miR-22 Suppresses Epithelial-Mesenchymal Transition by Modulating Snail and MAPK1 in Hepatocellular Carcinoma

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Abstract

**Background:** Recently studies have reported that miR-22 plays an important role in epithelial-mesenchymal transition (EMT) of many human cancers. However, the involvement of miR-22 in hepatocellular carcinomas (HCC) EMT progression has not been investigated.

**Methods:** We measured miR-22 expression level in 38 paired of HCC and matched normal tissues by real-time quantitative RT-PCR. Then, we performed morphological analysis and immunofluorescence to observe the role of miR-22 in HCC EMT progression. The expression of EMT markers were detected by real-time RT-PCR and western blot. The regulation role of miR-22 on Snail, mitogen-activated protein kinase 1 (MAPK1) and slug were determined by luciferase reporter assay. The expression of Snail and MAPK1 were also detected by real-time quantitative RT-PCR in HCC and normal tissues.

**Results:** We found that the expression of miR-22 in HCC tissues were much lower than that in normal control. The expression of miR-22 was inversely correlated with HCC metastatic ability. Then, we found that overexpression of miR-22 could inhibit HCC EMT. Importantly, miR-22 is found to inhibit cell motility by directly targeting both Snail and MAPK1. Furthermore, the suppression role of miR-22 in HCC EMT could be blocked by Snail and MAPK1 overexpression. Additionally, the expression of Snail and MAPK1 were inversely correlated with miR-22 expression in HCC tissues.

**Conclusion:** Our results suggested that miR-22 was downexpressed in HCC tissues and inhibited HCC EMT through downregulating Snail and MAPK1 which may provide a new bio-target for HCC therapy.

Background

Hepatocellular carcinoma (HCC) is one of the most common neoplasms worldwide. In China, about 466,100 cases are newly diagnosed and causes about 422,100 deaths in 2015 [1]. The high recurrence and low five-year survival rate of HCC is mainly due to the intrahepatic and extrahepatic metastases [2], and the rate of recurrence is 86.5% for intrahepatic metastasis and 13.5% for extrahepatic metastasis [3]. The epithelial-mesenchymal transition (EMT) plays a pivotal role in local invasion and distant metastasis during HCC progression [4]. However, the mechanism underlying the EMT of HCC is largely unknown.

Members of Snail family (Snail/Snail1 and Slug/Snail2) are critical inducers of EMT progression [5, 6]. The expression of Snail is closely associated with cancer metastasis [7] Slug was found to induce EMT progression by enhancing vimentin expression and migration in pre-malignant breast epithelial cells [8]. MAPK1 (ERK2) is an important member of MAPK/ERK pathway and known to regulate the transcription of target genes [9].

MiR-22 is a 22-nt non-coding RNA and originally identified in HeLa cells as a tumor-suppressing miRNA. Subsequently, miR-22 was identified to be ubiquitously expressed in a variety of tissues [10]. Recently, some paper reported that miR-22 suppresses EMT by targeting MMP, snail family and MAPK1 in many
cancers such as bladder, lung and gastric cancer, etc [11–13]. In this study, we determined whether miR-22 was involved in Snail and MAPK1 regulation and whether it participated in HCC EMT progress.

In this study, we first found that miR-22 could inhibit EMT in HCC cells. Then, we identified the regulation mechanism of miR-22 in EMT through the Snail and MAPK1 pathway. Thus, our data suggested important roles for miR-22 in HCC EMT and implicated miR-22 as a potential target for HCC therapies.

**Methods**

**HCC tissue specimens collection.**

Thirty-eight paired tissue specimens of HCC and adjacent non-tumor (ANT) tissues were obtained from Tangdu Hospital. Ethical approval was obtained from the Ethics Committee of the Fourth Military Medical University, and informed consent was obtained from each patient. The thirty-eight HCC tissues were divided into 2 groups: metastatic HCC tissues (patients with intrahepatic metastasis or portal vein cancer embolus, n = 22) and non-metastatic HCC tissues (n = 16). All the tissues were obtained at the time of surgery and immediately stored in liquid nitrogen until use.

**Cell Lines And Culture Conditions**

The HCC cell line MHCC-97H (HCC cells with high metastatic potential) was used in this study.[14] All cell lines were purchased from Shanghai Institute for Biological Sciences (Shanghai, China). All cell lines were routinely cultured in RPMI-1640 medium (Hyclone Laboratories, Logan, UT) supplemented with 10% fetal calf serum (Gibco BRL, Rockville, MD, USA) at 37 °C in a humidified atmosphere of 5% CO₂.

**Immunofluorescence**

Cells were seeded in 4-well 35-mm dishes (Greiner Bio-One North America Inc., Monroe, NC, USA) at a density of 1,000 cells/well and grown for 48 h in culture medium. Then cells were fixed in 4% paraformaldehyde for 20 min and permeabilized in phosphate-buffered saline (PBS) supplemented with 0.5% Triton X-100. After blocking, cells were incubated with the indicated antibodies for 2 h. Cells were washed in PBS, incubated with their corresponding FITC-labeled secondary antibodies (Pierce, Rockford, IL, USA) for 1 h at room temperature and stained with DAPI (Vector Labs, Burlingame, CA, USA). Finally, the cells were mounted using glycerol and observed using a Nikon A1 laser scanning confocal microscope (Tokyo, Japan).

**Western Blot**

Western blot
Cell samples were lysed with RIPA buffer (Beyotime, China). Equal amounts (10 µg) of total protein were loaded, and then subsequently immunoblotted with the primary antibodies, including anti-E-cadherin (BD Biosciences, Franklin Lakes, USA), Vimentin (Invitrogen, Carlsbad, CA, USA), Snail, MAPK1, slug and tubulin (Santa Cruz, CA, USA). Proteins were detected using the Amersham enhanced chemiluminescence system (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

**Real-time Rt-pcr**

Real-time RT-PCR was performed as described previously [15]. Expression data were uniformly normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, and the relative expression levels were evaluated using the ΔΔCt method [16, 17]. Primers were used as described previously [11, 18, 19].

**Vector Construction And Luciferase Reporter Assay**

The miR-22 overexpression vector was constructed according to previous [20]. Cultured cells were transfected with miR-22 expression vector, antisense miR-22 (anti-miR-22), scramble miRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The sequence were described as before [21].

The 3'UTR segment of Snail and MAPK1 were subcloned into the pmirGLO vector (Promega, Madison, WI, USA), respectively [11, 12]. The mutant constructs were generated using a QuickChange mutagenesis kit (Stratagene, La Jolla, CA, USA). All constructs were further confirmed by sequencing. Snail and MAPK1 overexpression plasmids were constructed as previous [19] Cell transfection and dual luciferase reporter assay were performed as described previously.

**Statistical Analysis**

All statistical analyses were performed using the SPSS statistical software package (SPSS, Chicago, IL, USA). The significance of the data was determined using Student's t test. All the statistical tests were two-sided, and a P value < 0.05 was considered significant.

**Results**

**The expression of miR-22 is downexpressed in HCC tissues**

The expression levels of miR-22 were first evaluated in thirty eight pairs of HCC and normal tissues by real-time RT-PCR. As shown in Fig. 1A, we found that the tumor tissues showed aberrant down-regulation of miR-22 compared with adjacent nontumor tissues (P< 0.05). Furthermore, the expression of miR-22 in
metastatic HCC tissues was much lower than in no metastatic HCC tissues (Fig. 1B) which indicated that the miR-22 expression was negatively correlated with the HCC metastatic ability.

**miR-22 inhibits EMT in HCC cells.**

Morphological analysis showed that MHCC-97H cells transfected with miR-22 expression plasmid exhibited a lower number of mesenchymal cells (Fig. 2A). Then, the epithelial and mesenchymal markers were detected. We performed an immunofluorescence using E-cadherin and Vimentin as epithelial and mesenchymal markers, respectively. As shown in Fig. 2B, the expression of E-cadherin was upregulated, and the mesenchymal marker Vimentin was downregulated after transfection of miR-22 compared with that of control. Furthermore, we observed a increase of E-cadherin at both the protein and mRNA levels transfected with miR-22 in response to negative control (Fig. 2C, 2D). On the contrary, Vimentin was decreased in protein and mRNA (Fig. 2C, 2D). Altogether, these results demonstrate that miR-22 inhibits EMT in HCC cells.

**miR-22 regulates Snail, MAPK1 and slug expression to inhibit EMT.**

Because previous studies have shown that Snail, MAPK1 and slug participate in EMT progress, we explored whether miR-22 affects EMT progress by modulating them expression. As shown in Fig. 3A and 3B, Snail, MAPK1 and slug protein and mRNA expression levels decreased after transfected with miR-22. So, we speculated that miR-22 might inhibit HCC EMT through regulating Snail, MAPK1 and slug passway. Next, we want to explore whether miR-22 directly bind to Snail, MAPK1 and slug 3’UTR to regulate them expression.

**Snail and MAPK1 are direct targets of miR-22.**

To verify whether miR-22 directly targeted Snail, MAPK1 and slug, luciferase reporter assays were conducted. We constructed the genes 3’UTR/pmirGLO plasmids and the miR-22 binding sites mutation pmirGLO plasmids (Fig. 4A, 4B and 4C). Co-transfection of MHCC-97H cells with Snail-3’UTR/pmirGLO and miR-22/pcDNA3.1 caused a 59.3% decrease in the luciferase activity compared with the negative control \((P < 0.05)\). This suppression was rescued by the four-nucleotide substitution in the core binding sites (Fig. 4A). The similar effect was also found transfected with MAPK1-3’UTR/pmirGLO and miR-22/pcDNA3.1 (58.3% decrease compared with the negative control, \(P < 0.05\), Fig. 4B). With the binding sites mutation plasmid the suppression was rescued (Fig. 4B). All these results indicated that miR-22 exerts inhibitory effects on Snail and MAPK1 expression via directly binding to the 3’UTR of Snail and MAPK1.

We also performed the same luciferase reporter assay cotransfected with the slug-3’UTR/pmirGLO and miR-22/pcDNA3.1 in MHCC-97H cells. There was no decrease in the luciferase activity compared with the negative control \((P > 0.05,\) Fig. 4C). The similar result was found in transfection with mutation plasmid \((P > 0.05,\) Fig. 4C). So, we found that there was no direct binding of miR-22 to slug 3’UTR. Our above results showed that miR-22 decreased the slug expression in mRNA and protein levels. Taken together, we
speculated that there was a regulation mediator factor between miR-22 and slug. Preview studies revealed that the MAPK1 could regulate the slug expression [22]. Therefore, our results indicated that miR-22 exerts inhibitory effects on slug expression via regulating the MAPK1 expression. Thus, miR-22 regulates Snail, MAPK1 and slug expression to inhibit EMT.

**miR-22 inhibits EMT in HCC cells via regulation of Snail and MAPK1.**

To confirm the role of miR-22 in HCC EMT via its regulation on Snail and MAPK1 expression, we upregulated Snail and MAPK1 expression through transfecting overexpression plasmids to block miR-22 regulation. As expected, transfection of the miR-22 expression plasmid resulted in decreased Snail expression. By contrast, resuming Snail expression eliminated the downregulation induced by miR-22. The EMT markers E-cadherin and vimentin were also detected (Fig. 5A). Our results showed that the inhibition role of miR-22 on EMT was blocked by Snail overexpression. The real-time RT-PCR also confirmed the western blot results (Fig. 5B). The similar results were found transfected with MAPK1 overexpression plasmid (Fig. 5C and 5D). Our results indicated that miR-22 significantly inhibited HCC EMT by directly downregulating Snail and MAPK1 expression. Altogether, we identified the regulation mechanism of miR-22 in EMT through the Snail and MAPK1 pathway. A summary diagram that outlines the above-described regulatory network is shown in Fig. 6.

**The expression of miR-22 were inversely correlated with Snail and MAPK1 expression in HCC tissues.**

Furthermore, we also detected the Snail and MAPK1 expression in 38 pairs of HCC and normal tissues by real-time quantitative RT-PCR. The miR-22 expression was inversely correlated with Snail and MAPK1, respectively (correlation coefficient \( r = -0.5355, R \text{ squared} = 0.2868; r = -0.6124, R \text{ squared} = 0.3751; \) Fig. 7A and 7B). This correlation indicates that miR-22 could negatively regulate Snail and MAPK1 expression in HCC tissues.

**Discussion**

In our study, we found that the expression of miR-22 in HCC tissues were much lower than that in normal control. The expression of miR-22 was inversely correlated with HCC metastatic ability. Overexpression of miR-22 could inhibit HCC EMT through directly targeting both Snail and MAPK1. The expression of Snail and MAPK1 were inversely correlated with miR-22 expression in HCC tissues. Our study presents the regulation mechanism of miR-22 in HCC EMT through the Snail/MAPK1 pathway for the first time.

Hepatocellular carcinoma is one of the most frequently occurring cancers with poor prognosis. Since clinical symptoms are not easily observed during the early stage, the prognosis is poor at the time of diagnosis, which, in most cases, is during the advanced stage [23]. Thus, it is of much significance to explore new diagnostic and therapeutic molecular targets for HCC. miRNAs have been demonstrated to have close relationship with HCC. miR-22, originally identified in HeLa cells, has been found to be overexpressed in prostate cancer, but down-regulated in breast cancer, cholangiocarcinoma, multiple myeloma, and hepatocellular carcinoma [24]. miR-22 inhibits cell growth and induces cell-cycle arrest,
apoptosis and senescence in breast cancer, colon cancer and lung cancer [25]. In our study, we found that the tumor tissues showed aberrant down-regulation of miR-22 compared with adjacent nontumor tissues. Furthermore, the expression of miR-22 in metastatic HCC tissues was much lower than in no metastatic HCC tissues.

EMT is a key process driving cancer metastasis and the loss of E-cadherin and increase in Vimentin expression are considered to be the most important molecular markers of EMT. Recent studies have revealed that miRNAs act as crucial modulators of EMT through the regulation of E-cadherin and other molecules such as Vimentin and ZEB [26]. In this study, we found that transfected with miR-22 expression plasmid exhibited a lower number of mesenchymal cells. The expression of E-cadherin was upregulated, and the mesenchymal marker Vimentin was downregulated after transfection of miR-22 compared with that of control. These findings demonstrated that miR-22 inhibits EMT in HCC cells.

Next, we explored the underlying mechanisms involved in the regulation of EMT by miR-22. Members of Snail family (Snail/Snail1 and Slug/Snail2) are critical inducers of EMT progression. In the present study, we found that the expression of miR-22 were inversely correlated with Snail and MAPK1 expression in HCC tissues. Our results also shown that Snail and MAPK1 are negatively regulated by miR-22 at the posttranscriptional level, via a specific target site within the 3'UTR. So, we identified the regulation mechanism of miR-22 in EMT through the Snail and MAPK1 pathway. Currently, the emergence of new technologies that use synthetic miRNA mimics or anti–miRNA oligonucleotides holds great promise for clinical miRNA therapy [27]. Synthetic miR-22 mimic treatments for cancer will become a significant scientific and therapeutic challenge.

In conclusion, miR-22 down-expressed in HCC tissues and the overexpression of miR-22 inhibited EMT in HCC cells. Snail and MAPK1 contain the binding sites for miR-22 and are negatively regulated by miR-22, respectively. Down-regulation of Snail and MAPK1 mediated the inhibition EMT function of miR-22. This newly identified miR-22/Snail/MAPK1 link provides a new, potential therapeutic target to treat HCC.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| HCC          | hepatocellular carcinomas |
| ANT          | adjacent nontumor tissues |
| EMT          | Epithelial-mesenchymal transition |
| miRNA        | microRNA |
| ECM          | extracellular matrix |
| UTR          | extracellular matrix |

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Declarations

Ethics approval and consent to participate

This study was conducted with the approval of the Ethics Committee of Tangdu Hospital, Fourth Military Medical University.

Consent for publication

Not applicable.

Availability of data and material

All the data supporting our findings can be found in the “Results” section of the paper.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

CGL carried out the molecular genetic studies, animal studies and drafted the manuscript. ZQ participated in the histologic study. XHL participated in cell biology experiments. CGL performed the statistical analysis. LMK conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figures
miR-22 is down-expressed in HCC tissues. (A) Analysis of the expression pattern of miR-22 in adjacent non-tumor and HCC tissues using real-time RT-PCR. (B) The expression of miR-22 in non-metastasis tumors and metastasis tumors of HCC were detected by real-time RT-PCR. Student's t test was used to analyze the significant differences, *, P < 0.05.
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Figure 2

miR-22 inhibits EMT in HCC cells. (A) In MHCC-97H cells transfected with miR-22 overexpression plasmid, morphological changes were observed under a light microscope. Scale bars, 50 μm. (B) Expression of E-cadherin and Vimentin in MHCC-97H cells with miR-22 overexpression were examined by immunofluorescence. Fluorescence was observed by confocal laser-scanning microscopy. (C) and (D) The E-cadherin, Vimentin protein and mRNA levels were detected by western blot and real-time RT-PCR after overexpression of miR-22 in MHCC-97H cells. *, P < 0.05, **, P < 0.01.
miR-22 inhibits EMT in HCC cells. (A) In MHCC-97H cells transfected with miR-22 overexpression plasmid, morphological changes were observed under a light microscope. Scale bars, 50 μm. (B) Expression of E-cadherin and Vimentin in MHCC-97H cells with miR-22 overexpression were examined by immunofluorescence. Fluorescence was observed by confocal laser-scanning microscopy. (C) and (D) The E-cadherin, Vimentin protein and mRNA levels were detected by western blot and real-time RT-PCR after overexpression of miR-22 in MHCC-97H cells. *, P < 0.05, **, P < 0.01.
miR-22 regulates Snail, MAPK1 and slug expression in HCC cells. (A) The protein expression levels of Snail, MAPK1 and slug were measured in MHCC-97H cells transfected with miR-22 plasmid by western blot. (B) mRNA expression levels of Snail, MAPK1 and slug as above were detected by real-time RT-PCR.
Figure 3

miR-22 regulates Snail, MAPK1 and slug expression in HCC cells. (A) The protein expression levels of Snail, MAPK1 and slug were measured in MHCC-97H cells transfected with miR-22 plasmid by western blot. (B) mRNA expression levels of Snail, MAPK1 and slug as above were detected by real-time RT-PCR.
Figure 4

Snail and MAPK1 are direct targets of miR-22. (A) and (B) Upper panel, predicted duplex formation between Snail and MAPK1 3’UTR and miR-22. Diagram of the luciferase reporter plasmids: plasmid with the full length Snail and MAPK1 3’UTR insert and plasmid with a mutant Snail and MAPK1 3’UTR which carried a substitution of four nucleotides within the miR-22 binding site, respectively. Lower panel, the relative luciferase activity in MHCC-97H cells were determined after the Snail and MAPK1 3’UTR or mutant plasmids were co-transfected with miR-22 overexpression vector or negative control, respectively. (C) The relative luciferase activity in MHCC-97H cells were determined after the slug 3’UTR or mutant plasmids were co-transfected with miR-22 overexpression vector or negative control. *, P < 0.05.
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Figure 5

miR-22 inhibits EMT in HCC cells via regulation of Snail and MAPK1. (A) and (C) Snail, MAPK1, E-cadherin and Vimentin expression in HCC cells treated with miR-22 or miR-22 + Snail and MAPK1 overexpression plasmids by Western blotting. (B) and (D) Real-time RT-PCR analyses of Snail, MAPK1, E-cadherin and Vimentin mRNA levels as above were transfected. *, P < 0.05.
Figure 5

miR-22 inhibits EMT in HCC cells via regulation of Snail and MAPK1. (A) and (C) Snail, MAPK1, E-cadherin and Vimentin expression in HCC cells treated with miR-22 or miR-22 + Snail and MAPK1 overexpression plasmids by Western blotting. (B) and (D) Real-time RT-PCR analyses of Snail, MAPK1, E-cadherin and Vimentin mRNA levels as above were transfected. *, P < 0.05.
Figure 6

A schematic representation of the major molecular mechanism of miR-22 inhibits EMT through the Snail and MAPK1 pathway.
Figure 6

A schematic representation of the major molecular mechanism of miR-22 inhibits EMT through the Snail and MAPK1 pathway.
The expression of miR-22 were inversely correlated with Snail and MAPK1 expression in HCC tissues. (A) and (B) Analysis for correlation of miR-22 mRNA and Snail and MAPK1 mRNA expression in HCC tissues, respectively.