Overexpression of Rhophilin Rho GTPase-binding protein 2 promotes hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma is a serious public health problem in China. The mortality rate associated with the majority of cancer types has decreased as a result of targeted therapy. However, the mortality rates associated with hepatocellular carcinoma have not improved; therefore, the identification of new molecular targets is required for the development of novel targeted therapies. In the present study, a new molecular target, Rhophilin Rho GTPase-binding protein 2 (RHPN2), was identified. The levels of RHPN2 protein in tumor tissues were assessed via immunohistochemistry, while the mRNA levels were analyzed via reverse transcription-quantitative PCR. Additionally, cell viability was tested via MTT analysis. RHPN2 expression was upregulated in hepatocellular carcinoma tissues compared with that of matched adjacent normal tissues. More importantly, low expression of RHPN2 in patients with hepatocellular carcinoma was associated with an improved prognosis rate compared with patients with high expression. Downregulation of RHPN2 reduced the proliferation of hepatocellular carcinoma cells and increased the rate of apoptosis, whereas overexpression of RHPN2 demonstrated the opposite effects. Hepatocyte nuclear factor 1α was implicated in the mechanism of RHPN2. Overall, these data indicated that overexpression of RHPN2 may promote hepatocellular carcinoma.

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Introduction

Hepatocellular carcinoma is the second leading cause of cancer-associated mortality worldwide, with a morbidity rate of 782,000 and a mortality rate of 746,000 in 2012 (1). Liver cancer has several types based on the types of cells that become cancerous, including hepatic angiosarcoma, cholangiocarcinoma and hepatocellular carcinoma (2). hepatocellular carcinoma accounts for >80% of liver cancer cases worldwide (3), and more than half of these occur in China (4). In the past years, the mortality rate of the majority of liver cancer types has decreased with the development of targeted therapy (5,6). However, the hepatocellular carcinoma mortality rates in China did not decrease (7), thus the identification of new molecular targets for hepatocellular carcinoma treatment is required. Therefore, this was the aim of the present study.

Rhophilin Rho GTPase binding protein 2 (RHPN2) belongs to the rhophilin family of Ras-homologous (Rho)-GTPase-binding proteins (8). RHPN2 has been implicated in the organization of actin cytoskeleton (8). RHPN2 is reported to drive mesenchymal transformation by triggering RhoA activation in malignant glioma (9). However, the function of RHPN2 in hepatocellular carcinoma remains unknown.

Previous studies have demonstrated a significant reduction of hepatocyte nuclear factor 1α (HNF1α) and therapeutic effects in hepatocellular carcinoma tissues (10,11). Therefore, the present study aimed to investigate the role of RHPN2 in hepatocellular carcinoma, whether there was a correlation between RHPN2 and HNF1α, and whether RHPN2 could represent a novel therapeutic target.

Materials and methods

Bioinformatics analysis. The cBio Cancer Genomics Portal (http://cbioportal.org) was used to explore the role of RHPN2 in cancer genomic data. The portal is an open-access resource for interactive exploration of multidimensional cancer genomics data sets, and currently provides access to the data of >5,000 tumor samples from 20 cancer studies (12,13). The Cancer Genome Atlas (14) PanCancer Atlas Studies (10,953 patients/10,967 samples in 32 studies) were selected and queried by RHPN2. The PanCancer Atlas figures were

obtained and 372 liver cases were selected and queried by RHPN2. The survival of patients with liver cancer was then obtained. The possible microRNAs (miRs) that may target RHPN2 were bioinformatically predicted using TargetScan Human (http://www.targetscan.org/vert_72/) (15,16).

Tissue samples. A total of 31 hepatocellular carcinoma tissues and the matched adjacent normal tissues were acquired from the Sichuan Provincial Cancer Hospital (Sichuan, China). All tissues were obtained from surgery. The histology of the 31 hepatocellular carcinoma tissues was confirmed by the senior pathologist of the Department of Pathology of the hospital. The inclusion criteria included: ≥18 years of age, histologically confirmed diagnosis of hepatocellular carcinoma and patients who underwent first surgery. The exclusion criteria included: Secondary liver cancer, hepatocellular carcinoma recurrence and patients who received surgery before chemotherapy or sorafenib. The distance between the carcinoma and adjacent normal tissues was 0.5-1 cm. The mean age of the 31 patients (27 male and 4 female) with hepatocellular carcinoma was 59±11.4 years (age range, 39-72 years). The present study was approved by The Ethics Committee of Sichuan University (Chengdu, China) and written informed consent was obtained from all patients enrolled in the present study. For survival analysis, patients were then divided into two groups (high and low RHPN2 expression groups) according to the RHPN2 mRNA expression levels in the tumor tissues, and the median value (expression=2.28; expression SEM=0.51) of the 31 hepatocellular carcinoma tissues was chosen as the cut-off point. Patients were followed up for 60 months.

Immunohistochemical (IHC) analysis and point-scoring system. A total of ten hepatocellular carcinoma tissues were processed as per the standard protocol of IHC analysis (17). The tumor tissues were fixed in 10% neutral buffered formalin at room temperature for at least 5 days. After fixation, the tissues were dehydrated by immersion in increasing concentrations of alcohol (75% alcohol for 2 h, 80% for 2 h, 85% for 2 h, 90% for 2 h and twice, 95% for 1 h and 100% for 1 h). Subsequently, the alcohol was cleared by incubation in xylene prior to paraffin embedding. Paraffin is typically heated to 60˚C and then allowed to harden overnight. For IHC staining, tissue samples were cut into 4-μm-thick sections, deparaffinized, hydrated (at 70˚C in xylene) and microwaved at full power for 20 min for antigen retrieval (using sodium citrate pH 6.0). BSA (cat. no. 37520; Thermo Fisher Scientific, Inc.) was used as the blocking reagent overnight in 4˚C. The slides were incubated overnight at 4˚C with anti-RHPN2 primary antibody (1:500; cat. no. PA5-62469; Thermo Fisher Scientific). The slides were washed three times with PBS and incubated with goat anti-rabbit polyclonal horseradish peroxidase-conjugated secondary antibody (1:50; cat. no. 32260; Thermo Fisher Scientific, Inc.) at room temperature for 2 h. The slides were developed by diaminobenzidine staining as described previously (18). IHC staining was scored according to the following criteria: - (0-10% of the nucleated cells were stained), + (11-40% stained), ++ (41-70% stained) and +++ (71-100% stained). Tissues were analyzed using a light microscope (Leica DM750; Leica Microsystems GmbH) and images were captured at a magnification of x200.

Cell culture and reagents. HepG2 hepatocellular carcinoma and THLE-2 normal liver cell lines were purchased from the Cell Bank of the Third Military Medicine University. The HepG2 hepatocellular carcinoma cell lines were authenticated by short tandem repeat profiling. These cell lines and the hepatocellular carcinoma cells obtained from patients were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.). Cisplatin was purchased from Shanghai Yaji Biological Technology Co., Ltd. The final concentration of cisplatin used in experiments was 2.0 μg/ml. Cells were treated with cisplatin for 24 h.

Detection of RHPN2 and hepatocyte nuclear factor 1 (HNF1) α using reverse transcription-quantitative (RT-q)PCR. Total RNA from the tumor or normal tissues or cells was extracted using TRIzol® reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cDNA was synthesized using SuperScript™ IV First-Strand Synthesis System (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.). Reactions were incubated at 42˚C for 50 min, followed by heat inactivation for 5 min at 80˚C. The gene expression levels were assessed via qPCR using the $2^{-\Delta\Delta Cq}$ method (19). The PCR amplification was performed using SYBR™ Green PCR Master Mix (cat. no. 4334973; Thermo Fisher Scientific, Inc.). GAPDH was used as an internal control. RT-qPCR was performed using the following primers: RHPN2 Forward, 5'-AAGGGCTGTAATCCCCTTGC-3' and reverse, 5'-CCGCACCTTTGAGTTTGTGG-3'; HNF1α forward, 5'-AGCCGAGCCATGGTTTCTAA-3' and reverse, 5'-GGCTCGTTAGGAGCTGAGGG-3; GAPDH forward, 5'-CTGACTTCAACAGCGACACC-3' and reverse, 5'-TAGCCAAATTCGTTGTCATACC-3. Thermocycling conditions consisted of 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. RHPN2 mRNA expression levels in THLE-2 cells were arbitrarily defined as 100%.

Small interfering (si) RNA-RHPN2 and pcDNA3.1-RHPN2 transfection. siRNA-RHPN2 (si-RHPN2) and pcDNA3.1-RHPN2 were designed and constructed by Shanghai Shengong Biotechnology Co., Ltd. Scrambled siRNA (si-NC) and pcDNA3.1 were used as controls. Cells were seeded into 24-well plates at a density of $5x10^4$ cells/well overnight. Transfection was performed using Lipofectamine® 2000 (cat. no. 11668027; Thermo Fisher Scientific, Inc.). siRHPN2 (0.6 μg) or pcDNA3.1-RHPN2 (1 μg) were used separately. RHPN2 mRNA expression levels of the si-NC transfection group and those of the pcDNA3.1 transfection group were arbitrarily defined as 100%. MTT analysis was performed at 0, 24, 48 and 72 h following transfection. The cell apoptosis analysis was performed 24 h following transfection.

Cell proliferation assay. Cell proliferation was analyzed using an MTT-based colorimetric assay (20-24). Briefly, $5x10^5$/well cells were placed into 96-well plates. MTT reagent was then added into the medium at a final concentration of 0.1 mg/ml. After formation of insoluble formazan, 100 μl DMSO was added to each well to solubilize formazan. The optical density

was then measured using a microplate reader equipped with a 570 nm filter.

Apoptosis analysis. Cells ($5x10^5$ cells/ml) were suspended in Annexin V-fluorescein isothiocyanate-binding buffer (FITC; Abcam), and incubated for 15 min at room temperature followed by addition of propidium iodide (PI; Abcam) to each sample. All samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) at 488 nm excitation (Argon-ion laser or solid-state laser). Emission was detected at 530 (green for FITC) and 575-610 nm (orange for PI). The early apoptosis rate (Annexin V-$FITC^+/PI^-$) was calculated. The data were analyzed using the BD FACSuite™ version 1.01 (BD Biosciences).

Network analyses. The network of RHPN2 was analyzed using Cytoscape software v3.8.0 (25). The complete analysis was performed by the Department of Bioinformatics of Sichuan University.

Statistical analysis. Data were presented as the mean ± standard deviation of three independent repeats. Kaplan-Meier analysis was used for survival curves, and the log-rank test was used to compare the difference between two groups. Paired two-tailed Student's t-tests were used to analyze the mean values of paired groups (tumor and non-tumor tissue from the same patient). Unpaired two-tailed Student's t-tests were used to analyze the mean values of THLE and HepG2 cells. One-way ANOVA followed by Tukey's post hoc test was used to analyze the mean values ≥3 groups. Correlation analysis was performed using two-tailed Pearson's correlation coefficient analysis. All analyses were performed using SPSS software (version 16.0; SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Bioinformatics analysis of patients' survival associated with RHPN2 expression. Initially, the role of RHPN2 was investigated using bioinformatics analysis and cBioPortal. The results revealed that amplified RHPN2 is the most common mutation in various types of cancer, including uterine, ovarian, stomach, esophageal, lung and liver cancer (Fig. 1A). Next, the overall survival of patients with numerous types of cancer according to RHPN2 mutation were studied. The results demonstrated that the altered group exhibited a lower survival rate compared with that of the unaltered group (Fig. 1B; log-rank P=5.260e-4). Additionally, the overall survival rate of patients with hepatocellular carcinoma was analyzed according to RHPN2 alterations, and no significant differences were observed between the altered and unaltered group; however, this may have been due to inclusion of a limited number of patients (Fig. 1C). Thus, 31 hepatocellular carcinoma tissues and their matched adjacent tissue samples were collected for patient's survival analysis.

RHPN2 expression levels in hepatocellular carcinoma tissues and patient survival analysis. The protein levels of RHPN2 in 31hepatocellular carcinoma tissues and their matching adjacent tissue samples were analyzed using IHC. Hepatocellular carcinoma tissues consistently exhibited higher protein expression levels of RHPN2 compared with those of matched healthy tissues (Fig. 2A-C). Next, the RHPN2 mRNA expression levels were analyzed in the 31 hepatocellular carcinoma tissues and matched samples. The results demonstrated higher RHPN2 mRNA expression levels in the 31 hepatocellular carcinoma tissues compared with those of matching healthy tissues (Fig. 2D and E). The patients were then divided into two groups (RHPN2 high expression group and RHPN2 low expression group) according to RHPN2 mRNA expression levels in the tumor tissues, and the median value of the 31 hepatocellular carcinoma tissues was chosen as the cut-off point. These patients were followed up for 60 months, and the RHPN2 low expression group demonstrated an improved prognosis compared with that of RHPN2 high expression group (Fig. 2F).

Downregulation of RHPN2 decreases hepatocellular carcinoma cell proliferation and increases the apoptotic rate. RHPN2 mRNA expression levels were determined in THLE-2 and HepG2 cells. HepG2 cells displayed significantly higher RHPN2 mRNA expression levels compared with those of THLE-2 cells (Fig. 3A). The HepG2cells were then transfected with si-RHPN2 or si-NC. RT-qPCR was used to analyze RHPN2 mRNA levels after 24 h. The results demonstrated that the levels of RHPN2 mRNA were significantly decreased following si-RHPN2 transfection compared with si-NC (Fig. 3B). In addition, HepG2 cells transfected with si-RHPN2 exhibited significantly decreased cellular proliferation compared with the control group (si-NC; Fig. 3C). Additionally, apoptosis was analyzed using Annexin V/PI double-staining. The results demonstrated that downregulation of RHPN2 significantly increased the apoptotic rate of HepG2 cells compared with that of cells transfected with si-NC (Fig. 3D).

Overexpression of RHPN2 promotes hepatocellular carcinoma cell proliferation and reduces the apoptotic rate. RHPN2-overexpression was induced by transfection with an overexpression plasmid, pcDNA3.1-RHPN2, in HepG2 cells. The RHPN2 mRNA expression levels in HepG2 cells were analyzed after 24 h using RT-qPCR. Transfection with pcDNA3.1-RHPN2 resulted in upregulation of RHPN2 mRNA expression levels in HepG2 cells compared with that of HepG2 cells transfected with pcDNA3.1 (Fig. 4A). MTT analysis demonstrated that overexpression of RHPN2 significantly promoted hepatocellular carcinoma cell proliferation compared with the blank and negative controls (Fig. 4B). After 12 h of pcDNA3.1-RHPN2 or pcDNA3.1 transfection, cisplatin was applied, and then the apoptotic rates were assayed 12-h later. The results demonstrated that, compared with the blank group, cisplatin significantly increased the apoptotic rate, and that overexpression of RHPN2 significantly reduced the cisplatin-induced increased apoptotic rate compared with cisplatin-treated cells (Fig. 4C).

HNF1α is involved in the mechanism of RHPN2. A network view of RHPN2 demonstrated the association between HNF1α and RHPN2 (Fig. 5A). HNF1α is a liver-enriched

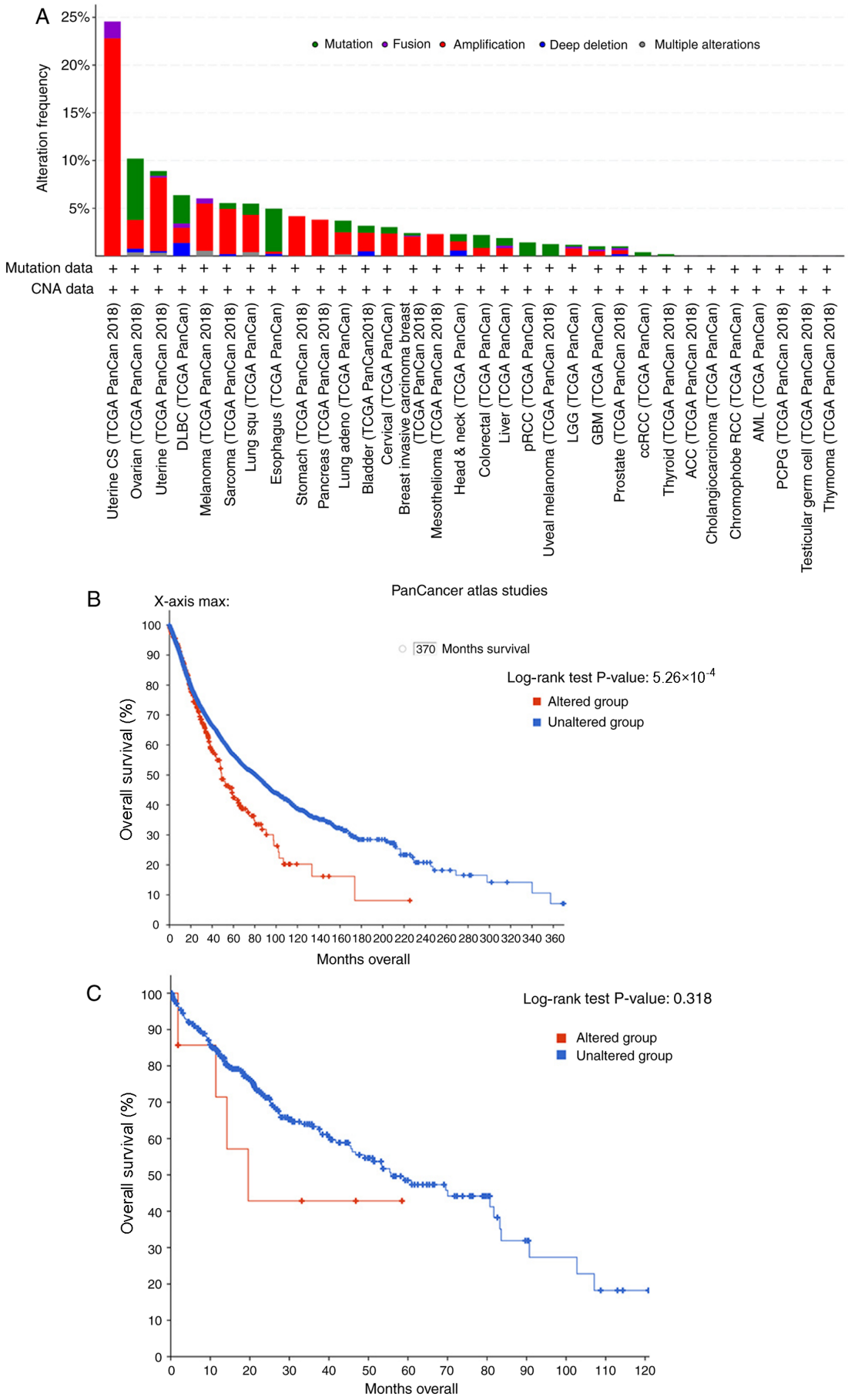

Figure 1. Association between RHPN2 expression levels and survival of patients with various types of cancer. (A) Amplification of RHPN2 across various types of cancer. (B) Log-rank test of the overall survival rate of all types of cancer according to RHPN2 alterations. (C) Log-rank test of overall survival rate of patients with hepatocellular carcinoma according to RHPN2 alterations. RHPN2, Rhophilin Rho GTPase-binding protein 2; TCGA, The Cancer Genome Atlas.

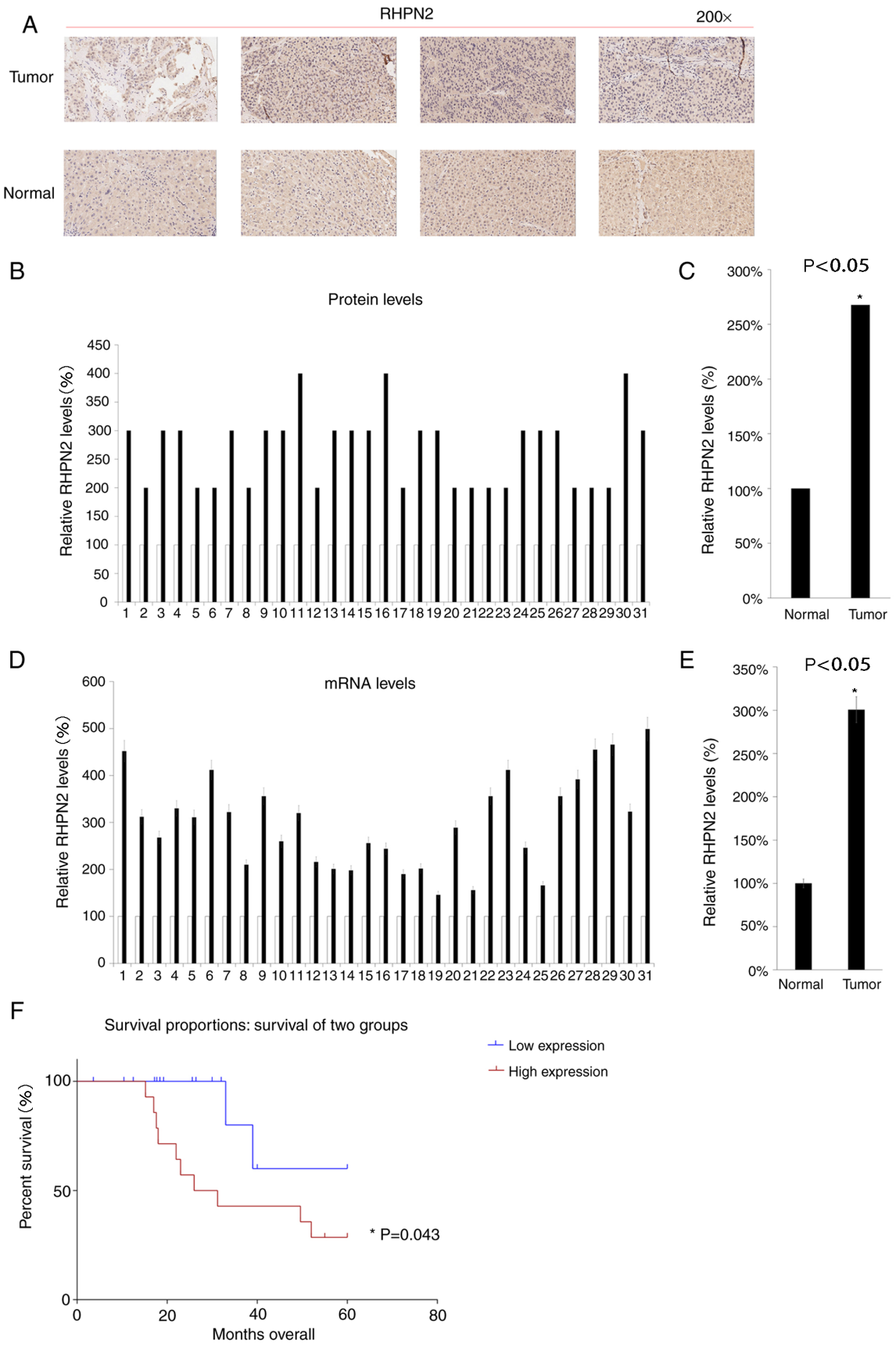

Figure 2. Hepatocellular carcinoma tissues display higher levels of RHPN2 mRNA and protein. (A) Representative images of hepatocellular carcinoma and adjacent normal tissues stained for RHPN2. (B) RHPN2 protein levels in 31 hepatocellular carcinoma samples and matched normal adjacent tissues were quantified. The RHPN2 protein levels in tumor and normal tissues were compared using paired two-tailed Student's t-tests. (C) Mean values of RHPN2 protein levels in hepatocellular carcinoma samples. (D) RHPN2 mRNA levels in hepatocellular carcinoma samples were analyzed using reverse transcription-quantitative PCR. The RHPN2 mRNAs levels in tumor and normal tissues were compared using paired two-tailed Student's t-tests. (E) Mean values of RHPN2 mRNA expression in 31 hepatocellular carcinoma tissues and their matched adjacent normal tissues. (F) Patient survival according to RHPN2 expression was analyzed by using log-rank test. *P<0.05. RHPN2, Rhophilin Rho GTPase-binding protein 2.

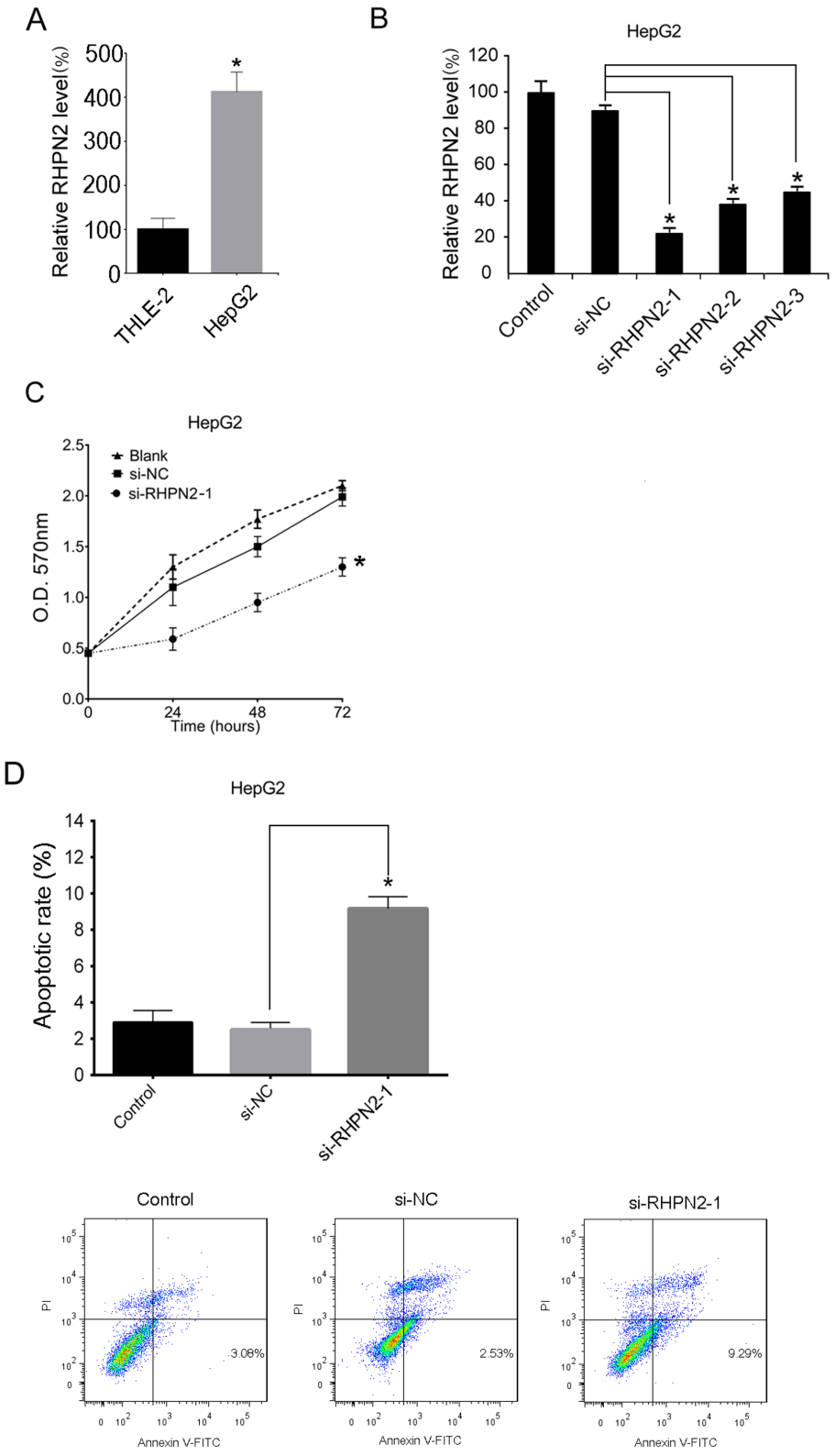

Figure 3. Downregulation of RHPN2 reduces hepatocellular carcinoma cells proliferation and increases the apoptotic rate. (A) mRNA expression levels of RHPN2 in THLE-2 and HepG2 cells were analyzed by RT-qPCR. RHPN2 mRNA expression levels in THLE-2 cells were arbitrarily defined as 100%. (B) HepG2 cells were transfected with si-RHPN2 or si-NC. RHPN2 mRNA expression levels were detected using RT-qPCR 24 h post-transfection. RHPN2 mRNA expression levels of the si-NC transfection group were arbitrarily defined as 100%. (C) Proliferation of HepG2 cells was investigated using MTT analysis following si-RHPN2 and si-NC transfection. (D) Apoptosis was investigated by Annexin V/propidium iodide double-staining 24 h post-transfection with si-RHPN2 or si-NC. *P<0.05 vs. si-NC. RHPN2, Rhophilin Rho GTPase-binding protein 2; si, small interfering; RT-qPCR, reverse transcription-quantitative PCR.

transcription factor that is considered critical for the maintenance of hepatocyte function (10). Previous studies have demonstrated a significant reduction of HNF1α and therapeutic effects in hepatocellular carcinoma tissues (10,11). Therefore, HNF1α mRNA expression levels were analyzed in tumor tissues using RT-qPCR, and the

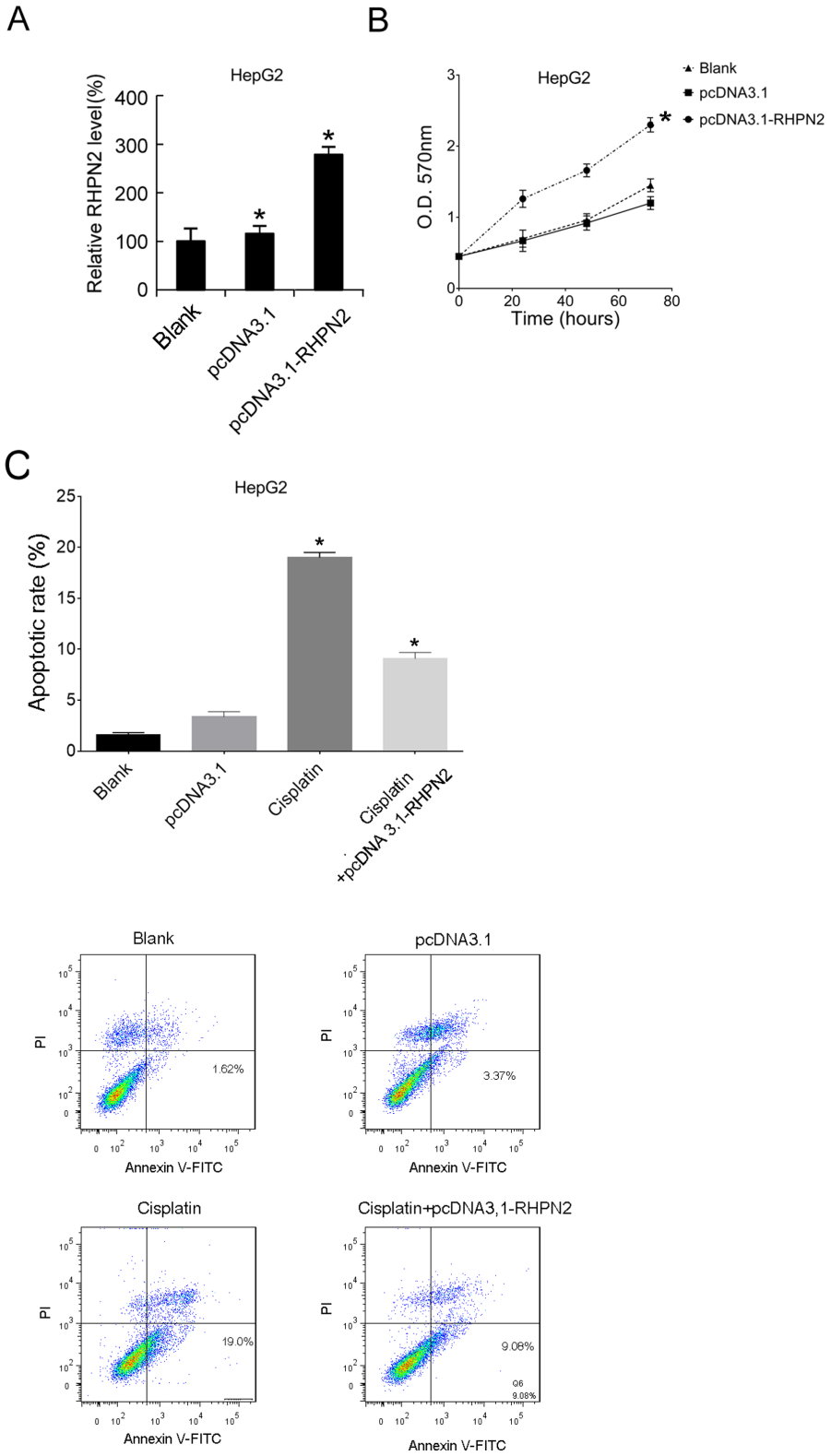

Figure 4. Overexpression of RHPN2 promotes hepatocellular carcinoma cell proliferation and reduces the apoptotic rate. (A) HepG2 cells were transfected with pcDNA3.1-RHPN2 and pcDNA3.1. RHPN2 mRNA expression levels were detected using reverse transcription-quantitative PCR 24 h post-transfection. RHPN2 mRNA expression levels of pcDNA3.1 transfection group were arbitrarily defined as 100%. (B) Proliferation of HepG2 cells was analyzed using MTT analysis following pcDNA3.1-RHPN2 or pcDNA3.1 transfection. (C) After 12 h of pcDNA3.1-RHPN2 or pcDNA3.1 transfection, cisplatin was applied. Apoptosis was investigated by Annexin V/propidium iodide double-staining. *P<0.05 vs. Blank. RHPN2, Rhophilin Rho GTPase-binding protein 2.

relative HNF1α mRNA expression levels were calculated (Fig. 5B). Analysis of HNF1α mRNA expression levels demonstrated a negative correlation between HNF1α and RHPN2 expression (Fig. 5C), suggesting that HNF1α may serve a role in the mechanism of RHPN2 in hepatocellular carcinoma. Additionally, bioinformatics analysis revealed RHPN2 as the target gene of miR-141 and miR-200a (data not shown).

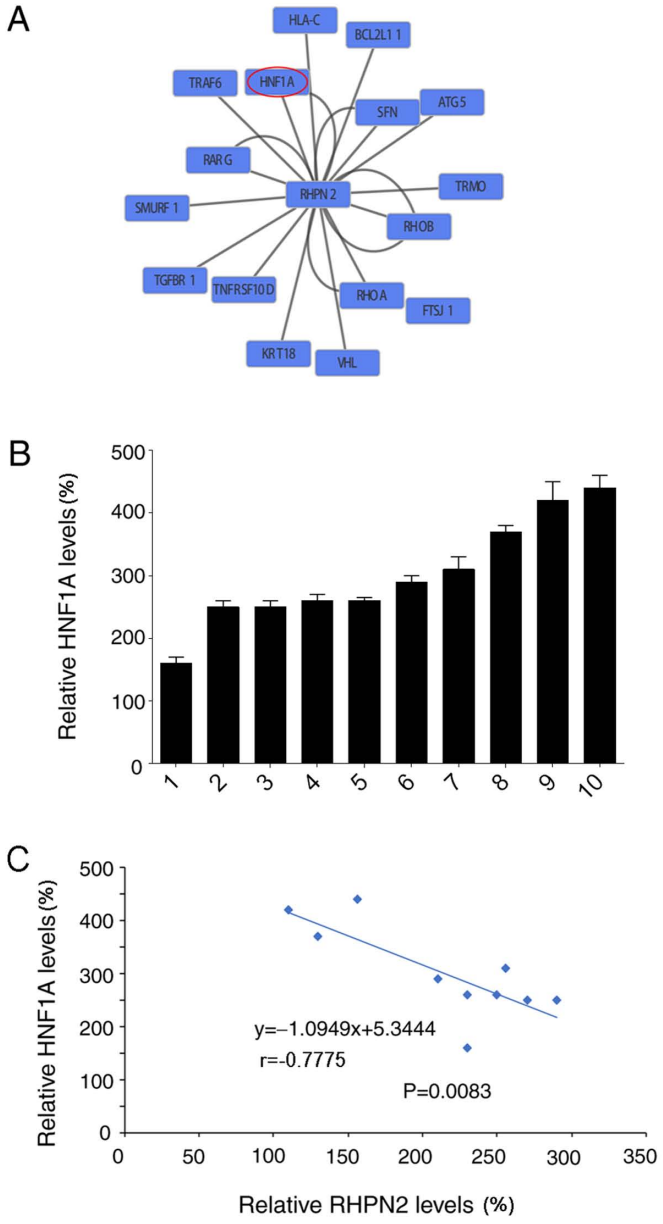

Figure 5. RHPN2 network and correlation analysis. (A) RHPN2 network was constructed using Cytoscape software. (B) mRNA expression levels of HNF1α were analyzed using reverse transcription-quantitative PCR. The relative HNF1α mRNA levels were calculated. (C) Correlation between HNF1α and RHPN2 was analyzed. Rhophilin Rho, GTPase-binding protein 2; HNF1α, hepatocyte nuclear factor 1.

Discussion

The present study analyzed the function of RHPN2 in hepatocellular carcinoma. IHC analysis revealed higher expression levels of RHPN2 protein in hepatocellular carcinoma tissues compared with those in adjacent normal tissues. RHPN2 promoted the proliferation of hepatocellular carcinoma cells and suppressed apoptosis. Gene network and correlation analyses revealed a negative correlation between HNF1α and RHPN2 expression.

To the best of our knowledge, the present is the study to investigate the oncogenic function of RHPN2 in hepatocellular carcinoma as the oncogenic function of RHPN2 has only been studied in malignant glioma (9). This previous study reported that RHPN2 drives mesenchymal transformation by triggering RhoA activation (9). The results of the present study demonstrated that RHPN2 promoted the proliferation of hepatocellular carcinoma cells and suppressed apoptosis, providing a possible explanation for the high expression levels of RHPN2 in hepatocellular carcinoma tissues. More importantly, low expression levels of RHPN2 in patients with human hepatocellular carcinoma were associated with an improved prognosis rate. These survival data highlighted on the importance of RHPN2 in hepatocellular carcinoma.

Notably, bioinformatics analysis revealed RHPN2 as the target gene of miR-141 and miR-200a by bioinformatics analysis (data not shown). miR-141 and miR-200a belong to miR-200 family (26) and both have been reported to suppress the growth of various tumors, such as colon, gastric, ovarian, lung and breast cancer (27-36). Thus, it may be possible to treat hepatocellular carcinoma with miR-141 and miR-200a targeting RHPN2.

The results of the present study demonstrated that there was a negative correlation between HNF1α and RHPN2 expression, therefore it is possible that HNF1α-downregulation may contribute to elevated RHPN2 levels in hepatocellular carcinoma. However, the interaction between HNF1α and RHPN2 and the molecular mechanism underlying this correlation remains unclear and requires further investigation in future studies.

In conclusion, the results of the present study suggested that overexpression of RHPN2 may promote hepatocellular carcinoma and therefore inhibition of RHPN2 may delay the progression of hepatocellular carcinoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BY collected the patient data. WB performed the bioinformatics analysis. BY and XF performed PCR and transfection. YH and WB performed the apoptosis analysis. BY and JZ contributed to the study design and manuscript writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of Sichuan University (Chengdu, China; approval no. 20160612) and written informed consent was provided by all patients enrolled.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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