

Long non-coding RNA SLNCR1 regulates non-small cell lung cancer migration, invasion and stemness through interactions with secretory phospholipase A2

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Abstract. Long non-coding RNA (lncRNA) SRA-like non-coding RNA (SLNCR1; also known as linc00673) is a recently identified oncogenic lncRNA. The role of SLNCR1 in non-small cell lung cancer (NSCLC), a common malignancy, remains poorly understood. The present study aimed to investigate the involvement of long non-coding RNA SLNCR1 in the pathogenesis of NSCLC. Reverse transcription-quantitative PCR (RT-qPCR) and ELISA were performed to measure the levels of lncRNA SLNCR1 and secretory phospholipase A2 (sPLA2) in lung biopsies, respectively. Correlations between expression levels of lncRNA SLNCR1 and sPLA2 were analyzed by Pearson's correlation analysis. Diagnostic value of lncRNA SLNCR1 for NSCLC was evaluated by receiver operating characteristic curve analysis. lncRNA SLNCR1 small interfering (si)RNA and sPLA2 overexpression vector were transfected into NSCLC cells. Cell migration, invasion and stemness were examined by Transwell migration assay, Matrigel invasion assay and flow cytometric assay, respectively. Following transfection, expression of lncRNA SLNCR1 and sPLA2 was detected by RT-qPCR and western blotting, respectively. The results demonstrated that lncRNA SLNCR1 expression levels were upregulated in tumor tissues compared with adjacent healthy tissues of patients with NSCLC. Plasma lncRNA SLNCR1 and sPLA2 expression levels were upregulated in patients with NSCLC compared with healthy

controls. Plasma lncRNA SLNCR1 and sPLA2 were positively correlated in patients with NSCLC, but not in healthy controls. lncRNA SLNCR1 siRNA silencing inhibited, whereas sPLA2 overexpression promoted cell migratory and invasive abilities and stemness. lncRNA SLNCR1 siRNA silencing led to downregulated sPLA2 expression, whereas sPLA2 overexpression did not significantly affect lncRNA SLNCR1 expression. sPLA2 overexpression attenuated the inhibitory effects of lncRNA SLNCR1 siRNA silencing on cell migration, invasion and stemness. In conclusion, lncRNA SLNCR1 may regulate cancer cell migration, invasion and stemness in NSCLC through interactions with sPLA2.

Introduction

Lung cancer is one of the most frequently diagnosed malignancies, with >14 million new cases and >8 million mortalities every year (1). The incidence of lung cancer has been predicted to further increase in the near future (2). In developing countries, such as China, lung cancer remains the most common cancer and the leading cause of cancer-related mortalities (3). This situation will not change unless air pollution in China is significantly reduced (4). With the efforts made on cancer treatment, such as the development of novel therapeutic approaches, survival of patients with lung cancer at early stages has been significantly improved in the past decades (5). However, once metastasis occurs, treatment outcomes become extremely poor (6). At present, early diagnosis and treatment are still crucial.

Phospholipase A2 (PLA2) enzymes catalyze the release of fatty acids from the second carbon group of glycerol. As an extracellular form of PLA2, secretory phospholipase A2 (sPLA2) widely participates in the development of different types of human cancers (7). sPLA2 is considered to be a promising therapeutic target for cancer treatment (8,9). sPLA2 has been demonstrated to participate in human disease through interactions with different signaling proteins (10,11), whereas its interactions with non-coding RNAs have rarely been studied.

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Long non-coding RNA (lncRNA) SRA-like non-coding RNA (SLNCR1; also known as linc00673) has been proven as an oncogenic lncRNA in several types of cancers, such as NSCLC and melanoma (12,13). However, its functionality in NSCLC remains to be further studied. In the present study, it was observed that lncRNA SLNCR1 may regulate NSCLC migration, invasion and stemness through interactions with sPLA2. To evaluate the early diagnostic value of SLNCR1 for non-small cell lung cancer (NSCLC), patients with stage I or II NSCLC were included in the study. The results demonstrated that altered lncRNA SLNCR1 expression may be used to effectively distinguish patients with early stage NSCLC from healthy controls.

Materials and methods

Human specimens and cell lines. A total of 66 patients with NSCLC (stage I and II) who were admitted to Jiangxi Provincial People's Hospital (Nanchang, China) between January 2016 and January 2018 were included in the study. Inclusion criteria: i) Diagnosed by biopsy; ii) stage I or II for early diagnosis analysis. Exclusion criteria: i) Combined with other disease; ii) received treatment within 6 months prior to admission. Tumor tissues and paired healthy tissues (within 5 cm from tumors) were collected from three sites. Blood was extracted from the patients with NSCLC and 42 healthy volunteers to prepare plasma by centrifuging blood samples in EDTA tube for 20 min at 1,200 x g at room temperature. The patient group included 35 males and 31 females, with an age range of 29-68 years (mean, 48.5±4.7 years). The control group included 22 males and 20 females, with an age range of 28-66 years (mean, 47.1±4.4 years). The two groups had similar age and sex distributions. The study was approved by the ethics committee of Jiangxi Provincial People's Hospital. All participants signed written informed consent.

Two human NSCLC cell lines, H1581 and H1993, were purchased from American Type Culture Collection (ATCC). ATCC-formulated RPMI-1640 medium containing 10% FBS (cat. no. 30-2020; ATCC) was used to cultivate cells in a 5% CO₂ incubator at 37°C.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA extraction from plasma (0.3 ml) and cells (10⁶) was performed using RNeasy Mini Kit (Qiagen China Co., Ltd.) according to manufacturer's instructions. Reverse transcription was carried out using Applied Biosystems™ High-Capacity cDNA Reverse Transcription kit to synthesize cDNA following DNase I (Sigma-Aldrich; Thermo Fisher Scientific, Inc.) digestion. Reverse transcription conditions were 55°C for 20 min and 80°C for 10 min. SuperScript III Platinum one-Step qRT-PCR SYBR kit (Thermo Fisher Scientific, Inc.) was used to prepare PCR reaction systems. The thermocycling conditions were 95°C for 55 sec, followed by 40 cycles of 95°C for 16 sec and 59.5°C for 25 sec. The primers were as follows: Human lncRNA SLNCR1, forward 5'-GGACCCCTTAACGTGGATTAC-3', reverse 5'-AAATACCTCCAGCTTGGCG-3'; β -actin, forward 5'-GACCTCTATGCCAACACAGT-3', reverse 5'-AGTACTTGCGCTCAGGAGGA-3'. The 2^{- $\Delta\Delta C_q$} method (14) was used to normalize expression levels to β -actin.

Cell transfection. Vectors (pcDNA3.1) expressing sPLA2, lncRNA SLNCR1 siRNA (5'-AAGAGGATGGGAAGGACTGAT-3') and Scrambled siRNA (5'-UUCUCCGAACGUGUCACGUdTdT-3') were synthesized by Shanghai GenePharma Co., Ltd. NSCLC cells were cultured to 70-80% confluence, and transfection was performed using Lipofectamine® 3000 Transfection Reagent (Thermo Fisher Scientific, Inc.) with 10 nM vectors or 30 nM siRNAs. Incubation of cells with transfection mixtures were performed at 37°C for 6 h. Cells treated with Lipofectamine® 3000 alone were used as an untransfected control. Cells transfected with empty vectors or Scramble siRNAs were used as a negative control. The transfections were considered successful if the knockdown rate of lncRNA SLNCR1 reached 50% and the overexpression rate of sPLA2 reached 200%. Three biological replicates were included in all subsequent experiments. All subsequent experiments were performed at 24 h post-transfections.

Enzyme-linked immunosorbent assay (ELISA). Plasma levels of sPLA2 were measured using a human sPLA2 ELISA kit (cat. no. MBS265046; MyBioSource, Inc.). Plasma levels of sPLA2 were expressed as pmol/l.

Transwell migration and Matrigel invasion assays. RPMI-1640 medium containing 1% FBS was used to prepare suspensions of transfected cells (5x10⁴ cells/ml). The upper chamber (Corning HTS Transwell 96 well, 8.0 μ m pore, cat. no. CLS3374, Sigma-Aldrich; Merck KGaA) was filled with 100 μ l cell suspension, whereas the lower chamber was filled with RPMI-1640 medium containing 20% FBS. Following 24-h incubation, the membranes were subjected to staining with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) at 25°C for 30 min. Matrigel (cat. no. 356234; EMD Millipore) was used to pre-coat the upper chamber prior to the invasion assay. Cells in 5 randomly selected visual fields (magnification, x40) were counted under Olympus CX22 Microscope (Olympus Corporation). Cells were counted using Image J v1.46 software (National Institutes of Health). Control group was set to '100'. All other groups were normalized to control group.

Flow cytometry. Trypsinization was performed to harvest transfected cells. Cells (10⁵) were incubated with phycoerythrin (PE)-conjugated immunoglobulin G (IgG) 1 (cat. no. 130-112-760; Miltenyi Biotec GmbH) or CD133-PE antibody (cat. no. 130-093-193; Miltenyi Biotec GmbH) at 4°C for 15 min. Cells were then resuspended in PBS and signals were detected using FACS Aria system (BD Immunocytometry Systems) and processed by CellQuest software, version 5.1 (Becton, Dickinson and Company).

Western blotting. Total protein was extracted from 10⁵ transfected cells using ReadyPrep™ Protein Extraction kit (Bio-Rad Laboratories, Inc.). Protein samples were quantified using a BCA kit (Sangon Biotech Co., Ltd.). Different proteins were separated by 10% SDS-PAGE with 40 μ g per lane. Following gel transfer onto PVDF membranes and blocking in PBS containing 5% non-fat milk for 2 h at room temperature, Membranes were first blotted with rabbit anti-human primary antibodies sPLA2 (cat. no. ab47105; 1:1,200; Abcam) and GAPDH (cat. no. ab9485; 1:1,200; Abcam) for 12 h at 4°C, followed by incubation with

secondary goat anti-rabbit horseradish peroxidase-conjugated IgG antibody (cat. no. MBS435036; 1:1,000; MyBioSource, Inc.) for 2 h at 24°C. Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) was used to develop signals. Densitometric analysis was performed using ImageJ v.1.46 software (National Institutes of Health); expression data were normalized to GADPH.

Statistical analysis. All *in vitro* experiments were repeated three times and data are presented as the mean \pm standard deviation. Comparisons of lncRNA SLNCR1 expression levels in tumor tissues and adjacent tissues were performed using Student's paired t-test. Comparisons between patient and control groups were performed using Student's unpaired t-test. One-way ANOVA followed by Tukey's test was used for comparisons between cell transfection groups. Pearson's correlation coefficient was used for correlation analysis. Diagnostic analysis was performed by receiver operating characteristics (ROC) curve. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

lncRNA SLNCR1 expression is upregulated in NSCLC tumor tissues. lncRNA SLNCR1 expression in tumor tissues and adjacent healthy tissues of 66 patients with NSCLC was detected by RT-qPCR. Compared with adjacent healthy tissues, the expression levels of lncRNA SLNCR1 were significantly increased in tumor tissues ($P < 0.05$; Fig. 1).

Upregulation of plasma lncRNA SLNCR1 distinguishes patients with NSCLC from healthy controls. Expression levels of lncRNA SLNCR1 in plasma of 66 patients with NSCLC and 44 healthy controls were detected by RT-qPCR. Compared with healthy controls, expression levels of plasma lncRNA SLNCR1 were significantly increased in patients with NSCLC ($P < 0.05$; Fig. 2A). ROC curve analysis was performed to evaluate the diagnostic value of lncRNA SLNCR1 for patients with NSCLC using patients with NSCLC as true positive cases and healthy controls as true negative cases. The area under the curve was 0.8660, with standard error of 0.03377 and 95% confidence interval of 0.7998-0.9322 (Fig. 2B).

Plasma sPLA2 expression is upregulated in patients with NSCLC and positively correlates with plasma lncRNA SLNCR1. ELISA results demonstrated that, compared with healthy controls, plasma levels of sPLA2 were significantly upregulated in patients with NSCLC ($P < 0.05$; Fig. 3A). Pearson's correlation analysis revealed a positive correlation between plasma levels of sPLA2 and lncRNA SLNCR1 in patients with NSCLC (Fig. 3B). Conversely, no correlation was observed between plasma levels of sPLA2 and lncRNA SLNCR1 in healthy controls (Fig. 3C).

lncRNA SLNCR1 siRNA silencing leads to downregulation of sPLA2 in NSCLC cell lines H1581 and H1993. Compared with untransfected control cells and negative control cells, transfection with lncRNA SLNCR1 siRNA significantly reduced the expression levels of lncRNA SLNCR1 and sPLA2 in NSCLC cell lines H1581 ($P < 0.05$; Fig. 4A) and H1993 ($P < 0.05$; Fig. 4B). By contrast, sPLA2 overexpression exhibited no

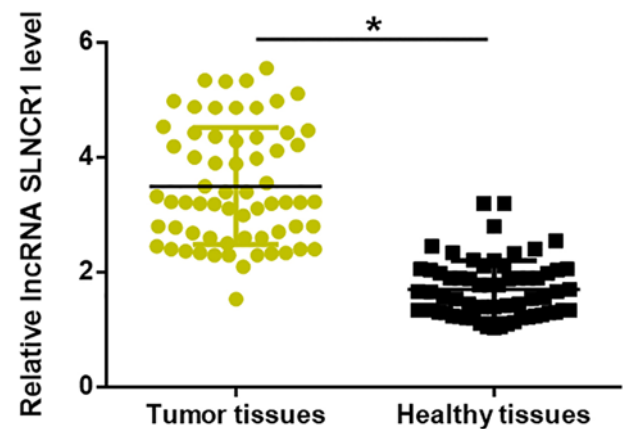


Figure 1. lncRNA SLNCR1 expression in tumors and healthy tissues from patients with NSCLC. lncRNA SLNCR1 expression was upregulated in tumor tissues compared with adjacent healthy tissues of 66 patients with NSCLC. * $P < 0.05$. lncRNA, long non-coding RNA; NSCLC, non-small cell lung cancer; SLNCR1, SRA-like non-coding RNA.

significant effects on lncRNA SLNCR1 expression in cells of H1581 (Fig. 4C) and H1993 (Fig. 4D) cell lines.

lncRNA SLNCR1 regulates NSCLC cell migration, invasion and stemness through sPLA2. Compared with the control groups, lncRNA SLNCR1 siRNA silencing and sPLA2 overexpression clearly affected the expression of sPLA2 protein (Fig. 5A). In addition, lncRNA SLNCR1 siRNA silencing significantly reduced, whereas sPLA2 overexpression significantly increased the migratory ability (Fig. 5B), invasive ability (Fig. 5C) and stemness (Fig. 5D) of H1581 and H1993 cells. sPLA2 overexpression partially attenuated the inhibitory effects of lncRNA SLNCR1 siRNA silencing on cell migration (Fig. 5B), invasion (Fig. 5C) and stemness (Fig. 5D) ($P < 0.05$). Notably, the changes in sPLA2 expression followed a similar pattern to the changes of cell migration, invasion and stemness (Fig. 5).

Discussion

The present study found that lncRNA SLNCR1 is an oncogenic lncRNA in NSCLC, which is the major pathological type of lung cancer. The data also revealed that lncRNA SLNCR1 may regulate NSCLC migration, invasion and stemness through interactions with sPLA2.

sPLA2 has oncogenic functions in cancer biology and usually exhibits upregulated expression in certain types of human cancers, including lung cancer (15,16). Consistent with previous studies (15,16), in the present study plasma sPLA2 was significantly upregulated in patients with NSCLC compared with healthy controls. overexpression of sPLA2 not only promotes cancer cell migration and invasion, but also participates in the maintenance of prostate cancer cell stemness (17,18). CD133 is a widely used marker for cancer stem cells (19). In the present study, increased NSCLC cell migration, invasion and stemness were also observed following sPLA2 overexpression. The results further supported a potential oncogenic role for sPLA2 in lung cancer and provided new insights to its functionality in this disease.

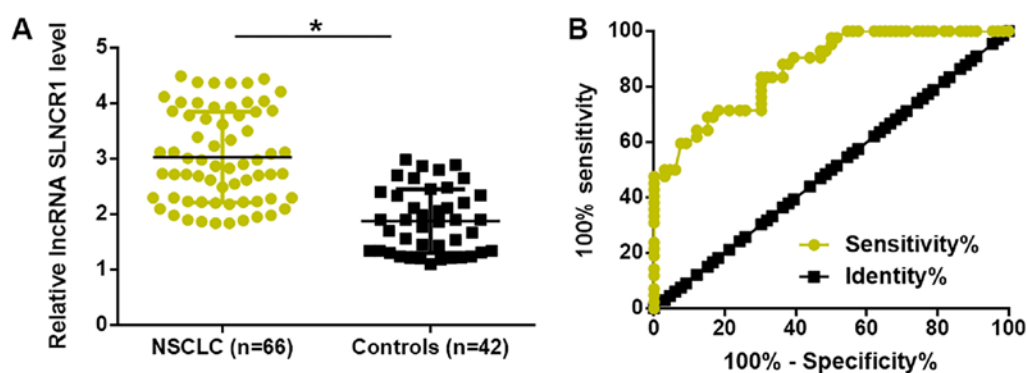


Figure 2. Upregulation of plasma lncRNA SLNCR1 expression levels distinguishes patients with NSCLC from healthy controls. (A) Compared with healthy controls, expression levels of plasma lncRNA SLNCR1 were significantly increased in patients with NSCLC; * $P < 0.05$. (B) Receiver operating characteristic curve analysis demonstrated that upregulation of plasma lncRNA SLNCR1 distinguished patients with NSCLC from healthy controls. lncRNA, long non-coding RNA; NSCLC, non-small cell lung cancer; SLNCR1, SRA-like non-coding RNA.

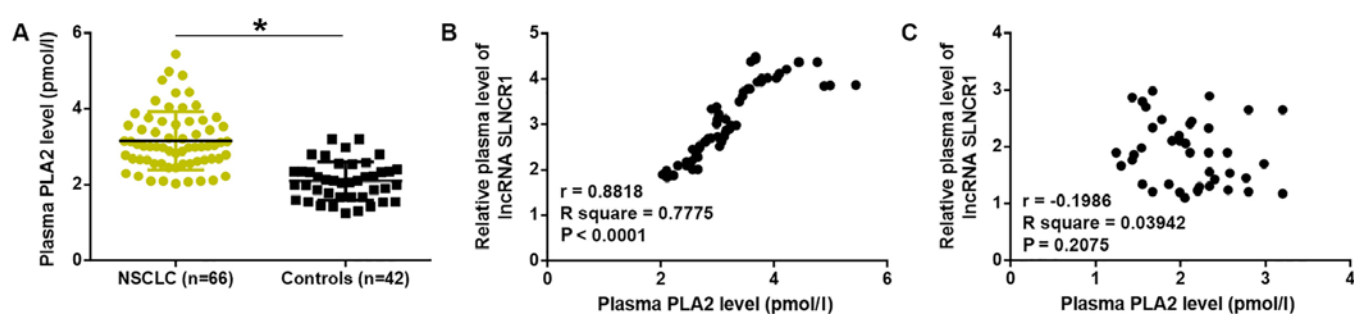


Figure 3. Plasma sPLA2 expression levels are upregulated in patients with NSCLC and are positively correlated with plasma lncRNA SLNCR1 expression levels. (A) Compared with healthy controls, plasma expression levels of sPLA2 were significantly upregulated in patients with NSCLC; * $P < 0.05$. (B and C) Pearson's correlation analysis revealed a positive correlation between plasma levels of sPLA2 and lncRNA SLNCR1 in (B) patients with NSCLC, but not in (C) healthy controls. lncRNA, long non-coding RNA; NSCLC, non-small cell lung cancer; sPLA2, secretory phospholipase A2; SLNCR1, SRA-like non-coding RNA.

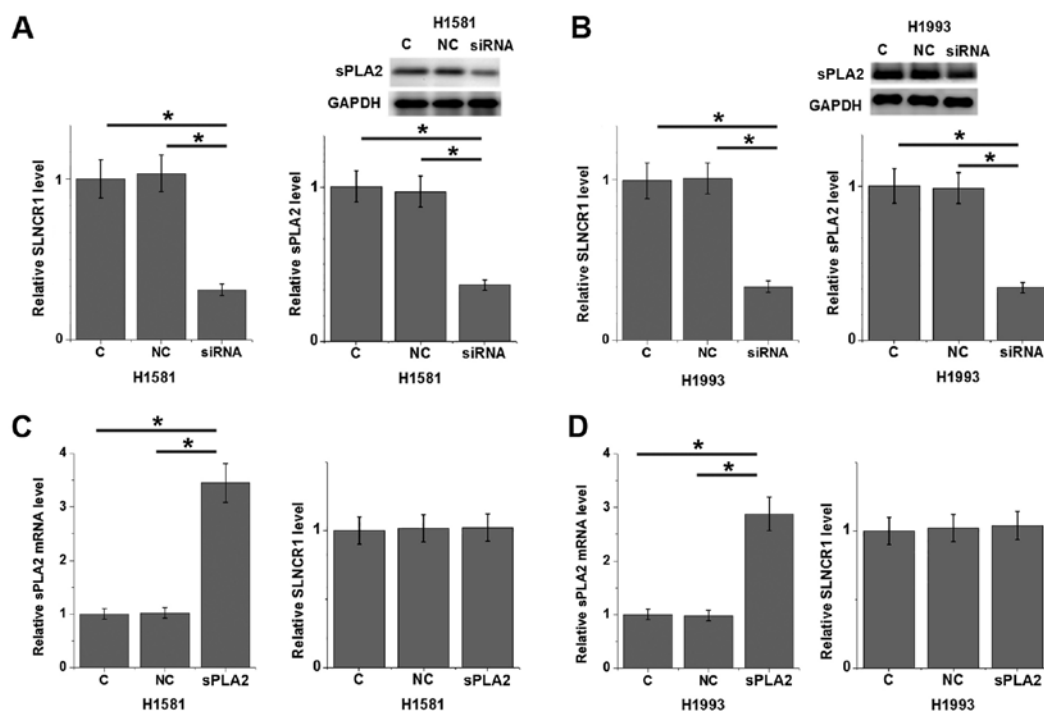


Figure 4. lncRNA SLNCR1 siRNA silencing mediates the downregulation of sPLA2 in NSCLC cell lines H1581 and H1993. (A and B) lncRNA SLNCR1 siRNA led to reduced mRNA and protein expression levels of sPLA2 in NSCLC cell lines (A) H1581 and (B) H1993 compared with control and NC. (C and D) sPLA2 overexpression did not significantly alter lncRNA SLNCR1 mRNA or protein expression levels in (C) H1581 and (D) H1993 cells; * $P < 0.05$. C, untransfected control; lncRNA, long non-coding RNA; NC, negative control; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; sPLA2, secretory phospholipase A2; SLNCR1, SRA-like non-coding RNA.

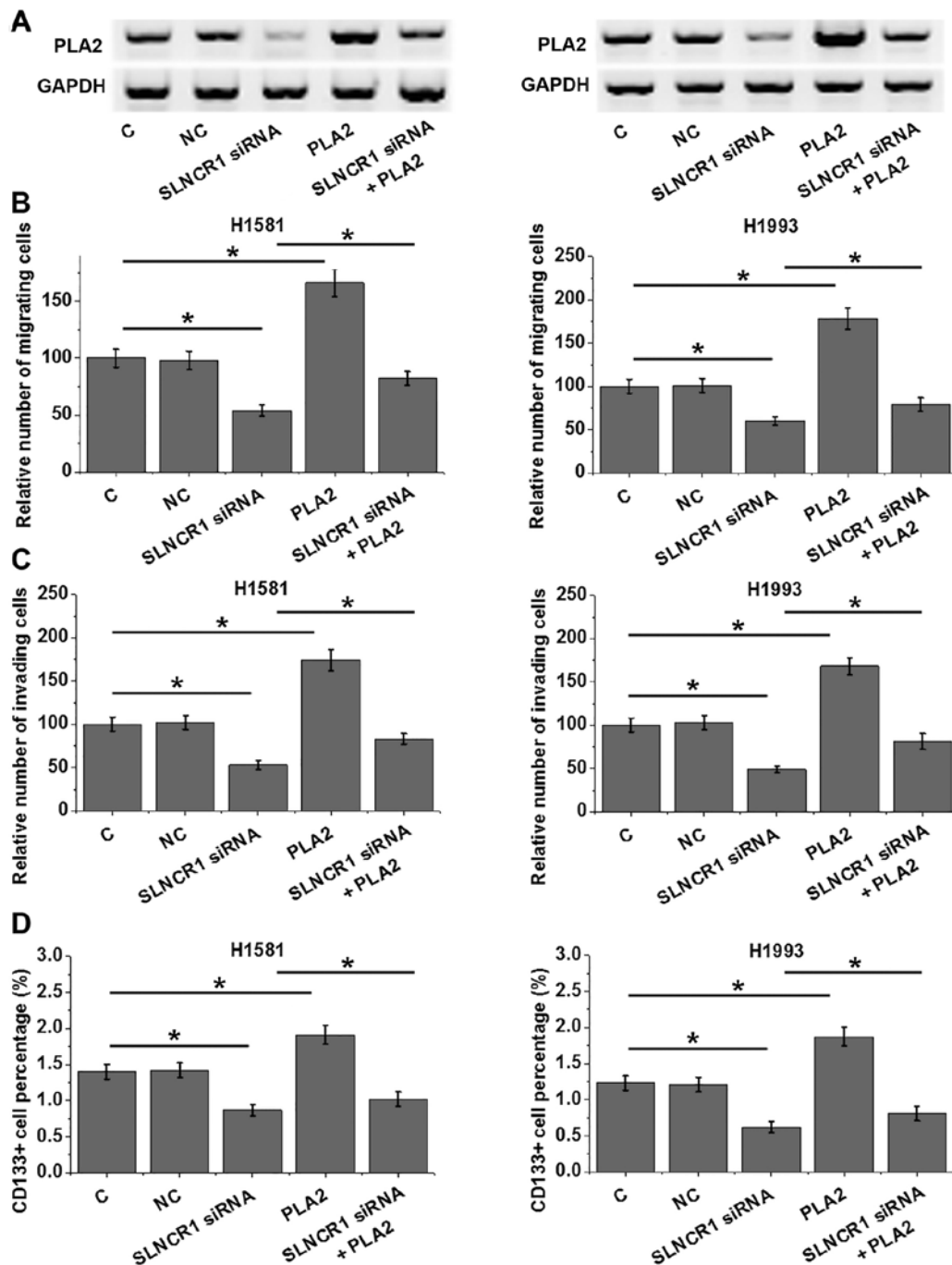


Figure 5. IncRNA SLNCR1 regulates NSCLC cell migration, invasion and stemness through sPLA2. (A) IncRNA SLNCR1 siRNA silencing and sPLA2 overexpression affected the expression of sPLA2 protein in H1581 and H1993 cells. (B-D) IncRNA SLNCR1 siRNA silencing reduced, whereas sPLA2 overexpression significantly increased the migratory ability (B), invasive ability (C) and stemness (D) of H1581 and H1993 cells vs. control and NC. In addition, sPLA2 overexpression attenuated the inhibitory effects of IncRNA SLNCR1 siRNA silencing on cell migration, invasion and stemness; * $P < 0.05$. C, untransfected control; IncRNA, long non-coding RNA; NC, negative control; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; sPLA2, secretory phospholipase A2; SLNCR1, SRA-like non-coding RNA.

IncRNA SLNCR1 is an oncogenic IncRNA in many different types of cancers, such as NSCLC and melanoma (12,13); the present study demonstrated an upregulation of IncRNA SLNCR1 expression levels in tumor tissues compared with adjacent healthy tissues of patients with NSCLC. Significantly higher plasma levels of IncRNA SLNCR1 were also observed in patients with NSCLC compared with healthy controls, which indicated a potential oncogenic role for IncRNA SLNCR1 in NSCLC. IncRNA SLNCR1 has been previously demonstrated to mediate

melanoma invasion (13). In another study, SLNCR1 was proved to serve roles in regulating multiple cell behaviors in NSCLC, such as invasion, migration and proliferation (12). Consistent with previous studies (12,13), the present study also demonstrated that IncRNA SLNCR1 may be involved in the regulation of cancer cell migration in NSCLC. The present study is the first to report the role of SLNCR1 in regulating cancer cell stemness in NSCLC. Therefore, the inhibition of IncRNA SLNCR1 expression may serve as a potential therapeutic target for NSCLC.

Notably, a significantly positive correlation between plasma lncRNA SLNCR1 and sPLA2 was identified in patients with NSCLC. *In vitro* experiments using NSCLC cell lines also demonstrated that lncRNA SLNCR1 may be an upstream activator of sPLA2 in the regulation of migration, invasion and stemness of NSCLC cells. To the best of our knowledge, this is the first reported interaction between sPLA2 and lncRNAs. However, the interaction between lncRNA SLNCR1 and sPLA2 is unlikely to be direct owing to the lack of correlation between plasma lncRNA SLNCR1 and sPLA2 in healthy controls. The development and progression of NSCLC are associated with pathological factors, which may mediate the interaction between lncRNA SLNCR1 and sPLA2. However, the present study failed to identify these pathological mediators. Future studies are needed to identify the mediators involved in this process.

In conclusion, lncRNA SLNCR1 and secretory sPLA2 were both upregulated in NSCLC. lncRNA SLNCR1 may regulate cancer cell migration, invasion and stemness in NSCLC through interactions with secretory sPLA2.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

WX designed the experiments. WX, QX, DK and ZW performed the experiments. QLu, QLi, HW and LC assisted with the experiments and analyzed data. WX drafted the manuscript. All authors approved the manuscript.

Ethics approval and consent to participate

The study was approved by the ethics committee of Jiangxi Provincial People's Hospital. All participants signed written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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