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LncRNA TUG1 is upregulated and promotes cell proliferation in osteosarcoma

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Abstract: Objective: To examine the expression and function of long non-coding RNA *taurine up-regulated 1* (*TUG1*) in human osteosarcoma cells. Methods: Real-time quantitive PCR was used to detect the transcription level of *TUG1* in a series of osteosarcoma cell lines. Knockdown of *TUG1* in U2OS cells was carried out by transient transfection of siRNAs. MTT assay was performed to access the cell growth rates. Afterwards, RNA and protein of these cells were extracted to analyze the transfection efficient as well as the expression of other molecules. Results: Compared to the normal cell line, *TUG1* exhibited a significant upregulation in osteosarcoma cells. Phenotyping analysis showed the growth-promotion activity of *TUG1*, since knockdown of *TUG1* resulted in declined proliferation. We also found that AKT phosphorylation was impaired after *TUG1* was inhibited, suggesting that the AKT pathway was involved in the regulation of *TUG1* in U2OS cells. Conclusion: Our data provided evidence that *TUG1* was upregulated and acted as a possible oncogene via positively regulating cell proliferation in osteosarcoma cells.

Keywords: *TUG1*, osteosarcoma, proliferation, AKT

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1 Introduction

Long non-coding RNA (LncRNA) is a group of transcribed non-coding RNAs that contain more than 200 nt in length and not encoding any proteins [1,2]. They constitute a major but still poorly characterized part of human transcriptome. They were initially considered as genomic noise. Thanks to the highly thorough sequencing as well as functional technologies in recent years, lncRNAs have been demonstrated to play important roles in basic biological processes [3-5], like DNA replication, gene transcription and epigenetic modification. Recently, we gradually recognized that lncRNAs were closely correlated with human carcinogenesis since they had been proved to be abnormally expressed and act as either oncogenes or tumor suppressors [6-9]. In addition, it is a hot topic to discuss the clinical application of specific lncRNAs as a potential novel class of biomarkers for cancer diagnosis and prognosis [10-12].

Taurine up-regulated 1 (*TUG1*), a 7.1-kb lncRNA, was a long non-coding RNA that was characterized first two years ago in a paper exploring differential expression of lncRNAs in DNA damage-induced cell death in Hela cells [13]. In the following studies, it was clear that *TUG1* was increased in bladder urothelial carcinomas, and promote cell proliferation and apoptosis-inhibition [14]. It exhibited similar high expression and pro-tumorous effects in esophageal squamous cell carcinoma, in which *TUG1* had the ability to promote cell migration [15]. However, in other types of cancers, including non-small cell lung cancer [16], *TUG1* was reported to be downregulated and inhibits cell proliferation as well as inducing apoptosis. This cancer type-specific activity of *TUG1* illustrated the importance of the microenvironment. Although it was already found that in osteosarcoma *TUG1* was upregulated [17], the related mechanisms were still lacking. In our study, experiments were designed to access its expression, function and possible signaling pathway that TUG1 regulated.

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2 Materials and Methods

2.1 Cell Culture

Human normal osteoblastic cell line hFOB1.19 and human osteosarcoma cell line SaoS2, MG63, U2OS and HOS were ordered from the Cell Line Resource Center, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences (Shanghai, China) or ATCC (American Type Culture Collection, USA). All these cells lines were cultured in 1640 or DMEM supplemented with 10% fetal bovine serum, penicillin (100units/ml) and streptomycin (100μg/ml) at 37 ◦ C in a humidified 5% CO_2 atmosphere.

2.2 Reagents and Antibodies

Both the control siRNA and LncRNA *TUG1* siRNA were purchased by GenePharma (Shanghai, China). The cells were transfected with mimics or inhibitors using Lipofectamine RNAiMAX as described below. PCNA and β-actin antibodies were purchased from Santa Cruz Biotechnology Inc. Phosphated-Akt and total-Akt antibodies were purchased from Cell Signaling Technology Inc.

2.3 Transient Transfection

Transfections were performed using Lipofectamine RNAiMAX Reagent (Invitrogene) as per the manufacturer's instructions. After 48 hours, cells were harvest for the other experiments.

2.4 Real-Time PCR Analysis

Total RNAs was extracted by TRIZOL from the cell lines of human osteosarcoma. Real-time PCRs were performed using an ABI7900 real-time PCR system (Applied Biosystems, Carlsbad, CA) and the SYBR Premix Ex Taq reagent kit (Takara Bio Inc., Shiga, Japan) using Ct quantization method. Ct value was the number of PCR cycles at which the fluorescence signal exceeded the threshold. ΔCt was the difference in Ct values between the control (*GAPDH*) and test targets (LncRNA *TUG1*). ΔΔCt was the difference in ΔCt values between the experimental group and paired control group, which represents the fold change in long non-coding RNA expression. The primers were designed by Primer premier 5.0, and the sequences were as follows:

TUG1:

Forward 5'-CTGAAGAAAGGCAA CATC-3';
Reverse 5'-GTAGGCTACTACAGGATTTG-3';

GAPDH:
Forward 5'-GTCAACGGATTTGGTCTGTATT-3';
Reverse 5'-AGTCTTCTGGGTGGCAGTGAT-3'.

2.5 Western Blot

For western blot analysis, in brief, the cell lysate was run on SDS-PAGE in 9% acrylamide gels and transferred onto nitrocellulose membranes. After blocking, blots were incubated with mAb. β-actin was used to normalise protein loading. A total of 30μg of cell lysate was loaded in each lane for western blot analysis.

2.6 MTT Assay

Cellular growth ability was determined by the MTT assay. In brief, 3×10^3 U2OS cells/well were plated into 96-well plates. At the indicated time point, 5μl MTT solution (5mg/ml) was added into each well and incubated for 2h at room temperature (RT). Then the reaction was terminated by 100μl DMSO and the absorbance at 590nm was measured on a microplate-reader.

2.7 Statistic Analysis

Data are presented as mean ± sd of at least triplicate experiments. Statistical analyses were performed by using SPSS version 18.0 (SPSS, Chicago, IL) and GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA). For all statistical analyses, a P value of <0.05 was considered statistically significant.

3 Results

Increased expression of TUG1 in osteosarcoma cell lines

Firstly, we applied real-time PCR to quantify the expression of *TUG1* in osteosarcoma cell lines. As shown in Figure 1A, when compared to the normal hFOB1.19 cell line, the expression of *TUG1* was significantly upregulated in a panel of osteosarcoma cells, including SaoS2, MG63, U2OS and HOS cell lines. This finding was consistent with

the previous conclusion that supported its upregulation in clinical osteosarcoma tissue samples [17].

3.1 TUG1 has a growth-promotion activity in osteosarcoma

Afterwards, TUG1 siRNA was designed and transiently transfected into the U2OS cells to knockdown the endogenous expression of *TUG1*. The knockdown efficiency was revealed to be effective, as approximately 80% of *TUG1* expression was knockdown (Figure 2A). The MTT assay was performed to visualize the proliferation of cells. As shown in Figure 2B, after *TUG1* was inhibited, the growth rate of U2OS cells had decreased compared with the cells in the control group. These results confirmed the previous conclusion that regards *TUG1* as a potential oncogene via regulating cell proliferation.

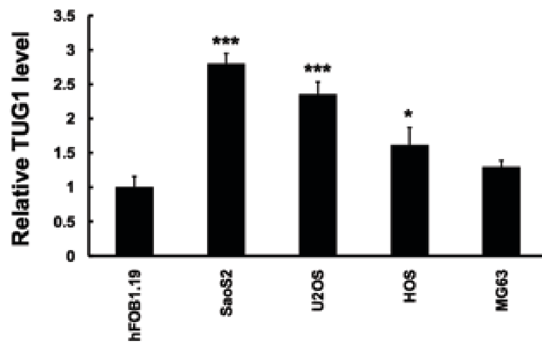

Figure 1: LncRNA TUG1 is upregulated in osteosarcoma cell line. QRT-PCR analysis performed to detect expression of LncRNA *TUG1* expression in human normal osteoblastic cell line hFOB1.19 and human osteosarcoma cell lines. LncRNA *TUG1* expression levels were calculated by the $2^{-\Delta CT}$ method and normalized to GAPDH. ΔCt is the difference in Ct values between LncRNA and *GAPDH*. The plot shows the *TUG1* expression was elevated.

3.2 TUG1 modulated the AKT signaling

To further understand the possible mechanisms that govern the pro-growth function of *TUG1* in U2OS cells, we detected proliferation-related molecules including proliferating cell nuclear antigen (PCNA) and the well known AKT signaling pathway. As shown in Figure 3, after *TUG1* was knocked down, the expression of PCNA was decreased, reflecting the growth inhibition at the molecule level. In addition, we found that the phosphor-AKT (at Ser 473) was also decreased in the *TUG1*-siRNA group, whereas the total AKT expression was unchanged. Since the phosphorylated AKT was an important transducer of AKT signaling pathway, this result suggested that the oncogenic activity of *TUG1* might act through this pathway at least partially.

4 Discussion

Osteosarcoma is the most common primary bone malignant tumor in children and young adults. Although it has small incidence, the high degree of malignancy makes it a second leading cause of cancer-related death in this age group [18]. The 5-year survival rate is about 65% for patients with localized osteosarcoma, however, it decreases to only about 20% for these cases with metastasis [19-20].

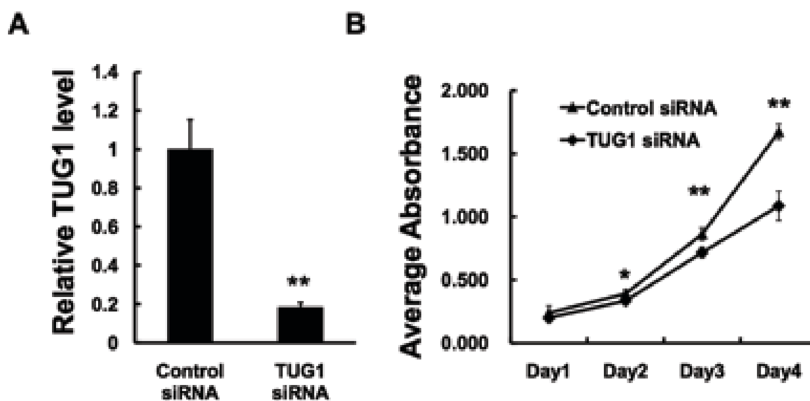

Figure 2: TUG1 promotes tumor proliferation in U2OS cells. (A) U2OS cells were transfected with control siRNA or lncRNA *TUG1* siRNA. In 48h, the cells were harvested for examining knockdown effect. (B) U2OS cells were transfected with control siRNA or lncRNA *TUG1* siRNA. In 24h, cells were trypsinized and seeded into four 96-well plates at a density of 3×10^3 cells for MTT assay as described under Materials and Methods. Data are expressed as the mean ± s.d. of the experiments performed in triplicate. **P*<0.05, ***P* <0.01.

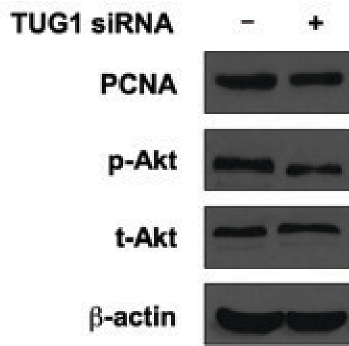

Figure 3: Knockdown of TUG1 reduces survival markers *in vitro*. U2OS cells were transfected with control siRNA or lncRNA *TUG1* siRNA. Forty-eight hours later, cells were harvested for western blotting. Protein level of PCNA, p-Akt (S473) and t-Akt were tested. β-actin was used as the loading control in western blotting. The results showed that inhibited expression of *TUG1* suppressed cell proliferation marker PCNA, and also suppressed cell survival marker p-Akt.

Moreover, objective and reliable diagnostic biomarkers and effective targeted therapeutic agents are also lacking. Thus, further elucidating the underlying mechanisms of osteosarcoma is urgently required. According to the recent studies, lncRNAs emerge as a new frontier of translational research from molecular biology to cancer in the clinic.

TUG1 has been reported in osteosarcoma to be abnormally expressed and accelerates cell proliferation. However, the precise mechanisms or the specific signaling pathways it involves remain unknown. In the present study, we similarly demonstrated its upregulation in four osteosarcoma cell lines, compared to the normal control cells. In *in vitro* experiments, we found that after *TUG1* was inhibited, the growth rate of U2OS cells was reduced. These findings once again confirmed the former study [17]. Besides, we found that *TUG1* might regulate the expression of PCNA, and phosho-AKT (at Ser 473), but the total level of AKT remains unchanged after *TUG1* was knocked down. The AKT pathway or PI3K-AKT pathway is a signal transduction pathway that promotes survival and growth in response to extracellular signals. AKT is activated by phosphorylation at several sites. Activated AKT mediates downstream responses, including cell survival, growth, proliferation, cell migration and angiogenesis, by phosphorylating a range of intracellular proteins. Our results indicated that *TUG1* might promote cell proliferation in osteosarcoma cells through AKT signaling pathway.

In summary, we demonstrated that TUG1 was increased and promoted cell proliferation in osteosarcoma cells. Mechanically, we proved that AKT signaling was involved in this oncogenic regulation. Further studies need to be carried out to verify its pro-tumor activity and clarify the mechanisms in detail.

Conflict of interest statement: Authors state no conflict of interest.

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