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Original Article

TBL1XR1 promotes migration and invasion in osteosarcoma cells and is negatively regulated by miR-186-5p

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Abstract: TBL1XR1 has been reported to play promoting roles in various malignances, yet little is known about its role in osteosarcoma, and the up-stream molecules regulating TBL1XR1 are also unclear. In the present study, we investigated the clinical significance of TBL1XR1 and its biological role in osteosarcoma, and further explored up-stream miRNAs regulating its expression. The results showed that TBL1XR1 was significantly up-regulated in osteosarcoma cells and tissues by using western blot and immunohistochemical staining. Overexpression of TBL1XR1 was positively related to adverse clinicopathological features and poor prognosis, and which may be an independent prognostic marker for osteosarcoma patients. Functional experiments revealed that down-regulation of TBL1XR1 inhibited proliferation, migration and invasion of osteosarcoma cells. Further studies indicated that TBL1XR1 was a direct target of miR-186-5p, and miR-186-5p negatively regulated TBL1XR1 expression. Moreover, TBL1XR1 was involved in miR-186-5p-repressed migration and invasion in osteosarcoma cells. Taken together, miR-186-5p/TBL1XR1 may be a novel therapeutic candidate target in osteosarcoma treatment.

Keywords: TBL1XR1, osteosarcoma, migration, invasion, miR-186-5p

Introduction

Transducin-(β) like 1 X-linked receptor 1 (TBL1XR1), also known as transducin β-like-related protein 1(TBLR1), was a core component of the SMRT/N-CoR corepressor complexes [1, 2]. It was thought to be involved in multiple biological processes and played important roles in various cellular functions including cell growth, anti-apoptosis, inflammation and gene transcription [2-5]. In addition, accumulating studies suggested that TBL1XR1 was a candidate oncogene which contributed to carcinogenesis and tumor progression. In esophageal squamous cell cancer [6], cervical cancer [7], breast cancer [8], nasopharyngeal cancer [9], colorectal cancer [10] and other malignances [11], TBL1XR1 was consistently up-regulated in cancer tissues and was closely associated with malignant behaviours and poor prognosis. Our group [12] and others [13] also separately demonstrated that TBL1XR1 was highly expressed in gastric cancer and predicted poor prognosis. More importantly, in hepatocelluar carcinoma, an MRI-visible and folate-targeted theranostic small interfering RNA (siRNA) nanomedicine Fa-PEG-gPEI-SPION/psiRNA-TBLR1 could effectively suppressed hepatocellular carcinoma growth and metastasis in vitro and in vivo [14], suggesting that TBL1XR1 was a potential diagnostic marker and an effective therapeutic target. However, little is known about its role in osteosarcoma, a mesenchymal sarcoma, which is the most common malignant bone tumor in children and adolescents with a poor prognosis. Therefore, it is necessary to clarify its clinical and biological significance in osteosarcoma.

MicroRNAs (miRNA) were endogenous, noncoding, single-stranded RNAs, which negatively regulated gene expression by repressing translation via binding to the 3'-untranslated region (3'-UTR) of their target mRNAs [15, 16]. MiRNAs have been explored to act as proto-oncogenic or tumor suppressive role through directly down-regulating the expression of target genes in various tumors including osteosarcoma [17, 18]. For instance, miR-92b promoted osteosarcoma cell proliferation, migration and invasion by targeting RECK [19]. While miR-199a-3p exerted its suppressive effect in the progression of osteosarcoma through targeting AXL [20]. On the other hand, growing evidence indicated that more than 30% of human genes were regulated by miRNAs [21, 22]. A multitude of oncogenes or anti-oncogenes, such as MYC [23], EGFR [24] or PTEN [25], were under control of miRNAs. Our previous studies also found that TMEM16A promoted gastric cancer invasion and metastasis, and which was suppressed by microRNA-381 (miR-381) [26, 27]. As for TBL1XR1, a candidate oncogene, which was indicated as a direct target of miR-130a-3p in gastric cancer, and which was involved in miR-130a-3p mediated suppression of tumorigenesis and metastasis [28]. However, the upstream miRNAs regulating TBL1XR1 expression in cancer cells remain unclear.

In this study, we investigated the clinical and biological significance of TBL1XR1 in osteosarcoma, and explored its up-stream regulating miRNA. TBL1XR1 was up-regulated in osteosarcoma, and high expression level of TBL1XR1 was notably associated with adverse clinicopathological features and poor prognosis. Inhibition of TBL1XR1 abrogated proliferation, colony formation, migration and invasion of osteosarcoma cells. Additionally, we identified microRNA-186-5p (miR-186-5p) as a negative regulator of TBL1XR1 and validated that TBL1XR1 was a direct target of miR-186-5p. Moreover, TBL1XR1 was involved in miR-186-5p-repressed migration and invasion in osteosarcoma cells.

Materials and methods

Tissue specimens and patients

Paraffin-embedded tissue blocks were retrieved from the Department of pathology, the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, which including 72 osteosarcoma and 20 matched adjacent non-tumor tissues. None of the patients had received preoperative radiotherapy or chemotherapy before biopsy. Standard postsurgical chemotherapies were performed depending on the severity of the disease and according to the National Comprehensive Cancer Network (NCCN) guidelines. The clinical and pathological parameters are shown in **Table 1**. A written informed consent was obtained from all the patients at the time of admission, with which the tissue, blood and other samples might be used for scientific research, but it had not association with patient's privacy. The study's protocol was approved by the Institute Research Medical Ethics Committee of the First Affiated Hospital, Sun Yat-sen University.

Cell lines and transfection

All the osteosarcoma cell lines (U2-OS, 143B, MG63, HOS) and human osteoblast cell (hFOB) were obtained from Cell Banks at the Department of pathology, the First Affiliated Hospital of Sun Yat-sen University. Cells were grown in Minimum Essential Medium (MEDM) or Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, US) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C with humidified 5% CO_2.

Cells transfected with SiRNA, miR-mimic, miR-Control (miR-NC) or pcDNA3.1 vector by using Lipofectamine RNAiMax Transfection Reagent (Invitrogen, US). The SiRNA-TBL1XR1, miR-186-5p mimic and miR-NC were synthesized by Ribobio (Guangzhou, China). The sequence of SiRNA-TBL1XR1 is: sense 5'-CCUCUCAGAUGAUGAUAAATT-3', anti-sense 5'-CUUUAUCAUCAUCUGAGACCTT-3'. The coding sequences of TBL1XR1 were amplified by PCR and inserted into pcDNA3.1 vector (Invitrogen, US) to generate TBL1XR1 overexpression vectors.

RNA extract and quantitative real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, US) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA by using RevertAid First Strand cDNA Synthesis Kit (Thermo, US), and quantitative real time PCR was performed using SYBR Green PCR Master Mix (Thermo, US). The sequences of the PCR primers were as follows: TBL1XR1 sense, 5'-CGAGAAGACCCCAAACCAAATG-3', and anti-sense, 5'-TGGTGGACCTGCTTTGCTGTT-3'; miR-186-5p sense, 5'-AAACAAACAUGGUGCACUUCUU-3', and anti-sense, 5'-GAAGUGCACCAUCUUUGUUUUU-3'.

Immunohistochemistry and evaluation

Immunohistochemical (IHC) staining was performed using a standard streptavidin-biotin-

Table 1. Correlation between TBL1XR1 expression with clinicopathological parameters of osteosarcoma patients

Variable	All Cases	TBL1XR1 expression		P value[1]
		Low	High	
Gender				
Male	42	11 (26.2%)	31 (73.8%)	0.964
Female	30	8 (26.7%)	22 (73.3%)	
Age at surgery				
< 20 y	57	13 (22.8%)	44 (77.2%)	0.179
≥ 20	15	6 (40.0%)	9 (60.0%)	
Tumor size				
≥ 5 cm	48	10 (20.8%)	38 (79.2%)	0.719
< 5 cm	24	9 (37.5%)	15 (62.5%)	
Metastasis				
Present	29	3 (10.3%)	26 (89.7%)	0.011
Absent	43	16 (37.2%)	27 (62.8%)	
Enneking staging				
IA+IIA	27	11 (40.7%)	16 (59.3%)	0.032
IIB+IIIA	45	8 (17.8%)	37 (82.2%)	
ALP level				
< 135	23	7 (30.4%)	16 (69.6%)	0.594
≥ 135	49	12 (24.5%)	37 (75.5%)	
Histology type				
Conventional OS	56	13 (23.2%)	43 (76.8%)	0.253
Others	16	6 (37.5%)	10 (62.5%)	

[1]Chi-Square test; OS osteosarcoma.

peroxidase complex method as described previously [12]. The primary antibody was rabbit monoclonal antibody against human TBL1XR1 (1:100, Abcam). Brown nuclear immunoreactivity for TBL1XR1 protein was regarded as positive staining. TBL1XR1 expression level was evaluated by integrating the percentage of positive tumor cells and the intensity of positive staining. The intensity of staining was scored as follows: negative (score 0), weak (score 1), moderate (score 2) and strong (score 3). We scored the staining percentage according to the percentage of positive stained cells in the field: 0-5% (score 0), 6-25% (score 1), 26%-50% (score 2), 51%-75% (score 3) and 76%-100% (score 4). The sum of the intensity and extent score was considered as the overall IHC score (values from 0 to 12). Then, we classified osteosarcoma patients into two groups according to their TBL1XR1 expression: the high-expression group with a scoring index ≥ 6 (median score of TBL1XR1 expression; n=53) and the low-expression group with a scoring index < 6 (n=19).

Western blot assay

Total protein from cell lines was extracted in RIPA lysis buffer, and then size-fractionated using SDS polyacrylamide gel electrophoresis. After that, protein was transferred to polyvinylidene difloride membrane. The membrane was then blocked and incubated with primary antibody against TBL1XR1 (1:1000, Abcam), After blotting overnight at 4°C, the membrane was incubated with secondary antibody for 1 h at room temperature. The expression of proteins was then tested by enhanced chemiluminescence system. GAPDH was used as the internal reference.

Plate colony formation assay

Cells were trypsinized, counted and plated onto 6-well plates and cultured for 2 weeks. At the end-point, the cells were washed and stained with crystal violet. The number of colonies in 10 random view fields was counted under a microscope and the average number of colonies was achieved.

Cell proliferation assay

Cell proliferation rates were measured using Cell Counting Kit-8 (CCK-8) (Boster, China). Cells were seeded in each 96-well plate for 24 h, transfected with the indicated siRNA, and further incubated for 1 d, 2 d, 3 d respectively. 10 μl CCK-8 reagent was added to each well at 1 h before the endpoint of incubation. OD450 nm value in each well was detected by a microplate reader.

Cell migration and invasion assays

Cell migration and invasion were assessed by wound healing assays and transwell assays as previously described [26]. Briefly, for wound healing assay, cells were cultured in 6-well plates with serum-free culture medium. A scratch lesion was created using a 200 μl pipette tip. Then, culture medium was removed and wells were washed gently with PBS to avoid dislodged cells. The wound was photographed immediately and again at 24 h and 36 h after scraping to document cellular migration across the wound. For migration assay, 1×10^5 transfected cells were plated in serum-free culture

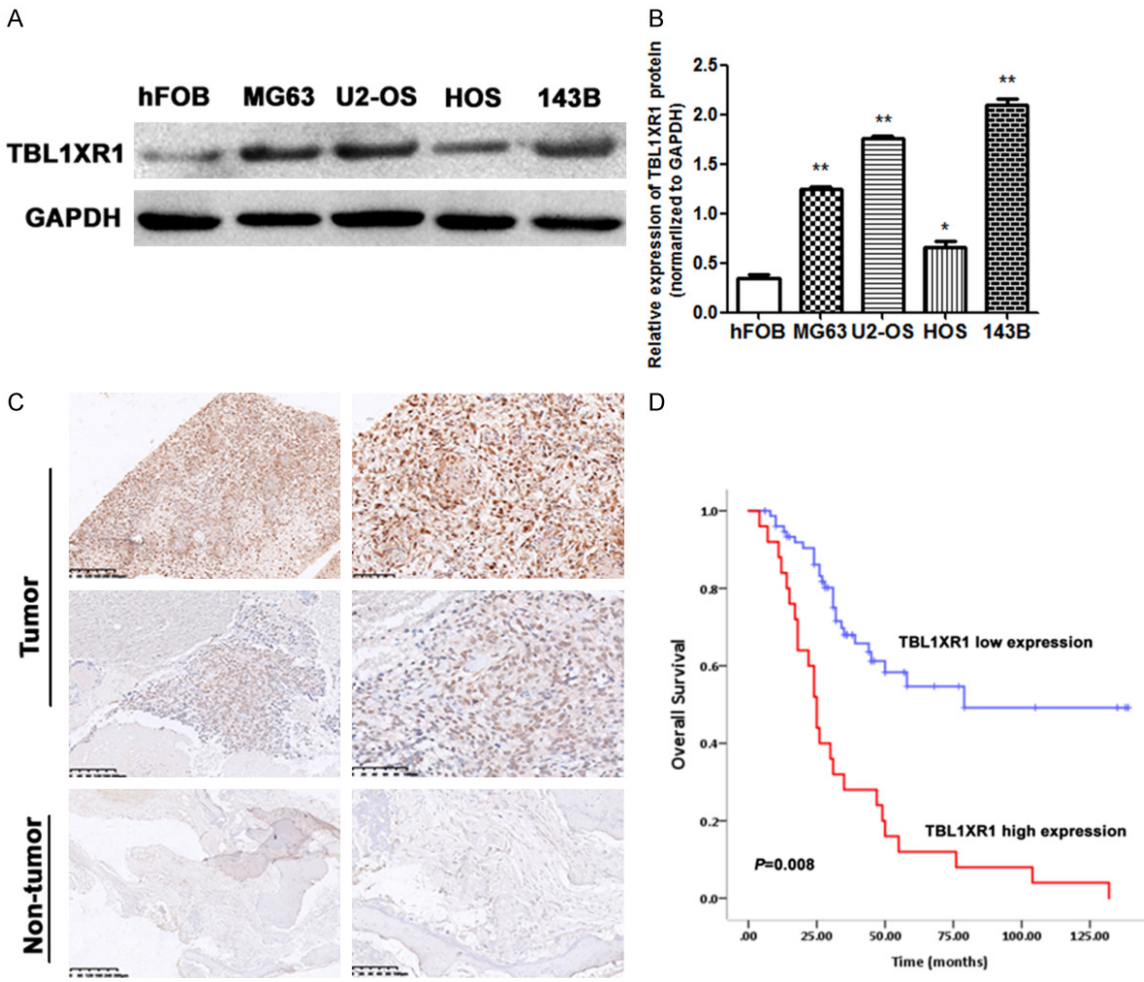

Figure 1. TBL1XR1 is highly expressed in osteosarcoma and predicts poor prognosis. TBL1XR1 protein (A) and mRNA (B) level was elevated in osteosarcoma cells. $^{**}P < 0.01$, $^{*}P < 0.05$. (C) Immunohistochemical staining indicated that TBL1XR1 protein was up-regulated in osteosarcoma tissues. (D) Overexpression of TBL1XR1 predicts poor prognosis of osteosarcoma patients. (P=0.008).

medium in the upper chamber of 24-well Transwell Chambers (Corning, USA), while medium containing 10% fetal bovine serum were added to the lower chamber as a chemoattractant. After the cells were incubated for 48 h, cells adhering to the lower surface were stained by crystal violet, then was counted. For cell invasion assay, the procedure was similar to the cell migration assay, except that the Transwell membranes were precoated with 0.25 mg/mL Matrigel (Corning, USA).

Luciferase reporter assay

The wild-type TBL1XR1-3'UTR (WT) and mutant TBL1XR1-3'UTR (MUT) containing the putative bingding site of miR-186-5p were chemically synthesized and cloned into the pmiR-RB-Report vector (Ribobio, China) respectively. Osteosarcoma cells co-transfected with the constructed vector and miR-186-5p mimic. Then, cells were subjected to luciferase reporter assay as previously described [27].

Statistical analysis

Statistical analysis was conducted by using SPSS Standard version 21.0 and GraphPad Prism 6. We assessed the association of TBL1XR1 protein expression with clinicopathologic features by the Chi-square test or Fisher's exact test. For univariate survival analysis, Kaplan-Meier analysis was employed. We used the log-rank test to compare different survival curves. The multivariate Cox proportional hazards regression model was utilized to assess

Table 2. Multivariate analysis on overall survival (Cox regression model)

Variable	Hazard ratio	95% confidence interval	*P* value
Tumor size[a]	1.780	1.238-1.872	0.015
Metastasis[b]	1.427	1.344-2.322	0.013
Enneking staging[c]	1.322	1.658-2.763	0.001
TBL1XR1 expression[d]	0.366	0.349-0460	0.021

a ≥ 5 cm vs < 5 cm; b prsent vs absent; c IA+IIA vs IIB+IIIA; d high expression vs low expression.

the potential independent prognostic factors and 95% CI of hazard ratios. *P* value of < 0.05 was considered statistically significant.

Results

TBL1XR1 is highly expressed in osteosarcoma cell lines and tissues

To explore the expression type of TBL1XR1 in osteosarcoma, we first detected the protein and mRNA levels of TBL1XR1 in osteosarcoma cell lines (MG63, U2-OS, 143B, HOS) and osteoblast cells (hFOB) by using western blot and qRT-PCR assay. As shown in **Figure 1A**, **1B**, TBL1XR1 was highly expressed in almost all osteosarcoma cells at both protein and mRNA levels compared to osteoblast cells. Particularly, its expression was found to be most elevated in U2-OS and 143B cells. Then, we validate the expression type of TBL1XR1 in 72 osteosarcoma tissues and corresponding adjacent non-tumor tissues. As expected, high expression of TBL1XR1 protein was observed in 53/72 (74.5%) cases of osteosarcoma tissues, which was markedly higher than that in adjacent non-tumor tissues (**Figure 1C**). The expression of TBL1XR1 was mainly located in the nucleus, and in just a few cases (4/72) in the cytoplasm. All the above results indicated that TBL1XR1 was highly expressed in osteosarcoma.

TBL1XR1 expression is associated with adverse clinicopathological features and poor prognosis

We next examined the potential clinical significance of TBL1XR1 in osteosarcoma. Based on the results of immunohistochemical staining of TBL1XR1 in osteosarcoma tissues, the association between TBL1XR1 expression and clinicopathological features was analyzed. As shown in **Table 1**, high expression level of TBL1XR1 was positively associated with present metastasis and advanced Enneking stage (*P*=0.011, *P*=0.032, respectively). However, the expression level of TBL1XR1 was not significantly associated with gender, age at surgery, tumor size, ALP level and histological type. Kaplan-Meier survival analyses showed that osteosarcoma patients with high TBL1XR1 expression had a significantly shorter overall survival time than those patients with low TBL1XR1 expression (*P*=0.008) (**Figure 1D**). Moreover, multivariate cox regression analysis identified TBL1XR1 expression as an independent prognostic factor for osteosarcoma patients (hazard ratio, 0.366; 95% confidence interval, 0.349-0.460; *P*=0.021) (**Table 2**). In addition, cytoplasmic staining of TBL1XR1 had no significant association with clinicopathologic parameters and patients' prognosis (data not shown). Taken together, we speculated that TBL1XR1 might play a critical role in osteosarcoma progression.

Knockdown of TBL1XR1 expression inhibits osteosarcoma cells proliferation and colony formation

To further investigate the biological function of TBL1XR1 in development and progression of osteosarcoma, we first down-regulated the expression of TBL1XR1 in 143B and U2-OS cells, which expressed higher level of TBL1XR1, by using SiRNA-TBL1XR1 (Si-TBL1XR1). The efficiency of Si-TBL1XR1 was confirmed by western blot (Figure S1). Then, CCK-8 assay was employed to check the influence of Si-TBL1XR1 on the proliferation of osteosarcoma cells. The results showed that TBL1XR1 knockdown significantly suppressed 143B and U2-OS cells proliferation compared with the control groups (**Figure 2A**). Then, colony formation assay demonstrated that the mean number of colony formation in 143B and U2-OS cells transfected with Si-TBL1XR1 was significantly less than that in control groups (**Figure 2B**).

Downregulation of TBL1XR1 abrogates invasion and migration of osteosarcoma cells

Scratch wound healing and transwell chamber assay were conducted to clarify the effect of TBL1XR1 expression on migration and invasion of osteosarcoma cells. As shown in **Figure 3A**,

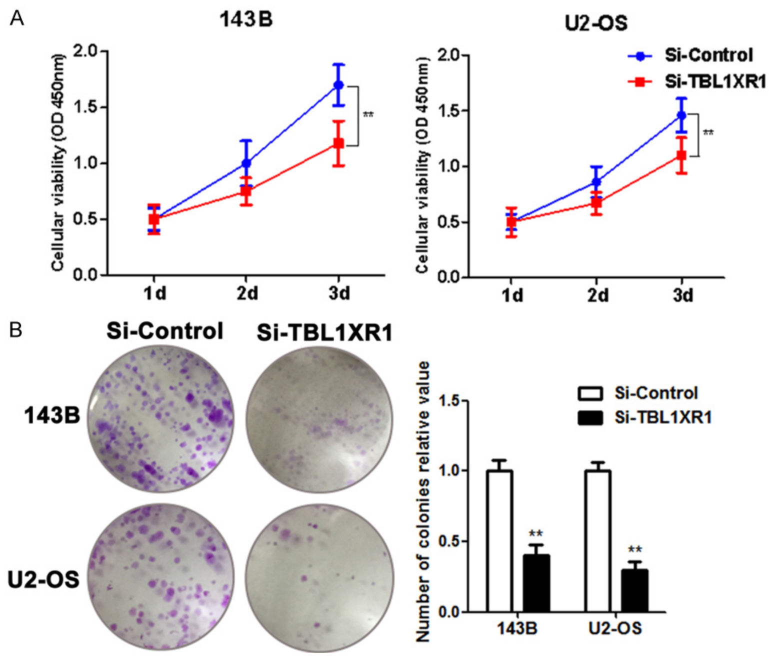

Figure 2. Knockdown of TBL1XR1 expression inhibits osteosarcoma cells proliferation and colony formation. A. CCK-8 assay showed that down-regulation of TBL1XR1 by using Si-TBL1XR1 abrogated proliferation in 143B and U2-OS cells. $^{**}P < 0.01$. B. Plate colony formation assay demonstrated that the mean number of colony formation in 143B and U2-OS cells transfected with Si-TBL1XR1 was significantly less than that in control groups. $^{**}P < 0.01$.

scratch wound healing assay showed that cell migration was dramatically inhibited in 143B and U2-OS cells transfected with Si-TBL1XR1 compared with control groups at 24 and 36 hours, respectively. To further confirm the role of TBL1XR1 in osteosarcoma cell migration, we carried out a transwell migration assay, and found that the mean number of migrated cells was significantly lower in Si-TBL1XR1 transfected 143B cells than that in control group. Furthermore, transwell matrix penetration assay showed that downregulation of TBL1XR1 expression notably reduced the ability of 143B cells to invade through the Matrigel matrix and the number of 143B cells from Si-TBL1XR1 group that invaded through the Matrigel matrix was significantly decreased as compared with control (**Figure 3B**). Similar results were found in U2-OS cells (**Figure 3C**).

TBL1XR1 is a direct target of miR-185-5p

To explore the candidate miRNAs regulating TBL1XR1, we first predicted the putative target sites of miRNAs in 3'-UTR of TBL1XR1 using target prediction algorithms, TargetScan, miRanda and Starbase (Figure S2A). Based on the data from different prediction software, we focused on miR-186-5p which had 8 potential binding sites in 3'-UTR of TBL1XR1 mRNA (Figure S2B). Moreover, overexpression of miR-186-5p induced a strong decrease of TBL1XR1 expression in 143B and U2-OS cells (**Figure 4A**). To further validate interaction between TBL1XR1 and miR-186-5p, we performed luciferase reporter assay with a vector encoding the total sequence of the 3'-UTR of TBL1XR1 mRNA, or a vector encoding the mutant 3'-UTR ofTBL1XR1 mRNA lacking the predicted miR-186-5p target site. The putative target site of miR-186-5p in the 3'-UTR of TBL1XR1 is illustrated in **Figure 4B**. The results showed that the presence of miR-186-5p led to a significant reduction in the relative luciferase activity of the wild-type (WT) construct of TBL1XR1 3'-UTR in 143B and U2-OS cells. However, the mutant (MUT) construct of TBL1XR1 3'-UTR abolished such the suppressive effect of miR-186-5p (**Figure 4C**). The above results demonstrated that TBL1XR1 is the direct target of miR-186-5p.

MiR-186-5p is downregulated in osteosarcoma cells and inhibits cell proliferation, migration and invasion

Based on our results that TBL1XR1 was a direct target of miR-186-5p, we attempted to evaluate the expression level of miR-186-5p in osteosarcoma cells. First, we downloaded microRNA array expression profile datasets GSE28-423 from the open Gene Expression Omnibus (GEO) database. Bioinformatics analysis found that miR-186-5p was significantly down-regulated in osteosarcoma cell lines compared with normal bones ($P < 0.001$) (**Figure 5A**). The results were further validated through using qRT-PCR (**Figure 5B**).

To further assess the biological roles of miR-186-5p in osteosarcoma, CCK8 assay and transwell chamber assay was conducted in 143B and O2-OS cells which transfected miR-186-5p mimic or miR-NC (control group). The

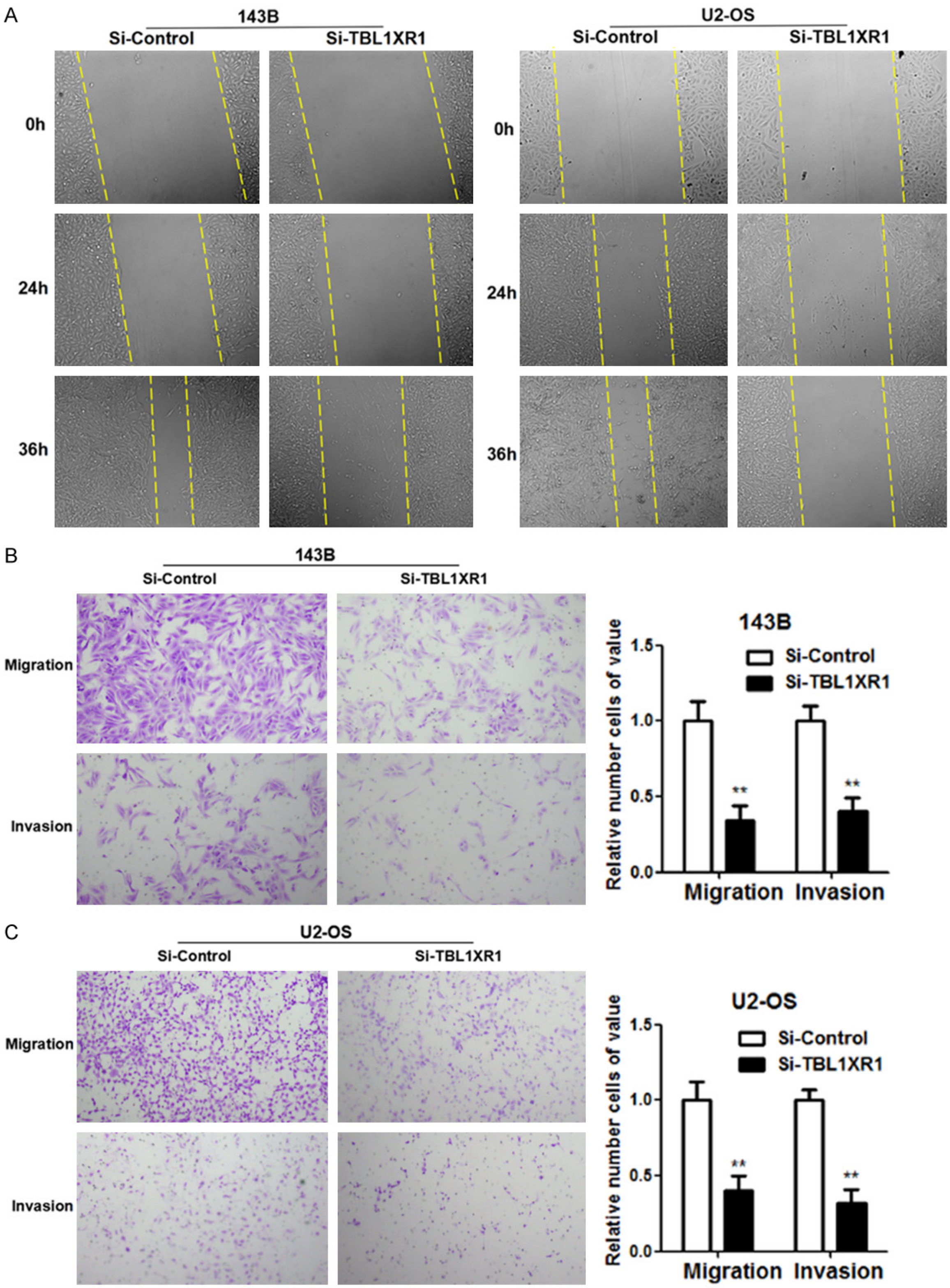

Figure 3. Downregulation of TBL1XR1 inhibits invasion and migration of osteosarcoma cells. A. Wound healing assay showed that cell migration was dramatically inhibited in 143B and U2-OS cells when TBL1XR1 was downregulated. B, C. Transwell assay revealed that the ability of migration and invasion was significantly inhibited in 143B and U2-OS cells when transfected with Si-TBL1XR1. ***P* < 0.01.

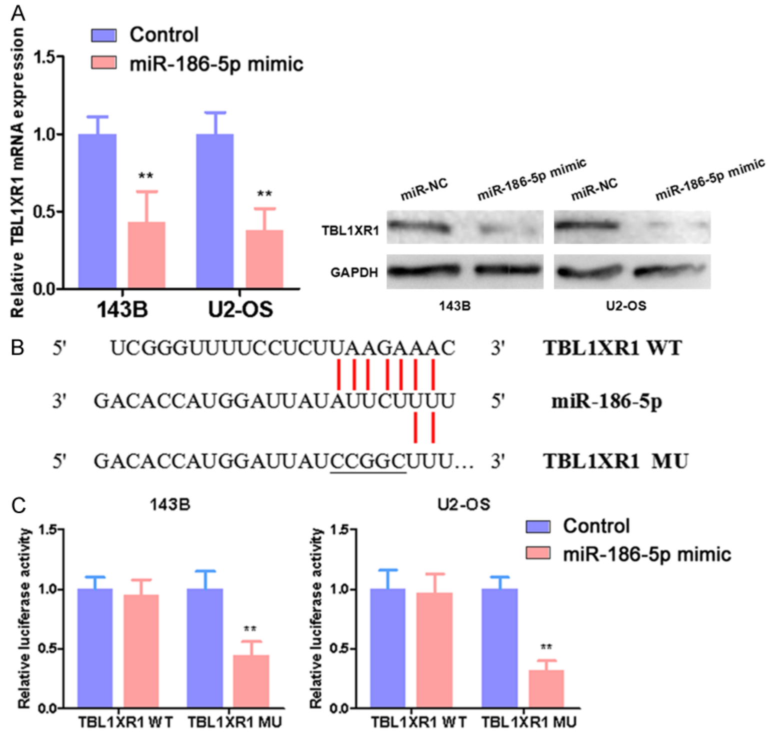

Figure 4. TBL1XR1 is a direct target of miR-185-5p in osteosarcoma cells. A. Overexpression of miR-186-5p markedly suppressed the mRNA and protein levels of TBL1XR1 in osteosarcoma cells. $**P < 0.01$. B. MiR-186-5p and its putative binding sequence in the wild-type (WT) and mutant (MU) 3'-UTR of TBL1XR1. C. Overexpression of miR-186-5p significantly decreased the luciferase activity that carried wild-type (WT) but not mutant type (MU) 3'-UTR of TBL1XR1 in osteosarcoma cells. $**P < 0.01$.

results showed that the proliferation of osteosarcoma cells were notably suppressed in miR-186-5p mimic group compared to miR-NC group (**Figure 5C**). Moreover, migrated and invaded cell numbers were significantly decreased in miR-186-5p mimic-transfected 143B and U2-OS cells in comparison with the controls (**Figure 5D**).

TBL1XR1 mediates the functional effects of miR-186-5p on migration and invasion in osteosarcoma cells

Given that the negative regulatory effect of miR-186-5p on TBL1XR1, we further to ask whether miR-186-5p exert its negative role in osteosarcoma through downregulating TBL1-XR1. First, we transduced miR-186-5p mimic or negative control and overexpression of TMEM16A plasmids. As expected, overexpression of miR-186-5p reduced the TBL1XR1 protein expression, while co-transfection of TBL1-XR1-overexpressing plasmids could restore the TBL1XR1 protein expression (**Figure 6A**). Furthermore, transfection of TBL1XR1-overexpressing plasmid significantly reversed the inhibition of migration and invasion in 143B and U2-OS cells induced by the miR-186-5p overexpression (**Figure 5D**). These results indicated that miR-186-5p inhibits osteosarcoma cell migration and invasion partially by downregulating TBL1XR1.

Discussion

TBL1XR1, a candidate oncogene, have been widely investigated as a potential prognostic marker and therapeutic target in various solid tumors [11]. In the current study, the clinical and biological role of TBL1XR1 has been detected in osteosarcoma, and the up-stream miRNA regulating TBL1XR1 also has been uncovered. The results showed that: (1) TBL1XR1 was highly expressed in osteosarcoma cells and tissues, (2) overexpression of TBL1XR1 protein was closely related with adverse clinicopathlogical features, such as present metastasis and advanced Enneking stage, (3) high expression level of TBL1XR1 predicted poor prognosis, and which may be severed as an independent prognostic factor for osteosarcoma patients, (4) inhibition of TB-L1XR1 abrogated proliferation, colony formation, migration and invasion in osteosarcoma cells, (5) TBL1XR1 was a direct target of miR-186-5p, which negatively regulating TBL1XR1 expression, (6) miR-186-5p suppressed osteosarcoma cells migration and invasion, (7) TBL1XR1 was involved in miR-186-5p induced decreasing ability of migration and invasion of osteosarcoma cells.

Accumulating evidence indicated that TBL1-XR1 expression was up-regulated in multiple tumors, and functioned as a tumor promoter by regulating proliferation, apoptosis, angiogenesis, migration and invasion in various cancer

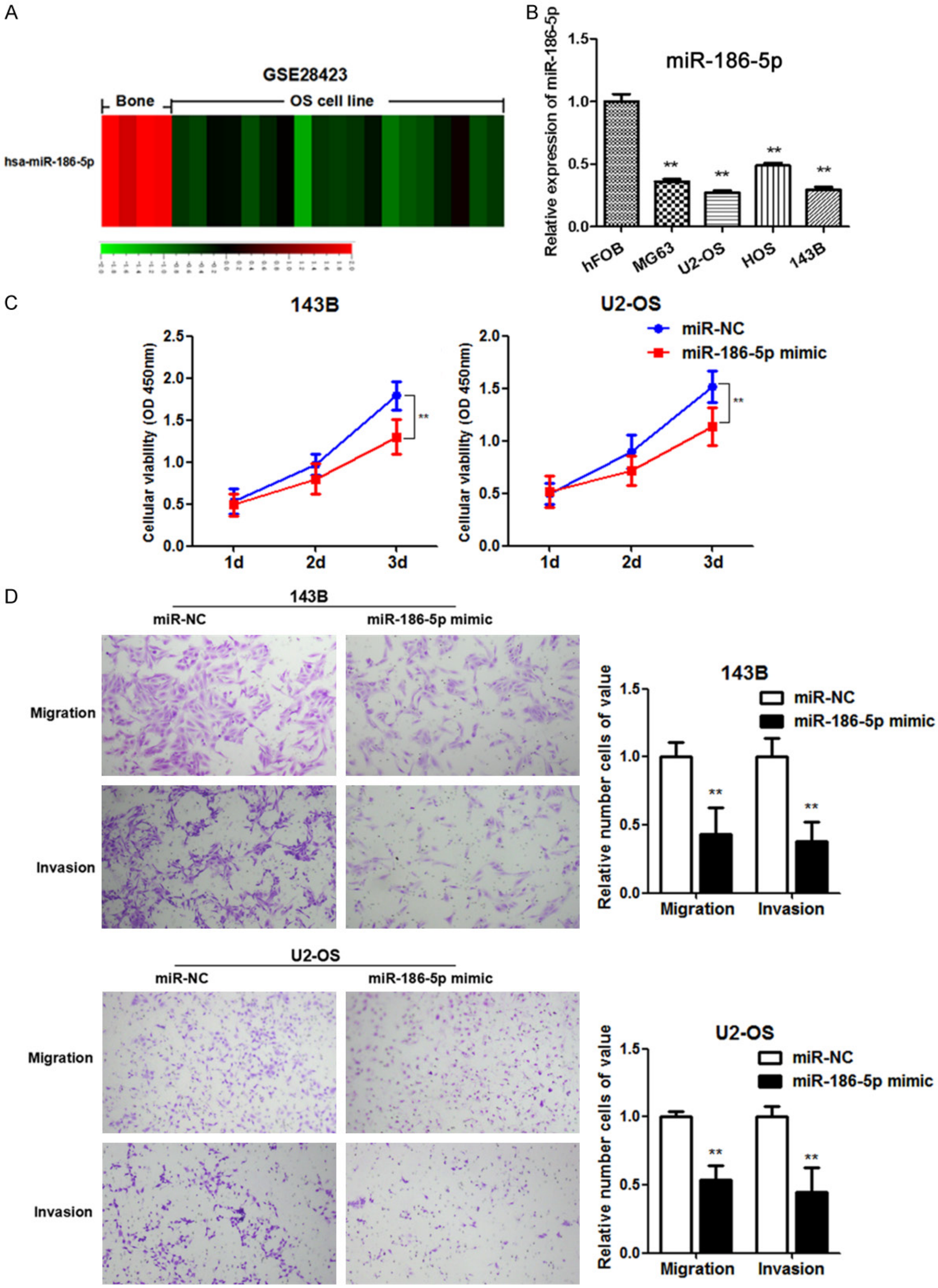

Figure 5. MiR-186-5p is downregulated in osteosarcoma cells and inhibits cell migration and invasion. A. Bioinformatic analysis of the level of miR-186-5p expression of GSE2842 dataset showed that miR-186-5p was significantly lower in osteosarcoma cell lines (T) than in non-tumor bone tissues. $P < 0.01$. B. QRT-PCR analysis validated the lower expression of miR-186-5p in osteosarcoma cells. $^{**}P < 0.01$. C. MiR-186-5p mimic significantly abrogated the proliferation of 143B and U2-OS cells by using CCK assay. $^{**}P < 0.01$. D. Overexpression of miR-186-5p inhibited migration and invasion of 143B and U2-OS cells through transwell assay. $^{**}P < 0.01$.

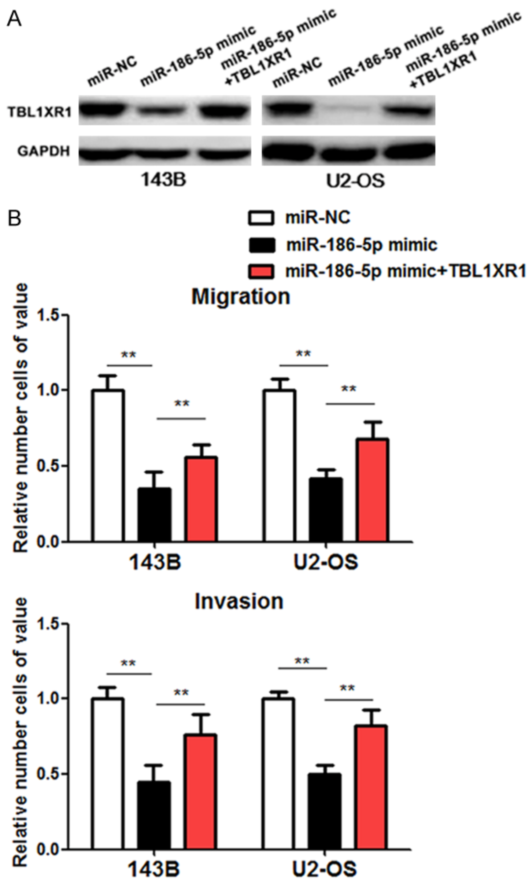

Figure 6. TBL1XR1 mediates the functional effects of miR-186-5p on migration and invasion in osteosarcoma cells. A. Western blot analysis showed that co-transfection of TBL1XR1 overexpression plasmids could recover decreased TBL1XR1 expression which was induced by ectopic expression of miR-186-5p. B. Overexpression of TBL1XR1 could partially revert the suppressive effects of miR-186-5p overexpression on the migration and invasion of osteosarcoma cells. $^{**}P < 0.01$.

types. For example, in hepatocellular carcinoma, TBL1XR1 (TBLR1) positively regulated cancer cell proliferation, antiapoptosis, and angiogenesis, and which was proved to be a great potential prognostic marker and gene therapeutic target [14]. In esophageal squamous cell carcinoma, TBL1XR1 expressed higher in cancer tissues and promoted lymphangiogenesis and lymphatic metastasis in vitro and in vivo [6]. Similar results were illustrated in breast cancer [8, 29], cervical cancer [7] and gastric cancer [13]. Consistent with previous studies, we demonstrated that TBL1XR1 was up-regulated in osteosarcoma, a malignant mesenchymal sarcoma, and inhibition of TBL1XR1 suppressed proliferation, migration and invasion of osteosarcoma cells. These data suggested that overexpression of TBL1XR1 may act as a common mechanism to promote tumorigenesis in different types of malignancies. However, in prostate cancer, nuclear TBL1XR1 was a transcriptional coactivator of androgen receptor and functioned as a tumor suppressor [30]. Whereas the subsequent study from the same group indicated that cytoplasmic TBL1XR1 was elevated in prostate cancer cells and promoted growth, migration and invasion, paralleled with reducing apoptosis [31]. These findings revealed that TBL1XR1 may be a multifunctional protein depending on its subcellular localization. In our present study, cytoplasmic staining was detected in just a few of cases, and which has no association with clinical features. The role of cytoplasmic TBL1XR1 in osteosarcoma needs to be further investigated.

Although the role of TBL1XR1 and its related mechanism have been well explored, the upstream regulators of TBL1XR1 remain largely unknown. MiRNAs played critical role in post-transcriptional or translational regulation of gene expression [21]. To date, just a few studies focus on the miRNAs regulating TBL1XR1. For instance, in lung squamous cell carcinoma, TBL1XR1 was predicted to be one of targets of miR-205, but it was needed to be further validated [32]. Wang et al [28] first reported that TBL1XR1 expression was suppressed by miR-130a-3p in gastric cancer, and which was involved in miR-130a-3p mediated suppression of tumorigenesis and metastasis. However, each gene might be regulated by multiple miRNAs, while one given miRNA could target many genes [33]. In this regard, we conducted searches and validation of miRNAs that regulating TBL1XR1 based on bioinformatic analysis, and found that miR-186-5p negatively regulated the expression of TBL1XR1 in osteosarcoma cells. In fact, besides miRNAs, several potential long noncoding RNA (lncRNA) sequences were identified in the region encompassing the TBL1XR1 gene [34]. In addition, the active status of TBL1XR1 was also modulated by posttranslational modifications, including phosphoration and sumoylation [35, 36]. Nevertheless, further works are needed to uncover the mechanism involved in regulating TBL1XR1.

MiR-186-5p, located in chromosomal 1p31.1, has been reported to be dysregulated in various cancer types and contribute to the development of cancers. In glioblastoma multiforme [37] and hepatocellular carcinoma [38], miR-186-5p was down-regulated in tumor cells and acted as a tumor suppressor to inhibit tumorigenesis. On the contrary, the expression of miR-186-5p was elevated in prostate cancer [39] and colorectal cancer [40], and which played oncogenic role in cell proliferation and invasion. Therefore, the functional role of miR-186-5p is cancer type-dependent. Indeed, large numbers of studies have found that miR-186 played its promoting or inhibting role in cancer depending on the cancer type and stage of the disease. For instance, miR-186 suppressed cancer progression in lung cancer [41], renal cell carcinoma [42], whereas it exerted oncogenic role in pancreatic carcinoma [43] and bladder cancer [44]. Although the specific form of miR-186 has not been clarified in these studies, the predominant form of miR-186 is miR-186-5p. In our study, we investigated the expression type and functional role of miR-186-5p in osteosarcoma, and found that miR-186-5p expression was decreased in osteosarcoma cells, and abrogated tumor cells migration and invasion, suggesting that miR-186-5p may serve as a tumor suppressor in osteosarcoma.

Conclusions

In this study, we demonstrated that TBL1XR1 is highly expressed in osteosarcoma and its upregulation is closely related to poor prognosis of osteosarcoma patients. Functional studies showed that TBL1XR1 may serve as a tumor promoter to stimulate proliferation, migration and invasion in osteosarcoma. Additionally, we identified that TBL1XR1 is a direct target of miR-186-5p, and which mediates the suppressive role of miR-186-5p in migration and invasion in osteosarcoma cells. Collectively, miR-186-5p/TBL1XR1 pathway may be a novel therapeutic candidate target in osteosarcoma treatment.

Disclosure of conflict of interest

None.

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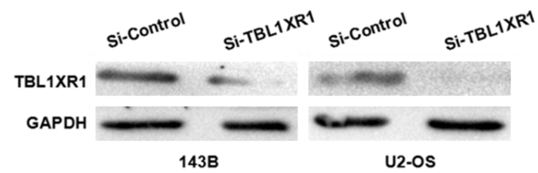
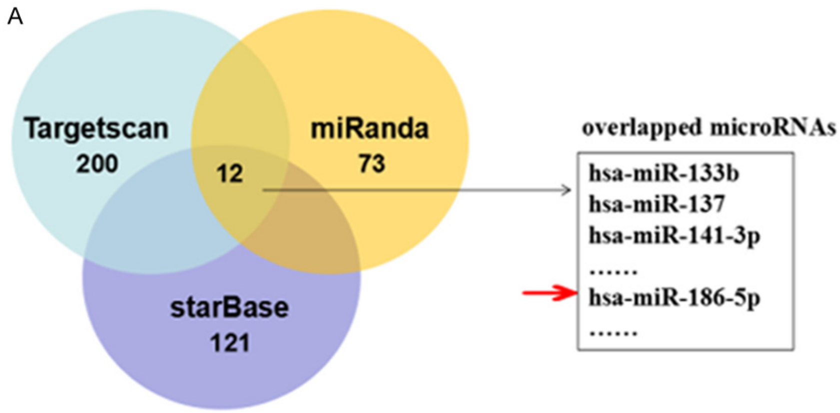


Figure S1. SiRNA-TBL1XR1 could effectively suppress expression of TBL1XR1 in osteosarcoma cells. Western blot assay cofirmed that transfection of SiRNA-TBL1XR1 (Si-TBL1XR1) inhibited TBL1XR1 expression in 143B and U2-OS cells.

A



B

	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 383-389 of TBL1XR1 3' UTR hsa-miR-186-5p	5' ...GACACCAUGGAUUAUAUUCUUUU... 3' UCGGGUUUUCCUCUUAAGAAAC
Position 563-570 of TBL1XR1 3' UTR hsa-miR-186-5p	5' ...CCUUUUAAACAAAAAAUUCUUUA... 3' UCGGGUUUUCCUCU-UAAGAAAC
Position 585-591 of TBL1XR1 3' UTR hsa-miR-186-5p	5' ...AAAAUAUUUUAAUGCAUUCUUUU... 3' UCGGGUUUUCCUCUUAAGAAAC
Position 1141-1147 of TBL1XR1 3' UTR hsa-miR-186-5p	5' ...AUCUCUCAUGUUGUUUUCUUUAU... 3' UCGGGUUUUCCUCUUAAGAAAC
Position 2480-2486 of TBL1XR1 3' UTR hsa-miR-186-5p	5' ...UUUUUUUUCUUUUUUUUUCUUUAG... 3' UCGGGUUUUCCUCUUAAGAAAC
Position 4403-4409 of TBL1XR1 3' UTR hsa-miR-186-5p	5' ...AAAUGUCUGCAUCAUUUCUUUAG... 3' UCGGGUUUUCCUCUUAAGAAAC
Position 5174-5180 of TBL1XR1 3' UTR hsa-miR-186-5p	5' ...GGAUUGUAAUUUUUAAUUCUUUU... 3' UCGGGUUUUCCUCUUAAGAAAC
Position 6095-6101 of TBL1XR1 3' UTR hsa-miR-186-5p	5' ...GGAGUUCCUUGUAAUUUCUUUAC... 3' UCGGGUUUUCCUCUUAAGAAAC

Figure S2. MiR-186-5p is one of the candidate miRNAs regulating TBL1XR1 expression. A. Candidate miRNAs predicted by using 3 different prediction software (TargetScan, miRanda and Starbase), and miR-186-5p was included in overlapped miRNAs. B. miR-816-5p had 8 putative binding sites in 3'-UTR of TBL1XR1 mRNA.