Original Paper

TGF- β 1 Promotes Osteosarcoma Cell Migration and Invasion Through the miR-143-Versican Pathway

Fengfeng Li^a Shaohua Li^b Tao Cheng^a^aDepartment of Orthopedics, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai^bDepartment of Orthopedics, Tenth People's Hospital of Tongji University, Shanghai, China**Key Words**Osteosarcoma • TGF- β 1 • Versican • MiR-143 • Migration • Invasion**Abstract**

Background & Aims: TGF- β 1 is an abundant cytokine present in the tumour microenvironment. It has been shown to trigger versican expression in human osteosarcoma cells, which may account for the metastatic potential of these cells. However, the underlying mechanism of TGF- β 1-mediated metastasis remains unclear. The aim of this study was to evaluate the roles of versican in TGF- β 1-induced osteosarcoma cell migration and invasion. **Methods:** Sixty paired osteosarcoma tumour tissues and adjacent normal tissues were obtained, and the relationship between Enneking stage and versican expression was tested by ANOVA analysis. Real-time PCR or Western blot was used to detect versican, Smad and miR-143 expression. Osteosarcoma cell migration and invasion was assessed using Boyden chambers. A luciferase reporter assay was employed to validate the miR-143-versican interaction. **Results:** Both versican isoforms, V0 and V1, were significantly differentially expressed in tumours at different stages. TGF- β 1 promoted osteosarcoma cell migration and invasion in vitro by up-regulating versican. Furthermore, TGF- β 1 suppressed miR-143 expression through a Smad 2/3-dependent pathway. miR-143 directly targets the versican 3'-UTR, and anti-miR-143 or versican knockdown blocked the effects of TGF- β 1. **Conclusion:** Our results suggest that TGF- β 1 up-regulates versican expression by suppressing miR-143, and this pathway is important for osteosarcoma cell migration and invasion.

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Introduction

Osteosarcoma is the most common primary bone cancer, and it is characterised by a high propensity for local invasion and early metastasis [1]. Because of advances in chemotherapy and surgery, patients with localised disease have a 65–70% chance of 5-year relapse-

free survival [2]. However, patients with metastatic disease or relapse have less chance of survival, and treatment options have not greatly improved over the past several decades [3]. Therefore, a clearer understanding of metastasis biology is required to develop novel strategies to improve cancer mortality and outcomes.

Osteosarcoma growth and development are influenced by the tumour microenvironment, which is filled with various cytokines. Transforming growth factor- β (TGF- β) is one of the most abundant cytokines in the tumour microenvironment, and it is important in tumour initiation and progression [4]. Notably, high-grade human osteosarcomas express higher levels of TGF- β 1 compared to low-grade variants [5]. TGF- β 1 induces cell cycle progression and proliferation [6, 7] and may establish a positive feedback loop to promote extracellular matrix (ECM) remodelling and tumour progression [8]. In addition to the effects of TGF- β 1, TGF- β 2 triggers versican expression in human osteosarcoma cells and may account for their metastatic potential [9].

Versican is an ECM-related gene that encodes a large chondroitin sulphate proteoglycan [10]. Alternative splicing of versican mRNA generates four isoforms, designated V0, V1, V2 and V3 [11, 12]. Increased versican levels have been reported in many malignancies and are associated with cancer relapse and poor patient outcome in breast cancer [13], non-small cell lung cancer [14], oral squamous cell carcinoma [15], and other cancer types [12]. In addition to regulating adhesion [16], proliferation [17] and apoptosis [17, 18], versican has been shown to bind hyaluronan, accumulate in the pericellular matrix and promote cancer cell motility [19]. Despite these findings, however, the functional role of versican in osteosarcoma progression has yet to be thoroughly evaluated.

In the present study, we studied the effect of versican expression in osteosarcoma cell migration and invasion and explored the mechanism by which TGF- β 1 up-regulates versican expression. We found that versican expression correlated with osteosarcoma progression and TGF- β 1 promoted osteosarcoma cell motility in vitro by up-regulating versican. Furthermore, TGF- β 1 suppressed miR-143 expression, which directly targets the versican 3'-UTR. Thus, our data suggest that the TGF- β -miR-143-versican pathway plays an important role in osteosarcoma progression and suggests its potential application in cancer therapy.

Materials and Methods

Patients and tumour samples

Surgically resected paired osteosarcoma tumour tissues and adjacent normal tissues were obtained from 60 primary osteosarcoma patients between 2006 and 2011 at the Sixth Affiliated People's Hospital of Shanghai Jiaotong University (Shanghai, China) with informed consent. All tumour samples were classified according to Enneking surgical stage [20] (stage I, 9 cases; stage II A, 21 cases; stage II B, 20; stage III, 10 cases), and normal tissues were confirmed to be normal. The experiments were approved by the ethics committee of Shanghai Jiaotong University, Shanghai, China.

Cell culture

The human osteosarcoma cell lines MG63 and U2OS were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (Invitrogen) supplemented with 10% (v/v) foetal bovine serum (Invitrogen), 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified incubator containing 5% CO₂.

For TGF- β 1 treatment, cells were then treated with recombinant human TGF- β 1 (R&D systems, Minneapolis, MN, USA) or TGF- β 1-neutralising antibody (TGF- β RII Antibody, R&D systems) at the indicated concentrations for 3 d.

Real-time PCR

Total RNA was isolated from osteosarcomas or cells using Trizol (Invitrogen), and reverse transcription was performed with the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Expression of mature miRNA was determined using TaqMan miRNA

Table 1. Primer sequences for Real-time PCR

Primers	sequences
Versican V0-F	5'-GAC CTC AGG CGC TTT-3'
Versican V0-R	5'-CAG TGG TAA CGA GAT GCT TC-3'
Versican V1-F	5'-CTTTAAACGTGCAAT-3'
Versican V1-R	5'-CAG TGG TAA CGA GAT GCT TC-3'
β -actin-F	5'-CACTCTTCCAGCCTTCCTTC-3'
β -actin-R	5'-GTACAGGTCTTTGCGGATGT-3'

assay (Applied Biosystems, Foster City, CA, USA), and mRNA was quantified by TaqMan-qRT-PCR (Applied Biosystems) with primers listed in Table 1. The relative expression level of miRNA or mRNA was normalised using the $2^{-\Delta\Delta CT}$ method [21] relative to U6-snRNA or β -actin. All TaqMan-PCRs were performed in triplicate.

Western blotting

Cells were lysed in M-PER Protein Extraction Reagent (Pierce) containing protease inhibitors (Roche Applied Science). For versican analysis, protein lysates were first digested using chondroitinase ABC (Sigma-Aldrich) for 3 h at 37°C. Proteins were separated by polyacrylamide gels and transferred to PVDF membranes (Merck Millipore, Billerica, MA, USA). Blots were blocked for 1 h at room temperature and incubated with antibodies against versican (Sigma-Aldrich), p-Smad2, Smad2, p-Smad3, Smad3 or β -actin (all from Santa Cruz) for 1 h at room temperature. After incubation with horseradish peroxidase-conjugated secondary antibody for 1 h, the blots were visualised by enhanced chemiluminescence (ECL, GE Healthcare).

Lentiviral versican shRNA vector construction

Multiple short hairpin RNAs (shRNA) targeting versican (V0/V1 isoform) were designed and screened for effective versican knockdown. For lentivirus construction, oligonucleotides with the following sequences were cloned into the pHLV-U6-Puro lentiviral RNAi vector (Hanbio, Shanghai, China): 5'-GACCTATACCACAGTGAAGTTCAAGAGACTTCACTGTGGTATAGG -3'. Recombinant lentivirus containing versican shRNA was produced by co-transfection of 293T cells with PSPAX2 and PMD2G plasmids with LipoFiter (Hanbio). Lentivirus-containing supernatants were harvested 48 h after transfection, and recombinant lentiviruses were concentrated by ultracentrifugation.

miRNA and shRNA transfection

For transient overexpression or suppression of miR-143, 30 nM miR-143 mimic or anti-miR-143 (Ambion) was transfected using LipoFiter (Hanbio). Cells were used for further assays 3 d after transfection. Cells transfected with Mimic NC or Anti-NC (Ambion) were used as negative controls.

For versican or Smad 2/3 knockdown, cells were transduced with lentiviral RNAi vector containing versican shRNA or Smad 2/3 shRNA lentiviral particles (Santa Cruz) in the presence of 5 μ g/ml polybrene. After 24 h, culture medium was removed and fresh medium was added. Three days after transduction, 5 mg/ml puromycin was added to the medium. Empty lentivector lenti-puromycin or Control shRNA lentiviral particles (Santa Cruz) were used as negative controls (Lv-NC). After 3 weeks of antibiotic selection, stable versican or Smad 2/3 knockdown cells were obtained.

In vitro migration and invasion assay

For migration assays, 3×10^4 cells were trypsinised and seeded into the upper chamber of Boyden chambers (8 μ m pores, BD Biosciences). For invasion assays, Boyden chambers were precoated with Matrigel (BD Bioscience, USA). In both assays, cells were plated in chambers containing serum-free medium, and chambers were immersed in medium containing 10% foetal bovine serum. After 24 h incubation, non-migrating or invading cells were removed from the upper chamber and cells on the bottom surface of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. Five random fields per chamber were imaged on a microscope, and the number of migrated cells was quantified. Each experiment was repeated three times.

Luciferase reporter assay

Full-length versican 3'-UTR was amplified by PCR from genomic DNA using the following primers: 5'-TCCCTAAAATGGCGAACATGTGT-3' and 5'-GTGTAGTAAAAGAAGGATTTTAGGTT-3'. miR-143 targeting sites

Fig. 1. Versican expression correlates with patient osteosarcoma progression. Relative versican V0 (A) and V1 (B) expression was detected in 60 human osteosarcoma tumour tissues at different stages and matched adjacent normal tissues using Real-time-PCR. Differences in Versican (VCAN) expression among different Enneking stages were analysed by ANOVA analysis. * $p < 0.05$, compared to normal tissues.

in the versican 3'-UTR were mutated using the QuikChange site-directed mutagenesis kit (Stratagene). Wild-type or mutant versican 3'-UTR was inserted into the firefly luciferase pMir-report vector (Ambion). Reporter vectors were co-transfected with miR-143 mimics or anti-miR-143 using LipoFiter (Hanbio). After 48 h, luciferase activity was measured using the Dual Luciferase Assay System (Promega) according to the manufacturer's protocol. Luciferase activity was normalised to the internal control Renilla luciferase activity.

Statistical analysis

Data are presented as the means $\pm$ SD and were analysed with an unpaired two-tailed Student's t-test or ANOVA followed by S-N-K test. $P < 0.05$ was considered statistically significant.

Results

Versican expression correlates to osteosarcoma progression

We first compared V0 and V1 versican isoform expression in various stages of human osteosarcoma and adjacent normal tissues by real-time-PCR. As shown in Fig. 1, V0 and V1 expression was statistically significantly different among different stage osteosarcomas ($p < 0.001$). V0 expression was significantly higher in stage II A, II B and III, and V1 expression was significantly higher in stage III compared to normal tissues ($p < 0.05$).

TGF- β 1 promotes osteosarcoma cell motility by up-regulating versican expression

Previous studies have indicated that TGF- β 2 induces versican V0 and V1 expression [9]. To validate whether TGF- β 1 also regulates versican V0 and V1 expression in osteosarcoma, we treated two osteosarcoma cell lines with various concentrations of TGF- β 1 or TGF- β 1-neutralising antibody (TGF- β 1 Ab) and measured versican V0 and V1 expression by Real-time-PCR. TGF- β 1 treatment (from 0.1 ng/ml) significantly increased versican V0 and V1 expression in both MG63 and U2OS cells (Fig. 2A and B). In contrast, TGF- β 1-neutralising antibody (10 μ g/ml), which block endogenous TGF- β 1, down-regulated versican V0 and V1 expression (Fig. 2A and B). Versican V0 and V1 protein expression showed similar trends after TGF- β 1 or TGF- β 1 Ab treatment (Fig. 2C and D).

To further understand the effect of TGF- β 1 and versican V0 and V1 on osteosarcoma cell motility, we generated stable knockdowns of versican (V0 and V1) in both MG63 and U2OS cells. Both versican V0 and V1 expression significantly decreased, as measured by Real-time PCR (Fig. 3A and B). Treatment of cells with exogenous TGF- β 1 dramatically increased MG63 and U2OS cell migration and invasion, while TGF- β 1 Ab treatment decreased cell migration

Fig. 2. TGF- β increases versican expression. MG63 (A and C) and U2OS (B and D) cells were treated with the indicated concentration of TGF- β 1 or TGF- β 1-neutralising antibody (TGF- β 1 Ab) for 3 d. Versican (VCAN) V0 and V1 expression was detected by Real-time-PCR (A and B) and Western blotting (C and D). * p <0.05, compared to control.

Fig. 3. TGF- β promotes osteosarcoma cell motility by up-regulating versican. MG63 (A) and U2OS (B) cells were transfected with versican shRNA lentivector or negative control lentivector (Lv-NC), and versican (VCAN, V0 and V1) expression was detected by Real-time PCR. MG63 (C) and U2OS (D) cells transfected with versican shRNA or Lv-NC were treated with TGF- β 1 (5 ng/ml) or TGF- β 1 Ab (10 μ g/ml). Migration and invasion was measured across Transwell chambers. * p <0.05, compared to control, # p <0.05, compared to TGF- β 1 group.

Fig. 4. TGF- β suppresses miR-143 expression in osteosarcoma cells. MG63 (A) and U2OS (B) cells were treated with the indicated concentration of TGF- β 1 or TGF- β 1 Ab for 3 d. miR-143 expression was detected by Real-time-PCR. MG63 (C and E) and U2OS (D and F) cells transfected with Smad 2/3 shRNA lentivector or negative control lentivector (Lv-NC) were treated with TGF- β 1 (5 ng/ml). (C and D), Phosphorylated Smads (Smad2 and Smad3) were analysed by Western blotting. (E and F), miR-143 expression was detected by Real-time-PCR. * p <0.05, compared to control.

and invasion (Fig. 3C and D). Versican knockdown significantly abrogated the effect of TGF- β 1 on migration and invasion in MG63 and U2OS cells (Fig. 3C and D), suggesting that versican acts downstream of TGF- β 1 to induce cell migration and invasion.

TGF- β 1 suppresses miR-143 expression in osteosarcoma cells

To explore the mechanism by which TGF- β 1 up-regulates versican expression, we examined miR-143, which has been reported to directly repress versican expression [22]. To determine whether TGF- β 1 regulates versican expression through miR-143 in osteosarcoma cells, we examined miR-143 expression after exogenous TGF- β 1 expression or inhibition of TGF- β 1. As shown in Fig. 4A and B, miR-143 expression significantly decreased upon exogenous TGF- β 1 addition, while its expression increased upon TGF- β 1 Ab treatment in a concentration-dependent manner. We next examined whether TGF- β 1-mediated miR-143 downregulation required Smad2 and Smad3 phosphorylation. TGF- β 1 caused a marked increase in Smad2 and Smad3 activation, which was significantly repressed by Smad 2/3 shRNA transfection (Fig. 4C and D). Real-time PCR showed that Smad 2/3 knockdown rescued the TGF- β 1-mediated inhibition of miR-143 expression (Fig. 4E and F), suggesting that TGF- β 1 suppresses miR-143 expression through Smad 2/3-dependent pathways.

Fig. 5. Versican is a target gene of miR-143 in osteosarcoma. **A**, Schematic diagram of luciferase reporters and wild type (wt) or mutated (mut) putative miR-143 binding site in the versican 3'-UTR. **B**, MG63 cells were co-transfected with miR-143 or anti-miR-143 (anti-143) and the luciferase reporter plasmid harbouring the wild type or mutated predicted miR-143 binding site of versican 3'-UTR, as indicated. Firefly luciferase activity was measured and normalised to Renilla luciferase activity. MG63 (**C** and **E**) and U2OS (**D** and **F**) cells were transfected with miR-143 or anti-miR-143. (**C** and **D**), miR-143 expression was detected by Real-time-PCR. (**E** and **F**), Versican (VCAN) expression was examined by Western blotting. * $p < 0.05$, compared to control.

TGF- β 1 promotes osteosarcoma cell motility through miR-143-versican interactions

MiR-143 has been shown to repress versican expression in smooth muscle cells by targeting the versican 3'-UTR [22]. We validated whether the same interaction exists in osteosarcoma cells. Luciferase reporter assays revealed that luciferase activity induced by the wild-type versican 3'-UTR significantly decreased or increased in the presence of miR-143 or anti-miR-143, respectively, whereas luciferase activities of constructs containing a mutated miR-143 binding site were not affected by miR-143 or anti-miR-143 (Fig. 5C and D). These data suggest that miR-143 modulates versican expression by directly targeting the versican 3'-UTR. Furthermore, transfection of a miR-143 mimic significantly reduced versican protein expression in both MG63 and U2OS cells (Fig. 5C and D). In contrast, anti-miR-143 increased versican protein expression (Fig. 5C and D). We confirmed the effect of miR-143 or anti-miR-143 transfection on miR-143 expression in MG63 and U2OS cells by Real-time PCR (Fig. 5E and F).

To assess whether miR-143 is responsible for the TGF- β -dependent up-regulation of versican, we treated osteosarcoma cells transfected with miR-143, anti-miR-143 or versican shRNA with TGF- β 1 or TGF- β 1 Ab, and examined versican expression by Real-time PCR. As shown in Fig. 6A-D, miR-143 abrogated TGF- β 1-induced versican up-regulation and anti-miR-143 significantly attenuated TGF- β 1 Ab-induced versican down-regulation in both osteosarcoma cell lines.

We next sought to determine whether the miR-143-versican pathway is responsible for TGF- β 1-enhanced osteosarcoma cell motility. MiR-143 or anti-miR-143 significantly attenuated the effect of TGF- β 1 or TGF- β 1 Ab. Versican knockdown abrogated the effect of TGF- β 1 Ab and anti-miR-143 co-treatment on cell migration and invasion (Fig. 6E and H). These data suggest that regulation of versican by miR-143 is essential for TGF- β 1-induced osteosarcoma cell migration and invasion.

Fig. 6. TGF- β promotes osteosarcoma cell motility through the miR-143-versican pathway. Osteosarcoma cells transfected with miR-143 or mimic NC were treated with TGF- β 1 (5 ng/ml). (A and B), Versican (VCAN, V0 and V1) expression was detected by Real-time PCR. Osteosarcoma cells transfected with versican shRNA were re-transfected with anti-miR-143 or anti-NC in the presence of TGF- β 1 Ab (10 μ g/ml). (C and D), Versican (VCAN, V0 and V1) expression was detected by Real-time PCR. (E-H), Osteosarcoma cells were transfected and treated as described in A-D. Migration and invasion was measured with Transwell chambers. A, C, E, G for MG63 cells and B, D, F, H for U2OS cells. * p <0.05, compared to control.

Discussion

Although it is one of the most important factors in the bone environment [23], the role of TGF in osteosarcoma is far from clear. Here, we found that TGF- β 1 promotes osteosarcoma

cell migration and invasion through the miR-143-versican pathway, providing a new perspective for the function of TGF in osteosarcoma metastasis.

Versican belongs to the family of large aggregating proteoglycans, which primarily localise within the ECM [24]. Versican has been described in a number of tumour types, and can regulate many cellular processes [12]. In mammals, versican exists as four possible splice variants (V0-V3), and V0 and V1 are the most common isoforms expressed in cancer tissues [13, 25]. In this study, we found that versican V0 and V1 isoforms were over-expressed in high-stage osteosarcoma tissues compared to normal tissues, suggesting that versican may contribute to osteosarcoma metastasis.

Previous studies have shown that TGF- β regulates versican expression in various cells [25-27], however, the underlying mechanism is largely unknown. In the current study, we found that exogenous TGF- β 1 significantly increased versican V0 and V1 expression in both MG63 and U2OS cells. In contrast, TGF- β 1 Ab down-regulated versican expression, indicating that TGF- β 1 Ab blocked the effects of endogenous TGF- β 1. These data are consistent with recent studies showing that osteosarcoma cells secrete TGF- β 1 [28]. In further support of this, we observed a significant attenuation in TGF- β 1-induced migration and invasion in versican-knockdown osteosarcoma cells (Fig. 3C and D).

Furthermore, TGF- β 1 and versican were linked by miR-143, which has previously been shown to suppress versican expression in smooth muscle cells [22]. We found that miR-143 inhibited versican expression in osteosarcoma cells by binding its 3'-UTR. Similar to the effect of TGF- β 3 in mesenchymal stem cells (MSCs) [29], TGF- β 1 treatment suppressed miR-143 expression in osteosarcoma cells.

MiR-143 has been shown to be downregulated in several cancers, and it functions as a tumour suppressor [30-32]. To our knowledge, ours is the first study to demonstrate that miR-143 mediates TGF- β 1-induced osteosarcoma cell migration and invasion by targeting versican. In addition, miR-143 and versican were shown to be necessary for TGF- β 1-enhanced migration and invasion of osteosarcoma cells, suggesting that the miR-143-versican pathway is a critical regulator of osteosarcoma metastasis.

Previous functional studies indicated that versican promotes cell motility by binding HA to subsequently activate CD44-mediated downstream signalling [13, 24]. Both HA and CD44 have been reported to enhance osteosarcoma metastasis [33, 34]. Further investigation is required to comprehensively understand the roles of these three factors in osteosarcoma progression.

Taken together, our results demonstrate that versican V0 and V1 are relevant markers of osteosarcoma progression and are involved in TGF- β 1-induced osteosarcoma cell migration and invasion. Furthermore, TGF- β 1 up-regulates versican V0 and V1 expression by suppressing miR-143, which can directly bind the versican 3'-UTR. Understanding the role of the TGF- β 1-miR-143-versican pathway in osteosarcoma progression may aid the development of new treatment strategies for osteosarcoma.

Disclosure Statement

The authors report no conflicts of interest.

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