Research article

SNHG20 promotes the development of laryngeal squamous cell carcinoma via miR-342-3p/MTDH axis

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ABSTRACT

Long noncoding RNAs (lncRNAs) are important players in laryngeal squamous cell carcinoma (LSCC). However, the function of the long noncoding RNA small nucleolar RNA host gene 20 (SNHG20) in LSCC is hardly known. We therefore analyzed the role of this lncRNA in LSCC. Our data showed that SNHG20 was significantly over-expressed in LSCC cell lines and human LSCC tissue. SNHG20 significantly promoted cell proliferation, migration and invasion of LSCC cells. The actions of SNHG20 are likely mediated by miR-342-3p expression, which results in increased expression of MTDH. Finally, the results of in vivo models confirmed that SNHG20 promotes LSCC progression through modulating miR-342-3p and MTDH expression. Taken together, our study demonstrates that SNHG20/miR-342-3p/MTDH axis participates in LSCC progression.

1. Introduction

Laryngeal squamous cell carcinoma (LSCC) is a type of highly aggressive head and neck squamous cell carcinoma [1]. Although the incidence of LSCC is relatively low compared with other common malignant tumors, LSCC is still considered as a major cause of head and neck cancer-related deaths worldwide due to its aggressive nature, low early detection rate and lack of effective treatment [2, 3]. The age-standardized mortality rate of LSCC is about 2.3/100,000 [4]. With the advances in LSCC treatment, patients at early stages can usually retain swallowing and vocal functions after proper treatment. However, in most cases, patients lose their natural voice and be conducted with permanent tracheostomy [5]. The progression of LSCC is characterized by locoregional or distant metastasis [6]. Inhibition of tumor cell migration and invasion is considered as a promising approach to improve the survival rate of LSCC patients. Although the occurrence of LSCC is affected by many factors, genetic variation is still the main cause of LSCC [7, 8, 9]. Therefore, the study of molecular pathways involved in LSCC may provide useful information to guide the diagnosis, prognosis and treatment of LSCC.

Long noncoding RNAs (lncRNAs) are transcripts over 200 nucleotides in length and have no protein-coding potential [10]. The imbalance of lncRNAs is related to the proliferation, invasion and metastasis of cancer cells [11]. SNHG20 involves in many types of cancer, such as cervical cancer and liver cancer [12, 13]. However, the role and molecular mechanism of SNHG20 in LSCC are still unknown.

MicroRNAs (miRNAs/miRs), ~22 nucleotides, exert crucial roles via inhibiting translation or promoting degradation [11]. Multiple studies demonstrated that miRNAs participated in the progression of various cancer types, including non-small cell lung cancer [14], colorectal cancer [15], thyroid cancer [16] and esophageal cancer [17]. A recent study suggested that miR-342-3p level was decreased in miR-342-3p, and upregulation of miR-338-3p repressed miR-342-3p cell proliferation [18]. Moreover, Jiang et al [19] demonstrated that has an anti-tumor effect, is low expressed in glioma and is associated with good prognosis of patients. These data revealed that miR-342-3p played an important role in cancers. But, the biological function of miR-342-3p in CRC remains unclear. Thus, the present explored the underlying mechanism of miR-342-3p in LSCC.

Here, we determined the levels of SNHG20, miR-342-3p and MTDH in LSCC tissues and cells. Furthermore, the function of SNHG20 was investigated in LSCC in vitro and in vivo. Besides, the underlying mechanism of SNHG20 in LSCC progression was explored.
2. Methods

2.1. Tissue sample and cells

This study included 80 LSCC patients recruited from the Third Xiangya Hospital, and inclusion criteria for patient selection were larynx carcinoma operation conducted between September 2018 and July 2021. No chemotherapy or neoadjuvant therapy was performed before surgery. All procedures complied strictly with the laryngeal carcinoma staging criteria of the American Joint Committee on Cancer (AJCC) staging system (8th version) in 2017. The collected tissues were stored in liquid nitrogen and stored at −80 °C until use. The Ethics Committee of the Third Xiangya hospital approved this study, and all patients signed the informed consent. Human LSCC cell lines TU177, TU686, LSC-1 and Third Xiangya hospital approved this study, and all patients signed the informed consent. Human LSCC cell lines TU177, TU686, LSC-1 and AMC-HN-8 as well as human bronchial epithelial cells 16HBE cell line (Shanghai Academy of Sciences) were used in this study. All cells were incubated in RPMI-1640 medium ( Gibco, NY, USA) with 10% fetal bovine serum (Gibco, NY, USA) at 37 °C with 5% CO2.

2.2. RNA extraction and RT-qPCR

TRizol reagent (Invitrogen, CA, USA) was mixed with tissue powder orcells to extract total RNA. The RNA amount and quality were detected using Nanodrop 2000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). A total of 500 ng RNA was reverse transcribed into cDNA in a volume of 10 μl using by using PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). PrimeScript RT reagent (Takara, Kusatsu, Japan) was used to perform reverse transcription. SYBR Green Master Mix II (Takara) was used to perform PCR mixtures. PCR reactions were conducted on the ABI 7900 fast real-time PCR system (ABI, CA, USA). The internal standard controls for lncRNA/mRNA and miRNA were GAPDH and U6, respectively. After the Ct values of GAPDH or U6 in each sample were obtained by RT-qPCR, the stability of the reference genes was analyzed by internal reference screening software (geNorm and NormFinder) (Table S1). Reactions were repeated 3 times, and data were processed utilizing 2−ΔΔCT methods. The sequences of the primers were as follows:

SNHG20-sense: 5′-CCAATTGGGCTTCCAGGCTA-3′;
SNHG20-antisense: 5′-GGGGGGATCTATCCACCA-3′;
MiR-342-3p-sense: 5′-CAGGAGGGCTTGGATCAGATG-3′;
MiR-342-3p-antisense: 5′-GGGCATACCCCTCGTAGATG-3′;
GAPDH-sense: 5′-ACGUGACACGUUCGGAGAATT-3′;
GAPDH-antisense: 5′-UUGAGUUGGGUAUGCA-3′.

2.3. Cell transfection

Lentiviral vectors were purchased from GenePharma (Shanghai, China). LV- SNHG20 refers to SNHG20 lentivirus, and the empty lentiviral vector LV-NC was utilized as a control. SNHG20-siRNA was synthesized by Genema (Shanghai, China). NC-siRNA was synthesized by Genema (Shanghai, China). NC-siRNA was synthesized by Genema (Shanghai, China). SNHG20-siRNA (antisense sequence): 5′-GGUGACAGUGGAGGAGAATT-3′.

2.4. CCK-8 cell proliferation assay

After transfection, cells were transferred to 96-well plates with 1 × 10^3 cells/well. Three replicate wells were set up for each experiment. CCK-8 (10 μl, Dojindo, Tokyo, Japan) was added to each well at 2 h before the end of cell incubation. Proliferated cell number was reflected by measuring the absorbance at 450 nm.

2.5. Colony formation assay

Trypsinization was conducted for colony formation. Cells were transferred to 6-well plates with 500 cells/well. Cells were cultivated for 10 d, and cell colonies were observed. Then, 4% paraformaldehyde was used to fix the cells for 15 min, and crystal violet (0.1%, Sigma, Germany) staining was conducted for 30 min. Colony-forming efficacy (%) was determined by calculating the ratio of colony number to cell seeding number.

2.6. Wound healing analysis

Wound healing analysis and transwell migration were conducted to analyze cell migration. In the wound healing analysis, cells were plated in 6-well plates after scratching the plate with a pipette tip. Images were taken under an optical microscope (Olympus, Japan) at 0 and 24 h. Cells were then incubated with serum-free medium for 24 h, and images were taken with the same view. ImageJ v1.46 software (National Institutes of Health, Bethesda, Maryland, USA) was used to analyze the images.

2.7. Transwell invasion assay

For transwell assay, 5 × 10^4 cells in non-serum culture medium were transferred to the upper chamber, and the lower chamber was filled with a culture medium with 20% FBS. The upper chamber was coated with Matrigel (BD Biosciences). Crystal violet (0.1%) was used to stain the cells, which were observed under IX71 inverted microscope (Olympus, Tokyo, Japan).

2.8. Luciferase reporter assay

Mutant (mut) miR-342-3p and putative wild-type (wt)-binding sites in the 3′-UTR of SNHG20 or MTDH mRNA, termed pmirGLO-SNHG20-wt or pmirGLO- SNHG20-mut and pmirGLO-MTDH-wt or pmirGLO-MTDH-mut, were cloned to a pmirGLO-Report luciferase vector (Genearray Biotechnology, China). The reporter plasmid was transfected into TU177 cells in the presence of either LV- SNHG20 and/or miR-342-3p mimic. Luciferase activity was assessed using a dual-luciferase reporter-assay system (Promega, USA) at 48 h post-transfection.

2.9. In vivo tumor formation assay

BALB/c nude mice (SPF grade) aged 4–6 weeks were reared without pathogen. The nude mice were fed at 22.25 ± 3.14 °C and 52.56 ± 2.03% humidity. The nude mice were divided into three groups: experimental group, control group and blank group. In each group, LV-NC, LV-SNHG20 or LV-SNHG20 + miR-342-3p was injected into the second pair of breast fat pads on the right chest wall of nude mice. The growth of the tumor was observed and recorded every day. The mice were euthanized, and the tumor was removed at 28 d after the first injection. The tumor was weighed, and the long and short diameter of the tumor were measured with Vernier caliper. The volume of the tumor was calculated as (long diameter × short diameter^2)/2, once every 7 d for a total of 4 times. The average value was calculated, and the analysis of variance was made. Animal experiments were conducted in accordance with guidelines for the Protection and Use of Experimental Animals issued by the
National Institutes of Health. Animal research was carried out following the scheme approved by the Animal Protection and Use Committee of the Third Xiangya Hospital, Central South University.

2.10. Western blot

Cells were mixed with RIPA reagent (Beyotime, Shanghai, China) to extract total proteins. Proteins were separated on 10% SDS-polyacrylamide gel. Gel transfer to polyvinylidene difluoride (PVDF) membrane was conducted. After blocking for 2 h in 5% skim milk, the membranes were incubated at 4 °C for overnight with primary antibodies of MTDH (Cell Signaling Technology) and GAPDH (Cell Signaling Technology). After that, incubation with HRP-conjugated anti-rabbit IgG (1:2000) was conducted for 2 h at room temperature.

2.11. Statistical analysis

All experimental data were analyzed using GraphPad software 7.0 and SPSS19.0. One-Sample Kolmogorov–Smirnov test was performed to analyze whether the data are normally distributed or skewed distributed. For normally distributed data, comparisons between two groups were performed using student’s t test. For skewed distributed data, the comparison between two groups was performed by paired sample Wilcoxon signed-rank test. Pearson’s correlation coefficient was used for correlation analysis. $P < 0.05$ was statistically significant.

3. Results

3.1. The expression of SNHG20 is upregulated in LSCC

The expression of SNHG20 in 80 pairs of LSCC tissues and adjacent non-tumor tissues was determined by RT-qPCR. The results showed that the expression of SNHG20 in LSCC tissues were markedly up-regulated compared with non-tumor tissues (Figure 1A, $p < 0.01$). Moreover, the expression of SNHG20 in the four LSCC cell lines (TU177, TU686, LSC-1 and AMC-HN-8) was remarkably enhanced compared with human bronchial epithelial cells line (16HBE) (Figure 1B, $p < 0.05$, $p < 0.01$, $p < 0.001$). Besides, the expression of SNHG20 in colon cancer cell lines (HCT116, SW480, SW620 and LoVo cells) was markedly increased compared with normal colonic cell line (NCM460) (Figure 1B, $p < 0.05$, $p < 0.01$, $p < 0.001$). Furthermore, the 80 patients were divided into two groups according to the median expression levels of SNHG20 (high versus low, $n = 40$). The sample size and took the average of the two middle values as the median (1.752). As shown in Table 1, the high expression levels of SNHG20 were markedly correlated with positive lymph node metastasis ($p = 0.010$), advanced T category ($p = 0.006$) and advanced clinical stage ($p = 0.017$). The expression levels of SNHG20 were higher in patients with positive lymph node metastasis, advanced T category and advanced clinical stage (Figure 1C, D, and E, $p < 0.01$, $p < 0.05$). These results suggest that SNHG20 may play an important role in LSCC development.

![Figure 1](image-url)
3.2. SNHG20 promotes the proliferation, migration and invasion of LSCC cells

The si-SNHG20 and LV-SNHG20 lentiviral vector were designed, synthesized and transfected to TU177 cells. Overexpression and knockdown of SNHG20 in LSCC cells were confirmed by qRT-PCR (Figure 2A, \( p < 0.001 \)). The results showed that the proliferated TU177 cell number was markedly decreased in the siRNA-SNHG20 group compared with the control group, while it was markedly increased in the LV-SNHG20 group (Figure 2B and C, \( p < 0.01 \)). Moreover, migration and invasion of LSCC cells were also significantly affected (Figure 2D and E, \( p < 0.05, ***p < 0.001 \)).

Figure 2. SNHG20 promotes the proliferation, migration and invasion of LSCC cells. A. RT-qPCR was conducted to test the transfection efficiency of si-SNHG20 or LV-SNHG20 in TU177 cells. B. CCK-8 assay to test cell viability. C. Colony formation assay to test proliferated cell number. D. Wound healing assay to test cell migration. E. Transwell assay to test cell invasive ability. (* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \)).

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Figure 3. SNHG20 directly targets miR-342-3p in LSCC. A. Bioinformatics analysis illustrated the predicted potential base pairing formed through SNHG20 and miR-342-3p. B. Luciferase activity was determined in TU177 cells co-transfected with miR-342-3p and luciferase reporters with SNHG20 or mutant transcript. C. RT-qPCR was conducted to detect the expression of miR-342-3p in LSCC tissue samples and matched normal tissue samples. D. RT-qPCR was used to detect the expression of miR-342-3p after LV-SNHG20 or si-SNHG20 transfection. E. Pearson’s correlation coefficient revealed a negative correlation between SNHG20 and miR-342-3p in LSCC tissue samples. (** \( p < 0.01 \)).
transwell assay revealed that the colony formation, migration and invasion of LSCC cells transfected with si-SNHG20 were markedly decreased compared with that transfected with si-NC, while they were markedly enhanced after LV-SNHG20 transfection (Figure 2C, D, and E, \( p < 0.01 \)).

Therefore, these results suggest that SNHG20 promotes the proliferation, migration and invasion of LSCC cells.
3.3. **SNHG20 directly targets miR-342-3p in LSCC cells**

To investigate the binding between SNHG20 and miR-342-3p, the bioinformatics analysis by TargetScan and miRDB was used to identify the potential MRE binding to miR-342-3p in the 3'UTR region of SNHG20 mRNA, which was named mut-SNHG20 (Figure 3A). Moreover, dual luciferase reporter assay illustrated that miR-342-3p mimic markedly lowered the luciferase activity of SNHG20-WT compared with negative control in TU177 cells (Figure 3B, \( p < 0.01 \)). The mRNA and protein expression of MTDH was examined through RT-qPCR and western blot in TU177 cells treated with si-SNHG20 and miR-342-3p mimics. The mRNA and protein expression of MTDH were determined in TU177 cells treated with LV-SNHG20 and miR-342-3p. G. Positive correlation between SNHG20 and MTDH expression in LSCC tissues. H. Negative correlation between MTDH and miR-342-3p in LSCC tissues. The non-adjusted images for western blot were in supplementary material. (*\( p < 0.05 \), **\( p < 0.01 \)).

3.4. **SNHG20 suppresses miR-342-3p to promote cell proliferation, migration and invasion of LSCC cells**

TU177 cells were transfected with miR-342-3p mimic and LV-SNHG20 or miR-342-3p mimic and si-SNHG20. As shown in Figure 4A and B, miR-342-3p mimic further reduced the cell viability induced by SNHG20 knockdown, while enhanced the cell viability induced by SNHG20 overexpression (Figure 4A and B, \( p < 0.01 \)).
Similarly, as shown in Figure 4B, C, and D, miR-342-3p mimic enhanced the effects of SNHG20 knockdown and inhibited the effects of SNHG20 overexpression on the proliferation, migration and invasion of TU177 cells (p < 0.01, or p < 0.05). In addition, colony formation assay and transwell assay were also conducted to analyze the effect of miR-342-3p inhibitor on the proliferation and invasion of TU177 cells transfected with si-SNHG20 (Figure 4E and F, p < 0.01). The results showed that 342-3p inhibitor reversed the effects of SNHG20 knockdown on the proliferation and invasion of TU177 cells. Taken together, SNHG20 promotes cell proliferation, migration and invasion through suppressing miR-342-3p in LSCC.

3.5. SNHG20 upregulates the expression of MTDH by inhibiting miR-342-3p

To investigate the direct binding between microRNA-342-3p and MTDH, the bioinformatics analysis by TargetScan and miRDB was conducted to predict one potential MRE binding to miR-342-3p in the 3'-UTR region of MTDH mRNA, which was named MTDH-MUT (Figure 5A). The results showed that the MTDH-WT decreased the reporter activities (Figure 5B, p < 0.01), but not the MTDH-MUT. Moreover, the mRNA and protein expression of MTDH was also markedly decreased after transfection of miR-342-3p mimic into TU177 cells (Figure 5C, p < 0.01). These data indicated that MTDH was a direct target of miR-342-3p. Next, we further investigated whether SNHG20 promoted LSCC development through regulating the miR-342-3p/MTDH axis. As expected, knockdown of SNHG20 downregulated the expression of MTDH, which was further decreased after co-transfection with miR-342-3p mimic. In contrast, overexpression of SNHG20 markedly upregulated the expression of MTDH, while miR-342-3p mimic inhibited the effect of SNHG20 overexpression on the expression of MTDH (Figure 5D and F, p < 0.05, p < 0.01). RT-qPCR and Western blot were conducted to evaluate the effect of miR-342-3p inhibitor on the expression of MTDH in TU177 cells (Figure 5E, p < 0.01). The results showed that miR-342-3p inhibitor could further decrease after co-transfected with miR-342-3p inhibitor. In addition, the expression of MTDH was positively correlated with the expression of SNHG20 and negatively correlated with the expression of miR-342-3p in LSCC tissues (Figure 5G and H, p < 0.01). These results suggest that SNHG20 can inhibit miR-342-3p to promote the expression of MTDH in LSCC.

3.6. SNHG20 promotes LSCC development in vivo through regulating the miR-342-3p/MTDH axis

As shown in Figure 6A, B, and C, overexpression of SNHG20 increased the tumor size (p < 0.01) and weight (p < 0.01), while overexpression of miR-342-3p inhibited the effect of SNHG20 overexpression on the tumor size (p < 0.05) and weight (p < 0.05). Moreover, overexpression of SNHG20 markedly promoted the expression of MTDH (p < 0.01), while overexpression of miR-342-3p inhibited the effect of SNHG20 overexpression on the expression of MTDH in xenograft tumors (Figure 6D, p < 0.05). These results illustrate that SNHG20 promotes LSCC development through the inhibition of the miR-342-3p/MTDH axis.

4. Discussion

Our study investigated the involvement of SNHG20 in LSCC. We found that SNHG20 was upregulated in LSCC and promoted the development of LSCC by regulating the miR-342-3p/MTDH axis.

It is frequently observed that lncRNAs play similar roles in different types of cancer. For example, lncRNA HOTAIR and H19 promoted the development of different types of cancer by regulating cancer cell behaviors, such as promoting cell migration, invasion and proliferation or inhibiting cell apoptosis [20, 21, 22]. However, lncRNAs could also exert different functions in different types of cancer. For example, lncRNA TUG1 was downregulated in glioma and induces cell apoptosis as a tumor suppressor gene [23]. In contrast, lncRNA TUG1 was upregulated in osteosarcoma and could accelerate cancer development through promoting cell proliferation [24]. LncRNA SNHG20 played oncogenic roles in many types of cancer, such as cervical cancer and liver cancer [12, 13]. In this study, we found that the expression of SNHG20 was also upregulated in LSCC, and it promoted LSCC cell migration, invasion and proliferation. These results revealed its oncogenic role in LSCC.

LncRNAs exert multiple functions in cancer biology [25]. LncRNAs also interact with other non-coding RNAs, such as miRNAs [26]. LncRNAs might sponge miRNAs to attenuate their functions [27]. In this study, we illustrated that SNHG20 was likely a sponge of miR-342-3p in LSCC cells. MiR-342-3p was a well-characterized tumor-suppressive miRNA in many types of cancer [28, 29]. MiR-342-3p inhibited tumor development through directly targeting oncogenes, such as AGR2 and E2F1 [28, 29]. In this study, knockdown of SNHG20 inhibited cell proliferation, migration and invasion. MiR-342-3p could strengthen the inhibitory effect of SNHG20 overexpression on the expression of MTDH, which was further decreased after co-transfection with miR-342-3p mimic. In addition, the expression of MTDH was positively correlated with the expression of SNHG20 and negatively correlated with the expression of miR-342-3p in LSCC tissues (Figure 5G and H, p < 0.01). These results suggest that SNHG20 can inhibit miR-342-3p to promote the expression of MTDH in LSCC.

Figure 6. SNHG20 promotes LSCC development in vivo via regulating the miR-342-3p/MTDH axis. A. The tumor size was directly tested in indicated groups. B. Tumor volume was tested in indicated groups. C. Tumor weight was tested in indicated groups. D. The expression of MTDH protein was determined through western blot. The non-adjusted images for western blot were in supplementary material. (*p < 0.05, **p < 0.01).
SNHG20 knockdown cell behavior. Moreover, overexpression of SNHG20 promoted the proliferation, migration, and invasion of LSCC cells in vitro as well as tumor growth in vivo by competitively binding to miR-342-3p.

MTDH is a multifunctional oncoprotein that has been confirmed to be activated in many malignant tumors, including gastric cancer [30], prostate cancer [31] and glioma [32]. Moreover, it has been reported that MTDH accelerated EMT in non-small cell lung cancer cells [33]. He et al. revealed that MTDH facilitated migration and invasion of clear cell renal cell carcinoma by regulating SND1-mediated ERK signaling and EMT [34]. In this study, through bioinformatic prediction and dual-luciferase reporter assays, MTDH was demonstrated to be a direct target gene of miR-342-3p in LSCC cells. Moreover, downregulation of SNHG20 resulted in a decrease in MTDH expression, which was further inhibited by miR-342-3p overexpression. SNHG20 overexpression led to increased expression of MTDH, which could be partially reversed by miR-342-3p overexpression, indicating a SNHG20/miR-342-3p/MTDH axis in LSCC.

This research only included 80 LSCC patients. This small sample size is a major limitation of this research. Our future study will include more patients to further detect the expression and role of SNHG20 in LSCC.

5. Conclusion

In summary, we identified SNHG20 as a tumor-promoter in LSCC, and the higher expression of SNHG20 was associated with tumor proliferation, migration and invasion. LncRNA SNHG20 acted as a sponge for miR-342-3p to attenuate its repressive effect on MTDH. Our results provide a better understanding of the role of SNHG20 in LSCC progression and a potential therapeutic target and prognostic predictor against this malignancy.

Declarations

Author contribution statement

Zuozhong Xie: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Hong Xiang, Jingkun Li, Xiaowei Zhang, Wei Li: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Guolin Tan: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

No data was used for the research described in the article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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