CHINESE
MEDICAL SCIENCES
JOURNAL

ORIGINAL ARTICLE

microRNA-18a Promotes Cell Migration and Invasion Through
Inhibiting Dicer I Expression in Hepatocellular
Carcinoma *In Vitro*[△]

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Key words: hepatocellular carcinoma; microRNA-18a, Dicer I; migration; invasion

Objective To investigate the effects of microRNA-18a (miR-18a) on migration and invasion of hepatocellular carcinoma (HCC) cells, and its possible mechanism associated with Dicer I.

Methods HepG2 and HepG2.2.15 cells were transfected with miR-18a inhibitor using Lipofectamine. Cell invasion was evaluated by transwell invasion assay, and cell migration was detected by transwell migration and wound-healing assays. Moreover, luciferase reporter assay was used to identify whether Dicer expression was regulated by miR-18a. Real-time RT-PCR and western blot were performed to analyze Dicer 1 expression. In addition, a functional restoration assay was performed to investigate whether miR-18a promotes HCC cell migration and invasion by directly targeting Dicer 1.

Results miR-18a inhibitor can suppress the migration and invasion of HCC cells. Furthermore, suppression of Dicer I expression by small interfering RNA essentially abolished the inhibition of cell migration and invasion induced by miR-18a inhibitor, restoring these activities to levels similar to the parental HCC cells. Interestingly, suppression of miR-18a in HCC cells resulted in enhanced expression of Dicer I. In addition, the results of a luciferase assay demonstrated targeted regulation of Dicer I by miR-18a.

Conclusion Our findings suggest that miR-18a promotes migration and invasion of HCC cells by inhibiting Dicer I expression.

Chin Med Sci J 2017; 32(1):34-43

Received for publication May 15, 2016.

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△Supported by the National Natural Science Foundation of China (81472485), the Project of Six Talent Peaks of Jiangsu Province (WSW-014), and Project of Hospital Management Center of Wuxi (YGZXQ1311, YGZ1106, and YGZ1007).

HEPATOCELLULAR carcinoma (HCC) is one of the most prevalent cancers and the third most common cause of cancer mortality worldwide.¹⁻² More than half of HCC is caused by chronic hepatitis B virus (HBV) infection. Despite a great improvement in the treatment of HCC, relapse or metastasis is frequently occurred. Therefore, it is necessary to identify the molecular mechanisms participated in HCC metastasis, serving as a tool to diagnose HCC, predict clinical outcomes, and guide treatment of HCC.

MicroRNAs (miRNAs) are a class of the conserved, endogenous, single-stranded, non-coding RNA molecules that integrate with argonaut to form a multi-protein complex, RNA-induced silencing complex (RISC). miRNAs in the RISC can bind to complementary sequences in the 3'-untranslated region (3'-UTR) of the target mRNAs to regulate mRNA stability and translation.³⁻⁴ miRNAs have been identified to be involved in the initiation and progression of various cancers.⁵⁻⁸ microRNA-17-92 (miR-17-92) cluster has been found to be abnormally expressed in B-cell lymphoma and breast, colorectal, lung, pancreatic, prostate and stomach cancers.⁹⁻¹² miR-18, a member of miR-17-92 cluster, has been revealed aberrant expression in human and rodent HCCs.¹³⁻¹⁴ We previously reported that the serum miR-18a levels were substantially higher in patients with HBV-related HCC than those in the healthy controls.¹⁵ Liu *et al*¹⁶ reported that miR-18a could prevent oestrogen receptor alpha expression and promote proliferation of HCC cells.

To date, various studies have indicated the miR-17-92 cluster plays a role in HCC metastasis. Over-expression of miR-17-5p leads to marked cell proliferation and intrahepatic metastasis of HCC.¹⁷ It was also postulated and verified that increased miR-17-5p expression led to downregulation of its target, E2F1, thereby triggering activation of the p38 (MAPK) pathway and increasing phosphorylation of heat shock protein 27 (HSP27).¹⁷ As a result, HCC cell migration was enhanced, and increased expression of miR-106b correlated with a high HCC tumor grade. Over-expression of miR-106b has been shown to activate the epithelial-mesenchymal transition, leading to cell migration and metastasis.¹⁸

Dcr-1 homologue (Dicer I) is a highly conserved, multi-domain RNase type III enzyme that has a critical function in the processing and maturation of miRNAs.¹⁹⁻²⁰ Indeed, Dicer I is responsible for processing precursor miRNAs (pre-miRNAs) into mature miRNAs,²¹ and the role of Dicer I in tumorigenesis has recently attracted increasing interest. Interestingly, Dicer I is a conserved target of miR-18a, and it has been reported that inhibition of Dicer I expression may contribute to suppressing

carcinoma malignant progression.²²⁻²³ Although downregulation of Dicer I and its association with poor prognosis in HCC have been reported,²⁴⁻²⁵ the function and regulation of Dicer I in HCC remains unclear.

Here, we analyzed the effects of miR-18a on migration and invasion of HCC cells, and its possible mechanism associated with Dicer I.

MATERIALS AND METHODS

Cell culture

HepG2 and stably HBV expressing HepG2.2.15 cells were purchased from the American Type Culture Collection (ATCC, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum at a final concentration of 10%. All the cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Oligonucleotide synthesis and transfection

The nucleotide sequences of miR-18a inhibitor and negative control (NC), Dicer I small interfering RNA (siRNA) and NC, and miR-18a mimic and NC were synthesized by Gene Pharma (Shanghai, China). The sequences are as follows: miR-18a inhibitor 5'-CUAUCUGCACUAGAUGCACCUUA-3'; inhibitor NC 5'-CAGUACUUUUGUGUAGUACAA-3'; Dicer I siRNA 5'-GGGCACCAUCUCUAAUUATT-3', 5'-UAAUUAGAG-AUGGGUGCCCTT-3'; siRNA NC 5'-UUCUCCGAACGUGUCAC-GUTT-3', 5'-ACGUGACACGUUCGGAGAATT-3'; miR-18a mimic sense 5'-UAAGGUGCAUCUAGUGCAGAUAG-3', mimic anti-sense 5'-AUCUGCACUAGAUGCACCUUAUU-3'; mimics NC 5'-UUCUCCGAACGUGUCACGUTT-3', 5'-ACGUGACACGUUC-GGAGAATT-3'. All the cell transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Each transfection was performed in three replicates.

Transwell migration and invasion assays

A total of 5×10⁴ transfected cells were resuspended in 100 µl of serum-free medium and placed in the upper compartment of a transwell chamber (Corning, New York, USA; 24-well insert, pore size: 8 µm). The lower chamber was filled with 10% fetal bovine serum as a chemoattractant. The cells were incubated for 48 hours for migration assays and 72 hours for invasion assays. For the invasion assay, the inserts were pre-coated with extracellular matrix gel (BD Biosciences, Sparks, MD, USA). At the end of each experiment, the cells on the upper surface of the membrane were removed, and ones on the lower surface were fixed and stained with Giemsa. The number of cells in 5 randomly selected microscopic fields (x200) was counted. And then the invasion and migration

rates were calculated as follows: invasion/migration rate (%)=(the average transmembrane cell number of the miR-18a inhibitor group/the average transmembrane cell number of the inhibitor NC) x100%.

Wound-healing assay

Cells (1×10^6 /well) were seeded in 6-well plates, cultured overnight and transfected with miR-18a inhibitor, inhibitor NC, Dicer I siRNA or NC, respectively. Upon reaching the appropriate confluence, the cell layer was scratched with a sterile plastic tip, washed twice with culture medium, and cultured again up to 48 hours with serum-free medium. Images were captured at the different time points (0, 24, 48 and 72 hours) under a microscope to assess the rate of gap closure. The distance between the wound edges was measured. The wound healing rate=[(wound edges distance at 0 hour–wound edges distance at the indicated time)/wound edges distance at 0 hour] x100%.

Luciferase reporter assay

We then performed a bioinformatics study to explore the underlying molecular mechanism through which miR-18a promotes HCC cell migration and invasion. A schematic diagram of the miR-18a-binding sequence in the Dicer I mRNA 3'-UTR is shown in Figure 1; two putative binding sites for miR-18a are present at 1085-1091 bp and 4184-4191 bp. To further verify the targeting of Dicer I by miR-18a, reporter constructs in which the 3'-UTR of Dicer I, either wild type (WT) or mutant type, were cloned downstream of the luciferase open reading frame. In addition, miR-18a or control miRNA was co-transfected with the luciferase construct.

HEK293 cells were purchased from ATCC and plated in a 48-well plate and transfected with 20 ng of reporter plasmid, miR-18a mimic or its NC using Lipofectamine™ 2000. Each sample was also co-transfected with 4 ng of Renilla luciferase plasmid (Promega, Madison, WI, USA) as an internal control. The luciferase assay was performed at

48 hours after transfection using the Dual-Luciferase® Reporter Assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well, and each assay was repeated 4 times.

Real-time RT-PCR assay

Total RNA was extracted from cells using Trizol reagent and reverse transcribed using ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. Real-time RT-PCR was carried out using SYBR® Green Real-time PCR Master Mix (TOYOBO). The primers for Dicer I mRNA analysis were synthesized by Shanghai Bioengineering (Shanghai, China). The sequences are forward 5'-AAGGAA-GCTGGCAAACAAGA-3' and reverse 5'-AAAACGAACCACCA-AGTTGC-3'. The GAPDH primers are forward 5'-CAACAGC-CTCAAGATCATCAGC-3' and reverse 5'-CTCATGACCACAG-TCCATGCCA-3'. The relative amount of gene transcripts was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH.

Western blot analysis

Whole-cell lysates were separated by 8% SDS-PAGE, and proteins were blotted onto PVDF membranes. The membranes were hybridized with a monoclonal antibody at 4°C overnight followed by incubation with a secondary antibody for 1 hour at room temperature. Antibodies specific for Dicer I and GAPDH were purchased from Santa Cruz Biotechnology (1:200, Santa Cruz, CA, USA). GAPDH was used as a loading control. Signals were visualized using the Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) by exposure to film.

Statistical analysis

Statistical analysis was performed using SPSS 17.0. Data from at least three separate experiments are presented as the means $\pm$ SD. Student's *t*-test was used for comparisons between groups, unless otherwise noted. Differences were considered significant at *P*-values less than 0.05.

Figure 1. Scheme of putative microRNA-18a (miR-18a) binding sites in the Dicer I mRNA 3'-untranslated region (3'-UTR). Two putative binding sites for miR-18a are present. Mutations were generated in the Dicer I 3'-UTR sequence in the complementary sites for the miR-18a seed regions.

RESULTS

Downregulation of miR-18a inhibited HCC cell migration and invasion

To investigate the influence of miR-18a on the metastasis and invasion of HCC cells, we first constructed low miR-18a expression cells. Transwell migration and invasion assays

showed a significant reduction in cell migration and invasion after HepG2 and HepG2.2.15 cells were transfected with miR-18a inhibitor compared with miR-18a inhibitor NC groups. Inhibiting miR-18a expression reduced the migration and invasive rates of both HepG2 cells (0.446 and 0.410, $P_{\text{migration}}=0.0002$, $P_{\text{invasion}}=0.0009$; Fig. 2A and 2C) and HepG2.2.15 cells (0.602 and 0.565, $P_{\text{migration}}=0.0008$, $P_{\text{invasion}}=0.0013$; Fig. 2B and 2D).

Figure 2. Reducing miR-18a expression degrades migration and invasion of HCC cells.

Transwell migration assays of HepG2 cells (A) or HepG2.2.15 cells (B) expressing miR-18a inhibitor or inhibitor negative control (inhibitor NC). Transwell matrigel invasion assay of HepG2 cells (C) or HepG2.2.15 cells (D) expressing miR-18a inhibitor or inhibitor NC. Representative light microscopy images ($\times 200$ magnification) are shown on the left, and the histogram showing the migrated cell number is shown on the right. The values shown are expressed as mean $\pm$ SD.

* $P < 0.01$ compared with the inhibitor NC ($n=3$).

To further evaluate the role of miR-18a on HCC cell migration, wound-healing assays were performed. The results showed substantially reduced cell migration by miR-18a inhibitor transfectants compared with inhibitor NC transfectants at 24, 48 and 72 hours after wounding (HepG2 cells: $P_{24h}=0.0045$, $P_{48h}=0.0004$, $P_{72h}=0.0017$; HepG2.2.15 cells: $P_{24h}=0.0024$, $P_{48h}=0.0033$, $P_{72h}=0.0000$) (Fig. 3A and 3B).

Dicer I is a direct target of miR-18a

As shown in Figure 4, the relative luciferase activity was reduced in cells co-transfected with the Dicer I 3'-UTR-Wt luciferase reporter and the miR-18a mimic compared to the

negative control cells ($P_{Wt1}=0.00089$, $P_{Wt2}=0.0034$). In contrast, the luciferase activity of the mutant 3'-UTR was not significantly affected in miR-18a-overexpressing cells.

Downregulation of Dicer I eliminates the miR-18a-inhibitor-induced inhibition of cell migration and invasion in HCC cells

To further confirm that miR-18a post-transcriptionally downregulates Dicer I expression, we detected the expression level of Dicer I in different transfectants. Real-time RT-PCR and western blot analysis showed that compared with the inhibitor NC group, the mRNA and protein expression levels of Dicer I were significantly upregulated

Figure 3. Wound healing assays of HepG2 cells (A) and HepG2.2.15 cells (B) after treatment with miR-18a inhibitors or inhibitor NC. The wound healing rates of wound closure per field is shown.

* $P<0.01$ compared with the inhibitor NC ($n=3$).

Figure 4. miR-18a post-transcriptionally downregulates Dicer I expression by directly targeting the Dicer I mRNA 3'-UTR.

A human Dicer I 3'-UTR fragment containing either the wild-type (WT) or mutant (Mut) miR-18a-binding sequences was cloned downstream of the luciferase reporter gene. Luciferase assays verified direct binding of miR-18a to the predicted sites. HEK293 cells were co-transfected with the luciferase construct and miR-18a mimics or control miRNA. The Renilla construct was also co-transfected as an internal control. Luciferase activity was normalized to Renilla luciferase activity. The data are presented as the mean $\pm$ SD of two experiments with four replicates each.

* $P<0.01$ compared with the mimic NC ($n=4$).

after transfection of HepG2 and HepG2.2.15 cells with miR-18a inhibitor for 48 hours (HepG2: $P_{\text{mRNA}}=0.001$, $P_{\text{protein}}=0.00037$; HepG2.2.15: $P_{\text{mRNA}}=0.001$, $P_{\text{protein}}=0.00002$; Fig. 5). Compared with the miR-18a inhibitor+siRNA NC co-transfected group, the Dicer I mRNA and protein levels were significantly downregulated after co-transfection with Dicer I siRNA+miR-18a inhibitor for 48 hours in both HepG2 and HepG2.2.15 cells (HepG2: $P_{\text{mRNA}}=0.001$, $P_{\text{protein}}=0.00036$; HepG2.2.15: $P_{\text{mRNA}}=0.002$, $P_{\text{protein}}=0.00003$).

We also performed transwell migration, invasion, and wound-healing assays to determine whether Dicer I silencing may eliminate the effects of miR-18a inhibition on cell migration and invasion. The migration and invasion of HepG2 and HepG2.2.15 cells transfected with miR-18a inhibitor were significantly reduced compared with inhibitor NC groups (HepG2: $P_{\text{migration}}=0.0007$, $P_{\text{invasion}}=0.0035$; HepG2.2.15: $P_{\text{migration}}=0.0024$, $P_{\text{invasion}}=0.0049$). In contrast, significantly enhanced cell migration and invasion were observed after HepG2 and HepG2.2.15 cells were co-transfected with miR-18a inhibitor+Dicer I siRNA when compared with miR-18a inhibitor+siRNA NC transfectants (HepG2: $P_{\text{migration}}=0.0017$, $P_{\text{invasion}}=0.0059$; HepG2.2.15: $P_{\text{migration}}=0.0012$, $P_{\text{invasion}}=0.0095$). The number of migrating or invasive HCC cells co-transfected with miR-18a inhibitor+Dicer I siRNA was comparable with cells transfected with

the inhibitor NC transfectants (Fig. 6).

We reproduced the migration-suppressing effects of miR-18a inhibition by knocking down Dicer I with siRNA. A wound-healing assay showed significantly increased cell migration following Dicer I siRNA+miR-18a inhibitor co-transfection compared with the siRNA NC+miR-18a inhibitor transfectant at 24, 48 and 72 hours after wounding in both HepG2 and HepG2.2.15 cells (HepG2: $P_{24\text{h}}=0.02$, $P_{48\text{h}}=0.01$, $P_{72\text{h}}=0.0003$; HepG2.2.15: $P_{24\text{h}}=0.02$, $P_{48\text{h}}=0.01$, $P_{72\text{h}}=0.0004$) (Fig. 7). This observation suggests that silencing Dicer I may eliminate the effects of miR-18a inhibitor. These results indicate that miR-18a-induced pro-migration and pro-invasion effects may be partially attributed to its suppression of Dicer I.

DISCUSSION

Metastasis and invasion, two of the most important hallmarks of cancer, are the leading lethal factors for malignant cancer, particularly for HCC. As metastasis is still the primary contributor to liver failure, tumour recurrence and death in HCC patients, identifying metastatic factors and understanding the molecular mechanisms involved in the process of metastasis are crucial issues. In the present study, we demonstrated that miR-18a, a member of the

Figure 5. Endogenous Dicer I expression levels were detected in HepG2 and HepG2.2.15 cells transfected with miRNA-18a inhibitor or Dicer I small interfering RNA (siRNA) for 48 hours by real-time RT-PCR (A) and western blotting (B).

a. inhibitor NC; b. miRNA-18a inhibitor; c. miRNA-18a inhibitor + si-RNA NC; d. miRNA-18a inhibitor + si-Dicer I.

The data represent the mean $\pm$ SD of three independent experiments.

* $P<0.01$ compared with the inhibitor NC ($n=3$); # $P<0.01$ compared with the miRNA-18a inhibitor + si-RNA NC ($n=3$).

Figure 6. Downregulation of Dicer I promotes cell migration and invasion in HCC cells.

The effects of miRNA-18a inhibitor or restoration of miRNA-18a function through downregulation of Dicer I on cell migration and invasion were confirmed by transwell migration and invasion assays in HepG2 (A and C) and HepG2.2.15 (B and D) cells transfected with miRNA-18a inhibitor or si-Dicer I.

The data represent the mean $\pm$ SD of three independent experiments.

* $P < 0.01$ compared with the inhibitor NC ($n = 3$); # $P < 0.01$ compared with the miRNA-18a inhibitor + si-RNA NC ($n = 3$).

Figure 7. Wound healing assays of HepG2 cells (A) and HepG2.2.15 cells (B) treated with miRNA-18a inhibitor or si-Dicer I. The wound healing rate of wound closure per field is shown.

$P < 0.05$, * $P < 0.01$ compared with the miRNA-18a inhibitor + siRNA NC ($n=3$).

oncogenic miR-17-92 cluster that is commonly upregulated in HCC tissues and serum, can promote cell migration and invasion and inhibition of miR-18a can suppress migration and invasion in HCC cells.

Tao *et al*²³ reported that miR-18a suppressed Dicer expression *via* mRNA degradation as well as translational inhibition and repressed cell growth in bladder cancer T24 cells. Here, we found that Dicer I was a direct and functional target of miR-18a in HCC. Furthermore, we found that silencing Dicer I eliminated the effects of miR-18a inhibitor on the suppression of cell migration and invasion in HCC cells. The data suggest that miR-18a promotes cell migration and invasion by silencing Dicer I.

Dicer is an essential component of the miRNA-producing machinery. Dicer expression directly influences the maturation of miRNAs, ultimately affecting gene expression patterns in cells. One study has demonstrated that dysregulation of Dicer I resulted in expression changes of many miRNAs and the disrupted expression balance of tumour-suppressing *versus* tumour-promoting miRNAs promotes oncogenesis.²⁶ Sekine *et al*²⁷ considered that Dicer I and miRNAs play critical roles in hepatocyte survival, metabolism, developmental gene regulation, and tumor suppres-

sion *in vivo*. Loss of Dicer primarily impairs hepatocyte survival but can promote hepatocarcinogenesis when coupled with additional oncogenic stimuli. Moreover, germline mutations of Dicer have been identified in familial pleuropulmonary blastoma,²⁸ nonepithelial ovarian cancers,²⁹ and other cancers.³⁰⁻³² Indeed, low expression of Dicer I is frequently detected in HCC,²⁴⁻²⁵ ovarian cancer,³³ neuroblastoma,³⁴ lung adenocarcinoma³⁵ and primary gastrointestinal diffuse large B-cell lymphoma,³⁶ and low Dicer I expression is significantly associated with poor prognosis.^{25, 33-36}

In conclusion, we found that miR-18a promotes HCC cell migration and invasion and downregulation of its expression could reduce these effects. Consistent with our bioinformatics analysis, we also found that miR-18a post-transcriptionally could suppress the Dicer I expression and that knockdown of Dicer I could abolish these effects of miR-18a inhibitor in HCC cells. These results indicated that miR-18a might play a fundamental role in HCC, especially in the process of HCC metastasis.

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