microRNA-539 suppresses tumor growth and tumorigenesis and overcomes arsenic trioxide resistance in hepatocellular carcinoma

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A B S T R A C T
Aims: Dysregulation of microRNAs (miRNAs) plays a critical role in tumor growth and progression. In this study, we sought to explore the expression and biological roles of miR-539 in hepatocellular carcinoma (HCC).

Main methods: The expression of miR-539 in human HCC tissues and cell lines was examined. The effects of miR-539 overexpression on cell growth, tumorigenicity, arsenic trioxide resistance of HCC cells were determined. The signaling pathways involved in the action of miR-539 in HCC were also investigated.

Key findings: miR-539 was downregulated in HCC tissues and cells, relative to corresponding controls. Overexpression of miR-539 inhibited HCC cell viability and colony formation in vitro and impaired tumorigenesis of HCC cells in vivo. Transfection with miR-539 mimic significantly induced apoptosis in HepG2 cells, which was coupled with reduced expression of anti-apoptotic proteins Bcl-2 and Bcl-xL and decreased phosphorylation of Stat3. Overexpression of a constitutively active form of Stat3 partially blocked miR-539-mediated apoptosis. Enforced expression of miR-539 resensitized arsenic trioxide-resistant HCC cells to arsenic trioxide. Intratumoral delivery of miR-539 mimic significantly retarded the growth of xenograft tumors from arsenic trioxide-resistant HCC cells by about 35%, compared to delivery of control miRNA (P<0.05). In combination with arsenic trioxide, miR-539 mimic yielded about 80% decrease in tumor burden.

Significance: miR-539 functions as a tumor suppressor in HCC and reexpression of this miRNA offers a potential therapeutic strategy for this disease.

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1. Introduction
Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [1]. For HCC at an early stage, several potentially curative treatment options are available, including surgical resection, transplantation, and local ablation with radiofrequency. For advanced unresectable HCC, transcatheter arterial chemoembolization (TACE) and systemic chemotherapy are frequently used but offer limited survival benefits [2,3]. Arsenic trioxide is an effective chemotherapeutic agent for acute promyelocytic leukemia and other hematopoietic malignancies [4]. Its therapeutic potential in solid tumors is gaining increasing interest [5,6]. A randomized control study reported that TACE combined with arsenic trioxide significantly prolonged the survival time for primary HCC patients, compared to TACE alone [6]. Another randomized case-control study demonstrated that arsenic trioxide transarterial chemoembolization and intravenous administration were effective in unresectable HCC with lung metastasis, yielding an increased median overall survival [7]. However, the clinical application of arsenic trioxide in HCC is hindered by its high toxicity and acquired drug resistance [8]. Activation of signal transducer and activator of transcription 3 (Stat3) signaling is causally linked to development of chemoresistance in HCC cells [9]. Dovitinib, a multiple tyrosine kinase inhibitor has been shown to overcome sorafenib resistance in HCC cells by inhibiting Stat3 activity [10]. Therefore, understanding the mechanisms of tumor growth and chemoresistance is of significance for successful treatment of HCC.

microRNAs (miRNAs) are a class of endogenous small noncoding RNAs that can regulate gene expression by incompletely binding to the 3′-untranslated region (UTR) of target mRNAs [11]. miRNAs are regarded as an important player in various biological processes, such as differentiation, proliferation, survival, tumorigenesis, and metastasis [12]. Several miRNAs have been identified to be implicated in chemoresistance of tumor cells. For instance, downregulation of miR-122 confers resistance to sorafenib in HCC cells [13]. Another study showed that miR-26b increases the chemosensitivity of HCC cells to doxorubicin [14]. miR-539 is a less characterized miRNA and has shown tumor-suppressive activity in osteosarcoma [15], thyroid cancer [16], and prostate cancer [17]. However, few studies have determined the biological functions of miR-539 in HCC.
In this study, we examined the expression of miR-539 in human HCC tissues and cells and explored its roles in HCC growth, tumorigenesis, as well as resistance to arsenic trioxide. The signaling pathways involved in the action of miR-539 in HCC were also investigated.

2. Materials and methods

2.1. Tissues and cell culture

We collected surgically resected tumors and adjacent non-cancerous liver tissues from 54 patients with pathological diagnosis of HCC. These patients were not given any anti-cancer therapy before surgery. Tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until gene expression analysis. The study protocol was approved by the Ethical Committee of Shanghai Jiaotong University School of Medicine (Shanghai, China).

Human HCC cell lines (PLC/PRF/5, HepG2, Huh7, Sk-Hep-1, and Hep3B) and primary human hepatocytes were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), streptomycin (100 μg/mL), and penicillin (100 U/mL).

2.2. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA from tissues and cells was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR analysis of miR-539 expression was done with TaqMan MicroRNA assay kits (Applied Biosystems) on a Roche LightCycler 480 Sequence Detection System (Roche Applied Science, Foster City, CA, USA). Relative miR-539 expression was normalized against U6 snRNA as internal standard.

2.3. miR-539 mimic and plasmids

miR-539 mimic and negative control miRNA were obtained from Thermo Scientific (Waltham, MA, USA). A fragment containing human miR-539 precursor was amplified from genomic DNA by PCR and inserted into pcDNA3.1 (+) vector (Invitrogen). A plasmid expressing constitutively active Stat3 (CA-Stat3) was obtained from Addgene Inc. (Cambridge, MA, USA).

2.4. Cell transfection

HCC cells were transfected with miR-539 mimic or control miRNA at a final concentration of 50 nM using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). For rescue experiments, cells were co-transfected with miR-539 mimic (50 nM) together with CA-Stat3 or empty vector (1 μg) using Lipofectamine 2000. At 48 h post-transfection, if not stated otherwise, cells were collected for further analyses. For generation of miR-539 stably expressing HCC cells, cells were transfected with pcDNA3.1-miR-539 plasmid or vector using Lipofectamine 2000 and selected in medium containing 600 μg/mL G418 (Sigma-Aldrich) for 2 weeks.

2.5. Cell viability assay

Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. In brief, cells were plated at 4 × 10^3 cells per well in 96-well plates. After treatment, MTT solution (final concentration of 0.5 mg/mL, Sigma-Aldrich) was added to each well. After incubation for 4 h at 37 °C, dimethyl sulfoxide was added. The absorbance was recorded on a microplate reader at the wavelength of 570 nm.

2.6. Colony formation assay

Stably transfected HCC cells were seeded in 6-well plates (500 cells per well) and cultured for 10 days. Cells were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldrich). Colonies consisting of > 50 cells were counted.

2.7. Apoptosis detection by flow cytometry

To quantify apoptosis, a commercially available apoptosis detection kit (BD Bioscience, San Jose, CA, USA) was used. In brief, cells were harvested by trypsinization and suspended in binding buffer containing Annexin V-FITC and propidium iodide (PI). Stained cells were analyzed by a flow cytometer (FACSCalibur; BD Bioscience).

2.8. Western blot analysis

Primary antibodies for Western blot analysis were as follows: anti-Bcl-2, anti-Bcl-xl, anti-phospho-Stat3(Tyr705), anti-Stat3, and anti-β-actin antibodies (Abcam, Cambridge, MA, USA). Whole cell extracts were prepared with RIPA buffer (Thermo Scientific) containing a mixture of protease and phosphate inhibitors. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were probed with the primary antibody, followed by horseradish peroxidase-conjugated antibodies (Cell Signaling Technology, Danvers, MA, USA). Proteins were visualized using an enhanced chemiluminescence detection system (Cell Signaling Technology). Densitometric quantification was done using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

2.9. Preparation of arsenic trioxide-resistant HCC cells

Arsenic trioxide-resistant HCC cell lines (HepG2-ATR and PLC-ATR) were generated as described previously [18]. In brief, HepG2 and PLC cells were exposed to increasing concentrations of arsenic trioxide (Sigma-Aldrich) up to a final concentration of 2 μM. For assessment of arsenic trioxide sensitivity, cells were treated with different concentrations of arsenic trioxide for 48 h and tested for cell viability.

2.10. Animal studies

Male athymic nu/nu mice (5–6-week old; Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were used in this study. All procedures involving animals were approved by the Animal Care and Use Committee of Shanghai Jiao tong University School of Medicine. For tumorigenesis assay, HepG2 and PLC cells stably expressing miR-539 or control cells were implanted subcutaneously in the right flank of mice (3 × 10^6 cells per mouse). Tumor volumes were measured every week for 4 consecutive weeks. Growth curves were plotted using average tumor volume at different time points. To examine the effects of miR-539 on arsenic trioxide resistance in vivo, nude mice received subcutaneous injections of HepG2-ATR cells (3 × 10^6) into the flanks. When tumors reached an average volume of about 100 mm³, animals were randomly divided into 4 groups (n = 4): control group treated with vehicle, arsenic trioxide group (i.p. 5 mg/kg, once every 3 days), miR-539 group, and arsenic trioxide group (i.p. 5 mg/kg, once every 3 days) + miR-539 group. According to the methods described by Gabriely et al. [19], miR-539 mimic (5 μg) mixed with in vivo jet polyethyleneamine transfection reagent (Invitrogen) was administered intratumorously every 3 days for 18 days. Animals were sacrificed after the last treatment, and tumors were excised and weighed.
2.11. Statistical analysis

Results are expressed as mean ± standard deviation. Statistical differences between two groups were determined by the Student’s t-test. One-way analysis of variance (ANOVA) analysis was applied for multiple group comparison. A significant difference was considered as \( P < 0.05 \).

3. Results

3.1. miR-539 is downregulated in HCC tissues and cells

As shown in Fig. 1A, miR-539 expression was significantly decreased in HCC tissues, relative to adjacent normal liver tissues \( (P = 0.0146) \). To confirm the downregulation of miR-539 in HCC, we examined its expression in a panel of HCC cell lines. Compared to primary human hepatocytes, all the HCC cell lines tested exhibited a significantly \( (P < 0.05) \) lower level of miR-539 (Fig. 1B). These results indicate that miR-539 is underexpressed in HCC.

3.2. Restoration of miR-539 impedes HCC cell growth and tumorigenicity

To determine the biological roles of miR-539 in HCC, we overexpressed this miRNA in HepG2 and PLC cells. MTT assay revealed that compared to control cells, delivery of miR-539 mimic inhibited the viability of HepG2 and PLC cells by 68% and 54%, respectively, after 3-day culturing (Fig. 2A). Colony formation assay confirmed that miR-539 overexpression significantly suppressed the growth of HCC cells \( (P < 0.05 \) vs. corresponding controls; Fig. 2B). To further clarify the function of miR-539 in vivo, a xenograft tumor model was established in nude mice with HepG2 and PLC cells stably overexpressing miR-539. At 4 weeks after cell transplantation, the xenograft tumor volumes were significantly smaller in the miR-539 group than in the control group.
These results collectively indicate that miR-539 overexpression impedes HCC growth.

### 3.3. miR-539 induces apoptosis in HCC cells via inactivation of Stat3 signaling

Next, we checked whether the suppressive activity of miR-539 is associated with induction of apoptotic death in HCC cells. To this end, HepG2 cells were transfected with miR-539 mimic or control miRNA and tested for apoptosis using Annexin V-FITC/PI staining. Flow cytometry analysis showed that transfection with miR-539 mimic significantly induced apoptosis at 48 h after transfection, compared to control cells (23.6 ± 1.8 vs. 5.3 ± 0.7%, *P* < 0.05; Fig. 3A). Western blot analysis revealed that enforced expression of miR-539 remarkably reduced the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL (Fig. 3B). Moreover, the phosphorylation of Stat3 at Tyr705 was inhibited by the delivery of miR-539 mimic (Fig. 3B). However, the level of total Stat3 was not altered by miR-539 mimic. To validate whether inactivation of Stat3 signaling accounts for the pro-apoptotic activity of miR-539, we co-transfected miR-539 mimic along with CA-Stat3. CA-Stat3-transfected HepG2 cells displayed marked Stat3 phosphorylation relative to vector-transfected cells, which was accompanied by a decrease in apoptosis (Fig. 3D).
by increased expression of Bcl-2 and Bcl-xL (Fig. 3C). Most interestingly, miR-539-mediated apoptosis in HepG2 cells was partially reversed by the transfection with CA-Stat3 (Fig. 3D). These results demonstrate that miR-539-induced HCC cell apoptosis is mediated, at least partially, through inhibition of Stat3 signaling.

3.4. Overexpression of miR-539 increases chemosensitivity to arsenic trioxide

Given the link between Stat3 signaling and HCC chemoresistance [9], we next explored the role of miR-539 in the sensitivity of HCC cells to arsenic trioxide. We established 2 arsenic trioxide-resistant HCC cell lines (HepG2-ATR and PLC-ATR) by chronic exposure to arsenic trioxide. As shown in Fig. 4A and B, HepG2-ATR and PLC-ATR cells were 5–8-fold more resistant to arsenic trioxide than parental control cells. Both resistant cell lines had significantly higher expression of phosphorylated Stat3 than control cells (Fig. 4C), suggesting that Stat3 signaling is related to acquired arsenic trioxide resistance. In contrast to Stat3 phosphorylation, miR-539 expression was significantly reduced in HepG2-ATR and PLC-ATR cells relative to parental controls (P < 0.05; Fig. 4D). Notably, overexpression of miR-539 significantly reduced the IC50 dose of arsenic trioxide by about 45% and 60% in HepG2-ATR and PLC-ATR cells, respectively (Fig. 4E).

3.5. Intratumoral injection of miR-539 mimic potentiates the antitumor activity of arsenic trioxide in established HCC xenografts

To examine the therapeutic effect of miR-539 in vivo, HepG2-ATR cells were injected into nude mice to form xenograft tumors and miR-539 mimic and arsenic trioxide alone or in combination were administered. We found that arsenic trioxide alone caused a modest reduction in tumor size at 18 days after treatment, which was not statistically significant (P > 0.05 relative to vehicle-treated mice; Fig. 5A).

Fig. 4. Overexpression of miR-539 increases chemosensitivity to arsenic trioxide. (A and B) Arsenic trioxide-resistant HepG2-ATR and PLC-ATR cells and their parental controls were exposed to different concentrations of arsenic trioxide for 48 h and tested for cell viability by MTT assay. The 50% inhibitory concentration (IC50) value was calculated from concentration-response curves. (C) Western blot analysis of phosphorylated and total Stat3 proteins in HepG2-ATR and PLC-ATR cells and their parental controls. Bottom, bar graphs represent the results from three independent experiments. (D) qRT-PCR analysis of miR-539 expression in HepG2-ATR and PLC-ATR cells and their parental controls. (E) HepG2-ATR and PLC-ATR cells were transfected with control or miR-539 mimic and treated with different concentrations of arsenic trioxide. IC50 values of arsenic trioxide were calculated. *P < 0.05.

Fig. 5. Intratumoral injection of miR-539 mimic enhances the antitumor activity of arsenic trioxide (As2O3) in established HCC xenografts. (A) HepG2-ATR xenograft tumors (about 100 mm3) were treated with vehicle, arsenic trioxide, miR-539 mimic, or arsenic trioxide plus miR-539 mimic for 18 days. Tumor growth curves were plotted based on tumor volumes. (B) Final tumor weight was determined at 18 days after treatment. *P < 0.05.
Administration of miR-539 mimic alone significantly reduced tumor size by about 30%, compared to delivery of control miRNA (P < 0.05). When combined with arsenic trioxide, miR-539 mimic caused about 63% decrease in tumor burden, compared to controls (P < 0.05). Final tumor weight of mice with combined treatment was only 26% that of vehicle-treated mice, which was also significantly lower than that of mice treated with miR-539 mimic or arsenic trioxide alone (Fig. 5B). Together, these data demonstrate that miR-539 can overcome arsenic trioxide resistance in HCCs.

4. Discussion

In this study, we showed that miR-539 expression was downregulated in HCC tissues and cell lines. Restoration of its expression led to a significant decline in cell viability and colony formation capacity in HCC cells. Consistent with the in vitro findings, miR-539-overexpressing HCC cells displayed significantly slower growth in xenograft tumor mouse models than control cells. These observations point toward that miR-539 acts as a tumor suppressor in HCC. Similarly, miR-539 also plays a negative role in tumor growth and progression in several other types of malignancies such as osteosarcoma [15], thyroid cancer [16], and prostate cancer [17].

Induction of apoptosis is a common mechanism for the regulation of tumor growth by tumor suppressors [20,21]. In this study, we found that delivery of miR-539 mimic significantly promoted apoptosis in HCC cells. Mechanistic studies further revealed that miR-539 caused apoptotic death in HCC cells by inhibiting Stat3 activation, as overexpression of CA-Stat3 significantly reversed miR-539-mediated apoptosis. miR-539 has been proved to target many genes, including SPAG5 [17], miR-539 need to be further identified.

The direct target genes mediating the tumor suppressive activity of miR-539 overexpression have had no significant impact on total Stat3 protein levels. Therefore, miR-539 influences Stat3 signaling activation via an indirect manner. At the molecular level, miR-539 overexpression resulted in a marked decline in the amounts of anti-apoptotic proteins Bcl-2 and Bcl-xL, confirming the pro-apoptotic activity of miR-539. Luciferase reporter assays demonstrated that overexpression of miR-539 failed to inhibit the activity of the reporter gene harboring the entire 3'-UTR of Bcl-2 or Bcl-xL mRNA (data not shown). Since Bcl-2 and Bcl-xL are known as target genes of Stat3 [24,25], it was speculated that the down-regulation of Bcl-2 and Bcl-xL in miR-539-overexpressing HCC cells might be a consequence of inhibition of Stat3 activity. Nevertheless, the direct target genes mediating the tumor suppressive activity of miR-539 need to be further identified.

Stat3 signaling is implicated in chemoresistance of various cancers [26,27]. Inhibition of Stat3 activity has been documented to induce synergistic cytotoxicity with arsenic trioxide [28]. Augmented Stat3 inhibition has been shown to account for enhanced cytotoxicity of arsenic trioxide and bortezomib in myeloma [29]. Since miR-539 had the ability to inhibit Stat3 activation in HCC cells, we hypothesized that this miRNA may modulate chemosensitivity in this malignancy. In support of this hypothesis, enforced expression of miR-539 significantly decreased the IC50 dose of arsenic trioxide in arsenic trioxide-resistant HCC cells, which was accompanied by inhibition of Stat3 phosphorylation. In vivo studies confirmed that adenoviral delivery of miR-539 significantly reduced tumor burden in mice injected with arsenic trioxide-resistant HCC cells, and to a greater extent, in combination with arsenic trioxide. Taken together, our data suggest a novel approach to overcome arsenic trioxide resistance in HCC cells, which involves restoration of miR-539 expression and suppression of constitutive Stat3 activity.

5. Conclusions

Downregulation of miR-539 is causally linked to tumor growth, tumorigenesis, and arsenic trioxide resistance in HCC cells. The tumor-suppressive activity of miR-539 is associated with inhibition of Stat3 activation and modulation of Bcl-2 and Bcl-xL expression. Reexpression of miR-539 may represent a promising therapeutic strategy for HCC.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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