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miR-502 inhibits cell proliferation and tumor growth in hepatocellular carcinoma through suppressing phosphoinositide 3-kinase catalytic subunit gamma

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ABSTRACT

MicroRNAs (miRNAs) play a key role in carcinogenesis and tumor progression in hepatocellular carcinoma (HCC). In the present study, we demonstrated that miR-502 significantly inhibits HCC cell proliferation *in vitro* and tumor growth *in vivo*. G1/S cell cycle arrest and apoptosis of HCC cells were induced by miR-502. Phosphoinositide 3-kinase catalytic subunit gamma (PIK3CG) was identified as a direct downstream target of miR-502 in HCC cells. Notably, overexpression of PIK3CG reversed the inhibitory effects of miR-502 in HCC cells. Our findings suggest that miR-502 functions as a tumor suppressor in HCC via inhibition of PI3KCG, supporting its utility as a promising therapeutic gene target for this tumor type.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common aggressive hepatobiliary disease worldwide, which mainly occurs in men [1]. Despite clinical implementation of numerous treatment approaches, HCC continues to be associated with high mortality rates of over 90% [2]. To develop effective strategies for diagnosis, treatment, and prognosis of the disease, the mechanisms that regulate HCC development and progression require clarification.

MicroRNAs (miRNAs) are a type of small non-coding RNAs that regulate the translation of numerous target genes [3–5]. As miRNAs play vital roles in regulating pathogenesis, including tumor growth and metastasis, they are promising targets for diagnosis and prognosis of different cancers [6,7]. The expression patterns of miRNAs differ between HCC and corresponding noncancerous liver tissues. Specific miRNAs, such as miR-199, miR-26a and miR-7, have been correlated with proliferation and survival of HCC [8–10]. Human miR-502 is reported to be associated with several human malignancies, including colon and breast cancer [11,12]. Zhai and colleagues showed that miR-502 inhibits autophagy and tumor growth in colon cancer, and expression levels of miR-502 and p53 are mediated through a negative feedback loop in colon cancer cells. Based on the results, the authors suggested that miR-502 is a potential tumor suppressor and therefore a novel candidate for developing therapeutic strategies [11]. However, the function of miR-502 in the context of HCC remains largely unknown.

In the present study, we investigated the effects of miR-502 on HCC and mechanisms of action. Our data showed that miR-502 significantly inhibits HCC cell proliferation *in vitro* and tumorigenicity *in vivo*. Furthermore, miR-502 induced G1/S cell cycle arrest and apoptosis in HCC cells. Interestingly, phosphoinositide 3-kinase catalytic subunit gamma (PIK3CG) was identified as a novel miR-502 target. Overexpression of PIK3CG reversed the suppressive effects of miR-502 in HCC cells. Our data collectively indicate that miR-502 acts as a tumor suppressor via regulation of PIK3CG expression and may thus be an efficacious therapeutic target for HCC.

2. Materials and methods

2.1. Cells and clinical tissues

Human HCC cell lines, SMMC-7721 and Huh-7, were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Both cell lines were

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propagated in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, and cultured at 37 °C in a humidified incubator of 5% CO_2. Human HCC and corresponding adjacent non-tumorous liver tissues were obtained from 20 HCC patients during hepatic resection at Heping Hospital Attached to Changzhi Medical College. All tissues were immediately preserved in liquid nitrogen after surgical resection. Informed consent was obtained from each patient, and use of human tissues approved by the Medical Ethics and Human Clinical Trial Committee of the Changzhi Medical College.

2.2. RNA extraction and qRT-PCR

Total RNA was extracted from tissues or cells using the miRNeasy Mini Kit (QIAGENE, Shanghai, China), according to the manufacturer's instructions. Complementary DNA was synthesized from total RNA using the PrimeScript RT Reagent Kit (Takara, Dalian, China). qRT-PCR was conducted using Express SYBR greenER qPCR supermix Universal Kit (Invitrogen) in a Rotor-gene 2000 Real-time Cycler detection system (Corbett Research, Sydney, Australia). Relative miR-502 and PIK3CG levels were calculated using the $2^{-\Delta\Delta CT}$ method. U6 and β-actin mRNA levels were used for normalization.

2.3. miRNA mimics and plasmids

The miR-502 mimics, miR-502 inhibitor, agomiR-502, and the corresponding negative control were purchased from Ribobio (Guangzhou, China). Oligonucleotide transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The final concentration of the miR-502 mimics, miR-502 inhibitor, agomiR-502 or negative control in the transfection mixture was 100 nM. The PIK3CG-3′UTR (WT) sequence was amplified from human genomic DNA and cloned into psiCHECK-2 luciferase reporter vector. Mutant 3′UTR of PIK3CG containing a mutated sequence within the miR-502 target site was generated. Constructs were verified by DNA sequencing. PIK3CG without the 3′UTR region was cloned into the multiple cloning site of pcDNA3.1 to generate the PIK3CG overexpression plasmid.

2.4. Cell viability assay

Cells were seeded in 96-well plates at a density of 5000 cells/well in a final volume of 100 μl. CCK-8 solution (10 μl) was added to each well after culture for 24, 48, 72, and 96 h. Absorbance at 490 nm was measured after incubation for 2 h at 37 °C with a microplate reader (Bio-Rad, Richmond, CA, USA) to calculate the viable cell number. All experiments were performed in triplicate.

2.5. Cell cycle analysis

Cells were harvested and fixed in 70% ice-cold ethanol in PBS. Before staining, cells were pelleted using a chilled centrifuge and resuspended in cold PBS. Next, cells were treated with bovine pancreatic RNase (Sigma, St Louis, Mo, USA) and incubated at 37 °C for 30 min, followed by treatment with 20 μg/ml propidium iodide (PI, Sigma) for 20 min in the dark at room temperature. Cell cycle assays were performed using a FACS flow cytometer (BD Biosciences, Bedford, MA, USA).

2.6. Annexin V staining

To detect apoptotic cells, the ApopNexin FITC Apoptosis Detection Kit (Beyotime, Jiangsu, China) was used according to the manufacturer's instructions [13]. Briefly, 3×10^5 cells were seeded in 6-well plates in triplicate, followed by transfection with miR-502 mimics, miR-502 inhibitor or NC for 48 h. Cells were washed with Annexin V binding solution and incubated with Annexin V for 15 min. Next, cells were stained with 50 μg/ml PI, incubated for 5 min, and analyzed using the FACSCalibur flow cytometer (BD Biosciences).

2.7. Xenograft assay in nude mice

All animal procedures were performed in accordance with protocols approved by the Institute Research Ethics Committee at Changzhi Medical College. For the xenograft assay, 2×10^6 SMMC-7721 cells transfected with agomiR-502 or negative control were subcutaneously injected into 5 week-old female BALB/C nude mice (five in each group). Tumor volumes were measured every 7 days and calculated using the formula: volume $= 0.5 \times$ tumor length $\times$ tumor width^2. After 35 days, mice were killed, and tumors removed and weighed.

2.8. Luciferase assay

HEK-293T cells (4×10^4) were seeded in 24-well plates and cultured for 24 h psiCHECK-PIK3CG-3′UTR-WT or psiCHECK-PIK3CG-3′UTR-mut (500 ng) and 50 nM miR-502 mimics or negative control were co-transfected into cells using Lipofectamine 2000 (Invitrogen), in keeping with the manufacturer's instructions. Luciferase and Renilla signals were measured 48 h after transfection using a Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA), according to the manufacturer's protocol.

2.9. Protein extraction and western blot

Cells in culture were lysed using RIPA buffer, along with a Protease Inhibitor Cocktail (Pierce, Appleton, WI, USA). Lysates were analyzed using a BCA Protein Assay Kit (Pierce). Proteins were separated via 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were subsequently blocked with 5% non-fat milk and incubated overnight at 4 °C with primary antibodies against p110γ, p21, cleaved caspase 3 and β-actin (Santa Cruz Laboratories, Santa Cruz, CA, USA). After washing with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween20), membranes were incubated with secondary antibody for 1 h at room temperature. Signals were detected using the Enhanced Chemiluminescence system (Pierce), as described previously [14]. β-actin was used as the loading control.

2.10. Statistical analysis

SPSS 16.0 software was used for statistical analyses. All data are presented as means ± standard deviation (SD). The two-tailed Student *t*-test was performed to analyze differences between the two groups. The relationship between miR-502 and PIK3CG expression was explored via Spearman's correlation analysis. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. miR-502 suppresses HCC cell proliferation in vitro and tumorigenicity in vivo

To establish the biological function of miR-502 in HCC, two HCC cell lines, SMMC-7721 and Huh-7, were transfected with miR-502 mimics or negative control (NC), and increased expression of miR-502 verified using qRT-PCR (Fig. 1A). Data from the CCK-8

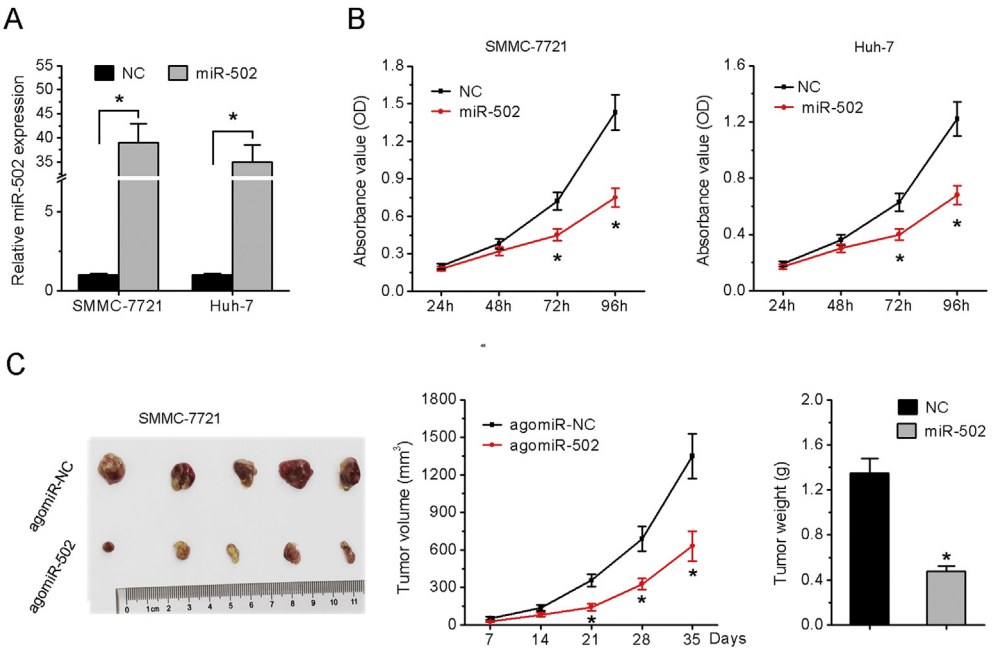

Fig. 1. miR-502 suppresses HCC cell proliferation *in vitro* and tumorigenicity *in vivo*. (A) miR-502 expression was detected via qRT-PCR in SMMC-7721 and Huh-7 cells transfected with miR-502 mimics or negative control, with U6 as the loading control. (B) Examination of cell viability with the CCK-8 assay in the indicated cells. (C) SMMC-7721 cells transfected with agomiR-502 or negative control were injected subcutaneously into mice. Tumor volumes were measured every 7 days. At 35 days after implantation, mice were killed, and tumors were removed and weighed. $^{*}P < 0.05$.

assay revealed significantly decreased cell viability in both HCC cell lines transfected with miR-502, compared with negative control (Fig. 1B). In contrast, the viability of these cells increased when endogenous miR-502 was knocked down with antisense oligonucleotides (Supplemental Fig. S1A and B). Next, we explored the effect of miR-502 on tumorigenicity *in vivo*. SMMC-7721 cells transfected with agomiR-502 or negative control (agomiR-NC) were subcutaneously injected into BALB/C nude mice. Tumors were stripped 35 days after implantation. As shown in Fig. 1C, tumors from mice bearing agomiR-502-transfected SMMC-7721 cells were smaller than those bearing agomiR-NC transfected cells. The inhibitory growth effect of miR-502 was further confirmed by calculation of survival curves and tumor weights. Our collective results clearly showed that miR-502 inhibits HCC cell proliferation and tumor growth ability.

3.2. *miR-502 regulates cell cycle and apoptosis in HCC cells*

To explore the mechanism underlying the inhibitory effects of miR-502 on HCC cell proliferation and tumor growth, we examined the distribution of cells in different stages of the cell cycle using flow cytometry. The results disclosed a significant increase in G0/G1 phase cells and decrease in S phase cells in miR-502 mimics-transfected SMMC-7721 and Huh-7 cells compared to those transfected with negative control (Fig. 2A). However, miR-502 knockdown cells led to the opposite results (Supplemental Fig. S1C). Annexin V staining was additionally performed to detect apoptotic cells. The percentage of apoptotic cells was significantly higher in SMMC-7721 and Huh-7 cells overexpressing miR-502, and lower in cells treated with the miR-502 inhibitor, compared to those transfected with control cells (Fig. 2B, Supplemental Fig. S1D). In addition, expression levels of p21 and cleaved caspase 3, related to cell cycle and apoptosis, respectively, were assessed via western blot. Notably, miR-502-overexpressing cells displayed upregulation of p21 and cleaved caspase 3 levels, as shown in Fig. 2C. Based on these findings, we propose that miR-502 induces cell cycle arrest and apoptosis in HCC cells.

3.3. *PIK3CG is a direct target of miR-502*

To establish the molecular mechanisms by which miR-502 suppresses hepatocarcinogenesis, we employed a combination of two public bioinformatic algorithms, Targetscan and miRanda, to identify potential target genes of miR-502 [15,16]. Phosphoinositide 3-kinase catalytic subunit gamma (PIK3CG), which plays a crucial role in the PI3K signaling pathway [17], was identified as a potential target gene of miR-502, with the predicted binding site at the base from positions 508 to 515 (Fig. 3A). To ascertain whether PIK3CG is a miR-502 target gene, two luciferase reporters, psiCHECK2-3′UTR-WT (containing the wild-type binding site within the 3′UTR of PIK3CG) or psiCHECK2-3′UTR-MUT (containing a mutation within the binding site), were constructed and co-transfected with the miR-502 mimics or NC into HEK-293T cells. Data from the luciferase assay showed significant suppression of the luciferase activity of PIK3CG 3′UTR by miR-502, compared with the negative control. Upon mutation of the predicted binding site, this inhibitory effect disappeared (Fig. 3B). Furthermore, overexpression of miR-502 significantly suppressed PIK3CG expression (Fig. 3C and D), while silencing of miR-502 increased PIK3CG expression in SMMC-7721 and Huh-7 cells (Supplemental Fig. S1E). Since PIK3CG is involved in the AKT signaling pathway, protein levels of phosphorylated and total AKT were additionally examined. We observed an inhibitory effect of miR-502 on p-AKT (Ser473), but not total AKT, as shown in Fig. 3D, and a positive effect of miR-502 inhibitor on p-AKT (Supplemental Fig. S1E).

To determine whether the inhibitory role of miR-502 is mediated through repression of PIK3CG, SMMC-7721 cells were co-transfected with miR-502 mimics and PIK3CG plasmid (without

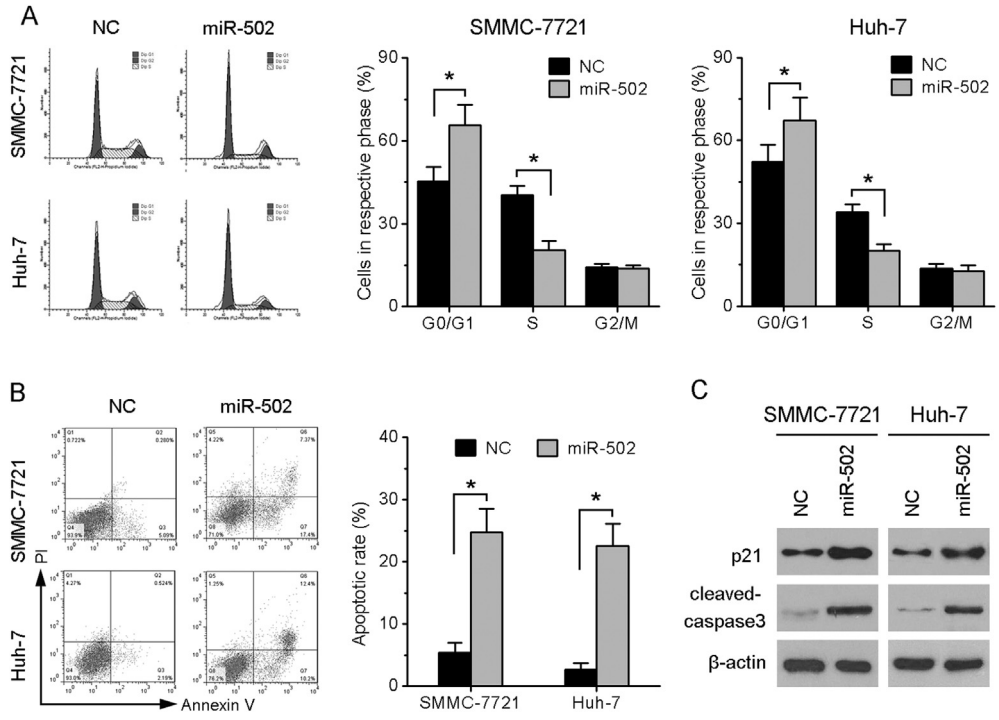

Fig. 2. miR-502 inhibits cell cycle progression and promotes apoptosis in HCC cells. (A) Cell cycle profiles of SMMC-7721 and Huh-7 cells treated with miR-502 mimics or NC detected via flow cytometry. (B) Flow cytometry analysis of apoptotic cells stained with Annexin V-FITC/PI. (C) Western blot analysis of p21 and cleaved caspase 3 expression in the indicated cells. $^{*}P < 0.05$.

3′UTR). As shown in Fig. 3E, miR-502-mediated PIK3CG downregulation was counteracted following co-transfection. Moreover, overexpression of PIK3CG promoted HCC cell proliferation and cell cycle progression and inhibited apoptosis (Fig. 3F), effectively reversing the effects of miR-502 on HCC. Accordingly, we propose that PIK3CG is a direct and functional downstream target of miR-502 in HCC cells.

3.4. *miR-502 and PIK3CG expression patterns are inversely correlated in HCC tissues*

In view of the above finding that miR-502 directly binds to the 3′UTR of PIK3CG and suppresses its expression, we further explored the expression patterns of miR-502 and PIK3CG in HCC tissue samples. As shown in Fig. 4A, expression of miR-502 was significantly decreased in HCC tissues, compared with matched normal tissues. Conversely, PIK3CG mRNA levels were markedly increased in HCC tissues (Fig. 4B). Correlation analyses revealed that miR-502 expression is inversely correlated with that of PIK3CG in HCC tissues (Fig. 4C).

4. Discussion

In the present study, we explored the biological role of miR-502 in the pathogenesis and development of HCC. Our experiments disclosed significant downregulation of miR-502 in HCC tissues, compared with matched normal tissues. miR-502 suppressed cell proliferation and progression of the cell cycle, increased cell apoptosis *in vitro* and suppressed tumorigenicity *in vivo* through targeting the PIK3CG oncogene.

Previous studies have documented that single-nucleotide polymorphisms in the miR-502–binding site at the 3′-UTR of SET8 modulate SET8 expression and contribute to the development of several cancers, including HCC [18–20]. Furthermore, aberrant miR-502 expression has been shown to be correlated with carcinogenesis, and the miRNA identified as a tumor suppressor in breast and colon cancer [11,12]. However, the specific roles and underlying molecular mechanisms of miR-502 in HCC have remained unclear to date. We observed significant downregulation of miR-502 in HCC clinical samples. In functional analyses, transfection of miR-502 mimics induced marked inhibition of cell proliferation and cell cycle progression, and increased apoptosis in HCC cells, whereas miR-502 inhibitor caused significant promotion of cell proliferation and cell cycle progression, and decreased apoptosis in HCC cells. These data indicates that miR-502overexpression inhibits HCC carcinogenesis and progression.

Among the signaling pathways, PI3K-Akt/PKB is believed to play critical roles in cell survival, proliferation, migration and invasion [21,22]. PIK3CG (PI3K p110γ), one of the four isoforms of mammalian type 1 PI3K, is activated via interactions with G-protein-coupled receptors [23,24]. Accumulating evidence indicates that PIK3CG is involved in human cancer progression [25–28]. A high level of p110γ expression is positively correlated with the proliferation marker, Ki-67, in HCC tissues [27]. Knockdown of p110γ is reported to inhibit HCC cell proliferation, and induce arrest of the cell cycle at the G0-G1 phase and apoptosis [27,28]. Our findings indicate that PIK3CG promotes cancer cell proliferation, opposite to the effects of miR-502. PIK3CG was further identified as a direct and functional downstream target gene of miR-502. Specifically, miR-502 directly targeted the binding site on PIK3CG mRNA 3′UTR and suppressed both mRNA and protein expression. Upon mutation of the miR-502 binding site of PIK3CG, the inhibitory effects of miR-502 overexpression on cell proliferation, cell cycle and apoptosis were rescued. Moreover, we observed a negative correlation between miR-502 and PIK3CG expression patterns in HCC tissues. These results collectively demonstrate that miR-502 inhibits proliferation, cell cycle progression and tumorigenesis of HCC cells via repression of PIK3CG.

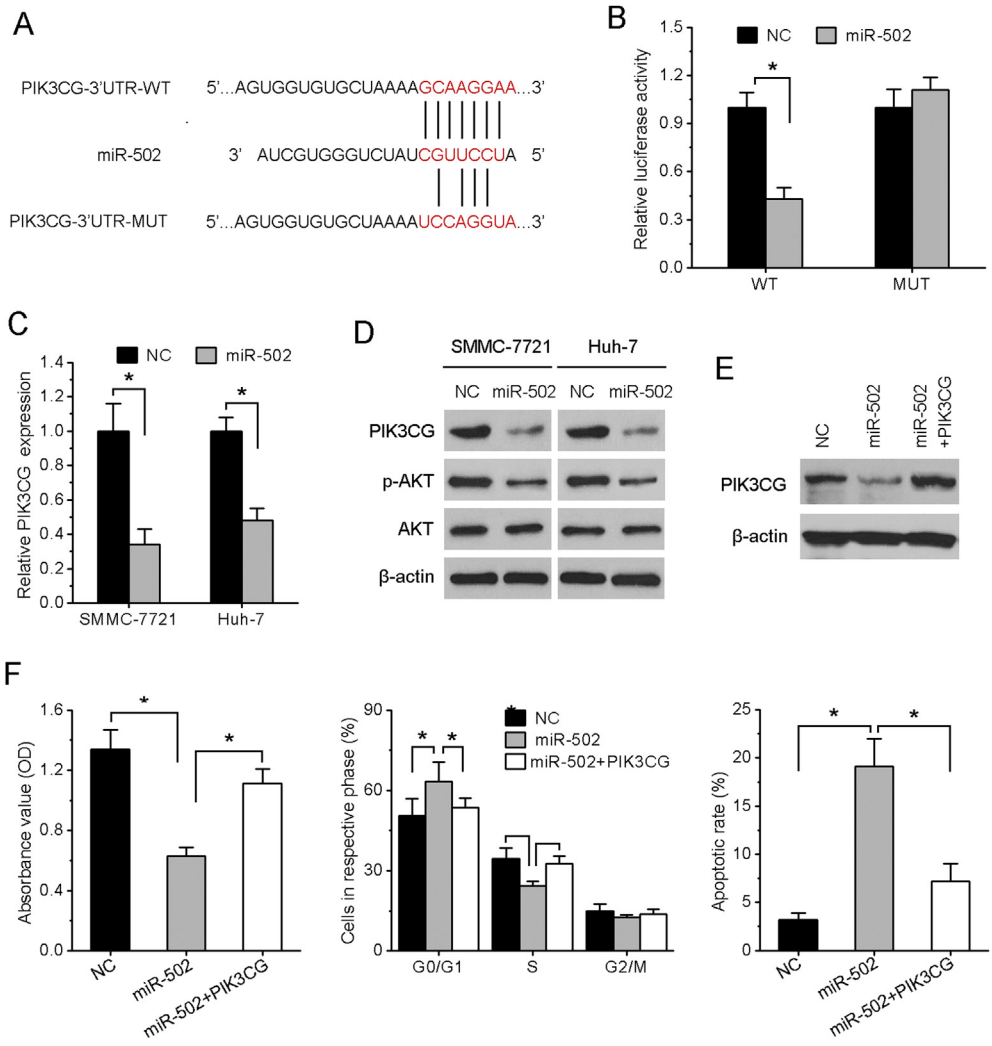

Fig. 3. miR-502 directly targets PIK3CG. (A) Predicted target sequences in PIK3CG 3′UTR that bind to miR-502. (B) Luciferase assay of HEK-293T cells co-transfected with pGL3-PIK3CG-3′UTR-WT or pGL3-PIK3CG-3′UTR-MUT reporter, along with miR-502 mimics or NC. (C) PIK3CG mRNA expression determined via qRT-PCR in SMMC-7721 and Huh-7 cells transfected with miR-502 mimics or NC. (D) Western blot analysis of protein levels of PIK3CG, phosphorylated and total AKT. (E) Western blot of PIK3CG protein expression in SMMC-7721 cells transfected with NC or the miR-502 mimics or co-transfected with miR-502 mimics and PIK3CG plasmid without 3′UTR. (F) Overexpression of PIK3CG reversed the effects of miR-502 on HCC cell proliferation, cell cycle and apoptosis. $^{*}P < 0.05$.

In conclusion, miR-502 functions as a novel tumor suppressor in HCC, and its downregulation may be necessary for carcinogenesis, at least in part, through upregulation of the PIK3CG oncogene. The current findings facilitate our understanding of the molecular pathogenesis of HCC and support the potential of miR-502 as an effective novel therapeutic target for treatment of the disease.

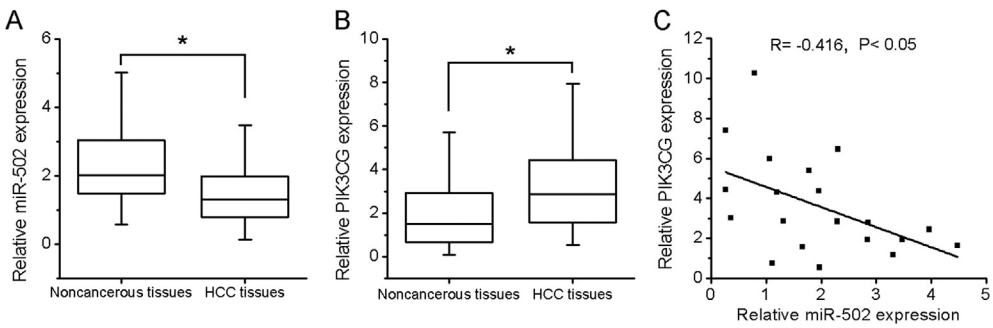

Fig. 4. miR-502 expression is inversely correlated with that of PIK3CG in HCC tissues. (A and B) qRT-PCR analysis of miR-502 and PIK3CG expression in 20 pairs of HCC and matched normal samples. (C) Statistically significant negative correlation between miR-502 and PIK3CG mRNA levels in HCC specimens (Spearman's correlation analysis). $^{*}P < 0.05$.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.168.

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