Original Article

miR-506 regulates cell proliferation and apoptosis by affecting RhoA/ROCK signaling pathway in hepatocellular carcinoma cells

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Abstract: Background: Hepatocellular carcinoma (HCC), is the third leading cause of cancer-related death. MicroRNA-506 (miR-506) has been reported to exhibit abnormal expression in HCC; however, the role of miR-506 in HCC and the molecular mechanisms underlying miR-506 in HCC remain unclarified. Methods: Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay was performed to detect the expression of miR-506 and Rho associated coiled-coil containing protein kinase 2 (ROCK2). Cell proliferation and apoptosis were evaluated by MTT assay and flow cytometry, respectively. Bioinformatics analysis and luciferase reporter assays were performed to identify the regulation between miR-506 and ROCK2. Western blot assay was performed to detect the expression of ROCK2, RhoA, and Ras-related C3 botulinum toxin substrate 1 (Rac1). The tumor growth in vivo was evaluated in a HCC xenograft mice model. Results: The mRNA levels of ROCK2 were significantly upregulated, while miR-506 levels were significantly downregulated in HCC tissues and cells. The expression of ROCK2 was negatively correlated with miR-506 in HCC tissues. In vitro, upregulation of miR-506 inhibited proliferation and induced apoptosis, and downregulation of miR-506 promoted proliferation and blocked apoptosis in HepG2 and Hep3B cells. ROCK2 was a target gene of miR-506 and miR-506 regulated the expression of ROCK2 in HepG2 and Hep3B cells. Furthermore, downregulation of miR-506 partially attenuated the tumor-suppressive effect of ROCK2 knockout on HepG2 and Hep3B cells, and upregulation of miR-506 partially attenuated the oncogenic effect of ROCK2 overexpression on HepG2 and Hep3B cells; Overexpression of ROCK2 increased and ROCK2 knockdown decreased the expression of Rac1, which were attenuated by upregulation of miR-506 or downregulation of miR-506, respectively. In addition, ROCK2 overexpression or knockdown had no significant effect on RhoA expression. In vivo, upregulation of miR-506 suppressed tumor growth, while downregulation of miR-506 promoted tumor growth. Conclusion: miR-506 was involved in cell proliferation and apoptosis by affecting RhoA/ROCK signaling pathway in HCC cells. Our results provide a novel mechanism of miR-506-mediated suppressive effects on HCC tumorigenesis.

Keywords: ROCK2, miR-506, hepatocellular carcinoma, apoptosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide with a high mortality rate [1-3]. Thus, it is pivotal to clarify the underlying molecular mechanism of HCC initiation and progression and to find novel treatment for HCC.

MicroRNAs (miRNAs) are small, non-coding, single-stranded RNAs that have been described to play critical roles in various essential biologic processes, such as development, metabolism, and apoptosis [4-7]. Accumulating evidence indicates that microRNA-506 (miR-506) plays an important role in tumorigenesis [8]. Extensive research in recent years has demonstrated that miR-506 functions as a tumor suppressor in cancer development and progression. miR-506 has been reported to be involved with cell proliferation, migration, and apoptosis in a variety of cancer cells, including ovarian cancer [9], lung cancer [10], osteosarcoma [11], cervical cancer [12, 13], and gastric cancer [14]. For example, miR-506 was significantly downregulated in human cervical cancer (CC).
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cell lines (HeLa and C33A) and clinical CC specimens, and upregulated expression of miR-506 inhibited CC cells proliferation both in vitro and in vivo and suppressed the expression of Multidrug resistance-associated protein 4 (MRP4, ABCC4) by directly targeting its 3'-UTR [12]. Suppression of tumor metastasis by miR-506 was also found in HCC [15, 16]. miR-506 was significantly downregulated in HCC tissues and cell lines, and upregulation of miR-506 suppressed HCC cell migration, invasion, and metastasis by targeting interleukin 8 (IL-8) both in vitro and in vivo [16], indicating that miR-506 could exert its antitumor activity in HCC. However, the function of miR-506 in HCC has not been clearly elucidated and should be intensively investigated.

Rho associated coiled-coil containing protein kinase 2 (ROCK2) is serine/threonine kinases that is downstream targets of the small GTPases RhoA [17]. ROCK2 has been reported to be critical for diverse cellular activities including actin cytoskeleton organization, cell adhesion and motility, cell migration and invasion, proliferation, and apoptosis [18, 19]. It has been reported that miR-139-5p inhibited cell proliferation and invasion by targeting ROCK2 in ovarian cancer [20]. However, the molecular mechanism of miR-506/ROCK2 axis-mediated tumor progression in HCC is poorly understood, and extensive research is necessary to explore the molecular basis of miR-506/ROCK2 axis in HCC.

In the present study, we investigated the biological functions of miR-506 in HCC in vitro and in vivo. We also validated whether miR-506 directly targeted ROCK2 to inhibit HCC progression. Our study showed that miR-506 exhibited a tumor-suppressive function by directly targeting ROCK2 in HCC cells. Moreover, we also found that miR-506 regulated the RhoA/ROCK signaling pathway, revealing that miR-506 may be involved in HCC progression by affecting the RhoA/ROCK signaling pathway.

Materials and methods

Clinical samples and cell culture

This study has acquired informed consents from the guardians of all patients, and it has been approved by the Research Ethic Committee of Renmin Hospital, Hubei University of Medicine. HCC tissues and adjacent tissues were obtained from twenty HCC patients at Renmin Hospital, Hubei University of Medicine. All patients involved gave signed, informed consent and had not undergone any other therapy. HCC cell lines HepG2, Hep3B, QSG-7701, SMMC-7721, Huh-7 and human liver cell line LO2 were purchased from American Type Culture Collection (ATCC). All cells were cultured in the Dulbecco’s Modified Eagle’s medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% of fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% of penicillin/streptomycin stock solution (Sigma, St. Louis, MO, USA). All cells were incubated at 37°C in 5% CO₂.

Reagent and cell transfection

The miR-506 mimic, mimic negative control (NC mimic), miR-506 inhibitor, inhibitor negative control (NC inhibitor), pcDNA3.0 (pc-NC), pcDNA-ROCK2 (pc-ROCK2), small interfering RNA ROCK2 (si-ROCK2), and negative control si-NC were purchased from Jin Wei Zhi Biotechnology Co., Ltd (Suzhou, China). HepG2 and Hep3B which has reached 70% confluence in one well of a 6-well plate were transfected with abovementioned plasmids or RNAs using Lipofectamine 3000 (Thermo Fisher Scientific), respectively. The transfected cells were prepared for the following experiments.

qRT-PCR assay

Total RNAs were extracted from tumor tissues, adjacent tissues or cells individually using Trizol (Thermo Fisher Scientific). miRNAs were isolated using miRNeasy Mini Kits (Qiagen, Hilden, Germany). Subsequently, complementary DNA (cDNA) was obtained by reverse transcription using the miScript Reverse Transcription Kit (Qiagen). To quantify miRNAs, reverse transcription was performed using Prime Script™ RT reagent kit (Takara, Shiga, Japan). qPCR was performed using the TaqMan® Universal PCR Master Mix II (Biosystems, Forster City, CA, USA), The primers used were as follows: miR-506-F, 5'-TAAGGCACCCTTCTGAGTAGA-3', miR-506-R, 5'-GCGAGCACAGAATTAATACGAC-3'; U6-F, 5'-CTCGCTTCGAGCAGCACA-3', U6-R, 5'-AACGCTTCAAGAATTCGCG-3'; ROCK2-F, 5'-TCTCCGCTTGGCAGAC-3', ROCK2-R, 5'-CTCCTGCAGATGAAAGGC-3'; β-actin-F, 5'-GGACCTGACTGACTACCT-3', β-actin-R, 5'-TACT-
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CCTGCTTGCTGAT-3'. miR-506 and ROCK2 expression were normalized according to U6 and β-actin, respectively.

**MTT assay**

HepG2 and Hep3B cells transfected with above-mentioned plasmids or RNAs were seeded in 96-well plates for 24 h, 48 h and 72 h, 20 μL/well of MTT solution (5 mg/mL, Sigma) was added and incubated for another 4 h. Then the supernatants were removed and formazan crystals were solubilized in 150 μL dimethylsulfoxide (DMSO, Sigma). The crystals were dissolved at room temperature for 10 min. The cell proliferation was evaluated by measuring the absorbance at 450 nm using a microplate reader (Bio Tek, Winooski, VT, USA).

**Cell apoptosis assay**

Cell apoptosis was analyzed using FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA). HepG2 and Hep3B cells transfected with above-mentioned plasmids or RNAs were collected and digested with trypsin, washed with phosphate buffer saline (PBS), Then cells were labeled with 5 μl annexin V-FITC and 5 μl propidium iodide (PI) in dark for 15 min at room temperature. Cell apoptotic rate was detected by a FACS Calibur flow cytometer with Cell Quest software (BD Biosciences).

**Western blot assay**

Total protein obtained from cultured cells was lysed using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) supplemented with protease inhibitors (Thermo Fisher Scientific). Protein lysates (20 μg/lane) of cells were separated using SDS-PAGE Gel Quick Preparation Kit (Beyotime, Shanghai, China). Subsequently, the separated products were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and blocked with 5% nonfat milk. Western blotting was performed using the following specific antibodies: ROCK2 (1:5000, Abcam, Cambridge, UK), RohA (1:5000, Abcam), Rac1 (1:5000, Abcam), β-actin (1:5000, Abcam). Membranes were incubated overnight at 4°C and then probed for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, Beyotime). Finally, protein bands were visualized using Pierce™ ECL Western Blotting Substrate (Beyotime) with the intensity analysis via Quantity One software (Bio-Rad Laboratories, Philadelphia, PA, USA).
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Luciferase reporter assay

A wild-type (WT) fragment of ROCK2 harboring the miR-506 binding site and its mutated (MUT) seed sequence were purchased from Jin Wei Zhi Biotechnology Co., Ltd and cloned into the pMirGLO reporter vector (Promega, Madison, WI, USA), namely ROCK2-WT and ROCK2-MUT. ROCK2-WT or ROCK2-MUT and NC mimic or miR-506 mimic were transfected into HepG2 and Hep3B cells using Lipofectamine 3000 (Thermo Fisher Scientific). Cells were harvested at 48 h after transfection. The luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to protocols.

Tumor xenografts in vivo

The experiments were approved by the Animal Care and Experiments committee of Renmin Hospital, Hubei University of Medicine. The 32 female BALB/c nude mice (20-22 g, 4-6 weeks) were purchased from Guangzhou Sino biotech
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HepG2 cells were transfected with miR-506 mimic, NC mimic, miR-506 inhibitor, or NC inhibitor, respectively. The cells were subcutaneously injected into nude mice. The tumor volume was measured and calculated by 0.5 × length × width × height every 5 days, and mice were euthanized 30 days after cells injection. Tumor weight was measured and qRT-PCR was performed to detect the expression of miR-506.

Statistical analysis

Each experiment was performed in triplicates. All data are shown as mean ± standard deviation (SD). All statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Statistical differences were analyzed by two-tailed Student’s t-test or one-way analysis of variance (ANOVA). A value of P less than 0.05 was considered significant.

Results

miR-506 and ROCK2 expression are negatively correlated in HCC

To investigate the expression correlation between miR-506 and ROCK2 in HCC, qRT-PCR was performed to detect the expression levels of miR-506 and ROCK2 in HCC tissues and cells. First, we analyzed the expression of miR-506 and ROCK2 in 20 tissue samples from HCC patients (Figures 1A and 2B). ROCK2 expression was markedly increased while miR-506 expression was significantly decreased in tumor tissues compared with adjacent tissues. The same phenomenon was found in HCC cell lines. miR-506 expression was significantly downregulated while the expression levels of ROCK2 was significantly upregulated in HCC cell lines compared with human liver cell line L02 (Figure 1C and 1D). A significant negative correlation between miR-506 and ROCK2 was also observed in HCC tissues (Figure 1E).

miR-506 regulated HCC proliferation and apoptosis in vitro

To examine the function of miR-506 in HCC cells, we transfected miR-506 mimic or miR-506 inhibitor into HepG2 and Hep3B cells (Figure 2A). MTT assay demonstrated that the proliferation of HepG2 and Hep3B cells transfected with miR-506 mimic was inhibited, which was significantly promoted in the miR-506 inhibitor group (Figure 2B). In addition, the percentage of apoptotic cells was significantly increased in HepG2 and Hep3B cells transfected with miR-506 mimic compared with NC mimic group, which was significantly decreased in miR-506 inhibitor group (Figure 2C and 2D).

miR-506 was involved in tumor growth in vivo

To further explore the role of miR-506 in tumorigenesis, HepG2 cells transfected with miR-506 mimic or miR-506 inhibitor were subcutaneously injected into nude mice. The mean tumor volume in the miR-506 mimic group was significantly smaller than in NC group, and was significantly bigger in the miR-506 inhibitor group (Figure 3A). A same phenomenon was found in tumor weight (Figure 3B). These results supported that miR-506 was involved in tumor growth in vivo, consistent with findings in vitro.
In addition, qRT-PCR indicated that miR-506 were obviously increased in miR-506 mimic group, and miR-506 was significantly decreased in miR-506 inhibitor group compared with NC mice (Figure 3C).

**ROCK2 was a target gene of miR-506**

To better understand the underlying mechanism of miR-506, we analyzed the expression of ROCK2 in the HepG2 and Hep3B cells transfected with miR-506 mimic or miR-506 inhibitor. ROCK2 was significantly upregulated in miR-506 inhibitor-transfected cells and downregulated in miR-506 mimic-transfected ones (Figure 4A). To further evaluate whether ROCK2 is a functional target of miR-506, bioinformatics software suggested that ROCK2 was a putative target gene of miR-506 (Figure 4B). The luciferase reporter assay was performed to confirm the relationship between miR-506 and ROCK2 with the results showing that transfection with miR-506 mimic significantly decreased the luciferase activity of ROCK2-WT group, and showed no significant impact on the luciferase activity of ROCK2-MUT group (Figure 4B and 4C). In addition, to further verify the influence of miR-506 on ROCK2 expression, we analyzed the expression of ROCK2 in HepG2 and Hep3B cells transfected with pcDNA ROCK2+miR-506 mimic or si-ROCK2+miR-506 inhibitor. We found that ROCK2 was significantly upregulated...
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ROCK2 regulated HCC proliferation and apoptosis and miR-506 partly reversed the effect of ROCK2 on HCC cells in vitro

To confirm whether miR-506 affects HepG2 and Hep3B cells through ROCK2, HepG2 and Hep3B cells were treated as described in Figure 4D and 4E. MTT and flow cytometry results showed that ROCK2 overexpression promoted the proliferation and blocked apoptosis in HepG2 and Hep3B cells, which was undermined by miR-506 mimic transfection (Figure 5A, 5C, and 5E). In addition, ROCK2 knockdown inhibited proliferation and induced apoptosis in HepG2 and Hep3B cells, which was attenuated by miR-506 inhibitor transfection (Figure 5B, 5D, and 5F).

**Discussion**

miR-506 is aberrant in various cancers and associated with progression and metastasis. For instance, miR-506 was downregulated in glioblastoma tissues and cell lines and repressed cell proliferation, blocked G1/S transition, and suppressed cell invasion by targeting insulin-like growth factor-2 mRNA-binding protein 1 (IGF2BP1) in glioblastoma cells (21). Consistently, miR-506 was downregulated in retinoblastoma (RB) tissues and cells, and miR-506 directly targeted mitosis Gene A (NIMA)-
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related kinase 6 (NEK6) and overexpression of miR-506 significantly suppressed cell proliferation, induced G0/G1 cell cycle phase arrest and apoptosis in RB cells [22]. In a recent study, Wang et al. found that overexpression of miR-506 suppressed HCC cell migration, invasion, and metastasis both in vitro and in vivo, and miR-506 acted as a tumor suppressor by targeting IL8 in HCC cells [16]. Nevertheless, the exact function of miR-506 in HCC was largely unclear. According to our present study, miR-506 was downregulated in HCC tissues and cells, and miR-506 and ROCK2 has a significant negative correlation in HCC tissues, which was consistent with previous reports. Furthermore, miR-506 regulated the tumor growth in vivo, supporting that miR-506 plays an important role in HCC development. We also found that miR-506 has a tumor-suppressive effect on HCC proliferation and apoptosis by targeting ROCK2. In addition, miR-506 regulated the RhoA/ROCK signaling pathway in HCC cells.

Ras homolog A (RhoA) plays a crucial role in the proliferation, apoptosis and migration of cancer cells [23-25]. ROCK family includes two subtypes, ROCK1 (ROKβ, p160-ROCK) and ROCK2 (ROKo), was involved in the cancer progression by regulating cell proliferation, migration, and invasion [26-31]. For example, miR-129-5p/miR-101/miR-202-5p suppressed cell proliferation, migration and invasion by targeting ROCK1 in osteosarcoma [26-28]. Moreover, miR-506 suppressed cell proliferation and tumor growth by targeting ROCK1 in HCC [15]. Also, ROCK2 was frequently overexpressed in a variety of human cancers, including lung cancer, breast cancer and HCC [18, 31-33]. However, whether miR-506 is involved in cell proliferation and apoptosis by targeting ROCK2 in HCC has not been clarified. It is believed that the clarification of the question will provide an insight into how HCC cells are integrated with various signaling pathways, which includes RhoA/ROCK pathway.

In the present study, we found that miR-506 has binding sites with ROCK2, and luciferase reporter assay confirmed the relationship between miR-506 and ROCK2. Furthermore, our study also analyzed the effect of miR-506/ROCK2 axis on proliferation and apoptosis. We showed that ROCK2 attenuated the suppressive effect of miR-506 on HCC cells, suggesting that miR-506/ROCK2 axis was involved in the development of HCC. Previous studies have demonstrated that the ROCK signaling pathway was involved in a variety of cancers. For instance, FMNL2, acting as upstream of RhoA...
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by interacting with LARG, can promote actin assembly and colorectal cancer (CRC) cell invasion through activating Rho/ROCK signaling pathway [34]. Rho/ROCK was also reported to be closely correlated with the progression and dedifferentiation of gastric cancer [35]. In addition, it has been reported that miR-200b/200c/429 subfamily inhibited HCC cell migration through modulating Rho/ROCK mediated cell cytoskeletal reorganization and cell-substratum adhesion [36], suggesting that RhoA/ROCK signaling pathway may be involved in the development of HCC. Members of the Rho small GTPases family, prototype RhoA and Ras-related C3 botulinum toxin substrate 1 (Rac1), were involved in the regulation of cell polarity, proliferation, migration, and angiogenesis in diverse cancer cells [37, 38]. It has been reported that overexpression of Rac1 was linked to aggressive growth and other malignant characteristics of tumor [39]. The potential role of Rac1 in tumor development has drawn extensive attention. In our study, we found that ROCK2 knockdown decreased the expression of Rac1 and downregulation of miR-506 partly reversed the effect of ROCK2 knockdown on the expression of Rac1. In line with this, upregulation of miR-506 abolished the ROCK2 overexpression on the expression of Rac1. Those results suggested that miR-506 may play an important role on proliferation and apoptosis through RhoA/ROCK signaling pathway by regulating ROCK2 and Rac1 in HCC cells.

Taken together, our data show that miR-506 inhibited ROCK2 expression in vivo and vitro and affected RhoA/ROCK signaling pathway in HCC. Our findings provided a novel mechanism insight into the role of miR-506/RhoA/ROCK axis in the HCC development and progression. Targeting miR-506/RhoA/ROCK axis may be a potential effective therapeutic approach for HCC patients. However, we only explored the roles of miR-506/RhoA/ROCK axis in the proliferation and apoptosis of HCC cells (HepG2 and Hep3B). The influence of miR-506, and ROCK2, alone or in combination, on the development (including invasion, migration, proliferation and apoptosis) of HCC needs to be further investigated in other HCC cells.

Disclosure of conflict of interest

None.

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References

[12] Gong M, Chen C, Zhao H, Sun M and Song M. miR-506 suppresses cervical cancer cell prolif-
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