

Tim-3 facilitates osteosarcoma proliferation and metastasis through the NF- κ B pathway and epithelial-mesenchymal transition

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Genet. Mol. Res. 15 (3): gmr.15037844

Received October 19, 2015

Accepted June 15, 2016

Published September 2, 2016

DOI <http://dx.doi.org/10.4238/gmr.15037844>

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ABSTRACT. The aim of this study was to investigate the expression of T-cell immunoglobulin mucin domain molecule-3 (Tim-3) in osteosarcoma tissues, and analyze its effect on cell proliferation and metastasis in an osteosarcoma cell line. Tim-3 mRNA and protein expression in osteosarcoma tissue was detected by reverse transcriptase-polymerase chain reaction and immunohistochemistry, respectively. Additionally, the cell viability, apoptosis rate, and invasive ability of the osteosarcoma cell line MG-63 were tested using the methyl thiazolyl tetrazolium assay, Annexin V-propidium iodide flow cytometry, and a Transwell assay, respectively, following Tim-3 interference using small interfering RNA (siRNA). We also analyzed the expression of Snail, E-cadherin, vimentin, and nuclear factor

(NF)- κ B in the cells by western blot. We observed that Tim-3 mRNA and protein was significantly overexpressed in osteosarcoma tissues, compared to the adjacent normal tissue ($P < 0.01$). Moreover, MG-63 cells transfected with the Tim-3 siRNA presented lower cell viability, a greater number of apoptotic cells, and decreased invasive ability ($P < 0.01$), compared to control cells. Additionally, we observed a decrease in Snail and vimentin expression, an increase in the E-cadherin level, and an increase in NF- κ B p65 phosphorylation ($P < 0.01$) in Tim-3 siRNA-transfected MG-63 cells. Based on these results, we concluded that Tim-3 is highly expressed in osteosarcoma tissue. Moreover, we speculated that interfering in Tim-3 expression could significantly suppress osteosarcoma cell (MG-63) proliferation and metastasis via the NF- κ B/Snail signaling pathway and epithelial-mesenchymal transition.

Key words: Tim-3; Osteosarcoma; Cell proliferation; Metastasis; NF- κ B/Snail signaling pathway

INTRODUCTION

Osteosarcoma is the leading type of primary malignant tumor that (primarily) affects the bone tissues of adolescents. Osteosarcoma has a fatality rate of up to 80%, as well as high malignancy and invasive ability. Most cases are diagnosed during the late stage of the disease (Aycan et al., 2015; Liu et al., 2015b). Recent advances in chemotherapy, radiation therapy, biological therapy, and surgical technology have allowed a vast majority of osteosarcoma patients to receive limb salvage treatment. However, patient satisfaction with this method remains low because of the 40% recurrence and 22% 5-year survival rates (Zhang et al., 2014). Therefore, it is highly important to explore the mechanism of osteosarcoma occurrence and development.

The T-cell immunoglobulin mucin domain molecule (Tim) gene family was first identified in mice by McIntire in 2001; subsequent studies have confirmed its location in the 5th chromosome in humans. Tim-3, one of the most important members of this family, is primarily expressed in CD8⁺ T cells, monocytes, dendritic cells, and mast cells. Tim-3 is a specific surface marker of Th1 cells, and regulates autoimmune and allergic disease by mediating the immune response of Th1 cells (Han et al., 2013; Anderson, 2014). Furthermore, Tim-3 was found to be closely related to the occurrence and development of several types of cancer, including prostate cancer, non-small cell cancer, cervical cancer, and melanoma (Geng et al., 2006; Cao et al., 2013). Geng et al. (2006) reported, in a study wherein C57BL/6 mice were inoculated with melanoma cells transfected with a soluble Tim-3 vector, that melanoma transfected with the plasmid grew significantly faster in mice compared to the negative control. Cao et al. (2013) found that siRNA Tim-3 suppressed the invasive abilities of cervical cancer cell lines HeLa and SiHa, and concluded that disrupted Tim-3 expression could lead to suppressed tumor cell metastasis (Cao et al., 2013). Another study reported that Tim-3 is co-expressed with epithelial-mesenchymal transition (EMT)-related proteins in the cytoplasm and cell membrane of the osteosarcoma tissue (Shang et al., 2013). Based on the results of these studies, we speculated that Tim-3 was closely associated with osteosarcoma occurrence and metastasis.

MATERIAL AND METHODS

Specimens

Twenty pairs of osteosarcoma tissue and adjacent normal muscle tissue were collected from patients diagnosed by a pathological examination at our hospital between June 2014 and June 2015. None of these patients received chemotherapy or radiotherapy prior to surgery.

Reagents and instruments

The human osteosarcoma cell line MG-63 was purchased from the cell bank of the Chinese Academy of Sciences (catalog No. TCHu124; Beijing, China). Tim-3 siRNA and the control were synthesized by Gene Pharma (Shanghai, China). Primary antibodies against Snail, E-cadherin, and vimentin were obtained from Epitomics (AbCam, Burlingame, CA, USA). Antibodies against NF- κ B p65 and pp65 were purchased from Cell Signaling Technology (Danvers, MA, USA). Methyl thiazolyl tetrazolium (MTT) was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA) and fetal calf serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone (GE Healthcare, Buckinghamshire, UK). Transwell chambers were obtained from Corning (Corning, NY, USA), the electrophoresis system was purchased from Six One (Beijing, China), and the ChemiDocTM XRS gel imaging system was obtained from Bio-Rad (Hercules, CA, USA).

RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen; Life Technologies, Carlsbad, CA, USA) according to the manufacturer protocol. This was reverse-transcribed and the cDNA samples were amplified by polymerase chain reaction (PCR) using a one-step RT-PCR kit (Takara, Dalian, China). The cycling conditions were set as follows: 35 cycles of denaturation at 95°C for 45 s, annealing at 59°C for 45 s, and extension at 72°C for 60 s. The primers used for PCR amplification are listed in Table 1. The PCR products (5 μ L) were separated by agarose gel electrophoresis.

Table 1. Primers used in reverse transcriptase polymerase chain reaction.

Gene	Primer (5'-3')	Base pairs
<i>Tim-3</i>	Forward: CCAGAGACTGTTAATCAT	749
	Reverse: ATGATTAACAGTCTCTGG	
<i>GADPH</i>	Forward: AGCCACATCGCTCAGACA	314
	Reverse: TGGACTCCACGACGTACT	

Immunohistochemistry

The tissue sections were de-paraffinized using dimethyl benzene and dehydrated using an alcohol gradient (absolute ethyl alcohol, 95 and 80% ethanol). The sections were subjected to antigen repair, and subsequently blocked by horse serum. The section was then subjected to hybridization and hematoxylin staining, sealed, and observed. Tim-3-positive cells were stained a faint yellow or claybank color, and were located in the cytoplasm.

MTT assay

The osteosarcoma cell line MG-63 was seeded on a 96-well plate and transfected with siRNA Tim-3 or negative control with Lipofectamine 2000 for 48 h. The cells were then incubated with 20 μ L (5 mg/mL) MTT (per well) for 4 h, and the optical density at 560 nm was read after adding 150 μ L dimethyl sulfoxide (DMSO).

Annexin V-propidium iodide (PI) flow cytometry

MG-63 cells were seeded on a 6-well plate and transfected with siRNA Tim-3 or the negative control using Lipofectamine 2000. The cells were digested with 0.25% trypsin (without EDTA) and centrifuged at 600 *g* for 5 min. Annexin V-fluorescein isothiocyanate (FITC) and PI (5 μ L each) were added to each of the wells, and the plate was incubated for 15 min. The cells were analyzed by flow cytometry within 1 h.

Cell invasion assay

Matrigel was added to the membranelle of a Transwell chamber. MG-63 cells were seeded on a 6-well plate and transfected with siRNA Tim-3 or a negative control using Lipofectamine 2000. The cells were then seeded onto the upper chamber; DMEM supplemented with 5% fetal bovine serum was added to the lower chamber. The chamber was incubated for 24 h, and the membranelle was fixed with paraformaldehyde and stained with crystal violet. The cell numbers in five random fields were counted and analyzed for cell invasion.

Western blot

Total protein was extracted from the cells using the RIPA lysis buffer (Sangon, Shanghai, China), and quantified using a standard BCA kit. Total protein was separated by a denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The bands were transferred to a PVDF membrane; the membrane was blocked with milk and subsequently incubated with primary and secondary antibodies. The bands were detected by chemiluminescence and analyzed using the Quantity One software.

Statistical analysis

All data were statistically analyzed using SPSS v.17.0 (SPSS Inc., Chicago, IL, USA). Numerical data are reported as means and standard deviations. Differences between means were analyzed using the *t*-test. $P < 0.05$ was considered as a significant difference.

RESULTS

Tim-3 expression in osteosarcoma and adjacent tissues

As shown in Figure 1A, Tim-3 expression was significantly higher in osteosarcoma tissue, compared to that in the adjacent normal tissue (control; 1.38 ± 0.21 vs 10.94 ± 1.02 , $P < 0.01$). RT-PCR quantification of Tim-3 expression revealed that Tim-3 mRNA expression was

significantly higher in the osteosarcoma tissue compared to the para-carcinoma tissue (0.02 ± 0.00 vs 0.94 ± 0.02 , $P < 0.01$; Figure 1B).

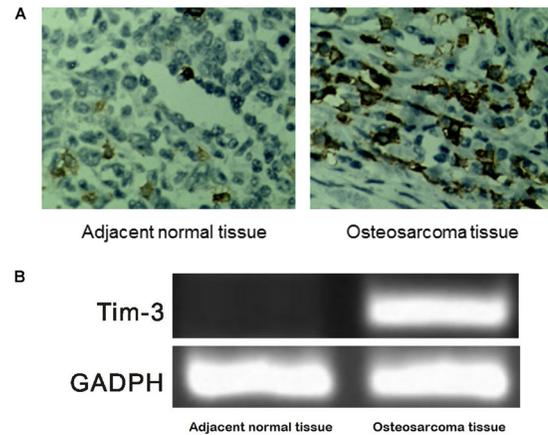


Figure 1. Tim-3 expression in osteosarcoma and adjacent tissues. **A.** Immunohistochemistry detection of Tim-3 protein expression in osteosarcoma tissue. **B.** RT-PCR detection of Tim-3 mRNA expression in osteosarcoma tissue.

Impact of Tim-3 on MG-63 cell viability, apoptosis, and invasion

Transfection with the Tim-3 siRNA led to a significant decrease in the MG-63 cell viability compared to the control ($P < 0.01$; Figure 2A). Annexin V-PI flow cytometry revealed a significant elevation in the MG-63 cell apoptosis rate following Tim-3 siRNA transfection ($P < 0.01$; Figure 2B). Moreover, we observed an apparent decrease in the invasive MG-63 cell number after Tim-3 siRNA transfection (208.19 ± 28.35 vs 60.94 ± 9.23 , $P < 0.01$; Figure 2C).

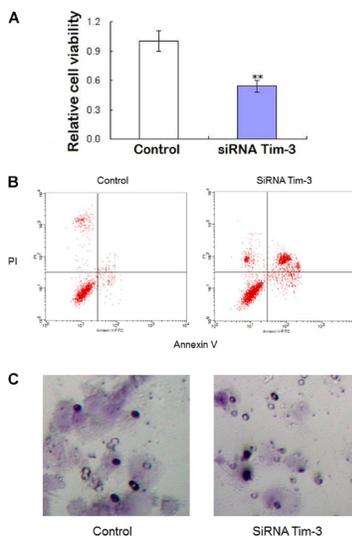


Figure 2. Impact of Tim-3 on MG-63 cell behavior. **A.** Cell viability after siRNA Tim-3 transfection. **B.** Cell apoptosis after siRNA Tim-3 transfection. **C.** Cell invasion after siRNA Tim-3 transfection.

Effect of Tim-3 on EMT and NF- κ B activity in MG-63 cells

Western blot analysis of Tim-3 siRNA-transfected MG-63 cells showed a significant decrease in Snail and vimentin expression and a significant increase in the E-cadherin level ($P < 0.01$) in the siRNA-transfected cells (Figure 3A). As shown in Figure 3B, we also observed a significant decrease in the NF- κ B p65 phosphorylation level in the Tim-3 siRNA-transfected MG-63 cells ($P < 0.01$).

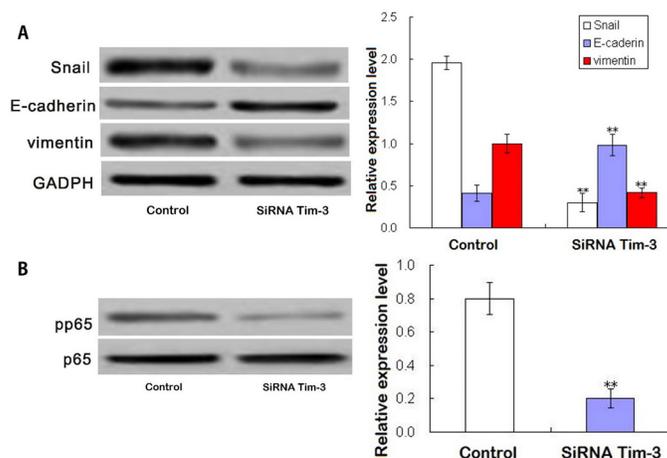


Figure 3. Effect of Tim-3 on EMT and NF- κ B activity in MG-63 cells. **A.** EMT-related protein expression after siRNA Tim-3 transfection. **B.** NF- κ B activity after siRNA Tim-3 transfection.

DISCUSSION

Osteosarcoma is a common primary malignant bone tumor that originates in the marrow cavity. Osteosarcoma is a highly malignant disease with a higher prevalence in children and adolescents, and features such as early metastasis and poor prognosis. Pulmonary metastasis has been reported as the most important factor affecting the survival of osteosarcoma patients. Osteosarcoma metastasis is influenced by many factors and biological processes including changes in proto-oncogenes and tumor suppressor genes, disorders in cell apoptosis and proliferation and multiple signaling pathways.

As with all tumors, osteosarcoma is characterized by unlimited growth, which leads to faster protein synthesis as compared to catabolism, in turn inducing cancer progression. Therefore, the inhibition of cell proliferation and induction of cancer cell apoptosis could be effective methods for restraining infinite tumor proliferation. EMT is the key for the invasion and metastasis of osteosarcoma cells. Cancer cells lose their epithelial phenotype and develop a mesenchymal cell phenotype during EMT. EMT further induces the loss of polarity in epithelial cells, reduces cell-cell interactions, and increases cell migration and movement (Yang et al., 2011; Ogunwobi and Liu, 2012). Molecular characteristics of EMT include the downregulation of E-cadherin expression and upregulation of vimentin and fibronectin expression (Yamada et al., 2014; Hou et al., 2015). E-cadherin downregulation promotes tumor cell infiltration and diffusion. Therefore, reversing the expression of EMT-related proteins could inhibit cancer cell invasion and metastasis. Snail, a member of the Snail superfamily, was first identified

in *Drosophila melanogaster*. The human Snail gene is located on chromosome 20, and has been reported to suppress E-cadherin expression by interacting with the E-box sequence in the promoter area of E-cadherin. Transfection of Snail-1 shRNA into the osteosarcoma cell line SaOS2 has been shown to increase the E-cadherin expression and decrease cell migration and invasion. On the other hand, Snail overexpression led to a reduction in E-cadherin expression, as well as improved cell migration and invasion, suggesting that Snail induces the invasiveness of osteosarcoma cells by suppressing the E-cadherin expression (Yang et al., 2014). Snail expression is regulated by the NF- κ B signaling pathway. NF- κ B enhances Snail transcription by interacting with the promoter area of Snail (from 194 to -78 bp). NF- κ B is a nuclear transcription factor that is closely associated with cancer cell apoptosis, invasion, and metastasis. External stimuli can separate these factors from I κ B, transfer them to the nucleus, and allow them to interact with their target genes to promote cancer cell motility and invasion. Inhibition of the EMT and NF- κ B/Snail signaling pathway has been shown to significantly suppress metastasis in multiple cancer cell types, such as gastric adenocarcinoma cells SGC-7901 (Zhu et al., 2015) and breast cancer cells A2780 (Liu et al., 2015a). Moreover, suppression of the NF- κ B activity and EMT-related proteins, such as vimentin, Snail, and Slug, has been shown to suppress metastasis in osteosarcoma cell lines MG-63, Saos-2, and U-2OS (Chang et al., 2015), indicating that cancer metastasis can be effectively suppressed by inhibiting the EMT and NF- κ B/Snail signaling pathway.

Tim-3 is a specific surface marker of Th1 cells, and is therefore chiefly studied in autoimmune and allergic diseases (Han et al., 2013; Anderson, 2014). Recently, Ji et al. (2015) and Komohara et al. (2015) reported an increase in Tim-3 expression in non-small cell lung cancer infiltrated lymphocytes by immunohistochemistry and flow cytometry and in renal clear cell carcinoma tissue and CD204⁺ tumor-associated macrophages, respectively. Piao et al. (2014) proved that Tim-3 expression was highly elevated in CD4⁺ and CD8⁺ T cells of prostate cancer patients, and not in patients with benign prostatic hyperplasia, by flow cytometry and immunohistochemistry, whereas Cao et al. (2013) reported a significantly higher Tim-3-positive expression rate in cervical cancer tissues than that in cervical intraepithelial neoplasia and chronic cervicitis. Tumors with high Tim-3 expression have been reported to present a higher metastatic potential and lower overall survival, suggesting a close association between Tim-3 expression and tumor occurrence and metastasis. (Shang et al., 2013) Moreover, immunohistochemical analysis and double-immunofluorescence staining in 9 human osteosarcoma tissues revealed that Tim-3 was distributed in the cytoplasm and cell membrane of these tissues (Shang et al., 2013), and was also shown to be widely expressed in CD68⁺ macrophages, CD31⁺ endothelial cells, CK-18⁺ epidermal cells, and PCNA⁺ cancer cells.

Therefore, in this study, we tested for Tim-3 expression in osteosarcoma tissue samples by immunohistochemistry and RT-PCR. Our results were in accordance with those obtained by Shang et al. (2013); that is Tim-3 mRNA and protein expression was obviously higher in osteosarcoma tissues than that in the adjacent normal tissue, suggesting a close association between Tim-3 expression and the occurrence of osteosarcoma. Dardalhon et al. (2010) reported that breast tumor growth was restrained in Tim-3-deficient mice, and that the Tim-3 monoclonal antibody significantly inhibited the EL4 subcutaneous lymphoma growth in mice. Cao et al. (2013) reported that siRNA interference of Tim-3 expression in cervical cancer cell lines HeLa and SiHa significantly suppressed tumor metastasis. In this study, we investigated the effect of Tim-3 on osteosarcoma cell (MG-63) proliferation and metastasis using siRNA interference. The results indicated a significant decrease in the proliferation and

invasive capacities of MG-63 cells. Taken together with the results obtained by Shang et al. (2013), this revealed that Tim-3 is co-expressed with various EMT-related proteins, including vimentin, Slug, Snail, and Smad, in osteosarcoma tissues, which contributes to the increased invasiveness of cancer cells. Tim-3 promotes melanoma cell (B16) invasion by elevating the NF- κ B activity, leading to melanoma metastasis (Wu et al., 2010). Our results revealed that Tim-3 siRNA transfection induced a decrease in the expression of Snail, E-cadherin, and vimentin, as well as NF- κ B phosphorylation, in MG-63 cells. Therefore, we speculated that Tim-3 promotes EMT and induces osteosarcoma metastasis by activating the NF- κ B/Snail signaling pathway. This study showed that the Snail-1 siRNA and the NF- κ B inhibitor BAY11-7082 can significantly inhibit E-cadherin and N-cadherin expression in the osteosarcoma cell line U2OS, indicating that Tim-3 mediates osteosarcoma proliferation and metastasis by regulating EMT-related proteins, via the NF- κ B/Snail signaling pathway.

In summary, Tim-3 was found to be overexpressed in osteosarcoma tissues. We theorized that Tim-3 might regulate the proliferation and metastasis of MG-63 cells via the NF- κ B/Snail signaling pathway and EMT.

Conflicts of interest

The authors declare no conflict of interest.

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