

Diagnostic and Biological Significance of Immunohistochemical Expression of Osteopontin and Ki67 in Fibro-osseous lesions of jaws

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Abstract

Background and Aim: Fibrous dysplasia (FD) and ossifying fibroma (OF) are the most important fibro-osseous lesions (FOLs) of the jaws with similarities in radiographic and morphological features while showing completely different biological behavior. Limited studies have been evaluated immunohistochemistry markers, such as osteopontin (OPN) and osteocalcin to help differentiate these two lesions. This study aimed to assess the immunoeexpression of OPN and Ki67 as potential markers for differentiation of different FOLs.

Materials and Methods: This cross-sectional study was conducted on 12 FD, 19 OF, and eight FOL samples retrieved from the archives of the department of oral pathology. The specimens were examined immunohistochemically using streptavidin-biotin method for OPN and Ki67. The intensity score (IS), proportional score (PS) and total score (TS) were assessed in hard and soft tissue matrix and in mesenchymal cells for Ki67. The data were analyzed by independent samples Kruskal-Wallis.

Results: Osteopontin showed positive immunoreaction in both stromal and trabecular components of all FDs and OFs. Among the scores, PS and TS of bone trabeculae were significantly different in FD and OF ($P=0.005$). Nevertheless, no significant difference was observed in Ki67 expression in mesenchymal cells ($P=0.880$) and OPN scores in soft tissue matrix between the lesions; their P-value were 0.336, 0.340 and 0.415 for IS, TS, and PS, respectively.

Conclusion: Osteopontin can serve as a useful marker for differential diagnosis of FD and OF. However, we suggest evaluation of other NCMPs, especially functionally similar molecules such as bone sialoproteins (BSPs) in FOLs for differential diagnosis.

Key Words: Osteopontin (OPN), Ki67, Fibrous dysplasia (FD), Ossifying fibroma (OF)

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Introduction

Fibro-osseous lesions (FOLs) of the jaws are a diverse group of bone disorders that microscopically characterized by replacement of normal bone with connective tissue containing proliferated fibroblast-like cells and sparse collagen bundles in addition to variable amounts of newly produced mineralized substances [1,2]. Hence, the term FOL refers to a diverse group of

different entities [3], which show similarities, especially in histopathological features. Among these lesions, fibrous dysplasia (FD) and ossifying fibroma (OF) are clinically distinct lesions with similarities in histopathological features while demonstrating completely different biological behaviors and consequently require different management and treatment planning [4,5]. Fibrous dysplasia is a developmental or hamartomatous

condition [1,2]. According to the World Health Organization [6], it is a genetically-based sporadic disease, which probably arises from an activating point mutation of the stimulatory G protein gene associated with cell differentiation into mature osteocytes [4,7].

Ossifying fibroma (OF) is a benign neoplasm with a slow and progressive growth [2,8]. Some researchers have found mutation of HRPT2 gene in a few cases of OF [3]; however, its role in the pathogenesis of OF has not been understood.

Osteopontin (OPN) is a non-collagenous multifunctional phosphorylated glycoprotein (NCMPs), which bonds to the bone matrix and causes bone remodeling and repair [3,9-11]. It has been shown that OPN is an essential factor responsible for the increase in osteoclastic bone resorption and a decrease in osteoblastic bone formation usually associated with skeletal unloading [12].

Ki67 is one of the cellular proliferation markers, which is expressed in the nuclei of growing cells and shows significantly different expression in reactive, benign and malignant neoplastic lesions [13-15].

The aim of this study was to assess expression of OPN in hard tissue and soft tissue matrix in three groups of FOLs to evaluate its possible role in differential diagnosis of the lesions. We also assessed the expression of Ki67 in mesenchymal cells in FD as a hamartomatous non-neoplastic lesion and compared it with OF and FOLs, as a neoplastic lesion and an osseous dysplasia respectively, to evaluate the possible difference biologically.

Materials and Methods

Tissue specimens:

This study was conducted in Department of Oral Pathology, School of Dentistry, Shahid Beheshti University of Medical Sciences. All cases which diagnosed with FD, OF, and FOL from 2003 to 2014, were selected. There were some cases of FOL, which could not be distinguished as FD or OF by the pathologists using clinical, radiographic and histopathologic features; probably due to the mature phase of the lesions. Patient information such as age, sex, the location of the lesion and histopathological diagnosis were recorded in a

datasheet. Histopathological features were examined on hematoxylin and eosin-stained tissue sections under the light microscope (Leica, CMS GmbH, Wetzlar, Germany, Model: DM 500).

Immunohistochemistry:

Formalin-fixed paraffin-embedded tissue blocks were used for immunohistochemical analysis. Tissue blocks were decalcified using 10% formic acid. After deparaffinization of sections with xylene, they were rehydrated in graded ethanol series (80%, 90%, 95%, and three 100%) and immersed in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature to block endogenous peroxidase activity. In the next step, for antigen retrieval, sections were incubated with retrieval solution (Tris 1/21 code 8382E510221 + EDTA 0.37 gr, Merck, Germany) with a pH of 6 for 15 minutes in a microwave. The sections were separately incubated with primary antibodies for one hour at room temperature including lyophilized monoclonal mouse anti-human OPN antibody (Leica Biosystems UK Novocastra Liquid Osteopontin Clone OP3N Code NCL-L-O-PONTIN) diluted to 1:20, and monoclonal mouse anti-human Ki67 antibody (Dako, Cytomation Denmark Clone: MIB-1 code IR626/IS 626 Ready to use). The EnVision kit (Dako Real Envision + system+ HRP Rabbit/ Mouse K 3468) indirect peroxidase system was used. The sections were reacted with 3,3'-diaminobenzidine (DAB, Dako Denmark Code: K3468) in order to visualize staining. The sections were subsequently counterstained with Mayer's hematoxylin and mounted with Entellan (Merck Ltd, Darmstadt, Germany). Results of staining for OPN were analyzed in cytoplasm/nucleus of mesenchymal cells and hard tissue [16], and the intensity score (IS) and proportional score (PS) were calculated and reported [17].

The IS was given as follows: 0= No positively stained cell or weak staining, 1= moderate staining, and 2=strong staining [17]. To determine PS, we used the following cutoff points: 0= Stained tissue <5%, 1= stained tissue between 5% up to 25%, 2= stained tissue between 25% up to 50%, 3= stained tissue more than 50% [18].

The total score (TS) of OPN expression was defined as IS plus PS. Results of staining intensity for Ki67 were evaluated in the nuclei of

fibroblast-like cells at high power field and categorized as negative, weakly positive, or strongly positive and scored 1, 2 and 3, respectively [19-21]. All stained slides were observed under a light microscope (LEICA DM 500, Germany) by two pathologists blinded to the group allocation of samples.

Statistical analysis:

Data were analyzed with SPSS version 21 (SPSS Inc., IL, USA). Independent samples Kruskal-Wallis test was used to compare the expression of OPN and Ki67 in FD, OF, and FOL due to the OPN and Ki67 expression and presence of three independent groups. $P < 0.05$ was considered statistically significant.

Results

Forty-one specimens including 19 OF, 12 FD, eight FOL with indefinite diagnosis, and two cemento-osseous dysplasia (COD) samples were retrieved from the archives of the Department of Oral Pathology. Of 12 FDs, eight cases were males, and four were females with a mean age of 29.91 ± 15.49 years (Table 1). Of 19 OFs, three cases were males, and 16 were females with a mean age of 40.58 ± 15.44 years. Among eight FOLs, two cases were males, and six were females with a mean age of 34.13 ± 9.26 years. Both cases of COD were females aged between 61 and 64 years. Among 12 cases of FDs, Seven cases of were located in the maxilla, two in the mandible, one in orbit, one in the maxillary sinus, and the

location of the last lesion was unknown. Five cases of OFs were located in the maxilla, 10 in the mandible, and locations of four cases were not registered. According to the patients' files, among eight cases of FOLs, five cases were located in the mandible, one in the maxilla, and one was found in orbit. Both two cases of CODs were located in the mandible.

All cases of FD and OF revealed immunohistochemical staining of OPN in both bone trabeculae and stromal cells (Figure 1). In hard tissue, Kruskal-Wallis test revealed no statistically significant differences ($P=0.094$) in IS of OPN between FD and OF (Table 2). However, PS of OPN in hard tissue of OFs showed a significant increase in immunostaining ($P=0.005$, Table 2). Moreover, TS of OPN immunopositivity in hard tissue revealed a statistically significant higher expression ($P=0.009$) in OF group (Table 2, Figure 1).

Immunoreactivity of OPN in mesenchymal cells showed variable expression in FD and OF and even in different cases within each group. Nevertheless, IS, PS, and TS of OPN in mesenchymal cells did not reveal any statistically significant differences between groups ($P=0.336$, $P=0.340$, and $P=0.415$, respectively, Table 3, Figure 1). The expression of Ki67 in different groups is summarized in Table 4. Kruskal-Wallis test revealed no statistical differences between groups ($P=0.880$) in Ki67 expression in the mesenchymal cell (Figure 1).

Table 1. Demographic data of patient with FD, OF and FOL

Parameter	FD (n=12)	OF (n=19)	FOL (n=8)	P-value	
Age (mean \pm SD)	29.91 \pm 15.49	40.58 \pm 15.44	34.13 \pm 9.26	0.160	
Gender	Male N(%)	8(66.7)	3(5.8)	2(25)	0.014
	Female N(%)	4(33.3)	16(84.2)	6(75)	
Location	Mandible N(%)	2(18.2)	10(66.7)	5(71.4)	0.078
	Maxilla N(%)	7(63.6)	5(33.3)	1(14.3)	
	Orbit N(%)	1(9.1)	0	1(14.3)	
	Maxillary sinus N(%)	1(9.1)	0	0	
	Undefined	1	4	1	

FD: Fibrous dysplasia, OF: Ossifying fibroma, FOL: Fibro-osseous lesion

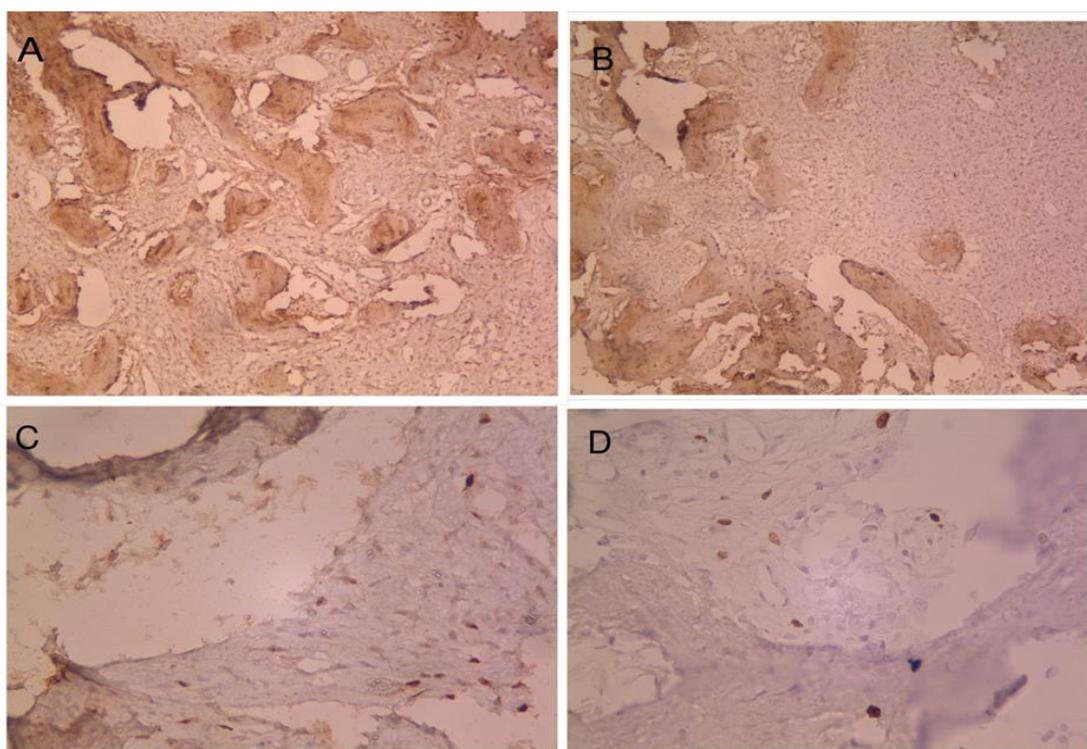


Figure 1. Immunoexpression of osteopontin (OPN) and Ki67 in fibrous dysplasia (FD) and ossifying fibroma.

Strong hard tissue and mesenchymal staining were noted for osteopontin in FD (A) and OF (B).

Immunostaining of Ki67 in nuclei of mesenchymal cells in FD (C) and OF (D).

Table 2. Scores of osteopontin expression in hard tissue matrix; values are presented as number (percentage)

	IS Scores			PS Scores				TS Scores					
	0	1	2	0	1	2	3	0	1	2	3	4	5
FD	3 (25)	3 (25)	6 (50)	3 (25)	1 (83)	3 (25)	5 (41.7)	3 (25)	0 (0)	0 (0)	4 (33.3)	0 (0)	5 (41.7)
OF	1 (5.3)	6 (31.6)	12 (63.2)	1 (5.3)	1 (5.3)	6 (31.6)	11 (57.9)	1 (5.3)	0 (0)	1 (5.3)	5 (26.3)	1 (5.3)	11 (57.9)
FOL	1 (12.5)	6 (75)	1 (12.5)	3 (37.5)	3 (37.5)	2 (25)	0 (0)	1 (12.5)	2 (25)	2 (25)	3 (37.5)	0 (0)	0 (0)

IS: Intensity score; PS: Proportional score; TS: Total score; FD: Fibrous dysplasia; OF: Ossifying fibroma; FOL: Fibro-osseous lesion

Table 3. Scores of osteopontin expression in soft tissue matrix; values are presented as number (percentage)

	IS			PS				TS					
	0	1	2	0	1	2	3	0	1	2	3	4	5
FD	2 (16.7)	9 (75)	1 (8.3)	6 (50)	5 (41.7)	1 (8.3)	0 (0)	2 (16.7)	4 (33.3)	4 (33.3)	2 (16.7)	0 (0)	0 (0)
OF	4 (21.1)	6 (31.6)	9 (47.7)	7 (36.8)	3 (15.8)	8 (42.1)	1 (5.3)	4 (21.1)	3 (15.8)	3 (15.8)	0 (0)	8 (42.1)	1 (5.3)
FOL	1 (12.5)	5 (62.5)	2 (25)	4 (50)	1 (12.5)	1 (12.5)	2 (25)	1 (12.5)	3 (37.5)	1 (12.5)	1 (12.5)	0 (0)	2 (25)

IS: Intensity score; PS: Proportional score; TS: Total score; FD: Fibrous dysplasia; OF: Ossifying fibroma; FOL: Fibro-osseous lesion

Table 4. Scores of Ki67 expression in mesenchymal cells; values are presented as number of cases (percentage)

	1	2	3	Total
FD	5(41.6)	4(33.4)	3(25)	12(100)
OF	9(47.3)	6(31.5)	4(21)	19(100)
FOL	4(50)	1(12.5)	3(37.5)	8(100)

FD: Fibrous dysplasia; OF: Ossifying fibroma; FOL: Fibro-osseous lesion

Discussion

In many cases of FOLs of the jaws, especially in cases of FD and OF, it is impossible to reach a definite diagnosis despite all the clinical, imaging and histopathological assessments. Therefore, there have been many attempts to resolve this diagnostic uncertainty [2-5]. Since it has been shown that lack of osteoblastic rim in FD is not a reliable finding, many studies have been conducted to find other microscopic features to differentiate between the two entities [2]. Some researchers proposed peritrabecular clefting in FD as a reliable feature for differential diagnosis [22], while other pathologists questioned it as a dependable finding [23]. Molecular and immunohistochemical assessments of FD and OF could resolve this diagnostic dilemma and uncover the real biological nature of these lesions. Most of the studies focused on bone matrix proteins especially osteocalcin and

OPN [7,16,19,20]. In the present study, we evaluated the immunoexpression of OPN and Ki67 in FD and OF cases and found that the extent of bone matrix, which showed immunoreactivity to OPN with or without intensity reaction (PS and TS) was significantly higher in OF than in FD samples.

Formation of hard tissue is a complex process requiring the coordinated interaction of cell function and extracellular matrix [24]. Some of the extracellular matrix proteins play roles in bone morphogenesis [25,26]. Interestingly, studies have shown that specific noncollagenous matrix proteins (NCMPs) control the extracellular deposition of mineral salts [27], and mediate cell biological behaviors such as cell attachment and migration during mineralization and in fully mineralized tissues [28,29]. One such multifunctional extracellular matrix molecule that has been

recently considered is OPN, a phosphorylated sialoprotein [30]. In a physiological bone formation, OPN is secreted by osteoblasts as an NCMP near the mineralization front, where the first mineralization foci appear within the osteoid tissue [31]. Moreover, upon termination of mineralization, OPN will not be diffused; instead, it is appropriately concentrated along the cement line at the interface of new and old bone [31]. On the other hand, OPN and bone sialoproteins (BSP), as members of arginine-glycine-aspartic acid-containing molecules [31], have been termed cell-binding motifs [32] and facilitate osteoblast adhesion to extracellular matrix [32] and bone surface [31]. Therefore, NCMPs regulate hard tissue turnover via initiation, stabilization, and inhibition of mineralization [31]. Generally, NCMPs are present in bone, cementum, and dentin, and OPN is upregulated in reparative dentin [31].

Furthermore, OPN and BSP are expressed in the initial phase of bone mineralization; whereas, osteocalcin and osteonectin are detectably expressed at the final stage and fully mineralized bone [32]. BSP is a crystal nucleator [33] necessary for increasing the number of hydroxyapatite crystals [34]. However, expression of OPN increases prior to initiation of mineralization and prevents the formation of premature calcium phosphate crystals that do not have the well-organized crystal structure of hydroxyapatite [32]. This mechanism can be explained by the lower expression of OPN in FD, which is composed of woven bone with different crystal organization. Woven bone contains intertwined collagen fibrils organized in different manners with relatively wide interfibrillar spaces [35]. Also, OPN and BSP can be found, either focally or diffusely, between the calcified collagen fibrils [31]. Additionally, collagen is a very slow initiator of mineralization [36]. Thus, the disorganized orientation of collagen fibrils in the woven bone of FD could be another reason for lower expression of OPN in FD. Moreover, the structural similarity of the calcified structures of OF to normal bone in general, and composition of cementum structure, in particular, could be additional factors describing an increase OPN expression in OF. Evidence shows that OPN

regulates bone growth and turnover and its expression is related to the maturity of osteoblasts or bone matrix [31]. Therefore, it could be assumed that higher expression of OPN in OF might be related to the more developed characteristics of soft and hard tissue components of OF. Also, formation and structure of the hard tissue in OF share more similarities with normal bone and cementum, and this could be another probable reason for higher expression of OPN in OF. Moreover, some studies demonstrated higher expression of OPN in peripheral OF compared to other reactive lesions which are not related initially to periodontal ligament [37,38]. Also, it could explain its overexpression in FD of the jaws compared to osteofibrous dysplasia, a FOS of long bones. It has been shown that OPN is expressed in tooth development especially during cementogenesis [7]. Since the expression of OPN was higher in OF compared with FD, it could be an emphasize on the cementoblastic differentiation of mesenchymal cells and periodontal ligament origin of OF.

Despite limited studies on the expression of OPN in FOLs, most studies focused on osteocalcin (OC) and had reported controversial results. A previous study showed higher expression of OC in FD than in OF [4], whereas, another study demonstrated that expression of OC in the bone matrix of OF was more than that in FD [16]. However, considering the presence of fibrous stroma in addition to bone matrix, expression of OC was significantly higher in FD than in OF [16]. This discrepancy might indicate the interspecies diversity of OC expression, and its cross-reaction with other closely related matrix proteins, which control mineralization process [38]. This variation in OC expression in different studies was one of the reasons to focus on OPN rather than OC in the current study.

The exact process of calcified tissue formation in biologically different bone lesions is not entirely understood. However, various growth rates and behaviors of different neoplastic and non-neoplastic diseases of bone indicate a wide range of diversities in the mechanism of pathological bone formation. Therefore, proteins expressed for instance in osteogenic sarcoma, might not be expected to express differently in FOLs. Hence,

the present study focused on OPN expression rather than OC.

Excessive mineralization could be prevented by OC and osteonectin (ON) [32], this might probably be the reason for higher expression of OC in some bone producing lesions, such as FOLs and osteosarcoma, which are not entirely calcified.

We also assessed the expression of Ki67 (MIB-1) in FD and OF specimens to assess possible differences due to neoplastic nature of OF compared with the hamartomatous origin of FD. There were no significant differences in Ki67 expression in stromal cells of the two lesions. This finding was different from the results of a study on PCNA (proliferating cell nuclear antigen) expression in peripheral ossifying fibroma (POF) and OF [19]. We believe that the proliferative activity of stromal cells in OF is high enough to show a difference to a reactive lesion. However, FD is a progressive lesion, especially in the initial phase which might originate a high proliferative rate similar to the proliferation activity of stromal cells in OF, a slow-growing neoplastic lesion.

As a limitation, it is important to know that the antibody binding sites of proteins are thoroughly preserved in fully mineralized tissues, and decalcification may remove them significantly [36]. Glutaraldehyde fixation resolves this problem [39], but it is not a conventional way for processing and decalcifying bone lesions in pathology laboratories due to its time consuming and costly nature. We propose that a study should be conducted to compare two methods to find the best technique for decalcification of bone lesions.

The restricted sample size was a limitation of this study. However, the rarity and limited number of FDs of the jaws make it challenging to design studies in this field [16,40]. Only one study in which three centers have collaborated had sufficient cases of FD and OF of the jaws [6], therefore, multicenter studies to obtain a larger sample size, is recommended.

Conclusion

Despite the different nature of different types of FOLs, hard tissue is produced in all of these lesions. Therefore, the study on the bone morphogenesis helps to understand the biological behavior of these lesions. NCMPs appears to be

the most important factors in this process. Our results showed both "PS" and "IS" of OPN are significantly more expressed in OF, but future studies are needed to evaluate other members of NCMPs.

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