Original Paper

MicroRNA-214-5p Inhibits the Invasion and Migration of Hepatocellular Carcinoma Cells by Targeting Wiskott-Aldrich Syndrome Like

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Key Words

MicroRNA-214-5p • Hepatocellular carcinoma • Invasion • Migration • WASL

Abstract

Background/Aims: This study aims to explore the effects of microRNA-214-5p (miR-214-5p) on the invasion and migration of Hepatocellular Carcinoma cells (HCC). **Methods:** Hepatocellular Carcinoma tissues and adjacent normal tissues from 44 hepatocellular carcinoma patients were prepared for this study. The HepG2 and BEL-7402 cells were transfected with miR-214-5p mimic and inhibitor. qRT-PCR was performed to detect the expressions of miR-214-5p. Transwell assays were used to detect the invasion and migration assays in HepG2 and BEL-7402 cells. A dual-luciferase reporter assay was conducted to examine the effect of miR-214-5p on Wiskott-Aldrich Syndrome Like (WASL/ N-WASP). Western blot and qRT-PCR were used to measure the expressions of the E-cadherin, N-cadherin and Vimentin proteins. Transwell chamber assays were performed to detect cell invasion and migration. **Results:** Compared with normal tissues, HCC tissues demonstrated significantly lower expression of miR-214-5p. Overexpression of miR-214-5p significantly inhibited the migration and invasion of HCC cells and inhibition of miR-214-5p promoted the migration and invasion. Additionally, miR-214-5p suppressed the epithelial-mesenchymal transition (EMT). Further study showed WASL was a putative target gene of miR-214-5p. Up-regulating the expression of WASL could reverse the inhibition effect of miR-214-5p on invasion and migration. **Conclusion:** Our data suggested that miR-214-5p inhibited the invasion and migration of HepG2 and BEL-7402 by targeting WASL in Hepatocellular carcinoma.

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Introduction

Hepatocellular Carcinoma is the sixth most common cancer and the third-leading cause of cancer-related death worldwide [1]. Recently, the incidence of HCC has increased [2]. After curative resection [3, 4], HCC frequently breaks out tumor recurrence and metastasis

[5, 6]. Although much effort has been made, there are no effective therapeutic strategy to cure Hepatocellular carcinoma [7, 8]. Therefore, it is of great significance to identify the mechanism of migration and invasion and develop targeted therapeutics.

N-WASP is a scaffold that links upstream signals to the activation of the Arp2/3 complex, leading to a burst of actin polymerization [9]. Cancer cell movement requires dynamic remodeling of the cytoskeleton and cell membrane and is controlled by multiprotein complexes including focal adhesion kinase (FAK) or the Neural Wiskott-Aldrich Syndrome Protein (N-WASP) [9]. MicroRNAs (miRNAs) are widely accepted to have a profound effect on gene expression, cellular homeostasis, and expressions of multiple oncogenes and tumor suppressor genes in cancer cells [10, 11]. Modulating the functions of miRNAs might be effective in developing novel adjuvant therapeutics. MiR-214-5p is a product of the 110 bp miR-214 gene in the intron of the Dynamin-3 gene on human Chromosome 1-NC_000001.10, which produces a mature miRNA with a sequence of ugccugucacacugcugugc [12]. In 2012, Iizuka M and his colleagues revealed miR-214-5p may play crucial roles in the activation of stellate cells and the progression of liver fibrosis [13]. Moreover, in serum, Elemeery MN, et al. found miR-214-5p could be considered as early biomarkers for tracking the progress of liver fibrosis to HCC [14]. Zhang, et al. showed that miR-214-5p suppresses proliferation and invasion of human osteosarcoma cells by targeting ROCK1 [15]. Li et al. found in osteoblastic MC3T3-E1 cells, inhibition of microRNA-214-5p promotes survival and extracellular matrix formation [16]. Chandrasekaran et al. showed in human cervical and colorectal cancer cells, microRNA-214 suppresses growth, migration and invasion through AT-hook 1 [17]. Yang et al. [18] and Li et al. [19] also found miR-214 acts as a tumor suppressor in liver cancer proliferation and cell cycle. There also some different view, many researchers showed miR-214 promoted tumor pathogenesis, such as in gastric cancer cell [20, 21], ovarian cancer cells [22]. However, the roles of miR-214-5p on HCC invasion remain unknown.

Here, we report the upregulation of miR-214-5p inhibits the invasion and migration in HCC. Importantly, the functional effect of miR-214-5p is exerted by epithelial-mesenchymal transition (EMT). And miR-214-5p inhibited the invasion and migration by targeting WASL. Thus, our findings provide a novel mechanistic role for miR-214-5p in HCC metastasis and a rationale for clinically exploring the use of this microRNA in curbing HCC.

Materials and Methods

Ethics statement

This study was approved by the Ethics Committee of Jinzhou medical university. All samples were collected based on the informed consent of the study subjects.

Cell culture

Human HCC cells lines (HepG2 and BEL-7402) were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The two cell lines were cultured in an incubator (37°C, 5% CO₂ and saturated humidity) with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The culture medium was replaced every two days. When the cells reached 90% confluence, they were passaged at a ratio of 1:4.

Cell transfection

For the cell transfection experiments, the cells were divided into the following groups: (1) miR-214-5p mimics group (transfected with artificially synthesized miR-214-5p mimics); (2) miR-214-5p-NC group (transfected with a scrambled sequence); (3) miR-214-5p inhibitor (transfected with miR-214-5p inhibitors); (4) control group (no sequence transfected); (5) WASL group (transfected with WASL over-expression vector); (6) Vector group (transfected with empty plasmid vector); and (7) miR-214-5p mimics + WASL group (transfected with miR-214-5p mimics and WASL over-expression vector). The chemically synthesized miR-214-5p mimics, miR-214-5p inhibitors and negative control sequences were all purchased from Guangzhou RiboBio Co., Ltd., China. The cells were inoculated into a 6-well plate 24 h before

transfection. When the cells reached approximately 50% confluence, the human HepG2 and BEL-7402 cell line was transfected using Lipofectamine2000 (Invitrogen Inc., Carlsbad, CA, USA). After 6 hours, the culture medium was replaced. After 48 h in culture, the cells were then collected for further experiments.

Transwell chamber assay

Cell migration and invasion were measured using 24-well Transwell plate cell culture inserts (Corning, Cambridge, MA, USA) with or without Matrigel coating (BD Biosciences, San Jose, CA, USA). Forty-eight hours after transfection, the HepG2 and BEL-7402 cells were collected for serum starvation, where the cells were cultured for 24 h in FBS-free RPMI 1640 cell culture medium. The collected cells were suspended in FBS-free RPMI 1640 cell culture medium, which was followed by adjustment of the cell concentration to 10^5 cells/mL. The serum-free cell suspension (200 μ L) was added to the upper chamber of a Transwell invasion chamber, and RPMI 1640 culture medium containing 10% FBS (600 μ L) was added to the lower chamber; the cells were then cultured for 48 h at 37°C with 5% CO₂. After the liquid in the upper chamber was absorbed, the cells that did not pass through the micropores on the membrane were removed with cotton swabs, which was followed by staining with 4% crystal violet for 10 min. Then, cells that had passed through the membrane and infiltrated the lower chamber were then imaged under a microscope. After the dye on the membrane was dissolved with acetic acid, the cells were transferred to a 96-well plate. The absorbance value of each well at a wavelength of 570 nm was detected. The experiment was repeated 3 times for each group.

Dual-luciferase reporter assay.

Plasmids encoding a portion of the 3'-untranslated region (3'UTR) of WASL linked to the firefly luciferase protein. Firefly luciferase constructs were co-transfected with Renilla luciferase vector control (TK) into HepG2 cells. Where indicated, HepG2 cells were stably expressing miR-214-5p. Twenty-four hours after co-transfection with 3'UTR of target gene and TK (ratio 1:10), HepG2 cells were detached, washed, and dissolved in passive lysis solution (PLS) in 15min, at room temperature. Luciferase activities were measured consecutively (Dual-Luciferase Assay; Promega), and the relative luciferase activity was assessed: (firefly activity)/ (Renilla activity).

RNA isolation and qRT-PCR

Total RNA was prepared from tissues or cells using TRIzol reagent (TaKaRa, Dalian, China). cDNA was synthesized using a PrimeScript RT Reagent Kit (TaKaRa) according to the manufacturer's instructions. Quantitative real-time PCR was carried out using SYBR Premix Ex Taq (TaKaRa) to determine the expression level of gene transcripts for miR-214-5p, U6, E-cadherin, N-cadherin, Vimentin, and β -actin. The sequences of real-time PCR primers were as follows:

WASL forward 5'- GAACGAGTCCCTCTTCACTTTC -3'; reverse 5'- GTTCCGATCTGCTGCATATAACT -3;
E-cadherin forward 5'- GGACAGCCTATTTTCCCTCGAC -3'; reverse 5'- ACCCAGTCTCTTCTGTCTTCT -3;
N-cadherin forward 5'- ATCCCTCCAATCAACTTGCCAGA -3'; reverse 5'- CCCATTCCAACCTGGTGTAAGA -3;
Vimentin forward 5'- CAGGACTCGGTGGACTTCTCGCT -3'; reverse 5'- TTCTCCGGAGGCGCATGATGTC -3;
 β -actin forward 5'- ATCCAGGCTGTGCTGTCCCGTA -3'; reverse 5'- GAAGTCCAGGGCAACATAGCACA -3;

Quantitative PCR was conducted in triplicate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s (7300 Fast Real-Time PCR System; Stratagen). Cycle thresholds were normalized to an internal control: U6 for precursor of miRNA and β -actin for mRNA assays. The amount of RNA was calculated using the $2^{-\Delta\Delta CT}$ method; the level of expression of an RNA was normalized to the adapted internal control (denoted "relative expression").

Western blot

The total protein of cells and tissues was extracted with RIPA lysis solution (Beyotime biotechnology, USA) containing PMSF 1%; the protein concentration was detected by BCA protein analysis kit. After 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred onto PVDF membrane and then incubated for 1 h, 300 mA. Blocked with Tris-buffered saline and Tween (TBST) containing 5% skim milk. Using β -actin as an internal reference, the membrane was subsequently incubated at 4°C overnight with primary antibodies against WASL, E-cadherin, N-cadherin, Vimentin diluted 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and β -actin diluted 1:5000 (Santa

Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Next, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies at room temperature for 2 h, followed ECL. The image analysis software Gel-Pro Analyzer 4.0 was used to analyze the gray scale images. The relative ratio was calculated by a comparison with the expression of the internal reference gene. The experiment was conducted 3 times for each group.

Results

miR-214-5p is significantly decreased in HCC

To explore whether the expression of miR-214-5p has difference between hepatocellular carcinoma tissue and para-carcinoma tissue. We collected 44 pairs of hepatocellular carcinoma and para-carcinoma tissues from the First affiliated hospital of Jinzhou medical university. By qRT-PCR, we found the expression of miR-214-5p was significantly lower in HCC tissues than in adjacent normal tissues ($P < 0.05$). Moreover, to further explore whether miR-214-5p was lower expressed in cancer cell lines compared to a normal hepatic cell line [23, 24], we detected the expression of miR-214-5p in cell lines. The results showed that miR-214-5p was significantly lower in Hepatic cell lines (HepG2, BEL-7402) than in normal hepatic cell line (Chang liver cells) (Fig. 1).

Overexpression of miR-214-5p suppressed invasion and migration of HCC

To detect the role of miR-214-5p in HCC cell invasion and migration. HepG2 and BEL-7402 cells were transfected with miR-214-5p mimic, miR-214-5p inhibitor and corresponding controls. qRT-PCR showed that the expression level of miR-214-5p was significantly up-regulated by miR-214-5p mimic and down-regulated by miR-214-5p inhibitor (Fig. 2A). In terms of cell migration and invasion, miR-214-5p overexpression remarkably decreased cells migration and invasion in HepG2 and BEL-7402 cells compared to its control group. Meanwhile, these effects were reversed by miR-214-5p suppression (Fig. 2B-2C).

Fig. 1. A: Expression of miR-214-5p in HCC tissues and normal hepatic tissues. qRT-PCR analysis of miR-214-5p levels in 44 pairs of hepatocellular carcinoma and para-carcinoma tissues. Normal, para-carcinoma tissue. Cancer, hepatocellular carcinoma. *: $P < 0.05$; B: miR-214-5p was significantly lower in HepG2 and BEL-7402 than in Chang liver cells. *: $P < 0.05$.

Fig. 2. miR-214-5p inhibited cell invasion and migration. HepG2 and BEL-7402 were transfected with miR-214-5p mimic, mimic control, miR-214-5p inhibitor and inhibitor control. A: miR-214-5p expression was up-regulated in miR-214-5p mimic transfected cells but down-regulated in miR-214-5p inhibitor transfected cells; B: Overexpression of miR-214-5p inhibited cell invasion, but suppression of miR-214-5p promoted invasion in HepG2 and BEL-7402 cells; C: Overexpression of miR-214-5p inhibited cell migration, but suppression of miR-214-5p promoted migration in HepG2 and BEL-7402 cells.

miR-214-5p suppressed EMT in HCC cells

To explore the effect of miR-214-5p on EMT process, HepG2 and BEL-7402 were transfected with miR-214-5p mimic, miR-214-5p inhibitor and corresponding controls. The protein levels of EMT-related factors were detected by western blot. The results showed that miR-214-5p promoted E-cadherin expression but inhibited N-cadherin, Vimentin expression (Fig. 3).

WASL was a putative target of miR-214-5p

To explore the target gene of miR-214-5p, the biological information online analysis softwares (TargetScan, miRDB, and microrna) were used. WASL was confirmed to be a target gene of miR-214-5p. The luciferase reporter vector, which contained WASL 3'UTR fragments with wildtype or mutant, miR-214-5p complementary sites, is shown in Fig. 4A. The dual-luciferase reporter assay (Fig. 4B) demonstrated a significant difference in the luciferase activity among the miR-214-5p-NC + WASL-3'UTR, miR-214-5p-NC + WASL-3'UTR mut (the WASL-3'UTR was deleted), miR-214-5p mimics + WASL-3'UTR and miR-214-5p mimics + WASL-3'UTR mut groups ($P < 0.001$). Compared with the miR-214-5p-NC + WASL-3'UTR, miR-214-5p-NC + WASL-3'UTR mut and miR-214-5p mimics + WASL-3'UTR mut groups, the luciferase activity was significantly decreased in the miR-214-5p mimics + WASL-3'UTR group ($P < 0.05$). qRT-PCR revealed a significant difference in miR-214-5p expression among the four groups ($P < 0.001$). The expression of miR-214-5p mimics + WASL-3'UTR mut groups compared with the miR-214-5p-NC + WASL-3'UTR and miR-214-5p-NC + WASL-3'UTR mut groups (all $P < 0.05$). To explore the relationship between miR-214-5p and WASL, HepG2 was transfected with miR-214-5p mimic, miR-214-5p inhibitor and corresponding controls. qRT-PCR and western blot assays were used to examine the mRNA and protein levels of WASL in different groups. The results displayed that mRNA expression of WASL were

Fig. 3. miR-214-5p inhibited EMT process. HepG2 and BEL-7402 cells were transfected with miR-214-5p mimic, miR-214-5p inhibitor and corresponding controls. A and B: Western blot showed overexpression of miR-214-5p promoted E-cadherin expression but inhibited N-cadherin, Vimentin expressions in HepG2 and BEL-7402 cells. C and D: qRT-PCR showed overexpression of miR-214-5p promoted E-cadherin expression but inhibited N-cadherin, Vimentin expressions in HepG2 and BEL-7402 cells.

Fig. 4. WASL was a target of miR-214-5p. A: Schematic diagram of putative binding sites of miR-214-5p in the 3'UTR of WASL; B: Dual-luciferase reporter assay showed WASL is the target genes of miR-214-5p in human HepG2 cells; C and D: miR-214-5p negatively regulated the mRNA and protein expression of WASL in HepG2 cells.

Fig. 5. miR-214-5p inhibit cell invasion and migration by regulating WASL. A: Invasion ability of HepG2 and BEL-7402 cells 48 h after transfection was detected by Transwell chamber assay in each group; B: migration ability of HepG2 and BEL-7402 cells 48 h after transfection was detected by Transwell chamber assay in each group.

signally down-regulated by miR-214-5p overexpression but up-regulated by miR-214-5p inhibitor compared to corresponding controls (Fig. 4D). Western blot analysis with a similar trend to mRNA level of WASL (Fig. 4C).

miR-214-5p inhibit cell invasion and migration by regulating WASL

As shown by the invasion and migration assay (Fig. 5), remarkable significant differences were found in the invasion ability of HCC cells among the different groups 48 h after transfection ($P < 0.001$). Compared with the control, mimic control, Vector and miR-214-5p mimics + WASL groups, the invasion ability of cells in the miR-214-5p mimics and miR-214-5p mimics + Vector group were significantly decreased ($P < 0.05$). No significant difference was seen in the invasion ability of cells among the control, mimic control, Vector and miR-214-5p mimics + WASL groups (all $P > 0.05$).

Discussion

Invasion and metastasis has been well acknowledged as one of acquired capacities of cancer cells [25]. The exceedingly complex process involved many molecular mechanisms [26-28]. Among them, the proteins of the WASP family regulate Arp2/3-related pathway [29-31]. WASL, which belongs to WASP family, was found to be expressed in colorectal cancer liver metastases and esophageal squamous cell carcinoma [32-34]. WASL and WAVE-2 expression were shown to be extensive in HCC [35]. Researchers try to regulate the expression of WASP family proteins to inhibit the invasion of cancer cells [36-38]. Among them, microRNAs gain many attention for their function as gene expression regulators. Just recently, miR-214-5p has been found to influence tumor process [15]. However, the effect of miR-214-5p on HCC remains unknown.

In the present study, we found, by qRT-PCR that miR-214-5p was low expressed in HCC tissues compared with parental tissues, indicating that miR-214-5p might be involved in carcinogenesis of HCC. MiR-214-5p is lower expressed in cancer tissues than parental tissues in 44 pairs of HCC. Additionally, we overexpressed miR-214-5p in HepG2 and BEL-7402 cells and found miR-214-5p suppressed invasion and migration of HCC. Not only that, miR-214-5p also suppressed EMT, promoted expression of E-cadherin, and inhibited the expression of N-cadherin and Vimentin. By bioinformation software, we found WASL was a putative target of miR-214-5p. Then, we examined the correlation between miR-214-5p and WASL, and the results showed miR-214-5p could down-regulate the expression of WASL by qRT-PCR and Western blot. Dual-luciferase reporter gene analysis showed WASL is the target gene of miR-214-5p. The over-expression of miR-214-5p can inhibit invasion and migration of HCC, and targeting WASL activates this process. So, up-regulating the expression of WASL could

reverse the inhibition effect of miR-214-5p on HCC. These data indicated that miR-214-5p could inhibit invasion and migration through targeting WASL in HCC.

Taken together, our study demonstrated that miR-214-5p as a tumor suppressor that inhibited HCC cells invasion and migration and blocked the process by targeting WASL. These findings suggested that miR-214-5p might be considered as a putative target in the treatment of Hepatocellular carcinoma.

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Disclosure Statement

No conflict of interests exists.

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