

## Original Research Article

# LncRNA TUG1 facilitates the development of endometrial cancer via interaction with FXR1

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### Abstract

**Purpose:** To investigate the potential influence of long non-coding RNA (lncRNA) TUG1 on the development of endometrial cancer (EC).

**Methods:** A total of 24 paired EC species and paracancerous species were collected, and the differential expressions of TUG1 in them were determined. The regulatory effects of TUG1 on proliferative and migratory potential in Ishikawa and HEC-1A cells were assessed using cell counting kit-8 (CCK-8) and Transwell assay, respectively. Potential protein binding TUG1 was predicted by bioinformatics analysis and subsequently verified using RIP (RNA-Binding Protein Immunoprecipitation) assay. Rescue experiments were conducted to uncover the mechanism of TUG1 in regulating the development of EC.

**Results:** TUG1 was highly expressed in EC species and cell lines. Higher levels of TUG1 was observed in EC patients with metastases than those without metastatic cancer ( $p < 0.05$ ). Overexpression of TUG1 markedly facilitated proliferative and migratory potential in EC cells. Taurine-upregulated gene 1 (TUG1) directly bound Fragile X-related protein 1 (FXR1) and positively regulated its level ( $p < 0.05$ ). Through interaction with FXR1, TUG1 stimulated the malignant development of EC.

**Conclusion:** LncRNA TUG1 is upregulated in EC species, which facilitates proliferative and migratory potentials in EC cells by interacting with FXR1.

**Keywords:** Endometrial cancer, Taurine-upregulated gene 1, Fragile X-related protein 1, Proliferative and migratory potential

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## INTRODUCTION

Endometrial cancer (EC) is a malignant tumor that is frequently found in the female reproductive system. Its incidence is second only to cervical cancer. It is reported that approximately 320,000 women are diagnosed with EC, and 76,000 die of EC annually [1]. In

developed countries, the incidence of EC ranks first place in reproductive tract malignancies. The incidence of EC amongst the younger people is on the increase in developing countries [2]. Currently, most EC patients are diagnosed in the early stage through screening and symptoms of abnormal vaginal bleeding. Nevertheless, therapeutic efficacy for advanced, recurrent and metastatic EC is far from satisfactory. It is

therefore essential to clarify the molecular mechanism of EC, thus seeking for effective hallmarks and therapeutic targets.

With rapid development in-molecular biological research and genetic diagnosis, long non-coding RNAs (lncRNAs) have been identified to be involved in tumor development. lncRNAs are non-coding RNAs with 200 bp to 10 kb long [3]. They were initially considered as meaningless transcriptional by-products [4]. lncRNAs were later found to regulate life activities through diverse mechanisms *via* interaction with DNAs, RNAs and proteins [5,6]. Seeking abnormally expressed lncRNAs in EC species may contribute to the development of precise treatment, thus improving prognosis in EC patients. lncRNA TUG1 was initially found to be upregulated in taurine-treated retinal cells [7]. More and more evidence has shown that TUG1 is abnormally expressed in many types of tumors. Overexpression of TUG1 stimulates the proliferative and invasive capacities of esophageal squamous cell carcinoma [8]. In human glioma, TUG1 induces tumor cell apoptosis as an anti-tumor factor [9]. Highly expressed TUG1 presents a poor prognosis in gastric cancer [10]. This study is aimed at determining the role of TUG1 in EC, and the underlying mechanism.

## METHODS

### Sample collection

Endometrial cancer tissues and paired paracancerous tissues were collected from 24 EC patients. None of the included patients had preoperative anti-tumor treatments. The study followed the guidelines of Declaration of Helsinki. Clinical data and postoperative pathology were completely recorded. This study got approval from the Ethics Committee of The 3rd Affiliated hospital of Guangdong pharmaceutical University (approval no. 08-CN-GD-30521) and conducted after informed consent was obtained on each subject.

### Cell culture

Epithelial cell of uterus (EMC) and EC cell lines (HEC-1A, HEC-1B, Ishikawa and KLE) were purchased from Cell Bank (Shanghai, China).

The cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10 % fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 µg/mL streptomycin in a 5 % CO<sub>2</sub> incubator at 37 °C.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs in cells and tissues were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription of RNAs was performed using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan), and complementary deoxyribose nucleic acid (cDNA) was sent for qRT-PCR. Relative level of the target was calculated using 2<sup>-ΔΔCt</sup> method. Primer sequences are shown in Table 1.

### Cell transfection

Transfection plasmids were provided by Genechem, Co. Ltd (Shanghai, China). The cells were cultured to 60 – 70 % confluence, and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in a serum-free medium. Six hours later, the complete medium was replaced.

### Cell counting kit-8 (CCK-8) assay

The cells were inoculated into 96-well plates with 2 × 10<sup>3</sup> cells per well. At the appointed time points, 10 µL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added in each well. The absorbance of each sample at 450 nm per sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

### Transwell assay

Cell suspension (100 µL, 1×10<sup>5</sup> cells/mL) was inoculated on the upper side of the Transwell chamber (Millipore, Billerica, MA, USA), and inserted into a 24-well plate with 500 µL of medium containing 10% FBS at the bottom. After 48-h incubation, bottom cells were reacted with 15-min methanol and 20-min crystal violet, and captured using a microscope. Migratory cells were counted in 10 random fields per sample (magnification 200×).

**Table 1:** Primers used for qRT-PCR

Gene	Forward	Reverse
TUG1	5'-TAGCAGTTCCCAATCCTTG-3'	5'-CACAAATTCATCATTCCC-3'
FXR1	5'-GAGAGAAGATTTAATGGGCCTGG-3'	5'-GCTCAATGGCGGTAAGTCCA-3'
GAPDH	5'-ACAACCTTTGGTATCGTGAAGG-3'	5'-GCCATCACGCCACAGTTTC-3'

## Subcellular distribution

Cytoplasmic and nuclear RNAs were extracted using PARIS kit (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR. The internal references of nucleus and cytoplasm were set as U6 and GAPDH.

## RIP (RNA-Binding Protein Immunoprecipitation) assay

RIP assay was performed following the procedures of Millipore Magna RIP (RNA-Binding Protein Immunoprecipitation) Kit (Millipore, Billerica, MA, USA). The cells were incubated with anti-Ago2 or anti-IgG at 4°C overnight. A protein-RNA complex was obtained after capturing intracellular specific proteins, which were digested by proteinase K to extract RNAs. During the experiment, the magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. The immunoprecipitant RNAs were quantified using qRT-PCR.

## Western blot

The cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5 % skimmed milk for 2 hours.

## Statistical analysis

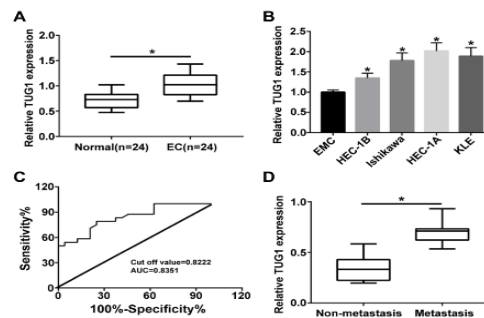
Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) was used for data analysis. Data are expressed as mean  $\pm$  standard deviation. Differences between the two groups were analyzed using the *t*-test. Receiver operating characteristic (ROC) curves were depicted for assessing the diagnostic potential of TUG1 in EC. The relationship between expression levels of TUG1 and FXR1 in EC tissues was analyzed using Pearson correlation test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### TUG1 was upregulated in EC species

Compared with paracancerous tissues, TUG1 was highly expressed in EC species (Figure 1 A). Similarly, it was also upregulated in EC cell lines (Figure 1 B). EC patients with tumor metastasis expressed higher abundance of TUG1 than the non-metastatic patients (Figure 1 C). ROC curves showed that TUG1 was capable of

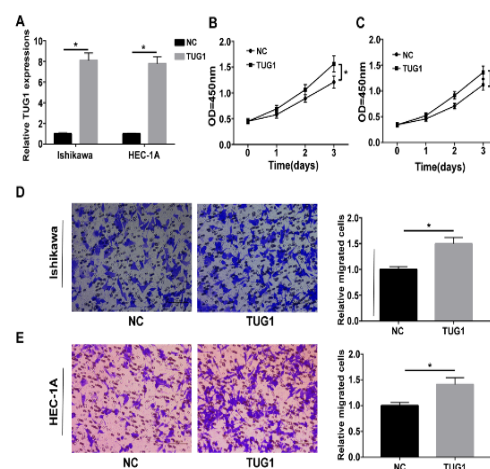
distinguishing EC from normal species (cut-off value = 0.8222, AUC = 0.8351) (Figure 1 D). It is suggested that TUG1 become a potential hallmark in EC.



**Figure 1:** TUG1 was upregulated in EC species and EC cell lines. (A) TUG1 expressions in EC tissues (n=24) and paracancerous tissues (n = 24). (B) TUG1 expressions in EC cell lines. (C) Diagnostic potential of TUG1 in EC. (D) TUG1 expressions in EC patients either with metastasis or not. \* $P < 0.05$

### TUG1 facilitated the proliferative and migratory potentials of EC cells

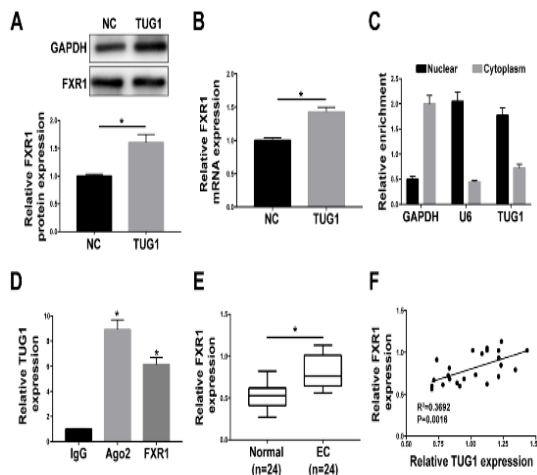
Among the four tested EC cells, Ishikawa and HEC-1A cells expressed the highest abundance of TUG1, and they were selected for the following experiments. Here, transfection of pcDNA-TUG1 markedly upregulated TUG1 in EC cells (Figure 2 A). Viability (Figure 2 B and C) and migratory cell number increased in Ishikawa (Figure 2 D) and HEC-1A cells (Figure 2 E) overexpressing TUG1.



**Figure 2:** TUG1 facilitated proliferative and migratory potential in EC cells. (A) TUG1 expression in Ishikawa and HEC-1A cells transfected with NC or pcDNA-TUG1. Viability of Ishikawa (B) and HEC-1A cells (C) transfected with NC or pcDNA-TUG1. Migration in Ishikawa (D) and HEC-1A cells (E) transfected with NC or pcDNA-TUG1. ( $\times 200$ ) \* $p < 0.05$

### TUG1 directly bound FXR1

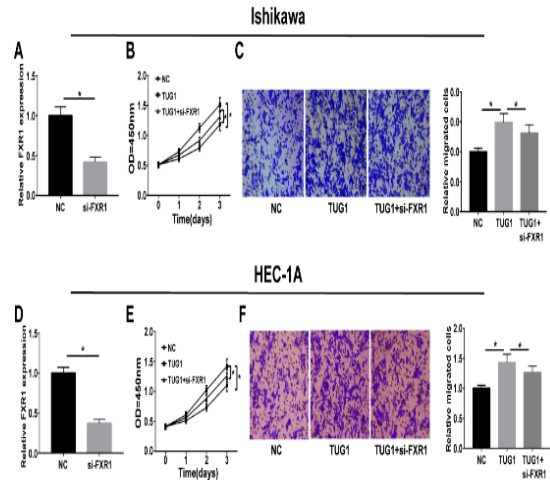
Bioinformatics analysis predicted that FXR1 may be the target binding TUG1. Both protein and mRNA levels of FXR1 were upregulated in HEC-1A cells overexpressing TUG1 (Figure 3 A and B). TUG1 was identified to be mainly enriched in cell nuclei (Figure 3C). Subsequently, RIP assay confirmed the interaction between TUG1 and FXR1 (Figure 3 D). Compared with paracancerous tissues, FXR1 was highly expressed in EC tissues (Figure 3 E) and its level was positively correlated with TUG1 expression (Figure 3 F).



**Figure 3:** TUG1 directly bound FXR1. (A, B) Protein (A) and mRNA level (B) of FXR1 in HEC-1A cells transfected with NC or pcDNA-TUG1. (C) Subcellular distribution of TUG1. GAPDH and U6 were the internal references of cytoplasm and nuclei, respectively ( $\times 200$ ) (D) Enrichment of TUG1 in anti-Ago2 or anti-FXR1. (E) FXR1 expressions in EC tissues ( $n = 24$ ) and paracancerous tissues ( $n = 24$ ). (F) A positive correlation between expression levels of TUG1 and FXR1 in EC tissues ( $\times 200$ );  $*p < 0.05$

### TUG1 regulated phenotypes of EC cells via interaction with FXR1

Next, we explored the involvement of FXR1 in the development of EC. Transfection efficacy of si-FXR1 was first verified in Ishikawa and HEC-1A cells (Figure 4 A and D). Interestingly, increased viability in EC cells overexpressing TUG1 was abolished by silencing FXR1 (Figure 4 B and E). Enhanced migratory potential after TUG1 overexpression in EC cells was also reversed by co-transfection of si-FXR1 (Figure 4 C and F).



**Figure 4:** TUG1 regulated phenotypes of EC cells via interaction with FXR1. (A) FXR1 expression in Ishikawa cells transfected with NC or si-FXR1. (B) Viability of Ishikawa cells transfected with NC, pcDNA-TUG1 or pcDNA-TUG1+si-FXR1. (C) Migration of Ishikawa cells transfected with NC, pcDNA-TUG1 or pcDNA-TUG1+si-FXR1. (D) FXR1 expression in HEC-1A cells transfected with NC or si-FXR1. (E) Viability in HEC-1A cells transfected with NC, pcDNA-TUG1 or pcDNA-TUG1+si-FXR1. (F) Migration in HEC-1A cells transfected with NC, pcDNA-TUG1 or pcDNA-TUG1+si-FXR1.  $*P < 0.05$

## DISCUSSION

It is generally accepted that the incidence of type I EC is related to long-term estrogen stimulation, while type II EC often occurs in postmenopausal women, and it is non-estrogen dependent [11]. The clinical significance of assessing estrogen and progesterone receptor levels in type II EC patients remains controversial [12]. It has been reported that mutation and loss of PTEN gene and microsatellite instability are caused by DN mismatch. The mutation of K-ras and upregulation of estrogen receptor subtype ER $\alpha$  are potential pathogenic factors of type I EC. Type II EC is featured by upregulated mutant mTOR or P53 in serous cancer [13]. The pathogenesis of metastasis and recurrence of EC remain largely unknown.

LncRNAs are capable of regulating gene expressions at epigenetic, transcriptional and post-transcriptional levels. They are involved in tumor development as well as in tumor diagnosis and treatment [14,15]. The findings of the present study showed that TUG1 was upregulated in EC patients, especially metastatic EC patients. In addition, ROC curves illustrated the diagnostic potential of TUG1 in EC. *In vitro* experiments demonstrated that TUG1 stimulated proliferative and migratory potential in EC cells.

LncRNAs participate in tumor development through diverse mechanisms [16]. They regulate gene expressions by interacting with histones, DNAs or chromatin-modified proteins, or specifically targeting DNA sequences (especially cis-regulation elements) [17,18]. Meanwhile, both activators and repressors can bind lncRNAs.

FXR1 locates the human chromosome 3q26-27, which belongs to fragile X-related transcription factor family [19]. It is reported that FXR1 is upregulated in colorectal cancer tissues and cell lines, which stimulates cancer cells to proliferate and migrate [20]. In oral squamous cell carcinoma, upregulated FXR1 alleviates cell senescence by stabilizing lncRNA TERC. Through bioinformatics prediction and RIP assay, the interaction between TUG1 and FXR1 was verified. Silencing of FXR1 was able to reverse the effects of TUG1 in regulating phenotypes of EC cells.

## CONCLUSION

LncRNA TUG1 is upregulated in EC species, which facilitates proliferative and migratory potentials in EC cells by interacting with FXR1. Taurine-upregulated gene 1 may be a new therapeutic target in EC. However, the findings require validation in *in vivo* experiments.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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