A novel long non-coding RNA LSAMP-1 is down-regulated in non-small cell lung cancer and predicts a poor prognosis

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Abstract

Background

There is very little known about how long non-coding RNAs (lncRNAs) are associated with membrane proteins in lung cancer. The limbic system-associated membrane protein (LSAMP) has been reported to play a tumor suppressor role in a variety of cancers.

Methods

We aimed to explore the lncRNA associated with LSAMP and explore its effects on lung cancer.

Results

We found that Lnc-LSAMP-1 was significantly down-regulated in 170 cases of lung tumor tissues when compared to the adjacent non-cancerous tissues (p<0.001). Our results indicated that low expression of Lnc-LSAMP-1 was significantly correlated with stage (TNM) (p=0.006), N status (p=0.009) and poor prognosis (p=0.004). Further investigation showed that overexpression of Lnc-LSAMP-1 significantly inhibited lung cancer cell proliferation, viability, invasion and migration ability, arrested cell cycle and facilitated apoptosis. Meanwhile, the expression of Lnc-LSAMP-1 is highly correlated with the expression of nearby tumor suppressor gene LSAMP in our samples (r=0.7074, p<0.001) and TCGA database (r=0.78, p<0.001). It was found that overexpression of Lnc-LSAMP-1 can slow down the degradation rate of LSAMP gene by mRNA protection experiments. By knocking down of LSAMP gene, it was found that overexpressed Lnc-LSAMP-1 cells showed a high proliferation rate. Chemotherapy sensitization experiments showed that overexpression of Lnc-LSAMP-1 enhanced TKI inhibition of lung cancer cell proliferation, which is probably related to affecting the prognosis of patients.

Conclusions

Consequently, the above data suggested that Lnc-LSAMP-1 functions as a tumor
suppressor and provides a new potentially therapeutic and prognostic target for non-small cell lung cancer.

**Background**

Lung cancer is one of the most common malignant tumors and has become the number one cause of cancer-related deaths in the world[1, 2]. The past 50 years saw a significant increase in the incidence and mortality due to lung cancer in many countries[3]. There are 1.8 million people diagnosed with lung cancer each year, of which in 1.6 million people, it leads to death[4]. Non-small cell lung cancer (NSCLC) is responsible for approximately 85% of all new lung cancer cases[5]. Despite current technological advancement in clinical and experimental oncology for NSCLC, the overall five-year survival rate for the disease in different regions and countries is between 4–17%, which is still at a low level[6-8]. Molecular biomarkers for potential lung cancer diagnosis and potential gene therapy targets are a recent research focus in the field of lung cancer[9, 10]. An in depth understanding of the carcinogenesis is crucial for the development of diagnostic markers and new treatment options for anticancer therapy[11].

Recent studies have shown that non-coding RNA (ncRNA) plays a pivotal regulatory role in gene expression[12-15]. LncRNAs (long noncoding RNA), important new members of the ncRNA family, are not only modulators of apoptosis and invasion, but also participate in the occurrence and development of cancer[16-18]. For example, LncRNA FEZF-AS1 is associated with advanced clinical stages and family history of lung cancer in patients with NSCLC[19]. LncAGER-1 has an inhibitory effect on lung cancer development by regulating gene AGER[20]and LncRNA IGFBP4-1 reprograms energy metabolism to promote lung cancer progression[21]. MicroRNAs (miRNAs) are also a class of ncRNAs. There are a lot of LncRNA promoting lung cancer by regulating miRNAs[22].

With the help of bioinformatics analysis, we discovered that the low expression of Lnc-
LSAMP-1 in lung cancer tissues was associated with poor survival rate of NSCLC patients. We also found that Lnc-LSAMP-1 was down-regulated in 170 cases of lung cancer tissues compared to that in adjacent non-cancerous tissues (p < 0.001). Lnc-LSAMP-1 is located on the human chromosome 3q13.32 (chr3: 117391001–117716439) approaching LSAMP gene, which is related to the onset of cancer[23–25]. However, the specific functions of Lnc-LSAMP-1 in lung cancer are not yet clear. Mounting evidence suggested that both self-sufficiency in growth signals and insensitivity to antigrowth signals are among the ten characteristics of tumor[26] and it is known that signal transduction between cells plays an important role in the development of cancer[27]. Membrane proteins are also important mediators of cell signaling[28]. Consequently, we suspected that the Lnc-LSAMP-1 suppresses the tumor genesis of lung cancer by regulating LSAMP gene and the following results supported our hypothesis.

Materials And Methods

Patients and tissue samples

The protocol was fully approved by the Institutional Medical Ethics Committee of Guangzhou Medical University, Guangzhou Medical University First Affiliated Hospital, Guangzhou Medical University Affiliated Tumor Hospital, and First Affiliated Hospital of Suzhou University. The purpose of this study was explained and written informed consent forms were obtained from all subjects. A total of 170 NSCLC and adjacent non-tumor tissue specimens were obtained from Guangzhou Medical University First Affiliated Hospital, Guangzhou Medical University Affiliated Tumor Hospital, and First Affiliated Hospital of Suzhou University. 170 patients diagnosed with NSCLC, who had never received any therapy before surgery, were recruited. All clinical data, including age, gender, clinical stage, smoking history, infiltration degree, lymph node metastasis and distant metastasis,
of these patients were recorded in a database. In addition, tissue specimens were immediately preserved in RNA later Solution (Thermo Fisher Scientific, US) after removal from the body and were stored at – 80°C until use.

Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA from 170 paired NSCLC tissues and 11 cell lines were extracted using TRIzol reagent (Invitrogen, Carlsbad, California, USA). The total RNA was reversely transcribed by using commercial kits according to the manufacturer’s instructions (TaKaRa, Japan). RT-qPCR reaction (DBI, Germany) was performed in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, CA, USA). β-actin was used as the endogenous control. We found the Lnc-LSAMP-1 sequence from Lncipedia (http://www.lncipedia.org/).

With the help of UCSC genome bioinformatics public database (http://genome.ucsc.edu/cgi-bin/hgGateway), we gained access to other related gene sequences. Primer3Plus online primer design software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and NCBI online software were used for primer design. The primers were synthesized by Sangon Biotech Ltd (Shanghai, China). The primer sequences of Lnc-LSAMP-1 were 5´- GTGTTTACACCAGGCACGGG-3´ (forward) and 5´-AACCCGGACAGACAAGCCTC-3´ (reverse) respectively. The primer sequences of β-actin were 5´-GGCGGCACCACCATGTACCCT-3´ (forward) and 5´-AGGGGCCGGACTCGTCATACT-3´ (reverse). The primer sequences of LSAMP gene were 5´-AGAGTTCAGCCGGATCGGAA-3´ (forward) and 5´-CGTGCTCGGTAAAATCCAC-3´ (reverse). The 2^−ΔΔCT was used to demonstrate the expression levels of Lnc-LSAMP-1 and LSAMP. All the experiments were conducted in triplicate. The data was analyzed by using the comparative cycle threshold (Ct) method.

Cell culture
A549 and PC-9 human lung cancer cell lines used in this study were purchased from Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai Institute of Cell Biology, China), in which PC-9 derived from human adenocarcinomas of the lung[29].

Both cell lines were tested and authenticated using a panel of genetic and epigenetic markers before being used in this study. All cells were cultured in RPMI 1640 medium (Gibco, life technologies, California, USA) with 10% fetal bovine serum (FBS). Cells were placed in a CO\(_2\) incubator (SANYO Electric Co., Ltd., Japan) with constant 90% humidity and 5% CO\(_2\).

**Transfection of lentiviral vector**

To generate clones, stably overexpressing Lnc-LSAMP-1, A549 and PC-9 cells were first infected with a lentiviral vector (iGeneBio Co, Ltd, Guangzhou, China) encoding full-length human Lnc-LSAMP-1 sequence and an empty lentiviral vector as the control. Stable clones were screened for 2 weeks using puromycin and the expression level of Lnc-LSAMP-1 was determined by quantitative RT-PCR (qRT-PCR).

**Cell proliferation assay**

Cell proliferation assay was performed with Cell Counting Kit-8 (CCK-8, Corning Corporation, USA). Logarithmic phase cells were seeded into 96-well plates and were cultured for 12, 24, 36, and 48 h, respectively. The absorbance of each well was read on a Thermo Scientific™ VarioskanTM LUX plate reader (Thermo Instruments, USA) (detection wavelength was 450 nm, reference wavelength was 600 nm). We also used a dynamic cell viewer for detection abiding by the manufacturer’s protocols (IncuCyte ZOOM. Essen BioScience Co., Ltd., USA).

**Flow cytometric analysis of cell cycle and apoptosis**

The flow cytometry analysis was used to identify whether Lnc-LSAMP-1 influenced cell
cycle and apoptosis. For cell cycle analysis, stable transfected cells in the logarithmic growth phase were harvested 48 h after transfection by trypsinization and the propidium iodide staining solution was used for staining. Then the flow cytometric assays (FACScan; BD Biosciences, Shanghai, China) were performed according to the manufacturer's instructions.

For cell apoptosis analysis, Annexin V/7-AAD apoptosis detection kit was used (MultiSciences, HangZhou, China) following the protocol. Cells were washed twice in PBS and re-suspended in 1 × Binding Buffer to achieve a cell concentration of 1.0 × 10^6 cells/ml. Subsequently, 10 ul of 7-AAD reagent and 5 ul of Annexin V reagent were added into cell suspension and stored for 30 min at room temperature in dark place. Apoptotic cells were examined and quantified using flow cytometry (Becton Dickinson, Lincoln Park, NJ, USA).

**Colony-formation assay**

For plate colony formation assay, cells were trypsinized into single cells and seeded into 6-well plates at a density of 200 cells/well. After 10 days of culture, cell clones that had formed from individual cells were directly observed by eye and then the colonies were fixed and stained with Crystal violet, followed by air-drying. The plate colony formation efficiency equals \( \frac{\text{number of colonies}}{\text{inoculated cells}} \times 100\% \). These experiments were performed in triplicate.

**Transwell assays**

For the Transwell migration assay, a total of 2 × 10^4 post-transfection A549 and PC-9 cells were plated in a serum-free medium in the top chamber with a non-coated membrane (24-well insert, pore size 8 µm; Corning, NY, USA) and a medium supplemented with 10% serum was in the lower chamber. The invasion experiment was done using the Corning BD
BioCoat Matrigel Invasion chamber with a cell diameter of 8 µm according to the manufacturer's protocol. After 48 hours, the bottom of the chamber was fixed using formaldehyde for 30 minutes and then stained with 0.1% crystal violet for 30 minutes. Ten fields were randomly selected under a 100x microscope and the number of cells that migrated to the lower layer was counted. The migration procedure is identical to the invasion experiment procedure without coating with Matrigel matrix. Each assay was performed in triplicate.

**Tumorigenicity assay in nude mice**

Animal experiments were approved by the Animal Ethics Committee of Guangzhou Medical University. The number of mice in each group was 5. 200 µl of stably transfected lung cancer cells and control cells (1 × 10^7 cells/ml, A549, PC-9) were injected into the flanks of four-week-old female nude mice (Beijing Huarongkang Biotechnology Co. Ltd). Tumor growth was examined every 3 days for at least one month by measuring the length and width of the tumor mass.

The experimental procedure for tumor metastasis in nude mice is similar to tumor growth model in nude mice. But the injection site of tumor cells is in the tail vein of nude mice. After continuous observation for 6 weeks, the nude mice were killed, and the number of lung cancer metastasis was observed. HE slices were made following relevant protocols and strict operating procedures after soaking and fixing with 4% paraformaldehyde. The program was approved by the Animal Ethics Committee of Guangzhou Medical University.

**Hematoxylin-eosin (HE) staining**

Tumor tissues were fixed in 10% formalin, embedded in paraffin and then cut into 3-µm-thick sections that were baked at 45 °C for 5 h. Sections were then stained with HE (artificial hematoxylin and eosin) according to the following steps: 30 min of xylene
dewaxing, treated with ethanol at different concentrations (100%, 90%, 70%), hydrated in distilled water, stained with hematoxylin (15 min), differentiated in hydrochloric acid ethanol and ammonia water, dehydrated with ethanol at 70% and 90% concentrations (10 min), stained with eosin ethanol (3 min), dehydrated with ethanol and cleared with xylene, and tumor tissue sections were then observed under a microscope.

**Actinomycin D inhibits RNA synthesis experiments**

Over-expressed A549 and PC-9 cells and control were seeded at 10mm*24-well plates at 5 × 10⁴ per well. After 24 hours, the cells were treated with actinomycin D (the concentration of actinomycin D was 2 mg/L). After 30 minutes, 1 hour, 2 hours, 3 hours and 4 hours, the expression levels of LSAMP gene was detected by qRT-PCR.

**Rescue experiment**

The Rescue experiment was performed to validate that Lnc-LSAMP-1 regulated lung cancer cell biology functions by targeting LSAMP. LSAMP inhibitor and blank inhibitor were transferred into A549 and PC-9 cells. The efficiency of siRNA was measured by qRT-PCR. It was observed in Incucyte zoom (Essen BioScience Co., Ltd., USA) that the proliferation of A549 and PC-9 cells led to the inhibition of LSAMP.

**Drug sensitivity test of Lnc-LSAMP-1 on cisplatin and TKI (Tyrosine kinase inhibitors)**

The IC50 of A549 and PC-9 cells was first screened using cisplatin concentration gradient (10 ug / ml, 5 ug / ml, 2.5 ug / ml, 1.25 ug / ml, 0.625 ug / ml, 0.3125 ug / ml). A549: IC50 of 5ug / ml; PC-9: IC50 2.5ug / ml. Nilotinib (TKI) was used as mean plasma drug concentrations such as Nilotinib = 3.6umol / L. High expression of Lnc-LSAMP-1 and control cells were treated with cisplatin and Nilotinib. The high expression group and the control group were inoculated into a 96-well plate, and the number of cells per well was 5.0 × 10³.
Nilotinib was added after adhering to the wall and growing to about 10%, cisplatin was added up to 30%-40%, and the 96-well plate was placed in a dynamic cell observer for detection (IncuCyte ZOOM. Essen BioScience Co. Ltd. USA). The rate of cell proliferation was tested.

**Statistical analysis**

All statistical data were analyzed using the SPSS 16.0 software (SPSS, Chicago, USA). The paired-t test was used to assess the differences between lung cancer tissues and adjacent lung normal tissues in regards to gene expression and frequency of methylation level. The $\chi^2$ test was applied to analyze gene expression levels in Table 1 and table S1. The association between Lnc-LSAMP-1 expression and the clinical pathological parameters of NSCLC was assessed by the one-way analysis of variance (ANOVA). Kaplan-Meier and Cox regression analyses were used to assess the association between Lnc-LSAMP-1 and overall survival rate as well as the prognosis of NSCLC. Additionally, $P < 0.05$ was considered statistically significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).
Table 1
Associations between the expression of Lnc-LSAMP-1 and clinical features as well as demographics among lung cancer patients

| Character                      | Southern samples N (%) | Eastern samples N (%) | Total N (%) |
|--------------------------------|-------------------------|-----------------------|-------------|
|                                | Low expression | High expression | P value | Low expression | High expression | P value | Low expression | High expression | P value |
| Age                            |             |                |   |             |                |   |             |                |   |             |                |   |
| < 60                           | 40(69.0)    | 18(31.0)      | 0.155 | 19(63.3)    | 11(36.7)      | 0.311 | 59(67.0)    | 29(33.0)      | 0.508 |
| >= 60                          | 32(56.1)    | 25(43.9)      |   | 19(76.0)    | 6(24.0)       |   | 51(62.2)    | 31(37.8)      |   |
| Gender                         |             |                |   |             |                |   |             |                |   |             |                |   |
| Female                         | 21(67.7)    | 10(32.3)      | 0.489 | 10(62.5)    | 6(37.5)       | 0.722 | 31(66.0)    | 16(34.0)      | 0.833 |
| Male                           | 51(60.7)    | 33(39.3)      |   | 28(71.8)    | 11(28.2)      |   | 79(64.2)    | 44(35.8)      |   |
| Family tumor history           |             |                |   |             |                |   |             |                |   |             |                |   |
| No                             | 62(60.8)    | 40(39.2)      | 0.407 | 36(70.6)    | 15(29.4)      | 0.767 | 98(64.1)    | 55(35.9)      | 0.593 |
| Yes                            | 10(76.9)    | 3(23.1)       |   | 2(50.0)     | 2(50.0)       |   | 12(70.6)    | 5(29.4)       |   |
| Smoking                        |             |                |   |             |                |   |             |                |   |             |                |   |
| No                             | 25(64.1)    | 14(35.9)      | 0.813 | 11(73.3)    | 4(26.7)       | 0.929 | 36(66.7)    | 18(33.3)      | 0.715 |
| Yes                            | 47(61.8)    | 29(38.2)      |   | 27(67.5)    | 13(32.5)      |   | 74(63.8)    | 42(36.2)      |   |
| Stage (TNM)                    |             |                |   |             |                |   |             |                |   |             |                |   |
| I + II                         | 35(74.5)    | 12(25.5)      | 0.029 | 16(84.2)    | 3(15.8)       | 0.078 | 51(77.3)    | 15(22.7)      | 0.006 |
| III + IV                       | 37(54.4)    | 31(45.6)      |   | 22(61.1)    | 14(38.9)      |   | 59(66.7)    | 45(34.3)      |   |
| T status                       |             |                |   |             |                |   |             |                |   |             |                |   |
| 1 + 2                          | 47(66.2)    | 24(33.8)      | 0.312 | 13(54.2)    | 11(45.8)      | 0.035 | 60(63.2)    | 35(36.8)      | 0.635 |
| 3 + 4                          | 25(56.8)    | 19(43.2)      |   | 25(80.6)    | 6(19.4)       |   | 50(66.7)    | 25(33.3)      |   |
| N status                       |             |                |   |             |                |   |             |                |   |             |                |   |
| 0                              | 34(75.6)    | 11(24.4)      | 0.021 | 22(75.9)    | 7(24.1)       | 0.251 | 56(75.7)    | 18(24.3)      | 0.009 |
| 1 + 2 + 3                      | 38(54.3)    | 32(45.7)      |   | 16(61.5)    | 10(38.5)      |   | 54(56.3)    | 42(43.8)      |   |
| M status                       |             |                |   |             |                |   |             |                |   |             |                |   |
| 0                              | 54(65.1)    | 29(34.9)      | 0.382 | 23(62.2)    | 14(37.8)      | 0.111 | 77(64.2)    | 43(35.8)      | 0.820 |
| 1                              | 18(56.3)    | 14(43.7)      | 15(83.3) | 3(16.7) | 33(66.0) | 17(34.0) |   |             |                |   |
| Histological classification    |             |                |   |             |                |   |             |                |   |             |                |   |
| Adenocarcinoma                 | 39(65.0)    | 21(35.0)      | 0.632 | 15(68.2)    | 7(31.8)       | 1.000 | 54(65.9)    | 28(34.1)      | 0.752 |
| Squamous carcinoma             | 16(55.2)    | 13(44.8)      | 13(68.4) | 6(31.6) | 29(60.4) | 19(39.6) |   |             |                |   |
| Other types                    | 17(65.4)    | 9(34.6)       | 10(71.4) | 4(28.6) | 27(67.5) | 13(32.5) |   |             |                |   |

a Large cell carcinoma, small cell carcinoma and hybrid or undifferentiated carcinoma.

Results
Lnc-LSAMP-1 and LSAMP gene expression in lung cancer

The demographics and clinical features of studied patients were listed in Table S1. The expression of Lnc-LSAMP-1 at 9 lung cancer cell lines and 4 human immortalized lung normal cell lines (p = 0.0007) has been shown in Fig. 1a. We found that Lnc-LSAMP-1 was down-regulated in tumor tissues compared to that in non-tumor tissues in 170 lung cancer cases (p < 0.001, as shown in Fig. 1b) and LSAMP gene was also down-regulated in 86 cases of lung tumor tissues when compared to that of the adjacent non-cancerous tissues (p = 0.0314, as shown in Fig. 1c). Meanwhile, the expression of Lnc-LSAMP-1 was positively associated with LSAMP gene expression in 143 cases of NSCLC tissues (r = 0.7074, p < 0.001, Fig. 1d). Both A549 and PC-9 expressed a relevant portion of Lnc-LSAMP-1 in nucleus (Fig. 1e). As shown in Fig. 1f, Kaplan-Meier analysis indicated that down-regulation of Lnc-LSAMP-1 expression in tissues was associated with poor survival rate of NSCLC patients (p = 0.004), which is also consistent with TCGA database (Fig. S2d). The A549 and PC-9 cells that overexpressed Lnc-LSAMP-1 showed significantly higher levels of LSAMP and Lnc-LSAMP-1 expression compared with that of control groups (Fig. 1g and h).

Lnc-LSAMP-1 expression is correlated with Stage (TNM) and N status

We analyzed the relationship between Lnc-LSAMP-1 expression level and clinical features. The expression status of Lnc-LSAMP-1 was classified as “High” or “Low” based on the expression of LNC-LSAMP-1 in lung cancer tissues and in adjacent normal lung tissues. We found that low expression of Lnc-LSAMP-1 was significantly correlated with stage (TNM)(p = 0.006) and N status (p = 0.009), which is in accordance with TCGA database (Fig. S2f and g). However, Lnc-LSAMP-1 down-expression was not associated with age, gender, family tumor history, smoking, stage, T status and histological classification (all p > 0.05), as shown in Table 1.
The correlation of Lnc-LSAMP-1 and LSAMP gene was investigated in the GEPIA database

TCGA database (From GEPIA) analysis revealed that the expression levels of Lnc-LSAMP-1 and LSAMP gene were significantly down-regulated in NSCLC compared with that of adjacent normal tissues (all P < 0.05, Fig. S2a and b). To further search the target gene for Lnc-LSAMP-1, we analyzed the correlation between Lnc-LSAMP-1 and target gene from GEPIA dataset. We found that the LSAMP gene is the most relevant gene (R = 0.78 P < 0.001, Fig. S2c and S1a). We further analyzed the relationship between Lnc-LSAMP-1 expression and clinical analysis in TCGA and found that Lnc-LSAMP-1 expression was associated with T status (p = 0.0349), N status (p = 0.0012), and stage (TNM) (p = 0.0049) (Fig. S2e, S2f and g). We also found that Lnc-LSAMP-1 is located in the downstream of the LSAMP gene and has partial sequence overlap regions (Fig. S1b). The above results indicate that Lnc-LSAMP-1 is highly correlated with the LSAMP gene.

Down-regulation of Lnc-LSAMP-1 predicts a poor prognosis in NSCLC

To evaluate the potential prognostic value of Lnc-LSAMP-1, we analyzed RNA-seq data from GEPIA. From a cohort of 472 NSCLC patients, Lnc-LSAMP-1 was highly expressed in the lung cancer tissues of 238 patients and lung cancer tissues of the other 234 patients showed low Lnc-LSAMP-1 expression. We assessed the overall survival rate of patients who were Lnc-LSAMP-1-high or Lnc-LSAMP-1-low using the Kaplan-Meier method. As shown in Fig. S2d, the 20-year survival rate was lower in Lnc-LSAMP-1-low patients comparing to Lnc-LSAMP-1-high patients, indicating that a high level of Lnc-LSAMP-1 was correlated with a long survival time (Log rank p = 0.007, HR(high) = 0.66 p(HR) = 0.0076), supporting the argument for its utility as a biomarker for NSCLC progression and its potential function for early detection of NSCLC.
Lnc-LSAMP-1 suppresses cell proliferation

To determine the effect of Lnc-LSAMP-1 on the viability and proliferation of lung cancer cell in vitro, CCK-8 and colony formation assay showed that the overexpressed Lnc-LSAMP-1 suppressed the viability of A549 and PC-9 in a time dependent manner (P < 0.05; Fig. 2b). The same results were observed in the dynamic cell viewer (Fig. 2a) and plate colony formation assay (Fig. 2c).

Lnc-LSAMP-1 affects migration and invasion of A549 and PC-9 cells

To detect the effect of Lnc-LSAMP-1 on cell migration, the Transwell migration assay was carried out in A549 and PC-9 cell lines. As shown in Fig. 2d, the overexpressed Lnc-LSAMP-1 resulted in attenuated migration of lung cancer cells. The Transwell invasion assay results also showed that the cell invasive ability was suppressed in A549 and PC-9 cell lines when cells were transfected with Lnc-LSAMP-1 compared with the results from the control groups (Fig. 2f).

Lnc-LSAMP-1 affects cell cycle and induces apoptosis

As shown by flow cytometry analysis in Fig. 2e, overexpressed Lnc-LSAMP-1 caused significantly increased apoptosis in A549 and PC-9 cells compared with that of the control groups. Accordingly, flow cytometric analysis showed a decrease in the percentage of cells in the S phase and a marked accumulation in the percentage of cells in the G0/G1 phase in A549 and PC-9 cells (Fig. 2g), compared with that of the control groups.

Lnc-LSAMP-1 inhibits tumor growth in vivo

As shown in Fig. 3a, b and c, tumor growth in nude mice injected with overexpressed Lnc-LSAMP-1 A549 and PC-9 cells was slower than that in control groups and the tumors were smaller. Accordingly, the number of suspected tumors in the lung of nude mice is less than that of control groups, when mice were injected with overexpressed Lnc-LSAMP-1.
A549 and PC-9 cells (Fig. 3d and e). Figure 3f showed a subcutaneous tumor-forming HE section of nude mice, which is consistent with lung cancer tissue morphology. Figure 3g showed a HE slice of suspicious lung nodules in nude mice injected with tail vein injection, which can be observed as lung cancer cell morphology from the enlarged part of the figure (red boxes in Fig. 3g).

The knock down of LSAMP gene promotes cell proliferation rate in vitro

The efficiency of si (LSAMPgene) was measured by qRT-PCR and the results showed that si-1 could achieve 70 percent of inhibition (Fig. 4a and b), so we selected si-1 for the following experiments. The results showed that A549 and PC-9 cells were proliferating faster after knocking down of LSAMP gene (Fig. 4c and d). Overexpressed A549 and PC-9 cells and control groups were treated with actinomycin D (the concentration of actinomycin D was 2 mg/L). The results suggested that the degradation rate of LSAMP gene in A549 and PC-9 cells of over-expressed Lnc-LSAMP-1 was lower than that of the control groups (Fig. 4e and f), which means that Lnc-LSAMP-1 may have the function of protecting LSAMP gene.

Lnc-LSAMP-1 enhances the susceptibility of TKI

We compared the sensitizing effect of Lnc-LSAMP-1 on TKI and cisplatin by inhibition rate. Proliferation inhibition rate = (experimental group (dosing) - experimental group) / (control group (dosing) - control group) × 100%. In the Nilotinib group, the over-expressed Lnc-LSAMP-1 A549 cell inhibition rate in test group was 1.01 times that of the control group. It’s 0.8 times in the PC-9 cells (Fig. 5a, b, c and d). However, the effect was not obvious in the carboplatin group. The over-expressed Lnc-LSAMP-1 A549 cell inhibition rate was 0.10 times that of the control group, and in PC-9 there was no effect (Fig. 5e, f, g and h).
Discussion

Mounting evidence indicates that IncRNAs are involved in the development of cancer even if some of their roles in lung cancer were poorly understood[30]. The Lnc-LSAMP-1 is closely related to the development of lung cancer from TCGA database. Firstly, the different level of expression of Lnc-LSAMP-1 in lung cancer tissues and adjacent normal tissues was up to 50 (0.04:2.04. from GEPIA database) times. Secondly, the expression level of Lnc-LSAMP-1 was closely related to the development and prognosis of lung cancer. Encouraged by these results, we highly suspected that Lnc-LSAMP-1 is involved in the development of lung cancer. In our experiments we also discovered that Lnc-LSAMP-1 is markedly down-regulated in lung tumor tissues and cell lines. The expression levels of Lnc-LSAMP-1 in patients with NSCLC were associated with stage (TNM) and N status. Additionally, the low expression of Lnc-LSAMP-1 in lung cancer tissues was associated with poor survival rate of NSCLC patients. Thus, we constructed overexpressed Lnc-LSAMP-1 vector in lung cancer cells (A549, PC-9) using lentivirus. We found that overexpression of Lnc-LSAMP-1 inhibited proliferation, viability, invasion and migration ability, arrested cell cycle and facilitated apoptosis. Therefore, these results indicated that Lnc-LSAMP-1 might be a potential tumor suppressor in lung cancer cells. Moreover, survival analysis suggested that down-regulation of Lnc-LSAMP-1 expression in tissues was associated with poor survival rate of NSCLC patients, so we conducted chemotherapy sensitization experiments. The results showed that overexpression of Lnc-LSAMP-1 enhanced TKI inhibition of lung cancer cell proliferation, which is probably related with the prognosis of the lung cancer patients.

There is still an abundant amount of uncharted IncRNAs remaining to be elucidated in terms of their effects on cancer risk[31]. The chromosome location can provide a meritorious hint to the function of IncRNAs as a lot of IncRNAs have the potential to
regulate neighbor-coding genes[32]. When we examined the Lnc-LSAMP-1 gene information, we found that there is a sense of mRNA, which is "LSAMP". The LSAMP gene is located on chr3:115528941-117716095 in front of Lnc-LSAMP-1 and some of their sequences overlap. Pearson correlation analysis showed that the expression of Lnc-LSAMP-1 in lung cancer tissues is highly related to the expression of the gene LSAMP. The correlation is the highest in the TCGA database (figure S1a).

LSAMP (limbic system-associated membrane protein) is a self-binding, antibody-like cell surface adhesion protein[33]. The findings of SanzRL showed that overexpression of the LSAMP gene and NTM suppresses outgrowth from DRG neurons. The LSAMP gene locus is activated through recurrent deletions and has been reported in osteosarcoma[34, 35], acute myeloid leukemia[36], renal carcinoma[37] and ovarian carcinoma[38]. Single nucleotide polymorphism within the first intron of LSAMP gene has recently been shown to be a predictor of prostate cancer-specific mortality[39]. Furthermore, alterations of ZBTB20, GAP43 and GSK3B adjacent to LSAMP genes are less clear but they are suspected to have suppressed cancerous functions[40, 41]. Tale Barøy, et al. also reported that re-expression of LSAMP inhibits the growth of osteosarcoma cells and confirmed that LSAMP has tumor suppressor function[42].

Previous studies showed that LSAMP found in the cortical and subcortical areas of the limbic system play a pivotal role in the regulation of emotional behaviors[43]. LSAMP is one of the four IgLONs that constitute the immunoglobulin superfamily. The IgLONs as cell adhesion molecules, are positively involved in modification of cell-cell recognition[44]. Chen et al. found that LSAMP has been recognized as a translocation breakpoint-spanning gene in familiar clear cell renal cell carcinoma. In addition, he pointed out that the expression of LSAMP protein in cell lines with LSAMP promoter methylation inhibited cell proliferation[37]. Based on this information, LSAMP may function as a tumor suppressor
gene in cancer development.

However, the molecular mechanisms behind how Lnc-LSAMP-1 suppresses cell proliferation, cell migration, cell invasion and tumor metastasis remain unclear. To examine the relationship of LSAMP and Lnc-LSAMP-1, we found that LSAMP is the most relevant target gene for Lnc-LSAMP-1 in TCGA database, which is consistent with our data. The correlation analysis indicated that the expression of LSAMP is highly related to Lnc-LSAMP-1 in lung cancer tissues. Hence, we suspected that Lnc-LSAMP-1 suppresses proliferation, viability, invasion and migration ability, arrests cell cycle and facilitates apoptosis by regulating LSAMP gene.

Conclusions

In conclusion, our study demonstrated that Lnc-LSAMP-1 is one of the down-regulated LncRNAs in NSCLC, and showed that Lnc-LSAMP-1 was involved in the prognosis of NSCLC for the first time. Low Lnc-LSAMP-1 expression was associated with poor survival rate of NSCLC patients. Furthermore, the co-expression of Lnc-LSAMP-1 and LSAMP genes in lung cancer cells may affect the lymph node metastasis of lung cancer through cell adhesion. We believe that our results may provide a potentially therapeutic and prognostic target for NSCLC.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Guangzhou Medical University and Soochow University.

Consent for publication

We have obtained consents to publish this paper from all the participants of this study.

Availability of data and materials
The datasets are available from TCGA (http://cancergenome.nih.gov/).

**Competing interests**

The authors have declared no conflicts of interest.

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**Authors’ contributions**

Jiachun Lu designed the study. Wei Gong performed the most experiments. Yinyan Li analyzed the qRT-PCR results. Fuman Qiu, Mingzhu Lin Jianfeng Xian and Xin Zhang collected the tissue samples. Yuanyuan Wang wrote the paper. Lei Yang, Xiaoxiao Lu, Soham Datta and Yifeng Zhou revised the paper writing.

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**Abbreviations**

IncRNA: long non-coding RNA; LSAMP: limbic system-associated membrane protein; RNA-seq: RNA sequencing; ncRNA: non-coding RNA; TCGA: The Cancer Genome Atlas.
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Supporting Information

**Fig. S1** (a) Predictive analysis of *Inc-LSAMP-1* target genes in the GEPIA data. (b) The positions of *LSAMP* gene and *Inc-LSAMP-1* overlap partially in the UCSC data.

**Fig. S2**. (a) (b) *Lnc-LSAMP-1* was down regulated in LUAD and LUSC similar to *LSAMP* gene by GEPIA data. (c) The correlation between *LSAMP* gene and *Lnc-LSAMP-1* in lung cancer tissues by GEPIA data (d) Kaplan-Meier analysis of patients in low expression of *Inc-LSAMP-1* group and control group. (e) (f) (g) Low expression of *Lnc-LSAMP-1* was significantly correlated with T status, N status and Stage(TMN) by TCGA data. Significance was defined as p<0.05 (*, p<0.05; **, p<0.01; ***, p<0.001).

**Figures**
(a) The expression of Lnc-LSAMP-1 in lung cancer and normal cell lines (b) Lnc-LSAMP-1 expression levels in 170 pair-matched tumor tissues and adjacent normal tissues measured by qRT-PCR. (c) LSAMP gene expression levels in 86 pair-matched tumor tissues and adjacent normal tissues measured by qRT-PCR. (d) The correlation between Lnc-LSAMP-1 and LSAMP gin 143 pairs of lung cancer tissues. (e) Subcellular localization of Lnc-LSAMP-1 expression. (f) Kaplan-Meier analysis of patients in low expression of Lnc-LSAMP-1 group and control group. (g) High expression of Lnc-LSAMP-1 and LSAMP gene in A549 and PC-9 cells that transfected with overexpressed Lnc-LSAMP-1. Significance was defined as p<0.05
Overexpression of endogenous Lnc-LSAMP-1 inhibits lung cancer cell proliferation in vitro. A549 and PC-9 cells were transfected with overexpressed Lnc-LSAMP-1 vector (pLNC-LSAMP-1) and empty vector as control. (a) Cell proliferation assay in Incucyte zoom (Essen BioScience Co., Ltd., USA). (b) CCK8 assay was performed to determine the cell proliferation. (c) Clone formation was performed to
determine the cell proliferation. Migration (d) and invasion (f) capacities determined by Transwell assays. (e) The flow cytometry was conducted to determine the cell cycle of A549 and PC-9 cells. (g) The flow cytometry was conducted to determine the cell apoptosis of A549 and PC-9 cells. The results from three independent experiments, showed as mean ± s.d. Significance was defined as p<0.05 (*, p<0.05; **, p<0.01; ***, p<0.001).
Overexpression of Lnc-LSAMP-1 inhibited proliferation and metastasis of A549 and PC-9 cells in vivo. (a) Nude mice inoculated subcutaneously with A549 and PC-9 cells. (b) (c) Tumor growth rate and tumor volume are compared in Lnc-LSAMP-1 overexpression group and control group. (d) Lung images observed after treatment of nude mice in different groups of the two cell lines and the black circles in the figure refer to suspected metastases. (e) The number of suspected cancerous lesions in the naked eyes of nude mice after sacrificed in nude mice. (f) A subcutaneous tumor-forming HE section of nude mice. (g) A HE slice of suspicious lung nodules in nude mice injected with tail vein injection. Red box
shows potential cancer areas. Significance was defined as $p<0.05$ (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).
Figure 4

(a) (b) The efficiency of si (LSAMP gene) measured by qRT-PCR. (c) (d) The cell proliferation rate was compared with or without si-1. (e) (f) Over-expressed A549 and PC-9 cells and control were treated with actinomycin D (the concentration of actinomycin D was 2 mg/L).
(a) (b) (c) (d) The inhibition rate of Nilotinib in A549 and PC-9 cells. (e) (f) (g) (h) The inhibition rate of carboplatin in A549 and PC-9 cells.

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