The positive feedback loop of RNASEH1-AS1/has-miR-218-5p/NET1 mediated by POU2F1 contributes to the development and progression of human lung squamous carcinoma

CURRENT STATUS: UNDER REVIEW

BMC Cancer  BMC Series

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DOI: 10.21203/rs.3.rs-18125/v1

SUBJECT AREAS
- Cancer Biology
- Oncology

KEYWORDS
- LUSC, IncRNA, RNASEH1-AS1, has-miR-218-5p, NET1, POU2F1
Abstract

Background In molecular level, competing endogenous RNAs (ceRNAs) regulates other RNA transcripts through competing for shared microRNAs (miRNA). miRNA negatively regulate gene expression at the levels of mRNAs stability and translation suppression.

Methods We tested the mRNA level of miR-218-5p and RNASEH1-AS1 in clinical lung squamous cell carcinoma tissues by qRT-PCR. In the exploring of the role of miR-218-5p and RNASEH1-AS1 in the malignant phenotype of NCI-H520 cells, colony formation and MTT assay were used to test the cell viability and proliferation capability, trans-well invasion and wound healing assay were performed to examine the cell migration and invasion. ChIP assay was conducted to confirm the direct interact of POU2F1 and RNASEH1-AS1 promoter.

Results In this investigation, we found that LncRNA RNASEH1-AS1 is up-regulated in human lung cancer, and serves as a miRNA sponge for hsa-miR-218-5p in human lung squamous carcinoma cells. IncRNA RNASEH1-AS1 facilitates growth and motility of lung squamous carcinoma cells, while miR-218-5p does the opposite. NET1 and POU2F1 are validated as direct and functional targets of miR-218-5p. The downregulation of miR-218-5p releases the suppression of NET1 and POU2F1. POU2F1 binds directly to the IncRNA-RNASEH1-AS1 promoter and acts as transcription factor to enhance the promoter activity of RNASEH1-AS1.

Conclusion Above all, the positive feedback loop of RNASEH1-AS1/ hsa-miR-218-5p/ NET1/ POU2F1 can help us to understand the regulatory mechanism behind genesis and progression of human lung squamous carcinoma, possibly providing new biomarkers for its diagnosis and treatment.

Background

Lung cancer is the most regular and fatal cancer throughout of the world [1] and is recognized as a cluster of distinct diseases with high genetic, cellular and molecular heterogeneity [2–4]. In general, 80–85% of the human lung cancers are non small cell lung cancer (NSCLC), of which lung adenocarcinoma (LUAD) and lung squamous carcinoma (LUSC) are the main subtypes. Moreover, the genetic and epigenetic alterations between LUSC and LUAD are extremely different. The exploitation of targeting drugs for particular gene mutations has dramatically improved the treatment for
advanced LUAD patients. However, only a small part of LUSC contains driver gene mutations, leading to an extremely low five-year survival rate because of compromised efficacy of platinum-based chemotherapy in LUSC [5]. Thus, it is crucial to further address the molecular mechanisms underlying the pathogenesis of LUSC for more effective therapy options.

Recent investigations indicate unexpectedly large group of non-protein-coding transcripts consist in mammalian cells, which called non-coding RNA (ncRNA) [6-9]. Different from small ncRNAs, such as miRNAs, siRNAs and piRNAs, long ncRNAs (lncRNAs) are a new class of mRNA-like transcripts with size longer than 200 nucleotides [10]. Mature miRNAs regulate the expression of most protein-coding genes by binding to the 3’-UTR of its target gene thereby leading to degradation of target mRNA and depression of protein translation [11, 12]. In contrast, lncRNAs play their roles through acting as molecular decoys for miRNAs in the cytoplasm and cell nucleus [13, 14]. lncRNA dysregulation was demonstrated in many kinds of cancers, along with its importance in key cancer signaling networks and manlignant behaviors [15], such as in prostate cancer [16], breast cancer [17] and liver cancer [18, 19]. Further study concerning lncRNAs and miRNAs dysregulated in LUSC is still in urgent need to be expanded. RNASEH1-AS1 (RNASEH1 antisense RNA 1) located at chromosome 2p25.3. RNASEH1-AS1 was highly expressed in lung cancer in our early stage screening. However, the role of RNASEH1-AS1 in LUSC progression and the possible target miRNAs were previously not well investigated in other reports. In order to explor the effect of RNASEH1-AS1 in LUSC, we conducted the in vitro cell experiment and in vivo xenograft assay. miR-218 function as lncRNA targets and influence cancer cell malignant behaviors of many cancers, such as hepatocellular carcinoma [20], pancreatic cancer [21] and breast cancer [22]. We found that the expression of miR-218-5p was suppressed in clinical LUSC tissues.

POU2F1 (POU class 2 homeobox 1) also known as OCT1, OTF1 or oct-1B. The POU2F1 transcription factor was among the first identified members of the POU transcription factor family [23]. It is a ubiquitous transcription factor that regulates transcription of genes involved in inflammation and cell cycle by binding to cis-acting octamer elements [24]. In our study, we investigated the role of miR-218-5p on POU2F1 expression and the relationship between POU2F1 and RNASEH1-AS1 expression in
LUSC cells. NET1 (neuroepithelial cell transforming 1) gene is part of the family of Rho guanine nucleotide exchange factors. The protein encoded by this gene interacts with RhoA within the cell nucleus and may play a role in repairing DNA damage after ionizing radiation. NET1 is reported overexpressed in many human cancers, including non small cell lung cancer [25–27]. We further examined its expression and its upstream regulation network in LUSC.

Methods

Bioinformatic prediction and screening.

The long non-coding RNA highly expressed in lung cancer was screened by the StarBase V3.0 (http://starbase.sysu.edu.cn/). The miRNA combined with the IncRNA and the possible downstream targets of miR-218-5p were predicted by the most usually used algorithms of TargetScan, miRDB and PicTar, and the overlap part of the three algorithms came into our notice. The putative promoter of miR-218-5p was predicted through Promoter Scan (http://www-bimas.cit.nih.gov/molbio/proscan/).

Cell line and human LUSC tissue

The NCI-H520 cells were cultured with the RPMI-1640 (Gibco, Grand Island, NY, USA) contained 10% fetal bovine serum and 1,000 U/mL P/S. The human LUSC cells were cultured at 37℃ in a humidified atmosphere with 5% CO2. The cell transfection was performed with Lipofectamine 2000 Reagent (Invitrogen, USA) following the manufacturer’s instructions. The NCI-H520 cells and three pairs of human lung squamous cell carcinoma tissues were provided by the Department of Cancer Institute, North China University of Science and Technology Affiliated People’s Hospital. The number, gender, classification and age of the LUSC patients were shown in Table 1. Informed consent was obtained from all subjects or their direct relatives. The cell and tissue studies were submitted to and approved by both the Ethics Committee of North China University of Science and Technology and the Ethics Committee of Hebei Medical University.

| Number   | Gender | Classification | Age | Date    |
|----------|--------|----------------|-----|---------|
| 370823   | Male   | LUSC           | 63  | 2019.2.26 |
| 371406   | Male   | LUSC           | 50  | 2019.3.6  |
| 376552   | Male   | LUSC           | 62  | 2019.4.29 |

qRT-PCR.
The total RNA of lung cancer tissues and cell line were extracted with the mirVana miRNA Isolation Kit (Ambion, USA) following the manufacturer’s instruction. One µg of RNA was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Takara, Japan). The qRT-PCR was conducted with a SYBR® Taq™ kit (Takara Bio, Japan) and the iQ5 Real Time PCR Detection System. The relative fold-change of hsa-miR-218-5p in the transcripts was calculated using U6 as the internal control. The relative fold-change of RNASEH1-AS1, NET1 and POU2F1 were calculated using β-actin as the internal control. All the RT and qPCR primer sequences were listed in the Table 2.

Table 2
The PCR Primers Used in This Work.

| Primer Name | Primer Sequence (5’ →3’) |
|-------------|---------------------------|
| hsa-miR-218-5p-RT | GTCGTATCCAGTGCAAGGATCGCTGCCACGGATAC |
| hsa-miR-218-5p-Fwd | TGCGGTTGTGCTCTGGTAC |
| U6 RT | GTCGTATCCAGTGCAAGGATCGCTGCCACGGATAC |
| U6 Forward | TGGCGGTGGTCCTGCCTGGCAC |
| Reverse | CACGCTAGGAGGTGTGCTG |
| RNASEH1-AS1-qPCR-S | GAAAGCACCCGCACCTGG |
| RNASEH1-AS1-qPCR-AS | GCCCTCAATCACCACACT |
| NET1-qPCR-S | GAAAGGTAATCCAGGATG |
| NET1-qPCR-AS | GTGCCGTTGCTGCCTGGT |
| POU2F1-qPCR-S | TGGAGGCTAGGGCAGAGG |
| POU2F1-qPCR-AS | CGTGCATATAGGAGAAGCTG |
| β-actin-qPCR-S | CTAGAAAGCATTGAGCAGGAC |
| β-actin-qPCR-AS | CTAGAAAGCATTGAGCAGGAC |
| RNASEH1-AS1-Chip-S | TCTGAGGGAGACTGATTC |
| RNASEH1-AS1-Chip-AS | ACAGGTGTAGAGTCTGACGTGC |
| RNASEH1-AS1-target-Top | AAACCTAGGAATATGAATAAATAGCACAAAGAGG |
| RNASEH1-AS1-target-Bot | CTAGAAGCTATGAGAATAAATAGCACAAAGAGG |
| RNASEH1-AS1-target-mut-Top | AAACCTAGGAATATGAATAAATAGCACAAAGAGG |
| RNASEH1-AS1-target-mut-Bot | CTAGAAGCTATGAGAATAAATAGCACAAAGAGG |
| NET1-3’UTR-Top | AAACCTAGGAATATGAATAAATAGCACAAAGAGG |
| NET1-3’UTR-Bot | CTAGAAGCTATGAGAATAAATAGCACAAAGAGG |
| NET1-3’UTR-mut-Top | AAACCTAGGAATATGAATAAATAGCACAAAGAGG |
| NET1-3’UTR-mut-Bot | CTAGAAGCTATGAGAATAAATAGCACAAAGAGG |
| POU2F1-3’UTR-Top | AAACCTAGGAATATGAATAAATAGCACAAAGAGG |
| POU2F1-3’UTR-Bot | CTAGAAGCTATGAGAATAAATAGCACAAAGAGG |
| POU2F1-3’UTR-mut-Top | AAACCTAGGAATATGAATAAATAGCACAAAGAGG |
| POU2F1-3’UTR-mut-Bot | CTAGAAGCTATGAGAATAAATAGCACAAAGAGG |
| sh-POU2F1-Top | GATCCCACGTCACACACAAAGAGCAGATCTCGAGATTC |
| sh-POU2F1-Bot | GATCCCACGTCACACACAAAGAGCAGATCTCGAGATTC |
| Sh1-RNASEH1-AS1-top | GATCCCAGAGAGCACCAGCAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTG4
Plasmid and miRNA mimics.

The miR-218-5p overexpression mimics and ASO-miR-218-5p were commercially synthesized. The sequence was listed in Table 2. The sh-RNASEH1-AS1 and sh-POU2F1 pSilencer vector were generated by annealing the sense and antisense strands of the hairpin RNA following by insertion onto the pSilencer 2.1-U6 neo vector between the BamHI and HindIII sites.

The two strands of both wildtype and mutant 3'-UTR of the NET1 or POU2F1 gene harboring the predicted miR-218-5p binding area were annealed followed by insertion into the upstream of the reporter gene of pmirGLO vector. Similarly, the putative promoter area and miR-218-5p binding region of lnc-RNASEH1-AS1 were amplified by PCR and inserted into the upstream of the reporter gene of pGL3-basic-luciferase vector.

All the insertions mentioned above were verified by DNA sequencing. All primers used in this work were shown in Table 2.

Dual-luciferase reporter assay.

NCI-H520 cells were cultivated in 48 well plate at a density of $6 \times 10^4$ every well. miR-218-5p transfection reagents were prepared at the final density of 20 µM and incubated for 4 h. After 24 h, pmirGLO-targetgene-3'UTR /mut transfection regents were added into cultured cells at the final density of 0.5 µg. After transfection for 4 h, the transfection mixture was replaced with 300 µL of new complete 1640 medium. After cotransfection for 48 hours, the luciferase activity was tested following the instructions of the Dual-Luciferase Reporter System (Thermo, USA).

Cell viability and proliferation assay.

In the MTT assay, NCI-H520 (1000 per well) cells were planted into 96 well plates. 24, 48, and 72 h after transfection, MTT was added into every well, and the plates were cultured for 4 h. The absorbance at a wavelength of 570 nm was measured to evaluate the cell viability.

In the colony formation assay, NCI-H520 (200 per well) cells after transfection were trypsinized, and planted into 12 well plates and cultured for 7 to 14 days at 37 °C. The colonies were dyed with dyeing solution with 0.2% crystal violet and 20% methanol. Colonies of cell number over 50 were counted and analyzed. Colony formation rate calculating: colony formation rate = (number of colonies /
number of seeded cells) × 100%. All cell experiments were conducted for over three times.

Trans-well invasion and wound healing assay.

In the trans-well invasion assay, NCI-H520 cells (1 × 10^5 per well) were put into the upper chamber of every insert (Milipore, USA) containing 50 µl of matrigel (Milipore, USA). 800 µl of DMEM medium supplemented with 20% FBS (JIBCO, USA) was added onto the lower chambers. After 72 h, cells attached to the lower surface were stained for 15 min with crystal violet. After that we take pictures for counting.

In the wound-healing assay, NCI-H520 cells were cultured in 12 well plates. When cell confluence reached 70%-80%, scratches were made by a 50 ml pipette tip, and non-adherent cells were removed by three times of PBS wash. Wounded cells were cultured in medium containing non FBS for 0, 24, and 48 hour. Three fields of view were randomly captured for every well.

Western blot.

NCI-H520 cells were collected and lysed with lysis buffer (100 mM Tris-HCl, 2% SDS, 1 mM Mercaptoethanol and 25% Glycerol). Cell extracts were heated in loading buffer and the same amount of cell extracts were run on a 10% SDS-PAGE gels. After being electrophoretically transferred to a PVDF membranes (Millipore, USA), the protein bands were probed with its corresponding primary antibodies (anti-NET1, anti-POU2F1, Saier Biotechnology, china), and cultured overnight at 4 °C. The secondary antibody (anti-GAPDH, Saier Biotechnology, Tianjin, China) was added and incubated at room temperature for 1.5 hour. The PVDF membranes were washed in PBS for four times and the immunoreactive target bands were visualized by the chemiluminescence imaging system (Huqiu Image Instruments, Suzhou, China). The band intensities were quantified by Lab Works image analysis software (UVP, USA).

Animal model.

Twenty BALB/c-nu mice (female) of 5 to 6 week old were purchased from the institute of experimental animals, Chinese academy of medical sciences (Beijing). They were randomly divided into two groups. In total, 2 × 10^7 NCI-H520 cells were transfected with siR- RNASEH1-AS1 or siR-NC and were
suspended in 100 µl of serum free RPMI1640 for every nude mouse. The cells suspension was injected directly into the left back of the mice. The tumor volume was measured every three days after injection. Four weeks later, the mice were sacrificed by the method of cervical dislocation after anesthesia, and their tumors mass were harvested. No mice dead before this. The tumor weight was measured and the average tumor weight was calculated. The tumor tissues were stored in -80°C or used to perform Hematoxylin-Eosin (HE) and immunohistochemical staining. All studies were performed under the American Association for the Accreditation of Laboratory Animal Care guidelines and adhered to national and international standards. All animal works were approved by the Ethics Committee of Hebei Medical University.

CHIP assay.

The combination of POU2F1 to promoter of Inc- RNASEH1-AS1 was confirmed by chip assay following the instructions of Chromatin Immunoprecipitation Kit (milipore, USA). Primers flanking the predicted POU2F1 binding site in the Inc- RNASEH1-AS1 promoter were used in PCR. DNAs were purified using the Chromatin Immunoprecipitation Kit (milipore, USA). The primers used in this study were shown in Table 2.

Statistical evalution.

The data was analyzed by GraphPad Prism 6 Software (GraphPad Software, USA) with the two-tailed Student’s t test. The results were presented as mean ± S.D. of three separate experiments. Unpaired Student’s t-test was used to compare the two groups. p value of less than 0.05 was regarded as statistically significant (* p<0.05, ** p<0.01, ***p<0.001).

Results

LncRNA RNASEH1-AS1 is highly expressed and acts as miRNA sponge for miR-218-5p in human lung cancer.

To find the LncRNA dysregulated in human lung cancer, we used the StarBase V3.0. The screening results reviewed that RNASEH1-AS1 was highly expressed in 526 cancer samples compared to 59 normal samples (Fig. 1A). We also detected the mRNA expression level of RNASEH1-AS1 in three pairs of clinical lung squamous cell carcinoma tissues, and find that RNASEH1-AS1 expression was higher in
LUSC tissues compared to the adjacent normal tissues (Fig. 1B). Base-pairing complementation showed that lnc-RNASEH1-AS1 contains a putative binding site that has obvious complementarity with the seed region of miR-218-5p (Fig. 1C). When the wildtype of lncRNA RNASEH1-AS1 was cotransfected with miR-218-5p mimics or ASO-218-5p, the luciferase activity of NCI-H520 cells were obviously weaken or enhanced than their corresponding control (Fig. 1C). However, both overexpression and blocking of miR-218-5p did not have apparent effect on the luciferase intensity of cells transfected with lnc-RNASEH1-AS1 mutant (Fig. 3C).

To check the influence of miR-218-5p on endogenous lncRNA RNASEH1-AS1, the mRNA level of RNASEH1-AS1 was examined using qRT-PCR. The data showed that the expression level of RNASEH1-AS1 was reduced after miR-218-5p overexpression (Fig. 1D). Meanwhile, a small interfering RNA (siRNA) for RNASEH1-AS1 was synthesized and the effect of pSilencer/shRNA-RNASEH1-AS1 was verified by qRT-PCR (Fig. 1E). Knockdown of RNASEH1-AS1 could result in 2.9-fold increases in endogenous miR-218-5p mRNA level of NCI-H520 cells (Fig. 1F). In contrast to lncRNA RNASEH1-AS1, the miR-218-5p expression in clinical lung squamous cell carcinoma samples was lower than in adjacent normal samples (Fig. 1G).

miR-218-5p suppresses proliferation and motility of LUSC cells in vitro, while lncRNA-RNASEH1-AS1 does the opposite.

To explore the impact of miR-218-5p in the malignant phenotype of LUSC cells, we transfected its over-expressing and ASO mimic into NCI-H520 cells and performed some functional experiment. In the MTT assay, miR-218-5p obviously reduced the OD value of NCI-H520 cells at 48 and 72 hour after transfection, while its ASO mimics enhanced cell viability at 24, 48 and 72 hour respectively (Fig. 2A). As is shown in the colony formation assay, miR-218-5p apparently decreased while its ASO mimics increased the colony formation rate of NCI-H520 cells compared to its corresponding control (Fig. 2C). In other words, miR-218-5p could inhibit both the viability and the growth of LUSC cells in vitro.

In order to investigate the function of miR-218-5p in cell motility, we also conducted trans-well invasion and wound-healing assay in NCI-H520 cells. The data revealed that miR-218-5p could lessen the number of invasive cell (Fig. 2E) and enlarge the distance of cell gaps at both 24 and 48 hours.
(Fig. 2G) compared with the control group, while its ASO mimic does the opposite (Fig. 2E and 2G).

Our study focused on Inc-RNASEH1-AS1 at the same time. The results reviewed that the cell viability (Fig. 2B), growth (Fig. 2D), migration (Fig. 2G) and invasion (Fig. 2F) of NCI-H520 cells was suppressed after interfering of Inc-RNASEH1-AS1. miR-218-5p ASO mimics could counteract the growth inhibition effect of sh-RNASEH1-AS1 on LUSC cells (Fig. 2D), but have no significance difference on cell viability, migration and invasion (Fig. 2B 2F and 2G).

NET1 and POU2F1 were identified as direct targets of miR-218-5p. To know the mechanism how miR-218-5p regulates the oncogenesis of lung squamous carcinoma cells, we screened the potential downstream targets of miR-218-5p by TargetScan, miRDB and PicTar (Fig. 3A). Among the overlapping target genes, NET1 and POU2F1 were selected for further investigation.

Both the 3’ UTR of NET1 and POU2F1 contain putative sites that are conserved among many species and the sites have apparent complementarity with the seed region of miR-218-5p according to the base-pairing rules (Fig. 3B and 3C). The 3’UTR of NET1 or POU2F1 was co-transfected with miR-218-5p into NCI-H520 cells. miR-218-5p led to reduced fluorescence intensity while its ASO mimics enhanced the fluorescence intensity by dual-luciferase reporter assay (Fig. 3B and 3C). On the contrary, neither overexpression nor knockdown of miR-218-5p had obvious influence on the fluorescence intensity of NCI-H520 cells transfected with 3’UTR of NET1 or POU2F1 that contains mutated miR-218-5p binding sequence (Fig. 3B and 3C). These results discovered that miR-218-5p directly binds to the 3’-UTR of NET1 and POU2F1. qRT-PCR reviewed that miR-218-5p downregulates mRNA level of endogenous NET1 and POU2F1 in NCI-H520 cells (Fig. 3D). Western analysis revealed that miR-218-5p apparently reduced the protein expression level of endogenous NET1 and POU2F1 (Fig. 3E and 3F), meaning that miR-218-5p negatively regulates NET1 and POU2F1 expression by binding to their 3’-UTR.

We examined the mRNA expression of NET1 in three pairs of clinical lung squamous cell carcinoma samples subsequently. As our expected, NET1 was higher in LUSC tissues than in corresponding normal tissues (Fig. 3G), which is contrary to miR-218-5p and similar to Inc-RNASEH1-AS1.

Downregulation of IncRNA-RNASEH1-AS1 inhibites tumor growth of LUSC cells in vivo.
To know the effect of lncRNA- RNASEH1-AS1 in vivo, we conducted tumorgenicity assay on nude mice. Twenty mice were randomly divided into two groups for ten mice per group. NCI-H520 cells transfected with siR- RNASEH1-AS1 or siR-NC were subcutaneous injected into the left back of the nude mice. Figure 4A showed the two groups of nude mice 4 weeks after injection, and Fig. 4B is the tumor mass after sacrificed. The tumor in nude mice grew slower in the Inc-RNASEH1-AS1 interfering group than its control group (Fig. 4C), and the average tumor volume of the Inc-RNASEH1-AS1 interfering group was smaller than control group (Fig. 4D). The weight of tumor mass was lighter than its control group (Fig. 4E). Figure 4F showed the loci of tumors derived from transfected cells by Hematoxylin-Eosin (HE)-staining and the NET1 or POU2F1 expression by immumohistochemical staining. NET1 and POU2F1 were significantly reduced after interfering of RNASEH1-AS1 by immumohistochemical staining (Fig. 4F). The mRNA expression level of miR-218-5p was apparently increased and the targets of miR-218-5p were decreased in xenograft tumors after interfering of RNASEH1-AS1 by qRT-PCR (Fig. 4G). Moreover, the protein level of its targets also decreased by western-blot (Fig. 4H). These data revealed that downregulation of Inc- RNASEH1-AS1 promoted the up-regulation of miR-218-5p and down-regulation of its target genes, and suppressed the growth of lung squamous carcinoma cells in vivo.

POU2F1 binds directly to the IncRNA-RNASEH1-AS1 promoter and stimulates its promoter activity. To verify whether POU2F1 is potential transcription factor of RNASEH1-AS1 gene, we constructed a POU2F1 interfering plasmid and validated its efficiency by qRT-PCR (Fig. 5A). As can be seen in Fig. 5A and 5B, knockdown of POU2F1 effectively reduced the mRNA expression and promoter luciferase activity of RNASEH1-AS1. ChIP assay was conducted with anti-POU2F1 antibody using NCI-H520 cell lysates, followed by PCR with primers which can amplify RNASEH1-AS1 promotor. The band indicated the direct interact and positive relationship of POU2F1 with the RNASEH1-AS1 promotor (Fig. 5C and 5D). Figure 5E is a sketch map of the regulatory mechanism of IncRNA-RNASEH1-AS1/hsa-miR-218-5p/ NET1/ POU2F1 in human lung squamous carcinoma cell.

Discussion
Recent reports have discovered that the combination of the miRNA seed sequence with mRNA is not
uni-directional, but that the pool of pseudogenes, mRNAs, long non-coding RNAs (IncRNA), circular RNA (circRNA) compete for the same pool of miRNA [28, 29]. These competitive endogenous RNAs (ceRNAs) serve as molecular sponges for a miRNA by their miRNA binding sites, consequently derepressing all targets of the corresponding miRNA family. More and more researchers pay close attention to IncRNA as competing endogenous RNA for miRNA during tumorigenesis and progression in recent years. Numerous of IncRNAs are abnormally expressed or mutated in many types of cancers [30]. HULC was upregulated in both tumors and plasma of hepatocellular carcinoma patients, and was a possible biomarker for HCC [31]. Abnormal X inactivation caused by aberrantly expressed XIST promotes carcinogenesis of leukemia [32]. PCA3 is over-expressed in ninety-five percent of prostate cancer clinical samples and is examined with high specificity in the urine of patients with benign and malignant prostate cancer [33, 34]. In our research, we firstly discovered that IncRNA-RNASEH1-AS1 was highly expressed in 526 lung cancer compared to 59 normal samples by screening on StarBase, and was higher in LUSC tissues than in the adjacent normal tissues. Moreover, the cell viability, proliferation, invasion and migration capacity of NCI-H520 cells was suppressed after interfering of RNASEH1-AS1, suggesting the oncogenic character of RNASEH1-AS1 in LUSC. We observed the same oncogenic effect of RNASEH1-AS1 in LUSC in the in vivo xenograft animal model as in vitro cell experiment.

Our study also discovered that RNASEH1-AS1 acts as a ceRNA by directly interaction with the seed region of miR-218-5p in LUSC. The expression of miR-218-5p in LUSC cells was suppressed due to the up-regulation of RNASEH1-AS1. Contrary to RNASEH1-AS1, miR-218-5p could inhibit LUSC cell growth and motility and function as a tumor suppressor gene in lung squamous carcinoma. Yu et al [35] recently found that circRNA-104718 acts as ceRNA and promotes HCC progression by the microRNA-218-5p-TXNDC5 signaling pathway. Li et al [36] found that oncogenic KSHV-encoded interferon regulatory factor up-regulates HMGB2 and CMPK1 to promote cell invasion through disrupting the IncRNA-OIP5-AS1-miR-218-5p network. Ye et al [37] also discovered that E2F1-mediated MNX1-AS1-miR-218-5p-SEC61A1 feedback loop involved in the progression of colon adenocarcinoma. Our results expand the recognition on miR-218-5p and its competitive endogenous RNA in the development and
progression of LUSC.

It is estimated that half of all genes of the genome are targets of miRNA, spanning a large layer of regulation on a post-transcriptional level [11]. We illustrated that both NET1 and POU2F1 were direct targets of miR-218-5p. miR-218-5p negatively regulates NET1 and POU2F1 expression by directly binding to their 3′-UTR in LUSC cells. The mRNA and protein level of NET1 or POU2F1 were decreased after interfering of RNASEH1-AS1 in xenograft tumor tissue. In other words, down-regulation of Inc-RNASEH1-AS1 led to the up-regulation of miR-218-5p, thereby resulting in the down-regulation of its target genes. POU2F1 also regulated by miR-665 and miR-9-5p in human osteosarcoma [38, 39], and by miR-449 in liver cancer [40]. It is the first evidence that identified POU2F1 and NET1 as the downstream moleculars of miR-218-5p and RNASEH1-AS1 in the lung squamous cell carcinoma. More importantly, our data revealed that interfering of POU2F1 attenuates the activity of RNASEH1-AS1 promoter in LUSC cells. The direct combination of POU2F1 and RNASEH1-AS1 promoter was validated by Chromatin immunoprecipitation. POU2F1 serves not only the downstream target gene of miR-218-5p but also the upstream pro-transcription factor of RNASEH1-AS1.

Conclusion

Taken together, our results revealed that RNASEH1-AS1 functions as an oncogene through acting as molecular sponge for miR-218-5p. The down-regulation of miR-218-5p releases the suppression of NET1 and POU2F1. POU2F1 binds directly to the RNASEH1-AS1 promoter to facilitate its expression, thereby forming a close regulatory loop of IncRNA-miRNA-GENE-TF in LUSC. These findings expand our understanding of the positive feedback loop of RNASEH1-AS1/ hsa-miR-218-5p/ NET1/ POU2F1 and the mechanism behind genesis and progression of human lung squamous carcinoma, possibly providing new biomarkers for its diagnosis and treatment.

Abbreviations

ceRNAs
Competing endogenous RNAs

miRNA
microRNA
LncRNA
Long non-coding RNA
CircRNA
Circular RNA
NSCLC
Non-small cell lung cancer
LUSC
Lung squamous cell carcinoma
LUAD
Lung adenocarcinoma
RNASEH1-AS1
RNASEH1 antisense RNA 1
POU2F1
POU class 2 homeobox 1
NET1
Neuroepithelial cell transforming 1

Declarations

**Ethics approval and consent to participate**

All studies were performed under the American Association for the Accreditation of Laboratory Animal Care guidelines and adhered to national and international standards. For obtaining the tissue specimen, informed consent was obtained from all subjects or their direct relatives. All cell and tissue studies were submitted to and approved by both the Ethics Committee of North China University of Science and Technology and the Ethics Committee of Hebei Medical University. All animal works were submitted to and approved by the Ethics Committee of Hebei Medical University.

**Consent for publication**

Not applicable.

**Availability of data and materials**

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

All authors declare that no conflicts of interest exist.

Funding

This work was supported by the Young Top-Notch talent Project of Hebei province (No. JI2016(10), Talent Project of Hebei province (A201801005), Academician Workstation Construction Special Project Of Tangshan People’s Hospital (199A77119H), Natural Science Foundation of Outstanding Youth of Hebei Province (H2019105026), and Basic Research Cooperation Project of Beijing-Tianjin-Hebei [H2019105143,19]CZDJC64500(Z)]. No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this study.

Authors’ contributions

GF S designed and supervised the completion of the experiment. GG S designed, directed the experiment and revised the manuscript. JH J wrote and revised the manuscript, and was a major contributor in performing the experiments and analyzing data. JW prepared the clinical samples and participated in performing the experiment and manuscript preparation. JR Y analyzed and interpreted the data. PG performed part of the experiment. YK L and YF L participated in the experimental coordination. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures
LncRNA RNASEH1-AS1 is ceRNA of miR-218-5p. (A) RNASEH1-AS1 expression in cancer and normal samples by StarBase V3.0 screening. (B) RNASEH1-AS1 expression in three pairs of clinical tissue samples by qRT-PCR. (C) The direct interaction between RNASEH1-AS1 and miR-218-5p was confirmed by luciferase report assay. (D) The influence of miR-218-5p on endogenous IncRNA RNASEH1-AS1 level by qRT-PCR. (E) The interfering effect of sh-RNASEH1-AS1 plasmid. (F) The influence of IncRNA RNASEH1-AS1 on endogenous miR-218-5p level by qRT-PCR. (G) miR-218-5p expression in three pairs of clinical tissue samples by qRT-PCR.
Figure 2

The function of miR-218-5p and RNASEH1-AS1 in LUSC cells. (A and B) MTT assay performed in NCI-H520 cells. (C and D) Colony formation assay performed in NCI-H520 cells. (E and F) Transwell invasion assay conducted with NCI-H520 cells. (G) Wound-healing assay of NCI-H520 cells
miR-218-5p directly targets to NET1 and POU2F1. (A) Target prediction by Target Scan, miRDB and Pic Tar. (B) The direct interaction between miR-218-5p and NET1 confirmed by luciferase report assay. (C) The direct interaction between miR-218-5p and POU2F1 confirmed by luciferase report assay. (D) The influence of miR-218-5p on endogenous NET1 or POU2F1 mRNA level by qRT-PCR. (E and F) The influence of miR-218-5p on endogenous NET1 and POU2F1 protein level by western blot. The full-length blots are presented in Supplementary Figure S1 and Supplementary Figure S2. (G) NET1 expression in three pairs of clinical tissue samples by qRT-PCR.
Figure 4
The tumor promoting effect of RNASEH1-AS1 in tumorigenicity assay in vivo. (A and B) The tumor mass generated from transfected NCI-H520 cells. (C) The tumor volume was measured every three days. (D and E) The average tumor volume and tumor weight was measured. (F) H-E staining reveals the interface of the metastatic tumor and the adjacent liver tissue. Bar, 200 µm. Immunohistochemical staining showed the NET1 and POU2F1 expression in xenograft tumor. (G) The NET1, POU2F1 and miR-218-5p mRNA level in xenograft tumor by qRT-PCR. (H) The NET1 and POU2F1 protein level in xenograft tumor by western blot. The full-length blots are presented in Supplementary Figure S3.
POU2F1 is transcription factor of RNASEH1-AS1. (A) The interfering effect of sh-POU2F1 plasmid. The decreased mRNA level of RNASEH1-AS1 after knockdown of POU2F1. (B) The decreased promoter activity of RNASEH1-AS1 after knockdown of POU2F1. (C and D) The positive relationship of POU2F1 with RNASEH1-AS1 expression in chip assay. The full-length blots are presented in Supplementary Figure S4. (E) The summary of this work.

Supplementary Files
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