A Novel Long Noncoding RNA (lncRNA), LL22NC03-N64E9.1, Promotes the Proliferation of Lung Cancer Cells and is a Potential Prognostic Molecular Biomarker for Lung Cancer

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

AE 1 **Hongyu Jing**
BF 1 **Xinglong Qu**
BDF 2 **Lingyun Liu**
CDG 1 **Huan Xia**

1 Department of Respiratory Medicine, First Hospital of Jilin University, Changchun, Jilin, P.R. China
2 Department of Andrology, First Hospital of Jilin University, Changchun, Jilin, P.R. China

Corresponding Author: Huan Xia, e-mail: xiaohuanzi@163.com
Source of support: Departmental sources

Background: The aim of this study was to investigate the expression of a novel long noncoding RNA (lncRNA), LL22NC03-N64E9.1, and its effect on the phenotype of lung cancer cells and tissues using The Cancer Genome Atlas (TCGA) RNA sequencing data and other publicly available profiling data.

Material/Methods: The lung cancer dataset GSE30219 was downloaded from the Gene Expression Omnibus (GEO) repository. Differentially expressed lncRNA, LL22NC03-N64E9.1, in 48 lung cancer tissue samples and adjacent normal lung tissues, normal lung cell lines BEAS-2B and A549, and lung cancer cell lines, H1703, and H292, were detected by quantitative reverse transcription polymerase chain reaction (PCR) (qRT-PCR). Interference efficiency was performed using small interfering RNA (siRNA). Tumor levels of lncRNA, LL22NC03-N64E9.1, and clinicopathological parameters were statistically analyzed.

Results: Analysis of the GSE30219 test cohort showed that lncRNA, LL22NC03-N64E9.1 expression was significantly increased in lung cancer. In clinical tissue samples, the level of LL22NC03-N64E9.1 in patients with lung cancer was significantly increased compared with adjacent normal lung tissues (P<0.001). The level of LL22NC03-N64E9.1 in patients with lung cancer was significantly correlated with tumor size and TNM stage (P<0.05), but not with age, sex and the presence of lymph node metastasis (P>0.05). In the H292 cells, following knockdown of LL22NC03-N64E9.1, cell proliferation and cloning were reduced.

Conclusions: Expression of lncRNA, LL22NC03-N64E9.1, promoted proliferation of lung cancer cells *in vitro*, was highly expressed in lung cancer tissues and was associated with increased overall survival (OS), tumor size, and tumor stage in patients with lung cancer.

MeSH Keywords: **Cell Proliferation • Lung Neoplasms • RNA, Long Noncoding**

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/908359
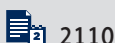
📄 2110   🗂 1   📊 3   📖 17

Background

Lung cancer is a malignant tumor originating from bronchial epithelial cells or bronchial glands. Worldwide, of all types of cancer, lung cancer the highest incidence and mortality rate of all forms of cancer and is a serious threat to human health and life [1]. The pathogenesis of lung cancer continues to be studied, and many aspects of its etiology and pathogenesis remain unclear, but lung cancer is known to be associated with smoking, occupational exposure and environmental exposure to carcinogens, ionizing radiation, previous chronic lung infections, air pollution, genetic, and other factors. The early stages of lung cancer may be difficult to diagnose, and research continues to develop biomarkers that may improve the early diagnosis of lung cancer, to improve treatment and patient prognosis in lung cancer.

Long non-coding RNAs (lncRNAs) are a class of RNA molecules with transcript lengths greater than 200 nt [2]. The lncRNAs have no function in encoding proteins but are capable of regulating the expression of genes at the epigenetic, and transcription levels [3,4], Studies have shown that lncRNA was widely involved in cell proliferation, differentiation, and apoptosis, and played an important role in the pathogenesis of many diseases [5], and in malignancy, the lncRNAs have been shown to be associated with tumor development, metastasis, patient prognosis, and response to treatment of cancer [6,7].

The LL22NC03-N64E9.1 gene is located at the chromosome locus 22q11.1, transcribes a 1388nt transcript, and has been reported to be involved in the occurrence and development of tumors [8]. Currently, no study has been performed on the relationship between expression of the lncRNA, LL22NC03-N64E9.1 and the incidence and prognosis of lung cancer.

Therefore, the aim of this study was to investigate the expression of the novel lncRNA, LL22NC03-N64E9.1 on lung cancer cells *in vitro* and human lung tissue, and its effect on the phenotype of lung cancer cells and tissues using The Cancer Genome Atlas (TCGA) RNA sequencing data and other publicly available profiling data.

Material and Methods

Acquisition of data and patient information

The lung cancer dataset GSE30219 was downloaded from the Gene Expression Omnibus (GEO) repository, corrected using the robust multi-array average (RMA) function, and analyzed differentially using the linear models for microarray (LIMMA) function. Lung cancer expression profiles were also downloaded from The Cancer Genome Atlas (TCGA) database and analyzed by EdgeR function. The study included 48 cases of lung cancer resection specimens and adjacent normal tissues, which were sampled.

The patient clinical data regarding the tumor diagnosis, clinical data, and patient survival data were collected for further analysis.

Cell culture of normal lung cell lines, BEAS-2B and A549 and lung cancer cell lines, H1703 and H292

Normal lung cell lines, BEAS-2B and A549 and lung cancer cell lines, H1703 and H292 were used, having been stored in liquid nitrogen. The frozen cells were quickly taken out of storage and mixed in a 37°C water bath with gently agitation to completely and rapidly thaw the cells, creating a cell suspension that was moved to a clean centrifuge tube. Then, 10 ml of fetal bovine serum (FBS) was added (Siji Qing, Hangzhou, China) containing 10% high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA). The cells were cultured in 5% CO_2, in a 37°C cell culture incubator.

Cell transfection

The lung cancer H292 cells were plated in 6-well plates and transfected with small interfering RNA (siRNA), including si-NC and si-LL22NC03-N64E9.1 sequences, according to the manufacturer's instructions for the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced six hours after transfection. The interference sequences were si-NC UUCUCCGAACGUGUCACGUTT; si- LL22NC03-N64E9.1 1 # UAGCUGGAGCAGUACAUCUUCAAUU; si- LL22NC03-N64E9.1 2 # AAGUAAUCAAGUUCCUGCUUUCUUG.

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Twenty-four hours after transfection, cells were collected in 1 ml of TRIzol, the total cellular RNA was extracted, and cDNA was reverse transcribed, according to the manufacturer's instructions. The expression levels of the novel long noncoding RNA (lncRNA), LL22NC03-N64E9.1 were detected by quantitative reverse transcription polymerase chain reaction (PCR) (qRT-PCR) and the reaction system used 5 µl of extracted RNA. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control, and each sample was performed in triplicate.
The lncRNA, LL22NC03-N64E9.1 primer sequences were:
LL22NC03-N64E9.1 (Forward) AAGCCATGTAAAGGGGGCTC;
LL22NC03-N64E9.1 (Reverse) TGGTAGTCTGACCATTCTGCAT;
GAPDH (Forward) GAAGAGAGAGACCCTCACGCTG;
GAPDH (Reverse) ACTGTGAGGAGGGGAGATTCAGT.

Cell proliferation assay using the cell counting kit-8 (CCK-8) method

Two groups of transfected H292 cells were placed in a 96-well plate and the serum-free medium was replaced after cultured
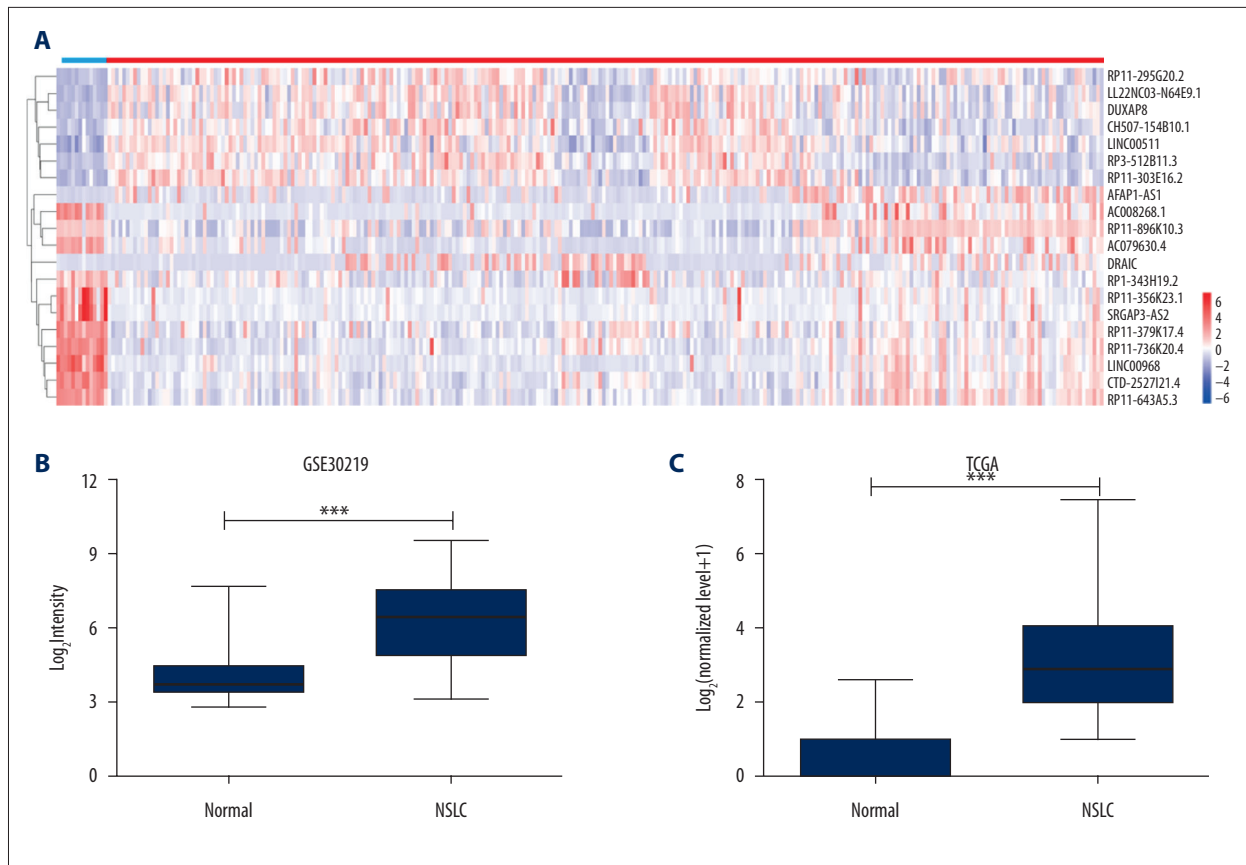
Figure 1. The long noncoding RNA (lncRNA), LL22NC03-N64E9.1, is highly expressed in lung cancer. (**A**) Heat map of differential expression between lung cancer tissues and adjacent normal lung tissues. (**B**) The analysis of the lung cancer dataset GSE30219, downloaded from the Gene Expression Omnibus (GEO) repository showed that the long noncoding RNA (lncRNA), LL22NC03-N64E9.1 was highly expressed in lung cancer tissues.

for 24, 48, 72 and 96 hours. Then, 10 μL of cell counting kit-8 (CCK8) medium was added to each well. After incubation at 37°C and 5% CO_2 for 1 hour, the optical density (OD) value was measured at 450 nm. Each measurement was repeated in triplicate.

Colony formation assay

Two groups of H292 cells, transfected for 24 hours, were placed in the medium plate at a density of $3 \times 10^3/100$ μl for a further 24 hours in an incubator at 37°C, with 5% CO_2. Culture medium was replaced every two days, and the culture was terminated after 14 days. The medium was removed and cells were washed twice with phosphate buffered saline (PBS), the cells were fixed with 5% paraformaldehyde for 30 min, the remaining culture fluid was removed, and 1 ml of 0.1% crystal violet solution was added to each well. The crystal violet solution was removed after 30 min, and the cells were washed with PBS until the solution was clear. The viable cell colonies were counted.

Statistical analysis

The SPSS version 22.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. GraphPad Prism 5.0 version X (GraphPad, La Jolla, CA, USA) was used for image editing. Measurement data were compared using a t-test and presented as the mean ± standard deviation (SD), categorical data were compared with the chi-squared test (χ^2 test). $P<0.05$ indicated a significant difference; * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

Results

Relationship between the expression of the long noncoding RNA (lncRNA), LL22NC03-N64E9.1 in lung cancer and patient clinical data

Analysis of the lung cancer dataset GSE30219, downloaded from the Gene Expression Omnibus (GEO) repository showed that differentially expressed long noncoding RNA (lncRNA), LL22NC03-N64E9.1, was significantly increased in lung cancer
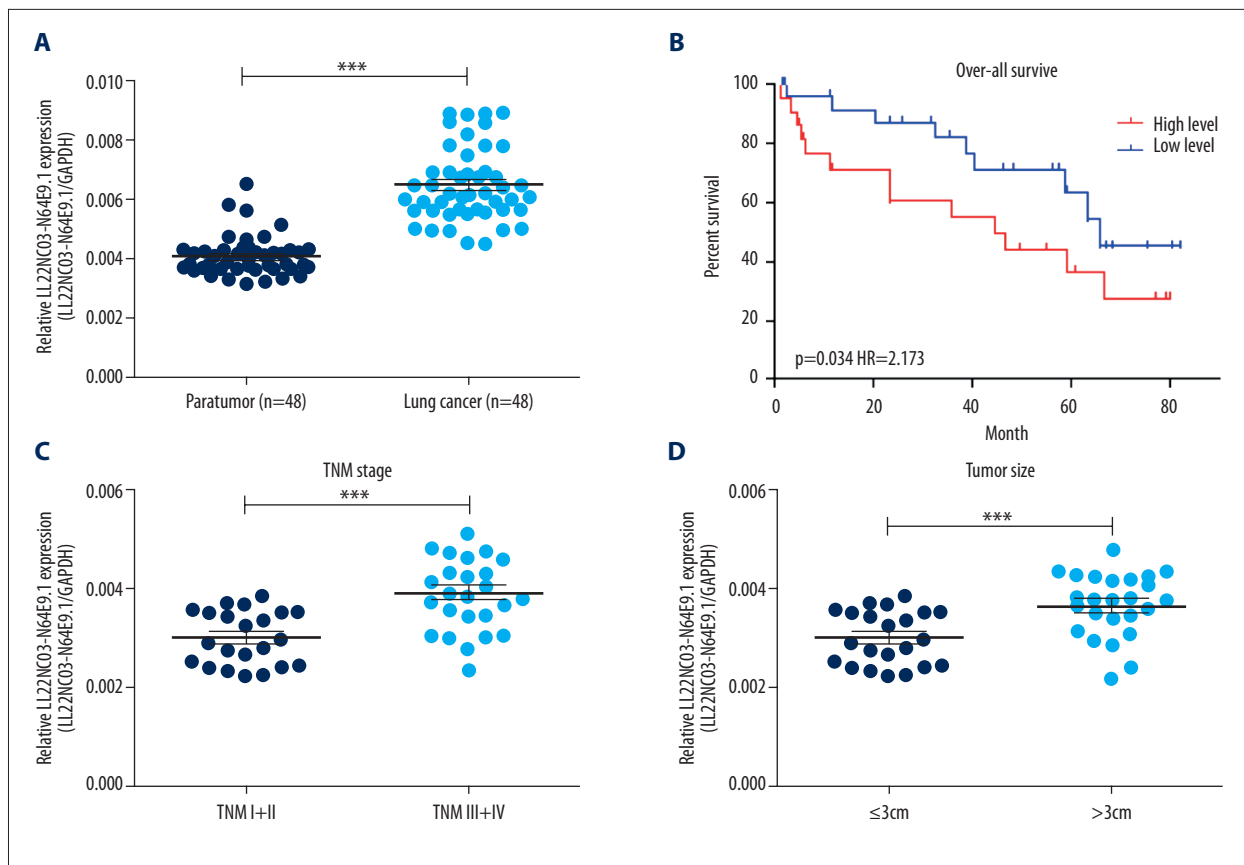
This work is licensed under Creative Common Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0)

4319

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Figure 2. The long noncoding RNA (lncRNA), LL22NC03-N64E9.1, promotes proliferation of lung cancer tissue. (**A**) The long noncoding RNA (lncRNA), LL22NC03-N64E9.1, in 48 patients with lung cancer tissues was significantly increased compared with the expression in adjacent normal lung tissue. (**B**) The overall survival (OS) rate of lung cancer patients with overexpression of the lncRNA, LL22NC03-N64E9.1, was significantly lower compared with the group showing lower expression of the lncRNA, LL22NC03-N64E9.1. (**C**) The expression of the lncRNA, LL22NC03-N64E9.1, was positively correlated with TNM lung cancer staging. (**D**) The expression of the lncRNA, LL22NC03-N64E9.1, was positively correlated with tumor size.

tissue compared with normal lung (Figure 1A, 1B) (P<0.001). The lncRNA, LL22NC03-N64E9.1 expression using The Cancer Genome Atlas (TCGA) RNA sequencing data was significantly increased compared with normal lung tissues. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the expression of the lncRNA, LL22NC03-N64E9.1 in lung cancer tissues and adjacent normal lung tissues in 48 patients (Figure 2A) (P<0.001).

The clinical data of patients were analyzed. The overall survival (OS) rate of the patient groups showed that increased expression levels of lncRNA, LL22NC03-N64E9.1 were lower compared with the groups showing reduced OS (Figure 2B) (P=0.034, HR=2.173). The lncRNA, LL22NC03-N64E9.1 was upregulated in patients with advanced tumors and tumors with a large volume (Figure 2C) (P<0.001) and Figure 2D (P<0.001). The chi-squared test analysis showed that tumor stage of the group that overexpressed lncRNA, LL22NC03-N64E9.1 was increased (Table 1).

Screening of cell lines and interference efficiency using small interfering RNA (siRNA) in lung cancer cells

The normal lung cell line BEAS-2B was used as the control cell line. Total RNA was extracted from cell lines A549, H1703, H292, and the relative expression of the lncRNA, LL22NC03-N64E9.1 in cells was measured by qRT-PCR. The expression of the lncRNA, LL22NC03-N64E9.1 was greatest in the H292 cell line (Figure 3A). Therefore, the H292 cell line was chosen for the subsequent interference experiments. Using small interfering RNA (siRNA), a corresponding interference sequence was constructed and transfected into H292 cell line. The results of the transfection are shown in Figure 3B. The interference effect of si-LL22NC03-N64E9.1 1 # was the best.

Table 1. Relevance of LL22NC03-N64E9.1 expression to clinicopathological features in patients with lung cancer (n=48).

| Clinicopathologic features | Number of cases | LncRNA LL22NC03-N64E9.1 expression | | P value |
		Low (n=24)	High (n=24)	
Age (years)				
≤56	22	10	12	0.5623
>56	26	14	12	
Gender				
Male	26	11	15	0.2466
Female	22	13	9	
Tumor size				
≤3CM	23	8	15	0.0431*
>3CM	25	16	9	
TNM stage				
I~II	21	7	14	0.0417*
III~IV	27	17	10	
Lymph node metastasis				
Absent	25	12	13	0.7726
Present	23	12	11	

Knockdown of the lncRNA, LL22NC03-N64E9.1 expression regulated the proliferation of lung cancer cells using a cell counting kit-8 (CCK-8) assay

The results of the cell counting kit-8 (CCK-8) assay showed that the optical density (OD) 450 value of cells transfected with si-LL22NC03-N64E9.1 1 # was decreased compared with cells transfected with si-NC negative controls, which indicated that the knockdown of LL22NC03-N64E9.1 inhibited the proliferation of H292 cells (Figure 3C). The CCK8 assay for cell proliferation showed that the lncRNA, LL22NC03-N64E9.1 could promote proliferation of lung cancer cells.

Knockdown of the lncRNA, LL22NC03-N64E9.1 expression regulated cloning ability of lung cancer cells

The cloning assay showed that the ability of cells to form a cloned cell mass was significantly lower compared with the negative control after transfection with si-LL22NC03-N64E9.1, which indicated that interference with the lncRNA, LL22NC03-N64E9.1 inhibited the cloning ability of H292 cells (Figure 3D). The cloning assay showed that the lncRNA, LL22NC03-N64E9.1 could increase the cloning ability of lung cancer cells.

Discussion

The expression of a novel long noncoding RNA (lncRNA), LL22NC03-N64E9.1, has been shown to have a role in the normal physiological activities and diseases, including the development and progression of tumors, and can be involved in gene expression, epigenetic regulation, transcription regulation, and post-transcriptional regulation [9,10]. Previously published studies have shown that the proportion of tumor-associated lncRNAs (18%) was approximately twice that of tumor-associated proteins (9%) [11].

Currently, several lncRNAs related to lung cancer have been reported in lung cancer, including lung adenocarcinoma metastasis-related transcript 1 (MALAT-1), HOX transcriptional antisense RNA and the lncRNA, DQ786227, which have been shown to be involved in the regulation of the development of lung cancer and metastasis [12,13]. The lncRNAs are abnormally expressed in lung cancer [14], and expression of certain lncRNAs have been shown to be significantly associated with the progression of lung cancer [15]. The expression of lncRNAs is tissue-specific and spatiotemporally specific and might be a potential marker for the early diagnosis and progression of tumors. Therefore, the identification of lung cancer-associated lncRNAs might have a potential role in the diagnosis and prognosis lung cancer in patients.
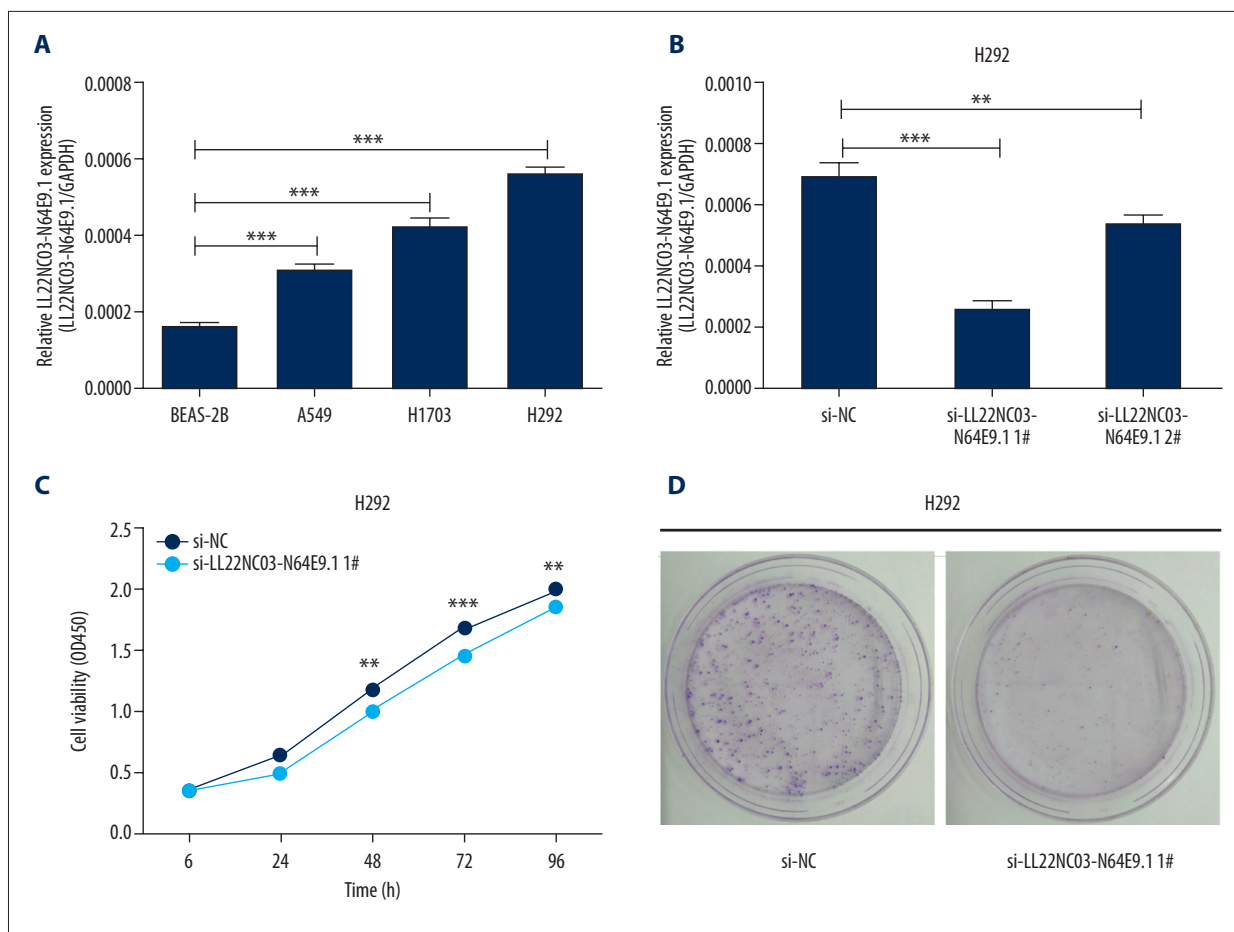
Figure 3. The effect of knockdown of the long noncoding RNA (lncRNA), LL22NC03-N64E9.1 on cell phenotype in normal lung cell lines BEAS-2B and A549, and lung cancer cell lines, H1703 and H292. (**A**) Expression of the long noncoding RNA (lncRNA), LL22NC03-N64E9.1, in normal lung cell lines BEAS-2B and A549, and lung cancer cell lines, H1703 and H292. (**B**) Interference efficiency using small interfering RNA (siRNA) in H292 lung cancer cells. (**C**) Cell counting kit-8 (CCK-8) assay showed that interference with si-LL22NC03-N64E9.1, inhibited the viability of H292 lung cancer cells. (**D**) Interference with si-LL22NC03-N64E9.1 1 enhanced the invasive properties of H292 lung cancer cells.

The lncRNA, LL22NC03-N64E9.1 is relatively newly discovered and located in the 22q11.1 region, and few studies have been previously undertaken on the role of lncRNA, LL22NC03-N64E9.1 in malignancy. Lian et al. found that the lncRNA, LL22NC03-N64E9.1 in colorectal cancer inhibited the transcription of the *KLF2* gene by binding to the enhancer of zeste homolog 2 (EZH2), a histone-lysine N-methyltransferase enzyme, thereby promoting the development of colorectal cancer [8]. However, no previous studies on the role of the lncRNA, LL22NC03-N64E9.1 in lung cancer have been conducted.

With the wide application of gene chip analysis, the Gene Expression Omnibus (GEO) repository is increasingly used in medical research because of the ease of chip data sharing, the large sample size, and the detailed patient prognostic information in the database. Therefore, the lncRNA map of lung cancer and adjacent normal lung tissues was compared

by the chipset GSE30219 downloaded from GEO database to select the lncRNA, LL22NC03-N64E9.1, which was highly expressed in lung cancer. Therefore, it is possible that the lncRNA, LL22NC03-N64E9.1 might be a lung cancer oncogene. In the present study, a further 48 lung cancer tissue samples and adjacent normal tissue samples were examined, and the findings showed that the lncRNA, LL22NC03-N64E9.1 was highly expressed in lung cancer tissues, which was consistent with the GEO data analysis.

In this study, the clinical data analysis of the lung cancer patients showed that the lncRNA, LL22NC03-N64E9.1 was related to the patient's total survival time, tumor size, and tumor stage, but was not related to the age, sex, grade, lymph node metastases, and distant metastases. These findings support the possibility that the lncRNA, LL22NC03-N64E9.1 might provide the basis for the future prevention and treatment of lung

cancer. Previously published studies have shown that lncRNA can have a role in the regulation of the malignant phenotype of cancer cells [16,17]. The present study showed that the lncRNA, LL22NC03-N64E9.1 was associated with increased proliferation of lung cancer cells.

To our knowledge, currently, this study was the first to demonstrate that the lncRNA, LL22NC03-N64E9.1 was highly expressed in lung cancer, and promoted the proliferation of lung cancer cells. The high expression of the lncRNA, LL22NC03-N64E9.1 might also be related to the progression of lung cancer, as an independent indicator for the prognosis of lung cancer. Further studies are recommended on the role of the lncRNA, LL22NC03-N64E9.1 and its regulatory mechanisms in lung cancer and other types of human cancer.

Conclusions

This study showed that the novel long noncoding RNA (lncRNA), LL22NC03-N64E9.1, was overexpressed in lung cancer tissues, and would promote the proliferation of lung cancer cells. The expression of the lncRNA, LL22NC03-N64E9.1 was significantly correlated with the patient survival, tumor size and tumor stage in patients with lung cancer. Further studies are required to determine whether the lncRNA, LL22NC03-N64E9 might be a new target for therapy in lung cancer.

Conflict of interest

None.

References:

1. Torre LA, Bray F, Siegel RL et al: Global cancer statistics, 2012. Cancer J Clin, 2015; 65: 87–108

2. Hauptman N, Glavac D: MicroRNAs and long non-coding RNAs: Prospects in diagnostics and therapy of cancer. Radiol Oncol, 2013; 47: 311–18

3. Quinn JJ, Chang HY: Unique features of long non-coding RNA biogenesis and function. Nat Rev Genet, 2016; 17: 47–62

4. Di Gesualdo F, Capaccioli S, Lulli M: A pathophysiological view of the long non-coding RNA world. Oncotarget, 2014; 5: 10976–96

5. Melissari MT, Grote P: Roles for long non-coding RNAs in physiology and disease. Pflugers Arch, 2016; 468: 945–58

6. Malek E, Jagannathan S, Driscoll JJ: Correlation of long non-coding RNA expression with metastasis, drug resistance and clinical outcome in cancer. Oncotarget, 2014; 5: 8027–38

7. Wang PQ, Wu YX, Zhong XD et al: Prognostic significance of overexpressed long non-coding RNA TUG in patients with clear cell renal cell carcinoma. Eur Rev Med Pharmacol Sci, 2017; 21: 82–86

8. Lian Y, Yan C, Ding J et al: A novel lncRNA, LL22NC03-N64E9.1, represses KLF2 transcription through binding with EZH2 in colorectal cancer. Oncotarget, 2017; 8: 59435–45

9. Gupta RA, Shah N, Wang KC et al: Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature, 2010; 464: 1071–76

10. Yap KL, Li S, Munoz-Cabello AM et al: Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell, 2010; 38: 662–74

11. Khachane AN, Harrison PM: Mining mammalian transcript data for functional long non-coding RNAs. PLoS One, 2010; 5: e10316

12. Liu XH, Liu ZL, Sun M et al: The long non-coding RNA HOTAIR indicates a poor prognosis and promotes metastasis in non-small cell lung cancer. BMC Cancer, 2013; 13: 464

13. Gao L, Mai A, Li X et al: LncRNA-DQ786227-mediated cell malignant transformation induced by benzo(a)pyrene. Toxicol Lett, 2013; 223: 205–10

14. Weber DG, Johnen G, Casjens S et al: Evaluation of long noncoding RNA MALAT1 as a candidate blood-based biomarker for the diagnosis of non-small cell lung cancer. BMC Res Notes, 2013; 6: 518

15. Han L, Kong R, Yin DD et al: Low expression of long noncoding RNA GAS6-AS1 predicts a poor prognosis in patients with NSCLC. Med Oncol, 2013; 30: 694

16. Lian Y, Cai Z, Gong H et al: HOTTIP: A critical oncogenic long non-coding RNA in human cancers. Mol Biosyst, 2016; 12: 3247–53

17. Evans JR, Feng FY, Chinnaiyan AM: The bright side of dark matter: LncRNAs in cancer. J Clin Invest, 2016; 126: 2775–82