LncRNA SNHG15 promotes hepatocellular carcinoma progression by sponging miR-141-3p

Junfeng Ye | Ludong Tan | Yu Fu | Hongji Xu | Lijia Wen | Yu Deng | Kai Liu

Abstract
Small nucleolar RNA host gene 15 (SNHG15) is a long noncoding RNA (lncRNA), which promotes progression of multiple cancers. Its specific function in hepatocellular carcinoma (HCC), however, is uncertain. The aims of our study were, therefore, to explore the role of SNHG15 in HCC. SNHG15 and miR-141-3p expression were assessed via quantitative real-time PCR (qRT-PCR) in 58 paired HCC samples and adjacent matched adjacent normal tissues. CCK-8 assay, flow cytometric examination, and wound healing/invasion assays were used to respectively assess how SNHG15 influences cell proliferation, the cell cycle, and the migratory and invasive potential of HCC cells. MicroRNA (miRNAs) that targeted SNHG15 was screened by Starbase2.0 and identified by RNA immunoprecipitation and luciferase reporter assays. SNHG15 expression was markedly increased, whereas miR-141-3p expression was substantially reduced in HCC cells and tissue samples relative to normal controls. When SNHG15 was knocked down, this resulted in a significant disruption to the proliferation, as well as the invasive and migratory ability of these HCC cells. miR-141-3p was also found to be an SNHG15 target in HCC cells. Furthermore, miR-141-3p inhibitor partially reversed the observed SNHG15 depletion-mediated reduction in HCC proliferation, migration, and invasion. By repressing miR-141-3p, SNHG15 could modulate zinc finger E-box binding homeobox 2 (ZEB2) and E2F transcription factor 3 (E2F3) expression, both of which are miR-141-3p targets. These finding suggested that SNHG15 promoted HCC progression via negative regulation of miR-141-3p, thus identifying a potential novel HCC treatment pathway.

KEYWORDS
hepatocellular carcinoma, lncRNA, miR-141-3p, SNHG15

1 | INTRODUCTION
Hepatocellular carcinoma (HCC) is encountered with the fifth highest frequency of tumor types, and it has high morbidity and high mortality. It was well known that genetic or epigenetic change was involved in HCC progression. Although many studies have explored pathways governing HCC progression, the outcomes of
patients with HCC remain unsatisfactory. There is thus an urgent need for the identification of novel HCC-related therapeutic targets.

Long noncoding RNAs (lncRNA) play roles in the initiation and progression of various cancers including HCC. lncRNAs are highly conserved noncoding RNAs without protein-coding abilities greater than 200 nucleotides in length. Importantly, lncRNAs play crucial functions as regulators of various processes, such as cellular differentiation and proliferation, apoptotic death, or cancer cell migratory and metastatic activity. lncRNAs were reported to implicate in regulating carcinogenesis, progression, and metastasis, and therefore have been suggested as novel regulators in the HCC.

Small nucleolar RNA host gene 15 (SNHG15), an important lncRNA, can promote the development and proliferation of epithelial ovarian cancer, lung cancer, colorectal cancer, pancreatic ductal adenocarcinoma, papillary thyroid carcinoma, breast cancer, glioma, and osteosarcoma. Although recently a study revealed that the HCC tissues exhibit increased SNHG15 expression, with expression being correlated to histological grade, TNM stage, vein invasion, as well as poor overall survival. However, biological functions and the underlying mechanism of SNHG15 in HCC remain uncovered.

Herein, we assessed SNHG15 expression in HCC cell lines and tissues, exploring its potential biological function in the proliferation, cell cycle status, and invasive and migratory potential of HCC cells. We also investigate the regulatory mechanism played by SNHG15 that in HCC progression.

2 MATERIALS AND METHODS

2.1 Samples collection

Patients with primary HCC who were undergoing primary surgical resection in the Department of Hepatopancreaticobiliary Surgery, the First Hospital of Jilin University from 2015-2016 served as the donors for 58 paired tumor and adjacent healthy tissue samples. Liquid nitrogen was used to snap freeze tissues upon isolation, and they were then stored at −80°C before use. The research was conducted in a manner consistent with the Declaration of Helsinki, with patients providing written informed consent. The Ethics Committees of the First Affiliated Hospital of Jilin University (Changchun, China) approved this study.

2.2 Cell lines and transfection

In total, four HCC cell lines (SMMC-7721, Hep3B, HepG2, and Huh-7), as well as a normal human LO2 liver cell line, came from the American Type Culture Collection (VA). DMEM (Gibco, NY) containing 10% FBS (Gibco) and penicillin/streptomycin (Invitrogen) was used for cell culture in a humid 37°C environment with 5% CO2 atmosphere.

Small interfering RNAs (siRNAs) against SNHG15 (si-SNHG15) and an appropriate nontargeting control (si-NC) bought from Sigma-Aldrich. miR-141-3p, a non-targeting miR control (miR-NC), and the miR-141-3p inhibitor (miR-141-3p in) bought from RiboBio (Guangzhou, China). Transfection was conducted using lipofectamine 2000 (Invitrogen) on the basis of provided protocols.

2.3 Quantitative real-time PCR

TRIlzol (Invitrogen) served to extract total RNA from cultured cells and tissues. Then, PrimeScriptRT Reagent Kit (Takara, Shiga, Japan) was used to synthesize the complementary DNA from extracted total RNA. miR-141-3p and SNHG15 levels were examined using the miR-ValaTMqRT-PCR microRNA (miRNA) Detection (Ambion, TX) and SYBR Premix ExTaq II (Takara) kits and using the ABI 7500 PRISM 7500 Platform (Applied Biosystems, CA), respectively. Relative miR-141-3p expression was compared to U6 for normalization, while that of SNHG15, ZEB2, and E2F3 was normalized to GAPDH according to $2^{-\Delta\Delta C_t}$ method. Primers from these analyses are listed at Table 1.

2.4 Cell proliferation

Cell proliferation was assessed via Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Kuma-moto, Japan). The cells that had been transfected were plated in 96-well plates (5 x 10³ /well) for between 24 and 72 hours, with 10 µL/well of CCK-8 solution at

| Target gene | Prime(5'-3') |
|-------------|-------------|
| U6          | F-TCCGATCGTGAAGCGTTTC R-GTCCAGGTCCAGGTGCAGAT |
| miR-141-3p  | F-GGCTGTAACTTATGTGCAGAC R-GACTTCTTATGACCCAGGAC |
| SNHG15      | F-GCTGAGGTGACCGTGCTCAT T-RGGAGGTTGAGGAGGAGGCA |
| ZEB2        | F-GGAGGAGGGAGGTGGTGGTGAAGAC R-GCCTCCACCTCTTATGGAGCA |
| E2F3        | F-TCACTGCTCTTTGCTCTTATGGGAGAC R-CACTGTTTCCATGCCCAACAC |
| GAPDH       | F-CGGAGGTCACGAGGATGTCAGTGGTGAAGAC R-AGGTGTCAGGAGGAGGAGGCA |

Abbreviations: F, forward; mRNA, messenger RNA; PCR, polymerase chain reaction; R, reverse.
appropriate time point (24, 48, or 72 hours) being added for 4 hours. Absorbance (450 nm) was assessed via an ELISA reader (Thermo Labsystems, Helsinki, Finland).

2.5 | Flow cytometry analysis

HCC cell cycle arrest was assessed via flow cytometry. Transfected cells were stained for 15 minutes using propidium iodide (PI; 50 μg/mL; Sigma-Aldrich) plus RNase A (200 μg/mL) at 4°C. A FACS Calibur machine (Beckman Coulter, CA) was used to collect the data, which was then used to assess G0/G1, S, and G2/M phase cell frequencies.

2.6 | Wound healing assay

Transfected cells were plated into six-well plates (5 × 10⁴ cells/well) until 100% confluent, and the monolayer was wounded with a sterile pipette tip, followed by 24 hours culture using the serum-free medium. Wound size both at baseline and following 24 hours were imaged via microscopy (Olympus Corporation, Tokyo, Japan) to determine to what extent cells had migrated.

2.7 | Transwell assay

A total of 1 × 10⁵ transfected cells in serum-free media were placed into the upper portion of a Matrigel-coated transwell chambers (Corning Inc, Corning, NY), whereas the lower portion of the chamber was filled using DMEM containing 10% to promote chemotaxis. After 48 hours, 4% paraformaldehyde was used to fix invasive cells, which were then stained using 1% crystal violet. Five random fields of stained cells were assessed via light microscopy (Olympus Corporation).

2.8 | Dual-luciferase reporter assay

Regions of SNHG15 believed to contain miR-141-3p binding sites, as well as a mutated version of this same sequence, were synthesized and cloned into a psiCHECK2 (Promega, WI) luciferase reporter vector, named SNHG15-Wt and SNHG15-Mut. The vectors were sequenced and cotransfected into SMMC-7721 cells along with miR-NC or miR-141-3p. After being cotransfected for 48 hours, a Dual-Luciferase Reporter Assay Kit (Promega, Shanghai, China) was used to examine luciferase activity.

2.9 | RNA-binding protein immunoprecipitation

RNA-binding protein immunoprecipitation (RIP) was assessed via a Magna RNA-RIP Kit (Millipore) on the basis of provided directions. Briefly, complete RNA lysis buffer (Millipore) was used to lyse SMMC-7721 cells, and magnetic beads conjugated to either control mouse IgG or human anti-Argonaute2 (Ago2) were then mixed with this lysate, after which immunoprecipitated RNA was collected for purification. Quantitative real-time PCR (qRT-PCR) was then conducted as above.

2.10 | Statistical analysis

The data are means ± standard deviation (SD), with SPSS v19.0 (SPSS Inc, IL) used for all analyses. The groups were compared via one-way ANOVA and the Student t tests. The correlation was analyzed on the basis of Pearson correlation coefficients. A P < .05 was the significance threshold. Potential lncRNA–miRNA interactions were identified with Starbase2.0 (http://starbase.sysu.edu.cn/).

3 | RESULTS

3.1 | Elevated SNHG15 expression was evident in HCC

We first used qRT-PCR to assess SNHG15 expression in HCC cells and tissues. As expected, SNHG15 was upregulated in HCC tissues relative to healthy controls (Figure 1A). Consistent with this result, HCC cell lines also exhibited increased SNHG15 expression compared with the LO2 control liver cells (Figure 1B). This suggested that SNHG15 might be important in the context of HCC.

3.2 | SNHG15 knockdown inhibited the proliferation of HCC cells and resulted in cell cycle arrest at the G1/G0 phase

We next performed SNHG15 knockdown in the SMMC-7721 cells using an appropriate siRNA to explore how SNHG15 functions in the context of HCC (Figure 2A). Then, the CCK-8 assay was performed to assess the importance of this lncRNA for HCC proliferation, revealing that SNHG15 knockdown markedly decreased HCC cell proliferation over 48 to 72 hour time periods following siRNA transfection (Figure 2B). SNHG15 knockdown also increased G1/G0 phase cell cycle arrest and reduced the S phase arrest, as determined via flow cytometry (Figure 2C).

3.3 | Knockdown of SNHG15 inhibited HCC cell migratory and invasive ability

We further assessed how SNHG15 affected HCC cell migratory/invasive potential abilities using respective
wound healing and transwell-based invasive assays. We found knocking down SNHG15 to significantly decrease both migratory and invasive capabilities in SMMC-7721 cells (Figure 3A,B).

3.4 | SNHG15 in HCC cells bound miR-141-3p

A hypothesis identifying lncRNAs as competing endogenous RNAs (ceRNAs) has proposed that they can...
regulate miRNA expression via binding them in a competitive fashion. Thus, we measure the expression of SNHG15 in SMMC-7721 cell nuclear and cytoplasmic fractions via qRT-PCR. We found SNHG15 to be mainly located in the cytoplasm, suggesting that SNHG15 might exert the functions of ceRNA in HCC cells (Figure 4A). Next, starbase2.0 was employed for prediction of SNHG15 targeting miRNAs, with miR-141-3p being identified as a target of relevance on the basis of its biological function in tumor progression (Figure 4B). Luciferase reporters confirmed miR-141-3p binding to SNHG15 in SMMC-7721 cells (Figure 4C). To assess if SNHG15 and miR-141-3p interact in a manner that is dependent upon Ago2, we conducted RIP, revealing SNHG15 and miR-141-3p to be preferentially enriched among Ago2-containing microribonucleoproteins (Figure 4D). SNHG15 knockdown significantly increased SMMC-7721 cell miR-141-3p expression (Figure 4E), whereas overexpressing miR-141-3p declined SNHG15 expression (Figure 4F). Relative miR-141-3p expression in the HCC cell lines and tissues was also measured, revealing it to be markedly decreased in HCC tissues and cell lines relative to corresponding controls (Figure 4G and H). SNHG15 was also found to negatively correlate with miR-141-3p (Figure 4I). On the basis of all the above results, we concluded that SNHG15 targets miR-141-3p in HCC cells.

3.5 | SNHG15 modulates miR-141-3p to promote HCC progression

We next sought to determine whether SNHG15-mediated phenotypes pertaining to HCC proliferation and invasion were miR-141-3p-dependent via conducting rescue assays by transfecting miR-141-3p into SNHG15-depletion-SMMC-7721 cells. We found miR-141-3p inhibitors to reverse the SNHG15 knockdown-mediated increase in miR-141-3p levels (Figure 5A). Furthermore, SNHG15 knockdown-mediated inhibitory effects on cell proliferation, cycle arrest, migration/invasion were partially

![Figure 3](image-url)
rescued using miR-141-3p inhibitors in SMMC-7721 cells (Figure 5B-E). These results suggested that SNHG15 promoted HCC cells progression via modulating miR-141-3p.

3.6 SNHG15 modulated ZEB2 and E2F3 expression by repressing miR-141-3p

miR-141-3p is known to suppress HCC by regulating multiple genes, including E2F3 and ZEB2.21,22 Thus, we explored the relationship between ZEB2/E2F3 and SNHG15 in HCC. The correlation analysis detected SNHG15 expression to be positively correlated with ZEB2 and E2F3 expression in HCC tissues (Figure 6A,B). Rescue assays showed that downregulation of SNHG15 expression notably decreased ZEB2 and E2F3 expression in SMMC-7721 cells, whereas miR-141-3p could partially reverse this trend (Figure 6C,D). These results suggested that SNHG15 modulated the expression ZEB2 and E2F3 through sponging miR-141-3p in human HCC cells.
4 | DISCUSSION

lncRNAs act either as a tumor suppressor or oncogenes in HCC via regulating tumor-related gene or miRNA expression in tumorigenesis.9,10 Bai et al reported the lncRNA A1BG antisense RNA 1 (A1BG-AS1) to act as a ceRNA, inhibiting HCC proliferation and invasion via sponging miR-216a-5p.23 Zhang et al. found KTN1-AS1 to drive HCC growth by targeting miR-23c/ERBB2IP axis.24 Zhou et al. revealed that lncRNA-H19 targeted miR-15b to activate CDC42/PAK1 signaling to facilitate HCC cell proliferation, migration, and invasion.25 We found that SNHG15 drove the progression of HCC through its ability to act as a ceRNA, directly sponging miR-141-3p to regulate E2F3 and ZEB2, highlighting SNHG15 as a possible HCC therapeutic target.

SNHG15, encoded at position 7p13, reportedly acts as an oncogene linked with progression of multiple types of cancer. Although SNHG15 expression has been shown to be increased in HCC tissues, and upregulation SNHG15 was closely associated with histologic grade, TNM stage, vein invasion as well as overall survival,19 how it affects the underlying biology remained uncertain. Herein, we showed that HCC tissues have a high expression of SNHG15, which was consistent with the previous study.19 SNHG15 knockdown had a clear inhibitory effect on cell proliferation, resulting in the G1/G0 phase cell cycle arrest. Furthermore, SNHG15 knockdown significantly decreased HCC cell migratory and invasive abilities. Our findings suggested that SNHG15 could exert an oncogenic role in HCC progression.

lncRNAs can mediate their biological roles by serving as ceRNAs and, thereby, sequestering miRNAs.20,26 In this study, SNHG15 primarily localized to the cytoplasm of HCC cells, suggesting that SNHG15 might exert the functions of ceRNA. Next, Starbase2.0 was employed as a means of predicting SNHG15 targeting miRNAs, with the identified miR-141-3p being of particular interest based on its biological function in HCC. miR-141-3p was downregulated in HCC tissues, serving as a tumor suppressor that targets several genes to inhibit proliferation and invasion in these cancer cells.21,22,27 Luciferase reporter and RIP assays further confirmed miR-141-3p was a SNHG15 target in HCC. miR-141-3p expression was substantially decreased in HCC tissues, and its expression was negatively correlated with miR-141-3p. Moreover, changes to cell proliferation, cycle arrest,
migration and invasion upon SNHG15 knockdown could be partially rescued by miR-141-3p inhibitors in SMMC-7721 cells. These results suggested that SNHG15 promoted HCC progression via modulating miR-141-3p.

It was well known that lncRNAs could harbor miRNA to indirectly regulate its target genes. ZEB2 and E2F3 were known to be miR-141-3p targets in HCC. In addition, ZEB2 and E2F3 were reported to play an oncogenic role in HCC. Thus, we investigate whether SNHG15 could regulate ZEB2 and E2F3 in HCC. Rescue assays showed that downregulation of SNHG15 expression notably decreased ZEB2 and E2F3 expression in SMMC-7721 cells, whereas miR-141-3p could partially reverse this trend. Moreover, the SNHG15 expression was positively correlated with ZEB2 and E2F3 levels in HCC tissues. This suggested that SNHG15 modulated the expression ZEB2 and E2F3 through sponging miR-141-3p in human HCC cells.

Together, our results indicate that SNHG15 is upregulated in HCC cell lines and tissues. Molecular mechanism exploration reveals that SNHG15-mediated oncogenic effect in HCC through acting as a sponge for miR-141-3p to control ZEB2 and E2F3 expression. Future efforts will be necessary to explore other miRNAs targeted by SNHG15 in HCC, but our findings offer valuable insights regarding HCC pathology and offer a theoretical basis for developing novel treatment of patients with HCC.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.
Kai Liu  http://orcid.org/0000-0002-8840-0054

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