Effect of Lncrna FER1L4 Overexpression on Prognosis and Cell Proliferation in Non-Small Cell Lung Cancer

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Research Article

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Abstract

Background: The purpose of this study was to investigate the clinical significance and biological function of lncRNA FER1L4 in NSCLC.

Methods: A total of 114 cases of NSCLC tissues and matched normal lung tissues were obtained from The first hospital of Jilin University between January 2009 and December 2016. The clinicopathological characteristics of patients were collected, including age, gender, tumor size, etc. The expression levels of LncRNA FER1L4 were detected by qRT-PCR. Non-small cell lung cancer cell lines (A549, H292 and H1299) were cultured with LncRNA FER1L4 mimics and LncRNA FER1L4 inhibitor. Cell proliferation was evaluated by CCK-8 and colony formation assay.

Results: The expression levels of lncRNA FER1L4 were significantly lower in NSCLC tissues compared with those in matched normal lung tissues (P<0.05). Decreased level of lncRNA FER1L4 was significantly correlated with tumor size, TNM stage and lymph node metastasis (P<0.05). Moreover, lncRNA FER1L4 over-expression predicted favorable prognosis in NSCLC (P<0.05). Furthermore, CCK-8 and colony formation assay showed that lncRNA FER1L4 over-expression significantly suppressed cell proliferation.

Conclusions: Our results suggested that LncRNA FER1L4 overexpression predicted favorable prognosis and suppressed cell proliferation in NSCLC, which may serve as a promising therapeutic target.

Background

Non-small cell lung cancer (NSCLC), the most common histopathological type of lung cancer, accounts for nearly 85% of all lung cancers.[1,2] Despite a great improvement has been made in the early diagnosis and clinical therapy of NSCLC, the survival rate of NSCLC patients is still not satisfactory.[3,4] NSCLC remains to be the predominant cause of cancer-induced death in the world, and the 5-year survival rate was less than 16%.[5,6] Therefore, identifying effective molecular targets is urgently required to improve the prognosis of NSCLC.

Recently, many studies have demonstrated that long non-coding RNAs (lncRNAs) are expressed in many kinds of tumors and regulate gene expression at transcriptional, posttranscriptional and epigenetic levels.[7–9] Moreover, accumulating evidence have demonstrated that lncRNAs play key roles in tumor processes, including cell proliferation, invasion and metastasis.[10–12] Fer-1-like protein 4 (FER1L4), a novel tumor-associated lncRNA, is reported to be down-regulated in osteosarcoma,[13,14] esophageal squamous cell carcinoma,[15] endometrial carcinoma,[16] gastric cancer,[17,18] hepatocellular carcinoma,[19] and colon cancer,[20] while it is over-expressed in human glioblastoma.[21] LncRNA FER1L4 is correlated with prognosis in patients with tumors.[13,17,19,20] However, the clinical significance and biological function of lncRNA FER1L4 in NSCLC remain unknown.

In this study, the expression levels of lncRNA FER1L4 and its clinical significance were evaluated in 114 patients with non-small cell lung cancer. Moreover, the effect of lncRNA FER1L4 on cell proliferation was investigated.

Methods

Patients and samples

A total of 114 NSCLC and matched adjacent normal lung tissues were obtained from the First Hospital of Jilin University during January 2009 to December 2016. No patient received chemotherapy, radiotherapy or immunity therapy before surgery. Adjacent normal lung tissues were greater than 3 cm away from tumor. Clinical pathological characters were collected from hospitalized records, including age, gender, smoking history, histological type, tumor size, tumor differentiation, lymph node metastasis and TNM stage. Follow-up time was recorded from the surgery day. The median survival time was 41.7 months (range: 10–74 months). All diagnosis was confirmed by the pathologists in the First Hospital of Jilin University. This study was approved by the Ethics Committee of the First Hospital of Jilin University in accordance with the Declaration of Helsinki. All patients signed the informed consents.

Cell culture and transfection.

Non-small cell lung cancer cell lines (A549, H292 and H1299) were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 (Gibco, USA) with 10% fetal bovine serum (FBS, Hyclone) at 37˚C in a humidified incubator with 5% CO_2. Cell transfection was performed according to the manufacturer's instructions. The sh-RNA sequence (5’-CAGGACAGCUUCGAGUUAATT-3’) was cloned into the lentivirus vector (Jima Company, Shanghai, China) and transferred into A549 cells to knock down lncRNA FER1L4. The over-expression of lncRNA FER1L4R was performed by transferring the recombinant lncRNA FER1L4 lentivirus vector. At 8 h after transfection, the medium was replaced with fresh medium containing 10% FBS. Non-target shRNA (5’-GCGACAAACGGGTTGATG-3’) lentivirus vector was used as the negative control (NC).

qPCR

Fresh tissues were collected and stored at liquid nitrogen after surgical resection. The total RNA of tissues and cells were obtained by using RNAisoTM PLUS (Invitrogen, California, USA) and reversely transcribed into cDNA by cDNA Synthesis Kit (TaKaRa Corp, Dalian, China). 7500 SYBR Green Fast Real-Time PCR System (ABI Corp, USA) was used to quantitatively analyze the expression levels of lncRNA FER1L4 according to the manufacturer's instructions. Reaction conditions were 95°C for 10 min, 95°C for 15 s by 40 cycles and 60°C for 60 s. The primer sequences were as follows. LncRNA FER1L4: forward primer: 5'-CCGTGTTGAGGTGCTGTTC-3'; reverse primer: 5'-GGC AAGTCCACTGTCAGATG-3'. GAPDH: forward primer: 5'-CTGAACGGGAAGCTCACTGG-3'; reverse primer: 5'-TGAGGTCCACCACCCTGTTG-3'.

GAPDH was considered as the internal control. Each test was performed for three times at the same reaction.

Cell counting kit (CCK)-8 assay

Cells were seeded in 96-well plates (200 cells/well) and cultured in RPMI-1640 with 8% FBS at 37˚C in a humidified incubator with 5% CO_2 for 5 days. Then, cell was incubated with CCK-8 solution for 4 h and then absorbance of each well was measured at 450 nm using a spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Colony formation assay

After transfection, cells were seeded in a 24-well plates at a density of 1000 cells per well and cultured in RPMI-1640 with 8% FBS for 14 days. Fresh culture medium was replaced per 3 days. After fixed with 4% paraformaldehyde, cells were stained with Wright-Giemsa (Ruigen company, Beijing, China) at room temperature for 2h. Colonies with > 50 cells were counted under the confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis

All data were expressed as means ± standard deviation (SD). All experiments were repeated three times with the same sample. Statistical analysis was made by software SPSS software (version 19.0; SPSS, Chicago, IL, USA). Paired t-test was used to analyze the level of lncRNA FER1L4 in NSCLC and matched normal lung tissues. Kaplan-Meier method and Cox's proportional hazards model were used for survival analysis. $P < 0.05$ was considered as statistical significant.

Results

LncRNA FER1L4 was down-regulated in NSCLC tissues and correlated with clinicalpathological characters

To investigate the clinical role of FER1L4 in NSCLC, the expression levels of FER1L4 in NSCLC tissues and matched adjacent normal lung tissues were measured by qPCR. As shown in Figure 1A, the expression levels of FER1L4 in NSCLC tissues were significantly reduced than those in matched adjacent normal lung tissues (P<0.001). Then, NSCLC tissues were divided into high-expression and low-expression groups based on the median level of FER1L4. There were 53 NSCLC cases with FER1L4 over-expression and 61 NSCLC cases with FER1L4 low-expression. Moreover, lncRNA FER1L4 low-expression was closely associated with tumor size, TNM stage and lymph node metastasis (Table 1, P<0.05). Nevertheless, the expression level of FER1L4 was not significantly connected with age, gender, smoking history, histological type and tumor differentiation (Table 1, P>0.05).

LncRNA FER1L4 correlated with prognosis and cell proliferation

Subsequently, the correlation between FER1L4 expression and prognosis of NSCLC was further analyzed. Kaplan-Meier analysis showed that NSCLC patients with FER1L4 over-expression had statistically favorable survival compared with those with FER1L4 low-expression (P<0.001; Figure 1B). TNM stage and lymph node metastasis were also correlated with patients' survival (Table 2, *P*<0.05). Cox's analysis showed that FER1L4, TNM stage and lymph node metastasis were independent prognostic factors in NSCLC (Table 3). However, patients' survival rates were not correlated with tumor size, age, gender, smoking history, histological type and tumor differentiation (Table 2, Table 3, *P*>0.05).

In addition, the effect of lncRNA FER1L4 on cell proliferation was investigated. As shown in Figure 2A, the expression levels of lncRNA FER1L4 in A549 cells were higher compared with those in H292 and H1299 cells. Therefore, A549 cells were selected to knock down the expression levels of FER1L4, and FER1L4 over-expression vectors were transferred into H1299 cells. Satisfactory transfection efficiency was confirmed by qRT-PCR (Figure 2B). CCK-8 assay showed that down-regulation of lncRNA FER1L4 significantly promoted cell proliferation in A549 cells (Figure 3A, P<0.001). While over-expression of FER1L4 significantly suppressed cell proliferation in H1299 cells (Figure 3B, P<0.001). Moreover, colony formation assay showed that knock-down lncRNA FER1L4 significantly promoted colony formation in A549 cells (Figure 3C and 3D, P<0.001). While FER1L4 over-expression significantly inhibited colony formation in H1299 cells (Figure 3C and 3D, P<0.001). These data indicated that lncRNA FER1L4 was significantly related with cell proliferation of NSCLC.

Discussion

LncRNA is a type of RNA that is more than 200 nucleotides in length and does not code proteins.[22,23] Nowadays, many studies have confirmed that lncRNAs play crucial roles in the occurrence and development of NSCLC, which may be considered as diagnostic and prognostic markers.[24,25] Fer-1-like protein 4 (FER1L4), a novel long non-coding RNA, is reported to be implicated with the initiation and progression of multiple cancers by regulating cell proliferation, apoptosis, migration and invasion.[14–16] Moreover, FER1L4 is correlated with prognosis.[13,14,17,19,20] For example, Fei, et al. reported that FER1L4 upregulation significantly suppressed cell proliferation, colony formation, migration and invasion in osteosarcoma and associated with TNM stage, lymph node metastases and poor overall survival.[14] Ma et al. showed that FER1L4 overexpression could inhibit cell proliferation and invasion, and promote apoptosis in esophageal squamous cell carcinoma.[15] Qiao et al. revealed that FER1L4 suppressed cell proliferation and cycle in endometrial carcinoma.[16] Chen et al. showed that FER1L4 was significantly associated with disease stage, metastasis and tumor differentiation, and might be a prognostic marker in osteosarcoma.[13] Wu et al. reported that FER1L4 acted as a tumor suppressor and predicted good prognosis in hepatocellular carcinoma.[19] Yue et al. reported that FER1L4 suppressed oncogenesis and exhibited prognostic value in colon cancer.[20]

In this study, our data showed that the expression levels of FER1L4 in NSCLC tissues was significantly decreased compared with those in matched adjacent normal lung tissues. It was consistent with the

observations in other cancers.[13–20] These data indicated lncRNA FER1L4 depletion was implicated with the formation of NSCLC, which might be helpful for the diagnosis of NSCLC. Moreover, lncRNA FER1L4 downregulation was closely associated with tumor size, TNM stage and lymph node metastasis, indicating that lncRNA FER1L4 depletion might correlate with tumor progression. NSCLC patients with FER1L4 over-expression had more favorable survival compared with those with low-expression, suggesting that lncRNA FER1L4 was a prognostic biomarker in NSCLC. In addition, our results showed that lncRNA FER1L4 knockdown significantly promoted cell proliferation, which further supported that lncRNA FER1L4 down-regulation might be connected with tumor progression. Therefore, these data further supported that lncRNA FER1L4 was as a tumor suppressor and correlated with the formation and progression of NSCLC.

However, there are also some limitations in present study. Firstly, the effect of lncRNA FER1L4 on cell proliferation was only confirmed *in vitro*, which needed to be further validated *in vivo*. Secondly, the exact mechanism of lncRNA FER1L4 in NSCLC remains unknown. Therefore, further investigations are needed to validate our findings.

In conclusion, these data indicated that lncRNA FER1L4 was lowly expressed in NSCLC tissues and affected cell proliferation in NSCLC. Moreover, overexpression of lncRNA FER1L4 predicted favorable prognosis in NSCLC patients, which might serve as a potential biomarker.

Abbreviations

Non-small cell lung cancer: NSCLC; Fer-1-like protein 4: FER1L4; standard deviation: SD;

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the First Hospital of Jilin University in accordance with the Declaration of Helsinki. All patients signed the informed consents.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Confirmation

XJ and XL: Substantial contributions to the conception or design of the work;

XL and YG: Drafting the work or revising it critically for important intellectual content;

YG: Final approval of the version to be published;

XJ,HZ, YG: Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Tables

Table 1 LncRNA FER1L4 correlated with clinicalpathological characters in NSCLC

Clinicalpathological characters	N	LncRNA FER1L4 over-expression	LncRNA FER1L4 low-expression	*P* value
Age (years)				
≤49	64	29	35	0.851
>49	50	24	26	
Gender				
Male	61	25	36	0.259
Female	53	28	25	
Smoking history				
Negative	65	30	35	1.000
Positive	49	23	26	
Histological type				
Adenocarcinoma	60	30	30	0.725
Squamous cell carcinoma	30	12	18	
Adenosquamous carcinoma	19	8	11	
Large-cell carcinoma	5	3	2	
Tumor size (cm)				
≤ 3	39	31	8	<0.001
>3	75	22	53	
Tumor differentiation				
High-middle grade	76	35	41	1.000
Low grade	38	18	20	
Lymph node metastasis				
Negative	64	38	26	0.002
Positive	50	15	35	
TNM stage				
I-II	75	41	34	0.018
III	39	12	27	

Table 2 The prognostic factors were analyzed in NSCLC by Kaplan-Meier method

Variables	N	Survival time (Month, 95% CI)	P value
LncRNA FER1L4 expression			
Over-expression	53	66 (61-71)	<0.001
Low-expression	61	46 (42-50)	
Age (years)			
≤50	64	54 (50-68)	0.124
>50	50	62 (55-59)	
Gender			
Male	61	54 (50-58)	0.224
Female	53	61 (54-68)	
Smoking history			
Negative	65	58 (53-63)	0.874
Positive	49	55 (50-60)	
Histological type			
Adenocarcinoma	60	58 (53-63)	0.561
Squamous cell carcinoma	30	57 (52-62)	
Adenosquamous carcinoma	19	47 (41-53)	
Large-cell carcinoma	5	47 (35-59)	
Tumor size (cm)			
≤ 3	39	64 (56-72)	0.144
>3	75	54 (50-58)	
Tumor differentiation			
High-middle grade	76	59 (54-64)	0.548
Low grade	38	50 (46-54)	
Lymph node metastasis			
Negative	64	63 (58-68)	0.006
Positive	50	49 (45-53)	
TNM stage			
I-II	75	62 (57-67)	0.009

III	39	47 (42-52)

Table 3 The prognostic factors were analyzed in NSCLC by Cox's proportional hazards model

Variables	Hazard rate	95% CI	P value
LncRNA FER1L4	3.096	1.718-5.555	<0.001
Lymph node metastasis	1.969	1.188-3.264	0.009
TNM stage	1.862	1.143-3.034	0.012
Age	3.218	0.952-10.886	0.060
Gender	0.333	0.071-1.571	0.165
Smoking history	1.025	0.561-1.874	0.935
Tumor size	1.396	0.515-3.784	0.513
Tumor differentiation	1.326	0.713-2.466	0.373
Histological type	0.701	0.387-1.267	0.239

Figures

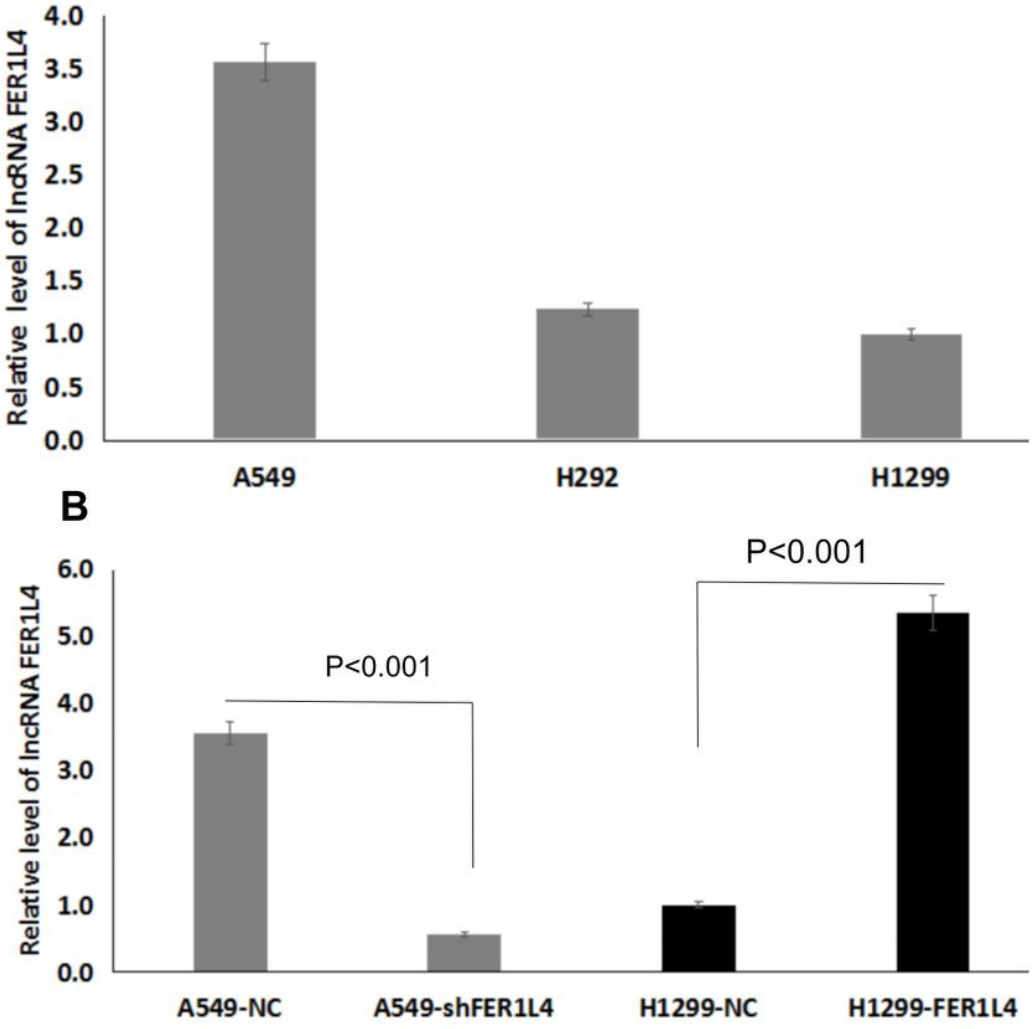

Figure 1

LncRNA FER1L4 was lowly expressed in NSCLC tissues and correlated with prognosis of NSCLC patients. A: The levels of lncRNA FER1L4 were tested in NSCLC and matched normal lung tissues by qRT-PCR. B: NSCLC patients with lncRNA FER1L4 over-expression (N=53) presented favorable prognosis compared with those with lncRNA FER1L4 low-expression (N=61).

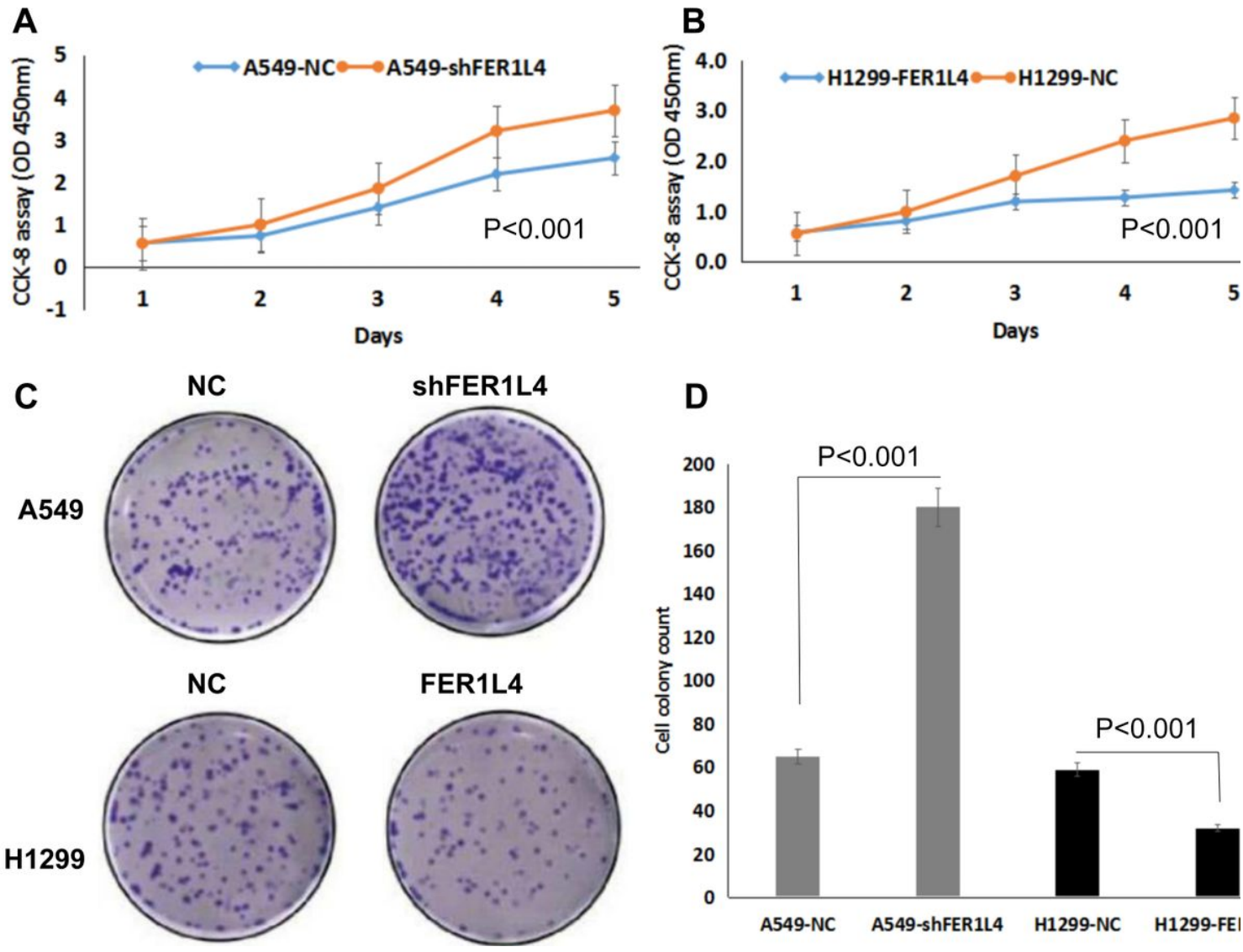

Figure 2

LncRNA FER1L4 over-expression and knockdown were successfully performed in lung cancer cells. A: The levels of lncRNA FER1L4 were examined in lung cancer cell lines by qRT-PCR. B: The transfection efficiency of lncRNA FER1L4 over-expression and knockdown was validated by qRT-PCR.

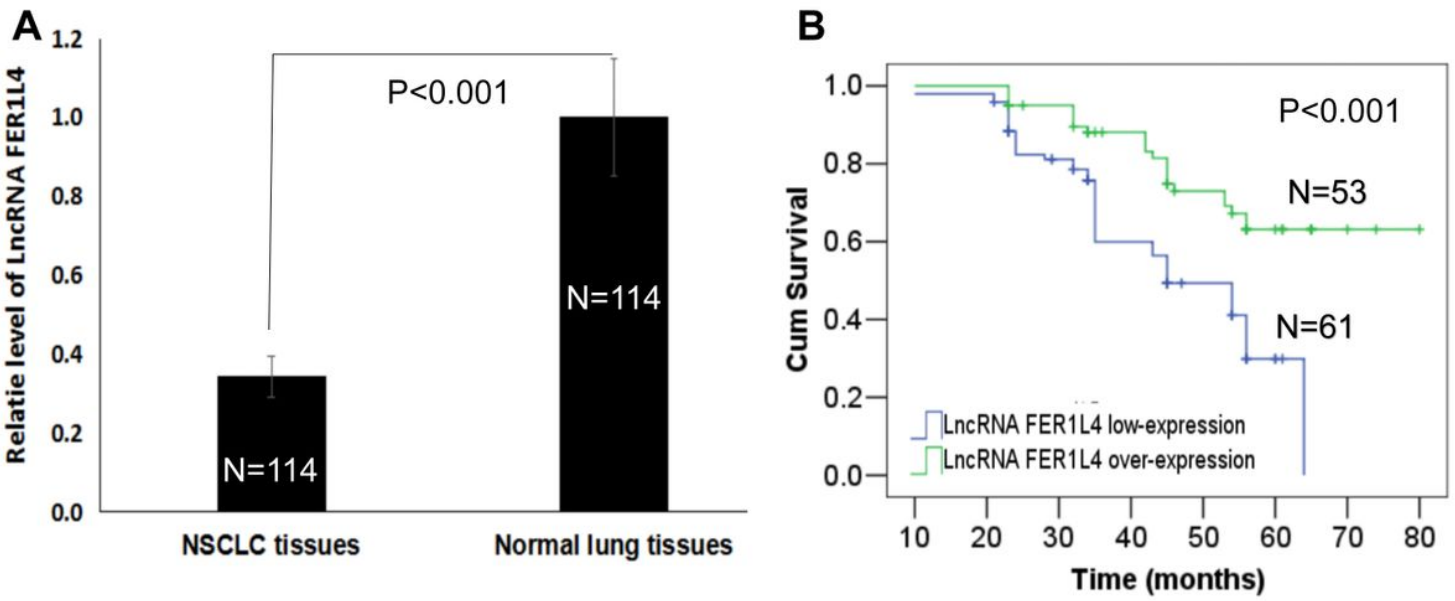

Figure 3

LncRNA FER1L4 overexpression inhibited cell proliferation. A: CCK-8 assay showed lncRNA FER1L4 knockdown significantly promoted cell viability in A549 cells. B: CCK-8 assay showed lncRNA FER1L4 overexpression significantly inhibited cell viability in H1299 cells. C: Colony formation assay showed that lncRNA FER1L4 overexpression significantly suppressed colony formation. D: Cell colony count in A549 cells and H1299 cells.