Original Article miR-34c-3p inhibits cell proliferation, migration and invasion of hepatocellular carcinoma by targeting MARCKS

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Abstract: Recent studies have shown that microRNA-34c-3p (miR-34c-3p) is down-regulated in various types of cancers and involved in tumor growth, invasion and metastasis. However, the roles of miR-34c-3p in hepatocellular carcinoma (HCC) are poorly understood. In this study, the expression profile of miR-34c-3pin HCC tissues and cell lines were examined by quantitative real-time polymerase chain reaction (qRT-PCR). The correlations of miR-34c-3p expression and clinicopathological characteristics were analyzed. The biological role of MiR-34c-3pin cell proliferation, migration and invasion was examined. In addition, the targets of miR-34c-3p were identified. The results showed that miR-34c-3p expression was significantly down-regulated in HCC tissues and cell lines; low expression level of miR-34c-3p was correlated with vascular invasion and advanced TNM stage. *In vitro* functional assays showed that overexpression of miR-34c-3pin HepG2 and Huh7 cells significantly reduced cell proliferation, migration and invasion. Furthermore, target analysis and luciferase assay identified myristoylated alanine-rich protein kinase c substrate (MARCKS) as a specific target of miR-34c-3p. Knockdown of MARCKS in HepG2 cells reduced cell migration and invasion, but not cell proliferation. Taken together, our findings implicate the potential application of miR-34c-3p as a tumor suppressor in cancer therapy.

Keywords: MiR-34c-3p, hepatocellular carcinoma, tumor suppressor, MARCKS

Introduction

MicroRNAs (miRNAs) are a class of non-coding small RNAs that regulate expression of target genes at the post-transcriptional level through imperfect with the 3'untranslated region (3' UTRs) of specific target mRNAs [1]. Increasing evidence has shown that miRNA scan function as oncogenes or tumor suppressors in cancer development [1]. Many studies have demonstrated that miRNAs play critical roles in hepatocellular carcinoma (HCC) progression and directly contribute to cell proliferation and metastasis by targeting a large number of critical genes. For example, miR-188-5p suppresses HCC cell proliferation and metastasis by directly targeting FGF5 [2]. miR-128-3p suppresses HCC proliferation by regulating PIK3R1 and is correlated with the prognosis of HCC patients [3]. MiR-141 suppresses the migration and invasion of HCC cells by targeting Tiam1 [4]. Although a large number of miRNAs have been identified, their roles in HCC development and the underlying mechanisms remain largely unknown.

miR-34 family (miR-34a, miR-34b and miR-34c) are critical modulators of the p53 pathway and potential tumor suppressors in human cancers [5]. Their expression shows a global decrease in many different human cancers, including ovarian cancer, prostate cancer and cervical carcinoma [6, 7]. MiR-34c-3p is one of the mature miRNAs of miR-34c. Previous studies have showed that it was down-regulated in human NSCLC [8, 9], glaucoma [10] and cervical carcinoma [11], and inhibited tumor proliferation, migration and invasion through targeting multiple signaling pathway. A recent study has shown that miR-34c-3p was over expressed in liver cancer stem cells in diethylnitrosamine (DEN) treated rats [12]. However, the biological roles

Characteristics	Cases (60)	miR-34c-3p expression		Dvolue
		High (24)	Low (36)	Pvalue
Age (years)				
≤55	43	17 (70.8%)	26 (72.2%)	0.907
>55	17	7 (29.2%)	10 (27.8%)	
Gender				
Male	46	19 (79.2%)	27 (75%)	0.709
Female	14	5 (20.8%)	9 (25%)	
Hepatitis history				
Yes	37	18 (75%)	19 (52.8%)	0.083
No	23	6 (25%)	17 (47.2%)	
Liver cirrhosis				
Yes	35	16 (66.7%)	19 (52.8%)	0.285
No	25	8 (33.3%)	17 (47.2%)	
AFP (ng/mL)				
≤20	33	15 (62.5%)	18 (50%)	0.340
>20	27	9 (37.5%)	18 (40%)	
ALT (U/mI)				
≤75	31	14 (58.3%)	17 (47.2%)	0.399
>75	29	10 (41.7%)	19 (52.8%)	
Tumor size (diameter)				
≤5 cm	32	18 (75%)	14 (38.9%)	0.006
>5 cm	28	6 (25%)	22 (61.1%)	
Vascular invasion				
Yes	23	4 (16.7%)	19 (52.8%)	0.005
No	37	20 (83.3%)	17 (47.2%)	
Tumor differentiation				
1-11	41	15 (62.5%)	26 (72.2%)	0.428
III-IV	19	9 (37.5%)	10 (27.8%)	
TNM stage				
I	35	19 (79.2%)	16 (44.4%)	0.024
II	17	4 (16.7%)	13 (36.1%)	
111	8	1 (4 1%)	7 (19 5%)	

 Table 1. Correlation between miR-34c-3p expression and
 clinicopathological characteristics of HCC patients

of miR-34c-3p in HCC are still poorly understood. Therefore, the aim of the present study was to examine the function role of miR-34c-3p in human HCC. We showed that miR-34c-3p was down regulated in HCC tissues and cell lines, and low expression level of miR-34c-3p was associated with advanced tumor stage and metastasis. Restoration of miR-34c-3p expression in HCC cell lines suppressed cell proliferation, colony formation and also limited migration and invasion in HCC cells. In addition, MARCKS was confirmed as a direct target of miR-34c-3p, and the tumor suppressor function of miR-34c-3p may be associated with the downstream gene MARCKS. Thus, our data suggest that miR-34c-3p might be a promising therapeutic target for HCC.

Materials and methods

Tissue specimens

In this study, 60 paired HCC tissues and adjacent non-tumor tissues were collected from The First Affiliated Hospital of Wenzhou Medical University. All tissue samples were flash-frozen in liquid nitrogen immediately after collection and stored at -80°C until use. Both tumor and non-tumor samples were confirmed by pathological examination. No patients received chemotherapy or radiotherapy prior to surgery. Tumor stages were determined by TNM classification according to the 2002 International Union against Cancer guidelines. Tumor differentiation was graded according to Edmondson-Steiner classification. The demographic and clinical characteristics of all patients are given in **Table 1.** The study was approved by the Ethics Committee of The First Affiliated Hospital of Wenzhou Medical University, and informed consent was obtained from each patient according to the committee's regulations.

Cell culture

Four human HCC cell lines, including HCCLM3, Huh7, SK-Hep1, HepG2, and one normal liver cell line LO2 were used in this study. These cell lines were all purchased from American Type Culture Collection (ATCC, Rock-

ville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified incubator containing 5% CO₂.

RNA extraction and real-time quantitative PCR

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA concentration was assessed by measuring absorbance at 260 nm using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA). 2 µg of total RNA was reversely transcribed using the Prime Script RT reagent kit with gDNA Eraser (TaKaRa, Japan) and miRNA-specific stem-loop RT primer (Applied Biosystems, USA). Stem-loop RT primer for miR-34c-3p was: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA-CTGGATACGACCCTGGC-3'. Gene-specific amplification was performed using ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The following gene-specific primers were used in this study: forward, 5'-GGTG-GAATCACTAACCACG-3' and reverse 5'-GTGC-AGGGTCCGAGGT-3' for miR-34c-3p; forward, 5'-AGAGCCTGTGGTGTCCG-3' and reverse, 5'-CATCTTCAAAGCACTTCCCT-3' for internal control U6 small nuclear RNA. The relative expression level of miR-34c-3p was normalized to that of internal control U6 using the comparative delta CT (2-DACt) method. Each sample was analyzed in triplicate and the mean expression level was calculated.

Cell transfection

Hsa-miR-34c-3p mimics and mimic negative control RNA were designed and synthesized by RiboBio (Guangzhou, China). siRNA to MARCKS or negative control siRNA were designed and chemically synthesized by Shanghai Gene Pharma Company (Shanghai, China). For transfection, 2×105 HepG2 and Huh7 cells were seeded into 6-well plate in growth medium without antibiotics at a density of 30%-40% and incubated overnight, then transfected with negative control (NC) RNA or miR-34c-3p mimic using HiPerFect Transfection Reagent (Qiagen, Hilden, German) according to the manufacturer's protocol. The mixture was added to cells at a final concentration of 100 nM. RNA oligonucleotides were transfected by using Lipofectamine RNAi-MAX (Invitrogen, Carlsbad, CA, USA) and medium was replaced 6 hours after transfection.

Cell proliferation assay

The in vitro cell proliferation of HCC cells transfected with NC or miR-34c-3p was measured using CCK-8 (Beyotime Biotechnology, Haimen, China) assay according to manufacturer's instructions. Briefly, Forty eight hours after transfection, the transfected cells were harvested, seeded into 96-well plates at 2×10^3 cells per well and cultured for 1, 2, 3, 4, days before addition of 10 μ I CCK-8 (5 mg/ml) to the culture medium in each well. After 1 hour incubation at 37 °C, OD values were read using a microplate reader (Bio-Tek Company, Winooski, VT, USA) at the 450-nm wavelength. Each time point was repeated in three wells and the experiment was independently performed for three times.

Cell apoptosis assay

Cell apoptosis was evaluated by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotech Co. Roche, Nanjing, China). Briefly, Forty eight hours after transfection, spent cell culture medium was replaced by fresh serum free DMEM and cells were cultured for an additional 48 h, cells were harvested, washed, resuspended in the staining buffer. A volume of 5 µL Annex in V-FITC and 5 µL propidium iodide was added and mixed gently, and the cells were stained in the dark for 10 min at room temperature. The cells were analyzed immediately by flow cytometry (BD FACSCalibur, BD Bioscience, San Diego, CA, USA) and analyzed using Flowjo software (FlowJo, Ashland, OR, USA). The Annexin V-positive and propidium iodide (PI)-negative cells were regarded as apoptotic cells. The experiment was repeated three times.

Cell migration and invasion assays

Cell migration and invasion were assessed using Boyden chamber assay with 24-well transwells (8-µm pore size; Minipore, Billerica, MA, USA). For the migration assay, 1×10⁵ transfectedcells were suspended in 200 µL serum-free medium and were added to the upper chamber. For the invasion assays, the membrane was precoated with 45 µg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) to forma matrix barrier. 600 µL medium with 10% fetal bovine serum was placed in the lower chamber. After 12 hours of incubation, the cells in the upper membrane were removed carefully with cotton wool. Cells that had migrated or invaded through the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Shanghai, China) for 30 min, rinsed in phosphate-buffered saline, and cells in five microscopic fields (at 200× magnification) were counted and photographed. Experiments were independently repeated three times.



Figure 1. MiR-34c-3p is downregulated in HCC tissues and cell lines. A. The relative expression of miR-34c-3p in HCC tissues and paired adjacent normal liver tissues was examined by qRT-PCR. B. The relative expression of miR-34c-3pin four HCC cell lines HCCLM3, Huh7, HepG2 and SK-Hep1 and one normal liver cell line LO2 was examined by qRT-PCR. **P*<0.05, ****P*<0.001.

Prediction of miR-34c-3p target genes

Putative miR-34c-3p targets were predicted using several different algorithms, including Target Scan (http://www.targetscan.org/), Pictar (http://pictar.bio.nyu.edu/) and miRanda (http://microrna.sanger.ac.uk/).

Dual-luciferase reporter assay

Luciferase reporter gene assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Cells of 90% confluence were seeded in 24-well plates. For MARCKS luciferase reporter assay, wild type or mutant reporter constructs (termed WT or Mut) were co-transfected into HepG2 or Huh7 cells in 24-well plates with 100 nM miR-34c-3p or 100 nM miR-NC and Renilla plasmid by using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Reporter gene assays were performed 24 hours post-transfection using the Dual luciferase assay system (Promega, Madison, WI, USA). Firefly luciferase activity was normalized for transfection efficiency using the corresponding Renilla luciferase activity. All experiments were performed at least three times.

Western blotting

About 20 µg of total protein was extracted and separated by 10% SDS-PAGE, transferred onto

polyvinylidene fluoride membranes. The primary antibodies to MARCKS (Santa Cruz, CA, USA) and β -actin (Santa Cruz, CA, USA) were incubated with the blot overnight at 4°C. After being extensively washed with PBS containing 0.1% Triton X-100, the membranes were incubated with HRP-conjugated goat anti-rabbit antibody for 30 min at room temperature. The bands were visualized using the ECL system (Millipore, Billerica, WI, USA).

Statistical analysis

Statistical Package of the Social Sciences 19.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical analyses. Differences in MiR-34c-3p expression were evaluated with the pairedsamples t-test or Mann-Whitney U test. The relationships between MiR-34c-3p expression and various clinicopathological parameters were analyzed by chi-square test. Differences between experimental groups were assessed using the two-tailed unpaired Student's t test. P<0.05 was considered as statistical significance.

Results

MiR-34c-3p expression is down-regulated in human HCC tissues and cell lines

Previous studies have showed that the expression level of miR-34c-3p was down regulated in



Figure 2. MiR-34c-3p suppresses the proliferation, migration and invasion of HCC cell. HepG2 and Huh7 cells were transfected with hsa-miR-34c-3p mimics and mimic negative control, respectively. A, B. Cell proliferation was analyzed by time course CCK-8 assay. C, D. Cell apoptosis was analyzed by flow cytometry using an AnnexinV-FITC Apoptosis Detection Kit. E, F. Cell migration and invasion ability were performed by Transwell or Matrigel precoated Transwell assay. Data shown are mean ± SD from three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001.

human NSCLC [8, 9], glinoma [10] and cervical carcinoma [11]. To further investigate the roles

of miR-34c-3p in the development of HCC, we analyzed the expression level of miR-34c-3p in



Figure 3. MARCKS is a direct target ofmiR-34c-3p. A. Sequence alignment of wild-type (WT) and mutated (Mut) putative miR-34c-3p-binding sites in the 3'-UTR of MARCKS. B. Relative luciferase activities of plasmids carrying WT or mutant MARCKS 3'UTR in HepG2 and Huh7 cells co-transfected with miR-NC versus miR-34c-3p. C. The endogenous expression levels of MARCKS were examined in HepG2 and Huh7 cells by western blot. Data shown are mean \pm SD from three independent experiments. **P<0.01.

60 paired clinical HCC tissues and adjacent non-tumor tissues by qRT-PCR. As shown in **Figure 1A**, the relative expression of miR-34c-3p was significantly down regulated in HCC tissues compared with the adjacent normal tissues (P<0.001). Meanwhile, the expression levels of miR-34c-3p were decreased in 4 HCC cell lines relative to 1 normal liver cell lines (**Figure 1B**). These data suggest that the expression level of miR-34c-3p is down regulated in human HCC.

MiR-34c-3p down regulation is correlated with advanced tumor stage and metastasis

To determine the effects of miR-34c-3p expression on tumor progression, comparisons of the clinical pathological variables with miR-34c-3p expression in HCC were made. MiR-34c-3p expression levels less than the mean expression level ($2^{-\Delta\Delta Ct}$ =0.47) were assigned to the low expression group (n=36), and those samples with expression above the mean value were assigned to the high expression group (n=24). As shown in **Table 1**, low levels of miR-34c-3p expression were associated with tumor size, vascular invasion and advanced TNM stage. However, miR-34c-3p expression was not cor-

related with other or pathological parameters, including age, gender, hepatitis history, liver cirrhosis history, AFP level, ALT level and tumor differentiation (**Table 1**).

MiR-34c-3p inhibits HCC cell proliferation, migration and invasion and promotes cell apoptosis

Based on the findings mentioned above, we hypothesized that miR-34c-3p was implicated in HCC growth and metastasis. To investigate the biological function of miR-34c-3pin HCC, HepG2 and Huh7 cells were transfected with miR-34c-3p mimic or negative control (termed miR-NC). Using these transient transfection cell lines, we evaluated the role of miR-34c-3p on cell proliferation, migration, and invasion. As shown in Figure 2A and 2B, cell proliferation of HepG2 and Huh7 cells transfected with miR-34c-3p was significantly slower than those transfected with miR-NC. In addition, restoration of miR-34c-3p promoted cell apoptosis upon serum deprivation in HepG2 and Huh7 cells (Figure 2C and 2D). Furthermore, In vitro migration and invasion assay showed that over expression of miR-34c-3p decreased HepG2 and Huh7 cells migration and invasion (Figure



Figure 4. MiR-34c-3p targeted MARCKS contribute to migration and invasion of HCC cells. A. The expression levels of MARCKS were examined by western blot in HepG2 cells treated with siRNA targeting MARCKS (termed si-MARCKS) and control siRNA (termed siRNA-NC). B. Cell proliferation was analyzed by time course CCK-8 assay. C, D. Cell migration and invasion ability were performed by Transwell or Matrigel precoated Transwell assay. Data shown are mean \pm SD from three independent experiments. **P*<0.05, ***P*<0.01.

2E and **2F**). Taken together, these results suggest that miR-34c-3p suppressed the proliferation, migration, and invasion abilities of HCC cells *in vitro*.

MiR-34c-3p directly targeted MARCK Sin HCC cells

To explore the mechanisms of miR-34c-3pinduced cell growth and metastasis inhibition, we searched for the target genes of miR-34c-3p. As shown in **Figure 3A**, MARCKS was predicted to be a target of miR-34c-3p. To validate whether the 3'-UTR of MARCKS is a functional target of miR-34c-3p, a dual-luciferase reporter system was employed. We cloned 3'UTR sequences containing the predicted target site (wild type, WT) of miR-34c-3p or mutated sequences (mutant type, Mut) into the pGL3 vector, respectively. The results showed that the co-expression of miR-34c-3p mimics significantly suppressed the firefly luciferase activities of the reporter with wild type 3'UTR but not that of the mutant reporter, indicating that miR- 34c-3p can directly target the 3'UTR of MARCKS (**Figure 3B**). In addition, transfection of miR-34c-3p resulted in an obvious down regulation of MARCKS at the protein levels in HepG2 and Huh7 cells (**Figure 3C**). Taken together, these data demonstrate that MARCKS is a direct target of miR-34c-3p.

MiR-34c-3p targeted MARCKS contribute to migration and invasion of HCC cells

It has been showed that down regulation of MARCKS could increase the migration of human hepatic stellate cells. To explore whether the effects of miR-34c-3p on HCC cell growth and invasion are due to MARCKS, we examined the effects of knockdown of MARCKS on the cell proliferation, migration and invasion in HepG2 cells, MARCKS silencing effect was confirmed by western blot (Figure 4A). CCK-8 assay showed that knockdown of MARCKS did not decrease the proliferation ability in HepG2 cells transfected with MARCKS siRNA relative to control (Figure 4B). However, transwell assay showed that silencing of MARCKS led to a significant reduction of migration and invasion in HepG2 cells (Figure 4C and 4D). These data suggested that MARCKS partly mediated miR-34c-3p effect on migration and invasion of HCC cells.

Discussion

miRNA dysregulation has been observed in various types of malignancies, and their differential expressions are closely related to tumorigenesis, metastasis and prognosis [1]. Recently, miR-34c-3p was identified as a tumor suppressor that is down-regulated in many types of cancers [8-10]. However, up to now, there is no relevant report about the relationship between miR-34c-3p and the progression of HCC.

In the present study, by using quantitative RT-PCR, we showed that the expression of miR-34c-3p was down regulated in both HCC tissues and hepatoma cell lines. Notably, miR-34c-3p expression was found to be significantly higher in patients with advanced tumor stage and in patients that had undergone vascular invasion. In agreement with our findings, Zhou et al. and Liu et al. together showed that miR-34c-3p functions as a tumor suppressor in non-small cell lung cancer (NSCLC) partially by inhibiting PAC1/MAPK pathway and eIF4E [8, 9]. In glioma, Wu et al. found that the over expression of miR-34c-3p suppressed proliferation and invasion of OS cells by targeting Notch pathway [10]. In the present study, we found that forced over expression of miR-34c-3p inhibited cell proliferation, migration, and invasion, and promoted cell apoptosis in HepG2 and Huh7 cells. Collectively, these data, together with ours, support that miR-34c-3p functions as a tumor suppressor and inhibits HCC malignant progression, miR-34c-3p may represent a novel therapeutic target for HCC treatment.

My ristoylated alanine-rich protein kinase c substrate (MARCKS) is a ubiquitously expressed protein kinase C (PKC) substrate that binds both actin and calmodulin (CaM) and regulates actin dynamics [13]. Phosphorylation of MAR-CKS by PKC, or CaM binding, results in the release of MARCKS from the plasma membrane into the cytosol in a process called the "myristoyl-electrostatic switch" mechanism [13, 14]. MARCKS is involved in multiple cellular processes, including cell adhesion, migration, metastasis, membrane trafficking and motility through regulation of the actin cytoskeletal structure [15-18]. The expression of MARCKS has shown to be down-regulated in HCC tissues, and down regulation of MARCKS could increase the migration of human hepatic stellate cells [19, 20]. It was also involved in TPA-mediated migration of neuroblastoma cells [21]. Recent studies showed that elevated MARCKS phosphorylation contributes to unresponsiveness of breast cancer to paclitaxel treatment [22]. In this study, we predicted that MARCKS was the precise intracellular target of miR-34c-3p by using miRanda, Target Scan and PICTAR databases. Our data show that miR-34c-3p was able to directly target the 3'UTR of MARCKS. Furthermore, Inhibition of MARCKS showed similar tumor-suppressive effects with miR-34c-3p. Taken together, our data suggest that the effects of miR-34c-3p on HCC migration and invasion observed in the current study may be partly due to its regulation of MARCKS.

In summary, our study for the first time demonstrated that miR-34c-3p was downregulated in HCC tissues and cell lines, and its downregulation was correlated with advanced TNM stage and vascular invasion. In addition, our finding certified that miR-34c-3p is able to inhibit HCC cell proliferation, migration and invasion partly through regulation of MARCKS. Our data demonstrate that miR-34c-3p may serve as a suppressor in HCC progression and represent a valuable prognostic marker and potential therapeutic target for HCC.

Disclosure of conflict of interest

None.

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