Original Article

Long noncoding RNA *SNHG6* promotes papillary thyroid cancer cells proliferation via regulating miR-186/CDK6 axis

Jian Xu, Miaomiao Liao

Department of General Surgery, The Seventh Affiliated Hospital of Sun Yat-sen University, Shenzhen, China

Contributions: (I) Conception and design: J Xu; (II) Administrative support: M Liao; (III) Provision of study materials or patients: J Xu; (IV) Collection and assembly of data: Both authors; (V) Data analysis and interpretation: J Xu; (VI) Manuscript writing: Both authors; (VII) Final approval of manuscript: Both authors.

Correspondence to: Jian Xu. Department of General Surgery, The Seventh Affiliated Hospital of Sun Yat-sen University, 628 Zhenyuan Road, Guangming District, Shenzhen 518107, China. Email: jianjian19880314@163.com.

Background: Papillary thyroid cancer (PTC) is a common endocrine malignancy, and its incidence rate has been increasing in recent years. Long noncoding RNAs (lncRNAs) participate in cell biological processes through a variety of regulatory ways, and play an essential role in tumor development.

Methods: This study explored the expression of lncRNA small nucleolar RNA host gene 6 (*SNHG6*) in PTC by bioinformatics analysis, and quantitative real-time PCR (qRT-PCR). Cell counting kit-8 (CCK-8) assay, colony formation assay, and 5-ethynyl-2'-deoxyuridine (EdU) assay were used to study the effect of *SNHG6* on the proliferation of PTC cells. Luciferase reporter gene assay and western blot were used to study the mechanism.

Results: *SNHG6* was highly expressed in PTC tissue samples and cell lines. *In vitro*, overexpression of *SNHG6* promoted the proliferation of PTC cells, while silencing *SNHG6* inhibited the proliferation of PTC cells. miR-186 is the downstream target of *SNHG6*. *SNHG6* regulates the proliferation of PTC cells through miR-186. In addition, *CDK6* is the target gene of miR-186, which can inhibit the expression of CDK6 protein. *SNHG6* can promote the expression of CDK6 by regulating miR-186.

Conclusions: *SNHG6* is highly expressed in PTC and can promote the proliferation of PTC cells by regulating the miR-186/CDK6 axis, which is expected to become a potential therapeutic target for PTC.

Keywords: CDK6; long noncoding RNAs (lncRNAs); miR-186; papillary thyroid cancer (PTC); small nucleolar RNA host gene 6 (*SNHG6*)

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Introduction

In recent years, the incidence rate of thyroid cancer has been increasing and becoming the most common endocrine malignancy (1,2). Papillary thyroid cancer (PTC) is the most common subtype, accounting for about 80% of all thyroid cancers (3). Although the degree of malignancy of PTC is low and the prognosis is usually good, many PTC patients will have tumor recurrence after operation (4,5). Moreover, the prognosis of advanced PTC is still not ideal (4,5). The pathogenesis of PTC is not completely clear, although many risk factors are known to be closely related to the occurrence and development of PTC, such as genetic factors, environmental exposure, and epigenetic changes (6,7). Therefore, it is of great significance to study the molecular mechanism of PTC progression for finding new therapeutic targets.

Long noncoding RNAs (lncRNAs) have a length of >200 nucleotides, and participate in cell biological processes through a variety of regulatory ways (8). Many studies have shown that lncRNAs play a critical role in the occurrence and development of tumors (9). In general, lncRNAs are

involved in tumor proliferation, metastasis, differentiation, metabolism, and angiogenesis by regulating key tumor suppressor genes or oncogenes (10,11). There are many reports about lncRNAs in PTC. For example, lncRNA *GAS8-AS1* is downregulated in PTC tissues and cell lines. Its low expression is associated with higher TNM stage and lymph node metastasis. *GAS8-AS1* can upregulate the expression of ATG5 and ATG7 through miR-187-3p and miR-1343-3p, which significantly promote autophagy and inhibit the proliferation of PTC cells and tumorigenesis (12). Small nucleolar RNA host gene 15 (*SNHG15*) is highly expressed in PTC. As a competitive endogenous RNA, it regulates the YAP1-Hippo signaling pathway by binding miR-200a-3p, thus promoting the occurrence and development of PTC (13).

SNHG6 is a newly discovered lncRNAs that is highly expressed and plays a carcinogenic role in many human malignant tumors, such as lung cancer, colorectal cancer, hepatocellular carcinoma, and gastric cancer (14-18). However, the role of *SNHG6* in PTC remains to be studied, and its expression, biological function and molecular mechanism in PTC are still unclear. So we explored the role of *SNHG6* in the progress of PTC and its regulatory mechanism.

We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/gs-21-586).

Methods

Bioinformatics analysis

This study used the Starbase database (http://starbase.sysu.edu.cn) and the UALCAN database (http://ualcan.path.uab.edu/index.html) to perform a bioinformatics analysis, thus identifying the expression differences of SNHG6 and miR-186 in thyroid cancer samples and normal tissues. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell culture and transfection

Nthy-ori 3-1 and PTC cell lines (TPC-1 and K1) were purchased from American Type Culture Collection (VA, USA). All cell lines used in the study were cultured in Dulbecco's modified Eagle's medium (Gibco, MD, USA) containing 10% fetal bovine serum (Hyclone, UT, USA). The cell incubator was set at 37 ℃ with 5% CO_2. According to the manufacturer's instructions, Lipofectamine 3000 reagent (Invitrogen, Waltham, MA, USA) was used to transfect PTC cells.

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

Trizol reagent (Invitrogen) was used to extract total RNA from cultured cells, and the purity and concentration of RNA were determined by spectrophotometer. Using PrimeScript RT reagent kit (Takara, Dalian, China), 1 μg RNA was reversely transcribed into cDNA. SYBR Green Premix EX Taq (Takara) and specific primers were used for the PCR reaction. β-actin was used as an internal reference. The relative expression level of mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequence was as follows: *SNHG6*-F, 5'-ATACTTCTGCTTCGTTACCT-3'; *SNHG6*-R, 5'-CTCATTTTCATCATTTGCT-3'; *β-actin*-F, 5'-AGCGAGCATCCCCCAAAGTT-3'; *β-actin*-R, 5'-GGGCACGAAGGCTCATCATT-3'.

Cell counting kit-8 (CCK-8) assay

A total of 3,000 transfected PTC cells were seeded in 96-well plates. At the designated time point, 10 μL CCK-8 solution (Beyotime, Shanghai, China) was added to each well. After incubation for 1 h, the optical density value at 450 nm was detected by spectrophotometer (Bio-Rad, Hercules, CA, USA).

Colony formation assay

The transfected cells were seeded in a 6-well plate with a density of 500 cells per well. After 2 weeks of culture, the cells were fixed with 4% paraformaldehyde for 30 min, and stained with 0.5% crystal violet for 15 min. The images were photographed, and the number of cell clones was counted.

5-ethynyl-2'-deoxyuridine (EdU) assay

After transfection, a BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime) was used to detect PTC cells' proliferation. The cells were photographed by fluorescence microscope (Olympus, Tokyo, Japan) [EdU—red fluorescence, 4',6-diamidino-2-phenylindole (DAPI)—blue fluorescence] to calculate the positive rate.

Luciferase reporter assay

The *SNHG6* or *CDK6* 3'UTR sequence of the wild-type (WT) or mutation (MUT) containing the miR-186 binding site were cloned into pGLOvector (Promega, Madison, WI, USA). miR-186 mimics and luciferase reporter plasmid were cotransfected into the cells and cultured for 48 h. The luciferase activity was detected by a dual-luciferase reporter assay system (Promega).

Protein extraction and western blot

The total protein was extracted from the cells with RIPA lysis buffer containing protease inhibitor. The protein concentration was determined by the bicinchoninic acid (BCA) method. Next, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the total protein and transfer the protein to the polyvinylidene fluoride (PVDF) membrane, which was blocked with 5% skim milk for 2 h at room temperature. After that, the membrane was mixed with anti-CDK6 antibody (ab124821, Abcam, USA), and anti-β-actin antibody (ab8226, Abcam) was incubated overnight at 4 ℃. After washing, the membrane was incubated with Rabbit anti-Human IgG H&L (HRP) (ab6759, Abcam, USA) at room temperature for 1 h. The protein bands were detected by Novex™ ECL Chemiluminescent Substrate Reagent Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Statistical analysis

The data were analyzed by GraphPad Prism 5.0 software (GraphPad, CA, USA). Each experiment was repeated at least three times independently, and the data were expressed as mean ± standard deviation (SD). Independent sample *t*-test was used to compare the differences between groups. One-way analysis of variance (ANOVA) and Bonferroni post-test were used to analyze the differences among three groups or more. P value <0.05 was statistically significant.

Results

Expression of SNHG6 in PTC

Using the UALCAN database, we found that the expression of *SNHG6* in thyroid cancer tissues was significantly higher than in normal tissues (*Figure 1A*), and its expression was closely related to tumor stage (*Figure 1B*). Moreover, the expression of *SNHG6* in PTC cell lines TPC-1 and K1 was significantly higher than in Nthy-ori 3-1 cells (*Figure 1C*). These results indicated that SNHG6 is highly expressed in PTC. Subsequently, we overexpressed *SNHG6* in TPC-1 cells and knocked down *SNHG6* in K1 cells (*Figure 1D,1E*).

Effect of SNHG6 on proliferation of PTC cells

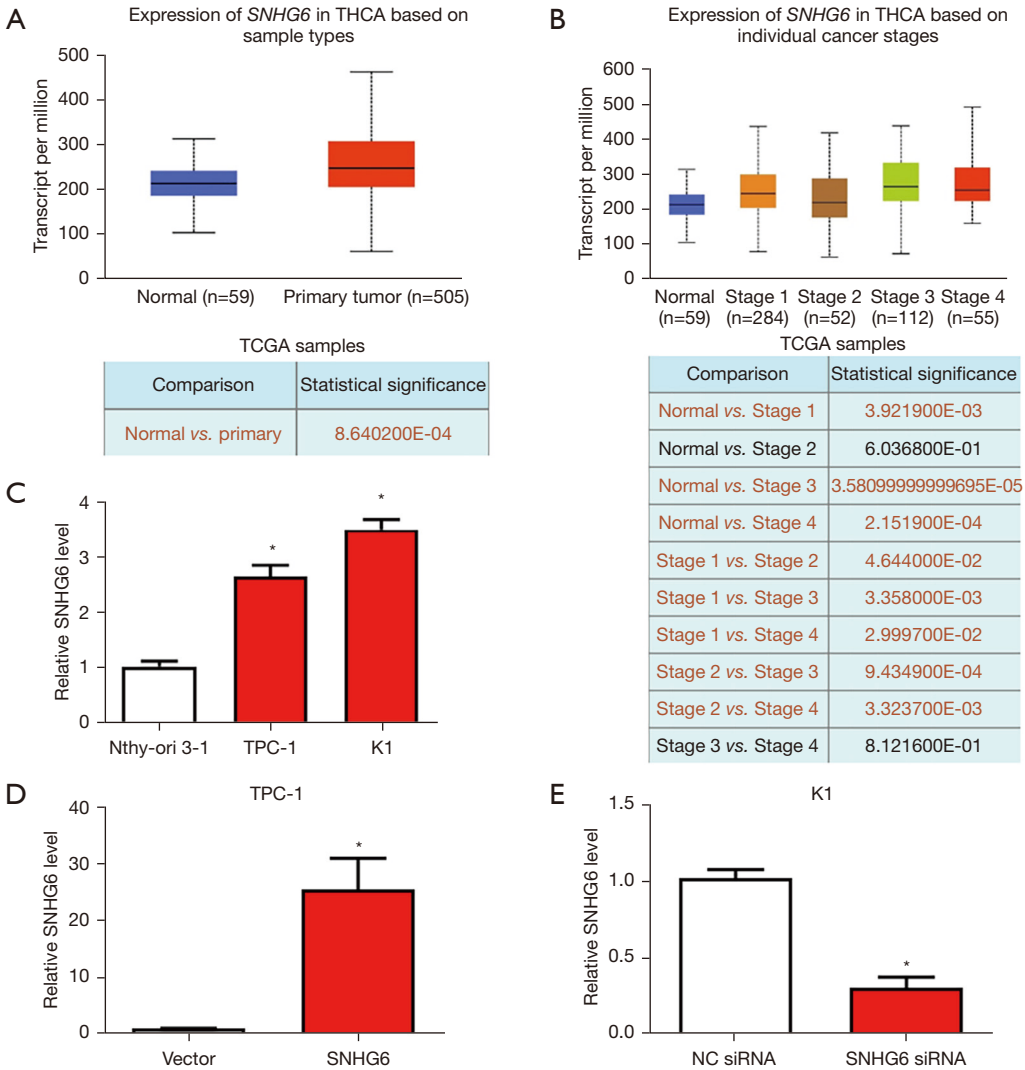
We studied the effect of *SNHG6* on the proliferation of PTC cells. The results of the CCK-8 experiment showed that overexpression of *SNHG6* significantly increased the activity of TPC-1 cells, and silencing *SNHG6* inhibited the activity of K1 cells (*Figure 2A,2B*). The colony formation assay showed that overexpression of *SNHG6* significantly enhanced the colony formation ability of TPC-1 cells, and silencing *SNHG6* inhibited the colony formation ability of K1 cells (*Figure 2C,2D*). The EdU assay showed that overexpression of *SNHG6* significantly promoted the proliferation of TPC-1 cells, and silencing *SNHG6* inhibited the proliferation of K1 cells (*Figure 2E,2F*). These results indicated that *SNHG6* can promote the proliferation of PTC cells.

Downstream target of SNHG6

Using the Starbase database (http://starbase.sysu.edu.cn), we predicted a potential miR-186 binding site in *SNHG6* (*Figure 3A*). Subsequently, we used a luciferase reporter gene experiment to verify this. The results showed that miR-186 mimics could significantly inhibit the luciferase activity of the plasmid containing the WT *SNHG6* sequence but did not affect the mutant plasmid (*Figure 3B*). These results indicated that miR-186 can bind to *SNHG6* and is the downstream target of *SNHG6*. Moreover, using the UALCAN database, we found that the expression of miR-186 in thyroid cancer tissues was significantly lower than in normal tissues (*Figure 3C*). Moreover, the expression of miR-186 in the PTC cell lines TPC-1 and K1 was significantly lower than in Nthy-ori 3-1 cells (*Figure 3D*).

Mechanism of SNHG6 regulation of PTC cell proliferation

To investigate the mechanism of *SNHG6* enhancing the proliferation of PTC cells, we transfected miR-186 mimics into *SNHG6*-overexpressing TPC-1 cells, and miR-186 inhibitor into *SNHG6* knockdown K1 cells. CCK-8, colony formation, and EdU experiments showed that miR-186 mimics could reverse the proliferation of TPC-1 cells induced by overexpression of *SNHG6*, and miR-186



Comparison	Statistical significance
Normal *vs.* primary	8.640200E-04

Comparison	Statistical significance
Normal *vs.* Stage 1	3.921900E-03
Normal *vs.* Stage 2	6.036800E-01
Normal *vs.* Stage 3	3.58099999999695E-05
Normal *vs.* Stage 4	2.151900E-04
Stage 1 *vs.* Stage 2	4.644000E-02
Stage 1 *vs.* Stage 3	3.358000E-03
Stage 1 *vs.* Stage 4	2.999700E-02
Stage 2 *vs.* Stage 3	9.434900E-04
Stage 2 *vs.* Stage 4	3.323700E-03
Stage 3 *vs.* Stage 4	8.121600E-01

Figure 1 *SNHG6* is highly expressed in PTC (A,B) The UALCAN database was used to analyze the expression of *SNHG6* in THCA and normal tissues and its correlation with tumor stage. (C) Expression of *SNHG6* in PTC and Nthy-ori 3-1 cell was analyzed by qRT-PCR. (D,E) qRT-PCR verification of *SNHG6* overexpression and silencing in PTC cells. *, P<0.05. *SNHG6*, small nucleolar RNA host gene 6; PTC, papillary thyroid cancer; THCA, thyroid carcinoma; qRT-PCR, quantitative real-time PCR; TCGA, The Cancer Genome Atlas; NC, normal control.

inhibitor partially eliminated the inhibition of proliferation induced by silencing *SNHG6* (*Figure 4*). These results indicated that *SNHG6* regulates the proliferation of PTC cells through miR-186.

Mechanism of SNHG6 promotion of CDK6 expression

Using the Starbase database, we predicted a potential miR-186 binding site in the 3'UTR sequence of *CDK6* (*Figure 5A*), suggesting that it may be a downstream target gene of miR-186. We used a luciferase reporter gene experiment to verify this. The results showed that miR-186 mimics could significantly inhibit the luciferase activity of the plasmid containing the WT *CDK6* 3'UTR sequence, but did not affect the mutant plasmid (*Figure 5B*). Western blot analysis showed that overexpression of miR-186 significantly inhibited the expression of CDK6 protein, and knockdown of miR-186 promoted the expression of CDK6 protein (*Figure 5C,5D*). These results indicated that miR-186 can target *CDK6* 3'UTR and inhibit the

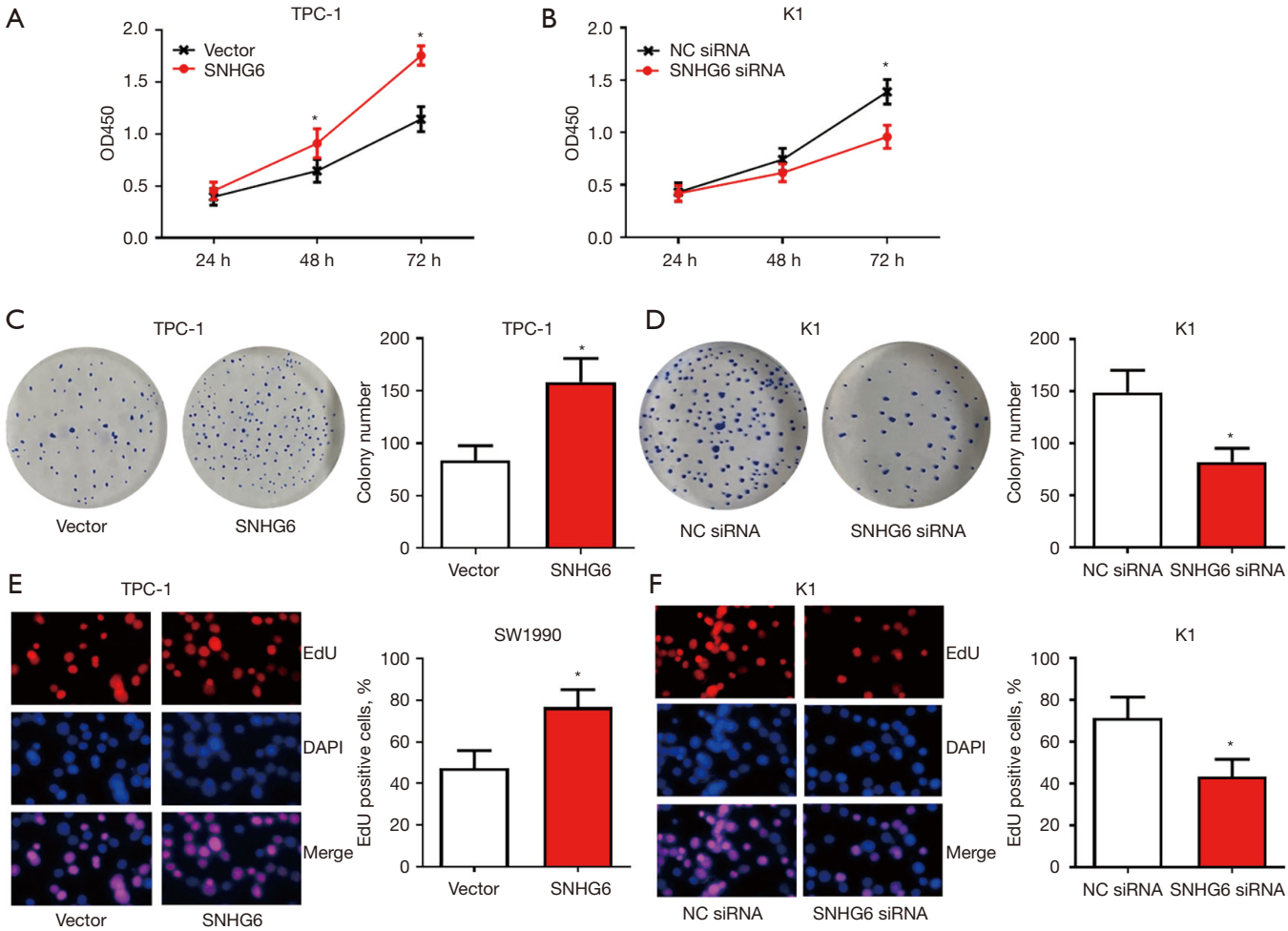


Figure 2 *SNHG6* promotes the proliferation of PTC cells (A,B). CCK-8 assay was used to study the effect of *SNHG6* overexpression and silencing on the activity of PTC cells. (C,D) Colony formation assay was used to study the effects of *SNHG6* overexpression and silencing on colony formation of PTC cells. Cells were stained with 0.5% crystal violet solution. (E,F) EdU assay was used to study the effect of *SNHG6* overexpression and silencing on the proliferation of PTC cells (magnification 100×). *, P<0.05. *SNHG6*, small nucleolar RNA host gene 6; PTC, papillary thyroid cancer; CCK-8, cell counting kit-8; EdU, 5-ethynyl-2'-deoxyuridine; OD, optical density; NC, normal control; DAPI, 4',6-diamidino-2-phenylindole.

expression of CDK6 protein. Furthermore, we found that overexpression of *SNHG6* promoted the expression of CDK6 protein, whereas miR-186 mimics reversed this effect (*Figure 5E*). Similarly, silencing *SNHG6* inhibited the expression of CDK6, and miR-186 inhibitor reversed this effect (*Figure 5F*). These results indicated that *SNHG6* promotes the expression of CDK6 by regulating miR-186.

Discussion

Thyroid cancer is the most common cancer in the endocrine system. With the deepening of research, many molecular pathogenesis of thyroid cancer have been discovered. For example, RAS mutations, BRAF mutations, and activation of the mitogen-activated protein kinase pathway play an important role in tumorigenesis (18). Moreover, some molecular markers, such as TERT promoter mutation and TP53 mutation, are closely related to tumor progression (18).

In recent years, miRNAs and lncRNAs have been confirmed to play a crucial role in the occurrence and development of various cancers, and may be effective targets for the treatment

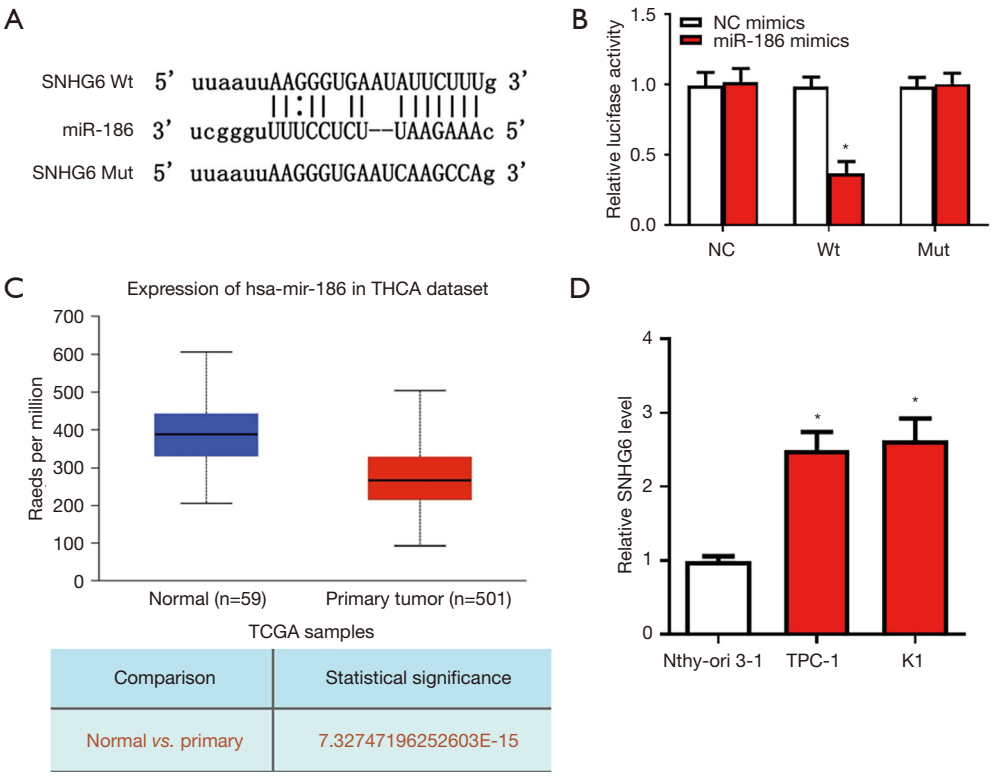


Figure 3 miR-186 as the downstream target of *SNHG6*. (A) Binding sites of *SNHG6* and miR-186 were predicted by the Starbase database. (B) Luciferase reporter gene assay was used to verify the binding sites of *SNHG6* and miR-186. (C) Expression of miR-186 in thyroid cancer and normal tissues analyzed by the UALCAN database. (D) Expression of miR-186 in PTC cells and Nthy-ori 3-1 cells analyzed by qRT-PCR. *, P<0.05. *SNHG6*, small nucleolar RNA host gene 6; PTC, papillary thyroid cancer; qRT-PCR, quantitative real-time PCR; WT, wild-type; MUT, mutation; NC, normal control; THCA, thyroid carcinoma; TCGA, The Cancer Genome Atlas.

of malignant tumors (19,20). In this study, we explored the important role of lncRNA *SNHG6* in the malignant process of PTC through regulating miR-186/CDK6, and we determined that *SNHG6* is a carcinogen in PTC.

First, bioinformatics analysis revealed *SNHG6* was highly expressed in PTC tissue, and its expression was closely related to the tumor stage of PTC. We also confirmed that *SNHG6* was highly expressed in a PTC cell line. The expression and clinical significance of *SNHG6* in many types of tumor have been reported, and it is suggested that the high expression of *SNHG6* in a tumor is related to malignant phenotype and poor clinical prognosis of the tumor (14-17,19). The expression of *SNHG6* in PTC and its clinical significance are revealed for the first time.

In addition, we found that overexpression of *SNHG6* promoted proliferation of PTC cells, which was inhibited by silencing *SNHG6*. The biological role of *SNHG6* as an oncogene has been reported in other tumors (14-17,19), but for the first time in PTC. Our mechanism studies showed that *SNHG6* can bind to miR-186 and affect its function, thus regulating the proliferation of PTC cells. lncRNA miRNA mRNA competitive endogenous RNA regulatory network is a common molecular regulatory mechanism for lncRNA (21,22). In this mode, lncRNAs can competitively bind miRNAs with mRNAs, thus affecting the inhibitory effect of miRNAs on mRNA translation and ultimately promoting mRNA translation (21,22). In this study, we demonstrated that *SNHG6* promotes the expression of CDK6 by binding to miR-186.

The expression of miR-186 is low in many tumors, such as colorectal cancer, liver cancer, breast cancer, osteosarcoma, and esophageal cancer. It is a tumor suppressor gene that inhibits malignant progression (23-27). Those studies also found many target genes of miR-186, such as *SMAD6/7*, *PTPN11*, *ZEB1*, *GPRC5A*, *PARP9*, etc., indicating that miR-186 plays a biological role by inhibiting the expression of

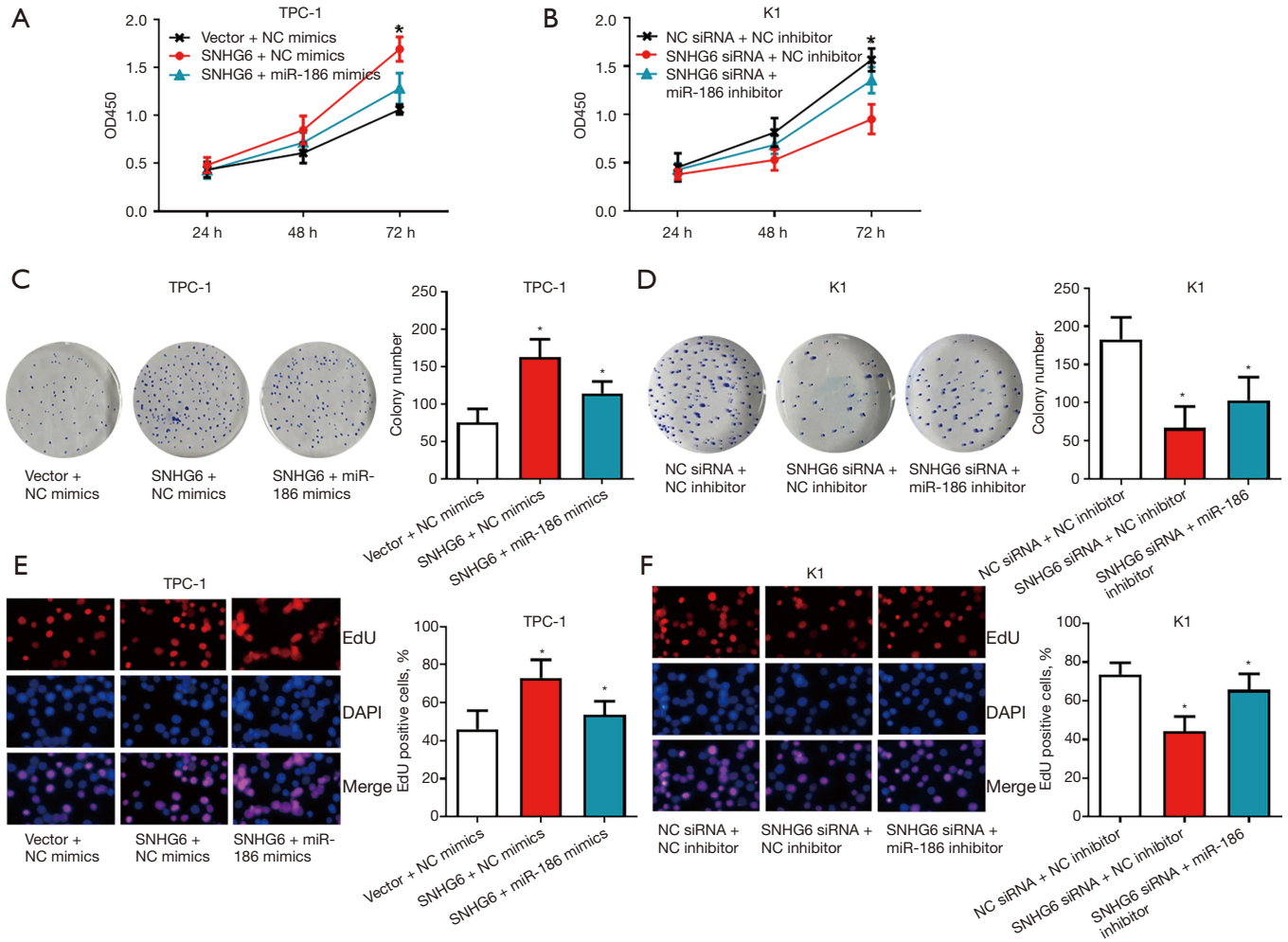


Figure 4 *SNHG6* regulates PTC cell proliferation through miR-186. (A,B) CCK-8 experiment was used to study the effect of *SNHG6* regulating miR-186 on the activity of PTC cells. (C,D) Colony formation assay to study the effect of *SNHG6* regulating miR-186 on the colony formation of PTC cells. Cells were stained with 0.5% crystal violet solution. (E,F) EdU assay was used to study the effect of *SNHG6* on the proliferation of PTC cells (magnification 100×). *, P<0.05. *SNHG6*, small nucleolar RNA host gene 6; PTC, papillary thyroid cancer; CCK-8, cell counting kit-8; EdU, 5-ethynyl-2'-deoxyuridine; OD, optical density; NC, normal control; DAPI, 4',6-diamidino-2-phenylindole.

these genes (23-27). In this study, we found a new target gene of miR-186, viz., *CDK6*. CDK6 is a cell cycle-dependent kinase and a transcription regulator, which plays a vital role in G1 phase progression and G1/S cell cycle transition (28-30). Studies have shown that it is highly expressed in many malignant tumors and promotes the proliferation of tumor cells (28-30). Inhibitors targeting *CDK6* can slow down tumor growth (28-30). In this study, we confirmed that *SNHG6* promotes the expression of CDK6 by regulating miR-186. Therefore, we believe that *SNHG6* promotes cell proliferation by regulating the miR-186/CDK6 axis in PTC. Moreover, if a targeted drug is designed for SNHG6 as a target, it may have potential application value in the clinical treatment of PTC, and it has potential significance for improving the prognosis of PTC.

In conclusion, *SNHG6* was highly expressed in PTC, and miR-186 was minimally expressed in PTC. *SNHG6* can promote the proliferation of PTC cells by regulating the miR-186/CDK6 axis, which is expected to become a potential therapeutic target for PTC.

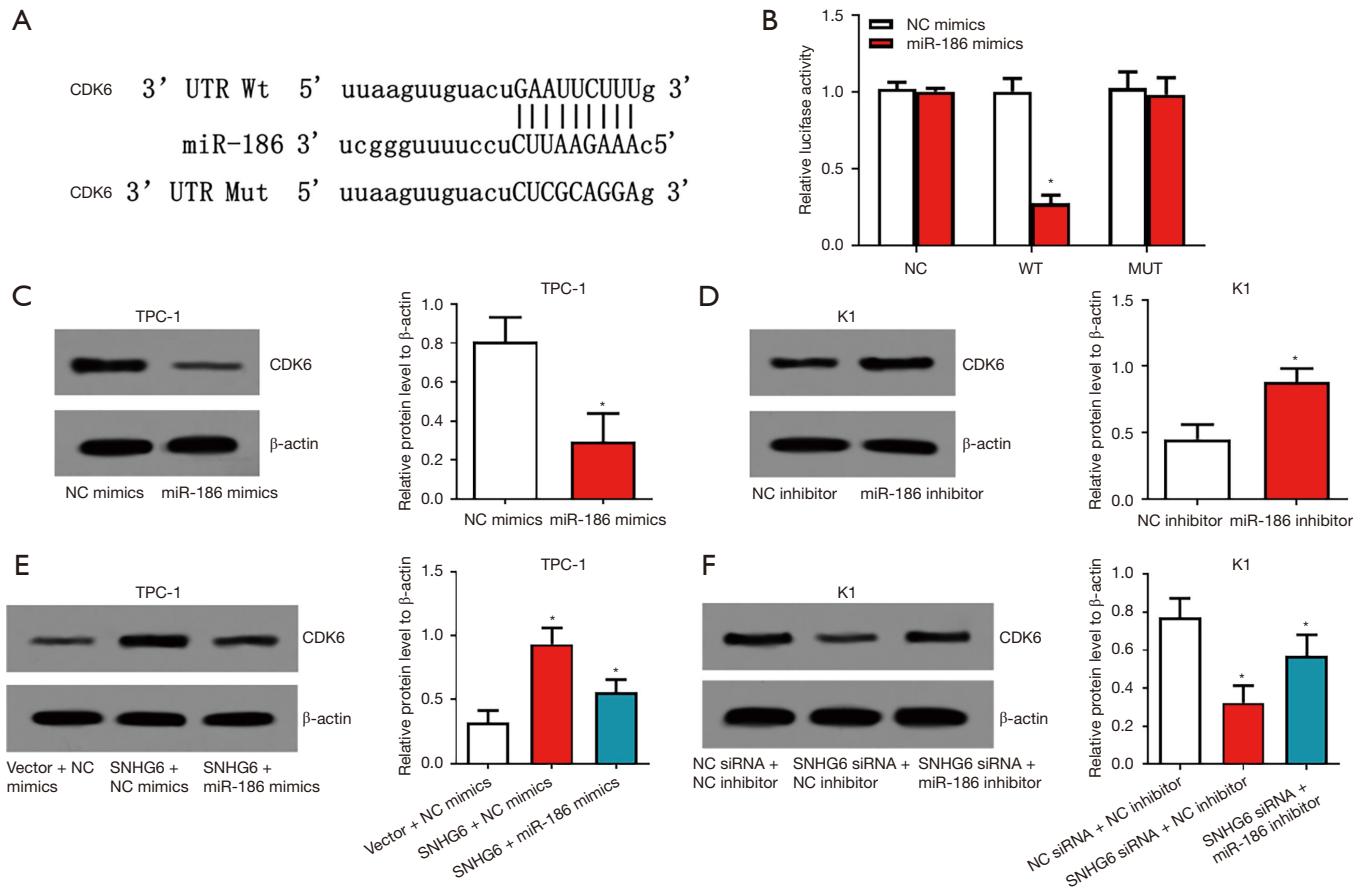


Figure 5 *SNHG6* promotes CDK6 expression by regulating miR-186. (A) Starbase database predicted the binding sites of CDK6 3'UTR and miR-186. (B) Luciferase reporter gene assay was used to verify the binding sites of CDK6 3'UTR and miR-186. (C,D) Western blot analysis shows that overexpression and silencing of miR-186 affected CDK6 protein expression. (E,F). Western blot was used to study the effect of *SNHG6* on the expression of CDK. *, P<0.05. *SNHG6*, small nucleolar RNA host gene 6; WT, wild-type; MUT, mutation; NC, normal control.

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Footnote

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Data Sharing Statement: Available at https://dx.doi.org/10.21037/gs-21-586

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/gs-21-586). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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