MicroRNA-199 suppresses cell proliferation, migration and invasion by downregulating RGS17 in hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC), the most common primary tumor of the liver, has a poor prognosis and shows rapid progression. MicroRNAs (miRNAs) play important roles in carcinogenesis and tumor progression. Regulators of G-protein signaling (RGS) are critical for defining G-protein-dependent signal fidelity. RGS17 plays an important role in the regulation of cancer cell proliferation, migration and invasion. Here, we showed that miR-199 was downregulated in a hepatocarcinoma cell line. Overexpression of miR-199 significantly suppressed HCC cell proliferation, migration, and invasion in vitro. RGS17 overexpression promoted HCC cell proliferation, migration, and invasion, and reversed the miR-199 mediated inhibition of proliferation, migration, and invasion. Dual-fluorescence reporter experiments confirmed that miR-199 downregulated RGS17 by direct interaction with the 3′-UTR of RGS17 mRNA. In vivo studies showed that miR-199 overexpression significantly inhibited the growth of tumors. Taken together, the results suggested that miR-199 inhibited tumor growth and metastasis by targeting RGS17.

1. Introduction

Hepatocellular carcinoma (HCC), one of the most common and aggressive malignancies in the world (Zucman-Rossi, 2010; Costentin et al., 2017). HCC is difficult to detect at onset, and it is characterized by a high degree of malignancy, a poor prognosis, and rapid progression. Since the diagnosis of HCC at its early stage is particularly difficult, only 10–20% of patients with HCC are eligible for surgical treatment and even then, some of these patients experience recurrence (El-Serag et al., 2008; Han and Park, 2008). The poor prognosis of HCC is mainly associated with its high propensity for metastasis and recurrence.

MicroRNAs (miRNAs) are a class of short, evolutionarily conserved, endogenous, single-stranded, non-coding RNA molecules with a length of 18–24 nucleotides. Increasing evidence indicates that miRNAs are involved in developmental and progression of many cancers mainly by binding to the 3′ untranslated region (3′-UTR) of specific mRNAs (Krol et al., 2010; Ha and Kim, 2014; Shenoy and Blelloch, 2014). miRNAs also regulate tumor cell metabolism by modulating gene expression at the post-transcriptional level (Qian et al., 2017; Pan et al., 2018). They can act as both tumor suppressors and oncogenes (He et al., 2017; Huang et al., 2017b; Jiang et al., 2017).

Emerging evidence indicates that certain tumor-specific miRNAs are downregulated or upregulated in HCC and closely associated with the occurrence and development of HCC (Qadir and Rizvi, 2017; Zhang et al., 2017). A recent study showed that miR-199 plays an important role in HCC diagnosis and progression (Amr et al., 2016; Zhan et al., 2017). However, to the best of our knowledge, the expression pattern and the role of miR-199 in NSCLC remain to be determined.

Regulators of G-protein signaling (RGS) proteins include > 20 different proteins and are divided into eight subfamilies (RZ/A, R4/B, R7/C, R12/D, RA/E, RGEF/F, RGRK/G, and RSNX/H) based on the homology of RGS domains and protein structure (Hurst and Hooks, 2009; Li et al., 2015). RGS17, the most recently discovered member of the RZ/A subfamily, is overexpressed in human lung adenocarcinomas, prostate cancer, and HCC (Sokolov et al., 2011). However, the relationship between RGS17 and miR-199 remains unexplored. In the present study, we investigated the regulatory roles of miR-199 and RGS17 in HCC by examining cell migration, invasion, and proliferation and investigated the relationship between miR-199 and RGS17.

Abbreviations: RGS17, G-protein signaling 17; HCC, hepatocellular carcinoma; 3′UTR, 3′-untranslated region
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2. Materials and methods

2.1. Ethics statement

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals, and all experiments were approved and performed according to the guidelines of the Ethics Committee of Affiliated Zhongshan Hospital of Fudan University, Shanghai, China. All surgical procedures were performed under anesthesia, and every effort was made to minimize suffering.

2.2. Animal experiments

For tumor growth assays, HepG2 cells transfected with miR-199 mimics or the negative control were injected subcutaneously into the right scapula of nude mice (5-week-old BALB/c-nude, six per group, 2.0 × 10⁶ cells for each mouse). The mice were observed for 30 days for tumor formation. Tumor volume (V) was monitored every 5 days and calculated using the formula: \[ V = 0.5 \times \text{length} \times \text{width}^2. \]

2.3. Cell lines and cell culture

The liver cancer cell lines HepG2, Hep3B, Huh-7, and SMMC-7721 were provided by the cell bank of the Chinese Academy of Sciences, and the normal human hepatic cell line (LO2) was preserved in our laboratory and maintained in RPMI-1640 supplemented with 10% FBS (GIBCO, New York, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a 5% CO₂ incubator.

2.4. Cell transfection

The miR-199 mimics and the negative control were synthesized by GenePharma (Shanghai, China) and transfected into HepG2 cells to a final oligonucleotide concentration of 20 nmol/l. The full length RGS17 obtained from a human cDNA library was ligated into the pCDNA3.1 vector. All cell transfections were performed using Lipofectamine® 3000 (Invitrogen Life Technologies, USA) according to the manufacturer’s instructions. For each cell transfection, three replicates were performed.

2.5. Transwell assay

Cells were transfected with miR-199p mimic or the RGS17 overexpression vector. After 48 h, cells were starved in medium without serum for another 12 h, digested with trypsin, and then seeded on the top chambers of 24 well Transwell culture inserts (Promega, Wisconsin, USA). The medium supplemented with 20% serum was used as chemotactrant. After 24 h, cells were fixed for 10 min with 4% formalin.

Invasion assays were performed using the BD Bio-Coat Matrigel invasion assay system (BD Biosciences, New Jersey, USA) according to the manufacturer’s instructions. Noninvasive cells were removed, and the lower side of the filter was stained with 0.005% crystal violet and invaded cells were counted.

2.6. Qualitative RT-PCR

Total RNA was extracted from tissues and cells using the TRIzol reagent (Invitrogen, California, USA) following the reagent kit protocol. The cDNA was synthesized and amplified using the TaqMan miRNA reverse transcription kit. The mRNA levels of RGS17, miR-199, and U6 were determined by qRT-PCR using the qRT-PCR kit. The 2^{-ΔΔCT} method was used to calculate the relative fold difference. The primers for PCR were designed by GenePharma (Shanghai, China) and the primer sequences were as follows: miR-199, 5'-GGGAAGAGTTATGTAGTTGGAAAGA-3' (Forward), 5'-AAGCATCAGGAATTGTT-3' (Reverse); RGS17, 5'-GGGTCCCTGAAGTACGCTGAAT GCAGAAA-3' (Forward), 5'-CCCACTCCAGCTCCCTCAAAATGATTGTT-3' (Reverse); U6, 5'-CTGCGTTCCCAGACA-3' (Forward), 5'-AACGCTCAGAATTGCGT-3' (Reverse).

2.7. CCK-8 assay

HCC cell proliferation activity was detected by the CCK-8 assay according to the protocol’s instruction. HepG2 cells transfected as indicated were cultured in 96-well plates. At 0, 24, 48, and 72 h, 90 μl of fresh culture media and 10 μl CCK-8 solution were added to each sample. Cells were incubated at 37°C for 2h, and absorbance was measured using a microplate reader at 450 nm.

2.8. Dual-luciferase reporter assay

Reporter plasmids containing wild-type Luc-RGS17 (WT) and mutant Luc-RGS17 3′-UTR were constructed. miR-199 mimics were synthesized by GenePharma Co., Ltd. (Shanghai, China). Luciferase activity was assessed in the indicated cells using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) 24 h after transfection, according to the manufacturer’s instructions.

2.9. Western blot analysis

Cells or tissues were lysed in lysis buffer, and total protein content was quantified by the BCA assay. Protein samples from cells were separated by SDS-PAGE using a 10% (w/v) acrylamide gel and transferred to a PVDF membrane. The bands were visualized using an enhanced chemiluminescence kit after incubation with primary and secondary antibodies. The intensities of individual bands were analyzed by densitometry using IMAGEJ (National Institutes of Health Software, Bethesda, MD, USA) and normalized to the GAPDH level.

2.10. Statistical analysis

Continuous variables were expressed as the mean ± standard deviation (SD). One-way ANOVA was performed for multiple comparisons using GraphPad Prism software, version 5.0 (GraphPad, La Jolla, CA, USA). P-values ≤ 0.05 indicated a statistically significant difference.

3. Results

3.1. Overexpression of miR-199 inhibits hepatocellular carcinoma cell proliferation, migration and invasion

To examine the role of miR-199 in HCC, the expression of miR-199 was measured in a normal human hepatic cell line (LO2) and HCC cell lines (SMMC-7721, Hep3B, HepG2, and Huh 7) by qRT-PCR. The results showed that miR-199 expression was lower in all the HCC cell lines than in LO2 cells (Fig. 1A). The function of miR-199 in HCC was investigated by transfecting HepG2 cells with miR-199 mimics or a negative control. miR-199 was significantly upregulated by miR-199 mimic transfection compared with its expression in the control or NC group (Fig. 1B). Analysis of HepG2 cell proliferation using the CCK-8 assay showed that miR-199 overexpression reduced cell viability compared with that in untransfected cells and the NC group (Fig. 1C). To evaluate the effect of miR-199 on HepG2 cell metastasis, Transwell™ migration (Fig. 1D) and invasion (Fig. 1E) assays were performed using HepG2 cells treated with miR-199 mimics compared with untransfected controls and the NC group. The results showed that miR-199 overexpression significantly suppressed HCC cell migration and invasiveness compared with those in the control or NC group. Taken together, these results indicated that miR-199 expression was significantly decreased in hepatic carcinoma cell lines, and overexpression of miR-199
suppressed HCC cell proliferation, migration, and invasion in vitro.

3.2. RGS17 overexpression promotes hepatocellular carcinoma cell proliferation, migration and invasion

Previous studies showed that RGS17 is involved in different processes during tumorigenesis. To determine whether RGS17 was involved in the miR-199-mediated regulation of tumor cell proliferation, an RGS17 overexpression vector (RGS17) and a negative control (NC) were constructed and successfully transfected into HepG2 cells. qRT-PCR (Fig. 2A) and western blot analysis (Fig. 2B) showed that the expression of RGS17 was significantly increased in HepG2 cells transfected with RGS17 overexpression vector compared with that in the control or NC group. CCK-8 analysis showed that RGS17 overexpression significantly promoted HepG2 cell proliferation (Fig. 2C). Transwell\(^{\#}\) migration (Fig. 2D) and invasion (Fig. 2E) assays in HepG2 cells showed that RGS17 overexpression significantly promoted cell migration and invasiveness. Taken together, the results showed that RGS17 and miR-199 had opposing effects on the regulation of HCC tumorigenesis.

3.3. RGS17 is a direct target of miR-199

To determine whether there was an interaction between RGS17 and miR-199, bioinformatics analysis (http://www.genecards.org/) was performed, which identified RGS17 as a possible target of miR-199. The luciferase reporter assay showed that the 3\(^{\prime}\)-UTR of RGS17 was a downstream binding target of miR-199 (Fig. 3A-C). The results showed that miR-199 inhibited the luciferase activity of the wild type but not the mutant 3\(^{\prime}\)-UTR of RGS17 (Fig. 3D). qRT-PCR detection further showed that miR-199 overexpression significantly inhibited RGS17 expression (Fig. 3E).

3.4. RGS17 overexpression reversed the effect of miR-199 on inhibiting hepatocellular carcinoma cell proliferation, migration, and invasion

miR-199 targeted RGS17 by interacting with the 3\(^{\prime}\)-UTR of RGS17 mRNA. However, in vitro transfected RGS17 lacks the 3\(^{\prime}\)-UTR. Therefore, an RGS17 overexpression vector was transfected into miR-199 overexpressing HepG2 cells to determine whether the effect of miR-199 on inhibiting HCC cell proliferation, migration, and invasion was mediated by the direct suppression of RGS17. The results showed that upregulation of RGS17 expression significantly reversed the effect of miR-199 on inhibiting HCC cell proliferation (Fig. 3F), migration (Fig. 3G and I), and invasion (Fig. 3H and J). Taken together, these results indicated that the antitumor effect of miR-199 on HCC cells was suppressed by overexpression of RGS17.

3.5. miR-199 decreased tumor growth in an animal experiment

The results obtained in vitro indicated that overexpression of miR-199 plays an important role in HCC cell growth. To determine whether miR-199 had a similar antitumor effect in vivo, HepG2 cells stably expressing miR-199 or not were subcutaneously inoculated into nude mice (n = 6 for each group). The size of tumors in the mice was...
measured using a caliper every 5 days and tumor volume was calculated. The groups treated with miR-199 mimics showed slower tumor growth than the control groups (Fig. 4A). At the end of the experiments, the tumors were isolated (Fig. 4B) and weighed. Tumors from nude mice transfected with miR-199 mimics weighed significantly less than those of the control mice (Fig. 4C). These results were consistent with the antiproliferative effect of miR-199 in vitro and indicated that miR-199 overexpression elicited a strong anti-tumor effect in HCC in vivo.

The expression of RGS17 in xenograft tumors was determined by western blotting (Fig. 4D) and qRT-PCR (Fig. 4E). The results showed that RGS17 expression was downregulated in the xenograft tumors from the miR-199 mimic group compared with the xenograft tumors of the control groups. Taken together, the results indicated that upregulation of miR-199 inhibited HCC cell growth in vivo, and that this inhibition may be related to the regulation of RGS17 levels.

4. Discussion

HCC is one of the most common cancers in the world and has an extremely poor prognosis (Tschoatzis et al., 2012; Huang et al., 2017a). Its underlying molecular mechanism remains largely unknown. Accumulating evidence indicates that dysregulation of miRNAs may contribute to tumorigenesis (Liu et al., 2013). Increasing numbers of studies show that miR-199 has an anti-tumor function, and ectopic expression of miR-199 inhibits cancer cell aggressiveness (Byrnes et al., 2016; Gui et al., 2016; Zhou et al., 2016). For example, miR-199 expression significantly inhibits cell migration and invasion in bladder cancer by targeting ITGA3 (Sakaguchi et al., 2017). In the present study, we found that miR-199 was downregulated in HCC cell lines, and overexpression miR-199 suppressed HCC cell proliferation, migration, and invasion. G-protein-dependent signaling is critical for normal hepatocyte function, liver development, hepatic regeneration, and HCC. In recent years, RGS proteins have received increasing attention as potential targets for therapeutic intervention (Sokolov et al., 2011). High expression levels of the RGS family protein RGS17 are found in various types of cancer including non-small-cell lung cancer (Chi et al., 2017), prostate cancer (Bodle et al., 2017), colorectal carcinoma (Li and Luo, 2017), ovarian cancer (Hooks et al., 2010), breast cancer (Li et al., 2015), and HCC (Sokolov et al., 2011) and play an potential role in tumor growth and metastasis. In the present study, we found that RGS17 plays an important regulatory role in HCC cell proliferation, migration and invasion. Upregulation of RGS17 promoted HCC cell proliferation, migration, and invasion. In addition, RGS17 overexpression reversed the effect of miR-199 on inhibiting carcinoma cell proliferation, migration, and invasion. A dual fluorescence reporter experiment confirmed that miR-199 targets the 3′-UTR of RGS17. However, the exact regulatory mechanism underlying the RGS17-mediated promotion of cell proliferation, invasion, and migration in HCC remains unknown and needs further study.

In conclusion, the present study showed that miR-199 inhibited cell proliferation, migration, and invasion in HCC cells by targeting the 3′-UTR of RGS17. miR-199 may therefore be a novel diagnostic and therapeutic option for the treatment of patients with HCC.

Disclosure statement

The authors have no conflicts of interest to declare.

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Fig. 3. RGS17 overexpression reversed miR-199 mediated hepatocellular carcinoma cell proliferation, migration and invasion inhibition in vitro. (A) The RGS17 3′-UTR sequence was analyzed for miRNA target sites. The miR-199 site is printed in yellow. (B) Bioinformatics prediction of the interaction between RGS17 and miR-199. (C) miR-199 and its predicted binding sequence for WT (wild type) in the 3′-UTR of RGS17. (D) miR-199 decreased the luciferase reporter activity of RGS17 in the wild-type but not in the mutant RGS17 3′-UTR. The data are expressed as the mean ± SD, n = 5. **P < 0.001 versus other groups. (E) qRT-PCR detection showed that miR-199 expression significantly downregulated RGS17 expression at the mRNA level. The data are expressed as the mean ± SD, n = 5. ***P < 0.001 versus the control. (F) The CCK-8 assay showed the proliferation of miR-199 overexpressing HepG2 cells transfected with or without the RGS17 overexpression vector. The data are expressed as the mean ± SD, n = 5. *P < 0.05, **P < 0.001 versus the control. ***P < 0.001 versus the mimics. (G-J) Transwell migration (G and I) and invasion (H and J) assay of HepG2 cells. The data are expressed as the mean ± SD, n = 5. *P < 0.05, **P < 0.001 versus the control. ***P < 0.001 versus the mimics. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. miR-199 suppressed tumor growth in vivo. (A) Tumor volume was measured after subcutaneous transplantation with miR-199 overexpressing HepG2 cells or wild-type HepG2 cells. The data are expressed as the mean ± SD, n = 6. *P < 0.05, **P < 0.001 versus the control. (B) Images of tumor tissues from the different groups on day 30. (C) Tumor weight was measured at 30 days after subcutaneous transplantation. The data are expressed as the mean ± SD, n = 6. *P < 0.05, **P < 0.001 versus the control. (D and E) Western blot (D) and qRT-PCR (E) detection show the expression of RGS17 at the protein and mRNA levels. The data are expressed as the mean ± SD, n = 5. **P < 0.001 versus the control.

References


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