Original Article

LncRNA TUG1 accelerates cervical cancer progression through miR-29b

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Abstract: Objective: To probe into the regulatory mechanism of long non-coding RNA Taurine up-regulated gene 1 (TUG1) in cervical cancer (CC). Methods: The expression of TUG1 and miR-29b in CC tissues, serum, human normal cervical epithelial cell line HUCEC and CC cell lines (HeLa, SiHa, CaSki, C33A) were detected by qRT-PCR. Further, TUG1 and miR-29b were up-regulated or down-regulated to measure the levels of apoptosis-related proteins Caspase9, Caspase3, Bax and Bcl-2, and to investigate the mechanisms of cell proliferation, invasion and apoptosis. Results: In CC tissues and serum, TUG1 was highly expressed while miR-29b was lowly expressed. The area under the curve (AUC) for TUG1 and miR-29b in the diagnosis of CC were over 0.800, and their expression in CC serum was a significant negative correlation. Overexpression of miR-29b and silencing of TUG1 inhibited CC cell proliferation and invasion, while it reduced the apoptosis rate and improved apoptosis-related protein levels. After TUG1-inhibitor+miR-29b-inhibitor co-transfection, the proliferation and invasion capacity of SiHa and CaSki cells were more evidently inhibited, and the increase in apoptosis rate was more significant. Inhibition of TUG1 could increase miR-29b expression, thereby reducing the cell proliferation and invasion ability of CC cells and increasing the rate of apoptosis. Conclusion: TUG1 can reduce the proliferation and invasion and promote apoptosis of CC cells by targeting miR-29b, which indirectly proves that it may function as a novel biomarker and a feasible treatment target for CC.

Keywords: TUG1, miR-29b, cervical cancer, cell survival

Introduction

Cervical cancer (CC), a malignant tumor occurring in the cervical canal of the uterus and vagina, is the third most pervasive cancer among women globally, with nearly 85% of cases occurring in developing countries [1, 2]. However, due to the absence of obvious clinical symptoms of early CC, many patients have progressed into advanced stages before cervical cytology screening, bringing certain troubles to clinical treatment [3, 4]. Despite clinical progress in screening and effective surgery or radiotherapy, the prognosis of patients with CC is still unfavorable due to recurrence or metastasis, and there is currently no effective biomarker to identify high-risk patients [5, 6]. Therefore, it is high time to identify valid biomarkers for CC patients with poor prognosis.

Widely distributed in the genome, IncRNAs are believed to be involved in many biological processes, cell cycle control, translation, splicing, transcription and chromosome imprinting, and the dysregulation of IncRNAs leads to various diseases by altering the expression of target genes [7]. Of these, TUG1 was first found to be highly expressed in the retina and nerve tissues and is extensively expressed in human tissues and cells. Experimental studies have confirmed that, as a proto-oncogene, overexpression of TUG1 can accelerate tumor cell proliferation and metastasis, while reducing cell apoptosis [8]. For example, in the study of Xu Y et al., TUG1 was down-regulated in placental samples of patients with preeclampsia, and TUG1 gene knockout could inhibit the tumorigenic phenotype of trophoblast cells and induce apoptosis, thus affecting the reconstruction process of spiral arteries [9]. According to Duan W et al., TUG1 was statistically up-regulated in CC tissues, especially in patients with poor TNM stage and large tumor volume, which indicated that TUG1 overexpression was correlated to the enhancement, proliferation and migration of CC cells [10]. These reports provide
potential ideas for molecular diagnosis and treatment of TUG1 in tumors. In order to understand the clinical value and mechanism of TUG1 in patients with CC, we predicted through use of the starbase3.0 database that miR-29b was among the potential targets of TUG1. MiR-29b, a tumor suppressor, can regulate cell autophagy and enhance ovarian cancer cisplatin sensitivity by reducing cell viability and promoting apoptosis [11, 12]. Although it is known that both TUG1 and miR-29b play important roles in a variety of cancers, their mechanisms of action in CC need further study.

In this study, we explored the effect of TUG1 regulation of miR-29b on the biological cell functions of patients with CC, aiming to provide a new therapeutic target for clinical treatment of this disease.

Materials and methods

Cell source

Human normal cervical epithelial cell line (HUVEC) and CC cell lines (HeLa, SiHa, CaSki, and C33A) were all purchased from American Type Culture Collection (ATCC; Cat. Nos. BNCC353405, BNCC246354, BNCC102118, BNCC102119, BNCC337882).

Sample collection

This study was approved by the ethics Committee of our hospital. The participants and their families had been informed of the details of this study and signed a fully informed consent. With the consent of the patients and the hospital, the cancer tissues and adjacent normal tissues of 98 patients with CC were collected and stored in liquid nitrogen. In addition, 5 mL venous blood from the elbow was drawn from 98 patients with CC and 76 healthy subjects who underwent physical examination in the same period. The blood was centrifuged at 1500×g at 4°C for 10 min, and stored in a cryogenic freezer at -70°C for subsequent research. Cervical cancer patients were included in the research group with an average age of (53.23±5.54) years and an average course of disease of (2.54±0.16) years. While the healthy controls that were allocated into the control group had an average age of (52.67±5.37) years. There were no significant differences in gender and age between the two groups.

Cell culture and transfection experiments

TUG1 and miR-29b inhibition plasmids (TUG1-inhibitor, miR-29b-inhibitor), overexpression plasmids (TUG1-mimics, miR-29b-mimics) and blank control groups (si-NC, miR-NC) were established, and the established drug resistant cell lines were transferred into 24-well plates. The cell plasmids were transfected 48 h later with Lipofectamine 2000 kit. Next, 100 nM overexpression, suppression, and blank control were transfected into cells in strict accordance with the instructions of the kit. All primers were transfected into the cells with the greatest difference in expression.

QRT-PCR detection

As instructed by the manufacturer’s instructions, Trizol reagent was applied for total RNA isolation from CC tissues and cell lines (Simgen Biological Reagent Development Co., Ltd., Hangzhou, China, 5301100), and an ultraviolet spectrophotometer (Spectral Laboratory Equipment Technology Co., Ltd., Dongguan, China, SPCC) was used to detect its concentration and purity. Then, 5 μg total RNA was processed for reverse transcription of cDNA in line with the instructions provided with the kit. On a PCR instrument (Microsep Biotechnology Co., Ltd., Wuxi, China, TC9639), SYBR Premix Ex Taq TM kit (Saihonggrui Biotechnology Co., Ltd., Nanjing, China, Cat. No.: DRR041A) was used for PCR reaction. PCR amplification cycle conditions (40 cycles): first to pre-denature at 95°C for 10 min, then to denature at 95°C for 15 s, and finally to anneal/extend at 60°C for 60 s. There were 3 replicate wells for each sample, and the experiment was carried out 3 times. The data were analyzed using 2-ΔΔct [13]. The primer sequence is detailed in Table 1, with U6 as the internal reference of miR.

Western blot (WB)

RIPA lysate (Shifeng Biological Technology Co., Ltd., Shanghai, China, R1614) was added to each group of cells after culture to extract total protein. Then the supernatant was obtained by centrifuging the lysates at 12000×g at 4°C for 15 min, and its protein concentration was determined by bicinchoninic acid (BCA) kit (Abbkine Scientific Co., Ltd., Wuhan, China, KTD3001). Next, the protein was isolated with 10% sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE; Huzhen Industrial Co., Ltd., Shanghai, China, HZ101-451), and transferred to a Polyvinylidene Fluoride (PVDF) membrane (Kairuiji Biotechnology Co., Ltd., Beijing, China, ISEQ00011), which was soaked in Phosphate Buffered Saline add Tween-20 (PBST: Jingke Chemical Technology Co., Ltd., Shanghai, China, JKL1525) for 5 min for washing and then sealed with 5% skim milk powder (Xinyu Biotechnology Co., Ltd., Shanghai, China, 232100) at room temperature for 4 h. At 4°C, FOXO3 (1:1000), caspase-3 (1:1000), Bax (1:1000), Bcl-2 (1:1000) and β-catenin (1:1000) primary antibodies (Abcam, USA) were added for an overnight binding. The next day, after washing to discard the primary antibodies, the membrane was added with goat anti-rabbit second antibody (1:4000; Qunji Biotechnology Co., Ltd., Shanghai, China, MAB-19500) and cultured at 37°C for 1 hour, and then rinsed with TBST 3 times for 5 min each time. Enhanced chemiluminescence (ECL) reagent (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China, ECL-0113) was used for luminescence and development. With the aid of Quantity One software, the grayscale values of the protein bands were scanned and analyzed, and their expression profiles were calculated.

Cell viability test

Cell Counting Kit-8 (CCK-8; Hubei Biotechnology Co., Ltd.) was applied for cell viability determination, and the operation steps were performed according to the kit instructions. The details were as follows: The cells collected 24 h after transfection were cultivated in 96-well plates at 2.5×10^4 per well. At 24 h, 48 h, 72 h and 96 h, CCK-8 (10 μL) was added into the wells respectively for another 4 hours of culture at 37°C. The absorbance at 450 nm was measured using a microplate reader (Haisian Biotechnology Co., Ltd., Shenzhen, China, HR801). All the experiments were repeated three times.

Cell invasion test (Transwell)

After 24 h of transfection, the collected cells were adjusted to 5×10^4 and seeded on 6-well plates. They were then rinsed with PBS twice and inoculated in the upper chamber where 200 μL Dulbecco's Modified Eagle Medium (DMEM; Mengya Biological Technology Co., Ltd., Shanghai, China, Y10166-500 ml) was added, and 500 mL DMEM (containing 20% FBS) was put into the lower chamber. After 48 h of culture at 37°C, the substrates and cells that failed to cross the membrane surface of the upper chamber were wipped off, washed 3 times with PBS, immobilized with paraformaldehyde for 10 min, and rinsed with double distilled water 3 times. After drying, the cells were stained with 0.5% crystal violet for the observation of cell invasion with the help of a microscope.

Apoptosis assay

AnnexinV-fluorescein isothiocyanate-propidium iodide (AnnexinV-FITC/PI; Fushen Biotechnology Co., Ltd., Shanghai, China, 556547) detected the apoptosis ability of SiHa and CaSki cells. Forty-eight hours after transfection, the cells were digested with 0.25% trypsin (Lian-shuobaowei Biotechnology Co., Ltd., Shanghai, China, N/A-797), and adjusted to 1×10^6 mL suspension, which was then cultivated for 5 minutes at room temperature in the dark. Apoptosis detection was carried out using FC500MCL flow cytometer (Beamdiag Biotechnology Co., Ltd., Changzhou, China, 1026).

Target gene detection

Starbase3.0 was used to predict the down-stream target genes of TUG1. SOX1'UT wild type (Wt), SOX1-3'UTR mutant (Mut), TUG1678-mimics, and si-NC were transferred into target cells using the Lipofectamine 2000 kit (We-gene Biotechnology Co., Ltd., Shanghai, China, 11668-019). Determination of luciferase activi-
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The expression of TUG1 and miR-29b in serum was conducted 48 h later using the dual luciferase reporter (DLR) gene assay kit.

Statistical methods

The data were analyzed by SPSS 22.0 (EasyBio Co., Ltd., Beijing, China) and illustrated using GraphPad 6. All the data were expressed in the form of mean ± standard deviation (mean ± SD). The inter-group and multi-group comparisons were carried out by independent sample t-test and one-way analysis of variance (ANOVA) respectively, and LSD-t test was utilized for post-hoc pairwise comparisons. Multi-time expression profiles were analyzed by repeated measures ANOVA, and the post hoc test was done by Bonferroni. P<0.05 meant that the difference was statistically significant.

Results

Expression of TUG1 and miR-29b in the serum and tissues of patients with CC

QRT-PCR results showed that compared with the control group, TUG1 was statistically higher while miR-29b was statistically lower in the serum and tissues of patients with CC, with statistical significance (P<0.05) (Figure 1).

Diagnostic value of TUG1 and miR-29b in patients with CC

By drawing the ROC curve of serum TUG1 and miR-29b in the diagnosis of CC patients, we found that the AUC value of serum TUG1 in the diagnosis of CC patients was 0.878, the sensi-
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Activity was 65.31, the specificity was 96.15, and the optimal cutoff was 0.813. While the AUC value, sensitivity and specificity of serum miR-29b in diagnosing CC were 0.840, 79.59, 79.48 and 0.793 respectively. Further, Pearson test showed that there was a negative correlation between TUG1 and miR-29b in serum of patients with CC (P<0.05) (Table 2; Figure 2).

**Effects of TUG1 expression in transfected cells on the biological function of patients with CC**

Detection of TUG1 expression showed that TUG1 in HeLa, SiHa, CaSki and C33A cells of patients with CC was notably higher than that of HUCEC (P<0.05), and SiHa and CaSki, with the greatest difference in expression profiles, were selected for transfection. After transfection with TUG1-inhibitor, TUG1 was dramatically reduced in the TUG1-inhibitor group compared to that in the si-NC group (P<0.05). After transfection, the proliferation and invasion ability of TUG1-inhibitor group were statistically decreased compared with that of si-NC group, while the apoptosis rate was noticeably increased (P<0.05). In addition, we also investigated the effect of TUG1 on apoptosis-related proteins and found that inhibition of TUG1 could increase Caspase9, Caspase3 and Bax, and decrease Bcl-2 (Figure 3).

**Effects of miR-29b expression in transfected cells on the biological function of patients with CC**

After detection, it was found that miR-29b was remarkably lower in HeLa, SiHa, CaSki and C33A cells than in HUCEC, and SiHa and CaSki cells, which showed the greatest difference in expression profiles, were selected for transfection. After transfection, it was found that miR-29b was statistically higher in the miR-29b-
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Figure 3. Effects of TUG1 expression in transfected cells on the biological function of patients with CC. A. TUG1 expression in human normal cervical epithelial cell line HUCEC and CC cell lines HeLa, SiHa, CaSki, C33A cell lines. B. TUG1 expression in SiHa and CaSki cells after transfection. C. Proliferation of SiHa cells after transfection. D. Proliferation of CaSki cells after transfection. E. Invasion of SiHa and CaSki cells after transfection. F. Apoptosis of SiHa and CaSki cells after transfection. G-J. Effects of TUG1 inhibition on SiHa and CaSki cell apoptosis-related proteins. K. FC diagram of apoptosis. L. Apoptosis-related protein map. Note: ** indicated P<0.05.
mimcs group than in the miR-NC group (P<0.05). Further detection showed that after transfection, the proliferation and invasion ability of SiHa and CaSki cells in the miR-29b-mimcs group was markedly decreased compared with the miR-NC group, and the apoptosis rate was notably increased (P<0.05). We also explored the effect of miR-29b on apoptosis-related proteins and found that inhibition of miR-29b increased Caspase9, Caspase3, Bax, and reduced Bcl-2 (Figure 4).

Gene identification of TUG1

To verify the correlation between TUG1 and miR-29b, we first predicted TUG1's downstream target genes through starbase3.0 and found its targeted binding sites with miR-29b. Therefore, we performed dual luciferase activity detection. It was found that after miR-29b overexpression, the luciferase activity of TUG1-3'UTR Wt reduced significantly (P<0.05), but that of TUG1-3'UTR changed little (P>0.05). According to PCR results, miR-29b was down-regulated significantly in HeLa and SiHa cells after transfection of TUG1-mimics, while that in SiHa and CaSki cells was significantly up-regulated after transfection of TUG1-inhibitor (P<0.05) (Figure 5).

Rescue experiment

We detected the biological functions of SiHa and CaSki cells through co-transfection of TUG1-inhibitor+miR-29b-inhibitor. The results showed that the proliferation, invasion and apoptosis of cells transfected with TUG1-inhibitor+miR-29b-inhibitor were not significantly different from si-NC transfected ones, but the proliferation and invasion ability of cells transfected with TUG1-inhibitor increased significantly, while the apoptosis rate decreased remarkably (P<0.05) (Figure 6).

Discussion

For the pressing need of developing new and effective treatments for CC, we need to better understand the underlying mechanisms of CC development and progression. Disorders of TUG1 can affect genes related to the growth and migration of a variety of cell types in cancers, such as human esophageal squamous cell carcinoma, osteosarcoma, and non-small cell lung cancer [14-17]. In this study, we found that TUG1 was a key factor in the proliferation, invasion and apoptosis of CC cells, and inhibiting its expression or the overexpression of its target gene miR-29b could inhibit CC cell proliferation, invasion and promote apoptosis.

Previous studies have reported that TUG1 is involved in the development of several malignancies [18-20]. For example, research has identified that TUG1 is highly expressed in prostate cancer patients, and those with high TUG1 expression accompany with low survival rate and poor prognosis; while TUG1 gene knockdown can reduce prostate cancer cell proliferation and invasion and enhance apoptosis [21]. Other evidence has revealed that TUG1 plays a vital carcinogenic role and can promote cell malignant phenotypes in ovarian cancer by regulating the miR-29b-3p/MDM1 axis [22]. Whether TUG1 can regulate the biological function of CC cells by targeting miR-29b is unclear. Therefore, this study first analyzed the expression of TUG1 and miR-29b in the tissues and serum of patients with CC. Compared with the control group, TUG1 was statistically higher in the serum and tissues of patients in the research group, while miR-29b was noticeably lower. Through the ROC curve analysis, we found that the AUCs of TUG1 and miR-29b were 0.878 and 0.840 respectively, indicating that both of them enjoyed high diagnostic efficiency for CC. Subsequently Pearson correlation analysis identified that TUG1 and miR-29b were negatively correlated in the serum of patients with CC. We speculated that TUG1 and miR-29b are involved in the occurrence and development of CC, so we conducted cell biology experiments for further verification.

Also, there are numerous studies on the cellular biological functions of TUG1 and miR-29b in human diseases. For example, the dysregulation of TUG1 has been shown to be related to the proliferation, migration, cell cycle changes, drug resistance and inhibition of apoptosis of tumor cells [23]. Lei L and Mou Q reported that in endometrial cancer, TUG1 was up-regulated in tissues and cell lines, and it could enhance VEGFA expression by competing with miR-299 and miR-34a-5p, thereby mediating cell proliferation [24]. Li Y et al. also showed that in CC, chemotherapy-mediated miR-29b inhibited CC cell proliferation, EMT process and angiogenesis by targeting the STAT3 signaling pathway.
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A. The relative expression of miR-29b

B. The relative expression of miR-29b

C. OD value

D. OD value

E. Cell invasion

F. Apoptosis rate (%)

G. Caspase-9 expression level

H. Caspase-3 expression level

I. Bax expression level

Figure 4. Effects of miR-29b expression in transfected cells on the biological function of patients with CC. A. MiR-29b expression in human normal cervical epithelial cell line HUCEC and CC cell lines HeLa, SiHa, CaSki, C33A cell lines. B. MiR-29b expression in SiHa and CaSki cells after transfection. C. Proliferation of SiHa cells after transfection. D. Proliferation of CaSki cells after transfection. E. Invasion of SiHa and CaSki cells after transfection. F. Apoptosis of SiHa and CaSki cells after transfection. G-J. Effects of miR-29b overexpression on apoptosis-related proteins in SiHa and CaSki cells. K. FC diagram of apoptosis. L. Apoptosis-related protein map. Note: ** indicated P<0.05.
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The effects of TUG1 and miR-29b on the biological function of CC cells were then observed, and we found their same expression profiles as those in tissues and serum, that is, TUG1 was statistically higher while miR-29b was dramatically lower in CC cell lines compared with normal cells. In the study of Zhu J et al. [26], TUG1 inhibition significantly reduced the proliferation and invasion ability of HeLa and CaSki cells, which is similar to the results of this study. Subsequently, by transfecting TUG1-inhibitor and miR-29b-mimcs into SiHa and CaSki cells, it was found that TUG1 inhibition limited proliferation and invasion of CC cells and promoted apoptosis, and miR-29b overexpression could also yield the same effects. This is also similar to the results of Zhu J's study, indicating that inhibiting TUG1 can effectively reduce the proliferation, invasion and apoptosis of cervical cancer cells. Therefore, we hypothesize that TUG1 and miR-29b can be used as potential targets for the treatment of CC.

Further, we explored the correlation between TUG1 and miR-29b. The results showed that the proliferation, invasion and apoptosis of CC cells in the TUG1-inhibitor+miR-29b-inhibitor group were not statistically different from those in the si-NC group, but the proliferation and invasion ability of the cervical cells in the TUG1-inhibitor group were higher than those in the TUG1-inhibitor group, while the apoptosis rate was decreased, which indicated that there was a certain regulatory relationship between the two. Therefore, we further verified the regulatory relationship between them by DLR. The results showed that miR-29b overexpression markedly decreased the luciferase activity of TUG1-3′UTR Wt, but had little effects on the luciferase activity of TUG1-3′UTR Mut. Besides, TUG1-mimics transfection led to dramatically down-regulated miR-29b in CC cells, while the transfection of TUG1-inhibitor resulted in remarkably up-regulated miR-29b levels. This indicates that there is a targeted regulatory relationship between TUG1 and miR-29b; that is, TUG1 can increase miR-29b, thus reducing the proliferation and invasion ability of CC cells.

Figure 5. Gene identification of TUG1. A. The binding sites between TUG1 and miR-29b. B. DLR results. C. MiR-29b expression in SiHa and CaSki cells after transfection. Note: ** indicated P<0.05.
Figure 6. Rescue experiment. A, B. Cell proliferation of TUG1-inhibitor+miR-29b-inhibitor group was not different from that of si-NC group, while was statistically increased compared with that of TUG1-inhibitor group. C. The cell invasion of TUG1-inhibitor+miR-29b-inhibitor group was not different from that of si-NC group, but was statistically increased compared with that of TUG1-inhibitor group. D. Apoptosis of TUG1-inhibitor+miR-29b-inhibitor group was not statistically different from that of the si-NC group, while was statistically reduced compared with that of TUG1-inhibitor group. E. FC diagram of apoptosis. Note: ** indicated P<0.05.
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and increasing the rate of apoptosis. Therefore, TUG1 is expected to be a new marker for the treatment of CC. This is the first report confirming that TUG1 can directly target CC progression through miR-29b, but there are still some shortcomings. First of all, this experiment was only constructed in vitro, with the absent of in vivo experiments to verify the anti-tumor effect of TUG1 in CC, or were there nude mouse experiments, so it is still unknown whether TUG1 injection can improve tumor formation in rodents. Secondly, the clinical application of TUG1 needs to be verified in clinical practice.

In conclusion, this study shows that by targeting miR-29b, TUG1 can inhibit CC cell proliferation and invasion and enhance apoptosis, which indirectly proves its potential as a novel biomarker and a feasible treatment target for CC.

Disclosure of conflict of interest

None.

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