European Review for Medical and Pharmacological Sciences 2020; 24: 9522-9531

LncRNA SNHG16 drives proliferation, migration, and invasion of lung cancer cell through modulation of miR-520/VEGF axis

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Abstract. – OBJECTIVE: Long non-coding RNAs (lncRNAs) have been shown to have important effects on various biological behavior of human diseases. Although increasing lncRNAs have been explored in human cancers, there are still countless lncRNA to be mined. The purpose of this study was to investigate the effect of lncRNA SNHG16 on the proliferation and metastasis of lung cancer cells.

PATIENTS AND METHODS: RT-qPCR was used to analyze the expression patterns of SNHG16, miR-520 and VEGF. MTT and transwell methods were used to detect the effect of SNHG16 on cell migration. The association between SNHG16, miR-520 and VEGF was analyzed by bioinformatics analysis and Dual-Luciferase verification reporter analysis. Finally, lung cancer cells have demonstrated the role of SNHG16-miR-520-VEGF in the cell biological behavior axis.

RESULTS: Compared with normal cells, SNHG16 is highly expressed in lung cancer cells. Silent SNHG16 has a negative effect on the migration of lung cancer cells.

CONCLUSIONS: LncRNA SNHG16 as ceRNA up-regulates VEGF in lung cancer cells by binding to miR-520. LncRNA SNHG16 as ceRNA promotes the migration of lung cancer cells by regulating the miR-520/VEGF axis.

Key Words:
SNHG16, NSCLC, MiR-520, VEGF.

Introduction

Lung cancer is the main cause of cancer deaths in the world[1]. According to the differences and morphological characteristics of different degrees, lung cancer is divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), of which NSCLC accounts for about 85% of all lung cancer patients. When clinical symptoms appear, it has developed to the middle and advanced stage[2,3]. Although great efforts have been made to improve the clinical management of lung cancer, the 5-year survival rate of lung cancer patients is still low. Over the years, research has revealed a complex genetic network, involving the occurrence and progression of lung cancer[4]. The number of noncoding RNAs in the transcriptome is amazing. They were thought to be redundant genes. Lately, they have been found to be an important factor in carcinogenesis and progression[5]. In noncoding RNA, long noncoding RNA (lncRNA) plays an increasingly important role as a broad-spectrum regulator in cancer. They have been shown to be involved in biophysiological and pathological processes. Moreover, lncRNA can regulate cell growth and differentiation, reprogramming and stress response[6-8]. LncRNA is an mRNA like transcript, ranging in length from 200 nt to 100 kb. The specific molecular mechanism of lncRNA enables to influence various biological processes. LncRNA can directly bind to some specific DNA or RNA chains to regulate transcription or translation. In addition, they may form functional complexes by collecting RNA and protein in the cytoplasm or nucleus[9]. Some expression disorders of lncRNA may affect epigenetic information and provide cell tumor growth advantage. Their roles in cancer are to regulate

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the microRNAs. Therefore, lncRNA has gradually become a new biomarker for cancer diagnosis and cancer target treatment. The regulation of lncRNA is an attractive strategy to prevent cancer progression and overcome cancer resistance. LncRNA is involved in the development of NSCLC[10]. SnoRNA Host Gene 16 has been demonstrated to be upregulated in malignant tumors. It plays a carcinogenic part in diversified mankind cancers. It's worth noting that lncRNA SNHG16 improves bladder cancer by epigenetic silencing p21[11]. SNHG16 as ceRNA promotes the development of cervical cancer[12]. SNHG16 is a detrimental prognostic element for gastric cancer[13]. Although previous studies have announced the molecular mechanism and biological function of SNHG16 in cancer, its specific role in lung cancer remains unknown. The aim of this study was to investigate the effect of SNHG16 disorder on biological behavior of lung cancer cells. MicroRNA (miRNA) is a small non coding RNA molecule encoded in genome. The mature miRNA interacts with the 3 '-untranslated region (3'-UTR) of the target mRNA and negatively regulates gene expression by degrading the target mRNA, thus inhibiting gene translation[14,15]. Overexpression of miR-520a-3p in breast tumor cells may control cell metastasis. At the same time, miR-520a-3p can lead to breast cancer cell apoptosis[16]. Lately, it has been covered that miR-520a-3p can restrain the apoptosis of NSCLC. MiR-520a-3p has been discovered to control proliferation and reverse Gefitinib by targeting miR-520a-3p NSCLC cells[17]. MiR-520b may serve as an emerging therapeutic target that may be further developed for the intervention of refractory head-neck cancer (HNC). Zhou et al[18] found that different doses of Oxymatrine can up-regulate miR-520, selectively inhibit VEGF and thus inhibit the proliferation and migration of lung cancer. Here, we found that the level of lncRNA SNHG16 in lung cancer cells was exceeded than that in normal cells. According to bioinformatics, SNHG16 is expected to be ceRNA of VEGF through immediately binding miR-520.

Patients and Methods

Tissue Samples

30 pairs of clinical lung cancer and paracancer tissue samples were taken from Zhangqiu District People's Hospital. After surgical removal of the tumor, histopathological examination was performed to determine all lung cancer and adjacent tissue samples. None of the patients received preoperative treatment for cancer. All patients signed written informed consent. The investigation was approved by the Ethics Committee of Zhangqiu District People's Hospital. Table I shows the detailed characteristics of 26 patients.

Inclusion criteria: (1) patients over 18 years of age with lung cancer confirmed by pathology or cytology are free of gender, race and nationality. (2) There was no chemotherapy before treatment, no other complications, no significant abnormalities in liver and kidney function, hematology or electrocardiogram. (3) The informed consent of the patient and his/her family members is signed and approved by the hospital ethics.

Exclusion criteria: (1) patients who do not meet the diagnostic criteria for lung cancer, allergic constitution or allergic to drugs. (2) Patients who cannot actively and seriously cooperate with medical staff in treatment.

Cell Culture

NSCLC cell lines NCI-H1299 and A549 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS (Hyclone, South Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were cultured in 37°C, 5% CO_2, and statured humidity incubator. The logarithmic growth phase cells were used for the experiment.

Cell Transfection

First, lung cancer cells were seeded into 96 well plates at a density of about 1×10^6 / well. Add DMEM medium to each well and incubate for 24 hours. After the cell adhering to the wall, the cells were transfected. miR-520 mimic or overexpression of VEGF vector was transfected into cells according to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The related indexes were detected 48 hours later. Three groups were established: NC group (negative control), miR-520 mimic group (NCI-H1299 and A549 cells transfected with miR-520 mimic) and miR-520 mimic + VEGF group (NCI-H1299 and A549 cells transfected with miR-520 mimic and VEGF).

MTT Determination

On the basis of the manufacturer's illustrations, the MTT assay kit was used to check cell proliferation. Lung cancer cells were cultured (8000 cells/well) in 96 well plate. At different time points, 10

μl MTT reagent (10 mg/ml) was added to each pore and incubated for about 4 h. The reaction was terminated by the addition of 100 μl cracking reagent and incubated in darkness at 22°C for 2 hours. The absorbance was measured at 570 nm on Bio-Rad (Hercules, CA, USA).

Cell Migration Assay

The chamber system was used to measure the cross whole migration. These cells were inserted into 24 well plates and 30 μl of 3×10^4 cells in serum-free DMEM were added to the upper chamber. DMEM (supplemented with 10% FBS) was added to the inferior chamber of each well and the cells were incubated for 24 hours. The cells in the lower side of the membrane were fixed with methanol and stained with 0.1% crystal violet. Count the migrating cells in at least three different areas.

Real Time Quantitative PCR Analysis

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cells. The extracted RNA inverse transcribed into complementary DNA (cDNA). RT-qPCR was used for fluorescent RT-qPCR instrument. The specific reaction conditions were as follows: pre-denaturation at 95°C for 10 minutes, denaturation at 95°C for 30 minutes. After renaturation for 30 s at 55°C, it was extended for 2 min at 72 °C for a total of 35 cycles. The relative expression of miR-203 and RGS17 were quantified by the $2^{-\Delta\Delta CT}$ method. The primer sequences: SNHG16, forward: 5'-CCC AAG CTT GCG TTC TTT TCG AGG TCG GC -3', reverse: 5'-CCG GAA TTC TGA CGG TAG TTT CCC AAG TT-3'; U6: forward: 5'-GCA ACG GCA GCA CAT ATA CTA AAA T-3', reverse: 5'-CGC TTC AGA ATT TGC GTG TCA T-3'; GAPDH, forward: 5'-CGC TCT CTG CTC CTC CTG TTC-3', reverse: 5'-ATC CGT TGA CTC CGA CCT TCA C-3'.

Western Blotting (WB) Analysis

The protein was extracted from cells using protein lysates. The concentration of the extracted protein was quantified by the analysis of bicinchoninic acid assay (BCA). The extracted protein was transferred to PVDF membrane. The membrane was then incubated overnight at 4°C with rabbit anti-human VEGF primary antibody and 3-phosphoglyceraldehyde dehydrogenase (GAPDH). After washing with Tris Buffered Saline and Tween-20 (TBST), the membrane was incubated with the secondary antibody for 1 hour at 37°C. After washing, enhanced chemiluminescence (ECL) method was used to develop color (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Student's *t*-test or *F*-test was used for statistical analysis. All *p*-values were positive and negative, $p < 0.05$ was considered statistically significant.

Results

Up-regulation of SNHG 16 can Promote the Migration of Lung Cancer Cells

We measured the expression of SNHG16 in a group of lung cancer tissues, the expression level of SNHG16 in tumor tissues was higher than that in adjacent non cancer tissues (Figure 1A). SNHG16 expression was detected in BEAS-2B, NCI-H1299 and A549 cell lines. SNHG16 was significantly higher in NCI-H1299 and A549 cells (Figure 1B). Then NCI-H1299 and A549 cells were knockdown SNHG16 through siRNA. NCI-H1299 and A549 cells were used for overexpression experiments. RT-qPCR showed that siRNA could reduce the expression of SNHG16 (Figure 1C), but pcDNA3.1-SNHG16 (SNHG16) might significantly enhance the level of SNHG16 (Figure 1D). SNHG16 knockdown by siRNA obviously reduced the migration ability (Figure 1E, F), while overexpressing SNHG16 by pcDNA3.1-SNHG16, it was significantly enhanced cell migration (Figure 1G, H).

SNHG16 Is Expected to be ceRNA of VEGF by Direct Combinding MiR-520

Recent research has shown that lncRNA can play its regulatory role as ceRNA. To further probe the latent mechanism of SNHG16 in lung tumor, we applied Starbase v2.0 program to examine a group of predicted competitive binding miRNA with SNHG16 (http://starbase.sysu.edu.cn). Bioinformatics analysis showed that there was an ordinary combination site of miR-520 between lncRNA SNHG16 and VEGF (Figure 2A). After SNHG16 was silenced in NCI-H1299 and A549 cells, the expression of miR-520 increased significantly (Figure 2B), and after overexpression of SNHG16, the expression of miR-520 decreased in NCI-H1299 and A549 cells (Figure 2C). Verify that the immediate combination between SNHG16 and miR-520 is at the endogenous level. We have RNA immunoprecipitation (RIP) detection with MS2 binding protein (MS2bp) binding RNA including the MS2 binding sequence

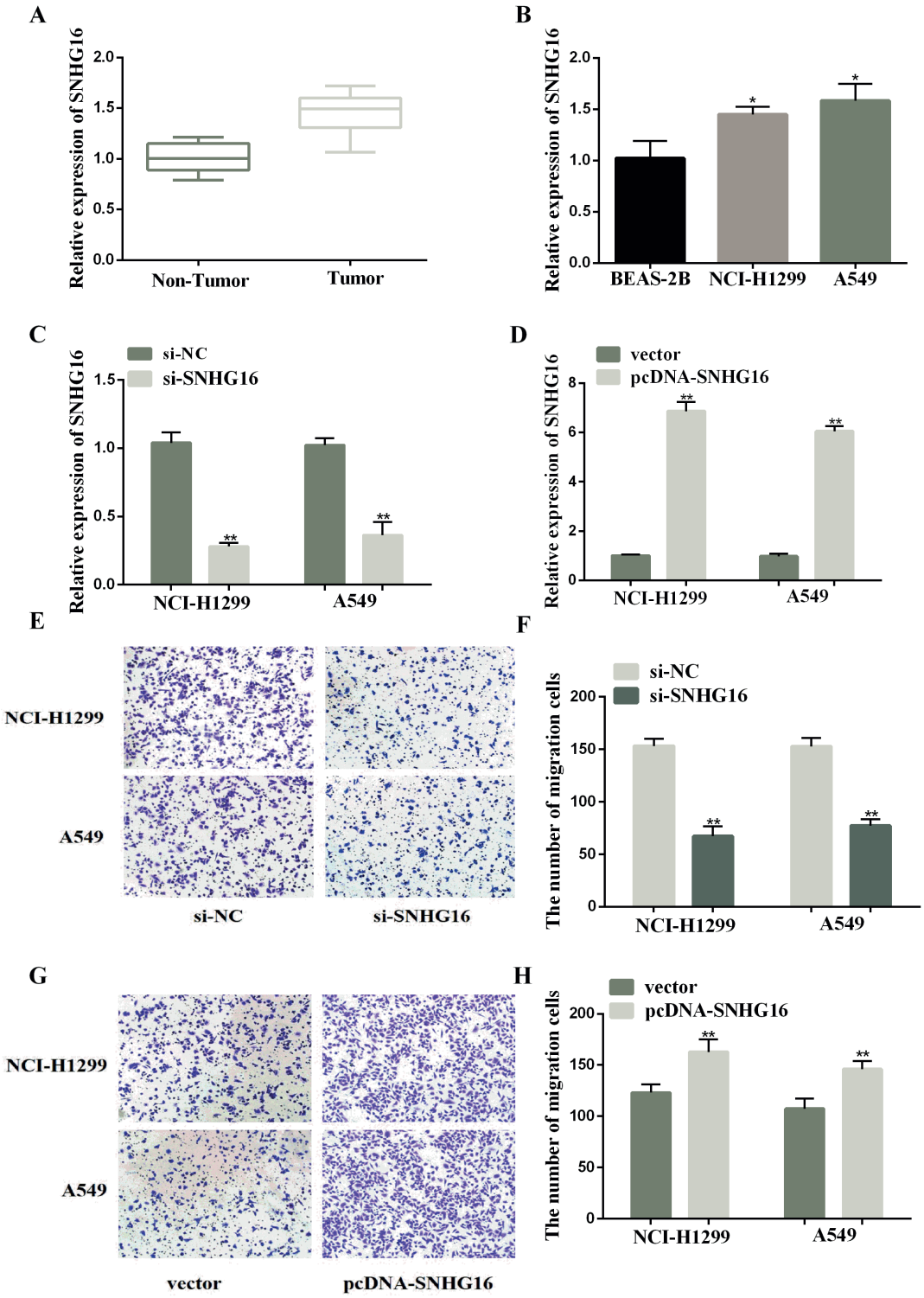

Figure 1. The expression and function of SNHG16 in lung cancer. **A**, The different expression levels of SNHG16 lung cancer tissues and non-cancer tissues were detected by RT-qPCR. **B**, The relative SNHG16 expression was checked by RT-qPCR in NCI-H1299 and A549 cells. **C**, when knockdown SNHG16, the level was checked by RT-qPCR. **D**, After pcDNA3.1-SNHG16 was transfected, SNHG16 was overexpressed in NCI-H1299 and A549 cells. **E-F**, Migration analysis of NCI-H1299 and A549 cells after knockdown of SNHG16 in lung cancer cell (100X). **G-H**, Cell migration in NCI-H1299 and A549 cells was checked after overexpression of SNHG16 (100×).

Table I. The information characteristics of the 30 patients.

	Groups	No.
Sex	Male	16
	Female	14
Age	≤ 60	12
	>60	18
TNM stage	I and II	8
	III and IV	22
Lymph node metastasis	Negative	10
	Positive	20
Tumor size	≤3 cm	20
	>3 cm	8
History of smoking	Ever	16
	Never	10

(MS2bs) to pull down endogenous miRNA related to SNHG16. We produced a SNHG16 transcripts that we were integrated with MS2bs elements and co-transfected into NCI-H1299 and A549 cells by MS2bp-GFP containing constructs. Then, GFP antibody (IgG as negative control) was used for immunoprecipitation, and miR-520 expression was analyzed by RT-qPCR. Figure 2D shows that miR-520 is significantly enriched compared with MS2. There was no significant difference between SNHG16-MUT (miR-520) and MS2. In addition, we structured a Luciferase reporter vector containing SNHG16 or its mutant form and detected

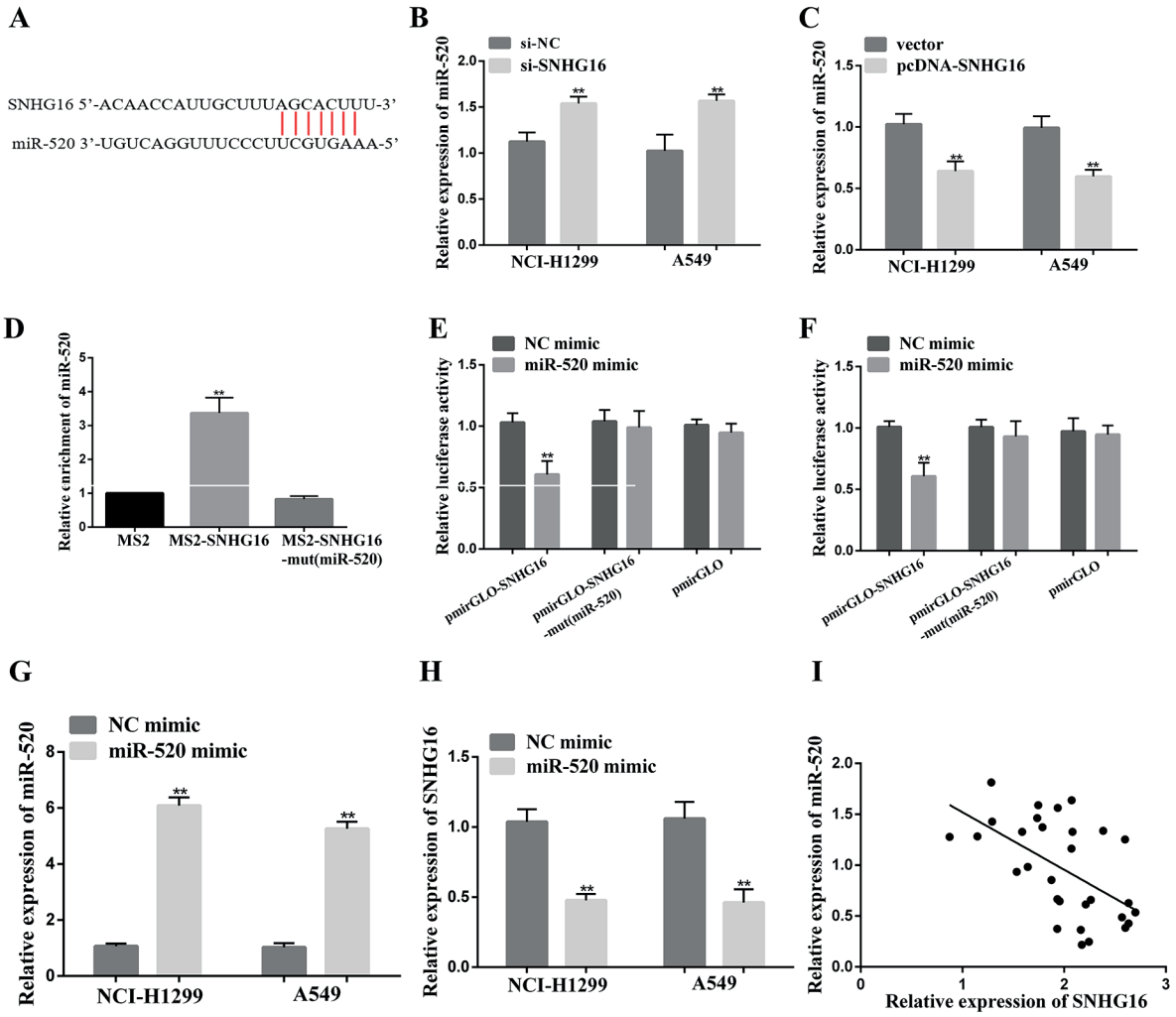

Figure 2. Target and connection between SNHG16 and miR-520. **A**, Prediction of miR-520 co-binding sites on SNHG16 transcripts. **B-C**, Relative to RT-qPCR analysis showed that SNHG16 silencing significantly raised the expression of miR-520 in NCI-H1299 and A549 cells, but the overexpression of SNHG16 in NCI-H1299 and A549 cells decreased the expression of miR-520. **D**, Ms2-RIP analysis was followed by miR-520 RT-qPCR analysis of miR-520, which is related to SNHG16 endogenous. **E-F,** Luciferase activity in NCI-H1299 and A549 cells co transfected with miR-520 containing SNHG16 or mutant transcripts and Luciferase Report vector. **G**, The expression of miR-520 in lung cancer cells was confirmed by RT-qPCR. **H**, The expression of SNHG16 in lung cancer cell lines transfected with miR-520 or NC was analyzed by RT-qPCR. **I**, The negative regulation of miR-520 by SNHG16 was measured in 30 lung cancer tissues.

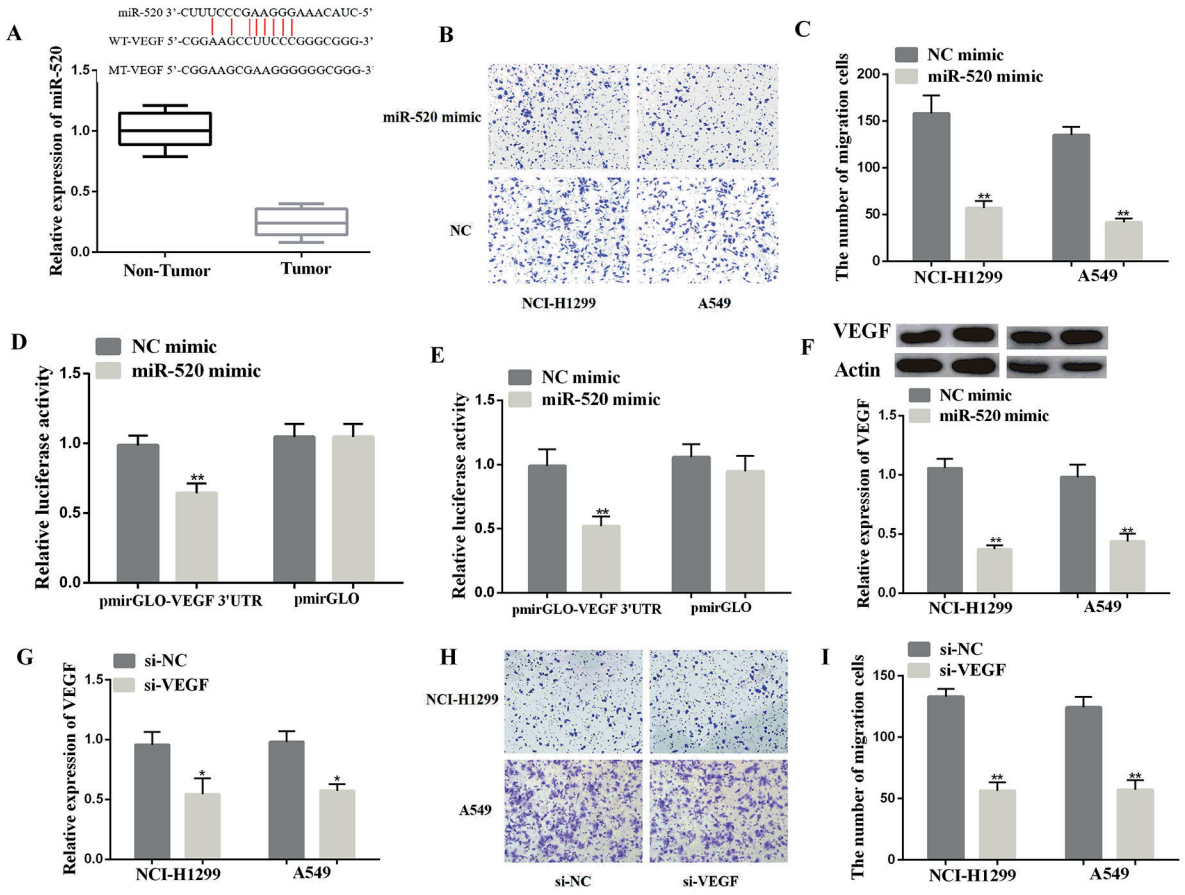

Figure 3. MiR-520 inhibited cell migration by targeting VEGF. **A**, The binding site of miR-520 in the 3'UTR of VEGF. The expression of VEGF in 30 lung tissues was analyzed by RT-qPCR. **B**, Cell migration analysis of NCI-H1299 and A549 cell lines were checked after transfected miR-520 mimic (100X). **C**, The migration of these cells was analyzed quantitatively. **D-E**, Luciferase reporter assay in NCI-H1299 and A549 cells showed that miR-520 inhibited luciferase activity of VEGF 3'UTR. **F**, The expression level of endogenous VEGF in NSCLC cells after transfected miR-520 mimic was analyzed by RT-qPCR and Western blot. **G**, Compared with the negative control group, VEGF expression in cells decreased when VEGF siRNA transfection. **H-I**, After VEGF silencing, the migration number of lung cancer cells decreased significantly contrasted with the NC (100×).

NCI-H1299 and A549 cells with Dual-Luciferase reporter genes. Figure 2E shows that overexpression of miR-520 significantly reduced the Luciferase activity of WT reporting vectors, rather than empty vectors or mutant reporting vectors. They were expressed in NCI-H1299 and A549 cells respectively (Figure 2E-F). In order to determine whether SNHG16 negatively regulates miR-520, miR-520 mimic was transfected into lung cancer cells. After treatment with miR-520 mimic, the expression level of miR-520 in lung cancer cells increased significantly (Figure 2G). Significant inhibition of SNHG16 by miR-520 mimic was watched in lung cancer, indicating that SNHG16 and miR-520 inhibit each other (Figure 2H). In addition, the negative correlation between miR-520 and SNHG16 was tested in 30 lung tumor (Figure 2I).

MiR-520 Inhibits Cell Migration by Targeting VEGF

To determine the possible interaction between miR-520 and VEGF, we first carried out a bioinformatics screen for its possible target genes, using an online 3'-UTR binding site prediction database. We detected the RNA level of VEGF in lung tumor tissues and non-cancer tissues by RT-qPCR, revealing the up-regulation of VEGF expression in lung cancer tissues (Figure 3A). Transwell analysis indicated that the overexpression of miR-

520 significantly inhibited lung cancer cell migration (Figure 3B-C). According to the target sites predicted by bioinformatics, we structured a luciferase reporter vector including VEGF of 3'UTR. As displayed in figure 3D-E, miR-520 restrained the luciferase activity of VEGF 3'UTR reporter gene in NCI-H1299 and A549 cells, respectively. As we expected, miR-520 significantly inhibited the expression of VEGF in lung cancer cells at RNA and protein levels (Figure 3F-G). Then we studied the function of VEGF in NCI-H1299 and A549 cells. After VEGF silencing (Figure 3I), the number of cell migration decreased significantly (Figure 3H-I), and the influence of miR-520 mimic on lung tumor cells.

SNHG16 Positively Regulates VEGF Expression and Promotes Migration of MiR-520-Dependent Lung Cancer Cells

Knockdown of SNHG16 reduced the expression of VEGF in lung cancer cells (Figure 4A, B). In contrast, the ectopic expression of SNHG16 led to the up regulation of VEGF in NCI-H1299 and A549 cells (Figure 4C, D). In the rescue test, the overexpression of miR-520 significantly eliminated the overexpression of SNHG16 (Figure 4E) and the up regulation of VEGF induced by SNHG16 (Figure 4F), which strongly indicated that SNHG16 monitored the expression of VEGF in a miR-520 dependent way. Then, we measured the expression of SNHG16 and VEGF in two lung cancer cells and tumor tissues. Cells with high levels of endogenous SNHG16 expression showed a high level of VEGF as determined by RT-qPCR (Figure 4G). Figure 4H showed that there was a positive connection between SNHG16 transcription level and VEGF level. Because SNHG16 regulated VEGF expression by controlling miR-520 expression level, we were interested in whether the recovery of miR-520 can overturn the SNHG16 mediated acceleration of lung cancer cell migration. The outcome displayed that the overexpression of miR-520 may notably reduce the SNHG16 mediated migration of lung cancer cell (Figure 4H- I).

Discussion

Competitive endogenous RNA (ceRNA) as a substitute for long noncoding RNA has been widely concerned[19,20]. The study of tumor suppressive lncRNA provides a new way to elucidate the pathogenesis and development of NSCLC, which provides a unique platform for more effective treatment of NSCLC[21]. SNHG16 expression level is significantly up-regulated in malignant tumors, and it has potential oncogene effect[22-24]. In our study, different expression levels of SNHG16 and its partial role in tumor were detected in human tissues. We showed that SNHG16 could serve as a ceRNA by competitively sponging miR-520 and regulating the target gene VEGF. Compared with lung cancer, the expression level of lung cancer is higher in normal tissues. Then, the results of different expression levels of SNHG16 in regulating tumor cell migration were studied. SNHG16 has been found to increase the migration of NSCLC cells, which might lead to bad prognosis of lung tumor. In NSCLC studies, many down regulated miRNAs have been shown to regulate cell functions[25,26]. MiR-520 expression in lung cancer tissues is significantly down-regulated, which is significantly negatively correlated with normal tissues adjacent to the cancer[18,27]. NORAD can monitor the proliferation of NSCLC through adjusting miR-520a-3p/PI3K/Akt/mTOR signaling pathway, so as to promote its occurrence and progression[28]. LncRNA SNHG16 acts as ceRNA, which drives the proliferation, angiogenesis, migration and invasion of lung cancer cells by regulating miR-520d-3p/STAT3 axis[29]. In our research, we demonstrated that SNHG16 contained some binding site of miR-520 by bioinformatics analysis. Our RIP experiment and fluorescein enzyme analysis showed that SNHG16 could significantly increase miR-520, but SNHG16 had no such relationship when there was mutation in the target site of miR-520. On the other hand, VEGF is the target gene of miR-520, which can play a role in lung cancer[30]. Liu et al[31] found that the serum VEGF level of lung cancer patients was significantly higher than that of lung cancer patients by detecting the serum VEGF level of lung cancer patients, benign lung diseases and normal controls. Buttigliero et al[32] found that the increased expression of VEGF at protein level was closely related to brain metastasis, and the survival period was short. In agreement, our results confirm the expression analysis of miR-520 and VEGF in lung cancer. In this study, we showed that VEGF expression was up-regulated with miR-520 and negatively correlated with lung cancer. Human intervention of VEGF low expression can inhibit the migration of lung cancer cells, the same results appear in the overexpression of miR-520 in lung cancer cells. Our current outcome showed that the overexpression of miR-520 can signifi-

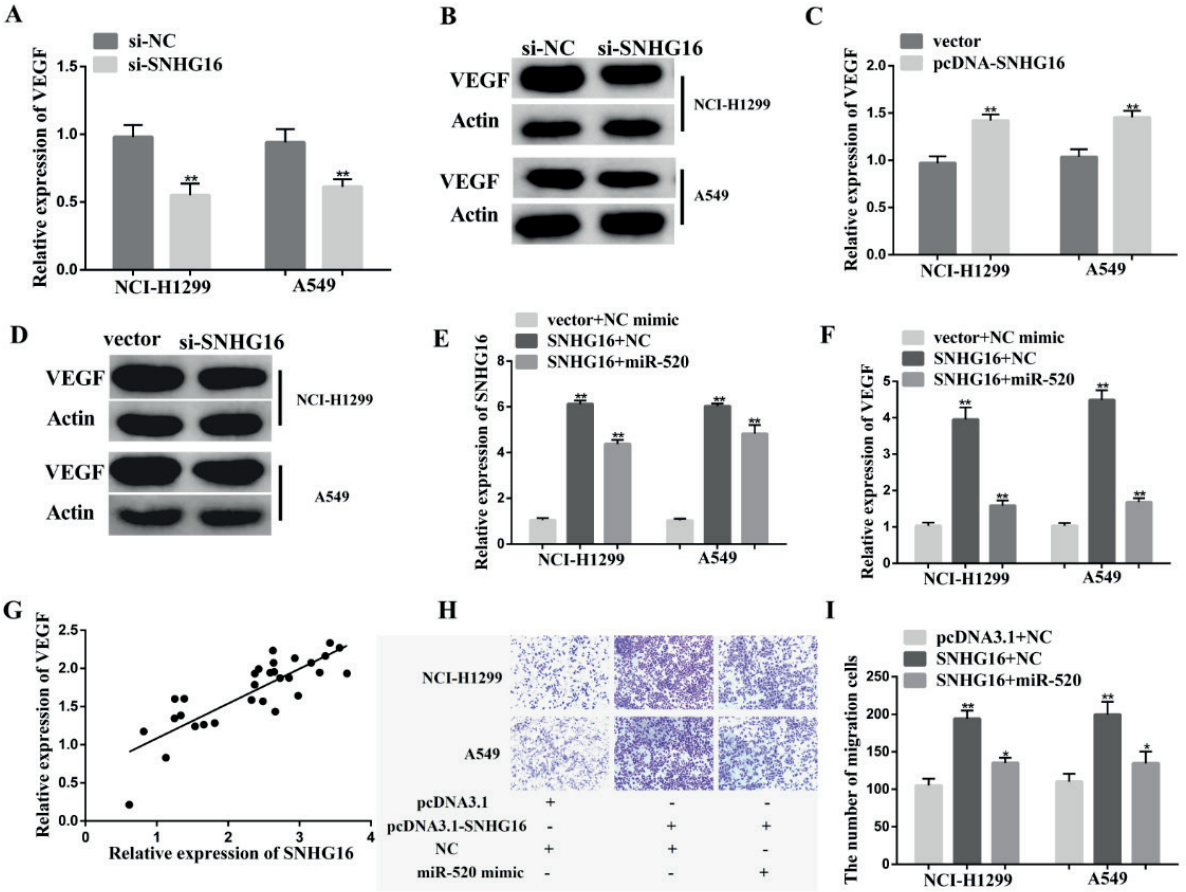

Figure 4. SNHG16 regulated VEGF and promoted lung cancer cell migration through miR-520 expression. **A**, The relative expression of VEGF in NCI-H1299 and A549 cells after SNHG16 knockout were analyzed by relative RT-qPCR and (**B**) Western blot. **C**, The expression of VEGF in NCI-H1299 and A549 cells after overexpression of SNHG16 was analyzed by relative RT-qPCR and (**D**) Western blot. **E**, To carry out the rescue experiment, pcDNA3.1-SNHG16, miR-520 mimic or the control vector were transfected with miR-520 to NCI-H1299 and A549 cells, and SNHG16 expression simulation or NC appeared. **F,** In NCI-H1299 and A549 cell lines, the forced expression of miR-520 decreased the up regulation of VEGF induced by SNHG16. **G**, SNHG16 was positively correlated with VEGF mRNA level in 30 lung cancer tissues. **H**, The migration of lung cancer cells transfected with SNHG16 or miR-520 mimic was measured (100X). **I**, The relative migration was analyzed quantitatively.

cantly inhibit the migration of lung cancer cells by directly targeting VEGF expression in tumor cells. After that, we detected whether SNHG16 might modulate the expression of miR-520 in tumor cells. The overexpression of SNHG16 can increase the expression of VEGF, while the overexpression of miR-520 can decrease the expression level of VEGF induced by SNHG16. Our results displayed that the expression of VEGF in lung cancer samples was higher than that in normal non-cancer tissues, which was consistent with the up-regulation of SNHG16 expression. In addition, SNHG16 transcription level was positively correlated with VEGF mRNA level. These results indicated that SNHG16 can isolate miR-520 and interfere with the interaction between miR-520 and VEGF. In this study, we demonstrated that SNHG16 can act as ceRNA to regulate VEGF through competitive spongization of miR-520. In this study, we demonstrated that SNHG16 can act as ceRNA through competitive spongization of miR-520 and regulation of targeted gene VEGF. In general, we demonstrated that SNHG16 was up-regulated in lung cancer cell lines. Our experiments demonstrate that silenced SNHG16 affects the proliferation, invasion and migration of lung cancer cells. This interaction between SNHG16 and miR-520 highlighted the vital role of RNA-RNA interaction and afforded a novel perception, that is, lncRNA-based mechanisms underlying diverse aspects of tumorigenesis.

Conclusions

We displayed that the overexpression of SNHG16 enhanced the number of cell migration, while miR-520 forced expression reversed the process, indicating that SNHG16 might promoting lung cancer metastasis by competitively combining miR-520 with VEGF. As the most important tumor inducing cytokine, VEGF has proved to be a key target for the treatment of cancer angiogenesis[30]. Specific analysis of the relationship between VEGF and SNHG16 expression regulation, VEGF in lung cancer cells using three different siRNA was knocked down. The above evidence suggested that lncRNA can adjust the level of VEGF and affect the migration in cells. According to this result, it is necessary for us to further explore the deeper discovery and a series of new discoveries. These findings indicate that there is a positive significance.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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