ORIGINAL RESEARCH

Knockdown of lncRNA TDRG1 Inhibits Tumorigenesis in Endometrial Carcinoma Through the PI3K/AKT/mTOR Pathway

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Background and objective: Endometrial carcinoma (EC) is one of the most frequently diagnosed malignancies in females. Dysregulation of lncRNA TDRG1 has been widely documented in several cancers, including EC. However, the mechanism of this lncRNA involving in EC progression remains to be further elucidated.

Materials and methods: The enrichment levels of TDRG1 in EC tissues and cell lines were examined by RT-qPCR. Flow cytometry, cell counting kit-8 (CCK-8), transwell, and Western blot assays were conducted to assess whether TDRG1 knockdown could affect cell cycle arrest, proliferation, migration, invasion, and apoptosis of EC cells. The phosphorylation levels of mTOR, AKT and PI3K that associated with PI3K/Akt/mTOR pathway were determined by Western blot assay.

Results: TDRG1 expression was markedly upregulated in EC tissues and cell lines. Knockdown of TDRG1 significantly induced cell cycle arrest and apoptosis, inhibited cell proliferation, restrained the invasion and migration abilities in EC cells. Moreover, TDRG1 silencing decreased the protein levels of p-AKT, p-PI3K, and p-mTOR of EC cells.

Conclusion: Our data underlined the implication of TDRG1 in EC progression, proposing that targeting TDRG1 might be a potential therapeutic avenue in EC.

Keywords: endometrial carcinoma, TDRG1, PI3K/AKT/mTOR pathway

Introduction

Endometrial carcinoma (EC) is a common malignancy occurring in the female genital system with almost 63,230 new cases and 11,350 deaths in the United States just in 2018.[1] In recent years, this disease exhibits a trend in younger women attributed to the increased obesity and reduced physical activity.[2,3] Surgery is considered as the mainstay for EC treatment, with radiotherapy and chemotherapy having established places in the adjuvant treatment setting.[4] Nevertheless, these treatments fail to be effective for those patients with distant metastases and advanced TNM stage.[5] Over the past decades, clinicopathological, epidemiological and genetic studies have enhanced our understanding of EC. Previous studies have highlighted a series of genes, including PTEN, COX-2, survivin, and PIK3CA,[6–8] that are linked with the occurrence and development of EC.[9,10] However, the clinical application of these markers remains controversial. Thus, identification of novel therapeutic targets and further clarification of the potential molecular mechanisms involving in the emergence and progression of EC are of great significance for EC treatment.

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Recent data obtained from the ENCODE project revealed that a large proportion of the human genome could be transcribed into non-coding RNAs (ncRNAs).[11,12] LncRNAs are a group of ncRNA transcripts with a length greater than 200 nucleotides, have emerged as essential regulators in diverse cellular processes, including cell cycle, proliferation, differentiation, apoptosis, invasion, and migration.[13] Dysregulation of lncRNAs has been reported in a vast range of human cancers.[14] In addition, the involvement of some lncRNAs in EC tumorigenesis has also been confirmed. For instance, the elevation of lncRNA H19 actively contributes to tumorigenesis in EC via regulation of epithelial-mesenchymal transition (EMT).[15] LncRNA CCAT2 exerts a critical regulatory function in EC metastasis and cell growth.[16] LncRNA GAG5 and FER1L4 act as a tumor suppressor by enhancing the expression of PTEN in EC.[17,18]

Human testis developmental related gene 1 (TDRG1) is a novel human testis-specific lncRNA that is differentially expressed in testicular germ cell tumor tissue and normal testicular tissue.[19] Abnormal expression of TDRG1 may be associated with the malignant processes of diverse cancers, including seminoma,[20] epithelial ovarian carcinoma,[21] and EC.[22] However, the underlying mechanism of TDRG1 involving in EC progression remains to be further investigated.

In this study, elevated abundances of TDRG1 were observed in EC tissues and cell lines. Knockdown of TDRG1 by transient transfection with si-TDRG1 induced cell cycle arrest and apoptosis, while hampered cell proliferation, migration, and invasion. Furthermore, we also confirmed that lncRNA TDRG1 functioned as an activator of the PI3K/Akt/mTOR signaling pathway.

Materials and Methods
Reagents
Human endometrial epithelial cell (hEEC) was purchased from the American Type Culture Collection (ATCC, Rockefeller, MD, USA). EC cell lines (HEC-1A and Ishikawa) were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and Cell Resource Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China), respectively. RPMI-1640 medium, McCoy's medium, minimum Eagle's medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA, USA). Penicillin-streptomycin, cell counting kit-8 (CCK-8), and BCA protein quantification kit were obtained from Solarbio (Beijing, China). Lipofectamine 2000, TRIzol® reagent, M-MLV Reverse Transcriptase Kit,

SYBR Green PCR Master Mix, PCR primers, siRNA targeting TDRG1 (si-TDRG1), siRNA targeting control (si-NC), TDRG1 expressing plasmid (TDRG1) and its negative control (vector) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Cell Cycle and Apoptosis Analysis Kit and Annexin V-FITC Apoptosis Detection Kit were bought from Beyotime (Shanghai, China). Polyvinylidene fluoride (PVDF) membrane was obtained from Millipore (Billerica, MA, USA). Specific antibodies against proliferating cell nuclear antigen (PCNA), MMP-9, MMP-2, Bcl-2, Bax, cleaved caspase 3, p-mTOR, mTOR, p-AKT, AKT, p-PI3K, PI3K, β-actin, and HRP-conjugated secondary antibody were obtained from Abcam (Cambridge, MA, USA). Enhanced chemiluminescence reagent (ECL) was purchased from GE Healthcare (Giles, UK). Matrigel was provided by BD Biosciences (San Jose, CA, USA).

Clinical Specimens
EC tissues (n=35) and normal tissues (n=15) were collected from patients who underwent surgical resection at the Affiliated Hospital of Weifang Medical University between Feb 2015 and Oct 2017, and were affirmed by histopathological diagnosis. No patient received preoperative treatment, including chemotherapy and radiotherapy. After surgery, these samples were immediately frozen and stored at −80°C until further use. This study was performed with the approval of the Research Ethics Committee of the Affiliated Hospital of Weifang Medical University, and written informed consent was obtained from all participants.

Cell Culture and Transfection
hEEC cells were cultured in RPMI-1640 medium, HEC-1A cells were cultured in McCoy's medium, and Ishikawa cells were cultured in MEM. All the medium contained 10% FBS and 1% penicillin-streptomycin, and all cells were cultured at 37°C with 5% CO_2 incubator. The medium was changed every 48 h until cells reached the confluence of 80–85%. Then, Lipofectamine 2000 was used to transfected si-TDRG1, TDRG1, or their negative controls into cells.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA from EC tissues and cell lines were extracted using TRIzol® reagent. Then, M-MLV Reverse Transcriptase Kit was applied for RNA reverse transcription on the basis of the manufacturer's instructions. For TDRG1 expression, qPCR was conducted using SYBR Green PCR Master Mix on Applied Biosystems 7500 real-time PCR system. Primer sequences for TDRG1 were 5′-GAAGAGGAGGGAGGC

AGTCT-3′ (forward) and 5′-GGGAACCTAGACCTGGGA
AG-3′ (reverse); GAPDH were 5′-GGAGCGAGATCCCTC
CAAAAT-3′ (forward) and 5′-GGCTGTTGTCATACTTCT
CATGG-3′ (reverse). The average value from three indepen-
dent experiments was used to calculate the relative expres-
sion of TDRG1 using $2^{-\Delta\Delta Ct}$ method, and GAPDH was used
as an internal control.

Cell Cycle Analysis
Cell cycle assay was performed by PI single staining method
using Cell Cycle and Apoptosis Analysis Kit. Briefly, after
transfecting with si-TDRG1 or si-NC for 48 h, Ishikawa and
HEC-1A cells were washed with PBS, centrifuged at
1000 rpm for 5 min, and fixed with 75% cold ethanol at 4°C
overnight. Afterward, cells were re-suspended with cold PBS
and stained with cell cycle staining solution containing PI and
RNase A at 37°C for 30 min. The percentage of cells in each
cell cycle phase was analyzed on a flow cytometer using Cell
Quest Pro software.

Cell Proliferation Analysis
Cell viability was determined using CCK-8 according to
the manufacturer's instructions. Generally, Ishikawa and
HEC-1A cells transfected si-NC or si-TDRG1 were seeded
into 96-well plates at a density of 3000 cells/well. At
different points after transfection (0 h, 24 h, 48 h, and
72 h), 10 µL CCK-8 solution was added into each well for
2 h at 37°C, and the absorbance at 450 nm was measured
by a microplate reader.

Western Blot
Total proteins were extracted using RIPA buffer and quantified
using the BCA protein quantification kit. Then, an equal
amount of proteins were denatured at 98°C for 5 min, separated
by SDS-PAGE and transferred onto PVDF membranes. After
blocked with skim milk for 2 h, the membranes were incubated
with specific antibodies overnight at 4°C, with β-actin as an
internal reference. Then, the membranes were washed with
TBST buffer and exposed to horseradish peroxidase (HRP)-
conjugated secondary antibody for 2 h. Protein bands were
detected using ECL reagent and quantified by Image
J software.

Migration and Invasion Assays
Cell migration and invasion assays were implemented
using transwell chambers with 8.0 µm pore-size polycar-
bonate membranes. For migration assay, 1×10^5 cells sus-
pended in serum-free medium were loaded onto the upper

chambers, and the lower chambers were filled with 500
µL complete medium containing 10% FBS. After cul-
tured for 16 h, non-migratory cells on the top surface of
the membranes were removed using cotton swabs. The
cells of the lower chambers surface were fixed with 4%
paraformaldehyde and stained with 0.1% crystal violets.
Next, the number of migrated cells was recorded for
randomly selected five fields of view. For invasion
assay, the upper chambers were pre-coated with 100 µL
of Matrigel, and cell invasion assay was performed as
described above.

Cell Apoptosis Analysis
Annexin V-FITC/PI double-staining method was applied
for cell apoptosis analysis using Annexin V-FITC
Apoptosis Detection Kit. In brief, collected Ishikawa and
HEC-1A cells were washed with PBS and re-suspended
with binding buffer. After double-stained with Annexin
V and PI avoid of light for 15 min, cell apoptosis in each
well was analyzed by a flow cytometer.

Statistical Analyses
SPSS 20.0 software was applied for all statistical analyses.
The data from more than 3 independent experiments were
displayed as the mean ± standard deviation (SD). Difference
analysis between two or more groups was carried out using
Student's t-test or one-way ANOVA test. $P < 0.05$ was
considered to be statistically significant.

Results
TDRG1 Was Upregulated in EC Tissues and Cell Lines
The expression pattern of TDRG1 in EC tissues and cell
lines was determined by RT-qPCR. As shown in
Figure 1A, TDRG1 was highly expressed in EC tissues
(35 cases) compared to normal tissues (15 cases).
Likewise, the expression of TDRG1 was markedly ele-
vated in EC cell lines (HEC-1A and Ishikawa) compared
with that in normal hEEC cells (Figure 1B). Furthermore,
the relationship between TDRG1 and the clinicopatholo-
gical parameters of EC patients showed that high TDRG1
expression was positively correlated with the tumor grade
and stage of EC patients ($P < 0.05$, Table 1). These find-
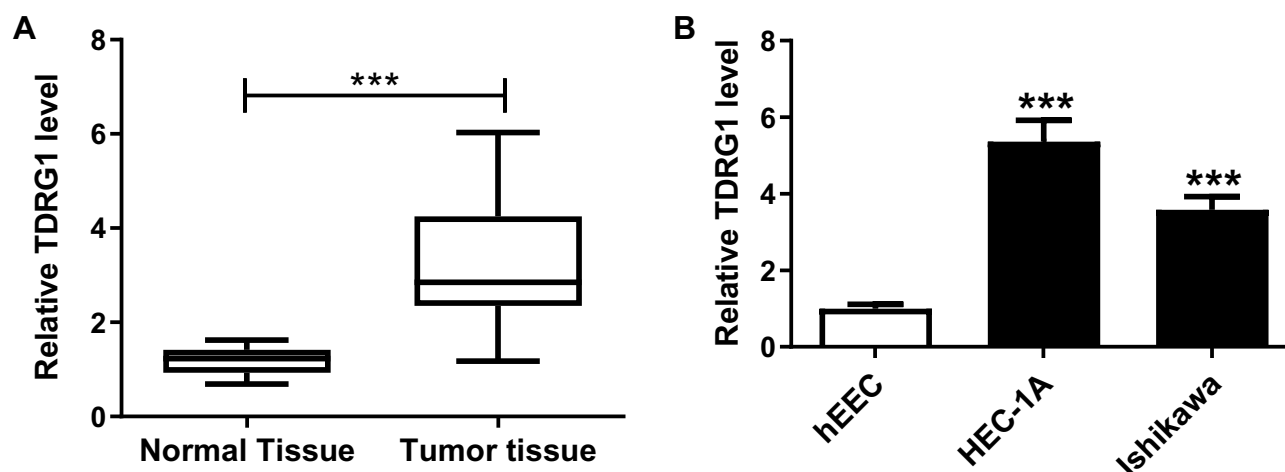ings provided evidence that TDRG1 might play a central
role in EC.

Figure 1 TDRG1 expression was upregulated in EC tissues and cell lines. (**A**) The expression levels of TDRG1 in EC tissues (n=35) and normal tissues (n=15) were measured by RT-qPCR assay. (**B**) TDRG1 expression in EC cell lines (HEC-1A and Ishikawa) and normal hEEC cells was examined by RT-qPCR assay. ***$P< 0.001$.

Knockdown of TDRG1 Induced Cell Cycle Arrest and Hindered Proliferation in EC Cells

To investigate the biological significance of TDRG1 in EC, loss-of-function assay was performed via knockdown of TDRG1 in Ishikawa and HEC-1A cells. The results of RT-qPCR assay showed that transfection of si-TDRG1 resulted in a significant reduction of TDRG1 expression in Ishikawa and HEC-1A cells compared to control (Figure 2A), indicating that si-TDRG1 could be used for the subsequent loss-of-function study. Flow cytometry for cell cycle assay demonstrated that TDRG1 downregulation

triggered cell cycle arrest at G0/G1 in Ishikawa and HEC-1A cells (Figure 2B). CCK-8 assay revealed that si-TDRG1 significantly inhibited cell viability compared to the si-NC group (Figure 2C). In addition, Western blot assay results indicated that the protein level of PCNA was obviously reduced in Ishikawa and HEC-1A cells after si-TDRG1 transfection (Figure 2D).

Knockdown of TDRG1 Restrained Cell Migration and Invasion in EC Cells

Transwell assay revealed that the silencing of TDRG1 significantly impeded the migration and invasion abilities of Ishikawa and HEC-1A cells (Figure 3A and B). MMP-2 and MMP-9, known as key enzymes in the degradation of type IV collagen, are closely correlated with tumor invasion and metastatic potential.[23,24] Western blot assay results displayed that the protein levels of MMP-9 and MMP-2 were markedly increased by the knockdown of TDRG1 in Ishikawa and HEC-1A cells (Figure 3C), which further demonstrated that TDRG1 depletion relieved the aggressive phenotype of EC cells.

The Stimulatory Effect of TDRG1 Knockdown on EC Cell Apoptosis

Flow cytometry for apoptosis assay showed that the apoptotic rate of Ishikawa and HEC-1A cells in the si-TDRG1 group was higher than that in the si-NC group (Figure 4A). Next, we also performed Western blot assay to detect the protein levels of anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax, and cleaved caspase-3 in EC cells. Results showed that knockdown of TDRG1 suppressed Bcl-2

Table 1 The Relationship Between TDRG1 Level and the Clinicopathological Parameters of 35 EC Patients

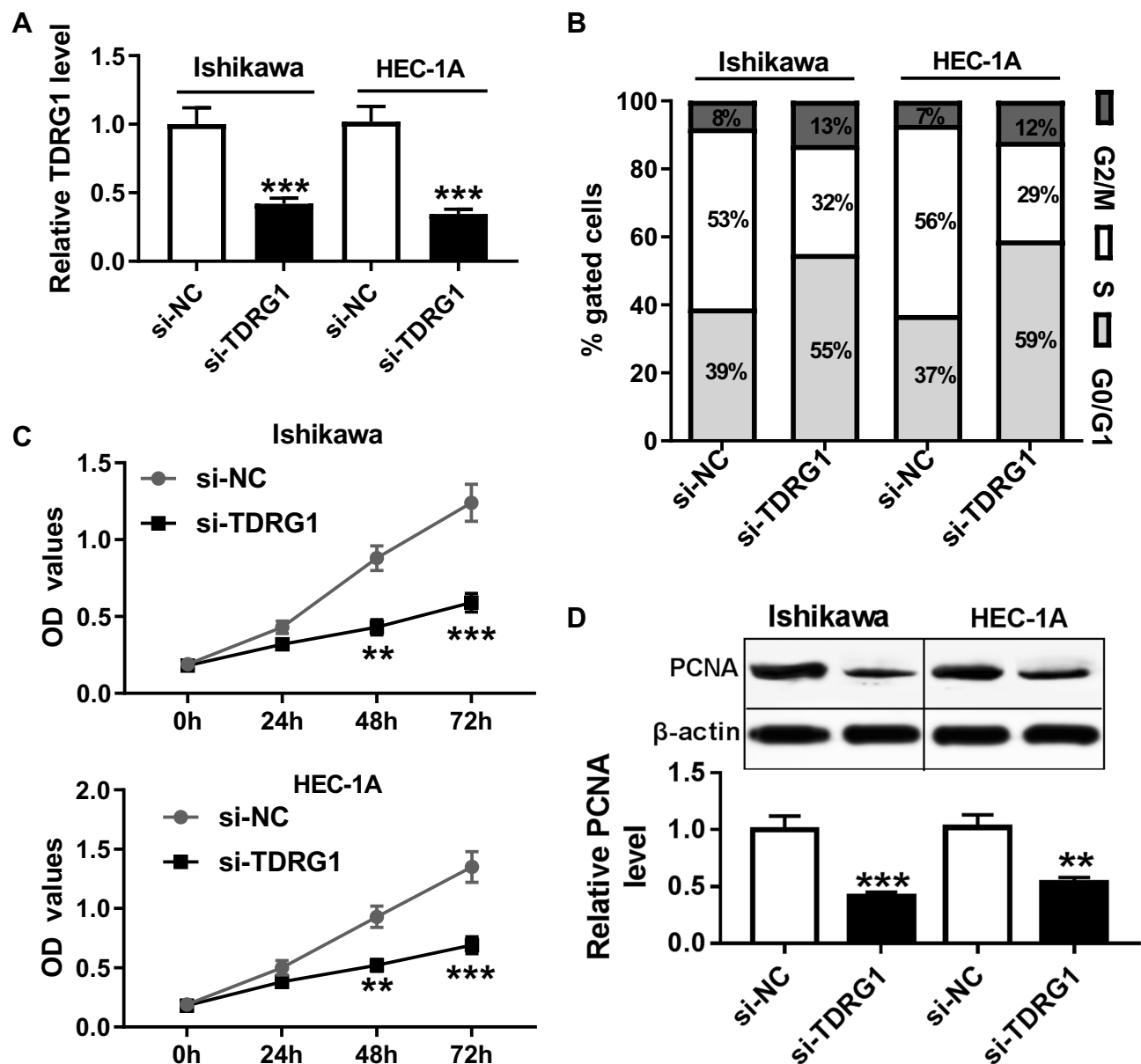| Clinicopathologic Parameters	Relative TDRG1 Level		P Value
	High (%)	**Low (%)**	
Age(years)			0.6405
≥55	11 (57.9)	8 (42.1)	
<55	8 (50.0)	8 (50.0)	
Grade			0.0082*
G1+G2	7 (35.0)	13 (65.0)	
G3	12 (80.0)	3 (20.0)	
Stage			0.0127*
I - II	9 (39.1)	14 (60.9)	
III-IV	10 (83.3)	2 (16.7)	
Lymph node metastasis			0.387
No	13 (50.0)	13 (50.0)	
Yes	6 (66.7)	3 (33.3)	

Note: *$P<0.05$.

Figure 2 TDRG1 silencing induced cell cycle arrest and inhibited proliferation in EC cells. Ishikawa and HEC-1A cells were transfected with si-NC or si-TDRG1. At 48 h thereafter, TDRG1 expression was detected by RT-qPCR (**A**), cell cycles were evaluated by flow cytometry using PI single staining method (**B**), cell proliferative activity was determined by CCK-8 assay (**C**), and the protein level of PCNA protein was measured by Western blot assay (**D**). $**P < 0.01$, $***P < 0.001$.

expression, while induced Bax and cleaved caspase 3 expressions compared with the control group (Figure 4B). These results indicated that TDRG1 silencing contributed to EC cell apoptosis.

TDRG1 Activated PI3K/Akt/mTOR Signaling Pathway in EC Cells

To investigate whether TDRG1 was involved in the progression of EC cells via regulating PI3K/AKT/mTOR signaling pathway, the levels of relevant proteins associated with this pathway were examined by Western blot assay. As shown in Figure 5A and B, TDRG1 overexpression increased the protein levels of p-mTOR, p-AKT and p-PI3K in Ishikawa and HEC-1A cells, while TDRG1 silencing decreased their levels, indicating that knockdown of TDRG1 exerted its antitumor property possibility via the blockade of PI3K/AKT/mTOR signaling pathway.

Discussion

LncRNAs have emerged as critical regulators in cellular behaviors and many of them are identified to be implicated in the formation and progression of human cancers. Researchers
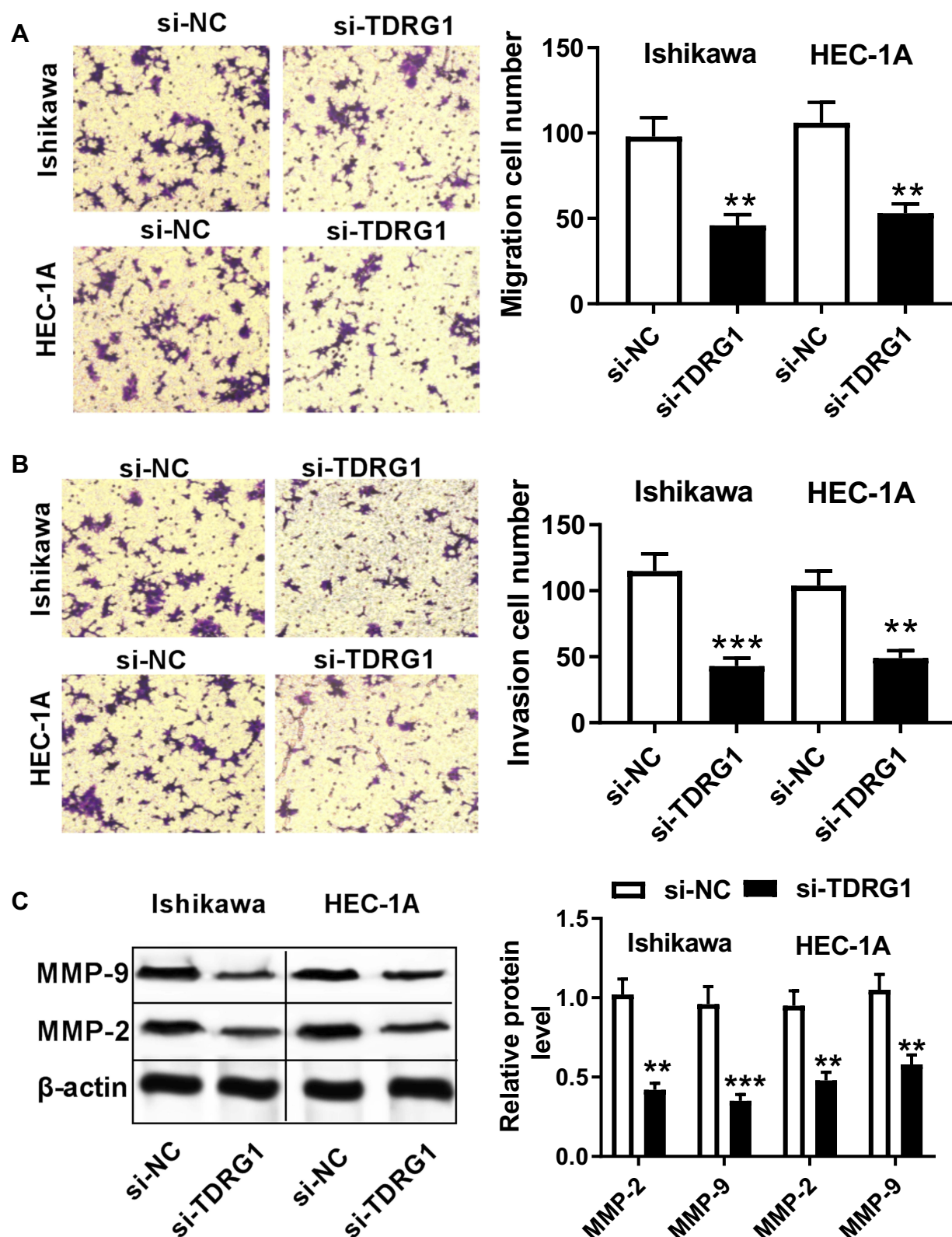
Figure 3 Knockdown of TDRG1 prevented migration and invasion in EC cells. Ishikawa and HEC-1A cells were transfected with si-NC or si-TDRG1 for 48 h. Cell migration and invasion abilities were assessed by Transwell assay (**A** and **B**), and the protein levels of MMP-9 and MMP-2 were measured by Western blot assay (**C**). **P< 0.01, ***P< 0.001.

have found that ectopic expressions of lncRNAs, such as H19, MALAT1, and HOTAIR, are pervasively overexpressed in

most solid cancers and correlate with tumorigenesis and development.[25–27] The low-expressed pattern of lncRNA
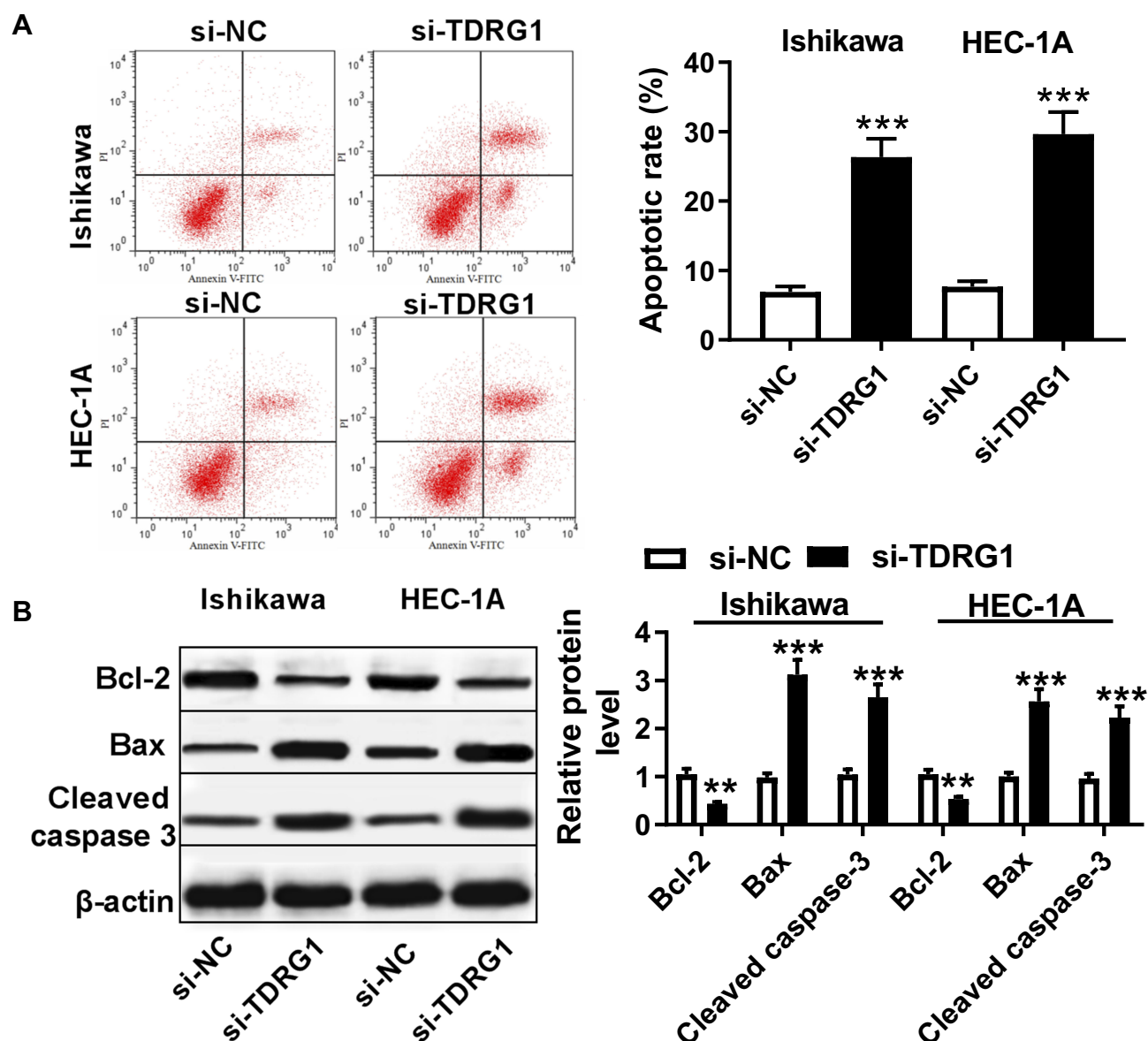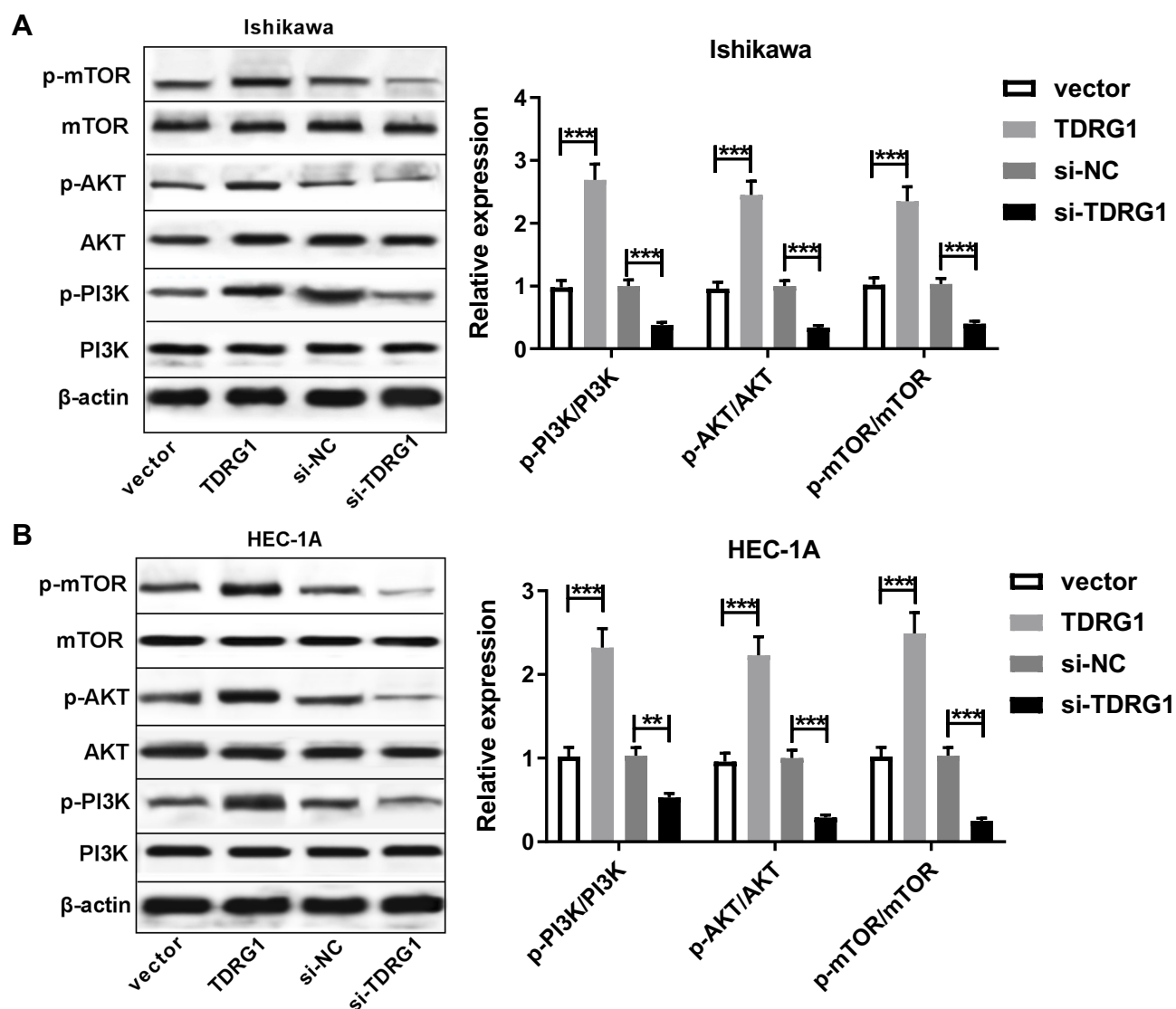
Figure 4 Knockdown of TDRG1 induced EC cell apoptosis. Ishikawa and HEC-1A cells were transfected with si-NC or si-TDRG1. Then, cell apoptotic rate was detected by flow cytometry using Annexin V-FITC/PI double-staining method (**A**), protein levels of Bcl-2, Bax and cleaved caspase 3 were examined by Western blot assay (**B**). **$P < 0.01$, ***$P < 0.001$.

GAS5 heightens the proliferative capacity of tumor cells and signifies a poorer prognosis.[28] Cell apoptosis usually involves the activation, expression and regulation of a series of genes, including the decrease of Bcl-2 expression and the increase of Bax and caspase-3 expression.[29,30] Lnc-SOX6-1,[31] HOST2[32] and lnc-Sox5[33] have been shown to inhibit apoptosis of cells in many diseases and thus promote disease progression, including pediatric acute myeloid leukemia, osteosarcoma and tongue carcinogenesis. Besides, some lncRNAs are reported to be greatly implicated in the tumorigenesis of EC by serving as tumor suppressors or oncogenes, such as FER1L4,[18] BANCR,[34] and TUG1.[35] However, the clinical application of

these biomarkers remains controversial. Thereby, identifying a potential biomarker that associated with the carcinogenesis has vital practical significance for EC diagnosis and treatment.

As a newly characterized human testis-specific gene, lncRNA TDRG1 has the potential to encode a 100-amino acid protein that lacks characterized protein domains.[36] Early study indicates that TDRG1 contributes to the occurrence and development of testicular seminoma.[20] Additionally, elevated expression of TDRG1 is identified as an oncogene in epithelial ovarian carcinoma and positively correlates with the poor prognosis and malignant progression.[21] The present study revealed that TDRG1 expression was markedly higher in EC

Figure 5 TDRG1 regulated the activity of PI3K/AKT/mTOR signaling pathway. (**A** and **B**) Ishikawa and HEC-1A cells were transfected with TDRG1 expression plasmid, si-TDRG1, or relative control for 48 h, followed by the detection of phosphorylated and un-phosphorylated mTOR, AKT, PI3K using Western blot assay. **P< 0.01; ***P< 0.001.

tissues and cell lines than that in normal tissues and hEEC cells, indicating the possible involvement of this lncRNA in EC progression. Next, TDRG1 was knocked down by the transfection of si-TDRG1 into EC cells, followed by a series of functional researches by flow cytometry, CCK-8, transwell, and Western blot analyses. We observed that knockdown of TDRG1 increased the distribution of G0/G1, and impeded cell proliferation in Ishikawa and HEC-1A. Furthermore, the migration and invasion abilities were suppressed and apoptosis was enhanced in EC cells after TDRG1 knockdown. Our findings were fully consistent with the previous study reported by Chen et al, who highlighted the promotive effects of TDRG1 on EC cell tumorigenicity and tumor growth.[22]

PI3K/AKT/mTOR signaling pathway regulates several normal cellular processes, including cell survival, proliferation, growth, and death.[37] PI3K is a heterodimer that is composed of an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110).[38] Activated PI3K has the ability to recruit AKT to the plasma membrane, leading to the phosphorylation of various proteins, including mTOR.[39] mTOR functions as a catalytic subunit of mTORC1 complex, the activation of which affects cell proliferation and survival by repressing autophagy.[40] Until now, a large amount of evidence has confirmed the critical role of PI3K/AKT/mTOR signaling pathway in various tumorigenesis.[41] Aberrant expression or mutation of many components in this pathway are associated

with the malignant processes of several types of human cancers, such as cervical, ovarian cancer, colorectal cancer, and breast cancer.[42,43] Also, activated PI3K/AKT/mTOR signaling is associated with the aggressive progression and poor prognosis in EC.[44] Thus, identifying a novel biomarker that connected with PI3K/AKT/mTOR pathway is of great significance for cancer therapy.

Early research by Li et al showed that abnormally elevated lncRNA UCA1 played a pivotal role in the tumorigenesis of gastric cancer through the PI3K/AKT/mTOR pathway.[45] Yang et al stated that lncRNA SNHG7 was associated with the poor prognosis and triggered colorectal cancer progression via activating PI3K/AKT/mTOR pathway.[46] In our study, we observed that the protein levels of p-mTOR, p-AKT and p-PI3K were decreased in EC cells after TDRG1 knockdown. Thus, we proposed the conclusion that TDRG1 contributed to the progression of EC by the activation of PI3K/AKT/mTOR signaling pathway.

Lymph node involvement is one of the most important adverse prognostic factors of EC, which seriously affects the survival rate of patients. Preoperative risk assessment of lymph node metastasis can effectively predict the incidence of lymph node metastasis after surgery in order to develop the best treatment for patients.[47,48] Nevertheless, once lymph node metastasis occurs in postoperative patients, the mortality rate will be very high. Therefore, the proposal of EC biomarkers can also provide certain clinical diagnostic value for the prognosis of EC patients.

As a regulator of cancer progression, the role of many lncRNAs has been confirmed. This study explored the role of TDRG1 in EC tumorigenesis, confirmed its existence as a cancer-promoting factor of EC, emphasized the important role of TDRG1 in EC progression, and added a new reference for the study of TDRG1. However, there are some deficiencies in this study. We only explore the function of TDRG1 in EC, but its mechanism is not well understood. In addition, the experimental results in this study have only been confirmed at the cellular level, and there may be some limitations. Therefore, further experiments may be around to explore the mechanism of TDRG1 in EC and further validation of our experimental results in vivo.

In conclusion, our data indicated that knockdown of TDRG1 restrained EC development via regulating the PI3K/AKT/mTOR signaling pathway. These findings contributed to a better understanding on the regulatory mechanism of TDRG1 in EC progression, which might provide a novel therapeutic avenue for EC treatment.

Disclosure

The authors report no conflicts of interest in this work.

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