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Original Article

Long noncoding RNA LINC00337 accelerates the non-small-cell lung cancer progression through inhibiting TIMP2 by recruiting DNMT1

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Abstract: Accumulating evidence reveals the essential roles of long noncoding RNAs (lncRNAs) in the non-small-cell lung cancer (NSCLC) tumorigenesis. Here, our research investigated the biological roles of novel lncRNA LINC00337 in the NSCLC tumorigenesis and discover the potential mechanism. In the NSCLC tissue and cell lines, LINC00337 was found to be remarkedly up-regulated, and the ectopic LINC00337 overexpression indicated the poor survival of NSCLC patients. In vitro, gain and loss of functional assays showed that LINC00337 promoted the progression of NSCLC cells, including proliferation and invasion. In vivo, LINC00337 knockdown inhibited the tumor growth of NSCLC cells. Mechanically, LINC00337 could recruit the epigenetic repressor DNMT1 to the promoter region of TIMP2 to silence its expression. In conclusion, our study found the critical regulation of lncRNA LINC00337 for the NSCLC through epigenetic regulation, which may serve as a predictive biomarker and potential therapeutic target.

Keywords: NSCLC, LINC00337, DNMT1, TIMP2, promoter methylation

Introduction

Lung cancer has been the most concerning malignancy worldwide due to the high morbidity for the sufferers both in developing and developed country [1]. Non-small-cell lung cancer (NSCLC) accounts for a proportion near 80% of the all events of lung cancer [2]. NSCLC includes several pathological classifications, including squamous cell carcinoma or squamous cell carcinoma, adenocarcinoma, and large cell carcinoma [3, 4]. Chemotherapy resistance, distant metastasis and recurrence are the main reason for the poor therapeutic effect of NSCLC patients. Therefore, this pressing situation calls for more precise diagnose and more effective targeted therapy.

More and more researches uncover the critical roles of long non-coding RNAs (lncRNAs) in the human diseases, including cancers, cardiovascular disease and endocrine metabolic diseases [5-7]. The characteristics of lncRNAs typically represent the non-protein-coding potential with more than 200 nucleotides in length. Although lncRNAs are short of the functional protein coding capacity, they could bind with other vital elements to wildly modulate the biological or pathological process of human cancers [8]. For example, lncRNA LINC00460 is upregulated in the gefitinib-resistant NSCLC tissue and cells, and LINC00460 promotes the 50% inhibitive concentration of gefitinib in gefitinib-resistant NSCLC cells (A549/GR) and the multidrug-resistant-related proteins (P-gp, MRP1, and BCRP) [9].

The role of lncRNA LINC00337 has been identified in previous literature. In gastric cancer, LINC00337 was found to be up-regulated in the cells and tissue specimens and the ectopic LINC00337 overexpression indicates the poor clinical outcome [10]. In present research, our results illustrated that LINC00337 was remarkedly up-regulated in the NSCLC tissue and cells. In the molecular mechanism, LINC00337 could recruit the DNMT1 to the promoter region of TIMP2 and induce its promoter methylation.

Materials and methods

Human specimens

The clinical project had been approved by Ethics Committee of Human Research at the Nantong

Tumor Hospital. These NSCLC tissue samples were surgically archived at Nantong Tumor Hospital. All these written consents had been obtained from all patients. The lung cancer specimens were treated with liquid nitrogen and stored at a -80°C refrigerator for following analysis.

Cell culture and cell transformation

Normal bronchial epithelial cells (NHBE) and NSCLC cell lines (A549, H460, H1299, H322) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (FBS), 100 μg/ml Penicillin and Streptomycin in humidified incubator containing 5% CO_2 at 37°C.

Cell transfection

The required plasmids and shRNA targeting LINC00337 and TIMP2 were purchased from RiboBio (Guangzhou, China). NSCLC cells were seeded into a six-well plate at the density of 3 × 10^5 cells/well at confluence of 90%. Subsequently, NSCLC cells were transfected with Lipofectamine 2000 kit (Invitrogen Inc., Carlsbad, CA, USA). Cells were then cultured in a 5% CO_2 incubator at 37°C for 6 h.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RT-qPCR was performed to measure the relative level of these genes in NSCLC tissues and cell lines. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The purity was measured by the NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA). cDNA was reversely conducted using Reverse Transcription System Kit (Applied Biosystems, Darmstadt, Germany), which was performed under the manufacturer's protocol. The PCR reaction was conducted using SYBR qPCR Mix. The primer sequences of mRNA for candidate genes and housekeeping gene were listed in Table S1. The comparative Ct method was calculated for the results through $2^{-\Delta\Delta Ct}$ method.

Cell counting kit-8 and colony formation assay

For the CCK-8 assay, the transfected cells were cultured in six groups and then washed twice with PBS. The cells were administrated with cell counting kit-8 (CCK-8) reagent (Dojindo Laboratories, Kumamoto, Japan) after 24 h. Cells were cultured again in a 5% CO_2 incubator and the optical density (OD) value of wells was detected to measure cell activity. For the colony formation assay, NSCLC cells were placed in a fresh 6-well plate with 1640 medium containing 10% FBS. After 14 days, the clones were fixed with methanol and stained with 0.1% crystal violet.

Invasive assay

For invasion assays, 1 × 10^5 cells seeded on the top of the membrane that pre-coated with Matrigel (BD, Franklin Lakes, NJ, USA) in the 24-well Transwell chambers (8-μm-pore size). On the upper floor, the RPMI-1640 without 10% fetal bovine serum was added and the RPMI-1640 containing with 10% fetal bovine serum was added to lower chamber floor. After 24 hours, the invaded cells were fixed and then stained with 0.5% Crystal violet solution.

Western blotting

RIPA lysis buffer adding with 1% protease inhibitor (Solarbio, Beijing, China) was used to extract the total protein from NSCLC cell lines. 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate equal amounts of protein. Protein was transferred onto polyvinylidene difluoride (PVDF, Millipore, Massachusetts, USA) membranes and blocked using the non-fat milk, and then membranes were incubated overnight at 4°C with primary antibody (anti-TIMP2, Abcam, ab180630, 1:1000). The membranes were washed with TBST and incubated with the GAPDH secondary antibodies. Signals were visualized using Gel Doc 2000 imaging scanner (Bio-Rad).

Subcellular cytoplasmic and nuclear isolation

The PARIS Kit (Life Technologies, CA, USA) was used to isolate the nuclear and cytosolic fractions of A549 and H1299 cells according to the manufacturer's instructions. The qRT-PCR assay was carried out to detect the relative expression.

RNA-binding protein immunoprecipitation (RIP)

RNA immunoprecipitation was performed using the Magna RIP kit (EMD Millipore Co., Billerica,

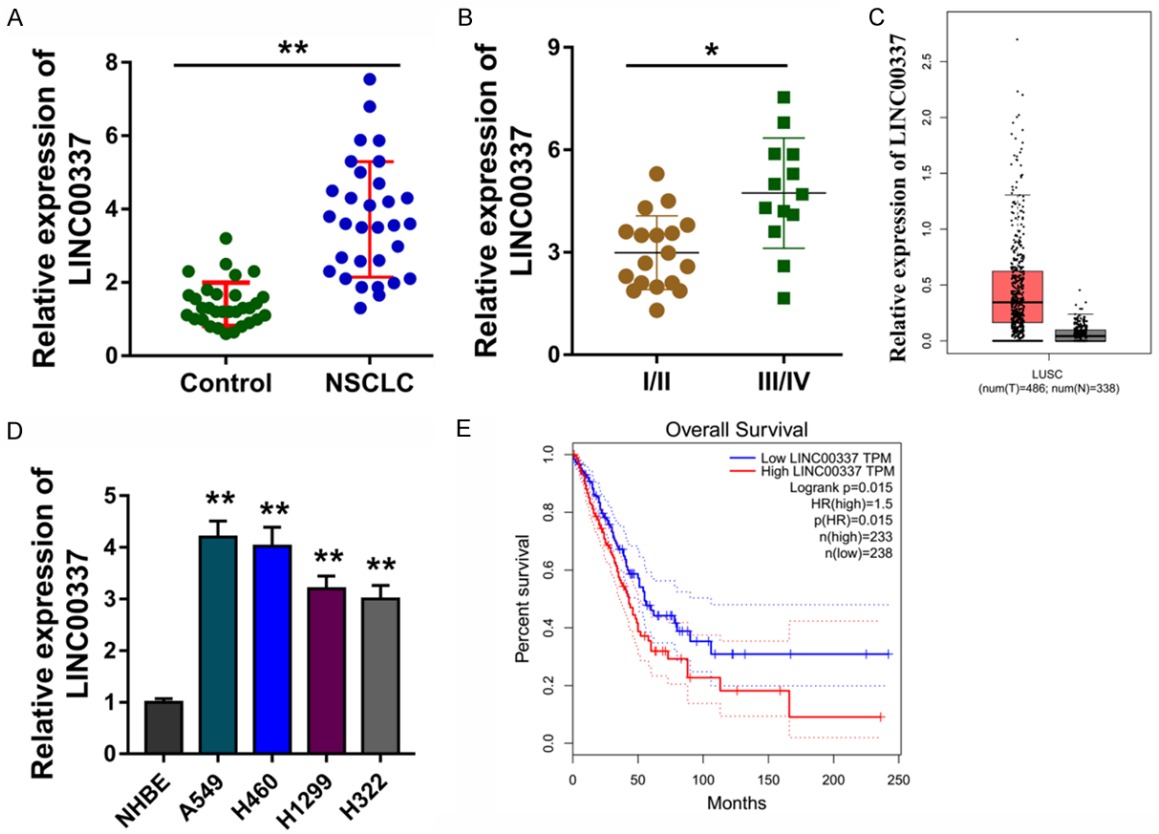

Figure 1. LncRNA LINC00337 acts as the oncogenic factor for NSCLC individuals. A. In the enrolled NSCLC patients' specimens, RT-PCR showed the up-regulation of lncRNA LINC00337 compared to the normal tissue. B. Level of LINC00337 in the advanced NSCLC pathological grading stage and the primary stage. C. In the TCGA database and GEPIA (http://gepia.cancer-pku.cn/index.html), expression of LINC00337 in the NSCLC cohort compared to the normal cohort. D. The enrichment of LINC00337 in the NSCLC cells was measured by RT-PCR. E. Overall survival rate showed the survival rate of NSCLC cohort with higher or lower LINC00337. Error bars denote SD of technical triplicates. *P < 0.05, **P < 0.01.

MA, USA). The antibody for DNMT1 and EZH2 were transfected into A549 and H1299 cells and then incubated at 4°C overnight. Monoclonal anti-Ago2 antibody and IgG was used as the control. The RNAs were extracted and assessed by qPCR as fold enrichment in Ago2 relative to input.

Chromatin immunoprecipitation (ChIP) assay

EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Millipore) was used for the ChIP assay. The antibody (anti-DNMT1, Millipore) was performed according to the manufacturer's protocols. Cross-linked chromatin was sonicated to be 200-1000 bp fragment length. The isolated DNA was then quantified with SYBR Green PCR Kit (Takara) to measure enrichment. Normal mouse immunoglobulin G (IgG) was used as negative control.

In vivo mice xenograft assay

The in vivo mice xenograft assay was carried on male BALB/C nude mice and approved by the Animal Experimental Committee of Nantong Tumor Hospital. The assay processes were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory. About 1×10^6 A549 transfected with shRNA cells were subcutaneously implanted into the flank of nude mice. Three week later, the volume of xenografts was assessed and calculated through the formula: Volume = $(\text{Width}^2 \times \text{Length})/2$.

Statistical analysis

Statistic data of each group were determined by SPSS (18.0 version, SPSS, Inc., USA) and GraphPad (6.0 version). Data were examined

Table 1. Correlation within LINC00337 level and clinicopathological feature of NSCLC patients

	Num	LINC00337		*P*-Value
		Low = 15	High = 16	
Gender				0.467
Male	18	8	10	
Female	13	7	6	
Age (years)				0.511
≥ 65	19	10	9	
< 65	12	5	7	
Tumor size				0.122
≥ 4 cm	16	5	11	
< 4 cm	15	10	5	
Differentiation				0.002*
well, moderate	10	7	3	
poor	21	8	13	
TNM				0.030*
I-II	18	7	11	
III/IV	13	8	5	
Lymph metastasis				0.568
No	16	8	8	
Yes	15	7	8	

*P < 0.05 represents statistical difference.

for the normality and equal variance across groups and represented as mean ± SD. Intergroup difference was assessed using Student's t-tests, and multivariate statistical analysis was assessed using variance (ANOVA) analysis. Three replicates were done for the final data. P < 0.05 was considered statistical significance.

Results

LncRNA LINC00337 acts as the oncogenic factor for NSCLC individuals

In the enrolled NSCLC patients' specimens, RT-PCR showed that lncRNA LINC00337 was evidently up-regulated in the NSCLC individuals compared to the normal tissue (**Figure 1**, **Table 1**). Besides, the level of LINC00337 was much higher in the advanced NSCLC pathological grading stage than the primary stage (**Figure 1B**). In the TCGA database and GEPIA (http://gepia.cancer-pku.cn/index.html), we found the similar results that LINC00337 was up-regulated in the NSCLC cohort compared to the normal cohort (**Figure 1C**). The enrichment of LINC00337 in the NSCLC cells was measured by RT-PCR, illustrating that LINC00337 was up-regulated (**Figure 1D**). Overall survival rate showed that the higher LINC00337 in the NSCLC patients suggested the lower survival rate (**Figure 1E**). These finding concludes that lncRNA LINC00337 acts as the oncogenic factor for NSCLC individuals.

LINC00337 promotes the oncogenesis of NSCLC cells in vivo and vitro

The potential functions of LINC00337 for the NSCLC oncogenesis were investigated by the gain and loss of functional assays. To obtain the LINC00337 overexpression and the silencing, plasmids and short hairpin RNA were respectively transfected in to A549 and H1299 cells (**Figure 2A**, **2B**). Proliferative potential of A549 and H1299 cells was measured by the CCK-8 assay, unveiling the promoting effects by LINC00337 over-expression and the repressive effects by LINC00337 silencing (**Figure 2C**, **2D**). The colony formation experiments elucidated that LINC00337 silencing inhibited the A549 cells clone number and LINC00337 over-expression promoted the H1299 cells clone number (**Figure 2E**). Transwell invasion elucidated that LINC00337 silencing inhibited the A549 cells invasion and LINC00337 over-expression promoted the H1299 cells invasion (**Figure 2F**). In vivo animals of nude mice assay elucidated that LINC00337 silencing inhibited the tumor growth using A549 cells (**Figure 2G**). Above all, LINC00337 promotes the oncogenesis of NSCLC cells in vivo and vitro.

LINC00337 represses the TIMP2 via binding DNMT1

The subcellular location analysis for LINC00337 was performed using the subcellular fractionation assays, showing the dominating nuclear location of LINC00337 in the NSCLC cells (**Figure 3A**). Several potential targets for the LINC00337 were measured using RT-PCR in the A549 cells transfected with sh-LINC00337, including p21, p57, PTEN, LATS2 and TIMP2. Among them, TIMP2 was significantly up-regulated in the transfection (**Figure 3B**). RNA binding protein immunoprecipitation (RIP) showed that LINC00337 could interact with the DNMT1, instead of EZH2, in the NSCLC cells (**Figure 3C**). Western blot assay showed that LINC00337 knockdown and DNMT1 knockdown could bo-

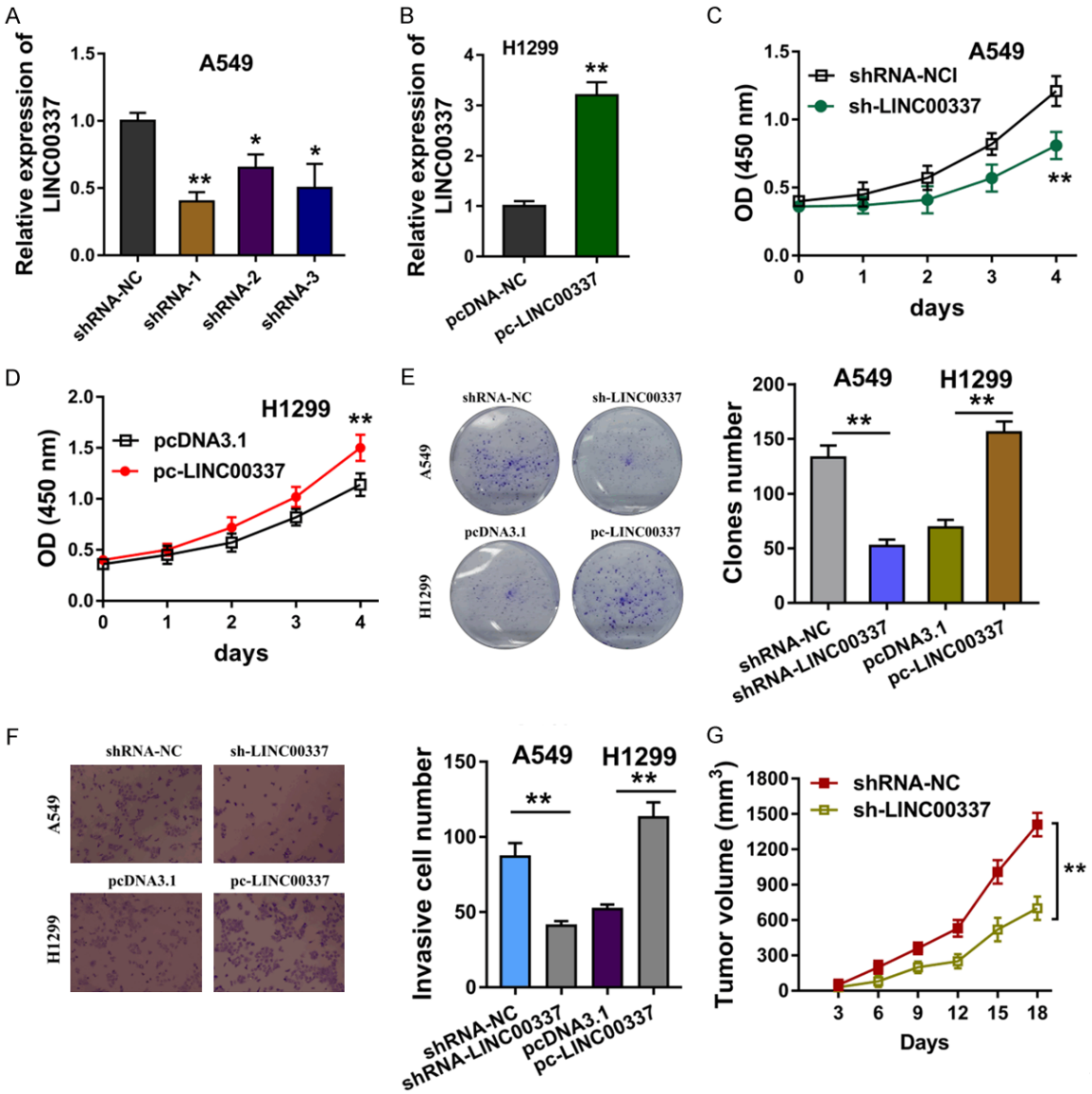

Figure 2. LINC00337 promotes the oncogenesis of NSCLC cells in vivo and vitro. A, B. Plasmids and short hairpin RNA were respectively transfected in to A549 and H1299 cells to obtain the LINC00337 overexpression and the silencing. C, D. Proliferative potential of A549 and H1299 cells was measured by the CCK-8 assay. E. The colony formation experiments elucidated the clone number of A549 cells and H1299 cells. F. Transwell invasion elucidated the invasion of A549 cells and H1299 cells. G. In vivo animals of nude mice assay elucidated the tumor growth using A549 cells with LINC00337 silencing transfection. Error bars denote SD of technical triplicates. *P < 0.05, **P < 0.01.

th promote the TIMP2 protein (**Figure 3D**). Chromatin immunoprecipitation (ChIP) illustrated that LINC00337 knockdown inhibited the incorporation of LINC00337 with DNMT1 in the precipitated complex (**Figure 3E**). Correlation analysis within LINC00337 and TIMP2 using Pearson's analysis indicated that LINC00337 was negatively correlated with TIMP2 (**Figure 3F**). Overall, LINC00337 represses the TIMP2 via binding DNMT1.

TIMP2 could eliminate the functions of LINC00337 in NSCLC cells

It had been found that LINC00337 could repress the TIMP2 via binding DNMT1. Rescue assay was performed to identify the combined roles of LINC00337 and TIMP2. The overexpression plasmid for TIMP2 was transfected in the H1299 cells, which could significantly overexpress the TIMP2 mRNA (**Figure 4A**). CCK-8

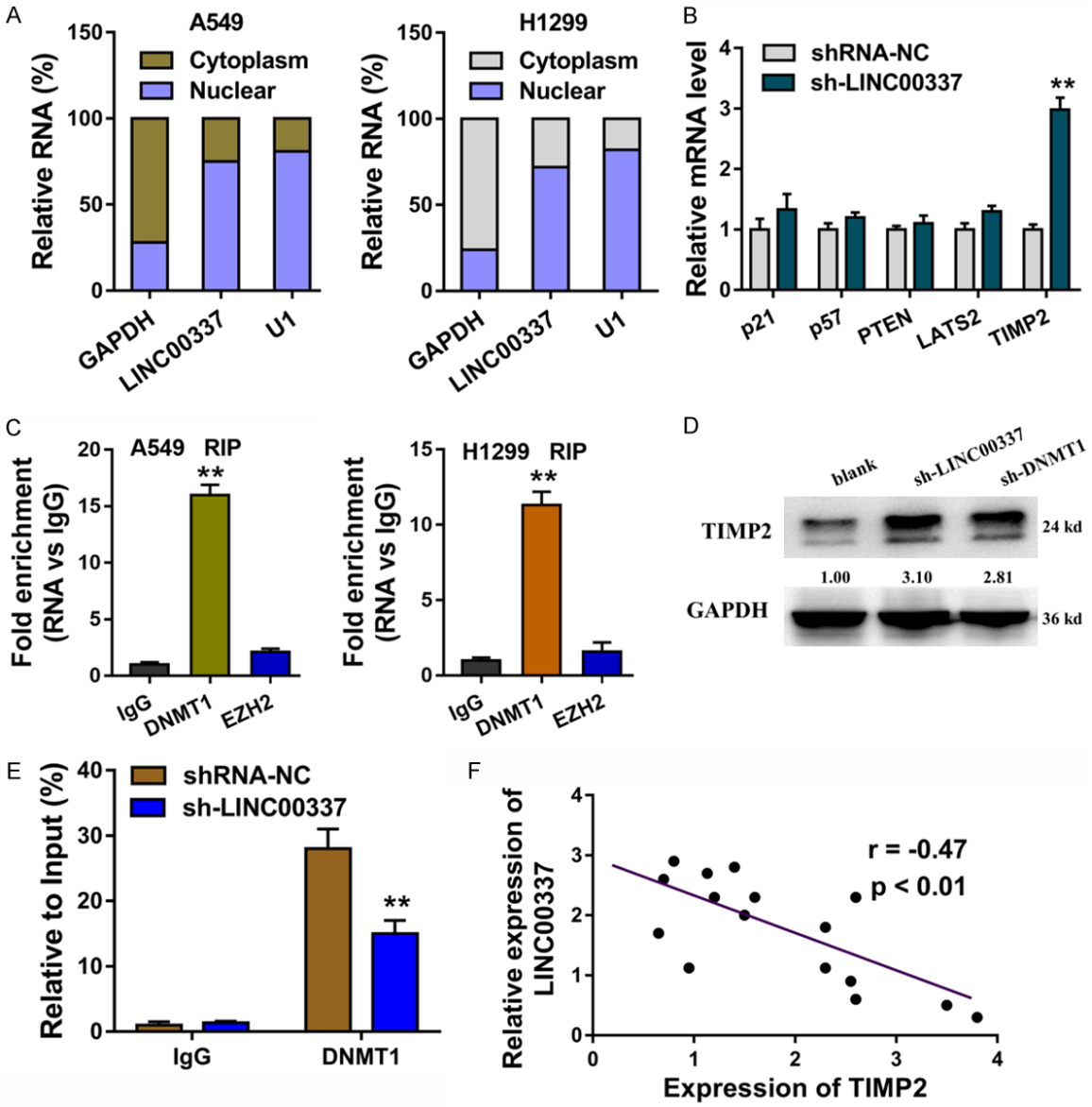

Figure 3. LINC00337 represses the TIMP2 via binding DNMT1. A. The subcellular location analysis for LINC00337 was performed using the subcellular fractionation assays. B. Potential targets for the LINC00337 were measured using RT-PCR in the A549 cells transfected with sh-LINC00337, including p21, p57, PTEN, LATS2 and TIMP2. C. RNA binding protein immunoprecipitation (RIP) showed the interaction of LINC00337and DNMT1. D. Western blot assay showed the TIMP2 protein in A549 cells with LINC00337 knockdown and DNMT1 knockdown. E. Chromatin immunoprecipitation (ChIP) illustrated the incorporation of LINC00337 with DNMT1 in the precipitated complex. F. Correlation analysis within LINC00337 and TIMP2 was performed using Pearson's analysis. Error bars denote SD of technical triplicates. *P < 0.05, **P < 0.01.

assay illustrated that TIMP2 overexpression transfection decreased the proliferative ability of NSCLC cells (**Figure 4B**). Moreover, colony formation assay and transwell assay both showed that TIMP2 overexpression transfection reduced the colony formation and invasion of NSCLC cells (**Figure 4C**, **4D**). The public tumor database of TCGA (www.tcga.org/) and GEPIA (http://gepia.cancer-pku.cn/index.html) showed that TIMP2 expression level was decreased in the LUSC and LUAD subgroup (**Figure 4E**), and the higher TIMP2 expression indicated the higher survival rate of NSCLC patients (**Figure 4F**). In conclusion, these findings elucidated that TIMP2 could eliminate the functions of LINC00337 in NSCLC cells.

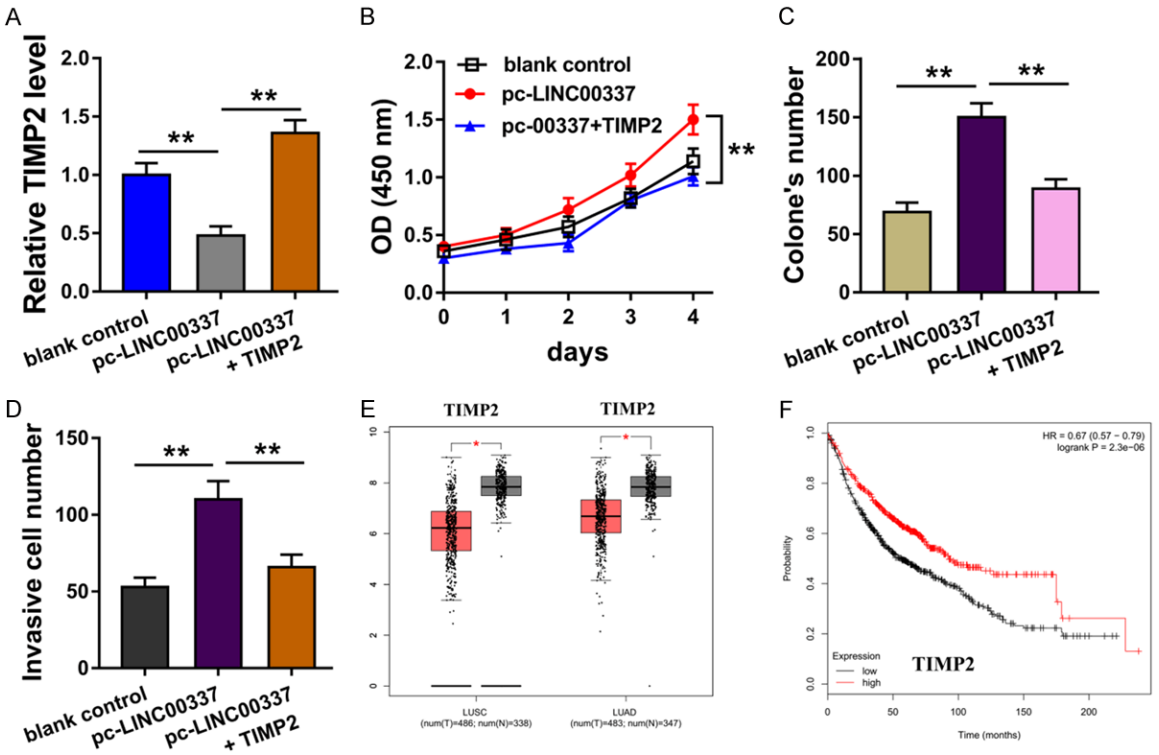

Figure 4. TIMP2 could eliminate the functions of LINC00337 in NSCLC cells. A. The overexpression plasmid for TIMP2 was transfected in the H1299 cells to over-express the TIMP2 mRNA. B. CCK-8 assay illustrated the proliferative ability of NSCLC cells with TIMP2 overexpression transfection and/or LINC00337 knockdown. C. Colony formation assay showed the colony formation of NSCLC cells. D. Transwell assay showed the invasion of NSCLC cells. E. The public tumor database of TCGA (www.tcga.org/) and GEPIA (http://gepia.cancer-pku.cn/index.html) showed the decreased level of TIMP2 in the LUSC and LUAD subgroup. F. The survival rate of NSCLC patients with higher or lower TIMP2 expression. Error bars denote SD of technical triplicates. *P < 0.05, **P < 0.01.

Discussion

The essential molecular roles and clinical relevance of ncRNAs in human cancers, especially NSCLC, have been demonstrated by more and more published report [11, 12]. LncRNAs not only participate the biological regulation of cancer cells, including proliferation, apoptosis and metastasis, but also function as the clinical predictive biomarker indexes for NSCLC [13, 14].

LINC00337 is a relatively new lncRNA in the high-throughput sequencing with fewer reports [15]. In the gastric cancer, researchers find that LINC00337 is up-regulated in the gastric cancer cells and tissue specimens, which indicates the poor clinical outcome for gastric cancer patients. The functional experiments illustrated that LINC00337 silencing repressed the proliferation, invasion and tumor growth in vitro and in vivo. The mechanical experiments showed that LINC00337 epigenetically repressed the p21 via EZH2 [10]. These results conclude that LINC00337 might function as an oncogene for the human cancer.

To investigate the potential roles of LINC00337 in the NSCLC, we measured its enrichment in the tissue specimens and cell lines, unveiling the over-expression of LINC00337 in both samples. More significantly, the higher LINC00337 level indicates the poor overall survival of NSCLC individuals. This shows that the LINC00337 might act as an oncogene for NSCLC. Gain and loss of functional investigation showed that LINC00337 promoted the proliferation, invasion in vitro and the knockdown of LINC00337 repressed the tumor growth in vivo. We herein identify the tumor promoting roles of LINC00337 in the NSCLC carcinogenesis.

In the following research, we discovered that LINC00337 primarily located in the nuclear of NSCLC cells, being capable of regulating NSCLC

phenotype via transcriptional regulation [16, 17]. The downstream target of LINC00337 was critical for mechanism research and we selected several potential targets for LINC00337. Then, we found that TIMP2, a remarkable tumor repressor for NSCLC, was significantly regulated by LINC00337. Certainly, the mediating factor by which LINC00337 regulates TIMP2 was DNMT1, a vital DNA methyltransferase in the epigenetic regulation.

In the multiple pathogenic factors of NSCLC, DNMT1 has been wildly identified to function as an oncogene which promotes the expressional inhibition of tumor suppressor gene, thereby accelerating the tumor progressions [18-20]. For instance, EZH2-recruited DNMT1 epigenetically silenced the miR-142-3p and suppressed nasopharyngeal carcinoma cell metastasis and epithelial-mesenchymal transition [21]. In NSCLC, microRNA-9 enhanced the methylation status of the microRNA-9 promoter and promotes radiosensitivity via its promoter methylation status [22]. DNMT1 was verified as an EPAS1 target gene and could be activated by stabilized EPAS1 proteins in NSCLC cells to decrease EPAS1 mRNA expression by promoting methylation [23].

In conclusion, our study found the critical regulation of lncRNA LINC00337 for the NSCLC through the recruiting the epigenetic repressor DNMT1 to the promoter region of TIMP2. The dysregulation of lncRNAs could be one of the leading factors for NSCLC progression. The novel finding about LINC00337 might bring new ideas for the NSCLC clinical treatment strategy.

Disclosure of conflict of interest

None.

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Table S1. Primers sequences for qRT-PCR and sequences of shRNA

	Sequences
LINC00337	forward, 5'-TGAGCAAACCCGCGCCTGCTTG-3'
	reverse, 5'-TTGCCATTCGGGGTGCGCGAAG-3'
TIMP2	forward, 5'-AAGCGGTCAGTGAGAAGGAAG-3'
	reverse, 5'-GGGGCCGTGTAGATAAACTCTAT-3'
sh-LINC00337-1	5'-ACCTAAGGTTGGAATTCGGT-3'
sh-LINC00337-2	5'-ACAGTATTCCGGGCTTATGT-3'
sh-LINC00337-3	5'-ATGCCGGGTACCAGACTAATC-3'
GAPDH	forward, 5'-AGAAGGCTGGGGCTCATTTG-3'
	reverse, 5'-AGGGGCCATCCACAGTCTTC-3'