

Overexpressing lncRNA *SNHG16* inhibited HCC proliferation and chemoresistance by functionally sponging hsa-miR-93

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Background: Long noncoding RNAs (lncRNAs) have been identified as prognostic biomarkers and functional regulators in human cancers. The present study aimed to determine the expressions and functions of an lncRNA, *Small Nucleolar RNA Host Gene 16 (SNHG16)*, in hepatocellular carcinoma (HCC).

Patients and methods: *SNHG16* expressions were tested by quantitative real-time PCR (qRT-PCR) in HCC cell lines, as well as 43 pairs of HCC tissues and pair-matched healthy hepatic tissues. It was overexpressed in Hep3B and HuH7 cells. The effects of *SNHG16* overexpression in HCC in vitro proliferation, 5-fluorouracil (5-FU) chemoresistance, and in vivo tumor growth were tested. A potential microRNA (miRNA) sponge target of *SNHG16*, hsa-miR-93, was tested by luciferase reporter assay and qRT-PCR. In addition, hsa-miR-93 was upregulated in *SNHG16*-overexpressed HCC cells to examine its effect on *SNHG16*-mediated cancer cell functional regulation in HCC.

Results: *SNHG16* levels were markedly downregulated in both HCC cell lines and HCC tissues. Lentivirus-mediated *SNHG16* overexpression inhibited HCC cell proliferation, 5-FU chemoresistance, and in vivo tumor growth. Hsa-miR-93 was confirmed to be directly sponging on *SNHG16*. Its upregulation in HCC cells reversed *SNHG16* overexpression and induced tumor-suppressing effects in HCC cells.

Conclusion: Our data demonstrate that *SNHG16* plays a critical role in HCC development via functionally sponging hsa-miR-93.

Keywords: lncRNA, *SNHG16*, miRNA, hsa-miR-93, proliferation

Introduction

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed human cancers in the world.^{1,2} In developed countries, HCC is the sixth leading cause of cancer-related death in men.¹ However, in less developed countries, including China, HCC has become the second leading cause of cancer-related death in men.¹ Every year, nearly 1 million new HCC cases and more than ¾ million HCC-associated deaths occur worldwide, with China accounting for >50% of the total number of cases and deaths.^{1,2} The underlying pathological mechanisms of HCC may be very complicated, though most HCC cases may be attributed to chronic infection with hepatitis B or C virus.^{3,4} Unfortunately, despite recent advances in HCC early diagnosis and specialized HCC treatments, cancer patients still suffer from poor prognosis and high recurrence rate.⁵⁻⁷ Therefore, it is of crucial importance to understand the pathophysiological conditions contributing to HCC carcinogenesis and development, in order to seek new diagnosis and treatment strategies to improve the overall prognosis of HCC patients.

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Long noncoding RNAs (lncRNAs) are families of non-coding molecular transcripts with more than 200 nucleotides in length, which have been identified as having significant functional roles in various human diseases.^{8–10} Specifically, lncRNAs have been shown to be deregulated in many types of human cancers, and the deregulation of lncRNAs was demonstrated to induce aberrant gene expression, thus contributing to the development, progression, or apoptosis of human cancers, including HCC.^{11–15} Notably, our recent study revealed that a novel lncRNA colon cancer associated transcript-1 (CCAT1) was aberrantly upregulated in human HCC, and its overexpression significantly promoted HCC cell progression and migration.¹¹

MicroRNAs (miRNAs) are families of small-length (8–22 nt long) noncoding RNAs that may modulate gene and protein expressions in various cancer types via either oncogenic or tumor-suppressive activities.^{16–18} Recently, it has been demonstrated that many RNA transcripts, including lncRNAs, may function as competing endogenous RNAs (ceRNAs) by binding miRNAs and preventing their targeted transcripts from being degraded.^{19,20} In human HCC specifically, our recent study demonstrated that CCAT1 could competitively sponge on miRNA *let-7* to affect its downstream cancer-related signaling pathways.¹¹ Yet, whether lncRNAs other than CCAT1 may also be dysregulated and functioning as miRNA-sponging epigenetic regulators in human HCC is not clear.

It was recently identified that another lncRNA, *Small Nucleolar RNA Host Gene 16 (SNHG16)*, might act as a potential cancer biomarker or functional cancer development modulator in various human cancers.^{21–25} Thus in this study, we explored the expression and functions of *SNHG16* in HCC. We hypothesized that *SNHG16* may be dysregulated in HCC cell lines and human HCC tumors. Also, we hypothesized that it may have functional roles in regulating HCC cell biological function, possibly through competitive sponging on certain miRNA candidates.

Patients and methods

Ethics statement

This study was approved by the Ethics Committees of the First and Fifth Affiliated Hospitals of Guangzhou Medical University (#903-GD-452-21) in Guangzhou, Guangdong Province, China. Written informed consents were acquired from each participating patient. For animal experiments, all procedures were reviewed and approved by the Animal Study Committee of the First and Fifth Affiliated Hospitals of Guangzhou Medical University in Guangzhou, Guangdong Province, China, and in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of

Laboratory Animals and Animal Welfare Act. In addition, all protocols were conducted in accordance with the principles of the Declaration of Helsinki.

Cell culture and clinical samples

HCC cell lines (Hep3B, HuH7, SNU398, SNU423, SNU429, Hep3G2, SK-HEP-1, and PLC/PRF/5) were all purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). In addition, two healthy liver cell lines, THLE2 and THLE3, were purchased from the American Type Culture Collection, Manassas, VA, USA. All cells were maintained in a 6-well plate in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific), 100 µg/mL streptomycin (Thermo Fisher Scientific), and 100 U/mL penicillin (Thermo Fisher Scientific) in a humidified 37°C incubator with 5% CO₂.

Clinical samples, including HCC tissues and adjacent noncancerous hepatic tissues, were collected from 43 patients with HCC during surgeries from June 2010 to January 2018. Participating patients did not receive any chemotherapy or radiation treatment prior to surgery. Upon surgical removal, all tissues were preserved in liquid nitrogen prior to further processing.

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA extraction and qRT-PCR were performed according to the methods in our previous publications.^{11,26} Briefly, total RNAs were extracted from HCC cell lines or clinical samples using a TRIzol reagent (Takara Bio Inc., Shiga Prefecture, Japan) according to the manufacturer's instruction. First-strand cDNA was generated using the Reverse Transcription System Kit (Thermo Fisher Scientific) according to the manufacturer's instruction. For *SNHG16*, qRT-PCR was performed using the SYBR Green PCR kit protocol in a StepOne Plus system (Thermo Fisher Scientific). The 18S rRNA was used as an endogenous control. For hsa-miR-93, qRT-PCR was performed using a mirVana™ qRT-PCR miRNA Detection Kit (Thermo Fisher Scientific). U6 snRNA was used as an endogenous control. All qRT-PCRs were performed on a Biosystems 7300 Real-Time PCR system (Thermo Fisher Scientific). Relative gene expression levels were calculated using the 2^(-ΔΔC_t) method.

SNHG16 upregulation assay

A lentiviral vector containing human lncRNA *SNHG16*, *SNHG16(L)*, was purchased from GenePharma (Shanghai, China). In addition, a nonspecific lentiviral vector, NS(L), was

also purchased from GenePharma. Hep3B and HuH7 cells were transduced with NS(L) or *SNHG16*(L) in the presence of polybrene (8 $\mu\text{g}/\text{mL}$; GenePharma) for 48 hours. After a selection procedure using blasticidin (10 $\mu\text{g}/\text{mL}$; GenePharma) for 72 hours, surviving cells were collected and reseeded in six-well plates. After 3–6 passages, qRT-PCR was performed to examine endogenous *SNHG16* expression in Hep3B and HuH7 cells.

Cell proliferation assay

HCC in vitro proliferation was measured according to the method in our previous publication.¹¹ Briefly, $\sim 5 \times 10^3$ Hep3B or HuH7 cells were plated in 96-well plates for 96 hours. Every 24 hours, cell proliferation was assessed using the cell counting kit-8 ([CCK-8]; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. The cell proliferation curves were plotted using the absorbance (570 nm) reading at each time point.

Chemoresistance assay

Approximately 5×10^3 Hep3B or HuH7 cells were plated in 96-well plates and treated with 5-fluorouracil (5-FU) at concentrations of 0, 2, 10, and 20 μM for 48 hours. Then, a CCK-8 assay was performed according to the manufacturer's protocol. At each 5-FU concentration, viable cells were measured using the absorbance (570 nm) and then normalized to the absorbance at 0 μM 5-FU.

In vivo tumorigenicity assay

HCC in vivo tumor growth was evaluated according to the method in our previous publication.²⁶ Briefly, 100 μL , 1×10^6 HuH7 cells were inoculated subcutaneously into the dorsal flanks of 5-week-old female athymic nude mice ($n=8$ for each group). In vivo tumor volume (V) was monitored each week by measuring the subcutaneous length (L) and width (W) and then calculated according to the formula $V=(L \times W^2/2)$. All nude mice were killed after 5 weeks. HuH7 tumors were extracted and examined under a broad-light microscope.

Luciferase assay

A luciferase assay was performed according to the method in our previous publications.^{11,26} Briefly, a pmirGLO dual-luciferase plasmid with the insertion of wild-type (WT) *SNHG16* (*SNHG16*[WT]) was purchased from GenePharma. The putative hsa-miR-93 binding sites on *SNHG16* were mutated. The mutant (MU) *SNHG16* (*SNHG16*[MU]) was also inserted in a pmirGLO plasmid, also from GenePharma. A total of 5×10^4 293T cells/well were seeded into 24-well plates and co-transfected with *SNHG16*(WT) or

SNHG16(MU) and a synthetic hsa-miR-93 mimics ([miR93-mimics]; GenePharma) or a nonspecific miRNA mimics ([NS-mimics]; GenePharma) using lipofectamine 2000 (Thermo Fisher Scientific). After 48 hours, relative luciferase activities were measured using a dual luciferase reporter assay system (Promega Corporation, Fitchburg, WI, USA), and normalized to the luciferase activity measured in cells co-transfected with miR93-mimics and *SNHG16*(WT).

Statistical analysis

All experiments were performed in at least three biological repeats. For comparisons, Student's *t*-test or Wilcoxon signed-rank test was performed. All *P*-values were two-sided and were obtained using the SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA). Differences were defined as statistically significant for *P*-values < 0.05 .

Results

Downregulation of *SNHG16* and inhibition of HCC in vitro proliferation on upregulation

To investigate the role of *SNHG16* in HCC, we first examined the endogenous expression of *SNHG16* in eight human HCC cell lines (Hep3B, HuH7, SNU398, SNU423, SNU429, Hep3G2, SK-HEP-1, and PLC/PRF/5) and two normal liver cell lines (THLE2 and THLE3). Downregulated expression of *SNHG16* was observed in all eight HCC cell lines compared with that in THLE2 cells (Figure 1A; $*P < 0.05$, $\Delta P > 0.05$). We then measured *SNHG16* expression level in 43 pairs of HCC tissues and pair-matched noncancerous (healthy) hepatic tissues. This demonstrated that *SNHG16* expression was significantly decreased in HCC tissues as compared with that in pair-matched healthy hepatic tissues (Figure 1B; $*P < 0.005$, Wilcoxon signed-rank test).

Then, we stably overexpressed *SNHG16* by transducing lentiviruses of *SNHG16*(L) or NS(L) into the HCC cell lines, Hep3B, and HuH7. Analysis of qRT-PCR showed that *SNHG16* gene levels significantly increased in HCC cells transduced with *SNHG16*(L) than in HCC cells transduced with NS(L) (Figure 1C, $*P < 0.05$). Then, a CCK-8 assay indicated that *SNHG16* upregulation significantly inhibited cell proliferation in Hep3B and HuH7 cells (Figure 1D, $*P < 0.05$).

SNHG16 upregulation inhibited HCC chemoresistance and in vivo tumorigenicity

To further investigate the functional roles of *SNHG16* in HCC, we examined chemoresistance to 5-FU in lentiviral-transduced Hep3B and HuH7 cells. The results showed that *SNHG16*

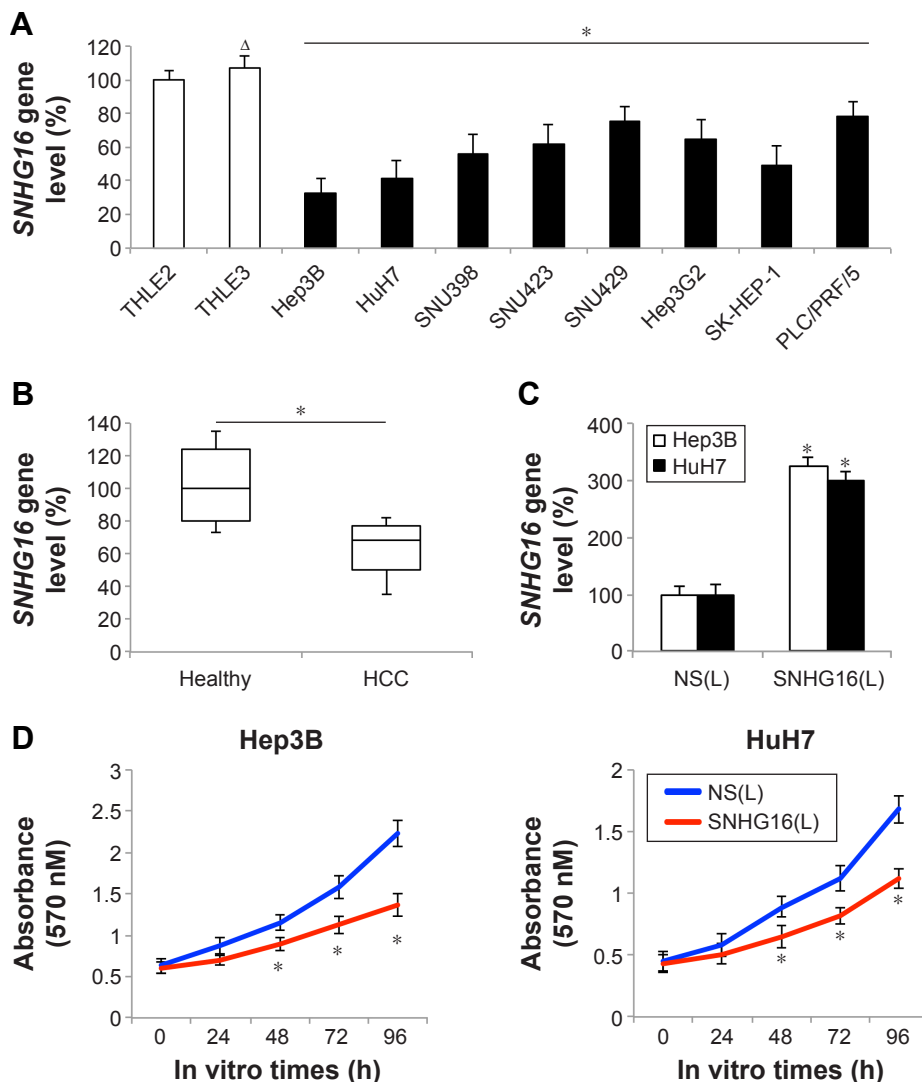


Figure 1 *SNHG16* expression in HCC and its role in HCC cell in vitro proliferation.

Notes: (A) Expression of *SNHG16* in eight HCC cell lines and two liver normal cell lines. Data are presented as mean \pm standard errors (* $P < 0.05$, $^{\Delta}P > 0.05$). (B) Differences in *SNHG16* expression levels between HCC tissues and pair-matched noncancerous hepatic tissues. Statistical differences between samples were analyzed using the Wilcoxon signed-rank test ($n = 43$, * $P < 0.005$). (C) Hep3B and HuH7 cells were transduced with *SNHG16(L)* or NS(L). The expression of *SNHG16* in lentiviral-transduced Hep3B and HuH7 cells was then measured by qRT-PCR (* $P < 0.05$). (D) In a CCK-8 cell proliferation assay, lentiviral-transduced Hep3B and HuH7 cells were seeded in 96-well plates for 96 hours. The absorbance was measured every 24 hours and normalized toward the absorbance at 0 hour (* $P < 0.05$).

Abbreviations: CCK-8, cell counting kit-8; HCC, hepatocellular carcinoma; NS(L), nonspecific lentivirus; *SNHG16*, *small nucleolar RNA host gene 16*; *SNHG16(L)*, *SNHG16*-overexpressing lentivirus; qRT-PCR, quantitative real-time PCR.

upregulation significantly inhibited 5-FU chemoresistance (between 2 and 20 μM) in Hep3B and HuH7 cells (Figure 2A, * $P < 0.05$).

We also examined in vivo tumorigenicity using lentiviral-transduced HuH7 cells. By measuring weekly subcutaneous tumor volumes, we discovered that 3–5 weeks after lentiviral-transduced HuH7 cells were transplanted, *SNHG16* upregulation significantly inhibited the in vivo growth of transplanted HCC tumors (Figure 2B, * $P < 0.05$). At the end of 5-week in vivo tumorigenicity assay, HuH7 transplants were extracted and directly compared under a microscope. This confirmed our in vivo discovery, showing

that *SNHG16*-overexpressed HuH7 transplants were significantly smaller than control HuH7 transplants (Figure 2C).

hsa-miR-93 sponges *SNHG16*

To investigate the mechanisms responsible for *SNHG16* upregulation-induced tumor suppression in HCC, bioinformatics analyses were performed to search for *SNHG16*-associated molecular regulators. By searching through online miRNA-lncRNA database of StarBase v2.0,^{27,28} we noticed that human mature miRNA, hsa-miR-93, has a very strong potential to bind *SNHG16* (Figure 3A). To verify this hypothesis, we constructed two luciferase plasmids, one containing

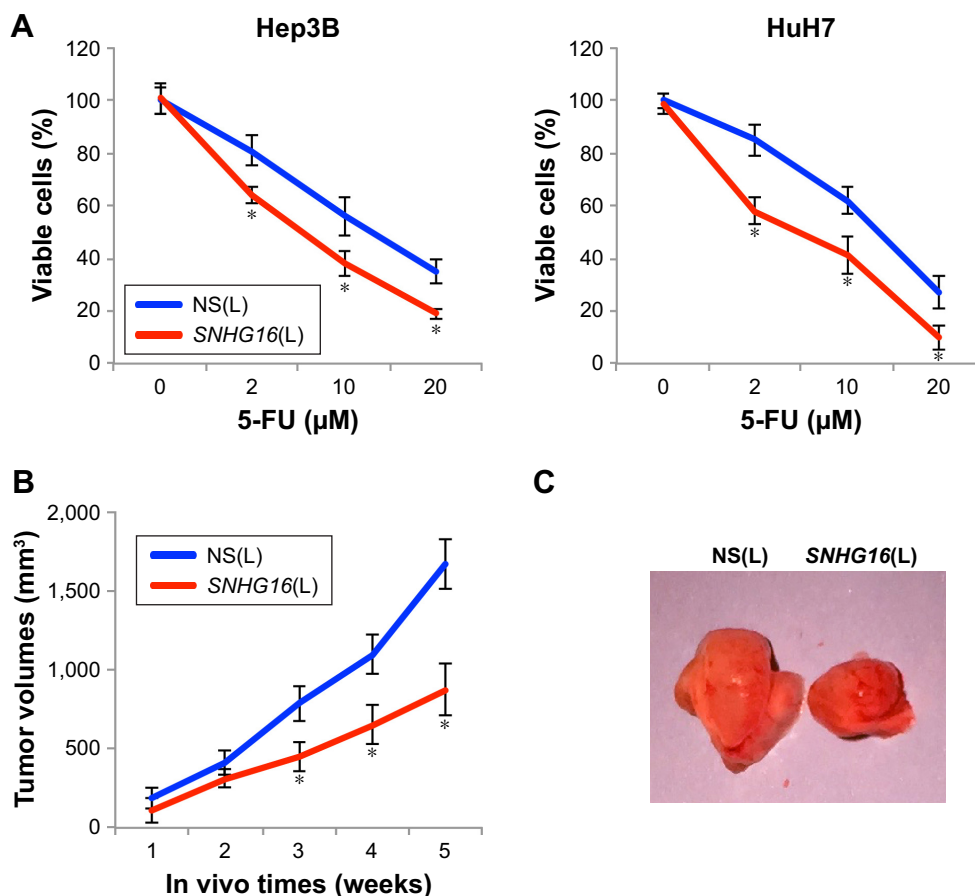


Figure 2 The role of *SNHG16* in HCC chemoresistance and in vivo tumor growth.

Notes: (A) Lentiviral-transduced Hep3B and HuH7 cells were treated with 5-FU at concentrations of 0, 2, 10, and 20 μM for 48 hours. A CCK-8 assay was then applied to measure cancer cell viability. The absorbance was measured for cells treated with each concentration of 5-FU and normalized toward the absorbance with 0 μM 5-FU treatment (* $P < 0.05$). (B) Lentiviral-transduced HuH7 cells were examined through a 5-week in vivo tumorigenicity assay. Subcutaneous tumor volumes were measured weekly and compared between NS(L)- and *SNHG16*(L)-transduced HuH7 transplants (* $P < 0.05$). (C) After in vivo tumorigenicity assay, lentiviral-transduced HuH7 transplants were extracted and inspected.

Abbreviations: CCK-8, cell counting kit-8; 5-FU, fluorouracil; HCC, hepatocellular carcinoma; NS(L), nonspecific lentivirus; *SNHG16*, *small nucleolar RNA host gene 16*; *SNHG16*(L), *SNHG16*-overexpressing lentivirus.

the DNA sequence of *SNHG16*(WT) and the other containing the DNA sequence of *SNHG16*(MU) with eliminated hsa-miR-93 binding site. Then, a luciferase assay demonstrated that only in 293T cells co-transfected with *SNHG16*(WT) and miR93-mimics, the relative luciferase activity was significantly suppressed, thus confirming that hsa-miR-93 could directly be sponged by *SNHG16* (Figure 3B, * $P < 0.05$).

In addition, we examined whether the expression of hsa-miR-93 was regulated by *SNHG16* in HCC cells. qRT-PCR then showed that *SNHG16* upregulation significantly decreased the mRNA level of endogenous hsa-miR-93 in both Hep3B and HuH7 cells (Figure 3C, * $P < 0.05$).

SNHG16 inhibits HCC functions by competitively binding hsa-miR-93

To explore whether hsa-miR-93 can functionally reverse the roles of *SNHG16* in HCC cells, we overexpressed

hsa-miR-93 in Hep3B and HuH7 cells with upregulated *SNHG16* expression (Figure 4A, * $P < 0.05$).

Then, functional assays were used to clarify the role of hsa-miR-93 binding in *SNHG16*-mediated HCC regulation. CCK-8 assays indicated that in *SNHG16*-upregulated Hep3B and HuH7 cells, overexpressing hsa-miR-93 significantly promoted HCC proliferation (Figure 4B, * $P < 0.05$). Chemoresistance assay also indicated that in *SNHG16*-upregulated Hep3B and HuH7 cells, overexpressing hsa-miR-93 significantly increased 5-FU resistance (Figure 4C, * $P < 0.05$). Thus, these results showed that *SNHG16* inhibited HCC functions via competitively binding hsa-miR-93.

Discussion

In this study, we evaluated the expression of lncRNA *SNHG16* in HCC and discovered that *SNHG16* was markedly downregulated in both HCC cancer cell lines

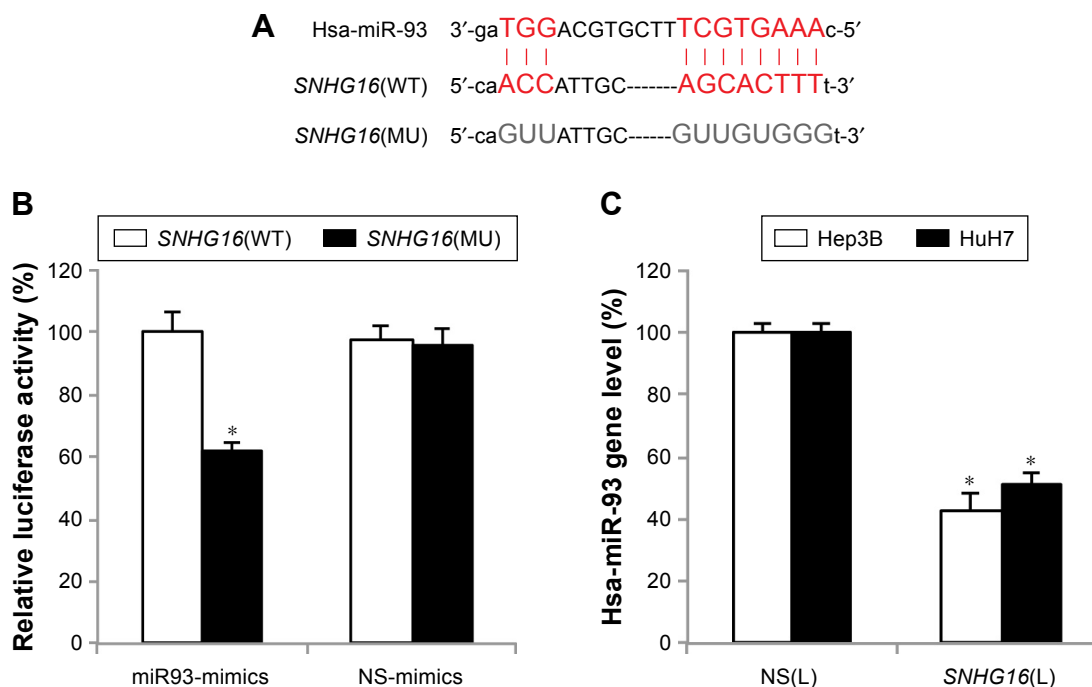


Figure 3 Hsa-miR-93 is directly modulated by *SNHG16* in HCC.

Notes: (A) Schematic representation of hsa-miR-93 sponging on *SNHG16*(WT). A *SNHG16*(MU) sequence was also generated to eliminate the binding by hsa-miR-93. (B) The WT and MU *SNHG16* DNA sequences were fused with luciferase reporter (*SNHG16*[WT] and *SNHG16*[MU]) and co-transfected with miR93-mimics or NS-mimics into 293T cells. The luciferase assay revealed that hsa-miR-93 binds to *SNHG16*(WT) (**P*<0.05). (C) The gene expression level of hsa-miR-93 was remarkably decreased when Hep3B and HuH7 cells were transfected with *SNHG16*(L), as compared to NS(L) (**P*<0.05).

Abbreviations: HCC, hepatocellular carcinoma; miR93-mimics, hsa-miR-93 mimics; NS-mimics, nonspecific miRNA mimics; NS(L), nonspecific lentivirus; *SNHG16*, *small nucleolar RNA host gene 16*; *SNHG16*(L), *SNHG16*-overexpressing lentivirus; MU, mutant; WT, wild-type.

and human tumors. Interestingly, our findings seem to be different from several previous studies in human cancers, which demonstrated that *SNHG16* was upregulated in colorectal cancer, gastric cancer, or breast cancer.^{22,24,29} Based on those previous findings and ours, it seems like the deregulating pattern of *SNHG16* in human cancers could be either upregulation or downregulation, possibly determined by the sites and microenvironments of specific cancer types.

We also evaluated the biological functions of *SNHG16* in HCC cells. Through lentiviral transduction, we successfully overexpressed *SNHG16* in HCC cell lines. Then, through several in vitro and in vivo cancer function assays, we identified that *SNHG16* overexpression had significant tumor-suppressing effects by inhibiting HCC cancer cell in vitro proliferation, 5-FU chemoresistance, and in vivo tumor growth, indicating that *SNHG16* might function as a tumor suppressor in HCC. Interestingly, *SNHG16* was demonstrated to function as oncogene in human breast cancer, gastric cancer, or bladder cancer.²²⁻²⁴ The findings in our study thus presented new evidence that *SNHG16* may exert both oncogenic and tumor-suppressing effects on human

cancer development or progression. Furthermore, our recent study identified another lncRNA, *CCAT1*, to function as oncogene in HCC.¹¹ Therefore, the epigenetic regulations of lncRNAs may be very complex, involving both oncogenic and tumor-suppressing ones in HCC.

In studies on human cancers, it has been demonstrated that *SNHG16* might act as ceRNAs to sponge on another group of epigenetic regulators, miRNAs. In human esophagus cancer, *SNHG16* was shown to directly sponge on miR-140-5 p, thus inversely upregulating *ZEB1* gene to promote cancer cell progression.²¹ In addition, *SNHG16* was demonstrated to induce breast cancer cell migration by competitively binding miR-98 with E2F5.²⁴ In this study, we presented several lines of evidence to demonstrate that *SNHG16* acted as a direct molecular sponge of hsa-miR-93, a known oncogenic transcript in HCC.³⁰ First, our luciferase assay provided convincing evidence that *SNHG16* may directly bind with hsa-miR-93. Second, qRT-PCR assay demonstrated that hsa-miR-93 was inversely downregulated in *SNHG16*-overexpressed HCC cell lines. Third and most importantly, in double-transfected HCC cells, upregulating hsa-miR-93 was able to reverse the tumor-suppressing effects

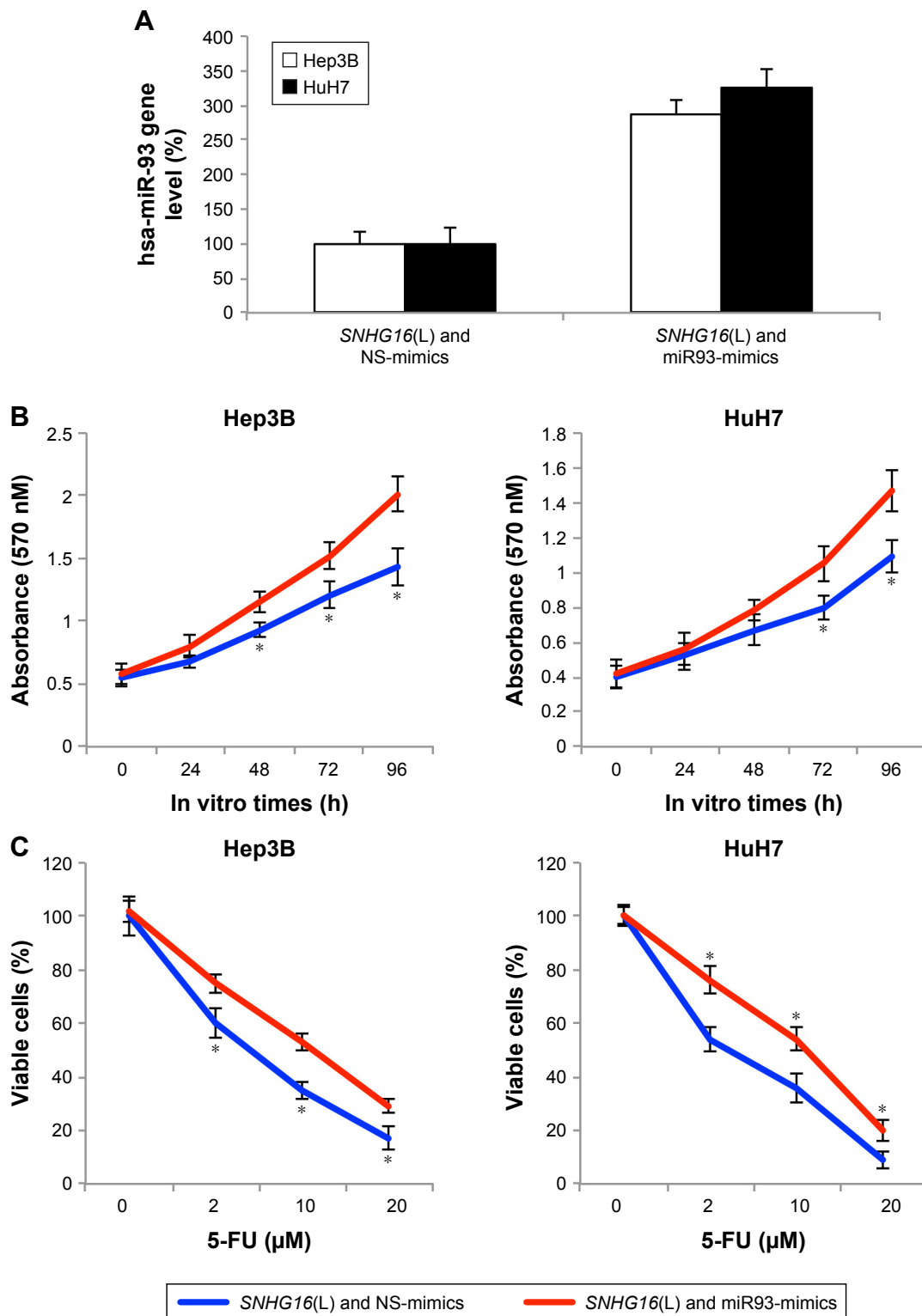


Figure 4 hsa-miR-93 reverses the inhibitory effect of *SNHG16* on HCC cell proliferation and chemoresistance.

Notes: (A) Hep3B and HuH7 cells transfected with *SNHG16*(L) were double transfected with miR93-mimics or NS-mimics. The expression of hsa-miR-93 in transfected Hep3B and HuH7 cells was then measured by qRT-PCR (* $P < 0.05$). (B) In a CCK-8 cell proliferation assay, double-transfected Hep3B and HuH7 cells were seeded in 96-well plates for 96 hours. The absorbance was measured every 24 hours, and normalized toward the absorbance at 0 hour (* $P < 0.05$). (C) Double-transfected Hep3B and HuH7 cells were treated with 5-FU at concentrations of 0, 2, 10, and 20 μM for 48 hours. A CCK-8 assay was then applied to measure cancer cell viability. The absorbance was measured for cells treated with each concentration of 5-FU and normalized toward the absorbance with 0 μM 5-FU treatment (* $P < 0.05$).

Abbreviations: CCK-8, cell counting kit-8; 5-FU, fluorouracil; HCC, hepatocellular carcinoma; miR93-mimics, hsa-miR-93 mimics; NS-mimics, nonspecific miRNA mimics; *SNHG16*, small nucleolar RNA host gene 16; *SNHG16*(L), *SNHG16*-overexpressing lentivirus; qRT-PCR, quantitative real-time PCR.

induced by *SNHG16* upregulation on cancer cell proliferation and 5-FU chemoresistance. Therefore, our results support the notion that tumor-suppressing functions of *SNHG16* may very possibly act through competitively binding on hsa-miR-93. Intriguingly, it was demonstrated that hsa-miR-93 exerted oncogenic regulations on HCC development by activating c-Met/PI3K/Akt pathway in HCC.³⁰ The possibility of *SNHG16* sponging on hsa-miR-93 to inversely inhibit c-Met/PI3K/Akt pathway would certainly require further investigations.

Conclusion

Overall, our studies indicate that *SNHG16* is markedly downregulated in HCC and may act as a tumor suppressor to inhibit HCC development, possibly by functioning as a competitive sponge of hsa-miR-93. Furthermore, based on the findings in our study, future clinical studies focusing on the correlation between *SNHG16* and HCC patients' clinicopathological characteristics may help to further define the translational implication of *SNHG16* as it may be developed as a novel prognostic marker or therapeutic target to benefit HCC diagnosis and therapy.

Disclosure

The authors report no conflicts of interest in this work.

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