microRNA-34a overexpression inhibits cell migration and invasion via regulating SIRT1 in hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) remains one of the most common types of malignancy with high mortality and morbidity rates. Previous studies have suggested that microRNAs (miRs) serve pivotal functions in various types of tumor. The aim of the present study was to assess the association between miR-34a expression and HCC cell migration and invasion, and the potential underlying mechanisms. The miR-34a overexpression vector or scramble control was transfected into human Hep3B and Huh7 cell lines. Transwell assays, and Matrigel and wound healing assays were used to detect the effects of miR-34a expression on HCC cell invasion and migration, respectively. The expression of miR-34a and the mRNA expression of other associated proteins were detected using quantitative reverse transcription polymerase chain reaction, and protein levels were measured using western blot analysis. Compared with the control, miR-34a expression was significantly downregulated in Hep3B and Huh7 cells, but this was reversed by the transfection with exogenous miR-34a (P<0.01). The number of migrated or invaded cells was significantly reduced by the overexpression of miR-34a in Hep3B or Huh7 cells (P<0.01). The expression of sirtuin 1 was upregulated, while the level of acetylate-p53 was downregulated by overexpression of miR-34a. Taken together, the results of the present study suggested that the overexpression of miR-34a may have suppressed HCC metastasis via inhibited cell migration and invasion.

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Introduction

Hepatocellular carcinoma (HCC) remains one of the most common malignancies, and is a leading cause of cancer-associated mortality due to its high mortality rate (1). Although previous studies have demonstrated that surgery is the first choice for HCC treatment, the diagnostic methods for HCC remain restricted due to hidden lesions and an increased metastatic rate (2,3). The 5-year survival rate of patients with HCC was 5-9% in the United States in 2009 (4). Therefore, it is important to evaluate biomarkers for HCC treatment.

microRNAs (miRNAs/miRs) are endogenous, highly conserved non-coding RNAs, 20-22 nucleotides in length, that function in a variety of biological processes at the transcriptional or post-transcriptional level by targeting the 3'-untranslated regions of genes (5). Previous evidence has suggested that various miRNAs are involved in the progression and underlying biology of HCC (6,7). For example, Tsai *et al* (8) indicated that the tumor suppressor miR-122 regulates HCC metastasis, and Meng *et al* (9) suggested that miR-21 expression is abnormal in HCC tissue and is correlated with HCC growth via regulation of cytochrome b6-f complex subunit 8, chloroplastic expression. Additionally, miR-34a is considered to be a tumor suppressor in various types of cancer, including colon and breast cancer, and to be involved in a variety of biological processes (9,10). Previous studies have revealed a significant correlation between miR-34a expression and cancer metastasis, including in HCC (11,12). However, few studies refer to the involvement of miR-34a in HCC metastasis. Li *et al* (13) indicated that miR-34a functions as an inhibitor for HCC cell migration and invasion via the downregulation of c-Met expression. Although several studies have suggested the involvement of miR-34a in HCC cell migration or invasion, the basic mechanism remains unknown.

In the present study, the potential effects of miR-34a expression on HCC cell migration and invasion were investigated using Hep3B and Huh7 cells. Comprehensive experimental methods were used to analyze the potential underlying mechanism.

Materials and methods

Cell lines and cell culture. The human hepatocellular carcinoma cell lines Hep3B and Huh7 and the normal hepatocyte

cell line THLE-2 were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO_2.

Cell transfection. Cells were plated onto a 60-mm dish, and following incubation for 24 h, the overexpression vector for miR-34a (sense, 5'-UGGCAGUGUCUUAGCUGG UUGU-3' and antisense, 5'-AACCAGCUAAGACACUGC CAUU-3') (Sangon Biotech Co., Ltd., Shanghai, China) or the scramble control miRNA (sense, 5'-UGUCAGCUU UGGAGCUGGUUGU-3' and antisense, 5'-AACCUA AGAUGCCACCAGCAUU-3') was transfected into the Hep3B and Huh7 cells using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Inc.). Following transfection for 48 h at 37°C, cells were prepared for additional analysis.

Cell invasion assay. For cell invasion assay, the Transwell with Matrigel method was performed as previously described (14). Briefly, following transfection for 48 h, hepatocellular cells (5x10^5/ml) were re-suspended in serum-free DMEM containing 0.01% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for an additional 24 h. The upper chamber of the Transwell plate was covered with serum-free DMEM supplemented with 50 mg/l Matrigel, and then air-dried at 4°C for 15 min. Then, 50 μl fresh serum-free DMEM medium containing 10 g/l BSA was added, and cultured for 30 min at 37°C. Following removal of this medium, 200 μl cell suspension was added into the upper chamber of the Transwell plate, and 600 μl complete DMEM culture supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA). After 48 h of incubation at 37°C in 5% CO_2, the Transwell plate was washed with PBS buffer to remove the cells on the upper side of the microporous membrane, followed with fixation in ice-cold alcohol for 30 min. Non-migratory cells were removed from the upper surface of the filter with a cotton swab. Finally, the cells were stained with 0.1% crystal violet for 30 min at room temperature, and then decolorized with 33% acetic acid. The absorbance of eluents was observed at an optical density of 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cells transfected with the control vector served as the negative control.

Cell migration assay. For the cell migration assay, the wound healing asssay was performed as previously described (15). Hep3B and Huh7 cells (1x10^5) transfected with miR-34a overexpression or scramble controls were seeded in 24-well plates and grown overnight to achieve confluence. The monolayer cells were scratched using a 20 μl pipette tip to create the wound. The floating cells were removed by washing twice with PBS (Sigma-Aldrich; Merck KGaA). Subsequently, serum-free DMEM was added to permit wound healing. The rate of wound closure was assessed using images captured after 24 h. Image analysis was performed using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Table I. Primers used for target amplification in the present study.

Name	Primer	Sequence (5'-3')
GAPDH	Sense	GGGTGGAGCCAAACGGGTC
	Antisense	GGAGTTGCTGTTGAAGTCGCA
SIRT1	Sense	CAGAGCAT CACACGCAAGC
	Antisense	CAGGAAACAG AAACCCCAGC
p53	Sense	TTCCTCTTCCTGCAGTACTC
	Antisense	ACCCTGGGCAACCAGCCCTGT

SIRT, sirtuin; p53, tumor protein 53.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR). Total RNA from the Hep3B or Huh7 cells was collected 48 h after transfection and isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., USA) as previously described (16). The samples were treated with RNase-free DNase I (Promega Corporation, Madison, WI, USA). Consequently, the concentration and purity for the isolated RNA were measured with SMA 400 UV-VIS (Merinton Instrument, Ltd., Beijing, China). Purified RNA at a density of 0.5 μg/μl with nuclease-free water was used for cDNA synthesis using the PrimerScript 1st Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.), with the following temperature protocol: 30°C for 10 min, 42°C for 50 min, and 95°C for 5 min. The expressions of targets in the cells were detected in an Eppendorf Mastercycler (Brinkmann Instruments, Westbury, NY, USA) using the SYBR ExScript RT-qPCR kit (Takara Bio, Inc., Otsu, Japan). The total reaction system of 20 μl volume was as follows: 1 μl cDNA from the above PCR, 10 μl SYBR Premix EX Taq, 1 μl each of the primers (10 μM), and 7 μl ddH$_2$O. The PCR thermocycler conditions were as follows: Denaturation at 50°C for 2 min; 95°C for 10 min; followed by 45 cycles of 95°C for 10 sec and 60°C for 1 min. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. These data were quantified using the $2^{-\Delta\Delta Cq}$ method (17). GAPDH was used as the internal control. Primers used for the amplification of the targets are listed in Table I.

Western blot analysis. Cells cultured for 48 h were lysed with radioimmunoprecipitation buffer (Sangon Biotech Co., Ltd.) containing phenylmethanesulfonyl fluoride (Sigma-Aldrich; Merck KGaA), and the lysates were then centrifuged at 12,000 x g for 10 min at 4°C. The supernatants were collected, and protein concentrations were determined using a bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). The proteins (30 μg/lane) were separated using SDS-PAGE on a 10% gel, as previously described (18) followed by transfer onto a polyvinylidene fluoride membrane (Merck KGaA). The membranes were blocked using Tris-buffered saline with 0.05% Tween-20 (TBST) containing 5% non-fat milk for 1 h at room temperature, and then incubated with rabbit anti-human antibodies [(purchased from Abcam (Cambridge, MA, USA)] against sirtuin 1 (SIRT1; cat. no. ab12193), tumor
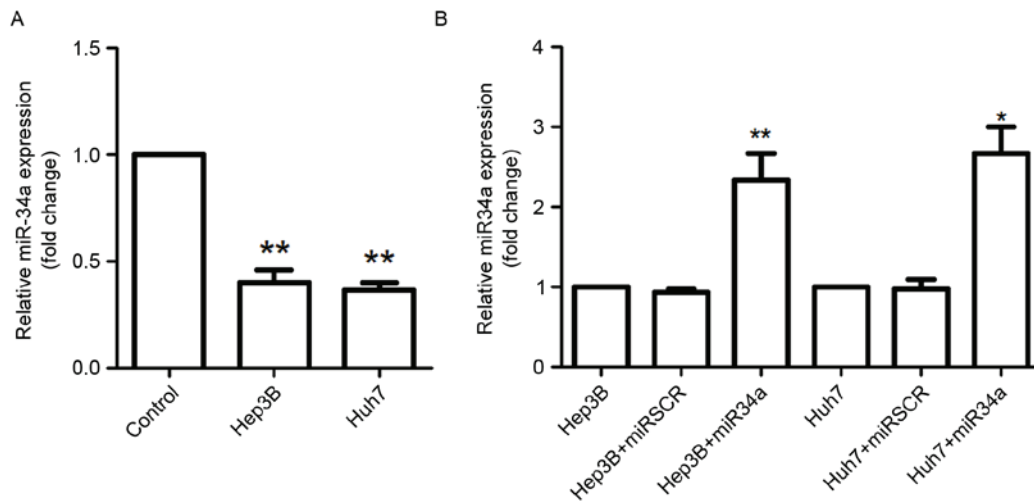
Figure 1. Expression of miR-34a in the two types of hepatocellular cells. (A) miR-34a expression was downregulated in Hep3B and Huh7 cells. (B) Relative expression of miR-34a was significantly upregulated in Hep3B and Huh7 cells following the transfection of miR-34a overexpression vectors. *P<0.05, **P<0.01 vs. control group. miR, microRNA.

protein 53 (p53; cat. no. ab131442), acetylate-p53 (Ac-p53; cat. no. ab75754) and GAPDH (cat. no. ab37168; all 1:100) overnight at 4˚C. Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (cat. no. ab205718; 1:5,000; Abcam) for 1 h at room temperature. Finally, the polyvinylidene fluoride membranes were washed 3 times with 1X TBST buffer for 10 min each time. The signals were detected following incubation of the membranes with a chromogenic substrate using the SuperSignal® West Pico chemiluminescent western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.). Analysis was performed using the Image Guage 4.0 program (Fuji, Tokyo, Japan). GAPDH served as the internal control.

Statistical analysis. All experiments were conducted independently 3 times. All data are presented as the mean ± standard deviation. The significant difference for the data was calculated using SPSS v19.0 statistical software. P-values were calculated using one-way analysis of variance followed by Duncan's multiple-range test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-34a in Hep3B and Huh7 cells. The relative expression levels of miR-34a in THLE-2, Hep3B and Huh7 cells were assessed using RT-qPCR analysis (Fig. 1). The results demonstrated that miR-34a expression was significantly decreased in Hep3B and Huh7 cells compared with that in the THLE-2 cells (P<0.01; Fig. 1A). The present study further analyzed the expression of miR-34a in Hep3B and Huh7 cells following transfection with miR-34a overexpression vectors, and the results suggested that miR-34a was highly expressed in these cells by miR-34a overexpression transfection compared with miR-34a expression in scramble control cells (Huh7 cells, P<0.05; Hep3B cells, P<0.01; Fig. 1B).

miR-34a overexpression suppresses hepatocellular cell migration. When cells were transfected with miR-34a
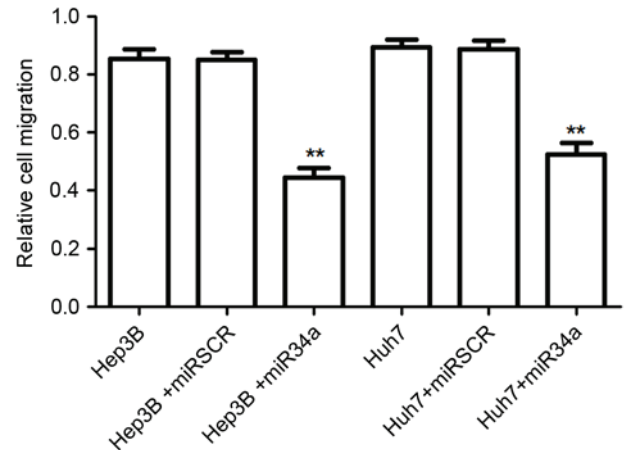

Figure 2. Effects of miR-34a on hepatocellular cell migration. Compared with the control cells, overexpression of miR-34a significantly decreased the number of migrated Hep3B and Huh7 cells. **P<0.01 vs. control cells. miR, microRNA; miRSCR, scramble miR-34a.

overexpression vectors, the number of migrated cells was significantly decreased compared with that for the scramble control group (P<0.01; Fig. 2). There was no significant difference between the number of migrated cells for the negative control group and that for the scramble treated cells.

miR-34a overexpression suppresses hepatocellular cell invasion. The effect of miR-34a expression on hepatocellular cell invasion was also detected. The results suggested that the number of invasive cells was significantly decreased by the overexpression of miR-34a in Hep3B and Huh7 cells compared with that in the control group (P<0.01; Fig. 3). However, there was no significant difference in the number of invasive cells between control cells and the scramble treated cells.

Effects of miR-34a expression on cell metastasis-associated protein expressions. To further investigate the potential
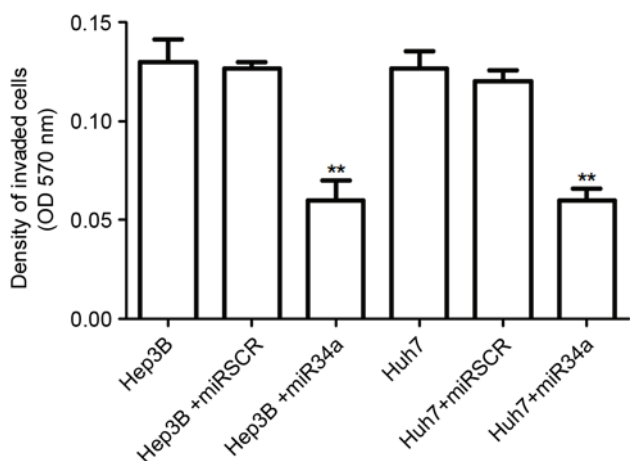
Figure 3. Effects of miR-34a on hepatocellular cell invasion. Compared with the control cells, the overexpression of miR-34a significantly decreased the number of invaded Hep3B and Huh7 cells. **P<0.01 vs. control cells. miR, microRNA; miRSCR, scramble miR-34a; OD, optical density.
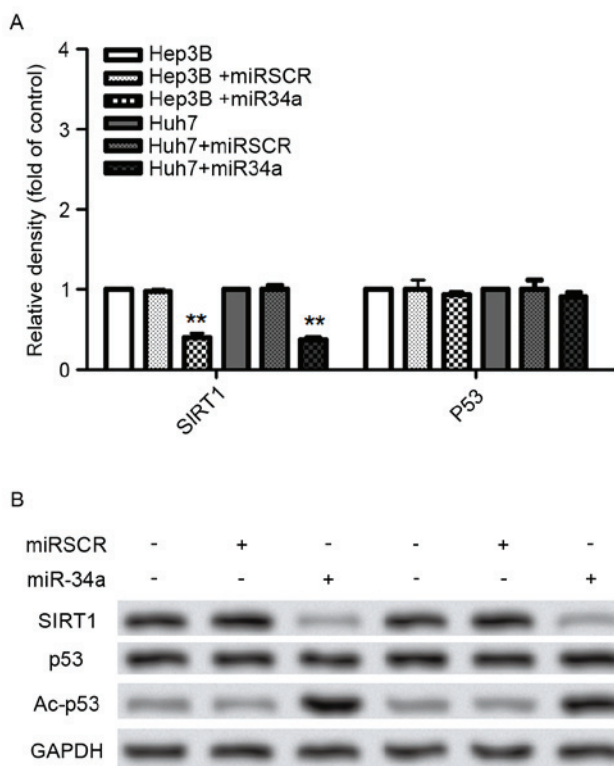

Figure 4. Effects of miR-34a expression on cell metastasis-associated protein expressions in hepatocellular cells. (A) Overexpression of miR-34a significantly decreased the mRNA levels of SIRT1. (B) Overexpression of miR-34a decreased the protein expression of SIRT1, but increased the expression of Ac-p53. **P<0.01 vs. control cells. miR, microRNA; miRSCR, scramble miR-34a; SIRT, sirtuin; p53, tumor protein 53, Ac-p53, acetylated p53.

mechanism underlying the effect of miR-34a on hepatocellular cell migration and invasion, the expression of proteins including SIRT1, p53 and Ac-p53 were analyzed (Fig. 4). The results demonstrated that the SIRT1 mRNA and protein levels were significantly decreased by the overexpression of miR-34a (P<0.01; Fig. 4A and B). The Ac-p53 protein level was significantly increased by the overexpression of miR-34a in Hep3B

and Huh7 cells, compared with that in control cells (P<0.05; Fig. 4A and B).

Discussion

Previous studies have demonstrated that miRNAs serve crucial functions in tumor biology, and miR-34a has been suggested to be associated with HCC cell migration and invasion (6,19). The present study analysed the function of miR-34a expression in HCC cell migration and invasion, and its potential underlying mechanism. In agreement with previous data (20), the results indicated that miR-34a was downregulated in the two types of HCC cells. Following transfection with the miR-34a overexpression vector, the numbers of migrated and invaded cells were significantly decreased by the overexpression of miR-34a in Hep3B and Huh7 cells. Additionally, the protein and mRNA levels of SIRT1 were suppressed, while the protein levels for Ac-p53 were increased by overexpression of miR-34a.

These results demonstrated that the number of the migrated and invaded Hep3B or Huh7 cells was significantly reduced by the overexpression of miR-34a, suggesting that miR-34a expression was associated with HCC cell migration and invasion. Cell migration and invasion are important biological processes associated with tumor metastasis (21). Previous studies have demonstrated that miR-34a functions as a tumor suppressor in various tumors, including colon cancer and neuroblastoma (9,22). miR-34a has been suggested to be involved in the venous metastasis of Hepatitis B virus-positive HCC (23). Except for the study conducted by Li *et al* (13), the involvement of miR-34a expression in HCC cell migration and invasion has not been fully discussed. Based on the results of the present study, it was hypothesized that the overexpression of miR-34a may inhibit HCC metastasis through suppressing cell migration and invasion.

Concurrently, SIRT1 is an nicotine adenine dinucleotide-dependent deacetylase that is involved in multiple biological processes, including DNA damage, apoptosis and proliferation (24). A previous study demonstrated that SIRT1 expression is correlated with breast cancer invasion and metastasis (25), and Hao *et al* (26) suggested that the overexpression of SIRT1 promotes HCC metastasis through the epithelial mesenchymal transition. In the present study, SIRT1 expression was downregulated by the overexpression of miR-34a, indicating that miR-34a may suppress HCC metastasis via the downregulation of SIRT1. Conversely, Lewis *et al* (27) demonstrated that the absence of p53 promotes HCC metastasis in a mouse model. It has been revealed that deacetylated p53 is associated with cell growth (28). Ac-p53 is associated with breast cancer cell migration and invasion through the targeting of the *SMAR1* gene (29). The results of the present study demonstrated that the overexpression of miR-34a increased Ac-p53 expression, suggesting that miR-34a may suppress HCC cell migration and invasion by increasing Ac-p53 expression.

Taken together, the results of the present study revealed that the overexpression of miR-34a may be a suppressor of HCC metastasis. The overexpression of miR-34a inhibits Hep3B and Huh7 cell migration and invasion, and downregulates SIRT1 expression while increasing Ac-p53 expression. The present study may provide a theoretical basis for studies investigating

the mechanisms of HCC pathogenesis and metastasis, and for the potential application of miR-34a in HCC metastasis diagnosis. However, additional studies are needed to explore the basic underlying mechanisms.

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