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Small Nucleolar RNA Host Gene 12 (SNHG12) Promotes Proliferation and Invasion of Laryngeal Cancer Cells via Sponging miR-129-5p and Potentiating WW Domain-Containing E3 Ubiquitin Protein Ligase 1 (WWP1) Expression

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The clinical significance and biological function of long noncoding RNA SNHG12 have not been identified in laryngeal squamous cell carcinoma (LSCC).

Material/Methods: Expression levels of SNHG12, miR-129-5p, and WWP1 in LSCC tissues or cells were tested by RT-qPCR. MTT assay, flow cytometry, and Transwell assay were used to identify the progression of LSCC cells *in vitro*. Luciferase reporter assay was used to assess the associations among SNHG12, WWP1, and miR-129-5p.

Results: SNHG12 was significantly overexpressed in LSCC tissues compared with adjacent normal tissues. The expression level of SNHG12 was significantly associated with T classification, lymph node metastasis, and cancer stage of LSCC. High expression of SNHG12 predicted shorter disease-free survival. Suppressing SNHG12 using siRNA inhibited proliferation and invasion and promoted apoptosis in the AMC-HN-8 LSCC cell line. SNHG12, mainly located in cytoplasm of AMC-HN-8 cells, was validated by dual luciferase reporter test and RT-qPCR to directly interact with miR-129-5p. Inhibition of miR-129-5p significantly increased proliferation and invasion of AMC-HN-8 cells and ameliorated the suppressive effects of si-SNHG12. Luciferase assay showed that miR-129-5p was able to combine with the 3'UTR region of WWP1, which is generally regarded as an E3 ubiquitin protein ligase. RT-qPCR and Western blot showed that WWP1 was positively regulated by SNHG12 and negatively regulated by miR-129-5p at the mRNA level and protein level. Overexpression of WWP1 significantly increased proliferation and invasion of laryngeal cancer cells. Moreover, when SNHG12 was suppressed, rescue of WWP1 restored the proliferation and invasion abilities of AMC-HN-8 cells.

Conclusions: Our study demonstrated that SNHG12 promoted LSCC cells progression via sponging miR-129-5p and potentiating WWP1 expression.

MeSH Keywords: Laryngeal Neoplasms • MicroRNAs • RNA, Long Noncoding

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Background

Laryngeal squamous cell carcinoma (LSCC) is the second most common head and neck squamous cell carcinoma (HNSCC), with a reported 177 422 new cases and 94 771 deaths occurring in 2018 [1]. Although multidisciplinary treatment, including radiotherapy, surgery, and chemotherapy, achieved much progress in improving survival of LSCC patients, some of these patients die of tumor recurrence and metastasis. It was reported that in the last 10 years, the incidence of LSCC showed a downward trend, with a 1.27% annually decrease in China, while the overall mortality rate of LSCC remained stable [2]. Thus, understanding the molecular mechanisms of carcinogenesis and exploring predictive biomarkers for LSCC are still urgent, which may benefit early diagnosis and recurrence monitoring.

Long noncoding RNAs (lncRNAs), which are over 200 nucleotides in length and without protein-coding capability, are well recognized as critical regulators of gene transcription and chromosome modification [3]. Their clinical applications as diagnostic and prognostic biomarkers and vital biological roles in cancer cells have been reported in many malignances [4]. Previous studies have indicated that small nucleolar RNA host gene 12 (SNHG12), which is a long noncoding RNA located at chromosome 1p35.3 and is 675 nucleotides in length, can promote proliferation and metastasis of cancer cells, including gastric cancer [5], triple-negative breast cancer [6], and cervical cancer [7]. It also has been reported to predict worse survival of patients with osteosarcoma [8], hepatocellular carcinoma [9], and glioma [10]. However, to the best of our knowledge, the prognostic role, biological effects, and regulatory mechanism of SNHG12 in laryngeal squamous cell carcinoma have not been revealed.

The most common regulatory mechanism of lncRNAs is to function as competitive endogenous RNAs (ceRNAs), in which lncRNAs bind to targeted miRNA, resulting in downstream gene silencing [11]. miR-129-5p has recently been identified as a tumor suppressor in many types of cancer, including ovarian cancer [12], breast cancer [13], and osteosarcoma [14]. However, its biological role in LSCC has not been explored. WW domain-containing E3 ubiquitin protein ligase 1(WWP1) is an E3 ubiquitin protein ligase and WWP1 gene frequently amplified in many types of human cancer. To the best of our knowledge, its biological effects on proliferation and invasion of LSCC have not been explored.

In this study, we explored the relationship between SNHG12 expression and clinical parameters of LSCC patients. We found that SNHG12 and WWP1 promote proliferation and invasion of laryngeal cancer cells, and the relationships among SNHG12, miR-129-5p, and WWP1 were preliminary defined.

Material and Methods

Tissue samples

Fifty LSCC patients undergoing surgery at the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology from April 1, 2015 to May 31, 2017 were enrolled in this study, with follow-up visits for disease-free survival to June 1, 2018. Disease-free survival was defined as the time from the date of surgery to LSCC recurrence or last visit. The longest follow-up time was 36 months and the shortest was 12 months. A total of 50 tumor and 22 adjacent normal tissues were immediately frozen with liquid nitrogen, and kept in a freezer at –80°C. The histological diagnosis, confirmed by the Pathology Department of our hospital, and TNM stage (according to AJCC version 7) were verified and recorded. None of the enrolled patients had received chemotherapy or radiotherapy before surgery. Written informed consent was signed by all enrolled patients. This study was approved by the Ethics Committee of the Central Hospital of Wuhan in accordance with the ethics guidelines of the Declaration of Helsinki.

Cell culture and transfection

The LSCC cell line AMC-HN-8 was purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in 90% DMEM (high-glucose) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin and streptomycin. Cells in culture dishes were placed in a 37°C incubator with 5% CO_2 atmosphere. SNHG12 siRNA (si-SNHG12: 5'-GUGCUGCAAUCAACUUUAAUU-3') [15], WWP1 siRNA (si-WWP1: 5'-GGAGGCGCUUAUAUGUAAU-3') and control siRNA (si-NC: 5'-UUGUACUACACAAAAGUACUG-3') [16] were purchased from RiboBio (Guangzhou, China). miR-129-5p inhibitor (anti-miR-129-5p) and its negative control (anti-miR-NC) and miR-129-5p mimic and its control (miR-NC) were obtained from GenePharma (Shanghai, China). The cDNA encoding WWP1 was amplified by PCR and then cloned into the vector pcDNA3.1 (Invitrogen, CA, USA), generating the vector pcDNA-WWP1. The empty pcDNA3.1 vector was used as a control. Lipo3000 reagent (Thermo Fisher Scientific, Waltham, USA) was used to assist transfection according to the manufacturers' protocols. The transfection efficiency was assessed and subsequent assays were performed 48 h after transfection.

RNA extraction and real-time quantitative PCR

Trizol reagent (Invitrogen, Carlsbad, USA) was used to extract total RNA of cells and tissue samples. Then, the PrimeScript™ RT reagent kit and gDNA Eraser kit (Takara, Ohtsu, Japan) were used to perform reverse transcription reaction and to obtain cDNA. Primers used were purchased from Sangon Biotech (Shanghai, China). The sequences were:

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SNHG12 (forward), 5'-GGTGCTCCAGGCAATAACT-3',
SNHG12 (reverse), 5'-CTCCCATACAGTCCGAACAT-3';
WWP1 (forward), 5'-GAAGGCACGAATGGAATAG-3',
WWP1 (reverse), 5'-GTGACAGACGCATTATCAG-3';
miR-129-5p (forward), 5'-CGGCGGTTTTTTGCGGTCTGGGCT-3',
miR-129-5p (reverse), 5'-AGCCCAGACCGCAAAAAACCGCCG-3';
GAPDH (forward), 5'-CCTGCCTCTACTGGCGCTGC-3',
GAPDH (reverse), 5'-GCAGTGGGGACACGGAAGGC-3';
U6 (forward) 5'-CTCGCTTCGGCAGAACA-3',
U6 (reverse), 5'-ACGCTTCACGAATTTGCGT-3'.
Real-time quantitative PCR (RT-qPCR) was conducted in the Applied Biosystems 7900 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, USA) using SYBR® Premix Ex Taq™II Kit (Takara, Ohtsu, Japan) according to the manufacturer' instructions. The relative expression levels of RNAs were evaluated using the $2^{-\Delta\Delta CT}$ calculation method [17].

Subcellular fractionation assay

Nuclear and cytoplasmic extraction reagents (Thermo Scientific, Waltham, USA) were used to separate nuclear RNAs and cytoplasmic RNAs in AMC-HN-8 cells. Then, RT-qPCR analysis was conducted to identify the relative expression levels of SNHG12, U6, and GAPDH.

Cell viability assay

We placed 96-well plates containing 1.0×10^3 transfected AMC-HN-8 cells per well into the previously described incubator and cells were allowed to grow for 24, 48, and 72 h. Then, 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution with a final concentration of 0.5 mg/ml in culture medium was added into wells. After incubation for 4 h at 37°C, culture medium in each well was replaced by 100 µL formazan solubilization solution. Plates were gently mixed for 10 minutes to dissolve the formazan crystals. OD values at 570 nm of the wells were measured using a microplate reader (Bioteck Instrument, Vermont, USA).

Transwell invasion assay

Transwell invasion assay was conducted as previously described [18] with some modifications. Briefly, 2×10^4 treated LSCC cells in FBS-free medium were seeded onto the Matrigel-coated membrane in the upper chamber, while 600 µl medium with 10% FBS was added into the lower chamber. Twenty-four hours later, invading cells on the bottom of the filter were fixed, stained, air dried, photographed at 100×, and counted manually.

Flow cytometry test

Transfected LSCC cells at a concentration of 1×10^6 cells/ml were resuspended and labeled with Annexin V-APC (5 µl) and PI (1 µl) for every 100 µl suspension in the dark. After adding 400 µl 1× binding buffer to terminate the reaction, cell apoptosis was analyzed using a FACSort flow cytometer and FlowJo software (BD Biosciences, San Jose, USA).

Luciferase reporter test

The direct combination of miR-129-5p with WWP1/SNHG12 was evaluated by dual luciferase reporter assay. To achieve this, the sequence of WWP1/SNHG12, including bonding sites of miR-129-5p (WWP1-WT/SNHG12-WT), was cloned into the pmirGLO Dual Luciferase Expression Vector (Promega, Madison, WI, USA), while WWP1/SNHG12 mutated type (WWP1-MUT/SNHG12-MUT) contained an unmatched binding sequence of miR-129-5p. Then, miR-129-5p mimics were co-transfected into AMC-HN-8 cells with WWP1-MUT/SNHG12-MUT or WWP1-WT/SNHG12-WT with Lipofectamine 3000. Luciferase activities standardized to Renilla were calculated using the Dual Luciferase Reporter Assay System (Promega, Madison, USA).

Western blot analysis

Protein extraction and Western blot analysis were performed as previously described [19]. The primary antibodies used were WWP1 (1: 1000, 13587-1-AP, Proteintech, Rosemont, USA) and GAPDH (1: 10000, 10494-1-AP, Proteintech, Rosemont, USA). HRP-conjugated goat anti-rabbit antibody (BA1054, Boster, Wuhan, China) was used as the second antibody for incubation at a dilution of 1: 10 000.

Statistical analysis

Differences between 2 groups were calculated by *t* test, while the comparison of multiple groups was conducted by one-way ANOVA. The chi-square test was used to test the association of SNHG12 expression with clinical parameters. The relationship between RNA expression levels was analyzed using Pearson's correlation test. Data are shown as the mean ±SD and were statistically analyzed using SPSS version 19.0 (IBM Company, NY, USA).

Results

SNHG12 was overexpressed and predicted worse survival in LSCC

LSCC shares similar mechanisms of carcinogenesis with HNSCC. We preliminarily explored the role of SNHG12 in HNSCC using the TCGA database. Using the GEPIA online tool (*http://gepia.cancer-pku.cn/index.html*), we found that expression levels of SNHG12 in HNSCC were significantly higher than in adjacent normal tissues ($P<0.05$, Figure 1A). Then, RT-qPCR was applied

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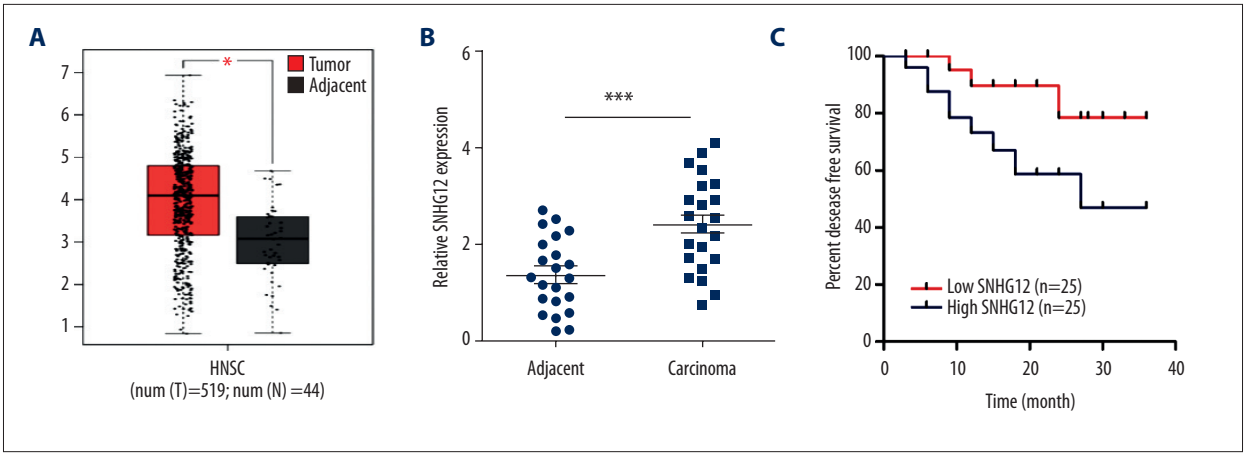

Figure 1. SNHG12 was overexpressed and predicted worse survival in LSCC. (**A**) SNHG12 expression in LSCC tissues and adjacent normal tissues was analyzed in the TCGA database using GEPIA tools. (**B**) Expression levels of SNHG12 in the collected 22 LSCC and paired 22 normal tissues were evaluated by RT-qPCR. (**C**) Kaplan-Meier survival analysis revealed the association of SNHG12 expression with disease-free survival. * P<0.05, *** P<0.001.

Table 1. Relationship between SNHG12 expression levels and clinicopathologic characteristics of LSCC patients.

Parameters	Group (n)	SNHG12 expression		P value
		Low	High	
Age, years	<60 (27)	11	16	0.256
	≥60 (23)	14	9	
Gender	Male (32)	14	18	0.239
	Female (18)	11	7	
Primary location	Supraglottic (20)	8	12	0.248
	Glottic (30)	17	13	
T classification	T1–2	17	7	0.005
	T3–4	8	18	
Lymph node metastasis	N0	16	8	0.023
	N1–2	9	17	
Clinical stage	I–II	14	7	0.045
	III–IV	11	18	

to validated this result in 22 LSCC tissues and 22 paired adjacent normal tissues (Figure 1B). For the 50 enrolled LSCC cases, SNHG12 expression was not significantly correlated with age, sex, or tumor location, but was significantly associated with clinical parameters such as T classification, lymph node metastasis, and cancer stage (Table 1). Kaplan-Meier survival analysis showed that high expression level of SNHG12 predicted shorter disease-free survival (DFS) of LSCC patients, while low expression indicated longer DFS (Figure 1C).

miR-129-5p was a direct target of SNHG12 in LSCC

Subcellular fractionation test results validated that SNHG12 was mainly located in the cytoplasm of AMC-HN-8 cells (Figure 2A). As interaction with mRNA via miRNA is the most common functional mechanism for lncRNA in the cytoplasm, we used the ceRNA theory to reveal its biological mechanism. Bioinformatic analysis using miRDB (*http://mirdb.org/custom.html*), mirDIP (*http://ophid.utoronto.ca/mirDIP/*) and miRcode (*http://mircode.org/*) predicted that miR-129-5p is a mutual target of SNHG12 (Figure 2B). We then explored the association of SNHG12 with miR-129-5p in LSCC tissues, showing a negative correlation between them (Figure 2C, r=0.412, P=0.04).

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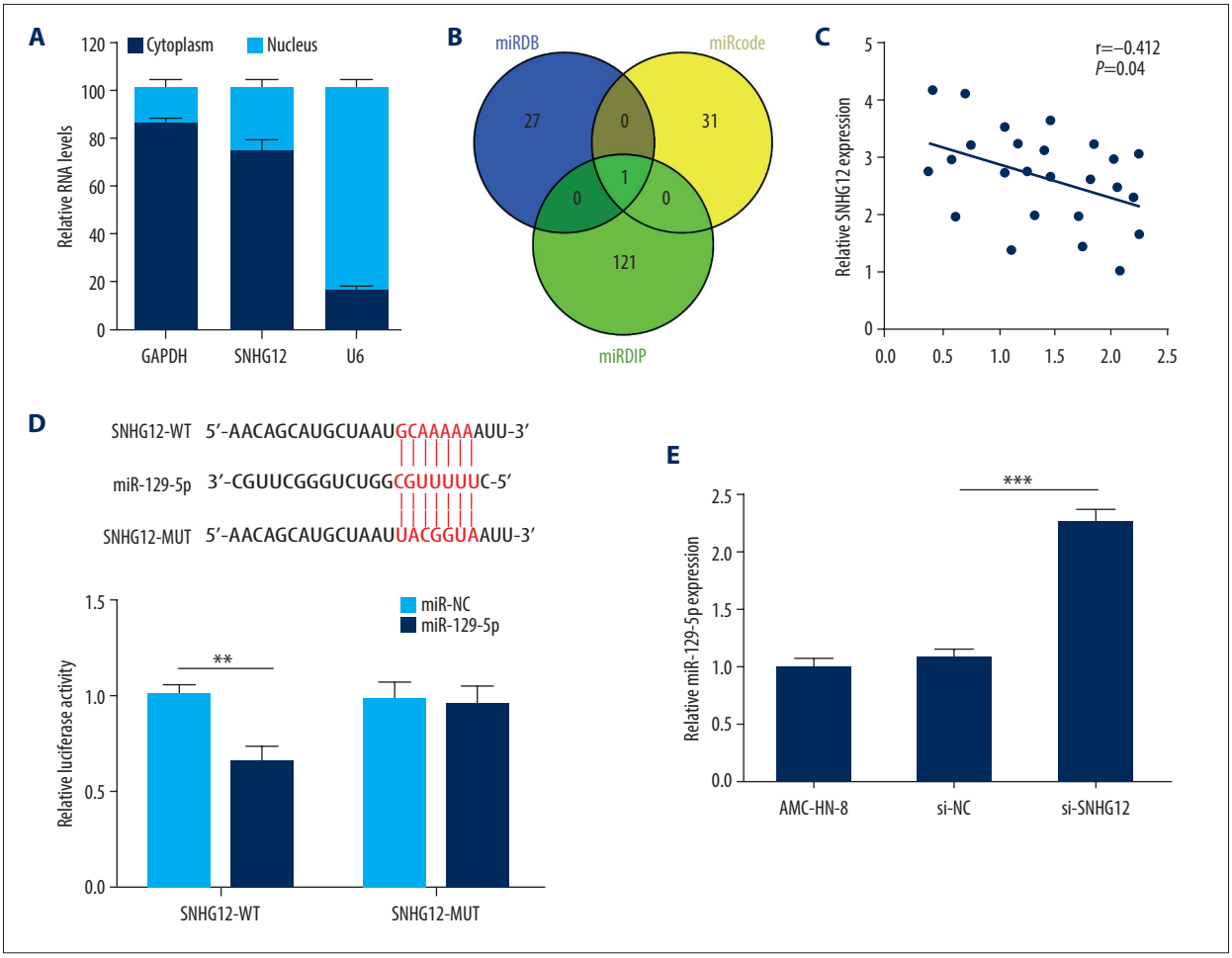

Figure 2. miR-129-5p is a direct target of SNHG12 in LSCC. (**A**) Subcellular fractionation test was conducted to evaluate the distribution of SNHG12 in AMC-HN-8 cells. (**B**) Online bioinformatic prediction of SNHG12 targeted miRNA using miRDB, mirDIP, and miRcode. These 3 online tools shared 1 predicted miRNA in common – miR-129-5p. (**C**) Expression levels of miR-129-5p and SNHG12 were evaluated by RT-qPCR, and the relationship between them was assessed using Pearson's correlation test. (**D**) Sequences contained in SNHG12-MUT/SNHG12-WT and the targeted sites of miR-129-5p are shown. miR-NC/miR-129-5p mimics were co-transfected with SNHG12-MUT/SNHG12-WT into AMC-HN-8 cells, and luciferase activity was analyzed. (**E**) The expression of miR-129-5p in AMC-HN-8 cells was inhibited by transfection of si-SNHG12.

According to the predicted binding sites (Figure 2D), we constructed SNHG12-MUT and SNHG12-WT vectors to perform dual luciferase reporter testing. The luciferase activity of SNHG12-WT was strongly repressed by upregulated miR-129-5p, while the luciferase of the SNHG12-MUT group was not influenced by miR-129-5p in AMC-HN-8 cells (Figure 2D). Moreover, suppressing SNHG12 significantly repressed the expression level of miR-129-5p (Figure 2E). All these results indicate that SNHG12 interacts with miR-129-5p and modulates its expression in LSCC cells.

SNHG12 interacted with miR-129-5p to promote LSCC progression

As shown in Figure 3A, SNHG12 was successfully silenced by siRNA transfection as evaluated by RT-qPCR. Cell viability assay demonstrated that SNHG12 silencing inhibited AMC-HN-8 proliferation while miR-129-5p inhibition promoted cell viability. When compared to the si-SNHG12+anti-miR-NC group, co-transfection of si-SNHG12 with anti-miR-129-5p significantly ameliorated the inhibiting effect of si-SNHG12 on cell proliferation (Figure 3B, 3C). These results revealed that the viability-promoting effects of SNHG12 were modulated by miR-129-5p, an anti-tumor miRNA. Similar findings were also found in the apoptosis assay and Transwell assay. Transfection of si-SNHG12 actuated cell apoptosis, which could be reversed by anti-miR-129-5p co-transfection (Figure 3D, 3E). Restraining SNHG12 significantly suppressed AMC-HN-8 cell invasion, while anti-miR-129-5p co-transfection almost neutralized the effect of SNHG12 (Figure 3F, 3G). Taken together, these results indicated that SNHG12 could interact with miR-129-5p to promote LSCC progression.

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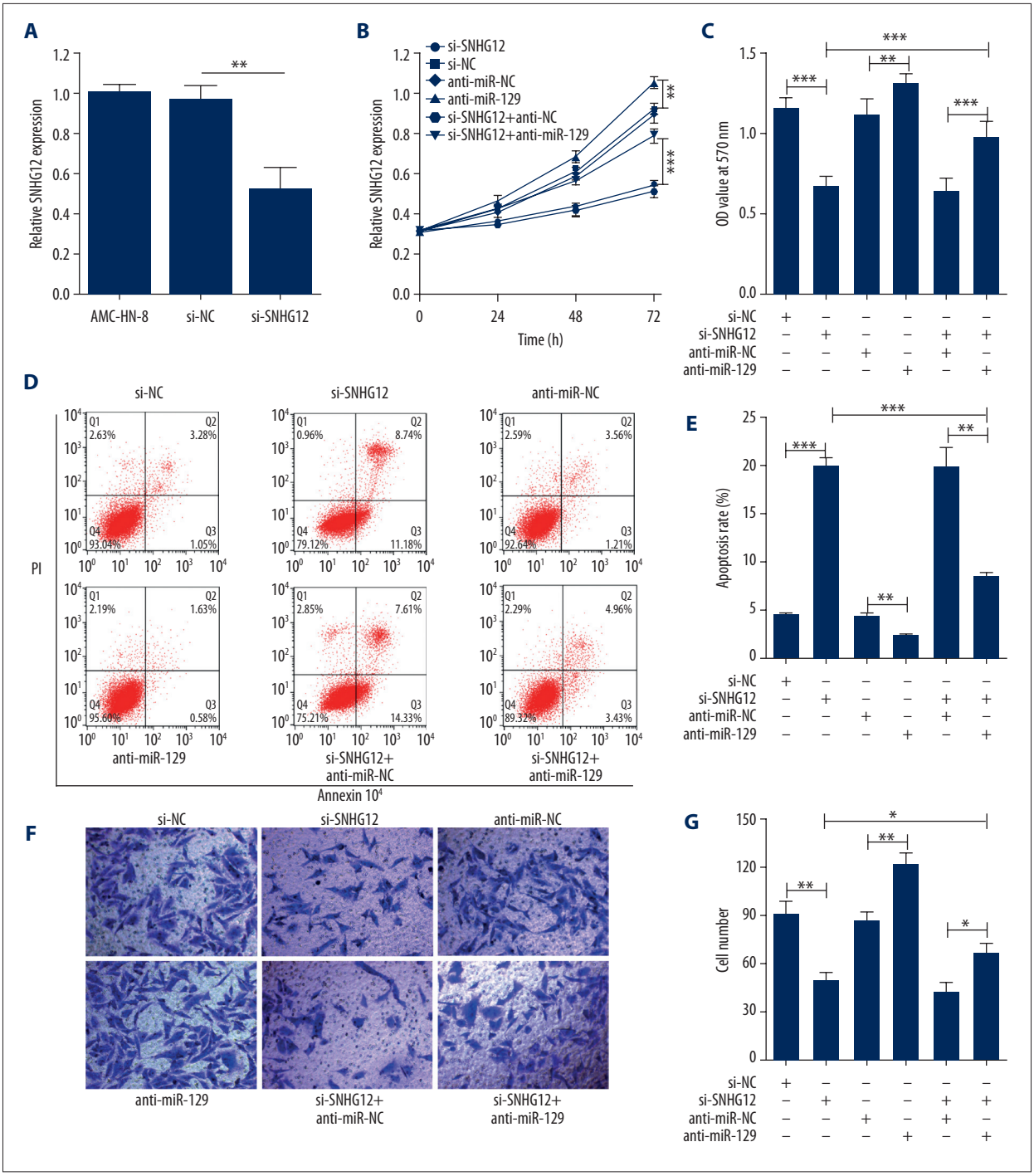

Figure 3. SNHG12 interacted with miR-129-5p to promote LSCC progression. (**A**) Silencing efficiency of si-SNHG12 and si-NC were evaluated by RT-qPCR. (**B**) Proliferation curves of transfected AMC-HN-8 cells showed OD values of different groups at 24 h, 48 h, and 72 h after transfection. (**C**) OD values of different groups at 72 h are shown. Si-SNHG12 inhibited AMC-HN-8 proliferation while anti-miR-129-5p promoted cell viability. Co-transfection of si-SNHG12 with anti-miR-129-5p reversed the inhibiting effect of si-SNHG12 on AMC-HN-8. (**D**) Results of apoptosis assay in different groups are shown. (**E**) Transfection of si-SNHG2 promoted apoptosis rate of AMC-HN-8 and anti-miR-129-5p inhibited apoptosis. Co-transfection of si-SNHG2 with anti-miR-129-5p reversed the apoptosis-promoting effects of si-SNHG12 on AMC-HN-8. (**F**) Representative images of Transwell invasion in different groups are shown. (**G**) Transfection of si-SNHG2 suppressed invasive cell number of AMC-HN-8, while anti-miR-129-5p increased invasive cell numbers. Co-transfection of si-SNHG2 with anti-miR-129-5p reversed the invasion-inhibiting effect of si-SNHG12 on AMC-HN-8.

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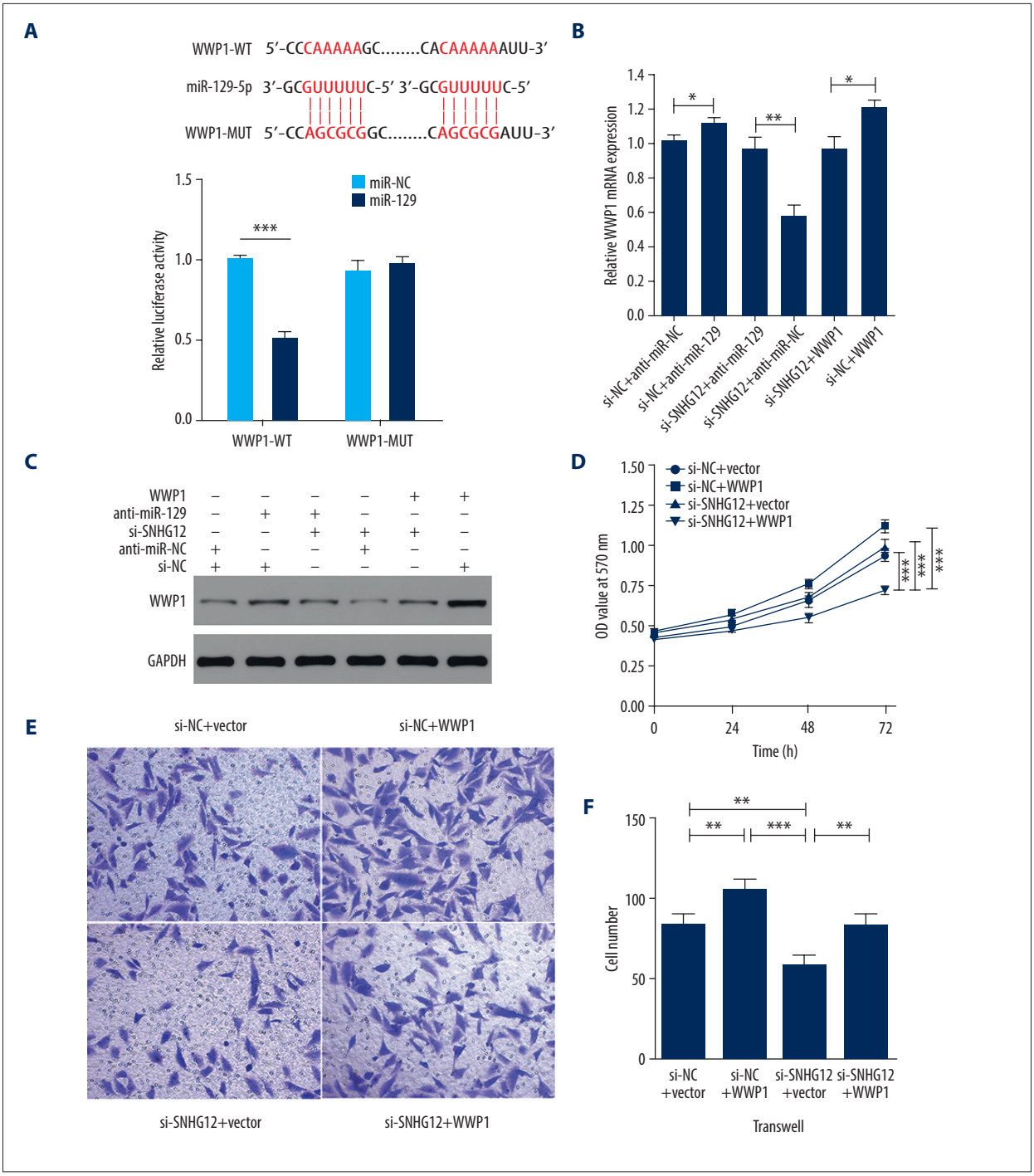

Figure 4. WWP1 promotes progression of LSCC cells and is regulated by SNHG12. (**A**) Sequences contained in WWP1-MUT/WWP1 -WT and the targeted sites of miR-129-5p are shown. Dual luciferase assay revealed WWP1 is a direct target of miR-129-5p. (**B**) RT-qPCR showed mRNA of WWP1 is positively regulated by SNHG12 and negatively regulated by miR-129-5p. Co-transfection of pcDNA-WWP1 and si-SNHG12 reduced the mRNA expression level of WWP1 compared to the si-NC+pcDNA-WWP1 group. (**C**) Western blot showed that WWP1 at the protein level was positively regulated by SNHG12 and negatively regulated by miR-129-5p. Co-transfection of pcDNA-WWP1 and si-SNHG12 reduced the protein expression level of WWP1 compared to the si-NC+pcDNA-WWP1 group. (**D**) WWP1 overexpression promoted proliferation of AMC-HN-8 cells and rescued the proliferation activity of the cells when SNHG12 was suppressed. (**E**) Representative images of Transwell assay showing cell invasiveness in si-NC+vector, si-SNHG12+vector, si-NC+WWP1, and si-SNHG12+WWP1 groups. (**F**) WWP1 overexpression promoted invasion of AMC-HN-8 cells and rescued the invasive ability of the cells when SNHG12 was suppressed.

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WWP1 promoted progression of LSCC cells and was regulated by SNHG12

Through publication searching and review, we found WWP1 was reported as a downstream target of miR-129-5p in gastric cancer cells [20]. Accordingly, we tried explored the regulatory relationship between miR-129-5p and WWP1 in laryngeal cancer cells. As shown in Figure 4A, co-transfection of miR-129-5p along with WWP1-WT vector significantly decreased luciferase activity compared to other groups, indicating the direct interaction between miR-129-5p and WWP1. Further, RT-qPCR and Western blot showed that WWP1 was positively regulated by SNHG12 and negatively regulated by miR-129-5p at the mRNA level (Figure 4B) and protein level (Figure 4C). Co-transfection of pcDNA-WWP1 and si-SNHG12 could to some extent reduce the expression level of WWP1 compared to the si-NC+pcDNA-WWP1 group. These results indicated WWP1 was a direct target of miR-129-5p and a potential target of SNHG12. Further phenotypic experiments were conducted to determine whether SNHG12 regulates proliferation and invasion through WWP1. The results showed that compared to the si-NC+vector group, WWP1 overexpression significantly increased proliferation (Figure 4D) and invasion of laryngeal cancer cells (Figure 4E, 4F). Moreover, when SNHG12 was suppressed, rescue of WWP1 restored the proliferation (Figure 4D) and invasion abilities of AMC-HN-8 cells (Figure 4E, 4F), indicating SNHG12 functions in laryngeal cancer cells through targeting WWP1. These results suggest that the SNHG12/miR-129-5p axis regulates the expression of WWP1, which has an oncogenic role in LSCC.

Discussion

Many studies have demonstrated that a large number of lncRNAs play critical roles in essential regulatory processes of carcinogenesis. The present study, for the first time, identified the relationship between expression levels of SNHG12 and clinical parameters in LSCC patients. Its biological effects on AMC-HN-8 cells and functional mechanism through interacting with miR-129-5p/WWP1 to promote LSCC progression were validated. These results were in accordance with previous studies reporting that SNHG12 actuated progression of colorectal cancer [21], cervical cancer [7,22], and nasopharyngeal carcinoma [23] cells. Furthermore, Zhou et al. reported that SNHG12 mediates doxorubicin resistance of osteosarcoma [24] and Wang et al. uncovered its role in mediating multidrug resistance through activating the MAPK/Slug pathway [25]. All these results revealed that SNHG12 is a tumor-promoting molecule in many cancers. Clarifying its biological effects and underlying mechanism could potentially benefit clinical practice.

The lncRNA-miRNA-mRNA regulatory network has been extensively studied, showing that lncRNAs competitively sponge miRNAs at the post-transcriptional level, thus leading to depression of targeted genes. In this study, we identified a new lncRNA-miRNA regulatory model in LSCC, the SNHG12-miR-129-5p-WWP1 axis. The miR-129-5p is reported to be a tumor-suppressor miRNA in several cancer types, with many validated targeted genes. For example, in gastric cancer it targets HMGB1 to restrain cell proliferation and invasion [26]. Here, we proved in LSCC miR-129-5p was regulated by SNHG12 and further modulated the expression of WWP1.

Ubiquitination participates in a series of biological processes and plays a pivotal role in post-translational modification. It has been reported to modulate receptor and signal transduction, govern cell cycle course, influence DNA repair, maintain protein stability, and regulate immune response [27]. Ubiquitination signaling transduction depends on a cascade of reactions catalyzed by 3 classes of enzymes: ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2), and ubiquitin protein ligases (E3) [28]. WWP1 is an E3 ubiquitin protein ligase reported to function as an oncogene in different types of malignancies, including breast cancer [29], prostate cancer [30], and gastric cancer [31]. However, its role in LSCC has not been reported. Here, we for the first report the tumor-promoting role of WWP1 regulated by the SNHG12/miR-129-5p axis in LSCC.

Our study has several limitations. First, the expression level of WWP1 was not detected in the collected tissue samples; therefore, the relationship between WWP1 and miR-129-5p in laryngeal cancer tissue could not be identified. Second, longer follow-up times are needed to explore the relationship between overall survival of LSCC patients and SNHG12 expression. Third, the number of LSCC samples was insufficient to reach strong conclusions. Fourth, due to limited financial support, we did not perform *in vivo* experiments.

Conclusions

In summary, this study demonstrated that SNHG12 promotes proliferation and invasion of laryngeal cancer cells by targeting miR-129-5p and WWP1. These results may provide a good biomarker for early diagnosis and recurrence monitoring for LSCC in clinical practice.

Conflict of interest

None.

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