, angiogenesis and invasion in cancers.<sup>[6]</sup>

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A number of studies have revealed the miRNA profile of HCC. Many miRNAs are aberrantly expressed in HCC and function as oncogenes or tumor suppressors.<sup>[7-9]</sup> Emerging data showed that miR-27a was up-regulated and could serve as a potential oncogene in distinct cancer types, including pancreatic, gastric, ovarian, breast, and lung cancers, as well as in HCC.<sup>[10,11]</sup> However, the effects of miR-27a in HCC have not been completely elucidated. Therefore, it is of great significance to further study the function and mechanism of miR-27a in HCC. The purpose of this study was to investigate the potential function of miR-27a in the development and progression of HCC.

## METHODS

### Reagents

Rosiglitazone and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM, high glucose) was obtained from GIBCO (Carlsbad, CA, USA). The Bradford Protein Assay Kit, radio immunoprecipitation assay (RIPA) lysis buffer and annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit were obtained from Beyotime Institute of Biotechnology (Beijing, China). Fetal bovine serum (FBS), sodium dodecyl sulfate (SDS), tetramethylethylenediamine, glycine, ammonium persulfate, acrylamide, Tris, agarose, Tween-20 and protease inhibitors were purchased from Beijing Dingguo Biological Technology Co., Ltd. (Beijing, China). Antibodies against peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ) (mouse, monoclonal, E-8, sc-7273) were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). All of the reagents were used without further purification. Deionized water was used in all experiments.

### Tissue samples and cell culture

This study was approved by the Ethics Review Committees of China-Japan Union Hospital, Jilin University, and informed consent was obtained from all patients. A total of 40 patients with HCC had undergone routine surgery at China-Japan Union Hospital, Jilin University. HCC samples and the matched pericarcinomatous tissues taken from these 40 patients were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  or fixed in 10% formalin for paraffin embedding. The human liver cancer cell lines HepG2, Bel-7402, and Bel-7404 and the normal human hepatic embryo cell line HL-7702 were obtained from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at  $37^{\circ}\text{C}$  in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ .

### RNA extraction and quantitative real-time polymerase chain reaction

Total RNA and miR fractions were isolated from tissue samples and the HepG2, Bel-7402, Bel-7404 and HL-7702 cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed into cDNA

using the TransScript First-Strand cDNA Synthesis SuperMix (TransScript) (Invitrogen), following the manufacturer's instructions. miRNA extraction was performed using the miRNA Extraction Kit (Tiangen, Beijing, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on the FastStart Universal STBR Green Master (ROX) (Roche, Basel, Switzerland). Primers for miR-27a and U6 were obtained from RiboBio (Guangzhou, China). The expression of miR-27a was normalized to that of U6 using the  $2^{-\Delta\Delta\text{Ct}}$  method.

### Immunohistochemistry

Immunohistochemical staining for PPAR- $\gamma$  in the above-mentioned 40 HCC samples were performed and evaluated as reported.<sup>[12]</sup> Briefly, after deparaffinization and blocking, the sections were incubated with the antibody overnight at  $4^{\circ}\text{C}$ . The sections were counterstained with Meyer's hematoxylin. PPAR- $\gamma$  expression, defined as the presence of specific staining in the cytoplasm of cancer cells, was evaluated as either positive or negative.

### Cell viability assay

Cells were seeded in 96-well plates at  $5 \times 10^3$  cells per well and transfected with 100 nmol/L miR-27a mimics, miR-27a inhibitor or PPAR agonist rosiglitazone, and were further incubated for 24 h. Thereafter, cells were incubated in 0.1 mg/ml MTT at  $37^{\circ}\text{C}$  for 3 h and lysed in dimethyl sulfoxide at room temperature for 30 min. The absorbance in each well was measured at 490 nm using a microplate reader. Each experiment was performed in triplicate.

### Cell cycle and apoptosis analysis

For cell cycle analysis, cells were harvested after transfection for 24 h, washed with precooled phosphate buffered saline (PBS), fixed in 70% ethanol, and stained with a freshly prepared solution containing 50  $\mu\text{g}/\text{ml}$  propidium iodide (PI), 100  $\mu\text{g}/\text{ml}$  RNase A, and 0.2% Triton X-100 for 30 min in the absence of light. Cell cycle distributions were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cell apoptosis was detected using an annexin V-FITC/PI apoptosis detection kit, following the manufacturer's instructions, and was analyzed using a FACSCalibur flow cytometer. Each experiment was performed in triplicate.

### Western blot analysis

Cells were collected and lysed in 0.1 ml cold RIPA lysis buffer containing 0.02% phenylmethanesulfonyl fluoride. The cell lysates were then centrifuged at  $12,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ . Protein concentrations were determined with the Bradford Protein Assay Kit, using bovine serum albumin as the standard. The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After nonspecific binding sites had been blocked with 5% nonfat dry milk in PBS for 60 min, the transferred membranes were incubated overnight at  $4^{\circ}\text{C}$  with primary antibodies. Following repeated washes with Tris buffered saline with Tween (TBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated mouse anti-rabbit

secondary antibody or HRP-conjugated rabbit anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc., USA) for 1.5 h at room temperature prior to additional washes with TBST. Detection of antibody binding was performed using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Amersham, UK). Equal loading was verified using antibodies against glyceraldehyde-3-phosphate dehydrogenase. All Western blot analyses were repeated 3 times.

### Statistical analysis

Data were expressed as the mean  $\pm$  standard deviation (SD). Statistical analysis was performed using nonpaired *t*-test or one-way analysis of variance, and a *P* < 0.05 was considered to be statistically significant.

## RESULTS

### miR-27a up-regulated significantly in hepatocellular carcinoma tissues and cell lines

To investigate the role of miR-27a in HCC, its expression levels between clinical HCC and matched pericarcinomatous tissues from 40 HCC patients were compared by qRT-PCR. As shown in Figure 1a, the expression of miR-27a ( $3.12 \pm 0.57$ ) was significantly higher in HCC than that in the non-tumor liver samples ( $1.00 \pm 0.21$ , *P* < 0.05); the overall expression of miR-27a increased by about three-fold in the HCC samples. We further evaluated the expression of miR-27a in the HepG2, Bel-7402, and Bel-7404 cell lines, as well as in the normal human hepatocyte HL-7702 cell line. MiR-27a was up-regulated overall but had different expression levels in all tested HCC cell lines ( $2.98 \pm 0.55$  in HepG2,  $1.63 \pm 0.37$  in Bel-7402, and  $2.28 \pm 0.49$  in Bel-7404), compared with the HL-7702 cell line ( $1.00 \pm 0.17$ , all *P* < 0.05) [Figure 1b]. Taken together, these results indicated that the up-regulation of miR-27a may be involved in HCC progression.

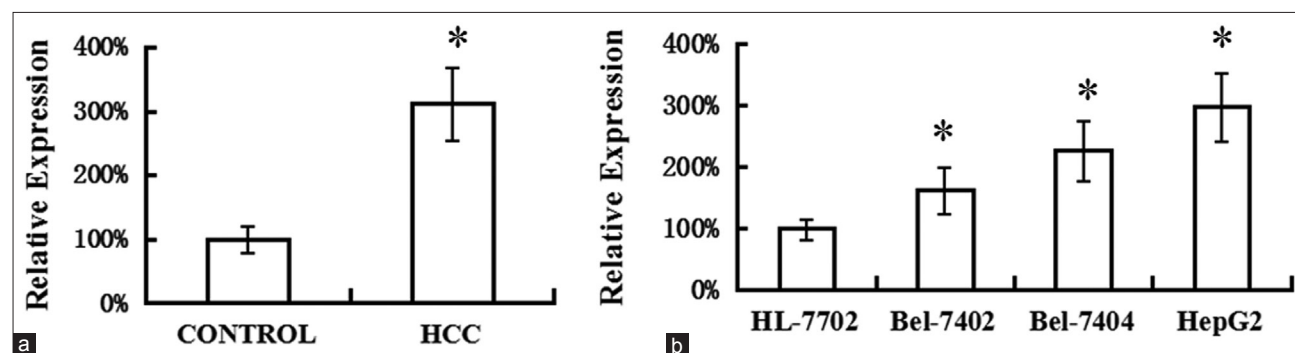
### In vitro effects of miR-27a on hepatocellular carcinoma cell proliferation, cell cycle, and apoptosis

To demonstrate the effect of miR-27a on HCC growth, we performed an HCC cell proliferation assay. As shown in Figure 2a-2d, transfection of miR-27a mimics

significantly up-regulated miR-27a expression, resulting in significantly increased proliferation compared with the control group of HepG2 cells at 12 h ( $1.34 \pm 0.07$  vs.  $1.00 \pm 0.02$ , *P* < 0.05), 24 h ( $1.93 \pm 0.19$  vs.  $1.00 \pm 0.05$ , *P* < 0.05), and 48 h ( $2.79 \pm 0.23$  vs.  $1.00 \pm 0.04$ , *P* < 0.05), respectively. However, HepG2 cells transfected with the miR-27a inhibitor showed reduced cell growth compared with the control at 12 h ( $0.82 \pm 0.03$  vs.  $1.00 \pm 0.04$ , *P* < 0.05), 24 h ( $0.61 \pm 0.04$  vs.  $1.00 \pm 0.04$ , *P* < 0.05), and 48 h ( $0.31 \pm 0.02$  vs.  $1.00 \pm 0.03$ , *P* < 0.05), respectively. To further elucidate the mechanism of growth inhibition by miR-27a down-regulation, flow cytometry was used to analyze the cell cycle and apoptotic rate in HepG2 cells. Analysis of the cell cycle distribution showed that compared with the control, transfection of HepG2 cells with the miR-27a inhibitor significantly increased the number of cells in G1 phase ( $0.67 \pm 0.04$  vs.  $0.50 \pm 0.02$ , *P* < 0.05) and decreased the number of cells in S phase ( $0.19 \pm 0.02$  vs.  $0.33 \pm 0.03$ , *P* < 0.05) [Figure 2e]. Moreover, analysis of the apoptotic rate showed that down-regulation of miR-27a led to a significant increase in the apoptotic rate of HepG2 cells ( $0.35 \pm 0.03$  vs.  $0.05 \pm 0.01$ , *P* < 0.05) [Figure 2f]. Taken together, these data suggested that down-regulation of miR-27a inhibited HCC cell proliferation by promoting apoptosis and inducing G1-phase cell cycle arrest.

### Peroxisome proliferator-activated receptor $\gamma$ , a direct target of miR-27a in hepatocellular carcinoma cells

To study the carcinogenic function of miR-27a on HCC, we searched for putative miR-27a targets using TargetScan. Combinational prediction using TargetScan revealed that the 3'UTR of PPAR- $\gamma$  contains the conserved putative miR-27a binding sites. To explore whether PPAR- $\gamma$  is a target gene of miR-27a in HCC cells, we constructed luciferase reporter vectors with the putative PPAR- $\gamma$  3'-UTR target sites for miR-27a downstream of the luciferase gene (pGL3-PPAR- $\gamma$ -3'-UTR). The luciferase reporter vectors were transfected into HepG2 cells together with miR-27a mimics or miRNA mimics control. A significant decrease in the relative luciferase activity was observed when pGL3-PPAR- $\gamma$ -3'-UTR was cotransfected with



**Figure 1:** MiR-27a expression in hepatocellular carcinoma (HCC) cell lines and tissues. (a) MiR-27a expression was examined by quantitative real-time polymerase chain reaction (qRT-PCR) in human HCC tissues and adjacent normal tissues (control group); (b) qRT-PCR analysis of miR-27a expression in normal liver cells (HL-7702, as control group) and three HCC cell lines (HepG2, Bel-7402 and Bel-7404). MiR-27a expression in each sample was normalized to that of U6, the internal control (\**P* < 0.05 vs. control group).

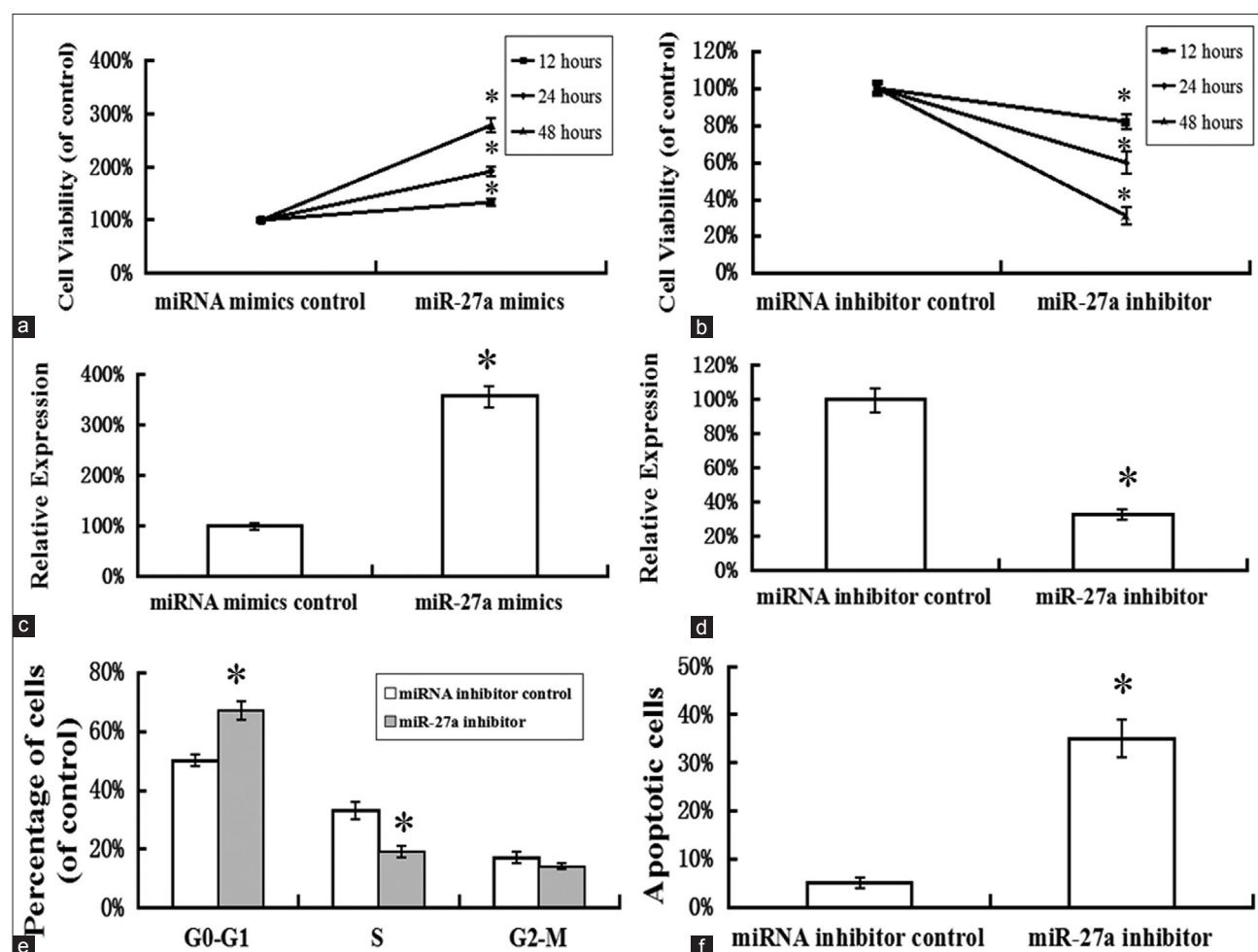
miR-27a mimics but not with the control ( $0.46 \pm 0.03$  vs.  $1.00 \pm 0.04$ ,  $P < 0.05$ ). These results showed that PPAR- $\gamma$  was a target gene of miR-27a in HepG2 cells [Figure 3a]. The effect of miR-27a on the endogenous PPAR- $\gamma$  expression was further examined. We found that ectopic expression of miR-27a in HepG2 cells significantly suppressed both the mRNA ( $0.57 \pm 0.04$  vs.  $1.00 \pm 0.02$ ,  $P < 0.05$ ) and protein ( $0.37 \pm 0.05$  vs.  $1.00 \pm 0.06$ ,  $P < 0.05$ ) levels of PPAR- $\gamma$  [Figure 3b and 3c]. In addition, we examined PPAR- $\gamma$  expression by immunohistochemistry using the clinical specimens from 40 patients. Immunohistochemistry staining revealed that the PPAR- $\gamma$  protein was localized in the nucleus, and PPAR- $\gamma$  expression was lower in the HCC tissues compared with the matched pericarcinomatous tissues [Figure 3d]. There was a significant association between PPAR- $\gamma$  expression and miR-27a expression. Taken together, these data demonstrated that PPAR- $\gamma$  is a direct target of miR-27a.

## Overexpression of peroxisome proliferator-activated receptor $\gamma$ attenuates the effect of miR-27a on hepatocellular carcinoma cells

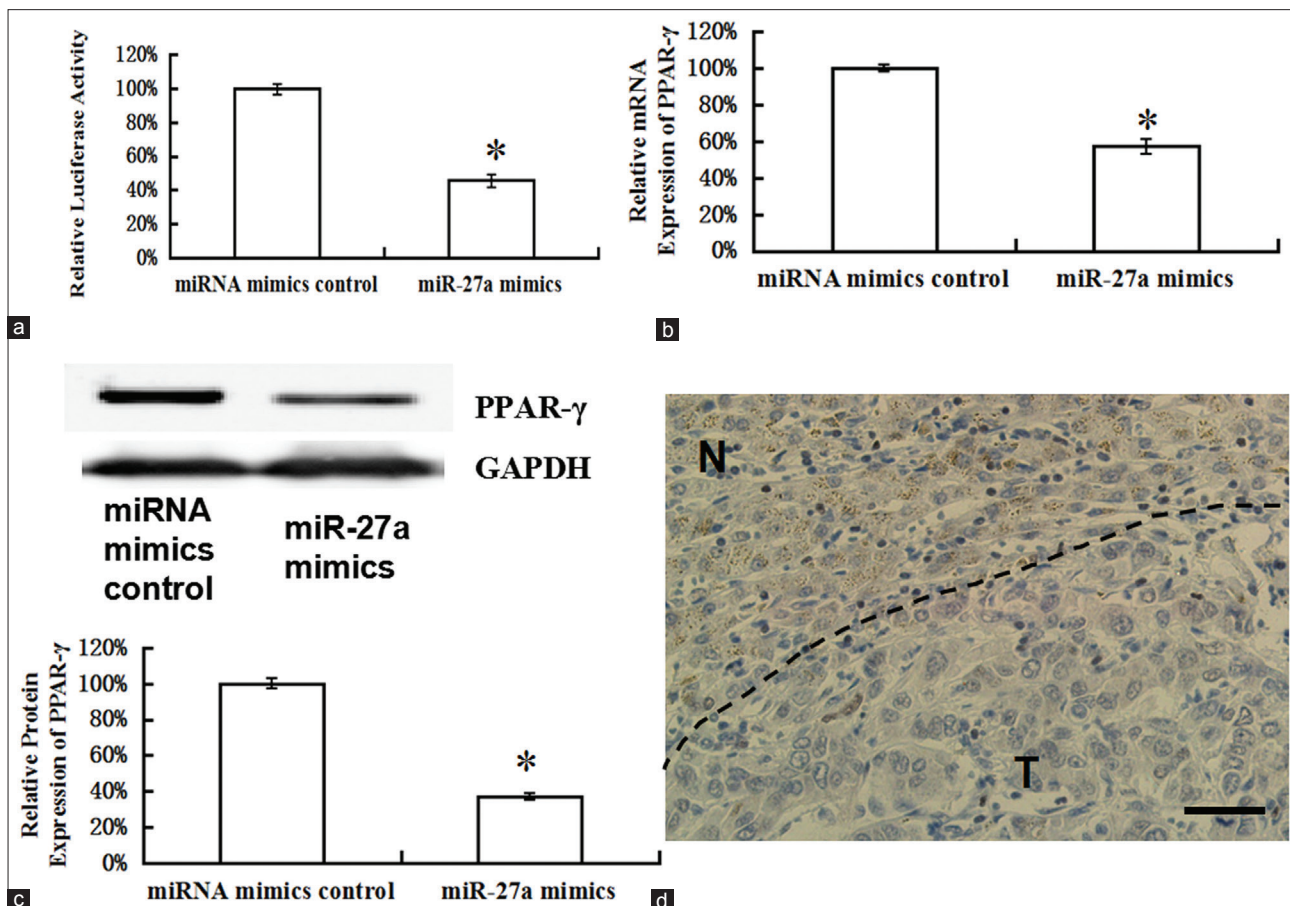
Further experiments were performed to investigate whether overexpression of PPAR- $\gamma$  could inhibit the effect of miR-27a on proliferation. MTT assays showed that complementation of PPAR- $\gamma$  by the PPAR- $\gamma$  agonist rosiglitazone could partially block the increased cell viability caused by miR-27a [Figure 4a]. In addition, the down-regulation of PPAR- $\gamma$  induced by miR-27a mimics can be partly counteracted by rosiglitazone-induced increased expression of PPAR- $\gamma$  [Figure 4b and 4c]. Taken together, these data suggested that miR-27a induces HCC cell proliferation, partially by targeting PPAR- $\gamma$ .

## DISCUSSION

The prevalence and severity of HCC are increasing



**Figure 2:** MiR-27a promoted hepatocellular carcinoma cell proliferation by activating the cell cycle and inhibiting apoptosis. (a) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of HepG2 cells transfected with miR-27a mimics or miR-27a mimics control for 12, 24 and 48 h *in vitro*; (b) MTT assay of HepG2 cells transfected with miR-27a inhibitor or miR-27a inhibitor control for 24 and 48 h *in vitro*; (c) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of miR-27a expression in HepG2 cells transfected with miR-27a mimics or miR-27a mimics control for 24 h *in vitro*; (d) qRT-PCR analysis of miR-27a expression in HepG2 cells transfected with miR-27a inhibitor or miR-27a inhibitor control for 24 h *in vitro*; (e) The flow cytometry analysis of the cell cycle distribution of HepG2 cells transfected with miR-27a inhibitor or miR-27a inhibitor control for 24 h; (f) Quantitative analysis of apoptotic cell death induced by miR-27a in HepG2 cells, as measured by an annexin V-fluorescein isothiocyanate/propidium iodide apoptosis detection kit. MiR-27a expression in each sample was normalized to that of U6, the internal control (\* $P < 0.05$  vs. the control).

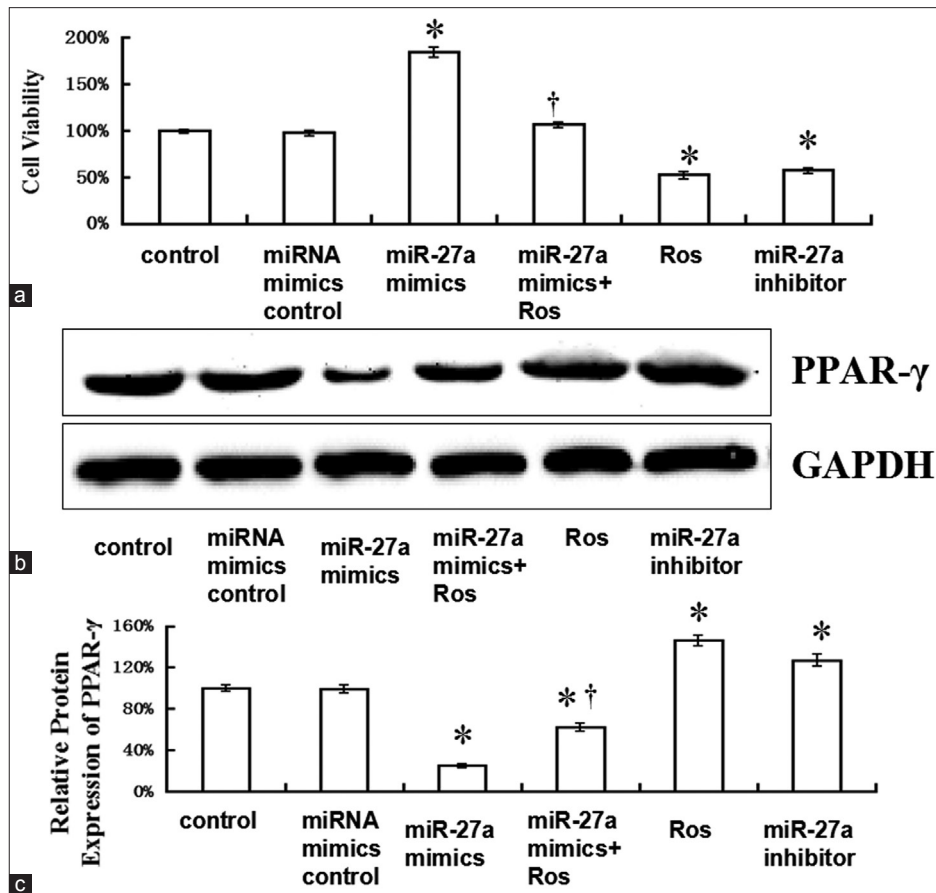


**Figure 3:** Peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ), a direct target of miR-27a in hepatocellular carcinoma (HCC) cells. (a) The luciferase reporter assay showed a significant decrease in the relative luciferase activity when the cells were cotransfected with pGL3-PPAR- $\gamma$ -3'-UTR and miR-27a mimics but not when co-transfected with microRNA mimics control; (b) Quantitative real-time polymerase chain reaction analysis of PPAR- $\gamma$  expression in HepG2 cells transfected with miR-27a mimics or miR-27a mimics control for 24 h; (c) Western blotting analysis of PPAR- $\gamma$  expression in HepG2 cells transfected with miR-27a mimics or miR-27a mimics control for 24 h. Glyceraldehyde-3-phosphate dehydrogenase served as the loading control; (d) Representative images of immunohistochemical staining for PPAR- $\gamma$  in HCC tissues. Scale bar = 20  $\mu$ m (\* $P$  < 0.05 vs. the control).

worldwide, and the prognosis of HCC patients remains unsatisfactory due to the high rate of recurrence and metastasis. Therefore, improved therapeutic strategies for HCC patients are critical for the management of HCC. MiRNAs have recently emerged as new anticancer drugs because they exert antitumor properties in a wide range of tumor cell types, including HCC cells. The emerging role of dysregulated miRNAs in HCC has been shown in many studies. Therefore, a better understanding of the roles of miRNAs in the pathogenesis of malignancy may help in the search for more effective HCC therapies. Abundant evidence has been produced in support of the oncogenic role of miR-27a in cancer progression. In gastric adenocarcinoma, miR-27a inhibited the cancer cell proliferation by targeting prohibitin.<sup>[13]</sup> In breast cancer, miR-27a inhibited the G2-M cell cycle transition by suppressing myelin transcription factor 1 (Myt-1) and zinc finger and BTB domain-containing protein 10 (ZBTB10) and induced cell apoptosis by down-regulating forkhead box O1 (FOXO1).<sup>[14]</sup> MiR-27a also helped to modulate the anti-tumorigenic potential of the anticancer agent methyl 2-cyano-3, 11-dioxo-18beta-olean-1,

12-dien-30-oate (CDODA-Me) in colon cancer.<sup>[15]</sup> In addition, miR-27a has been reported to target microcephalin 1 (MCPH1) and regulate its expression in human renal carcinoma.<sup>[16]</sup> Consistently, we showed that transfection of the miR-27a inhibitor suppressed proliferation of HepG2 cell lines by promoting apoptosis and inducing G1-phase cell cycle arrest, indicating the oncogenic role of miR-27a in liver cancer cells. All of these observations suggested that miR-27a activity may be closely related to human tumors.

To understand the functional mechanism of miRNAs, it is important to identify targets involved in their regulation. PPAR- $\gamma$  was further identified as a direct functional target of miR-27a in HCC cells. First, we identified that the 3'UTR of PPAR- $\gamma$  contains a binding site matching the miR-27a seed sequence. Second, overexpression of miR-27a decreased the luciferase activity upstream of the wild type 3'UTR of PPAR- $\gamma$ . Thirdly, overexpression of miR-27a led to decreased PPAR- $\gamma$  expression at the transcriptional and translational levels. Finally, PPAR- $\gamma$  expression tended to inversely correlate with miR-27a expression in HCC tissues.



**Figure 4:** Overexpression of peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ) attenuated the effect of miR-27a in hepatocellular carcinoma cells. (a) HepG2 cells were transfected with miR-27a mimics and treated with PPAR- $\gamma$  agonist rosiglitazone (ros); an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to examine HepG2 cell proliferation at 24 h; (b and c) Western blotting analysis of PPAR- $\gamma$  expression in HepG2 cells transfected with miR-27a mimics and treated with ros (\* $P < 0.05$  vs. control group, † $P < 0.05$  vs. miR-27a mimics group).

Therefore, we found that miR-27a regulated expression of PPAR- $\gamma$  by directly binding to its 3'UTR.

PPAR- $\gamma$  is a ligand-activated transcription factor that belongs to the nuclear hormone receptor superfamily; its roles include the control of several biological processes related to growth, differentiation, the cell cycle, and apoptosis.<sup>[17]</sup> PPAR- $\gamma$  activation has been shown to inhibit proliferation in several cancers *in vitro* and *in vivo*. As the organ that controls metabolism, the liver in particular shows strong involvement of PPAR- $\gamma$  in states of hypernutrition such as fatty liver. It has been reported that PPAR- $\gamma$  inhibited HCC in many *in vitro* studies.<sup>[18]</sup> PPAR- $\gamma$  controls the epithelial-mesenchymal transition and prevents the invasion and metastasis of carcinoma. The overexpression of PPAR- $\gamma$  inhibited carcinoma metastasis by increasing E-cadherin through tissue inhibitor of metalloproteinase 3.<sup>[19]</sup> PPAR- $\gamma$  has also been revealed to be involved in cell cycle arrest. These mechanisms have been reported to act through p21 and p53.<sup>[20]</sup> In addition, PPAR- $\gamma$  induces apoptosis directly through Fas, resulting in an inhibitory effect on carcinoma.<sup>[21]</sup> One group recently reported that PPAR- $\gamma$  activation by its agonist or ectopic PPAR- $\gamma$  expression by Ad-PPAR- $\gamma$  transfection

inhibited HCC growth and progression by suppressing cell proliferation, inducing cell apoptosis, and causing cell cycle arrest.<sup>[22]</sup> Based on these data, we showed that, similarly to the effect of miR-27a inhibitors, overexpression of PPAR- $\gamma$  can promote cell proliferation. The down-regulation of PPAR- $\gamma$  has been reported to correlate with differentiation and poor prognosis in patients with HCC. Further work is warranted to evaluate the roles of miR-27a and to develop therapeutic strategies for targeting miR-27a *in vivo*.

In summary, our study suggested that miR-27a was up-regulated in HCC. In addition, down-regulation of miR-27a inhibited cell proliferation and induced cell apoptosis and cell cycle arrest by regulating PPAR- $\gamma$  expression. MiR-27a may provide a potential therapeutic strategy for HCC treatment.

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