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Long noncoding RNA small nucleolar RNA host gene 12 promotes papillary thyroid carcinoma cell growth and invasion by targeting miR-16-5p

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Abstract
Emerging evidence has shown that long noncoding RNA (lncRNA) plays an important role in various types of malignant cancer. Small nucleolar RNA host gene 12 (SNHG12) was found to be upregulated and to act as an oncogene in several cancers. However, the function and regulatory mechanism of SNHG12 remain unclear in papillary thyroid carcinoma (PTC). In this study, SNHG12 was found to be increased in PTC tissues and cell lines using quantitative real-time PCR. Knockdown of SNHG12 significantly inhibited PTC cell proliferation, migration and invasion and induced apoptosis in vitro. Mechanistic investigations revealed that SNHG12 functions as a competing endogenous RNA (ceRNA) to sponge miR-16-5p, which was downregulated in PTC tissues. In addition, rescue assays further confirmed that SNHG12 contributed to the progression of PTC through regulating miR-16-5p expression. These results indicated that SNHG12 might contribute to tumor progression in PTC by acting as a ceRNA to sponge miR-16-5p.

Key words: SNHG12, PTC, miR-16-5p

Running title: SNHG12 acts as a ceRNA to sponge miR-16-5p
Introduction

Thyroid cancer is the most common type of endocrine cancer, and the incidence of thyroid cancer is expected to further increase all over the world (Burman and Wartofsky, 2015; Chen et al., 2016). Thyroid cancer incidence rates have increased by 211% from 1975 to 2013, and approximately 90,000 patients were diagnosed in China in 2015 (Ito et al., 2013; Chen et al., 2016; Lim et al., 2017). Papillary thyroid carcinoma (PTC), the main form of thyroid cancer, accounts for between 80 and 90% of all thyroid malignancies in young women and children (Davies et al., 2014). Although PTC has a favorable prognosis for patients with an 87% 15-year survival rate, increased cancer recurrence and cancer-related mortality are observed in patients at an advanced stage (Toniato et al., 2008). Therefore, it is crucial to determine the potential mechanisms that regulate the initiation and progression of PTC.

Long noncoding RNAs (lncRNAs), a class of RNAs longer than 200 nucleotides in length with no protein-coding ability, have gained widespread attention due to their function in the regulation of gene interactions and biological processes (Fatica et al., 2014; Fang et al., 2016). Recent studies have shown that dysregulation of lncRNAs has been implicated in various types of human diseases, such as cancer, cardiovascular diseases, diabetes and neurodegenerative disorders (Wapinski et al., 2011; Huarte, 2015; Li et al., 2018). For instance, the lncRNA MALAT1 promotes proliferation, migration, and invasion in FTC133 cells by mediating FGF2 protein secretion (Huang et al., 2017). Recent studies have demonstrated that the lncRNA small nucleolar RNA host gene 12 (SNHG12) was increased and acted in an oncogenic role in the progression of several cancers, including triple-negative breast cancer, hepatocellular carcinoma, colorectal cancer, osteosarcoma and endometrial carcinoma (Zhai et al., 2015; Lan et al., 2017; Wang et al., 2017; Wang et al., 2017; Zhou et al., 2018). However, whether SNHG12 participates in the regulation of PTC remains largely unknown.

In this study, we found that SNHG12 was increased in PTC, and knockdown of SNHG12 inhibited cell proliferation and invasion. Moreover, mechanistic analysis revealed that SNHG12 acted as a ceRNA for miR-16-5p to regulate cell proliferation and invasion. Our study sheds new light on the mechanisms of PTC.

Materials and methods

Tissue specimens and ethics statement
A total of 54 PTC tissues and corresponding noncancerous thyroid tissues were collected from patients who underwent surgical resection at Sir Run Shaw Hospital Affiliated Zhejiang University. Written informed consent was obtained from all patients and this study was approved by the Ethics Committee of Zhejiang University and complied with the Declaration of Helsinki. All specimens were frozen in liquid nitrogen immediately and stored at −80°C until use.

RNA extraction and quantitative real-time PCR (qRT-PCR)
Total RNA from tissues and cells were extracted using TRIzol reagent (Invitrogen,
Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA was reverse transcribed to cDNA using a PrimeScript RT reagent kit (Takara, Dalian, China). qRT-PCR was performed using SYBR PremixEx Taq TM II (Takara) on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The primers for SNHG12 and GAPDH were used as previously described (Wang et al., 2017).

Cells culture and transfection
The immortalized human thyroid epithelial cell line (Nthy-ori 3-1, NTHY) (Catalog No: Sigma-Aldrich) was purchased from Sigma-Aldrich and maintained according to the supplier’s instructions. Three papillary thyroid carcinoma cell lines (NPA87 (Catalog No: SCSP-1635), B-CPAP (Catalog No: SCSP-543) and TPC-1 (Catalog No: SCSP-791)) were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS at 37°C in a humidified atmosphere with 5% CO2.

siRNA oligonucleotides targeting SNHG12 were purchased from Shanghai GenePharma Co., Ltd. as previously described (Wang et al., 2017). Mimic negative control (NC), miR-16-5p mimics, NC inhibitor and miR-16-5p inhibitor were purchased from Invitrogen. Cells were plated and transfected using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instruction. Transfection efficiency in the cell lines was confirmed by qRT-qPCR.

Cell proliferation and apoptosis assay
Cell proliferation was determined using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Japan). After transfection, cells were seeded into 96-well plates at a density of 3×10^3 cells in 100 µl medium per well. 10 µl CCK-8 solution was added into each well, and the absorbance was measured at 450 nm using a multiwavelength spectrophotometer (Bio-Tek, Taiwan).

Cell apoptosis was measured by flow cytometry using the Annexin V-FITC Apoptosis Detection kit (Sigma-Aldrich, Germany) according to the manufacturer’s instructions on a FACSARia flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell migration and invasion assay
Cell migration assays were performed as described previously (Huang et al., 2018). In brief, cells (4 × 10^5) in 200 µL of serum-free medium were seeded into the upper chamber of transwell plates (Corning Incorporated, Corning, NY, USA) and medium with 10% FBS was added to the lower chamber. 36 h after incubation at 37 °C, the nonmigrated cells were removed with cotton swabs, and the migrated cells were stained with 0.5% crystal violet. Then, the cells were imaged and counted using a CKX53 inverted microscope (Olympus, Tokyo, Japan).

Plasmid construction and luciferase reporter assay
The SNHG12 sequence containing the putative miR-16-5p binding site was inserted into a pMIR-Reporter plasmid (Invitrogen) to generate the wild-type reporter
A mutant plasmid (SNHG12-MUT), with the mutation sequence without the complementary sequence of miR-16-5p, was generated using the Quick-change mutagenesis kit (Stratagene, CA, USA). Luciferase activity was measured by the dual luciferase reporter assay system (Promega, WI, USA) 24 h after transfection.

Protein extraction and Western blot
Cells were lysed with RIPA buffer (Cell Signaling Technology, USA) containing a protease inhibitor cocktail (Sigma-Aldrich). Proteins were separated on 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, USA). The membrane was blocked with 5% nonfat milk and probed with primary antibodies against human GAPDH, PCNA, Bax, and BCL-2 (Cell Signaling Technology), MMP-9 and MMP-13 (Proteintech, Chicago, IL, USA) at 4°C overnight. The signals were visualized using an enhanced chemiluminescence kit (Millipore Co., Billerica, MA, USA) according to the manufacturer's instructions.

Statistical analysis
Data are presented as the mean ± standard deviation. Student's t-test was used for comparisons between two groups, and one-way ANOVA tests were performed to assess multiple groups. Statistical analysis was performed using GraphPad Prism 6.0 software, and p < 0.05 was considered statistically significant.

Results
SNHG12 is overexpressed in thyroid cancer
To investigate the role of SNHG12 in PTC development, the expression level of SNHG12 was analyzed in three human PTC cell lines (NPA87, B-CPAP and TPC-1) and a normal human papillary cell line (NTHY) using qRT-PCR. Our results indicated that SNHG12 expression was upregulated in PTC cells compared with NTHY cells (Figure 1A). In addition, we found that the expression of SNHG12 was obviously higher in PTC tissues than that in the corresponding normal papillary tissues (Figure 1B).

Knockdown of SNHG12 inhibits thyroid cancer cell proliferation, migration and invasion
To investigate the function of SNHG12, knockdown of SNHG12 gene expression with siRNA was performed in B-CPAP and TPC-1 cells, which had high endogenous SNHG12 expression. As shown in Figure 2A, SNHG12 was significantly downregulated in both PTC cells, as assessed by qRT-PCR. CCK-8 cell viability assays showed that SNHG12 silencing significantly attenuated the proliferation curves compared to the NC (Figure 2B). As the siRNA#2 had the better effectiveness in inhibition of cell proliferation, siRNA#2 was chosen for further experiments and named siSNHG12. To investigate the potential impact of SNHG12 on apoptosis in PTC cells, flow cytometry analysis was used and showed that the percentage of apoptotic Annexin V-positive cells increased after silencing SNHG12 (Figure 2C). In addition, transwell analysis illustrated that SNHG12 silencing remarkably restrained cell migration and invasion in
both B-CPAP (Figure 2D) and TPC-1 (Figure 2E) cells. To further confirm the alteration, we measured the protein levels of proliferation-, apoptosis- and metastasis-related markers by Western blot. As shown in Figure 2F, SNHG12 silencing significantly decreased the expression of PCNA, BCL-2, MMP-9 and MMP-13, while it increased the expression of the proapoptotic protein Bax. These data suggested that SNHG12 played an oncogenic role in PTC.

SNHG12 directly targets miR-16-5p in thyroid cancer cells

Accumulating evidence has suggested that IncRNA could interact with microRNAs (miRNAs) to exert its regulatory functions (Tay et al., 2014). To further investigate the underlying molecular mechanism of SNHG12 in PTC, a bioinformatics database (http://starbase.sysu.edu.cn/mirLncRNA.php) was used, and we suspected that miR-16-5p might act as a potential target of SNHG12 (Figure 3A). Dual luciferase reporter experiments displayed that luciferase activity was significantly decreased in B-CPAP (Figure 3B) and TPC-1 (Figure 3C) cells cotransfected with wild-type SNHG12 and miR-16-5p mimics, while miR-16-5p-mediated repression of luciferase activity was abolished after the predicted binding sites were mutated. The expression of miR-16-5p was observed to dramatically increase after SNHG12 knockdown (Figure 3D). Meanwhile, miR-16-5p was significantly downregulated in PTC tissues compared to those in adjacent normal tissues of the same cohort of patients (Figure 3E). In addition, a significant reverse correlation was found between miR-16-5p and SNHG12 expression levels in PTC tissues (Figure 3F). These results indicated that SNHG12 directly targeted and regulated the expression of miR-16-5p.

MiR-16-5p is functionally important for the biological effects of SNHG12

To further determine whether SNHG12 promoted cell proliferation, migration and invasion and inhibited cell apoptosis in PTC through negatively regulating miR-16-5p, we knocked down miR-16-5p in SNHG12-knockdown B-CPAP and TPC-1 cells by transfection with an miR-16-5p inhibitor, which resulted in a significant decrease of miR-16-5p expression (Figure 4A). CCK-8 and apoptosis assay results revealed that the miR-16-5p inhibitor largely attenuated SNHG12 knockdown-induced cell growth inhibition and apoptosis in B-CPAP and TPC-1 cells (Figure 4B, C and D). Similarly, transwell assays showed that the miR-16-5p inhibitor partially rescued the inhibiting effects induced by SNHG12 knockdown in B-CPAP and TPC-1 cells (Figure 4E and F). Western blot also showed that the expression of PCNA, BCL-2, Bax, MMP-9 and MMP-13 was rescued by the miR-16-5p inhibitor in SNHG12 knockdown cells (Figure 4G). These results suggested that miR-16-5p potentially mediated the oncogenic function of SNHG12 in PTC.

Discussion

It is now increasingly acknowledged that IncRNAs have been associated with tumor initiation, progression, invasion and metastasis by regulating a variety of biological processes, including cell growth, apoptosis, differentiation and metastasis (Fatica et al., 2014; Huarte, 2015; Salmena et al., 2011). In our present study,
SNHG12 was found to be upregulated in PTC tissues and cells. Moreover, knockdown of SNHG12 inhibited proliferation and migration and induced apoptosis of PTC cells. Furthermore, our data showed that SNHG12 functioned as an oncogene through sponging the tumor suppressor miR-16-5p.

Previous studies have shown that SNHG12 could be implicated in the tumorigenesis and progression of several cancers and may be a useful diagnostic and prognostic cancer biomarker (Lan et al., 2017; Wang et al., 2017; Zhou et al., 2018). Consistent with previous studies, our data have shown that the relative expression of SNHG12 was significantly upregulated in PTC tissues compared with adjacent noncancerous tissues. Inhibition of SNHG12 led to proliferation suppression and apoptosis induction in endometrial cancer cells (Zhai et al., 2015). Further investigation revealed that SNHG12 promoted HCC cell proliferation and tumorigenicity by targeting miR-199a/b-5p (Lan et al., 2017). SNHG12 was activated by c-MYC to promote cell proliferation and migration by regulating MMP-13 expression in breast cancer (Wang et al., 2017). In the present study, knockdown of SNHG12 inhibited cell proliferation, migration and invasion in B-CPAP and TPC-1 cells accompanied with alterations in PCNA, BCL-2, Bax, MMP-9 and MMP-13 expression.

Emerging evidence has supported that IncRNAs may exert their roles by competitively binding to cancer-related miRNAs (Salmena et al., 2011). It is proposed that SNHG12 could accelerate the development of osteosarcoma by sponging miR-195-5p to modulate the expression of Notch2 (Zhou et al., 2018). SNHG12 was reported to act as an oncogene by negatively regulating the expression of miR-199a/b-5p and miR-138 in HCC (Lan et al., 2017) and non-small cell lung cancer (Wang et al., 2017). In this study, SNHG12 was identified to interact with miR-16-5p and negatively correlated with miR-16-5p expression. As a famous tumor suppressor, miR-16-5p has been verified to be decreased in many human cancer tissues, and overexpression of miR-16-5p inhibited cell proliferation and survival by targeting oncogenes, such as BCL-2, CCND1, CCNE1 and CDK4-6 (Aqeilan et al., 2010; Bonci et al., 2016; Cimmino et al., 2005). Our results indicated that SNHG12 could regulate BCL-2 expression by targeting miR-16-5p. Additionally, inhibition of miR-16-5p reversed the effects of SNHG12 silencing on B-CPAP and TPC-1 cells. Taken together, these findings implied that SNHG12 might function as an oncogene by acting as a ceRNA for miR-16-5p in PTC. A new report has also found SNHG12 was upregulated in PTC tissues and SNHG12 promoted the proliferation and metastasis of PTC cells through modulating the Wnt/β-catenin signaling pathway (Ding et al., 2018, p.), consistent with our data.

In conclusion, for the first time, our study provides evidence to clarify the expression and roles of SNHG12 and illustrates that SNHG12 promotes cell proliferation and invasion through competitively binding with miR-16-5p and inhibiting miR-16-5p expression.

Additional Files
None
Conflict of Interest
The authors declare that they have no conflict of interest.

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References:

**Figure legends**

**Figure 1** SNHG12 expression in thyroid cancer cell lines and tissues. (A) Expression levels of SNHG12 in Nthy cells and PTC cell lines determined by qRT-PCR. (B) Expression level of SNHG12 in paired thyroid cancer tissues and their adjacent nontumor tissues. **P < 0.01.

**Figure 2** SNHG12 silencing impaired PTC cells’ proliferation, migration and invasion in vitro. (A) SNHG12 expression was quantified in B-CPAP and TPC-1 cells transfected with siRNAs targeting SNHG12 by qRT-PCR analysis. (B) Cell proliferation of PTC cells was evaluated by CCK-8 assay after transfection with SNHG12 siRNA. (C) The effect of SNHG12 silencing on PTC cell apoptosis was assessed by flow cytometry. (D) B-CPAP and (E) TPC-1 cell migration and invasion were tested after transfection with SNHG12.
siRNA. Magnification, × 100. (F) Protein expression of PCNA, Bax, BCL-2, MMP-9 and MMP-13 was assessed using Western blotting. ** P < 0.01.

Figure 3 miR-16-5p is a target gene of SNHG12. (A) Predicted miR-16-5p binding sites in the wild-type and mutant SNHG12 sequences. (B, C) Dual luciferase reporter assay was performed to analyze the binding between miR-16-5p and the predicted binding sites in SNHG12 in B-CPAP (B) and TPC-1 (C) cells. (D) qRT-PCR assay analysis of miR-16-5p expression in B-CPAP and TPC-1 cells transfected with SNHG12 siRNA. (E) MiR-16-5p expression was measured in cancer tissues and adjacent nontumor tissues. (F) The negative association between SNHG12 and miR-16-5p expression levels was determined in PTC tissues. ** P < 0.01.

Figure 4 An miR-16-5p inhibitor attenuated the inhibitory effects of SNHG12 silencing in vitro. (A) qRT-PCR analysis for SNHG12 expression with indicated treatments. (B, C) CCK-8 was performed to assess cell proliferation of B-CPAP (B) and TPC-1 (C) cells. (D, E) Flow cytometry (D) and transwell assays (E) were conducted to determine cell apoptosis, migration and invasion with the indicated treatment. (G) Western blot analysis of PCNA, Bax, BCL-2, MMP-9 and MMP-13 expression in PTC cells with the indicated treatment. *P < 0.05, ** P < 0.01.
A

B

Relative LncRNA SNHG12 expression

NTHY  NPA87  B-CPAP  TPC-1

Relative LncRNA SNHG12 expression

Adjacent  Cancer tissues
A. LncRNA SNHG12-MUT 5'-ccCGCTAGTCGC-TG GCCGTg-3'
   hsa-miR-16-5p miRNA 3'-gcGGTATAATGCACGACGAt-5'
   LncRNA SNHG12-WT 5'-ccCGCTAGTCGC-TGCTGCTg-3'

B. Relative luciferase activity

C. Relative luciferase activity

D. Relative miR-16-5p expression

E. Box plot of relative miR-16-5p expression

F. Scatter plot of miR-16-5p expression vs SNHG12 expression

$r^2 = 0.19, P = 0.009$