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Research paper

Investigating SNHG3 as a potential therapeutic approach for HCC stem cells

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ABSTRACT

Introduction: Hepatocellular Carcinoma (HCC) is a common malignant tumor worldwide. Long Non-Coding RNA (lncRNA) has gained attention in tumor biology, and this study aims to investigate the role of lncRNA SNHG3 in HCC, specifically in the self-renewal and maintenance of liver cancer stem cells.

Methods: The expression of lncRNA SNHG3 was analyzed in HCC and adjacent normal tissue using the TCGA database. The expression levels of SNHG3 in HCC cell lines (Hep3B, HepG2, Huh7) were detected using qRT-PCR and Western blot techniques. Functional assays, including CCK-8, soft agar colony formation, and tumor sphere formation, were performed to evaluate the impact of SNHG3 on HCC stem cell functionality. MeRIP-qPCR was also used to investigate the regulatory role of SNHG3 in m6A modification of ITGA6 mRNA mediated by METTL3.

Results: The study found that SNHG3 was significantly upregulated in HCC tissue and cell lines compared to normal liver tissue. SNHG3 expression correlated with the pathological stage, metastasis status, and tumor size of liver cancer. Inhibiting SNHG3 reduced proliferation, colony formation, and tumor sphere formation ability in HCC stem cells. SNHG3 also played a role in regulating the m6A modification and expression of ITGA6 through METTL3.

Conclusion: This study emphasizes the upregulation of lncRNA SNHG3 and its role in HCC stem cell self-renewal. SNHG3 may regulate the m6A modification of ITGA6 mRNA through its interaction with METTL3, impacting the function of liver cancer stem cells. These findings support the potential of targeting SNHG3 as a therapeutic approach for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a prevalent malignant tumor worldwide, particularly in Asia and Africa, with increasing incidence and mortality rates (Zuo et al., 2020; Peng, 2022; Gonzalez-Sanchez et al., 2021). Often associated with chronic liver diseases such as cirrhosis, HCC development is closely related to risk factors such as hepatitis B or C virus infection, alcoholic liver disease, and non-alcoholic fatty liver disease (Roehlen et al., 2020; Gilles et al., 2022; Peiseler et al., 2022). Unfortunately, the majority of HCC cases are diagnosed at advanced stages, limiting treatment options and resulting in a poor prognosis (Krishnamurthy et al., 2021). Despite recent progress in surgical, radiation, chemotherapy, and targeted therapies, the overall survival rate for HCC patients remains low (Wei et al., 2021). Hence, it is crucial to understand the molecular mechanisms underlying HCC occurrence and development and identify novel therapeutic targets to

Abbreviations: HCC, Hepatocellular Carcinoma; lncRNA, Long Non-Coding RNA; SNHG3, Small Nucleolar RNA Host Gene 3; TCGA, The Cancer Genome Atlas; RNA, Ribonucleic Acid; CCK-8, Cell Counting Kit-8; ITGA6, Integrin Subunit Alpha 6; METTL3, Methyltransferase Like 3; M6A, N6-Methyladenosine; TPM, Transcripts Per Million; NCBI, National Center for Biotechnology Information; SRAMP, A predictive model for m6A sites; DMEM, Dulbecco's Modified Eagle Medium; EGF, Epidermal Growth Factor; FGF-β, Fibroblast Growth Factor-beta; qRT-PCR, Quantitative Real-Time PCR; MeRIP-qPCR, Methylated RNA Immunoprecipitation Quantitative PCR; ANOVA, Analysis of Variance.

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improve treatment outcomes and patients' quality of life.

Long non-coding RNA (lncRNA), characterized by its length of more than 200 nucleotides, has emerged as an essential player in tumor biology (Hao et al., 2020; Talebi et al., 2022). Understanding its role in tumor initiation and development has garnered significant attention recently. LncRNA influences various mechanisms, including gene expression regulation, chromosomal remodeling, RNA splicing, and translational regulation (Zuo et al., 2022; Fu et al., 2020; Dai et al., 2022). Moreover, the abnormal expression of lncRNA is closely associated with hepatocellular carcinoma, affecting its occurrence, development, metastasis, and drug resistance (Huang et al., 2020; Gupta et al., 2020; Ge et al., 2023). Some lncRNAs have been shown to regulate liver cancer stem cell characteristics, such as self-renewal capacity and pluripotency, thereby promoting tumor progression and treatment resistance (Mahpour and Mullen, 2020). Therefore, in-depth research on the mechanisms of lncRNA in hepatocellular carcinoma is crucial for discovering novel diagnostic markers and therapeutic targets.

Among the identified lncRNAs, SNHG3 (Small Nucleolar RNA Host Gene 3) has recently been dysregulated in various tumor types (Chen et al., 2022). Upregulation of SNHG3 has been associated with tumor progression, metastasis, and poor prognosis in gastric cancer, colorectal cancer, breast cancer, and other malignancies (Hu et al., 2022). Mechanistically, SNHG3 is believed to promote tumor cell proliferation, inhibit cell apoptosis, drive angiogenesis, and sustain tumor stem cell characteristics (Shan et al., 2022; Li et al., 2020a). However, more research has yet to be conducted regarding the specific role and mechanisms of SNHG3 in hepatocellular carcinoma. Given the significant role of liver cancer stem cells in the pathogenesis, progression, and therapy resistance of hepatocellular carcinoma, investigating the involvement of SNHG3 in maintaining liver cancer stem cell characteristics holds both theoretical and practical significance.

In recent years, the m6A modification, a common RNA epigenetic modification, has garnered significant attention in tumor biology (Song et al., 2022; Li et al., 2020b; Liu et al., 2022a). This modification, through its influence on RNA stability, post-transcriptional processing, and translation efficiency, plays a crucial role in regulating gene expression and consequently contributes to tumor development (Ye et al., 2023). METTL3, the primary catalytic enzyme responsible for m6A modification, displays aberrant expression in various tumors and is closely associated with tumor onset, progression, metastasis, and resistance to chemotherapy (Zhang et al., 2020; Zhu et al., 2023; Wang et al., 2023a). METTL3 regulates multiple biological processes, particularly in hepatocellular carcinoma, including those related to liver cancer stem cell traits, by mediating m6A modification (Zhang et al., 2022a; Zhang and Huang, 2023). Therefore, it is essential to investigate the role of SNHG3, mediated by METTL3-induced m6A modification, in hepatocellular carcinoma as it can provide deeper insights into the molecular mechanisms underlying liver cancer.

As a cell surface receptor, Integrin α6 (ITGA6) plays a crucial role in interacting with the extracellular matrix and the cytoskeleton. It has been extensively studied in the context of tumor invasion and metastasis (Guo et al., 2024). Research indicates that METTL3-mediated m6A modification of ITGA6 mRNA can promote tumor cell invasion and metastasis by increasing its mRNA stability and translation efficiency (Ying et al., 2024). Furthermore, high ITGA6 expression is closely associated with poor prognosis in cancer patients (Jin et al., 2019). Therefore, investigating the mechanism of METTL3-mediated m6A modification of ITGA6 mRNA in cancer stem cells is of great significance for elucidating the maintenance mechanisms of cancer stem cells and identifying new therapeutic targets.

This study investigates the expression pattern and function of the long non-coding RNA (lncRNA) SNHG3 in hepatocellular carcinoma (HCC). Specifically, we aim to understand its role in HCC stem cell self-renewal and stemness maintenance. To achieve this, we utilized the TCGA database for data analysis and performed laboratory experiments to explore the role and mechanism of SNHG3 in HCC. Our focus is mainly on the impact of SNHG3 on m6A modification of ITGA6 mRNA, which METTL3 mediates. Furthermore, we aim to elucidate how this modification affects the function of liver cancer stem cells. The findings of this study will enhance our understanding of HCC pathogenesis and provide a theoretical foundation for developing novel diagnostic and therapeutic strategies for liver cancer. Importantly, these results have significant scientific and clinical implications for improving the accuracy of diagnosis and treatment outcomes in patients with liver cancer.

2. Materials and methods

2.1. Bioinformatics analysis

The Materials and Methods section outlines our comprehensive research method, which utilizes multiple database resources for bioinformatics analysis. Initially, we extracted high-throughput RNA sequencing (RNAseq) data and clinical information from the TCGA database (The Cancer Genome Atlas) for the hepatocellular carcinoma (HCC) project. To ensure sample representativeness and dataset integrity, we selected 424 HCC samples and 50 standard liver tissue samples while excluding samples without clinical information and duplicate samples from the same patient.

We processed the downloaded data using R software (version 4.2.1) and its statistical package (stats, 4.2.1), employing appropriate statistical methods that fit the characteristics of the data. The transcript count (TPM) data was logarithmically transformed using the log2(value + 1) formula to address skewness and heterogeneity. To investigate the correlation between SNHG3 expression and the stem cell marker CD44 in liver cancer samples, expression data for SNHG3 and CD44 were extracted. Using R software (version 4.2.1) and the R packages dplyr (version 1.0.7) and tidyverse (version 1.3.1), the data were filtered and organized to ensure that each sample contained expression values for both SNHG3 and CD44. The Pearson correlation coefficient between SNHG3 and CD44 expression levels was calculated, and a scatter plot illustrating the relationship between SNHG3 and CD44 expression was generated using the R package ggplot2 (version 3.3.5). Additionally, to investigate the impact of RNA modifications on gene expression regulation, we obtained the mRNA sequence of ITGA6 from the NCBI database. Using the SRAMP database, which employs a machine learning method for predicting m6A sites on mRNA, we predicted potential m6A modification sites on the ITGA6 mRNA.

The integration of these various database resources and analysis tools lays a robust methodological foundation for conducting an in-depth exploration of SNHG3's expression patterns and the potential functional and clinical significance of m6A modification on ITGA6 in HCC.

2.2. Cell culture and processing

We experimented using human liver cell lines L02 and hepatocellular carcinoma (HCC) cell lines Hep3B, HepG2, and Huh7, obtained from the Cell Bank of Shanghai Institute of Life Sciences, Chinese Academy of Sciences. The cells were cultured in DMEM medium supplemented with 10 % fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5 % CO_2. All culture media and reagents were provided by Gibco (USA). The cells were passaged every three days using 0.5 % trypsin. After 3–4 passages, we selected cells in the logarithmic growth phase for real-time quantitative PCR (qRT-PCR) to detect the relative expression level of the long non-coding RNA (lncRNA) SNHG3.

For the experiments involving Huh7 cells, cells in the logarithmic growth phase were seeded into a 96-well plate at a density of 5000 cells per well and cultured until they reached 70–80 % confluence. Once the desired confluence was achieved, the cells were used for subsequent experiments. The cells were then divided into five groups using the transfection reagent Lipofectamine 2000 (Invitrogen, USA): control

group, si-SNHG3 group, si-NC group, si-SNHG3 + oe-METTL3 group, and si-SNHG3 + oe-NC group. The control group received no treatment, while the si-SNHG3 and si-NC groups were transfected with 200 ng of si-SNHG3 interference RNA (si-SNHG3) and negative control (si-NC), respectively. The si-SNHG3 + oe-NC group and si-SNHG3 + oe-METTL3 group were co-transfected with 200 ng of si-SNHG3 and 1 μg of METTL3 overexpression plasmid (oe-METTL3) or negative control empty plasmid (oe-NC), respectively. Jiangsu Genomedicine Co., Ltd synthesized the si-SNHG3, oe-METTL3, and corresponding negative controls.

Through this series of cell culture and transfection experiments, we aim to investigate the functions and interactions of SNHG3 and METTL3 in human liver cancer cells.

2.3. CCK-8

To determine the proliferative activity of Huh7 liver cancer cells in response to different treatments, we utilized the CCK-8 cell proliferation assay kit (Solarbio Biotechnology Co., Ltd., Beijing, China). Huh7 cells were seeded at a density of 5000 cells per well in 96-well plates and subjected to various treatment conditions. Following 24, 48, and 72 h of incubation, the culture medium was replaced, and 100 μL of the CCK-8 working solution was added to each well. The plates were then incubated at 37 degrees Celsius for 2 h. Subsequently, the optical density (OD value) at a wavelength of 450 nm was measured using a microplate reader (Thermo Fisher Scientific, USA). This approach allowed for quantitative analysis of changes in cell proliferation capacity at multiple time points, providing valuable experimental data to support further investigations into the biological characteristics of Huh7 cells.

2.4. Assessment of Colony-Forming ability of Huh7 cells based on soft agar plate method

To assess the growth ability of Huh7 liver cancer cells, we performed a soft agar colony formation assay. First, the Huh7 cells were treated with various conditions and then digested into single cells using 0.5 % trypsin. Cell counting was then conducted. Next, the cell density was adjusted to 1×10^6 cells/mL using a DMEM medium containing 20 % fetal bovine serum. We prepared a mixture of 1 % agar powder (Shanghai Yuanyang Biotechnology Co., Ltd., China) and 2 × DMEM medium with double antibiotics and fetal bovine serum at a ratio of 1:1. We added three milliliters of this mixture to a 6 cm diameter dish and allowed it to cool and solidify. After solidification, we added 0.4 mL of a preheated mixture of 1 % agarose and Huh7 cell suspension to the dish. The cell suspension contained approximately 1000 Huh7 cells and was preheated to a 1:1 mixture at 37 °C. The dish was then incubated at 37 °C and 5 % CO_2 for 2–3 weeks.

During the incubation period, visible colonies formed in the dish. Once colonies were visible, we stained them with 0.1 % crystal violet (Beijing Yida Biotechnology Co., Ltd.) for 30 min. Finally, we observed and counted the number of more significant colonies than 2 mm in diameter using an inverted microscope (OLYMPUS, Japan). This method allowed us to quantitatively analyze the proliferation and growth ability of Huh7 cells under different treatments.

3. Research method for Evaluating the Self-Renewal ability of Huh7 stem cells based on tumor sphere formation assay

This study aimed to assess the self-renewal ability of Huh7 stem cells using a tumorosphere formation assay. Initially, Huh7 cells were seeded in ultra-low attachment 24-well plates (Corning, USA) at a density of 1×10^4 cells per well in a single-cell suspension format. The cells were then cultured in serum-free DMEM-F12 medium (Gibco, USA) supplemented with 10 ng/mL epidermal growth factor (EGF) and 10 ng/mL fibroblast growth factor-beta (FGF-β). The culture medium was refreshed every 2–3 days to maintain cell viability. After a continuous culture period of 10–14 days, the tumorospheres were visualized and documented using an optical microscope (OLYMPUS, Japan). The number of tumorospheres was subsequently quantified. This experimental approach enabled the quantitative analysis of Huh7 stem cells in terms of their ability to generate tumorospheres under specific growth factor conditions, signifying their self-renewal potential and maintenance of an undifferentiated state.

3.1. Detection method for ITGA6 MRNA M6A modification level based on M6A MeRIP-qPCR Technology

In this study, we utilized the m6A MeRIP kit (BersinBio, Guangzhou, China) and the MeRIP-qPCR method to investigate the enrichment of m6A modification on ITGA6 mRNA. Initially, we isolated total RNA from differently treated Huh-7 cells and fragmented it into 100–200 nucleotides using ultrasound. Subsequently, we performed RNA immunoprecipitation using an m6A antibody and protein A/G magnetic beads, followed by RNA extraction using the phenol–chloroform method. We subjected equivalent amounts of RNA from each sample to reverse transcription and real-time quantitative PCR (qPCR) experiments to ensure consistency. By following this series of procedures, we could quantitatively assess the abundance of m6A modification on ITGA6 mRNA. This analysis provides an experimental foundation for further investigations into the biological functions and regulatory mechanisms of m6A modification in Huh-7 cells.

3.2. qRT-PCR

To determine the relative expression levels of the target gene in cells subjected to different treatment conditions, we utilized the real-time quantitative PCR (qRT-PCR) technique. Initially, cells from various treatments were collected, and the total RNA was extracted using the total RNA extraction reagent kit provided by TianGeng Biotechnology Co., Ltd. (Beijing, China). The concentration and purity of the extracted total RNA were assessed using a Nanodrop 2000 spectrophotometer (1011U, Nanodrop, USA).

Afterward, cDNA synthesis was performed using the reverse transcription reagent kit from TaKaRa Co., Ltd. (Japan). Real-time quantitative PCR experiments were conducted using the ABI 7500 quantitative PCR instrument (7500, ABI, USA). The reaction conditions were set as follows: an initial pre-denaturation step at 95 °C for 10 min, followed by 40 cycles containing denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s.

The relative expression level of the target gene was determined using the $2^{-\Delta\Delta Ct}$ method, with β-actin serving as the internal reference. Shanghai Sangon Biotech Co., Ltd synthesized the primers used in the experiment (Table 1).

3.3. Western blot

We performed a western blot to analyze changes in protein expression in Huh7 cells under different treatment conditions. Huh7 cells were subjected to various treatments, and total protein was extracted from the cells using RIPA lysis buffer (BB-3209, Bestbio, Shanghai, China). The protein concentration was then determined using the BCA protein quantification kit (Beyotime, Shanghai, China).

Subsequently, the extracted protein was separated on a 10 % SDS-PAGE gel and transferred onto a PVDF membrane (Solarbio, Beijing,

Table 1
Primer sequences for PCR.

Gene	Forward 5′-3′	Reverse 5′-3′
LncRNA SNHG3	TACTGGCTGCGCACTTCG	TACCCTGCACAAACCCGAAA
ITGA6	GGCGGTGTTATGTCCTGAGTC	AATCGCCCATCACAAAAGCTC
β-actin	CTCGACACCAGGGCGTTATG	CCACTCCATGCTCGATAGGAT

China). The membrane was incubated with 5 % skim milk powder to block non-specific binding for 1 h. Primary antibodies used for incubation were as follows: anti-CD44 (ab51037, Abcam, UK), anti-CD133 (ab216323, Abcam), anti-Nanog (ab109250, Abcam), anti-Sox2 (ab97959, Abcam), anti-METTL3 (ab195352, Abcam), anti-ITGA6 (ab181551, Abcam), and anti-β-actin (ab8227, Abcam). The incubation was carried out overnight at 4 degrees Celsius.

Following incubation, the membrane was gently shaken at room temperature for 2 h and then incubated with an HRP-conjugated secondary antibody (ab205718, Abcam), specifically goat anti-rabbit IgG H&L. After washing the membrane three times with PBS at room temperature, an ECL staining kit (Shanghai Yuan Yang Biotechnology Co., Ltd., China) was used for visualization. Image processing software Image J (National Institutes of Health, USA) was utilized to determine protein band density, with β-actin serving as the internal reference protein. These steps allowed us to accurately evaluate the impact of different experimental treatments on protein expression levels in Huh7 cells.

3.4. Statistical analysis

This study used the statistical software SPSS 22.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA) for data analysis and visualization. The normality of the data was assessed using the Shapiro-Wilk test. Data that exhibited a normal distribution were presented as mean ± standard deviation. To compare multiple groups, we employed a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test for post-analysis. The resulting p-values were derived from a two-tailed test, with statistical significance set at $p < 0.05$.

For analysis of the RNA sequencing (RNAseq) data obtained from the Liver Hepatocellular Carcinoma (TCGA-LIHC) project, which was downloaded from the TCGA database along with its clinical data, we utilized R software (version 4.2.1) and the corresponding stats package. The data processing steps included selecting appropriate statistical methods based on the characteristics of the data and transforming expression values using the log2(value + 1) formula to facilitate analysis. Data filtering strategies were also employed to exclude standard samples and samples lacking clinical information. This comprehensive data processing and analysis approach allowed us to accurately assess and interpret our biomedical experiments' results.

4. Results

4.1. LncRNA SNHG3 is significantly upregulated in hepatocellular carcinoma cells

Our research has focused on investigating the molecular mechanisms underlying the development of hepatocellular carcinoma (HCC), with particular emphasis on the long non-coding RNA (lncRNA) SNHG3. By analyzing data from the TCGA database, we sought to determine the expression pattern of lncRNA SNHG3 in HCC. Our findings revealed a significant increase in the expression level of lncRNA SNHG3 in HCC tissues compared to normal liver tissues (Fig. 1A, $P < 0.05$). This observation suggests that lncRNA SNHG3 may be crucial in developing HCC.

To validate our results, we performed real-time quantitative polymerase chain reaction (qRT-PCR) to assess the expression of lncRNA SNHG3 in both normal liver cells (L02) and HCC cell lines (Hep3B, HepG2, Huh7). Our analyses demonstrated a significant upregulation of lncRNA SNHG3 expression in all three HCC cell lines (Fig. 1B, all $P < 0.01$), with the highest expression level observed in Huh7 cells. As a result, we selected Huh7 cells as the focus of our subsequent studies.

Our investigation revealed a marked upregulation of lncRNA SNHG3 in HCC tissues and cell lines, indicating its potential regulatory role in developing HCC. The exceptionally high expression of lncRNA SNHG3 in Huh7 cells highlights its significance in HCC and provides a basis for further exploration into its role in hepatocellular carcinoma.

4.2. Expression characteristics of SNHG3 in hepatocellular carcinoma and its correlation with clinical pathological Parameters

Based on data from the Liver Hepatocellular Carcinoma (TCGA-LIHC) project in the TCGA database, we conducted a comprehensive analysis to investigate the long non-coding RNA SNHG3 expression pattern in hepatocellular carcinoma (HCC). We aimed to evaluate the association between SNHG3 expression and the pathological stages of liver cancer and explore its potential role in HCC development.

Our analysis revealed a significant correlation between SNHG3 expression levels and the pathological stages of HCC (Fig. 2A), with notably higher expression observed in patients at advanced stages (III/IV) compared to early stages (I/II). Furthermore, we observed a significant difference in SNHG3 expression levels between cases with and without distant metastasis (M stage) (Fig. 2B), with higher SNHG3

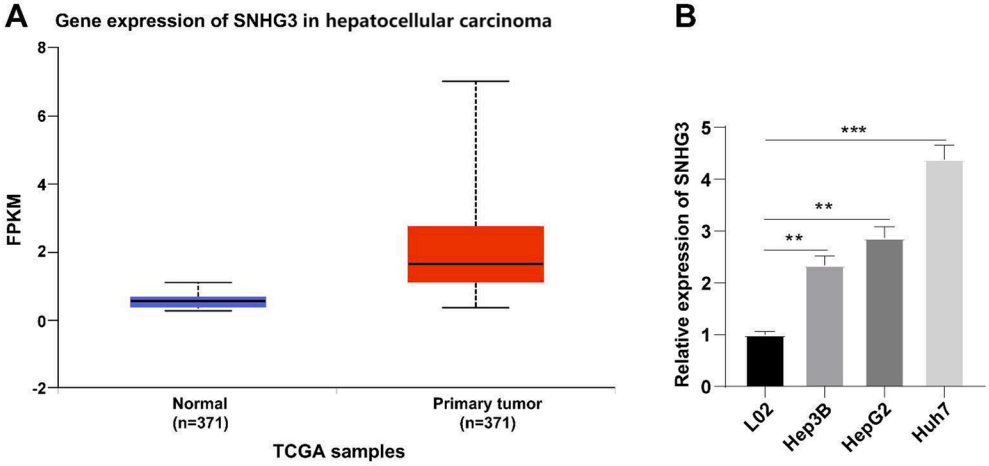

Fig. 1. Evidence of upregulated expression of lncRNA SNHG3 in hepatocellular carcinoma. Note: (A) The expression levels of the long non-coding RNA (lncRNA) SNHG3 were analyzed in hepatocellular carcinoma (HCC) tissue and normal liver tissue using the TCGA database. (B) To validate these findings, the expression of lncRNA SNHG3 was measured in normal liver cells (L02) and HCC cell lines (Hep3B, HepG2, Huh7) using quantitative real-time polymerase chain reaction (qRT-PCR). The experiment was performed in triplicate, presenting the results as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (One-way ANOVA), followed by Tukey's multiple comparison test to compare the data among multiple groups.

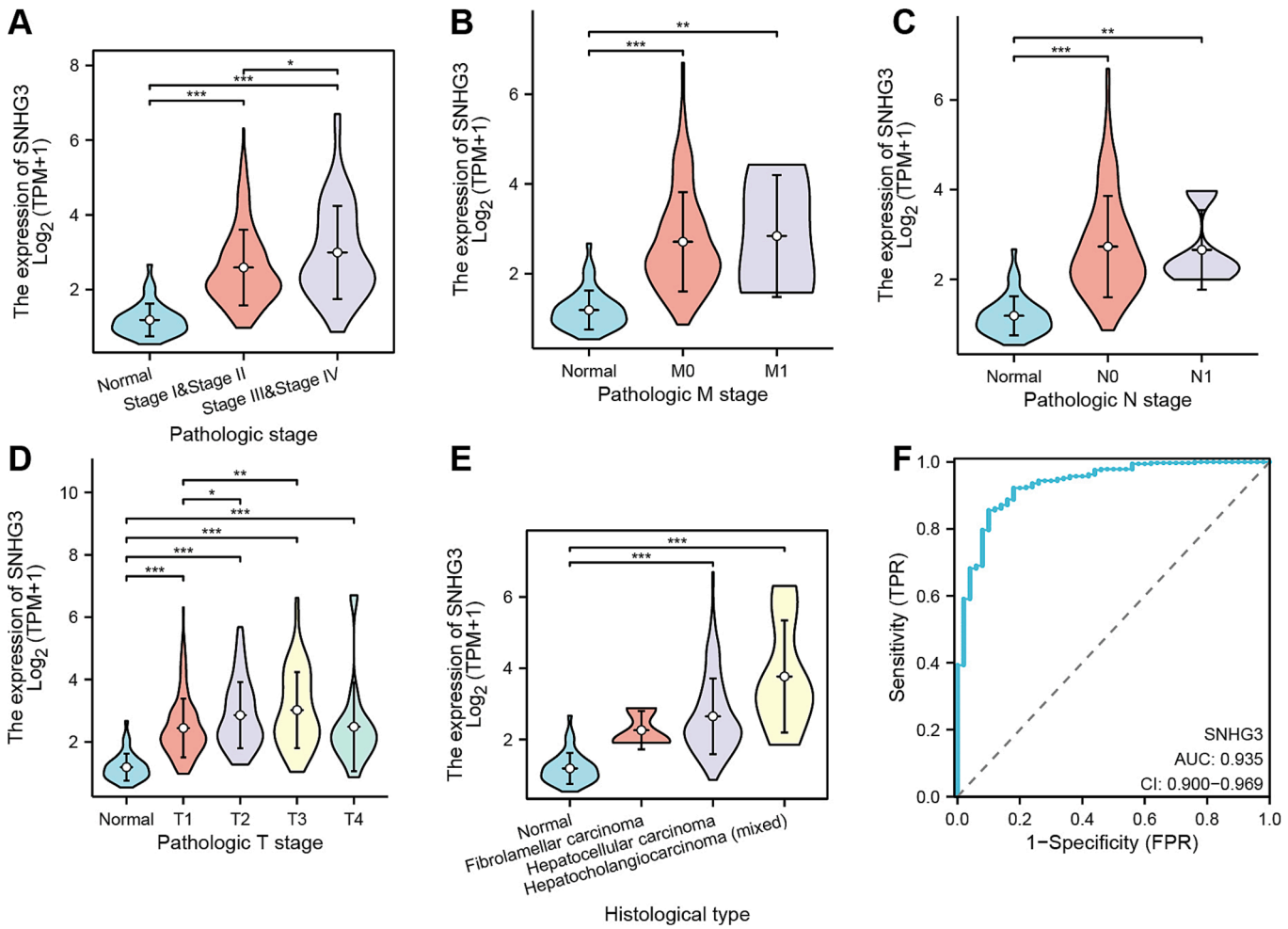

Fig. 2. Temporal differences in expression of SNHG3 in hepatocellular carcinoma and its diagnostic value. Note: (A) This violin plot depicts the expression of SNHG3 in liver cancer patients across different pathological stages (I, II, III, IV) compared to the expression levels in normal tissues. The y-axis represents logarithmically transformed expression values, demonstrating various expressions. (B) A boxplot illustrates the expression of SNHG3 in normal tissues and different pathological M stages (M0 and M1). M0 indicates the absence of distant metastasis, while M1 indicates the presence of distant metastasis. (C) This graph compares SNHG3 expression levels between normal tissues and pathological N stages (N0 and N1). N0 signifies the absence of regional lymph node metastasis, while N1 indicates the presence of regional lymph node metastasis. (D) The expression of SNHG3 in liver cancer is presented using a violin plot based on the different pathological T stages (T1 to T4), which describe the size and extent of the primary tumor. The y-axis represents logarithmically transformed expression values, and significant differences between stages are denoted by asterisks. (E) In this figure, the expression of SNHG3 is profiled in normal liver tissue and various histological types of liver cancer, including fibrolamellar carcinoma, hepatocellular carcinoma, and mixed hepatocellular-cholangiocarcinoma. (F) This receiver operating characteristic (ROC) curve demonstrates the sensitivity and 1-specificity of liver cancer diagnosis using SNHG3 expression. The area under the curve (AUC) and confidence interval (CI) are provided, indicating the high predictive value of SNHG3 expression. Statistical significances are denoted by *P < 0.05, **P < 0.01, and ***P < 0.001 as appropriate.

expression in patients at the M1 stage (with distant metastasis). Similarly, SNHG3 expression was significantly higher in cases with regional lymph node metastasis (N1) compared to those without regional lymph node metastasis (N0) (Fig. 2C). Additionally, higher T tumor size and invasion stages were associated with higher SNHG3 expression levels (Fig. 2D). We also observed significant differences in SNHG3 expression among histological subtypes, including fibrolamellar carcinoma, hepatocellular carcinoma, and mixed types (Fig. 2E). Finally, ROC curve analysis demonstrated that SNHG3 expression had high sensitivity and specificity for diagnosing HCC, with an AUC value of 0.935 and a CI range of 0.900–0.969 (Fig. 2F). For detailed data, please refer to Table 2.

Our findings indicate that SNHG3 expression levels exhibit distinct patterns in different clinical and pathological stages of hepatocellular carcinoma, particularly in pathological stages, metastatic status, and tumor size. These results suggest that SNHG3 may hold potential as a biomarker for the progression of liver cancer and could be of clinical significance in the diagnosis of HCC.

4.3. Downregulation of LncRNA SNHG3 in hepatocellular carcinoma stem cells and its impact on stemness markers

This study aimed to investigate the role of lncRNA SNHG3 in maintaining stem cell properties in hepatocellular carcinoma (HCC). We analyzed the correlation between SNHG3 expression and the stem cell marker CD44 in liver cancer samples using data from the TCGA database. The results showed a significant positive correlation between the expression levels of SNHG3 and CD44 (Fig. 3A, $P < 0.001$). The Pearson correlation coefficient (R) between log2(SNHG3 TPM) and log2(CD44 TPM) was 0.59, with a p-value of 1.6e-11, indicating a highly significant correlation between the two. Specifically, we examined the mechanism by interfering with lncRNA SNHG3 expression in Huh7 cells. By transfecting interfering RNA into the cells, we successfully reduced the level of lncRNA SNHG3, as confirmed by real-time quantitative polymerase chain reaction (qRT-PCR) (Fig. 3B, $P < 0.001$).

Subsequently, we assessed the protein expression of stem cell markers CD44 and CD133, as well as the stemness-related transcription

Table 2
Baseline Data Obtained from the TCGA Dataset.

Characteristics	Low expression of SNHG3	High expression of SNHG3	P value
n	187	187	
Age, n (%)			0.043
<= 60	79 (21.2 %)	98 (26.3 %)	
> 60	108 (29 %)	88 (23.6 %)	
Gender, n (%)			0.581
Female	58 (15.5 %)	63 (16.8 %)	
Male	129 (34.5 %)	124 (33.2 %)	
Weight, n (%)			0.022
<= 70	83 (24 %)	101 (29.2 %)	
> 70	93 (26.9 %)	69 (19.9 %)	
Tumor status, n (%)			0.667
Tumor free	105 (29.6 %)	97 (27.3 %)	
With tumor	76 (21.4 %)	77 (21.7 %)	
Pathologic T stage, n (%)			0.001
T1	108 (29.1 %)	75 (20.2 %)	
T2	40 (10.8 %)	55 (14.8 %)	
T3	28 (7.5 %)	52 (14 %)	
T4	8 (2.2 %)	5 (1.3 %)	
Pathologic N stage, n (%)			0.550
N0	120 (46.5 %)	134 (51.9 %)	
N1	3 (1.2 %)	1 (0.4 %)	
Pathologic M stage, n (%)			1.000
M0	129 (47.4 %)	139 (51.1 %)	
M1	2 (0.7 %)	2 (0.7 %)	
Pathologic stage, n (%)			0.008
Stage I	99 (28.3 %)	74 (21.1 %)	
Stage II	37 (10.6 %)	50 (14.3 %)	
Stage III	31 (8.9 %)	54 (15.4 %)	
Stage IV	3 (0.9 %)	2 (0.6 %)	
Histological type, n (%)			0.139
Fibrolamellar carcinoma	2 (0.5 %)	1 (0.3 %)	
Hepatocellular carcinoma	184 (49.2 %)	180 (48.1 %)	
Hepatocholangiocarcinoma (mixed)	1 (0.3 %)	6 (1.6 %)	

factors Nanog and Sox2 in the Huh7 cells using Western blot. Importantly, we observed a significant decrease in the protein levels of these stemness markers upon the downregulation of lncRNA SNHG3 (Fig. 3C, all $P < 0.01$).

These findings indicate that lncRNA SNHG3 potentially plays a crucial role in supporting the maintenance of stemness in hepatocellular carcinoma stem cells. Moreover, our results suggest that the downregulation of lncRNA SNHG3 can effectively inhibit the expression of stemness-related markers.

4.4. Downregulation of LncRNA SNHG3 suppresses the Self-Renewal capacity of hepatocellular carcinoma stem cells

This study aims to investigate the impact of lncRNA SNHG3 on the functionality of stem cells in hepatocellular carcinoma (HCC). Specifically, we focus on its regulatory effects on cell proliferation activity and self-renewal ability. To achieve this, we conducted several assays, including the CCK-8 assay, soft agar colony formation assay, and tumor sphere formation assay. These assays allowed us to analyze the proliferation activity of Huh7 cells as well as the self-renewal ability of HCC stem cells.

The results obtained from these experiments revealed that downregulation of lncRNA SNHG3 expression led to a significant decrease in the proliferation activity of Huh7 cells (Fig. 4A, $P < 0.01$). Additionally, we observed a significant reduction in the number of colony formations (Fig. 4B, $P < 0.01$) and tumor sphere formation ability (Fig. 4C, $P < 0.01$) following the downregulation of lncRNA SNHG3 expression. These findings strongly suggest that lncRNA SNHG3 inhibits the proliferation activity of HCC stem cells and plays a critical role in promoting self-renewal ability. Thus highlighting the vital role of lncRNA SNHG3 in the biology of HCC stem cells.

4.5. LncRNA SNHG3 promotes the expression of liver cancer stem cell markers by regulating METTL3

This study aimed to investigate the role of lncRNA SNHG3 in hepatocellular carcinoma (HCC) stem cells, specifically focusing on its influence on the expression of METTL3 and stemness markers. Initially, we evaluated the protein level of METTL3 and observed a significant decrease upon downregulation of lncRNA SNHG3 (Fig. 5A, $P < 0.01$). To validate this finding further, we overexpressed METTL3 in Huh7 cells and inhibited lncRNA SNHG3. Western blot analysis revealed a significant increase in METTL3 expression in the si-SNHG3 + oe-METTL3 group compared to the control group (Fig. 5A, $P < 0.01$). Additionally, we quantified the levels of stemness-related proteins, including CD44, CD133, Nanog, and Sox2. Interestingly, overexpression of METTL3 led to a noteworthy elevation in the protein levels of these stemness markers in Huh7 cells (Fig. 5B, all $P < 0.05$).

These results indicate that lncRNA SNHG3 plays a crucial role in expressing stemness markers in HCC stem cells through its regulation of METTL3, highlighting its significance in the biology of hepatocellular carcinoma stem cells.

4.6. LncRNA SNHG3 promotes Self-Renewal of liver cancer stem cells by regulating METTL3

This study aimed to investigate the role of long non-coding RNA (lncRNA) SNHG3 in the self-renewal of hepatocellular carcinoma (HCC) stem cells, specifically through its regulation of METTL3.

To assess the proliferative and self-renewal abilities of Huh7 cells, we conducted several assays: the CCK-8 assay, the soft agar colony formation assay, and the tumor sphere formation assay. In these experiments, we simultaneously downregulated the expression of lncRNA SNHG3 and overexpressed METTL3. The results of our study demonstrated that overexpression of METTL3 significantly enhanced the proliferation activity of Huh7 cells (Fig. 6A, $P < 0.01$). Moreover, there was a corresponding increase in soft agar colonies and tumor spheres (Fig. 6B-C, both $P < 0.01$).

These findings suggest that lncRNA SNHG3 plays a crucial role in the self-renewal process of HCC stem cells through its regulation of METTL3. The regulatory mechanism identified in our study has important implications for understanding the biological characteristics of HCC stem cells.

4.7. LncRNA SNHG3 promotes ITGA6 expression through regulating METTL3-Mediated M6A modification

This study investigates the impact of lncRNA SNHG3 on the self-renewal ability of cancer stem cells (CSCs), focusing on its regulation of the m6A modification of ITGA6.

Previous studies have demonstrated the crucial role of METTL3-mediated m6A modification of ITGA6 mRNA in the self-renewal of CSCs. Here, we further explored the regulatory role of lncRNA SNHG3 in this process. Using the SRAMP database, we predicted multiple potential m6A modification sites at the 3′ end of ITGA6 mRNA (Fig. 7A). Subsequent MeRIP-qPCR experiments revealed that decreased expression of lncRNA SNHG3 led to a decrease in m6A enrichment on ITGA6 mRNA, while low expression of lncRNA SNHG3 combined with overexpression of METTL3 increased m6A enrichment (Fig. 7B, all $P < 0.05$). Furthermore, analysis of ITGA6 mRNA and protein levels showed that downregulation of lncRNA SNHG3 expression resulted in decreased expression of ITGA6, whereas low expression of lncRNA SNHG3 combined with overexpression of METTL3 led to increased expression of ITGA6 (Fig. 7C, D, all $P < 0.05$).

In conclusion, our findings highlight the significant role of lncRNA SNHG3 in regulating the mRNA and protein expression of ITGA6 through the modulation of METTL3-mediated m6A modification. This provides valuable insights into the regulatory mechanisms governing

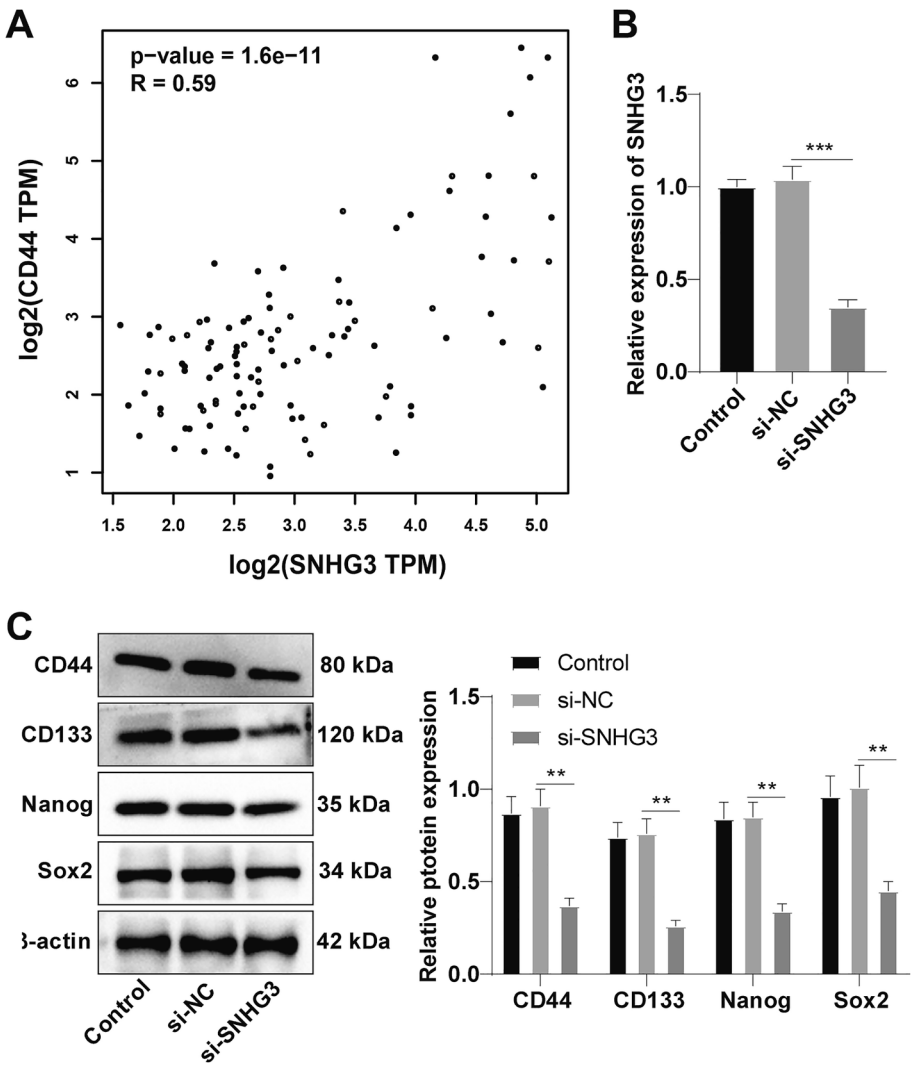

Fig. 3. Downregulation of lncRNA SNHG3 suppresses the expression of stemness markers in liver cancer stem cells. Note: (A) Analysis of the correlation between SNHG3 expression and the stem cell marker CD44 in liver cancer samples. (B) The expression levels of long non-coding RNA (lncRNA) SNHG3 in Huh7 cells were detected using quantitative real-time polymerase chain reaction (qRT-PCR). (C) The protein levels of stem cell markers, including CD44, CD133, Nanog, and Sox2, were detected in Huh7 cells by performing a Western blot. The experiment was repeated thrice, presenting the results as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The significance level was set at $^{**}P < 0.01$, with a lower threshold at $^{***}P < 0.001$.

the biology of CSCs.

5. Discussion

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths worldwide, particularly in China, where its incidence and mortality rates are increasing (Llovet et al., 2022; Chakraborty and Sarkar, 2022; Chen et al., 2020; Rinaldi et al., 2021; Xia et al., 2022). The poor clinical prognosis in HCC patients is mainly due to the development of drug resistance in cancer cells and tumor recurrence after remission (Lai et al., 2022; Fei et al., 2022; Wang et al., 2018). Liver cancer stem cells (CSCs) are a subset of cancer cells with self-renewal, unlimited proliferation, and multi-directional differentiation potential. These CSCs play critical roles in tumor development, invasion, metastasis, drug resistance, and post-treatment relapse (Yang et al., 2024; Yao et al., 2021; Walcher et al., 2020; French and Pauklin, 2021). Consequently, targeting HCC stem cells has emerged as a promising strategy to improve treatment outcomes in HCC.

Long non-coding RNAs (lncRNAs) have been found to regulate the stemness of HCC cells and promote HCC progression (Farzaneh et al., 2022; Rojas et al., 2022; Gong et al., 2020; Zhang and Zhu, 2021). One specific lncRNA, SNHG3, has been identified as a novel oncogenic lncRNA and is aberrantly expressed in various cancers, including osteosarcoma, HCC, and lung cancer (Xu et al., 2020). Moreover, elevated expression of SNHG3 has been associated with poor prognosis in HCC patients (Xie et al., 2023; Zhang et al., 2019; Yang et al., 2022). However, the role of SNHG3 in regulating self-renewal and stemness maintenance of HCC stem cells remains unclear. Therefore, this study aimed to investigate the role of SNHG3 in HCC stem cell self-renewal and stemness maintenance.

Using the TCGA database and conducting qRT-PCR experiments, we observed a significant increase in SNHG3 expression in HCC tissues and cells, consistent with previous research findings (Zhao et al., 2019). Subsequently, we employed small interfering RNA (siRNA) to downregulate SNHG3 expression in Huh7 cells. Consistent with the previous study by Zhao et al., our results demonstrated that downregulation of SNHG3 inhibited the proliferation of HCC cells. Additionally, SNHG3 downregulation decreased the expression levels of HCC stem cell markers CD44 and CD133 (Jeng et al., 2023). Furthermore, the protein levels of stem cell-associated transcription factors Nanog and Sox2 were

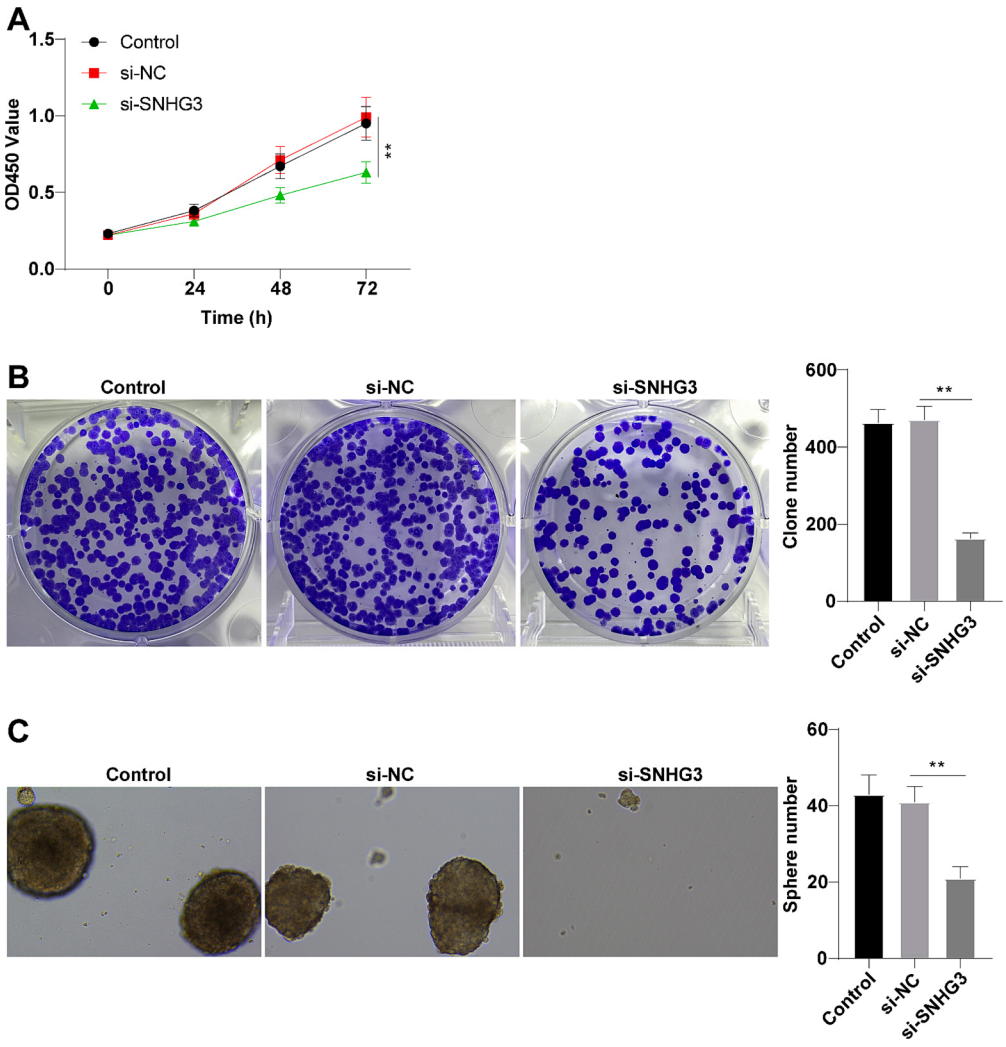

Fig. 4. The inhibitory effect of downregulation of lncRNA SNHG3 on the self-renewal capacity of HCC stem cells. Note: (A) We used the CCK-8 assay to assess the proliferation activity of Huh7 cells. (B) To examine the growth ability of Huh7 cells, we conducted the soft agar colony formation assay. (C) The self-renewal capability of Huh7 stem cells was evaluated through the tumor sphere formation assay. Each experiment was repeated three times, and the results are presented as mean ± standard deviation. We performed multiple group comparisons using a one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Statistical significance was defined as **P < 0.01, with *** representing P < 0.001.

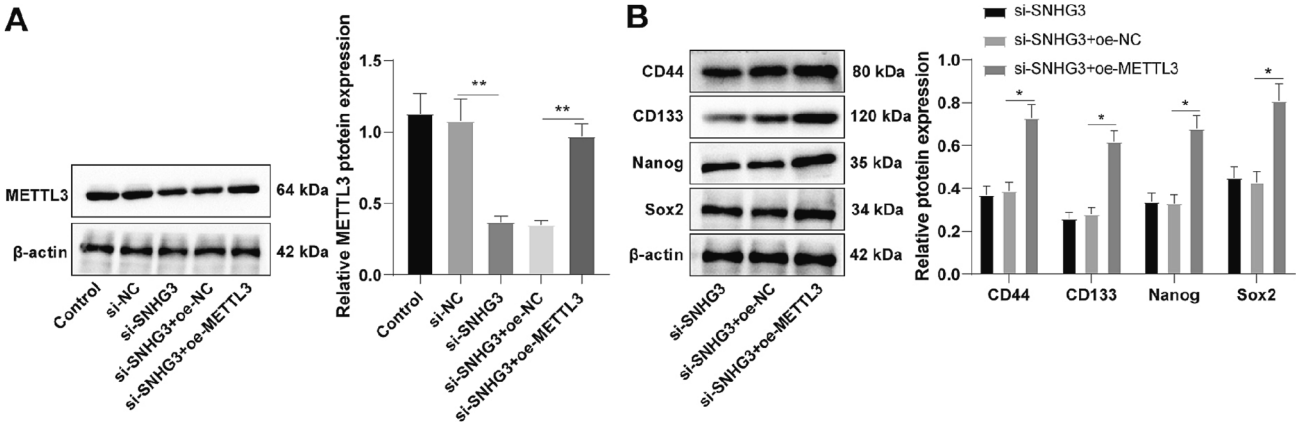

Fig. 5. LncRNA SNHG3 promotes the expression of HCC stem cell markers by regulating METTL3. Note: (A)Western blot was employed to assess the protein levels of METTL3 in Huh7 cells. (B) The expression of stemness-associated transcription factors, including CD44, CD133, Nanog, and Sox2, was also evaluated in these cells. The experiments were performed in triplicate, presenting the results as mean ± standard deviation. For statistical analysis, one-way analysis of variance (ANOVA) was utilized to compare multiple groups, followed by Tukey's multiple comparison tests for post-hoc analysis. Significance was set at *P < 0.05, with **P < 0.01 indicating higher significance.

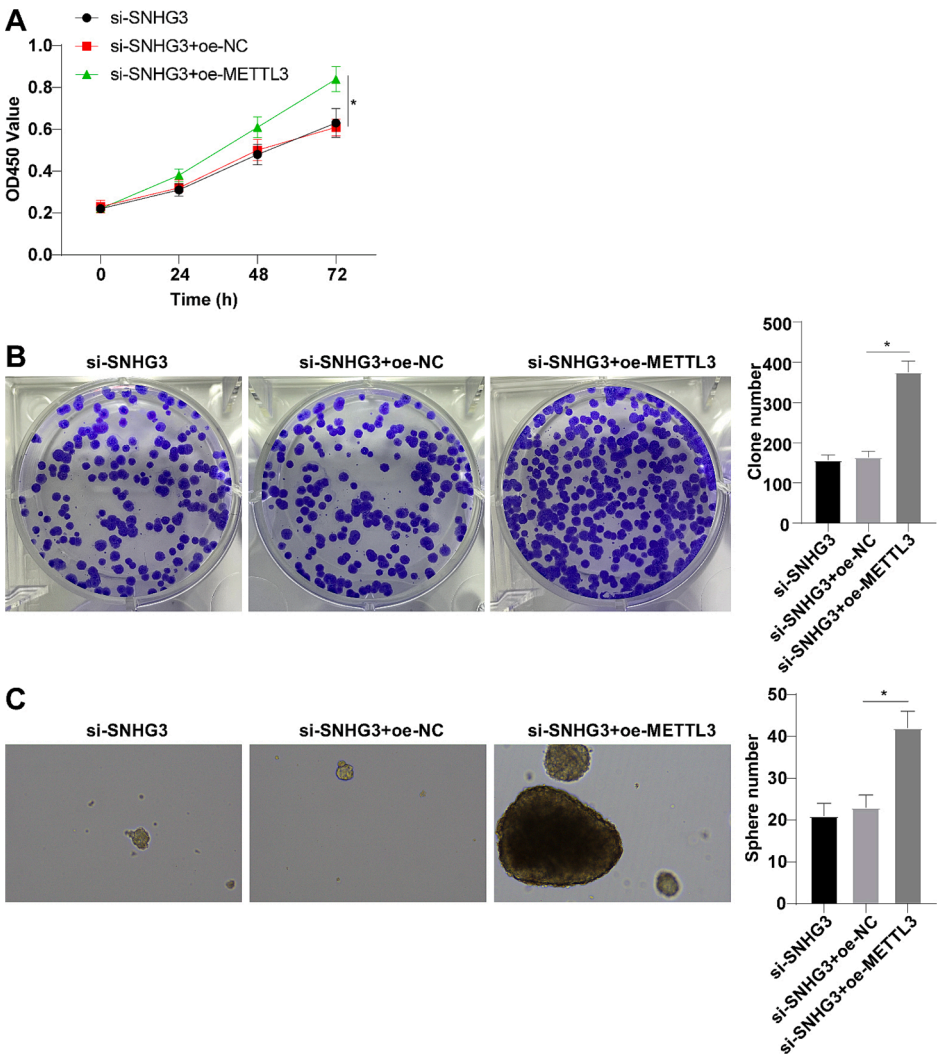

Fig. 6. LncRNA SNHG3 promotes self-renewal of HCC stem cells by regulating METTL3. Note: (A)The proliferation activity of Huh7 cells was assessed using the CCK-8 assay. (B) A soft agar colony formation assay was performed to evaluate the growth ability of Huh7 cells. (C) The self-renewal ability of Huh7 stem cells was evaluated using a tumor sphere formation assay. These experiments were repeated three times, and the results were expressed as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test for post-analysis. Statistical significance was set at *P < 0.05, with ** indicating P < 0.01.

significantly reduced (Li et al., 2022), decreasing stem cells' growth and self-renewal capacity.

ITGA6, a subunit of integrin α6, plays a crucial role in identifying and maintaining cancer stem cells (CSCs) in different tissues by interacting with the tumor microenvironment (Bigoni-Ordóñez et al., 2019). In particular, ITGA6 is a critical regulatory factor in HPV(+) head and neck squamous cell carcinoma and holds great promise as a therapeutic target (An et al., 2021). Moreover, ITGA6 serves as a membrane-bound stem cell marker in over 30 populations of stem cells, contributing to the regulation of stem cell fate determination and their interaction with the microenvironment (Struijk et al., 2020a; Struijk et al., 2020b; Zhou et al., 2018).

An essential post-transcriptional modification, RNA N6-methyladenosine (m6A) methylation, regulates gene expression. ITGA6 contains multiple m6A modification sites, and the m6A modification of ITGA6 is regulated by the methyltransferase METTL3 (Ying et al., 2024; Jin et al., 2019). In hepatocellular carcinoma (HCC), METTL3 is significantly upregulated and plays a critical role in m6A modification regulation (Zhang et al., 2022a; Zhang and Huang, 2023; Liu et al., 2020). Recent research has identified lncRNA SNHG3 as a marker associated with m6A modification in HCC cells (Yang et al., 2022; Zhang et al., 2022b). Therefore, lncRNA SNHG3 may enhance the self-renewal and maintenance of stemness in HCC stem cells by promoting METTL3-mediated m6A modification of ITGA6.

Our study showed a significant increase in cell proliferation when METTL3 was overexpressed in Huh7 cells with low lncRNA SNHG3 expression. This finding is consistent with Li et al., who demonstrated that METTL3 promotes HCC cell proliferation (Meng et al., 2022; Li et al., 2021). Additionally, overexpression of METTL3 resulted in increased self-renewal ability and levels of stem cell-related proteins in Huh7 stem cells. Previous studies have reported higher METTL3 expression in CSCs compared to non-stem cells, highlighting its role in enhancing stemness (Wang et al., 2023b; Wang and Tang, 2023; Ma and Ji, 2020; Kumari et al., 2023). Our findings further support the role of METTL3, regulated by lncRNA SNHG3, in promoting self-renewal and stemness maintenance in CSCs.

Using the SRAMP database, we predicted multiple potential m6A modification sites at the 3′ end of ITGA6 mRNA. MeRIP-qPCR experiments confirmed that METTL3 regulates the enrichment of m6A levels in ITGA6 mRNA, consistent with the findings of Jin et al. in bladder cancer research (Liu et al., 2022b; Sun et al., 2023; Zhang et al., 2022c; Jin et al., 2019). The relationship between SNHG3 and METTL3 has

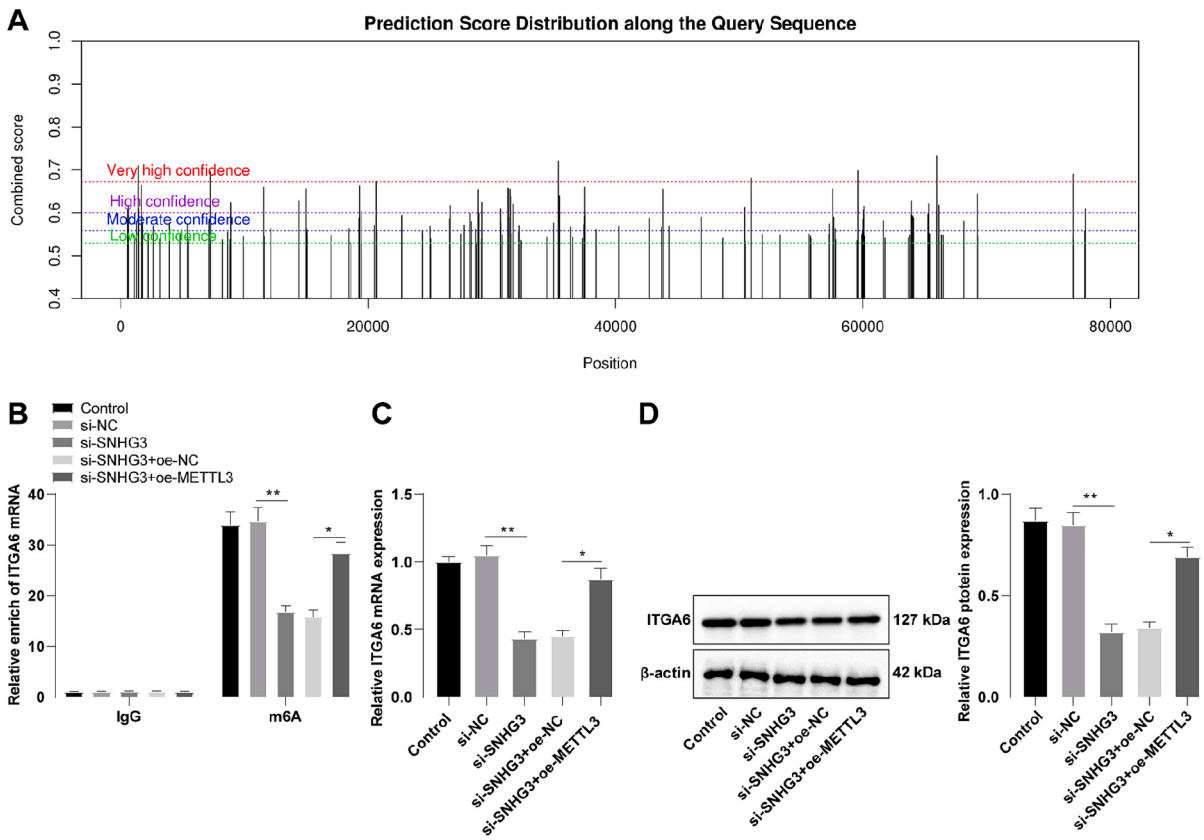

Fig. 7. LncRNA SNHG3 promotes ITGA6 mRNA expression through regulating METTL3-mediated m6A modification. Note: (A) The SRAMP database was utilized to identify potential m6A modification sites on the ITGA6 mRNA. (B) MeRIP-qPCR experiments were conducted to measure the enrichment level of m6A modification, specifically on the ITGA6 mRNA. (C) qRT-PCR was employed to evaluate the expression level of the ITGA6 mRNA. (D) Western blot analysis was performed to determine the protein level of ITGA6. The experiment was repeated three times, and the results were expressed as mean ± standard deviation. Multiple group comparisons were conducted using one-way ANOVA and post-hoc analysis using Tukey's multiple comparison test. Statistical significance was set at *$P < 0.05$, with ** indicating $P < 0.01$.

garnered widespread attention in various cancers. Literature indicates that SNHG3 regulates target gene expression by acting as a molecular sponge for miRNAs and enhances its stability and function through METTL3-mediated m6A modification. In esophageal cancer, SNHG3 regulates the expression of METTL3 by interacting with miR-186-5p, thereby affecting m6A modification levels (Zhang et al., 2021). Additionally, in melanoma and gastric cancer, METTL3 regulates SNHG3 expression through m6A modification, promoting tumor cell growth and invasion (Chu et al., 2023; Ji et al., 2023). These studies reveal the complex interplay between SNHG3 and METTL3 in cancer development and progression, suggesting they may serve as potential therapeutic targets. In HCC cells, we have discovered that lncRNA SNHG3 controls the self-renewal and maintenance of stemness in HCC stem cells by regulating METTL3-mediated ITGA6 m6A modification. Consequently, targeting METTL3-mediated ITGA6 m6A modification may hold potential for HCC stem cell therapy.

Although this study partially reveals the mechanism of SNHG3 in hepatocellular carcinoma (HCC), some limitations still need to be addressed. Firstly, the research relies on cellular experiments and lacks validation in animal models, which are crucial for understanding the in vivo interaction between SNHG3 and the tumor microenvironment. Future research should investigate the function of SNHG3 in animal models to better comprehend its role in HCC. Secondly, while this study explores the role of SNHG3 in HCC development through METTL3-mediated m6A modification of ITGA6 mRNA, SNHG3 may have other pathways and targets that need further exploration. Future studies should investigate additional mechanisms through which SNHG3 influences HCC.

Moreover, considering the pivotal role of the tumor microenvironment in tumor development, it would be essential to explore the impact of SNHG3 on the tumor microenvironment, particularly its effect on the immune microenvironment. Finally, exploring new therapeutic strategies targeting SNHG3, such as small molecule inhibitors or RNA interference techniques, will provide valuable insights for HCC treatment. Comprehensive investigations in these areas will contribute to further breakthroughs in treating hepatocellular carcinoma.

6. Conclusion

In summary, this study extensively examined the expression pattern and function of the long non-coding RNA (lncRNA) SNHG3 in hepatocellular carcinoma (HCC) and revealed its significant role in HCC development. The findings demonstrate that SNHG3 is notably upregulated in HCC tissues and cell lines, and its expression is closely associated with the pathological stage, metastatic status, and tumor size of HCC. Furthermore, reducing SNHG3 expression effectively inhibits the proliferation, colony formation, and tumor sphere formation of HCC stem cells. Notably, this study identifies that SNHG3 modulates the function of HCC stem cells through METTL3-mediated m6A modification of ITGA6 mRNA, providing insights into the pivotal role of SNHG3 in HCC stem cell biology (Fig. 8). These findings significantly impact our understanding of the molecular mechanisms underlying hepatocellular carcinoma. Firstly, by delineating the up-regulation of SNHG3 in HCC and its correlation with pathological features, this study introduces new biomarkers for the molecular classification and prognostic assessment of HCC. Moreover, it sheds light on the role of SNHG3 in regulating the

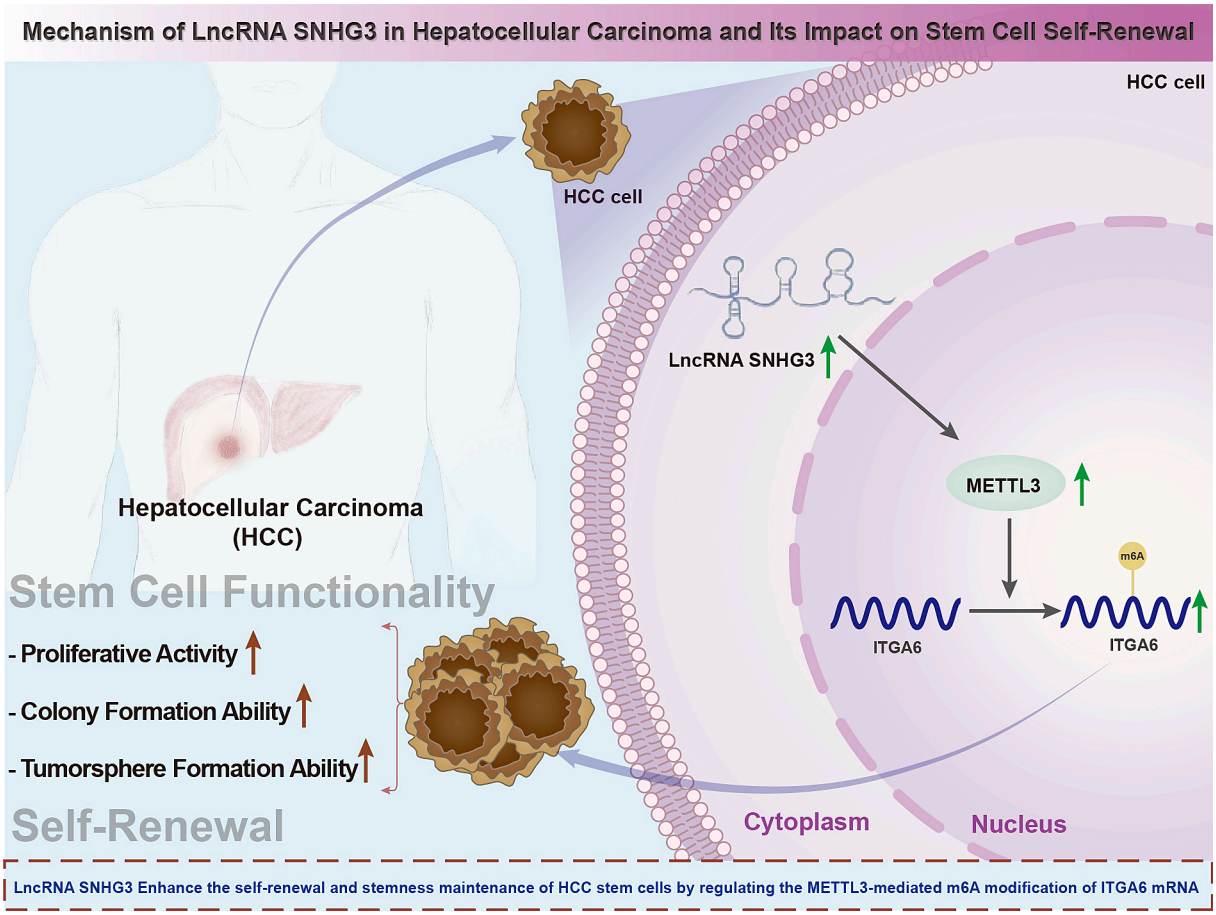

Fig. 8. Mechanisms of lncRNA SNHG3 in hepatocellular carcinoma and its impact on stem cell self-renewal.

function of HCC stem cells via METTL3-mediated m6A modification, presenting a novel perspective on the regulatory network of HCC stem cells. From a clinical standpoint, these findings offer potential therapeutic strategies targeting SNHG3, particularly for treating HCC stem cells.

7. Data availability statement

All data can be provided as needed.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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