doi: 10.4149/gpb_2024042

Small nucleolar RNA 42 facilitates the progression of hepatocellular carcinoma through PI3K/Akt signaling pathway

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Abstracts. Small nucleolar RNAs may serve as new potential biomarkers for the diagnosis and treatment of liver cancer. The purpose of our study was to reveal the mechanism small nucleolar RNA 42 (*SNORA42*) affects the proliferation and migration of liver cancer cells. Quantitative real-time PCR (qRT-PCR) was performed to detect the expression of *SNORA42* and its host gene. Cell proliferation and migration were measured using the CCK-8 and Transwell assays, respectively. Western blotting was performed to measure the expression of the proteins affected by *SNORA42*. *SNORA42* overexpression could reinforce the proliferation of hepatocellular carcinoma (HCC) cells, and promote the migration of hepatocellular carcinoma cells. In addition, *SNORA42* did not affect the expression of host genes *KIAA0907*. *SNORA42* is one of the most important components of the PI3K/Akt signaling pathway. *SNORA42* augmented phospho-Akt expression, which was reversed by PI3K and Akt inhibitors. Our study displayed that *SNORA42* may affect the proliferation and migration of HCC cells by interfering with the PI3K/Akt signaling pathway. Thus, *SNORA42* may be a new target for detecting or treating HCC.

Key words: Small nucleolar RNA 42 — Hepatocellular carcinoma — Metastasis — PI3K/Akt signaling pathway

Abbreviations: EMT, epithelial-to-mesenchymal transition; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR; siRNA, small interfering RNA; SNORA42, small nucleolar RNA 42.

Introduction

Liver cancer ranks seventh place of the majority of common cancer and leads to the second-leading cause of cancer-related mortality worldwide (Sung et al. 2021). Hepatocellular carcinoma (HCC) has a high mortality rate and constitutes approximately 80–90% of all primary liver cancer (Udoh et al. 2021). HCC is a kind of disease with different modalities of treatment (Ezzat et al. 2021). Surgical intervention is the most important therapy for HCC (Heimbach et al. 2018). However, this is not possible in all cases, for example, without the option for surgery is limited for the patients. Most patients are diagnosed at a late stage for the first time, indicating that radical surgery is not useful. Therefore, novel biomarkers are urgently required for early diagnosis and treatment of liver cancer.

Small nucleolar RNAs (snoRNAs) are a set of non-coding RNAs containing 60–300 nucleotides located in the nucleolus (Williams and Farzaneh 2012). According to their biological functions, two types of snoRNAs are classified. C/D box snoRNAs (SNORDs) play key roles in 2′-O-ribose methylation, while H/ACA box snoRNAs (SNORAs) play critical roles in pseudo-uridylation (Kiss et al. 2004; Dieci et al. 2009). *SNO-*

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Electronic Supplementary material. The online version of this article (doi: 10.4149/gpb_2024042) contains Supplementary material.

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RA42 belongs to H/ACA box snoRNA and is derived from *KIAA0907* (Mei et al. 2012). Recent studies have demonstrated that the abnormal expression of *SNORA42* is highly associated with tumorigenesis, progression, and prognosis (Barbosa et al. 2023). *SNORA42* plays an oncogenic role in non-small-cell lung cancer (NSCLC) through the p53-dependent manner (Mei et al. 2012). It has reported that *SNORA42* may be a promising biomarker in colorectal cancer (CRC) due to its oncogenic role (Okugawa et al. 2017). Another study revealed that increased expression of *SNORA42* is correlated to prostate cancer metastasis (Yi et al. 2018). *SNORA42* also promotes the progression of oesophageal squamous carcinoma cells through the DHX9/p65 pathway (Shan et al. 2021). Importantly, *SNORA42* serves as an oncogene in HCC *via* p53 signaling pathway (Wang et al. 2021). However, specific mechanism underlying SNORA42 promoting tumorigenic actions in HCC still needs further investigation.

In this study, we found that *SNORA42* played a vital role in the proliferation and migration of HCC cells. However, the overexpression or knockdown of *SNORA42* did not significantly alter the expression of its host gene *KIAA0907*. Subsequently, we verified that the PI3K/Akt signaling pathway was involved in *SNORA42*-mediated HCC progression.

Material and Methods

Cells and reagents

THLE-2, Huh7, MHCC97H, and PLC/PRF/5 cell lines were purchased from the cell collection of Chinese Academy of Sciences (Shanghai, China). Huh7, MHCC97H, and PLC/PRF/5 cell lines were cultured in high glucose DMEM (Hyclone, USA) containing 10% of fetal bovine serum (FBS) (Gibco, USA) and penicillin-streptomycin (100 U/ml, Hyclone, USA). THLE-2 was cultured using a special medium (Lonza, CC-3170) with an additional 5 ng/ml EGF, 70 ng/ml phosphoethanolamine and10% FBS. All cell lines incubated in the incubator at 37°C with 5% of CO_2.

Animals

Five-week-old BALB/c nude mice were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and were maintained at Shanghai Jiaotong University. Mice were randomly divided into four groups (n = 5 *per* group). Stable *SNORA42* overexpression cells, *SNORA42* knockdown MHCC97H cells and control cells (1×10^6 cells *per* mouse) were injected subcutaneously into BALB/c nude mice. Tumor volumes were measured every 3 days using Vernier calipers according the formula V (mm^3) = ($L\times W^2$)/2, where V is the volume, L means long dimensions, W means shorter dimensions. The maximum size of the tumor did not exceed 650 mm^3. Mice were euthanized with a CO_2 replacement rate of 30% of the cage volume *per* min (5 l/min), according to the AVMA Guidelines for Euthanasia. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiaotong University and Shanghai Fifth People's Hospital, affiliated with Fudan University.

Cell transfection assay

Small interfering RNA (siRNA) targeting *SNORA42* was synthesized by Genpharma (Shanghai, China) and the nucleotide sequence was 5′-GTACCCATGCCATAGCAAA-3'. Transient transfection was conducted according to Lipofectamine 2000 when the cell density reached 50–70%.

Quantitative real-time PCR (qRT-PCR)

TRIzol reagent (Invitrogen) was used according to the manufacturer's protocol to extract total RNAs from the cell lines. Then, inverse transcription was performed according to the instructions of the PrimeScript™ RT reagent Kit (Takara), and the qRT-PCR system was established according to the protocol of SYBR Green PCR Master Mix (Takara) in an Applied Biosystems QuantStudio 5. GAPDH was used as an internal reference gene to assess the relative expression of the genes using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used were *SNORA42* forward: 5′-TGGATTTATGGTGGGTCCTTCTCTG-3′ and reverse: 5′-CAGGTAAGGGGACTGGGCAATGGTT-3′; KIAA0907 forward: 5′- CCCTACGGAGTACCAAGCATAG-3′ and reverse: 5′-CAGGAGCAGCAGGAATAAAAGGA-3′; GAPDH forward: 5′-AGCCACATCGCTCAGACAC-3′ and reverse: 5′-GCCCAATACGACCAAATCC-3.'

Cell viability assay

One thousand cells transferred with *SNORA42* or si*SNORA42* were seeded into 96-well plates for detecting cell viability after seeding for 1, 2, 3, and 4 days, respectively, 10 μl CCK-8 (Sharebio, China) was added to the fresh medium in the dark. The plates were then incubated for 2 h to detecting OD450 nm absorbance a microplate reader (Tecan, Switzerland).

Transwell assay

For migration, the transferred cells were seeded in medium without FBS onto an 8 μm pore size Transwell upper chamber (BD Biosciences). Medium containing 10% FBS was added to the chamber. After culturing for 48 h, the chambers were washed three times and then, fixed in methanol for 30 min,

and stained with crystal violet solution for 30 min. Invading cells were captured at a magnification of 200× and the mean was counted.

Western blotting

Whole cell lysates samples were heated for 5 min at 100°C and subjected to SDS-PAGE. The proteins were transferred onto PVDF membranes (Millipore) and the PVDF membranes containing proteins were blocked with 5% w/v non-fat dry milk and incubated with primary antibodies at 4°C for 12 h. The primary antibodies used were Akt, p-Akt, E-cadherin, N-cadherin, Snail (Cell Signaling Technology, USA), p110α, p85α (Proteintech, China), and β-actin (Santa Cruz Biotechnology). Secondary antibodies (Cell Signaling Technology, USA) were incubated for 1 h at room temperature. PVDF membranes were detected using enhanced chemiluminescence (ECL).

Histopathology analysis and immunohistochemistry

Histopathology analysis and immunohistochemistry were performed as previously described (Liu et al. 2020).

Statistical analysis

All data were shown as mean ± SD. Differences between two groups were analyzed using the Student's *t*-test. Statistical significance was set at $p < 0.05$.

Results

SNORA42 expresses independently of its host gene

To corroborate the role of *SNORA42* in liver cancer, the expression level of *SNORA42* in liver cancer cells was first detected, and PLC/PRF/5 and MHCC97H cell lines were chosen for use in our study (Fig. 1A). The host gene of *SNORA42* was *KIAA0907*. qRT-PCR showed *KIAA0907* expression in hepatocytes THLE-2, HCC cell lines Huh-7, PLC/PPRF/5 and MHCC97H were comparable (Fig. S1 in Supplementary material). *SNORA42* in HCC cells, overexpressed or knockdown of *SNORA42* were performed by *SNORA42* or *SNORA42* siRNA. *SNORA42* was significantly upregulated or downregulated in PLC/PRF/5 and MHCC97H cells, respectively (Fig. 1B). We were not sure

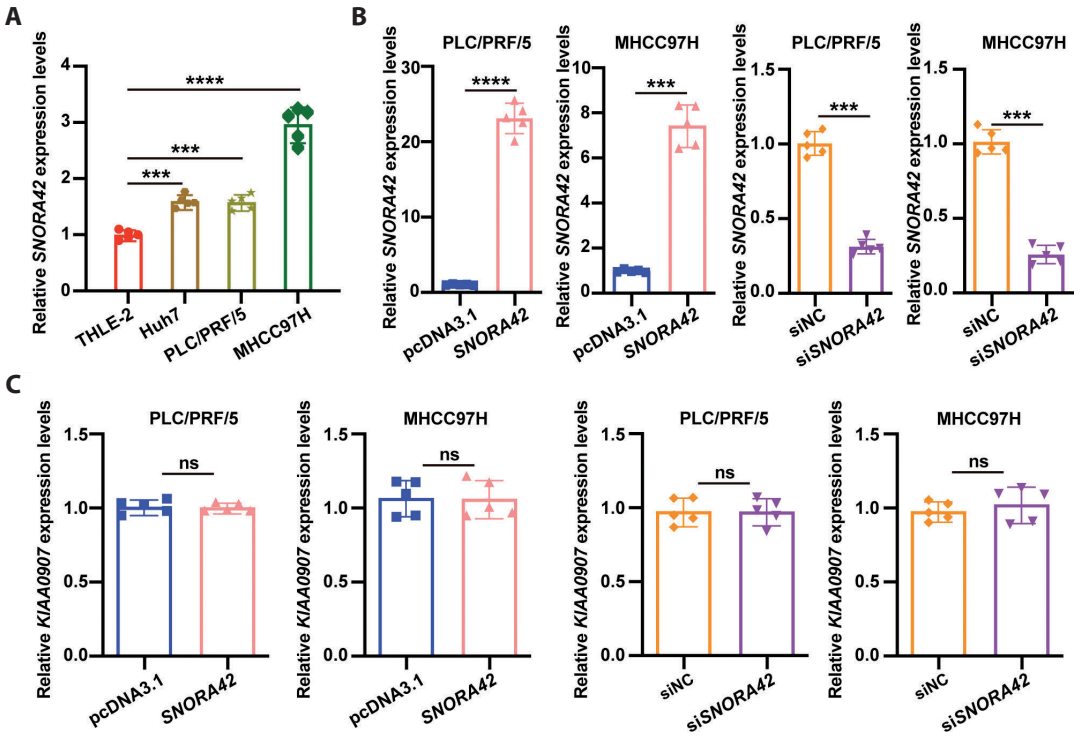

Figure 1. *SNORA42* does not regulate the expression of its host gene. **A.** qRT-PCR of *SNORA42* expression in hepatocytes THLE-2, hepatoma cell lines Huh-7, PLC/PPRF/5 and MHCC97H. **B.** *SNORA42* overexpressed plasmid and siRNA were used to construct liver cancer cell lines with overexpression and knockdown *SNORA42* in PLC/PRF/5 and MHCC97H. **C.** Overexpression of *SNORA42* in PLC/PRF/5 and MHCC97H cells did not alter *KIAA0907* expression as measured by qRT-PCR. Knockdown of *SNORA42* did not affect *KIAA0907* expression either. *** $p < 0.001$, **** $p < 0.0001$; $n = 5$.

whether the overexpression or knockdown of *SNORA42* affected the expression of *KIAA0907*. qRT-PCR experiments showed that siRNA only reduced the expression of *SNORA42* and had no effect on its host gene *KIAA0907*. The same was true for *SNORA42* overexpression (Fig. 1C). These data indicate that *SNORA42* does not affect the expression of host genes.

SNORA42 enhances cell growth and inhibits cell apoptosis in HCC cells

The CCK-8 assay indicated that upregulation of *SNORA42* promoted the growth of PLC/PRF/5 and MHCC97H cells, whereas downregulation of *SNORA42* hindered the growth of PLC/PRF/5 and MHCC97H cells (Fig. 2A). Next, we explored whether *SNORA42* affected cell apoptosis. The over-

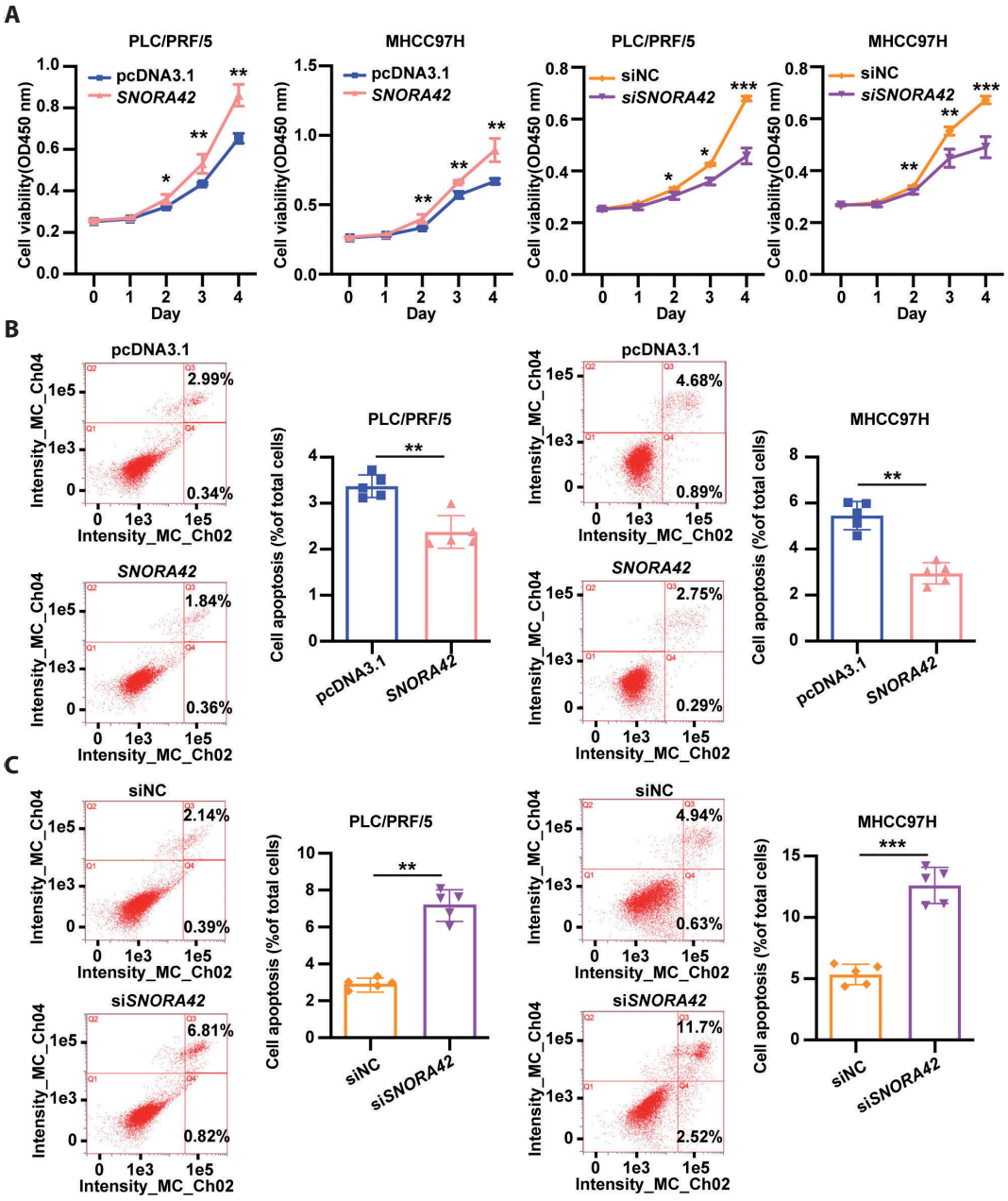

Figure 2. *SNORA42* promotes cell growth and inhibits cell apoptosis in HCC cells. **A.** The effect of *SNORA42* on hepatocellular carcinoma cell proliferation was detected by CCK8 assay. **B, C.** The effects of *SNORA42* on PLC/PRF/5 and MHCC97H apoptosis of liver cancer cells were detected by flow cytometry. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 5$.

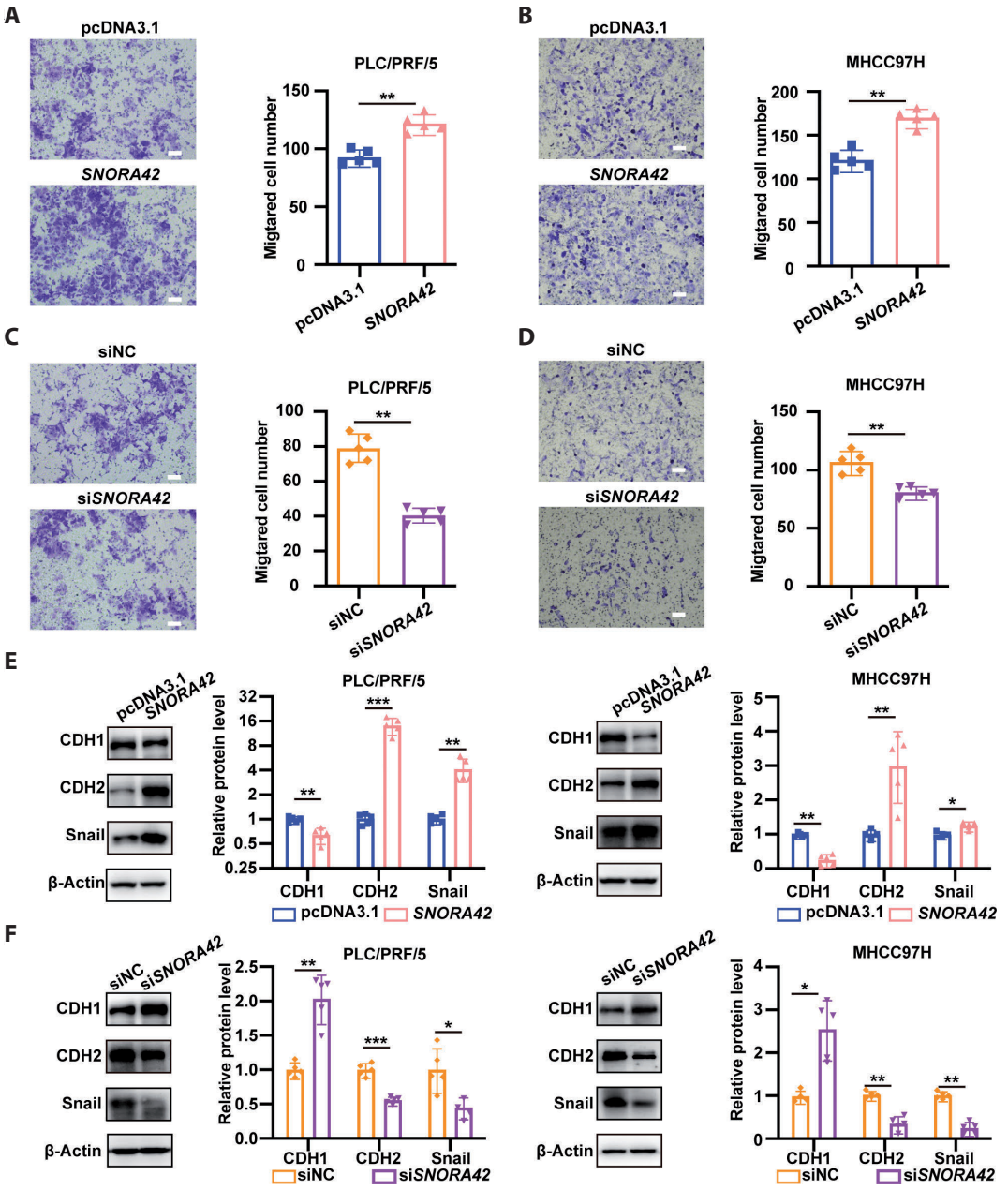

Figure 3. *SNORA42* promotes cells migration in HCC cells. **A, B.** Representative images of PLC/PRF/5 and MHCC97H migration in hepatocellular carcinoma cells by *SNORA42* overexpression. **C, D.** Representative images of PLC/PRF/5 and MHCC97H migration in hepatocellular carcinoma cells by *SNORA42* knockdown. And migration efficiency statistics. **E, F.** EMT-related proteins were determined by immunoblotting. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 5$.

expression of *SNORA42* diminished the percentage of early and late apoptosis in PLC/PRF/5 and MHCC97H cells (Fig. 2B). Consistently, early and late apoptosis were increased in PLC/PRF/5 and MHCC97H cells (Fig. 2C) transfected with *SNORA42* siRNA. Taken together, these results indicate that *SNORA42* promotes cell growth and inhibits apoptosis in HCC cells.

SNORA42 promotes cells migration in HCC cells

Metastasis is a vital biological process in HCC cancer cell adhesion, invasion and migration (Kummar and Shafi 2003). Therefore, we further studied the effect of *SNORA42* on HCC migration. *SNORA42* overexpression accelerated the migration of PLC/PRF/5 and MHCC97H cells (Fig. 3A,B).

Inhibition of *SNORA42* by siRNA reduced the migration of PLC/PRF/5 and MHCC97H cells (Fig. 3C,D). E-cadherin and N-cadherin are two important proteins that regulate epithelial-to-mesenchymal transition (EMT) during cancer metastatic progression (Lee et al. 2006). Snail is involved in EMT induction (Lin T et al. 2010; Lin Y et al. 2010). We then studied whether *SNORA42* regulated the expression of these proteins in PLC/PRF/5 and MHCC97H cells. *SNORA42* upregulation decreased E-cadherin protein levels and increased N-cadherin and Snail protein levels in PLC/PRF/5 and MHCC97H cells (Fig. 3E). Consistently, *SNORA42* downregulation had the opposite effect on the expression levels of these proteins in PLC/PRF/5 and MHCC97H cells (Fig. 3F). These data suggest that *SNORA42* enhances HCC cell migration.

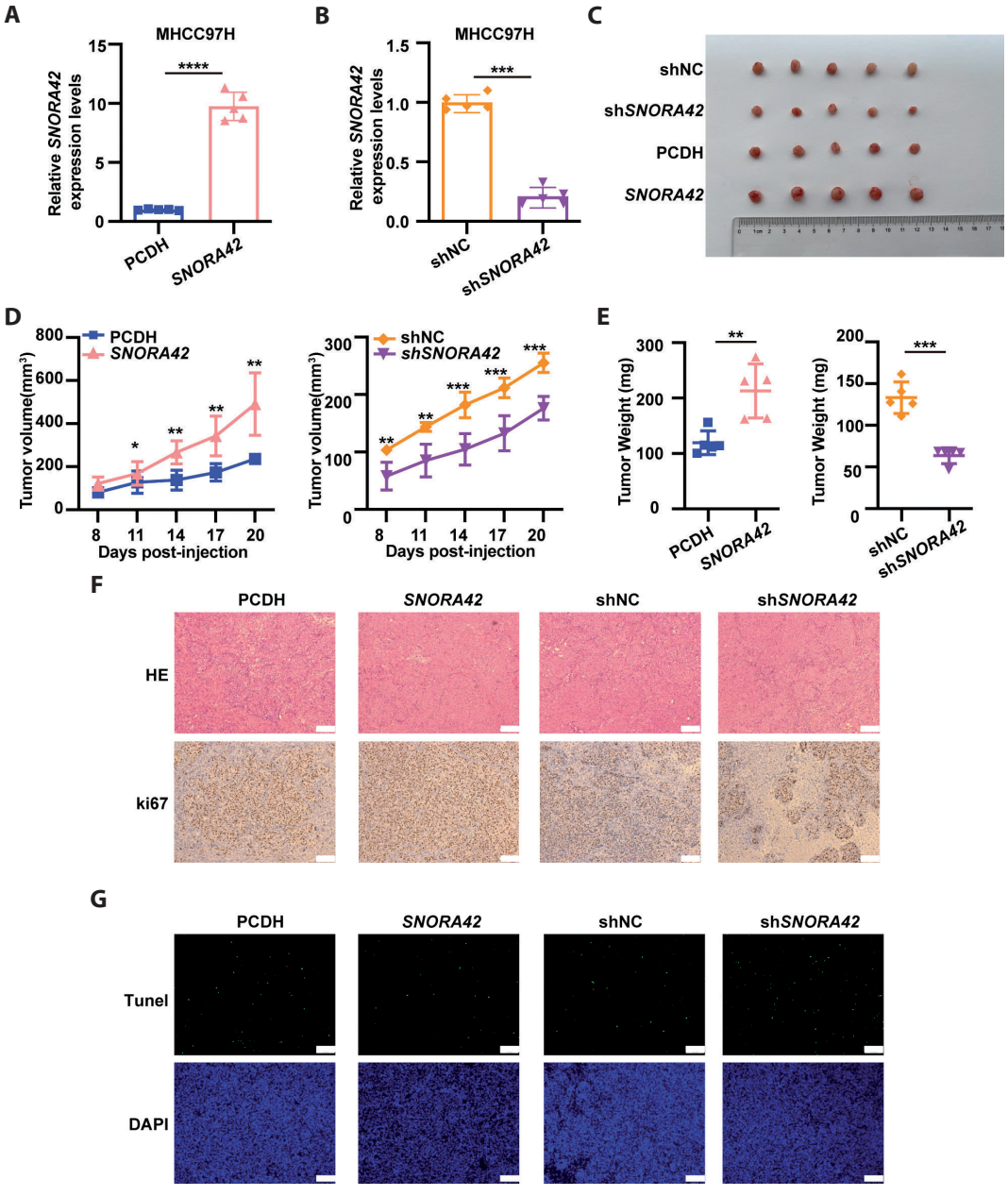

Figure 4. *SNORA42* promotes HCC xenograft growth *in vivo*. **A, B.** Construct stable *SNORA42* overexpression and *SNORA42* knockdown MHCC97 cells. **C.** A representative image of stable overexpression and knockdown MHCC97H in nude mice injected subcutaneously. **D.** Growth curve of subcutaneous graft tumor in nude mice. **E.** Statistical chart of weight of subcutaneous transplanted tumor in nude mice. **F, G.** Representative images dyed by HE, Ki67 and Tunel. Data were shown as the means ± SD with $n = 5$ *per* group, scale bar = 100 μm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; $n = 5$.

SNORA42 facilitates HCC xenograft growth in vivo

To further explore the oncogenic function of *SNORA42*, we established stable *SNORA42* overexpression and *SNORA42* knockdown MHCC97H cells (Fig. 4A,B). Nude mice were inoculated with control, *SNORA42* overexpression and *SNORA42* knockdown MHCC97H cells. Compared to control mice, mice received *SNORA42* knockdown MHCC97H cells showed lower tumor growth, which led to a smaller tumor size (Fig. 4C). Compared to control mice, *SNORA42* overexpression MHCC97H cells exhibited faster tumor growth, which resulted in a larger tumor volume (Fig. 4D). Notably, compared to the control mice, the tumor weight of mice received *SNORA42* knockdown MHCC97H cells was lower (Fig. 4E). Compared to the control mice, the tumor weight of mice received *SNORA42* overexpression MHCC97H cells was higher (Fig. 4E). Immunohistochemistry also indicated fewer Ki67-positive cells and more TUNEL-positive cells in tumors from mice received *SNORA42* knockdown MHCC97H cells than in tumors from control mice (Fig. 4F,G). Compared to tumors from control mice, more Ki67-positive cells and fewer TUNEL-positive cells were detected in tumors from mice received *SNORA42* overexpression MHCC97H cells (Fig. 4F,G). *In vivo* experiments further confirmed that *SNORA42* promoted the growth of liver cancer by regulating PI3K/AKT signaling pathway, and affected tumor metastasis by regulating EMT signaling pathway (Fig. S2 in Supplementary material). These results reveal that *SNORA42* acts as an oncogenic gene and promotes HCC xenograft growth *in vivo*.

SNORA42 accelerates the progression of HCC cells via regulating the PI3K/Akt pathway

The PI3K/Akt/mTOR pathway is an essential signal-exchanging pathway regulating the cell cycle, apoptosis, cell proliferation and angiogenesis (Manning and Cantley 2007). There are studies that show PI3K/Akt signaling pathway is involved in mediating the development and progression of HCC (Lai et al. 2021). Therefore, we further explored whether *SNORA42* could play the oncogenic role in HCC *via* PI3K/Akt signaling pathway. The results indicated that overexpression of *SNORA42* upregulated the protein levels of p-Akt and two subunits of its upstream PI3K (Fig. 5A). In addition, inhibition of *SNORA42* downregulated these proteins (Fig. 5B). To further validate our hypothesis, an inhibitor of PI3K was used in present experiment. Wortmannin, an inhibitor of PI3K, arrested the growth of *SNORA42*-overexpressing PLC/PRF/5 and MHCC97H cells (Fig. 5C), suggesting that PI3K participated in *SNORA42*-mediated cell growth. We also assessed the expression of p110α, AKT, and Snail proteins in PLC/PRF/5 cells using Western blotting. The results showed that wortmannin alleviated the expression of p110α, p-Akt, and Snail (Fig. 5D). Inactivation of Akt by the Akt inhibitor MK2206 in *SNORA42*-overexpressing PLC/PRF/5 and MHCC97H cells significantly suppressed cell growth (Fig. 5E). The Western blotting results indicated that MK2206 downregulated the expression of p-Akt, and Snail (Fig. 5F). These results indicate that the PI3K/Akt signaling pathway is involved in *SNORA42*-mediated hepatocellular carcinoma development.

Discussion

Previous studies on the pathogenesis of HCC have mainly focused on mutations in protein markers, and few studies have focused on non-coding RNAs, except miRNAs. The same is true in the treatment and development of HCC, most researchers focus on the mutation of protein markers for drug targets, while ignoring the importance of non-coding RNA in the diagnosis, treatment and prognosis of HCC. Alpha-fetoprotein (AFP) has long been used as a diagnostic marker for HCC, but AFP has not been significantly elevated in many patients with liver cancer. Small nucleolar RNA can be detected in the blood and is expected to be a new marker for the diagnosis of HCC (Liu et al. 2022).

SNORA42 acts as an oncogene in lung cancer (Liao et al. 2010), colorectal cancer (Okugawa et al. 2017), prostate cancer (Yi et al. 2018) and oesophageal squamous cell carcinoma (Shan et al. 2021), in which *SNORA42* is highly expressed. In our study, two HCC cell lines, MHCC97H and PLC/PRF/5, were used to validate the oncogenic role of *SNORA42*. Consistent with previous research (Wang et al. 2021), *SNORA42* overexpression promoted the proliferation and migration of MHCC97H and PLC/PRF/5 cells. In contrast, *SNORA42* knockdown by siRNA inhibited cell proliferation and migration. Furthermore, *SNORA42* did not affect the expression of the host genes *KIAA0907*. However, previous literature has not investigated whether it affects host genes (Wang et al. 2021). In addition, we conducted further validation on a subcutaneous transplanted tumor model in nude mice, and the results further demonstrated that *SNORA42* could promote the growth of subcutaneous transplanted tumors of liver cancer. The results were well confirmed by Ki67 staining and TUNEL assay.

However, the mechanism by which *SNORA42* affects the proliferation and migration of liver cancer cells remains unclear. Although one study has reported that SNORA42 may promote the progression of HCC by inhibiting p53 signaling pathway (Wang et al. 2021), but the evidence is insufficient, it is possible that *SNORA42* plays a significant role in promoting tumor proliferation by regulating other signaling pathways. Through the analysis of relevant data from public databases, this study found that liver cancer

patients with elevated PI3K and Akt have poor prognosis, which can play a role by affecting the proliferation, apoptosis and metastasis of liver cancer. The PI3K/Akt pathway is a crucial intracellular signal-exchange pathway that regulates metabolism, cell cycle, cell proliferation, apoptosis, and angiogenesis by exchanging message (Manning and Cantley 2007). Many studies have demonstrated that the PI3K/Akt signaling pathway plays a vital role in mediating the development and progression of HCC (Huang et al. 2018; Chen et al. 2019; Gong et al. 2019; Lai et al. 2021). Some studies have shown that small nucleolar RNAs promote the development of HCC and colorectal cancer (Fang et al. 2017; Wu et al.

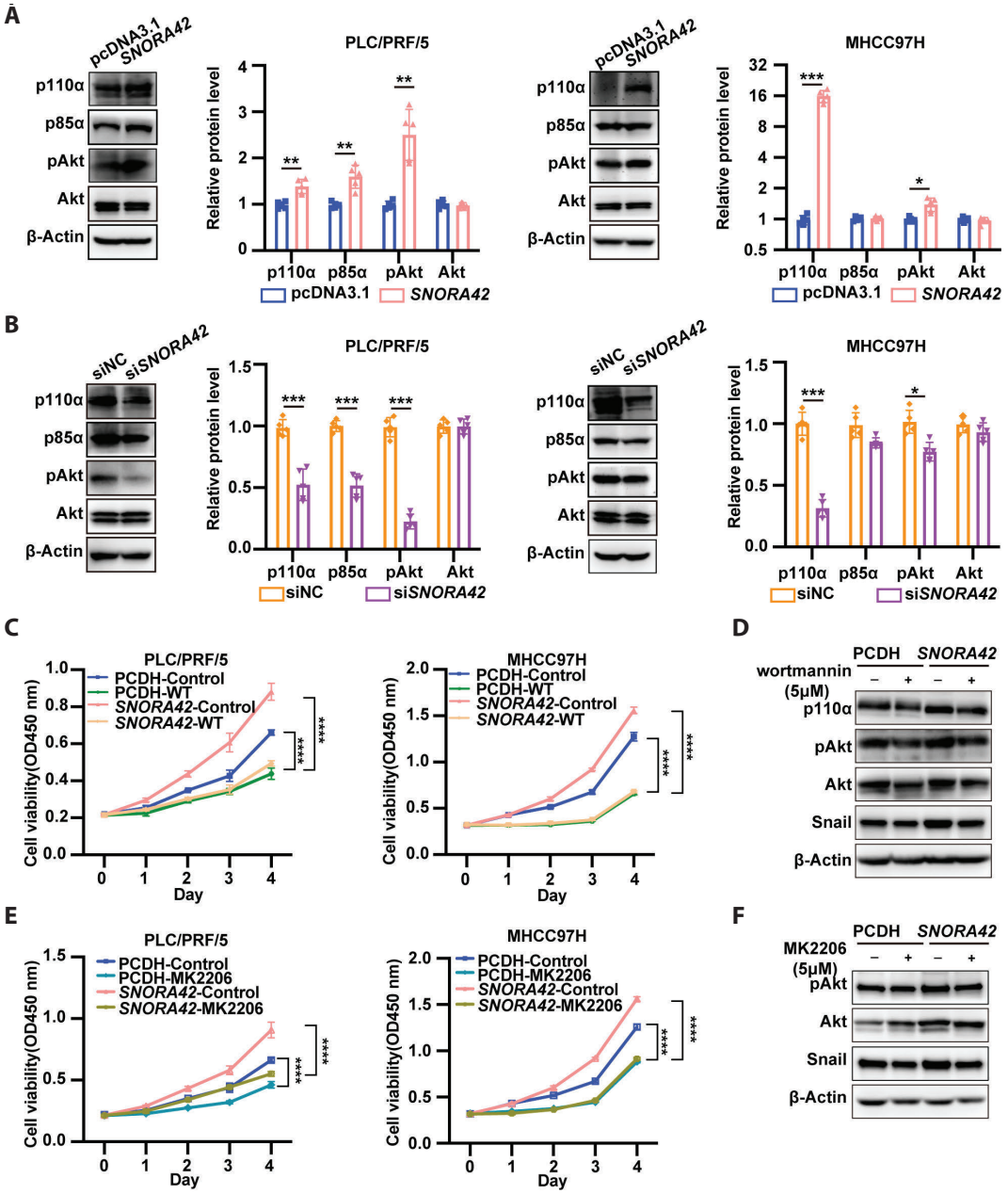

Figure 5. *SNORA42* promotes the progression of HCC cells *via* regulating the PI3K/Akt pathway. **A, B.** *SNORA42* could affect the expression of proteins related to PI3K/Akt signaling pathway by Western blotting. **C, D.** Wortmannin suppressed *SNORA42*-overexpressing PLC/PRF/5 and MHCC97H cell growth as measured by CCK8 assays. p110α, p-AKT, and AKT were analyzed by Western blotting. **E, F.** MK2206 suppressed *SNORA42*-overexpressing PLC/PRF/5 and MHCC97H cell growth as measured by CCK8 assays. p110α, p-AKT, and AKT were analyzed by Western blotting. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; $n = 5$.

2017). Therefore, we explored whether *SNORA42* affected the progression of liver cancer by influencing the PI3K/AKT signaling pathway. Consistent with our hypothesis, overexpression or downplaying of *SNORA42* caused upregulation or downregulation of both subunits of PI3K and p-AKT. To further confirm this mechanism, PI3K inhibitors were used in this study. PI3K inhibitor wortmannin inhibited the growth of HCC cells in PLC/PRF/5 and MHCC97H cells overexpressed with *SNORA42*, suggesting that PI3K was involved in *SNORA42*-mediated cell growth. Western blotting detected the expression of p110α, AKT and Snail proteins in PLC/PRF/5 cells. The results showed that wortmannin reduced the expression of p110α, p-Akt and Snail. Akt inhibitor MK2206 inhibited Akt expression in PLC/PRF/5 and MHCC97H cells overexpressing *SNORA42* and significantly inhibited the growth of HCC cells. Western blotting results showed that MK2206 downregulated the expression of p-Akt and Snail. These results suggest that the PI3K/Akt signaling pathway is involved in *SNORA42*-mediated HCC development.

The specific mechanism of *SNORA42* regulating liver cancer development through PI3K/Akt signaling pathway still needs to be further explored. *SNORA42* belongs to the box H/ACA snoRNAs, but it's still up to us to figure out exactly which domain is at work. In brief, *SNORA42* plays a very momentous role in the development of liver cancer, and *SNORA42* could be stably tested in blood, which is expected to become one of the markers for the diagnosis of liver cancer.

Conclusion

In conclusion, our study shows that *SNORA42* plays an important role in the proliferation, apoptosis, and migration of liver cancer cells. Furthermore, the expression of *SNORA42* does not affect the expression of the host genes. *SNORA42* may affect the proliferation, apoptosis, and migration of HCC cells by regulating the PI3K/Akt signaling pathway. Our study suggests that *SNORA42* plays an oncogenic role in HCC development, partly *via* its interaction with the PI3K/Akt signaling pathway.

Funding. This research was supported by the Scientific Research Project funded by Shanghai Municipal Science and Technology Commission (No.ZR1450400) and the Natural Science Research Funds of the Minhang District, Shanghai (No.2020MHZ016).

Author contributions. Conceptualization, GS and JL; methodology, JL and JD; software, YY and QL; validation, JD and XY; formal analysis, YY; investigation, JL; resources, JL; data curation, JD; writing –original draft preparation, JL; writing – review and editing, GS and QL; visualization, YY; supervision, XY; project administration, QL; funding acquisition, GS and JL. All authors have read and agreed to the published version of the manuscript.

Data availability statement. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest. The authors have no conflicts of interest to declare.

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Received: April 18, 2024
Final version accepted: October 16, 2024

doi: 10.4149/gpb_2024042

Supplementary Material

Small nucleolar RNA 42 facilitates the progression of hepatocellular carcinoma through PI3K/Akt signaling pathway

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Supplementary Figures

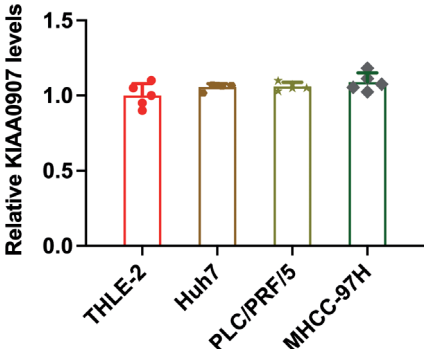

Figure S1. qRT-PCR of *KIAA0907* expression in hepatocytes THLE-2, HCC cell lines Huh-7, PLC/PPRF/5 and MHCC97H ($n = 5$).

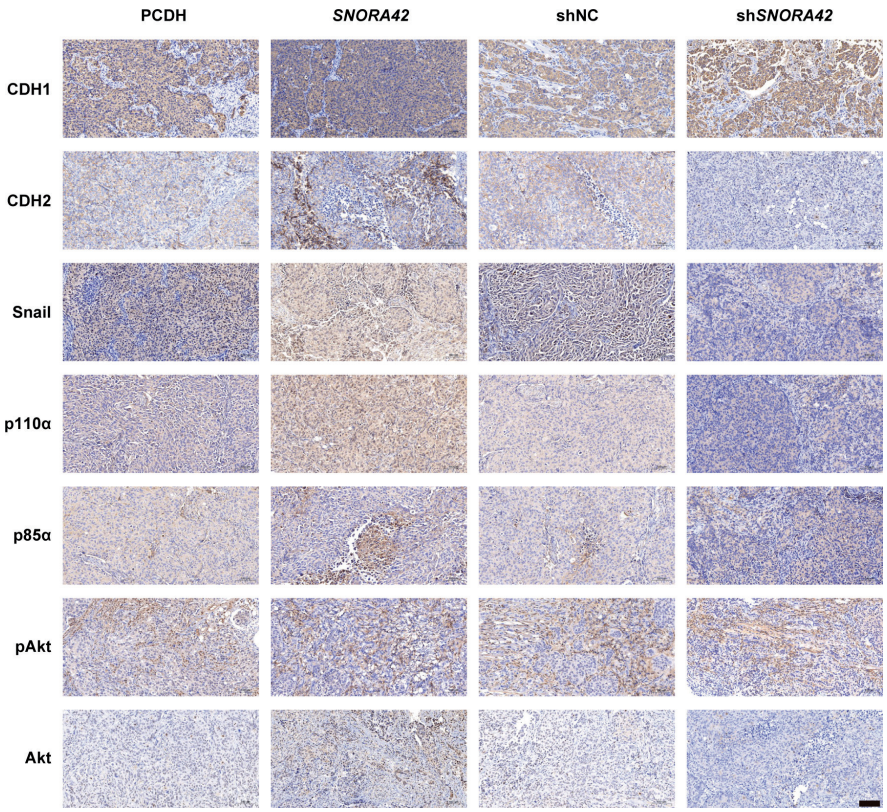

Figure S2. Representative images dyed by E-cadherin, N-cadherin, Snail, p110α, p85α, AKT and p-AKT. Scale bar = 100 μm, $n = 5$.