European Review for Medical and Pharmacological Sciences 2019; 23: 8211-8218

LncRNA TUG1 aggravates the progression of cervical cancer by binding PUM2

W. DUAN, L. NIAN, J. QIAO, N.-N. LIU

Department of Oncology, Yan'an University Affiliated Hospital, Yan'an, China

Abstract. – OBJECTIVE: To illustrate the role of long non-coding RNA (lncRNA) TUG1 in the influence of the progression of cervical cancer (CC) and its underlying mechanism.

PATIENTS AND METHODS: TUG1 level in CC tissues and adjacent normal ones was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Its level in CC patients with different tumor, node, and metastasis (TNM) staging and the tumor sizes were detected as well. The prognostic potential of TUG1 in CC was assessed by introducing the receiver operating characteristic (ROC) curves. The influences of TUG1 on proliferative and migratory abilities of HeLa and SiHa cells were evaluated. The subcellular distribution of TUG1 in CC cells was analyzed. Subsequently, the relative level of PUM2 (Pumilio2) in CC tissues and cell lines was examined. The prognostic potential of PUM2 in CC was assessed. RNA immunoprecipitation (RIP) and RNA pull-down were conducted to uncover the interaction between TUG1 and PUM2. Finally, the regulatory effect of TUG1/PUM2 axis on the viability of the CC cells was investigated.

RESULTS: TUG1 was upregulated in CC, especially in those with worse TNM staging and larger tumor size. The overexpression of TUG1 enhanced proliferative and migratory abilities of Hela and SiHa cells. TUG1 was mainly distributed in the cytoplasm. PUM2 interacted with TUG1 and its level was positively regulated by TUG1. The silence of PUM2 reversed the promotive effect of TUG1 on the viability of the CC cells.

CONCLUSIONS: TUG1 is upregulated in CC, which aggravates the progression of CC by interacting with PUM2.

Key Words:
Cervical cancer, LncRNA TUG1, PUM2.

Introduction

Cervical cancer (CC) is the third most common gynecological malignancy, and the fourth leading cause of female cancer deaths globally. It is reported that there are 530,000 newly onset cases of CC worldwide, and 275,000 CC patients die each year[1]. (Owing) Due to the advancement of cervical cytology and cervical biopsy, the detective rates of early-stage and locally advanced CC cases have been improved[2,3]. Nevertheless, the molecular mechanisms underlying the progression of CC are still poorly understood.

With the development of the genome-wide sequencing technology, non-coding RNAs (ncRNAs) are found to be the vast majority of mammalian genomes and they exert regulatory roles in tumorigenesis[4-6]. Long non-coding RNAs (lncRNAs) are transcribed by RNA polymerase II. Typically, they could not encode proteins except for a small number of polypeptides. LncRNAs are evolutionarily conserved lncRNAs which have crucial functions in cell proliferation, differentiation, and immune response[7,8]. In addition, lncRNAs are involved in chromatin modification[9], DNA methylation[10], gene imprinting[11], RNA processing[12], and genetic regulation[13]. Recent studies have identified that lncRNAs could be oncogenes or tumor suppressors that influence tumor progression.

LncRNA TUG1, also known as TI-227H, linc00080 or ncRNA00080, is a 7.1-kb gene located on 22q12.2. TUG1 was initially discovered by microarray screen in taurine-treated retinal cells[14]. The dysregulation of TUG1 would influence the progression of many types of tumors[15-19]. However, the role of TUG1 in CC has been rarely reported.

In this paper, we first determined the expression pattern of TUG1 in CC tissues and cell lines. The *in vitro* functions of TUG1 in the regulation of the proliferative and migratory abilities of CC cells were specifically explored.

Patients and Methods

Sample Collection and Ethical Statement

The CC tissues and adjacent normal tissues were surgically harvested from 64 CC patients

Corresponding Author: Ningning Liu, BM; e-mail: 283132024@qq.com

admitted in the Yan'an University Affiliated Hospital from April 2016 to December 2018. The pathological data of each patient were recorded, including TNM staging and tumor size. None of these patients were preoperatively treated. This study was approved by the Medical Ethics Committee of Yan'an University Affiliated Hospital and informed consent was given by each subject.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNAs in cells and tissues were extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA), and they were subjected to reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The RNA concentration was detected using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: TUG1: F: 5'-GAACTACTGCGGAACCTCAA-3'; R: 5'-ACTTGGTGAGCACCACTCC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5'-CTGCCAACGTGTCAGTGGTG-3'; R: 5'-TCAGTGTAGCCCAGGATGCC-3'.

Cell Culture

The CC cell lines (HeLa, C4-1, SiHa and CaSki) and the immortalized squamous cells of the human cervix (Etc1/E6E7) were provided by Cell Bank (Shanghai, China). Hela and CaSki cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and the others were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA). All cells were incubated in 5% CO_2 at 37°C.

Cell Transfection

The cells were seeded in a 6-well plate and cultured until 60-70% of confluence. They were incubated with the mixture containing 100 nmol/L oe-TUG1 and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Six hours later, the complete medium was replaced. Sequences of oeTUG1 were as the follows: oeTUG1 1#: F: 5'-GGGAUAUAGCCAGAGAACAAUUCUA-3'; R: 5'-UAGAAUUGUUCUCUGGCUAUAUCCC-3'; oeTUG1 2#: F: 5'-GCUUGGCUUCUAUUCUGAAUCCUUU-3'; R: 5'-AAAGGAUUCAGAAUAGAAGCCAAGC-3'.

Cell Counting Kit-8 (CCK-8) Assay

The transfected cells were seeded into 96-well plates with 5.0×10^3 cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo Molecular Technology, Kumamoto, Japan) was added in each well and the cells were incubated for 2 h. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell Migration Assay

The transfected cells for 48 h were adjusted to the dose of 1.0×10^5 cells/mL and subjected to serum starvation for 12 h. 200 μL/well suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA), while 700 μL of medium containing 10% FBS was applied in the bottom. After 48 h of incubation, the cells migrated to the bottom side were subjected to fixation in methanol for 15 min, crystal violet staining for 20 min, and cell counting using a microscope. The penetrating cells were counted in 5 randomly selected fields per sample.

Determination of Subcellular Distribution

The cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR. U6 was the internal reference of the nucleus and GAPDH was that of the cytoplasm.

RNA Immunoprecipitation (RIP)

The cells were treated according to the procedures of Millipore Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). The cell lysate was incubated with input, anti-PUM2, or anti-IgG antibody at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/mL proteinase K containing 0.1% sodium dodecyl sulphate (SDS) to extract RNA. The magnetic beads were repeatedly washed with RIP washing buffer to remove the non-specific adsorption as much as possible. Finally, the extracted RNA was subjected to mRNA level determination using qRT-PCR.

RNA Pull-Down

RNA pull-down was conducted using the Pierce™ RNA 3' End Desthiobiotinylation Kit (Thermo Fisher Scientific, Waltham, MA, USA). The purified proteins were subjected to Western blot analyses.

Western Blot

The total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membranes were blocked in 5% skim milk for 2 h and subjected to incubation with primary antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody after rinsing with the buffer solution (TBST). Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis. The data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The intergroup data were compared using the *t*-test. The receiver operating characteristic (ROC) curves were introduced for assessing the prognostic potential. The Pearson correlation analysis was conducted for evaluating the relationship between the two genes. In addition, the Kaplan-Meier methods were used for survival analysis. $p<0.05$ was considered the difference statistically significant.

Results

LncRNA TUG1 Was Upregulated in CC

As qRT-PCR data revealed, a higher abundance of TUG1 was observed in CC tissues compared to adjacent normal ones (Figure 1A). Its level in CC patients increased with the worsening of the TNM staging, manifesting as a higher level of TUG1 in CC patients with stage III-IV than those with stage I-II (Figure 1B). Moreover, TUG1 level was higher in CC tissues with larger tumor size (>4 cm) compared to those with a smaller one (≤4 cm) (Figure 1C). The ROC curves indicated the prognostic potential of TUG1 in CC (AUC=0.843, cut-off value=1.406, Figure 1D).

Overexpression of TUG1 Stimulated Viability and Migratory Rate of CC

Consistently, TUG1 was upregulated in CC cell lines (Figure 2A). HeLa and SiHa cell lines were chosen for the following *in vitro* experiments. Transfection of oeTUG1 greatly upregulated TUG1 level in CC cells, exhibiting an effective transfection efficacy (Figure 2B). In Hela and SiHa cells overexpressing TUG1, their viabilities were remarkably enhanced (Figures 2C, 2D). Besides, the number of migratory cells was elevated after the transfection of oeTUG1 (Figure 2E).

TUG1 Upregulated PUM2 (Pumilio2) Level

To uncover the molecular mechanism of TUG1 in regulating the viability and migratory ability of CC cells, PUM2, the target gene of TUG1 was found out. PUM2 was highly expressed in CC tissues relative to adjacent normal ones (Figure 3A). A positive correlation was identified between the expression levels of TUG1 and PUM2 in CC tissues (Figure 3B). After transfection of oeTUG1 in HeLa and SiHa cells, both protein and mRNA levels of PUM2 were upregulated (Figures 3C, 3D). Of note, the Kaplan-Meier curves revealed a worse overall survival in CC patients with a high level of PUM2 (Figure 3E). We therefore speculated about the important involvement of PUM2 in TUG1-regulated progression of CC.

Interaction Between TUG1 and PUM2

Through subcellular distribution analysis, TUG1 was found to be mainly expressed in the cytoplasmic fraction of the CC cells (Figures 4A, 4B). RIP assay showed much higher enrichment of TUG1 in anti-PUM2 than that of anti-IgG (Figure 4C). RNA pull-down assay also confirmed the interaction between TUG1 and PUM2 (Figure 4D). Interestingly, the transfection of si-PUM2 reversed the promotive effect of TUG1 on the viability of both HeLa and SiHa cells (Figure 4E). Therefore, it is considered that TUG1 mediated proliferative and migratory abilities of the CC cells by interacting with PUM2.

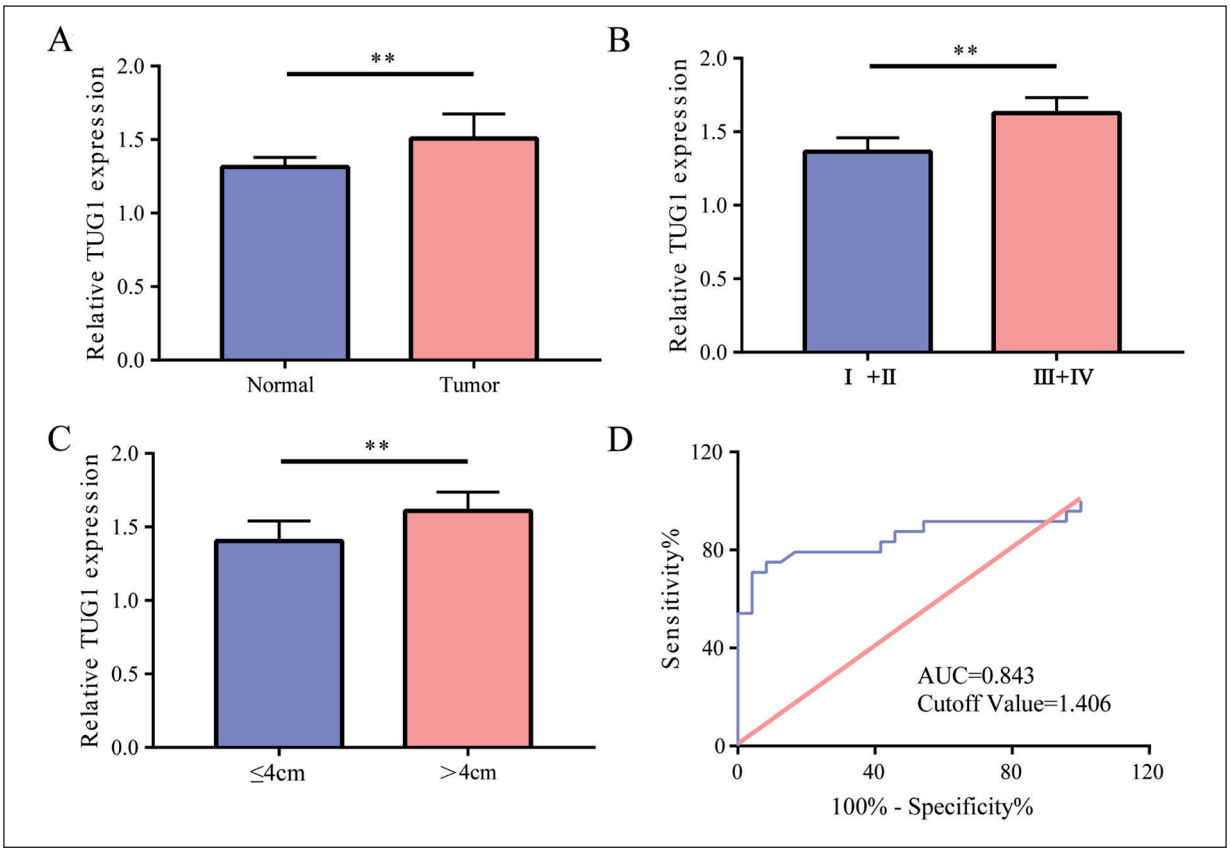

Figure 1. LncRNA TUG1 was upregulated in CC. **A,** Relative level of TUG1 in CC tissues and adjacent normal ones. **B,** The relative level of TUG1 in CC patients with stage I-II and stage III-IV. **C,** Relative level of TUG1 in CC tissues with >4 cm and ≤4 cm of tumor size. **D,** ROC curves introduced for assessing the sensitivity and specificity of TUG1 in diagnosing CC (AUC=0.843, cut-off value=1.406).

Discussion

Human papillomavirus (HPV) infection is considered to be a major risk factor of CC development, but the genetic and epigenetic changes also exert a crucial part. Recent studies have shown that epigenetic changes are common in the development and metastasis of CC. Abnormal DNA methylation and histone modifications in CC are well concerned nowadays. Non-coding RNAs, especially miRNAs and lncRNAs, are believed to serve as potential prognostic biomarkers in CC. In this paper, we focused on the biological function of lncRNA TUG1 in influencing the progression of CC, which may be used as a therapeutic target.

Mammalian genomes encode thousands of lncRNAs[6]. It is demonstrated that the dysregulation of cancer-related lncRNAs is closely related to the malignant progression of diseases[7,8]. The expression level of lncRNA TUG1 varies a lot in different types of cancers[15,17-19]. Our results illustrated the upregulation of TUG1 in CC tissues relative to adjacent normal ones. On the contrary, a previous study reported lowly expressed TUG1 in NSCLC, indicating the tissue-specificity of TUG1[15,20]. The pathological indexes of the CC patients were associated with TUG1 level, including tumor staging, tumor size, and overall survival. Additionally, TUG1 is able to mediate the cellular behaviors of tumor cells[15,16]. Here, the overexpression of TUG1 markedly enhanced the viability and migratory rate of HeLa and SiHa cells.

PUM2 is involved in the development of mammalian neural stem cells[21], epilepsy[22], and human germ cell development[23]. However, the role and target of PUM2 in tumor progression remain unclear. Several mechanisms underly the role of lncRNAs in regulating the gene expressions in

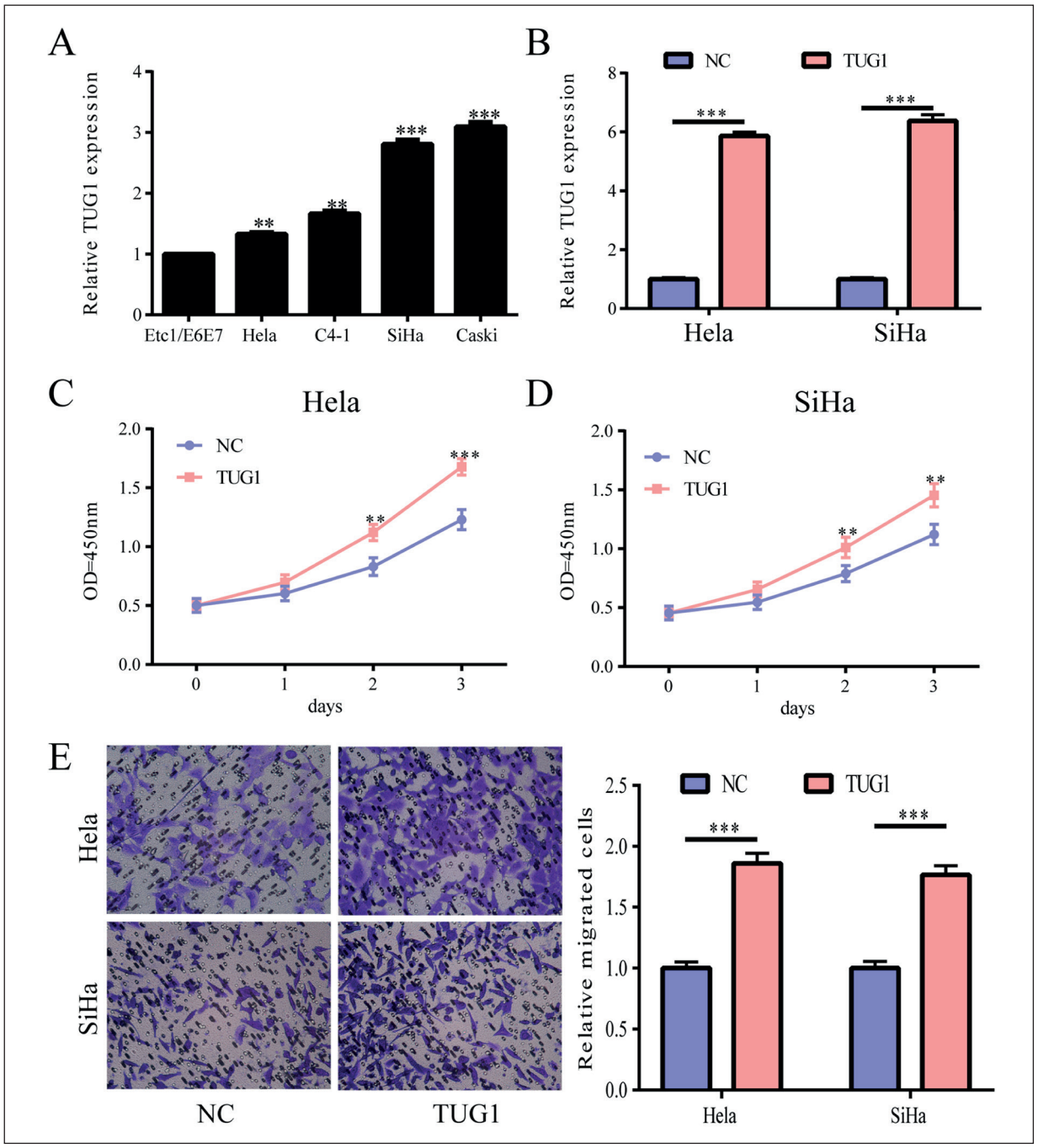

Figure 2. Overexpression of TUG1 stimulated viability and migratory rate of CC. **A,** The relative level of TUG1 in CC cell lines (HeLa, C4-1, SiHa and CaSki) and immortalized squamous cells of the human cervix (Etc1/E6E7). **B,** The transfection efficacy of oeTUG1 in HeLa and SiHa cells. **C,** CCK-8 assay showed the viability in HeLa cells transfected with NC or oeTUG1. **D,** CCK-8 assay showed the viability in SiHa cells transfected with NC or oeTUG1. **E,** The transwell assay showed the number of migratory number cells in HeLa and SiHa cells transfected with NC or oeTUG1 (magnification × 40).

cancer. Firstly, lncRNA binds to histones, DNAs or chromatin-modifying proteins through specific protein-binding domains to further mediate sequence-specific targeting of DNAs. Both activators and repressor proteins can bind to lncRNAs. Secondly, lncRNAs can establish contact with a target gene promoter and activate transcription by binding to a cis-regulatory element and pro-

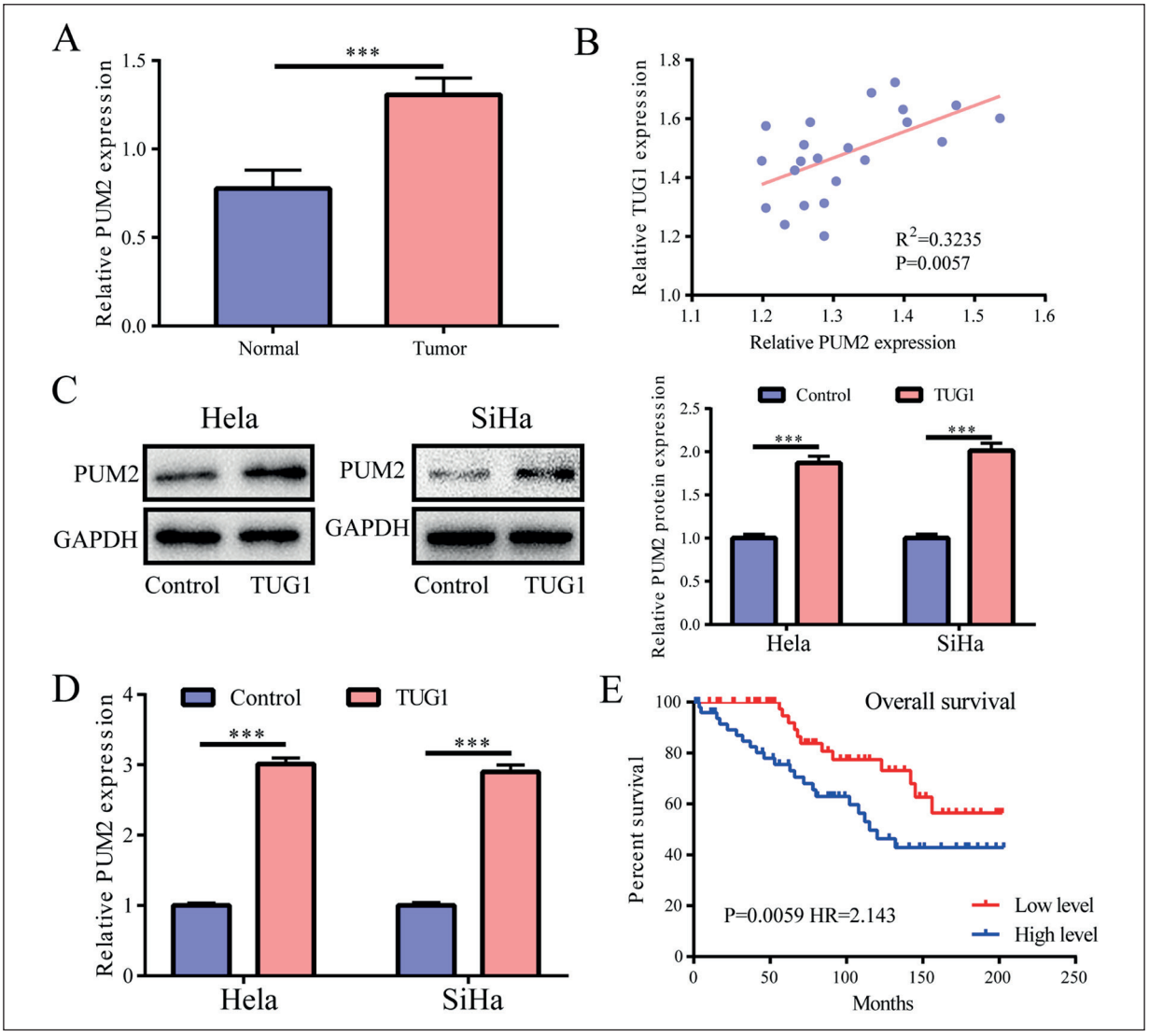

Figure 3. TUG1 upregulated PUM2 level. **A,** The relative level of PUM2 in CC tissues and adjacent normal ones. **B,** A positive correlation between the expression levels of TUG1 and PUM2 in CC tissues. **C,** The protein level of PUM2 in Hela and SiHa cells transfected with NC or oeTUG1. **D,** The mRNA level of PUM2 in HeLa and SiHa cells transfected with NC or oeTUG1. **E,** The Kaplan-Meier curves showed the overall survival in CC patients with a high or low level of PUM2.

moting chromatin cyclization. Thirdly, lncRNAs can be integrated into the RISCs (RNA-induced silencing complexes), resulting in the decline of mRNA stability and thus gene expression changes. In cancer diseases, the upregulated lncRNAs could competitively bind to a certain miRNA and further blocks its target genes. Meanwhile, lncRNAs can alter the expressions of metabolism-related genes, resulting in substrate availability changes of epigenetic modified enzymes. Our study demonstrated that TUG1 could bind to the PUM2 promoter region, thereafter, altering the expression level of PUM2 in CC. Notably, the silence of PUM2 reversed the promotive effect of TUG1 on the viability of the CC cells.

Conclusions

We showed that TUG1 is upregulated in CC, which aggravates the progression of CC by interacting with PUM2. TUG1/PUM2 axis may be utilized as an effective hallmark to improve the outcomes of CC patients.

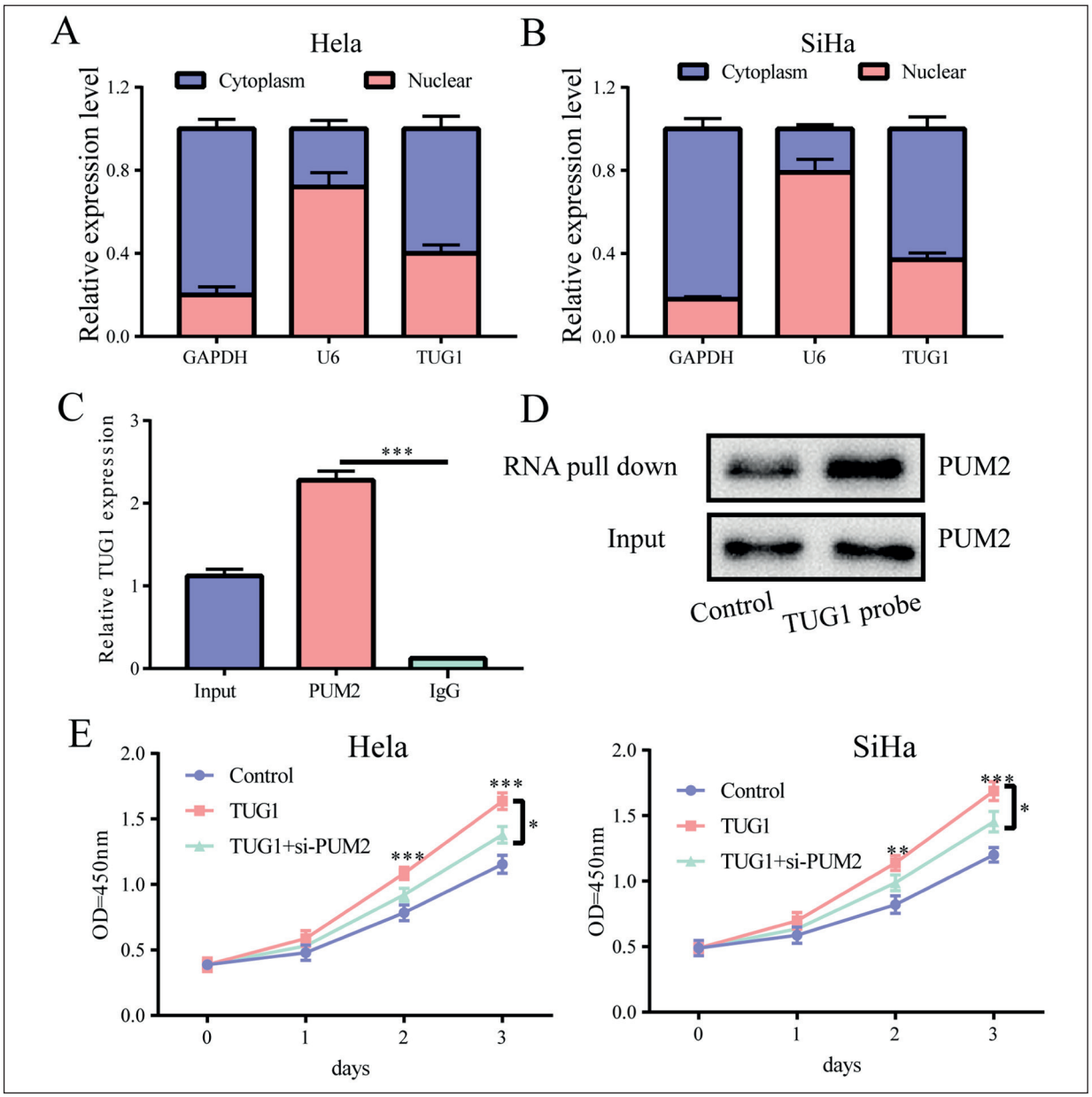

Figure 4. Interaction between TUG1 and PUM2. **A,** The subcellular distribution of TUG1 in nucleus and cytoplasm of Hela cells. GAPDH and U6 were served as cytoplasmic and nuclear internal reference, respectively. **B,** The subcellular distribution of TUG1 in nucleus and cytoplasm of SiHa cells. GAPDH and U6 were served as cytoplasmic and nuclear internal reference, respectively. **C,** RIP assay showed the enrichment of TUG1 in input, anti-PUM2 and anti-IgG. **D,** RNA pull-down assay showed the immunoprecipitant of PUM2 in control and TUG1 probe. **E,** CCK-8 assay showed the viability in Hela and SiHa cells transfected with control, oeTUG1 or oeTUG1 + si-PUM2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

2) Zhang HH, Li AH. Long non-coding RNA FEZF1-AS1 is up-regulated and associated with poor prognosis in patients with cervical cancer. Eur Rev Med Pharmacol Sci 2018; 22: 3357-3362.

3) Sakuragi N. Refining insight into cervical cancer progression. Lancet Oncol 2014; 15: 371-372.

4) Ponting CP, Belgard TG. Transcribed dark matter: meaning or myth? Hum Mol Genet 2010; 19: R162-R168.

5) Mattick JS. The genetic signatures of noncoding RNAs. PLoS Genet 2009; 5: e1000459.

6) Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassady JP, Cabili MN, Jaenisch R, Mikkelsen TS, Jacks T, Hacohen N, Bernstein BE, Kellis M, Regev A, Rinn JL, Lander ES. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 2009; 458: 223-227.

7) Gibb EA, Brown CJ, Lam WL. The functional role of long non-coding RNA in human carcinomas. Mol Cancer 2011; 10: 38.

8) Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.

9) Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 2007; 129: 1311-1323.

10) Kumegawa K, Maruyama R, Yamamoto E, Ashida M, Kitajima H, Tsuyada A, Niinuma T, Kai M, Yamano HO, Sugai T, Tokino T, Shinomura Y, Imai K, Suzuki H. A genomic screen for long noncoding RNA genes epigenetically silenced by aberrant DNA methylation in colorectal cancer. Sci Rep 2016; 6: 26699.

11) Gregg C, Zhang J, Weissbourd B, Luo S, Schroth GP, Haig D, Dulac C. High-resolution analysis of parent-of-origin allelic expression in the mouse brain. Science 2010; 329: 643-648.

12) Yang L, Lin C, Jin C, Yang JC, Tanasa B, Li W, Merkurjev D, Ohgi KA, Meng D, Zhang J, Evans CP, Rosenfeld MG. LncRNA-dependent mechanisms of androgen-receptor-regulated gene activation programs. Nature 2013; 500: 598-602.

13) Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004; 429: 457-463.

14) Young TL, Matsuda T, Cepko CL. The noncoding RNA taurine upregulated gene 1 is required for differentiation of the murine retina. Curr Biol 2005; 15: 501-512.

15) Zhang EB, Yin DD, Sun M, Kong R, Liu XH, You LH, Han L, Xia R, Wang KM, Yang JS, De W, Shu YQ, Wang ZX. P53-regulated long non-coding RNA TUG1 affects cell proliferation in human non-small cell lung cancer, partly through epigenetically regulating HOXB7 expression. Cell Death Dis 2014; 5: e1243.

16) Zhang Q, Geng PL, Yin P, Wang XL, Jia JP, Yao J. Down-regulation of long non-coding RNA TUG1 inhibits osteosarcoma cell proliferation and promotes apoptosis. Asian Pac J Cancer Prev 2013; 14: 2311-2315.

17) Huang MD, Chen WM, Qi FZ, Sun M, Xu TP, Ma P, Shu YQ. Long non-coding RNA TUG1 is upregulated in hepatocellular carcinoma and promotes cell growth and apoptosis by epigenetically silencing of KLF2. Mol Cancer 2015; 14: 165.

18) Xu Y, Wang J, Qiu M, Xu L, Li M, Jiang F, Yin R, Xu L. Upregulation of the long noncoding RNA TUG1 promotes proliferation and migration of esophageal squamous cell carcinoma. Tumour Biol 2015; 36: 1643-1651.

19) Wang L, Zhao Z, Feng W, Ye Z, Dai W, Zhang C, Peng J, Wu K. Long non-coding RNA TUG1 promotes colorectal cancer metastasis via EMT pathway. Oncotarget 2016; 7: 51713-51719.

20) Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, Rinn JL. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev 2011; 25: 1915-1927.

21) Martin-Broto J, Redondo A, Valverde C, Vaz MA, Mora J, Garcia DMX, Gutierrez A, Tous C, Carnero A, Marcilla D, Carranza A, Sancho P, Martinez-Trufero J, Diaz-Beveridge R, Cruz J, Encinas V, Taron M, Moura DS, Luna P, Hindi N, Lopez-Pousa A. Gemci ine plus sirolimus for relapsed and progressing osteosarcoma patients after standard chemotherapy: a multicenter, single-arm phase II trial of Spanish Group for Research on Sarcoma (GEIS). Ann Oncol 2017; 28: 2994-2999.

22) Follwaczny P, Schieweck R, Riedemann T, Demleitner A, Straub T, Klemm AH, Bilban M, Sutor B, Popper B, Kiebler MA. Pumilio2-deficient mice show a predisposition for epilepsy. Dis Model Mech 2017; 10: 1333-1342.

23) Ginter-Matuszewska B, Kusz K, Spik A, Grzeszkowiak D, Rembiszewska A, Kupryjanczyk J, Jaruzelska J. NANOS1 and PUMILIO2 bind microRNA biogenesis factor GEMIN3, within chromatoid body in human germ cells. Histochem Cell Biol 2011; 136: 279-287.