MicroRNA-139 suppresses hepatocellular carcinoma cell proliferation and migration by directly targeting Topoisomerase I

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Abstract. microRNAs (miRNAs) have been determined to be associated with cancer progression and metastasis. Mir-139 is located on 11q13.4 and exhibits anti- oncogenic and anti-metastatic activity in human cancers. It is downregulated in various malignant tumor types. In the present study, the potential functions and targets of miR-139 in hepatocellular carcinoma (HCC) were explored. Using a combinational analysis of four miRNA target prediction tools and biological experiments, it was determined that Topoisomerase I (TOP1) is a direct target of miR-139 in HCC. Several traditional topoisomerase inhibitors have demonstrated antitumor activity, but their side effects outnumbered their anticancer potential. The present study determined that overexpression of miR-139 significantly inhibits HCC cell proliferation (P<0.05) and migration (P<0.05), which is largely due to TOP1 downregulation. The present study indicated that miR-139 exerts a tumor-suppressive effect during hepatocarcinogenesis via the suppression of expression of TOP1; therefore, miR-139 is a promising target for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent and mortality-associated cancer types in developing and developed countries and accounts for 70-90% of primary liver cancer cases (1). Despite numerous years of basic and clinical research on HCC, the 5-year survival rate still remains at ~7% (2). An alternative approach for addressing the poor survival problem may rely on discovering novel targets for treatment. Hepatocarcinogenesis is a slow and complicated process that includes genomic changes that progressively alter the hepatocellular phenotype to produce abnormal cellular intermediates, finally resulting in HCC (3); however, the understanding of the underlying molecular mechanisms that drive hepatocarcinogenesis is still in its infancy. In the past 15 years, non-coding RNAs, particularly microRNAs (miRNAs), have received considerable attention regarding an elucidation of the molecular pathogenesis of cancer (4). Through recognizing the seed sequences in the 3’-untranslated region (3’-UTR) of target mRNAs, each miRNA has the ability to regulate the expression of numerous genes (5); therefore, miRNAs are frequently considered to efficiently coordinate and regulate multiple signaling pathways and biological processes in human diseases, particularly in cancers (5). Thus far, accumulating evidence has indicated that an abnormal miRNA expression profile is a hallmark of malignancies, including HCC (6,7). The previous study demonstrated that miR-139 was downregulated in HCC and could serve as a diagnostic and prognostic marker for HCC (8); however, the major targets and precise signaling pathways that miR-139 participates in in HCC are not fully understood. A number of studies determined that overexpression of miR-139 suppresses the proliferation, invasion and metastasis of HCC cell lines in vitro (9-11). miR-139 is also associated with the functions of particular genes; it is reported that miR-139 may target transcription factor 4 (TCF-4) 3’-UTR, regulate the expression of TCF-4 and inhibit the β-catenin/TCF-4 pathway in HCC cell lines (9). Wong et al (11) reported that miR-139 reduces the expression of Rho-kinase 2 (ROCK2) in HCC cell lines. c-fos may be another downstream gene responsible for the metastatic effect in HCC cell lines. Furthermore, miR-139 is also identified as one of the post-hepatectomy recurrence-associated miRNAs (12). The expression of zinc finger E-box binding homeobox 1 (ZEB1) and ZEB2 was also inhibited by miR-139 through recognizing the 3’-UTR of these two genes (13). Considering that miRNAs serve a crucial role in multiple genes’ expression and transcription regulation, it was hypothesized that miR-139 may have a major functional target gene and possibly acts as a key regulator of HCC progression.

In the present study, a combinational analysis of the data from four miRNA target prediction tools and biological experiments was applied to explore potential targets of
tumor-suppressive miR-139 in HCC. It was demonstrated that Topoisomerase I (TOPI) is a proven, direct target of miR-139 in HCC. Overexpression of miR-139 inhibits HCC cell proliferation and migration, largely due to TOPI down-regulation. The present study indicated that miR-139 exerts a tumor-suppressive effect during hepatocarcinogenesis via suppressing the expression of TOPI; therefore, miR-139 is not only a biomarker for diagnosis and prognosis but also a promising target for the biological treatment of HCC.

Materials and methods

Bioinformatics analysis of miRNA target prediction. The majority of model organisms have an miRNA target gene prediction database, including TargetScan (http://www.targetscan.org/) (14), miRanda (http://www.microrna.org/) (15), miRDB (http://www.mirdb.org/) (16) and CLIP-Seq (http://www.starbase.sysu.edu.cn/) (17). Through these databases, an analysis of miR-139 was performed using bioinformatics in HCC. Using the combinational analysis of the data from four miRNA target prediction tools, four groups of genes were selected from the database and were identified as screening objects.

Cell culture. Human liver cancer cell lines BEL-7404 and SMMC-7721 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). BEL-7404 and SMMC-7721 were maintained in RPMI-1640 medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences). HEK-293 was maintained in DMEM (GE Healthcare Life Sciences) supplemented with 10% FBS. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Cell transfection. BEL-7404 and SMMC-7721 cells were transfected with either miRNA mimics/inhibitors or plasmids using HiPerFect Transfection Reagent (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. The miRNA mimics were chemically synthesized, double-stranded RNAs that mimic mature endogenous miRNAs following transfection into cells, whereas the miRNA inhibitors were chemically modified antisense RNA oligonucleotides optimized to specifically target specific miRNA molecules in cells. miRNA mimics, inhibitors and negative control (NC) sequences were chemically synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China). miR-139-NC: 5'-ACGUAGACACGCUAGCAGAAGAUU3'; miR-139 mimics: 5'-UCUACAGUGACACGUUCUCGUCUCAGAUU3'-3' and miR-139 inhibitors: 5'-ACGUAGACACGUAGCAGAAGAUU3'-3'.

The reference miR-139 ID was MIMAT0000250 and the gene sequence was 5'-UCUACAGUGACACGUUCUCGUCUCAGAUU3'-3'. miRNAs and/or DNA plasmids were diluted in Opti-MEM I reduced serum medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Briefly, the day prior to transfection, BEL-7404 and SMMC-7721 cells (2x10⁶) were plated with medium of 100 µl DMEM medium containing 10% FBS, placed in an incubator with 5% CO₂ at 37°C. Transfection were performed when the cells were at 70-80% confluency and recorded as time 0. Recombination plasmid TOPI (Shanghai GenePharma Co., Ltd.) (0.1 µg) was added, and/or 0.5 µl 20 µM miR-139 mimics or inhibitors were added to Opti-MEM for a final volume of 10 µl. Subsequently, 0.4 µl Lipofectamine 2000 was added and the mixture were kept at room temperature for 15 min. Following this, the transfection mixture was added to each cell medium and mixed. The media were changed to RPMI-1640 medium supplemented with 10% FBS following incubation in the incubator with 5% CO₂ at 37°C for 5 h. Subsequently, the supernatant medium was removed following another incubation in an atmosphere containing 5% CO₂ at 37°C for 48 h. Following this, the Passive Lysis Buffer (100 µl; Promega Corporation, Madison, WI, USA) were added and the cells were lysed and collected following the mixture being shaken gently at room temperature for 15 min. The cells were then collected for other subsequent experiments.

Immunoblotting. Total cell lysates were obtained using a Triton X-100 lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate and 1 mM Na₃VO₄], supplemented with a protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany) and phenyl-methane sulfonyl fluoride (1 mM), and determined with the BCA Protein Quantification kit (cat. no. BLS21A; BioSharp, Hefei, China), according to the manufacturer's protocols.

Protein samples (20 µg) were separated respectively by 12, 10 and 8% SDS-PAGE, according to the following groups, which were divided by the protein molecular weight: BTG family member 3 (BTG3; 29 kDa); Casitas B-lineage lymphoma-transforming sequence-like protein 1 (CBLL1; 55 kDa); H2A Histone Family Member V (H2AFV; 14 kDa); Heterogeneous Nuclear Ribonucleoprotein F (HNRNPF; 46 kDa); Ligand Dependent Nuclear Receptor Corepressor (LCOR; 47 kDa); LIM Domain Only 4 (LMO4; 18 kDa); Protein Phosphatase 2 Catalytic Subunit Alpha (PPP2CA; 34 kDa), and β-actin (42 kDa) were separated by 12% SDS-PAGE; Mannosyl (Alpha-1,3-)Glycoprotein Beta-1,4-N-Acetylgalcosaminyltransferase, Isozyme A (MGAT4A; 62 kDa); Discoidin, CUB And LCCL Domain Containing 2 (DCBLD2; 78 kDa) and Intestinal Cell Kinase (ICK; 71 kDa) were separated by 10% SDS-PAGE; Eukaryotic Translation Initiation Factor 4 Gamma 2 (EIF4G2; 102 kDa); DNA Topoisomerase I (TOPI; 91 kDa) and Zinc Finger And BTB Domain Containing 10 (ZBTB10; 95 kDa) were separated by 8% SDS-PAGE and were transferred onto polyvinylidene fluoride membranes for western blotting. The membranes were blocked with 5% BSA (Solarbio Life Science, Beijing, China) at room temperature for 1 h, then probed with primary antibodies [anti-BTG3, (dilution, 1:500; cat. no. bs-7698R; BIOSS, Beijing, China); anti-H2AFV, (dilution, 1:200; cat. no. bs-17425R; BIOSS); anti-HNRNPF (dilution, 1:500; cat. no. bs-4205R; BIOSS); anti-LMO4, (dilution, 1:1,000; cat. no. bs-7698R; BIOSS); anti-ICL, (dilution, 1:1,000; cat. no. bs-15536R; BIOSS); anti-LCOR, (dilution, 1:200; cat. no. bs-18198R; BIOSS); anti-ZBTB10, (dilution, 1:1,000; cat. no. bs-13556R; BIOSS); anti-LMO4, (dilution, 1:1,000; cat. no. bs-5966R; BIOSS); anti-PPP2CA, (dilution, 1:1,000; cat. no. bs-0029R; BIOSS); anti-EIF4G2 (dilution, 1:500; cat. no. bs-1350R; BIOSS); anti-DCBLD2 (dilution, 1:500; cat. no. bs-5834R; BIOSS); anti-MGAT4A (dilution, 1:1,000; cat. no. bs-18907R; BIOSS); anti-TOPI (dilution, 1:1,000; cat. no. bs-18907R; BIOSS); anti-LMO4, (dilution, 1:500; cat. no. bs-18907R; BIOSS); anti-TOPI (dilution, 1:1,000; cat. no. bs-18907R; BIOSS).
cat. no. bs-10542R; BIOSS); anti-CBLL1 (dilution, 1:1,000; cat. no. bs-8386R; BIOSS) and anti-β-actin (dilution, 1:2,000; cat. no. 60008-1-Ig; Proteintech). The cells were seeded onto six-well dishes at 2x10^4 cells/well. A single scratch wound was created in confluent cells using a 10 µl micropipette tip. Cells were washed twice with PBS to remove cell debris. Monitored images were captured by fluorescence microscopy (magnification, x400) (IX71; Olympus Corporation, Tokyo, Japan) at 0 and 24 h following wounding.

**miRNA target prediction.** To define potential downstream targets of miR-139, candidate genes that were commonly predicted were matched by four publicly available algorithms: TargetScan version 7.0, miRanda and CLIP-Seq version 2.

**Statistical analysis.** Statistical analysis was performed using SPSS 11.0 for Windows (SPSS, Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation. A two-tailed Student's t-test was used to evaluate the statistical significance of differences between two groups of data in the luciferase, cell proliferation and wound-healing assays. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Prediction of miR-139 targets.** To identify the potential targets of tumor-suppressive miR-139 in HCC cells, a bioinformatics analysis was performed using miRNA target prediction tools. Computational predictions indicate that all human genes may be regulated by microRNAs, with each microRNA possibly targeting thousands of genes (18). As depicted in Fig. 1A, the four frequently used algorithms [TargetScan (14), miRanda (15), miRDB (16) and CLIP-Seq (17)] produced divergent sets of predicted targets of miR-139. To reduce bias caused by one method, the results predicted by the different algorithms were intersected and it was determined that a group of 28 genes are jointly identified by all four algorithms (Fig. 1). Some of these genes include: BTG3; CBLL1; DCBLD2; EIF4G2; H2AFV; HNRNPF; ICK; LCOR; LMO4; MGAT4A; PPP2CA; TOP1; and ZBTB10. These genes are reported to be aberrantly expressed in various cancer types and thus become a focus (Fig. 1B).
Figure 2. (A) miR-139 target screening by western blot analysis. (B) BEL-7404 cells were transfected with miR-139 mimics, miR-139 inhibitors or a negative control for 48 h. Endogenous β-actin was used as an internal control for protein loading.
miR-139 target screening by western blot analysis. To validate the potential targets of miR-139 in HCC cells, western blot analysis was performed to screen the predicted genes that are dysregulated in cancer. Two frequently used HCC cell lines, BEL-7404 and SMMC-7721, were selected and miR-139 mimics, miR-139 inhibitors or a negative control were transfected into these cells. Following 48 h, cells were lysed, and all samples were analyzed via semi-quantitative immunoblotting. As depicted in Figs. 2 and 3, increased miR-139 expression notably reduced TOP1 protein levels.
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Figure 3. (A) miR-139 target screening by western blot analysis. (B) SMMC-7721 cells were transfected with miR-139 mimics, miR-139 inhibitors or a negative control for 48 h. Endogenous β-actin was used as an internal control for protein loading. *P<0.05 and **P<0.01.
in both HCC cell lines, whilst miR-139 inhibitors had an opposite effect on TOP1 expression. These results indicated that miR-139 could negatively regulate TOP1 expression in HCC cells.

miR-139 directly targets and inhibits TOP1 expression. As western blot analyses could not discriminate between direct and indirect effects of miR-139 on TOP1 expression, a Dual-Luciferase reporter analysis was performed to determine...
if miR-139 targets TOP1 mRNA directly. The results demonstrated that miR-139 significantly repressed the luciferase activity of a reporter vector harboring the wild-type 3'-UTR of TOP1, whereas mutation of the putative miR-139-binding site in the 3'-UTR region abrogated the inhibitory effect of miR-139 (Fig. 4A, B and C). Similarly, inhibition of miR-139 significantly enhanced luciferase activity of the reporter vector harboring the wild-type 3'-UTR of TOP1, and this effect could be completely abolished by mutation of the miR-139-binding site (Fig. 4D and E). Taken together, these results indicate that miR-139 directly binds to the 3'-UTR region of TOP1 and inhibits its expression in HCC cells.

miR-139 suppresses HCC cell proliferation and migration through downregulation of TOP1. The previous study demonstrated that miR-139 is significantly downregulated in HCC tissues and is an independent risk factor for reduced survival (8); however, the biological function of this tumor-suppressive miRNA is largely unknown. To determine if miR-139 affects HCC cell proliferation, BEL-7404

Figure 4. miR-139 directly targets and inhibits TOP1 expression. (A) Schematic representation of the putative miR-139 binding site in the 3'-UTR of TOP1 mRNA. Mutations were generated in the miR-139 binding site of the TOP1 3'-UTR (indicated in red). (B) Relative luciferase activity (mean ± SD) mediated by reporter constructs harboring the wt or mut 3'-UTR of TOP1 upon transfection with 100 nM miR-NC or miR-139 in BEL-7404 cells. (C) Relative luciferase activity (mean ± SD) mediated by reporter constructs harboring the wt or mut 3'-UTR of TOP1 upon transfection with 100 nM miR-139 inhibitors or a negative control in BEL-7404 cells. (D) Relative luciferase activity (mean ± SD) mediated by reporter constructs harboring the wt or mut 3'-UTR of TOP1 upon transfection with 100 nM miR-139 inhibitors or a negative control in SMMC-7721 cells. (E) Relative luciferase activity (mean ± SD) mediated by reporter constructs harboring the wt or mut 3'-UTR of TOP1 upon transfection with 100 nM miR-139 inhibitors or a negative control in SMMC-7721 cells. wt, wild type; mut, mutant; UTR, untranslated region; TOP1, Topoisomerase I; miRNA, microRNA; SD, standard deviation. *P<0.05 and **P<0.01.

Figure 5. miR-139 suppresses HCC cell proliferation through downregulation of TOP1. BEL-7404 and SMMC-7721 cells were transfected with a negative control, miR-139 mimics or miR-139 inhibitors overexpressing vectors for 48 h. Proliferation rates of HCC cells were determined by Cell Counting Kit-8 assay. OD, optical density; miRNA, microRNA; NC, negative control miRNA, microRNA; HCC, hepatocellular carcinoma. *P<0.05 and **P<0.01.
and SMMC-7721 cells were treated with a negative control, miR-139 mimics or miR-139 inhibitors for 48 h. A CCK-8 assay demonstrated that enforced miR-139 expression significantly reduced the proliferation rate of both cell lines, whilst overexpression of TOP1 lacking the endogenous 3'-UTR completely abrogated the inhibitory effect of miR-139 (Fig. 5). Furthermore, a wound-healing assay was performed to test whether miR-139 had any effect on HCC cell migration. As depicted in Fig. 6, miR-139 overexpression notably repressed HCC cell migration, and miR-139-induced migration inhibition could be rescued by exogenous TOP1 expression. Overall, these data indicated that tumor-suppressive miR-139 inhibits cell proliferation and migration through downregulation of TOP1 in HCC.

Discussion

Since the beginning of this century, accumulating evidence has demonstrated that miRNAs serve pivotal roles in the process of tumorigenesis (19). An individual tumor type is characterized by a globally distinctive expression pattern of miRNAs (20-23). It has been demonstrated that miRNA expression patterns are closely associated with a number of important clinical events, including tumor diagnosis, treatment responses and prognosis (24,25). Furthermore, novel evidence has revealed more specific roles of miRNA in tumorigenesis (26). A number of functional studies have demonstrated that miRNAs serve an oncogenic or tumor suppressor role in different malignancies, in vitro and in vivo (27). MiRNA-139 was determined to be dysregulated in various cancer types, including breast and colon cancer (28,29), but the precise function of this miRNA still requires further exploration. Wong et al (11) determined that low expression of miR-139 is associated with metastatic HCC and overexpression of miR-139 suppresses metastasis and the progression of HCC by downregulating ROCK2 (11). In addition, a low-expression of miR-139 was also determined in colorectal cancer and breast cancer, which indicates that miR-139 may be a key regulator in malignancies (11,28). The previous study demonstrated that miR-139 is significantly downregulated in HCC tissues and could be used as an independent risk factor for predicting prognosis in patients with HCC (8).

Development of dysplastic hepatocytes in point foci and nodules dysplasia and formation emergence in HCC are associated with the build-up of an accumulation of irreversible structural alterations in genes and chromosomes (3); therefore, identification of key genes that promote genomic instability is of great importance to cancer gene therapy. In the present study, TOP1 was identified as a direct target of miR-139 in HCC. DNA topoisomerases are vital enzymes that solve DNA topological problems that result from strand separation during replication and transcription. TOP1 is a nuclear enzyme that cuts one of the two strands of DNA, relaxes the strand and reanneals the strand, thereby allowing moving DNA supercoils during DNA replication or gene transcription (30). Based on this function, topoisomerases are emerging as important factors in a wide range of fundamental biological processes in nuclear and mitochondrial genomes (31). Topoisomerases introduce transient DNA breaks using a transesterification mechanism, which is highly reversible and minimizes the risks of genome stability that would otherwise occur due to strand breakage (32); therefore, formation of a DNA-TOP1 complex is a crucial intermediate step in the transesterification mechanism. However, aberrant expression of TOP1 is potentially hazardous to the cell due to it mediating an illegitimate recombination that may lead to genomic instability and oncogenesis (33). Therefore, it is now established that topoisomerases can ensure and endanger genome integrity. Kim et al (32) indicated that TOP1 could provoke genome instability by action at sites of endogenous and exogenous DNA damage. The risks associated with strand breakage by topoisomerases indicate that there are aspects of fundamental processes, including transcription, that pose unique topological challenges and that cells require a wide repertoire of responses and specific repair pathways to safeguard the dangerous process of introducing transient DNA breaks. The importance of topoisomerases in genomic maintenance may also explain why cancer cells, which are high replicative and undergo transcriptional stress, frequently overexpress nuclear and mitochondrial topoisomerases (34).
Furthermore, it has been determined that \textit{TOP1} is highly expressed in a number of malignancy types, including colon cancer and breast cancer (35,36), indicating its potential role in tumorigenesis. The major effect of \textit{TOP1}-induced DNA lesions on cell survival has resulted in this enzyme being a prime target for cancer therapies to kill fast-growing cancer cells (37). To date, a number of \textit{TOP1} inhibitors have been developed, including camptothecins, irinotecan and topotecan. However, camptothecins and its water-soluble derivatives have several limitations. For example, camptothecins produce side effects (including leucopenia) that limit the dose that can be safely administered and, therefore, its anti-tumor efficacy (38). The diarrhea induced by irinotecan can be severe and is possibly due to ‘off-target’ effects that are associated with the bis-piperidine that confers water-solubility (38); therefore, novel therapeutic strategies targeting \textit{TOP1} are required to be developed by further increasing their antitumor activity and decreasing the side effects. However, miR-139 may be an alternative target for the same molecular signal pathway.

Due to the potential ability of miRNAs to influence multiple cellular behaviors, therapeutic strategies based on modulation of miRNA expression levels have demonstrated great promise (39). Recent studies indicated that enforced overexpression of individual miRNA exhibits a powerful antitumorogenic effect in lymphoma cells transformed by key oncogenes, including c-Myc and Bcl-2 (40). Furthermore, Kumar \textit{et al} (40) demonstrated that systemic administration of viral vectors expressing let-7 miRNAs impaired tumor growth in a mouse model with lung adenocarcinoma. In the present study, it was reported that \textit{TOP1} is inhibited by a tumor-suppressive miRNA miR-139 and increased expression of miR-139 impairs HCC cell proliferation and migration. The present study indicated that miR-139 could be a promising novel therapeutic option for targeting \textit{TOP1} and holds great potential in the treatment of HCC. It was speculated that miR-139 may be a safe and effective \textit{TOP1} inhibitor with fewer side effects.

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**Availability of data and materials**

The datasets used during the current study are available from the corresponding author on reasonable request. The raw data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

**Authors’ contributions**

PZ and JY proposed the study and wrote the first draft and analyzed the data. LY, QW, XD, RD, GZ and JL conceived and improved the manuscript. CW, QB and LJ performed the experiments. GZ and JL analyzed the data and approved the final version to be published. All authors read and approved the manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**