Long noncoding RNA TUG1 promotes renal cell carcinoma cell proliferation, migration and invasion by downregulating microRNA-196a

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Abstract. Long noncoding RNA taurine upregulated gene 1 (lncRNA TUG1) and microRNA-196a (miR-196a) have been reported to serve important roles in the development of renal cell carcinoma (RCC). However, their potential mechanisms have not been completely elucidated. The aim of the present study was to clarify the biological functions of lncRNA-TUG1 and miR-196a, in addition to investigating the interaction between lncRNA-TUG1 and microRNA-196a, providing a novel insight into RCC tumorigenesis. The present study comprised two parts. In the first part, lncRNA-TUG1 was confirmed as an oncogene, via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, MTT assay, flow cytometry analysis, and migration and invasion assays. In the second part, the association between lncRNA-TUG1 and miR-196a, and the molecular mechanism, was illustrated via RT-qPCR analysis, MTT assay, dual luciferase reporter assay and western blotting. The results of the present study demonstrated that lncRNA-TUG1 was able to promote RCC cell proliferation, migration and invasion in vitro by suppressing miR-196a. Additionally, lncRNA-TUG1 achieved its biological functions by regulating the expression levels of RAC-α serine/threonine-protein kinase, mitogen-activated protein kinase and extracellular signal-regulated kinase via inhibition of miR-196a. In conclusion, the present findings proposed a novel potential therapeutic target, the lncRNA-TUG1-miR-196a axis, which may be applicable to the treatment of RCC.

Introduction

Renal cell carcinoma (RCC) is the most common lethal urological carcinoma in adults, with an increasing global incidence rate (1). At present, surgery, chemotherapy and radiotherapy are the most frequently used therapies for RCC (2,3). Increasing studies have focused on minimizing damage while achieving total destruction of the tumor, to achieve decreased recurrence and a better prognosis for patients with RCC (4). Therefore, the present study was performed to examine novel therapeutic targets for RCC.

Long noncoding RNA (lncRNA) is a class of noncoding transcribed RNA molecules with >200 bases. Emerging evidence has demonstrated that lncRNAs appear to be associated with a number of malignancies, and act as tumor oncogenes or suppressors, which regulate gene expression through regulation of transcription, post-transcription, genomic imprinting and chromatin modification (5-7). MicroRNAs (miRs) are a class of small noncoding RNAs of 17-22 nucleotides in length (8). Previous studies have demonstrated that miRs regulate various cellular processes, and certain miRs are aberrantly expressed in cancer cells, acting as oncogenes or tumor suppressors by targeting mRNAs, and function as an RNA-induced silencing complex (9-12). Therefore, lncRNAs and miRs are potential diagnostic and prognostic biomarkers of cancer in clinical practice.

lncRNA taurine upregulated gene 1 (TUG1), an lncRNA located at chromosome 22q12, was first identified as a part of photoreceptors and during retinal development in mouse retinal cells (13). In further studies, TUG1 was considered to be involved in regulating carcinogenesis in a number of malignant tumors, and TUG1 has been reported to be a predictive biomarker of RCC, while the specific biological function and mechanism of lncRNA-TUG1 in RCC remains unclear (14-16). It has additionally been reported that miR-196a may be aberrantly expressed in numerous types of cancer, including RCC; certain studies reported that miR-196a expression is downregulated in RCC cells, although its function and mechanism remains to be elucidated (17-20). In the present study, the expression level of lncRNA-TUG1 and microRNA-196a was detected in RCC cells, and
the biological activity, and functional mechanisms, of lncRNA-TUG1 and microRNA-196a in regulating RCC cell proliferation, migration and invasion were investigated in vitro.

Materials and methods

Cell culture. Human renal cell carcinoma (RCC) cell lines 786-O, ACHN, A704, A498 and normal kidney cell line HK-2 were purchased from the Type Culture Collection of the Chinese Academy of Medical Sciences (Shanghai, China). All the cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 1% 100 µl/ml penicillin-streptomycin and 1% 100 U/ml glutamine (all Kailji, Nanjing, China) and grown in a humidified incubator containing 5% CO2 at 37°C.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses. Total RNA from cultured cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer’s protocol, and corresponding cDNA was transcribed from total RNA using primers and a Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer’s instructions. qPCR analysis was performed to detect relevant gene expression using a SYBR-Green PCR Master Mix kit (Takara Biotechnology Co., Ltd., Dalian, China) and the ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., USA), according to the manufacturer’s protocol. GAPDH was designated as the internal control. The primer sequences for GAPDH were forward, 5’-GGATGTGGCATATTGGGC-3’ and reverse, 5’-GGATTGTGGCATATTGGG-3’. The primer sequences for forward, lncRNA-TUG1 were 5’-TAGGAGTCTCCCATACTTCC-3’ and reverse, 5’-CACAAATTCACATGATTTGC-3’; and the primer sequences for miR-196a were forward, 5’-TGTTGCACTCACCTCA-3’ and reverse, 5’-GACTTCCCAACGCTTCC-3’. The PCR cycling conditions were as follows: 95°C for 5 min, followed by 45 cycles of 95°C for 10 sec and 60°C for 30 sec. The gene expression was normalized to GAPDH. All results were repeated three times and expressed as the mean ± standard deviation of three independent experiments. Results were analyzed using the 2-ΔΔCq method (21).

Lentiviral vector construction. Packaging vectors carrying green fluorescent protein (GFP), lncRNA-TUG1, and short hairpin (sh)RNA-TUG1 were constructed. Then the packaging vectors (pCMV-SVG, pMDLg/pRRE and pRSV-REV) and the lentiviral vectors (all Shanghai GenePharma Co., Ltd., Shanghai, China) were co-transfected into HK-2 cells (1x10^5 cells/well) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Following 8 h transfection, the medium was replaced with fresh DMEM containing 10% FBS and the cells were cultured for a further 48 h. Subsequently, the cell culture supernatant containing vectors was collected, centrifuged at 4,000 x g for 10 min at room temperature and filtered through a 0.45-µm filter. The lentiviral vectors, which are referred to as lenti-GFP, lenti-lncRNA-TUG1 or lenti-shTUG1, were stored at -80°C until further use.

Transfection. RCC 786-O cells (1x10^5 cells/well) were transfected with 20 nM lenti-GFP, lenti-lncRNA-TUG1 or lenti-shTUG1 in 8 µg/ml polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequently, G418 (Life Technologies; Thermo Fisher Scientific, Inc.; 0.8 mg/ml) was applied to screen and establish the stable expression of transfected genes. RCC 786-O cells (2x10^5 cells/well) were transfected with 10 nM negative control mimics (5’-UCCGAACGUGUCACGTT-3’), miR-196a mimic (5’-UAGGUAGUUCAUUGUGUGG-3’), or miR-inhibitor mimics (5’-TACGGAGTGGAGTGGTGGTGG-3’; Shanghai GenePharma Co., Ltd.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 72 h.

MTT assay. MTT assays were used to detect the proliferative ability of RCC 786-O cells with or without transfection. A total of 2x10^5 cells were seeded in a 96-well plate and incubated with 0.5 mg/ml MTT solution (Sigma-Aldrich; Merck KGaA) for 4 h at 37°C. Subsequently, the original culture medium was discarded and 100 µl dimethyl sulfoxide solution (Sigma-Aldrich; Merck KGaA) was added to each well. The absorbance was detected at a wavelength of 490 nm using a Tecan plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Flow cytometry analysis. Apoptotic rates of RCC 786-O cells with or without transfection of lenti-GFP, lenti-lncRNA-TUG1 or lenti-shTUG1 were analyzed using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BioVision, Inc., Milpitas, CA, USA), as per the manufacturer’s protocol. Briefly, 5x10^5 cells were harvested and washed with PBS twice, prior to being resuspended in 500 µl binding buffer and incubated with 5 µl Annexin V-FITC, and 5 µl PI, for 10 min at room temperature in the dark. The apoptotic rates of 786-O cells were examined using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and BD CellQuest™ Acquisition software version 2.9 (BD Biosciences). Cells were distinguished as viable, early apoptotic, apoptotic or dead.

Migration and invasion assays. Transwell assays were performed to determine the migratory and invasive ability of RCC 786-O cells transfected with lenti-lncRNA-TUG1 or lenti-shTUG1. To assess cell migration, 1x10^5 786-O cells in serum-free RPMI 1640 medium (Kaiji) were seeded onto Transwell inserts (pore size, 8 µm) Costar; Corning Incorporated, Corning, NY, USA). Subsequently, 600 µl RPMI medium supplemented with 10% FBS was added to the lower chambers. Cells were incubated for 24 h and non-migrated cells on the upper surface were removed. The inserts were washed with PBS, fixed with 4% methanol for 20 min at 37°C and stained with 0.1% crystal violet for 5 min at room temperature. The number of migratory cells was counted under a light microscope (magnification, x200; Nikon Corporation, Tokyo, Japan). To assess invasion, the same protocol for cell migration was used; however, the Transwell insert membranes were pre-coated with a layer of 200-300 µg/ml Matrigel (BD Biosciences) for 6 h at 37°C.

Western blot analysis. Western blot analysis was used to assess the expression levels of relevant proteins. RCC 786-O cells were
harvested in radioimmunoprecipitation assay lysis buffer (50 mM Tris/HCl, pH 7.4; 150 mM NaCl; 1% NP-40; and 0.1% SDS) containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Supernatants were collected by centrifugation at 6,000 x g for 5 min at 4°C and the protein concentration was detected using a Bio-Rad Protein Assay kit (cat. no. 5000002; Bio-Rad Laboratories, Inc.). A total of 30 µg protein was separated on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). After blocking in Tris-buffered saline containing 5% nonfat milk for 2 h at room temperature, membranes were incubated with primary antibodies at 4°C overnight, including anti-phosphorylated (p) RAC-α serine/threonine-protein kinase (1:1,000; cat. no. sc-16646; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-ERK antibody (1:1,000; cat. no. ab199380), anti-mitogen-activated protein kinase 9 (JNK2) antibody (1:1,000; cat. no. ab178953) and anti-GAPDH antibody (1:4,000; cat. no. ab9485; all Abcam). Horseradish peroxidase-conjugated IgG secondary antibodies (1:5,000; cat. no. ab7090) or anti (1:5,000; cat. no. ab8226; both Abcam) were incubated with goat anti-rabbit (1:5,000; cat. no. ab7090) or anti (1:5,000; cat. no. ab8226; both Abcam) horseradish peroxidase-conjugated IgG secondary antibodies for 1 h at room temperature. The enhanced chemiluminescence detection system (Amersham; GE Healthcare, Chicago, IL, USA) was used to develop and detect protein bands. BandScan software version 5.0 (Glyko, Inc., Novato, CA, USA) was used for densitometric analysis following western blotting.

**Dual luciferase reporter assay.** Wild type TUG1 gene with miR-196a binding sites or mutant TUG1 gene with target site deletions were constructed, amplified and cloned into the pRL-TK plasmid (Promega Corporation, Madison, WI, USA) vector. A total of 2x10^4 786-O cells/well were seeded into 96-well plates. Cells were co-transfected with the constructed plasmids using Lipofectamine® 2000, and Renilla luciferase was used as an internal control. The cells were harvested 48 h following transfection and the luciferase activities were detected by a Dual-Luciferase Reporter Assay kit (Promega Corporation), as per instructions provided by the manufacturer.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was tested by Student's t test or one-way analysis of variance followed by the Dunnett's post hoc test. All results are expressed as the mean ± standard deviation. *P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

**Results**

**lncRNA-TUG1 expression is increased in human RCC cell lines.** The present study examined the mRNA expression level of lncRNA-TUG1 in five cell lines, including the normal kidney cell line HK-2, and RCC cell lines 786-O, ACHN, A704 and A498, using RT-qPCR. The results demonstrated that the expression level of lncRNA-TUG1 was significantly increased in RCC cells compared with HK-2 cells (Fig. 1). Subsequently, the RCC cell lines 786-O was selected for further experiments, as it expressed the highest TUG1 mRNA level of the four RCC cell lines.

**lncRNA-TUG1 promotes RCC cell proliferation, migration and invasion in vitro.** To investigate the biological role of lncRNA-TUG1 in RCC, the present study analyzed the proliferative, migratory and invasive ability of RCC 786-O cells with or without transfection of lenti-GFP, lenti-lncRNA-TUG1 or lenti-shTUG1. RT-qPCR was used to determine the expression level of lncRNA-TUG1, from which it was demonstrated that lncRNA-TUG1 expression was significantly elevated in the lenti-lncRNA-TUG1 group, while significantly decreased in the lenti-shTUG1 group, compared with the control groups (Fig. 2A). An MTT assay and flow cytometry analysis were used to measure the effect of lncRNA-TUG1 on the cell viability and apoptosis rate of RCC cells. The results demonstrated that lncRNA-TUG1-overexpressing cells exhibited improved cell viability and a lower apoptosis rate, while lncRNA-TUG1-downregulated cells exhibited a poorer proliferative ability and a higher apoptosis rate, compared with the two control groups (Fig. 2B-D). Migration and invasion assays were performed to evaluate the impact of lncRNA-TUG1 on RCC migration and invasion. From these assays it was revealed that the migratory and invasive cell numbers were significantly increased in the lenti-lncRNA-TUG1 group, while significantly decreased in the lenti-shTUG1 group compared with the control (Fig. 2E-H). These results suggested that lncRNA-TUG1 may serve a tumor-promoting role in the development of RCC.

**lncRNA-TUG1 downregulates miR-196a in human RCC cells.** To elucidate the functional mechanisms of lncRNA-TUG1 as an oncogene, the present study detected the expression level of miR-196a by RT-qPCR in five cell lines, including the normal kidney cell line HK-2, and the RCC cell lines 786-O, ACHN, A704 and A498. The results demonstrated that miR-196a expression was significantly decreased in RCC cells compared...
Figure 2. lncRNA-TUG1 promotes RCC cell proliferation, migration and invasion. (A) The expression level of lncRNA-TUG1 was detected by reverse transcription-quantitative polymerase chain reaction in four groups, including a blank control group, negative control group, lenti-lncRNA-TUG1 group and lenti-shTUG1 group (value of $2^{-\Delta\Delta Cq}$). (B) The cell viability of RCC 786-O cells per day was measured by MTT assays with or without transfection of control vectors, lenti-lncRNA-TUG1 or lenti-shTUG1. The apoptosis rate of RCC 786-O cells was determined by (C) flow cytometry analysis with or without transfection of control vectors, lenti-lncRNA-TUG1 or lenti-shTUG1, and (D) the results were quantified. The migratory ability of RCC 786-O cells was measured by (E) migration assay, prior to and following transfection with lenti-lncRNA-TUG1 or lenti-shTUG1. Migratory cells were stained with crystal violet solution (magnification, ×200), and (F) the quantification of the relative migrated cell number is presented in the graph. The invasive ability of RCC 786-O cells was detected by (G) Matrigel invasion assay prior to and following transfection with lenti-lncRNA-TUG1 or lenti-shTUG1. Invasive cells were stained with crystal violet solution (magnification, ×200), and (H) the quantification of the relative invaded cell number is presented in the graph. *P<0.01 vs. Blank. lncRNA, long noncoding RNA; TUG1, taurine upregulated gene 1; sh, short hairpin; RCC, renal cell carcinoma; PE, phycoerythrin; FITC, fluorescein isothiocyanate; OD, optical density.
with HK-2 cells (Fig. 3A). To determine whether the low expression level of miR-196a was relevant to the overexpressed lncRNA-TUG1 in RCC cell lines, the present study determined the miR-196a expression level following transfection. The results demonstrated that the expression level of miR-196a significantly increased following transfection with miR-196a or lenti-shTUG1 compared with the controls, while it significantly decreased following transfection with miR-inhibitor or lenti-lncRNA-TUG1 (Fig. 3B). From these results, it was hypothesized that miR-196a may be involved in the lncRNA-TUG1 tumor-promoting function in RCC via a binding interaction. Therefore, a luciferase reporter assay was performed to verify this hypothesis, which demonstrated that luciferase activity was significantly decreased following co-transfection of miR-196a mimics and wild-type lncRNA-TUG1 (Fig. 3C).

The results of the present study indicated that there may be a binding site between lncRNA-TUG1 and miR-196a, through which miR-196a may serve an important role in the biological function of lncRNA-TUG1 in RCC.

**In conclusion,** lncRNA-TUG1 promotes RCC cell proliferation via suppression of miR-196a. Cell viability was determined using an MTT assay following transfection of RCC 786-O cells with lncRNA-TUG1 or shTUG1, or co-transfection with lncRNA-TUG1 and miR-196a, shTUG1 and miR-inhibitor. Consistent with the above results, it was observed that cells transfected with lncRNA-TUG1 or shTUG1 had markedly better or poorer cell viability, respectively, compared with the controls. Furthermore, this decrease or increase in cell viability was able to be reversed by co-transfection with miR-inhibitor or miR-196a (Fig. 4). In general, these results confirmed the above findings, which revealed that lncRNA-TUG1 promoted RCC cell proliferation by suppressing miR-196a.

**Conclusion**

With the number of patients with RCC increasing every year worldwide, there is an urgent requirement for more efficient and precise therapies (2,3). At present, a number of studies have reported that the abnormal expression of proteins is involved in regulating various RCC cell pathological processes, including proliferation, migration and invasion (22,23). Simultaneously, studies have detected noncoding RNAs in RCC cell genomes, which are considered to be important elements in modifying protein expression.
YANG et al: TUG1 PROMOTES RCC PROLIFERATION, MIGRATION AND INVASION VIA miR-196a (5,22,23). Therefore, analyzing noncoding RNAs may be an effective way to examine novel potential biomarkers and their molecular mechanisms in the development of RCC.

Noncoding RNAs are classified into two classes: Small noncoding RNAs (miRs) and lncRNAs. miRs target at mRNAs at the post-transcriptional stage, resulting in mRNA degradation (24,25). lncRNAs have been reported to serve an important role in gene regulation in multiple ways, including epigenetic, transcriptional, posttranscriptional and translational functions (26,27). miRs and lncRNAs are considered to be associated with the occurrence of numerous malignant diseases (28).

In present study, it was observed that the expression level of lncRNA-TUG1 was increased in human RCC cell lines. Therefore, lentiviral vectors were constructed that carried lncRNA-TUG1, shTUG1 or scramble genes, to artificially regulate lncRNA-TUG1 expression levels. By introducing
lentiviral vectors to RCC 786-O cells, the present study determined that lncRNA-TUG1 was able to promote RCC cell proliferation, migration and invasion in vitro. It was demonstrated that lncRNA-TUG1 acts as an oncogene. A similar finding was reported in previous studies. Han et al (29) reported that lncRNA-TUG1 was overexpressed in urothelial carcinomas, and that the high expression level was predictive for high grade and stage; additionally, downregulated lncRNA-TUG1 led to proliferation inhibition and a decrease in apoptosis. In another study, Zhang et al (16) reported that lncRNA-TUG1 was overexpressed in RCC, and silencing of it led to inhibition of proliferation, migration and invasion, in addition to the promotion of apoptosis.

In further experiments, the present study demonstrated that miR-196a expression was significantly decreased in RCC cell lines. Therefore, miR-196a, miR-inhibitor or scramble mimics were transfected into RCC 786-O cells. Via co-transfection of lentiviral vectors and microRNA mimics, an association between the expression levels of lncRNA-TUG1 and miR-196a was demonstrated. The dual luciferase reporter assay further confirmed that lncRNA-TUG1 and miR-196a had a binding site. Re-examination of cell viability revealed that lncRNA-TUG1 achieved its functions in RCC cells by suppressing miR-196a. The present study was the first, to the best of our knowledge, to discuss the association between lncRNA-TUG1 and miR-196a, and their functions in RCC. JNK and p-ERK are widely accepted as proteins that promote cell death. p-AKT is regarded as a regulator of cell survival (30,31). All of these proteins have been reported to serve important roles in cancer cell proliferation, growth and survival (32,33). In the present study, the expression of the aforementioned proteins was determined post-transfection, and it was observed that the lncRNA-TUG1/miR-196a axis may regulate these proteins to interfere with RCC cell proliferation.

In conclusion, although there remain further details to be elucidated with respect to the lncRNA-TUG1/miRNA-196a axis, the present study clarified the expression levels of lncRNA-TUG1 and miR-196a in RCC cells. In addition, the present study confirmed the function of lncRNA-TUG1 as an oncogene, which achieves its biological functions in RCC via regulation of protein expression by suppressing miR-196a. The results of the present study revealed a novel oncogenic pathway in the development of RCC, which may be a promising therapeutic target in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

YY, A-JL and D-MS designed the study. J-FY and MZ analyzed the data. CY, B-HD and RY performed the experiments. A-JL and YY wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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