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LncRNA TDRG1 enhances tumorigenicity in endometrial carcinoma by binding and targeting VEGF-A protein

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ABSTRACT

Endometrial carcinoma is one of the most frequently diagnosed cancers in females. Long non-coding RNAs (lncRNAs) have been associated with cancer; its role in endometrial carcinoma is an emerging area of research. In this article, lncRNA *TDRG1* expression in human endometrial carcinoma tissues and normal endometrial tissues was quantified by qRT-PCR. LncRNA TDRG1 was overexpressed or knocked-down in neither HEC-1B nor Ishikawa endometrial carcinoma cells, respectively, to assess cellular phenotype and expression of related molecules. Our results showed that lncRNA TDRG1 was significantly overexpressed in endometrial carcinoma tissues. Overexpression of lncRNA TDRG1 promoted endometrial carcinoma cell viability, invasion and migratory ability, inhibited apoptosis, and upregulated VEGF-A, PI3K, Bcl-2, MMP2 and survivin; knockdown of lncRNA TDRG1 had the opposite effects. LncRNA TDRG1 overexpression increased tumorigenicity in vivo and was associated with the upregulation of VEGF-A. RNA binding protein immunoprecipitation (RIP) assays confirmed that lncRNA TDRG1 directly binds to VEGF-A protein. Furthermore, knockdown of *VEGFA* in lncRNA TDRG1-overexpressing endometrial carcinoma cells reversed the effects of lncRNA TDRG1 on cell proliferation, invasion, migration and apoptosis. In conclusion, lncRNA TDRG1 may promote endometrial carcinoma cell proliferation and invasion by positively targeting VEGF-A and modulating relative genes.

1. Introduction

Endometrial carcinoma is the most common malignancy of the female reproductive tract. The incidence and mortality rates of endometrial cancer have increased gradually in recent years, with > 60,000 new cases and 10,000 deaths in the United States in 2016 [1, 2].

Long noncoding RNAs (lncRNAs) are a set of transcripts longer than 200 bp [3] that play important roles in regulation of the cell cycle, cell differentiation and other crucial functions [4, 5] via a number of mechanisms. LncRNAs may encode the upstream promoter region of genes to interfere with the expression of downstream genes; combine with the transcription of protein coding genes to form complementary double-stranded DNA complex, interfere with mRNA cleavage or lead to alternative splicing; bind to specific proteins to regulate their activity; or function as precursor molecules for small RNAs such as microRNAs (miRNA) or piwi-interacting RNAs (piRNAs) [6].

LncRNAs were previously considered to be genetically transcribed "noise" as a byproduct of Pol II transcription of specific genes. The newly identified human testis-specific gene testis developmental related gene 1 (*TDRG1*) encodes a 100 amino acid protein that does not possess any known protein domains [7]. TDRG1 may be involved in the development of testicular germ cell tumors by modulating the PI3K/Akt/mTOR signaling pathway [8]. Recent studies have shown a TDRG1 lncRNA exists, and may enhance the proliferation of bone marrow mesenchymal stem cells via a mechanism involving fibroblast growth factor 1 (FGF1) [9], our previous work also showed that lncRNA TDRG1 may contributes to epithelial ovarian carcinoma tumorigenesis and progression [11]. However, the function of lncRNA TDRG1 in endometrial cancer has not been investigated.

VEGF is a major growth and survival factor for vascular endothelial cells. There are six secretory isoforms in the VEGF family: VEGF-A, -B, -C, -D, -E and placental growth factor [13]. VEGF-A functions as a potent stimulator of angiogenesis during development, and carcinogenesis, and is overexpressed in many cancers including endometrial carcinoma [14]. Through bioinformatical prediction, we found that there were a series of microRNAs (miR-17, 20a, 20b, 93, 106a, -106b, 186, 299-3p, 519d), which have binding sites with both lncRNA TDRG1 and

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VEGF-A. Therefore, we put forward that there might be an interaction between lncRNA TDRG1 and VEGF-A.

In this study, we quantified the expression of lncRNA TDRG1 in human endometrial carcinoma tissues and normal endometrial tissues by qRT-PCR. Furthermore, lncRNA TDRG1 was stably overexpressed through TDRG1 plasmid transfection or knocked-down by transient transfection with TDRG1 siRNA in endometrial carcinoma cell lines to examine the function of lncRNA TDRG1 in endometrial carcinoma tumorigenesis and progression, and its relationship with VEGF-A.

2. Materials and methods

2.1. Tissue specimens

A total of 69 endometrial carcinoma and 16 normal endometrial specimens were collected from patients undergoing surgical resection at the First Affiliated Hospital of China Medical University (Shenyang, Liaoning, China). No patients received preoperative chemotherapy or radiotherapy. All specimens were confirmed by two pathologists. This research program (No. 2016-32-2) was approved by the Chinese Medical University Ethics Committee. All tissue samples were processed in accordance with ethical and legal standards.

2.2. Cell culture and transfection

Human HEC-1B and Ishikawa endometrial cancer cell lines were cultured in Dulbecco's modified Eagle's medium or RPMI-1640 (HyClone, Logan, UT, USA) supplemented with penicillin/streptomycin (100 U/mL) and 10% fetal bovine serum (FBS; HyClone). Ishikawa cells were purchased from Nanjing Keygen Biotech (Nanjing, China) and HEC-1B cells from the China Center for Type Culture Collection (CCTCC; Wuhan, China). Cells were incubated in a 5% CO_2 incubator at 37 °C and passaged routinely. LncRNA TDRG1 plasmid (details could be found in supplementary material) and siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The target sequences of the *VEGFA* siRNA were 5′-GAAGUUCAUGGAUGUCUAUdTdT-3′ (sense) and 5′-AUAGACAUCCAUGAACUUCdTdT-3′ (anti-sense), and for *Lnc-TDRG1* siRNA were 5′-CCUUCCCAGGUCUAGGUUCdTdT-3′ (sense); 5′-GAACCUAGACCUGGGAAGGdTdT-3′ (antisense).

2.3. Cell viability assays

Cells were trypsinized, seeded at 3000 cells/well into 96-well plates, allowed to adhere, cultured for 0, 24, 48 or 72 h, then 20 μL of MTT (5 mg/mL) was added, the cells were incubated at 37 °C for 2–4 h, the medium was discarded and 150 μL of dimethyl sulfoxide (DMSO) was added under dark conditions. OD values were measured at 490 nm using a spectrophotometer (BioTek Instruments, Winooski, VT, USA). Each experiment was performed in triplicate.

2.4. Apoptosis assays

For TDRG1 siRNA transfection, flow cytometry was performed after PI and FITC-labeled annexin V (BD Biosciences, New Jersey, USA) staining according to the manufacturer's protocol, while for lncRNA TDRG1 plasmid transfection, apoptosis was quantified using flow cytometry after staining with 7AAD and PE-labeled annexin V (BD Biosciences, New Jersey, USA). Briefly, after 48-h incubation, the cells were washed twice with ice-cold PBS, resuspended in 100 μL 1× binding buffer at 1×10^6 cells per mL, and incubated with 5 μL FITC–annexin V and PI or PE–annexin V and 7-AAD. The samples were gently vortexed and incubated for 15 min at room temperature in the dark, and then 400 μL 1× binding buffer was added to each tube, and the cells were subjected to flow cytometry analysis within 1 h.

2.5. Wound healing assay

Cells were seeded into 6-well plates at 10^6/cells per well, allowed to adhere, scratches were created in the monolayers using 200 μL pipette tips, then the cells were washed three times with PBS and cultured in normal culture medium lacking FBS and containing 20 μg/mL mitomycin C. Photomicrographs of the scratch wounds were captured in the same positions at 0, 24 and 48 h after transfection. The rate of cell migration was measured using Image J software.

2.6. Cell invasion assays

Matrigel Transwell Cell Culture chambers (BD Biosciences, San Jose, CA, USA) were used for the cell invasion assays. Matrix (30 μL; 1:15 dilution; BD Biosciences, San Jose, CA, USA) was placed into the upper chamber of the chamber and incubated for 4 h at 37 °C. Then 5×10^4 cells suspended in 200 μL of FBS-free culture medium was added to the upper chambers; 600 μL of complete culture medium (contain 10% FBS) was added to the lower chambers. After incubation for 2 days at 37 °C, the cells were washed, fixed with 4% paraformaldehyde, and the cells in the lower chamber were stained with crystal violet, sealed with resin adhesive film, imaged using an Olympus fluorescence microscope (Tokyo, Japan) and the numbers of cells in five independent fields of view in each well were counted.

2.7. Real-time PCR

Total RNA isolated from tissue specimens or cells was reverse transcribed into cDNA using avian myeloblastosis virus transcriptase and random primers (Takara, Shiga, Japan) according to the manufacturer's instructions. Target genes were amplified by real-time quantitative PCR using SYBR Premix Ex Taq™ II kit (Takara). The fold changes of lncRNA TDRG1 expression was determined by comparing the threshold cycle (*Ct*) values of the target genes to that of 18S rRNA (18S) using the $2^{-\Delta\Delta Ct}$ method. The primer sequences for lncRNA TDRG1 were F: 5′-TCTTCCCTGGCTTGGC-3′; R: 5′-TGGGCTCTTTCGTGGC-3′, and for 18 s were F: 5′-ACGGACAGGATTGACAGATT-3′; R: 5′-GGCGTAGGGTAGGCACA-3′.

2.8. Western blotting

Cell protein samples were extracted, quantified, diluted with 5× loading buffer to the same concentration, denatured at 95 °C, then separated by electrophoresis on 10% or 12% sodium dodecyl sulfate (SDS) polyacrylamide gels. Proteins were transferred to Hybond membrane (Amersham, Munich, Germany), and the membranes were blocked with 5% skimmed milk at room temperature for 1–2 h, incubated with primary antibodies against VEGF-A, Bcl-2, PI3K, survivin antibodies (1:1000; Proteintech, Rosemont, IL, USA) overnight at 4 °C. The membranes were washed three times with Tris-buffered saline (TBST), incubated with the corresponding secondary antibodies (1:5000) for 2 h, washed three times with TBST, and the protein bands were visualized using enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-actin (1:3000, Proteintech) was used as the internal control.

2.9. RNA binding protein immunoprecipitation (RIP) assay

The RIP assay was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) following the manufacturer's protocol. Briefly, HEC-1B cells at 80–90% confluency were lysed in RIP lysis buffer, and 100 μL of cell extract was incubated with RIP buffer containing magnetic beads conjugated to human anti-VEGF-A antibody or negative control normal mouse IgG. The samples were incubated with proteinase K to digest proteins, then the immunoprecipitated RNA was isolated and subjected to qRT-PCR