Nicotine promotes the development of non-small cell lung cancer through activating LINC00460 and PI3K/Akt signaling

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Objective: Nicotine, the main ingredient in tobacco, is identified to facilitate tumorigenesis and accelerate metastasis in tumor. Studies in recent years have reported that long intergenic non-protein coding RNA 460 (LINC00460) is strongly associated with lung cancer poor prognosis and nicotine dependence. Nonetheless, it is unclear whether nicotine promotes the development of lung cancer through activation of LINC00460.

Methods: We determined that LINC00460 expression in lung cancer tissues and the prognosis in patients with non-small cell lung carcinoma (NSCLC) using Gene Expression Profiling Interactive Analysis (GEPIA) website and The Cancer Genome Atlas (TCGA) database. Through in vitro experiments, we studied the effects of nicotine on LINC00460 in NSCLC cell lines using Cell Counting Kit-8 (CCK-8), transwell test, flow cytometry, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and Western blot assays.

Results: We identified the significant up-regulated expression level of LINC00460 in NSCLC tissues and cell lines, especially, the negative correlation of LINC00460 expression level with overall survival (OS). In in vitro experiments, LINC00460 was overexpressed in NSCLC cell lines under nicotine stimulation. Nicotine could relieve the effect of LINC00460 knockdown on NSCLC cell proliferation, migration and apoptosis. The same influence was observed on PI3K/Akt signaling pathway.

Conclusions: In summary, this is the first time to examine the potential roles of LINC00460 in lung cancer cell proliferation, migration and apoptosis induced by nicotine. This may help to develop novel therapeutic strategies for the prevention and treatment of metastatic tumors from cigarette smoke-caused lung cancer by blocking the nicotine-activated LINC00460 pathway.

Introduction

It is well known that mortality caused by lung cancer ranks first in cancer-related deaths worldwide [1]. Lung cancer mainly includes two types, namely non-small cell lung cancer (NSCLC) and small cell lung cancer, the former of which accounts for approximately 85% in lung cancer [2,3]. Moreover, cigarette smoking comprises almost 90% of all deaths from lung cancer [4,5]. In addition to its documented risks for lung carcinogenesis, cigarette smoking has also been implicated in cancer development. Recently, Izzotti et al. [6] investigated the expression of 484 microRNAs (miRNAs) in the rat lungs exposed to environmental cigarette smoke for 4 weeks, found that cigarette smoke down-regulates 126 miRNAs at least two-fold and 24 miRNAs more than three-fold. Their dysregulation is associated with the initiation and development of lung cancer, which suggested that tobacco smoke might exert their carcinogenic effects by deregulation of miRNAs [7,8]. Nicotine is a major alkaloid in tobacco plants. Its biosynthesis and regulation in tobacco (Nicotiana tabacum) have been well studied.
Long non-coding RNAs (lncRNAs) can participate in the occurrence and development of tumor functioning as oncogenes or tumor suppressor [9–11]. Long intergenic non-protein coding RNA 460 (LINC00460) is one of lncRNAs and shows a high expression level in squamous-cell carcinoma and esophageal cancer cells [12]. Besides, it is associated with poor prognosis in patients with squamous-cell carcinoma of the head and neck, as well as tumor node metastasis (TNM) staging and lymph node metastasis [13]. Similarly, the LINC00460 has a higher expression level in NSCLC tissues and cells and interference of LINC00460 can affect cell invasion and migration of NSCLC via regulating epithelial–mesenchymal transition (EMT) [14–16]. Many chemical and physical agents have been reported to induce or treat a disease via dysregulating miRNAs or restoration of aberrant miRNAs [17], respectively. However, there are few reports on the involvement of non-coding RNAs in alkaloid biosynthesis.

In the present study, we determined that LINC00460 was overexpressed in lung cancer tissues, which led to a poor prognosis in patients with NSCLC. Through in vitro experiments, we first examined the effects of nicotine on LINC00460 in NSCLC cell lines and investigated the underlying mechanism.

Materials and methods

Database analysis

The RNA sequencing expression data of LINC00460 was downloaded from Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/) [18], which analyzed 483 NSCLC tumors and 347 normal samples from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/) database. Besides, under the influence of different LINC00460 expression level, the overall survival (OS) of patients with NSCLC was also analyzed.

Cell culture

The NSCLC cell lines A549 and H1299 were all obtained from Chinese Academy of Sciences Shanghai Branch Cell Bank (Shanghai, China). A549 cells were cultured in F12K culture medium (Sigma–Aldrich, St. Louis, MO, U.S.A.) supplemented with 2.5 g/l NaHCO₃ and 10% (v/v) fetal bovine serum (FBS; Sigma–Aldrich), while H1299 cells were seeded in Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma–Aldrich) supplemented with 1.5 g/l NaHCO₃, 2.5 g/l glucose, 0.11 g/l sodium pyruvate and 10% (v/v) FBS at 37°C in 5% CO₂ atmosphere. Until the cells grew into logarithmic phase, they were washed three times by PBS, digested with trypsin and then repeatedly beaten to single cell suspension. Subsequently, cells were seeded into a six-well plate in preparation for the following experiments.

Nicotine was purchased from Sigma–Aldrich (product number N3876) and added to cells after dilution with a concentration of 0, 1, 10, 100 and 200 μg/ml. After 48 h, LINC00460 expression level in A549 and H1299 cells was detected using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Time-dependent analysis was also measured as above instructions using 100 μg/ml.

RNA interference

When cells grow to logarithmic phase, the medium was replaced with fresh medium without serum and antibiotics 2 h prior to transfection. The short hairpin RNA (sh-RNA) (5′-UCACCUUGACUACUGCUAUTT-3′) was chemically synthesized from Genepharma Co. Ltd. (Shanghai, China) and added with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) into cells according to the manufacturer’s instructions. After 24 h, the expression of pSUPER-LINC00460 (LINC00460-KD) mRNA in transfected cells could be observed using qRT-PCR.

QRT-PCR assay

Total RNA of cells was extracted using 1.0 ml TRIzol (Invitrogen) after transfection of 48 h. Synthesis of cDNA was conducted with reverse transcriptase M-MLV (Takara Bio Inc., Shiga, Japan). qRT-PCR was used to detect the expression level of LINC00460 using SYBR Premix ExTaq™ kit (Applied Biosystems, Foster City, CA, U.S.A.) following the manufacturer’s instructions and the parameters were as follows: hot start at 95°C for 5 min; followed by 40 cycles of 95°C for 30 s, 60°C for 45 s and 72°C for 30 min. The primer sequence was as follows: LINC00460, F: 5′-ATGCACACTTCTCGGCTAAG-3′, R: 5′-GGTCGTAACCTTCGTTCTCATC-3′; β-actin (NC, negative control), F: 5′-CCCAGGCCGTTCCCTTCTC-3′, R: 5′-GTCCCAGTTGGTGACGATGC-3′. The relative quantitation of the value was determined using the 2−ΔΔCt calculation method and each sample was assayed in triplicates.

Cell proliferation assay

According to the manufacturer’s protocols, cell viability was detected using Cell Counting Kit-8 (CCK-8; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) assay after transfection for 24 h. A total of 100 μl cell suspension...
Figure 1. Expression of LINC00460 in NSCLC tissues based on GEPIA data

(A) Expression levels of LINC00460 in NSCLC tissues (n=483) and normal tissue (n=347) from the TCGA database were analyzed. *P<0.05. (B) OS after 20 years of patients with low or high expression levels of LINC00460 was analyzed by Kaplan–Meier method and log rank test. P=0.0037.

(1000 cells/well) was placed in 96-well plates and after 10 μl of CCK-8 reagent was added to each well, their viabilities were detected at 0, 24, 48, 72 h. Optical density (OD) value was measured using a microplate reader (Bio-Rad, Hercules, CA, U.S.A.) at a wavelength of 450 nm. The experiments were performed in triplications.

**Cell migration assay**

Transwell assay was used to detect cell migration utilizing A549 and H1299 cell lines. Based on the manufacturer's instructions, 24-well transwell chamber with Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, U.S.A.) was performed for cell invasion. A density of 5 × 10³ cells after transfection for 24 h was added into the upper chamber and 500 μl complete medium was added into the bottom chamber. After overnight, cells were washed by PBS, fixed with 4% paraformaldehyde for 30 min and stained with 0.1% Crystal Violet for 20 min. The number of migrated cells was counted under a microscope in five random fields per well at 200× magnification.

**Cell apoptosis assay**

Flow cytometry was used to detect apoptotic cells. After transfection for 24 h, cells were trypsinized without ethylenediaminetetraacetic acid (EDTA) and collected in a centrifuge tube, then the cells were centrifuged at 1000 rpm for 5 min. Afterward, cells were resuspended in PBS pre-cooled at 4°C, centrifuged again and carefully aspirated the supernatant. After that we added 1× binding buffer to resuspend the cells and regulated the cell density of 1–5 × 10⁶/ml. One hundred microliters of the cells were gently mixed with 5 μl of Annexin V-FITC/PI (Beijing 4A Biotech Co., Ltd, Beijing, China) and incubated in the dark for 5 min at room temperature. Ten microliters of PI dye and 400 μl of PBS were added to the samples, and the results were analyzed in a flow cytometer using FlowJo. The experiment was repeated three times independently.

**Western blot analysis**

After transfection for 48 h, total proteins were extracted by a Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Inc., Shanghai, China) and quantitated by a BCA Protein Assay Kit (Beyotime Inc.) for the concentrations. Cell lysates (20 μg protein/lane) were separated by 8–12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and then transferred on to polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat dry milk for 1 h, the membranes were incubated with primary antibodies (1:1000; Cell Signaling Technology Inc.,
**Figure 2. Expression of LINC00460 in NSCLC cell lines under nicotine induced**

(A, B) Expression levels of LINC00460 in NSCLC cell lines (A549 and H1299) under nicotine stimulation were measured by qRT-PCR. *P<0.05, **P<0.01 vs. 0 μg/ml group. (C, D) The time-dependent analysis of LINC00460 expression levels in NSCLC cell lines (A549 and H1299). **P<0.01 vs. NC group. (E) The expression levels of LINC00460 knockdown mRNA were assessed using qRT-PCR, *P<0.05 vs. NC group. LINC00460-KD, small interfering LINC00460 RNA.

**Figure 3. Effect of LINC00460 knockdown on NSCLC cells proliferation**

(A) OD values in A549 cell were detected by CCK-8 assay in three groups for 0, 24, 48, 72 h. *P<0.05 vs. NC group. (B) OD values in H1299 cell were detected by CCK-8 assay in three groups for 0, 24, 48, 72 h. *P<0.05 vs. NC group.
Figure 4. Effect of LINC00460 knockdown on NSCLC cell migration
(A) Microscopic images of A549 cells passing though the microwells of the transwell chamber were detected by transwell assay. The number of migrated cells was counted. *P<0.05 vs. NC group, bar = 200 μm. (B) Microscopic images of H1299 cells passing though the microwells of the transwell chamber were detected by transwell assay. The number of migrated cells was counted. *P<0.05 vs. NC group, bar = 200 μm.

Figure 5. Effect of LINC00460 knockdown on apoptosis in NSCLC A549 cells
(A) Flow cytometric plots show specific cell populations in A549 cells. Q2 were late apoptotic cells, Q3 were live cells, and Q4 were early apoptotic cells. (B) Expression of activated Bcl-2, Bax and Cleaved Caspase-3 were determined by Western blot and shown by bar graphs in (C). *P<0.05 vs. NC group. Data were expressed as mean ± SD.

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Figure 6. Effect of LINC00460 knockdown on apoptosis in NSCLC H1299 cells

(A) Flow cytometric plots show specific cell populations in H1299 cells. Q2 were late apoptotic cells, Q3 were live cells, and Q4 were early apoptotic cells. (B) Expression of activated Bcl-2, Bax and Caspase3 were determined by Western blot and showed by bar graphs in (C). *P<0.05 vs. NC group.

Danvers, MA, U.S.A.) overnight at 4°C. The primary antibodies were used against protein kinase B (AKT), p-AKT, phosphorylated-serine-threonine protein kinase (p-P70S6K), Cyclin D1, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), Cleaved Caspase-3 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Followed by washing for 3–5 min, the membranes were incubated by anti-mouse/rabbit horseradish peroxidase–conjugated secondary antibodies (1:3000; Cell Signaling Technology Inc.) at room temperature and detected with electro-chemi-luminescence (ECL) imaging analysis system (Thermo Fisher Scientific Inc, Waltham, MA, U.S.A.). The bands were scanned with Quantity One software (Bio-Rad).

Statistical analysis
All statistical analyses were performed by SPSS 22.0 (SPSS Inc., Chicago, IL, U.S.A.) software. The comparison of two groups used Student’s t test and more than two groups used Analysis of variance (ANOVA). The data were presented as mean ± standard deviation (SD) and a value of P<0.05 was considered statistically significant.

Results
LINC00460 was overexpressed in NSCLC tissues and led to worse OS
First, we learned from the data of GEPIA that LINC00460 was overexpressed in lung adenocarcinoma tissues compared with the normal tissues (Figure 1A, *P<0.05). Besides, percent survival of NSCLC patients with LINC00460 high expression was significantly lower than that of patients with LINC00460 low expression (Figure 1B, P=0.0037).
Figure 7. Effect of LINC00460 knockdown on activation of PI3K pathway in NSCLC cell lines

(A,B) Represented bands of PI3K/Akt signaling associated proteins were assessed by Western blot in A549 and H1299 cells. (C,D) Relative protein expression levels of proteins were showed by column diagram. *P<0.05 vs. NC group.

Therefore, it could be remarkably found that overexpression of LINC00460 exerted poor prognosis of the NSCLC patients, implying LINC00460 may be a potential prognostic marker for patients with advanced NSCLC.

LINC00460 was up-regulated in NSCLC cell lines under the nicotine stimulation

In view of the previous analysis, we then explored the expression of LINC00460 in NSCLC cell lines, especially under the nicotine stimulation. We detected its expression in A549 and H1299 cell lines under different concentrations (0, 1, 10, 100, 200 μg/ml) of nicotine using qRT-PCR. As shown in the Figure 2A,B, LINC00460 expression was increased to various degrees, notably under the stimulation of 100 μg/ml nicotine in both the cell lines (*P<0.05, **P<0.01).

Besides, we also assessed the time-dependent analysis using 100 μg/ml nicotine to observe the expression level of LINC00460. The expression level of LINC00460 is significantly higher than that of the control group (Figure 2C,D, **P<0.01). Hence, we chose this concentration of 100 μg/ml to perform the following experiments.

Subsequently, in order to verify the direct effect of nicotine on LINC00460, sh-RNA was used to silence LINC00460 and the mRNA expression level was detected by qRT-PCR after 72 h transfection. In Figure 2E, the LINC00460 expression was both decreased markedly in A549 and H1299 cell lines (*P<0.05). Thus we could confirm that LINC00460 was induced by nicotine.
The effect of LINC00460 on cell proliferation was mediated by nicotine

The effect of LINC00460 knockdown on proliferation of A549 and H1299 cells was assessed using CCK-8 assay. As shown in Figure 3A, the OD values of A549 cells in LINC00460-KD group decreased at 72 h compared with those in NC or nicotine group (* \( P < 0.05 \)), suggesting knockdown of LINC00460 could inhibit the proliferation of NSCLC cells. However, the inhibition was escaped from the nicotine addition (* \( P < 0.05 \)). Similar results were observed in H1299 cells (Figure 3B, \( P < 0.05 \)). In general, these results indicated that LINC00460 could promote cell proliferation which could be encouraged by nicotine in NSCLC cells.

Nicotine restored the inhibitory effect of LINC00460 knockdown on NSCLC cell migration

To further confirm the role of LINC00460 on the migration of NSCLC cells, transwell assay was used to determine the cell migratory ability induced by the treatment of nicotine after silencing LINC00460 in A549 and H1299 cells. In Figure 4A, A549 cells significantly lost their migratory capability after LINC00460 knockdown (* \( P < 0.05 \)), while under the treatment of nicotine, cells once again acquired the migrated ability to some degree (* \( P < 0.05 \)). The nicotine-alone group was applied to show its promoted effect on cell migration. Likewise, the similar tendency in migration was revealed in H1299 cells because of nicotine interference (Figure 4B, \( * P < 0.05 \)). Collectively, LINC00460 knockdown plays an inhibitory role in NSCLC cell migration, which can be attenuated by nicotine.

Nicotine was involved in cell apoptosis mediated by LINC00460

Flow cytometry after Annexin V-FITC/PI staining and Western blot assay were used to explore whether LINC00460 knockdown was associated with cell apoptosis. Results in Figure 5 revealed that compared with NC group, the expression level of anti-apoptotic protein Bcl-2 was decreased after LINC00460 knockdown, conversely, pro-apoptotic protein Bax and Cleaved Caspase-3 expression were increased in A549 cells (* \( P < 0.05 \)). As expected, nicotine rescued the suppressive effect of LINC00460 knockdown on apoptosis. The same results were established in H1299 cells (Figure 6, all * \( P < 0.05 \)). As a consequence, silencing LINC00460 impaired cellular apoptotic prosperity in NSCLC cells but nicotine significantly relieved this impact.

Nicotine could relieve suppressive effect of LINC00460 knockdown on PI3K/Akt signaling pathway

In order to explore the underlying mechanism of LINC00460 in NSCLC cell, we determined the protein expression level of PI3K/Akt signaling-related markers by Western blot assay (Figure 7A,B). The results showed that expression levels of p-AKT, p-P70S6K and Cyclin D1 in LINC00460-KD group were significantly decreased compared with NC group (Figure 7C,D, * \( P < 0.05 \)), while the reduced expression was restored under nicotine stimulation. The data showed that the PI3K/Akt signaling pathway was significantly inhibited by LINC00460 knockdown, and the suppressive effect was eliminated by nicotine.

Discussion

In this present investigation, we identified that the effect of LINC00460 knockdown on NSCLC cells' activities can be restored by administration of nicotine and regulating the PI3K/Akt signaling pathway. All the findings established that the significance of LINC00460 activation induced by nicotine in NSCLC cell behaviors, enriching awareness of tumor treatments relying on IncRNAs.

Cigarette smoking has been found to enhance dissemination of tumor cells in the body of patients with various types of cancer [4,5,15]. Nicotine, one of the major components of cigarette smoking, has a role on cell proliferation of colorectal cancer, as well as of cell lines derived from several different cancer types [19]. It can suppress cell apoptosis induced by extracellular stress stimuli both in normal cells, such as vascular endothelial and smooth muscle cells [5], and in several human cancer cell lines derived from various organs [4,20]. Although there are a few studies on the mechanism of nicotine carcinogenesis, the effects of nicotine on lung cancer is still needed to fulfill in detail. In our in vitro experiments, LINC00460 was up-regulated in NSCLC cell lines owing to the nicotine stimulation. Nicotine can relieve the inhibition of LINC00460 knockdown on NSCLC cell proliferation and migration. Moreover, the effect of LINC00460 knockdown on NSCLC cell apoptosis was also related to nicotine, along with the alteration of Bcl-2, Bax and Cleaved Caspase-3. Furthermore, nicotine can make the suppressive effect of LINC00460 knockdown on PI3K/Akt signaling pathway worse. Thus, we boldly speculated that nicotine's regulation of LINC00460 might play an important role in the development of NSCLC.
As reported in various papers, nicotine activates multiple survival mechanisms, including PI3K/Akt and PKC/ERK1/2 pathways [9]. We also found that nicotine can make the suppressive effect of LINC00460 knockdown on PI3K signaling pathway worse. The PI3K/Akt signaling pathway is crucial for normal cell growth, and its deregulation influences various cellular responses that are associated with cancer phenotypes [21–26]. It runs as following steps: at first, PI3K activation phosphorylates AKT and active AKT can lead to a number of downstream effects including the activation of mTOR, ultimately mediate p-P70S6K activity, which in turn directly impacts cell growth and survival [27–29]. The main influence of the activation of Akt is the increase in survival in cell that normally undergoes death by apoptosis [19] and is involved in many other progressions, such as cell proliferation, angiogenesis, invasiveness and migration, modulating the initiation and progression of cancer [30,31]. Therefore, in accordance with above-mentioned reports, we hypothesized that LINC00460 might act as one upstream of PI3K/Akt pathway to control NSCLC progression. Furthermore, the consistent findings have been explained by Ma et al. [32], indicating that LINC00460 could promote tumor growth and gefitinib resistance via EMT [33,34].

In conclusion, to the best of our knowledge, this is the first study to reveal the potential roles of LINC00460 in lung cancer cell growth, migration and apoptosis induced by nicotine. This may help to develop novel therapeutic strategies for the prevention and treatment of lung cancer caused by cigarette smoke through inhibiting the nicotine-activated LINC00460 pathways.

Author Contribution
Hongying Zhao and Xiubao Ren conceived and designed the research, analyzed data and drafted the manuscript. Hongying Zhao and Yu Wang performed experiments. Xiubao Ren interpreted results of experiments. All authors read and approved the final manuscript.

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Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Abbreviations
AKT, protein kinase B; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; CCK-8, cell counting kit-8; EMT, epithelial–mesenchymal transition; FBS, fetal bovine serum; F12K culture medium, Kaighn's Modification of Ham's F-12 Medium; GEPIA, Gene Expression Profiling Interactive Analysis; LINC00460, long intergenic non-protein coding RNA 460; IncRNA, long non-coding RNA; miRNA, microRNA; NC, negative control; NSCLC, non-small cell lung carcinoma; OD, optical density; OS, overall survival; PI, Ppidium Iodide; PKC/ERK1/2, protein kinases C (PKC)/extracellular-regulated protein kinases 1/2; p-P70S6K, phosphorylated-serine-threonine protein kinase; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; sh-RNA, short hairpin RNA; TCGA, The Cancer Genome Atlas; TNM, tumor node metastasis.

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