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Original Article

MiR-200c suppresses cell proliferation and migration by targeting R-RAS2 in hepatocellular carcinoma

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Abstract: MicroRNAs (miRNAs) are involved in various biological functions, including regulating the degradation and suppressing the translation of their downstream target genes. The dysregulation of miR-200c has been found to be implicated in several types of cancer progression. However, the biological roles of miR-200c in hepatocellular carcinoma (HCC) remain unclear. Here, we demonstrated that miR-200c expression is significantly downregulated in HCC tissues and cell lines. Meanwhile, R-RAS2 mRNA is highly expressed in HCC tissues and cell lines, and an inverse relationship is found between miR-200c and R-RAS2 mRNA levels in HCC tissues. Moreover, we discovered that R-RAS2, as a direct target of miR-200c, and the overexpression of miR-200c cause a significant downregulation of both the R-RAS2 mRNA and protein. Functional investigations revealed that miR-200c inhibits the proliferation, cell cycle progression, and invasion of HCC cells by targeting R-RAS2. Furthermore, R-RAS2 overexpression reverses the effect of miR-200c on inhibiting the tumorigenesis of HCC cells *in vivo*. Taken together, our results show that miR-200c inhibits tumor growth and migration by targeting R-RAS2.

Keywords: miR-200c, R-RAS2, hepatocellular carcinoma, proliferation, migration

Introduction

Hepatocellular carcinoma (HCC), which accounts for 85%-90% of all primary liver cancers, is the second most common cause of cancer deaths in men and the fifth leading cause of cancer deaths in women [1, 2]. HCC is difficult to detect at its 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 [3, 4]. Therefore, investigating novel diagnostic biomarkers and novel therapeutic targets for HCC is urgently needed.

MicroRNAs (miRNAs) are small non-coding RNAs comprised of 19-24 nucleotides that negatively regulate the expression of target genes at the post-transcriptional level through interaction with the 3'-untranslated regions (3'UTRs) of target mRNAs, resulting in either mRNA degradation or the inhibition of translation [5, 6]. Emerging evidence indicates that certain tumor-specific miRNAs are downregulated or upregulated in HCC and closely associated with the occurrence and development of HCC [7-10].

Thus, the present study was performed to determine the level of miR-200c in HCC tissues and cells, its effects on cell proliferation and migration, and the mechanisms for the observed effects, including the potential targets of miR-200c. Our results show that miR-200c is downregulated in HCC and inhibits cell proliferation and migration by directly targeting R-RAS2. These findings provide the first evidence that miR-200c and R-RAS2 could be potential therapeutic targets for HCC treatment.

Materials and methods

Tissue samples

Tumors and the adjacent hepatocellular carcinoma tissues were obtained from patients with

HCC who underwent surgery at the Second Affiliated Hospital of Anhui Medical University. The study protocol was approved by the ethics committee of Anhui Medical University. All tissue samples were collected in compliance with the informed consent policy.

Cell culture and transfection

Human hepatocellular carcinoma cell lines were purchased from the American Type Culture Collection (ATCC, USA) and the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The pCDNA3.1 control and pCDNA3.1-R-RAS2 were purchased from Genewiz (Suzhou, China). The cell lines were cultured in DMEM medium (Gibco) supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2. The cells were transfected with pCDNA3.1-R-RAS2 using Lipofectamine 2000 (Thermo Fisher Scientific, USA), according to the manufacturer's protocol.

Transfection

The MiR-200c control and mimics were purchased from GenePharma (Shanghai, China). We harvested all the cells and seeded them at the rate of 5×10^5 cells per well of a six-well plate. After 18 h, we mixed 5 μL scramble, the miRNA mimics (20 μM), 91 μL RNase-free water, and 4 μl Lipofectamine 2000 DNA transfection reagent (Invitrogen, USA). After 20 min, we poured the mix into the wells of a six-well plate and cultured the mix for 24 or 48 hours.

Real-time PCR assay

The total RNA was extracted using TRIzol reagent (Invitrogen, USA), and 1 μg of total RNA was reverse-transcribed using the PrimeScript RT Reagent Kit (Perfect Real Time; Takara). The amplified transcript level of each specific gene was normalized to that of GAPDH. RT-PCR was performed using SYBR green (Takara, Dalian, China) on ABI Prism 7900HT PCR system (Applied Biosystems, CA, USA) according to the direction of the reagents. The $2^{-\Delta\Delta CT}$ method was used to calculate the gene expression. U6 small nuclear RNA and GAPDH were used as internal controls. The primers were obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/). Each sample was analyzed in triplicate.

Western blotting

The whole cells were washed in PBS and lysed in a RIPA lysis buffer supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). The total protein was quantified using a BCA Protein Assay Kit (Beyotime, Jiangsu, China). Equal amounts of cell lysates were subjected to a western blot assay. The cell protein lysates were separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then they were transferred to 0.22 μm PVDF membranes (Millipore). The blots were blocked with BSA (5% w/v in PBS) for 1 h at room temperature. Antibodies: Cell Signaling Technology: anti-RRAS2 (ab182264), anti-GAPDH (ab8245). The antibodies were used as 1:1,000 dilutions at 4°C overnight. The appropriate secondary antibodies (Santa Cruz, CA, USA) were used at 1:2,000-1:5,000 (v/v) dilutions in PBS + 0.1% Tween 20 for 1 h at room temperature and the blots were visualized using an ECL kit (Millipore, MA, USA).

Luciferase reporter assay

The wild-type (WT) and mutant (Mut) sequence of RRAS2 3'UTR was synthesized and purchased from Genewiz (Suzhou, China). The wild-type (WT) and mutant (Mut) of RRAS2 3'UTR were cloned into pGL3 firefly luciferase expression plasmid by Genewiz (Suzhou, China). The cells were seeded onto 24-well plate a day before transfection, 1×10^5 cells per well. The cells were co-transfected with pGL3 firefly luciferase plasmid and miR-200c mimic by Lipofectamine 2000 (Thermo Fisher Scientific, USA), pRL-TK Renilla luciferase report (Promega, USA), and plasmid was used as internal loading control. After transfection for 48 h, we poured 100 μL of passive lysis buffer into the lysis cells, and the luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega, USA).

Cell proliferation assay

The cell proliferation ability was assayed using CCK-8 (Dojin Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The cells were seeded at a density of 10^4 cells/well in a 96-well flat-bottom and respectively cultured for the CCK-8 assay according to the protocol provided by manufacturer. The OD 450 nm was measured at 0, 24, 48, and 72 h.

Transwell assay

Transwell assays were performed using 24-well transwells (8-μm pore size; Millipore) precoated with Matrigel (BD Biosciences). The cells were

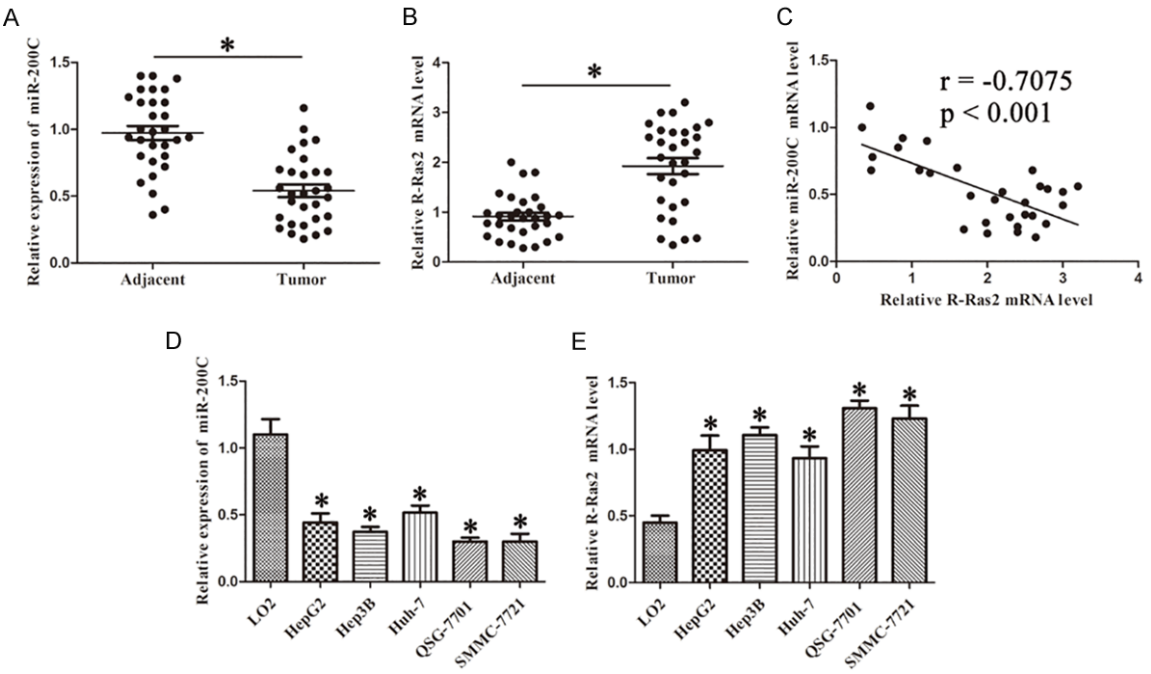

Figure 1. The expression levels of miR-200c and R-RAS2 in HCC tissues and cells. (A) The expression levels of miR-200c and R-RAS2 (B) were validated in 30 paired HCC tumor tissues and para-tumor counterparts with RT-PCR. The data are presented as the mean and plots, **P* < 0.05, Student's *t*-test. (C) The correlation analysis of miR-200c and R-RAS2 were observed in 30 HCC tumor tissues with RT-PCR. *P < 0.05, linear regression correlation analysis (F test). (D and E) The expression levels of miR-200c and R-RAS2 were studied in 5 HCC cell lines with RT-PCR. The data are presented as the mean ± SEM, n = 3, *P < 0.05, ANOVA.

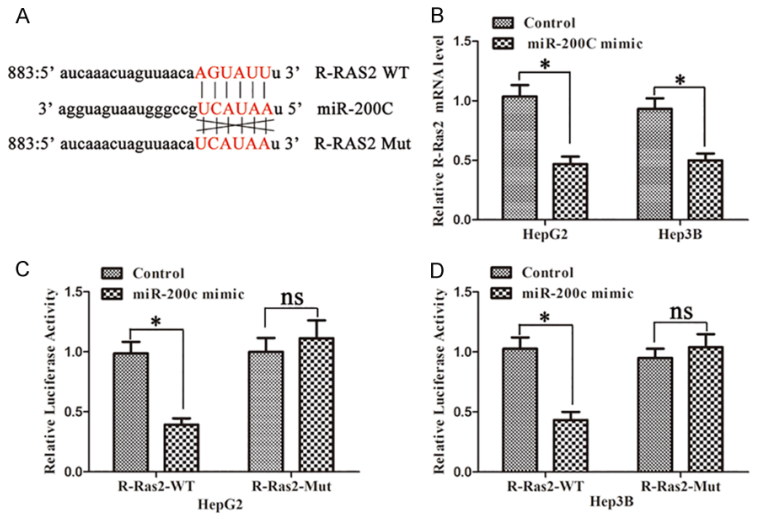

Figure 2. R-RAS2 is a direct target of miR-200c in HCC cells. A. The binding sites of R-RAS2 and miR-200c by a target microRNA (miRNA) prediction website. B. Upregulated expression of miR-200c decreased the R-RAS2 mRNA levels. Data are expressed as the mean ± SEM, n = 3, **P* < 0.05. C and D. A dual-luciferase reporter assay showed the binding activity between miR-200c and R-RAS2. Data are expressed as the mean ± SEM, n = 3, **P* < 0.05.

then harvested, and 105 cells were seeded in a serum-free medium into the upper chamber, and a medium supplemented with 20% FBS was applied to the lower chamber as a chemoattractant. After 24 hours of incubation, the migrated cells at the bottom surface of the filter were fixed with pre-cooled methanol, stained with 0.2% crystal violet solution, and counted under a microscope.

Cell cycle assay

The cells were stained using a BD Pharmingen™ Cell Cycle Kit (BD Biosciences, San Jose, USA) according to manufacture-supplied protocols (propidium iodide/RNase). The samples for the cell cycle were harvested by trypsin and fixed in 70% ethanol in PBS at -20°C overnight, washed twice with PBS and stained with propidium iodide (PI) with RNase (BD Biosciences, USA) for 30 min in the dark. Flow Cytometry

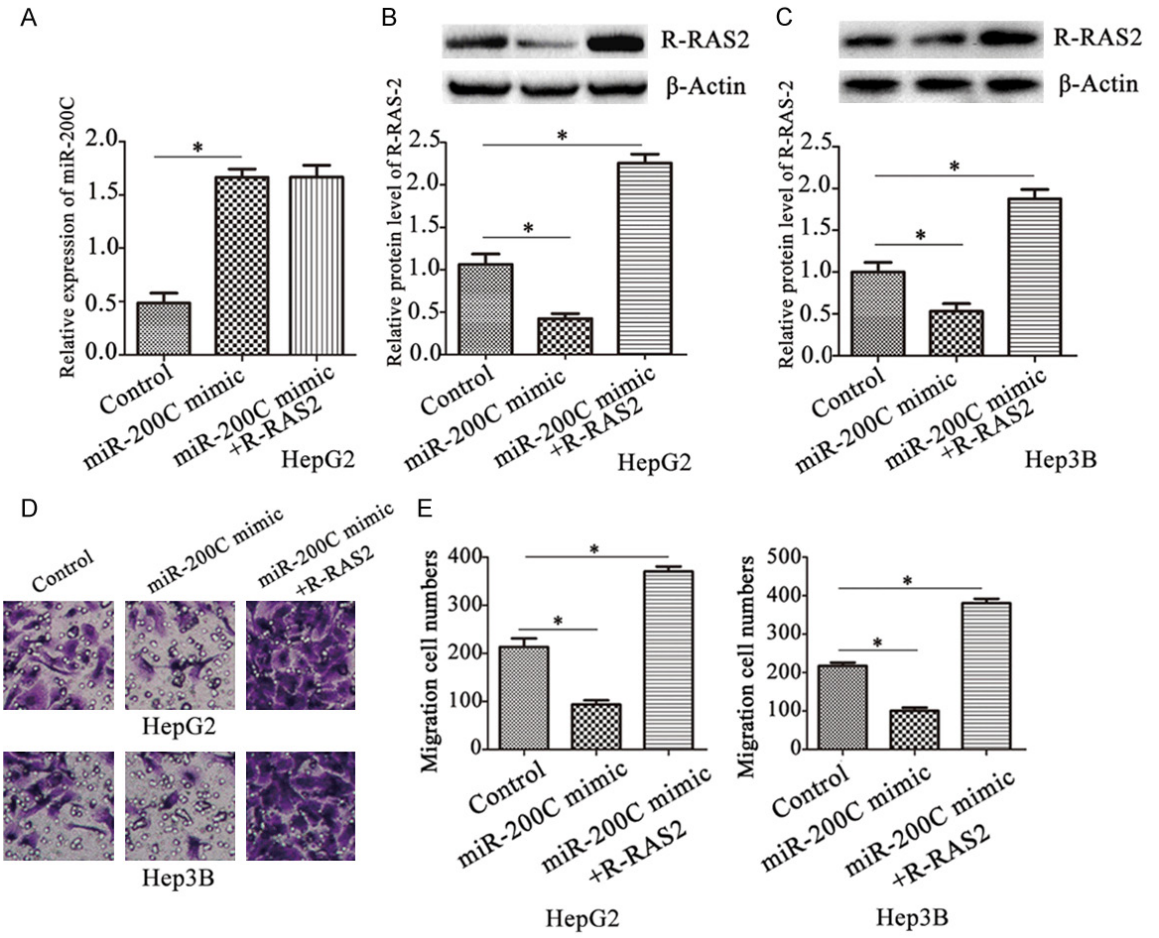

Figure 3. MiR-200c inhibited HCC cell invasion by targeting R-RAS2. The transfection of miR-200c mimics or the co-transfection of miR-200c mimics and the R-RAS2 vector in HCC cells. A. The expression levels of miR-200c were examined with RT-PCR. The data are presented as the mean ± SEM, n = 3, **P* < 0.05, ANOVA. B and C. The expression levels of R-RAS2 were examined with western blotting. The data are presented as the mean ± SEM, n = 3, **P* < 0.05, ANOVA. D and E. A transwell assay was used to determine the invasion of transfected-HCC cells. The images show the number of cells that penetrated the porous membrane. The data are presented as the mean ± SEM, n = 3, **P* < 0.05, ANOVA.

was performed using a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, USA), and the data were analyzed using MODIFIT software (Becton Dickinson, San Jose, USA).

The subcutaneous xenograft models

The animal care and euthanasia were approved by the Anhui Medical University animal studies committee. The indicated cells (10^5) were subcutaneously implanted into the bilateral axilla of the nude mice. Tumors were measured every week after the implantation, and the tumor width (W, mm) and length (L, mm) were measured using a caliper. The volume (V) of each tumor was calculated: V (mm^3) = 1/2 × L × W^2. All the mice were sacrificed 7 weeks afterwards, and the xenografts were peeled off subcutaneously.

Statistics

The experiment was independently repeated three times. The results are reported as the mean ± standard error of the mean (SEM) of at least three independent experiments. Comparisons were performed using Student's *t*-test or a one-way ANOVA (**P* < 0.05), as indicated in the individual figures. The F-test was employed for the statistical analyses of linear regression correlation analysis. All statistical analyses

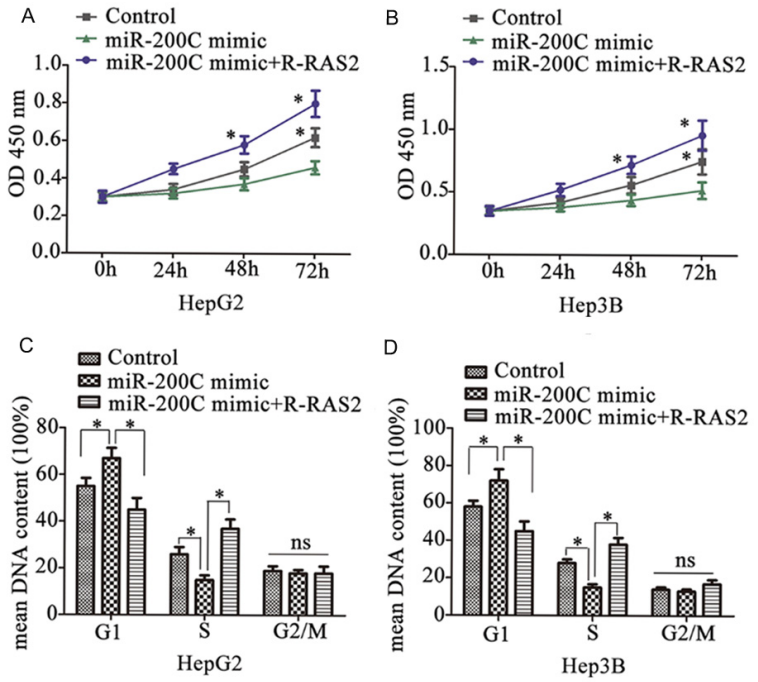

Figure 4. MiR-200c inhibited HCC cell proliferation and cell cycle via targeting R-RAS2. The transfection of miR-200c mimics or the co-transfection of the miR-200c mimics and R-RAS2 vector in HCC cells. A and B. The cell growth rates of transfected-HCC cells were determined by CCK-8 viability assay. The absorbance at 450 nm was measured at the indicated times. The data are presented as the mean ± SEM, n = 3, $*P < 0.05$, ANOVA. C and D. A cell cycle assay showed the phase proportion in the transfected-HCC cells. Data are expressed as the mean ± SEM, n = 3, $*P < 0.05$.

were presented with GraphPad prism 5 software.

Results

Expression of miR-200c and R-RAS2 in HCC tissues and cells

The results showed that miR-200c expression was significantly downregulated in HCC tissues compared to the corresponding adjacent normal tissues (**Figure 1A**), but the R-RAS2 expression was highly increased in the HCC tissues (**Figure 1B**). Moreover, an inverse relationship was found between the miR-200c and R-RAS2 mRNA levels in the HCC tissues (**Figure 1C**). Furthermore, we found that the expression levels of miR-200c in 5 HCC cell lines - HepG2, Hep3B, Huh7, QSG-7701, and SMMC-7221 - were remarkably reduced when compared to a normal liver cell line LO2 (**Figure 1D**), and in accordance with our expectations, the R-RAS2 expression was clearly upregulated in the HCC cell lines (**Figure 1E**).

R-RAS2 is a direct target of miR-200c in HCC cells

A bioinformatics analysis of TargetScan predicted that the 3' UTR of R-RAS2 was a potential target of miR-200c (**Figure 2A**), so to investigate whether miR-200c could directly interact with R-RAS2 3' UTR, luciferase reporter vectors containing wild type R-RAS2 3' UTR and mutant R-RAS2 3' UTR wereconstructed.Cellsco-transfected with miR-200c mimics and wild type R-RAS2 3' UTR indicated lower luciferase activity compared with the levels in the NC group, but it failed to work when the target site was mutated in HepG2 (**Figure 2B**) or Hep3B cells (**Figure 2C**). Moreover, the overexpression of miR-200c clearly inhibited the expression of R-RAS2 in the HCC cells (**Figure 2D**). Thus, these data suggest that R-RAS2 is the target gene of miR-200c in HCC cells.

MiR-200c inhibits HCC cell proliferation, invasion, and the cell cycle by targeting R-RAS2

As shown in **Figure 3A-C**, after the transfection of the miR-200c mimics, miR-200c was significantly up-regulated, and R-RAS2 was down-regulated in the mimics group compared with the control group. However, the expression of R-RAS2 showed an increase in co-transfection with the miR-200c mimics and the R-RAS2 vector in the HCC cells. Furthermore, the transwell assay revealed that the miR-200c mimics-transfected cells exhibited significantly decreased cell migration numbers compared with the control group in HepG2 and Hep3B HCC cells, but the miR-200c mimics and the R-RAS2 vector co-transfection showed an increase in the cell invasion ability of the HCC cells (**Figure 3D** and **3E**). Consistent with the reduction in cell invasion ability, both the cell proliferation and cell cycle arrest were markedly repressed in miR-200c mimic-transfected cells than in the control HepG2 and Hep3B HCC cells (**Figure 4A** and **4B**). Conversely, the overexpression of miR-

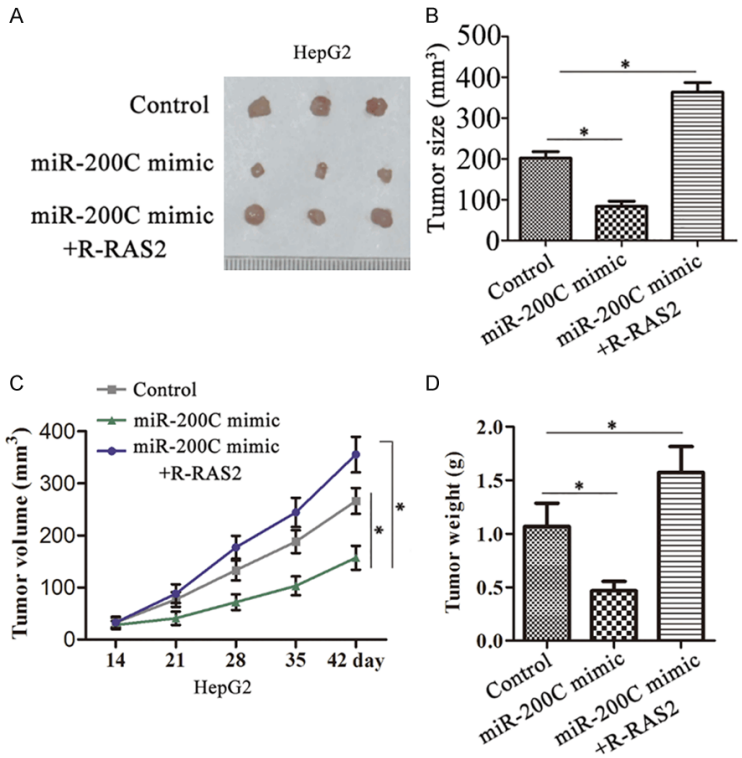

Figure 5. MiR-200c suppressed tumor growth of HCC cells *in vivo* by targeting R-RAS2. (A) The transfection of the miR-200c mimics or the co-transfection of the miR-200c mimics and the R-RAS2 vector in HepG2 HCC cells. The cells were injected subcutaneously into 6 nude mice per flank. The surgical resections of the HepG2 xenograft tumors on week 7 for animals are shown. (B and C) Measurements of tumor volumes and tumor weight (D) are shown. Data are expressed as the mean ± SEM, **P < 0.05*.

200c and R-RAS2 in the HepG2 and Hep3B HCC cells remarkedly increased both the cell proliferation and cell cycle compared with the control HCC cells (**Figure 4C** and **4D**). Therefore, our functional investigations revealed that miR-200c inhibits the proliferation, cell cycle progression, and invasion of HCC cells by targeting R-RAS2.

MiR-200c suppresses the tumor growth of HCC cells in vivo by targeting R-RAS2

To confirm the impact of miR-200c/R-RAS2 on gastric cancer cells' tumor growth in vivo, we generated xenograft models by implanting miR-200c mimics-transfected or miR-200c mimics and an R-RAS2 vector co-transfected HepG2 cells into nude mice. Six weeks after injection, the tumors formed in the miR-200c mimics-transfected HepG2 cells group were substantially smaller than those in the control group (**Figure 5A-C**). Moreover, the mean tumor weight at the end of the experiment was markedly lower in the miR-200c mimics-transfected HepG2 cells group compared to the control group (**Figure 5D**). Conversely, the tumor volumes and weights with the overexpression of miR-200c and R-RAS2 in the HepG2 cell group were substantially higher they were in the control group (**Figure 5A-D**). These data confirm the effect of miR-200c on R-RAS2 expression and that miR-200c contribute to the development of the malignant phenotype of HCC by targeting R-RAS2.

Discussion

RAS proteins play a causal role in human cancer and are binary switches, cycling between ON and OFF states during signal transduction [11]. R-Ras2 (also known as TC21) is a GTP-binding protein and is a member of the R-Ras GTPase subfamily, which also includes R-Ras1 and R-Ras3 [12]. Although Ras oncogenic proteins encoded by the H-Ras, K-Ras and N-Ras genes (Ras) have been comprehensively explored in various human cancers [11], the R-Ras also has oncogenic potential, yet its biological functions remain unclear. Recent studies have found that R-RAS2 expression is upregulated and contributes to the development of breast cancer, central nervous system cancer, esophageal cancer, and skin cancer [12-14]. In this study, we revealed that the expression of R-RAS2 is upregulated in HCC tumor tissues and cell lines. Furthermore, R-RAS2 overexpression promotes HCC cell proliferation, the cell cycle, and invasion both *in vitro* and *in vivo*.

As for miR-200c, an antitumor microRNA, several studies have shown that miR-200c plays important roles in all kinds of biological features in various cancer cells, including hepatocellular carcinoma [15-17]. Our study also discovered that the expression of miR-200c is downregulated and that miR-200c depletion inhibits HCC cell proliferation, the cell cycle, and invasion both *in vitro* and *in vivo*. Moreover, our results show that the expression of miR-

200c is negatively correlated to R-RAS2 expression in HCC tumor tissues. Furthermore, we searched and verified R-RAS2, a new target of miR-200c in the present study. Using a dual-luciferase reporter assay, an RT-PCR assay, and western blotting, we observed that miR-200c inhibits R-RAS2 expression in HCC cells. In our research, we also performed verification experiments including western blot, R-RAS2 overexpression, and a rescue experiment to confirm it is a real target of miR-200c. In nude mice xenograft models, HCC tumor formation was markedly repressed in the miR-200c overexpression group. However, the overexpression of R-RAS2 in miR-200c-overexpressed cells significantly increased the HCC tumor formation in the HepG2 cells group. In the present study, R-RAS2 promoted the tumorigenesis of HCC cells. Since miR-200c inhibited the oncogenic phenotypes of HCC cells and R-RAS2 levels simultaneously, R-RAS2 could be a possible mechanism mediated by miR-200c in liver tumorigenesis and malignant transformation.

In summary, we performed a series of functional experiments to demonstrate that R-RAS2 is a target gene of miR-200c and also a possible mechanism by which miR-200c regulates cell proliferation and invasion in HCC cells. These findings provide the first evidence that the miR-200c/R-RAS2 axis could be a potential therapeutic target for HCC treatment.

Disclosure of conflict of interest

None.

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