SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 28: 218, 2023

HIF-1α and RACGAP1 promote the progression of hepatocellular carcinoma in a mutually regulatory way

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Received May 9, 2023; Accepted September 12, 2023

DOI: 10.3892/mmr.2023.13105

Abstract. Hypoxia, a condition characterized by low oxygen levels, serves an important role in the progression of hepatocellular carcinoma (HCC). However, the precise molecular mechanisms underlying hypoxia-induced HCC progression are yet to be fully elucidated. The present study assessed the involvement of two key factors, hypoxia-inducible factor-1α (HIF-1α) and Rac GTPase activating protein 1 (RACGAP1), in HCC development under hypoxic conditions. HIF-1α and RACGAP1 genes were overexpressed and knocked down in Hep3B and Huh7 cells using lentiviral transduction and the levels of HIF-1α and RACGAP1 in the cells were assessed using quantitative PCR, western blotting and immunofluorescence. Co-immunoprecipitation experiments were performed to evaluate the interaction between HIF-1α and RACGAP1. Subsequently, the proliferation, apoptosis, migration and invasion of Hep3B and Huh7 cells were assessed using the Cell Counting Kit-8 assay, flow cytometry, Transwell assay and migration experiments. The expression levels of HIF-1α and RACGAP1 in normal and HCC tumor samples were analyzed utilizing the Gene Expression Profiling Interactive Analysis database. Furthermore, correlations between HIF-1α/RACGAP1 gene expression levels and patient survival outcomes were evaluated using the Kaplan-Meier plotter. Knockdown of HIF-1α resulted in a significant decrease in RACGAP1 expression, whilst overexpression of HIF-1α resulted in a significant increase in RACGAP1 expression. Moreover, overexpression and knockdown of RACGAP1 had the same effect on HIF-1α expression. Additionally, it was demonstrated that HIF-1α and RACGAP1 interacted directly within a complex. Overexpression of HIF-1α or RACGAP1 significantly increased proliferation, invasion and migration, and significantly decreased the proportion of apoptotic Hep3B and Huh7 cells. Conversely, knockdown of HIF-1α or RACGAP1 significantly decreased proliferation, invasion and migration, and significantly increased the proportion of apoptotic Hep3B and Huh7 cells. In addition, the combined knockdown or overexpression of HIF-1α and RACGAP1 had a more pronounced effect on HCC cell migration compared with knockdown of HIF-1α alone. Furthermore, there was a significant positive correlation between the expression levels of HIF-1α and RACGAP1 in HCC tissues and patients with HCC and upregulation of both HIF-1α and RACGAP1 demonstrated a lower overall survival probability. In conclusion, HIF-1α and RACGAP1 may synergistically contribute to the development of HCC, highlighting their potential as valuable targets for HCC therapy.

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Key words: hepatocellular carcinoma, hypoxia inducible factor-1-alpha, Rac GTPase activating protein 1, proliferation, apoptosis, invasion, migration

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and ranks as the sixth most common malignancy in the world (1). Early stages of HCC lack noticeable symptoms, resulting in ~80% of diagnoses occurring at an advanced stage when curative liver resection is not feasible (2). Consequently, there is an urgent need to develop novel and effective therapeutic targets for the prevention and treatment of HCC. Furthermore, HCC is typically linked with chronic liver diseases and primarily associated with hepatitis B and C virus infections, excessive alcohol consumption and metabolic syndrome (3-6). However, the molecular mechanism underling HCC is yet to be fully elucidated. Understanding the mechanisms involved in HCC carcinogenesis and progression is necessary for identifying new and more effective treatment strategies against this disease.

Hypoxia serves a crucial role in malignancy and is often observed in tumors as a result of poorly organized blood vessels and rapid tumor growth that outpaces vascularization (7). Hypoxic tumor cells are typically located away from blood vessels, making it difficult for conventional drugs to reach the hypoxic tissues (8). Heightened glycolysis in hypoxic tumors results in increased acidity, which is closely linked to tumor progression and drug resistance (9). Additionally, tumor hypoxia can induce genomic instability which results in the development of more aggressive tumors.

Tumor metastasis is responsible for >90% of cancer-related deaths (10).

Hypoxia-inducible factor-1α (HIF-1α) is the primary transcription factor involved in cellular adaptation to hypoxic conditions. HIF-1α contributes to tumor progression by modulating processes such as angiogenesis, energy metabolism, migration, invasion, proliferation and apoptosis (11,12). Consequently, inhibition of the expression of the HIF-1α gene in a hypoxic microenvironment may offer a novel approach for therapeutic interventions in malignant tumors such as HCC.

Rac GTPase activating protein 1 (RACGAP1), also known as MgcRacGAP and CYK4, is a type of GTPase activating protein that stimulates the intrinsic activity of Rho GTPases and enhances GTP hydrolysis (13). Abnormal expression of RACGAP1 has been implicated in the pathogenesis and progression of numerous malignant tumors (14,15). For example, recent studies have reported that upregulation of RACGAP1 can be an indicator of a worse overall survival rate in patients with HCC (16). The activation of RACGAP1 and demethylation of its promoter region, specifically at the H3K4me2 site, have been reported to promote early recurrence, metastasis and microvascular invasion in HCC (17,18). However, the specific role of RACGAP1 in HCC within a hypoxic microenvironment has not been extensively evaluated.

The objective of the present study was to assess the impact of hypoxia on gene expression and its role in HCC development and progression. Specifically, the gene expression levels of HIF-1 α and RACGAP1 were manipulated to assess their effects on numerous cellular processes, including proliferation, apoptosis, migration and invasion of HCC cells. These investigations are crucial to gain insight into the underlying mechanisms involved in the pathogenesis of HCC. Furthermore, such knowledge could aid in the identification of novel and effective therapeutic targets for the prevention and treatment of HCC.

Materials and methods

Cell culture. Human liver cancer Hep3B and Huh7 cell lines were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and cultured in DMEM medium (cat. no. 12430054; Gibco; Thermo Fisher Scientific, Inc.) which contained 10% FBS (cat. no. 10100147; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (cat. no. 15070063; Gibco; Thermo Fisher Scientific, Inc.). Cells were grown in a 37˚C incubator with 1% O_2, 5% CO_2 and 94% N_2 for 48 h.

Reverse transcriptase (RT)-quantitative polymerase chain reaction (qPCR). TRIzol (1 ml; cat. no. R1030; Applygen Technologies, Inc.) was added to the Hep3B and Huh7 cells pellets to extract the total RNA. The concentration and quality of extracted RNA were then assessed using a Nano Drop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.). The extracted RNA was reverse-transcribed into cDNA using the Promega M-MLV kit (cat. no. M1705; Promega Corporation) according to the manufacturer's protocol. qPCR was performed with the KAPA SYBR FAST qPCR kit (Kapa Biosystems; Roche Diagnostics) using SimpliAmp™ PCR System (Thermo Fisher Scientific, Inc.). Amplifications were performed using a two-step method as follows: 95˚C for 30 sec; 95˚C for 10 sec followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec. The relative mRNA expression of each sample was calculated using the $2^{-\Delta\Delta Cq}$ method (19). The sequences of the primers used were as follows: HIF-1α upstream: 5'-CAGCCA GATCTCGGCGAAG-3', downstream: 5'-CAGCATCCAGAA GTTTCCTCACA-3'; RACGAP1 upstream: 5'-CTGATGAAT CACTGGATTGGGACTC-3', downstream: 5'-GGTCTACTG CAGAGCCAATGG-3'; and GAPDH upstream: 5'-CCAGGT GGTCTCCTCTGA-3', downstream: 5'-GCGCCCAATACG ACCAAATC-3'.

Western blotting. Total proteins were extracted from Hep3B and Huh7 cells using RIPA lysis buffer (cat. no. P0013B, Beyotime Institute of Biotechnology) were semi-quantified by the BCA method (cat. no. 23225; Thermo Fisher Scientific, Inc.). Proteins were mixed with 5X SDS-PAGE protein loading buffer (cat. no. 20315ES05, Shanghai Yeasen Biotechnology Co., Ltd.). The mass of protein loaded per lane was 20 μg. Proteins were separated on 12% SDS-acrylamide gels and transferred onto PVDF membranes, which were incubated with 5% non-fat milk at room temperature for 2 h, followed by incubation with rabbit anti-HIF-1α (1:200; cat. no. 20960-1-AP; Proteintech Group, Inc.), rabbit anti-RACGAP1 (1:500; cat. no. 13739-1-AP; Cell Signaling Technology, Inc.) and mouse anti-GAPDH (1:5,000; cat. no. 66004-1-Ig; Proteintech Group, Inc.) primary antibodies overnight at 4˚C. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (1:5,000; cat. nos. BA1070 and BM2002; Boster Biological Technology) secondary antibodies for 1.5 h at room temperature. Immunoreactive protein bands were assessed using an ECL hypersensitive chemiluminescence kit (cat. no. P0018M; Beyotime Institute of Biotechnology) with an Odyssey Scanning System (version 3.0, LI-COR Biosciences).

Immunofluorescence. Hep3B and Huh7 cells were plated in a 35 mm confocal dish and cultured for 48 h followed by fixing with 4% paraformaldehyde for 20 min at room temperature. After rinsing with PBS, cells were permeabilized with 0.5% Triton X100 in PBS and blocked with 3% BSA (cat. no. AR1006; Wuhan Boster Biological Technology, Ltd.) at 37˚C for 1 h. Cells were then incubated overnight with anti-HIF-1α (1:50; cat. no. 20960-1-AP; Proteintech Group, Inc.) or anti-RACGAP1 (1:50; cat. no. 13739-1-AP; Cell Signaling Technology, Inc.) antibodies at 4˚C, followed by 2 h incubation with goat anti-mouse Alexa Fluor® 488 (1:200, cat. no. A28175, Thermo Fisher Scientific, Inc.) and goat anti-rabbit Alexa Fluor® 555 (1:200, cat. no. A27039; Thermo Fisher Scientific, Inc.) at room temperature. After three TBST (0.05% Tween-20) washing steps, cells were incubated with DAPI (cat. no. D1306; Thermo Fisher Scientific, Inc.) at 37˚C for 10 min. Finally, cells were observed using an LSM 510 META confocal microscope with a Plan Apochromat 63X oil/1.4 DIC objective (Zeiss GmbH).

Vector construction and lentiviral transfection. Overexpressed and knocked down HIF-1α or RACGAP1 lentiviral vectors were constructed based on human HIF-1α and RACGAP1 sequences from the Ensembl

Release 110 (July 2023) (gene nos. ENSG00000100644 and ENSG00000161800) and synthesized by the Shanghai GeneChem Co., Ltd. HIF-1α/RACGAP1 overexpressing sequences were cloned into GV358 vectors to produce HIF-1α- and RACGAP1-overexpressing vectors. HIF-1α-specific short-hairpin RNA (shRNA)-targeting coding sequences (sense (S): 5'-AAUGUGAGUUCGCAU CUUGAU-3'; antisense (AS): 5'-AUCAAGAUGCGA ACUCACAUU-3'), RACGAP1-specific shRNA-targeting coding sequences (S: 5'-CUAGGACGACAAGGCAA CUUU-3'; AS: 5'-AAAGUUGCCUUGUCGUCCUAG-3') and non-targeting negative control sequences (S: 5'-UUC UCCGAACGAGUCACGU-3'; AS: 5'-ACGUGACUC GUUCGGAGAA-3') (Shanghai GeneChem Co., Ltd) were cloned into GV248 vectors to produce HIF-1α- and RACGAP1-knockdown vectors.

A 3rd generation system was used to package the lentivirus. For lentiviral production, 293T human embryonic kidney cells (The Cell Bank of Type Culture Collection of The Chinese Academy of Medical Science) were used to generate lentiviral packaging and the lentivirus supernatant was collected and filtered using a 0.45 μm filter at 48 h after transfection. Hep3B and Huh7 cells were seeded in six-well plates at a density of 1 x10^5 cells/well and cultured in DMEM with 10% FBS at 5% CO_2 at 37˚C. The cells were transfected with 10 μg lentiviral plasmids, 7.5 μg packaging plasmid and 5 μg envelope plasmid at a multiplicity of infection (MOI) of 10 using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) the following day when the cells were ~70% confluent. Hep3B and Huh7 cells were cultured at 37˚C for 6 h followed by replacement of the medium. The cells were grown at 37˚C for 48 h and subsequently treated with puromycin (1 μg/ml; Invitrogen; Thermo Fisher Scientifics, Inc.) for 72 h to select transfected clones. Then the transfected cells were collected to assess the transfected efficiency using qPCR, western blotting and immunofluorescence according to the aforementioned methods.

Structure determination and refinement. The 3D protein structures of HIF-1α and RACGAP1 were modeled using AlphaFold 2 (www.hpc.caltech.edu/documentation/software-and-modules/alphafold-2), a protein structure prediction tool. Protein structures were prepared using AutoDockTools (version 1.5.7; https://autodock.scripps.edu/) to ensure the accuracy of the docking results (20). Water molecules were manually removed from the protein structures and polar hydrogen atoms were added. Protein-protein docking was performed using the Global RAnge Molecular Matching: Docking Web Server (release 306; Virtua Drug Ltd, http://www.dockingserver.com) (21). Finally, protein-protein interactions were predicted and visualized using PyMOL software (Version 2.5, Schrödinger, Inc.).

Cell Counting Kit-8 (CCK-8) assay. Hep3B and Huh7 were seeded at a density of 1,000 cells/well in 96-well plates and were cultured in an incubator at 37˚C with 5% CO_2 for four consecutive days. CCK-8 solution (10 μl; cat. no. 96992; MilliporeSigma) was added to each well and incubated for 2 h. Optical density at 450 nm was then measured with a Spectrafluor microreader plate (Molecular Devices, LLC). These experiments were repeated three times.

Apoptosis analysis. Apoptotic Hep3B and Huh7 cell frequencies were determined via staining with annexin V (cat. no. V13241; Thermo Fisher Scientific, Inc.) and propidium iodide (PI). After rinsing twice with PBS, Hep3B and Huh7 cells (1 x10^6 cells per sample) were centrifuged at 300 x g for 5 min at room temperature and resuspended using 195 μl annexin V-FITC/PI binding buffer, followed by incubation at room temperature with annexin V-FITC (5 μl) and PI (10 μl) for 40 min in the dark. Apoptosis levels were analyzed using a BD LSR II flow cytometer (BD Biosciences). Ten thousand events were collected per sample and data were processed using CXP analysis software (version 2.1; Beckman Coulter, Inc.).

Cell migration assay. The migration ability of Hep3B and Huh7 cells was assessed using a Transwell kit. A cell suspension prepared in DMEM (5x10^4 cells; 300 μl) was added to the top Transwell chamber (cat. no. 3422; Corning, Inc.) and 500 μl 30% FBS medium was added to the bottom. After incubating in a 37˚C incubator for 48 h, the cells that had transferred on to the surface of the bottom chamber were stained with 0.5% crystal violet for 20 min at room temperature, then the chamber was rinsed with PBS and air dried at room temperature. Images were obtained using a fluorescence microscope and the number of migrated cells were manually counted in six randomly selected fields per insert.

Wound healing assay. The motility of Hep3B and Huh7 cells were assessed using wound healing assays. Hep3B and Huh7 cells (1x10^5 cells/well) were seeded in 24-well culture plates and cultured until the confluence was ~90%. Then the culture medium was changed to serum-free medium for 12 h (22). A 96-Wounding Replicator (cat. no. VP408FH; V&P Scientific, Inc.) was subsequently used to scratch wounds across each cell layer. Each well was then rinsed three times with PBS and images were obtained at 0, 24 and 48 h using a fluorescence microscope. The width of the gap was quantified using ImageJ software (version 1.8.0, National Institutes of Health). Healing rate=(wound area at 0 h-wound area at 24/48 h)/wound area at 0 h.

Gene expression analysis using publicly available datasets. Gene expression levels of HIF-1α and RACGAP1 in normal and HCC tumor samples were obtained from The Cancer Genome Atlas Program (TCGA)-HCC dataset (accession no. 202208; HCC tissue samples, n=371 and normal tissue samples, n=226). Gene Expression Profiling Interactive Analysis (GEPIA; version 2.0, gepia.cancer-pku.cn/) was used for gene expression analysis. Median mRNA expression levels of the HIF-1α and RACGAP1 for all patients with HCC were calculated. Patients with expression levels in the fourth quartile were classified as high expression, whereas those with expression levels in the first quartile were classified as low expression. Gene expressions were compared using the Wilcoxon rank-sum test. Kaplan-Meier curves were created using GraphPad Prism software (version 8.0; Dotmatics). Spearman's rank correlation coefficient was used for correlation analysis and the log-rank

test was used to assess the significance of between-group differences.

Co-immunoprecipitation (Co-IP) experiments. A co-Immunoprecipitation kit (cat. No. 26149, Thermo Scientific) was used to perform co-immunoprecipitation according to the manufacturer's instructions. Briefly, 293T cells ($1x10^7$) were collected in 1 ml RIPA buffer supplemented with 1% protease inhibitor cocktail, and subsequently lysed by sonication on ice for 3 min (10 sec pulse at 50% amplitude with 10 s rest times). The cellular debris was pelleted by centrifugation at 15,000 x g for 15 min at 4˚C. The cleared protein lysates were then incubated with 80 μl Control Agarose Resin and anti-HIF-1α (1:1 dilution by coupling Resin; cat. no. 20960-1-AP; Proteintech Group, Inc.) antibodies with rotation overnight at 4˚C. The mixtures were then incubated with immobilized protein A/G beads (Thermo Fisher Scientific, Inc.) with rotation at 4˚C for 2 h. The beads were collected by centrifugation at 3,000 x g for 2 min at room temperature, and then washed five times with 0.5 ml IP wash buffer. SDS loading buffer was added to the beads, and the samples were denatured at 95˚C for 8-10 min. Finally, the supernatants were collected and stored at -80˚C or immediately analyzed by western blotting according to the aforementioned method.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (version 8.0; GraphPad Software; Dotmatics). Each experiment was repeated three times. Data are presented as the mean ± standard deviation. Unpaired Student's t-test was used for two-group comparisons and one-way ANOVA, followed by Tukey's test, was used for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HIF-1α positively regulates RACGAP1 expression. HIF-1α overexpression and knockdown lentivirus vectors were used to transfect Hep3B and Huh7 cells to assess the potential role of HIF-1α in HCC. The results from qPCR, western blotting and immunofluorescence staining demonstrated that HIF-1α mRNA and protein expression levels were significantly increased in the HIF-1α-overexpression group, compared with the empty vector control group, whereas the expression significantly decreased in the HIF-1α-knockdown group, compared with the non-targeting negative control group (Figs. 1 and 2A). These findings indicated that both overexpression and knockdown of HIF-1α in Hep3B and Huh7 cells was achieved. Additionally, it was demonstrated that RACGAP1 protein expression levels were significantly increased in the HIF-1α-overexpression group, compared with the empty vector control group, whereas the protein expression levels were significantly decreased in the HIF-1α-knockdown group, compared with the non-targeting negative control group (Figs. 1A, B and 2B). This suggested that HIF-1α positively regulated RACGAP1 expression in the HCC cells.

RACGAP1 positively regulates HIF-1α expression. Hep3B and Huh7 cells were transfected with RACGAP1 knockdown and overexpression lentivirus vectors. The results from qPCR, western blotting and immunofluorescence indicated that there was successful knockdown and overexpression of RACGAP1 in Hep3B and Huh7 cells. RACGAP1 mRNA and protein expression levels were significantly increased in the RACGAP1-overexpression group, compared with the empty vector control group, whereas the mRNA and protein expression levels were significantly reduced in the RACGAP1-knockdown group, compared with the non-targeting negative control group (Figs. 3A-C and 4A). Additionally, it was demonstrated that HIF-1α protein expression levels were significantly increased in the RACGAP1-overexpression group, compared with the empty vector control group, whereas the protein expression levels were significantly decreased in the RACGAP1-knockdown group, compared with the non-targeting negative control group (Figs. 3A, B and 4B). The 3D protein structures revealed interacting residues and nucleotides between HIF-1α and RACGAP1, suggesting the presence of binding sites between the two genes (Fig. 4C). Using CoIP with an anti-HIF-1α antibody in 293T cells, it was demonstrated that RACGAP1 was pulled down with HIF-1α, which indicated that HIF-1α and RACGAP1 interacted directly within a complex (Fig. 4D). These results indicated the existence of a mutual regulatory relationship between HIF-1α and RACGAP1.

HIF-1α promotes the progression of HCC cells. The CCK-8 assay was utilized to evaluate the impact of HIF-1α on the viability of HCC cells. Overexpression of HIF-1α significantly increased the viability of Hep3B and Huh7 cells, compared with the empty vector control, whereas knockdown of HIF-1α significantly reduced Hep3B and Huh7 cell viability, compared with the non-targeting negative control (Fig. 5A). Overexpression of HIF-1α significantly decreased the proportion of apoptotic Hep3B and Huh7 cells, compared with the empty vector control, whilst knockdown of HIF-1α expression significantly increased the proportion of apoptotic cells for both cell types, compared with the non-targeting negative control (Fig. 5B). In addition, cell migration assays demonstrated that overexpression of HIF-1α in Hep3B and Huh7 cells significantly increased cell migration, compared with the empty vector control, whereas knockdown of HIF-1α significantly decreased migration in both cell types, compared with the non-targeting negative control (Fig. 5C). Moreover, overexpression of HIF-1α resulted in a reduced scratch width and increased healing rate, indicating a significant increase in the rate of cell migration in Hep3B and Hih7 cells, compared with the empty vector control. Conversely, knockdown of HIF-1α resulted in a greater scratch width and decreased healing rate, indicating a significant decrease in the rate of cell migration in Hep3B cells and Huh7 cells, compared with the non-targeting negative control (Fig. 6A and B). Conversely, knockdown of HIF-1α led to a greater scratch width and decreased healing rate, which indicated a significant decrease in the rate of cell migration in Hep3B and Huh7 cells compared with non-targeting negative control.

RACGAP1 promotes the progression of HCC cells. The effect of RACGAP1 on cell viability and apoptosis was assessed to further evaluate the potential regulatory role of RACGAP1 in HCC progression. RACGAP1 overexpression significantly increased the viability of Hep3B and Huh7 cells,

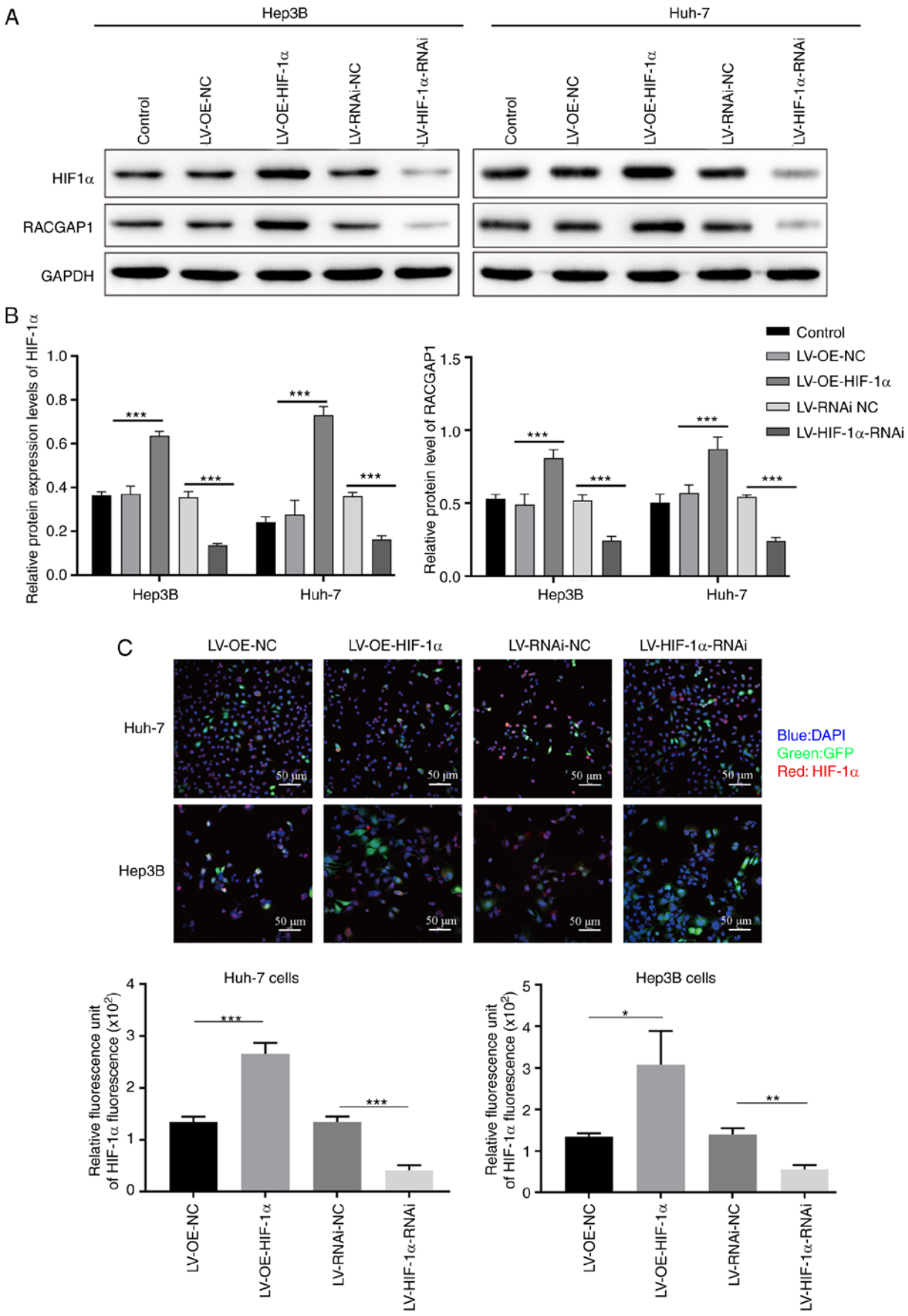

Figure 1. HIF-1α regulates RACGAP1 expression. Hep3B and Huh-7 cells were transfected with HIF-1α knockdown and overexpression lentivirus vectors. (A) and (B) Protein expression levels of HIF-1α and RACGAP1 in Hep3B and Huh-7 cells, determined using western blotting. Grayscale analysis performed using ImageJ. (C) Immunofluorescence analysis of GFP and HIF-1α in Hep3B and Huh-7 cells. n=3. *P<0.05, **P<0.01 and ***P<0.001. HIF-1α, hypoxia-inducible factor-1α; RACGAP1, Rac GTPase activating protein 1; GFP, green fluorescent protein; LV, lentivirus vector; OE, overexpression; NC, negative control; RNAi, RNA interference.

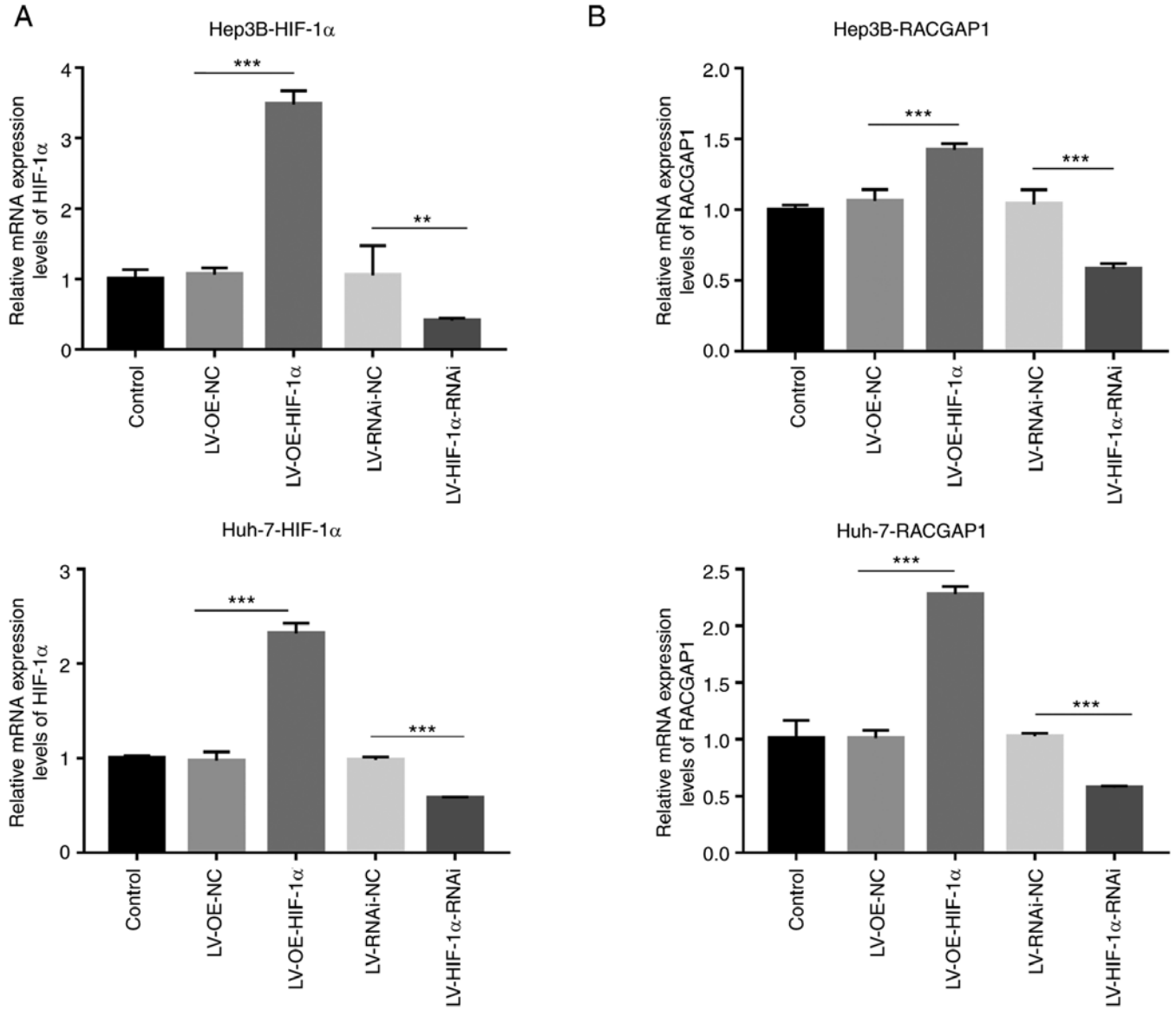

Figure 2. Transfection efficiency of HIF-1α knockdown and overexpression lentivirus vectors containing. mRNA expression levels of (A) HIF-1α and (B) RACGAP1 in transfected Hep3B and Huh-7 cells, determined by quantitative PCR. n=3. **P<0.01 and ***P<0.001. HIF-1α, hypoxia-inducible factor-1α; RACGAP1, Rac GTPase activating protein 1; LV, lentivirus vector; OE, overexpression; NC, negative control; RNAi, RNA interference.

compared with the empty vector control, whilst knockdown of RACGAP1 significantly decreased cell viability in Hep3B and Huh7 cells, compared with the non-targeting negative control (Fig. 7A). Conversely, overexpression of RACGAP1 significantly decreased the proportion of apoptotic Hep3B and Huh7 cells, compared with the empty vector control, whereas knockdown of RACGAP1 expression significantly increased the proportion of apoptotic cells for both cell types, compared with the non-targeting negative control (Fig. 7B). Furthermore, the interaction between HIF-1α and RACGAP1 was evaluated by assessing their combined effects on cell migration and apoptosis. After knockdown of HIF-1α, transfection with RACGAP1 knockdown lentivirus vectors had a superposition effect, resulting in a reduced cell migration ability (Fig. 8A) and increased proportion of apoptotic cell (Fig. 8B). These findings suggested the existence of a co-regulation network between HIF-1α and RACGAP1 in the HCC cells.

HIF-1α and RACGAP1 have a reciprocal regulatory relationship in HCC tissue. Clinical data from the TCGA database was utilized to further assess the potential regulatory relationship between HIF-1α and RACGAP1. Analysis of such data demonstrated a significant upregulation of HIF-1α expression in human HCC tissues that was significantly associated with a lower overall survival probability in patients with HCC (Fig. 9A). Additionally, significantly increased RACGAP1 expression was demonstrated in HCC tissues and this was also significantly associated with a decreased overall survival probability in patients with HCC (Fig. 9B). Significant upregulation of RACGAP1 was demonstrated in patients with HCC and high HIF-1α expression levels, compared with those with low HIF-1α expression levels (Fig. 9C). Moreover, there was significantly higher HIF-1α expression in patients with HCC and high RACGAP1 expression levels, compared with those with low RACGAP1 expression levels (Fig. 9D). Furthermore, Spearman's rank correlation analysis demonstrated a significant

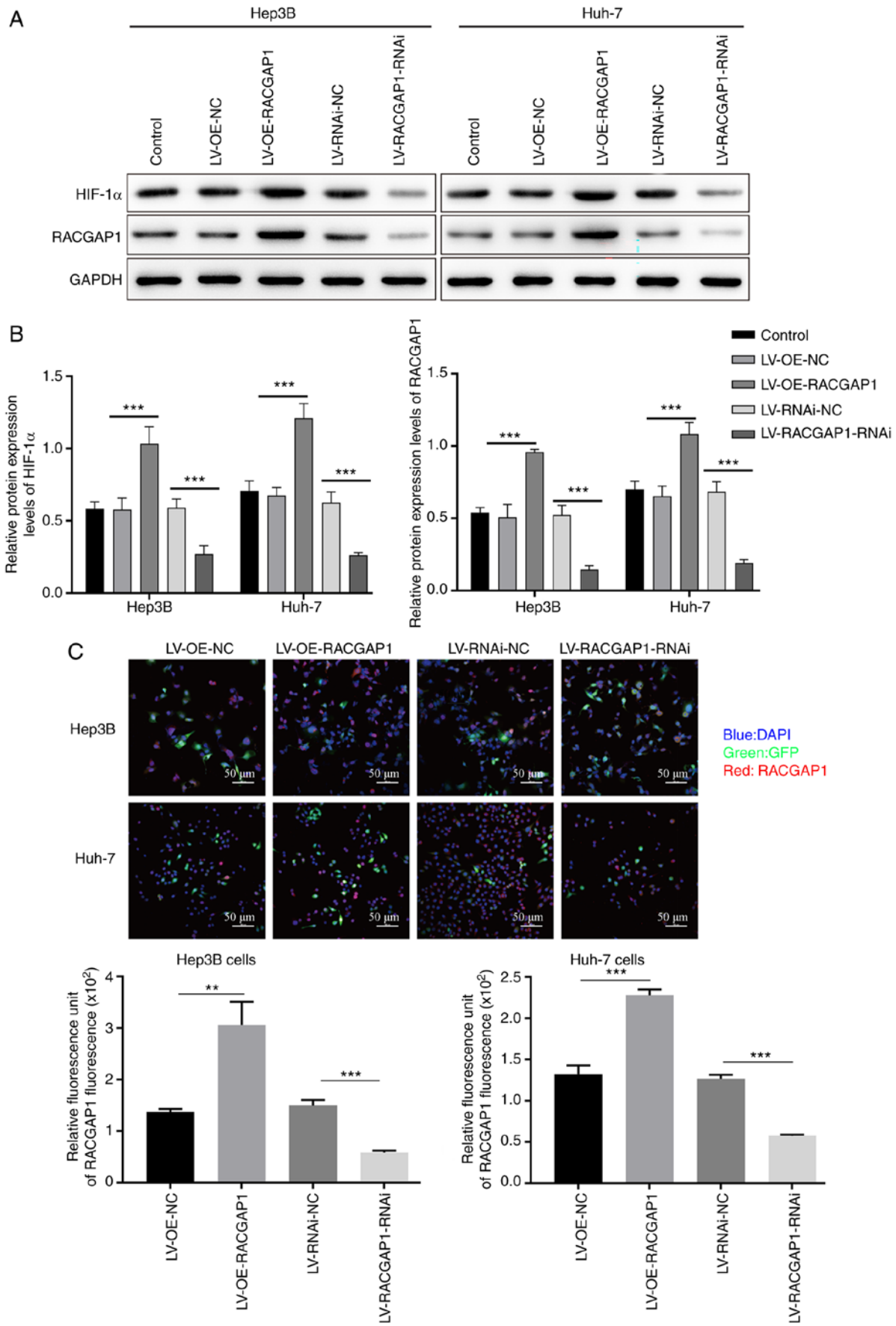

Figure 3. RACGAP1 regulates HIF-1α expression. Hep3B and Huh-7 cells were transfected with RACGAP1 knockdown and overexpression lentivirus vectors. (A) and (B) Protein expression levels of HIF-1α and RACGAP1 in Hep3B and Huh-7 cells, determined using western blotting. Grayscale analysis performed using ImageJ. (C) Immunofluorescence analysis of GFP and RACGAP1 in Hep3B and Huh-7 cells. n=3. **P<0.01 and ***P<0.001. RACGAP1, Rac GTPase activating protein 1; HIF-1α, hypoxia-inducible factor-1α; GFP, green fluorescent protein; LV, lentivirus vector; OE, overexpression; NC, negative control; RNAi, RNA interference; IP, immunoprecipitation.

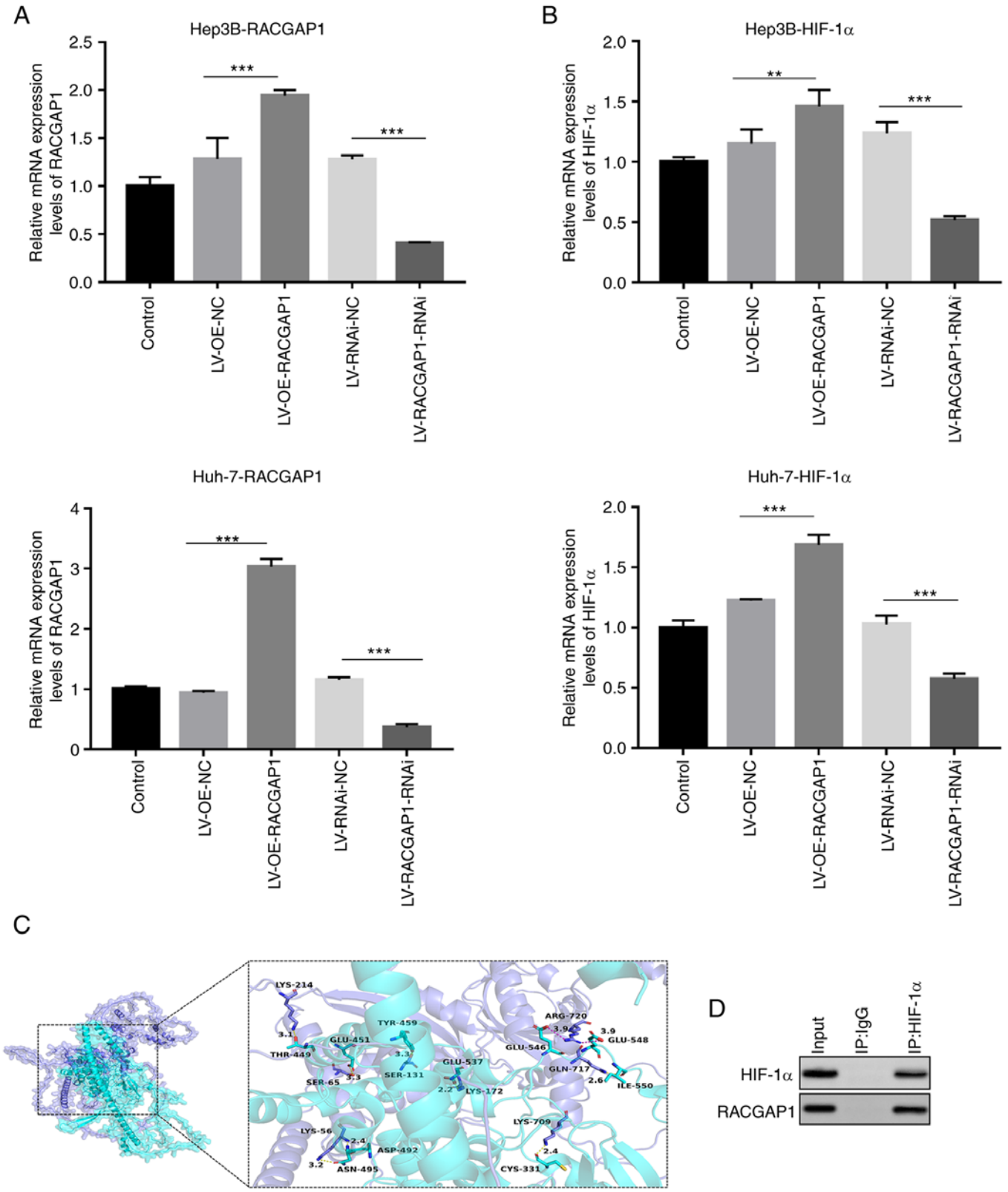

Figure 4. Transfection efficiency of RACGAP1 knockdown and overexpression lentivirus vectors. mRNA levels of (A) RACGAP1 and (B) HIF-1α in transfected Hep3B and Huh-7 cells, determined by quantitative PCR. n=3. **P<0.01 and ***P<0.001. (C) Predicted structures of HIF-1α and RACGAP1, visualized using PyMOL software. HIF-1α protein is represented as a slate-colored cartoon model, whilst RACGAP1 protein is shown as a cyan-colored cartoon model. The binding sites of each protein are depicted as corresponding colored stick structures. The binding site is highlighted and presented in the context of the respective protein within the binding region. (D) Co-immunoprecipitation analysis of HIF-1α and RACGAP1. RACGAP1, Rac GTPase activating protein 1; HIF-1α, hypoxia-inducible factor-1α; LV, lentivirus vector; OE, overexpression; NC, negative control; RNAi, RNA interference; IP, immunoprecipitation.

positive correlation between the expression levels of HIF-1α and RACGAP1 in HCC tissues (Fig. 10). These findings further suggested the involvement of HIF-1α and RACGAP1 in HCC progression through intricate reciprocal regulation.

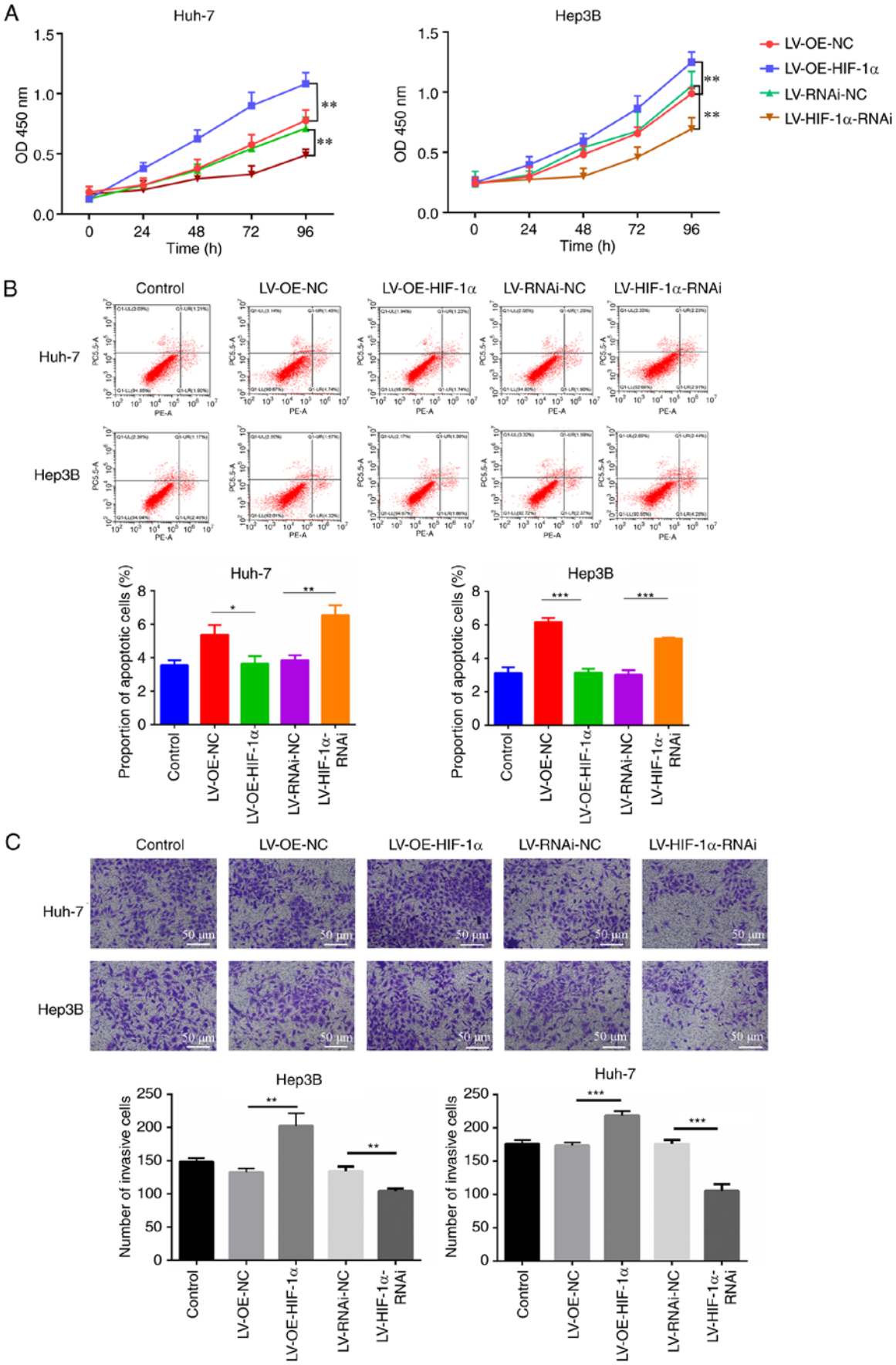

Figure 5. HIF-1α accelerates proliferation and migration and inhibits apoptosis of hepatocellular carcinoma cells. Hep3B and Huh-7 cells were transfected with HIF-1α knockdown and overexpression lentivirus vectors. (A) Viability of Hep3B and Huh-7 cells, assessed using the Cell Counting Kit-8. (B) Proportion of apoptotic Hep3B and Huh-7 cells, assessed using flow cytometry. (C) Migration ability of Hep3B and Huh-7 cells, evaluated using the Transwell migration assay. n=3. *P<0.05; **P<0.01 and ***P<0.001. HIF-1α, hypoxia-inducible factor-1α; OD, optical density; LV, lentivirus vector; OE, overexpression; NC, negative control; RNAi, RNA interference.

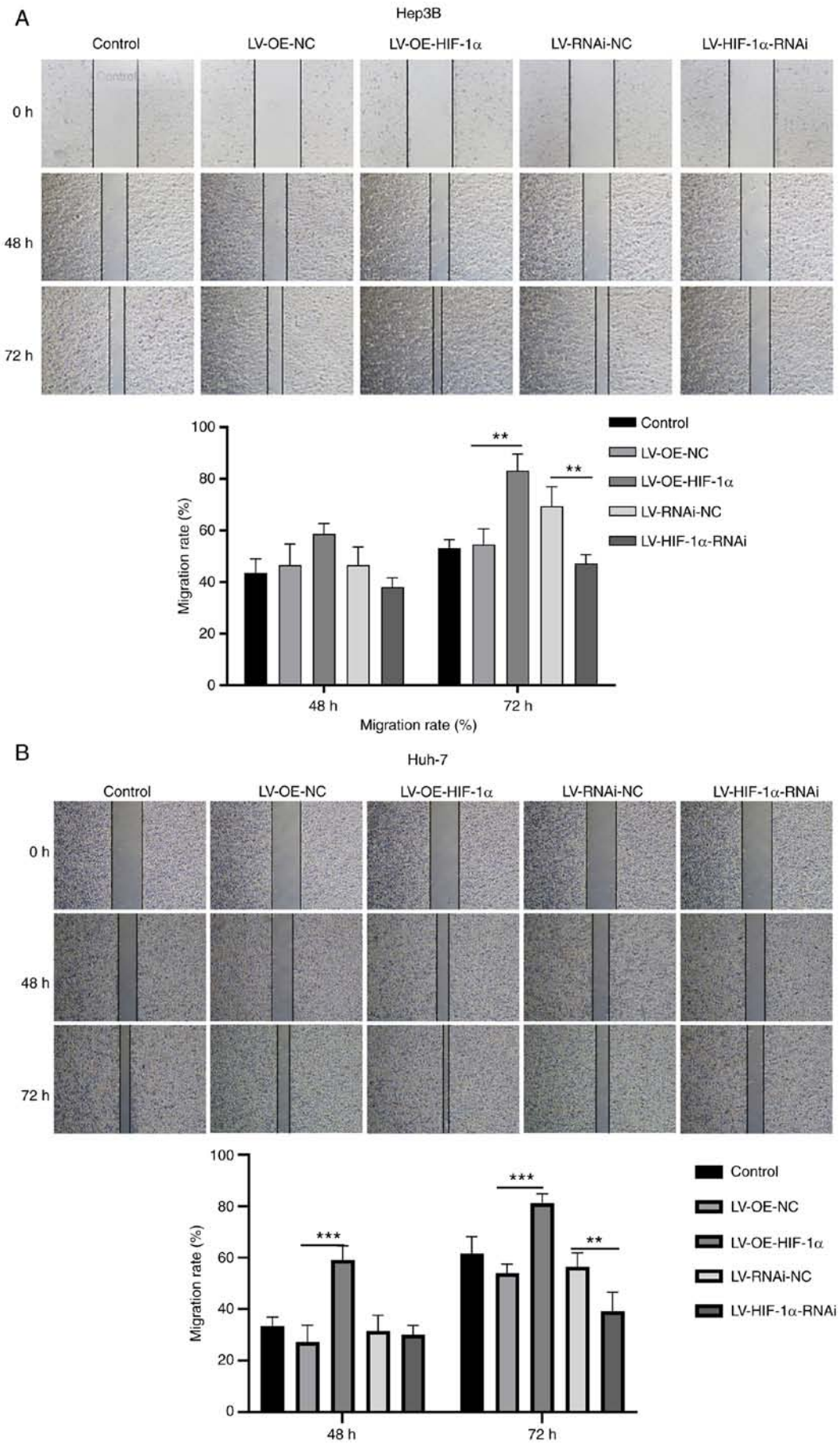

Figure 6. HIF-1α accelerates migration of hepatocellular carcinoma cells. Hep3B and Huh-7 cells were transfected with HIF-1α knockdown and overexpression lentivirus vectors. Migration ability of (A) Hep3B and (B) Huh-7 cells, assessed using scratch experiments. Magnification, x100. n=3. **P<0.01 and ***P<0.001. HIF-1α, hypoxia-inducible factor-1α; LV, lentivirus vector; OE, overexpression; NC, negative control; RNAi, RNA interference.

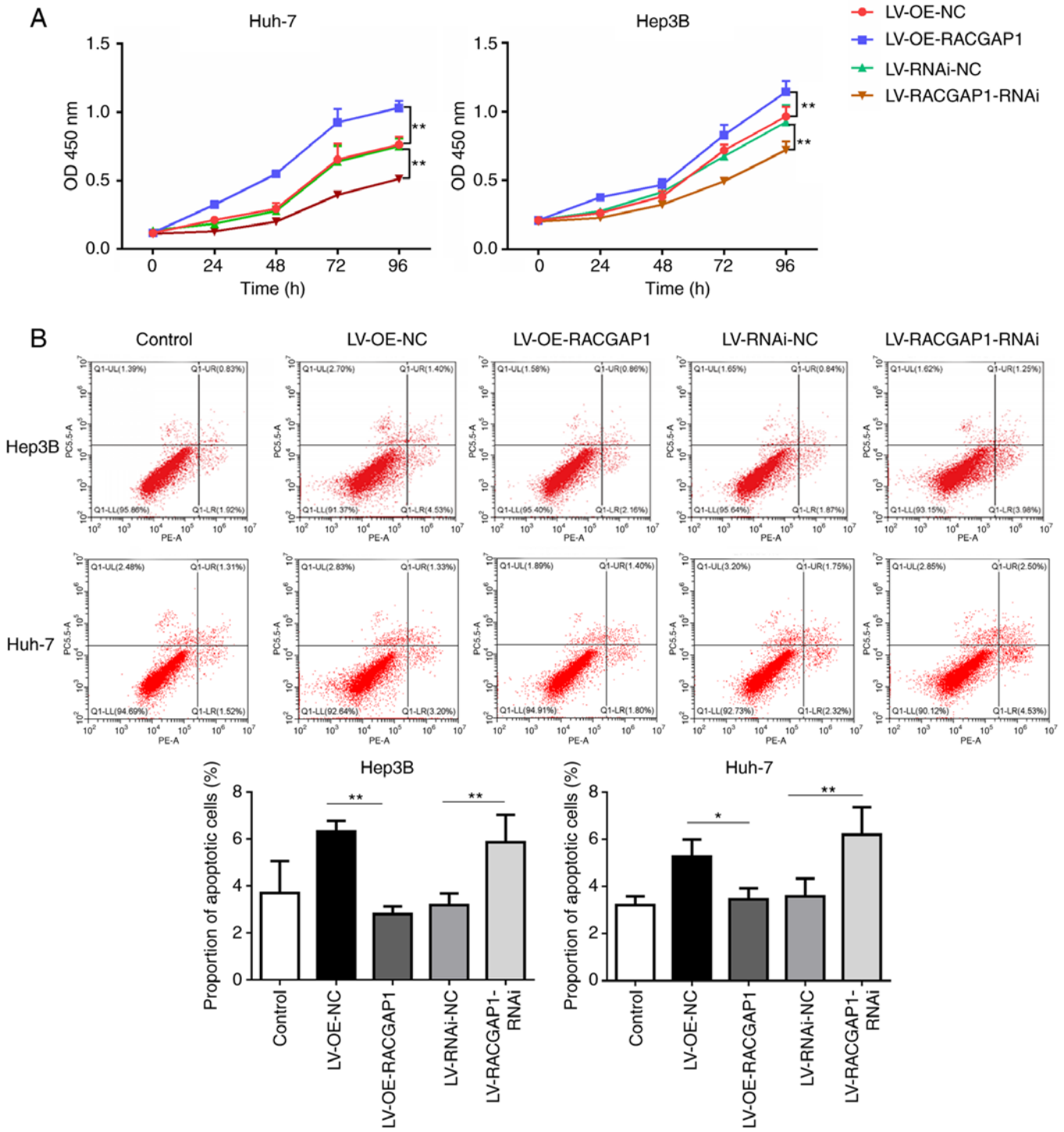

Figure 7. RACGAP1 accelerates proliferation and inhibits apoptosis of hepatocellular carcinoma cells. Hep3B and Huh-7 cells were transfected with RACGAP1 knockdown and overexpression lentivirus vectors. (A) Viability of Hep3B and Huh-7 cells, assessed using the Cell Counting Kit-8. (B) Proportion of apoptotic Hep3B and Huh-7 cells, assessed using flow cytometric analysis. n=3. *P<0.05 and **P<0.01. RACGAP1, Rac GTPase activating protein 1; OD, optical density; LV, lentivirus vector; OE, overexpression; NC, negative control; RNAi, RNA interference.

Discussion

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and ranks as the second leading cause of cancer-related deaths (23,24). Although several molecular and signaling pathways contributing to HCC have been elucidated (25,26), there is a need for increased early diagnoses and a lack of effective therapies targeting HCC in clinic (27).

HIF-1α is a major regulator of hypoxia-responsive genes in the tumor microenvironment and is a characteristic marker of solid tumors (28). HIF-1α overexpression has been reported to be a negative prognostic indicator for patients with HCC (29). It serves a crucial role in facilitating tumor migration, migration, metastasis, angiogenesis, as well as regulating glycolysis, epithelial-mesenchymal transition and lipid metabolism (30-35). RACGAP1, a member of the

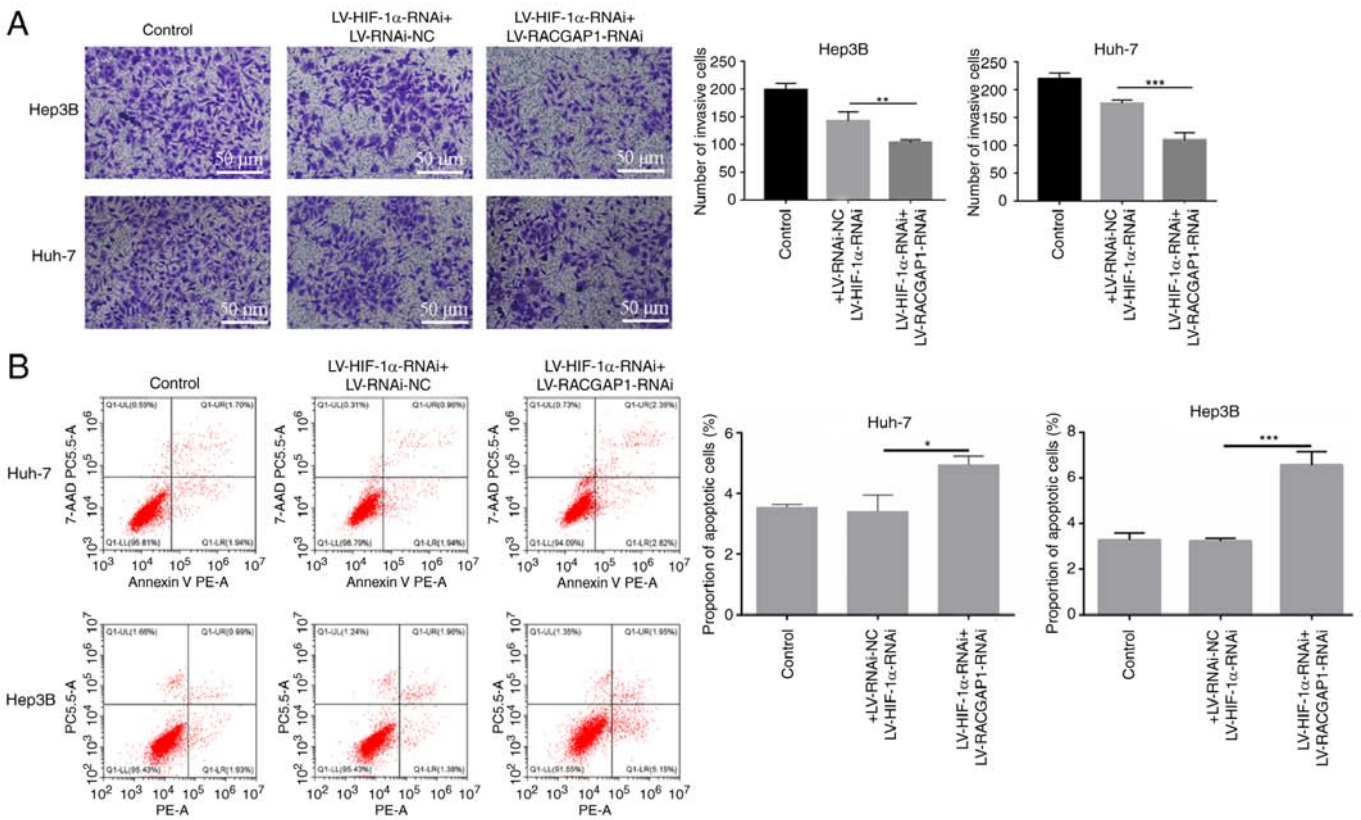

Figure 8. Simultaneous knockdown of HIF-1α and RACGAP1 exerts a synergistic effect on the migration and apoptosis of hepatocellular carcinoma cells. Hep3B and Huh-7 cells were simultaneously transfected with HIF-1α and RACGAP1 knockdown lentivirus vectors. (A) Migration ability of Hep3B and Huh-7 cells, assessed using the Transwell migration assay. (B) Proportion of apoptotic Hep3B and Huh-7 cells, assessed using flow cytometric analysis. n=3. *P<0.05; **P<0.01 and ***P<0.001. HIF-1α, hypoxia-inducible factor-1α; RACGAP1, Rac GTPase activating protein 1; LV, lentivirus vector; RNAi, RNA interference; NC, negative control.

GTPase-activating protein family, is a modulator of cytokinesis, migration and differentiation (14). RACGAP1 is frequently upregulated in HCC and is associated with shorter survival times in patients (36). Overexpression of RACGAP1 promotes HCC cell proliferation by inhibiting the activation of the Hippo and yes-associated protein pathways (32). Additionally, RACGAP1 acts as an oncogenic competing endogenous RNA by sequestering microRNA-15-5p, thereby promoting HCC recurrence (37). Therefore, HIF-1α and RACGAP1 exhibit similar functions in the progression of HCC under normal oxygen levels (28-36). In the present study, HIF-1α and RACGAP1 were demonstrated to promote HCC progression in a mutually regulatory manner under hypoxic conditions, providing evidence for their potential use in the clinical diagnosis and treatment of HCC.

Angiogenesis is tightly regulated in normal tissues, resulting in a well-organized vasculature that adequately supplies oxygen. By contrast, malignant tumors, including HCC, exhibit abnormal angiogenesis and an irregular vasculature, resulting in insufficient oxygen concentrations within solid tumors (38). This hypoxic microenvironment contributes to the metabolic reprogramming of cancer cells and triggers the production of numerous bioactive molecules that increase the resistance of cancer cells to radiation and chemotherapy (39-41). For example, in HCC cells, the activation of the FABP5/HIF-1α axis has been reported to lead to increased lipid uptake and storage, which results in lipid accumulation within the cells (35). Hypoxia has been reported to have significantly enhanced the resistance of human malignant mesothelioma cells to cisplatin by promoting epithelial to mesenchymal transition (41). Under hypoxic conditions, the degradation pathway of oxygen-dependent HIF-1α is inhibited, resulting in increased levels of HIF-1α within the tumor cells. HIF-1α serves a crucial role in promoting tumor progression by inducing sustained growth factor signaling, angiogenesis, epithelial-mesenchymal transition and replicative immortality (42,43). Hypoxia also leads to the selection of cancer cells that can evade growth suppressors or apoptotic triggers and disrupt cellular energetics (44,45). Additionally, HIF-1α is associated with genetic instability, tumor-promoting inflammation and evasion of the immune response (46,47). Therefore, HIF-1α represents an important therapeutic target, given its role in cancer development and progression. In the present study, overexpression of HIF-1α significantly promoted the proliferation, migration and metastasis of Huh-7 and Hep3B cells, whilst inhibiting their apoptosis under hypoxic conditions. Conversely, knockdown of HIF-1α effectively suppressed the proliferation, migration and metastasis of Huh-7 and Hep3B cells, whilst promoting apoptosis. These findings further support the role of HIF-1α as a promoter of cancer progression in HCC.

A previous study reported that RACGAP1 serves a crucial role in cell division and proliferation (37). RACGAP1 was initially identified in the testis and in germ cells and its

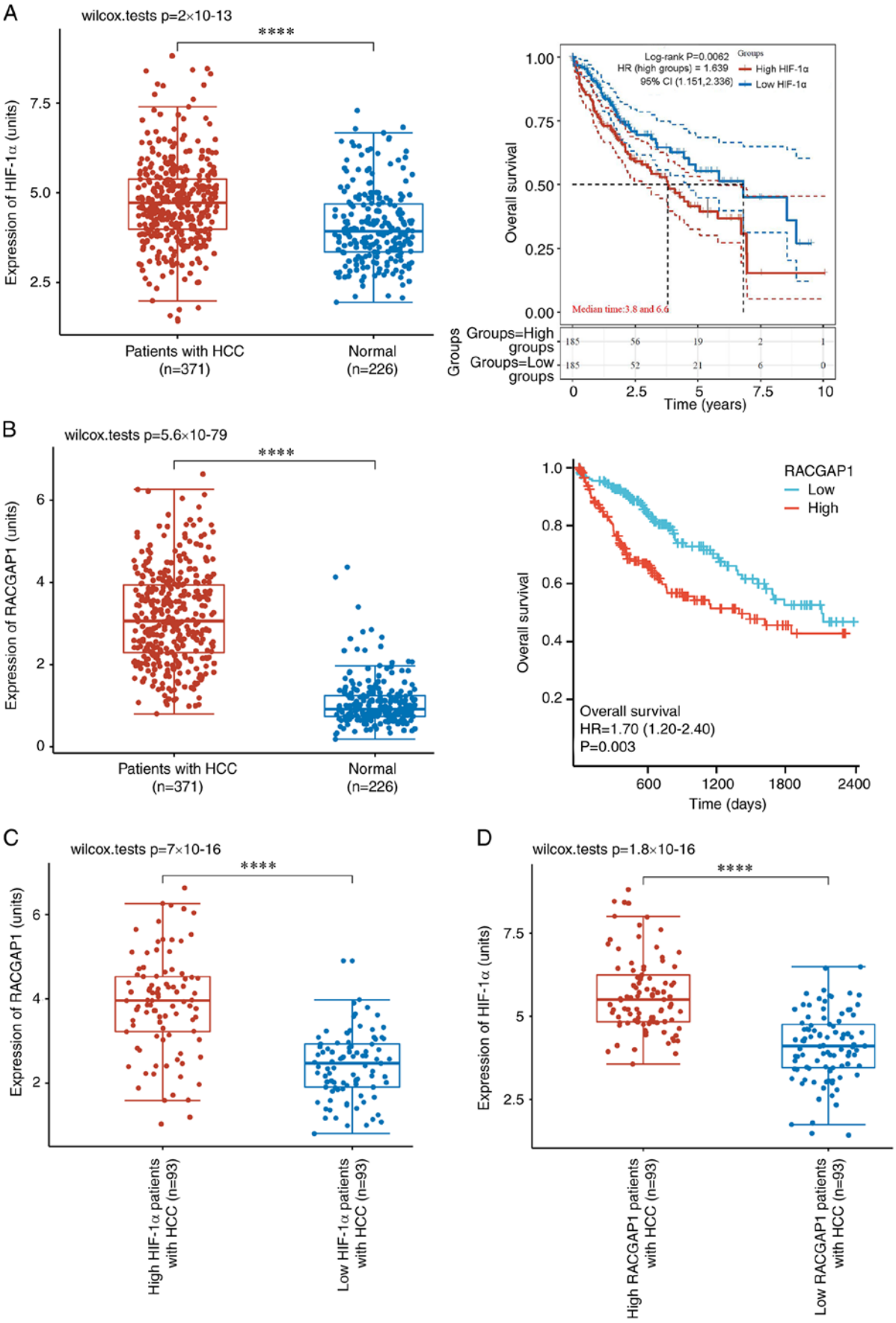

Figure 9. HIF-1α and RACGAP1 are upregulated and serves as a prognostic factor in HCC. mRNA expression levels of (A) HIF-1α and (B) RACGAP1 in HCC and normal tissues, utilizing data from the TCGA-HCC dataset and Kaplan-Meier analysis demonstrating the overall survival of patients with HCC based on high and low expression levels of HIF-1α/ RACGAP1. (C) RACGAP1 expression compared between HCC tissues with high and low levels of HIF-1α. (D) HIF-1α expression compared between HCC tissues with high and low levels of RACGAP1. ****P<0.0001. HIF-1α, hypoxia-inducible factor-1α; RACGAP1, Rac GTPase activating protein 1; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas Program; HR, hazard ratio.

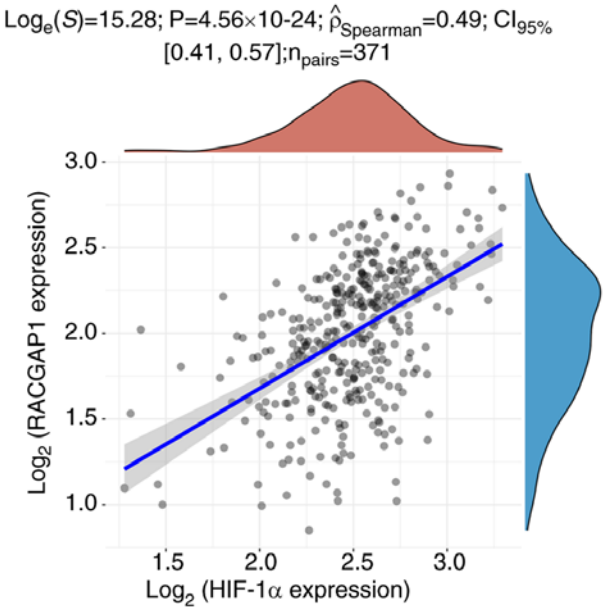

Figure 10. Patients with HCC and upregulation of both HIF-1α and RACGAP1 demonstrate a significantly worse prognosis. Correlation between expression of HIF-1α and RACGAP1 in HCC tissues, calculated by Spearman's rank correlation analysis. HIF-1α, hypoxia-inducible factor-1α; RACGAP1, Rac GTPase activating protein 1.

significance has been reported in numerous malignant tumors, including colorectal cancer, epithelial ovarian cancer, HCC, pancreatic and stomach cancer and meningiomas (37,48,49). Upregulation of RACGAP1 has been associated with doxorubicin resistance in squamous cell carcinoma and is associated with worse overall survival in patients (9). In esophageal carcinoma, high RACGAP1 expression has been reported to be significantly associated with worse overall survival, disease-free survival, lymphatic migration, vessel migration and advanced tumor, node, metastasis stage (50). Similarly, high nuclear RACGAP1 expression is associated with poor survival in patients with colorectal cancer (51). In HCC, RACGAP1 overexpression promotes disease progression and has been reported to be significantly associated with worse overall survival (16,17). In the present study, knockdown of RACGAP1 significantly suppressed cell proliferation and induced apoptosis, whilst overexpression of RACGAP1 significantly promoted cell proliferation and suppressed apoptosis in Huh-7 and Hep3B cells under hypoxic conditions. These findings demonstrated the oncogenic role of RACGAP1 in HCC progression and suggested that targeting the RACGAP1-mediated oncogenic pathway may hold therapeutic value for HCC.

However, the interaction between HIF-1α and RACGAP1 has not been previously reported in the literature and to the best of our knowledge, this is the first study that has demonstrated the interaction between HIF-1α and RACGAP1. In the present study, knockdown of HIF-1α resulted in a decrease in RACGAP1 expression, whereas overexpression of HIF-1α promoted RACGAP1 expression. Additionally, modulation of RACGAP1 expression had a reciprocal effect on HIF-1α expression. These findings suggested that HIF-1α and RACGAP1 mutually regulate each other and promote HCC progression under hypoxic conditions. Furthermore, simultaneous knockdown of HIF-1α and RACGAP1 had a more pronounced inhibitory effect on HCC cell migration compared with knockdown of HIF-1α alone. Similarly, simultaneous overexpression of HIF-1α and RACGAP1 had a more significant facilitation effect on HCC cell apoptosis than HIF-1α knockdown alone. Clinical data analysis in the present study also demonstrated RACGAP1 expression was significantly increased in HCC tissues with high HIF-1α expression levels and HIF-1α expression was significantly increased in HCC tissue with high RACGAP1 expression levels. Spearman's rank correlation analysis further demonstrated a significant positive correlation between the expression levels of HIF-1α and RACGAP1 in HCC tissues, emphasizing the reciprocal regulatory relationship between HIF-1α and RACGAP1.

The regulatory mechanism between HIF-1α and RACGAP1 may involve a feedback loop. HIF-1α, a transcription factor, can directly bind to specific DNA sequences, called hypoxia response elements (HREs), present in the promoter region of the RACGAP1 gene (52). Binding of HIF-1α to HREs promotes the transcription of RACGAP1, leading to increased expression of RACGAP1 (53). RACGAP1 may regulate HIF-1α through multiple mechanisms, including post-translational modifications and protein-protein interactions. RACGAP1 can modulate the stability of HIF-1α protein by inhibiting the ubiquitin-proteasome pathway (54,55). Additionally, RACGAP1 can inhibit the activity of prolyl hydroxylases, enzymes that regulate HIF-1α stability under normoxic conditions, leading to increased stabilization of HIF-1α (56). In the present study, AlphaFold 2 and PyMOL were utilized to predict the interactions between HIF-1α and RACGAP1 and the direct interaction between HIF-1α and RACGAP1 was further assessed using CoIP experiments. These reciprocal regulatory mechanisms between HIF-1α and RACGAP1 contribute to HCC progression.

The present study provided valuable insights into the roles of HIF-1α and RACGAP1 in HCC, however there are certain limitations to consider. Rescue experiments were not performed during the investigation into the reciprocal relationship between HIF-1α and RACGAP1 expression levels. The expression of HIF-1α and RACGAP1 were instead manipulated to assess their specific impact on the expression of the other. However, the inclusion of rescue experiments could have provided further support for the findings of the present study. Moreover, the absence of animal models limits the generalizability of the results. The present study utilized liver cancer clinical samples and cell information from the TCGA database to assess the regulatory effects of HIF-1α and RACGAP1. Future studies should utilize mouse and rat liver cancer models to evaluate the roles of HIF-1α and RACGAP1 in liver cancer progression.

In conclusion, HIF-1α and RACGAP1 cooperatively contribute to the pathogenesis of HCC, which may provide a valuable therapeutic target for HCC.

Acknowledgements

Not applicable.

Funding

This work was supported by Guangxi Clinic Medicine Research Center of Hepatobiliary Diseases (grant. no. AD17129025) and the Science Foundation of Youjiang Medical College for Nationalities (grant. no. yy2020ky022).

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

XW, ZX and JP conceived and designed the study. XW and YL performed experiments. WL performed the analyses and also participated in the study design. YL and JP wrote the manuscript. XW and JP confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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