

Lentivirus-Mediated Overexpression of MicroRNA-199a Inhibits Cell Proliferation of Human Hepatocellular Carcinoma

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Abstract microRNA-199a (miR-199a) is a highly conserved miRNA, always deregulated in numerous human tumors. The results of microarray analysis indicated that miR-199a was frequently downregulated in hepatocellular carcinoma (HCC). The expression levels of miR-199a in 11 pairs of matched HCC neoplastic and adjacent non-neoplastic tissues, 5 HCC cell lines and liver cell line L02 were examined by quantitative real-time PCR analysis. We found miR-199a was significantly down-regulated in HCC tissues when compared with pair-matched adjacent non-tumor tissues. We also found the expression level of miR-199a was also substantially decreased in several human HCC cell lines including SMMC-7721, BEL-7402, BEL-7701, MHCC97H, and HepG2. To investigate the role of miR-199a in tumorigenesis, we developed a lentiviral vector for the expression of pre-miR-199a (Lenti-miR-199a). Lenti-miR-199a inhibited HCC cell proliferation in vitro and in vivo. Compared to parental cells or cells

transfected with a control vector, the overexpression of microRNA-199a in the HCC cell lines HepG2 stably was showed to reduce cell proliferation in vitro and in vivo. Luciferase reporter assay revealed the regulation of miR-199a on 3'-UTR of HIF-1 α . Further investigation confirmed that miR-199a significantly reduced the endogenous protein level of HIF-1 α in hypoxia. MiR-199a inhibits cell proliferation in vitro and in vivo partly through down-regulation of HIF-1 α in human HCC. Thus, these studies provide an important new insight into the pathogenesis of human HCC and it may open a new perspective for the development of effective gene therapy for human HCC.

Keywords miR-199a · Hepatocellular carcinoma · Cell proliferation · Hypoxia inducible factor-1 α · HepG2

Abbreviations

miRNA	MicroRNA
HCC	Human hepatocellular carcinoma
HIF-1 α	Hypoxia inducible factor-1 α
CCK-8	Cell Counting Kit-8
RFP	Red fluorescent protein

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Introduction

With its increasing mortality rate in recent years, human hepatocellular carcinoma (HCC) has become one of the most frequently occurring malignancies world-wide, but curative options for HCC are limited [1, 2]. In its advanced stages, traditional therapy proves to be of low efficacy. Thus, the identification of new treatment options is needed. Cancer is the result of a complex multi-step process that involves the accumulation of sequential alterations of

several genes. In the last few years, emerging evidence suggests that miRNAs may also be involved in HCC tumorigenesis [3, 4].

MicroRNAs (miRNAs) are a non-coding family of genes involved in post-transcriptional gene regulation [5, 6]. They inhibit the expression of target genes by binding to a target site in the 3'-UTR of target mRNAs [7, 8]. Research indicates that miRNAs are involved in various biological processes, including cell proliferation, development, and differentiation [9]. Accumulating evidence suggests that alterations of their expression may play a role in the development of human cancers. miRNAs may function as regulatory molecules and act as oncogenes or tumor suppressors [10].

Microarray studies have identified a number of miRNAs that are up- or down-regulated in HCC. Murakami et al and other groups discovered several mature miRNAs, including miR-199a, which exhibit significant, differential expression pattern in HCC and other human neoplasms [11–14]. Chen R showed that regulation of IK κ B by miR-199a affects NF- κ B activity in ovarian cancer cells [15]. However, the exact role of miR-199a in carcinogenesis in HCC is still largely unknown.

By computational prediction, hypoxia inducible factor-1 α (HIF-1 α) may be a miR-199a target [16–18]. HIF-1 α is the major regulator of the adaptive responses of cells to hypoxia, which is a feature of solid tumors, promoting both cellular survival and apoptosis under different conditions [19]. Overexpression of HIF-1 α has been observed in a variety of tumor types [20, 21]. In addition, it has also been shown that inhibiting HIF-1 α can reduce tumor growth in vivo [22–24]. HIF-1 α , a key regulator of cellular adaptive responses to hypoxia, is important for tumor growth and metastasis [25].

In the present study, we examined the expression of miR-199a in HCC and found that miR-199a was significantly down-regulated in HCC tissues when compared with adjacent non-tumor tissues and hepatoma cell lines. The study provides experimental evidence that over-expression of miR-199a is able to inhibit HCC cell proliferation in vitro and in vivo partly through suppresses the expression of HIF-1 α protein at the post-transcriptional level in HCC. Our study suggests miR-199a may be involved in the pathogenesis of HCC.

Materials and Methods

Human HCC Tissue Samples and Cell Lines

HCC specimens and paired non-tumor liver tissues were obtained from patients with HCC (without previous anti-tumor treatment) diagnosed at the Second Affiliated

Hospital of Nanjing Medical University. Tissue samples were collected at surgery, immediately snap frozen in liquid nitrogen, and stored at -80°C until RNA extraction. All samples were thoroughly reviewed by two pathologists. The human hepatoma cell lines SMMC-7721, BEL-7402, BEL-7701, MHCC97H, HepG2 and liver cell line LO2 were purchased from the cell bank of type culture collection of Chinese Academy of Sciences (Shanghai, China).

Generation of Stable Cell Lines Expressing MiR-199a

The Lentiviral vector Lenti-miR-199a or Lenti-scramble and Lentiviral packaging plasmids (Open biosystems) arrest-in co-transfected 293FT packaging cells, 48 h later, the Lentivirus in supernatant were collected and filtered, used to infect cancer cells with polybreen. After antibiotic selection for 2 weeks, the stable clones were obtained.

qRT-PCR Assay

In this study, a stem-loop qRT-PCR assay was adopted to screen mature miR-199a expression in 11 pairs of matched HCC and adjacent non-tumor tissues samples. In accordance to the manufacturer's instructions, total RNA was extracted by using Trizol reagent (Invitrogen, USA). 1 μg of total RNA was reverse-transcribed to cDNA by using AMV reverse transcriptase (TaKaRa) and looped antisense primer mix. For mature hsa-miR-199a microRNA, the primers were as follows: forward, 5'-ACACTCCAGCTGG GCCAGTGTTTCAGACTAC-3' and reverse, 5'-CTCAAC TGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAACA GGT-3'. For U6, the primers were as follows: forward, 5'-CTCGCTTCGGCAGCACACA-3' and reverse, 5'-AACGC TTCACGAATTTGCGT-3'. The mix was incubated at 16°C for 15 min, 42°C for 60 min, 85°C for 5 min. Real-time PCR was performed using an Applied Biosystems 7300HT Sequence Detection system by standardized protocol. In each assay, 1 μl cDNA was used for amplification. The reactions were incubated in a 96-well optical plate at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60° for 1 min. To normalize the expression levels of target miRNAs, U6 was used as reference. The relative amount of each miRNA to internal control U6 was calculated by using the Eq. $2^{-\Delta\text{CT}}$, in which $\Delta\text{CT} = \text{CT miRNA} - \text{CT U6}$.

Western Blot Analysis

All mediums were supplemented with 10% fetal bovine serum. Cells were treated in serum-free media containing 100 μM CoCl_2 under normoxic conditions to mimic hypoxia. After culturing for 48 h, the cells were scraped off and harvested. The cells were washed twice with ice-cold

PBS and extracted in chilled lysis buffer for 30 min on ice. Approximately 50 μg of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and then electroblotted onto PVDF membranes. After blockage of non-specific binding sites, the membrane were incubated with a rabbit antibody against HIF-1 α overnight at 4°C. The membrane were then washed with PBS and incubated with horseradish peroxidase conjugated goat anti-rabbit antibody at 37°C for 1 h. The immunoblots were visualized using an enhanced chemiluminescence (ECL) kit.

Luciferase Reporter Assay

Using three computer-aided algorithms, including TargetScan, miRanda and PicTar, we have identified HIF-1 α as a possible target of miR-199a. The sites were predicted based on base-pairing seed sequence matches. The luciferase assay was validated using the pmiR-REPORT miRNA expression reporter vector system. To verify the putative, direct interaction between miR-199a and the HIF-1 α 3'-untranslated region (UTR), the regions of the 3'-UTR of human HIF-1 α mRNA, which included a seed sequence of mature miR-199a-binding sites, were cloned into the luciferase reporter plasmid. pmiR-REPORT vectors harboring HIF-1 α 3'-UTR sequences with miR-199a binding sites (wild type, WT) or mutated (MUT) miR-199a binding sites were generated by cloning the following oligonucleotides into the *Hind*III and *Spe*I restriction sites of pmiR-REPORT: Cells were co-transfected with (1) pmiR-REPORT vectors containing WT or MUT miR-199a binding sites; and (2) control plasmid (β -gal) (Promega) was introduced into HepG2 cells together for normalization. Cells were grown in high-glucose DMEM supplemented with 10% fetal bovine serum, and luciferase measurements were performed 48 h post-transfection using the Luciferase reporter assay system (Promega).

CCK-8 Assay

To determine the biological effect of miR-199a on cell proliferation, cell counting kit-8 (CCK-8, Dojindo, Japan) was used following manufacturer's protocol. 1×10^3 cells per well were plated into 96 well plates and grown for 24, 48 and 72 h. Then, 10 μl of CCK-8 solution was added to each well, and cells were incubated for 4 h at 37°C. Absorbance at 450 nm was read on a microplate reader (MultiSkan Spectrum). All experiments were performed for 3 times, and the average of the results was calculated.

Soft Agar Assay

2×10^3 cells were seeded on six-well plates at a density of cells in 2 ml of 0.3% agar layered onto 0.6% agar. The

cultures were grown at 37°C with 5% CO₂ atmosphere for 14 days. Cell colonies were stained with crystal violet and counted using conventional microscope. Colonies were scored in a blinded fashion by two independent observers.

Tumorigenicity

Athymic mice (4–5 weeks old) were purchased from Animal Center of Chinese Academy of Science (Shanghai, China) and maintained in laminar flow cabinets under specific pathogen-free conditions. The mice were treated with humanity and assigned to 3 groups, with each six. HepG2 cells stably transfectants expressing either miR-199a or the control were implanted into the flanks of mice in a total volume of 100 μl (1.0×10^6) cells resuspended by H-DMEM without FBS. The size of the tumors was measured at 1, 2, 3, 4, 5 and 6 weeks after inoculation. Tumor volume was calculated using the formula: $L \times S^2/2$, where L is the longest tumor diameter and S is the shortest tumor diameter. The tumor-bearing mice were sacrificed 6 weeks after inoculation and the tumors were incised and weighed.

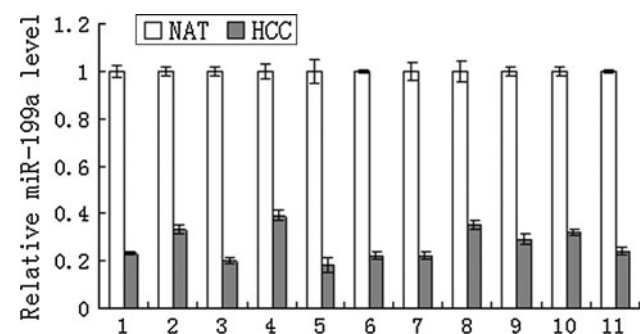


Fig. 1 The expression of miR-199a in hepatocellular carcinoma. Quantification of miR-199a was measured by stem-loop real-time PCR. Relative expression of miR-199a in HCCs was compared with that of their pair-matched adjacent non-tumor tissues ($P < 0.01$)

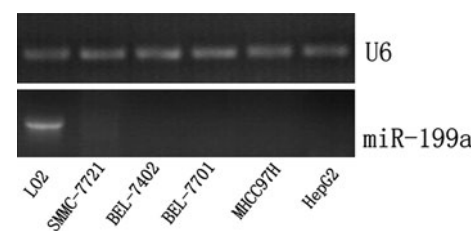


Fig. 2 Semi-quantitative RT-PCR analysis of the expression levels of miR-199a in HCC cell lines SMMC-7721, BEL-7402, BEL-7701, MHCC97H, HepG2 and liver cell line L02. U6 snRNA was regarded as endogenous control

Fig. 3 Stable cell lines Lenti-miR-199a-HepG2 and Lenti-scramble-HepG2 were generated. Cells were examined by phase contrast microscopy and fluorescent microscopy (original magnification $\times 200$). **a** Lenti-miR-199a-HepG2 cells examined by phase contrast microscopy; **b** Lenti-miR-199a-HepG2 cells examined by fluorescent microscopy; **c** Lenti-scramble-HepG2 cells examined by phase contrast microscopy; **d** Lenti-scramble-HepG2 cells examined by fluorescent microscopy

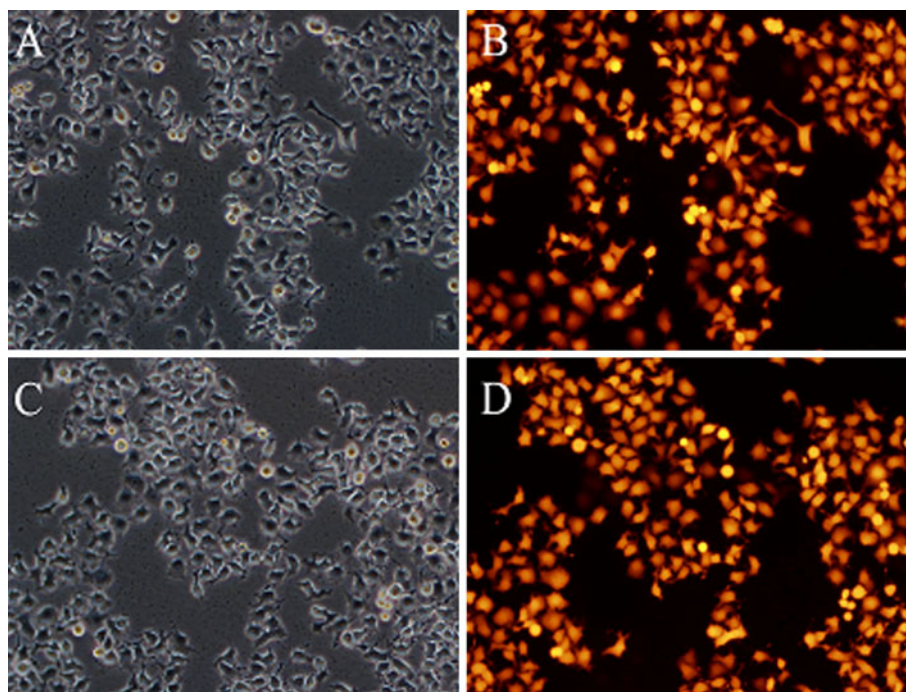
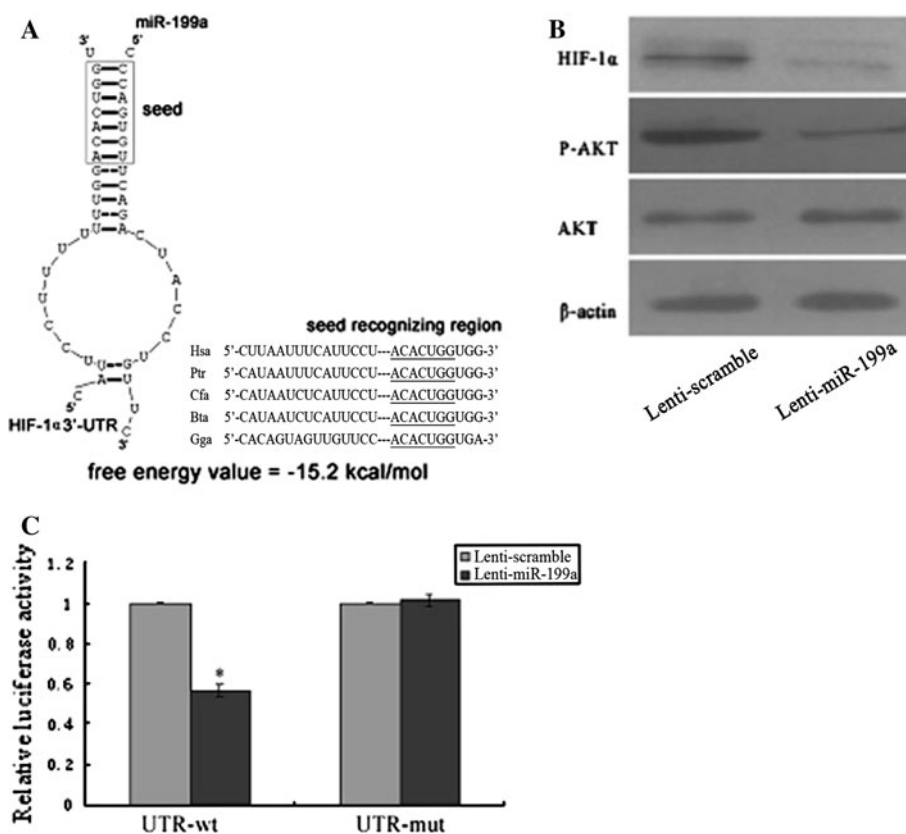


Fig. 4 HIF-1 α is a direct target of miR-199a. **a** Sequence alignment of miR-199a with 3'UTR of HIF-1 α . **b** Regulation of HIF-1 α expression by miR-199a at the translational level. HIF-1 α , AKT, P-AKT protein levels were determined by western blot analysis on 48 h after transfection. **c** Direct recognition of HIF-1 α 3'UTR by miR-199a. 530 bp sequence from the HIF-1 α 3'UTR containing the miR-199a target sequence (UTR-wt), or identical insert with a scrambled seed sequence (UTR-mut) were cloned into the p-MIR-report plasmid (Ambion)



Statistical Analysis

All values were presented as mean \pm standard deviation (SD). Statistical significance was evaluated using Student's *t*-test for unpaired comparison; $P < 0.05$ was considered statistically significant.

Results

Expression Pattern of Mature MiR-199a in Human HCC

In order to determine whether miR-199a was involved in the regulation of tumorigenesis of human HCC, we assessed the miR-199a expression level in HCC biopsy specimens and corresponding tissues. As shown in (Fig. 1), HCC samples showed down-regulation of miR-199a as compared with normal tissues ($P < 0.01$). The expression levels of miR-199a was examined in human HCC cells SMMC-7721, BEL-7402, BEL-7701, MHCC97H, HepG2 and liver cell line L02 by semiquantitative RT-PCR analysis. The result indicated that miR-199a expression was downregulated in the entire human hepatic carcinoma cell lines examined (Fig. 2).

Generation of Stable Cell Lines Expressing MiR-199a

Stable cell lines Lenti-miR-199a-HepG2 and Lenti-scramble-HepG2 were generated. RFP expression from the pLentiRTM vector allows measurement of transfection and transduction efficiency. High transduction efficiency is seen in these cells under fluorescent microscopy (Fig. 3).

Identification of HIF-1 α as a Target of MiR-199a in HCC

HIF-1 α was deduced to be a miR-199a target by computational prediction (Fig. 4a). To determine if miR-199a regulates HIF-1 α expression in hepatoma cell lines, HIF-1 α , AKT, P-AKT protein levels were analyzed by immunoblotting in Lenti-miR-199a-HepG2 cells and control cells. As shown in (Fig. 4b), miR-199a represses the expression of HIF-1 α , P-AKT protein in HepG2 cells in hypoxia. Overexpression of miR-199a in Lenti-miR-199a resulted in about 45% reduction of firefly luciferase reporter activity (normalized against β -gal activity) compared with the scrambled control cells. The observed alterations of luciferase activity are specific, because transfection of Lenti-miR-199a-HepG2 cells with the parental luciferase plasmid (with the mutant of the miR-199a binding site in HIF-1 α 3'-UTR) did not affect luciferase reporter activity. Taken together, our results

demonstrate that miR-199a directly recognize the 3'-UTR of HIF-1 α transcripts (Fig. 4c).

Effects of MiR-199a Overexpression in HepG2 Cell Proliferation

To explore the role of miR-199a in HCC carcinogenesis, we investigated the effect of miR-199a on cell proliferation in hepatocellular cell lines. The quantitative real-time PCR analysis showed that the miR-199a expression level in Lenti-miR-199a-HepG2 cells were significantly higher than those in the control stable cell lines (Fig. 5a). The MTT assays showed that HepG2 cells treated with pre-miR-199a resulted in a decrease in cell growth compared with the scrambled precursor and negative control

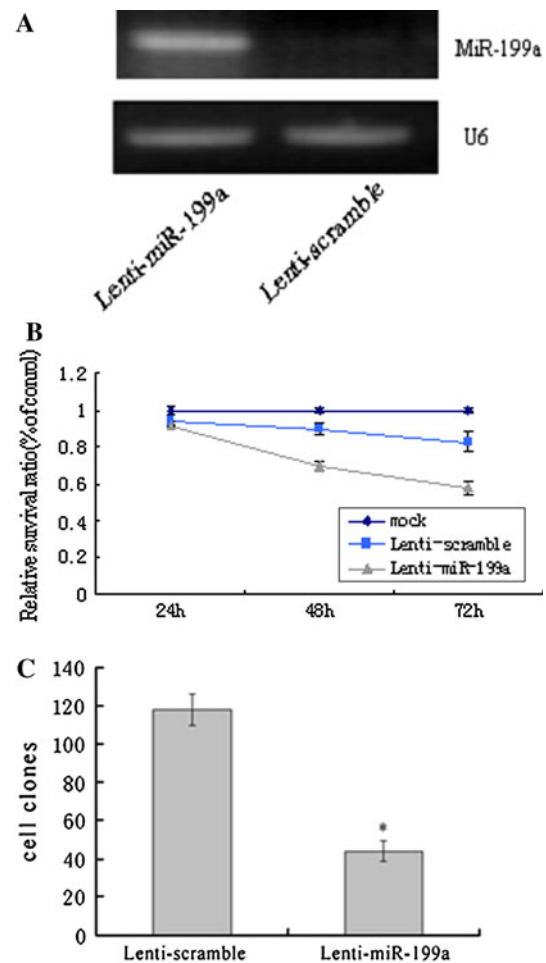
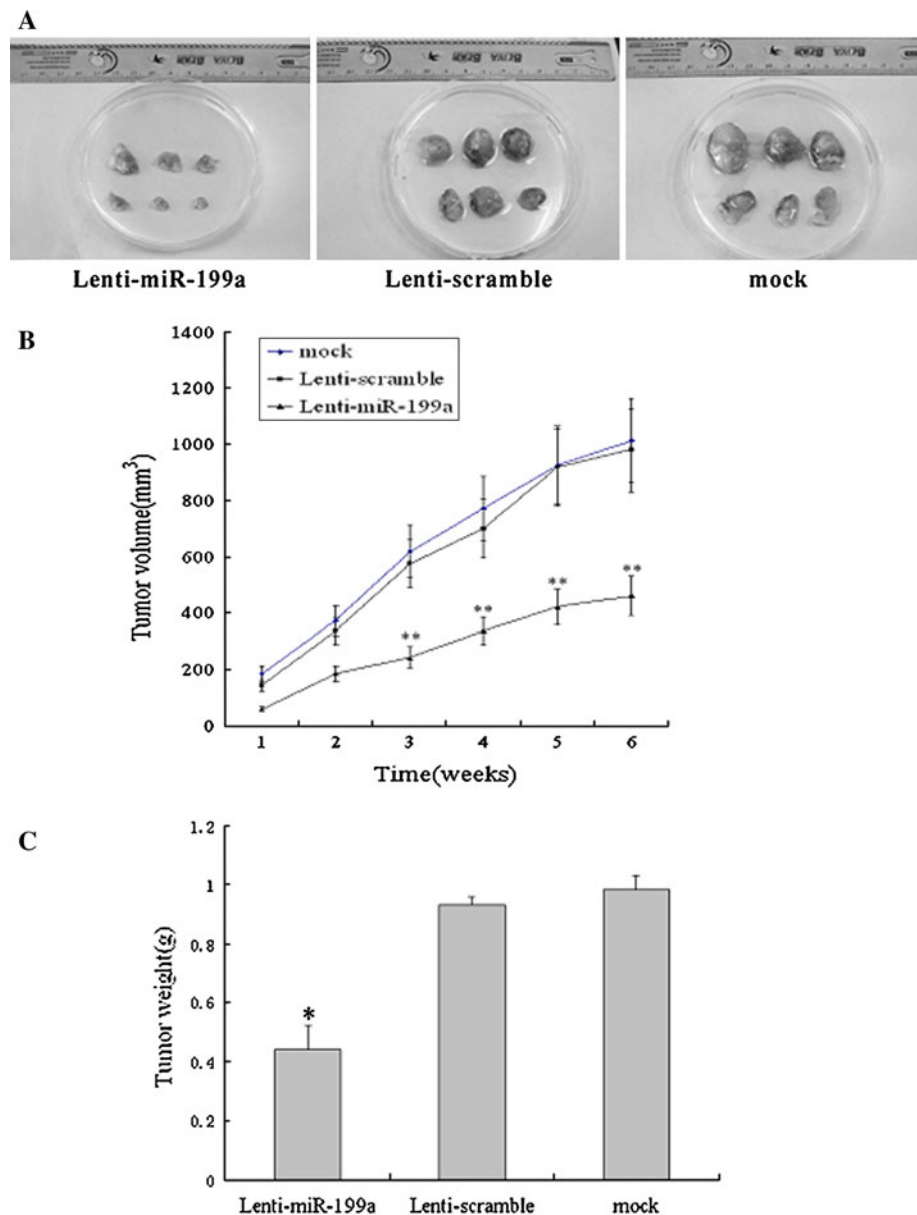


Fig. 5 The effect of miR-199a overexpression on HepG2 cells. **a** Relative expression of miR-199a in Lenti-miR-199a-HepG2 cells were measured by semi-quantitative real-time PCR. **b** The effects of miR-199a overexpression on the proliferation of HepG2 cells were measured by MTT assay at 24, 48, and 72 h. **c** The effects of miR-199a overexpression on the cell colony formation of HepG2 cells were measured by cells colony formation assay

Fig. 6 The effect of miR-199a overexpression on tumor growth. **a** Nude mice bearing Lenti-miR-199a or Lenti-scramble xenografts were sacrificed 6 weeks after inoculation. Tumors were excised and measured. **b** The volume of tumors in vivo are compared with the controls. **c** The tumors weight were investigated for tumor growth (* $P < 0.05$, ** $P < 0.01$)



(Fig. 5b). MiR-199a reduced HepG2 cells colony formation (Fig. 5c).

MiR-199a Inhibits Tumor Growth in Nude Mice Xenograft Model

Nude mice bearing Lenti-miR-199a or control xenografts were sacrificed in 6 weeks after inoculation. Tumors were excised and measured (Fig. 6a). The tumor volume of mice bearing Lenti-miR-199a tumors was 47% and 45% of mice bearing Lenti-scramble tumors and mock tumors (Fig. 6b). Furthermore, the weight of Lenti-miR-199a tumors was 45% and 43% less than Lenti-scramble tumors and mock tumors (Fig. 6c).

Discussion

Growing evidence indicates that deregulation of miRNAs contributes to tumorigenesis [26]. There are now numerous examples linking dysregulated expression of miRNAs to cancer, and miRNAs are increasingly viewed as potential therapeutic targets [27–30]. A miRNA is usually down-regulated in a particular human cancer (a “tumor suppressor” miRNA) and can have, in fact, tumor-suppressor-like effects if the main targets for that specific cell types are oncogenes. Numerous studies suggest that delivery of miRNAs that are lost in disease cells may provide a general strategy for miRNA replacement therapies [31].

In this study, to validate the down-modulation of miR-199a in HCC compared with normal matched tissue, we

first determined miR-199a expression in human HCC and its normal adjacent tissue (NAT) by quantitative reverse transcription PCR (qRT-PCR) assay. The results showed that miR-199a was significantly reduced in HCC when compared with normal tissues. In addition, miR-199a was down-regulated in HCC cell lines. With these findings, miR-199a seemed to be an interesting candidate for its involvement in HCC.

Lentiviral vectors encoding miRNAs are useful laboratory tools to study gene functions and some are now being considered for clinical gene therapy applications [32, 33]. Lentiviral vectors provide efficient gene delivery in vitro, can infect nondividing cells. To study the functions of miR-199a in HCC, HepG2 cells showing little endogenous expression of miR-199a were transduced by the use of lentiviral vectors leading to the forced expression.

Silico analysis has identified a panel of presumed target gene mRNAs that may interact with miR-199a. Among the potential mRNAs targeted by miR-199a, HIF-1 α is particularly interesting. Increasing evidences showed that HIF-1 α is a possible target for cancer treatment. Major efforts have been made to identify small molecules as selective HIF-1 α inhibitors [8, 34, 35]. To experimentally validate this computer prediction, we first investigated the modulation of HIF-1 α protein levels in hepatoma cell lines HepG2. Overexpression of miR-199a decreased HIF-1 α protein expression. Moreover, the mutation of the miR-199a binding site in HIF-1 α 3'-UTR prevented the down-regulation of luciferase expression, supporting the evidence that the effect of the miR-199a is exerted through direct interaction with the mRNA target, and that the "seed site" at the miR-199a is necessary for the binding of HIF-1 α 3'-UTR. In addition, overexpression of miR-199a with its precursors inhibited the proliferation of hepatocellular cancer cells.

In this study, we demonstrated a reduced accumulation of miR-199a in human HCC, implying that miR-199a may have a key function in HCC carcinogenesis. At the same time, we identified HIF-1 α , a key transcription factor, as a target for the miR-199a in HCC. The potential involvement of miR-199a as a suppressor of HCC tumorigenesis has been experimentally validated. These findings suggest an important role of miR-199a in the molecular etiology of HCC. A better understanding of these mechanisms will undoubtedly provide a new hope of therapeutic intervention in HCC. Restoration of miR-199a leads to the inhibition of tumor cell growth. Thus, these findings suggest that miR-199a may play an important role in regulating the tumorigenesis of HCC.

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