Deregulated WWOX is involved in a negative feedback loop with microRNA-214-3p in osteosarcoma

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Abstract. WW domain-containing oxidoreductase (WWOX) is frequently inactivated in human osteosarcoma, and the restoration of its expression can suppress tumorigenicity in WWOX-negative OS cells. However, its regulatory mechanisms remain to be fully elucidated. In the present study, we demonstrate that WWOX is downregulated and that it regulates proliferation and epithelial-to-mesenchymal transition (EMT)-associated protein expression in osteosarcoma. As shown by our results, WWOX overexpression by transfection with WWOX overexpression plasmids suppressed the proliferation, migration and invasion of osteosarcoma MG63 cells (as shown by MTT and migration and invasion assays). The silencing of microRNA (miR)-214-3p by transfection with anti-miR-14-3p upregulated WWOX protein expression and also inhibited the proliferation, migration and invasion of osteosarcoma cells. Additionally, we found that WWOX negatively regulated miR-214-3p and miR-10b expression. Our findings define a negative feedback pathway in control of WWOX and miR-214-3p expression, thus providing novel molecular targets for the treatment of osteosarcoma.

Introduction

Osteosarcoma is the most common type of malignant bone tumor, and it frequently originates in the metaphysis of the long bones (1). The prognosis of patients with osteosarcoma remains poor, as approximately 80% of patients eventually develop metastatic disease following surgical treatment (2) and pulmonary metastasis is the major cause of fatal outcome (3). The further elucidation of the molecular mechanisms responsible for the development of osteosarcoma will not only help us to understand the pathogenesis and progression of the disease, but may also provided novel targets for effective therapies.

Materials and methods

Tissue samples and cell lines. Osteosarcoma tissues and adjacent normal tissues (n=6) were collected from patients at surgery...
at Linyi People's Hospital, Linyi, China. The Medical Ethics Committee of Linyi People's Hospital approved the study, and all patients agreed to participate in the project and provided written informed consent prior to obtaining the samples. The human osteosarcoma cell line, MG63, was purchased from the Cell Bank of the Chinese Academy of Sciences (Beijing, China). The cells were maintained in RPMI-1640 medium (HyClone Co., Logan, UT, USA) supplemented with 10% fetal bovine serum and 1% penicillin G/streptomycin.

**Cell transfection.** All WWOX overexpression plasmids were purchased from Tiangen (Tianjin, China). The empty vectors were also obtained from Tiangen. Anti-miR-214-3p/scramble inhibitor was purchased from Ambion, Inc. (Austin, TX, USA). The cells were seeded into 6-well plates 24 h prior to transfection. When the MG63 cells reached 80% confluence, the expression plasmids were transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions.

**Western blot analysis.** The cells were collected and lysed using RIPA lysis buffer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto polyvinylidene difluoride (PVDF) membranes. The blots were blocked and incubated with antibodies against WWOX (ab137726), cyclin D1 (ab134175), p53 (ab109520), p27 (ab32034), c-myc (ab32072), E-cadherin (E-Cad; ab15148), zinc finger E-box-binding homeobox 1 (ZEB1; ab203829), vimentin (ab16700), Slug (ab27568) and β-actin (ab8227) (all from Abcam, Cambridge, MA, USA) overnight 4˚C. After washing, the membranes were incubated at 37˚C for 1 h with goat anti-rabbit IgG H&L (HRP) secondary antibody (ab6721; Abcam) and visualized using ECL detection reagent.

**MTT cell proliferation assay.** Following transfection, the cells were re-seeded at a density of 2x10^4 cells/well in 96-well plates containing 0.2 ml RPMI-1640 (with 10% FBS). Subsequently, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added followed by incubation for a further 4 h at 37˚C. A total of 150 µl of dimethyl sulfoxide was then added to each well and the absorbance was measured at 570 nm on an enzyme immunoassay analyzer (Bio-Rad, Hercules, CA, USA).

**BrdU assay.** Cell proliferation was also assessed using a colorimetric BrdU proliferation kit by following the manufacturer’s instructions (Roche; Cat. no. 11647229001). Briefly, the cells treated with the peptides were labeled with BrdU for 3-4 h. The genomic DNA was then fixed and denatured, then incubated with the peroxidase-conjugated anti-BrdU antibody for 90 min. The substrate of the conjugated peroxidase was then added and the reaction product was measured by the absorbance (A_{570 nm}-A_{492 nm}). The results were then normalized by the number of total viable cells, which was determined by a side-by-side cell viability assay as described above.

**Migration and invasion assays.** Following transfection, the cells were added to the chamber with the non-coated membrane (BD Biosciences, San Diego, CA, USA), and cells migrating to the lower sides of the filters were fixed in 4% paraformaldehyde and stained with crystal violet. Cell invasion assay was performed as the migration assay except that the chamber was coated with Matrigel (BD Biosciences) and the cells were observed under a microscope.

**Immunofluorescence assay.** The cells grown on polylysine-treated slides were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde on ice, and then blocked with 5% BSA at room temperature. Anti-WWOX antibody (ab137726; Abcam) were added at 4˚C overnight followed by incubation with goat anti-rabbit (Alexa Fluor®488) secondary antibody (ab150077; Abcam) at room temperature for 30 min. The samples were then washed 3 times with PBS-Tween-20, and the nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI).

**miRNA microarray analysis.** Microarray analysis was performed using Affymetrix GeneChip miRNA 3.0 arrays kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s instructions. After washing and staining, the arrays were scanned using GeneChip scanner 3000 (Affymetrix).

**Northern blot analysis.** For northern blot analysis of the cells, all RNA samples were prepared and separated in a denaturing urea-polyacrylamid gel and transferred to nylon membranes by semi-dry electroblotting. The RNA probes were labeled with [γ-32P] ATP complementary to the target miRNAs. U6 was used as a loading control.

**Bioinformatics analysis.** The analysis of potential microRNA target site using the commonly used prediction algorithms - miRanda (http://www.microrna.org/).

**Statistical analysis.** Data are expressed as the means ± SD, from at least 3 separate experiments and were analyzed using one-way ANOVA or the Student’s t-test. A value of p<0.05 was considered to indicate a statistically significant difference.
Results

WWOX regulates cell proliferation and epithelial to mesenchymal transition (EMT)-associated gene expression and its expression is downregulated in osteosarcoma. In an attempt to determine the role of WWOX in regulating cell proliferation and EMT in osteosarcoma, the MG63 cells were transfected with WWOX overexpression plasmids or empty vectors. Following stable transfection, WWOX expression was detected by western blot analysis and 9 proliferation- and EMT-associated markers were also examined by western blot analysis in the MG63 cells. The results revealed that transfection with WWOX overexpression plasmids markedly increased WWOX protein expression. It also promoted p53, p21 and E-Cad expression and suppressed cyclin D1, ZEB1, vimentin and Slug expression in the MG63 cells (Fig. 1A).

To assess the expression of WWOX in osteosarcoma, western blot analysis was conducted on 6 pairs of osteosarcoma tissues and matched adjacent normal tissues. The expression of WWOX was consistently lower in the osteosarcoma tissues compared with the normal tissues (Fig. 1B).

WWOX inhibits the proliferation of MG63 osteosarcoma cells. In order to determine the role of WWOX in regulating the proliferation of MG63 cells, the proliferation rates of MG63 cells transfected with WWOX overexpression plasmids or empty vectors were examined by MTT assay. The results revealed that the overexpression of WWOX significantly suppressed the proliferation of MG63 cells (Fig. 2A). This was confirmed by BrdU incorporation assay which revealed that transfection with WWOX overexpression plasmids resulted in decreased DNA synthesis per viable MG63 cell (Fig. 2B).

WWOX inhibits the migration and invasion of osteosarcoma MG63 cells. E-Cad, ZEB1, Vimentin and slug are EMT associated markers and they are associated with invasion and migration in cancer (27-31). Given that WWOX markedly inhibited the expression of EMT-associated markers and that of markers associated with invasion and migration (32,33), we then sought to determine whether WWOX has an effect on the migration and invasion of MG63 cells. The results from migration and invasion assay indicated that the overexpression of WWOX not only inhibited the migration of the MG63 cells, but also suppressed the invasion of these cells (Fig. 3).

Silencing of miR-214-3p upregulates WWOX protein expression in MG63 osteosarcoma cells. Having demonstrated that WWOX suppresses EMT, as well as the proliferation, migration and invasion of osteosarcoma cells, we then wished to determine the mechanisms regulating WWOX expression in...
miRNAs are a class of small non-coding RNAs (approximately 22 nucleotides in length) that negatively regulate protein-coding gene expression by targeting mRNA degradation or translation inhibition (34,35). To further confirm whether WWOX is regulated by miRNAs, we used the commonly used prediction algorithm, miRanda (http://www.microrna.org/microrna/home.do), to analyze the 3'UTR of WWOX. A dozen miRNAs were found by the algorithm. However, we were interested in miR-214-3p, as the expression of miR-214-3p has been confirmed to be significantly higher in osteosarcoma tissues than in normal tissues (25). It seems to play an important role in osteosarcoma. The target sites on the 3'UTR of WWOX are shown in Fig. 4A. We reasoned that miR-214-3p downregulates WWOX expression by targeting its 3'UTR and that WWOX protein is suppressed due to the overexpression of miR-214-3p in osteosarcoma. In an attempt to determine the role of miR-214-3p in regulating WWOX expression in osteosarcoma cells, the MG63 cells were transfected with anti-miR-214-3p and scramble inhibitor. Following transfection, miR-214-3p expression was detected by qPCR and the results revealed that miR-214-3p expression was significantly decreased by transfection of the cells with anti-miR-214-3p (Fig. 4B). We then performed RT-qPCR to detect WWOX mRNA expression in the MG63 cells transfected with anti-miR-214-3p or scramble inhibitor.
The results demonstrated that WWOX mRNA expression was not affected by transfection with anti-miR-214-3p compared with the scramble inhibitor-transfected groups (Fig. 4C and D). Moreover, western blot analysis was performed to detect WWOX protein expression in the MG63 cells transfected with anti-miR-214-3p or scramble inhibitor. The results revealed that WWOX protein (Fig. 4E) was significantly upregulated in the cells after the silencing of miR-214-3p. Consistent with the results of western blot analysis, we performed immunofluorescence assay of the MG63 cells transfected with anti-miR-214-3p or scramble inhibitor. The results revealed that WWOX protein expression was evidently upregulated in the cells transfected with anti-miR-214-3p (Fig. 4F). These data demonstrated that WWOX protein expression was suppressed by the overexpression of miR-214-3p in osteosarcoma cells.

Silencing of miR-214-3p inhibits the proliferation of MG63 osteosarcoma cells. To determine the role of miR-214-3p in regulating the proliferation of MG63 cells, the proliferation rates of the MG63 cells were examined by MTT assay. The results demonstrated that the silencing of miR-214-3p significantly suppressed the proliferation of the MG63 cells (Fig. 5A). This was further confirmed by BrdU incorporation assay, which indicated that transfection with anti-miR-214-3p resulted in decreased DNA synthesis per viable MG63 cell (Fig. 5B).

Silencing of miR-214-3p inhibits the migration and invasion of MG63 osteosarcoma cells. We then sought to determine whether the silencing of miR-214-3p affects the migration and invasion of MG63 cells. The results of migration and invasion assay indicated that the silencing of miR-214-3p inhibited the migration and invasion of MG63 cells (Fig. 6).

WWOX inhibits miR-214-3p and miR-10b expression in osteosarcoma MG63 cells. Tumor suppressor genes can exert their effects by regulating miRNA expression in cancer (36) and miRNAs are known to play a role in cancer pathogenesis and some of them function as tumor suppressor genes or oncogenes (37-39). Thus, we hypothesized that WWOX functions as tumor suppressor by regulating relevant miRNAs. Thus miRNA microarrays was performed. RNAs isolated from the MG63 cells were hybridized to a custom miRNA microarray platform. Following hybridization, quantification, and normalization 3 times, a dozen miRNAs were found to be altered in the MG63 cells. However, we were interested in miR-214-3p and miR-10b, as miR-214-3p and miR-10b appear to play the roles of oncogenes in various types of cancer (22,25,40-43). The results of microarray analysis demonstrated that miR-214-3p and miR-10b expression was decreased >50-fold in the cells transfected with the WWOX overexpression plasmids (Fig. 7A). To further confirm the regulatory role of WWOX, we performed
northern blot analysis to detect the expression of miR-214-3p and miR-10b. Consistent with the findings of microarray analysis, the results of northern blot analysis revealed that the overexpression of WWOX significantly downregulated miR-214-3p (Fig. 7B) and miR-10b (Fig. 7C) expression in the MG63 cells.

Discussion

Much research has been carried out in order to understand the pathogenesis of osteosarcoma and to provide novel therapeutic targets for the disease (44-46). miRNAs are small non-coding RNAs of 20-22 nucleotides in length that have been implicated in the development of various types of cancer (47). miR-214-3p expression is deregulated in several human tumors, including melanoma (48), breast cancer (49), ovarian cancer (22), gastric cancer (40), hepatocellular carcinoma (41) and osteosarcoma (25). Its pleiotropic and tumor-specific role in the formation and progression of various types of cancer is achieved by targeting its several target genes (22,25,40,41,48,49). miR-214-3p can function as a key and core regulator by regulating vital signaling networks, such as β-catenin, PTEN/AKT and the tyrosine kinase receptor pathways (42,43). Moreover, miR-214-3p can also regulate the expression of essential cancer-associated genes, for example, enhancer of zeste homolog 2 (Ezh2; the epigenetic repressor), p53, transcription factor AP-2 alpha (TFAP2) and miR-148b (22,48,50). Consistent with its role of regulating essential cancer-associated genes, miR-214 seems to play crucial roles in promoting tumor cell proliferation, stemness, angiogenesis, migration, invasiveness, extravasation, intravasation, metastasis, resistance to chemotherapy and in the microenvironment (22,25,40-43).

In the present study, we firstly observed that WWOX protein was downregulated in osteosarcoma and that it regulated cell proliferation and EMT-associated gene expression (Fig. 7D). Moreover, WWOX overexpression suppressed the proliferation, migration and invasion of MG63 osteosarcoma cells. These results, along with the fact that the deletion of the WWOX gene and the frequent loss of its protein expression are detected in human osteosarcoma, and the tumor suppressor, WWOX, inhibits osteosarcoma metastasis by modulating RUNX2 function (18,51), confirmed that WWOX is a tumor suppressor in osteosarcoma.

In line with the findings of previous studies demonstrating that miR-214-3p is an oncogene in various types of cancer (22,25,40-43), the silencing of miR-214-3p upregulated WWOX protein expression and inhibited the proliferation, migration and invasion of osteosarcoma cells. This finding may partly explain why WWOX protein is downregulated in osteosarcoma, since miR-214-3p is significantly upregulated in the disease. When we overexpressed WWOX in MG63 cells, it negatively regulated miR-214-3p. Thus, we concluded that the deregulation of microRNA-214-3p expression is involved in a negative feedback loop with WWOX in osteosarcoma. miR-10b has been proposed as an oncogene in various types of cancer (52-54). We found that WWOX significantly suppressed miR-10b expression in MG63 cells, although the role of miR-10b has not been reported in the disease, at least to the best of our knowledge.

In conclusion, the deregulation of microRNA-214-3p expression is involved in a negative feedback loop with WWOX in osteosarcoma, indicating that miR-214-3p has potential basic and clinical implications. On the one hand, WWOX may be a powerful osteosarcoma suppressor by exerting inhibitory effects on cell proliferation, migration and invasion, and the pharmacological restoration of WWOX may represent a promising therapeutic strategy. On the other hand, miR-214-3p as an oncogene is also associated with the pathogenesis and progression of osteosarcoma, and the targeting of miR-214-3p may also be apotential therapeutic strategy for patients with osteosarcoma.

References


