Evidence of molecular alterations in the tumour suppressor gene *WWOX* in benign and malignant bone related lesions of the jaws

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Abstract. WWOX is a tumour suppressor gene altered in various human neoplasms. Deletion of *WWOX* is associated with bone metabolic defects and development of osteosarcoma in mice. We hypothesized that alterations of this gene are associated with the development of benign and malignant mesenchymal bone related lesions of the jaws. We investigated *WWOX* mRNA by nested reverse transcription-PCR and direct sequencing and quantitative real-time PCR in two osteosarcoma, two fibrosarcoma, eight ossifying fibroma and two fibrous dysplasia fresh samples. Malignancy was associated with a decreased *WWOX* mRNA expression. Aberrant transcription pattern was found in five samples; however, the relative quantification (RQ) of the *WWOX* mRNA in such lesions was not different from those carrying only the wild-type. We provide new evidence of *WWOX* alterations in osteosarcomas and demonstrate for the first time alterations of this gene in fibrosarcomas as well as in ossifying fibromas of the jaws.

Introduction

Osteosarcoma is the most common non-hematologic bone malignancy in children and adults. Unlike other sarcomas, such as synovial sarcoma, alveolar rhabdomyosarcoma, and Ewing's sarcoma, no specific translocations or other genetic abnormalities have been identified in osteosarcoma (1). Loss of expression or mutations of the tumour suppressor genes retinoblastoma (RB1, MIM +180200), cyclin-dependent kinase inhibitor 2A (CDKN2A/TP16, MIM *600160), TP53 (MIM *191170), and activation of c-MYC, FOS, MDM2, among other genes have been described (1). However, the molecular pathogenesis of ostesarcoma remains to be established.

The fibrosarcoma, formerly a common subtype of soft-tissue sarcoma, is now a rather rare diagnosis, owing to advances in the classification of soft-tissue tumours (2). Although infantile fibrosarcoma shows a translocation (12;15) (p13;q26) that results in fusion of the *NTRK3* gene (located on chromosome 15) to *ETV6* gene (located on chromosome 12p), adult fibrosarcoma is not associated with specific translocations (3). Therefore, molecular changes related to fibrosarcomas are not fully understood.

Benign fibro-osseous lesions of the craniofacial complex comprise a diverse group of lesions characterized by replacement of bone by cellular fibrous tissue containing foci of mineralization that varies in amount and appearance (4). A member of this group is the fibrous dysplasia, a developmental condition arising as a result of activating missense mutations of the *GNAS1* gene, that encodes the GSα subunit of the heterotrimeric G protein complex (5,6). It can lead to facial deformity/asymmetry in variable degrees. Ossifying fibroma and juvenile ossifying fibroma are benign neoplastic lesions of the jaws, which can cause bone expansion. The presence of clonal aberrations in a subset of ossifying fibroma and fibrous dysplasias cases has been demonstrated, suggesting that molecular alterations are responsible for such tumours (7). Indeed, we have previously demonstrated that mutations of the *HRPT2* gene, a tumour suppressor gene, are associated with ossifying fibroma (8).

WWOX is a tumour suppressor gene that spans the common fragile site FRA16D (9-11). The *WWOX* mutations are rare and usually the gene is altered by deletions or translocations in many cancer types including prostate, breast, esophageal, pancreatic, lung, stomach, and oral squamous cell carcinomas (10,12-18). Mice carrying a target deletion of the *Wwox*$^{-/-}$ gene developed osteosarcoma spontaneously (19). Ludes-Meyers *et al* (20) demonstrated that *Wwox* hypomorphic mice displayed reduced levels of *Wwox*. Although these animals had a shorter life span than the wild-type mice, they survived. These results suggest that low levels of *Wwox* protein are enough to overcome the lethality observed in the *Wwox*$^{-/-}$ mice described by Aqeilan *et al* (21). In addition, a

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Table I. Diagnosis, gender and age of the patients of the samples included in the study.

Sample	Gender	Age	Diagnosis
1	F	48	Osteosarcoma
2	F	34	Osteosarcoma
3	M	56	Fibrosarcoma
4	F	51	Fibrosarcoma
5	F	56	Fibrous dysplasia
6	F	30	Ossifying fibroma (primary)
7	F	33	Ossifying fibroma (recurrence)
8	F	58	Ossifying fibroma
9	M	15	Ossifying fibroma
10	M	19	Juvenile ossifying fibroma
11	F	24	Ossifying fibroma
12	F	25	Ossifying fibroma
13	F	28	Ossifying fibroma
14	M	11	Fibrous dysplasia

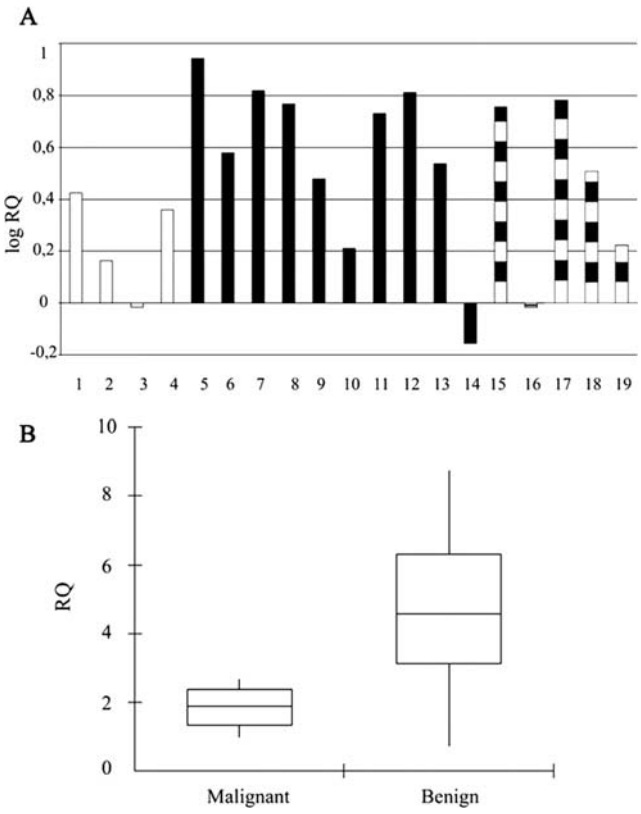

Figure 1. *WWOX* qPCR results. (A) Quantitative PCR showing higher transcription of the *WWOX* gene in malignant lesions (white bars), benign lesions (black bars) and normal oral mucosa (black and white bars) compared to normal blood (x-axis). Ostesarcoma (1 and 2), fibrosarcoma (3 and 4), fibrous dysplasia (5 and 14) and ossifying fibroma (6-13) and normal oral mucosa (15-19). (B) Despite expressing more *WWOX* than blood, decreased expression of *WWOX* was observed in malignant lesions (cases 1-4) compared to benign (cases 5-14) (P=0.047). RQ, relative quantification.

recent study in *Wwox* knock-out mice suggested that the *Wwox* has a significant role to bone tumour growth and the accompanying bone disease (21).

Considering all the available evidence regarding the importance of the *WWOX* in bone proliferation as well as the paucity of information regarding the expression of this gene in bone diseases involving the jaws, we conducted the present study.

Materials and methods

Human tissue. A total of 14 samples comprising two osteosarcomas, two fibrosarcomas, two fibrous dysplasia, seven ossifying fibromas and one juvenile ossifying fibroma were included in this study (Table I) according to criteria previously described (2). Five normal oral mucosa samples were also included as normal tissue reference. In each case, a portion of the lesion was resected, immediately snap frozen and stored at -80˚C. For immunohistochemistry and histopathological analyses, the surgical samples were fixed in 10% buffered formalin and paraffin embedded. The local Ethics Committee approved the present study.

Quantitative real-time PCR (qPCR). Total RNA was isolated using Reagente Tri-Phasis (BioAgency, São Paulo, Brazil) and treated with DNase (Invitrogen Life Technologies, Carlsbad, CA, USA). cDNA was synthesized with Superscript First-Strand Synthesis System Kit (Invitrogen Life Technologies) according to the manufacturer's instructions. For quantitative PCR analyses, wild-type transcripts of the *WWOX* cDNA were detected. We used 1X SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and primer sequences previously described (17). Reactions were performed in duplicate and ran on a Step One machine (Applied Biosystems). The cycling parameters were 10 min at 95˚C followed by 40 cycles at 95˚C for 15 sec and 57˚C for 1 min followed by melting curve analysis. *WWOX* expression was normalized to actin (internal control). The average threshold cycle (Ct) for two replicates per sample were used to calculate ΔCt. Relative quantification (RQ) of *WWOX* cDNA was calculated with the $2^{-\Delta\Delta Ct}$ method. As calibrator we used normal blood samples. Oral mucosa cDNA was used as normal tissue reference.

Nested reverse transcription-PCR (RT-PCR) and direct sequencing of transcripts. Total cDNA was used as a template for nested PCR amplification of the human *WWOX* cDNA as previously described (13). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was amplified as a control for cDNA quality. The amplified products were subjected to electrophoresis on a 6.5% polyacrylamide gel, followed by silver staining. cDNA bands corresponding to the normal and abnormal size transcripts were eluted and purified from agarose gel using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and sequenced. Briefly, 3-5 μl of purified PCR products were sequenced in both directions using two primer sets that amplify the whole open reading frame. DNA sequencing was performed on ABI PRISM 310 (Applied Biosystems) using the Big Dye terminator cycle sequencing kit (Applied

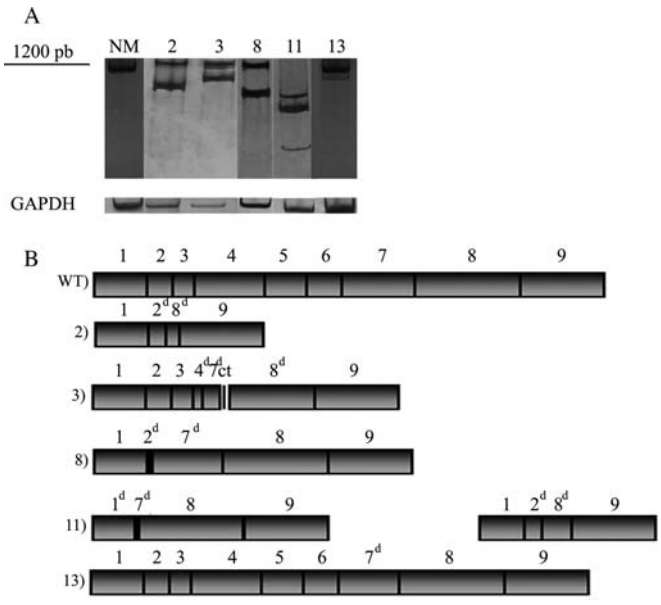

Figure 2. Analysis of *WWOX* aberrant transcripts. (A) Total RNA was subjected to Transcriptase Reverse PCR (RT-PCR) and *WWOX* transcripts were visualized in silver stained 6.5% polyacrylamide gel. The *WWOX* wild-type (WT) band runs at approximately 1200 bp. GAPDH was used as control of cDNA synthesis. (B) Aberrant products were purified from gel bands and direct sequenced. NM, normal oral mucosa; 2, osteosarcoma; 3, fibrosarcoma; 8, 11 and 13, ossifying fibroma; [d]indicates partial loss of the exon; ct, dinucleotide insertion. There was a polymorphism C969G located in the exon 8, nucleotide 969 (pro →ala), in the aberrant transcript of sample 13.

Biosystems). GenBank accession number NM_016373.1 was used as a reference sequence.

Statistical analysis. Statistical significance was analysed with the Mann-Whitney test. $P<0.05$ was considered to indicate statistical significance. These tests were performed with BioEstat software (Belém, Brazil), version 4.0.

Results

Quantitative PCR (qPCR) amplification was performed to determine only wild-type *WWOX* expression, as none of the aberrant transcripts conserved the sequences of qPCR primer annealing. As shown in Fig. 1A, on average, benign and malignant samples expressed more *WWOX* mRNA than the calibrator (blood of normal patients) (x-axis). However, there was a lower RQ (relative quantification) of *WWOX* mRNA in the malignant samples when compared to the RQ of the benign group (P=0.047), although the expression of both, malignant and benign groups, did not differ from the normal tissue control (Fig. 1).

As deletions are frequent in *WWOX*, in order to investigate the expression of aberrant *WWOX* mRNA, nested RT-PCR was carried out in all 14 tumour samples. Five samples (one osteosarcoma, one fibrosarcoma and three ossifying fibromas) showed aberrant transcripts with total or partial loss of exon length (Fig. 2). We found no statistically significant difference between the relative quantification (RQ) of the wild-type *WWOX* mRNA expression of samples showing aberrant products compared to those carrying only the wild-type (P=0.62). An interesting finding when observing only the benign lesions was that the median of the RQ of *WWOX* mRNA in the samples carrying only the wild-type transcript (median 3.39) is similar to the RQ of the normal oral mucosa (median 3). On the other hand, the median of the RQ of the benign samples carrying aberrant transcripts (median 4.63) is higher than the RQ of the normal oral mucosa (median 3).

Discussion

In 2007, Aqeilan and colleagues (19) developed a mouse strain lacking *WWOX* expression. In the absence of any carcinogenic treatment, about 30% of juvenile mice developed focal lesions in the femur resembling early chondroid osteosarcomas. Histological examination suggested proliferation of progenitor cells arising from the periosteum. These authors concluded that the formation of these bone lesions supported the notion that, at least in the bone, *WWOX* acts as a tumour suppressor. Two years after, Ludes-Meyers and colleagues (22) developed Wwox KO mice which presented bone metabolic defects. These animals showed smaller and thinner bones, having reduced bone volume as a consequence of a defect in mineralization, although no evidence of spontaneous neoplasia was observed in *Wwox* KO mice. In the present study we investigated the role of the *WWOX* gene in a set of human bone-related tumours.

First we investigated the transcription of the *WWOX* using quantitative real-time PCR (qPCR) reactions. Then, RT-PCR followed in order to investigate the presence of aberrant transcripts. The malignant samples showed a lower relative quantification of *WWOX* compared to the benign samples (Fig. 1B). The data support the idea that *WWOX* works as a tumour suppressor gene in bone lesions as previously proposed by Aqeilan *et al* (19). The decreased expression levels of *WWOX* in malignant neoplasias may be involved in the genetic regulation of many genes relevant to the malignant neoplasia pathogenesis, as *WWOX* interacts with other tumour suppressor proteins such as p53 and p73 (24,25). The decreased *WWOX* expression we demonstrated in the malignant bone related tumor is in line with the recent findings of Kurek and colleagues, that showed absent or reduced expression of *Wwox* protein in 58% of the osteosarcoma samples evaluated (28). In addition, a recent report using comparative genomic hybridization showed that the *WWOX* gene is deleted in three/ten osteosarcomas and also showed that the immunohistochemistry could not detect the protein in 34/55 samples (23).

The RT-PCR results demonstrated the existence of aberrant transcripts in one osteosarcoma, one fibrosarcoma and three ossifying fibroma samples. Whether this transcriptional alteration is an early event in the pathogenesis of such tumours or if it is just a result from the neoplasia progression remains to be elucidated. The biological role of such transcripts, if they have one, is speculative (26). As the *WWOX* gene is located in a fragile site, the aberrant transcripts are expected to be present in the neoplasias. Aqeilan and collegues (27) showed that tumours from *WWOX*$^{+/-}$ mice demonstrated Wwox protein expression. Their results suggested that the inactivation of one allele of W*WOX* accelerates the predisposition of normal cells to malignant

transformation (27). The presence of aberrant transcripts may be associated with reduced levels of the full length Wwox protein. Assuming that *WWOX* exhibits haplo-insufficiency (27) the reduced expression of the wild-type protein could contribute to neoplasia development. In the present study, the presence of the aberrant transcripts did not associate with different *WWOX* wild-type mRNA expression levels though. It is important to reinforce that the aberrant transcripts lacked the sequences of qPCR primers annealing, so that the qPCR detected only the normal size transcripts.

In conclusion, our study gives support to the notion that *WWOX* expression is altered in osteosarcomas and we provide additional evidence that it is also altered in fibrosarcomas. For the first time, we demonstrate the presence of *WWOX* aberrant transcripts in ossifying fibromas of the jaws. The meaning of such aberrant transcripts remains to be clarified.

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