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LncRNA TDRG1 functions as an oncogene in cervical cancer through sponging miR-330-5p to modulate ELK1 expression

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Abstract. – OBJECTIVE: Increasing evidence shows that long non-coding RNAs (lncRNAs) play important roles in the development and progression of human carcinoma. TDRG1 was a recently identified lncRNA which was reported to promote the progression of several carcinomas. However, its function in cervical cancer remains unknown.

MATERIALS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay was performed to determine the mRNA expression. siRNA for lncRNA TDRG1, miR-330-5p, and the corresponding negative control were conducted. The cell function analysis was evaluated by CCK-8 assay, colony formation assay, transwell assay, scratch assay, and flow cytometry analysis. RNA-binding protein immunoprecipitation (RIP), Dual-Luciferase reporter assay, and RNA pull-down assay were used to determine the potential targets of TDRG1 or miR-330-5p. Western blot and Immunohistochemistry (IHC) analysis were used to examine the protein expression. The effect of TDRG1 on tumor growth was evaluated *in vivo*.

RESULTS: LncRNA TDRG1 expression was notably increased in cervical cancer tissues and cancer cells. LncRNA TDRG1 promoted the proliferation and migration of cervical cancer cells. Mechanism investigation suggested that lncRNA TDRG1 up-regulated the expression of ELK1 by acting as a competing endogenous RNA (CeRNA) of miR-330-5p. Rescue experiments indicated that miR-330-5p-inhibitor reversed the si-TDRG1-induced cell activity changes. This *in vivo* study proved that the down-regulation of lncRNA TDRG1 inhibited cervical tumor growth by regulating miR-330-5p/ELK1.

CONCLUSIONS: The present study reveals that lncRNA TDRG1 promotes cervical cancer progression by acting as a CeRNA of miR-330-5p to modulate the expression levels of ELK1 and may be explored as a novel target for developing therapeutic strategies for the treatment of cervical cancer.

Key Words:
LncRNA TDRG1, Proliferation, Migration, Invasion, CeRNA, MiR-330-5p, Cervical cancer.

Introduction

Although the incidence of cervical cancer has been significantly reduced due to the HPV vaccine, cervical cancer is still the third leading cause of cancer-related deaths worldwide[1-3]. Despite the great advancements in therapeutic technology, the 5-year overall survival rate of patients with cervical cancer maintained at 60-70% without a significant decrease in the last 40 years[4,5]. Tumor formation and development are a complex process, regulated by a variety of biological molecules such as oncogenes, tumor suppressor genes, and non-coding RNAs[6-8]. Researches on the molecular mechanisms of the development and progression of cervical cancer will be of great benefit to find new diagnostic biomarkers and treatment strategies.

Long non-coding RNAs (lncRNAs) refer to RNAs with more than 200 nucleotides in length and lack protein-coding capacity, which accounts for at least 70% of human transcripts[9]. LncRNAs have been found to be involved in numerous physiological and pathological processes[10,11], especially in the tumorigenesis and progression of cancer[12,13]. We found that lncRNAs are frequently dysregulated in multiple tumor types[14,15]. Shi et al[16] have investigated the potential of lncRNAs as biomarkers for cancer diagnosis, and promising results have been achieved. In addition, lncRNA is found to act as competing for endogenous RNAs (CeRNAs). In this manner, lncRNA can indirectly regulate the post-transcriptional translation of mRNA target genes by

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competitively binding to miRNA and impeding its function[17,18]. LncRNAs such as HOTAIR[19], XIST[20], and MALAT1[21] have been identified as potential therapeutic targets in cervical cancer. However, there are stills numerous lncRNAs in cervical cancer which have not been fully explored. Human testicular development-associated gene 1 (TDRG1) is a recently identified lncRNA gene that is specifically expressed in testicular tissues and promotes the development of testicular seminoma[22]. The previous report[23] has showed that the lncRNA TDRG1 is highly expressed in bone marrow mesenchymal stem cells. Moreover, lncRNA TDRG1 was found to be up-regulated in epithelial ovarian cancer and endometrial cancer and acted as an oncogene which promoted the development and progression of the endometrial tumor[24,25]. The regulatory mechanisms of TDRG1 are mainly through direct interacting with functional proteins such as VEGF-A or acting as a CeRNA to modulate the expression of miRNA, such as miR-93/RhoC pathway[24,25]. However, the role of lncRNA TDRG1 in cervical cancer is still under investigation.

MiRNAs refer to small non-coding RNAs, which play important roles in tumorigenesis, development, and treatment outcome[26]. Previous researches[27,28] showed that miR-330-5p exerts anti-tumor activity in several tumor types such as cutaneous malignant melanoma and glioblastoma. However, there is no report about the functions and regulation mechanisms of miR-330-5p in cervical cancer.

We first detected the expression of lncRNA TDRG1 in cervical cancer tissues and cells. Then, lncRNA TDRG1 was knocked down by siRNA transfection to determine its function in human HeLa and SIHA cells. Next, we used luciferase reporter assay, RIP assay, and RNA pull-down assay to identify target miRNAs and corresponding regulatory proteins of lncRNA TDRG1 in cervical cancer cells. Finally, we verified our results *in vivo* by using a mouse xenograft model.

Material and Methods

Tissue Samples

Human cervical cancer tissue sections, including cervical tumor tissues (n=30) and para-carcinoma tissues (n=30), were obtained from the second affiliated hospital of the Zhengzhou University. The cervical cancer patients underwent no prior chemotherapy, immunotherapy, or radiotherapy before tumor excision. All experimental protocols were approved by the Second Affiliated Hospital of Zhengzhou University Ethics Committee**.**

Cell Culture and Transfection

Immortalized human cervical squamous cell line Ect1/E6E7 and human cervical cancer cell lines, HeLa, CASKI, and SIHA, were obtained from the Cell Bank of The Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

The cell transfection was performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol.

ORT-PCR Assay

Total RNA of tumor tissues, para-carcinoma tissues or cell lines was isolated using TRIzol reagent (Invitrogen, CA, USA) and cDNA was then synthesized using the reverse transcription kit (Takara, Otsu, Shiga, Japan). The mRNA expression was measured by using an FTC-3000 real-time quantitative thermal cycler (Funglyn Biotech Inc., Shanghai, China). The primer sequences were as follows: lncRNA TDRG1, 5'-TCTTCCCTGGCTTGGC-3'(-forward); 5'-TGGGCTCTTTCGT GGC-3' (reverse); GAPDH, 5'-GTCAACGGATTTGGTCTGTATT-3' (forward) and 5'-AGTCTTCTGGGTGGCAGTGAT-3' (reverse). All mRNA expression was normalized to GAPDH. The $2^{-\Delta\Delta Ct}$ method was used for gene expression quantification.

Cell Proliferation Analysis

Cell proliferation was performed by MTT assay and colony formation assay. After transfected with siRNAs for 24 h, cells were seeded into a 96-well plate with 5000 cells per well. At different time points, cells were added with MTT (5 mg/ml) solution and incubated for 4h, and OD values at 570 nm were measured to determine the cell viability. For colony formation assay, HeLa or SIHA cells (about 300 cells) were plated into a 6 cm dish. The cells were normally cultured for 14 days. Then, cell colonies were imaged and counted. All investigations were performed in triplicate with quadruplicates.

Cell Invasion and Migration Analysis

For transwell invasion assay, 2×10^5 cells in serum-free medium were added into transwell

inserts (8 μm; BD Biosciences, Franklin Lakes, NJ, USA) with Matrigel-coated membrane. A medium with 20% FBS was added to the lower chamber as a chemo-attachment. After 48 h, the residual cells on the upper surface of the transwell membrane were wiped off, and invasive cells were fixed with methanol, stained with 1% crystal violet. Next, the cells were imaged and counted under a microscope. For the scratch assay, 3.5×10^5 cells were cultured in a 6-well plate until 100% confluent. Then, a single-line scratch was created by using a 200 μl pipette tip, and detached cells were washed away. The cell monolayer was photographed at 0 h and 24 h

Flow Cytometry Analysis for Cell Cycle and Apoptosis

For the cell cycle, after transfection with siRNAs for 48 h, the cells were collected and incubated in pre-cooled 70% ethanol at –20°C. The cells were collected by centrifugation, resuspended in pre-cooled phosphate-buffered saline (PBS) to 1.0×10^6/ml and incubated with RNase A (final concentration 1 mg/ml) for 30 min. After incubation with the propidium iodide (PI) solution (final concentration 50 μg/mL) in the dark for 30 min, the cell cycle was analyzed by flow cytometry.

Cell apoptosis was measured by a FITC/PI Apoptosis Kit (Thermo Fisher Scientific, Waltham, MA, USA). After transfection with siRNAs for 48 h, the cells were collected and resuspended in Annexin V binding buffer (5×10^6/ml). Cells were then mixed with Annexin V/FITC for 5 min in the dark. Then, the cell solution was added with 10 μl PI dye and 400 μl PBS. Flow cytometry was performed for apoptosis analysis.

Dual-Luciferase Reporter Assay

For lncRNA TDRG1 and miR-330-5p, miR-330-5p mimic or NC mimic was co-transfected with pmirGlo-NC, pmirGlo-TDRG1-mut, or pmirGlo-TDRG1-wt into human HeLa cells. For miR-330-5p and ELK1, miR-330-5p mimic or NC mimic was co-transfected with pmirGlo-NC, pmirGlo-ELK1-3'UTR-wt or pmiRGlo-ELK1-3'UTR-mut into human HeLa cells. The relative luciferase activities were determined using a Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA).

RNA-Binding Protein Immunoprecipitation (RIP) Assay

RNA immunoprecipitation was performed using an RNA Immunoprecipitation Kit (Millipore, Billerica, MA, USA) following the manufacturer's protocol. The magnetic beads were combined with Anti-AGO2 or IgG (Millipore, Billerica, MA, USA). Immunoprecipitated RNAs were subjected to the qRT-PCR analysis. The total RNAs were the input controls.

RNA Pull-Down Assay

Human HeLa cells were transfected with biotin-labeled negative control, biotin-labeled miR-330-5p, biotin-labeled miR-330-5p-Mut for 48 h. The whole-cell extraction was incubated with M-280 streptavidin magnetic beads (Invitrogen, Carlsbad, CA, USA) at 4°C for 4 h. Then, the coprecipitated RNA was isolated by using lysis buffer containing proteinase K (Invitrogen, Carlsbad, CA, USA) and 10% sodium dodecyl sulphate (SDS) and detected by qRT-PCR analysis.

Western Blot

After transfection for 48 h, total proteins of cells were extracted using radioimmunoprecipitation assay (RIPA) buffer (Shenneng Bocai, Biotechnology, Shanghai, China). The protein concentration was measured using a bicinchoninic acid (BCA) method. Then, 20 μg of protein was separated by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunodetection was performed with standard techniques. Antibodies to ELK1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Leiden, the Netherlands). The visualization of protein bands visualized by an electrochemiluminescence (ECL) development system. The quantification of protein densities was performed using the ImageJ software.

Animal Studies

All animal studies were approved by the second affiliated hospital of the Zhengzhou University Ethics Committee. The female BALB/c nude mice (4-6 weeks) were purchased from Shanghai LAC Laboratory Animal Co. Ltd. (Shanghai, China). HeLa cells were transfected with shRNA-Ctrl or shTDRG1 and injected into the posterior flank of mice subcutaneously. The tumor volume was monitored at indicated time points. Finally, the mice were executed by cervical dislocation, and tumors were resected.

Immunohistochemistry (IHC) Analysis

Paraffin-embedded tumor blocks were cut into sections with 4 μm thickness. Deparaffinization,

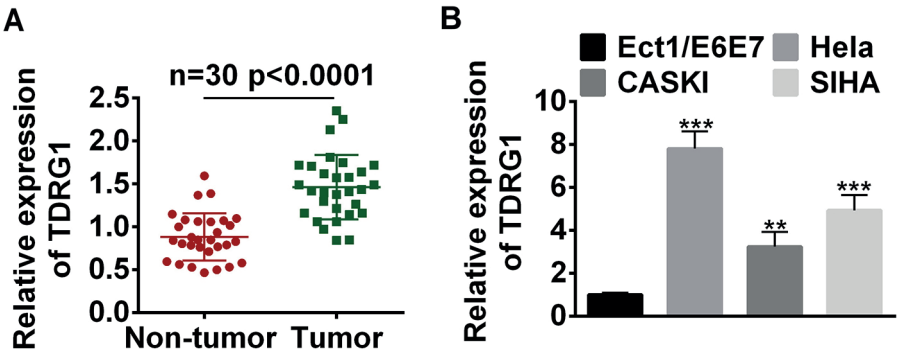

Figure 1. LncRNA TDRG1 is up-regulated in human cervical cancer tissues and cell lines. ***A***, LncRNA TDRG1 expression in human cervical cancer tissues (n=30) compared with paired para-carcinoma tissues (n=30). ***B***, Expression of lncRNA TDRG1 in HeLa, CASKI, and SIHA compared with normal cervical Ect1/E6E7 cells. LncRNA TDRG1 expression was detected by qRT-PCR assay. *p<0.05, **p<0.01 and ***p<0.001.

rehydration, and antigen retrieval (microwave, 30 min) were performed before incubation with anti-ALK1 or anti-Ki67 (20 min, 1:500). The tumor sections were next developed by using a DAB method. After doubled staining with hematoxylin, dehydration with gradient ethanol, and mounting with neutral resins, tissue sections were observed and imaged in 5 random fields at a microscope.

Statistical Analysis

Data are expressed as the mean ± SD from at least 3 independent repeats. Statistical analysis was performed using GraphPad Prism 7 software. Comparison between groups was made by using the double-sided Student's *t*-test. Comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA) with a Bonferroni's post-test. *p<0.05, **p<0.01 and ***p<0.001.

Results

LncRNA TDRG1 is Up-Regulated in Cervical Cancer Tissues and Cells

The qRT-PCR assay was used to detect the expression of lncRNA TDRG1 in cervical cancer tissues and cells. As shown in Figure 1A, the expression of lncRNA TDRG1 in cervical cancer tissues was significantly higher than that in normal controls (p<0.0001). Moreover, the expression of lncRNA TDRG1 was also significantly up-regulated in HeLa, CASKI, SIHA cells compared with normal Ect1/E6E7 cervical cells (Figure 1B).

Down-Regulation of LncRNA TDRG1 Inhibits Cervical Cancer Cell Proliferation and Migration

To investigate the function of lncRNA TDRG1 in cervical cancer, we down-regulated lncRNA TDRG1 in HeLa and SIHA cells using the siRNA transfection. QRT-PCR results showed that lncRNA TDRG1 was effectively knocked down by si-TDRG1 (Figure 2A). siTDRG1#3 for HeLa and siTDRG1#1 for SIHA were selected for the following experiments due to the higher interference efficiency. Cell proliferation analysis results indicated that the cell viability of HeLa and SIHA cells was significantly inhibited by the lncRNA TDRG1 silencing (Figure 2B and C). Scratch assay and transwell assay results also demonstrated that the migration and invasion rates were significantly decreased in lncRNA TDRG1 silenced cells compared with siNC groups (Figure 2D and E). Moreover, cells were also arrested in the G1 phase of the cell cycle by TDRG1 knockdown (Figure 2F), indicating by flow cytometry analysis. Cell apoptosis analysis by flow cytometry showed that the percentage of apoptotic cells was increased from (12.80±2.85)% in siNC group to (36.8±2.08)% in the siTDRG1 group in HeLa cells and (33.5±1.05)% to (53±2.15)% in SIHA cells (Figure 2G).

LncRNA TDRG1 Acts as a CeRNA by Competitively Binding to MiR-330-5p

Researches show that lncRNAs could competitively bind to miRNAs through complementary sequences to downregulate their expression,

which was defined as CeRNAs[29-31]. We used Starbase v2.0 and TargetScan to predict the possible target miRNA of lncRNA TDRG1. As shown in Figure 3A, miR-330-5p could potentially interact with lncRNA TDRG1. To verify the binding relation between miR-330-5p and lncRNA TDRG1, we conducted Dual-Luciferase reporter assays in HeLa cells. The results suggested that lncRNA TDRG1 was effectively up-regulated by lncRNA TDRG1 reporter vector (Figure 3B left). MiR-330-5p mimic reduced the luciferase activity of the lncRNA TDRG1 reporter vector compared with NC group, and this reduction disappeared when the target site on lncRNA TDRG1 was mutated (Figure 3B right). MiRNAs usually exert their function by forming RNA-induced silencing complex (RISC), of which Ago2 is a core component[32]. To determine whether lncRNA TDRG1 formed a RISC with miR-330-5p, we performed a RIP assay by using an Ago2 antibody. As shown in Figure 3C, the result showed that miR-330-5p was enriched by Ago2 antibody in lncRNA TDRG1 overexpressing cells. In addition, miRNA pull-down

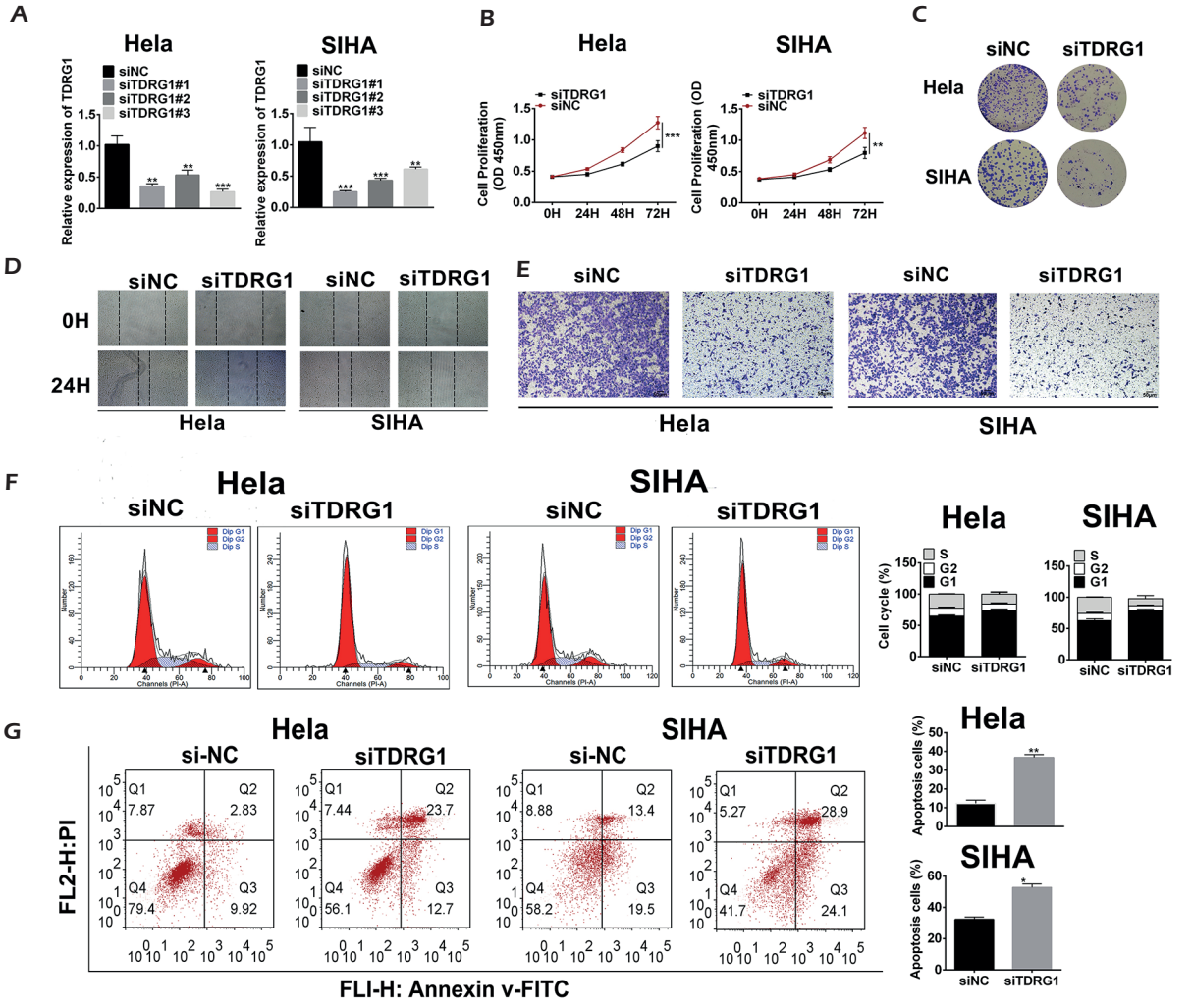

Figure 2. Knockdown of lncRNA TDRG1 inhibits human cervical cancer *in vitro*. ***A,*** LncRNA TDRG1 was knocked down in human HeLa and SIHA cells by transfection with si-TDRG1; The interference efficiencies were determined by qRT-PCR assay. (***B***) and (***C***) Effects of lncRNA TDRG1 on the proliferation of HeLa and SIHA cells were tested by MTT assay and colony formation assay (Magnification, 40X). ***D,*** Cell migration was tested by scratch assay in lncRNA TDRG1 silenced HeLa and SIHA cells (Magnification, 200X). ***E,*** Transwell assay was performed to detect cell invasion of HeLa and SIHA cells (Magnification, 200X). ***F***, Flow cytometry analysis was conducted for analysis of cell cycle of HeLa and SIHA cells. ***G,*** Apoptosis percentage was determined by using flow cytometry analysis. *p<0.05, **p<0.01 and ***p<0.001.

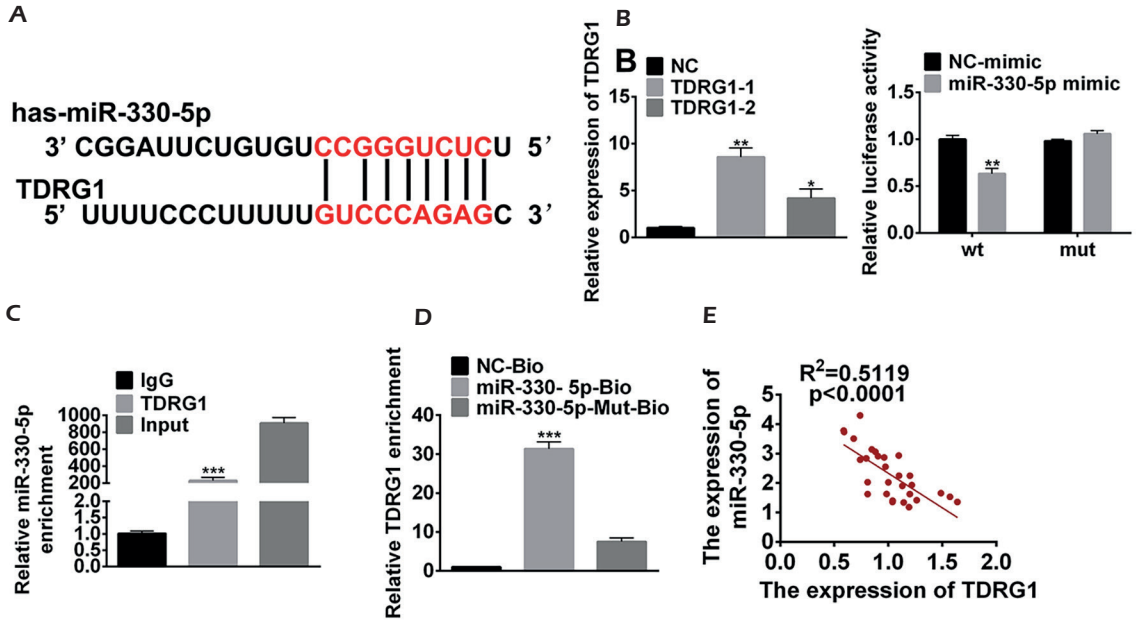

Figure 3. LncRNA TDRG1 functions as a CeRNA by competitively binding to miR-330-5p. ***A,*** Binding site of miR-330-5p in lncRNA TDRG1 was predicted by Starbase v2.0 and TargetScan. ***B,*** Dual luciferase reporter assays demonstrating that miR-330-5p was the target of lncRNA TDRG1; Left, lncRNA TDRG1 was effectively up-regulated by lncRNA TDRG1 reporter vector; Right, miR-330-5p mimic reduced the luciferase activity of the lncRNA TDRG1 wild type (TDRG1-wt) while had no effect on lncRNA TDRG1 mutant type (TDRG1-mut). ***C,*** RIP assay using anti-Ago2 antibody demonstrated that lncRNA TDRG1 and miR-330-5p formed a RNA-induced silencing complex (RISC). ***D,*** RNA pull-down assay indicated that lncRNA TDRG1 could be enriched by biotin-labelled miR-330-5p but not biotin-labelled miR-330-5p mutant type. ***E,*** Correlation between lncRNA TDRG1 expression and miR-330-5p expression in human cervical cancer tissues were determined by using qRT-PCR assay. *p<0.05, **p<0.01 and ***p<0.001.

assays indicated that lncRNA TDRG1 could be pulled down by miR-330-5p (Figure 3D). Since lncRNAs could down-regulate the expression of their target miRNAs via sponge effect, we performed a correlation analysis of the expression levels of miR-330-5p and TDRG1 in cervical cancer samples by using qRT-PCR. The results showed that lncRNA TDRG1 transcript level was negatively correlated with miR-330-5p transcript level (Figure 3E).

Taken together, these data suggest that lncRNA TDRG1 may act as a CeRNA via binding with miR-330-5p in cervical cancer.

MiR-330-5p targets ELK1 and Down-Regulates its Expression

We further used the bioinformatics algorithms TargetScan to predict the downstream target of miR-330-5p. As shown in Figure 4A, miR-330-5p could potentially bind to the 3' untranslated region (3' UTR) of ELK1. Luciferase reporter assays indicated that miR-330-5p mimic significantly reduced the luciferase activity of the wildtype 3'-UTR of ELK1 (ELK1-wt), but had no effect on the mutant 3'-UTR of ELK1 (ELK1-mut), which suggested a direct interaction between miR-330-5p and ELK1 (Figure 4A). To investigate the effect of miR-330-5p and lncRNA TDRG1 on ELK1 protein expression, we performed overexpression and knockdown assays. As shown in Figure 4B, the miR-330-5p mimic down-regulated ELK1 protein expression while miR-330-5p inhibitor up-regulates ELK1 in human cervical cancer HeLa and SIHA cells. In addition, ELK1 expression was increased by lncRNA TDRG1 (Figure 4C). The result in Figure 4D showed that the expression of ELK1 was positively correlated with lncRNA TDRG1 whereas negatively with miR-330-5p.

Taken together, these data suggest that ELK1 was a direct target of miR-330-5p, and its expression was down-regulated by miR-330-5p. Moreover, ELK1 expression was enhanced by lncRNA TDRG1in human cervical cancer cells.

LncRNA TDRG1 Promotes the Proliferation and Migration of Cervical Cancer Cells Through Regulation of MiR-330-5p

Since lncRNA TDRG1 functions as a CeRNA of miR-330-5p, we performed rescue experiments to investigate whether miR-330-5p was involved in lncRNA TDRG1-mediated cell activity change. To determine the function of miR-330-5p in human cervical cancer cells, we transfected miR-330-5p-inhibitor into HeLa and SIHA cells to down-regulation of miR-330-5p. For rescue experiments, miR-330-5p-inhibitor and si-TDRG1 were co-transfected into HeLa and SIHA cells. Intriguingly, miR-330-5p-inhibitor promoted cell proliferation, migration, invasion, and cell cycle arrest, as well as decreased cell apoptosis in both HeLa and SIHA cells (Figures 5 and 6). Co-transfection of si-TRDG1 and miR-330-5p-inhibitor reversed the si-TDRG1-INDUCED inhibition of

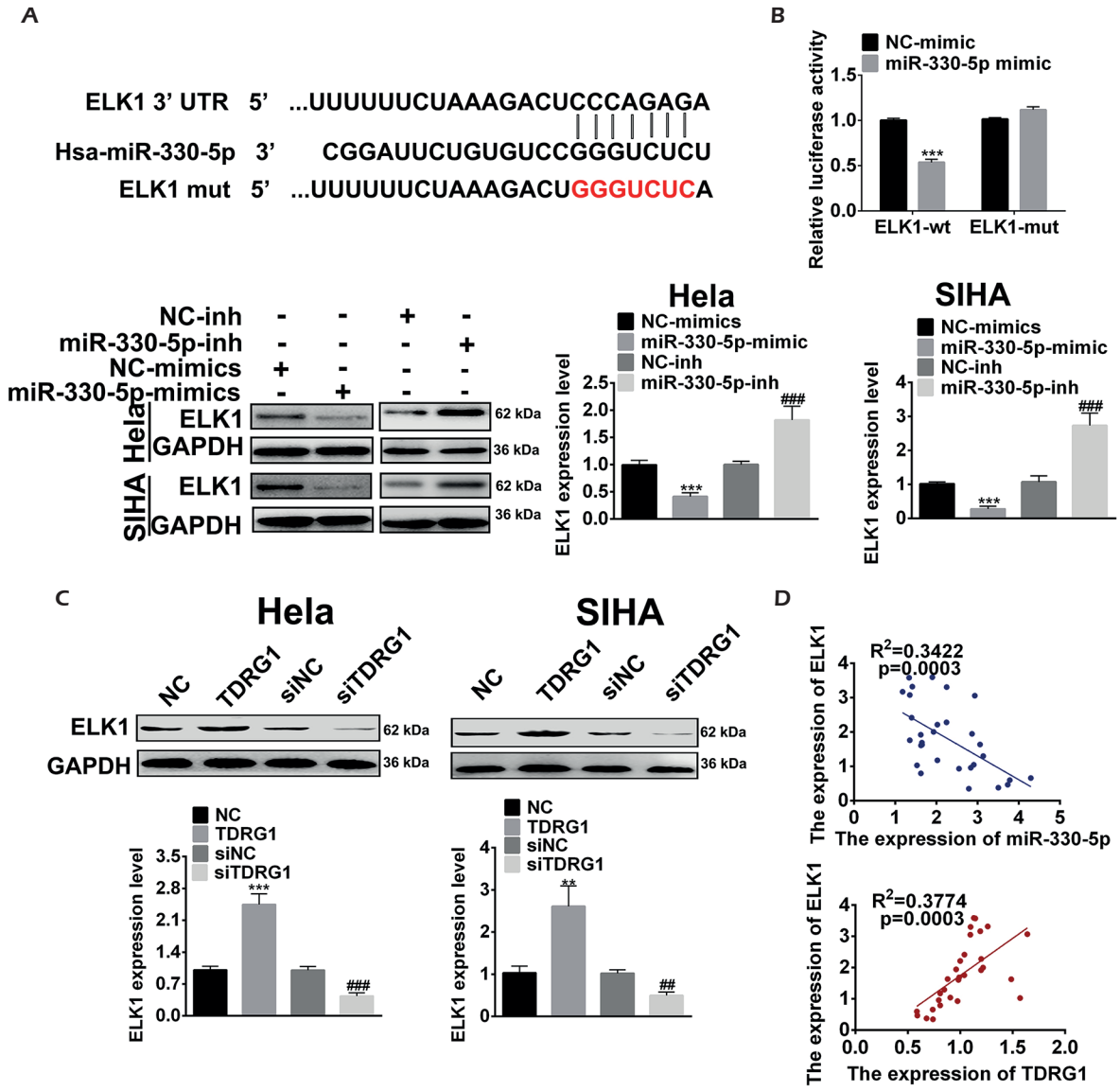

Figure 4. MiR-330-5p directly targets ELK1 and ELK1 expression was regulated by lncRNA TDRG1/miR-330-5p axis. ***A,*** Binding site of miR-330-5p and ELK1 3'UTR region was predicted by TargetScan. ***B,*** ELK1 expression was inhibited by overexpression of miR-330-5p and promoted by knockdown of miR-330-5p; ELK1 expression was detected by western blot. ***C,*** ELK1 expression was promoted by overexpression of lncRNA TDRG1 and down-regulated by knockdown of lncRNA TDRG1; ELK1 expression was detected by western blot. ***D,*** Correlation between ELK1 and lncRNA TDRG1 or miR-330-5p was analyzed in human cervical cancer tissues. *p<0.05, **p<0.01 and ***p<0.001.

proliferation and cell cycle arrest (Figure 5A-C). Moreover, cell apoptosis analysis indicated that miR-330-5p-inhibitor decreased cell apoptosis induced by siTDRG1 (Figure 6A). Scratch and transwell assays revealed that miR-330-5p-inhibitor significantly reversed the inhibitory effect of si-TDRG1 on cell migration and invasion in HeLa and SIHA cells (Figure 6B and C).

Altogether, these results demonstrated that lncRNA TDRG1 promotes proliferation, metastasis, cell cycle, and inhibits apoptosis in human cervical cancer cells through down-regulating of miR-330-5p.

Knockdown of LncRNA TDRG1 Inhibited Cervical Cancer Growth by Regulating MiR-330-5p/ELK1 axis In Vivo

To validate the function of lncRNA TDRG1 *in vivo*, a xenograft model was established in nude mice. shRNA-TDRG1 or shRNA-Ctrl transfected human HeLa cervical cancer cells were injected subcutaneously into the nude mice. Tumor growth and tumor size were monitored and measured regularly. We observed that knockdown of lncRNA TDRG1 significantly inhibited tumor growth compared with an shRNA-Ctrl group (Figure 7A and B, $p<0.05$). Moreover, the qRT-PCR analysis suggested that the expression of miR-330-5p was significantly elevated in the cervical tumor when lncRNA TDRG1 was down-regulated (Figure 7C). IHC staining showed that the expression of ELK1 and Ki67 was significantly inhibited by knockdown of lncRNA TDRG1 (Figure 7D). Taken together, these data suggest that knockdown of lncRNA TDRG1 inhibits cervical tumor growth by regulating miR-330-5p/ELK1 axis, which was consistent with our findings *in vitro*.

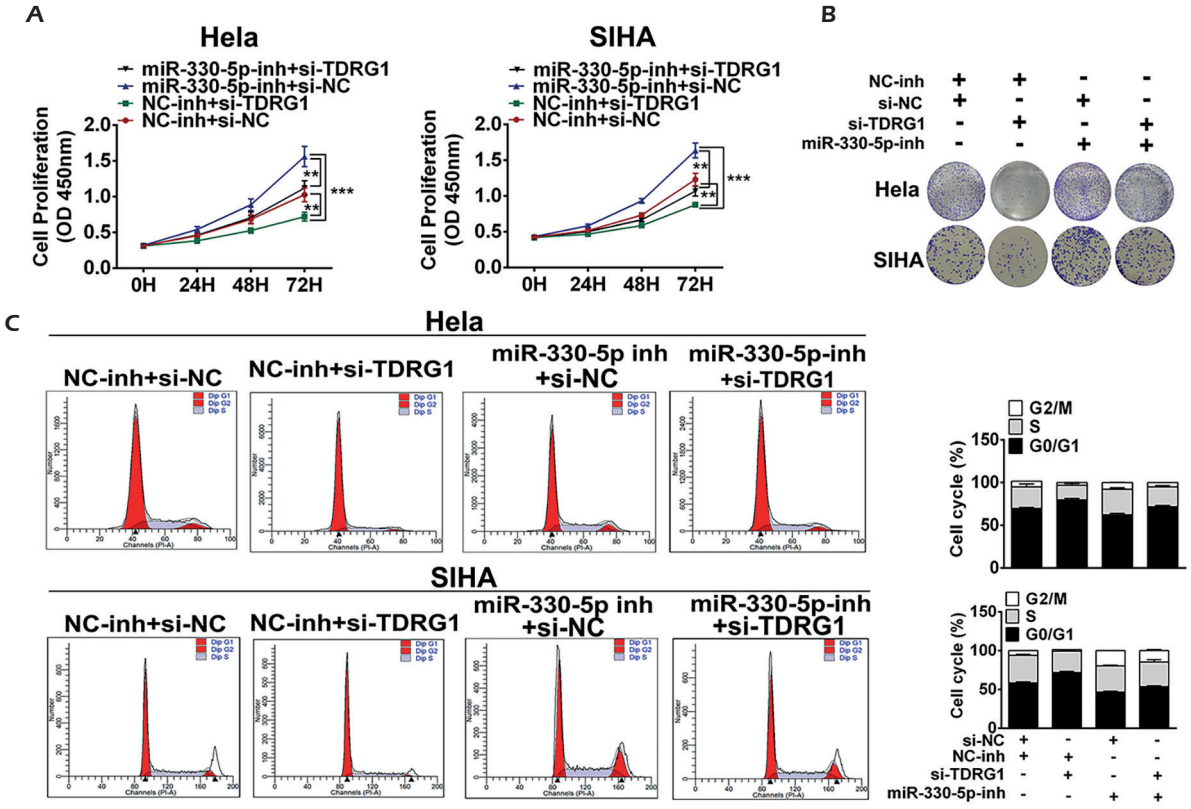

Figure 5. LncRNA TRDG1-induced cell activity changes ware mediated by miR-330-5p. HeLa and SIHA cells were co-transfected with NC-inh + siNC, NC-inh + si-TDRG1, miR-330-5p-inh + siNC or miR-330-5p-inh + si-TDRG1. Transfection with miR-330-5p-inh rescued the ability of cell proliferation (***A***), colony formation (***B,*** magnification, 40X), cell cycle (***C***) by si-TDRG1. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

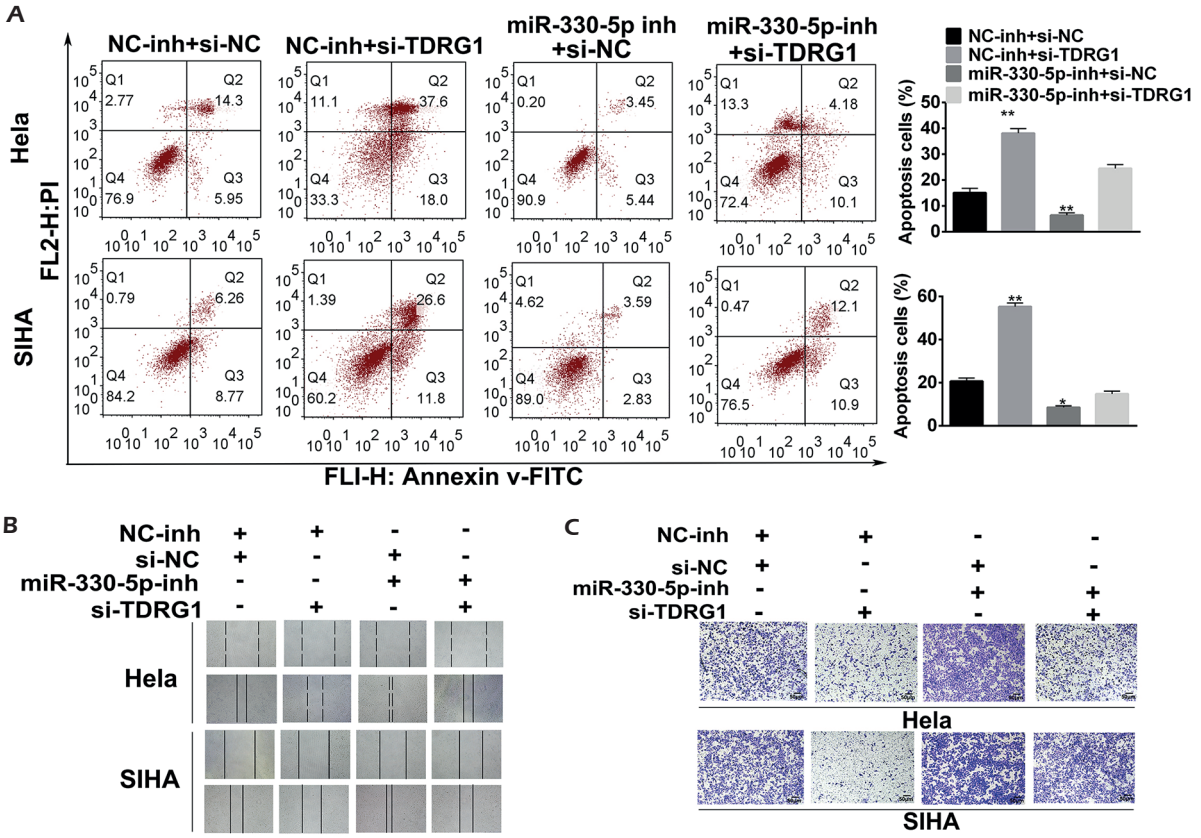

Figure 6. LncRNA TRDG1-induced cell activity changes were mediated by miR-330-5p**.** HeLa and SIHA cells were co-transfected with NC-inh + siNC, NC-inh + si-TDRG1, miR-330-5p-inh + siNC or miR-330-5p-inh + si-TDRG1. Transfection with miR-330-5p-inh rescued the ability of (***A***) Cell apoptosis induced by si-TDRG1. (***B***) cell migration (***C***) cell invasion inhibited by si-TDRG1. Magnification, 200X. **p*<0.05, ***p*<0.01 and ****p*<0.001.

Discussion

Cervical cancer is the second most common cancer among women[1]. Although great advancements have been made in the early diagnosis; however, the prognosis for patients with advanced cervical cancer still remains poor[33]. Scientists have been committed to identifying effective prognostic markers for cervical cancer. LncRNAs have been identified to be abnormally expressed and play important roles in the progression of numerous carcinomas, including cervical cancer[12,13]. For example, down-regulation of lncRNA UCA1 suppresses cell growth and movements of cervical cancer cells through regulation of miR-206 expression[34]. LncRNA TUG1 was significantly up-regulated in cervical tumor tissues and promoted proliferation and epithelial-mesenchymal transition (EMT) of cervical cancer cells[35]. LncRNA PCAT6 regulates cell growth and metastasis via the Wnt/beta-catenin pathway and serves as a prognosis marker in cervical cancer[36]. However, the function and regulating mechanism of lncRNA TDRG1 in cervical cancer remain unknown. Here we reported that lncRNA TDRG1 was up-regulated in cervical cancer tissues and cell lines. Function analysis suggested that down-regulation of lncRNA TDRG1 inhibited proliferation, migration, and invasion of cervical cancer cells by inducing apoptosis and cell cycle arrest. We further investigated the role of lncRNA TDRG1 in human cervical cancer *in vivo* by using a xenograft tumor model. The results suggested that lncRNA TDRG1 knockdown inhibits cervical tumor growth, which was consistent with our *in vitro* findings in the cell.

Studies[37,38] have found that lncRNAs contain miRNAs binding sites and act as CeRNAs to sponge miRNAs to antagonize their function.

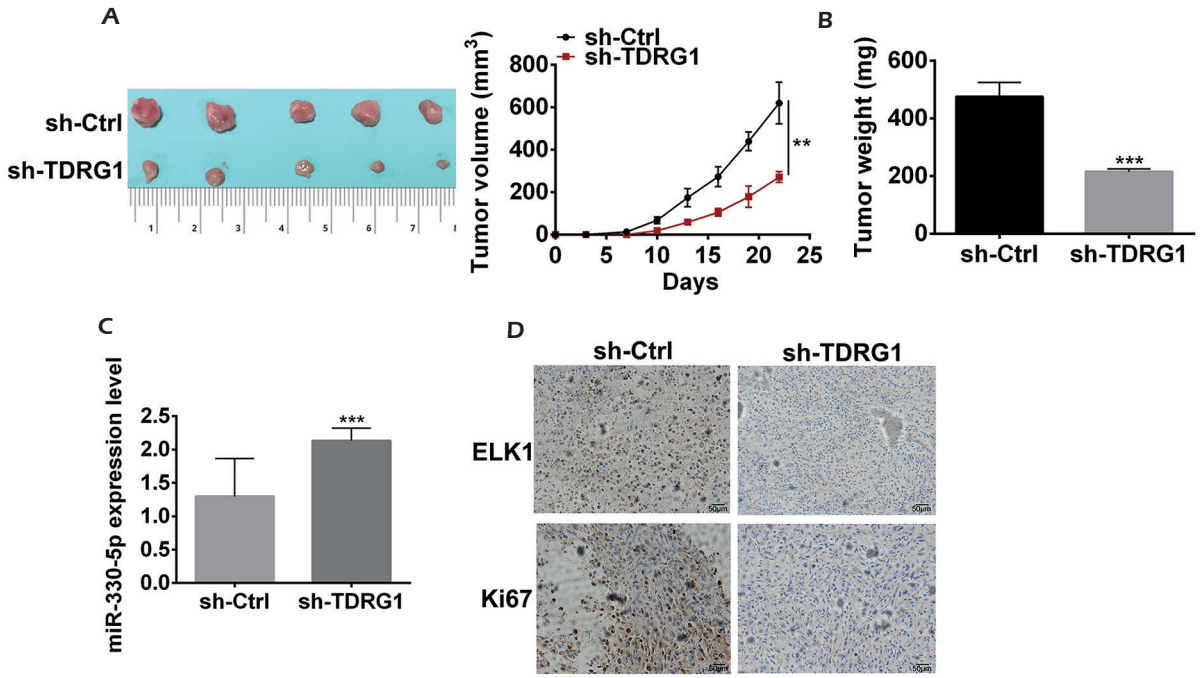

Figure 7. Knockdown of lncRNA TDRG1 inhibited cervical tumor growth by regulating miR-330-5p/ELK1 axis *in vivo*. shRNA-Ctrl or shRNA-TDRG1 transfected HeLa cells were implanted subcutaneously to establish a xenograft model in nude mice. ***A,*** Left, photograph of resected tumors; right, tumor growth curve. ***B,*** Average tumor weights. ***C,*** MiR-330-5p expression in xenograft tumors was detected by qRT-PCR assay. ***D,*** Expression of ELK1 and Ki67 in xenograft tumors was detected by IHC analysis. Magnification, 100X. *p<0.05, **p<0.01 and ***p<0.001.

For example, lncRNA WT1-AS has been found to inhibit cervical cancer metastasis via regulation of the miR-203a-5p/FOXN2 axis[39]. Chen et al[24] have found that lncRNA TDRG1 could down-regulate miR-93 by acting as a CeRNA mechanism and further induces RhoC expression in epithelial ovarian cancer. In this work, we predicted the potential binding site of miR-330-5p on lncRNA TDRG1 by bioinformatics software. Then, we identified miR-330-5p as the target miRNA of lncRNA TDRG1 in cervical cancer cells by dual luciferase assay and mRNA-pull down assay. Previous studies[27,28] show that miR-330-5p functions as a tumor suppress gene in several carcinomas such as cutaneous malignant melanoma and glioblastoma. Furthermore, miR-330-5p was also found to be down-regulated in cervical cancer tissues according to the Cancer Genome Atlas (TCGA) data. Therefore, we speculated that miR-330-5p also played a suppressive role in cervical cancer. Next, we further identified ELK1 as a target gene of miR-330-5p in cervical cancer cells through bioinformatics analysis. ELK1 is a transcription activator that modulates a variety of genes involved in proliferation and metastasis such as proto-oncogene c-fos, MMP9, and MMP2[40,41]. Indeed, ELK1 has been reported[42] to function as an oncogene in several carcinomas such as bladder cancer. In our studies, we found that lncRNA TDRG1 up-regulates miR-330-5p to increase ELK1 expression. Rescue experiments indicated that lncRNA TDRG1 promotes proliferation and migration of cervical cancer cells through down-regulation of miR-330-5p.

Conclusions

For the first time, these results demonstrated that lncRNA TDRG1 promoted proliferation, migration, and invasion of cervical cell *in vitro*. Also, it promoted tumorgenicity *in vivo* of cervical cancer by targeting miR-330-5p and up-regulation of ELK1, which revealed the molecular mechanism of lncRNA TDRG1 in the development and progression of cervical cancer. Thus, our results suggested that lncRNA TDRG1 has the potential to be explored as a prognostic biomarker for cervical cancer.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Ethics Committee Approval

The tissue sections were obtained from the Hospital. This investigation has been approved by the Ethical Committee at the Hospital. All patients provided written informed consent. All animal procedures were carried out under the guidelines approved by the Institutional Animal Care and Use Committee of the Hospital.

References

1) Hou T, Liang D, Xu L, Huang X, Huang Y, Zhang Y. Atypical chemokine receptors predict lymph node metastasis and prognosis in patients with cervical squamous cell cancer. Gynecol Oncol 2013; 130: 181-187.
2) Tewari KS, Sill MW, Long HJ, Penson RT, Huang H, Ramondetta LM, Landrum LM, Oaknin A, Reid TJ, Leitao MM, Michael HE, Monk BJ. Improved survival with bevacizumab in advanced cervical cancer. Obstet Gynecol Surv 2014; 69: 331-332.
3) Kim JJ, Campos NG, Sy S, Burger EA, Cuzick J, Castle PE, Hunt WC, Waxman A, Wheeler CM, New Mexico HPVPR. Inefficiencies and high-value improvements in US cervical cancer screening practice a cost-effectiveness analysis. Ann of Intern Med 2015; 163: 589-597.
4) Quinn M, Babb P, Jones J, Allen E. Effect of screening on incidence of and mortality from cancer of cervix in England: evaluation based on routinely collected statistics. Brit Med J 1999; 318: 904-908.
5) Jiang S, Wang HL, Yang J. Low expression of long non-coding RNA LET inhibits carcinogenesis of cervical cancer. Int J Clin Exp Pathol 2015; 8: 806-811.
6) Bhan A, Soleimani M, Mandal SS. Long noncoding RNA and cancer: a new paradigm. Cancer Res 2017; 77: 3965-3981.
7) Steenbergen RDM, Snijders PJF, Heideman DAM, Meijer CJLM. Clinical implications of (epi) enetic changes in HPV-induced cervical precancerous lesions. Nat Rev Cancer 2014; 14: 395-405.
8) Semenza GL. Regulation of cancer cell metabolism by hypoxia-inducible factor 1. Semn Cancer Biol 2009; 19: 12-16.
9) Qi P, Du X. The long non-coding RNAs, a new cancer diagnostic and therapeutic gold mine. Modern Pathol 2013; 26: 155-165.
10) Cech TR, Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. Cell 2014; 157: 77-94.
11) Geisler S, Coller J. RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. Nat Rev Mol Cell Biol 2013; 14: 699-712.
12) Liu B, Sun L, Liu Q, Gong C, Yao Y, Lv X, Lin L, Yao H, Su F, Li D, Zeng M, Song E. A cytoplasmic NF-kappa B interacting long noncoding RNA blocks I kappa B phosphorylation and suppresses breast cancer metastasis. Cancer Cell 2015; 27: 370-381.
13) Yang F, Zhang L, Huo XS, Yuan JH, Xu D, Yuan SX, Zhu N, Zhou WP, Yang GS, Wang YZ, Shang JL, Gao CF, Zhang FR, Wang F, Sun SH. Long noncoding RNA high expression in hepatocellular carcinoma facilitates tumor growth through enhancer of zeste homolog 2 in humans. Hepatology 2011; 54: 1679-1689.
14) Fu Z, Chen C, Zhou Q, Wang Y, Zhao Y, Zhao X, Li W, Zheng S, Ye H, Wang L, He Z, Lin Q, Li Z, Chen R. LncRNA HOTTIP modulates cancer stem cell properties in human pancreatic cancer by regulating HOXA9. Cancer Lett 2017; 410: 68-81.
15) Yang B, Zhang L, Cao Y, Chen S, Cao J, Wu D, Chen J, Xiong H, Pan Z, Qiu F, Chen J, Ling X, Yan M, Huang S, Zhou S, Li T, Yang L, Huang Y, Lu J. Overexpression of lncRNA IGFBP4-1 reprograms energy metabolism to promote lung cancer progression. Mol Cancer 2017; 16: 154.
16) Shi T, Gao G, Cao Y. Long noncoding RNAs as novel biomarkers have a promising future in cancer diagnostics. Dis Markers 2016; 2016: 9085195.
17) Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell 2011; 147: 358-369.
18) Cai Q, Wang Z, Wang S, Weng M, Zhou D, Li C, Wang J, Chen E, Quan Z. Long non-coding RNA LINC00152 promotes gallbladder cancer metastasis and epithelial-mesenchymal transition by regulating HIF-1alpha via miR-138. Open Biol 2017; 7:. pii: 160247.
19) Lee M, Kim HJ, Kim SW, Park SA, Chun KH, Cho NH, Song YS, Kim YT. The long non-coding RNA HOTAIR increases tumour growth and invasion in cervical cancer by targeting the Notch pathway. Oncotarget 2016; 7: 44558-44571.
20) Sun G, Wang C, Zhang H. Long non-coding RNA XIST promotes cervical cancer cell epithelial-mesenchymal transition through the Wnt/beta-catenin pathway. Int J Clin Exp Pathol 2017; 10: 2333-2339.
21) Liu S, Song L, Zeng S, Zhang L. MALAT1-miR-124-RBG2 axis is involved in growth and invasion of HR-HPV-positive cervical cancer cells. Tumor Biol 2016; 37: 633-640.
22) Jiang X, Li D, Yang J, Wen J, Chen H, Xiao X, Dai Y, Yang J, Tang Y. Characterization of a novel human testis-specific gene: Testis Developmental Related Gene 1 (TDRG1). Tohoku J Exp Med 2011; 225: 311-318.
23) Jiang S, Xia M, Yang J, Shao J, Liao X, Zhu J, Jiang H. Novel insights into a treatment for aplastic anemia based on the advanced proliferation of

bone marrow-derived mesenchymal stem cells induced by fibroblast growth factor 1. Mol Med Rep 2015; 12: 7877-7882.

24) Chen S, Wang LL, Sun KX, Xiu YL, Zong ZH, Chen X, Zhao Y. The role of the long non-coding RNA TDRG1 in epithelial ovarian carcinoma tumorigenesis and progression through miR-93/RhoC pathway. Mol Carcinog 2018; 57: 225-234.

25) Chen S, Wang LL, Sun KX, Liu Y, Guan X, Zong ZH, Zhao Y. LncRNA TDRG1 enhances tumorigenicity in endometrial carcinoma by binding and targeting VEGF-A protein. Biochim Biophys Acta Mol Basis Dis 2018; 1864: 3013-3021.

26) Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review (vol 4, pg 143, 2012). EMBO Mol Med 2017; 9: 852.

27) Su BB, Zhou SW, Gan CB, Zhang XN. MiR-330-5p regulates tyrosinase and PDIA3 expression and suppresses cell proliferation and invasion in cutaneous malignant melanoma. J Surg Res 2016; 203: 434-440.

28) Feng L, Ma J, Ji H, Liu Y, Hu W. MiR-330-5p suppresses glioblastoma cell proliferation and invasiveness through targeting ITGA5. Bioscience Rep 2017; 37. pii: BSR20170019.

29) Liang Y, Song X, Li Y, Sang Y, Zhang N, Zhang H, Liu Y, Duan Y, Chen B, Guo R, Zhao W, Wang L, Yang Q. A novel long non-coding RNA-PRLB acts as a tumor promoter through regulating miR-4766-5p/SIRT1 axis in breast cancer. Cell Death Dis 2018; 9: 563.

30) Wu XS, Wang F, Li HF, Hu YP, Jiang L, Zhang F, Li ML, Wang XA, Jin YP, Zhang YJ, Lu W, Wu WG, Shu YJ, Weng H, Cao Y, Bao RF, Liang HB, Wang Z, Zhang YC, Gong W, Zheng L, Sun SH, Liu YB. LncRNA-PAGBC acts as a microRNA sponge and promotes gallbladder tumorigenesis. EMBO Rep 2017; 18: 1837-1853.

31) Gou Q, Gao L, Nie X, Pu W, Zhu J, Wang Y, Liu X, Tan S, Zhou JK, Gong Y, He J, Wu K, Xie Y, Zhao W, Dai L, Liu L, Xiang R, Wei YQ, Zhang L, Peng Y. Long noncoding RNA AB074169 inhibits cell proliferation via modulation of KHSRP-mediated p21 expression in papillary thyroid carcinoma. Cancer Res 2018; 78: 4163-4174.

32) Karginov FV, Conaco C, Xuan Z, Schmidt BH, Parker JS, Mandel G, Hannon GJ. A biochemical approach to identifying microRNA targets. Proc Natl Acad Sci USA 2007; 104: 19291-19296.

33) Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; 136: E359-E386.

34) Yan Q, Tian Y, Hao F. Downregulation of lncRNA UCA1 inhibits proliferation and invasion of cervical cancer cells through miR-206 expression. Oncol Res. 2018 Mar 9. doi: 10.3727/096504018X15185714083446. [Epub ahead of print].

35) Gao Y, Di W, Li H, Chen C, Li Z. LncRNA TUG1 promotes cell growth and epithelial-mesenchymal transition in human cervical cancer. Int J Clin Exp Patho 2016; 9: 10327-10333.

36) Lv XJ, Tang Q, Tu YQ, Yan DD, Wei QC. Long noncoding RNA PCAT6 regulates cell growth and metastasis via Wnt/beta-catenin pathway and is a prognosis marker in cervical cancer. Eur Rev Med Pharmacol 2019; 23: 1947-1956.

37) Wu X, Cao X, Chen F. LncRNA-HOTAIR activates tumor cell proliferation and migration by suppressing miR-326 in cervical cancer. Oncol Res. 2017 Aug 31. doi: 10.3727/096504017X15037515496840. [Epub ahead of print]

38) Liu Q, Guo X, Que S, Yang X, Fan H, Liu M, Li X, Tang H. LncRNA RSU1P2 contributes to tumorigenesis by acting as a ceRNA against let-7a in cervical cancer cells. Oncotarget 2017; 8: 43768-43781.

39) Dai SG, Guo LL, Xia X, Pan Y. Long non-coding RNA WT1-AS inhibits cell aggressiveness via miR-203a-5p/FOXN2 axis and is associated with prognosis in cervical cancer. Eur Rev Med Pharmacol 2019; 23: 486-495.

40) Hipskind RA, Rao VN, Mueller CGF, Reddy ESP, Nordheim A. Ets-related protein Elk-1 is homologous to the c-fos regulatory factor p62TCF. Nature 1991; 354: 531-534.

41) Choi BD, Jeong SJ, Wang G, Park JJ, Lim DS, Kim BH, Cho YI, Kim CS, Jeong MT. Secretory leukocyte protease inhibitor is associated with MMP-2 and MMP-9 to promote migration and invasion in SNU638 gastric cancer cells. Int J Mol Med 2011; 28: 527-534.

42) Kawahara T, Shareef HK, Aljarah AK, Ide H, Li Y, Kashiwagi E, Netto GJ, Zheng Y, Miyamoto H. ELK1 is up-regulated by androgen in bladder cancer cells and promotes tumor progression. Oncotarget 2015; 6: 29860-29876.