LncRNA TUG1 promotes the progression of colorectal cancer via the miR-138-5p/ZEB2 axis

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Introduction

Colorectal cancer (CRC) is the third most prevalent cancer in the globe, and also one of the common digestive system malignant tumors in the gastrointestinal tract, which results in more than 1 million deaths every year [1,2]. As the social economy progresses, more and more youngsters suffer from CRC due to their unhealthy living and eating habits [3]. Despite a great advancement in the treatment methods of CRC including surgery, chemotherapy, radiation, and combined therapy, the prognosis of CRC patients is still poor according to clinical data [4,5]. In recent years, molecular targeting takes an increasingly crucial part in the diagnosis, staging, and comprehensive treatment of CRC. Some studies have revealed that CRC development is linked to long-chain non-coding RNA (LncRNA), and a deeper understanding of the molecular mechanism of CRC can provide novel insights into the pathogenesis of the disease, thus increasing treatment options [6–8].

LncRNAs affect various characteristics of cancers, such as proliferation, migration, and apoptosis [9], and they are confirmed to have abnormal expression in a variety of cancers [10]. According to a recent
study, lncRNA taurine up-regulated gene 1 (TUG1) is abnormally expressed in cancers, and it promotes the progression of hepatic carcinoma via the miR-216b-5p/DLX2 [11,12]. LncRNA TUG1 can act as a possible oncogene for CRC. Overexpressed TUG1 might accelerate the proliferation and migration of CRC cells [13,14]. However, there is a lack of research on the predictive value of lncRNA targeted therapy in CRC. Therefore, with the goal of providing a novel theoretical foundation for CRC diagnosis and treatment in molecular biology based on lncRNA targeted therapy, we detected the expression of lncRNA in CRC to explore the role of lncRNA TUG1 in promoting CRC via the miR-138-5p/zinc finger E-box-binding homeobox 2 (ZEB2) axis, so as to find reliable tumor markers and potential drug targets for clinical diagnosis and prognosis of CRC.

Materials and methods

Materials
A total of 84 CRC tissue specimens and 84 corresponding paracancerous tissue specimens were sampled from 84 patients with CRC admitted to the First Hospital of Jilin University from January 2018 to September 2019. The inclusion criteria of the study: patients diagnosed with CRC according to pathology, cytology, and imaging [15], and those who had not undergone related chemotherapy, immunotherapy, radiotherapy, and other anti-tumor treatment before treatment. The exclusion criteria of the study: patients with comorbid liver cirrhosis or blood coagulation dysfunction, patients without complete case data, patients reluctant to cooperate for the present study, patients with estimated survival shorter than 1 month, and those lost to follow-up. The study was carried out with permission from the ethics committee of the First Hospital of Jilin University and was in accordance with Helsinki Declaration, and all participants and their families signed informed consent forms after understanding the study.

Main instruments and reagents
Human CRC cells (LoVo) stored in the Stem Cell Bank and human colorectal mucosal cells (FHC) (BNCC100429 and BNCC338003) purchased from Beijing BeNa Culture Collection; ABI StepOne Plus real-time fluorescence quantitative PCR instrument, Lipofectamine™ 2000 Transfection Kit, and TRIzol Extraction Kit (Invitrogen); Apoptosis Detection Kit (TaKaRa); MTT Kit (C0009, Beyotime Biotechnology Co., Ltd., Shanghai, China), FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.); UV-2600 ultraviolet-visible spectrophotometer (SHI-MADZU (China) Co., Ltd, Shanghai, China). All primer sequences were provided by Invitrogen Company (Shanghai, China).

Determination methods

Cell culture and transfection experiments
Cell experiments: LoVo cells were transferred to DMEM containing 10% FBS and penicillin–streptomycin solution, followed by incubation in a constant-temperature 5% CO2 incubator with saturated humidity at 37°C. TUG1-siRNA (si-TUG1), TUG1-shRNA (sh-TUG1), and empty plasmid (siRNA-NC) were transfected, respectively, and primers were transfected into the cells with the largest TUG1 expression difference. Six hours later, the cells were incubated in the culture solution with 10% FBS continuously. The TUG1 siRNA sequence was 5′-CCAUCCACAGAGCUUCATT-3′. A qRT-PCR assay was carried out for verification of the cell transfection efficiency.

qRT-PCR assay
qRT-PCR was applied for the quantification of mRNA in tissues and cells. Total RNA was extracted from tissues according to the TRIzol reagent operating instructions, diluted in 20 μl diethylpyrocarbonate water, and then reversely transcribed via a reverse transcription kit in 15 μl total volume containing 1 μl Moloney murine leukemia virus (M-MLV), 1 μl Olig (dT), 0.5 μl RNAsin inhibitor, 1 μl nucleoside triphosphates (NTPs), and RNAs-free water added to adjust the volume. The RNA was incubated at 38°C for 60 min, and cDNA (1 μl) was sampled and synthesized at 85°C for 5 s. Reaction conditions were as follows: 95°C for 15 min, 35 cycles of 95°C for 15 s, and 58°C for 30 min, followed by 72°C for 15 min. Three duplicate wells were prepared for each sample for three times of the assay. After the reaction, the amplification curve and melting curve were confirmed. The relative quantification of the target gene was calculated using 2−ΔΔCt (Table 1).

Western blot assay
The harvested lysed cells were subjected to 10 min of 12000×g centrifugation at 4°C, and the supernatant was acquired as protein sample, followed by concentration determination via the Bicinchoninic Acid method, and lysis buffer was
added to dilute protein sample to prepare Tritonlysis buffer (spacer gel: pH = 6.8; separation gel: pH = 8.8), on which the protein sample was subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), and then transferred to a polyvinylidene fluoride (PVDF) membrane. Subsequently, the protein sample was added with primary antibody and internal reference and cultured at 4°C overnight, and then added with HRP-labeled secondary antibody, and cultured at 37°C for 1 h, followed by three times washing with TBST (10 min). Afterward, the sample was developed with the dark surroundings, absorbed to remove liquid, made luminescent with ECL, and developed. The protein bands were scanned, and the gray values were calculated using v4.6.6 Quantity One.

**Cell proliferation assay**

MTT assay was conducted for cell viability determination. After 24 h of transfection, the cells were transferred to a 96-well plate at 5 × 10^3 cells/well. Then the plate was added with 20 μl MTT solution (5 mg/ml, or 0.5% MTT) at 24, 48, and 72 h after being incubated at 37°C, separately, and then the cells were incubated at 37°C for 4 h. Each well was added with 200 μl dimethyl sulfoxide, and then the cell optical density of each group at 570 nm absorbance was determined via a spectrophotometer.

**Transwell invasion assay**

The transwell insert was covered with Matrigel glue, and allowed to stand at 37°C for 30 min, and the cells were resuspended in tissue-free DMEM at 4 × 10^5 cells/ml. The upper compartment and lower compartment were added with 200 μl cell suspension and 800 μl DMEM with 10% FBS. After being cultured for 24–48 h, the transwell insert was taken out, and the cells were immobilized in 4% paraformaldehyde (Bodi Company, Tianjin, China) for 15 min, and then dyed with Crystal Violet (0.1%). The number of cells penetrating the basement membrane of the transwell insert in ten randomly selected fields under an optical microscope was counted to evaluate the cell invasion ability.

**Cell apoptosis assay**

After 2 days of transfection, the cells were trypsinized through trypsin (0.25%), and washed with PBS twice. Subsequently, the cells were resuspended in 100 μl Annexin-V binding buffer to prepare 1 × 10^6 cells/ml suspension, and the suspension was added with 5 μl Annexin-V/FITC solution, and cultured with the dark surroundings for 15 min, followed by addition of PI staining solution (10 μl). The flow cytometry was used for detection. The experiment was repeated three times, and the results were averaged.

**Statistical analyses**

In the present study, data were processed statistically using SPSS 20.0 (SPSS, Inc., Chicago, Il, U.S.A.). Data in normal distribution were expressed as the mean ± standard deviation (means ± SD), and measurement data were compared between groups by the independent-samples t test, and the LSD method was adopted for pairwise intergroup comparison. Data at different time points were analyzed using the repeated-measures analysis of variance. P <0.05 indicates a significant difference.

**Results**

**Expression of IncRNA TUG1 in CRC**

The qRT-PCR assay was conducted to quantify TUG1 in CRC tissues and cell lines, and it came out that CRC tissues presented significantly higher TUG1 expression than normal paracancerous tissues, and the ROC area-under-the-curve (AUC) of TUG1 was larger than 0.8. The patients were assigned to high and low expression groups based on the median TUG1 expression, and it came out that TUG1 expression was linked to the differentiation of patients with CRC. In addition, CRC cells LoVo showed significantly higher TUG1 expression than human normal colorectal mucosal cells (Figure 1).
Influences of lncRNA TUG1 on the biological function of LoVo cells

LoVo cells were selected for transfection, and it came out that the siRNA-TUG1 group presented significantly lower TUG1 expression than the siRNA-NC group (P<0.01), and the shRNA-TUG1 group presented significantly higher TUG1 expression than the siRNA-NC group (P<0.01). The MTT assay revealed that the siRNA-TUG1 group presented significantly weaker proliferation ability than the siRNA-NC group (P<0.05), and the shRNA-TUG1 group presented stronger proliferation ability than the siRNA-NC group (P<0.05), and according to the flow cytometry results, the siRNA-TUG1 group presented a significantly higher apoptosis rate than the siRNA-NC group (P<0.001), and the shRNA-TUG1 group showed a significantly lower apoptosis rate than the siRNA-NC group (P<0.001). In addition, the Transwell assay revealed that in contrast with the siRNA-NC group, the siRNA-TUG1 showed significantly weaker invasion ability (P<0.001) and the shRNA-TUG1 group showed significantly stronger invasion ability (P<0.001) (Figures 2 and 3).

Targeting binding between lncRNA TUG1 and miR-138-5p

(1) The qRT-PCR assay was employed to quantify miR-138-5p in cell lines from each group, and it came out that LoVo cells showed significantly lower miR-138-5p expression than FHC cells (P<0.05). We selected LoVo cells for transfection, finding that in contrast with NC group, the miR-138-5p-mimics group showed significantly higher expression of miR-138-5p and the miR-138-5p-inhibition group showed significantly lower expression of it. The CCK-8 assay uncovered that in contrast with the NC group, the miR-138-5p-mimics group presented significantly weaker proliferation ability and the miR-138-5p-inhibition group showed significantly stronger proliferation ability (P<0.001). According to the flow cytometry, in contrast with the NC group, the miR-138-5p-mimics group presented significantly higher apoptosis rate (P<0.001) and the miR-138-5p-inhibition group presented significantly lower apoptosis rate (P<0.001). In addition, according to the Transwell assay, in contrast with the NC group, the miR-138-5p-mimics group showed significantly weaker invasion ability (P<0.001) and the miR-138-5p-inhibition group showed significantly stronger invasion ability (P<0.001).

(2) The dual luciferase reporter (DLR) gene assay was carried out for determination of the luciferase activity of cells transfected with pmirGLO-TUG1 WT/MUT and miR-138-5p mimics, and it came out that transfection of both pmirGLO TUG1 WT and miR-138-5p mimics strongly inhibited the dual luciferase activity of LoVo cells, while transfection of pmirGLO-TUG1 MUT and miR-138-5p mimics exerted no influence on that of LoVo cells (Figure 3).

LncRNA TUG1/miR-138-5p/ZEB2 molecular axis

(1) The qRT-PCR was carried out to quantify ZEB2 in cell lines from each group, and it came out that compared with FHC cells, LoVo cells showed significantly up-regulated ZEB2 (P<0.001). We selected LoVo for transfection and carried out a DLR gene assay for determination of the luciferase activity of cells transfected with pmirGLO-ZEB2WT/MUT and miR-138-5p mimics. It came out that transfection of pmirGLO-ZEB2WT
Figure 2. Expression of TUG1 in cells and its effects on the biological function of cells

Expression of TUG1 in cell lines from each group and expression of TUG1 in transfected LoVo cells (A). Proliferation of transfected LoVo cells (B). Apoptosis of transfected LoVo cells (C). Invasion of transfected LoVo cells (D). Expression of apoptosis-related proteins in transfected LoVo cells (E). a indicates \( P < 0.001 \).

and miR-138-5p mimics strongly inhibited the dual luciferase activity of LoVo cells, while transfection of pmirGLO-ZEB2MUT and miR-138-5p mimics exerted no influence on LoVo cells.

(2) The qPCR assay revealed that up-regulation of IncRNA TUG1 strongly inhibited the expression of ZEB2, and transfection of miR-138-5p-inhibitor reversed the inhibition on ZEB2 expression.

(3) Correlation analysis revealed that TUG1 was negatively correlated with miR-138-5p; miR-138-5p was negatively correlated with ZEB2, and TUG1 was positively correlated with ZEB2.

**Promotion of TUG1 on the development and metastasis of CRC by inhibiting the miR-138-5p/ZEB2 molecular axis in in vitro experiments**

Up-regulation of TUG1 strongly promoted LoVo reproduction, while down-regulation of ZEB2 or overexpression of miR-144-3p reversed the promotion caused by up-regulation of TUG1. Wb assay showed that after up-regulation of TUG1, β-catenin, c-myc, cyclin D1, vimentin, Snail, and N-cadherin were significantly up-regulated, while Bax, Caspase-3, E-cadherin, and ZO-1 were significantly inhibited, and down-regulation of ZEB2 or overexpression of miR-144-3p reversed the promotion on epithelial-to-mesenchymal transition (EMT) caused by up-regulation of TUG1, which indicated that TUG1 could inhibit the miR-138-5p/ZEB2 molecular axis to promote the occurrence and metastasis of CRC (Figures 4 and 5).
**Figure 3. Targeting binding between lncRNA TUG1 and miR-138-5p**

(A) Expression of miR-138-5p. The AUC of miR-138-5p for CRC diagnosis was larger than 0.8. (B) Expression of miR-138-5p in tissues with high/moderate/low differentiation. (C) Compared with FHC cells, LoVo cells showed significantly down-regulated miR-138-5p. (D) Expression of miR-138-5p in transfected LoVo cells. (E) Proliferation, apoptosis, and invasion of transfected LoVo cells. (F) Luciferase activity of LoVo cells. (G) Expression of apoptosis-related proteins in transfected LoVo cells. Cell apoptosis map. a indicates $P<0.001$.

**Discussion**

According to recent studies, lncRNA is closely linked to a variety of tumors, and lncRNA participates in carcinogenesis by changing the chromatin structure, sponging microRNA, and affecting the expression of some cancer-related pathways [16,17]. LncRNA plays a pivotal part in carcinogenesis and tumor progression. One study has uncovered that knockdown of TUG1 via shRNA can inhibit the formation of renal cell carcinoma *in vivo* and *in vitro* through the miR-299-3p/VEGF axis [18], and one other study by Yang et al. [19] has revealed that lncRNA TUG1 participates in pulmonary vascular remodeling of hypoxic pulmonary hypertension mice through miR-374c-mediated Foxc1. Our study was to explore the effect of lncRNA TUG1 on the progression and biological function of CRC by regulating the miR-138-5p/ZEB2 axis.

We performed a qRT-PCR to quantify lncRNA TUG1 in CRC, finding that TUG1 was abnormally up-regulated in the tissues of CRC patients, and LoVo cells showed significantly higher TUG1 expression than normal colorectal mucosal cells. We also conducted related analysis on clinicopathological features of the patients, and found that high expression of TUG1 was linked to differentiation of patients with CRC, and the AUC of TUG1 for the diagnosis of CRC was larger than 0.8. There have been reports indicating that lncRNA TUG1 is overexpressed in patients with CRC [20]. All in all, TGF-β promotes CRC metastasis through the TUG1/TWIST1/EMT signaling pathway. TUG1 might be a potential drug target for inhibiting the activation of TGF-β pathway activation in CRC therapy [21]. In addition, through TargetScan database, we found a targeted relation between lncRNA TUG1 and miR-138-5p and between miR-138-5p and ZEB2, and also found a correlation of miR-138-5p and ZEB2 with CRC differentiation.
Figure 4. LncRNA TUG1 up-regulated ZEB2 by targeting miR-138-5p

(A) Expression of ZEB2 in cancer tissues and paracancerous tissues. The AUC of ZEB2 for CRC diagnosis was larger than 0.8.
(B) Compared with FHC cells, LoVo cells showed significantly up-regulated ZEB2.
(C) Tissues with high/moderate differentiation showed significantly lower ZEB2 expression than those with low differentiation.
(D) Expression of ZEB2 in transfected LoVo cells.
(E) Luciferase activity of LoVo cells. (F) TUG1 was negatively related to miR-138-5p.
(G) MiR-138-5p was negatively correlated with ZEB2.
(H) TUG1 was positively correlated with ZEB2. a indicates $P<0.001$.

MiR-138-5p was significantly down-regulated in CRC tissue specimens and cell lines [22]. Human telomerase reverse transcriptase (hTERT) is a direct target of miR-138-5p in CRC cells, and miR-138-5p acts as a tumor suppressor in CRC by directly targeting hTERT [23]. One study by Xie et al. has pointed out that lncRNA TUG1 can act as ceRNA of miR-212-3p, promoting cell proliferation and inhibiting cell apoptosis in osteosarcoma by the miR-212-3p/FOXA1 axis [24]. However, it is not clear whether lncRNA TUG1 can be used as ceRNA to regulate CRC cell genes through the miR-138-5p/ZEB2 axis and affect CRC cell cycle.

Our cell experiments revealed that TUG1 and ZEB2 were overexpressed in LoVo cells, while miR-138-5p was poorly expressed in them. The correlation analysis uncovered that TUG1 was negatively correlated with miR-138-5p; miR-138-5p was negatively correlated with ZEB2, and TUG1 was positively related to ZEB2. Afterward, we up-regulated and down-regulated TUG1, miR-138-5p, and ZEB2 in LoVo cells, separately, finding that down-regulation of lncRNA TUG1 strongly inhibited the expression of ZEB2, while transfection of miR-138-5p-inhibitor reversed the inhibition on ZEB2 expression. One study by Zhu et al. [25] has verified that miR-138-5p inhibits the EMT, proliferation, and metastasis of lung adenocarcinoma cells by targeting ZEB2. In our study, analysis of the biological function of cells showed that inhibition of TUG1 expression and overexpression of miR-138-5p strongly inhibited cell proliferation and invasion, down-regulated apoptosis-related protein β-catenin, and up-regulated Bax and Caspase-3. LncRNA-ATB strengthens the inhibition on β-catenin degradation in the cytoplasm of non-small cell lung cancer cells through the miR-200a/β-Catenin axis to induce EMT conversion [26], which suggests that the lncRNA TUG1/miR-138-5p/ZEB2 axis inhibits CRC, but the specific influence of the lncRNA
Figure 5. Promotion of TUG1 on the development and metastasis of CRC by inhibiting the miR-138-5p/ZEB2 molecular axis in in vitro experiments

(A) Proliferation of LoVo cells. (B) Apoptosis of LoVo cells. (C) Invasion of LoVo cells. (D) Apoptosis-related proteins in the cytoplasm of LoVo cells. (E) EMT-related proteins in the cytoplasm of LoVo cells. (F) Wb map of apoptosis-related protein. (G) Wb map of EMT-related proteins.

TUG1/miR-138-5p/ZEB2 axis on the EMT of CRC is still under investigation. Finally, we carried out in vitro experiments, verifying that TUG1 inhibits the miR-138-5p/ZEB2 molecular axis to promote EMT of CRC cells. According to related reports, EMT is necessary for the growth and survival of tumor cells [27]. EMT is mainly regulated by EMT-transcription factors (EMT-TFs) including TWIST1, SNAI1, ZEB1, and ZEB2. ZEB2 plays a pivotal part in EMT and invasion of tumor cells, and can transform non-invasive cancer cells into cancer stem cells [28]. In this study, down-regulating TUG1 significantly inhibited the mRNA expression of ZEB2 in CRC, and strongly hindered the proliferation and EMT of CRC cells, while overexpressing miR-144-3p or down-regulating ZEB2 reversed the inhibition on the EMT caused by down-regulation of TUG1. TUG1 can inhibit the miR-138-5p/ZEB2 molecular axis to promote the EMT of CRC. Overexpression of miR-138-5p inhibits EMT in breast cancer cells through up-regulating E-cadherin and down-regulating N-cadherin and vimentin [29]. Through in vitro and in vivo experiments and researches on function acquisition and loss, Feng et al. [30] have revealed that IncRNA-CTS affects the cervical cancer through regulated miR-505 and ZEB2 that promotes metastasis and EMT of cells. Inhibition of TUG1 can inhibit the proliferation and invasion of cells by up-regulating miR-498 and down-regulating CDC42 in esophageal squamous cell carcinoma cells [31]. Moreover, one study by Qiu et al. [32] has revealed that lncRNA TUG1 alleviates the acute lung injury induced by septicemia by targeting miR-34b-5p/GAB1.

There are certain limitations in the present study. No animal experiments have been conducted and the regulatory network of carboplatin on TUG1 is still unclear. In addition, further research is needed to determine whether TUG1 can influence the development of tumors through other ways. Therefore, we hope to carry out bioinformatics analysis
to explore the regulatory network of chemical drugs combined with TUG1, so as to provide further references for our assays.

To sum up, lncRNA TUG1/miR-138-5p/VEGF axis inhibits the proliferation and EMT of CRC cells, and regulation of lncRNA TUG1 on the miR-138-5p/VEGF axis is a promising treatment method for CRC and is expected to be adopted in clinical practice.

Competing Interests
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Author Contribution
Zhenkun Yan performed the majority of experiments. Miaomiao Bi analyzed the data. Qiyu Zhang performed the molecular investigations. Yumei Song designed and coordinated the research. Sen Hong wrote the paper.

Abbreviations
AUC, area-under-the-curve; ceRNA, competing endogenous RNA; CRC, colorectal cancer; DLR, dual luciferase reporter; EMT, epithelial-to-mesenchymal transition; hTERT, human telomerase reverse transcriptase; IncRNA, long-chain non-coding RNA; MTT, MTT colorimetry; qRT-PCR, quantitative real-time PCR; TUG1, taurine up-regulated gene 1; Wb, western blot assay; ZEB2, zinc finger E-box-binding homeobox 2.

References


