

Gene Amplification-Driven Long Noncoding RNA SNHG17 Regulates Cell Proliferation and Migration in Human Non-Small-Cell Lung Cancer

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Lung cancer is the most common cancer all around the world, with high morbidity and mortality. Long noncoding RNA (lncRNA) has been reported to have a critical role in non-small-cell lung cancer (NSCLC) proliferation and migration. In the present study, we analyzed The Cancer Genome Atlas (TCGA) data, and we found that lncRNA Small Nucleolar RNA Host Gene 17 (SNHG17) was upregulated in NSCLC driven by the amplification of copy number, indicating the special role of SNHG17 in NSCLC. The full exact length of SNHG17 was determined by rapid amplification of cDNA ends (RACE). We modulated SNHG17 expression by RNAi and a series of functional assays were performed. Flow cytometry was used to explore the involvement of SNHG17 in NSCLC cell apoptosis. Results showed that the knockdown of SNHG17 inhibited the proliferation and migration and promoted the apoptosis of NSCLC cells. We acquired the global gene expression profile regulated by SNHG17 in A549 through RNA sequencing (RNA-seq) assays. We found 637 genes were upregulated while 581 genes were downregulated. We selected three genes (FOXA1, XAF1, and BIK) that were closely related to proliferation and apoptosis, and we confirmed their altered expression in A549 and PC-9 cells treated with small interfering RNA si-SNHG17. Our findings indicated gene amplification-driven lncRNA SNHG17 promotes cell proliferation and migration in NSCLC, suggesting its potential value as a biomarker in NSCLC.

INTRODUCTION

As the most common cancer all around the world, lung cancer is the leading cause of cancer-related death in the United States.¹ NSCLC accounts for 85% of all lung cancer.² In spite of recent advances in the comprehensive treatments like surgical operation, molecular targeted therapy, chemotherapy, and radiation treatment, the overall 5-year survival rate for NSCLC patients remains as low as 15%.³ Thus, a better understanding of the underlying mechanisms and molecular pathways in NSCLC development and progression is significant for the precise treatment of NSCLC.

With the development of high-throughput transcriptome analysis in the last few years, it was found that over 90% of the total mammalian genome can be transcribed but does not encode proteins.⁴ As a new class of noncoding RNA (ncRNA), long ncRNA (lncRNA) of >200 nt was found to be promoted or reduced in some diseases,^{5,6} particularly in cancer.⁷⁻¹⁰ Aberrant expressions of lncRNAs exert oncogenic and suppressive functions in cancer like breast cancer,^{11,12} gastric cancer,¹³ urothelial tract cancer,¹⁴ and NSCLC.^{15,16} SNP-mediated activation of lncRNA PCAT19 interacts with HNRNPAB to promote prostate cancer growth and metastasis.¹⁷ Growing evidence indicates that lncRNAs play a vital role in the proliferation^{18,19} and migration^{20,21} of NSCLC.²²⁻²⁴ For example, LINC0026 can regulate activation of the DNA damage response via phosphorylation in NSCLC.²⁵

Small Nucleolar RNA Host Gene 17 (SNHG17, a 1,186-nt lncRNA) is located on 20q11.23. It has been reported to be an unfavorable prognostic factor in colorectal cancer (CRC), and it promotes CRC cell proliferation by epigenetically silencing P57.²⁶ However, the expression and function of SNHG17 in NSCLC remain unclear. Also, the exact sequence of SNHG17 hasn't been reported yet. So we focused on its specific character in NSCLC. In this study, we found that gene amplification of SNHG17 could induce the overexpression of SNHG17 in NSCLC, by analyzing The Cancer Genome Atlas (TCGA) database; and, we acquired the exact sequence of SNHG17. Then, we found that knockdown of

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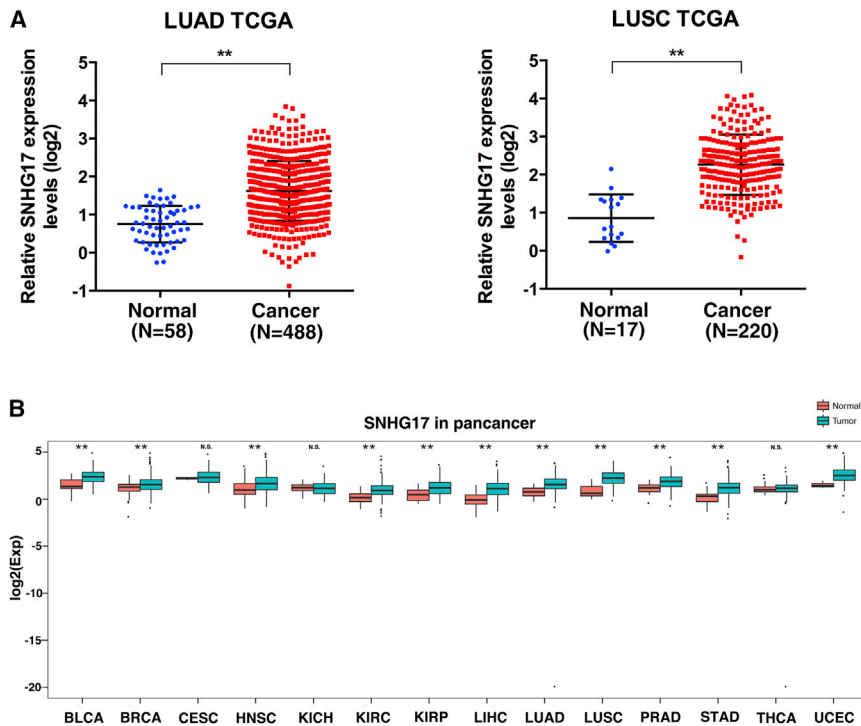


Figure 1. Expression of SNHG17 in NSCLC and Pancancer

(A) Data collected from TCGA database showed relative expression of SNHG17 in LUAD (n = 488) and LUSC (n = 220) tissues and nontumorous tissues (n = 58, n = 17). (B) Data collected from TCGA database of pan-cancer showed relative expression of SNHG17 in several cancers and nontumorous tissues. *p < 0.05 and **p < 0.01.

Downregulation of SNHG17 Inhibits NSCLC Cell Proliferation and Migration

We modulated SNHG17 expression by RNAi. After 48 h post-transfection, SNHG17 expression was knocked down in A549 and PC-9 cells when compared with control (Figure 3A). To investigate the influence of SNHG17 knockdown on NSCLC cell proliferation, we performed the methylthiazol tetrazolium (MTT) assay. The result of the MTT assay showed that the silencing of SNHG17 greatly inhibited both A549 and PC-9 cell proliferation (Figure 3B). Moreover, the colony formation assays also proved this result. The clonogenic survival of A549 and PC-9 cells was reduced by the SNHG17 decrease (Figure 3C).

Ethynyldeoxyuridine (EdU) staining assays also demonstrated that the proliferation capacity of A549 was reduced by the knockdown of SNHG17 (Figure 4A). Transwell assays were used to explore migration of NSCLC cells. As shown in Figure 3D, the migrations of A549 and PC-9 cells were remarkably inhibited after the knockdown of SNHG17. These results indicated that the inhibition of SNHG17 significantly reduced the NSCLC cell proliferation and migration.

SNHG17 Regulates NSCLC Cell Proliferation by Affecting the Cell Apoptosis

Apoptosis has crucial roles in the proliferation of cancer cells.²⁷ To figure out the role of apoptosis in NSCLC cell proliferation after the knockdown of SNHG17, we used flow cytometry to explore the involvement of SNHG17 in NSCLC cell apoptosis. Figure 4B shows that the percentages of apoptotic cells in A549 cells transfected with small interfering RNAs si-SNHG17 1 and si-SNHG17 2 were significantly higher compared to the cells treated with scrambled control. Our findings suggested that the downregulation of SNHG17 significantly increased NSCLC cell apoptosis in A549 cells. SNHG17 could regulate NSCLC cell proliferation by affecting the cell apoptosis.

The Global Gene Expression Profile Regulated by SNHG17 in NSCLC Cells

Although former reports have confirmed the function of SNHG17 in CRC,²⁶ the exact downstream global gene expression profile of SNHG17 still has remained unknown. To figure out the potential downstream gene of SNHG17, we used RNA-seq assays to acquire the global gene expression profile regulated by SNHG17. The RNA-seq experiment was performed after SNHG17 knockdown

SNHG17 could inhibit the proliferation and migration and promote the apoptosis of NSCLC cells. In addition, we screened out the potential downstream gene of SNHG17 by RNA sequencing (RNA-seq) assays after knockdown of SNHG17 in NSCLC cells. Our data demonstrated the important roles of SNHG17 in NSCLC oncogenesis, and it might serve as a target for NSCLC diagnosis and therapy.

RESULTS

Gene Amplification-Driven SNHG17 Overexpression in NSCLC

To identify the various expressions of SNHG17 between lung tumors and normal tissues, we analyzed TCGA data of NSCLC. We included 488 lung adenocarcinoma (LUAD) and 58 normal tissues; 220 lung squamous cell carcinoma (LUSC) and 17 normal tissues in TCGA were also analyzed. Overexpression of SNHG17 in NSCLC was confirmed, both LUAD and LUSC included (Figure 1A). In addition, the higher expression of SNHG17 in multiple cancers was proven by pan-cancer analysis, indicating that SNHG17 may play a comprehensive role in tumorigenesis (Figure 1B).

We also found that the expression of SNHG17 was significantly correlated with the copy number level in LUAD (N = 486, Cor = 0.39, p < 2.2e-16) and LUSC (N = 220, Cor = 0.30, p = 4.331e-6) (Figure 2A). It indicated that the increase in SNHG17 in NSCLC may be partly due to the amplification of copy number. As a lncRNA, the exact sequence of SNHG17 in NSCLC hasn't been identified yet. We performed rapid amplification of cDNA ends (RACE), and we determined its full exact length is 1,118 nt in the A549 cell (Figure 2B).

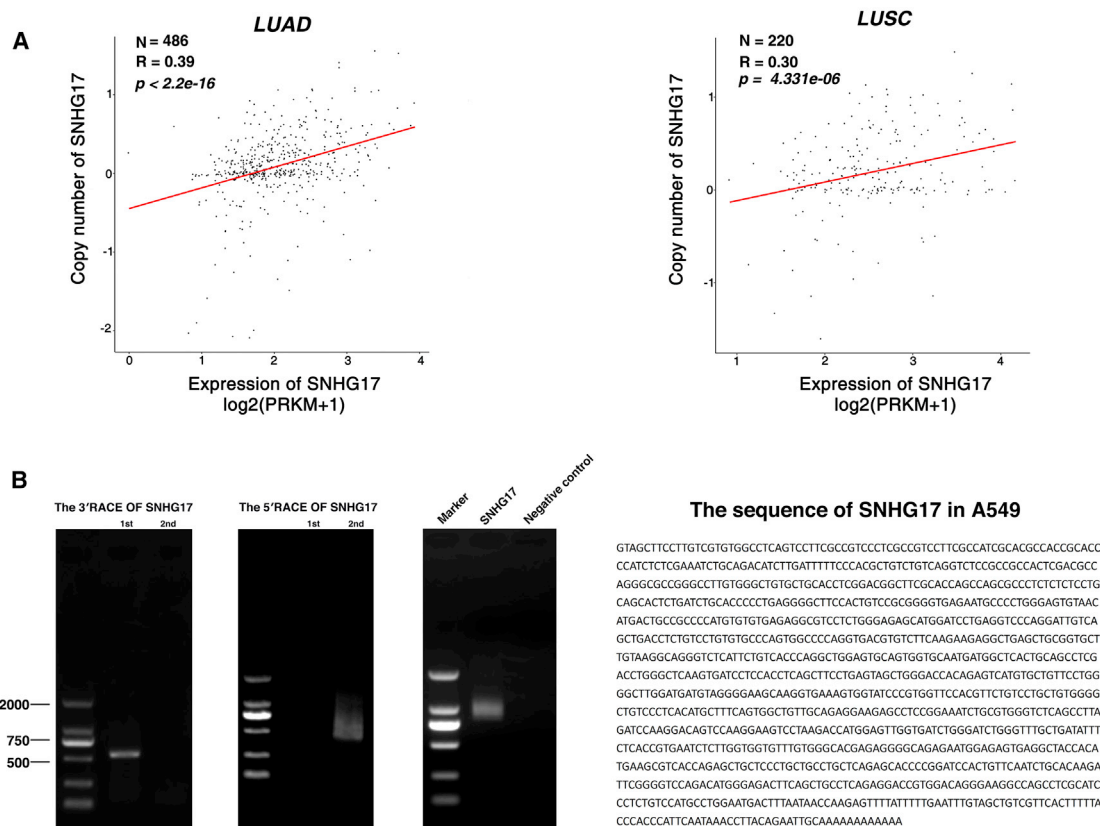


Figure 2. The CNV and Sequence of SNHG17 in NSCLC

(A) A positive correlation between SNHG17 gene copy number and its RNA expression was observed in TCGA data of LUAD (N = 486, Cor = 0.39, $p < 2.2 \times 10^{-16}$) and LUSC (N = 220, correlation coefficient [Cor] = 0.30, $p = 4.331 \times 10^{-6}$). (B) The 3' RACE of SNHG17 and the 5' RACE of SNHG17, the full-length amplification of SNHG17, and the full sequence of SNHG17 confirmed by RACE. * $p < 0.05$ and ** $p < 0.01$.

by si-SNHG17 in A549 cells. We found 637 protein-coding genes were upregulated while 581 protein-coding genes were downregulated ($p < 0.05$; data are available in Table S2).

Gene ontology (GO) terms of all significant genes demonstrated that most of these genes were related to the proliferation and apoptosis of cells (Figure 5A). We selected three genes (FOXA1,²⁸ XAF1,²⁹ and BIK³⁰) that were closely related to proliferation and apoptosis. Reports demonstrated that FOXA1³¹ was increased while XAF1³² and BIK³³ were decreased in lung cancer. FOXA1 could be upregulated by lncRNA LOC730100 to promote cancer proliferation.³⁴ Overexpression of XAF1 induced apoptosis in lung cancer cells.²⁹ BIK could antagonize the pro-proliferative activity of ERK1/2³³ in lung cancer. So we performed qRT-PCR and western blot to confirm their altered expression in A549 and PC-9 cells treated with si-SNHG17. FOXA1 was inhibited while XAF1 and BIK were promoted at the mRNA and protein expression levels after the SNHG17 knockdown (Figures 5B and 5C).

DISCUSSION

As the leading cause of death all around the world, lung cancer has attracted lots of attention, and it is urgent to figure out the underlying

mechanism. Recent studies reported the great significance of lncRNAs in the development of NSCLC, such as PVT1²⁴ and HOTAIR,³⁵ indicating their specific roles in NSCLC. In our study, we focused on lncRNA SNHG17. We confirmed the upregulation of SNHG17 in pan-cancer, especially in NSCLC. Then we identified its exact full-length sequence in NSCLC through RACE. The loss-of-function experiments proved that knockdown of SNHG17 inhibited the proliferation and migration of NSCLC cells. Apoptosis was promoted after the knockdown of SNHG17. We also acquired the global gene expression profile regulated by SNHG17 in A549 cells, and we confirmed several downstream genes that may contribute to the proliferation and apoptosis. The lncRNA SNHG17 has been reported to be an oncogene in CRC.²⁶ It contributed to the proliferation of CRC cells, which was consistent with our results. However, they didn't report the full length of SNHG17 and the global gene expression profile affected by SNHG17. Our findings can provide more information about SNHG17 in cancer cells.

Williams and Farzaneh³⁶ proposed that the SNHG family might contribute to the etiology of cancer. The SNHG family was proven to interact with classic tumor-related genes like MYC³⁷ and p53.³⁸

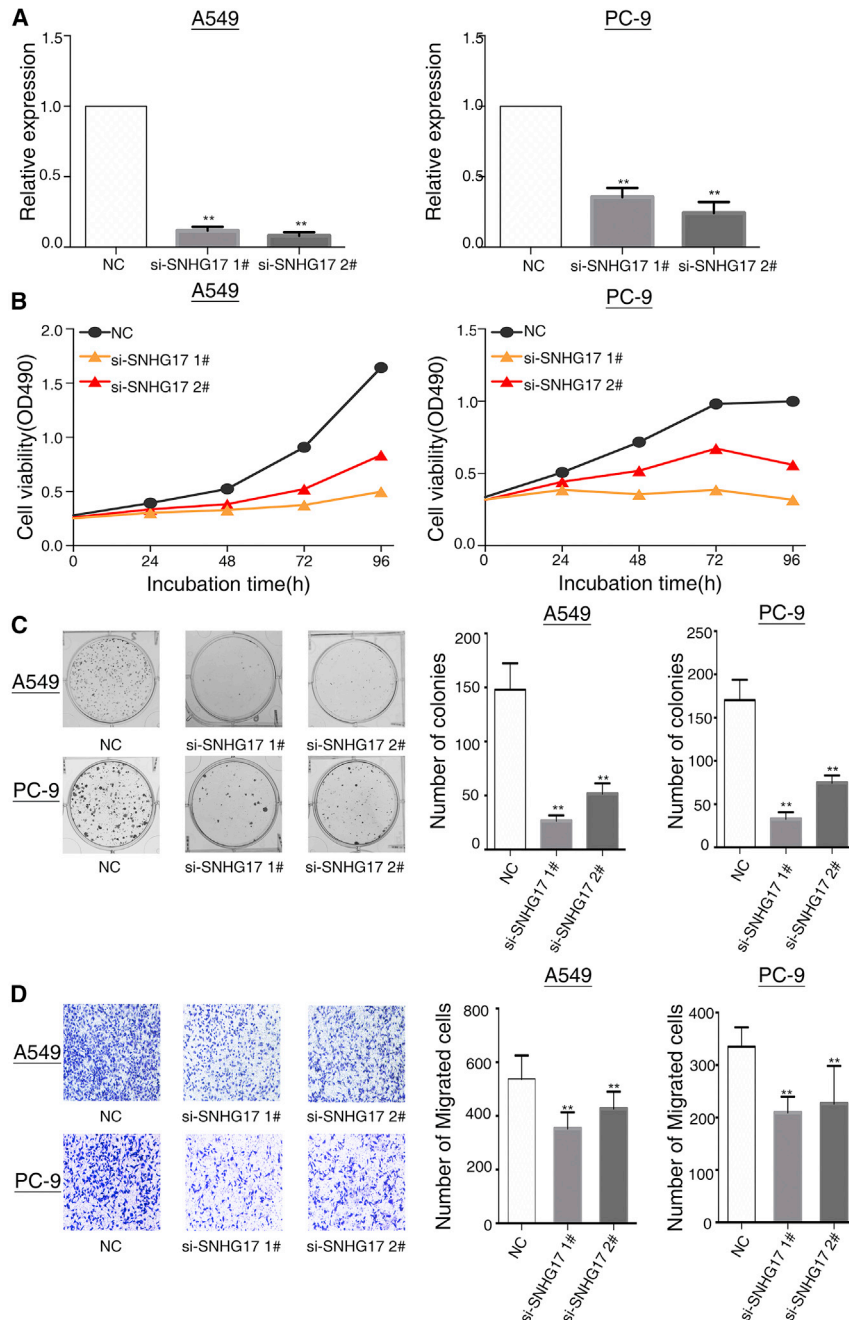


Figure 3. Downregulation of SNHG17 Inhibits NSCLC Cell Proliferation and Migration

(A) qRT-PCR assays examined the knockdown of SNHG17 in A549 and PC-9 cells by small interfering RNA (siRNA). (B) MTT assays were performed to detect the viability of A549 and PC-9 cells treated with si-SNHG17. (C) Colony formation assays were used to determine the proliferation of si-SNHG17-treated A549 and PC-9 cells. (D) The migration ability of A549 and PC-9 cells was tested by Transwell assays. * $p < 0.05$ and ** $p < 0.01$.

SNHG20 can interact with HER2, which is an important biomarker in breast cancer therapy, via miR-495.⁴² Our group also revealed that SNHG20 promotes the tumorigenesis of NSCLC by epigenetically silencing P21.⁴³ From the above, we can see the complicated molecular mechanism and clinical significance behind the SNHG family in cancer.

In our study, we have proven the high expression and the function of SNHG17 in NSCLC. The underlying downstream gene was screened through RNA-seq assays. We authenticated three potential downstream genes (FOXA1, XAF1, and BIK), which showed differential expressions after the transfection with si-SNHG17 by qRT-PCR and western blot. They may be the potential targets of SNHG17 in NSCLC for their contributions to the proliferation and apoptosis of cancer.^{28–30} FOXA1 has been well reported to be a promising prognostic marker in cancer like breast cancer.⁴⁴ It can also enhance the chemoresistance in NSCLC via interacting with non-coding RNAs.^{45,46} In our study, FOXA1 was also downregulated along with the knockdown of SNHG17. It indicated the synergistic effect between SNHG17 and FOXA1.

On the contrary, XAF1 and BIK were confirmed to be overexpressed after the inhibition of SNHG17. XAF1 has been well proven to be a tumor suppressor, which can promote the apoptosis of cancer cells.⁴⁷ Its expression was markedly reduced in NSCLC tumor samples.³² Previous study confirmed that XAF1 overexpression can promote the apoptosis of A549 cells both *in vivo* and *in vitro*.⁴⁸ Our result confirmed the upregulation of XAF1 induced by SNHG17 knockdown. SNHG17 may contribute to the carcinogenesis of NSCLC through the inhibition of XAF1. BIK, a member of the Bcl2 family, has been recognized as a tumor suppressor for its key role in pro-apoptosis.³⁰ The suppression of BIK can be seen in cigarette smokers, and it leads to the development of lung cancer via multiple mechanisms like ERK1/2.³³

SNHG1, a typical example of the SNHG family, can directly regulate various genes' expressions both *in trans* and *in cis*.³⁹ In common with other lncRNAs, SNHG1 also can act as a competing endogenous RNA (ceRNA) to interact with several microRNAs and, consequently, exert influence on cancer cells. Xiao et al.⁴⁰ demonstrated that a high expression of SNHG1 correlated with poor clinical outcomes in eight solid tumors, including NSCLC, osteosarcoma, and CRC. SNHG20 is another vital member of the SNHG family and acts as an oncogene. It can regulate malignant behaviors of tumor by sponging microRNAs.⁴¹

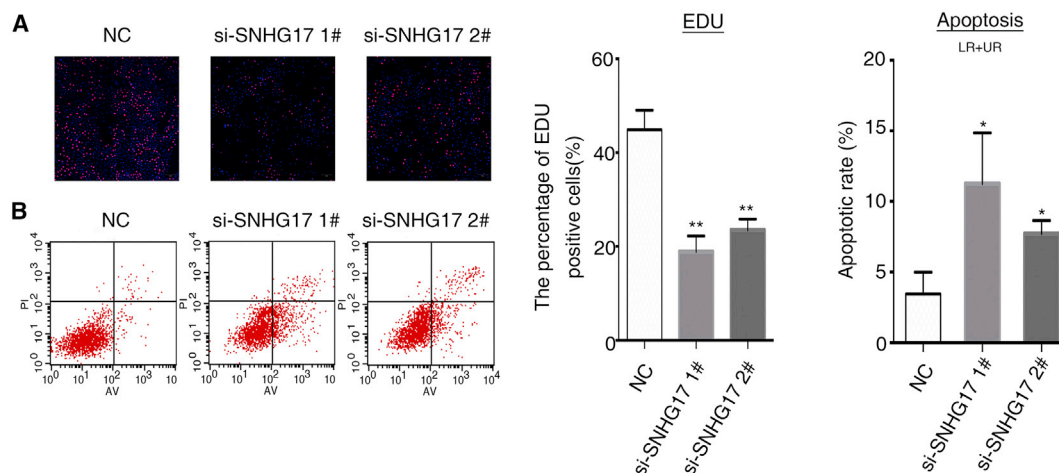


Figure 4. SNHG17 Regulates NSCLC Cell Proliferation by Affecting the Cell Apoptosis

(A) Proliferous A549 cells were displayed by EdU immunostaining assays. (B) Apoptotic rates of A549 cells were detected by flow cytometry assays. * $p < 0.05$ and ** $p < 0.01$.

Reports showed that classic chemotherapeutic agents such as doxorubicin could activate BIK expression.⁴⁹ The activation of BIK has been confirmed to induce significant cancer cell death both *in vivo* and *in vitro*.³⁰ Moreover, gene therapy targeted on BikDD, a constitutively active mutant form of BIK, caused immune anticancer response in lung cancer.⁵⁰ Since our results revealed significant upregulation of BIK after SNHG17 knockdown, they indicate that the SNHG17/BIK axis has great potential in NSCLC gene-targeting therapy.

In summary, we found that SNHG17 was upregulated in lung cancer driven by the amplification of copy number, and we identified its exact sequence. We demonstrated that the downregulation of SNHG17 inhibited cell proliferation and migration and promoted apoptosis. We also gained the global gene expression profile regulated by SNHG17 by RNA-seq assays, and we confirmed three underlying downstream genes. Hence, our study elucidates the function of SNHG17 and explores its potential target genes in NSCLC. lncRNA SNHG17 plays a vital role in NSCLC and needs further investigation to find out its specific underlying mechanism.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human NSCLC cell lines (A549 and PC-9) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in RPMI 1640 medium, and PC-9 cells were cultured in DMEM (Gibco-BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO₂.

RACE

5' RACE and 3' RACE were performed using SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA, USA), according to the manufacturer's instructions.

RNA Extraction and qRT-PCR Assays

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA (1,000 ng) was reverse transcribed in a final volume of 10 mL using random primers under standard conditions for the PrimeScript RT reagent kit (TaKaRa, Dalian, China). We used the SYBR Premix Ex Taq (TaKaRa, Dalian, China) to determine SNHG17 expression levels, following the manufacturer's instructions. Results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (Data are available in Table S1.) Results were analyzed and expressed relative to threshold cycle (CT) values, then converted to fold changes.

Cell Transfection

The small interfering RNAs si-SNHG17 and si-NC were transfected into PC-9 and A549 cells. A549 and PC-9 cells were grown in 6-well plates until confluent, then transfected with Lipofectamine 2000 (Invitrogen, Shanghai, China), according to the manufacturer's instructions. At 48 h post-transfection, cells were harvested for qRT-PCR. The sequences of siRNAs are shown in Table S1.

Cell Proliferation Assays

Cell proliferation was monitored using a Cell Proliferation Reagent Kit I (MTT) (Roche, Basel, Switzerland). A549 and PC-9 cells transfected with si-SNHG17 (2,000 cells/well) were grown in 96-well plates. Cell proliferation was assessed every 24 h following the manufacturer's protocol. For the colony formation assays, a certain number (1,000 cells/well) of transfected cells was placed into each well of a 6-well plate and maintained in media containing 10% FBS for 2 weeks, replacing the medium every 4 days. Colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 15 min. The colony formation was determined by counting the number of stained colonies. Triplicate wells were measured in each treatment group.

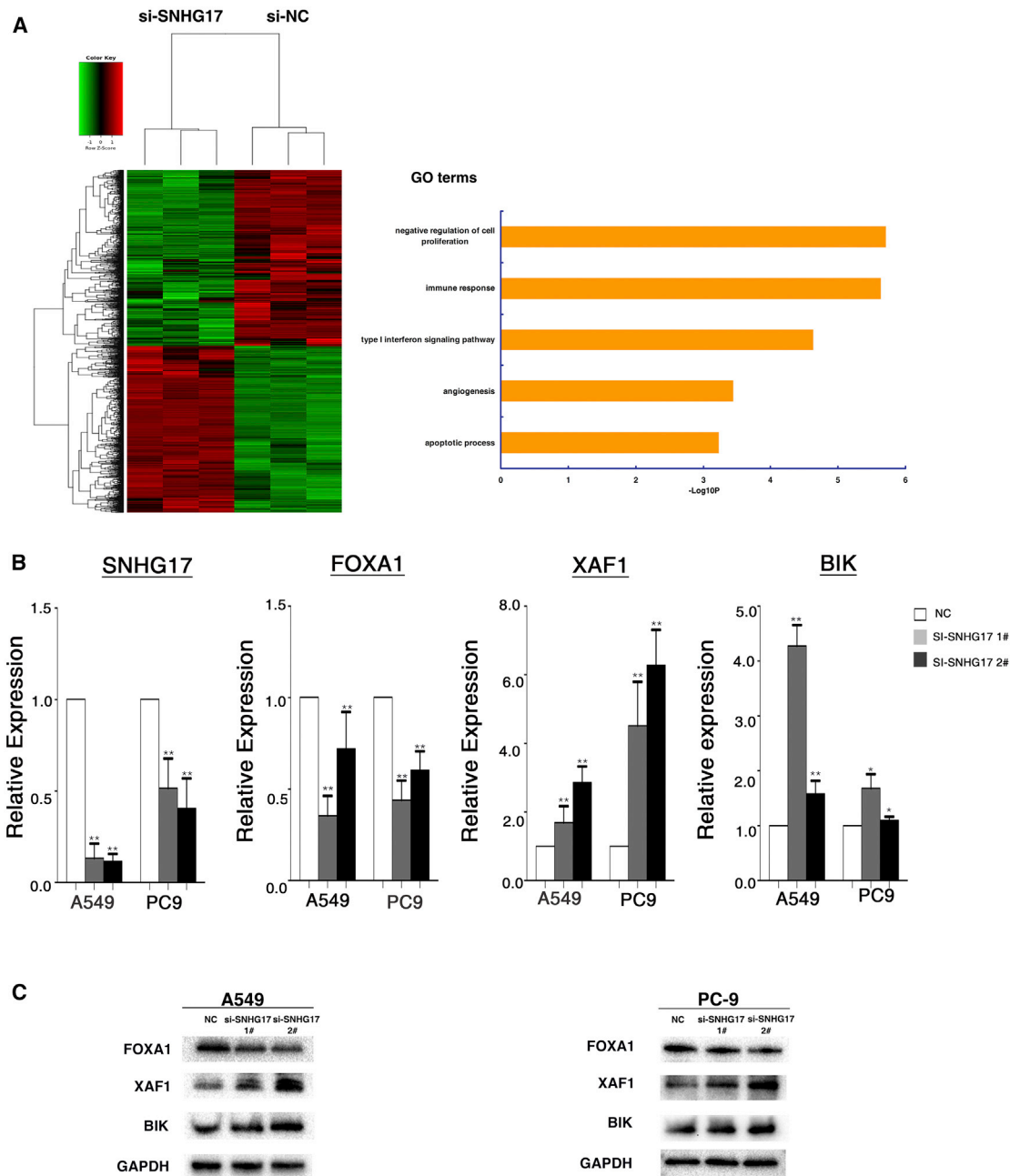


Figure 5. The Global Gene Expression Profile Regulated by SNHG17 and Potential Target Genes

(A) Mean-centered, hierarchical clustering of gene transcripts altered (≥ 2 -fold change) after the knockdown of SNHG17 in A549 cells, with three repeats. Gene ontology analysis was performed for all genes with altered expressions after the knockdown of SNHG17. (B) The altered mRNA levels of genes were selectively confirmed by qRT-PCR after SNHG17 knockdown in A549 and PC-9 cells. (C) The altered protein levels of genes were detected by western blot after SNHG17 knockdown in A549 and PC-9 cells. * $p < 0.05$ and ** $p < 0.01$.

Cell Migration Assays

For the migration assays, at 24 h post-transfection, 2×10^4 cells in serum-free medium were placed into the upper chamber of an insert (8 mm; BD Biosciences). Medium containing 10% FBS was added to the lower chamber. After incubation for 24 h, the cells remaining on

the upper membrane were removed with cotton wool. Cells that had migrated or invaded through the membrane were stained with methanol and 0.1% crystal violet, imaged, and counted using an IX71 inverted microscope (Olympus, Tokyo, Japan). Experiments were independently repeated three times.

EdU Analysis

Proliferating cells were assessed using an EdU-labeling/detection kit (Ribobio, Guangzhou, China), based on the manufacturer's protocol. Cells were grown in 24-well plates at 2×10^4 cells/well. At 48 h after transfection, 50 μ M EdU-labeling medium was added to cell culture and incubated for 2 h at 37°C under 5% CO₂. Next, the cultured cells were treated with 4% paraformaldehyde (pH 7.4) and then 0.5% Triton X-100 for 30 min and 20 min, respectively, at room temperature. Then the cells were stained with anti-EdU working solution and subsequently incubated with 300 μ L Hoechst 33342 (5 μ g/mL). The percentage of EdU-positive cells was observed under laser-scanning confocal microscopy.

Flow Cytometry Analysis

A549 cells were harvested 48 h post-transfection by trypsinization. Using the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (BD Biosciences), double staining with FITC-Annexin V and propidium iodide (PI) was performed following the protocol. Then the cells were analyzed by flow cytometry (FACScan, BD Biosciences) with CellQuest software (BD Biosciences). Cells were classified into viable, dead, early apoptotic, and apoptotic cells, and the ratio of early apoptotic cells was compared with the control for each experiment. All of the samples were assayed in triplicate.

Whole-Transcriptome Deep Sequencing

Total RNAs from the A549 cells with SNHG17 knockdown and control A549 cells were isolated and quantified. The concentration of each sample was measured by NanoDrop 2000 (Thermo Scientific, USA). The quality was assessed by the Agilent2200 (Agilent, USA). The sequencing library of each RNA sample was prepared by using Ion Proton Total RNA-Seq Kit version (v.)2, according to the protocol provided by the manufacturer (Life Technologies, USA). Mapping of the single-end was read. Before read mapping, clean reads were obtained from the raw reads by removing the adaptor sequences, reads with >5% ambiguous bases (noted as N) and low-quality reads containing more than 20% of bases with qualities of <13. The clean reads were then aligned to the human genome (version GRCh38) using the MapSplice program (v.2.1.8). HTseq⁵¹ was used to count genes and the reads per kilobase per million mapped reads (RPKM) method was used to determine the gene expression. We applied the DESeq⁵² algorithm to filter the differentially expressed genes after the significant analysis, p value, and FDR analysis under the following criteria:⁵³ (1) fold change >2 or <0.5 and (2) FDR < 0.05. We deposited the sequencing data in GEO: GSE131543 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131543>). Data are available in Table S2.

GO Analysis

GO analysis was performed to facilitate elucidating the biological implications of unique genes in the significant or representative profiles of the target gene of the differentially expressed mRNA in the experiment.⁵⁴ We downloaded the GO annotations from NCBI (<https://www.ncbi.nlm.nih.gov/>), UniProt (<https://www.uniprot.org/>), and Gene Ontology Resource (<http://geneontology.org/>). Fisher's exact

test was applied to identify the significant GO categories and FDR was used to correct the p values.

Western Blot

The cells were lysed using mammalian protein extraction reagent radioimmunoprecipitation assay lysis buffer (RIPA; Beyotime, Haimen, China), supplemented with protease inhibitor cocktail (Roche) and PMSF (Roche). 50 μ g of the protein extractions was separated by 10% SDS-PAGE, transferred to 0.22-mm nitrocellulose (NC) membranes (Sigma-Aldrich), and incubated with specific antibodies. The autoradiograms were quantified by densitometry (Quantity One software). Anti-FOXA1 (ab23738), Anti-BIK (ab52182), and Anti-XAF1 (ab17204) were from Abcam. Results were normalized to the expression of GAPDH.

Statistical Analysis

Student's t test (two tailed), paired t test, and one-way ANOVA test were used to analyze data with SPSS 16.0 software (IBM, Chicago, IL, USA); p values less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2019.06.008>.

AUTHOR CONTRIBUTIONS

Conception and Design, Z.W. and E.Z.; Development of the Methodology, T.X., S. Yan, L.J., S. Yu, T.L., and D.Y.; Acquisition of Data, T.X., B.L., and C.W.; Writing the Manuscript, T.X.; Administrative, Technical, and Material Support, Z.W. and E.Z. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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