LncRNA-SNHG6 promotes the progression of hepatocellular carcinoma by targeting miR-6509-5p and FKBP1A

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Abstract

Accumulating evidences has been reported that long noncoding RNAs play crucial roles in the progression of hepatocellular carcinoma (HCC). snoRNA host gene 6 (SNHG6) is believed to be involved in several human cancers, but the specific molecular mechanism of SNHG6 in HCC is not well studied. Here, we found SNHG6 was highly expressed in HCC tissues. Next, using Hep3B and Huh7 cells, we confirmed knockdown of SNHG6 could reduce the proliferation, migration and invasion abilities in vitro. Also, by bioinformatics analysis, further molecular and cellular experiments, we found miR-6509-5p bound to SNHG6 directly, and the expression level of FKBP1A was regulated through SNHG6 / miR-6509-5p axis. Finally, we found that down-regulation of SNHG6 could dramatically reduce the tumor growth ability of Huh7 cells in vivo. Taking together, we concluded that SNHG6/miR-6509-5p/FKBP1A axis functioned in the progression of hepatocellular carcinoma, and could be the promising therapeutic targets in hepatocellular carcinoma.

Keywords: SNHG6, miR-6509-5p, FKBP1A, hepatocellular carcinoma, proliferation, migration, invasion

Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies, accounting around 75% among all liver cancers [1]. The progression of HCC is contributed with several factors, involving hepatitis infection, environmental toxin, metabolic and genetic factors, which finally leads to cirrhosis and then HCC [2-7]. Increasing studies about the genetic factors have been reported,
however, the pathophysiologic mechanisms involved in the progression of HCC are complicated and still unclear, which limits the therapeutic effects.

Large amount of evidences reveals the genetic factors, especially the protein coding genes, contributes to the progression of HCC [8]. However, noncoding RNAs (ncRNAs), including long ncRNAs and small ncRNAs, are found to be involved in HCC progression in a competing endogenous RNA manner by directly binds to corresponding microRNAs [9-11]. Although the roles of IncRNAs are involved in diverse aspects in HCC progression, such as the proliferation, differentiation, and cell death, the identification of potential candidate IncRNAs remains at the early stage. SNHG6 (snoRNA host gene 6), a house keeping gene of the 5′ terminal oligopyrimidine family, is identified as a novel oncogene in diverse human cancers, including colorectal cancer, gastric cancer, ovarian clear cell carcinoma, human osteosarcoma and hepatocellular carcinoma [12-18]. For the complicated factors involved in HCC progression, the role of SNHG6 on the mechanisms of HCC initiation and progression still remains unknown.

Here, we found SNHG6 levels was remarkably increased in HCC tissues, especially in the aggressive cases of HCC. To further explore the the role of SNHG6 on HCC progression using Hep3B and Huh7 cells, we found SNHG6 functioned on the proliferation, migration and invasion abilities of HCC. Then, we identified miR-6509-5p as the corresponding binding partner of SNHG6, and confirmed SNHG6 indeed functioned as a sponge of miR-6509-5p. We further found the protein regulated by SNHG6/miR-6509-5p axis was FKBP1A. Finally, by tumor growth assays using nude mice, we confirmed down-regulation of SNHG6 could significantly reduce the tumor size by decreasing the proliferation ability in HCC tissues. Taking together, we suggested SNHG6/miR-6509-5p/FKBP1A functioned in the progression of HCC, and could be potential treatment targets in HCC.

Methods

Ethics for HCC samples collecting and animal experiments
HCC samples from patients were collected under the guidelines of the Ethics Committee. And the animal experiments were conducted under the guidelines of the Animal care and use committee. All nude mice were purchased from Shanghai SLAC Laboratory Animal Co.,Ltd, and housed under room temperature and a 12-h light-dark cycle.

The immune-deficient nude mice were injected with 5 x 10⁶ stable SNHG6 down-regulated Huh7 cells subcutaneously, and housed under specific-pathogen free environment. Following the surgery, the tumor size was checked daily, and the tumor size was analyzed.

Total RNA extraction and quantitative PCR
Total RNA from human HCC samples and cell lines were extracted using Beyozol (R0011, Beyotime, Shanghai) according to the standard guidelines. The extracted total RNA was the reverse transcribed to complementary DNA using BeyoRT cDNA synthesis kit (D7168L, Beyotime,
Shanghai). The expression levels of SNHG6 and FKBP1A were quantitatively quantified using qPCR SYBR Green Master Mix (DRR041A, Takara, China). GAPDH was selected as the internal reference gene.

**Culture and transfection of Hep3B and Huh7**

Hep3B and Huh7 cells were purchased from ATCC and cultured in supplemented Minimum Essential Medium with Earle's Balanced Salts (51415C, Sigma, America) supplemented with 10% FBS (fetal bovine serum) and 1% PS (penicillin and streptomycin). Cells were cultured at 37°C using a humidified incubator containing a 5% CO2 and 95% air. Huh7 cells were infected with lentivirus containing sh-control and sh-SNHG6 vectors to construct the SNHG6 stable down-regulated cell line. Cell transfection assays were conducted using Lipofectamine 3000 (L3000075, ThermoFisher, American) according to the standard procedures.

**Immunohistochemical staining**

The cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature, washed with PBS for 3 times, and incubated with 0.5% Triton X-100 for 20 minutes. The proliferation status of the cells were then measured using BeyoClick™ EdU Cell Proliferation Kit (C0071, Beyotime, Shanghai). The human samples were collected, incubated in 4% PFA for 12 hours, and sectioned into 20μm slices. The slices were further incubated in 1% Triton X-100, primary antibody solutions (Ki67, ab15580, Abcam, American) and secondary antibody solutions.

**Western blot**

The lysate from Hep3B and Huh7 cells were prepared using RIPA lysis buffer (radio-immunoprecipitation assay buffer), and the protein concentration was measured with Pierce™ BCA Protein Assay Kit (23225, ThermoFisher, American). Finally, the level of SOX2 was detected with the anti-SOX2 antibody (2748, Cell signaling, American).

**Migration and invasion assays**

To assess the migration and invasion abilities of cells after down-regulation of SNHG6 and up-regulation of miR-6509-5p, 12 mm Transwell® with 3.0 μm Pore Polycarbonate Membrane Insert (3402, Corning, American) were employed. For the migration ability assessment, Hep3B and Huh7 cells were respectively seeded in the lower chamber at the density of 3 x 10^5 cells. Cells were incubated till covering the entire bottom, and then scratched using the pipette tips, cultured for two days. For the invasion ability assessment, the chamber of the transwell insert was coated with Matrigel. And Hep3B and Huh7 cells were respectively seeded in the chamber at the density of 3 x 10^5 cells. Two days later, cells without migration were gently removed. Cells were fixed and then stained with 0.1% crystal violet. Finally, the wound-healing status of cells was imaged using the optical microscope.

**Luciferase assay**

For the purpose of confirm the direct binding between SNHG6 and miR-6509-5p, SNHG6 fragment with the miR-6509-5p binding site and SNHG6 with mutated miR-6509-5p binding site were subcloned into the luciferase reporter vectors. The SNHG-WT or SNHG6-MUT luciferase reporter
vectors and miR-6509-5p were co-transfected into Hep3B cells. To confirm the direct binding of miR-6509-5p and 3'UTR of FKBP1A mRNA, we constructed luciferase reporter vectors of miR-6509-5p using the same strategy. The dual-luciferase reporter assay system (E1910, Promega, American) were employed to measure the luciferase activities under the guidelines supplied by the manufacturer.

**RNA immunoprecipitation assay**
MiR-6509-5p mimics and miR-6509-5p-NC mimics were respectively transfected into Hep3B cells using Lipofectamine 3000, and the lysate of the collected cells were prepared with centrifugation. To conduct the RNA immunoprecipitation assay, anti-AGO antibodies (MA5-23515, ThemoFisher, American) were employed. And to confirm the direct binding of miR-6509-5p and SNHG6, the expression level of SNHG6 were measured using RT-quantitative PCR.

**Statistical analysis**
All statistical analyses were performed using GraphPad 8.0 under Student’s t test, and all data were presented as mean ± SEM from 3 to 6 independent experiments. Overall survival (OS) and progression-free survival (PFS) were presented using Kaplan-Meier curves. *P < 0.05, **P < 0.01, ***P < 0.001.

**Results**

**SNHG6 contributed to the progression of hepatocellular carcinoma (HCC)**
We first explored the expression levels of SNHG6 in hepatocellular carcinoma (HCC) patients, and found increased SNHG6 levels in HCC patients compared to the non-HCC controls, especially in aggressive HCC cases (Figure 1A). As SNHG6 level was positive correlated with HCC, we further conducted the analysis of overall survival (OS) and progression-free survival (PFS) based on Kaplan-Meier curves. The results shown that the expression level of SNHG6 was negatively correlated with abbreviated OS and PFS (Figure 1B, 1C), which indicated SNHG6 contributed to the progression of HCC.

**Down-regulation of SNHG6 inhibited the progression of HCC in vitro**
We then investigated the role of SNHG6 in the progression of HCC using two HCC cell lines, Hep3B and Huh7. First, we down-regulated SNHG6 using siRNAs in both cell lines, and found that SOX2 protein level was significantly decreased after knockdown of SNHG6, indicating the role of SNHG6 on the proliferation ability of HCC cells (Figure 2A). Also, knocking down of SNHG6 led to decreased proliferation ability of Hep3B and Huh7 cells, which was revealed using EdU labeling (Figure 2B, 2C, 2D). Furthermore, we explored the role of SNHG6 on the migration and invasion abilities in HCC by conducting transwell assays. The results shown that knockdown of SNHG6 weakened the migration and invasion abilities of both Hep3B and Huh7 cells (Figure 2E, 2F). In summary, the in vitro data collected using Hep3B and Huh7 cells confirmed that SNHG6 indeed contributed to HCC by affecting the proliferation, migration and invasion abilities of HCC cells.
SNHG6 functioned by directly binding to miR-6509-5p

Increasing evidence shown that IncRNAs played as a molecular sponge to directly binding to miRNAs, and further affected the expression level of the targeted protein by degrading corresponding mRNAs. Therefore, we conducted bioinformatics analysis and discovered miR-6509-5p as the potential partner of SNHG6. To test the directly binding of SNHG6 and miR-6509-5p, we constructed corresponding luciferase reporter vectors as shown (Figure 3A). The significantly decreased luciferase activity of the SHNG6-WT and miR-6509-5p mimics group confirmed the binding effect (Figure 3B). Furthermore, the directly binding effect in the AGO2-dependent manner was verified using the RNA immunoprecipitation assay (Figure3C). The expression level of miR-6509-5p was significantly increased after knockdown of SNHG6 (Figure 3D). Also, the expression level of miR-6509-5p in HCC patients was significantly higher compared to the health controls (Figure 3E). To verify whether up-regulated miR-6509-5p mimicked the si-SNHG6 phenotype, we analysed the role of miR-6509-5p on the progression of HCC. Results shown that corresponding to down-regulation of SNHG6, up-regulation of miR-6509-5p using mimics decreased the proliferation, migration and invasion abilities of Hep3B and Huh7 cells (Figure 3F, 3G, 3H, 3I, 3J). We concluded that SNHG6 functioned through binding to miR-6509-5p.

The expression of FKBP1A was regulated by SNHG6/miR-6509-5p axis

Next, to explore the potential protein effector regulated by SNHG6/miR-6509-5p axis, we used TargetScan analysis and identified that the 3’UTR of FK506-binding protein 12 (FKBP1A) mRNA. FKBP1A was reported to be involved in several cellular functions, including programmed cell death [19]. Consistent with our previous findings, increased expression of FKBP1A in HCC was found. To verify whether FKBP1A was regulated by SNHG6/miR-6509-5p axis, we constructed luciferase reporter vectors as illustrator (Figure 4A). The significantly reduced luciferase activities suggested the direct binding of miR-6509-5p and the 3’UTR of FKBP1A mRNA (Figure 4B). Also, up-regulation of miR-6509-5p using mimics significantly suppressed the expression of FKBP1A, and down-regulation of miR-6509-5p using inhibitors or SNHG6 increased the expression of FKBP1A (Figure 4C, 4D).

Down-regulation of SNHG6 inhibited the progression of HCC in vitro

After in vitro studies, we further explored the role of SNHG6 on the progression of HCC. We constructed stable down-regulated SNHG6 Huh7 cells, and injected into nude mice subcutaneously. The tumor size was observed daily. Results shown that down-regulation of SNHG6 led to significantly decreased tumor volume (Figure 5A, 5B). We further analyzed the HCC tissue by Ki67 labeling, and found the number of proliferating cells in the HCC tissue was significantly reduced (Figure 5C, 5D).

Conclusions

Hepatocellular carcinoma (HCC) remains to be a prevalent liver cancer for the poor prognosis and high recurrence rate [20]. HCC progression is contributed by diverse factor, especially genetic factor. Recently, including SNHG6, several long ncRNAs are identified as the potential targets responsible for HCC progression [18, 21-23]. However, the functional roles of SNHG6 in HCC progression
remains unclear. In this study, we found the expression level of SNHG6 was positively correlated with HCC progression using human HCC patient samples, and negatively correlated with shorter overall survival (OS) and progression-free survival (PFS) of HCC patients. Next, we further explored the role of SNHG6 on HCC progression on several aspects, including proliferation, migration and invasion ability assays, and found knockdown of SNHG6 led to slower HCC progression.

Long noncoding RNAs (lncRNAs) are noncoding RNAs which is longer than 200 nucleotides, and increasing data shown lncRNAs were involving in diverse cancers, including HCC [15, 18, 24-29]. lncRNAs typically functions in a ceRNA manner. Therefore, we conducted bioinformatics analyses and identified miR-6509-5p as the potential binding target of SNHG6. The luciferase assay and RIP AGO assay confirmed the directly binding between SNHG6 and miR-6509-5p. In addition, we up-regulated miR-6509-5p in Hep3B and Huh7 cells, and found the cancer progression of cells with miR-6509-5p mimics was slowed down, indicating SNHG6 indeed functioned as the sponge of miR-6509-5p.

Next, we conducted TargetScan analysis to investigate the potential protein targets of miR-6509-5p, and found the 3’UTR of FK506-binding protein 12 (FKBP1A) mRNA contained the binding sites for miR-6509-5p. FKBP1A is 12KD cis-trans peptidyl-prolyl isomerase, and expressed in a wide range of cell types, and plays important roles in the formation of ventricular walls and several human cancers [19, 30, 31]. The remarkably decreased luciferase activities indicated the direct binding between miR-6509-5p and the 3’UTR of FKBP1A mRNA. Also, the expression level of FKBP1A was down-regulated by using miR-6509-5p mimics and up-regulated using miR-6509-5p inhibitor, confirming FKBP1A was the protein target of miR-6509-5p.

Finally, we performed the tumor growth assay using stable down-regulated SNHG6 Huh7 cells, and decreased tumor volume indicated knockdown of SNHG6 inhibited HCC progression. To explore the proliferation status of HCC tissues, we collected the tumor tissue and stained with Ki67, a proliferation marker, and found the decreased proliferation in the HCC tissues.

To summarize, in this study, we discovered LncRNA-SNHG6 was highly expressed in HCC tissues and contributed to the proliferation, migration and invasion abilities of HCC. Furthermore, we identified the expression level of FKBP1A was regulated through SNHG6/miR-6509-5p axis in a ceRNA manner. Finally, based on both in vitro and in vivo data, we could conclude that LncRNA-SNHG6 promotes HCC progression by miR-6509-5p/FKBP1A axis, and the molecular pathway may provide novel potential targets for the development of HCC drugs.

Ethics approval and consent to participate
HCC samples from patients were collected under the guidelines of the Ethics Committee. And the animal experiments were conducted under the guidelines of the Animal care and use committee.

Consent for publication
All authors agreed the publication of this study.

**Availability of data and material**
The data in this study for supporting the results are included within the article.

**Competing interests**
None.

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**Authors' contributions**
ZZW, XM and JJS conceived and designed this study. ZZW, SJJ, ZDK, WFZ, TJF and XM conducted the experiments and analysis. JJS wrote the manuscript with information and critical comments supplied by ZZW, SJJ, ZDK, WFZ, TJF and XM. JJS is responsible for the supervision of this study and the acquisition of fundings.

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Figure 1. Increased expression level of SNHG6 was found in hepatocellular carcinoma (HCC) and led to poor prognosis. (A) The expression level of SNHG6 was increased in hepatocellular carcinoma patients compared to normal controls, especially in aggressive HCC cases. (B) Overall survival (OS) and (C) progression-free survival (PFS) using Kaplan-Meier analysis between low SNHG6 expression and low SNHG6 expression patients with HCC. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 2. Down-regulation of SNHG6 inhibited the progression of HCC in vitro. (A) The expression level of Sox2 of Hep3B and Huh7 cells after down-regulation of SNHG6 was measured with western blotting. The proliferation ability of HCC cell lines, (B) Hep3B and (C) Huh7, was detected with EdU labeling. (D) EdU positive cells per field were calculated. (E) The migration and (F) invasion abilities of HCC cell lines after down-regulation of SNHG6 was determined with the transwell assay. *P < 0.05, **P < 0.01, ***P < 0.001. Data were presented as mean ± SD from 6 independent experiments.
Figure 3. SNHG6 functioned as a sponge of miR-6509-5p. (A) The illustrator of the luciferase assay to test the binding between SNHG6 and miR-6509-5p. (B) SNHG6-WT or SNHG6-MUT was co-transfected with miR-6509-5p, and the luciferase activities of SNHG6 reporter vector were measured. (C) The binding between SNHG6 and miR-6509-5p was further confirmed using RNA immunoprecipitation (RIP) with the anti-AGO2 antibody. (D) The relative expression level of miR-6509-5p was detected using Hep3B and Huh7 cells. (E) The expression level of miR-6509-5p in
HCC patients was measured. (F, G) The proliferation ability of HCC cell lines after up-regulation of miR-6509-5p with miR-6509-5p mimics was detected using EdU labeling, and (H) EdU positive cells per field were calculated. (I, J) The migration and (F) invasion abilities of HCC cell lines after up-regulation of miR-6509-5p was evaluated using the transwell assay. *P < 0.05, **P < 0.01, ***P < 0.001. Data were presented as mean ± SD from 6 independent experiments.
Figure 4. The expression of FKBP1A was regulated by SNHG6/miR-6509-5p axis. (A) The illustrator of the luciferase assay to test the binding between miR-6509-5p and FKBP1A. (B) Luciferase activity of miR-6509-5p was measured to test the binding effect between miR-6509-5p and FKBP1A. (C) The expression level of FKBP1A in Hep3B and Huh7 cells after treating with up- and down-regulation of miR-6509-5p were detected with quantitative PCR. *P < 0.05, **P < 0.01, ***P < 0.001. Data were presented as mean ± SD from 3 independent experiments.
Figure 5. Down-regulation of SNHG6 suppressed the HCC growth in vivo. (A) The tumor growth ability of normal Huh7 cells and stable SNHG6 down-regulated Huh7 cells were evaluated using nude mice. (B) The tumor size was calculated. (C) The proliferated cells in the HCC tissues was labeled with Ki67 using immunohistochemistry methods. Ki67 positive cells were labeled brown. (D) Ki67 positive cells per field were calculated as the index of the proliferation status in the tumor tissues. ***P < 0.001. Data were presented as mean ± SD from 6 independent experiments.