European Review for Medical and Pharmacological Sciences 2019; 23: 2075-2083

LncRNA LINC00673 inhibits p53 expression by interacting with EZH2 and DNMT1 in papillary thyroid carcinoma

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Abstract. – OBJECTIVE: This study aims to elucidate the regulatory role of long noncoding RNA (lncRNA) LINC00673 in proliferative, invasive and metastatic capacities of papillary thyroid carcinoma (PTC), and to investigate the possible underlying mechanism.

PATIENTS AND METHODS: LINC00673 expression in PTC tissues and cell lines was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between LINC00673 expression and PTC prognosis was analyzed. By plasmid transfection, we constructed PTC cell lines with stable knockdown of LINC00673. The regulatory effect of LINC00673 on the proliferation of K1 and TPC-1 cells were determined by Cell Counting Kit-8 (CCK-8) assay and colony formation assay. The transwell assay was conducted to evaluate the effect of LINC00673 on the metastatic ability of PTC cells. The binding condition between LINC00673 with enhancer of zeste homolog 2 (EZH2) and DNA Methyltransferase 1 (DNMT1) was verified by RNA immunoprecipitation (RIP), chromatin immunoprecipitation (ChIP) and Western blot. The cellular mechanism of p53 in regulating biological behaviors of PTC cells was explored by Western blot. Finally, the gain-of-function experiment was performed to elucidate whether LINC00673 could regulate PTC development by inhibiting p53 expression.

RESULTS: LINC00673 expression in PTC tissues was significantly higher than that of the adjacent normal tissues. Besides, higher expression of LINC00673 indicated worse prognosis of PTC. The knockdown of LINC00673 in K1 and TPC-1 cells markedly reduced the proliferative rate. Meanwhile, LINC00673 down-regulation remarkably inhibited the migratory and invasive capacities of K1 and TPC-1 cells. RIP and ChIP assay demonstrated that LINC00673 could bind to EZH2 and DNMT1. Besides, Western blot analysis showed that LINC00673 negatively regulated p53 expression. In addition, the knockdown of p53 in K1 and TPC-1 cells partially reversed the inhibitory effect of LINC00673 deficiency on the proliferation and metastasis of PTC cells.

CONCLUSIONS: High expression of LINC00673 in PTC predicts a poor prognosis. LINC00673 remarkably promotes the proliferation and invasion of PTC cells by inhibiting p53 expression by binding to EZH2 and DNMT1.

Key Words:
LINC00673, PTC, Proliferation, Invasion, P53.

Introduction

Thyroid carcinoma (TC) is the most common endocrine malignant tumor in humans, accounting for 95% of all endocrine tumors and 1% of systemic malignant tumors. The pathogenesis of TC is closely related to iodine deficiency, local radiation, sex hormones, thyroid stimulating hormone, Hashimoto's disease, etc[1]. Due to increased morbidity and detection rate, the incidence of TC has sharply increased in recent years, which is the fastest-growing malignant tumor[2]. TC is pathologically classified into several subtypes, including papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), oncocytic carcinoma, medullary thyroid carcinoma (MTC) and anaplastic thyroid carcinoma (ATC). Among these subtypes of TC, PTC accounts for about 70%. PTC has a typical papillary structure and is often accompanied with local lymph node metastasis. Previous studies have found that the prognosis of PTC is usually well[3].

More than 90% of the genes in the mammalian genome do not have a protein-encoding function. These genes are previously thought as non-fun-

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ctional "transcriptional noise". With an advanced human genetic program, non-coding RNAs are found to exert regulatory roles in multiple cellular activities. Long noncoding RNAs (lncRNAs) are a type of non-coding RNAs with over 200 bases in length[4]. Although lncRNAs do not transcribe proteins, they participate in various physiological and life activities, such as cell development, gene imprinting, X chromosome silencing, transcriptional and post-transcriptional regulation[5]. In recent studies[6-8], the potential functions of lncRNAs have been identified in the progression of PTC.

Currently, the significant function of LINC00673 in tumorigenesis has been gradually revealed. Xia et al[9] have found that high expression of LINC00673 promotes the growth and metastasis of breast cancer cells by upregulating B7-H6 and accelerating EMT. LINC00673 promotes hepatocellular carcinoma development by suppressing miR-205 expression[10]. Meanwhile, LINC00673 activated by SP1 exerts carcinogenic properties in gastric cancer through interaction with LSD1 and enhancer of zeste homolog 2 (EZH2)[1]. However, the specific mechanism of LINC00673 in PTC remains to be fully elucidated.

In the present work, we demonstrated that LINC00673 expression significantly increased in PTC tissues. Meanwhile, higher expression of LINC00673 predicted worse prognosis of PTC. LINC00673 knockdown remarkably inhibited the proliferative, migratory and invasive rates of PTC. In addition, we demonstrated that LINC00673 promoted cell growth and metastasis by inhibiting p53 expression by binding to EZH2 and DNA Methyltransferase 1 (DNMT1). In conclusion, our findings indicated that LINC00673 might be a prognostic biomarker and therapeutic target for PTC.

Patients and Methods

Patients

Forty-five cases of PTC and adjacent normal tissues were surgically resected in the Shandong Weifang People's Hospital from July 2015 to December 2017. Tissue samples were immediately placed in liquid nitrogen and further preserved in a -80°C refrigerator. Collected PTC tissues were pathologically confirmed. None of these patients received I-131 treatment. Sample collection was approved by the subjects and their families. This study was approved by the Medical Ethics Committee of the Shandong Weifang People's Hospital.

Cell Culture and Transfection

Human thyroid epithelial cell line (Nthy-ori3-1) and PTC cell lines (K1, TPC-1 and BCPAP) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were placed in a 37°C, 5% CO_2 incubator.

Cells in logarithmic growth phase were selected and transfected with LINC00673 siRNA, EZH2 siRNA, DNMT1 siRNA, p53 siRNA or negative control (GenePharma, Shanghai, China) according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cellular RNAs were extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) according to the instructions. The concentration of extracted RNA was measured, and then qualified RNA samples were stored in a -80°C refrigerator. Reverse transcription was performed according to the instructions of the TaKaRa OneStep PrimeScript® miRNA complementary deoxyribose nucleic acid (cDNA) Synthesis Kit (TaKaRa, Otsu, Shiga, Japan). PCR detection was performed using the SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). Specific procedures were: 95°C for 3 min, denaturation at 95°C for 5 s and annealing at 60°C for 30 s, for a total of 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. Primers were listed as follows: LINC00673: F: 5'-TGCTGATGACACATACACA-3', R: 5'-GACAAGGATGAACCATGATAG-3'; P53: F: 5'-GGACTCTGCCCTGCCACCATTTA-3', R: 5'-CTTGTGCCCTGTGAGGTCGTTGA-3'; GAPDH: F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'.

Proliferation Determination

K1 and TPC-1 cells were seeded into 96-well plates at a density of 2×10^3 cells per well for 24, 48, 72, and 96 hours, respectively. After cell adherence, Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay was conducted to evaluate cell proliferation following the manufacturer's recommendations. Briefly, 10 μL of CCK-8 reagent was added, followed by incu-

bation for 2 hours in the dark. The absorbance at the wavelength of 450 nm was measured using a microplate reader (Tecan, Mechelen, Belgium).

Transfected K1 and TPC-1 cells were seeded into 12-well plates for 2-week incubation. Colonies were stained with crystal violet and captured under a microscope (magnification 10×) for colony calculation.

Cell Migration and Invasion Assays

Migratory ability was measured using the 8-μm transwell chamber (Millipore, Billerica, MA, USA). 100 μL of serum-free medium containing 5×10^4 cells were added to the upper chamber pre-coated with diluted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Meanwhile, 600 μL of medium containing 10% FBS was added to the bottom chamber. After incubation for 48 hours, un-penetrating cells were carefully wiped off. The remaining cells were fixed and stained with 0.1% crystal violet (Beyotime, Shanghai, China) for 15 min, and then photographed using a microscope (magnification 20×). Procedures of invasion assay were the same as the above except for Matrigel pre-coating (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blot

The proteins were extracted by radioimmunoprecipitation assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMSF; Beyotime, Shanghai, China). An equal amount of protein sample was loaded onto a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with skim milk, the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. At the other day, the membranes were incubated with the secondary antibody at room temperature for 2-3 h. Finally, the image of the protein band was captured by the Tanon detection system (Shanghai, China) using enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Chromatin Fractionation

Until cell growth to 1×10^6 cells/mL, 200 μL of Lysis Buffer J was added to the culture bottle to fully lyse the cells. After centrifugation, the supernatant contained cytoplasmic RNA, and the remaining contained nuclear RNA. The supernatant was transferred to a new tube. Subsequently, Buffer SK and absolute ethanol were added to cytoplasmic RNA and nucleus RNA, respectively, followed by extraction with column centrifugation. The cytoplasmic or nuclear expressions of LINC00673 were detected by qRT-PCR with GAPDH and U6 as internal controls, respectively.

RNA Immunoprecipitation (RIP) Assay

RIP assay was strictly performed according to the instructions of Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). After K1 and TPC-1 cells were lysed, they were incubated with the detection antibody. The working concentration of the antibody was 8 μg per reaction system. The mixture was incubated at 4°C for 4 hours, and then rewarmed at room temperature for 1 h. Protein G magnetic beads were added to capture the complex. After washing with buffer, RNA was extracted and quantified by qRT-PCR.

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed as previously described[12]. Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature. Subsequently, the cross-linked cells were lysed using lysis buffer and sonicated for 30 min. Finally, the sonicated lysate was immunoprecipitated with antibodies against EZH2, DNMT1, LSD1, SUZ12 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and IgG (Cell Signaling Technology, Danvers, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (SPSS Inc. Chicago, IL, USA) was used for all statistical analysis, and GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA) was introduced for figure editing. Data were represented as mean ± SD (standard deviation). The Student's *t*-test was used to compare the differences between the two groups. $p<0.05$ indicated the significant difference.

Results

LINC00673 Expression was Upregulated in PTC

We detected LINC00673 expression in 45 paired PTC and adjacent normal tissues by qRT-PCR. The results showed that LINC00673 was

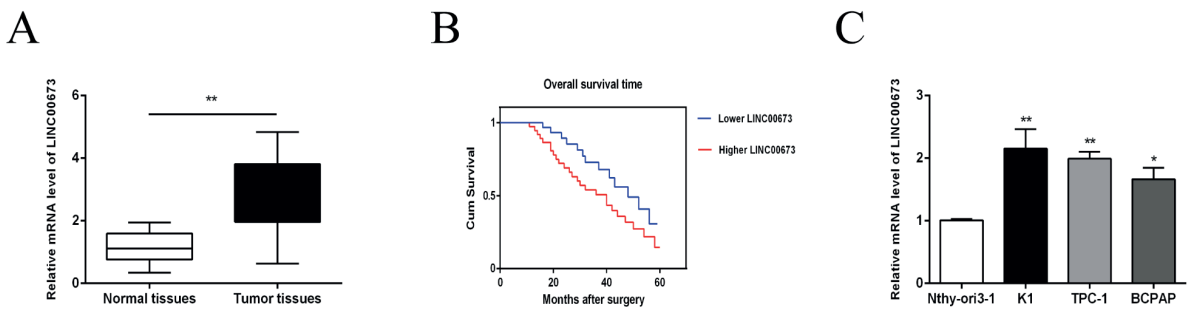

Figure 1. LINC00673 expression was upregulated in PTC. ***A,*** LINC00673 expression in PTC tissues and adjacent normal tissues detected by qRT-PCR (n=45). ***B,*** Correlation between LINC00673 expression and overall survival of PTC patients. ***C,*** LINC00673 expression in PTC cell lines and control cell line Nthy-ori3-1 detected by qRT-PCR. Data were evaluated from triplicates of a representative experiment, and were expressed as mean ± SD. *p<0.05 **p<0.01, ***p<0.001.

highly expressed in PTC tissues (Figure 1A). Besides, higher expression of LINC00673 predicted worse prognosis of PTC (Figure 1B). Subsequently, we examined LINC00673 expression in normal thyroid epithelial cells Nthy-ori3-1 and PTC cell lines K1, TPC-1 and BCPAP. QRT-PCR results demonstrated that LINC00673 expression in PTC cell lines was identically upregulated (Figure 1C). K1 and TPC-1 cells showed a pronounced expression of LINC00673, which were chosen for the following experiments.

LINC00673 Knockdown Inhibited Growth and Metastasis of PTC Cells

Three LINC00673 siRNAs (si-LINC00673-1, si-LINC00673-2 and si-LINC00673-3) were transfected into K1 and TPC-1 cells, respectively. Three siRNAs all markedly downregulated the LINC00673 expression (Figure 2A). Particularly, si-LINC00673-1 showed the highest interference efficiency, and was selected for subsequent experiments. We then tested the proliferative rate of K1 and TPC-1 cells transfected with si-LINC00673. Both CCK-8 and colony formation assay showed remarkably attenuated proliferative ability after interfering LINC00673 (Figure 2B and 2C). Subsequently, we examined the regulatory effect of LINC00673 on cell metastasis by transwell assay. The results found that the migratory and invasive rates of K1 and TPC-1 cells transfected with si-LINC00673 were significantly reduced compared with the control group (Figure 2D and 2E).

LINC00673 Inhibited p53 Expression by Binding to EZH2 and DNMT1

Previous studies have shown that lncRNAs can bind to RNA binding proteins, such as EZH2, DNMT1, LSD1PRC2 and STAU1[12]. To verify whether LINC00673 exerted biological functions through RNA binding, we first examined the subcellular localization of LINC00673 in PTC cells. Chromatin fractionation assay showed that LINC00673 was mainly distributed both in the nucleus of K1 and TPC-1 cells (Figure 3A). Subsequently, we examined the binding relationship between LINC00673 and RNA-binding protein in PTC cells by RIP assay. The results demonstrated that LINC00673 could bind to EZH2 and DNMT1 in K1 and TPC-1 cells (Figure 3B).

As a tumor-suppressor gene, p53 participates in the proliferation, migration and invasion of many tumor cells[13]. Previous studies have shown that DNMT1 binds to the promoter region of p53 and inhibits its expression[14]. Our results showed that p53 was lowly expressed in PTC tissues than that of adjacent normal tissues (Figure 3C). Meanwhile, LINC00673 expression was negatively correlated with p53 expression in PTC tissues (Figure 3D). Furthermore, we detected p53 expression after LINC00673 knockdown in K1 and TPC-1 cells. Both mRNA and protein levels of p53 were markedly upregulated by LINC00673 knockdown (Figure 3E and 3F). Similarly, the knockdown of EZH2 and DNMT1 in K1 and TPC-1 cells also upregulated p53 expression at mRNA and protein levels (Figure 3G-3J).

To verify whether EZH2 and DNMT1 could bind to the promoter region of p53, we constructed primers based on p53 promoter region. ChIP assay and qRT-PCR confirmed that EZH2 and DNMT1 could bind to the promoter region of p53 in PTC cells (Figure 3K). The ability of EZH2 and DNMT1 to bind to the p53 promoter region was significantly reduced after LINC00673 knockdown in PTC cells (Figure 3L). The above results demonstrated that LINC00673 inhibited

p53 expression by binding to EZH2 and DNMT1 in PTC cells.

P53 Reversed the Cancer-Promoting Effect of LINC00673

After LINC00673 knockdown in K1 and TPC-1 cells, CCK-8 and colony formation assay were conducted. The results showed that LINC00673 deficiency attenuated the proliferative ability, which was reversed by p53 knockdown (Figure 4A and 4B). Subsequently, transwell assay revealed that p53 knockdown could alleviate the inhibitory effect of LINC00673 knockdown on the migration and invasion of PTC cells (Figure 4C and 4D). These results indicated that LINC00673 could promote the growth and metastasis of PTC cells by inhibiting p53 expression.

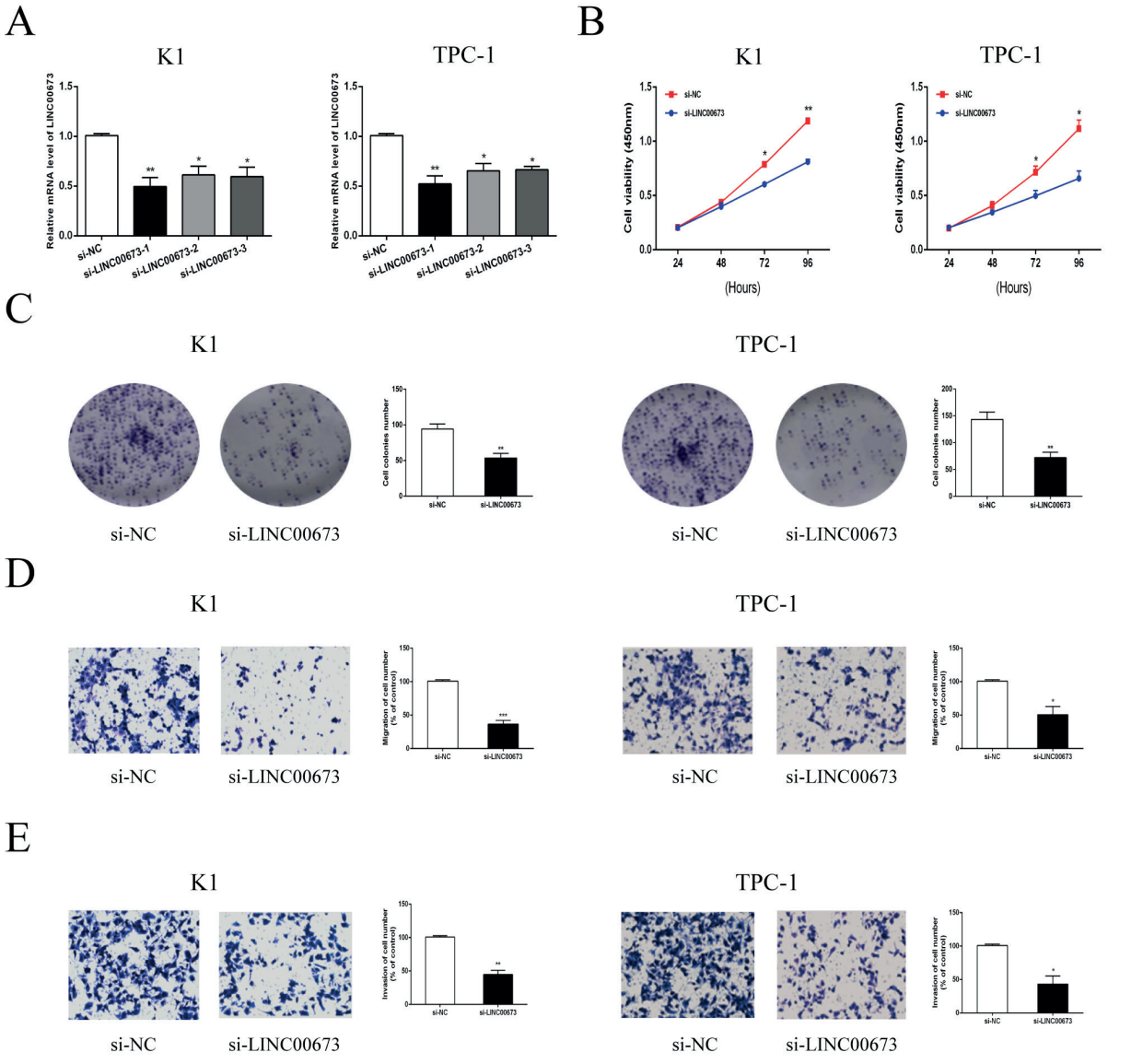

Figure 2. LINC00673 knockdown inhibited growth and metastasis of PTC cells. ***A,*** Transfection efficacy of si-LINC00673 in K1 and TPC-1 cells detected by qRT-PCR. ***B,*** Cell viability of K1 and TPC-1 cells transfected with si-LINC00673 detected by CCK-8 assay. ***C,*** The number of colonies in K1 and TPC-1 cells transfected with si-LINC00673 detected by colony formation assay (magnification: 10×). ***D,*** Cell migration of K1 and TPC-1 cells transfected with si-LINC00673 detected by transwell assay (magnification: 40×). ***E,*** Cell invasion of K1 and TPC-1 cells transfected with si-LINC00673 detected by transwell assay (magnification: 40×). Data were evaluated from triplicates of a representative experiment, and were expressed as mean ± SD. *p<0.05 **p<0.01, ***p<0.001.

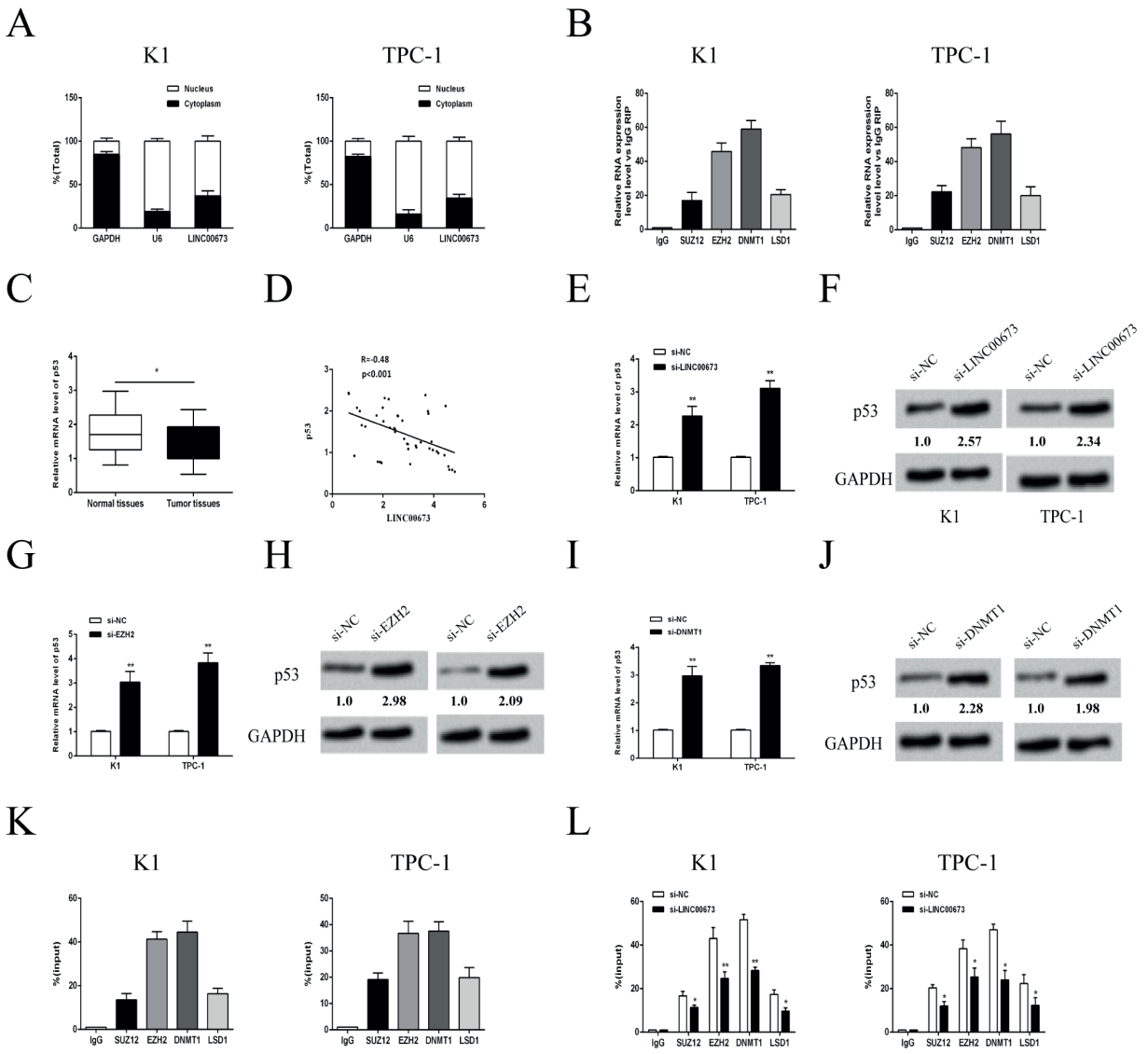

Figure 3. LINC00673 inhibited p53 expression by binding to EZH2 and DNMT1 in PTC cells. ***A,*** Cytoplasmic and nuclear levels of LINC00673 in K1 and TPC-1 cells relative to GAPDH and U6, respectively. ***B,*** RIP assay demonstrated that LINC00673 could bind to EZH2, LSD1, DNMT1 and SUZ12, especially EZH2 and DNMT1. ***C,*** P53 expression in PTC tissues and adjacent normal tissues detected by qRT-PCR (n=45). ***D,*** LINC00673 expression was negatively correlated with p53 expression in PTC tissues (R=-0.48, $p<0.001$). ***E,*** The mRNA level of p53 in K1 and TPC-1 cells with LINC00673 knockdown. ***F,*** The protein level of p53 in K1 and TPC-1 cells with LINC00673 knockdown. ***G,*** The mRNA level of p53 in K1 and TPC-1 cells with EZH2 knockdown. ***H,*** The protein level of p53 in K1 and TPC-1 cells with EZH2 knockdown. ***I,*** The mRNA level of p53 in K1 and TPC-1 cells with DNMT1 knockdown. ***J,*** The protein level of p53 in K1 and TPC-1 cells with DNMT1 knockdown. ***K,*** ChIP assay demonstrated that EZH2, LSD1, DNMT1 and SUZ12 could bind to DNA in p53 promoter region. ***L,*** The ability of EZH2 and DNMT1 to bind to the p53 promoter region was significantly reduced after LINC00673 knockdown in K1 and TPC-1 cells. Data were evaluated from triplicates of a representative experiment, and were expressed as mean ± SD. *$p<0.05$ **$p<0.01$, ***$p<0.001$.

Discussion

Papillary thyroid carcinoma is a common malignant tumor with a rapidly increased incidence. PTC patients usually lack significant manifestations or only have painless cysts on the neck. Quite a part of patients are diagnosed with B-ultrasound examination during a physical examination, and cysts found in the Isotope scanning are cold nodules[15]. At present, thyroid small mass puncture, especially B-guided puncture, contributes to early detection, diagnosis and treatment

of PTC. Typical histological and imaging features of most thyroid tumors can be diagnosed using cytological or histopathological examinations. However, atypical fibrous background or nodules originated from atypically cell papillary hyperplasia makes it difficult to distinguish between benign and malignant thyroid carcinoma. As a result, the diagnosis of PTC using frozen sections is still difficult[16]. Therefore, it is necessary to further explore the molecular mechanisms related to thyroid carcinoma, to discover sufficient diagnostic indicators[17].

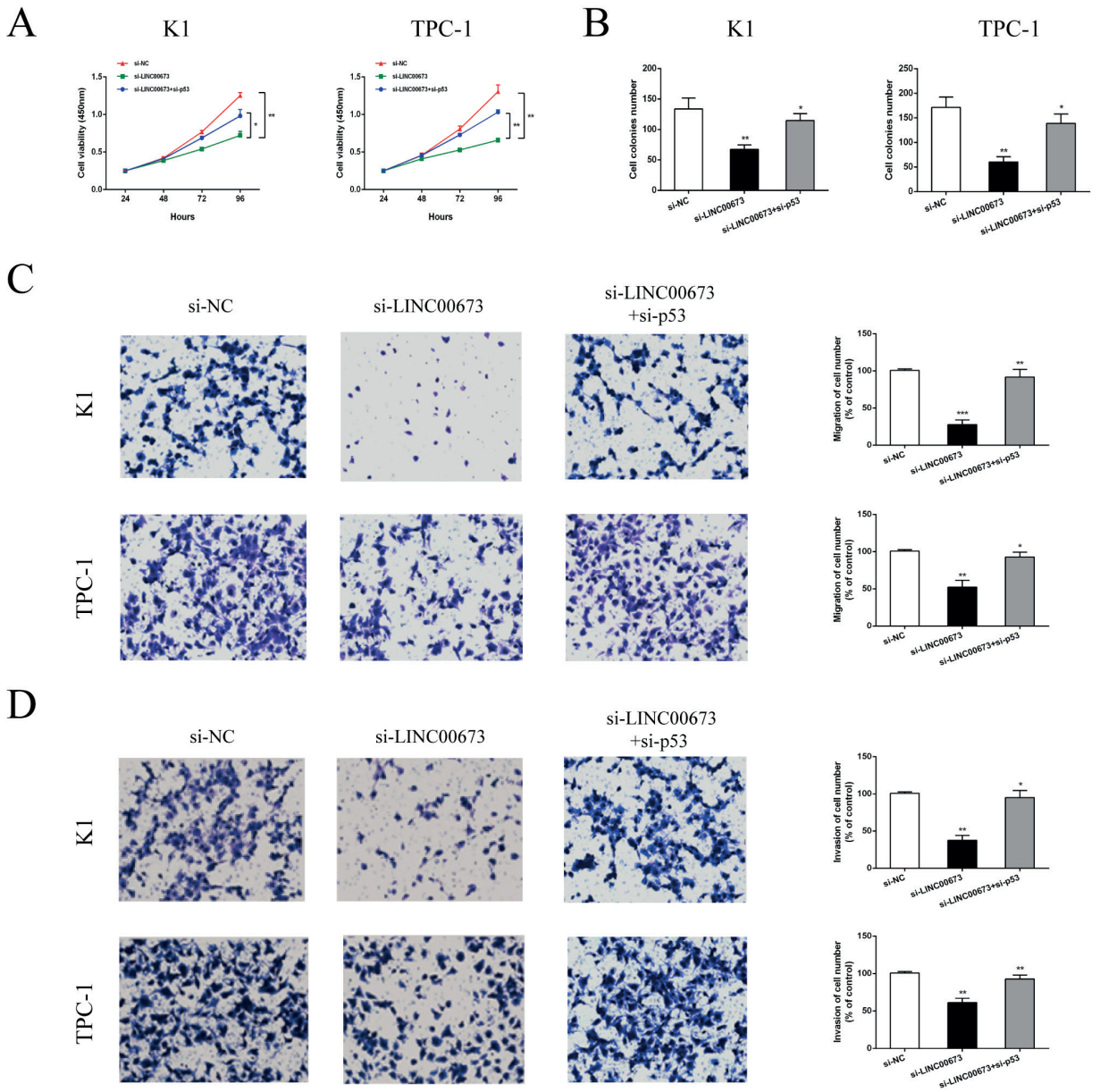

Figure 4. P53 reversed the cancer-promoting effect of LINC00673. ***A,*** Cell viability of K1 and TPC-1 cells transfected with si-NC, si-LINC00673, or si-LINC00673 and si-p53 detected by CCK-8 assay. ***B,*** The number of colonies of K1 and TPC-1 cells transfected with si-NC, si-LINC00673, or si-LINC00673 and si-p53 detected by colony formation assay. ***C,*** Cell migration of K1 and TPC-1 cells transfected with si-NC, si-LINC00673, or si-LINC00673 and si-p53 detected by transwell assay (magnification: 40×). ***D,*** Cell invasion of K1 and TPC-1 cells transfected with si-NC, si-LINC00673, or si-LINC00673 and si-p53 detected by transwell assay (magnification: 40×). Data were evaluated from triplicates of a representative experiment, and were expressed as mean ± SD. *p<0.05 **p<0.01, ***p<0.001.

Recent studies have shown the crucial regulatory roles of lncRNAs in the tumorigenesis, proliferation, metastasis and drug resistance of PTC. These certain lncRNAs may serve as new non-invasive tumor markers for early diagnosis and risk evaluation of PTC. For example, lncHIT000218960 promotes the tumorigenesis and progression of PTC by upregulating HMGA2 expression[18]. Lnc-ATB expression is associated with tumor size and lymph node metastasis of PTC, which can serve as a diagnostic marker for PTC. Lnc-ATB downregulation reduces the proliferative and metastatic capacities of PTC cells[19]. Lnc-H19 is lowly expressed in PTC tissues than those of controls. Moreover, its low expression promotes the proliferative and invasive rates of IHH-4 and K1 cells, thereafter accelerating PTC development[20].

P53 is a common tumor-suppressor gene that exerts various functions in cell cycle transition, cell differentiation, DNA repair and apoptosis[21]. Damaged intracellular DNA by genetic factors or environmental factors may arrest the cell cycle in G1 phase under the regulation of p53. Subsequently, DNA repair process initiates and allows damaged DNAs progress into cell apoptosis. Normal p53 protein encoded by p53 is difficult to be detected due to its short half-life. However, p53 protein encoded by p53 mutation is easily detected, which loses functions of cell cycle regulation and apoptosis promotion[22]. Furthermore, p53 mutation can be found in nearly 50% of human tumors, which is the most common mutation gene in malignancies[13].

In this work, we detected LINC00673 expression in PTC tissues, adjacent normal tissues and PTC cells by qRT-PCR. As the data illustrated, LINC00673 was highly expressed in PTC tissues and cell lines. Subsequently, biological functions of LINC00673 in PTC cells were explored through CCK-8, colony formation and transwell assay. The results showed that the proliferation and metastasis of PTC cells were remarkably promoted by LINC00673. Studies[13] have shown that lncRNAs exert their biological functions by inhibiting target genes. Based on this theory, we speculated that the role of LINC00673 in PTC was dependent on inhibiting p53 expression. Further qRT-PCR and Western blot results verified that both mRNA and protein levels of p53 were inhibited by LINC00673, suggesting that p53 was the target gene of LINC00673.

Studies have shown that lncRNAs inhibit the expressions of downstream genes by binding to RNA-binding proteins. The previous work has proved that LINC00673 inhibits downstream gene expressions in gastric cancer and non-small cell lung cancer by binding to EZH2 and DNMT1[11]. Through RIP assay, we found that LINC00673 could bind to EZH2 and DNMT1, and stabilize their expressions. DNMT1 is the most important methyltransferase in the human body. In tumor cells, high methylation of tumor-suppressor genes, as well as abnormal proliferation and differentiation are related to the increased activity of DNMT1[23]. Subsequently, ChIP assay disclosed that EZH2 and DNMT1 could bind to the promoter region of p53 and inhibit its expression. LINC00673 knockdown in PTC cells reduced the binding ability of EZH2 and DNMT1 to p53. Our results indicated that LINC00673 inhibited p53 expression by binding to EZH2 and DNMT1, thereby promoting the growth and metastasis of PTC cells.

Conclusions

We found that the high expression of LINC00673 in PTC predicts a poor prognosis. LINC00673 remarkably promotes the proliferation and invasion of PTC cells by inhibiting p53 expression *via* binding to EZH2 and DNMT1.

Conflict of interest

The authors declare no conflicts of interest.

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