Downregulation of miR-552 in hepatocellular carcinoma inhibits cell migration and invasion, and promotes cell apoptosis via RUNX3

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Abstract. Research conducted previously has indicated that microRNAs (miRs) have potential effects on the pathogenesis of hepatocellular carcinoma (HCC). The biological functions of miR-552 have been well documented in colon cancer; however, the role of miR-552 in HCC remains unclear. The present study evaluated the effects of miR-552 in HCC physiology, using HCC cell lines as model. An miR-552 inhibitor was transfected into HCC cell lines to knock down the expression of miR-552. Reverse transcription-quantitative PCR and western blot analysis were used to detect the expression of miR-552 and Runt-related transcription factor 3 (RUNX3), respectively. MTT assay was used to analyze cell viability, whilst Transwell and wound-healing assay were used to investigate cell migration. Flow cytometry was performed to measure cell apoptosis. The direct association between RUNX3 and miR-552 was evaluated using dual luciferase reporter assay. The expression of miR‑552 was significantly elevated in HCC tumor tissues compared with the adjacent healthy samples. Additionally, transfection with the miR-552 inhibitor decreased cell viability and migration. miR-552 knockdown also increased HCC cell apoptosis in vitro. In conclusion, these results suggest that miR-552 has an oncogenic function in HCC and is a potential biomarker for detecting HCC.

Introduction

The majority of hepatocellular carcinoma (HCC) cases occur in developing countries, and it is the most commonly diagnosed cancer in patients <60 years of age, particularly in males (1,2). One of the primary reasons for the high HCC mortality rate is late diagnosis. Although advances have been made in therapeutic strategies and surgical procedures, prognosis for patients with HCC remains unsatisfactory (3,4). Therefore, potential biomarkers of HCC are required to improve early detection and prognostic assessment.

MicroRNAs (miRNAs/miRs) are well conserved, small RNA molecules consisting of 18-22 nucleotides that do not encode proteins. The earliest report of an association between miRNAs and cancer was found in 2002 (5). In particular, certain miRNAs have been found to be upregulated in the serum or plasma samples in patients with HCC, including miR -21, miR-199 and miR -221, whilst others such as miR -122 have been found to be downregulated (6,7). These previous findings suggest that they can serve as potential biomarkers for the early diagnosis of HCC. Previous studies have reported that miR-142-3p inhibits cell viability and aerobic glycolysis (8) whereas miR -199 suppressed cell viability, migration and invasion in HCC (9), suggesting that the miRNAs serve roles in HCC physiology. One particular miRNA, miR-552, has been previous demonstrated to serve crucial roles in cell viability and migration of colorectal cancer, by targeting a number of mRNAs, including dachshund family transcription factor 1 and a disintergin and metalloprotease family member 28 (10,11). However, to the best of our knowledge, no research has investigated the role of miR-552 in HCC.

Run-related transcription factor 3 (RUNX3) is a tumor suppressor gene that regulates gene expression associated with cell viability and metastasis (12). Emerging evidence has indicated that RUNX3 is expressed in different types of cancer. It has been reported that miR-20a directly targeted RUNX3 expression, whereas miR-186 reversed RUNX3-induced inhibition of HCC cell metastasis, demonstrating functional interactions between miRNAs and RUNX3 (13); however, to the best of our knowledge, the association between RUNX3 and miR-552 has not been investigated previously.

In the present study, the role of miR-552 in HCC cell lines was investigated in vitro to determine whether an interaction between miR-552 and RUNX3 exists in this system. The
results of the present study may be useful in providing a potential biomarker for detection of HCC.

Materials and methods

Clinical samples. Fresh HCC and adjacent normal liver tissues were collected from patients that underwent hepatectomy at The First Affiliated Hospital of Xinjiang Medical University (Urumqi, China) from January 2017 to June 2018. Inclusion and exclusion criteria were as follows: i) The patients, including men and women, were 18-75 years old and all pathologically demonstrated to exhibit hepatocellular carcinoma; ii) no distant metastasis; iii) no chemotherapy prior to surgery. A total of 15 patients aged 27-74 years old, with 12 male and 3 female patients, were enrolled in the current study. The present study was approved by the Ethics Review Committees of The First Affiliated Hospital of Xinjiang Medical University and performed in accordance with Declaration of Helsinki. All patients provided informed consent.

Cell lines and culture. PLC/PRF/5 and Huh-7 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences. Huh-7 cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. PLC/PRF/5 cells cultured in minimum essential medium (MEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. All cells were maintained in a humidified atmosphere maintained at 37°C with 5% CO2.

Cell transfection. To knock down endogenous miR-552 expression, PLC/PRF/5 and Huh-7 cells, at ~30-50% confluence, were transfected with 20 μM miR-552 inhibitor (cat. no. miRB0026615-2-1; Guangzhou Ribobio Co., Ltd.) or miR-552 negative control (miR-NC; cat. no. miR2N0000001-1-5; Guangzhou Ribobio Co., Ltd.) for 48 h using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The transfected cells were performed to extract RNA, proteins or to detect cell viability, migration, apoptosis and luciferase activity immediately. Untransfected cells served as an additional negative control (NC).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from cells and tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. RNA quality and quantity were determined using Qubit™ 4 fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription and isolation of cDNA was performed using HiScript® II or III RT supermix plus gDNA wiper (Vazyme) according to the manufacturer's protocols. The levels of miR-552 and RUNX3 expression were measured using specific primers and SYBR® Green fluorophore probes (Vazyme). The sequences of primers used in this study are presented in Table 1. Relative expression was quantified by using the 2-ΔΔCq method (14). GAPDH and U6 were used as internal controls for mRNA and miRNA expression, respectively. The thermocycling conditions were as follows: Initial denaturation (50°C for 2 min), and enzyme denaturation (95°C for 10 min) followed by 40 cycles of denaturation (95°C for 15 sec), annealing (60°C for 30 sec), elongation (70°C for 1 min) and a final extension (72°C for 10 min).

Western blot analysis. Total protein was extracted from the PLC/PRF/5 and Huh-7 cells using cell lysis buffer (RIPA; Thermo Fisher Scientific, Inc.) containing protease inhibitors. Protein concentration was quantified using bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc.). Total protein (40 μg per lane) were separated by SDS-PAGE (10% for 30-205 kDa proteins; 12% for 14-66 kDa proteins) and subsequently transferred to polyvinylidene difluoride membranes (EMD Millipore). The PVDF membranes were blocked using 5% (m/v) skim milk powder (Thermo Fisher Scientific, Inc.) dissolved in TBST (Tris Buffered Saline Tween-20; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The PVDF membranes were incubated with specific primary antibodies (caspase-3; 1:1,000; cat. no. 9662; bcl-2; 1:1,000; cat. no. 4223; bax; 1:1,000; cat. no. 5023; RUNX3; 1:1,000; cat. no. 9647; All antibodies were purchased from Cell Signaling Technology) for 12 h at 4°C, before subsequent incubation with corresponding HRP-linked anti-rabbit secondary antibody (1:2,000; cat. no. 7074; Cell Signaling Technology) for 1 h at room temperature. Protein bands were visualized using Immobilon Western Chemiluminescent HRP substrate (Merck KGaA). Densitometric analysis was performed using ImageJ v1.8.0 (National Institutes of Health).

Cell viability analysis using MTT assay. PLC/PRF/5 and Huh-7 cells (1.5x10⁴ cells/ml) were seeded into 96-well plates with 100 μl medium/well and were allocated into three separate groups (miR-552 inhibitor, miR-552 NC and NC) and cell viability was measured after 24, 48 and 72 h. MTT solution was added at a concentration of 20 μl/well prior to incubation at 37°C for 4 h. Following incubation, 150 μl dimethylsulfoxide was added to each well to dissolve the formazan crystals and the absorbance values at 490 nm were detected using the Thermo Multiskan™ FC Microplate Reader for each well (Thermo Fisher Scientific, Inc.).

Cell migration analysis by wound-healing assay. PLC/PRF/5 and Huh-7 cells were first transfected with either miR-552 inhibitor or miR-NC and subsequently seeded at ~70-80% confluence into six-well plates and cultured to confluence. Following the attainment of 100% confluence, wounds were created by scratching using 200 μl pipette tips. The wound monolayers of PLC/PRF/5 and Huh-7 were cultured in serum-free MEM (Gibco; Thermo Fisher Scientific, Inc.) and 1640 culture medium (Gibco, Thermo Fisher Scientific) respectively. Cells were then treated with mitomycin c (10 μg/ml; Selleck Chemicals) for 30 min. The wound area was recorded using light microscopy (scale bar, 200 μm for the wound healing assay; 100 μm for the Transwell assay) at 0 and 24 h.

Transwell assay. PLC/PRF/5 and Huh-7 cell migration was measured using Transwell® chambers (8-μm pore size; Corning Inc.). PLC/PRF/5 and Huh7 cells (4x10⁴ cells/ml) were diluted, respectively, in 100 μl of MEM and 100 μl of 1640 culture medium supplemented with 10% FBS (transfected with miR-552 inhibitor, transfected with miR-NC and NC) and were seeded into the upper chamber; MEM or 1640 medium supplemented with 20% FBS was added into lower chamber. Following 12-h incubation, cells that
migrated through the membrane and adhered to the bottom of the chamber were stained using crystal violet and counted under a light microscope at x100 magnification (5 field of views).

Cell apoptosis assay. PLC/PRF/5 and Huh-7 cells (4x10^5 cells/ml) were seeded into six-well plates. Following adherence, cells were transfected with either miR-552 inhibitor or miR-552 NC in addition to NC. PLC/PRF/5 cell apoptosis was measured using Annexin V-FITC/7-AAD Apoptosis Detection kit (E-CK-A212; Elabscience Biotechnology Inc.) according to manufacturer's protocol. Huh-7 cell apoptosis was measured using Annexin V-FITC/PI apoptosis detection kit (Beijing Solarbio Science & Technology Co., Ltd.). Cell apoptosis detected using BD FACSCanto II (BD Biosciences). Viable cells were indicated by FITC-Annexin V and 7-AAD negative (left lower). Cells that were in early apoptosis were indicated by FITC-Annexin V-positive and 7-AAD negative (right lower). Cells that were in end stage apoptosis were indicated by FITC-Annexin V-positive and 7-AAD positive (right lower). Cells that were in early apoptosis were indicated by FITC-positive and 7-AAD positive (right up and left up). Viable Huh7 cells FITC-Annexin V and PI negative cells are indicated (left lower); Cells that are in early apoptosis are FITC-Annexin V-positive and PI negative (right lower); Cells that are in end stage apoptosis and death are FITC positive and PI positive (right up and left up). Cell apoptosis was detected using BD FACSCanto II (BD Biosciences) (15,16); (Figs. 4 and 5).

Dual luciferase reporter assay. To validate that RUNX3 mRNA is a target of miR-552 (miR-552 was validated using mirbase.org: Accession no. MIMAT0003215), a wild-type (WT) or a mutated (MUT) fragment of the human RUNX3 3'-untranslated region (3'-UTR) sequence encoding a potential miR-552 binding site was amplified using RT-qPCR. Subsequently, the WT and MUT 3'-UTR of RUNX3 was cloned into the pMIR-Report vector (BioVector NTCC, Inc.). PLC/PRF/5 cells were seeded in 96-well plate at a density of 1x10^5 cells/ml. A total of 1 µg pSicoR/miR-552 (EK-Bioscience Biotechnology Co., Ltd.) or 1 µg pSicoR/miR-NC (EK-Bioscience Biotechnology Co., Ltd.) was co-transfected with 0.5 µg pMIR-Report/RUNX3 WT or 0.5 µg pMIR-Report/RUNX3 MUT group, respectively. All plasmids were diluted into 25 µl diluted buffer (MEM). A total of 25 µl of MEM was added into pMIR-report/RUNX3 WT or MUT groups and was used as a control for the buffer. A total of 0.2 µg pRL-TK (EK-Bioscience Biotechnology Co., Ltd.) plasmids were added in each group to normalize the luciferase activity. Plasmids were transfected into cells using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Relative luciferase activity was detected by measuring relative activity of firefly luciferase unit at 48 h using a Dual-Luciferase Reporter assay kit (Promega Corporation). Luciferase activity= luc (firefly luciferase)/R-luc (Renin luciferase).

Statistical analysis. Experiments were performed in triplicate, with results presented as the mean ± standard deviation. The level of significance between two groups was determined using Student's t-test (used to analyze differences between tumor and adjacent tissues) and one-way ANOVA followed by Turkey's multiple comparisons for multiple group comparisons. Analyzes were performed using GraphPad Prism 6 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-552 is upregulated in HCC tissues and cell lines. However, an association between the endogenous miR-552 and HCC cell lines has not been reported. Therefore, in the present study the relative expression of miR-552 in HCC tumor tissues was validated using RT-qPCR. Compared with adjacent normal liver tissues, tumor tissues exhibited significantly higher miR-552 expression (Fig. 1).
Figure 2. Effect of miR-552 knockdown in PLC/PRF/5 cells. (A) miR-552 expression in PLC/PRF/5 cells following transfection with miR-552 inhibitor or its corresponding NC was analyzed using reverse transcription-quantitative PCR. (B) Cell viability measurements after 24, 48 and 72 h using MTT assay. (C) Cell migration as measured using wound-healing assays (scale bar, 200 µm). (D) Cell migration as measured using Transwell assays (scale bar, 100 µm). *P<0.05, **P<0.01 and ***P<0.001 vs. NC group. miR, microRNA; NC, negative control.

Figure 3. Effect of miR-552 knockdown in Huh-7 cells. (A) miR-552 expression in Huh-7 cells following transfection with miR-552 inhibitor or its corresponding NC was analyzed using reverse transcription-quantitative PCR. (B) Cell viability measurements after 24, 48 and 72 h using MTT assay. (C) Cell migration as measured using wound-healing assays (scale bar, 200 µm). (D) Cell migration as measured using Transwell assays (scale bar, 100 µm). *P<0.05, **P<0.01 and ***P<0.001 vs. NC group. miR, microRNA; NC, negative control.
Transfection efficiency of miR-552 inhibitor in HCC cell lines. In order to determine the role of miR-552 in HCC, PLC/PRF/5 and Huh-7 cells were transfected with a miR-552 inhibitor or corresponding miR-NC. miR-552 expression was significantly downregulated in cells transfected with miR-552 inhibitor compared with the miR-NC or NC group, whilst no significant differences were observed between the miR-NC and NC groups (Figs. 2A and 3A). These results suggest that miR-552 levels were successfully reduced by the miR inhibitor.

miR-552 knockdown reduces HCC cell viability. Viability of PLC/PRF/5 and Huh-7 cells was analyzed using MTT assay. miR-552 knockdown significantly decreased PLC/PRF/5 and Huh-7 cell viability compared with miR-NC and NC cells after 48 and 72 h (Figs. 2B and 3B) whilst no significant differences were observed between the miR-NC and NC groups. These results suggest that miR-552 regulated HCC cell viability.

miR-552 knockdown reduces HCC cell migration. Wound healing and Transwell assays were performed to determine the effect of miR-552 on HCC cell migration. miR-552 knockdown significantly inhibited PLC/PRF/5 (Fig. 2C and D) and Huh-7 migration (Fig. 3C and D) compared with the miR-NC group. These observations suggest that miR-552 promoted HCC cell migration.

Effect of miR-552 knockdown on HCC apoptosis. Bax, Bcl-2 and caspase-3, proteins associated with cell apoptosis, were measured to investigate the effects of miR-552 knockdown on HCC cell apoptosis. Flow cytometry analysis demonstrated that PLC/PRF/5 cells transfected with miR-NC or NC exhibited a low quantity of Annexin V-positive cells (Fig. 4A and B). However, apoptosis was significantly increased in cells transfected with the miR-552 inhibitor. Western blot analysis demonstrated that transfection with the miR-552 inhibitor increased the expression of caspase-3 and Bax compared with the miR-NC group and NC cells whilst significantly reducing Bcl-2 expression in PLC/PRF/5 cells (Fig. 4C and D). Similar results were obtained using Huh-7 cells (Fig. 5). These results suggest that miR-552 inhibits HCC cell apoptosis.

RUNX3 is directly targeted by miR-552 in HCC. Further analysis was performed to determine whether miR-552 regulates the expression of RUNX3, a protein previously found to be involved in the regulation of HCC physiology (17). Western blot and RT-qPCR analyzes revealed that miR-552 knockdown increased the expression of RUNX3 in PLC/PRF/5 (Fig. 6A and B) and Huh-7 cells (Fig. 6C and D). To confirm whether RUNX3 was directly targeted by miR-552, dual luciferase assay was performed. The transfection of miR-552 mimic was efficient in PLC/PRF/5 (Fig. 7A). miR-552 mimic or miR-NC with plasmids containing 3’untranslated regions (UTR) of
Figure 5. miR-552 knockdown induces apoptosis in Huh-7 cells. (A) Representative flow cytometry dot plots of Huh-7 cells transfected with miR-552 inhibitor or miR-NC and stained using Annexin V-FITC/PI. Viable Huh7 cells FITC-Annexin V and PI negative cells are indicated (left lower). Cells that are in early apoptosis are FITC-Annexin V-positive and PI negative (right lower). Cells that are in end stage apoptosis and death are FITC positive and PI positive (right up and left up). (B) Quantitative analysis of cell apoptosis rates. Caspase-3, Bax and Bcl-2 expression levels were (C) evaluated using western blot analysis and (D) quantified. *P<0.05, **P<0.01, ***P<0.001 vs. NC group. miR, microRNA; NC, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Figure 6. RUNX3 is regulated by miR-552. (A) Western blot and (B) reverse transcription-quantitative PCR analysis to detect the expression of RUNX3 protein and mRNA following miR-552 knockdown in PLC/PRF/5 cells, respectively. (C) Western blot and (D) reverse transcription-quantitative PCR analysis to detect the expression of RUNX3 protein and mRNA following miR-552 knockdown in Huh-7 cells, respectively. **P<0.01, ***P<0.001 vs. NC group. miR, microRNA; RUNX3, runt-related transcription factor 3; NC, negative control.
wt-RUNX3 or mut-RUNX3 were transfected into PLC/PRF/5 cells. miR-552 mimics and mimic-NC were transfected into RUNX3 wild-type and mutant reporter plasmids and subjected to luciferase activity, which exhibited that RUNX3 luciferase activity in WT instead of mutant group significantly reduced, compared with the control (Fig. 7B and C). Therefore, RUNX3 was directly targeted and downregulated by miR-552 in PLC/PRF/5 cells.

Discussion

HCC is the primary cancer of the liver (18). Although advances in HCC therapy have been made, overall survival has not improved over the previous decades (3). HCC is commonly accompanied by cirrhosis, which is associated with poor prognosis (19). miRNAs are involved in the post-transcriptional regulation of gene expression by binding to 3'-UTRs of target mRNAs, resulting in degradation of the mRNA targets (20). Aberrant miRNA expression is associated with a number of molecular pathways including cyclin-dependent kinases, Bel-2 family proteins, matrix metalloproteinase and the phosphatidylinositol 3-kinase (PI3K)-Akt kinase signaling pathway, and biological processes involved in the pathogenesis of HCC, including cell viability, apoptosis, angiogenesis and metastasis (21,22).

miR-552 expression is significantly increased in colon cancer and enhances proliferation, migration and clonogenicity (11). As the abnormal expression of miR-552 and subsequent downstream effects have been previously reported in colon, breast and lung cancer (23,24), it was hypothesized that miR-552 may also have an effect in HCC. In the present study, since aberrant expression of miR-552 was detected in HCC tissues and liver cancer cell lines, the role of miR-552 in HCC was investigated in more depth. To the best of our knowledge, the present study is the first report to demonstrate that miR-552 regulates cell viability, migration, invasion and apoptosis in HCC cell lines, suggesting that miR-552 can be used as a potential biomarker for HCC diagnosis and determining prognosis. A limitation of the present study is insufficient sample size for the validation of miR-552 as a biomarker for diagnosis and prognosis, and the lack of a correlation analysis between HCC and miR-552 expression, including age, gender, tumor size, lymph node and distance metastases, clinical stage and histological grade. The expression of miR-552 in the blood can be as a potential biomarker for the early diagnosis and prognosis of HCC. Larger sample sizes will need to be collected for further studies.

Caspase-3 is an important apoptotic mediator involved in endogenous and exogenous apoptotic pathways (25). Caspase-3 activation by the mitochondrial release of cytochrome c is a key mechanism that mediates cell apoptosis (26). Overexpression of Bcl-2 inhibits the dimerization of Bax which in turn inhibits the expression of cytochrome c, reducing caspase-3 activation and subsequent cell apoptosis (27). In the present study, inhibition of miR-552 significantly decreased the expression of Bcl-2 and increased the expression of caspase 3 and Bax. These results suggest that the inhibition of miR-552 may promote apoptosis by activating the mitochondrial apoptosis pathway.

In a previous meta-analysis, a screen for potential biomarkers in HCC was conducted, which resulted in identification of 17 aberrantly methylated genes (28). Within this list, RUNX3 DNA methylation was found to be significant higher in HCC tissues and serum of patients with HCC compared with normal controls (28). The findings of the present study demonstrated that miR-552 directly targeted RUNX3 mRNA, resulting in downstream effects on cell viability, metastasis and apoptosis. However, further research is required to validate the mechanism of miR-552 in HCC cell lines.

Taken together, the present study indicated that miR-552 may have oncogenic properties in HCC by enhancing cancer cell viability, migration and invasion whilst reducing cell apoptosis. miR-552 directly targeted RUNX3 in HCC and this mechanism may be involved in the mode of action of miR-552 and subsequent biological effects.

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MicroRNA -199 suppresses cell proliferation, \( \beta \)

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YM designed the design, drafted manuscript, performed the experiments and participated in data analysis. MM and LM collected the experimental samples and performed RT-qPCR, western blotting and cell apoptosis. XM and YL analyzed the data. XM also contributed to the study design and revised the manuscript critically. FZ produced the figures, performed data analysis and picture production, performed literature research for this investigation which is essential for the methods establishment and revised the draft manuscript. All authors have approved to publish the manuscript.

Patient consent for publication

All patients provided informed consent and approved the publication of data.

Availability of data and materials

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

Ethics approval and consent to participate

The present study was approved by the Ethics Review Committees of The First Affiliated Hospital of Xinjiang Medical University and performed in accordance with Declaration of Helsinki.

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