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Long non-coding RNA SNHG5 promotes human hepatocellular carcinoma progression by regulating miR-363-3p/RNF38 axis

P.-A. HU[1], Y.-Y. MIAO[2], S. YU[3], N. GUO[2]

[1]Department of Hepatopathy, the Luoyang Central Hospital Affiliated to Zhengzhou University, Luoyang, China
[2]Department of Hepatopathy, the Sixth People's Hospital of Qingdao, Qingdao, China
[3]Department of Hepatology, the Rizhao Hospital of Traditional Chinese Medicine, Rizhao, China

Ping-an Hu and Yanyan Miao contributed to this work equally as co-first authors

Abstract. – OBJECTIVE: The involvement of long non-coding RNA (LncRNAs) in HCC development has been widely recognized in recent decades. LncRNA small nucleolar RNA host gene 5 (SNHG5) has been identified to be implicated in the development of many tumors, and this study aimed to explore the role of SNHG5 in HCC tumorigenesis.

PATIENTS AND METHODS: The expression levels of SNHG5, miR-363-3p, and Ring Finger Protein 38 (RNF38) were measured by using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) or Western blot assay, respectively. Cell proliferation was analyzed by MTT assay. Flow cytometry was used to investigate cell apoptosis. Cell migration and invasion abilities were detected by transwell assay. The relationship among SNHG5, miR-363-3p, and RNF38 was confirmed using bioinformatics analysis and Luciferase reporter assay.

RESULTS: The expression of SNHG5 and RNF38 was elevated in HCC tissues and cell lines, and highly expressed SNHG5 and RNF38 could induce apoptosis and repress proliferation, migration, as well as invasion in HCC cells. Further investigations showed that SNHG5 might act as a competing endogenous RNA of miR-26a-5p and thereby cause the derepression of the downstream target RNF38. Moreover, rescue experiments indicated that SNHG5 silence inhibited the progression of HCC cells by regulating miR-363-3p, and the facilitated effects of RNF38 in the progression of HCC cells were regulated by miR-363-3p.

CONCLUSIONS: LncRNA SNHG5 may promote human HCC progression by regulating the miR-363-3p/RNF38 axis, providing a novel insight into the pathogenesis of HCC and therapeutic strategy for HCC treatment.

Key Words:
HCC, SNHG5, MiR-363-3p, RNF38, Progression.

Abbreviations

SNHG5=small nucleolar RNA host gene 5; RNF38=Ring Finger Protein 38; qRT-PCR=quantitative Real Time-Polymerase Chain Reaction; HCC=Hepatocellular carcinoma; ceRNAs=competing endogenous RNAs; RNF38=Ring Finger Protein 38; RING=really interesting new gene; BCA=bicinchoninic acid; MUT=mutant; WT=wild-type; SD=standard deviation; ANOVA= analysis of variance.

Introduction

Hepatocellular carcinoma (HCC), the fifth most common malignancy and the third cancer-related mortality worldwide, is the most common primary liver malignancy[1]. Although advancements in the development of early-stage diagnoses and curative therapy, there are not great benefits in terms of survival in more advanced stages patients because of metastatic and chemoresistant tumor[2,3]. The molecular pathogenesis of HCC is a complex process implicating a great number of events and genetic abnormalities, which contribute to oncogenic capacities to pre-neoplastic cells[4,5]. Thus, a better understanding of the molecular mechanisms and genetic alterations underlying HCC for finding the novel therapeutic strategies in treating HCC is of great importance.

Long non-coding RNAs (LncRNAs) are a group of non-coding RNAs that are more than 200 base pairs in length. LncRNAs were recognized earlier as "dark matter" or "transcriptional noise" with no biological functions[6]. However, lncRNAs play an important role in numerous cellular contexts and participate in

Corresponding Author: Na Guo, MD; e-mail: vofiux@163.com

almost every stage of gene expression, in both pathological and physiological cellular conditions[7,8]. Recently, emerging researches have pointed out the involvement of lncRNAs in epigenetic mechanisms in the pathogenesis of HCC, including in growth, chemotherapy resistant, cellular apoptosis, metastasis, angiogenesis, and oncogenesis[9-11]. Therefore, lncRNAs may be considered as potential regulators for HCC pathogenesis. Small nucleolar RNA host gene 5 (SNHG5), also called U50HG, is one of the well-defined cytoplasmic lncRNAs with 524 bp in length. SNHG5 has six exons and encodes for the snoRNAs U50 and U50'[12]. Aberrantly expressed SNHG5 has been reported in various human cancers and deregulated SNHG5 involved in the tumorigenesis of numerous cancers, such as colorectal cancer, gastric cancer, and osteosarcoma[13-15]. Recently, Li et al[16] displayed that SNHG5 could promote human HCC progression by regulating miR-26a-5p/GSK3β signal pathway, indicating the regulatory role of SNHG5in HCC development. However, the exact molecular mechanism of SNHG5 on tumorigenesis of HCC remains largely unclear.

LncRNAs may serve as competing endogenous RNAs (ceRNAs) by competitively binding microRNAs (miRNAs) to modulate the derepression of miRNA molecules on their targets and thereby imposing an additional regulation at post-transcriptional level[17]. MiRNAs are a class of small endogenous non-coding RNAs that negatively regulate protein synthesis and cleavage of mRNAs by binding to sequences with partial complementarity on target mRNAs, resulting in the repression of the target gene expression[18]. Various miRNAs, acting as tumor suppressors or oncogenes, are involved in the development of many cancers, including HCC[19,20]. Among these, microRNA-363-3p (miR-363-3p) functions as a tumor suppressor in HCC have also been identified[21]. Ring Finger Protein 38 (RNF38) mRNA is widely abundant in different human tissues, and the evolutionary conservation indicates a vital cellular function[22]. RNF38 is a member of the new interesting gene (RING) finger protein family, which exerts diverse effects on human physiology and pathology, especially on the oncogenesis of cancer[23]. In the current study, we aimed to investigate the expression pattern of SNHG5 in HCC patients and to explore the regulatory role of SNHG5 in the HCC progression, as well as its underlying molecular mechanisms.

Patients and methods

Clinical Specimens and Patients

HCC specimens and the matched adjacent non-tumor tissues (3-5 cm distal to the edge of tumor) were obtained from 31 HCC patients who underwent surgical excision at the sixth People's Hospital of Qingdao. None of the patients received any chemotherapy or radiotherapy treatments before surgery. HCC was diagnosed by histopathology. Tumor samples were immediately frozen in liquid nitrogen and stored at -80°C until further study. Informed consent was obtained from all patients and the study protocols were approved by the Ethics Committee of the Sixth People's Hospital of Qingdao.

Cell Culture and Transfection

The human HCC cell lines (Hep3B and Huh7) and the human immortalized hepatic cell line THLE-2 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin (Gibco, Carlsbad, CA, USA) in a humidified atmosphere at 37°C with 5% CO_2.

Small interfering RNA (siRNA) targeting SNHG5 (si-SNHG5), siRNA targeting RNF38 (si-RNF38), siRNA negative control (si-NC), pcDNA, pcDNA-RNF38 overexpression vector (RNF38), and pcDNA-SNHG5 overexpression vector (SNHG5), were synthesized by GenePharma (Shanghai, China). The miRNA mimic or inhibitor targeting miR-363-3p (miR-363-3p or anti-miR-363-3p) and their corresponding negative control (miR-NC or anti-miR-NC) were obtained from RiboBio (Guangzhou, China). All the oligonucleotides or vectors were transfected into Hep3B and Huh7 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Then cells were harvested for subsequent analysis.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from specimens and cultured cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then, the cDNA was synthesized with the help of PrimeScript™ RT Master Mix Kit (TaKaRa, Dalian, China) and

TaqMan MiRNA Reverse Transcript Kit (Applied Biosystems, Foster City, CA, USA). Then, quantitative Real Time-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq™ (TaKaRa, Dalian, China) on ABI 7500 fast system. β-actin and U6 were used as internal inference, respectively. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used in this study were presented as follow: SNHG5 Forward 5'-AGTAGCCAGTGAAGATAATG-3', SNHG5 Reverse 5'-ACAACAGTC AAGTAAACC-3'; β-actin Forward 5'-TCGTGCGTGACATTA-AGGAGAAG-3', β-actin Reverse 5'-GGAAG-GAAGGCTGGAAGAGTG-3'; miR-363-3p Forward, 5'-CGGCGAATTGCACGGTATCCA-3'; miR-363-3p Reverse, 5'-CAGTGCAGGGTC CGAGGT-3'; U6 Forward, 5'-CGCAAGGATG-ACACGCAAATTCG-3'; U6 Reverse 5'-CAGT-GCAGGGTCCGAGGT-3'. RNF38 Forward 5'-AACACGGAGAGCAG TTCCAC-3', Reverse 5'-CCTGGCATACGTCTTCAACA-3'.

Cell Proliferation Assay

MTT (3-(4,5)-dimethylthiahiazo(-z-yl)-3,5-diphenytetrazoliumromide assay (Sigma-Aldrich, St. Louis, MO, USA) was used to analyze cell proliferation. The transfected HepG2 and Huh7 cells were seeded in 96-well plates at a density of 5000 cells/well. After incubation for 0 h, 24 h, 48 h, and 72 h, 10 μL MTT was added into each well at the specific time points and the cells were incubated for additional 4 h. After that, the supernatant was discarded and 250 mL dimethyl sulfoxide (DMSO) was added. Cell viability was detected by measuring the optical density (OD) by using a microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm.

Cell Apoptosis Assay

Cell apoptosis was analyzed by the flow cytometry with the help of Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Solarbio, Beijing, China). Briefly, 5×10^5 cells were resuspended in the binding buffer at the specific time points, and then, incubated with 5 μL of FITC-Annexin V and 5 μL PI in the dark for 15 min according to the manufacturer's protocol. Finally, the cell apoptosis rate was assessed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Transwell Assay

The transwell assay was performed to analyze cell migration and invasion abilities. For migration assay, the transfected Hep3B and Huh7 cells were seeded in the upper chamber in DMEM medium without serum. DMEM medium containing 10% fetal bovine serum (FBS) was added into the lower chamber. After 24 h incubation, the cells on the upper surface were removed using a dry cotton swab, and then, the cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Finally, the number of five randomly selected fields was counted under a microscope (Olympus, Tokyo, Japan). For invasion assay, the philosophy of measurement was similar to the process of cell migration, except for the upper transwell chambers that were pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and the cells were incubated for 8 h with 5% CO_2 at 37°C.

Western Blot Assay

After transfection, the cells were harvested and lysed in RIPA lysis buffer (Beyotime, Shanghai, China) and quantified using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) in accordance with the manufacturer's instructions. Equal amounts of protein were separated by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membranes were incubated with primary antibodies against RNF38 or GAPDH overnight at 4°C after being blocked with 5% non-fat milk for 1 h at room temperature. Next, they interacted with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. Immunoreactive signal was visualized by commercial enhanced chemiluminescence chromogenic substrate (Beyotime, Shanghai, China) and quantitated by Image Lab software (Bio-Rad, Hercules, CA, USA).

Luciferase Reporter Assay

The SNHG5 mRNA and RNF38 3'-UTR with wild-type (WT) or mutant (MUT) binding sequence of miR-363-3p were cloned into the pMIR-Report plasmid (Promega, Madison, WI, USA), respectively. Then, the cells were cultured in 96-well plates and co-transfected with miR-363-3p mimic or miR-NC mimic, the corresponding Luciferase reporters, or a control Luciferase plasmid using Lipofectamine 2000. After transfection for 48 h, the Luciferase activity was analyzed by a Dual-Luciferase assay kit (Promega, Madison, WI, USA).

Statistical Analysis

Quantitative data were analyzed using the GraphPad Prism 7 (GraphPad Inc., San Diego, CA, USA) and presented as the mean ± standard deviation (SD) from at least three times independently experiment. The differences among multiple groups were analyzed using One-way analysis of variance (ANOVA) followed by Dunnett's test or two-tailed *t*-tests. The correlations analysis was conducted using Pearson's rank test. $p < 0.05$ was considered statistically significant.

Results

SNHG5 is Upregulated in HCC Tissues and Cell Lines

Expression of SNHG5 was measured by qRT-PCR in 31 HCC and matched adjacent non-tumor tissues and the results showed that SNHG5 expression was significantly elevated in HCC tissues compared to non-tumor tissues (Figure 1A). Besides, compared to the human immortalized hepatic cell line THLE-2, SNHG5 expression was also higher in human HCC cell lines, including Hep3B and Huh7 cells (Figure 1B). All the results indicated the potential regulatory role of SNHG5 in HCC.

SNHG5 Silence Inhibits the Proliferation, Migration, and Invasion but Induces Apoptosis in HCC Cells

To explore the potential biological function of SNHG5 in the progression of HCC cells, Hep3B and Huh7 cells were transfected with si-SNHG5 or si-NC. Then, the transfection efficiency was detected by qRT-PCR with the results of decreased SNHG5 expression in the cells transfected with si-SNHG5 (Figure 2A). Subsequently, MTT assay indicated that the knockdown of SNHG5 repressed the proliferation in Hep3B and Huh7 cells (Figure 2B, 2C). Then, flow cytometric analysis showed that the rate of apoptotic cells was significantly increased when silencing SNHG5 (Figure 2D, 2E). Moreover, we also found that SNHG5 deletion suppressed the migration and invasion abilities in HCC cells (Figure 2F, 2G). Thus, we identified that SNHG5 could regulate tumorigenesis by promoting proliferation, migration, and invasion but inhibiting apoptosis in HCC cells.

RNF38 is Elevated in HCC Tissues and Cell Lines and Downregulated RNF38 Inhibits the Progression of HCC Cells

To investigate the biological roles of RNF38 in HCC cell progression, we firstly detected the expression of RNF38 in HCC tissues and cells using qRT-PCR or Western blot. The results showed that compared with matched adjacent non-tumor tissues and normal cells, RNF38 was markedly upregulated in HCC tissues and cell lines (Figure 3A-3C). Next, cell proliferation, migration, and invasion, as well as apoptosis abilities, were analyzed after introducing specific siRNA against SNHG5 gene transcript into Hep3B and Huh7 cells. Then, an inhibition in the proliferation (Figure 3D, 3E), migration, and invasion (Figure 3H, 3I) abilities and a facilitation in the apoptosis rate (Figure 3F, 3G) induced by RNF38 deletion were observed in Hep3B and Huh7 cells. To sum up, RNF38 could promote cells progression by promoting the proliferation, migration, and invasion by inhibiting apoptosis in HCC cells.

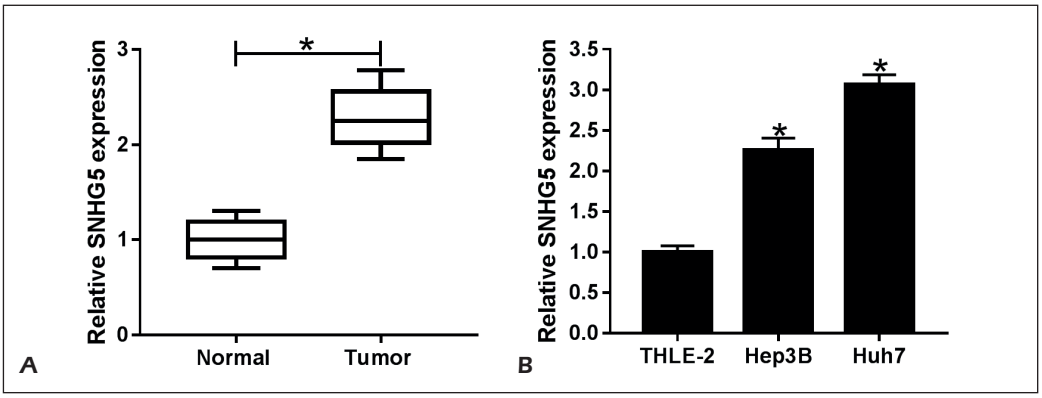

Figure 1. SNHG5 is upregulated in HCC tissues and cell lines. **A**-**B**, The expression of SNHG5 was detected in HCC and matched adjacent non-tumor tissues (**A**), as well as in the human immortalized hepatic cell line THLE-2 and human HCC cell lines (**B**), including Hep3B and Huh7 cells, using qRT-PCR. **p*<0.05.

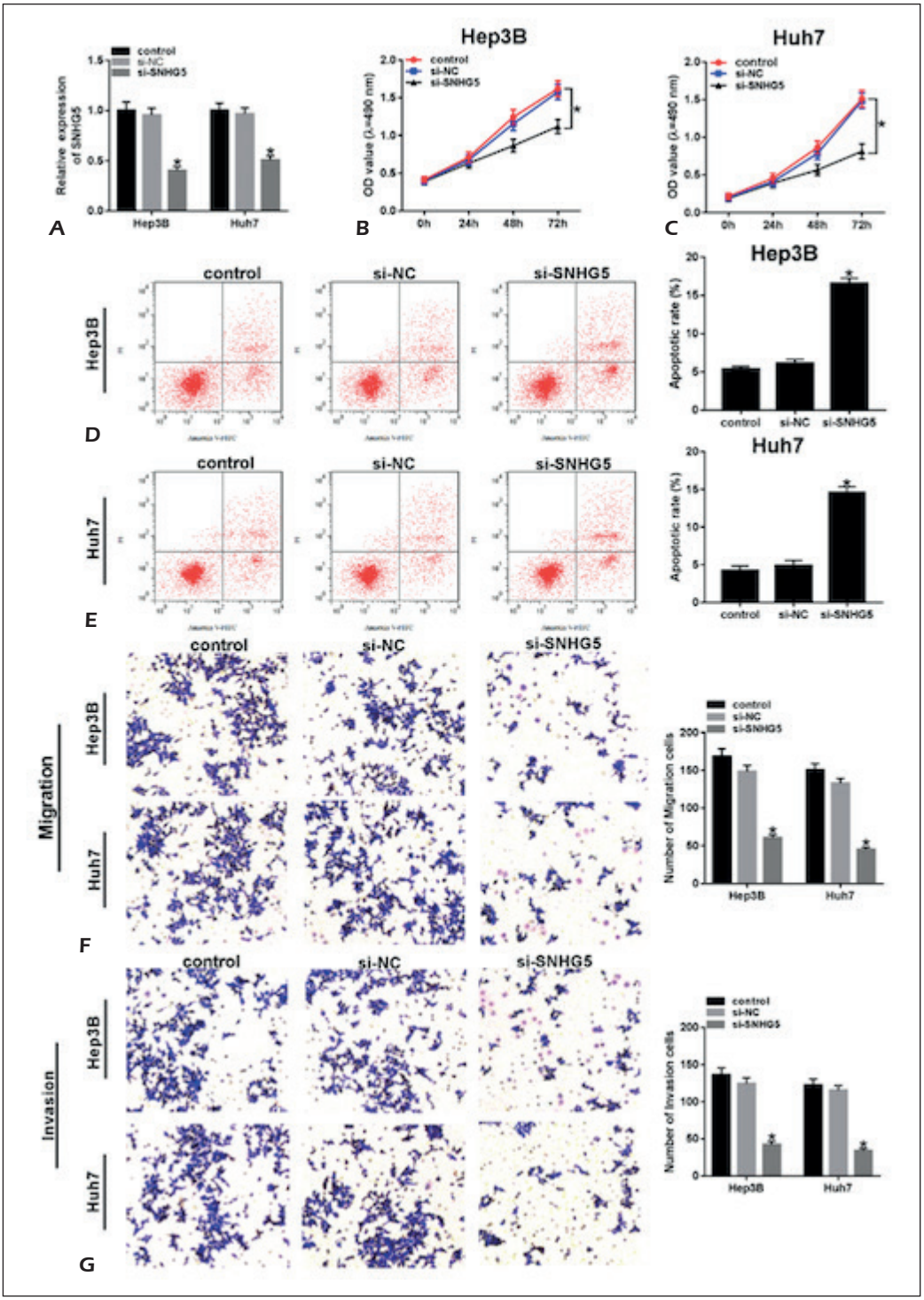

Figure 2. SNHG5 silence inhibits the proliferation, migration, and invasion and induces apoptosis in HCC. **A**, The expression of SNHG5 was measured by qRT-PCR in Hep3B and Huh7 cells transfected with si-SNHG5 or si-NC. **B-C**, Cell proliferation ability was detected by MTT assay in Hep3B and Huh7 cells. **D-E**, Cells apoptosis rate was analyzed using flow cytometry in Hep3B and Huh7 cells. **F-G**, The transwell assay was used to determine the migration and invasion abilities of Hep3B and Huh7 cells (magnification: x100). *p<0.05.

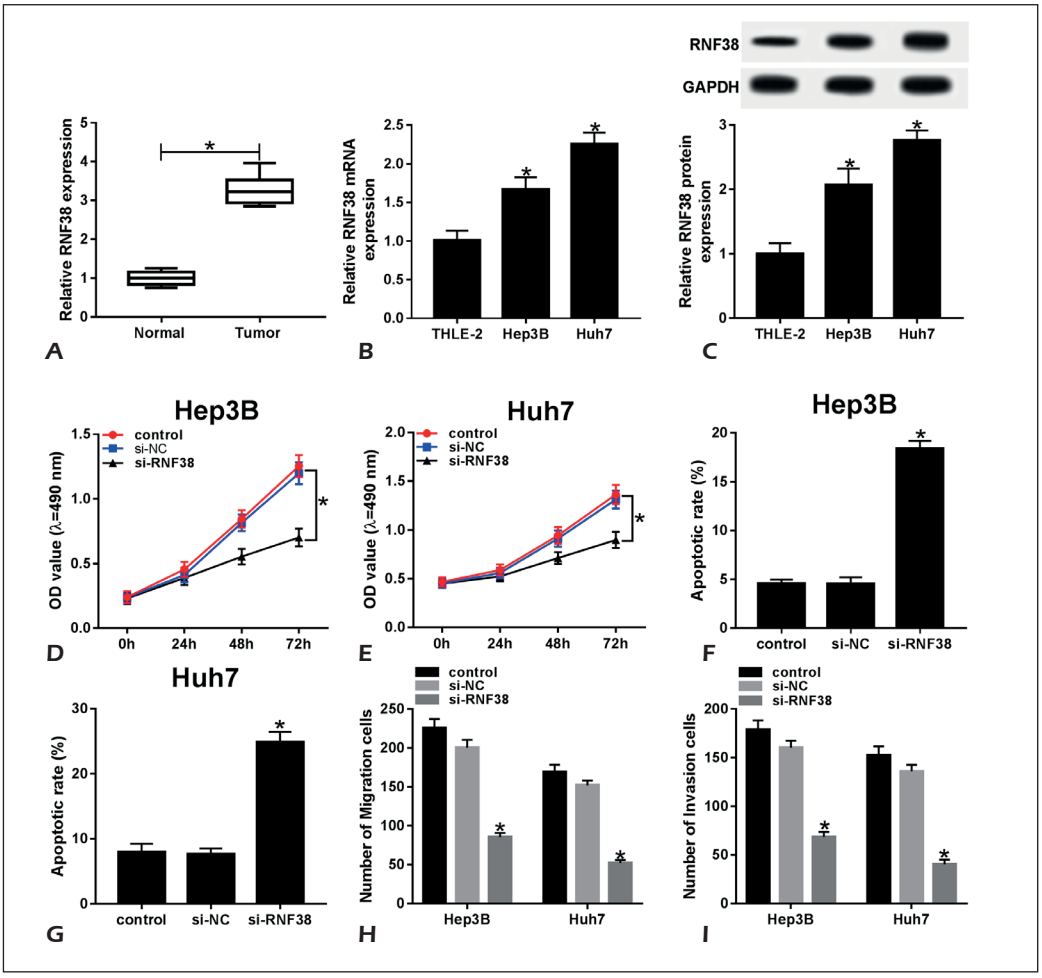

Figure 3. RNF38 is elevated and downregulated RNF38 inhibits the progression of HCC. **A**-**B**, The expression of RNF38 was detected in HCC and matched adjacent non-tumor tissues (**A**), as well as in THLE-2, Hep3B and Huh7 cells, (**B**) using qRT-PCR. **C**, The protein level of RNF38 was measured by Western blot in THLE-2, Hep3B, and Huh7 cells. **D**-**E**, Cell proliferation ability was detected by MTT assay in Hep3B and Huh7 cells transfected with si-RNF38 or si-NC. **F**-**G**, Cell apoptosis rate was analyzed using flow cytometry in Hep3B and Huh7 cells transfected with si-RNF38 or si-NC. **H**-**I**, The transwell assay was used to determine the migration and invasion abilities of Hep3B and Huh7 cells transfected with si-RNF38 or si-NC. *p<0.05.

SNHG5 is a Sponge of MiR-363-3p and Negatively Regulates its Expression

To further explore the underlying molecular mechanism of SNHG5 in HCC progression, the potential targets of SNHG5 were predicated using StarBase prediction program and SNHG5 contained the wild-type binding sequence of miR-363-3p (Figure 4A). To verify the prediction, the Luciferase reporter assay was performed, and the relative Luciferase activity of the SNHG5-WT reporter vector was dramatically inhibited by overexpressed miR-363-3p, whereas no significance was observed in SNHG5-MUT reporter vector co-transfection groups in Hep3B and Huh7 cells (Figure 4B, 4C). In addition, miR-363-3p expression was significantly inhibited by SNHG5 pcDNA transfection and enhanced by downregulated SNHG5 in Hep3B and Huh7 cells (Figure 4D). Furthermore, a perfect negative correlation between SNHG5 and miR-363-3p expression was determined in HCC (Figure 4E). These results confirmed that SNHG5 directly bound to miR-363-3p and targetedly suppressed its expression.

SNHG5 Silence Inhibits the Progression of HCC Cells by Regulating MiR-363-3p

To investigate whether miR-363-3p involved in the SNHG5 deletion-medicated suppression on HCC cells development, Hep3B and Huh7 cells were transfected with si-NC, si-SNHG5, si-SNHG5 + anti-miR-NC or si-SNHG5 + anti-miR-363-3p and the expression of miR-363-3p was found to be

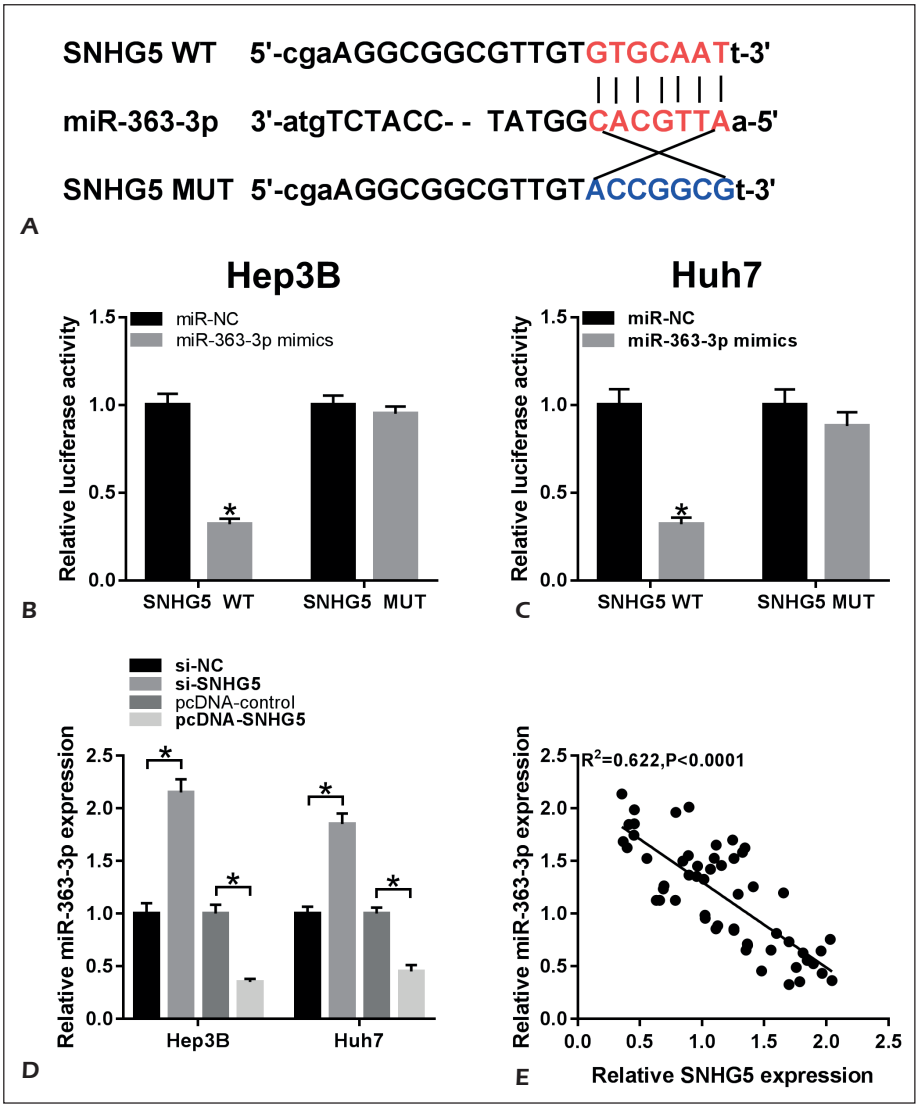

Figure 4. SNHG5 is a sponge of miR-363-3p. **A**, The putative binding site between SNHG5 and miR-363-3p was listed. **B-C**, The Luciferase activity was analyzed in Hep3B and Huh7 cells co-transfected with SNHG5-WT or SNHG5-MUT and miR-NC, miR-363-3p. **D**, The expression of miR-363-3p was determined using qRT-PCR in Hep3B and Huh7 cells transfected with si- SNHG5 or SNHG5 pcDNA. **E**, The correlation between SNHG5 and miR-363-3p was analyzed by Pearson's rank test in HCC tissues. *p<0.05.

decreased in si-SNHG5 transfection groups which could be restored by miR-363-3p inhibitor transfection (Figure 5A, 5B). Afterwards, cell proliferation, migration, and invasion, as well as apoptosis abilities in HCC were determined. The results showed that miR-363-3p inhibitor transfection could attenuate SNHG5 deletion-medicated suppression on the proliferation (Figure 5C, 5D), migration, and invasion (Figure 5G, 5H) abilities and the promotion on apoptosis (Figure 5E, 5F) abilities in Hep3B and Huh7 cells. Therefore, we confirmed that SNHG5 could promote HCC development *in vitro* by regulating miR-363-3p expression.

RNF38 is a Target of MiR-363-3p

According to the prediction of the TargetScan software, RNF38 was predicted to be a target of miR-363-3p with putative binding sites (Figure 6A). Then, the Luciferase reporter assay was performed to verify this prediction and the results indicated that miR-363-3p mimic transfection reduced the Luciferase activities of the RNF38-WT reporter vector but not RNF38-MUT reporter vector in Hep3B and Huh7 cells (Figure 6B, 6C). In the meanwhile, we discovered that miR-363-3p mimic transfection inhibited the expression of RNF38, while miR-363-3p inhibitor transfection promoted RNF38 expression, whether mRNA or protein, in Hep3B and Huh7 cells (Figure 6D-6F). Besides that, a negative correlation between RNF38 and miR-363-3p expression in HCC was discovered (Figure 6G). All the data suggested that miR-363-3p targetedly suppressed RNF38 expression.

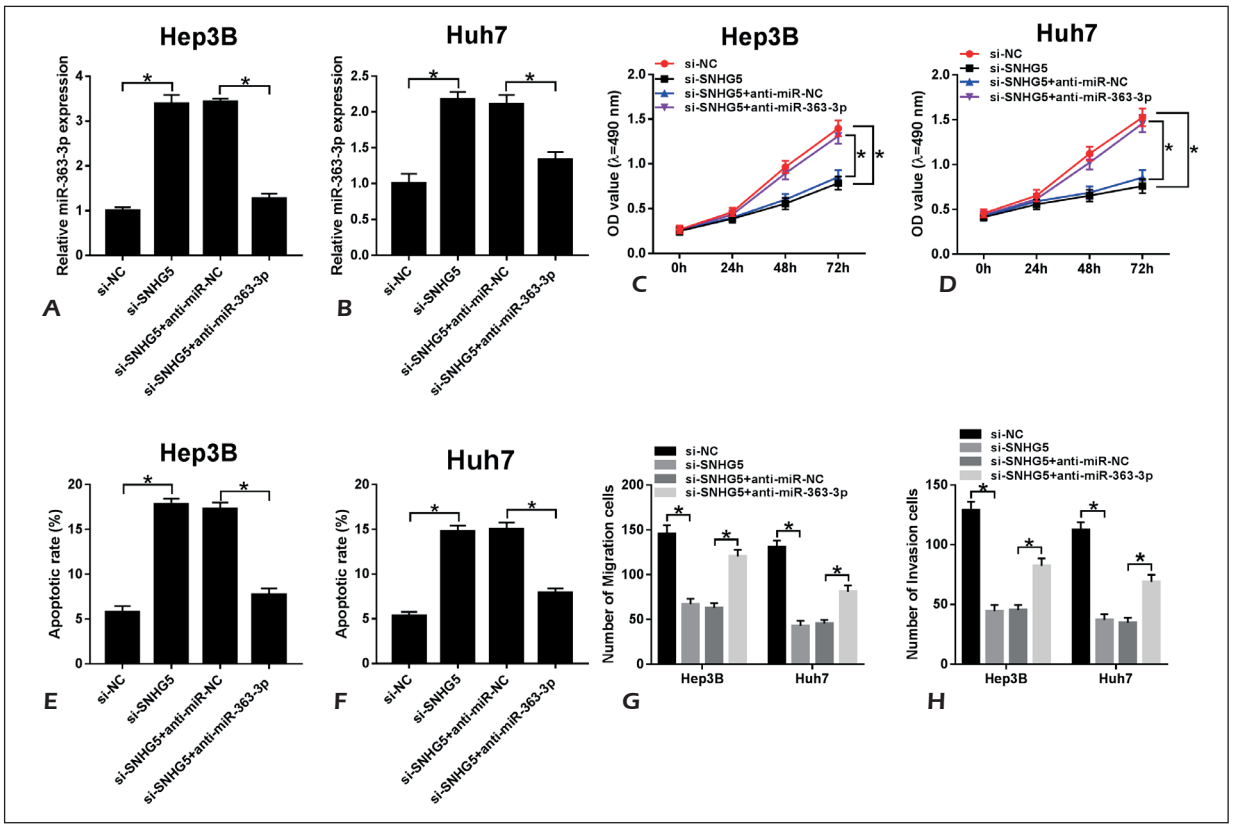

Figure 5. SNHG5 silence inhibits the progression of HCC cells by regulating miR-363-3p. **A-B**, The expression level of miR-363-3p was examined using qRT-PCR in Hep3B and Huh7 cells transfected with si-NC, si-SNHG5, si-SNHG5 + anti-miR-NCor si-SNHG5 + anti-miR-363-3p, respectively. **C-D**, Cell proliferation ability was detected by MTT assay in Hep3B and Huh7 cells. **E-F**, Cell apoptosis rate was analyzed using flow cytometry in Hep3B and Huh7 cells. **G-H**, The transwell assay was used to detect the migration and invasion abilities of Hep3B and Huh7 cells. *p<0.05.

The Facilitating Effects of RNF38 on the Progression of HCC Cells are Regulated by MiR-363-3p

To identify whether miR-363-3p associated with the effects of RNF38 on HCC cells development, Hep3B, and Huh7 cells were transfected with miR-NC, miR-363-3p, miR-363-3p + pcDNA or miR-363-3p + RNF38 and the expression levels of RNF38 were detected. The results showed that miR-363-3p mimic transfection inhibited RNF38 expression, which could be restored by RNF38 pcDNA transfection (Figure 7A-7D). Subsequently, rescue experiment was performed and the results showed that miR-363-3p repressed cell proliferation, migration, and invasion, but induced apoptosis abilities, which could be reversed by overexpressed RNF38 in Hep3B and Huh7 cells (Figure 7E-7J), suggesting that overexpressed miR-363-3p could inhibit the progression of HCC cells by regulating RNF38.

SNHG5 Regulates RNF38 Expression by Acting as a CeRNA of MiR-363-3p

Based on the above results, the regulatory relationship among SNHG5, RNF38, and miR-363-3p was investigated. Then, a positive correlation between SNHG5 and RNF38 expression was validated in HCC (Figure 8A). Furthermore, we determined that the expression of RNF38, whether mRNA or protein, was repressed by decreased SNHG5 but enhanced by miR-363-3p inhibitor in Hep3B and Huh7 cells (Figure 8B-8D), suggesting that SNHG5 could regulate RNF38 expression by targeting miR-363-3p.

Discussion

To date, many studies[24,25] suggests that dysfunction and alteration in lncRNAs lead to aberrant gene expression, thereby promoting tumor formation, progression, and metastasis in diverse

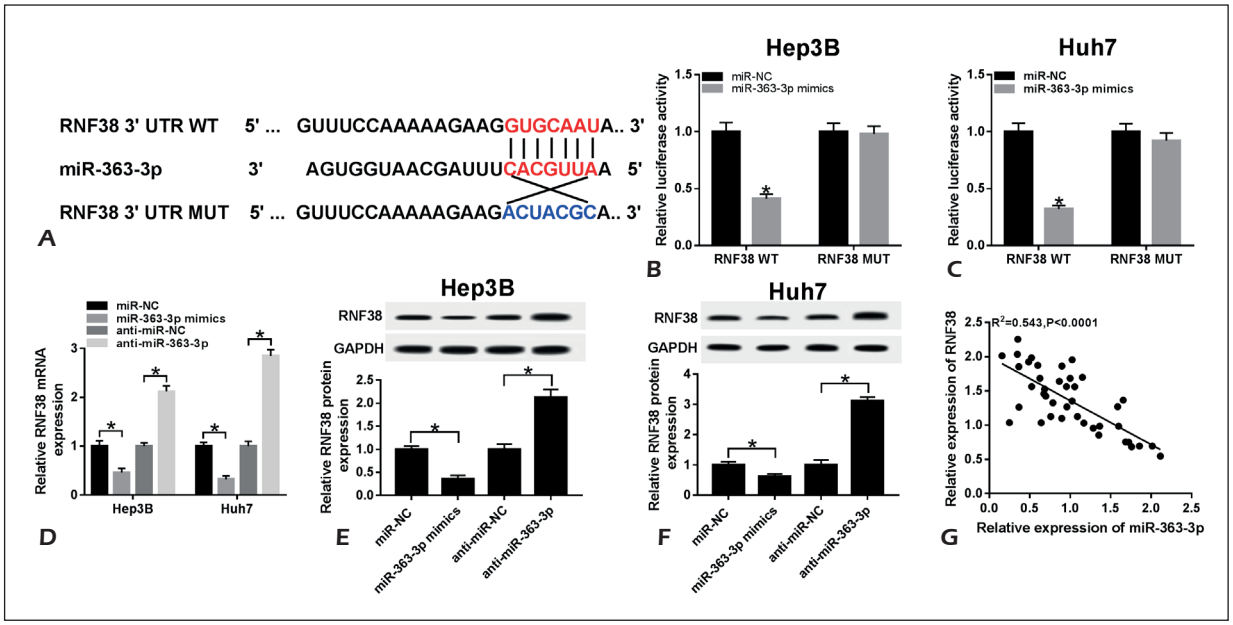

Figure 6. RNF38 is a target of miR-363-3p. **A**, The putative binding site between RNF38 and miR-363-3p was listed. **B-C**, The Luciferase activity was analyzed in Hep3B and Huh7 cells co-transfected with RNF38-WT or RNF38-MUT and miR-NC, miR-363-3p. **D**, The expression of RNF38 mRNA was determined using qRT-PCR in Hep3B and Huh7 cells transfected with anti-NC or anti-miR-363-3p. **E-F**, Western blot was used to demonstrate the protein expression of RNF38 in Hep3B and Huh7 cells transfected with anti-NC or anti-miR-363-3p. **G**, The correlation between RNF38 and miR-363-3p was analyzed by Pearson's rank test in HCC. *$p<0.05$.

cancer types. Some researchers have identified that some lncRNAs are often dysregulated in HCC, and the dysregulation of these lncRNAs is not only related to the clinicopathological features of HCC, but can regulate cell proliferation, apoptosis, migration, invasion autophagy, chemoresistance, and epithelial-mesenchymal transition (EMT) in HCC by modulating the target gene expression and cancer-related signaling pathways, thereby contributing to the onset and progression of HCC[26]. For example, lncRNA MIAT promotes proliferation and invasion of HCC cells *via* sponging miR-214[27]; LncRNA HANR promotes tumorigenesis and increases the chemoresistance in HCC[11]. SNHG5, a cancer-related lncRNA, has been investigated to be associated with the progression of several cancers by functioning as a tumor suppressor or oncogene. However, the role and exact regulatory mechanism of SNHG5 in the development of HCC remain pessimistic. In this study, we demonstrated that the expression of SNHG5 was significantly elevated in HCC tissues and cell lines compared with the non-tumor tissues and normal cell line, suggesting the potential regulatory role of SNHG5 in HCC. Then, to further explore the potential mechanism, the loss-of-function assay was performed using specific siRNA against SNHG5 gene transcript to investigate the function of SNHG5 in HCC cells. The results showed SNHG5 could regulate tumorigenesis by promoting the proliferation, migration, and invasion by inhibiting apoptosis in HCC cells. Thus, our findings indicated that SNHG5 acted as an oncogene to promote HCC progression *in vitro*.

The mechanism of ceRNAs has been proposed as a specific regulatory pathway of lncRNAs to affect the protein levels. The lncRNA-miRNA-mRNA axis plays an important role in the biological process of HCC[28]. In this study, the underlying regulatory mechanism of SNHG5 in the development of HCC was further investigated. According to the bioinformatics analysis prediction and Luciferase reporter assay confirmation, SNHG5 was a sponge of miR-363-3p and miR-363-3p directly targeted RNF38.

MiR-363-3p could destabilize c-Myc (Myc), which plays an important role in normal liver development and tumorigenesis by directly targeting and inhibiting ubiquitin-specific protease, thereby inhibiting G1 to S phase progression in hepatocarcinogenesis[29]. Yang et al[30] demonstrated that miR-363-3p could reverse sperm-associated antigen 5

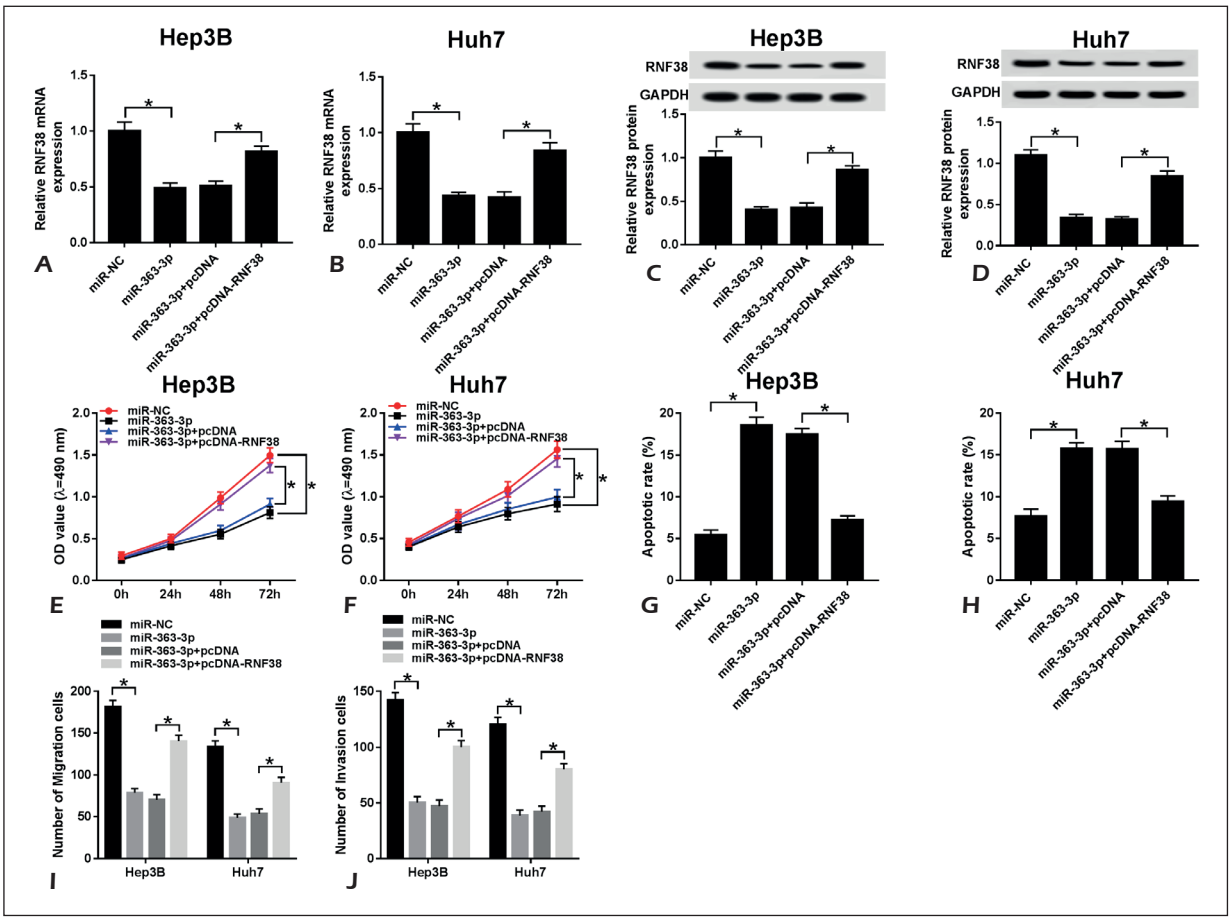

Figure 7. The facilitated effects of RNF38 on the progression of HCC cells are regulated by miR-363-3p. **A-B**, The levels of RNF38 mRNA were determined by qRT-PCR in Hep3B and Huh7 cells transfected with miR-NC, miR-363-3p, miR-363-3p + pcDNA or miR-363-3p + RNF38. **C-D**, The protein level of RNF38 was examined using Western blot in Hep3B and Huh7 cells after transfection. **E-F**, Cell proliferation ability was detected by MTT assay in Hep3B and Huh7 cells. **G-H**, Cell apoptosis rate was analyzed using flow cytometry in Hep3B and Huh7 cells. **I-J**, The transwell assay was used to detect the migration and invasion abilities of Hep3B and Huh7 cells. *$p<0.05$.

(SPAG5) which exhibited pro-HCC activities. All the reports indicated the tumor inhibition of miR-363-3p in HCC progression. In the current study, we also identified that miR-363-3p suppressed the progression of HCC by repressing cell proliferation, migration, and invasion and induced apoptosis, which was consistent with the previous observation. Besides that, a negative correlation between miR-363-3p and SNHG5 was investigated and rescue assay showed that SNHG5 could exert its promotional effects by regulating miR-363-3p.

RING finger proteins are implicated in various cellular processes, including cell cycle, DNA repair, apoptosis, signal transduction, tumor suppressor, vesicular transport, and peroxisomal biogenesis[31]. However, the functions of RNF38, a member of RNF family, remain unknown. Recently, RNF38 has been observed associated with the oncogenesis in several cancers. Sheren et al[32] found that RNF38 was a binding partner of p53, a tumor suppressor gene, and could ubiquitinate p53 *in vitro* and *in vivo*. Peng et al[33] reported that RNF38 induced gastric cancer cell growth by decreasing the stability of the protein tyrosine phosphatase SHP-1. In addition, overexpressed RNF38 facilitated TGF-β signaling by ubiquitinating and degrading AHNAK to promote HCC cell development[33]. In this study, RNF38 was found to be elevated in HCC tissues and cells and functional assay indicated that RNF38 could promote cell progression by promoting the proliferation, migration, and invasion and inhibiting apoptosis in HCC cells. Afterwards, a negative correlation between RNF38 and miR-363-3p expression in HCC was discovered and we demonstrated that the facilitated effects of RNF38 on

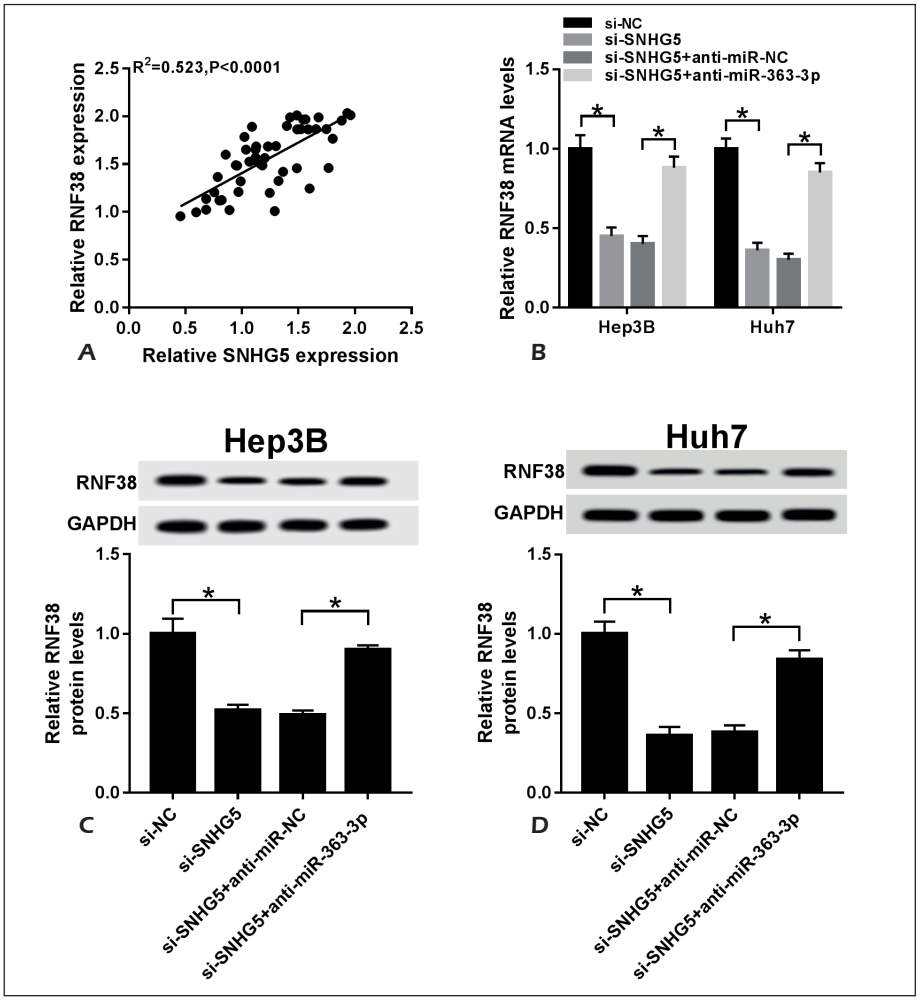

Figure 8. SNHG5 regulates RNF38 expression by acting as a ceRNA of miR-363-3p. **A**, The correlation between RNF38 and SNHG5 was analyzed by the Pearson's rank test in HCC. **B**, The RNF38 mRNA was analyzed by qRT-PCR in Hep3B and Huh7 cells transfected with si-NC, si-SNHG5, si-SNHG5 + anti-miR-NC or si-SNHG5 + anti-miR-363-3p. **C-D**, The RNF38 protein was measured by using Western blot in transfected Hep3B and Huh7 cells. *$p<0.05$.

the progression of HCC cells could be regulated by miR-363-3p. In the meanwhile, a positive correlation between RNF38 and SNHG5 was also confirmed; besides that, the expression analysis results also suggested that SNHG5 might act as a competing endogenous RNA of miR-26a-5p, thereby regulating the derepression of the downstream target RNF38.

Conclusions

Our study validated that SNHG5 was a novel oncogene, which promotes the tumorigenesis and progression of HCC by acting as a ceRNA of miR-26a-5p and regulating the expression of RNF38. The SNHG5/miR-363-3p/RNF38 axis in the development of HCC was firstly identified, providing a novel insight in the pathogenesis of HCC and therapeutic strategy on the treatment of HCC.

Ethics Approval and Consent to Participate

This study was supported by the Ethics Committee of the Sixth People's Hospital of Qingdao. The methods used in this study were performed in accordance with relevant guidelines and regulations.

Availability of Data and Materials

All original data and materials are available from the corresponding author upon request.

Conflict of Interests

The authors declare that they have no conflict of interests.

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