

MicroRNA-143 inhibits tumorigenesis in hepatocellular carcinoma by downregulating GATA6

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Abstract. MicroRNAs serve a critical role in human hepatocellular carcinoma (HCC) progression. However, the exact role of microRNA-143 (miR-143) in HCC remains unclear. The current study investigates the molecular mechanism of miR-143 in HCC. In cultured HepG2 and Bel7402 cell lines, miR-143 levels were raised by lentivirus transduction. This significantly inhibited HCC progression in terms of cell invasion and proliferation in both HepG2 and Bel7402 cell lines ($P < 0.05$). MiR-143 also significantly decreased tumor implantation *in vivo* ($P < 0.05$). Regulation of miR-143 on its direct target, GATA-binding factor 6 (GATA6), was investigated by multiple strategies, including dual-luciferase assay, quantitative polymerase chain reaction and western blot analysis. The results indicated that miR-143 was downregulated in both HCC cell lines and human tumors. GATA6 was identified as the downstream target of miR-143 in HCC, and overexpressing GATA6 was able to counter the tumor-suppressive effect of miR-143 on HCC in HepG2 and Bel7402 cells by significantly increasing proliferation and invasion rates ($P < 0.05$). Therefore, a novel epigenetic pathway was identified in which miR-143 may suppress the malignancy of HCC by targeting GATA6.

Introduction

Human hepatocellular carcinomas (HCC) are among the most aggressive types of tumor worldwide, with a notably low five-year survival rate of 18% (1). Most HCC patients are diagnosed in the late stages and the prognosis is poor, largely due to

a lack of known early markers. Identifying novel biomarkers, especially for early diagnosis, is an important research area, although treatment for HCC has improved substantially in the last 20 years (2).

MicroRNAs (miRNAs or miRs) are short, non-coding RNAs that can suppress gene expression through base pairing to the 3' untranslated regions (3'-UTR) of targets (3). An exact mapping of HCC oncogenesis and metastasis has not yet been established, but numerous previous studies have indicated that miRNAs serve key functions in HCC development (4-8). For instance, miR-1188 can directly target Bcl-2 and Sp1, inhibiting cell proliferation, invasion and migration, and resulting in attenuated growth of HCC cells *in vivo* (7). Another study reports that miR-148b can regulate cancer stem cell properties in HCC by directly targeting Neuropilin-1, which is a transmembrane receptor implicated as a key factor in initiating angiogenesis and metastasis (8).

MiR-143 commonly acts as a tumor suppressor miRNA in many tumor types. Numerous reports have identified significantly reduced miR-143 expression in various tumors such as non-small cell lung, colorectal, gastric, pancreatic and prostate cancers, osteosarcomas, cervical cancer and leukemia (9-16). However, to our knowledge, the current study is the first to investigate the function of miR-143 in regulating human HCC.

GATA-binding factor 6 (GATA6) has previously been described as a transcriptional factor with a zinc finger structure, which serves as an oncogenic factor in various types of tumor (17-19). For instance, it has been demonstrated that GATA6 can favor cancer progression by activating Wnt signaling in pancreatic cancer (17,19). In addition, GATA6 is reported to induce metastasis of colorectal carcinomas, possibly through urokinase-type plasminogen activator expression in colon cancer (18). However, little is known about the function of GATA6 in liver cancer.

The current study demonstrates that miR-143 is frequently downregulated in HCC cell lines and HCC human tumors. The function of miR-143 in modulating HCC proliferation and invasion was evaluated. Furthermore, using a bioinformatic strategy, the study investigated the targeting of miR-143 on GATA6 and the direct regulatory role of GATA6 in miR-143-induced HCC growth inhibition. The current study helps to elucidate the role of miRNA-mediated regulation in human HCC.

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Materials and methods

HCC cell lines and human specimens. The HCC cell lines (SMC-7721, Hep3B, HepG2, Huh7) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The following HCC cell lines were purchased from the Model Animal Research Center (Nanjing, China): Bel7402, MHCC97-H and SK-Hep1. A normal human liver cell line (L02; ATCC) was used as a negative control. All cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA USA), supplemented with 15% fetal bovine serum (FBS, Invitrogen; Thermo Fisher Scientific, Inc.), and 200 U/ml penicillin plus an additional 100 g/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in a culture chamber with 5% CO₂ at 37°C. 56 Human HCC specimens were obtained from the surgical specimen archives of the Affiliated Tumor Hospital of Xinjiang Medical University (Urumqi, China) between March 2014 and June 2015. A total of 56 patients were selected, 22:34 male:female (39-85 years) and demographics are presented in Table I. Tumorous HCC tissues and corresponding normal adjacent tissues were obtained in pairs. All patients signed consent forms and all experimental procedures in relation to human subjects were formally approved by the Research and Ethics Committee at the Affiliated Tumor Hospital of Xinjiang Medical University (no. 2014CB002).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Gene expression of miR-143 and GATA6 in HCC was measured by RT-qPCR. In brief, RNA was extracted from both HCC cells and HCC human specimens treated with Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and corresponding cDNA was obtained with an SYBR Premix Ex Taq™ kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocols. A TaqMan miRNA RT-qPCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was applied and 18 s rRNA was used as an internal control to monitor the expression level of miR-143. For GATA6 detection, we used the SYBR-Green PCR Master Mix kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 18s rRNA was used as the control. All kinetic reactions for RT-qPCR were carried out using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Gene expression was quantified as fold change relative to controls. The primer sequences were as follows: Forward, 5'-CACACGCTGACAGTGCTGG-3' and reverse, 5'-TACAGGGCGATACAAAGCAGGAGAA-3', for GATA6; forward, 5'-ACACTCGAGCTGGGGCTTCTCTGCTCTCC-3' and reverse, 5'-TGGTGTGGTGGAGTCG-3' for miR-143; forward, 5'-CTCGCTTGCGCAGCACA-3' and reverse, 5'-AACGCTTGACCAATTTGCGT-3' for 18s rRNA.

MiR-143 gene construction. Lentiviral transduction was utilized to overexpress miR-143 in HepG2 and Bel7402 cells. The lentiviruses containing human miR-143 mimics (Lenti-miR-143), or a negative control miRNA (Lenti-C) were purchased from Gene Tech Co., Ltd. (Nanjing, China). Transduction of lentiviruses into HepG2 and Bel7402 were implemented with Lipofectamine® 2000 reagent

Table I. Patient demographics.

| Features | n | miR-143 expression | | P-value |
|------------|----|--------------------|------------|--------------------|
| | | High, n (%) | Low, n (%) | |
| Age | | | | |
| <60 | 26 | 12 (46.2) | 14 (53.8) | 0.303 |
| ≥60 | 30 | 17 (56.7) | 13 (43.3) | |
| Gender | | | | |
| Male | 22 | 9 (40.9) | 13 (59.1) | 0.273 |
| Female | 34 | 18 (52.9) | 16 (47.1) | |
| Metastasis | | | | |
| Absent | 23 | 16 (69.6) | 7 (30.4) | 0.008 ^a |
| Present | 33 | 11 (33.3) | 22 (66.7) | |
| Serum AFP | | | | |
| >400 ng/ml | 24 | 9 (37.5) | 15 (62.5) | 0.188 |
| ≤400 ng/ml | 32 | 18 (56.2) | 14 (43.8) | |
| TNM stage | | | | |
| I | 17 | 13 (76.5) | 4 (23.5) | 0.006 ^a |
| II-IV | 39 | 14 (35.9) | 25 (64.1) | |

^aP<0.05. miR, microRNA; n, total patients; AFP, α-Fetoprotein; TNM, tumor node-metastasis.

(Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The culture medium was refreshed 48 h after transduction and RT-qPCR was performed to confirm the efficiency of transduction.

Proliferation assay. HepG2 and Bel7402 cells were seeded in 96-well plates (10⁴ cells per well) for five days. Cells were maintained in RPMI 1640 medium and supplemented with 10% FBS and 50 mg/ml streptomycin (all Invitrogen, Thermo Fisher Scientific, Inc.) in an incubator at 37°C with 5% CO₂. The Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was then used. At each 24 h interval, MTT solution was added into culture medium at a final concentration of 5 mg/ml. After 4 h, the medium was removed and crystalline formazan was dissolved in 100 μl SDS (15%) solution for 24 h. The plate was shaken for 5 min leading to complete solubilization. Finally, the optical density (490 nm) was evaluated using a Spectramax M5 microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) according to the manufacturer's protocols.

Invasion assay. A chemotaxis 96-well Transwell assay (Bioscience Research Agents; Merck KGaA, Darmstadt, Germany) was used to quantify the invasion. The upper chamber of the Transwell was covered with Matrigel (Sigma-Aldrich; Merck KGaA) overnight. Lentivirus-treated HepG2 or Bel7402 cells were seeded within the upper chambers (10⁴ cells per well) in RPMI-1640 medium without serum at 20°C. The lower chambers were then filled with RPMI-1640 medium plus 3% FBS. Cells that had migrated into the lower chambers after 24 h were fixed with 5% paraformaldehyde and stained with crystal violet. The image was obtained through

a Leica DM-IRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). The invasion was determined as fold change relative to the control.

In vivo implantation assay. HepG2 or Bel7402 cells transduced with lentivirus for 12 h were continuously cultured in RPMI-1640 medium (Invitrogen, Thermo Fisher Scientific, Inc.) for an additional 24 h at 37°C. They were then resuspended and 10⁶ cells were implanted subcutaneously into nude mice. A total of 12 mice (BALB/C; age, 5-6 weeks; average weight, 15.9 g; 6 male and 6 female) were used in this study. Mice were housed at 18-22°C, 50-60% humidity and a light-dark cycle of 10-14 h. Since an adult mouse consumes 5-7 g food and 4-7 ml fresh water per day, *ad libitum* access to food and water was provided. The nude mice were obtained from Model Animal Research Center (Nanjing, China). The Ethics Committee of The Affiliated Tumor Hospital of Xinjiang Medical University approved the animal experiments and procedures in the present study. The volumes (calculated as length×width×height) of the tumors *in vivo* were recorded by external caliper at weekly intervals. Five weeks later, all mice were sacrificed by an overdose of sodium pentobarbital (4%, 200 mg/kg with intraperitoneal injection; catalogue no. 1507002, Sigma-Aldrich; Merck KGaA) and the implants were immunostained with Ki-67 (Qiagen, Hilden, Germany). Formalin-fixed (35%, 5 days at 20°C), paraffin-embedded biopsy specimens obtained from the Affiliated Tumor Hospital of Xinjiang Medical University were used for immunostaining. The specimens were washed twice using PBS for 3 min and then blocked with 3% hydrogen peroxide for 10 min followed by 5% bovine serum albumin for 30 min at 20°C. The 2-μm sections were used in the current study and stained using the CINTec PLUS kit (MTM Laboratories, Heidelberg, Germany). The staining was performed according to the manufacturer's protocol. The slides were deparaffinized in xylene and rehydrated by passage through 70% alcohol. Following antigen retrieval in the supplied solution, the primary antibody, Ki-67 (catalogue no. P6834, 1:500, Sigma-Aldrich; Merck KGaA) was incubated for 30 min at 37°C. The staining is based on a rabbit monoclonal antibody directed against human Ki-67 (catalogue no. P6834, 1:500, Sigma-Aldrich; Merck KGaA). The image was visualized using a CX31-LV320 light microscope (Olympus Corporation, Tokyo, Japan).

Prediction of miR-143 target. The present study used algorithms for target gene prediction: TargetScan (<http://genes.mit.edu/targetscan>) and miRDB (www.mirdb.org) as previously described (20,21). Putative targets were ranked by Z scores. The top ranked targets that overlapped were selected for experimental verification.

Dual-luciferase reporter assay. Amplification of the GATA6 gene was obtained from a cDNA library (LIBEST_001365) and verified by DNA bidirectional sequencing using the ABI PRISM® 3730 DNA sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the BigDye® Terminator version 3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Inc.). Then, the 3'-UTR of GATA6 with a predicted binding site to human miR-143 (hsa-miR-143) was cloned into *Renilla*

luciferase reporter plasmid phRL-TK (Sigma-Aldrich; Merck KGaA) leading to the wild-type GATA6 luciferase reporter plasmid (GATA6 3'-UTR WT). The predicted binding site to hsa-miR-143 in the 3'-UTR region of GATA6 was mutated using a Quik-Change™ Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA, USA). The mutated GATA6 3'-UTR was then inserted into phRL-TK to create the mutated luciferase reporter plasmid for GATA6 (GATA6 3'-UTR MUT). Co-transfection was completed following 24 h in HEK293T cells (Shanghai Institute of Cell Biology, Shanghai, China). Relative luciferase units were recorded using the dual-luciferase reporter assay (Promega Corporation, Madison, WI, USA), following the manufacturer's instructions.

Western blot analysis. Cultured cell lysates were prepared by washing cells with PBS twice for 1 min. Then, HepG2 and Bel7402 cells were resuspended and harvested with a cell lysis buffer containing 20% glycerol and 2% NP-40 (Sigma-Aldrich; Merck KGaA) for 10 min at 4°C. The protein extracts (100 μg for each) were then dissolved in 5% SDS-PAGE gel and transferred to a nitrocellulose membrane (Sigma-Aldrich; Merck KGaA). The blot was blocked with 5% fat-free milk for 1 h at 20°C. The membrane was incubated with primary antibodies against human GATA6 (catalogue no. sc-517269, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight, and horseradish peroxidase-conjugated secondary antibodies (catalogue no. sc-280786, 1:1,000) at 20°C for 4 h. After being washed repeatedly in Tris-buffered saline (TBS) containing 0.1% Tween-20, the blots were monitored using a chemiluminescent method kit (Sino-American Biotechnology Company, Luoyang, China). The blots were quantified with gray analysis method using ImageJ software (22). Briefly, the backgrounds in each blot was first subtracted and then each band was located manually. The area and mean gray values were then quantified automatically using ImageJ software.

GATA6 overexpression. The GATA6 sequence was cloned into recombinant eukaryotic plasmid pcDNA3.1 (Sigma-Aldrich; Merck KGaA) to create pcDNA3.1/GATA6, following the manufacturer's instructions. Transfection of pcDNA3.1/GATA6, or an empty pcDNA3.1 plasmid (pcDNA3.1/+) into HepG2 and Bel7402 cells was implemented using Lipofectamine® 2000 kit (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Transfection persisted for 24 h before DMEM transfection medium with 5% FBS and 2 μg/ml Polybrene (Sigma-Aldrich; Merck KGaA) was replaced by fresh medium and incubated for additional 24 h at 37°C. RT-qPCR was performed to evaluate the transduction efficiency. The cells were then resuspended and seeded into 6-well plates (1×10⁵ cells/well, in RPMI-1640 medium), and subjected to proliferation and invasion assays, following the procedures described above.

Statistical analysis. All experiments were performed three times and results were presented as the mean ± standard error. Statistical differences were measured using the Student's t-test and analyzed using the statistical software SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Results were considered to be statistically significant if P<0.05.

Results

miR-143 is downregulated in a number of HCC cell lines and correlates with malignancy. The current study set out to determine whether the expression of miR-143 in *in vitro* HCC cell lines, as well as in *in vivo* HCC human samples was altered compared with normal liver cells. The RT-qPCR results showed that miR-143 expression was significantly reduced in seven HCC cell lines (SMMC-7721, Hep3B, HepG2, Huh7, Bel7402, MHCC97-H and SK-Hep1) compared with a normal liver cell line (L02; $P < 0.01$; Fig. 1A). The expression level of miR-143 was also found to have decreased significantly in tumorous tissues compared to corresponding normal adjacent tissues in 65 paired samples ($P < 0.01$; Fig. 1B). These results suggested that miR-143 was downregulated in HCC tissues and associated with malignancy.

Increasing miR-143 expression can inhibit HCC malignancy. The HCC cell lines HepG2 and Bel7402 were transfected with lentivirus to ectopically upregulate miR-143. After 24 h, transfection efficiency was measured using RT-qPCR. The results showed that miR-143 genes were expressed at significantly higher levels in both HepG2 and Bel7402 cells transfected with a lentivirus containing miR-143 mimics (Lenti-miR-143), compared with cells transfected with a control plasmid ($P < 0.05$, Fig. 2A).

In addition, HepG2 and Bel7402 cells were resuspended and seeded in a 96-well plate for 5 days. The proliferation was monitored every 24 h to investigate the role of miR-143 *in vitro* on HCC growth. The results demonstrated that miR-143 upregulation significantly inhibited HCC proliferation in both HepG2 and Bel7402 cells at 3-5 days ($P < 0.05$; Fig. 2B). Transwell assays were also performed to show the effect of miR-143 on invasion. After 24 h, the results showed miR-143 upregulation decreased the invasion rate in both HepG2 and Bel7402 cells (Fig. 2C, upper panel). Quantification results confirmed that upregulating miR-143 significantly reduced the invasive capabilities of both HCC cell lines ($P < 0.05$; Fig. 2C, lower panel).

Increasing miR-143 can inhibit HCC in vivo implantation. The *in vitro* effect of miR-143 was evident as demonstrated above. In order to investigate whether miR-143 could have a similar effect on HCC growth *in vivo*, HepG2 cells were transfected with Lenti-miR-143 or Lenti-C for 24 h, and 10^6 transfected cells were subcutaneously injected into the rear flank of null mice. The size of the HCC tumors was measured each week and the volumes were calculated (length \times width \times height). The results demonstrated that the rate of HCC implantation was significantly reduced when miR-143 was upregulated *in vivo* compared with normal miR-143 expression ($P < 0.05$; Fig. 3A). Five weeks later, HCC solid tumors were extracted and subject to Ki-67 immunostaining, where dark brown staining indicates increased proliferation. The present study demonstrated that the immunostaining in miR-143 transfected groups was significantly weakened compared with control groups ($P < 0.05$; Fig. 3B). The results suggested that HCC proliferation *in vivo* was also attenuated by miR-143 overexpression, compared with normal miR-143 expression (Fig. 3B).

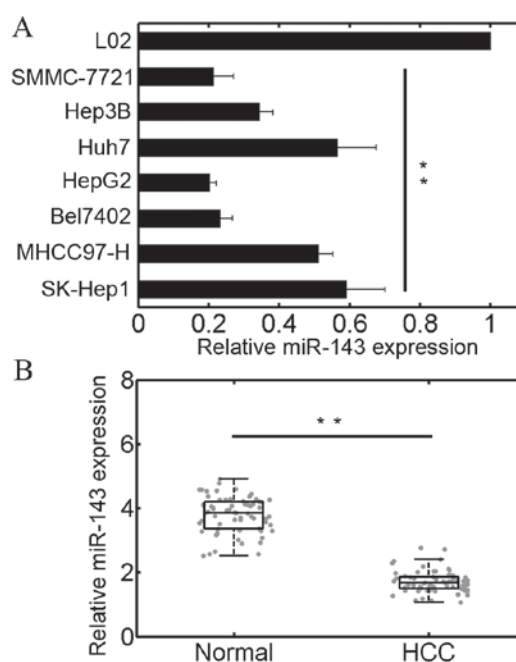


Figure 1. Rate of miR-143 expression in HCC cell lines. (A) RT-qPCR was used to measure expression levels of miR-143 in seven HCC cell lines (SMMC-7721, Hep3B, HepG2, Huh7, Bel7402, MHCC97-H and SK-Hep1) and a normal human liver cell line. (B) RT-qPCR was used to compare the miR-143 expression levels between paired tumorous tissues (HCC) and paired normal adjacent tissues (Normal). A paired t-test was used. ** $P < 0.01$ vs. control. HCC, hepatocellular carcinoma; miR-143, microRNA-143; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

GATA6 is a direct downstream target of miR-143 in HCC. Several miRNA target prediction websites, such as TargetScan (www.targetscan.org) and miRDB (www.mirdb.org), were consulted in order to identify the molecular targets of miR-143 in HCC. As a result, oncogene GATA6 was selected as a potential target for miR-143 (Fig. 4A). A dual-luciferase reporter assay was performed to assess the effect of miR-143 on GATA6 activity. MiR-143 had no significant effect on luciferase activity in the GATA6 3'-UTR (MUT) plasmids ($P > 0.05$), but produced a significant reduction in luciferase activity in the GATA6 3'-UTR (WT) plasmids compared with the control ($P < 0.01$ Fig. 4B). The results of a western blot analysis showed that the expression of GATA6 protein was downregulated by miR-143 overexpression in both HepG2 and Bel7402 cells (Fig. 4C). Results from RT-qPCR quantification confirmed that the level of GATA6 transcripts was significantly reduced by miR-143 upregulation in HepG2 and Bel7402 cells ($P < 0.05$; Fig. 4D). These results suggested that GATA6 was a direct downstream target of miR-143 in HCC.

GATA6 restored HCC proliferation and invasion after miR-143 upregulation. Given the targeting effect of miR-143 on GATA6, it was proposed that GATA6 may counteract miR-143-mediated inhibition on HCC *in vitro* progression. To test this, an overexpression plasmid pcDNA3.1/GATA6 was generated, in order to overexpress GATA6 in HepG2 and Bel7402 cells. The transfection efficiency was confirmed through RT-qPCR ($P < 0.05$; Fig. 5A). To investigate the role of GATA6 on miR-143 overexpression, HepG2 and Bel7402 cells were transfected with Lenti-miR-143 and either

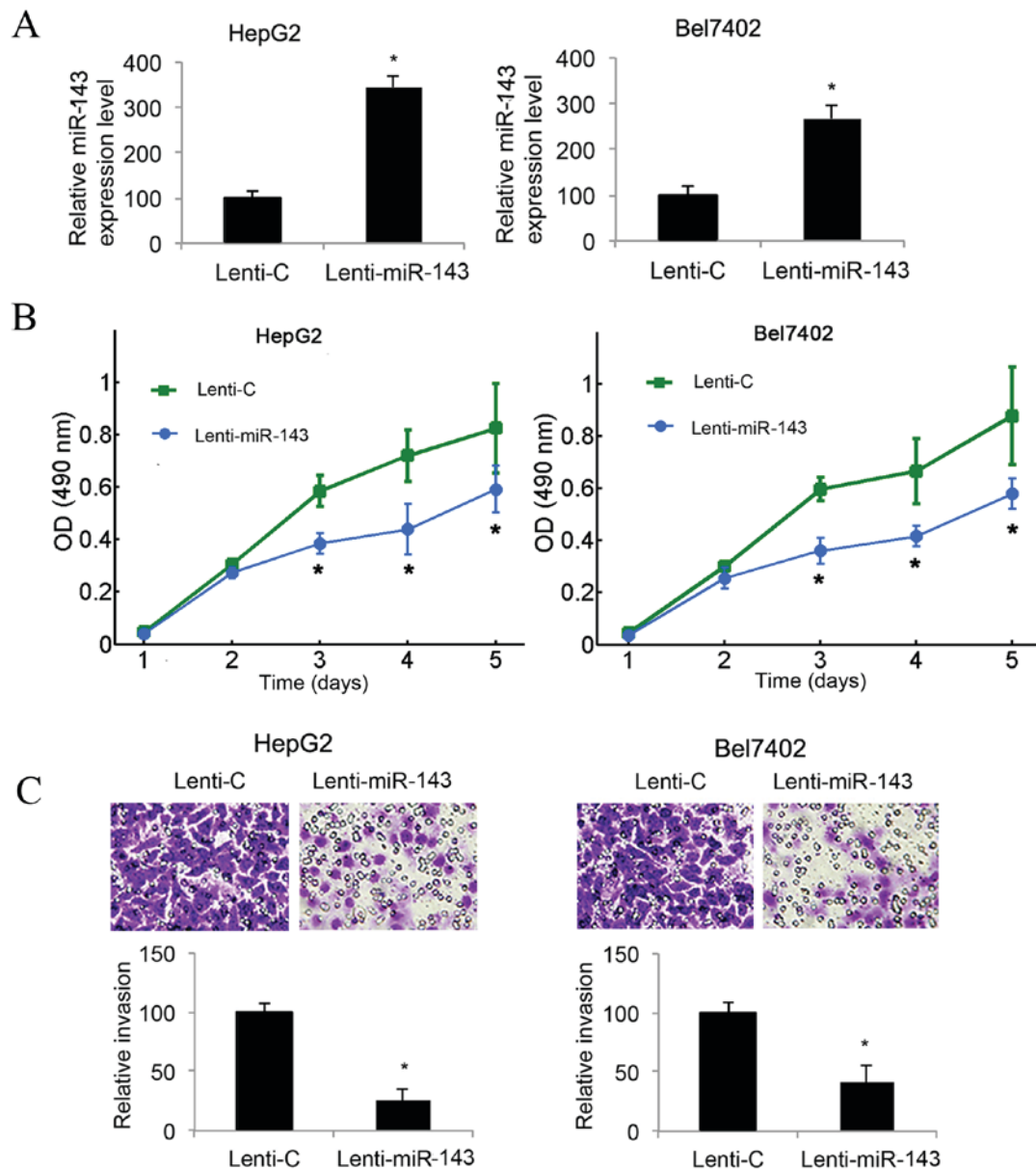


Figure 2. Effect of miR-143 upregulation on HCC proliferation and invasion *in vitro*. (A) HCC cell lines, HepG2 and Bel7402, were transfected with a lentivirus containing miR-143 mimics (Lenti-miR-143), or a lentivirus containing negative control miRNA (Lenti-C). After 48 h, RT-qPCR was performed to evaluate the transduction efficiency. (B) Post-transduction proliferation was evaluated in lentivirus-transduced HepG2 and Bel7402 cells using a five-day MTT assay. (C) Post-transduction invasion in HepG2 and Bel7402 cells was quantified using a Transwell assay. Cells that invaded into the lower chambers were stained with crystal violet (upper panel, x200 magnification). The relative invasion capabilities were also quantified (lower panel). * $P < 0.05$ vs. control. HCC, hepatocellular carcinoma; miR-143, microRNA-143; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; OD, optical density.

pcDNA3.1/GATA6 or with an empty pcDNA3.1 plasmid (pcDNA3.1/+). A five-day proliferation assay was performed. Results showed that GATA6 overexpression can reverse the inhibitory effect of miR-143 overexpression on HCC proliferation in both HepG2 and Bel7402 cells ($P < 0.05$; Fig. 5B). Transwell assays showed an increase in the relative level of cell invasion when GATA6 was overexpressed (Fig. 5C, upper panel). Furthermore, quantification of the invasion assay confirmed that GATA6 overexpression significantly increased the rate of invasion in both HepG2 and Bel7402 cells compared to cells with normal GATA6 expression levels ($P < 0.05$; Fig. 5C, lower panel). These results suggested that GATA6 is critically involved in miR-143-mediated regulation in HCC.

Discussion

Although miR-143 has been implicated in HCC metastasis, its precise functional role is not yet known. The current study was, to our knowledge, the first to evaluate the expression pattern of miR-143 in HCC cell lines. MiR-143 was found to be down-regulated in all seven HCC cell lines tested (Fig. 1). A low level of expression of miR-143 was also found in human HCC tumor specimens, as compared with adjacent non-tumor liver tissues. Furthermore, lentiviral transduction of miR-143 mimics was shown to upregulate miR-143 in HCC cell lines HepG2 or Bel7402 cells. Functional assays, including MTT and Transwell assays, demonstrated that miR-143 played a tumor-suppressing role in HCC by inhibiting cancer proliferation and invasion.

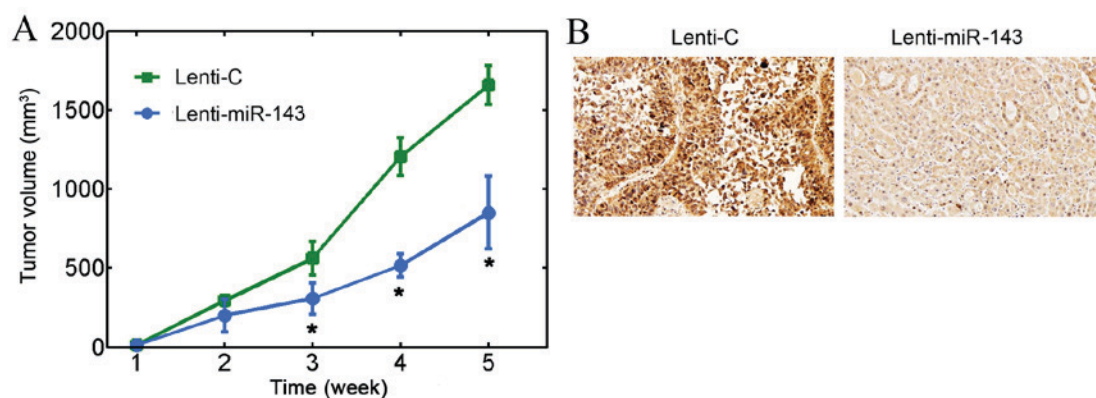


Figure 3. Effect of miR-143 upregulation on HCC implantation *in vivo*. (A) A total of 10^6 lentivirus-transduced HepG2 cells were subcutaneously implanted into null mice. The *in vivo* tumor volume was measured weekly for five weeks. (B) At the end of *in vivo* implantation, HCC tumors were retrieved and Ki-67 staining was carried out upon paraffin-embedded sections (x200 magnification). The dark brown staining indicates increased proliferation. * $P < 0.05$ vs. control. HCC, hepatocellular carcinoma; miR-143, microRNA-143.

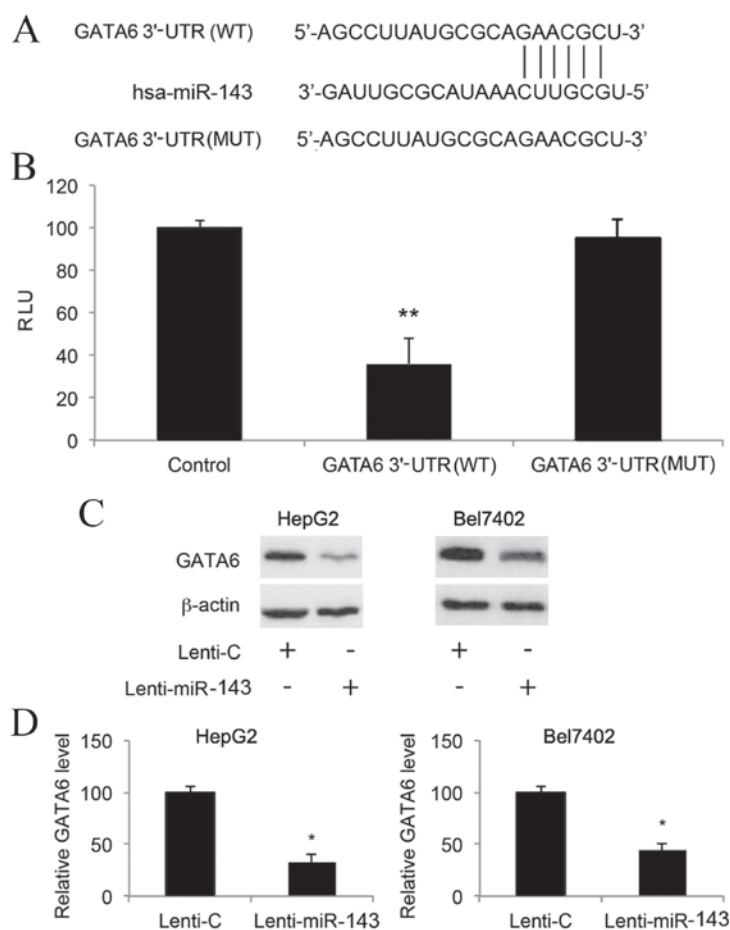


Figure 4. Confirming GATA6 as a downstream target of miR-143. (A) DNA sequences for the binding site of hsa-miR-143 on GATA6 3'-UTR (WT) and GATA6 3'-UTR (MUT). In GATA6 3'-UTR (MUT) plasmids, the DNA sequence at the binding site was mutated. (B) In a dual-luciferase reporter assay, GATA6 3' UTR (WT), GATA6 3'-UTR (MUT) or empty *Renilla* luciferase reporter (control) plasmids were co-transfected into HEK293T cells with a lentivirus containing miR-143 mimics (Lenti-miR-143) for 24 h. The luciferase activities of all three plasmids were measured, and normalized to the luciferase activity of the control plasmid. ** $P < 0.01$. (C) HepG2 and Bel7402 cells were transduced with Lenti-miR-143, or a lentivirus containing negative control miRNA (Lenti-C), for 24 h. The expression levels of GATA6 proteins were evaluated by western blot analysis. (D) The expression levels of GATA6 mRNAs were evaluated by RT-qPCR. * $P < 0.05$ and ** $P < 0.01$ vs. control. GATA6, GATA-binding factor 6; miR-143, microRNA-143; hsa-miR-143, human miR-143; UTR, untranslated region; WT, wild type; MUT, mutated; RLU, relative luciferase units; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Previous reports have described downregulated miR-143 levels in a series of human malignancies and its potential involvement by targeting many genes. For instance,

Noguchi *et al* suggested that miR-143 could induce apoptosis and negatively regulate proliferation in bladder cancer cells (23). Another report demonstrated significantly reduced

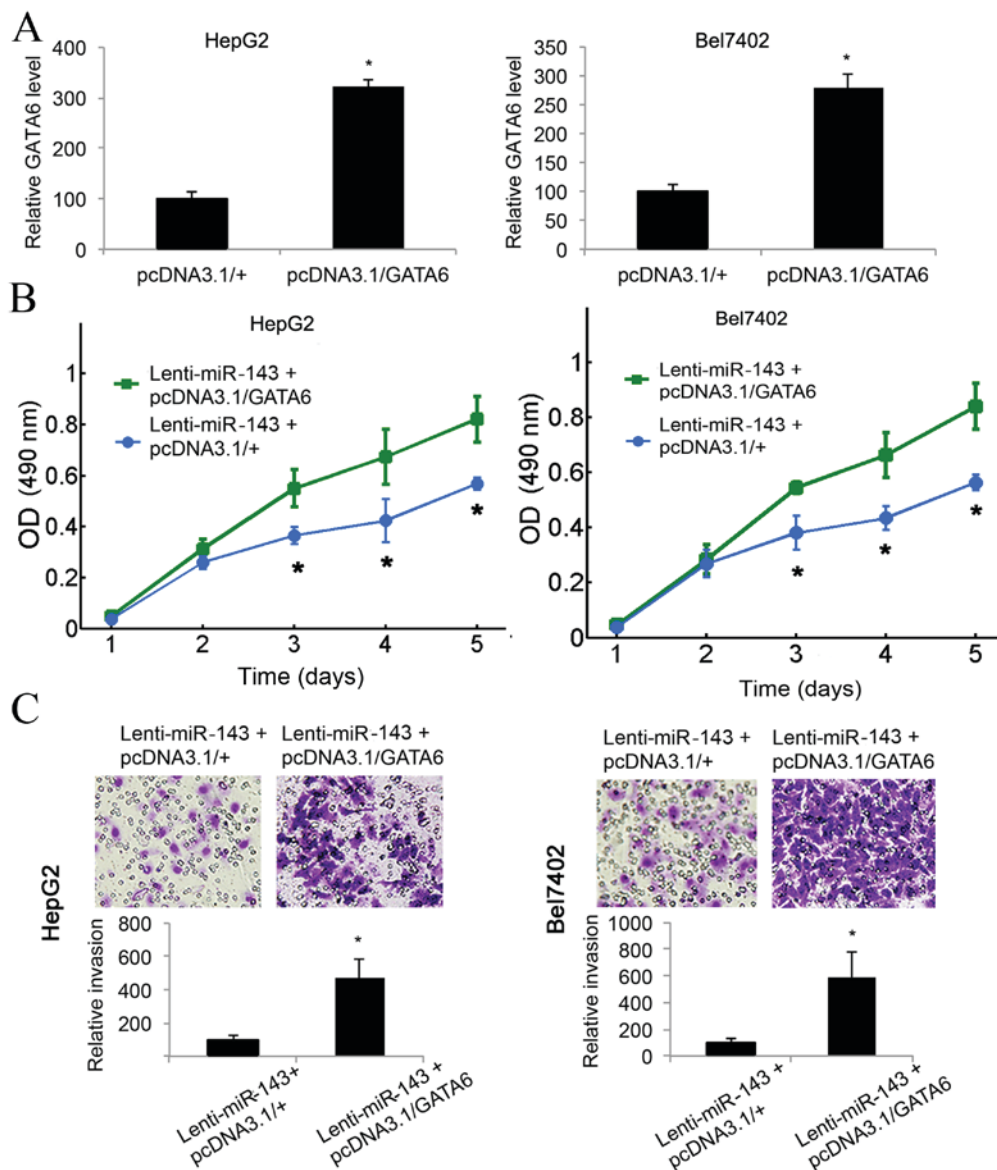


Figure 5. Effect of overexpressing GATA6 on the inhibitory effect of miR-143 in HCC cell lines. (A) HepG2 and Bel7402 cells were transfected with a GATA6 overexpression plasmid, pcDNA3.1/GATA6, or an empty plasmid pcDNA3.1/+ for 24 h. The GATA6 overexpression efficiency was verified using RT-qPCR. (B) HepG2 and Bel7402 cells were transduced with Lenti-miR-143 for 24 h, followed by transfection of pcDNA3.1/GATA6 or plasmid pcDNA3.1/+ for another 24 h. HCC *in vitro* proliferation was evaluated by a five-day MTT assay. (C) Invasive capabilities of HepG2 and Bel7402 cells after double-transfection were evaluated by a Transwell assay. Cells invaded into the lower chamber were immune-stained with crystal violet (upper panel). Relative invasive capabilities were also quantified (lower panel). * $P < 0.05$ vs. control. GATA6, GATA-binding factor 6; miR-143, microRNA-143; OD, optical density; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

miR-143 expression in lung cancer tissues and identified that CD44 might be a direct target of miR-143 (24). MiR-143 is also involved in the suppression of bladder cancer and osteosarcoma by targeting cyclooxygenase-2 and matrix metalloproteinase-13 (15,25), and the progression of colorectal cancer is reported to be significantly correlated with lower miR-143 expression (11). Xu *et al* overexpressed miR-143 in prostate cancer cells and observed that miR-143 transfection could effectively increase the sensitivity to docetaxel treatment, probably through RAS/MAPK signaling (26). Administration of miR-143 to nude mice harboring DLD-1 tumors also indicated that tumor volumes are decreased accordingly with injection doses (27). A systematic delivery of miR-143/145 also inhibited orthotopic and subcutaneous pancreatic cancer xenografts (12). These studies suggest that

miR-143 is a potential target for developing new therapeutic strategies for malignant liver tumors.

The current study identified that the zinc finger transcription factor GATA6 may be the potential target in HCC. Overexpression of miR-143 not only inhibited HCC proliferation and invasive capacity but also downregulated GATA6 expression, suggesting that GATA6 was directly involved in miR-143 mediated regulation in HCC. Further experiments demonstrated that miR-143-induced inhibition of *in vitro* proliferation and invasion in HCC cell lines was significantly restored by GATA6 overexpression (Fig. 5). GATA6 is reported to be an oncogene in various cancers such as colon (18), pancreatic (19) or colorectal cancer (28), but the function of GATA6 in liver cancer is still elusive. The current study showed direct involvement of GATA6 in miR-143-mediated HCC regulation,

suggesting that GATA6 also serves an oncogenic function in HCC, which may be related to miRNA dysregulation.

In conclusion, the current study presents a functional role of miR-143 in regulating human HCC. Upregulating miR-143 has tumor-suppressing effects on HCC proliferation and invasion. GATA6 is the downstream target of miR-143 in HCC, and its overexpression is able to counteract miR-143-induced HCC inhibition. The miR-143 and GATA6 signaling pathway requires further study in order to elucidate the epigenetic regulation mechanisms of miRNA in human HCC.

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