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#### **Research Article**

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# TUFT1 promotes osteosarcoma cell proliferation and predicts poor prognosis in osteosarcoma patients

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**Abstract:** Objective: This study is aimed at exploring the role of TUFT1 in osteosarcomas. Methods: We investigated the expression of TUFT1 in osteosarcoma cell lines and explored the correlation between TUFT1 expression and prognosis in osteosarcoma patients based on the expression data downloaded from Gene Expression Omnibus (GEO) website. The effects of TUFT1 on osteosarcoma cell proliferation, migration and invasion were investigated by silencing TUFT1 in osteosarcoma MG63 cell line. Finally, western blot was performed to determine the expression changes of MAPK signaling pathway related proteins after silencing TUFT1. Results: We found that the expression of TUFT1 was significantly up-regulated in osteosarcoma cell lines compared with the normal control. Using Kaplan-Meier analysis, we identified that high TUFT1 expression was positively correlated with poor prognosis in osteosarcoma patients. Furthermore, knockdown of TUFT1 remarkably inhibited MG63 cell proliferation, migration and invasion. Using western blot analysis, we found that the phosphorylation levels of MEK and ERK were reduced obviously in MG63 cells after silencing TUFT1 (p<0.01). Conclusions: Our results demonstrated that TUFT1 plays a promoting role in MG63 cell proliferation and metastasis and has the potential to be a predictor as well as a therapeutic target for osteosarcoma patients.

**Keywords:** Tuftelin, Osteosarcoma, proliferation, migration, MAPK signaling pathway

#### 1 Introduction

Osteosarcoma is the most common malignant bone sarcoma that originates from the mesenchymal tissue and most commonly affects adolescents and children [1, 2]. Until now, researchers have discovered many genes, proteins and signaling pathways that are changed in osteosarcoma [3]. However, due to the complicated pathogenesis of osteosarcoma, the disease process has not yet been clearly elucidated [4]. Currently, the treatment strategy for newly diagnosed osteosarcoma is as follows: neoadjuvant chemotherapy, resection of the primary tumor and all clinically evident metastatic disease by surgery, followed by postoperative chemotherapy [2, 5, 6]. The 5-year-survival-rate of osteosarcoma has somewhat increased due to the progress in treatment technology, but the survival-rate is still very low [7, 8]. Therefore, it is of great significance to explore the molecular mechanism and identify new gene targets of osteosarcoma.

Tuftelin (TUFT1) was firstly identified from developing and mature bovine enamel and is known to play a vital role in enamel mineralization [9, 10]. Furthermore, subsequent studies suggested that TUFT1 may be associated with individual predisposition to tooth hypomineralization [11]. In addition to mineralized tissues, TUFT1 was also found to express in many non-mineralizing soft tissues and cancer cells, suggesting that it may possess various functional roles [12-14]. The higher expression of TUFT1 in the deeper and mineralizing zones has been found in cartilage [15]. In 2017, Eeva Sliz et al. identified that TUFT1 is involved in chondrogenesis on a calcium-related pathway and has the potential to be a novel candidate gene for metatarsophalangeal osteoarthritis [16]. In pancreatic cancer (PC), TUFT1 was overexpressed and took a promoting part in PC cell migration and metastasis [16]. In mouse mesenchymal stem cells (MSCs), TUFT1 was suggested as a member of oxygen-sensitive genes, adjusting MSC function [17]. In the bone marrow of osteoporosis patients, the pool of human MSCs which

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differentiate into osteoblasts was often decreased [18]. However, the precise role of TUFT1 and whether TUFT1 involve in the progression of osteosarcoma remains unclear. In this research, we investigated the expression of TUFT1 in osteosarcoma and its correlation with the prognosis of osteosarcoma patients based on the data downloaded from the Gene Expression Omnibus (GEO) website. Furthermore, the effects of TUFT1 on osteosarcoma cell proliferation, invasion and migration were detected by knockdown of TUFT1. We found that high expression of TUFT1 was associated with poor prognosis in osteosarcoma patients and knockdown of TUFT1 could suppress osteosarcoma cell proliferation, invasion and migration.

#### 2 Methods

#### 2.1 Data collection

The GSE28424 dataset and GSE36001 dataset were all downloaded from the GEO website (http://www.ncbi.nlm. nih.gov/gds/) and used to analyze the expression of TUFT1 in osteosarcoma cells. There were a total of 23 samples in GSE28424 dataset, containing 19 osteosarcoma cell lines and 4 normal bone cell lines. GSE36001 dataset contains 25 samples, including 19 osteosarcoma cell lines and 6 normal samples (osteoblasts and bones). Gene expression profiles of human osteosarcoma and the corresponding prognosis data were also downloaded from GEO, with an access number of GSE16091. This dataset contains 34 human osteosarcoma samples and we used this dataset to analyze the relationship between TUFT1 expression and prognosis of osteosarcoma patients.

#### 2.2 Cell culture

Human osteoblasts cell line hFOB1.19 and human osteosarcoma cell lines MG63 and U2OS were all bought from Shanghai Life Science Cell Resource Center and cultured in RPMI-1640 Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% CO<sub>3</sub>. Single cell suspensions were prepared when the cell went into logarithmic growth phase followed by being seeded into six-well plate for the following experiments.

#### 2.3 Transfection

In order to knockdown TUFT1, cells were transfected with TUFT1 siRNA1 (5'- GUAGCAAGCUUGACAGGAA-3') or TUFT1 siRNA2 (5'- CCUAGGAGAUUCUCUCCAU-3') using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's description. Cells transfected with scramble siRNA (5' -GGACAGUUCGAACAGAUGA - 3') were used as control. Then the transfected cells were cultured for 6 h followed by replacing the medium with fresh complete medium. After 24 h culture, the cells were used to examine the inhibitory efficiency of TUFT1 siRNA or to perform the subsequent experiments.

#### 2.4 RNA extraction and qPCR

Total RNA of osteosarcoma cells was extracted using Ultrapure RNA kit (CwBio, Beijing, China) according to the manufacturer's description. Then reverse transcription was performed to form cDNA using a HiFiScript cDNA Synthesis Kit (CwBio, Beijing, China) following the manufacturer's protocol. The expression of TUFT1 at transcriptional level was detected by qPCR next. The PCR procedure was as follows: 95°C for 5 min, followed by 40 cycles of 5 sec at 95°C, 34 sec at 60°C, and then 72°C for 30 min. The primers for TUFT1 were TUFT1F: 5'-GAACTGGTGTACCCTGGTGG-3' and TUFT1R: 5'-GAATGACCAGCTGAGTGGC-3. Tubulin (F: 5' - GGAGCGAGATCCCTCCAAAAT -3'; R: 5' -GGCTGTTGTCATACTTCTCATGG-3') was used as an internal reference gene. Relative mRNA expression levels were calculated by 2-AACt method. All the experiments were done in triplicate and repeated for 3 independent times.

#### 2.5 Western blot

After 48 h transfection, cells were collected and lysed using RIPA lysate supplemented with protease inhibitor to extract the total proteins. Then 10% SDS-PAGE were performed to separate the proteins. The separated proteins on the gel were electric transferred to a PVDF membrane (Millipore, Bedford, MA). Subsequently, the membranes were blocked in 5% skimmed milk for 1h and incubated with the primary anti-body overnight at 4°C. After being washed by TBST for 3 times, the membrane was incubated with secondary anti-body for 1 h and washed with TBST again. Finally, the signals on the membrane were detected using an Enhanced chemi-luminescence (ECL) plus detection kit (Thermo Fisher Scientifc, Inc.). GAPDH was used as a reference gene. The density of the bands were

analyzed using Quantity One v4.6.9 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

#### 2.6 Colony formation assay

The colony formation ability of osteosarcoma cells transfected with TUFT1 siRNA or scramble siRNA were examined. About 500 cells were added to a 60 mm dish containing 5 mL culture medium. Then the cells were cultured at 37°C with 5%  $\rm CO_2$  for 1-2 weeks. When macroscopic colonies appeared, the colonies were washed by PBS carefully and fixed with 4% paraformaldehyde for 30 min. Next, the colonies were stained with 0.1% crystal violet for 30 min and washed using the running water. Finally, the number of the colonies formed were counted directly. All the experiments were performed in triplicate.

#### 2.7 Cell proliferation

Cell counting kit-8 (Dojindo, Tokyo, Japan) was used to further examine osteosarcoma cell proliferation. After being transfected for 24 h, cells were seeded into 96-well plates with 1,000 cells each well and cultured at 37°C with 5% CO $_2$ . Subsequently, each well was added with 10  $\mu$ l CCK-8 solution and the cells were incubated at 37°C for 1.5 h followed by detecting the optical density (OD) at 450 nm. Graph Pad Prism 5 was used to draw the proliferation curves.

#### 2.8 Scratch healing assay

Scratch healing assay was performed to explore the effect of TUFT1 on osteosarcoma cell migration. After 24 h transfection, cell suspensions were prepared and inoculated into six-well plate with  $5 \times 10^5$  cells/ well. After cultured for 24 h, the wounds were created using a sterile 200 ml micropipette tip. The widths of the wounds were measured and photographed before stimulation (time 0 h) and after 24 h scratching.

#### 2.9 Transwell invasion and migration assays

Transwell invasion assay was performed using transwell chambers ((Millipore, Bedford, MA, USA) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After 24 h transfection,  $1\times10^5$  cells were seeded into the upper chamber and 500  $\mu$ l complete medium were added to the lower chamber followed by incubated at 37°C for 24 h.

Then the cells left on the upper chamber were removed using cotton swabs and cells on the lower surface were fixed with 4% paraformaldehyde and stained by 0.1% crystal violet stain. Finally, the invaded cells were counted and captured under a light microscope.

Transwell migration assay was similar with transwell invasion assay, but no matrigel was needed. A number of 5, 000 transfected cells were added to the upper chamber. All the experiments were performed for three independent times.

#### 2.10 Statistical analysis

Kaplan-Meier method was used to evaluate the prognosis of osteosarcoma patients and log-rank test was used to assess the difference between groups. The samples were divided into high and low expression group according to the median of TUFT1 expression. All the data were presented as mean ± standard deviation. SPSS 22.0 software was used to perform the statistical analysis. Comparison between two groups were analyzed by Student's t test. The difference between multiple groups was compared using one-way ANOVA analysis. P<0.05 was considered as the statistical significance.

**Ethical approval:** The conducted research is not related to either human or animals use.

#### 3 Results

# 3.1 TUFT1 expression was up-regulated in osteosarcoma cell lines

At first, we analyzed the expression of TUFT1 based on the data sets (GSE28424 (containing 23 samples) and GSE36001 (containing 25 samples)) downloaded from the GEO database. We found that the expression of TUFT1 was significantly up-regulated in osteosarcoma cell lines compared with the normal bone cells (p<0.01, Figure 1A-B). We further investigated the expression of TUFT1 in human osteoblasts cell line hFOB1.19 and human osteosarcoma cell lines MG603 and U2OS by qPCR. As shown in Figure 1C, we found that the levels of TUFT1 in MG603 and U2OS cell lines were remarkably elevated compared with that in human osteoblasts hFOB1.19 cells (p<0.05). We choose MG603 cell line for the subsequent experiments due to the expression of TUFT1 in it was the highest among the OS cell lines tested.

## 3.2 High expression of TUFT1 correlated with poor prognosis in osteosarcoma patients

Gene expression profiles of human osteosarcoma and the corresponding prognosis data were downloaded from GEO database (Access number: GSE16091, containing 34 samples). The results of Kaplan-Meier and log-rank test analysis indicated that osteosarcoma patients with TUFT1 high expression showed a significant lower overall survival rate (%) compared with the patients with lower TUFT1 expression (p=0.019, Figure 1D), suggesting that high TUFT1 expression was associated with poor prognosis in osteosarcoma patients.

### 3.3 TUFT1 expression was significantly decreased after transfected with TUFT1 siRNA

TUFT1 siRNA1 and TUFT1 siRNA2 were transfected into MG63 cell line to suppress TUFT1 expression. We could

easily identify that the expression of TUFT1 at both RNA level and protein level were obviously declined in MG63 cells transfected with TUFT1 siRNA1 (si TUFT1 1) or TUFT1 siRNA2 (si TUFT1 2) compared with the scramble siRNA control (sicon, p<0.001, Figure 2). No significant difference was found between the control group and MG63 cells transfected with the scramble siRNA control (data not shown). We used MG63 cells transfected with TUFT1 siRNA2 for the subsequent experiments, due to its higher knockdown efficiency.

## 3.4 Knockdown of TUFT1 suppressed MG63 cell proliferation

After being transfected for 24 h, the effect of silencing TUFT1 on cell proliferation was investigated by colony formation assay and CCK8 assay. As shown in Figure 3B-C, the colony formation rate of MG63 cells was remarkably decreased after silencing TUFT1 (p<0.01). Furthermore, from the CCK8 assay, we found that the OD value in si

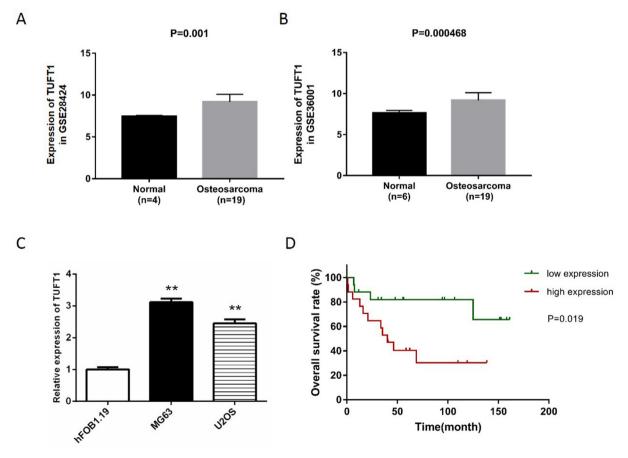


Figure 1. TUFT1 was up-regulated in osteosarcoma cell lines and correlated with poor prognosis of osteosarcoma patients. (A) Expression of TUFT1 in osteosarcoma cell lines based on GSE28424 dataset. (B) Expression of TUFT1 in osteosarcoma cell lines based on GSE36001 dataset. (C) Expression of TUFT1 in osteosarcoma cell lines determined by qPCR. (D) High TUFT1 expression was associated with worse prognosis in osteosarcoma patients. The median was used as the cutoff point of high TUFT1 expression (n=17) and low TUFT1 expression (n=17).

TUFT1 group was obviously lower than that in si-con group at 72 h and 96 h (p<0.01, Figure 3 A). Both the results of colony formation assay and CCK8 assay demonstrated that knockdown of TUFT1 could inhibit MG63 cell proliferation.

# 3.5 Knockdown of TUFT1 inhibited MG63 cell migration and invasion

A wound healing assay was used to investigate the effect of silencing TUFT1 on MG63 cell migratory distance. We found that silencing TUFT1 significantly decreased the migratory distance compared with the si-con group (p<0.01 Figure 4A). Moreover, we also performed a transwell migration assay to further explore the effect of silencing TUFT1 on migratory numbers of MG63 cells. Our results showed that the number of migrated cells in TUFT1 siRNA group was significantly reduced compared with

the si-con group (p<0.01, Figure 4 B-C). All these results suggested that silencing TUFT1 significantly inhibited MG63 cell migration.

The effect of TUFT1 on MG63 cell invasion was investigated using transwell invasion assay. As shown in Figure 4, the number of invaded cells was significantly decreased in TUFT1 siRNA group compared with si-con group (P<0.01), indicating that knockdown of TUFT1 significantly reduced the invasion potential of MG63 cells. Hence, TUFT1 may promote MG63 cell invasion and migration *in vitro*.

# 3.6 Silencing TUFT1 suppressed MAPK signaling pathway

To investigate the possible mechanism of TUFT1 promoted cell proliferation, invasion and migration, we analyzed the levels of proteins in MAPK signaling pathway in

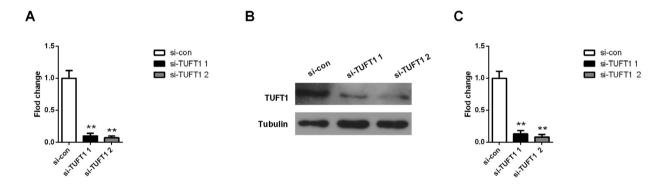


Figure 2. TUFT1 siRNA obviously decreased the expression of TUFT1 in MG63 cells. (A) The expression level of TUFT1 in MG63 cells transfected with TUFT1 siRNA1 and TUFT1 siRNA2 analyzed by qPCR. (B) TUFT1 expression at protein level in MG63 cells transfected with TUFT1 siRNA1 and TUFT1 siRNA2 analyzed by western blot. (C) Quantification of TUFT1 protein expression. Columns, mean (n= 6); bars, SD. \*\*P<0.01, vs. TUFT1 expression in MG63 cells transfected with scramble siRNA.

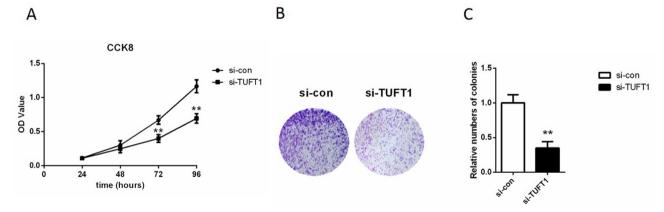


Figure 3. Silencing TUFT1 inhibited MG63 cell proliferation. (A) Colony formation assay was performed to investigate the effect of TUFT1 on MG63 cell proliferation. (B) Quantification of the colonies stained by crystal violet in figure (A). (C) The effect of TUFT1 on OS cell proliferation tested by CCK8 assay. Columns, mean (n= 6,); bars, SD. \*\*P<0.01, vs. MG63 cells transfected with scramble siRNA (si-con).

MG63 cells by western blot after transfected for 24 h. We found that knockdown of TUFT1 significantly reduced the phosphorylation levels of MEK and ERK (p<0.01) in MG63

cells (Figure 5). These data suggested that knockdown of TUFT1 suppressed the activation of MAPK signaling pathway.

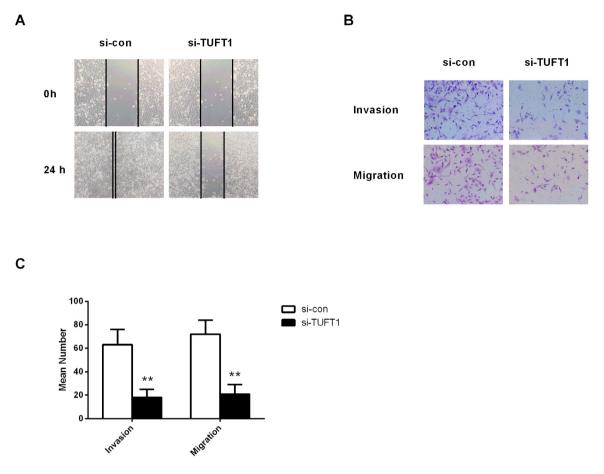


Figure 4. Knockdown of TUFT1 suppressed MG63 cell migration and invasion. (A) The effect of silencing TUFT1 on MG63 cell migration was assessed by wound healing assays after 0 h and 24 h scratching. (B) Representative images of Transwell invasion and migration assays. (C) Quantification of invaded and migrated cells. Columns, mean (n=6); bars, SD. \*\*P<0.01, vs. OS cells transfected with scramble siRNA (si-con).

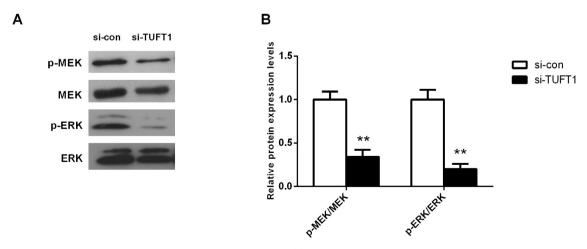


Figure 5. Silencing TUFT1 suppressed MAPK signaling pathway. (A) Western blot analysis of the expression levels of p-MEK, MEK, p-ERK, ERK. (B) Quantification of the ratio of p-MEK/MEK, p-ERK/ERK. Columns, mean (n= 6,); bars, SD. \*\*P<0.01, vs. expressions of these proteins in MG63 cells transfected with scramble siRNA (si-con)

## 4 Discussion

Osteosarcoma, also known as osteogenic sarcoma, is the most common bone sarcoma that originates from the mesenchymal tissue [1, 19, 20]. TUFT1 is a protein that is known to play a role in enamel mineralization and was indicated to possess various function roles since its wide expression in many different types of tissues, including tumor tissues [16]. In the present study, we identified that the expression of TUFT1 was up-regulated in osteosarcoma cell lines and osteosarcoma patients with high TUFT1 expression often exhibited shorter survival time. Furthermore, we found that silencing TUFT1 could inhibit MG63 cells proliferation, invasion and migration, indicating that TUFT1 may play a promoting role in osteosarcoma development. As far as we know, this is the first study to explore the role of TUFT1 in osteosarcoma.

In 2016, Bin Zhou et al. reported that the expression of TUFT1 was elevated in pancreatic cancer tissues compared with the corresponding adjacent normal pancreas tissues and high TUFT1 expression was associated with poor prognosis in pancreatic cancer patients [21]. Similarly, in breast cancer tissues, the level of TUFT1 was also up-regulated and high expression of TUFT1 was positively correlated with poor prognosis [22]. In this study, we found that TUFT1 was also overexpressed in osteosarcoma cell lines compared with the normal control (Figure 1). And osteosarcoma patients with high TUFT1 expression showed shorter survival time (Figure 2). These data suggested that TUFT1 may be involved in the development of osteosarcoma and possess the potential to serve as a prognostic factor for this disease.

To further investigate the biological effects of TUFT1 on MG63 cells, we silenced TUFT1 in MG63 cells by RNAi and explored the influence of TUFT1 depletion on MG63 cell proliferation, invasion and migration. Our results demonstrated that silencing TUFT1 significantly suppressed cell proliferation and metastasis (p<0.01, Figure 3-4). These findings were consistent with the previously observations that obtained in pancreatic cancer cells [21] as well as in T-47D and MDA-MB-231 breast cancer cells [22].

The mitogen-activated protein kinase (ERK/MAPK) signaling pathway has been indicated to involve in many cellular processes including proliferation, migration and apoptosis etc. [23-25]. In our study, we explored the effect of silencing TUFT1 on the expression of proteins in MAPK signaling pathway by western blot. Our results showed that the ratio of p-MEK/MEK and p-ERK/ERK reduced remarkably in TUFT1 silenced MG63 cells compared with that in MG63 cells transfected with scramble siRNA. This

result demonstrated that the MAPK signaling pathway in MG63 cells was significantly inhibited after silencing TUFT1. In breast cancer, TUFT1 was suggested could induce the activation of MAPK signaling pathway, which was in accordance with the results observed in our present study [22]. However, the specific molecular mechanism of how TUFT1 communicates with MAPK signaling pathway is still unclear and needs to be further investigated. Previously, it was demonstrated that treatment with MEK inhibitors (U0126 or PD98059) decreased osteosarcoma cell invasion [26, 27]. Furthermore, the inhibitory effect on cell proliferation of MEK inhibitors has been demonstrated in human melanoma cells [28], and breast cancer cells [29]. Hence, we supposed that TUFT1 may involve in osteosarcoma cell growth and metastasis partially by regulating MAPK signaling pathway.

In conclusion, we illustrated the up-regulation of TUFT1 in osteosarcoma cell lines and found that high TUFT1 expression was positively correlated with poor prognosis in osteosarcoma patients. Knockdown of TUFT1 showed an inhibitory effect on MG63 cell proliferation and motility. Our study indicated that TUFT1 may be a candidate prognosis factor as well as a potential therapeutic target for osteosarcoma. In addition, *in vivo* studies need to be carried out in the future to verify the results we obtained in this study.

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

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