SNHG16 indicates a poor prognosis and affects cell proliferation, migration and invasion in non-small cell lung cancer

Wei Han, Xuemei Du, Jing Wang, Lixin Sun, Yongchun Li

PII: S0014-4827(17)30508-6
DOI: http://dx.doi.org/10.1016/j.yexcr.2017.09.027
Reference: YEXCR10749

To appear in: Experimental Cell Research

Received date: 21 August 2017
Revised date: 15 September 2017
Accepted date: 16 September 2017

Cite this article as: Wei Han, Xuemei Du, Jing Wang, Lixin Sun and Yongchun Li, SNHG16 indicates a poor prognosis and affects cell proliferation, migration and invasion in non-small cell lung cancer, Experimental Cell Research, http://dx.doi.org/10.1016/j.yexcr.2017.09.027

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
SNHG16 indicates a poor prognosis and affects cell proliferation, migration and invasion in non-small cell lung cancer

Wei Han\textsuperscript{a1}, Xuemei Du\textsuperscript{a1}, Jing Wang\textsuperscript{a}, Lixin Sun\textsuperscript{b*}, Yongchun Li\textsuperscript{a*}

\textsuperscript{a}Department of Pulmonary Medicine, Qingdao Municipal Hospital, School of Medicine, Qingdao University, Qingdao 266011, China.
\textsuperscript{b}Department of Anesthesia, Qingdao Municipal Hospital, School of Medicine, Qingdao University, Qingdao 266011, China.

slixin1230@126.com
lyc5627@protonmail.com

\textsuperscript{*}Correspondence to: Qingdao Municipal Hospital, Qingdao University, No. 1, Jiaozhou Road, Qingdao 266011, Shandong Province, China.

Abstract

In this study, we report that long non-coding RNA (lncRNA) SNHG16 is upregulated in non-small cell lung cancer (NSCLC) tissues, and is correlated with tumor size, TNM stage and lymph node metastasis. Kaplan-Meier analysis shows that the patients with high SNHG16 expression have poorer disease-free survival (DFS) and overall survival (OS) than the patients with low SNHG16 expression. Multivariate Cox regression analysis reveals that SNHG16 expression could be regarded as an independent predictor for DFS and OS in NSCLC patients. \textit{In vitro} experiments show that SNHG16 knockdown inhibits NSCLC cell proliferation, migration and invasion, and SNHG16 overexpression promotes NSCLC cell proliferation, migration and invasion. We further identify and confirm that miR-146a is the target of SNHG16, and SNHG16 functions by targeting miR-146a. Subsequently, MUC5AC, a major mucin in the human respiratory tract correlated with post-operative metastasis and recurrence of NSCLC, is confirmed to be regulated by SNHG16 and miR-146a, and might be involved in the oncogenic activity of SNHG16-miR-146a axis in NSCLC. \textit{In vivo}

\textsuperscript{1} They contribute equally to this article.
experiments also confirm these conclusions. Taken together, the present results elucidate a potential mechanism underlying the carcinogenesis role of SNHG16 in NSCLC, and indicate that SNHG16 could act as a novel promising marker for prognosis, and a potential therapeutic target for NSCLC treatment in the future.

Keywords
Non-small cell lung cancer, long non-coding RNA SNHG16, miR-146a, proliferation, migration, invasion

1. Introduction
Lung cancer is the most common cause of cancer-related death worldwide, with about 1.8 million new cases and 1.59 million people dying from this disease per year [1,2]. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for about 80% of all cases. Despite the emergence of new adjuvant chemotherapy regimens and targeted biologic agents, the prognosis of the patients with NSCLC is not satisfactory with dismal 5-year survival rates and recurrence rate. Undoubtedly, better understanding of the underlying pathological mechanisms will contribute to develop more effective therapeutic strategies, thereby improving the clinical outcome of NSCLC patients.

Long non-coding RNAs (lncRNAs) represent a subgroup of non-coding RNAs, which own a length of more than 200 nucleotides and are unable to be translated into proteins [3,4]. They have been formed as a novel field of biology, with mounting evidence suggesting that they play a critical role in the development, genomic imprinting, cellular homeostasis and embryonic stem cell pluripotency [5-7]. In addition, the significance of lncRNA regulation is greatly emphasized by their roles in the etiology of human diseases, especially in cancer. For example in NSCLC, lncRNA HOTAIR, MALAT1 and MVIH indicate a poor prognosis and induce metastasis; low expression of lncRNA PANDAR predicts a poor prognosis and affects cell apoptosis by regulating Bcl-2; and lncRNA PVT1 promotes cancer cell proliferation through epigenetically regulating LATS2 expression [8-12]. LncRNA SNHG16, originally
identified as an oncogene in neuroblastoma, is associated with poor prognosis in neuroblastoma, and with metastasis of bladder cancer and breast cancer [13-15]. But, little is known about the role of SNHG16 in NSCLC.

MicroRNAs (miRNAs) belong to the small ncRNA group, usually 18-25 nucleotides in length, and are involved in various diseases, including cancer. miR-146a has been widely reported to exert its carcinostatic activity in a variety of cancers, including NSCLC [16-19]. In addition, miR-146a plays a negative role in the regulation of MUC5AC production [20]. MUC5AC is a major mucin in the human respiratory tract and correlates with post-operative metastasis and recurrence of NSCLC [21]. Kim et al. [22] demonstrate immunosensors for detection of Annexin II and MUC5AC for early diagnosis of lung cancer.

In this study, we determined the expression of SNHG16 in 66 paired NSCLC tissues and adjacent normal tissues, and found that SNHG16 was upregulated in NSCLC tissues, and was associated with poor prognosis. Loss- and gain-of-function experiments showed that SNHG16 promoted NSCLC cell proliferation, migration and invasion. We further explored the mechanism, and found that SNHG16 exerted its oncogenic activity by targeting miR-146a. In addition, the oncogenic activity of SNHG16-miR-146a axis might be also associated with MUC5AC expression.

2. Materials and methods

2.1. Tissue collection

Lung cancer tissues and adjacent normal tissues (at least 3 cm from the distal negative surgical margin to confirm the absence of malignancy) were obtained from 66 patients with NSCLC who had undergone surgery at the Qingdao Municipal Hospital, between 2008 and 2012. These NSCLC patients were diagnosed based on histopathological evaluation. None of the patients received local or systemic treatment before the operation. These tissue specimens were immediately snap frozen in liquid nitrogen, and stored at -80°C until use. The study was approved by the Qingdao Municipal Hospital ethics committee (Qingdao, Shangdong, China), and all patients signed informed consent.
2.2. Cell culture

Four NSCLC cell lines (A549, NCI-H292, NCI-H460, and NCI-H1703) and human bronchial epithelial (16HBE) cell were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cells were cultured in RPMI 1640 or DMEM (Gibco, Grand Island, NY, USA) medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), and antibiotics (Invitrogen) at 37°C in a 5% CO₂ atmosphere.

2.3. RNA extraction and qRT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen), and the First strand cDNA was synthesized by using a Reverse Transcription Kit (Takara, Dalian, China). The expression of SNHG16 and MUC5AC mRNA was quantified on ABI 7500 real-time PCR system, and β-actin was used as an internal control. The expression of miR-146a was quantified by using TaqMan miRNA assays in accordance with the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA), and U6 was used as an internal control. Fold change in gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method.

2.4. Cell transfection

Plasmid complementary DNA SNHG16 cDNA (pcDNA-SNHG16) was constructed by amplification and introduction of SNHG16 cDNA sequence into the pcDNA3.1 vector (Invitrogen). The siRNA (small interfering RNA) sequences targeting SNHG16 (si-SNHG16) and sequence-scrambled siRNA (si-control) were purchased from Invitrogen. miR-146a mimic, miR-146a inhibitor, or their respective controls were obtained from RiboBio (RiboBio Co. Ltd, Guangzhou, Guangdong, China). All transfection reactions were performed by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol.

2.5. Colony formation assay

The cells were plated at 300 cells per 35-mm tissue culture dish and cultured for
two weeks to allow colony formation. Colonies were fixed with methanol and stained with 0.1% crystal violet (E. Merck, AG, Darmstadt, BRD). Colonies were photographed, and the number of colonies was counted manually.

2.6. Cell proliferation assay

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Peptide Institute Inc., Osaka, Japan) in accordance with the manufacturer’s protocol. Briefly, the cells were plated in 96-well plates at a density of $1 \times 10^3$ cells per well, treated with 10 μl / well of CCK-8 solution for 4 h at indicated time points, and the cell proliferation curves were plotted using the absorbance (450 nm) at each time point.

2.7. Migration assay

Cells with $1 \times 10^5$ cells / well were seeded into 6-well plates and incubated until 85% confluence. Then similar sized scratches were made through the cell monolayer using 200-μl pipette tip, and free-floating cells and debris were removed by washing with PBS. The scraped monolayer was grown for additional 48 h in fresh serum-free medium. Scratch area was observed under a microscopy and the scratch length was measured at 0 and 48 h to calculate the migration rate according to the following formula: migration rate = $\frac{[(\text{scratch length at 0 h}) - (\text{scratch length at 48 h})]}{\text{(scratch length at 0 h)}} \times 100\%$.

2.8. Invasion assay

Matrigel-coated invasion chamber (8-μm pore membrane filters; Costar, Corning, NY, USA) was used to determine cell invasion. Briefly, $5 \times 10^4$ cells prepared in serum-free medium were loaded in the upper compartment of chamber, and complete medium was placed in the lower chamber as a chemo-attractant stimulus. After incubation for 24 h, the cells remaining on the upper surface of the filter were wiped out with a cotton swab, and the cells that migrated to the bottom surface of the filter were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet, and counted under a microscope in three random fields per filter.

2.9. Luciferase reporter assay

Two reporter plasmids were constructed by inserting SNHG16 fragments with wild-type (WT) or mutant (MUT) binding sites of miR-146a into the
pmiR-RB-REPORT™ (Ribobio, Guangzhou, China). Cells were cotransfected with the WT or MUT reporter plasmid and miR-146a mimic or mimic control. At 48 h post-transfection, luciferase activity was measured using dual-glo luciferase reporter system (Promega, Madison, WI, USA).

2.10. RNA-binding protein immunoprecipitation (RIP) assay

RIP experiment was performed by using the Magna RIP kit (Millipore, Bedford, MA, USA), following the manufacturer’s instructions. Briefly, cells were harvested and lysed in complete RIP lysis buffer at 50% confluency. Then, 100 μl of whole cell extract was incubated with RIP buffer containing magnetic beads conjugated with human anti-Argonaute2 (Ago2) antibody (Millipore) or normal mouse IgG (negative control) for 6-8 h at 4°C. After incubation with proteinase K at 55°C for 30 min, immunoprecipitated RNA was isolated, purified and subjected to qRT-PCR analysis.

2.11. Enzyme-linked immunosorbent (ELISA) assay

Then level of MUC5AC protein in tissues or cells was measured by Human MUC5AC ELISA kit (USCNK, Wuhan, China) according to the instructions of the manufacturer.

2.12. Tumor xenografts in nude mice

Athymic BALB/c mice (male, 4 weeks old) were purchased from Slac Laboratory Animal Co. Ltd. (Shanghai, China). They were housed in laminar airflow chambers under specific pathogen-free (SPF) conditions and manipulated according to protocols approved by the Animal Ethics Community of Qingdao Municipal Hospital. A549 cells transfected with si-SNHG16 or si-control were harvested and resuspended in phosphate-buffered saline (PBS), and the cell concentration was adjusted to $1 \times 10^8$ cells per ml. Then, 100 μl of suspending cells transfected with si-SNHG16 was subcutaneously injected into right side of the posterior flank of the nude mouse, while injection of 100 μl of suspending cells transfected with si-control was performed in the left side. The length and width of tumor in nude mouse were measured with calipers every 7 days, and tumor volume was calculated as length $\times$ width$^2$ / 2. Mice were sacrificed on day 35, and the tumors were excised and weighed.
2.13. **Statistical analysis**

All statistical analyses were performed using SPSS17.0 software (SPSS, Chicago, IL, USA). The comparisons between groups were performed via Student's t test, ANOVA or $\chi^2$ test. Disease-free survival (DFS) and overall survival (OS) rates were calculated by the Kaplan-Meier method, and were analyzed by log-rank test. Survival data were assessed by using multivariate Cox proportional hazards model. A value of $P < 0.05$ was considered to statistically significant.

3. **Results**

3.1. **SNHG16 is upregulated in human NSCLC tissues and cell lines**

SNHG16 expression in 66 pairs of NSCLC lung tissues and paired adjacent normal lung tissues was determined by using qRT-PCR. As shown in Fig. 1A and B, SNHG16 expression was elevated in tumor tissues compared with adjacent normal tissues. Specifically, 49 cases (74.2%) had significantly higher SNHG16 expression in tumor tissues than adjacent normal tissues, whereas only 8 cases (12.1%) had lower SNHG16 expression in tumor tissues than normal tissues (Fig. 1C). We then determined SNHG16 expression in NSCLC cell lines A549, NCI-H292, NCI-H460 and NCI-H1703, and human bronchial epithelial (16HBE) cells. The results showed that SNHG16 expression was significantly increased in NSCLC cell lines compared with 16HBE cells (Fig. 1D).

3.2. **SNHG16 expression is associated with tumor progression and poor prognosis**

To analyze the relationships between SNHG16 expression and clinical pathological features of NSCLC patients, we divided the NSCLC patients into high and low SNHG16 expression groups according to the median value of SNHG16 levels, each group with 33 patients. As shown in Table 1, SNHG16 level was remarkably correlated with tumor size, TNM stage and lymph node metastasis. No relationship between SNHG16 expression and other clinical factors, such as gender, age, histological grade and history of smoking, was found in this study. To further
investigate whether SNHG16 expression level was correlated with the prognosis of NSCLC patients, DFS and OS curves were plotted and analyzed by the Kaplan-Meier analysis and log-rank test. The results showed that NSCLC patients with high SNHG16 expression level had a shorter DFS and OS than those with low SNHG16 expression level (Fig. 2A and B). Moreover, in a multivariate Cox model, we found that SNHG16 expression and TNM stage could be regarded as independent predictors for DFS and OS in NSCLC patients (Table 2).

3.3. **SNHG16 promotes A549 cell proliferation, migration and invasion.**

To further explore the oncogenic activity and role of SNHG16 on NSCLC, we successfully established A549 cells with SNHG16 stable overexpression or transient knockdown (Fig. 3A). Then we performed colony formation, CCK-8, migration and invasion assays. Colony formation assay showed that SNHG16 knockdown decreased the clonogenic survival of A549 cells, while SNHG16 overexpression significantly increased the clonogenic survival of A549 cells (Fig. 3B). CCK-8 assay showed that SNHG16 knockdown significantly attenuated the vitality of A549 cells, while SNHG16 overexpression promoted A549 cell proliferation (Fig. 3C). Migration and invasion assays showed that the si-SNHG16 group had less migration distance and invasion cells than the si-control group, and the pcDNA-SNHG16 group had more migration distance and invasion cells than the pcDNA-control group, which suggested that SNHG16 knockdown obviously attenuated the migration and invasion ability, and SNHG16 overexpression increased the migration and invasion ability of A549 cells (Fig. 3D and E). Taken together, these results suggested that SNHG16 promotes A549 cell proliferation, migration and invasion.

3.4. **miR-146a is a target of SNHG16**

Accumulating evidence suggests that lncRNAs could act as a molecular sponge or a competing endogenous RNA (ceRNA) to regulate the accumulation of miRNA, thereby exerting its biological functions. We used the online software starBase2.0 to predict and select miRNAs interacted with SNHG16, and found that miR-146a can bind to complementary sequences in SNHG16 (Fig. 4A). It is well known that
miRNAs are present in the cytoplasm in the form of miRNA ribonucleoprotein complexes (miRNPs) containing Ago2 protein, the core component of the RNA-induced silencing complex (RISC). Moreover, lncRNAs can function as a molecular sponge or a ceRNA to regulate miRNA level by interacting with RISC. Thus, RIP assay was performed to test whether SNHG16 and miR-146a were in the same RISC. The results showed that SNHG16 and miR-146a were both preferentially enriched in Ago2-containing miRNPs relative to IgG immunoprecipitates, which suggested that SNHG16 and miR-146a were in the same RISC (Fig. 4B). We also performed dual-luciferase reporter assay to further verify whether SNHG16 could directly target miR-146a. The results showed that the luciferase activity of wild-type reporter was significantly decreased when transfection with miR-146a mimic in comparison with mimic control (Fig. 4C). In addition, miR-146a level was downregulated in NSCLC tissues and cell lines, and was negatively correlated with SNHG16 expression in NSCLC tissues (Fig. 4D-F). We also determined the effects of SNHG16 expression on miR-146a expression in A549 cells, and found that SNHG16 knockdown enhanced miR-146a expression, and SNHG16 overexpression decreased miR-146a expression (Fig. 4G). These results indicated that miR-146a is a target of SNHG16.

3.5. miR-146a inhibits A549 cell proliferation, migration and invasion.

Next, we further investigated whether SNHG16 promoted A549 cell proliferation, migration and invasion by targeting miR-146a. We transfected A549 cells with miR-146a mimic, miR-146a inhibitor or their respective controls, and then performed colony formation, CCK-8, migration and invasion assays. The results showed that miR-146a inhibited A549 cell proliferation, migration and invasion (Fig. 5A-D). Collectively, these results indicated that SNHG16 functions by targeting miR-146a.

3.6. SNHG16 regulates MUC5AC expression by targeting miR-146a

Several reports suggest that MUC5AC are overexpressed in NSCLC and correlated with disease progression. miR-146a has also been reported to negatively regulate MUC5AC production. Based on these findings, we further investigated whether SNHG16 could regulate MUC5AC expression by targeting miR-146a. We
first determined MUC5AC mRNA and protein expression in NSCLC lung tissues and paired adjacent normal lung tissues by qRT-PCR and ELISA assays, respectively. The results showed that both MUC5AC mRNA and protein were increased in tumor tissues compared with normal tissues (Fig. 6A and B). MUC5AC mRNA level had a significant positive correlation with SNHG16 expression, and a significant negative correlation with miR-146a expression (Fig. 6C and D). In addition, we also determined the effects of SNHG16 and miR-146a expression on MUC5AC protein expression in A549 cells. The results showed that miR-146a overexpression or SNHG16 knockdown significantly suppressed MUC5AC protein expression, while the co-transfection of miR-146a mimic and pcDNA-SNHG16 regained MUC5AC protein expression level (Fig. 6E).

3.7. SNHG16 promotes growth of NSCLC tumors in vivo

To further confirm the oncogenic activity of SNHG16, we inoculated A549 cells transfected with si-SNHG16 or si-control into nude mice. As shown in Fig. 7A and B, tumor volume and weight in si-SNHG16 group were significantly lower than those in si-control group. In addition, si-SNHG16 group had a significant increase in miR-146a expression and an obvious decrease in MUC5AC protein expression compared with si-control group (Fig. 7C and D). Taken together, these results indicated that SNHG16 exerted its oncogenic activity by targeting miR-146a, and MUC5AC might be involved in the oncogenic activity of SNHG16-miR-146a axis in NSCLC.

4. Discussion

Small nucleolar RNA host genes (SNHG) have been widely reported to be upregulated in NSCLC and promote NSCLC initiation and progression. For example, SNHG1 contributes to NSCLC progression through inhibition of miR-101-3p and activation of Wnt/β-catenin pathway; and SNHG7 promotes the proliferation, migration and invasion, and inhibits apoptosis of lung cancer cells by enhancing the FAIM2 expression [23,24]. SNHG16 is also an important member of SNHG, but little is known about its expression and role in NSCLC.

In this study, we determined SNHG16 expression in NSCLC samples and their
surrounding non-tumorous tissues, and analyzed the clinical significance of SNHG16 expression in NSCLC patients. SNHG16 was upregulated in NSCLC tissues in comparison with adjacent normal lung tissues, and that SNHG16 upregulation was correlated with tumor size, TNM stage and lymph node metastasis. Kaplan-Meier analysis showed that the patients with high SNHG16 expression had poorer DFS and OS than the patients with low SNHG16 expression. Moreover, multivariate analysis by Cox regression model showed that SNHG16 expression could be regarded as an independent predictor for DFS and OS in NSCLC patients. We also identified the role of SNHG16 in NSCLC cells by performing in vivo and in vitro experiments, and found that SNHG16 promoted NSCLC cell proliferation, migration and invasion. These findings suggested that SNHG16 plays a direct role in the regulation of multiple oncogenic properties and NSCLC progression and prognosis, stimulating new research directions and therapeutic approaches considering SNHG16 as a candidate for diagnosis and prognosis, and a potential therapeutic target in NSCLC.

The importance of lncRNAs in human disease is closely related with their ability to impact the expression of multiple genes via various mechanisms. In this study, SNHG16 has been suggested to act as an oncogene, but the underlying mechanism by which SNHG16-mediated gene expression participates in NSCLC progression remains unclear. Recently, growing evidence has proposed that lncRNAs can act as ceRNAs or molecular sponges, abolishing the endogenous suppressive effect of these miRNAs on their targeted transcripts. For example, IncRNA HOTAIR functions as a ceRNA to regulate HER2 expression by sponging miR-331-3p in gastric cancer; IncRNA H19 competitively binds miR-17-5p to regulate YES1 expression in thyroid cancer; IncRNA XIST promotes cell growth and invasion through regulating miR-497/MACC1 axis in gastric cancer; and SNHG16 contributes to breast cancer cell migration by competitively binding miR-98 with E2F5 [15,25-27]. Inspired by this notion, we performed bioinformatics analysis by starBase2.0 to predict potential lncRNA-miRNA interactions, and found that miR-146a is a potential SNHG16 binding miRNA. miR-146a has been widely reported to play a suppressive role in tumor initiation and progression. For example, miR-146a suppresses invasion of
pancreatic cancer cells; suppresses tumor growth and progression by targeting EGFR pathway and in ap-ERK-dependent manner in castration-resistant prostate cancer; and inhibits metastasis in gastric cancer by targeting WASF2 [16-18]. In addition, recent studies also showed that miR-146a inhibits NSCLC cell growth and migration, and induces apoptosis [19]. Based on these findings, it was tempting to speculate that SNHG16 functions by targeting miR-146a. We first validated the direct binding of the miR-146a to SNHG16 by performing RIP and luciferase assays. We also found that miR-146a level was downregulated in NSCLC tissues and cell lines, and was negatively correlated with SNHG16 expression in NSCLC tissues. And SNHG16 knockdown remarkably increased miR-146a expression, while SNHG16 overexpression decreased miR-146a expression. Function assays showed that miR-146a inhibited NSCLC cell proliferation, migration and invasion. Taken together, these results demonstrated that SNHG16 promoted NSCLC progression, at least partly, via inhibition of miR-146a.

Having shown the important inhibitory effect of miR-146a on NSCLC progression, we searched for the potential gene effectors involved in its function. In numerous potential gene effectors, MUC5AC caught our attention since it has previously been reported to be regulated by miR-146a and play an important role in NSCLC progression [20-22]. We first determined MUC5AC mRNA and protein expression in NSCLC tissues, and found that MUC5AC mRNA and protein were upregulated in NSCLC tissues. And MUC5AC mRNA level had a significant positive correlation with SNHG16 expression, and a significant negative correlation with miR-146a expression. In addition, SNHG16 knockdown or miR-146a overexpression significantly suppressed MUC5AC protein expression, and the co-transfection of miR-146a mimic and pcDNA-SNHG16 restored MUC5AC protein expression level. These results suggested that MUC5AC expression can be regulated by SNHG16 and miR-146a, and might be involved in the oncogenic activity of SNHG16-miR-146a axis. Next, we plan to investigate the molecular mechanism by which miR-146a regulates MUC5AC expression, and confirm that MUC5AC is involved in the oncogenic activity of SNHG16-miR-146a axis.
In summary, our study, for the first time, indicated that highly expressed SNHG16 is an oncogenic lncRNA that promotes tumor progression and poor prognosis of NSCLC by targeting miR-146a. In addition, the oncogenic activity of SNHG16-miR-146a axis might be associated with MUC5AC expression. The present results elucidate a potential mechanism underlying the carcinogenesis role of SNHG16 in NSCLC, and indicate that SNHG16 could serve as a novel promising marker for prognosis, and a potential therapeutic target for NSCLC treatment.

Acknowledgments
This work was supported by National Natural Science Foundation of China (grant No. 81400024), Natural Science Foundation of Shandong Province of China (grant No. ZR2014HM105) and Qingdao Outstanding Health Professional Development Fund.

Disclosure
The authors have stated that they have no conflicts of interest.

References
6. Guttman M, Donaghey J, Carey BW, Garber M, Grenier JK, Munson G,


24. She K, Huang J, Zhou H, Huang T, Chen G, He J, IncRNA-SNHG7 promotes
the proliferation, migration and invasion and inhibits apoptosis of lung cancer cells by enhancing the FAIM2 expression, Oncology Rep. 36 (2016) 2673-2680.


Figure caption
Fig. 1 SNHG16 is upregulated in human NSCLC tissues and cell lines. (A-C) Expression of SNHG16 in 66 pairs of NSCLC tissues and paired adjacent normal lung tissues. (D) Expression of SNHG16 in four NSCLC cell lines and human bronchial epithelial (16HBE) cells. *P < 0.05, **P < 0.01 and ***P < 0.001.

Fig. 2 SNHG16 promotes poor prognosis. (A and B) The Kaplan-Meier disease-free survival and overall survival curves by SNHG16 levels.

Fig. 3 SNHG16 promotes A549 cell proliferation, migration and invasion. (A) Knockdown or overexpression of SNHG16 in A549 cells analyzed by qRT-PCR. (B) The effects of SNHG16 knockdown or overexpression on the clonogenic survival of A549 cells measured using the colony formation assay. (C) The effects of SNHG16 knockdown or overexpression on the vitality of A549 cells measured using the CCK-8 assay. (D and E) The effects of SNHG16 knockdown or overexpression on A549 cell migration and invasion measured using the migration and invasion assay, respectively. *P < 0.05.

Fig. 4 SNHG16 can directly target miR-146a. (A) Putative miR-146a-binding sequence of SNHG16 RNA. (B) The association between SNHG16, miR-146a and
Ago2 was ascertained by analyzing A549 cell lysates by RNA immunoprecipitation with an Ago2 antibody. (C) The luciferase report assay demonstrates that the luciferase activity of wild-type reporter is significantly decreased when transfection with miR-146a mimic in comparison with miR-control. (D) Expression of miR-146a in 66 pairs of NSCLC tissues and paired adjacent normal lung tissues. (E) The correlation between SNHG16 RNA and miR-146a expression in 66 lung cancer tissues. (F) Expression of miR-146a in four NSCLC cell lines and 16HBE cells. (G) Expression of miR-146a in the si-SNHG16 or SNHG16 overexpression A549 cells compared with control group. *P < 0.05 and ***P < 0.001.

Fig. 5 miR-146a inhibits A549 cell proliferation, migration and invasion. (A-D) Colony formation, CCK-8, migration and invasion assays show that the effects of miR-146a decrease or increase on A549 cell proliferation, migration and invasion. *P < 0.05.

Fig. 6 SNHG16 regulates MUC5AC expression by targeting miR-146a. (A) Expression of MUC5AC mRNA in NSCLC tissues and normal tissues measured using qRT-PCR. (B) Expression of MUC5AC protein in NSCLC tissues and normal tissues measured using ELISA assay. (C) The correlation between MUC5AC mRNA and SNHG16 RNA expression in 66 lung cancer tissues. (D) The correlation between MUC5AC mRNA and miR-146a expression in 66 lung cancer tissues. (E) The effects of SNHG16 and miR-146a expression on MUC5AC protein expression. *P < 0.05 and ***P < 0.001.

Fig. 7 SNHG16 promotes growth of NSCLC tumors in vivo. (A and B) Tumor volume and weight of subcutaneous implantation models of A549 cells transfected with si-SNHG16 or si-control. (C) miR-146a expression in tumors isolated from si-SNHG16 and si-control group. (D) MUC5AC protein expression in tumors isolated from si-SNHG16 and si-control group. *P < 0.05.
Table 1 The relationship between SNHG16 expression and clinicopathological factors of 66 NSCLC patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Expression of SNHG16</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>&gt;60</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle or low</td>
<td>25</td>
<td>28</td>
</tr>
<tr>
<td>High</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3 cm</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td>&gt;3 cm</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I and II</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>III and IV</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smokers</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Smokers</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>22</td>
</tr>
</tbody>
</table>
Table 2 Multivariate Cox’s hazards analysis of different prognostic factors for disease-free survival and overall survival in 66 patients with NSCLC.

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>Disease-free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Gender</td>
<td>1.123</td>
<td>0.348-3.624</td>
</tr>
<tr>
<td>Age</td>
<td>0.735</td>
<td>0.352-1.534</td>
</tr>
<tr>
<td>Histological grade</td>
<td>2.148</td>
<td>0.863-5.348</td>
</tr>
<tr>
<td>Tumor size</td>
<td>0.592</td>
<td>0.292-1.198</td>
</tr>
<tr>
<td>TNM stage</td>
<td>0.035</td>
<td>0.011-0.115</td>
</tr>
<tr>
<td>Smoking history</td>
<td>0.881</td>
<td>0.285-2.718</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>1.224</td>
<td>0.558-2.683</td>
</tr>
<tr>
<td>SNHG16 expression</td>
<td>0.222</td>
<td>0.097-0.505</td>
</tr>
</tbody>
</table>

HR, hazard ratio; 95% CI, 95% confidence interval.

Figures:

Fig. 1

Fig. 2
Fig. 7

**Highlights**

1. SNHG16 is upregulated in human NSCLC tissues and cell lines.
2. SNHG16 expression is associated with tumor progression and poor prognosis.
3. SNHG16 promotes A549 cell proliferation, migration and invasion.
4. SNHG16 functions by targeting miR-146a.
5. SNHG16 regulates MUC5AC expression by targeting miR-146a.