MicroRNA-34a inhibits metastasis in liver cancer cells

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Abstract. MicroRNAs (miRNA/miRs) have the ability to target specific mRNAs, resulting in degradation of mRNA or inhibition of translation. Notably, miR-34a is able to regulate cell cycle and tumorigenicity. The level of miR-34a expression is usually low in tumors, and previous studies have indicated miR-34a to be an important tumor suppressor. In order to elucidate the association between miR-34a and metastasis, stable cell lines were established and transfected with miR-34a. Cell invasion assay was subsequently performed. The present study demonstrated that cell invasion was inhibited in cells that were transfected with miR-34a compared with the control group ($P<0.05$). Therefore, miR-34a was able to inhibit metastasis in liver cancer cells.

Introduction

MicroRNAs (miRNAs/miRs) exist in the majority of eukaryotes and consist of 21-25 nucleotides. miRNAs regulate gene expression via binding to target mRNAs, resulting in mRNA degradation or inhibition of translation (1-4). mRNAs acts as oncogenes or suppressor genes to affect tumor development (5-8). In 2013, Takahashi *et al* reported that hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver globally (9), and numerous miRNAs are abnormally expressed in HCC (9). miR-21 is one of the most prominently expressed miRNAs in a number of human cancer types, including pancreas, breast, prostate, colon, lung and stomach (10). The expression of miR-21 is increased in HCC tissues, compared with normal tissues (11). However, miR-146a has decreased expression in HCC tissues, compared with normal liver tissues (12). miR-34a is a member of the miR-34 family and has been demonstrated to modulate critical gene transcripts involved in tumorigenesis, but its role in tumorigenesis remains unknown. miR-34a may be activated by p53 to induce apoptosis and inhibit tumor growth (13-16). However, it often dysfunctions or mutates in tumors (17-21). Luo *et al* (22) has demonstrated that miR-34a was able to suppress the migration of breast cancer cells via targeting Fra-1. Wang *et al* (23) observed an inverse association between programmed death-ligand 1 (PD-L1) and miR-34a expression in a number of acute myeloid leukemia (AML) samples. miR-34a is a putative binder of the PD-L1-3' untranslated region (UTR), and overexpression of miR-34a in HL-60 and Kasumi-1 cells could block PD-L1 expression (23). The aim of the present study was to verify the effect of miR-34a on metastasis of liver cancer cells.

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Materials and methods

Materials. The miRNeasy Mini kit was purchased from Qiagen GmbH (Hilden, Germany). Real-time PCR detection kit was from GeneCopoeia, Inc., (Rockville, MD, USA). 5-fluorouracil (5-FU) and MTT were from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The concentration of 5-FU in the present study was 5 μg/ml, and cells were incubated at 37˚C for 48 h. Lipofectamine® 2000 was purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA). Minimum Essential Medium (MEM) was from Hyclone (GE Healthcare, Chicago, IL, USA), and fetal bovine serum was from Gibco (Thermo Fisher Scientific, Inc.).

Cell lines and cell culture. MHCC97H liver cancer cells were purchased from the Resource Center of Shanghai Institutes of Biological Sciences (Shanghai, China) were maintained in a monolayer culture at 37˚C and 5% CO_2 in MEM that was supplemented with 10% fetal bovine serum.

Cell transfection. MHCC97H cells were transfected with 5 mg/ml miR-NC and 5 mg/ml miR-34a mimic (Shanghai GenePharma Co., Ltd., Shanghai, China) using Lipofectamine® 2000 as follows. The cells ($4\text{-}5\times10^4$ cells/ml) were plated in a 6-well plate at 37˚C for 24 h. Prior to transfection, the miRNA-Lipofectamine solution was prepared by mixing MEM separately with Lipofectamine or miRNA and then mixing the solutions together. Finally, the miRNA-Lipofectamine solution was added to each well, and the cells were incubated at 37˚C for 24-72 h for subsequent analyses.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). All independent experiments were conducted in triplicate. Negative control [no complementary DNA (cDNA)] and RT control (no reverse transcriptase). Total RNA (including miRNA) was extracted using the miRNeasy Mini

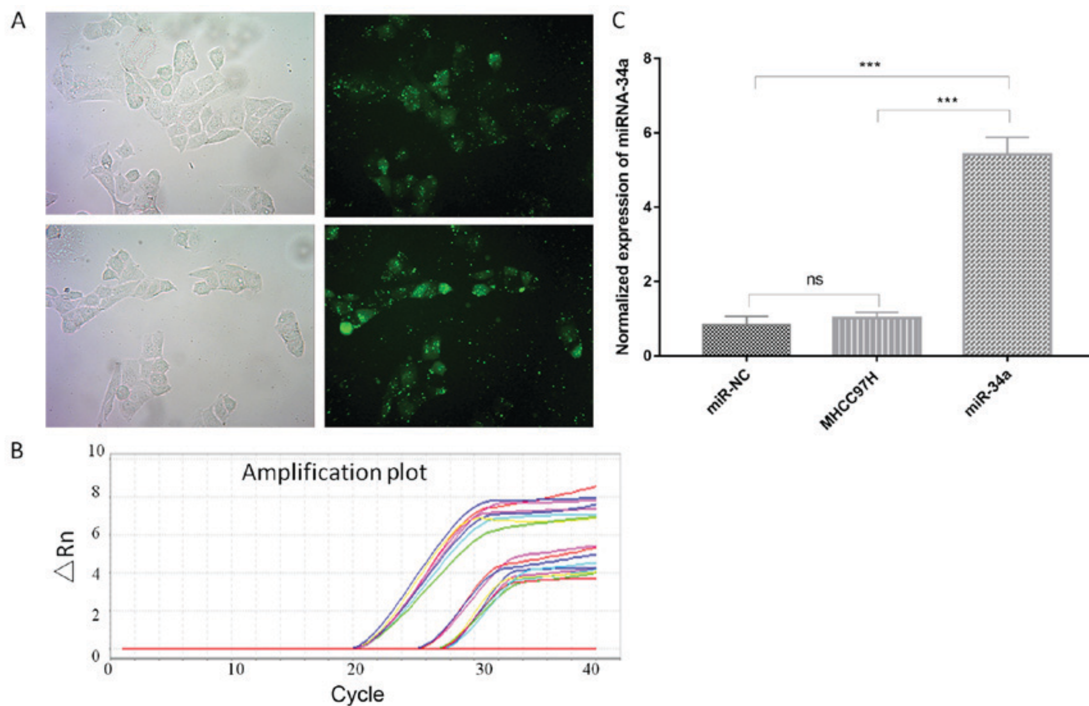

Figure 1. Cell transfection with miR-34a mimic. (A) The cells that were transfected with miR-NC (upper panel) and miR-34a mimic (lower panel) appeared green. The magnification is x20. (B) U6 and miR-34a were detected via quantitative polymerase chain reaction. Relative miR-34a expression was detected in transfected and non-transfected cells. (C) A graph of the results for quantitative polymerase chain reaction. ***P<0.05; miR, microRNA; NC, negative control; RQ, relative quantification.

kit (Qiagen GmbH), and DNA was removed using DNase I. Reverse transcription was conducted with cDNA Synthesis kit (cat. no. 6130; Takara Bio. Inc., Otsu, Japan). Reverse transcription was conducted with 2 μg total RNA, according to the manufacturer's instructions, and the negative control used diethyl pyrocarbonate (DEPC)-treated water instead of the total RNA, the positive control used the control RNA (contained within the cDNA Synthesis kit) instead of the total RNA. In brief, the following reagents were added in turn, 2 μl 10X reaction buffer, 1 μl RiboLock™ Ribonuclease Inhibitor (40 U/μl), DEPC-treated water up to 10 μl and incubated at 37˚C for 30 min, and then 1 μl reverse transcriptase (1 U/μl) was added and incubated at 72˚C for 5 min. All reagents were contained within the cDNA synthesis kit. qPCR was performed using a SYBR® Premix Ex Taq™ kit (Takara Bio, Inc., Otsu, Japan) on the QuantStudio™ 5 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), and with the following primers: miR-34a forward, 5'-TGGCAG TGTCTTAGCT-3' (10 μM) and reverse, 5'-TGGTGTCGT GGAGTCG-3' (10 μM); and U6 primer was used as the positive control, U6 forward, 5'-CTCGCTTCGGCAGCACA-3' (10 μM) and reverse, 5'-AACGCTTCACGAATTTGCGT-3' (10 μM; all BioSune, Shanghai, China). The final concentration of the primers was 0.4 μM. A 3-step PCR was performed using the SYBR Select Master Mix kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols, and conducted under the following conditions: Initial denaturation at 95˚C for 30 sec, followed by a total of 40 cycles were run with a initial denaturation at 95˚C for 5 sec, and an annealing temperature at 60˚C for 30 sec. Finally, 95˚C for 5 sec, and 60˚C for 1 min, and 95˚C for 5 sec. Relative quantification was analyzed using the $2^{-\Delta\Delta Cq}$ method (24).

Invasion assay. Each Transwell insert was coated with diluted BD Matrigel matrix coating solution (BD Biosciences, Franklin Lakes, NJ, USA) and then incubated at 37˚C for 30 min. Cell suspension ($5x10^4$ cells/ml) in Opti-MEM® I culture media (Thermo Fisher Scientific, Inc.) with no FBS was prepared. The negative control was cells transfected with miR-NC. Cell suspension (1 ml) was added to each 6-well invasion chamber. Culture media with 20% FBS was placed in each Transwell chamber and incubated in a humidified incubator (37˚C, 5% CO_2). Matrix and non-invading cells were gently scraped off following 24 h. The cells were fixed with 95% ethanol for 20 min and subsequently stained with hematoxylin at room temperature for 10 min. The membrane was washed with PBS and observed under a fluorescent microscope (Nikon Corporation, Tokyo, Japan). A total of 5 fields (x20) were selected randomly and the number of invaded cells were counted.

Statistical analyses. Experimental data are presented as the mean ± standard deviation. Multiple groups were compared using one-way analysis of variance with SPSS (version 17.0, SPSS, Inc., Chicago, IL, USA) statistical software. P<0.05 was used to indicate significant difference. All independent experiments were conducted in triplicate.

Results

miR-34a expression is promoted by 5-FU. To study the effect of miR-34a on cancer cells, cell lines were transfected with negative miR control (NC) and miR-34a vector. The transfected cells were green (Fig. 1A). The cells that were transfected with miR-34a exhibited a markedly increased miR-34a expression (5.35-fold) compared with non-transfected cells according to

Table I. Relative quantification of miR-34a expression in transfected and 5-FU-treated cells.

	U6		miR-34a				
Groups	Cq	Mean Cq	Cq	Mean Cq	ΔCq	ΔΔCq	$2^{-\Delta\Delta Cq}$
L-02	20.13	20.02	25.01	24.93	4.91	-2.97	7.84
	20.12		25.02				
	20.14		25.01				
	20.21		24.71				
	19.63		24.82				
	19.58		24.70				
	20.03		25.01				
	20.00		25.03				
	20.31		25.06				
MHCC97H	21.01	20.88	28.76	28.76	7.88	0.00	1.00
	21.03		28.82				
	21.00		28.79				
	20.53		28.69				
	20.47		28.80				
	20.70		28.77				
	21.13		28.70				
	21.02		28.77				
	21.00		28.71				
MHCC97H-miR-NC	20.14	20.49	28.67	28.67	8.18	0.30	0.81
	20.31		28.69				
	20.22		28.65				
	21.00		28.71				
	21.03		28.77				
	21.01		28.72				
	20.14		28.89				
	20.01		28.36				
	20.52		28.55				
MHCC97H-miR-34a	21.03	20.43	26.49	25.89	5.46	-2.42	5.35
	21.00		26.45				
	21.01		26.42				
	20.56		26.00				
	20.79		26.09				
	20.33		26.10				
	19.85		25.18				
	19.68		25.12				
	19.66		25.10				
MHCC97H+5-FU	18.76	19.30	25.03	25.54	6.24	-1.64	3.12
	18.42		25.02				
	18.62		25.05				
	19.01		25.42				
	19.10		25.32				
	19.24		25.40				
	20.20		26.33				
	20.01		26.15				
	20.32		26.11				

Table I. Continued.

Groups	U6		miR-34a				
	Cq	Mean Cq	Cq	Mean Cq	ΔCq	ΔΔCq	$2^{-\Delta\Delta Cq}$
MHCC97H-miR-34a+5-FU	19.97	19.78	25.00	24.92	5.14	-2.74	6.68
	19.83		25.02				
	19.70		25.03				
	21.02		26.15				
	21.01		26.09				
	21.11		26.10				
	18.15		23.62				
	18.57		23.63				
	18.63		23.61				

MHCC97H was used as the calibrator. miR, microRNA; 5-FU, 5-fluorouracil.

Table II. Effect of miR-34a or 5-FU on MHCC97H cells invasion.

Group	Mean number of invaded cells (mean ± SD)	P-value (compared with MHCC97H)
L-02	0	-
MHCC97H	105.14+15.87	-
miR-NC	103.26±20.59	-
miR-34a	51.36±8.43	<0.05
miR-NC+5-FU	52.11+9.42	<0.05
miR-34a+5-FU	30.24±7.85	<0.05

A total of 5 fields (x20) were selected randomly and the number of invaded cells were counted, and calculated the mean number of invaded cell. Group MHCC97H was the control group. miR, microRNA; NC, negative control; SD, standard deviation; 5-FU, fluorouracil.

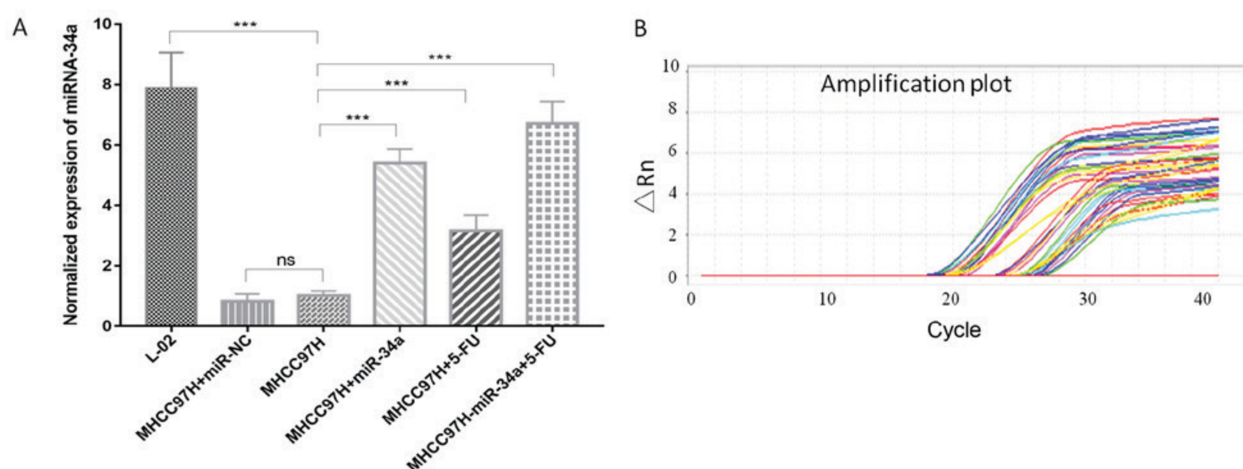

Figure 2. Relative quantification of miR-34a expression in transfected or 5-fluorouracil-treated cell lines as detected by quantitative polymerase chain reaction. (A) The mRNA expression of U6 and miR-34a was detected via quantitative polymerase chain reaction. Relative quantification of miR-34a was detected in transfected and 5-fluorouracil-treated cells. (B) The amplification plot of the mRNA expression of U6 and miR-34a. ***P<0.05; miR, microRNA; NC, negative control; 5-FU, 5-fluorouracil.

RT-qPCR analysis (Fig. 1B and C; Table I). The transfected cells were treated with 5 μg/ml 5-FU to observe miR-34a expression. 5-FU was able to promote miR-34a expression. The cells that were transfected with miR-34a and then treated

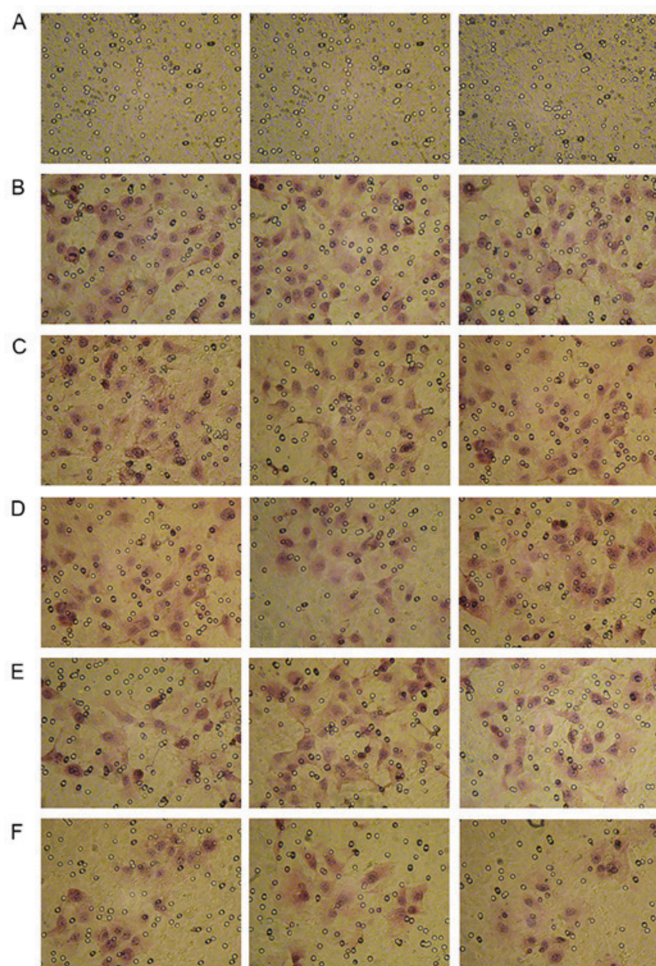

Figure 3. Cell invasion analysis of transfected and drug-treated cell lines: (A) Normal liver cell line L-02; (B) MHCC97H liver cancer cell line; (C) MHCC97H cell line that was transfected with miR-NC; (D) MHCC97H cell line that was transfected with miR-34a; (E) miR-NC-transfected MHCC97H cell line that was treated with 5-FU and (F) miR-34a-transfected MHCC97H cell line that was treated with 5-FU. A total of 5 fields (x20) were selected randomly and the number of invaded cells were counted. miR, microRNA; NC, negative control; 5-FU, 5-fluorouracil.

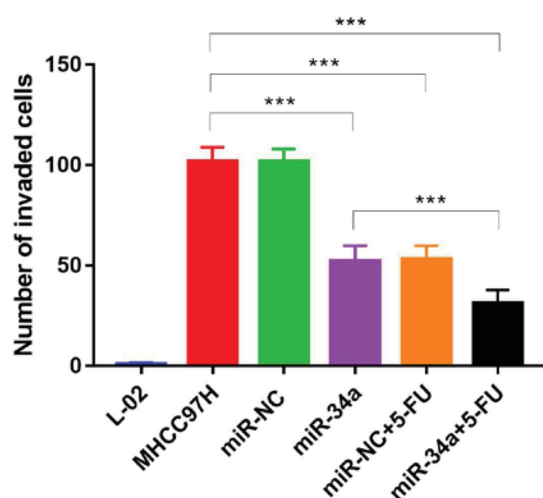

Figure 4. The statistical results of the number of invaded cells in each group. The MHCC97H group was the control. The number of invaded cells was significantly decreased when transfected with miR-34a, and further decreased following transfection with miR-34a and 5-FU. ***P<0.05. miR, microRNA; NC, negative control; 5-FU, 5-fluorouracil.

with 5-FU (MHCC97H-miR-34a+5-FU) were able to express a relatively increased level of miR-34a, compared with the control group (MHCC97H-miR-34a) (Fig. 2 and Table I).

miR-34a inhibits cell invasion. To study the effect of miR-34a on metastasis, invasion analysis of transfected and drug-treated cells was carried out using Transwell assay. The overexpression of miR-34a had a similar effect as 5-FU treatment as both treatments were able to inhibit cell invasion. The mean number of invaded cells in five random fields were 51.36±8.43 in the overexpressed miR-34a group. The mean number of invaded cells in five random fields were 52.11±9.42 in the drug-treated MHCC97H-miR-NC group. While the mean number of invaded cells in five random fields were 30.24±7.85 in the drug-treated MHCC97H-miR-34a group. Furthermore, overexpressing miR-34a was able to increase the effect of 5-FU, leading to reduced cell invasion (P<0.05) (Figs. 3 and 4; Table II).

Discussion

In recent years, miR-34a has been increasingly studied. Previous studies have revealed miR-34a to be decreased in tumors, compared with normal tissue (25). miR-34a is a member of the miR-34 family, and has been demonstrated to modulate critical gene transcripts involved in tumorigenesis, but its role in tumorigenesis remains unknown. miR-34a may be activated by p53 to induce apoptosis and inhibit tumor growth (13-16); however, it frequently causes dysfunctions or mutations in tumors (17-21). Luo *et al* (22) demonstrated that miR-34a was able to suppress the migration of breast cancer cells via targeting Fra-1. Wang *et al* (23) observed an inverse association between PD-L1 and miR-34a expression in a number of AML samples. miR-34a, as a putative binder of the PD-L1-3'UTR, overexpression in HL-60 and Kasumi-1 cells could block PD-L1 expression (23). There is hope to utilize miR-34a for diagnosis and therapy. Gallardo *et al* (26) reported that miR-34 was able to act as a prognostic marker of non-small-cell lung cancer, and Fang *et al* (27) indicated that miR-34a was able to be used to detect diffuse large B-cell lymphoma. Furthermore, Wiggins *et al* (28) reintroduced chemically synthesized miR-34a to cancer cells to drive a therapeutic response. The results of the present study also suggested that overexpressing miR-34a, which was depleted in cancer cells, was able to inhibit cell invasion. According to its importance in tumorigenesis, chemically synthesized miRNA mimic may be used to simulate endogenous miRNA to target genes and inhibit tumor development.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WW and HT contributed in the design of the study, developed the methodology, collected the data, performed the experiments, analysis and wrote the manuscript; LT contributed to the design of the study, critically revised the manuscript and approved the final version to be published. All authors agreed to be accountable for all aspects of the study.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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