European Review for Medical and Pharmacological Sciences 2020; 24: 1233-1242

LncRNA TRERNA1 promotes malignant progression of NSCLC through targeting FOXL1

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Abstract. – OBJECTIVE: Previous studies have shown the carcinogenic role of long-chain non-coding RNAs (lncRNA) TRERNA1. However, the role of TRERNA1 in non-small cell lung cancer (NSCLC) has not been reported. This research aims to explore the regulatory effect of TRERNA1/FOXL1 axis on the malignant progression of NSCLC.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the expression levels of TRERNA1 and FOXL1 in 39 pairs of tumor tissues and paracancerous ones collected from NSCLC patients. The potential relation between TRERNA1 expression and clinical indicators of NSCLC patients was analyzed. Meanwhile, expression levels of TRERNA1 and FOXL1 in NSCLC cell lines were also detected by qRT-PCR. In addition, TRERNA1 knockdown model was constructed in H358 and SPC-A1 cells. Cell counting kit-8 (CCK-8), cell colony formation assay, and flow cytometry were applied to analyze the influence of TRERNA1 on NSCLC cell biological functions. Finally, Dual-Luciferase reporter gene assay and cell reverse recovery experiments were performed to figure out the underlying mechanisms of TRERNA1 in regulating NSCLC progression.

RESULTS: QRT-PCR results indicated that the expression level of lncRNA TRERNA1 in tumor tissue samples of NSCLC patients was remarkably higher than that in adjacent tissues. Compared with NSCLC patients with low expression of TRERNA1, patients with high TRERNA1 expression had a worse pathological stage and overall survival. Similarly, compared with cells in sh-NC group, the proliferation ability of cells in sh-TRERNA1 group was remarkably attenuated. In addition, cell ratio in the G1 phase increased after knockdown of TRERNA1, suggesting the arrested G1/S cell cycle. Subsequently, FOXL1 was downregulated in NSCLC cell lines and tumor tissues. Meanwhile, FOXL1 level was verified to be negatively correlated with TRERNA1 level. Additionally, the binding between TRERNA1 and FOXL1 was confirmed by Dual-Luciferase reporter gene assay. Cell reverse investigation indicated the involvement of FOXL1 in TRERNA1-regulated malignant progression of NSCLC.

CONCLUSIONS: LncRNA TRERNA1 was up-regulated both in NSCLC tissues and cell lines. Its level was associated with pathological stage and poor prognosis in NSCLC. In addition, lncRNA TRERNA1 could promote the malignant progression of NSCLC *via* modulating FOXL1.

Key Words:
TRERNA1, FOXL1, NSCLC, Proliferation.

Introduction

As a malignant tumor with the highest morbidity and mortality, 80-85% of lung cancers belong to non-small cell lung cancer (NSCLC), including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma[1-3]. In recent years, significant progress on the diagnosis and treatment of lung cancer has been achieved. However, most patients have been in advanced stage at the time of diagnosis, losing the opportunity for radical surgery. The recurrence rate and metastasis risk of NSCLC patients are still high even after radical surgery, auxiliary chemoradiotherapy, targeting or biotherapy[4-6]. At present, biomarkers to accurately predict metastasis of NSCLC are lacked. Therefore, it is urgent to seek effective lung cancer biomarkers for predicting NSCLC metastasis, thus improving patients' prognosis[7-9].

In the past few decades, the function and mechanism of short-chain, non-coding RNAs, especially miRNAs and long-chain non-coding RNAs (lncRNA), have been fully elucidated. Abnormal regulation of lncRNA has recently been found in a variety of diseases, including tumors, and exhibits a greater tissue-specificity than that of mRNA-encoding proteins[10,11]. LncRNA is a class of RNA with a transcript of

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more than 200 nucleotides in length. Although lncRNA does not encode proteins, it can regulate gene expressions through a variety of pathways[12,13], such as apparent modification, transcription, and post-transcriptional regulation[13-15]. Since more and more lncRNAs have been discovered, their roles in malignant tumors have attracted widespread attention[16,17]. Abnormally expressed lncRNAs have been found in malignant tumors, such as lung cancer, breast cancer, gastric cancer, and liver cancer, suggesting that lncRNA may participate in the progression of malignant tumors[17,18].

Current research[19,20] has revealed that the occurrence and progression of lung cancer are especially relevant to the abnormal expressions and regulation of different genes. In recent years, the growing literature has demonstrated that a large proportion of lncRNAs are capable of interacting with adjacent protein-coding genes and function as a "lncRNA-mRNA" complex[21]. Therefore, in this work, we analyzed TRERNA1 and FOXL1 expressions in 39 pairs of tumor tissue specimens and paracancerous ones from NSCLC patients, and explored their influences on the biological functions of NSCLC cells. We aimed to investigate the role of TRERNA1 in influencing clinical parameters, prognosis, and malignant progression of NSCLC *via* adsorbing FOXL1.

Patients and Methods

Patients and NSCLC Samples

Tumor tissue specimens and paracancerous ones from 39 NSCLC patients were collected. All specimens were obtained from NSCLC patients admitted in the Department of Oncology, Thoracic Surgery, and Respiratory who accepted surgery and biopsy or bronchoscopy. In addition, none of the patients received pre-operative anti-tumor treatments, such as radiotherapy or chemotherapy. The research was approved by the Ethics Committee of the hospital, and all patients signed informed consent. All patients were followed up after discharge. Follow-up information included general conditions, clinical symptoms, and imaging examination.

Cell Lines and Reagents

Five human NSCLC cells (A549, H1299, PC-9, H358, SPC-A1) and one normal human bronchial epithelial cell (BEAS-2B) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). Cells were cultured with DMEM containing 10% FBS in a 37°C incubator with 5% CO_2.

Cell Transfection

The control plasmid (sh-NC) and the TRERNA1 interference sequence (sh-TRERNA1), as well as si-NC and FOXL1 knockdown sequence (si-FOXL1), were all purchased from GenePharma (Shanghai, China). Cells were inoculated in 6-well plates and grown to a cell density of 70%. Then, transfection was performed according to the manufacturer's instructions. After that, cells were collected 48 h later for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and cell function investigations.

Cell Counting Kit-8 (CCK-8) Assay

After 48 h of transfection, cells were collected and inoculated into 96-well plates with 2000 cells per well. Cells were cultured for 24 h, 48 h, 72 h, and 96 h respectively. Then, CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was applied. After incubation for 2 h, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

Colony Formation Assay

After 48 h of transfection, 200 cells were seeded in each well of a 6-well plate and cultured in complete medium for 2 weeks. The medium was changed once after one week and then twice a week. The medium should not be replaced as much as possible in the previous week to avoid cell adhesion. After 2 weeks, visible colonies were formed and then fixed in 2 mL of methanol for 20 min. After the methanol was aspirated, cells were stained with 0.1% crystal violet staining solution for 20 min, washed 3 times with phosphate-buffered saline (PBS), photographed, and counted under a light-selective environment.

Flow Cytometry Analysis

To determine changes in the cell cycle, transfected cells were stained with propidium iodide (PI) for 30 min. The analysis was performed in a BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometer, and FlowJo software.

QRT-PCR

Total RNA was extracted from tissue samples using the TRIzol (Invitrogen, Carlsbad, CA, USA) method. Then, 2 μg of total RNA was added to 20 μL system for complementary deoxyribose nucleic acid (cDNA) synthesis. Real-time PCR was subsequently performed using 2×SYBR Green PCR Master Mix (TaKaRa, Otsu, Shiga, Japan). An appropriate amount of cDNA was taken as a template, with 0.4 mol/l primer, 15 μL system for amplification, and β-actin as an internal reference. The PCR reaction was carried out on a quantitative PCR reactor. Data analysis was performed using ABI Step One software (Applied Biosystems, Foster City, CA, USA) and the relative expression levels of mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: TRERNA1, F: 5'-CCGAGCAACGTTCATAACGG-3', R: 5'-CGTCGGCTGCGTCGGACGCGA-3'; FOXL1, F: 5'-ACGCACGCCATTCTGCGACCACA-3', R: 5'-CGGTCGAATCAGCGCAGTCTCT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Dual-Luciferase Reporter Gene Assay

Luciferase vectors were constructed based on the binding sequences between TRERNA1 and FOXL1. Cells were co-transfected with NC/pcDNA-TRERNA1 and wild-type/mutant-type FOXL1 vector, respectively. After co-transfection for 48 h, cells were lysed for measuring the luciferase activity.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA). Differential expressions of TRERNA1 and FOXL1 in tumor tissue specimens and adjacent ones were analyzed using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). The relation between expression of TRERNA1 and FOXL1 and various clinicopathological parameters were analyzed by Chi-Square test. The relation between TRERNA1 expression and NSCLC prognosis was analyzed by the Kaplan-Meier method. Cox proportional hazard model was applied to analyze factors affecting the prognosis of NSCLC patients. Data were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

TRERNA1 was Up-Regulated in Human NSCLC Tissues and Cell Lines

The expression of TRERNA1 in NSCLC tissue samples and corresponding paracancerous ones, as well as in NSCLC cell lines, was examined through the qRT-PCR detection. Results indicated that, either in tumor tissues or NSCLC cell lines, TRERNA1 levels were found significantly higher than those in paracancerous tissues or the normal human bronchial epithelial cell line (Figure 1A, 1B).

TRERNA1 Expression Was Correlated with Lymph Node Metastasis, Distant Metastasis, and Overall Survival in NSCLC Patients

According to the qRT-PCR results, the above tissue samples were divided into TRERNA1 high expression group and low expression group. Chi-square test was applied to analyze the correlation between TRERNA1 expression with age, gender, pathological stage, lymph node, and distant metastasis of NSCLC patients. As shown in Table I, TRERNA1 level was not associated with age, gender, lymph node metastasis, and distant metastasis of NSCLC patients, while it was linked with the pathological stage (Figure 1C). In addition, relevant follow-up data were collected to figure out the relation between TRERNA1 and prognosis of NSCLC patients. Kaplan-Meier survival curves revealed that high TRERNA1 expression was closely related to the poor prognosis of NSCLC ($p<0.05$; Figure 1D), suggesting that TRERNA1 might serve as a new biological indicator for predicting the prognosis of NSCLC.

Down-regulation of TRERNA1 Inhibited Cell Growth

To explore the effect of TRERNA1 on NSCLC proliferation and cell cycle, TRERNA1 knockdown model was constructed. After knockdown of TRERNA1 in H358 and SPC-A1 cells, the qRT-PCR investigation was performed to verify the interference efficiency, and the difference was statistically significant (Figure 2A). The results of CCK-8, cell colony formation, and flow cytometry researches indicated that compared with controls, the proliferation ability of cells remarkably decreased, and the cell ratio in the G1 phase increased after knockdown of TRERNA1 (Figure 2B-2D).

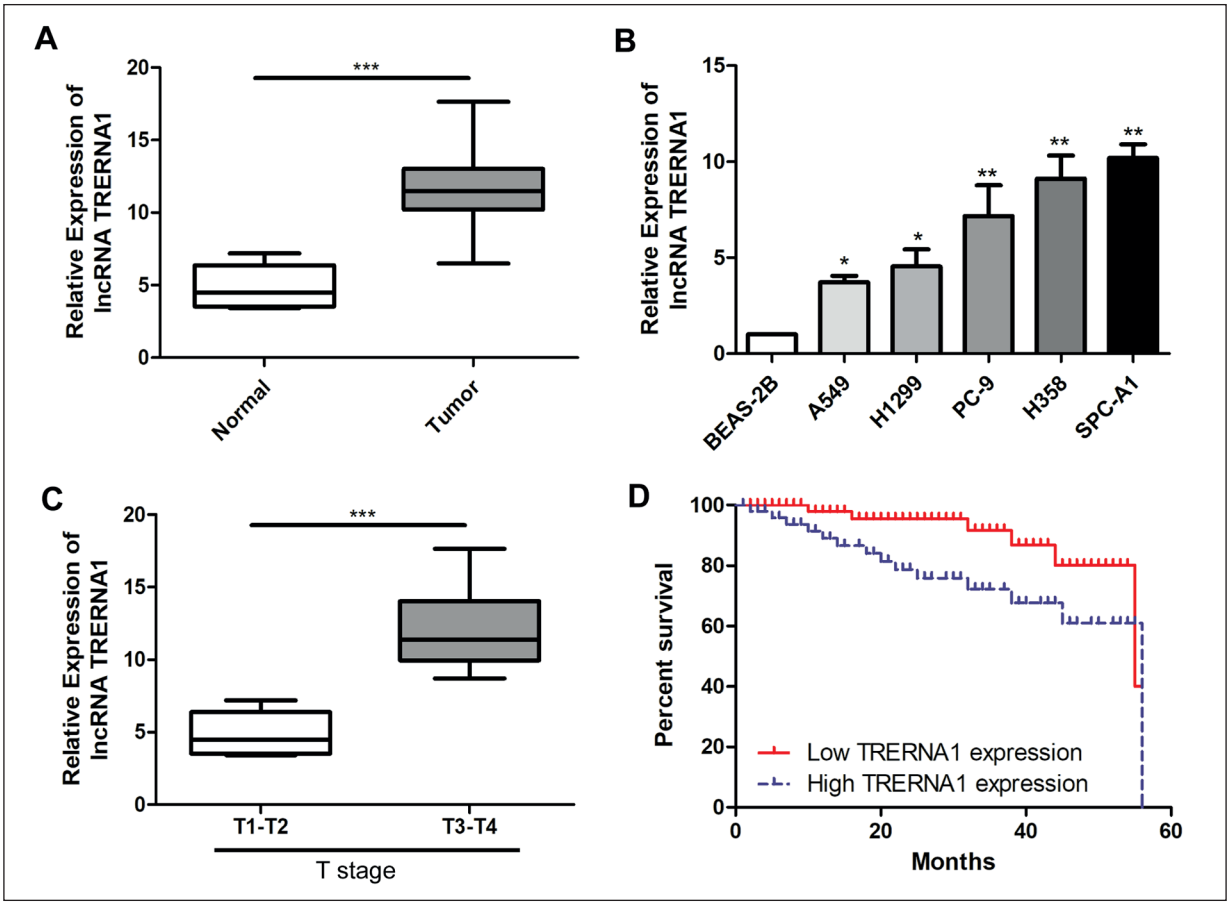

Figure 1. TRERNA1 is highly expressed in non-small cell lung cancer tissues and cell lines. **A,** QRT-PCR was used to detect the difference in TRERNA1 expression in non-small cell lung cancer tumor tissues and adjacent tissues; **B,** QRT-PCR was used to detect the expression level of TRERNA1 in non-small cell lung cancer cell lines; **C,** QRT-PCR was used to detect the difference in expression of TRERNA1 of NSCLC patients in different pathological stage; **D,** Kaplan-Meier survival curve of patients with non-small cell lung cancer based on TRERNA1 expression; the prognosis of patients with high TRERNA1 expression was significantly worse than that of low expression group. Data are expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Table I. Association of lncRNA TRERNA1 and FOXL1 expression with clinicopathologic characteristics of non-small cell lung cancer.

Parameters	No. of cases	TRERNA1 expression		p-value	FOXL1 expression		p-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.265			0.792
< 60	15	9	6		5	10	
≥ 6 0	24	10	14		9	15	
Gender				0.421			0.905
Male	20	11	9		7	13	
Female	19	8	11		7	12	
T stage				0.011			0.038
T1-T2	25	16	9		6	19	
T3-T4	14	3	11		8	6	
Lymph node metastasis				0.113			0.345
No	26	15	11		8	18	
Yes	13	4	9		6	7	
Distance metastasis				0.224			0.169
No	25	14	11		7	18	
Yes	14	5	9		7	7	

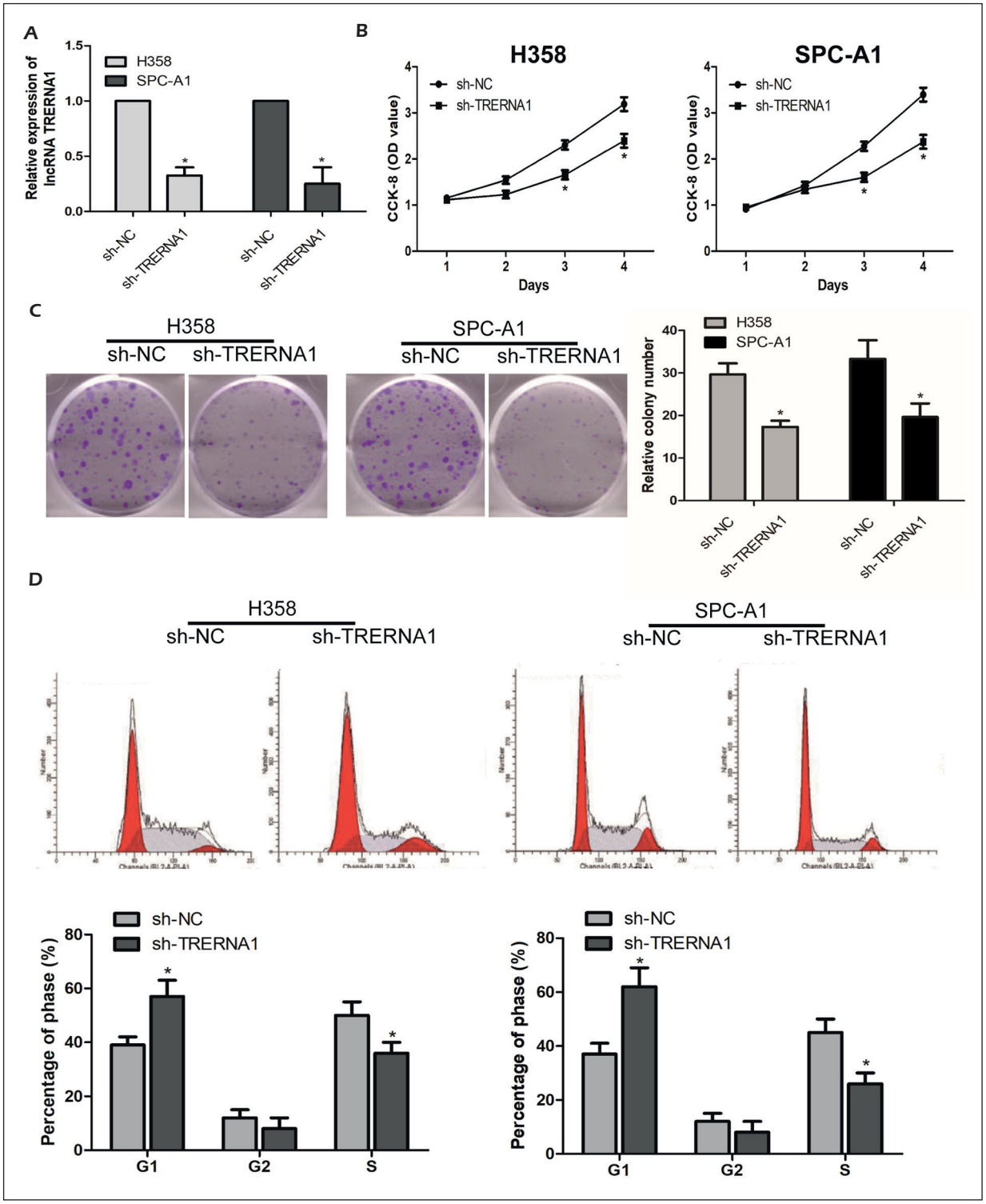

Figure 2. Silence of TRERNA1 inhibits the ability of non-small cell lung cancer cells to proliferate and cycle. **A,** QRT-PCR verified the transfection efficiency of sh-TRERNA1 in H358 and SPC-A1 cells; **B,** CCK-8 assay detected the effect of TRERNA1 on proliferation in H358 and SPC-A1 cells; **C,** Colony formation assay detected the effect of TRERNA1 on clonality in H358 and SPC-A1 cells (magnification: 20×); **D,** Flow cytometry assay was performed to detect the effect of TRERNA1 on cell cycle of H358 and SPC-A1 cells. Data are expressed as mean ± SD, *$p<0.05$.

FOXL1 Was a Direct Target of TRERNA1

To further validate the interaction between FOXL1 to TRERNA1, Dual-Luciferase reporter gene assay was conducted. Declined luciferase activity after co-transfection of pcDNA-TRERNA1 and wild-type FOXL1 vectors verified the binding between TRERNA1 and FOXL1 (Figure 3A). Furthermore, the FOXL1 level was remarkably down-regulated after silence of TRERNA1 (Figure 3B). In addition, the qRT-PCR result revealed that FOXL1 expression was remarkably lower in NSCLC tumor tissues or cell lines than in adjacent tissues or in BEAS-2B cells, (Figure 3C and 3D). Meanwhile, mRNA expressions of TRERNA1 and FOXL1 showed a negative correlation in NSCLC tissue specimens (Figure 3E).

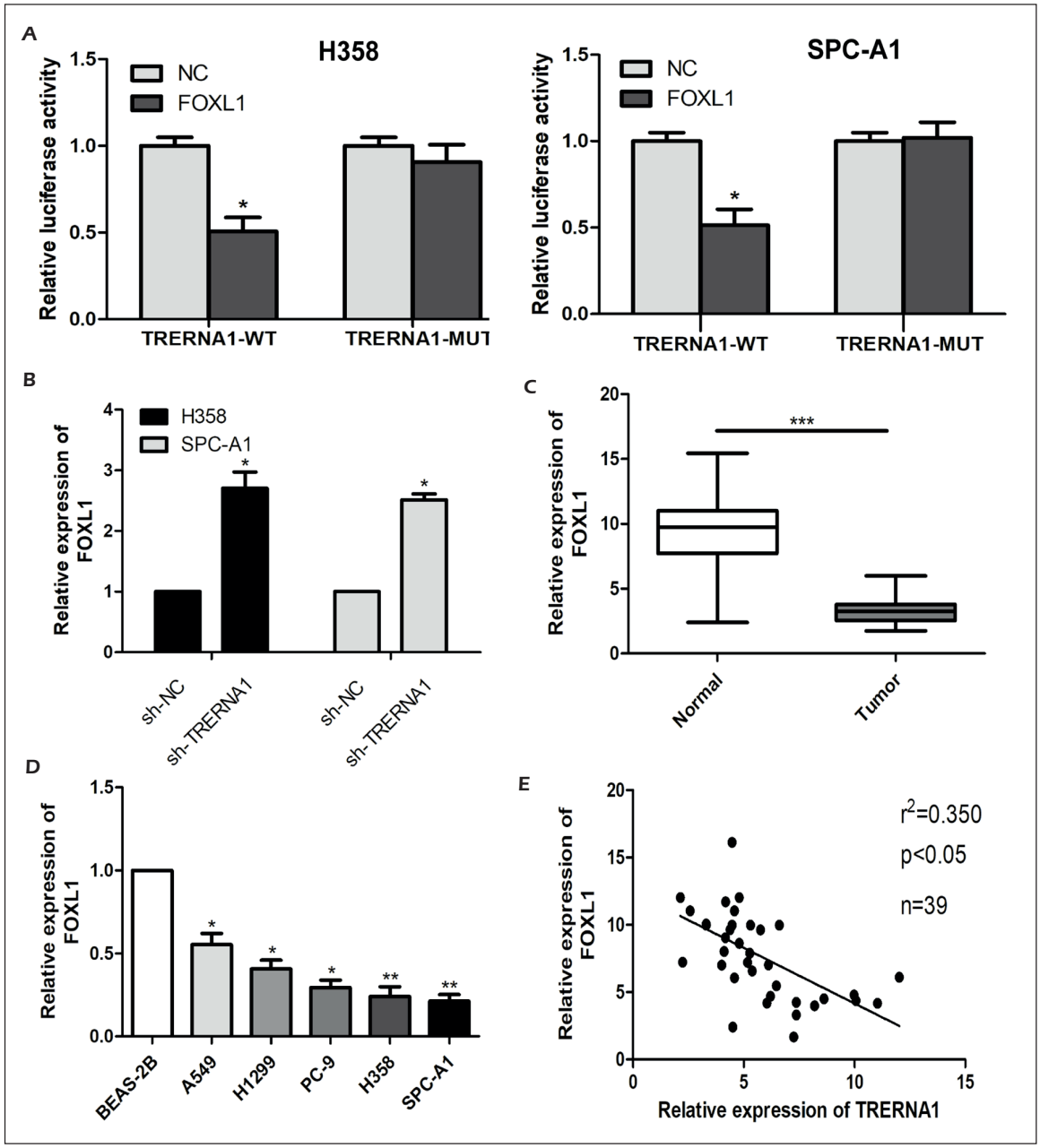

Figure 3. TRERNA1 can target FOXL1. **A,** Dual-Luciferase reporter gene assay validated the direct targeting of TRERNA1 to FOXL1; **B,** QRT-PCR detected differences in expression of FOXL1 after silencing TRERNA1; **C,** QRT-PCR was used to detect the difference in expression of FOXL1 in non-small cell lung cancer tumor tissues and adjacent tissues; **D,** QRT-PCR was used to detect the expression level of FOXL1 in non-small cell lung cancer cell lines; **E,** There was a significant negative correlation between TRERNA1 and FOXL1 expression in non-small cell lung cancer tissues. Data are expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Down-Regulation of FOXL1 Promoted Cell Growth

To investigate the effect of FOXL1 on NSCLC cell proliferation and cycle, FOXL1 knockdown model was constructed, and its interference efficiency was tested (Figure 4A). After knockdown of FOXL1 in H358 and SPC-A1 cells, CCK-8, colony formation assay, and flow cytometry illustrated the enhanced proliferation and arrested cell cycle in the G1 phase (Figure 4B-4D).

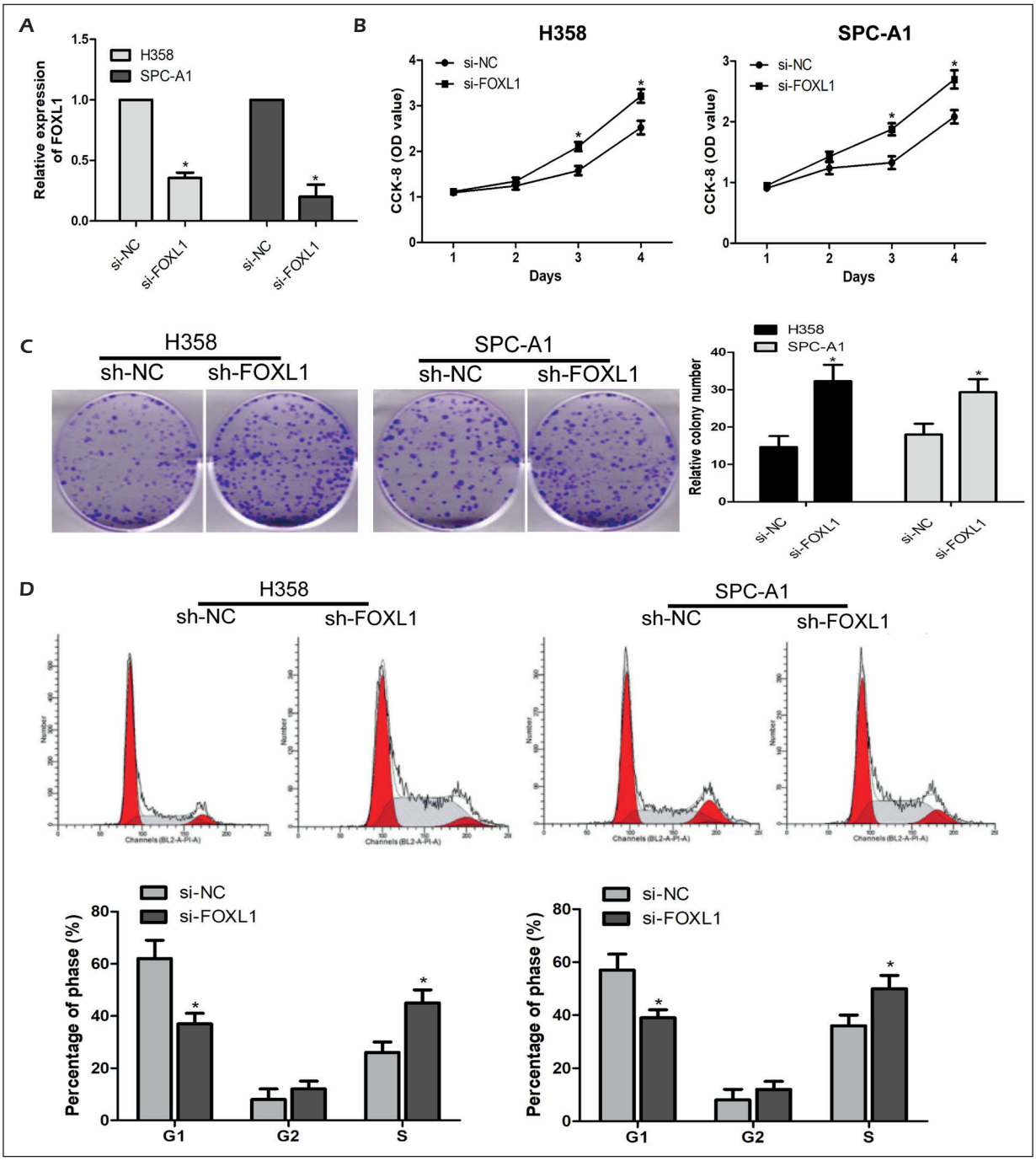

Figure 4. Silence of FOXL1 promotes proliferation of non-small cell lung cancer cells. **A,** QRT-PCR verified the transfection efficiency of TRERNA1 after knockdown of FOXL1 in the H358 and SPC-A1 cells; **B,** CCK-8 assay detected proliferation of H358 and SPC-A1 cells with FOXL1 knockdown; **C,** Colony formation assay detected the clonality in H358 and SPC-A1 cells with FOXL1 knockdown (magnification: 20×); **D,** Flow cytometry assay was performed to detect the effect of silencing FOXL1 on cell cycle in H358 and SPC-A1 cell lines. Data are expressed as mean ± SD, *$p<0.05$.

TRERNA1 Modulated FOXL1 Expression in Human NSCLC Cells

Subsequently, rescue investigations were conducted to analyze the role of TRERNA1/FOXL1 in NSCLC by cell co-transfection. TRERNA1 level in co-transfected cells was determined (Figure 5A). Moreover, they demonstrated that FOXL1 could counteract the influence of TRERNA1 on NSCLC progression (Figure 5B-5D).

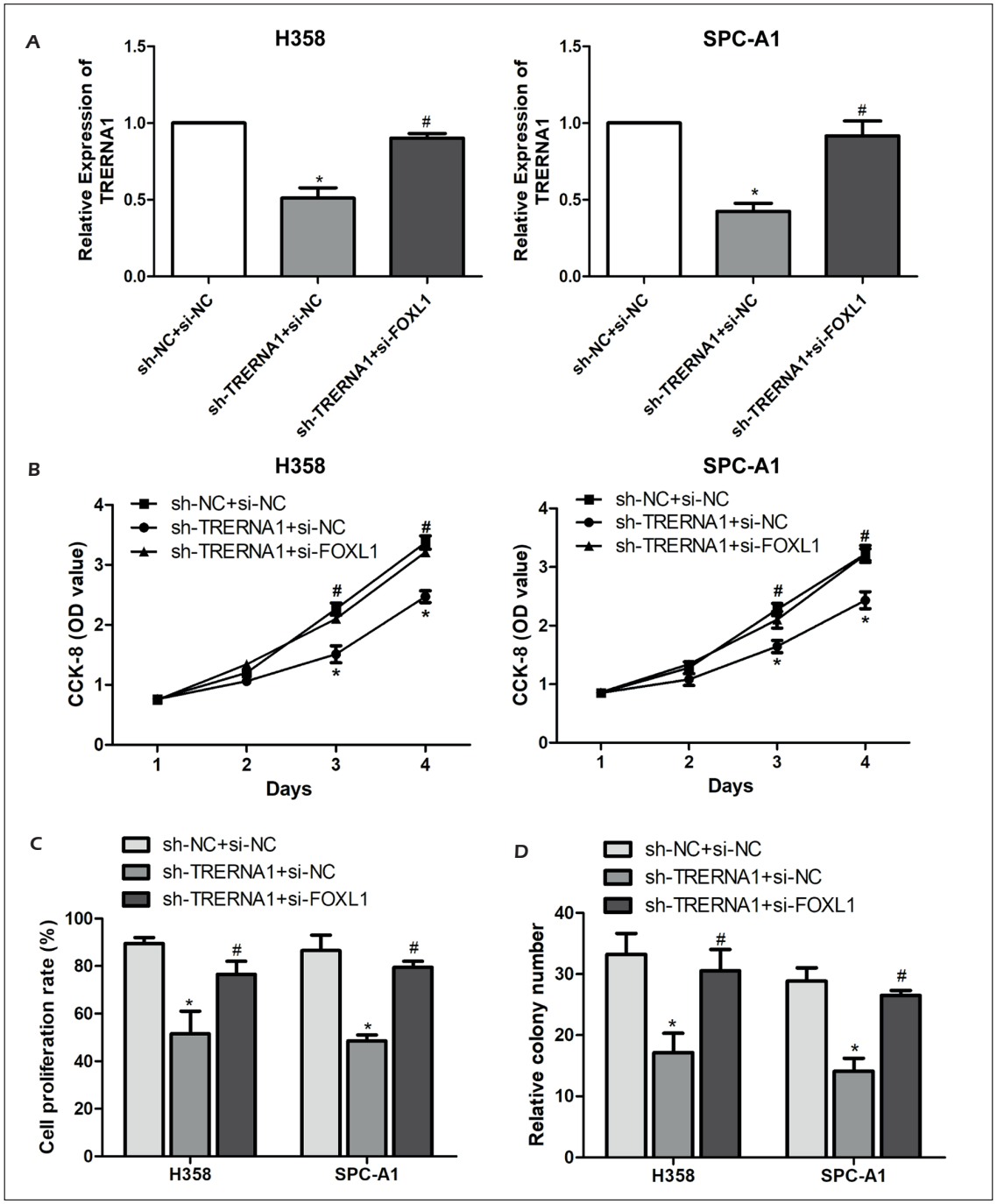

Figure 5. TRERNA1 regulates the expression of FOXL1 in non-small cell lung cancer. **A,** TRERNA1 expression in co-transfected cells detected by qRT-PCR; **B,** After co-transfection of TRERNA1 and FOXL1, CCK-8 assay was used to detect the cell proliferation in H358 and SPC-A1 cells; **C,** Cell proliferation assay detected the effect of co-transfection of TRERNA1 and FOXL1 on cell proliferation in H358 and SPC-A1 cell lines; **D,** Colony formation assay was performed to detect clonality in H358 and SPC-A1 cells after co-transfection of TRERNA1 and FOXL1. Data are expressed as mean ± SD, $*^{\#}p<0.05$.

Discussion

With the in-depth study of the human genome, the intricate gene regulatory networks are identified in the process of disease development. Tumor-associated genes could be utilized as diagnostic and therapeutic targets for tumor diseases[22]. Non-coding RNAs are the majority of human genome transcription, which are able to regulate disease progression even though they could not encode proteins[10-12].

With the deepening of research, it has been found that lncRNA, which is originally considered to be a transcriptional by-product and does not play a biological function, plays an equally important role in the occurrence and development of tumors. Its mechanism of tumor regulation is similar to that of miRNAs[13-15]. However, to date, only a few functional features of lncRNAs have been confirmed in oncology studies[16]. Reports[17-18] have demonstrated that lncRNAs play a critical regulatory role in many areas, including stem cell pluripotency and differentiation studies, molecular scaffolding, cell cycle, DNA methylation, histone modifications, and transcriptional gene silencing. It has been observed in several studies[16-18] that the abnormal expressions of lncRNAs are associated with the occurrence of various tumors. However, the specific biological roles they play in the processes of tumorigenesis, development, invasion, migration, etc., are still not very clear. In this work, TRERNA1 expression was remarkably up-regulated, and FOXL1 expression was down-regulated in NSCLC tissues. Meanwhile, their expression levels were verified to be significantly associated with pathological staging and poor prognosis of NSCLC patients. It is believed that TRERNA1 plays a role in promoting cancer in NSCLC, while FOXL1 plays a tumor-suppressing role. To further explore the effects of TRERNA1 and FOXL1 on the biological functions of NSCLC cells, the knockdown model of TRERNA1 was constructed. TRERNA1 was capable of promoting the progression of NSCLC.

Some reports[19-21] found a hypothesis about competitive endogenous RNA (ceRNA). Specifically, lncRNA acts as a miRNA sponge that recognizes the binding sequences in the promoter regions, and further regulates downstream genes[21]. Studies[15-17] have confirmed that the post-transcriptional regulation of lncRNA is correlated with the occurrence, proliferation, invasion, and migration of various tumors, as well as biological characteristics, such as apoptosis and autophagy. FOXL1 is a key tumor-suppressor gene. The results of our study disclosed that FOXL1 was lowly expressed in NSCLC tissues than in adjacent tissues, which inhibited cell proliferation in NSCLC cells. In addition, Dual-Luciferase reporter gene assay confirmed the binding relation between TRERNA1 and FOXL1. The above findings demonstrated that there may exist a positive feedback loop, which is, TRERNA1 leads to a rapid decline in FOXL1 expression through the ceRNA-related mechanism, thereby promoting the malignant progression of NSCLC.

Conclusions

We first observed that the expression of TRERNA1 was found remarkably enhanced in NSCLC, which was closely related to the pathological stage and poor prognosis of NSCLC patients. In addition, TRERNA1 might promote the malignant progression of NSCLC *via* modulating FOXL1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

State Key Laboratory of Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia Fund, SKL-HIDCA_2018-13.

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