

Original Article

Long noncoding RNA LINC00337 promote gastric cancer proliferation through repressing p21 mediated by EZH2

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Abstract: Gastric cancer is one of the most common human malignancies. Some long noncoding RNA (lncRNA) has been validated to be oncogene in gastric cancer. In this research, we found that LINC00337 was up-regulated in the gastric cancer cells and tissue specimens. Clinically, the ectopic LINC00337 overexpression indicates the poor clinical outcome. 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-mediated inhibition. Overall, this finding suggests that LINC00337 acts as the oncogene to promote gastric cancer cells proliferation through epigenetically repressing p21 mediated by EZH2, providing a new insight for gastric cancer.

Keywords: Gastric cancer, LINC00337, p21, EZH2

Introduction

Gastric cancer acts as the one of the most common malignancies and the primary cause cancer-related death worldwide [1, 2]. Despite the rapid improvements in diagnostic, surgical techniques and targeted chemotherapy, the 5-year overall survival rate of gastric cancer patients remains unsatisfactory due to advanced stage along with lymphatic metastasis [3, 4]. However, the underlying molecular mechanism of gastric cancer tumorigenesis and metastasis remains unclear [5]. Therefore, the understanding of gastric cancer tumorigenesis mechanisms will provide new treatment strategy for gastric cancer.

The noncoding RNA (ncRNA) include two major subgroups, microRNAs (20-22nt) and long non-coding RNAs (lncRNAs) longer than 200 nucleotides [6, 7]. Although these RNAs could not be translated to proteins, lncRNAs as well as microRNAs are much critical due to their extensive regulatory action in the tumorigenesis, metastasis and chemoresistance of gastric

cancer [8]. For example, in the gastric cancer cells and tissue, lncRNA NNT-AS1 expression level is significantly up-regulated compared with adjacent normal tissue and normal cell lines [9]. And, the overexpression of NNT-AS1 indicated the poor prognosis [10]. Moreover, lncRNA SNHG17 expression is upregulated in gastric cancer tissues and cells, and significantly correlated with lymphatic metastasis and advanced TNM stage [11].

In this research, we investigate the expression level of LINC00337 in the gastric cancer tissue and cells, and further explore the possible deepgoing mechanisms to unveil its pathogenesis. Moreover, we find that LINC00337 promotes the gastric cancer tumorigenesis via epigenetically repressing the p21.

Materials and methods

Tissue samples and ethics statement

Gastric cancer patients were enrolled for this study at Tianjin Medical University General

Hospital. The testing program in this study has been approved by the Ethics Committee of Tianjin Medical University General Hospital. Before the surgery extraction, every patient has signed the inform content and the tissue was stored and collected immediately in liquid nitrogen during the surgery.

Cell culture

Gastric cancer cells (SGC-7901, BGC-823, MGC-803, AGS) and the normal cell (GES-1) were provided Institute of the Chinese Academy of Sciences (Shanghai, China). Gastric cancer cells and normal cells were maintained in RPMI 1640 (Invitrogen, Shanghai, China) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin and 100 U/ml streptomycin (Invitrogen).

Oligo transfection

SGC-7901 and BGC-823 cells were seeded at six-well plates and instantaneously transfected with specific siRNA or scrambled siRNA controls were constructed. siRNA or controls were transfected with specific siRNA (100 nM) and control siRNA (100 nM) by using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The sequences were listed in the [Table S1](#).

RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, USA) based on the manufacturer's protocol. After being qualified by Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA), the reverse transcription was conducted using PrimeScript RT master mix (TaKaRa, Japan). Quantitative real-time PCR (qRT-PCR) analysis was conducted by SYBR Premix Ex Taq (TaKaRa, Japan) normalized to GAPDH. Results were analyzed using the $2^{-\Delta\Delta Ct}$ calculation method.

Western blot analysis

The global cellular lysates were isolated from cells and moved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF, Millipore). The PVDF members were blocked with milk (5% non-fat) and then incubated with primary antibodies against p21 (Abcam) following secondary antibodies. Blots were subjected to an electrophoresis gel imaging system (BD, Franklin Lakes, NJ, USA).

CCK-8 proliferation assay

siRNA was transfected into SGC-7901 and BGC-823 cells. Subsequently, cells were administrated with the CCK-8 reagents (EnoGene, Nanjing, Jiangsu, China) and the absorbance was evaluated (450 nm).

Invasive transwell assay

The invasion assay was performed by the transwell chamber (BD Biosciences, San Jose, CA, USA) after the starveling cells. The cells were trypsinized and seeded as single-cell suspension on the upper floor of Transwell chamber filter coated by Matrigel (BD). The lower chamber was supplied with culture medium added with 10% fetal bovine serum. After the culture of 24 hours, the invaded cells were fixed with 4% paraformaldehyde and stained with 0.025% crystal violet. The total invasive number is counted under microscope.

Nuclear and cytoplasm fractionation

To determine the nuclear and cytoplasm fractionation in the gastric cancer cells, PARIS Kit (Life Technologies, Carlsbad, CA, USA) was performed according to the manufacturer's instructions.

RNA immunoprecipitation (RIP)

RNA immunoprecipitation (RIP) assays were conducted by Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Boston, MA, USA) according to the manufacturer's instructions. Antibody for RIP (anti-EZH2, Abcam) assay was obtained.

Chromatin immunoprecipitation assay (ChIP)

Gastric cancer cells (2×10^6) were plated in plates and the DNA was dissociated into 300-1000 bp length. Then, the isolation was treated with 1% formaldehyde to cross-link the DNA and protein. After the cross-linking of protein-DNA complexes, Equal amounts of chromatin supernatants were incubated on a rocking bed at 4°C overnight, including anti-H3K27me (Abcam) or 1 µg anti-IgG antibody (Abcam). After the immunoprecipitation, the relative level was measured using the real-time PCR.

In vivo animal assay

After the transfection for gastric cancer cells, Nude mice (4-6 weeks old, male, BALB/c, 12

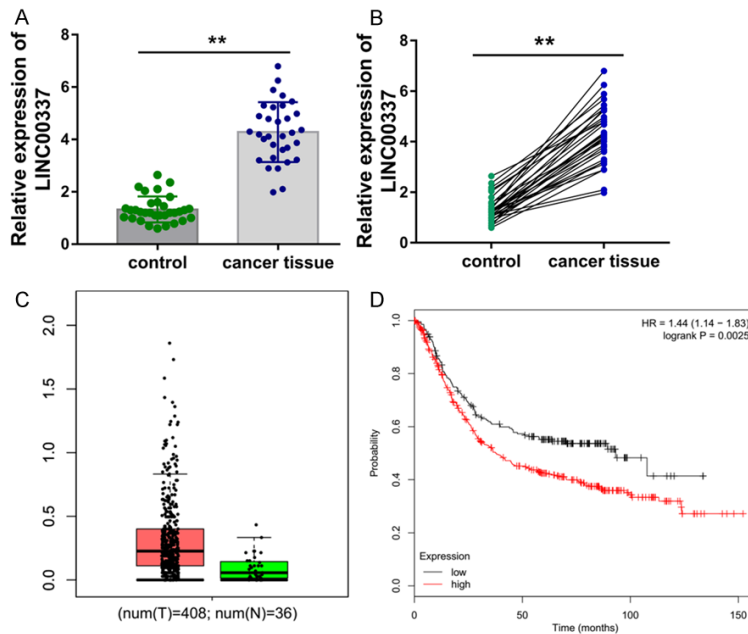


Figure 1. Ectopic LINC00337 overexpression indicates the poor clinical outcome. A. LINC00337 expression was tested in the gastric cancer tissue samples. B. The LINC00337 level in gastric cancer tissue and the adjacent normal tissue. C. LINC00337 level in the GEPIA database (<http://gepia.cancer-pku.cn/>) based on the TCGA. D. Kaplan-Meier plotter analysis (<http://kmplot.com/analysis/>) revealed the LINC00337 expression with the poor clinical outcome for gastric cancer patients. **indicates the *p* value less than 0.01.

mice) were injected subcutaneously with these cells at 3×10^6 cells per mice. After the transplantation, the tumor was measured using the vernier caliper every 3 days. The mice were sacrificed and tumor weight was tested. Animal experiments scheme was approved by the Animal Care and Use Committee of Tianjin Medical University General Hospital.

Statistical analysis

All data were calculated and expressed as mean \pm standard deviation (SD). After normality distribution testing, the intergroup difference was tested using Student's *t*-tests and ANOVA. Survival analysis was performed using Kaplan-Meier test. *P* < 0.05 was considered as statistical significance.

Results

Ectopic LINC00337 overexpression indicates the poor clinical outcome

In the gastric cancer tissue samples, LINC00337 expression was found to be highly increased (**Figure 1A**). Moreover, the LINC00337

level was higher than the adjacent normal tissue (**Figure 1B**). In the GEPIA database (<http://gepia.cancer-pku.cn/>) based on the TCGA, LINC00337 level was markedly higher in the gastric cancer tissue compared with the normal controls (**Figure 1C**). Then, the Kaplan-Meier plotter analysis (<http://kmplot.com/analysis/>) revealed that the higher LINC00337 expression indicates the poor clinical outcome for gastric cancer patients (**Figure 1D**). Overall, this data concludes that ectopic LINC00337 overexpression indicates the poor clinical outcome.

LINC00337 silencing represses the proliferation, invasion and tumor growth of gastric cancer cells in vitro and in vivo

In the gastric cancer cells, LINC00337 levels were proudly up-regulated (**Figure 2A**). In

order to investigate the role of LINC00337 in the gastric cancer cells' tumor phenotype, silencing expression oligonucleotides were synthesized in SGC-7901 and BGC-823 cells (**Figure 2B**). The proliferative ability of gastric cancer cells measured by CCK-8 showed that LINC00337 silencing impaired the proliferation (**Figure 2C, 2D**). Transwell invasion assay indicated that LINC00337 silencing impaired the invasive ability of SGC-7901 and BGC-823 cells (**Figure 2E**). In vivo xenograft mice assay showed that LINC00337 silencing reduced the tumor growth, volume and weight, comparing the controls (**Figure 2F, 2G**). Overall, this data suggests that LINC00337 silencing represses the proliferation, invasion and tumor growth of gastric cancer cells in vitro and in vivo.

LINC00337 epigenetically represses the p21 via EZH2-mediated inhibition

The subcellular location analysis showed that LINC00337 was mainly located in the nuclear, instead of the cytoplasm (**Figure 3A**). In the screening of cycle-related proteins, we found that p21 was increased when the LINC00337

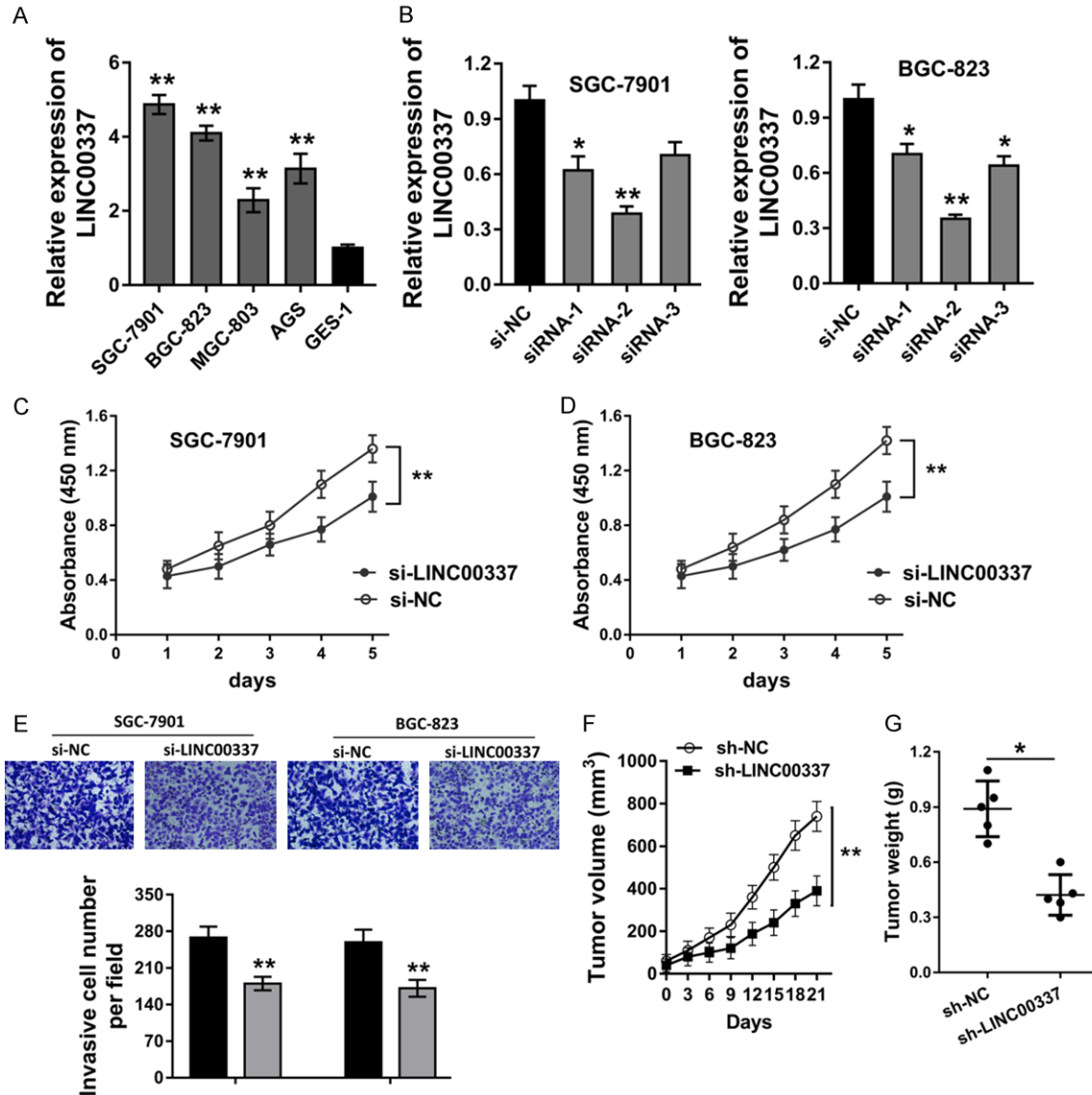


Figure 2. LINC00337 silencing repressed the proliferation, invasion and tumor growth in vitro and in vivo. A. LINC00337 expression level was tested in gastric cancer cells (SGC-7901, BGC-823, MGC-803, AGS) and the normal cell (GES-1). B. Silencing expression oligonucleotides were synthesized and transfected into SGC-7901 and BGC-823 cells. C, D. The proliferative ability of gastric cancer cells was measured by CCK-8. E. Transwell invasion assay indicated the invasive ability of SGC-7901 and BGC-823 cells. F, G. In vivo xenograft mice assay showed the tumor growth, volume and weight, comparing the controls. **indicates the *p* value less than 0.01. *indicates the *p* value less than 0.05.

silenced in the SGC-7901 cells (**Figure 3B**). In the SGC-7901 cells, the LINC00337 silencing transfection and EZH2 silencing transfection both up-regulated the p21 protein levels (**Figure 3C, 3D**). RNA binding protein immunoprecipitation (RIP) assay showed that LINC00337 could closely bind with the EZH2 (**Figure 3E**). EZH2 acts as the core of subunit of PRC2. It was assumed that LINC00337 could recruit EZH2 to p21 promoter region to silence p21 protein levels. Then, the chromatin immunoprecipitation (ChIP) was performed and showed that

EZH2 directly targeted p21 promoter region (**Figure 3F**). Overall, results show that LINC00337 directly bind with EZH2 to represses the p21 via EZH2-mediated inhibition.

p21 could recover the oncogenic role of LINC00337 on gastric cancer tumorigenesis

In the above findings, we recognized that LINC00337 epigenetically represses the p21 via EZH2-mediated inhibition. However, whether p21 could recover the oncogenic role of

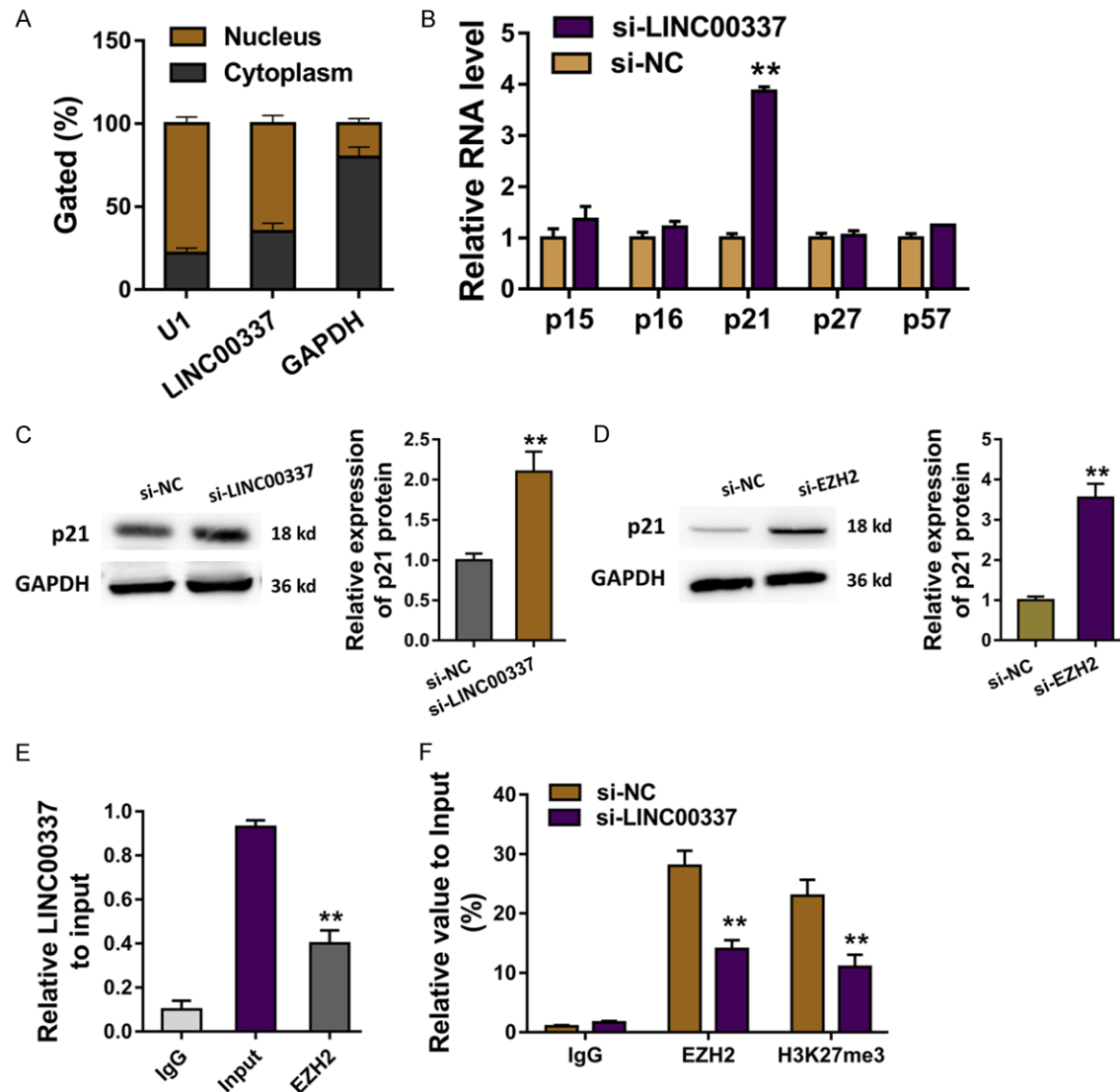


Figure 3. LINC00337 epigenetically represses the p21 via EZH2-mediated inhibition. A. The subcellular location analysis showed the main location of LINC00337 in the nucleus, instead of the cytoplasm. B. p21 mRNA was increased when the LINC00337 silenced in the SGC-7901 cells. C. In the SGC-7901 cells, the LINC00337 silencing transfection up-regulated the p21 protein levels. D. the EZH2 silencing transfection up-regulated the p21 protein levels. E. RNA binding protein immunoprecipitation (RIP) assay showed the closely binding within LINC00337 and the EZH2. F. Chromatin immunoprecipitation (ChIP) showed the direct targeting within EZH2 and p21 promoter region. **indicates the *p* value less than 0.01.

LINC00337 on gastric cancer tumorigenesis is still indeterminate (**Figure 4A**). CCK-8 assay showed that co-transfection of LINC00337 and p21 siRNAs recovered the inhibition of gastric cancer cells induced by LINC00337 knock-down (**Figure 4B**). Transwell invasion assay indicated that co-transfection of LINC00337 and p21 siRNAs recovered invasive ability of gastric cancer cells (**Figure 4C**). The Kaplan-Meier analysis from the TCGA database showed that the lower p21 expression and higher EZH2

expression was closely correlated with the poor prognosis of gastric cancer patients (**Figure 4D, 4E**). Therefore, data suggests that p21 could recover the oncogenic role of LINC00337 on gastric cancer tumorigenesis.

Discussion

The discoveries in recent decades indicated that long noncoding RNA (lncRNA) functions as vital regulators in the human cancers, as well

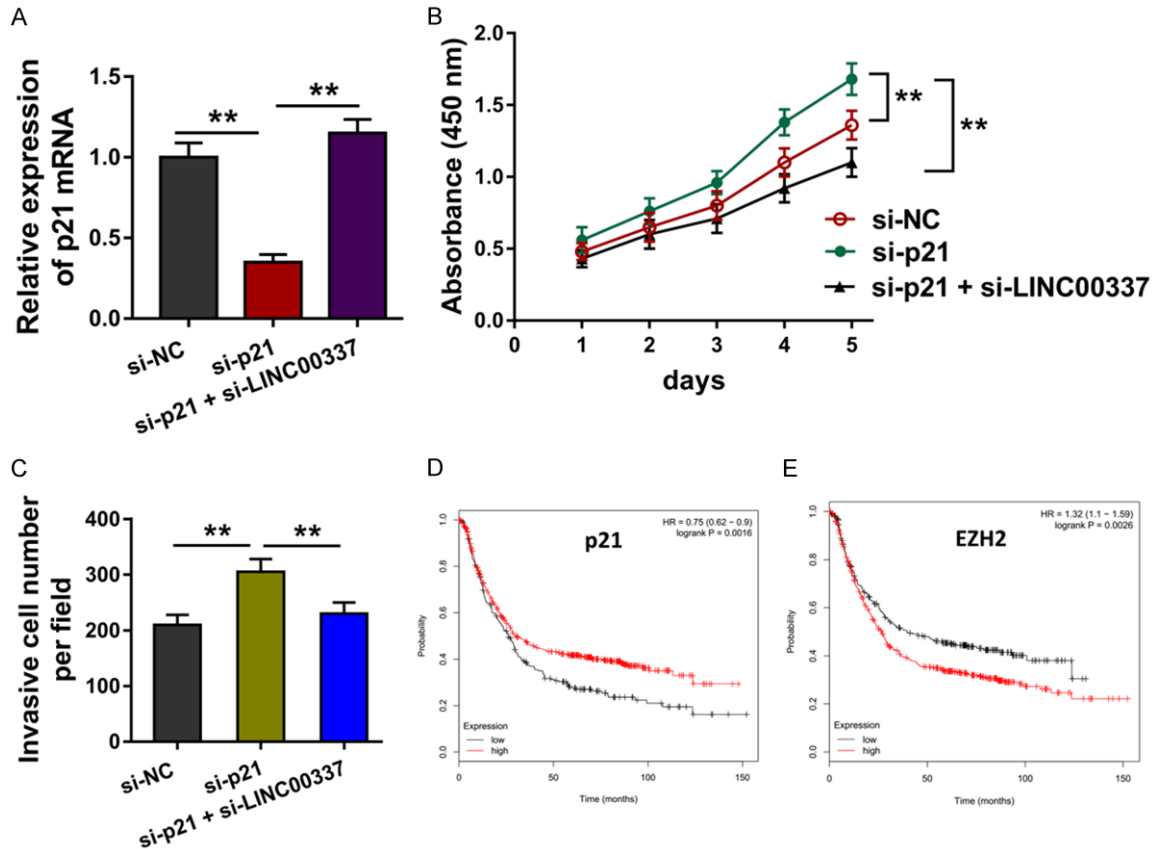


Figure 4. p21 could recover the oncogenic role of LINC00337 on gastric cancer tumorigenesis. A. LINC00337 siRNA could increase the p21 mRNA. B. CCK-8 assay showed the proliferation induced by LINC00337 knockdown and p21 siRNAs. C. Transwell invasion assay indicated the invasive ability of gastric cancer cells. D. The Kaplan-Meier analysis from the TCGA database showed the prognosis of gastric cancer patients with higher or lower p21 expression. E. The Kaplan-Meier analysis from the TCGA database showed the prognosis of gastric cancer patients with higher or lower EZH2 expression. **indicates the *p* value less than 0.01.

as the gastric cancer [12-14]. With the deepgoing research about the gastric cancer, more and more findings report the increasing quantity of oncogenic lncRNAs or anti-cancer lncRNAs in the specific period [15, 16].

In the gastric cancer, lncRNA could exert its functions via series of manners [17]. For example, NNT-AS1 expression level is significantly up-regulated in gastric cancer tissue and cell lines, and the overexpression of NNT-AS1 indicated the poor prognosis of GC patients, and NNT-AS1 knockdown suppressed the proliferation and invasion ability and induced the GC cell cycle progression arrest at G0/G1 phase [18]. LINC01296 is up-regulated in GC tissue and correlated with poor prognosis, and LINC01296 sponges miR-122 to recover its target MMP-9 [19]. Thus, this evidence support that lncRNA could participate the gastric cancer tumorigenesis via epigenetic regulation.

In this research, we found that lncRNA LINC00337 was markedly over-expressed in the gastric cancer tissue specimens and cells. Besides, this overexpression of LINC00337 was closely correlated with the clinical poor outcome in the gastric cancer patient individuals. LINC00337 is a novel identified lncRNA in the human cancer with few correlated literature [20]. In our previous experiments, we found that multiple lncRNAs are increased or decreased in the gastric cancer cells, such as MALAT1, HOTAIR, MEG3 et al. [21-23]. The mainstream views support that the increased level or overexpression of lncRNA functions as oncogenic factor in the human cancer genesis, while the decreased level or lower-expression of lncRNA could exert the anti-cancer effects.

The data in this work showed that LINC00337 could function as the nuclear scaffold for the EZH2 to recruit it to the promoter of p21. The

recruitment of EZH2 to the H3K27 of histone could trigger the transcriptional silencing p21, which as caused by the LINC00337. Therefore, this finding suggests that LINC00337 could function as the oncogenic role in the gastric cancer via the regulation of transcriptional perspective. The regulation of transcriptional dimension could give rise to the effective and exhaustive modulation of lncRNA for the gene transcription and protein translation. p21 acts as the anti-cancer gene in human cancer. For example, in the gastric cancer, p21 inhibits the cellular tumorigenesis, while the lncRNA HOXA-AS2 interact with EZH2/LSD1 and recruit them to p21 for the p21 silencing in order to promote the tumorigenesis [24].

Enhancer of zeste homolog 2 (EZH2) is encoded by EZH2 gene and functions as histone-lysine N-methyltransferase enzyme [25, 26]. In the gastric cancer, lncRNA could functions a critical regulator via recruiting the EZH2. For example, the SNHG17 acts as an oncogene via activating EZH2, which is required for epigenetic repression of p15 and p57 cyclin-dependent protein kinase inhibitors (CKIs) [27]. Similarly, FOXD2-AS1 promotes the gastric cancer progression and tumorigenesis partly via lysine (K)-specific demethylase 1A (LSD1) and EZH2 mediated EphB3 repression [28].

In conclusion, our study provides a new perspective for gastric cancer, in which LINC00337 acts as an oncogene in the cellular tumorigenesis via transcriptional regulation. Therefore, these discoveries bring new light to the mechanism of LINC00337 and provide candidate targets for gastric cancer.

Disclosure of conflict of interest

None.

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LINC00337 promote gastric cancer proliferation via p21

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

	Sequences
LINC00377	Forward, 5'-CGAAGACCCTGCTTGGAGG-3' Reverse, 5'-GGGGATTCTCCGAAATCAG-3'
si-LINC00377-1	TGTGGATTGGAAAGCACAC
si-LINC00377-2	GCATTTGTGGAGAACACGG
si-LINC00377-3	AAGACAGAGCCGTCTTGG
GAPDH	Forward, 5'-AGAAGGCTGGGGCTCATTTG-3' Reverse, 5'-AGGGGCCATCCACAGTCTTC-3'