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LncRNA SNHG20 promotes the development of laryngeal squamous cell carcinoma by regulating miR-140

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Abstract. – OBJECTIVE: This study aims to investigate the expression level of long non-coding RNA (lncRNA) SNHG20 in laryngeal squamous cell carcinoma (LSCC), and to explore further whether it can promote the development of LSCC by regulating microRNA-140 (miR-140).

PATIENTS AND METHODS: Expression levels of SNHG20 in 56 pairs of LSCC tissues and adjacent normal tissues were measured by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between SNHG20 expression with pathological parameters and the prognosis of LSCC was analyzed. Besides, the SNHG20 expression in LSCC cells was also analyzed by qRT-PCR. The SNHG20 knockdown and overexpression model were constructed by lentivirus transfection in AMC-HN-8 and Hep-2 cells. Cell counting kit-8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assay were used to analyze the effect of SNHG20 on the biological function of LSCC cells. Finally, the dual-luciferase reporter gene assay was performed to explore the potentials of SNHG20 and miR-140 in LSCC.

RESULTS: The SNHG20 expression in LSCC tissues or cells remarkably increased than controls, and the difference was statistically significant. The LSCC patients with the high expression level of SNHG20 were more likely to develop advanced tumor compared with patients with low expression of SNHG20. Moreover, the LSCC patients with the high expression level of SNHG20 had a shorter overall survival than those with low level. The cell proliferation ability significantly decreased in the SNHG20 knockdown group, while notably increased in SNHG20 overexpression group. MiR-140 was negatively correlated with SNHG20 in LSCC tissues and cells. Dual-luciferase reporter gene assay showed that SNHG20 could be targeted by miR-140 through a certain binding site. The cell rescue experiment also indicated that there was a mutual regulation between SNHG20 and miR-140, which could together affect the malignant progression of LSCC.

CONCLUSIONS: We showed that the expression levels of SNHG20 in LSCC tissues or cell lines significantly increased and was associated with advanced tumor staging and undesirable prognosis of LSCC. In addition, SNHG20 could promote the malignant progression of LSCC.

Key Words
SNHG20, MiRNA-140, Squamous carcinoma of larynx, Proliferation.

Introduction

Laryngeal cancer is derived from the epithelial tissue of the laryngeal mucosa, accounting for approximately 25% of head and neck malignancies[1-3]. About 95% of the pathological types of laryngeal cancer are laryngeal squamous cell carcinoma (LSCC), and the proportion of adenocarcinoma is small[4,5]. Laryngeal cancer is prone to metastasize through lymph nodes to the neck. The etiology of laryngeal cancer is still unclear. Its occurrence and development may be the result of multiple comprehensive factors, including smoking, alcoholism, papillomavirus infection, and poor eating habits[6-9]. Although early stage of laryngeal cancer is difficult to diagnose, it can't be effectively treated in time. Advanced laryngeal cancer would affect the swallowing, breathing and pronunciation functions due to the large scope of surgical resection. Patients often suffer from "difficult words", and their prognosis and life quality are still unsatisfactory, with the 5-year survival rate about 64.2%[10,11]. Therefore, in-depth study on the molecular mechanisms of laryngeal cancer helps to improve clinical outcome and life quality of affected patients.

Non-coding RNA (ncRNA) is transcribed from the genome but lack of protein-coding potential, including housekeeping non-coding RNA

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and regulatory non-coding RNA[12-14]. LncRNA has nucleotides longer than 200 nt but does not have a complete open reading frame to encode protein[15,16]. Studies[17-20] have shown that lncRNA is differentially expressed in tissues and cells. Abnormally expressed lncRNAs in tumors are related to the malignant behaviors of tumor cells. Some scholars[21,22] have found that lncRNA is involved in the occurrence and development of tumors as a cancer-promoting or tumor-suppressor gene. It is closely related to biological behaviors such as proliferation, apoptosis, metastasis, and cell cycle changes of tumor cells. SNHG20 plays a role in promoting some head and neck squamous cell carcinomas, but the specific mechanism is still unclear, and its role in laryngeal cancer is unknown[23-25].

Changes in miRNA expression affect tumorigenesis and are also closely related to tumor proliferation[26,27]. Pradillo and Santos[28] have shown that miRNAs can affect the tumor cell connection by altering expressions of specific genes, thereby affecting the proliferation of tumor cells. In the invasion and metastasis of many common tumors, miRNAs are very important as are shown to promote or inhibit the proliferation of tumor cells[28]. MiR-140 is involved in a variety of tumors, but no studies have reported its role in LSCC[29]. Therefore, this research investigated whether lncRNA SNHG20 can regulate miRNA-140-mediated proliferation of LSCC and thus provide experimental evidence for its clinical application.

Patients and Methods

Patients and Colon Cancer Samples

56 pairs of tissues were selected from LSCC patients undergoing surgery. Invasive LSCC tissues and their corresponding adjacent tissues were stored at -80°C. The collection of clinical specimens was approved by the Ethics Monitoring Committee. Patients and/or their families have been fully informed that their specimens will be used for scientific research, and all participating patients signed the informed consent.

Cell Lines and Reagents

AMC-HN-8 and Hep-2 cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in high-glucose Dulbecco's Modified Eagles's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) and placed at 37°C, 5% CO_2 incubator. Cells were passaged with 1% trypsin + Ethylene Diamine Tetraacetic Acid (EDTA; Thermo Fisher Scientific, Waltham, MA, USA) for digestion when grown to 80%-90% confluence.

Transfection

The negative control (NC or Anti-NC) and the lentivirus (SNHG20 or Anti-SNHG20) containing the SNHG20 overexpression and knockdown sequences were purchased from GenePharma (Shanghai, China). Cells were transfected according to the manufacturer's instructions when grown to 40% density and harvested at 48 hours for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and cell function experiments.

Cell Proliferation Assays

Transfected cells for 48 h were plated into 96-well plates at 2000 cells per well. After culturing for 24 h, 48 h, 72 h, and 96 h, cells were added with cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). After 2 hours of incubation, the optical density (OD) value of each well was measured at 490 nm absorption wavelength in the microplate reader.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

EdU proliferation assay (RiboBio, Nanjing, China) was performed according to the manufacturer's requirements. After transfection for 24 h, cells were incubated with 50 μM EdU for 2 h, and then stained with AdoLo and 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA). The number of EdU-positive cells was detected by fluorescence microscopy. The EdU-positive rate was calculated as the ratio of EdU-positive cell number to total DAPI chromogenic cell number (blue cells).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and repurify the RNA. RNA reverse transcription was performed according to the Prime Scirpt Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions. RT-PCR was performed according to the SYBR® Premix Ex TaqTM (TaKaRa, Otsu,

Shiga, Japan) kit instructions, and the PCR was performed using StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR reaction: SNHG20: forward: 5'-ATGGCTATAAATAGATACACGC-3', reverse: 5'-GGTACAAACAGGGAGGGA-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3', reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'; miRNA-140: forward: 5'-UGGCAGUGUCUUAGCUGGUUGU-3'; U6: forward: 5'-CGCAAGGATGACACGCAAATTC-3'. Each sample was subjected to a three-hole repeated experiment and repeated twice. The Bio-Rad PCR instrument was used to analyze and process the data with the software iQ5 2.0 (Hercules, CA, USA). GAPDH and U6 genes were used as internal parameters and calculated by the $2^{-\Delta\Delta Ct}$ method.

Dual-Luciferase Reporter Gene Assay

A reporter plasmid was constructed in which a specific fragment of the target promoter was inserted in front of the luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected with the reporter plasmid into AMC-HN-8 and Hep-2 cells or other related cells. Cells were lysed after transfection for determining the luciferase intensity.

Immunohistochemical Staining

After dewaxing and hydration of the wax block, tissues were incubated with 50 μL of I anti-rat ki-67 (1:100) at room temperature for 1 h. After washing with phosphate-buffered saline (PBS) for 3 times, with 5 min each, II anti-goat anti-rabbit 40-50 μL was applied for incubation. 1 h later, tissues were washed with PBS for 3 times and incubated with Diaminobenzidine (DAB) coloration (Sigma-Aldrich, St. Louis, MO, USA) for 5-10 min. Finally, tissues were observed and captured under the microscope.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Statistical differences between the two groups were analyzed using the Student's *t*-test. Independent experiments were repeated at least for three times. Data were shown as averaged ± standard. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, and ***$p<0.001$).

Results

SNHG20 Was Highly Expressed in LSCC Tissues and Cell Lines

56 pairs of LSCC and adjacent non-tumor tissues were collected for detecting the expression of SNHG20 by qRT-PCR. The results showed that SNHG20 level in LSCC was higher than controls (Figure 1A, 1B). It is suggested that SNHG20 may be an oncogene in LSCC. Simultaneously, we detected the ki-67 level in LSCC and adjacent non-tumor tissues by immunohistochemistry. The results showed that the positive expression of Ki-67 in LSCC significantly increased (Figure 1C).

SNHG20 Expression Was Correlated With Pathological Staging and Overall Survival in LSCC Patients

Based on the mRNA level of SNHG20 in 56 pairs of LSCC and adjacent tissues, LSCC patients were divided into high and low expression group. The correlation between SNHG20 expression and age, sex, clinical stage, lymph node metastasis, and distant metastasis was analyzed. SNHG20 was found to be positively correlated with tumor staging of LSCC, but not correlated with age, gender, distant metastasis or lymph node metastasis (Table I). In addition, to explore the function of SNHG20 in the prognosis of LSCC patients, we collected relevant follow-up data. Kaplan-Meier survival curves showed that SNHG20 was significantly associated with poor prognosis of LSCC. The higher the expression level of SNHG20, the worse the prognosis ($p<0.05$; Figure 1D).

Knockdown of SNHG20 Inhibited Cell Proliferation, and Overexpression of SNHG20 Promoted Cell Proliferation

To investigate the function of SNHG20 in LSCC, we constructed SNHG20 overexpression and knockdown cell model by transfection of lentiviral vector. After transfecting the SNHG20 lentiviral vector into the AMC-HN-8 and Hep-2 cell lines, we verified their transfection efficacies by qRT-PCR, and the difference was statistically significant (Figure 2A). CCK-8, EdU, and cell cloning experiments were performed to detect cell proliferation after overexpression or knockdown of SNHG20 in AMC-HN-8 and Hep-2 cells, respectively. The cell proliferation ability of the SNHG20 overexpressing group significantly increased, while the opposite result was observed in SNHG20 silencing group (Figure 2B and 2C).

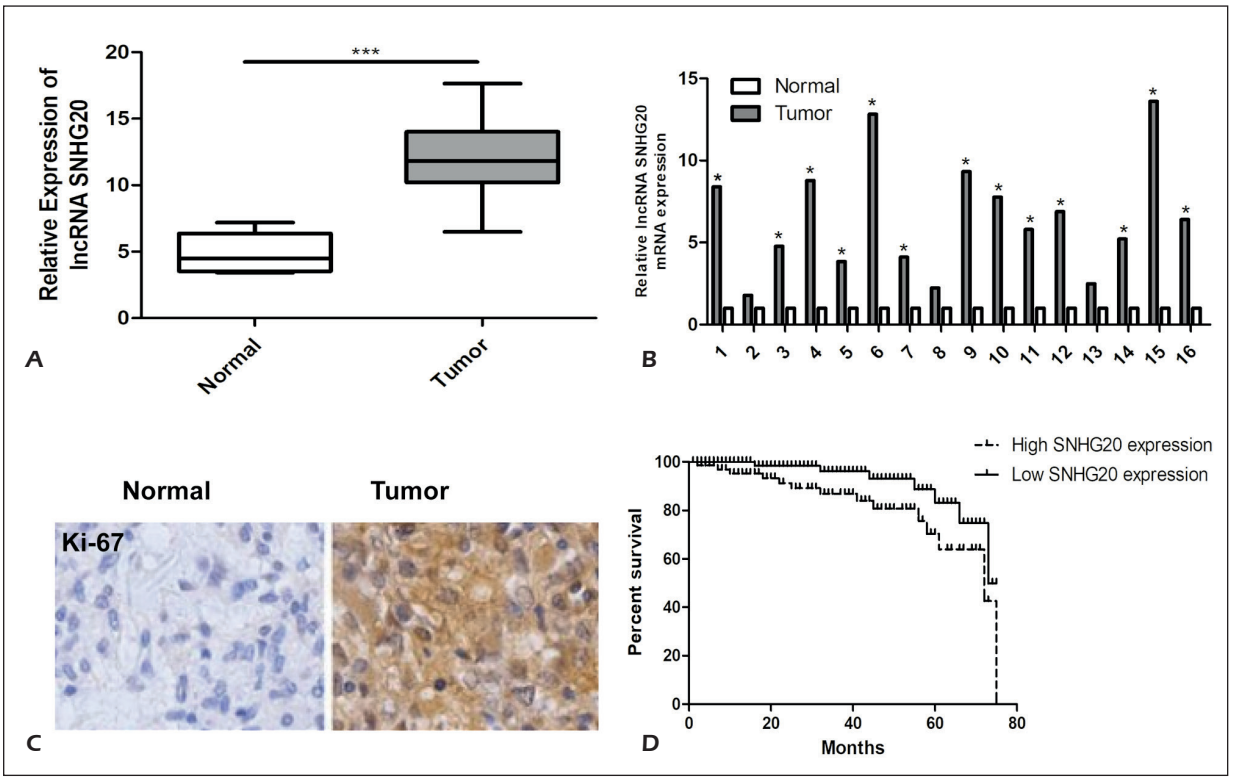

Figure 1. SNHG20 is highly expressed in LSCC and cell lines. **A**-**B**, qRT-PCR detection of SNHG20 mRNA in LSCC and adjacent non-tumor tissues. **C**, Immunohistochemical detection of ki-67 in LSCC and adjacent non-tumor tissue (magnification: 40×). **D**, Kaplan Meier survival curve of LSCC patients based on SNHG20 expression. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Table I. Association of lncRNA SNHG20 and miR-140 expression with clinicopathologic characteristics of laryngeal squamous cell carcinoma.

Parameters	Number of cases	SNHG20 expression		*p*-value	miR-140 expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.174			0.425
<60	23	14	9		8	15	
≥60	33	14	19		15	18	
Gender				0.285			0.415
Male	28	16	12		10	18	
Female	28	12	16		13	15	
T stage				0.014			0.012
T1-T2	33	21	12		9	24	
T3-T4	23	7	16		14	9	
Lymph node metastasis				0.094			0.114
No	36	21	15		12	24	
Yes	20	7	13		11	9	
Distance metastasis				0.057			0.159
No	33	20	13		11	22	
Yes	23	8	15		12	11	

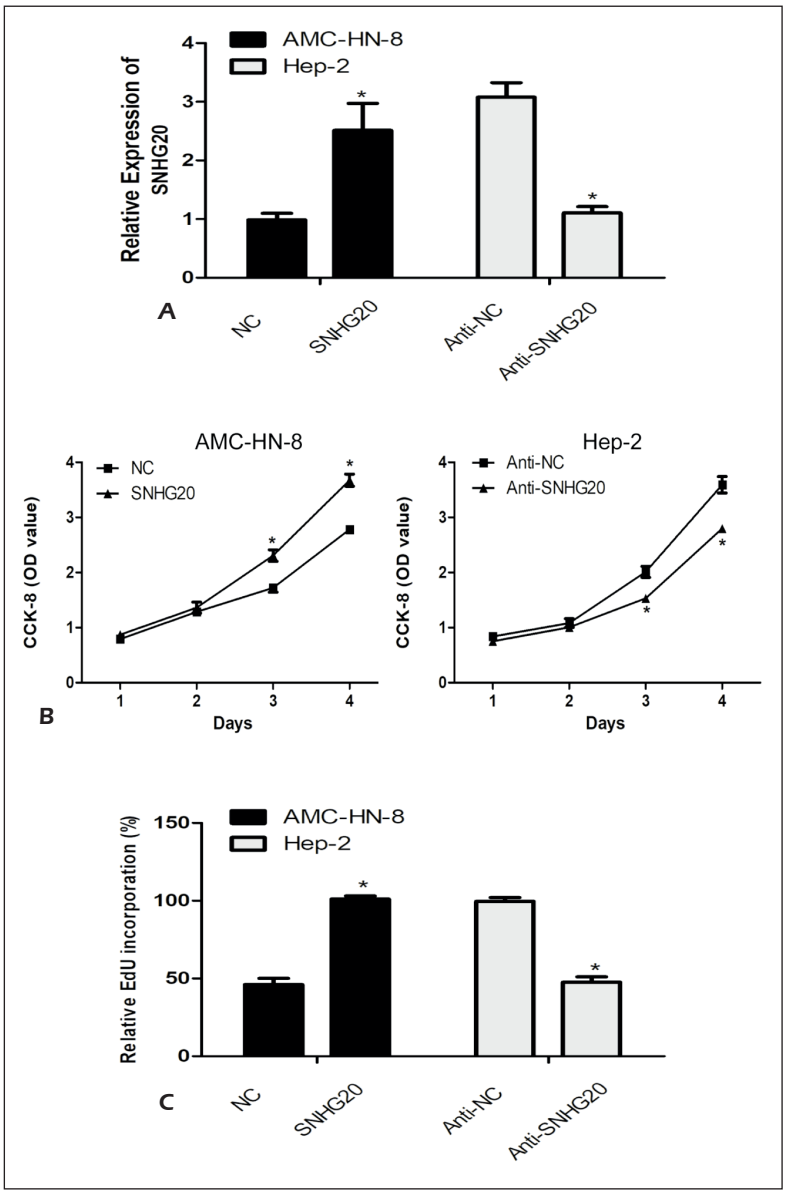

Figure 2. SNHG20 affects the proliferation of LSCC cells. **A**, qRT-PCR verified the efficiency of SNHG20 transfected with SNHG20 overexpression vector in AMC-HN-8 and SNHG20 knockout vector in Hep-2. **B**, CCK-8 assay Effects of AMC-HN-8 and Hep-2 on the proliferation of LSCC cells. **C**, EDU assay for the proliferation of LSCC cells in AMC-HN-8 and Hep-2 cell lines effect. Data are mean ± SD, *$p<0.05$.

Direct Targeting Effect of SNHG20 and Mir-140 was Displayed by Dual-Luciferase Reporter Gene Assay

To further verify the targeting of miR-140 to SNHG20, we cloned the SNHG20 sequence into the luciferase reporter plasmid pmirGLO, and also constructed the mutation vector pmirGLO-SNHG20-mut. AMC-HN-8 and Hep-2 cells were co-transfected with pmirGLO-SNHG20 or pmirGLO-SNHG20-mut and miR-140 mimics or negative control, respectively. Dual-luciferase reporter gene assay showed that the overexpression of miR-140 significantly attenuated the luciferase activity of the wild-type SNHG20 group ($p<0.05$). However, the luciferase activity in the mutant-type SNHG20 group did not change ($p>0.05$), further demonstrating that SNHG20 could bind to miR-140 (Figure 3A).

MiR-140 Was Lowly Expressed in LSCC Tissues and Cell Lines

MiR-140 was lowly expressed in 56 pairs of LSCC tissues and their corresponding adjacent normal tissues. Identically, miR-140 level in LSCC cells remained low (Figure 3B). Subsequently, it was found that miR-140 expression was downregulated by SNHG20 overexpression in AMC-HN-8 and Hep-2 cells, but was upreg-

ulated by SNHG20 knockdown (Figure 3C). Moreover, in 16 pairs of selected LSCC tissue samples, the SNHG20 expression was negatively correlated to the miR-140 expression (Figure 3D). In addition, we analyzed the effect of miR-140 on pathology and prognosis of LSCC. As shown in Table I, the low expression of miR-140 was positively correlated with the pathological stage of LSCC, but not correlated with age, gender, lymph node metastasis, or distant metastasis. In addition, Kaplan-Meier survival curve indicated that the downregulated miR-140 was significantly associated with the poor prognosis of LSCC patients (Figure 3E).

SNHG20 Modulated MiR-140 in LSCC Tissues and Cell Lines

To further explore the function of SNHG20 in the malignant progression of LSCC, we found a possible relationship between SNHG20 and miR-140 through bioinformatics analysis. Besides, in order to further understand the interaction between SNHG20 and miR-140 in LSCC cells, we co-overexpressed or co-silenced miR-140 and SNHG20 in LSCC cells, and the miR-140 expression in co-transfected cells was examined by qRT-PCR (Figure 4A). Subsequently, CCK-8 and EdU experiments revealed that miR-140 reversed the effect of SNHG20 on the proliferation of LSCC cells (Figure 4B and 4C).

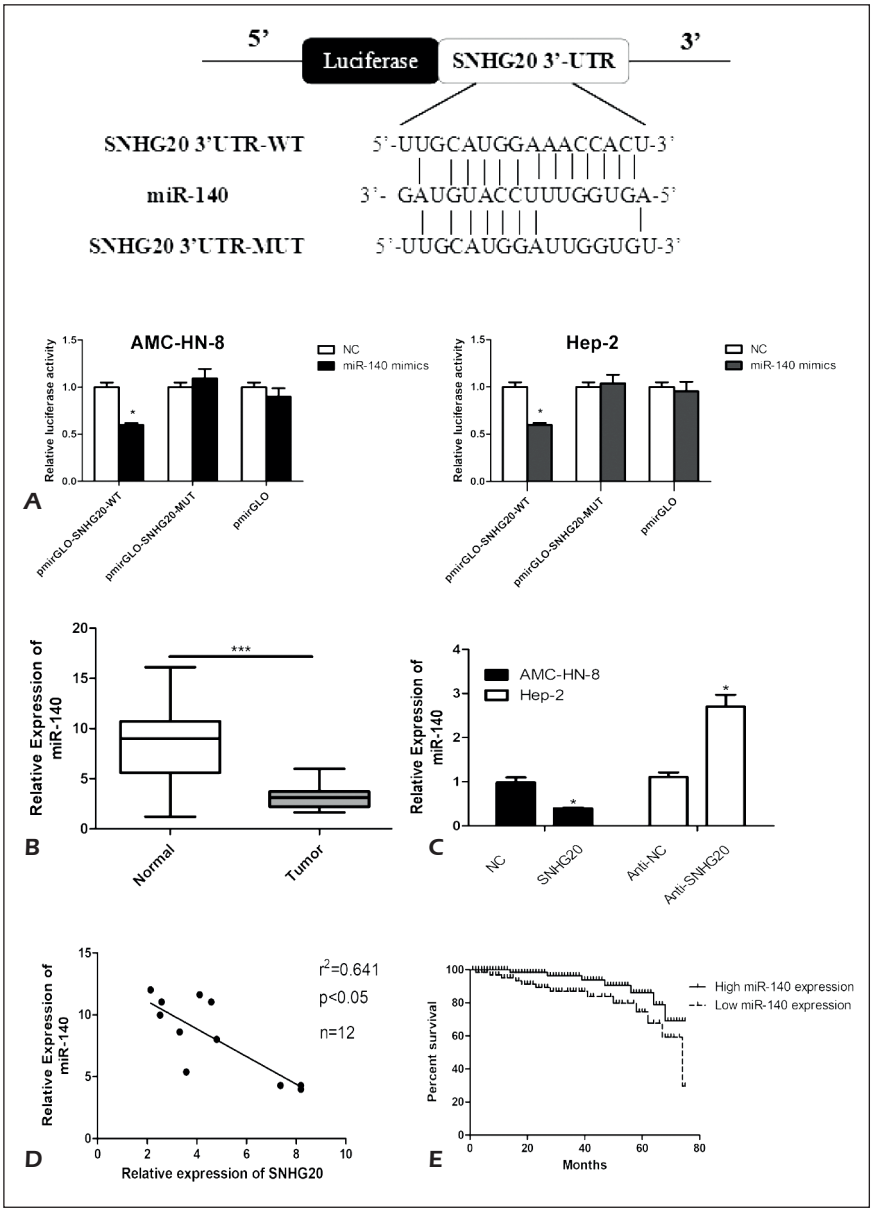

Figure 3. Direct targeting of miR-140 by SNHG20. **A**, Dual-luciferase reporter assay to demonstrate the direct targeting of SNHG20 to miR-140. The double luciferase reporter gene assay in AMC-HN-8 and Hep-2 cell lines showed that overexpression of miR-140 significantly attenuated luciferase activity ($p<0.001$) containing wild-type SNHG20 vector without decreased luciferase activity ($p>0.05$) containing mutant vector ($p>0.05$) or empty vector. **B**, MiR-140 expression in LSCC and adjacent non-tumor tissues detected by qRT-PCR. **C**, qRT-PCR verified the miR-140 mRNA level after transfection of SNHG20 in squamous cell carcinoma cell lines. **D**, The expression level of SNHG20 and miR-140 in LSCC was significantly negatively correlated. **E**, Kaplan Meier survival curve of LSCC patients based on miR-140 expression. Data are mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

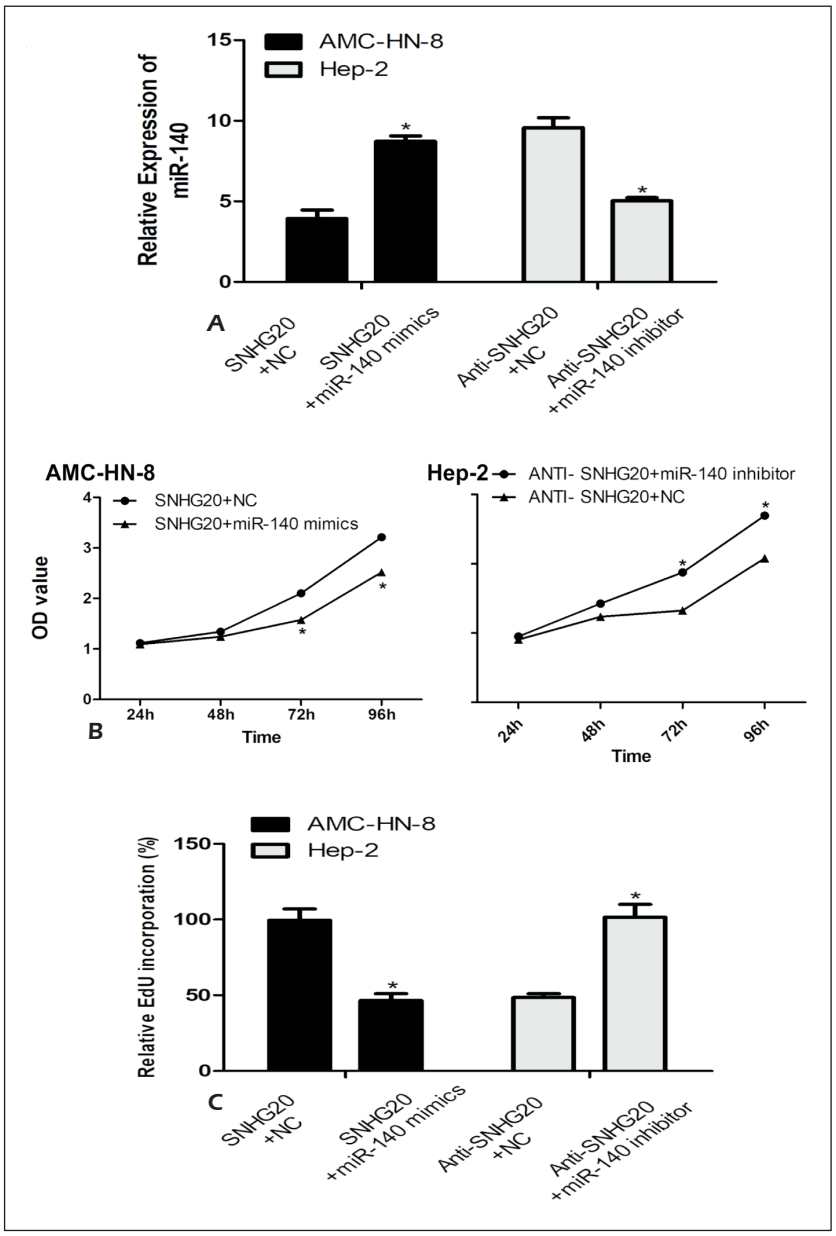

Figure 4. SNHG20 regulates the expression of miR-140 in LSCC and cell lines. **A**, Detection of miR-140 mRNA level in SNHG20 and miR-140 co-transfected cell lines by qRT-PCR. **B**, CCK-8 assay was done to explore the role of co-transfection of SNHG20 and miR-140 in laryngeal squamous cells proliferation. **C**, EDU assay to detect the role of SNHG20 and miR-140 after co-transfection in regulating the proliferation of LSCC cells. Data are mean ± SD, *$p<0.05$.

Discussion

Numerous studies have shown that less than 2% of the DNA sequences in the human genome can be translated into proteins, and more than 97% are transcribed into non-coding RNAs (ncRNAs). Most of the ncRNAs are transcribed to lncRNAs which are over 200 nucleotides in length[12-14]. Non-coding RNA was originally thought to be a non-functional noise accumulated during evolution[15,16]. With constant exploration, scientists found that lncRNA plays a particularly important role in the eukaryotic genome[17]. LncRNA is important in the development of various tumors, and its abnormal expression in tumors often leads to an imbalance in the expressions of oncogenes and tumor-suppressor genes[20]. At the same time, lncRNA has clinical value in predicting tumor prognosis and can be used as a molecular target for diagnosis and prognosis of various tumors[21]. However, whether lncRNA is involved in the development of LSCC still remains elusive[22]. SNHG20 is a recently discovered long non-coding RNA that is mapped on chromosome 17[23-25]. Previous studies have shown that SNHG20 is highly expressed in the liver cancer, and it is associated with a poor prognosis of osteosarcoma patients. Besides, the abnormally expressed SNHG20 is associated

with a poor prognosis in cervical cancer patients. However, researches on SNHG20 in the malignant progression of LSCC are rare. In the present work, SNHG20 overexpression or knockdown in LSCC cells was achieved by lentivirus transfection. Combined with bioinformatics analysis of the difference long non-coding RNAs expression between AMC-HN-8 and Hep-2 cell line, SNHG20 was selected as a candidate for evaluating tumor progression of LSCC. Up-regulated SNHG20 can promote the malignant progression of squamous cell carcinoma. Through tissue verification, we found that SNHG20 expression in LSCC was significantly higher, which could lead to an advanced tumor stage and a poor prognosis of LSCC. Therefore, we believed that SNHG20 may function in promoting squamous cell carcinoma.

According to the previous literature, the expression level of SNHG20 is up-regulated in osteosarcoma and cervical cancer tissues, and its up-regulation is closely related to metastasis and tumor resistance[23-25]. It is consistent with the high expression of SNHG20 in LSCC, illustrating that SNHG20 may be important in cancer-promoting process. To further study the molecular mechanism of SNHG20 in the development of laryngeal carcinoma and prove SNHG20 is a disease-related gene, we performed some cell experiments. In addition, to observe the role of SNHG20 in the proliferation of laryngeal squamous carcinoma cells, we performed CCK-8 and EdU experiments. We found that cell proliferation was significantly attenuated in SNHG20 silenced Hep-2 cell line compared with controls, revealing that SNHG20 knockdown could inhibit the proliferation of Hep-2 cell line. Compared with the NC group, the proliferation and cloning ability of AMC-HN-8 cell line overexpressing SNHG20 were significantly enhanced, displaying that SNHG20 can promote the proliferation of human LSCC cells. This study provides a theoretical basis for revealing the mechanism of laryngeal cancer development. However, the specific molecular mechanism of signal transduction needs further study.

LncRNA can play a role in promoting cancer or tumor suppression by competitively binding to the common miRNAs with mRNA. Previous studies have predicted by bioinformatics analysis that miRNA-140 may interact with SNHG20. MiRNA-140 is a key molecule in the miRNA family. The results of this experiment showed that miRNA-140 was lowly expressed in LSCC tissues, and inhibited proliferation of LSCC cells[29]. We used bioinformatics methods to analyze the SNHG20 sequence and found it contained a miRNA-140 binding site. Further experiments verified the direct binding of SNHG20 to downstream miRNA-140.

In LSCC cell lines, levels of SNHG20 and miR-140 were found to be negatively correlated. In addition, we performed a recovery experiment in LSCC cells and found that miRNA-140 counteracted the role of SNHG20 in LSCC cell lines. Evidence suggested that the transcriptional activity of SNHG20 may be regulated by miRNA-140. Dual-luciferase reporter gene assay demonstrated that SNHG20 could regulate the reporter gene activity of the miRNA-140 promoter. The above findings suggested that SNHG20 could inhibit the expression of miRNA-140 and promote the proliferation of LSCC cells.

Conclusions

We showed that lncRNA SNHG20 expression significantly increased in LSCC tissues or cells and significantly correlated with tumor stage and poor prognosis of LSCC patients. In addition, SNHG20 may promote the progression of LSCC by regulating miRNA-140.

Conflict of Interests

The authors declare that they have no conflict of interest.

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