RESEARCH ARTICLE

Down-regulation of Long Non-coding RNA TUG1 Inhibits Osteosarcoma Cell Proliferation and Promotes Apoptosis

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Abstract

Objective: To investigate the expression level of TUG1 and one of its transcript variants (n377360) in osteosarcoma cells and assess the role of TUG1 in proliferation and apoptosis in the U2OS cell line. Methods: TUG1 and n377360 expression levels in patients with osteosarcomas and the U2OS human osteosarcoma cell line were evaluated using real-time quantitative PCR. U2OS cells were transected with TUG1 and n377360 siRNA or non-targeting siRNA. MTS was performed to assess the cell proliferation and flow cytometry was applied to analyze apoptosis. Results: We found significantly higher TUG1 and n377360 expression levels in osteosarcoma tissues compared with matched non-tumorous tissues. In line with this, suppression of TUG1 and n377360 expression by siRNA significantly impaired the cell proliferation potential of osteosarcoma cells. Furthermore, inhibition of TUG1 expression significantly promoted osteosarcoma cell apoptosis. Conclusions: The overexpression of TUG1 and n377360 in osteosarcoma specimens and the functional role of TUG1 and n377360 regarding cell proliferation and apoptosis in an osteosarcoma cell line provided evidence that the use of TUG1 or n377360 may be a viable but an as yet unexplored therapeutic strategy in tumors that over express these factors.

Keywords: TUG1 - osteosarcoma - proliferation - apoptosis

Asian Pacific J Cancer Prev, **14 (4)**, 2311-2315

Introduction

Osteosarcoma is the most common primary bone malignant tumor. Osteosarcoma has lower incidence than many other malignant tumors, but it has high degree of malignancy, occurring mainly among children and adolescents with low 5-year survival rate, high amputation rate and poor postoperative function recovery (Qureshi et al., 2010). Similar to other tumors, osteosarcoma is a complicated disease with multi-genetic variations (Akiyama et al., 2008). In-depth studies on gene regulation networks are essential for further understanding the mechanism governing the origination and development of osteosarcoma.

Long non-coding RNA (lncRNA) is a transcribed RNA molecule greater than 200 nt in length and is not translated into a protein. LncRNAs regulate the gene expression in epigenetic level, transcriptional level and post-transcriptional level and are widely involved in the physiological and pathological process of human body (Ponting et al., 2009). Although Long non-coding RNAs (lncRNAs) are numerous, but the number of lncRNAs with known function is very small. In recent years, it has been found that a number of lncRNAs are closely correlated with various types of malignant tumors, acting as oncogenes or tumor suppressors (Ji et al., 2003; Matouk et al., 2007; Pasmant et al., 2007; Pandey et al., 2008; Gupta et al., 2010; Huarte et al., 2010; Kino et al., 2010; Poliseno et al., 2010; Zhang et al., 2010).

But research of lncRNA effects on osteosarcoma is still in the preliminary stage, and the related reports are rare (Pasic et al., 2010). LncRNA Taurine Up - regulated Gene 1 (TUG1) was initially identified as a transcript up - regulated by taurine, siRNA - based depletion of TUG1 suppresses mouse retinal development (Young et al., 2005). Previous studies suggest TUG1 can regulate cell cycle by binding to PRC2. Additionally, it has been reported over-expressed in bladder cancer (Khalil et al., 2009; Yang et al., 2011). However, investigations into the role of TUG1 in osteosarcoma have not been reported yet. In this study, we discussed the expression level of TUG1 and one of its different transcript variants in the sample including 19 cases of osteosarcoma tumor tissues and adjacent normal tissues. Moreover, this study investigated the impact of TUG1 knockdown on the proliferation and apoptosis of bone sarcoma cell U2OS.

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Materials and Methods

Patient Samples

Patients with osteosarcoma who underwent initial surgery at PLA General Hospital from 2010 to 2012 were retrospectively selected for this study. No patient had received therapy before resection. The utilization of the tumor material for research was approved by the ethical committee of PLA General Hospital.

Cell Culture and siRNA Transfection

The human osteosarcoma U2OS cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The U2OS cells were cultured in DMEM medium (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2. As for small interfering RNA (siRNA) analysis, siRNA for TUG1 common sequence, siRNA with specificity of n377360 and non-targeting siRNA were obtained from GenePharma (Shanghai, China). The siRNA sequences were as follow: targeting the common sequence of all the TUG1 transcript variants: Sense Strand:5'-rCrUrArCrArArCrUrArUrCrUrUrCrCrUrUrUrArCrCrArCCG -3', Antisense Strand:5'-rCrGrGrUrGrGrUrArArArGrGrArArGrArUrArGrUrUrGrUrArArGrCrA-3'; targeting the specific sequence of n377360: Sense Strand: 5'- rGrGrArArGrGrGrArGrGr ArGrUrUrUrArGrArGrArGrCrArGGC, Antisense Strand: 5'- rGrCrCrUrGrCrUrCrUrCrUrArArArCrUrCr CrUrCrCrUrUrCrCrUrA-3'. Cells cultured in 6-well plate 50-60% confluency were transfected with siRNA for TUG1 common sequence, siRNA for n377360specific sequence and non-targeting siRNA using X-tremeGENE siRNA transfection reagent (Roche) according to the manufacturer's instructions.

Real-Time Quantitative PCR

Total RNA was isolated from osteosarcoma tumor tissue, matched adjacent normal tissue and osteosarcoma cells using the Trizol Total RNA Reagent (Invitrogen, Carlsbad CA). cDNA synthesis was performed with 2 μg total RNA using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Takara, Ohtsu, Japan). The primers were designed by Primer Express 3.0 software (Applied Biosystems), and the sequences were as follows: TUG1 common sequence, forward 5'-CTGAAGAAAGGCAACATC-3'; reverse 5'-GTAGGCTACTACAGGATTTG-3'; n377360 specific sequence, forward 5'-TGCCCACATACACCACAACA-3'; reverse 5'-ATCCAGGTACCAGGTCTGTAGG-3'; β-actin, forward 5'-CCACTGGCATCGTGATGGA-3', reverse 5'-CGCTCGGTGAGGATCTTCAT-3'. Quantitative PCR was performed using the SYBR PrimeScript RT-PCR kit (Takara, Ohtsu, Japan) in an Applied Biosystems 7500 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA). The quantification of gene expression was performed by using the ΔΔCT calculation with CT as the threshold cycle. The reaction mixtures were incubated at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 3 min.

Table 1. Clinical Characteristics in Osteosarcoma Patients

Patient number	Sex	Age	Grade	Stage
1	F	27	High	T3N0M0
2	M	19	High	T3N0M0
3	F	33	Low	T1N0M0
4	F	21	High	T2N0M0
5	M	27	Low	T1N0M0
6	M	41	High	T3N0M0
7	F	15	Low	T1N0M0
8	M	36	Low	T1N0M0
9	M	29	High	T2N0M0
10	M	25	High	T3N0M0
11	F	29	High	T3N0M0
12	M	38	Low	T1N0M0
13	F	19	Low	T1N0M0
14	F	35	High	T2N0M0
15	F	24	Low	T1N0M0
16	M	38	Low	T1N0M0
17	F	32	High	T3N0M0
18	M	17	High	T2N0M0
19	F	24	NA	NA

M, male; F, female; NA, data not available

Cell Proliferation Assay

Cell proliferation was assayed by MTS assay (Promega) according to the manufacturer's protocol. U2OS cells (3,000 cells per well) were plated in 96-well plates. 20μl of the MTS reagent was added to each well containing 100μl culture medium. The plate was incubated for 2h at 37°C in a humidified, 5%CO_2 atmosphere. The plate was then read at 490 nm using a plate reader.

Cell Apoptosis Assay

U2OS cells were cultured and transfected with siRNA for TUG1 common sequence, siRNA with specificity of n377360 or negative control siRNA as described above. U2OS cells (10^6 cells per 6 cm^2 dish) were plated for the assay, and flow cytometry analysis was performed. Before harvesting, the cells were washed twice with cold PBS, and resuspended in 1×Binding Buffer at a concentration of 1×10^6 cells/ml. The cells were incubated in the Annexin V-FITC Apoptosis Detection Kit (BD, San Jose', USA) according to the manufacturer's instructions. Cells were then analyzed by using a FACS (Fluorescence-activated cell sorting) caliber flow cytometer (BD FACSCalibur, Becton, Dickinson and Company Biosciences, San Jose', USA).

Statistical Analysis

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, $P < 0.05$ was considered statistically significant.

Results

TUG1 and n377360 Was Up-Regulated in osteosarcoma tissue samples

The TUG1 and n377360 expression levels were assessed in a group of 19 patients with osteosarcoma (Table 1). Patients information was obtained from all

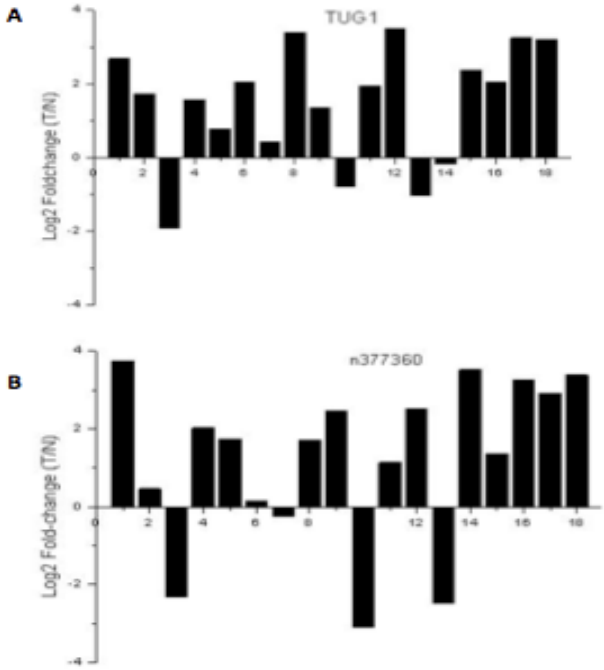

Figure 1. Expression of TUG1 and n377360. (A) The expression of TUG1 in osteosarcoma and matched nontumorous tissues. β-actin was used as an internal control. (B) The expression of n377360 in osteosarcoma and matched nontumorous tissues. β-actin was used as an internal control

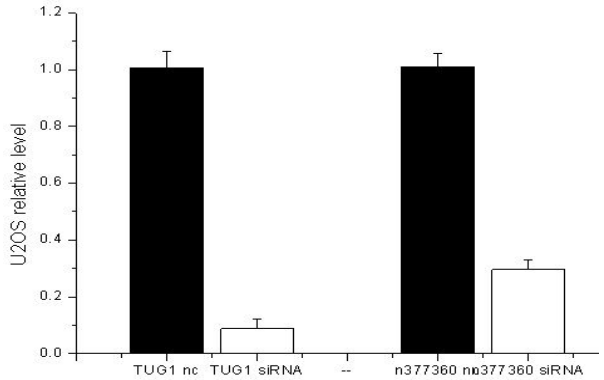

Figure 2. Knockdown of TUG1 and n377360 by siRNA. Relative TUG1 and n377360 expression levels was determined by qPCR. Values are means of 3 independent determinations. Actin expression was unaffected by either siRNA treatment

cases with the exception of one patient, for which the TUG1 and n377360 expression levels could not be ascertained due to insufficient original material. We analyzed a panel paired specimen obtained from patients with osteosarcoma. From each osteosarcoma patient, lncRNA was isolated from cancerous tissue and adjacent nontumorous osteosarcoma tissue. In four patients (nos. 3, 10, 13 and 14) TUG1 expression was lower than that of control tissue. In 14 osteosarcoma specimens, TUG1 expression was higher than matched nontumorous tissue. Only four osteosarcoma specimens (nos. 3, 7, 10 and 13) revealed degraded n377360 expression levels compared with nontumorous tissue. In other 14 osteosarcoma specimens, n377360 expression was elevated compared with matched nontumorous tissue (Figure 1).

Knockdown of TUG1 and n377360 expression by siRNA

TUG1 and n377360 siRNA was constructed and

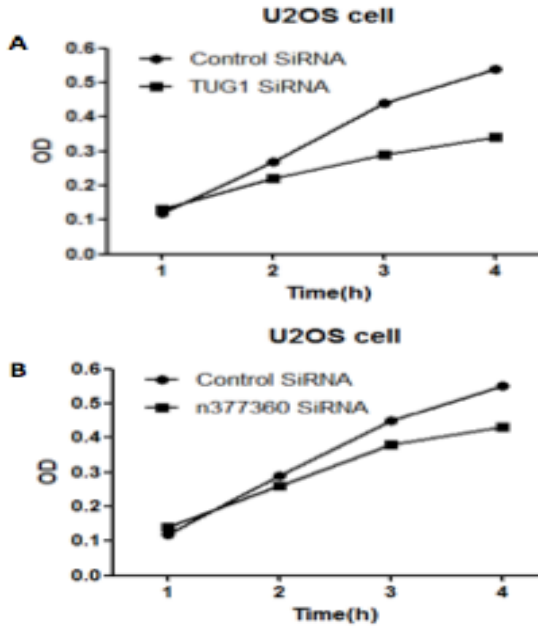

Figure 3. Analysis of Cell Proliferation in U2OS cell. (A) MTS Cell proliferation assay of U2OS cell following TUG1 siRNA. (B) MTS Cell proliferation assay of U2OS cell following n377360 siRNA

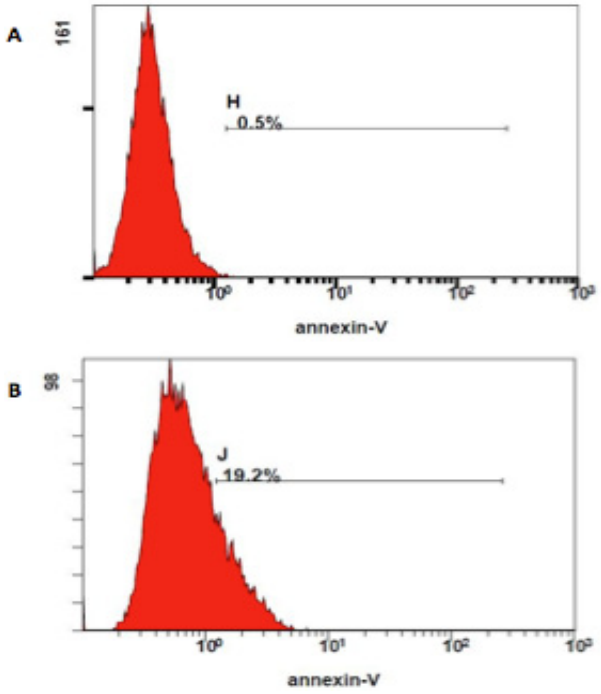

Figure 4. The status of cell apoptosis level was determined by flow cytometry. (A) negative control siRNA group. (B) TUG1 siRNA group

stably transfected into U2OS cells. TUG1 expression was markedly decreased, which was detected by qPCR. Quantification analysis showed that TUG1 expression level was knocked down by nearly 90% in TUG1 siRNA group (Figure 2A). Similarly, the expression level of n377360 was evidently inhibited by n377360 siRNA compared with the nonfunctional control siRNA in U2OS cell as detected by qPCR.

Quantification analysis showed that more than 77% ofn377360 expression was knocked down in n377360 siRNA group (Figure 2B).

TUG1 and n377360 Knocking Down Via siRNA Increased Osteosarcoma Cell proliferation

To further identify the role of TUG1 and n377360 in U2OS cells, we performed functional assays with U2OS cells by suppressing TUG1 and n377360 expression (TUG1 siRNA and n377360 siRNA) in comparison with U2OS cells transfected with nonfunctional control siRNA. The results showed that the U2OS cells in TUG1 or n377360 siRNA transfected group grew significantly slower compared with the cells in the controls group (Figure 3).

TUG1 expression negatively correlates with U2OS cell apoptosis

We compared the apoptosis of U2OS cell with suppressed TUG1 expression group and control group, Figure 4 showed that TUG1 down-regulation significantly promoted U2OS cell apoptosis. Results of these experiments demonstrated that U2OS cell with TUG1 suppression led to enhanced apoptosis in comparison to control group.

Discussion

Osteosarcoma is the most common malignant bone tumors occurring among children and adolescents (Nagarajan et al., 2011). Currently, although certain progress in clinical and basic research of osteosarcomas has been made (Akiyama et al., 2008), molecular genetic mechanism involved in osteosarcomas is still unclear. Moreover objective and reliable diagnostic biomarkers and effective targeted therapeutic agents are also lacking. Therefore, further researches for gene and molecular level remains to be conducted.

In recent years, with the increasing number of studies on the role of lncRNA in cancer, long non- coding RNA has become a new frontier of translational research from molecular biology to cancer clinical (Spizzo et al., 2012). Researchers began to seek for related lncRNA in the occurrence and progression of osteosarcoma, so as to develop new diagnostic markers and therapeutic targets. But at present, researches in this field are still rarely reported (Pasic et al., 2010).

A great number of lncRNA work via binding to polycomb repressive complex 2 (PRC2), such as frequently researched "HOTAIR". lncRNA TUG1 (Taurine Up-regulated Gene 1, TUG1), which is induced by p53, also binds to PRC2, and has a role in repressing specific genes involved in cell-cycle regulation (Khalil et al., 2009). TUG1 was originally identified as a transcript Up-regulated by Taurine, and it was found that knockdown of TUG1 in developing mouse eye blocks retinal development (Young et al., 2005).

Studies have indicated the over expression of TUG1 in bladder cancer, and TUG1 is connected to some characteristics of tumor cells, such as proliferation , apoptosis and so on (Khalil et al., 2009). But there have been no related TUG1 researches reported in osteosarcoma yet. Besides, TUG1 has multiple transcription variants (such as n343050, namely 343050; n377450, also known as TCONS_00029579l; n377360, also known as TCONS_00029410; n377310, also known as TCONS_00029336; n380155, also known as ENST00000521091; n380562, also known as ENST00000540687; n384135, also known as uc003ajf.1) (data from NONCODE website). Compared with other transcription variants above, n377360 contains an extra exon, with the length of about 100bp. So, is there any difference in the expression status of n377360 and of other transcription variants in osteosarcoma?

Therefore, we conducted an expression detection of the common exon sequence of all TUG1 transcription variants and unique exon sequence of n377360. Research results showed that the expression level of TUG1 in osteosarcoma tumor tissue was significantly higher than that of normal tissue adjacent to tumors. The expression of n377360 was in agreement with that of other TUG1 transcription variants. By down regulating TUG1 or n377360 expression in osteosarcoma cells U2OS with siRNA, the proliferation was inhibited. In addition, cells apoptosis was increased when TUG1expression was suppressed. In conclusion, TUG1 and n377360 may act as a new diagnostic marker and therapeutic target of osteosarcoma, and further studies need to be carried out.

Acknowledgements

This study is supported by National Natural Science foundation of China. The Grant number is 81101387. The authors have no conflicts to disclose.

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