

## POTENTIAL ROLE OF MYOFIBROBLASTS IN BEHAVIOR OF ORAL OSSIFYING FIBROMA

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### ABSTRACT

**Review:** Benign Fibro-osseous lesions is a generic term that includes a group of diseases affecting jaw and facial bones. Myofibroblasts (MFs) are cells that share properties of fibroblasts and smooth muscle cells. They have a main role in tissue growth, development and healing, and during organ fibrosis and cancers. Alpha smooth muscle actin ( $\alpha$ -SMA) is recognized to be present in smooth muscle cells, myoepithelial cells and pericytes. So it used to identify soft tissue neoplasms of smooth muscle, myoepithelial and myofibroblastic origin. Ki67 is one of the markers which is used to detect cellular proliferation in growing cells.

**Aim of study:** The present study aimed to examine the immunohistochemical labeling of  $\alpha$ -SMA in juvenile ossifying fibromas (JOFs), central ossifying fibromas (COFs) and peripheral ossifying fibromas (POFs) and to correlate its expression with their proliferative ability through Ki67 expression.

**Material and Methods:** Immunohistochemical expression of  $\alpha$ -SMA and Ki67 was evaluated in 10 samples of each of JOF, COF, and POF.

**Results:** JOFs showed the highest mean value of  $\alpha$ -SMA and Ki67 expression ( $16.69 \pm 5.25$ ) ( $17.04 \pm 2.75$ ) followed by COFs group ( $5.93 \pm 1.94$ ) ( $8.33 \pm 1.23$ ) while POFs showed the lowest mean value ( $1.54 \pm 0.64$ ) ( $3.14 \pm 0.95$ ), respectively, with a statistically significant positive correlation between  $\alpha$ -SMA and Ki67 in all groups together ( $P$ -value  $\leq 0.05$ ).

**Conclusion:** Overexpression and significant positive correlation between  $\alpha$ -SMA and Ki67 expression in JOFs, COFs, and POFs indicate the important role of MFs in the aggressive behavior of JOFs when compared with COFs and POFs through enhancing cellular proliferation, cellular migration, angiogenesis, and extracellular proteolytic activity and influencing matrix formation.

**KEY WORDS,**  $\alpha$ -SMA , Ki67, BFOLs, juvenile ossifying fibromas, central ossifying fibromas, and peripheral ossifying fibromas

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## INTRODUCTION

Benign Fibro-osseous lesions' (BFOLs) is a generic term that includes a group of diseases affecting jaw and facial bones (Slootweg et al., 2005) which are recognized histologically by the process in which normal bone is replaced by mesenchymal tissue with proliferating fibroblast-like cells and few collagen bundles together with variable quantity of newly formed calcified tissue (Sargolzaei et al., 2017). These include: fibrous dysplasia (FD), osseous dysplasia, and ossifying fibroma (OF) and their subtypes (Slootweg et al., 2005).

Ossifying Fibroma (OF) is considered the solely BFOL representing a true neoplasm with HRPT2 gene mutation reported in a few cases (Pimenta et al., 2006). There are classical and aggressive juvenile subtypes (Omer et al., 2008). OF is considered to originate from the periodontal membrane, it could occur both intrabony (central ossifying fibroma (COFs) and in soft tissues (peripheral ossifying fibroma (POFs) in the jaws (Liu et al., 2010). Microscopically, the lesion consists of variable amounts of newly formed and mature bony trabeculae, cementum-like tissue, dystrophic calcifications and variable quantity of collagen fibers with different amount of cells in their stroma (Hunasaki and Raghunath 2012)

COFs are expansile, well-circumscribed and bony lesions that tend to grow slowly usually occur in posterior regions of the mandible of adult in the third and fourth decade of life (Gondivkar et al., 2011, Urs et al., 2013). They originate from the endosteal layer or periodontal ligament (PDL), and grow outside from the medullary cavity of the bone (Alam et al., 2008).

POFs are sessile or pedunculated gingival diseases; they affect females more than men in their second decade of life and most commonly located in maxillary anterior region. The size of most of the lesions is usually smaller than 2 cm, sometimes larger cases occur, this destroys more amount of the surrounding bone causing marked facial disfigurement (Das and Azher 2009, Chatterjee et

al., 2010, Mishra et al., 2011) . POFs demonstrate a close relationship with the PDL, and usually occur only in the soft tissues overlying the alveolar process (Alam et al., 2008).

Juvenile ossifying fibromas (JOFs) are considered to be benign tumors, they characteristically grow progressively and destroy the maxillary and paranasal bones of young people and are considered as aggressive variant of COFs (Aggarwal et al., 2012, Patil et al., 2013, Ranganath et al., 2014). JOFs have a capsule and well limited with the production of variable amounts of mineralized tissue as bone and/or cementum-like material which represent two distinct histopathological patterns: trabecular and psammomatoid (El-Naggar et al., 2017, Abramovitch and Rice , 2016).

Myofibroblasts (MFs) are cells that have the ability to contract and share properties of fibroblasts and smooth muscle cells (Tomasek et al., 2002). During embryonic life, MFs originate from progenitor neural crest cells during the 13th week of pregnancy (Radisky et al., 2007) and are found in the PDL (Meng et al., 2010). They have a main role in the way tissues grow, develop and heal as well as during organ fibrosis and cancer progression. The function of MFs when the extracellular matrix (ECM) remodels in various intrabony diseases could influence the nature and growth of these lesions by affecting matrix production, cellular division, cellular movement, angiogenesis, and extracellular proteolytic activity (Goel et al., 2019). Vimentin, desmin and  $\alpha$ -SMA are the three filaments most commonly applied to classify MFs.  $\alpha$ SMA has been recognized as the most trusted marker of myofibroblast differentiation (Gabbiani 2003).

Actin is a 42-kDa microfilament protein involved in the cell skeleton and is nearly found in all cell types. Actin functions together with severable membrane and cytoplasmic cell skeleton proteins; it acts in the way how the cells contract, move and in cell adhesion (Gunst and Zhang 2008). The alpha isoform of smooth muscle actin ( $\alpha$ -SMA) is recognized as one of cytoskeletal proteins in smooth muscle cells, myoepithelial cells and pericytes. So

MFs particular monoclonal antibodies that identify  $\alpha$ -SMA are utilized to characterize soft tissue neoplasms of smooth muscle, myoepithelial and myofibroblastic origin (Folpe and Gown 2008).

$\alpha$ -SMA has been recognized in many primary bone neoplasms, such as leiomyosarcoma, malignant fibrous histiocytoma, osteosarcoma, giant cell tumor of bone and chondromyxoid fibroma (Salas et al., 2007, Ueda et al., 2002, Watanabe et al., 1997).

It was found also in leukemia, myeloma and some secondary bone tumors; the nature of cells which express  $\alpha$ -SMA in these neoplasms was not established (Papadopoulos et al., 2001).

Elevated proliferation ability of cells could indicate the aggressiveness of lesions. There are a lot of methodology procedures to detect proliferation potentiality. Recognition of Ki67 proliferation marker immunohistochemically is one of these (Omer et al., 2008). Ki67 is one of markers which is used to detect cellular proliferation, and is expressed in the nuclei of growing cells and is present in all cell cycle stages, except in G0 (Bohn et al., 2011). So it demonstrates a significant variable expression in reactive, benign and malignant neoplastic lesions (Thennavan et al., 2015, Sinanoglu et al., 2015, Humayun and Prasad, 2011).

Fibro-osseous lesions differential diagnosis is considered a true challenge, resulting in the need to search for useful biomarkers in cases of debate (Tabareau-Dalalande et al., 2015). Therefore, the following study was undertaken to evaluate the expression of  $\alpha$ -SMA and Ki 67 in COFs, POFs and JOFs and to correlate their expression with the pathogenesis or progression of these diseases.

## MATERIALS AND METHODS

### Case selection and immunohistochemical staining

In the present study, 10 samples of (POF, COF and JOF) were utilized. All cases were retrieved from the Oral Pathology archive, National Cancer Institute, Cairo University. Briefly, immunohistochemical procedures were as follows: paraffin wax blocks

were sectioned into four micrometer. Removal of wax from the sections was done with xylol and its water content was retrieved by placing sections in descending concentration of alcohol. The sample sections were immersed in citrate buffer before the immunohistochemical staining procedures. Peroxidase-antiperoxidase methodology using the biotin-streptavidin system was performed; we added 3% hydrogen peroxide to the tissue sections to inhibit endogenous peroxidase action. Primary antibodies  $\alpha$ -SMA (Lab Vision, Ferment CA, USA) and Ki 67 (Genome Me Lab) were added and then placed in the incubator at room temperature overnight. Then the sections were washed in phosphate buffer saline (PBS), and then link antibody was placed, followed by streptavidin labeling antibody. After washing with PBS, diaminobenzidine chromogen was applied to the sections followed by the counterstain.

The water content of the sections was removed by putting them in ascending concentration of alcohol, immersed in xylol and mounted. All the steps of immunohistochemical quantitative measurements were done on images taken at a magnification of X40. The photomicrographs are captured with a camera linked to the microscope and then the image analysis for the collected photomicrographs was done using the image software (Image J, 1.41a, NIH, USA).

### Statistical analysis

Analysis of the data was done using Statistical Package for Social Science software computer program version 26 (SPSS, Inc., Chicago, IL, USA). Shapiro-wilk test was used to find normal distribution of data. Quantitative data was parametric and represented in mean and standard deviation. One way ANOVA (Analysis of variance) followed by post-hoc tukey was utilized for the comparison of more than two different groups of parametric data. Pearson's correlation was applied to find the correlation between  $\alpha$ -SMA & Ki67. P value smaller than 0.05 was considered to be of statistical significance.

## RESULTS

### 1- Immunohistochemical Results

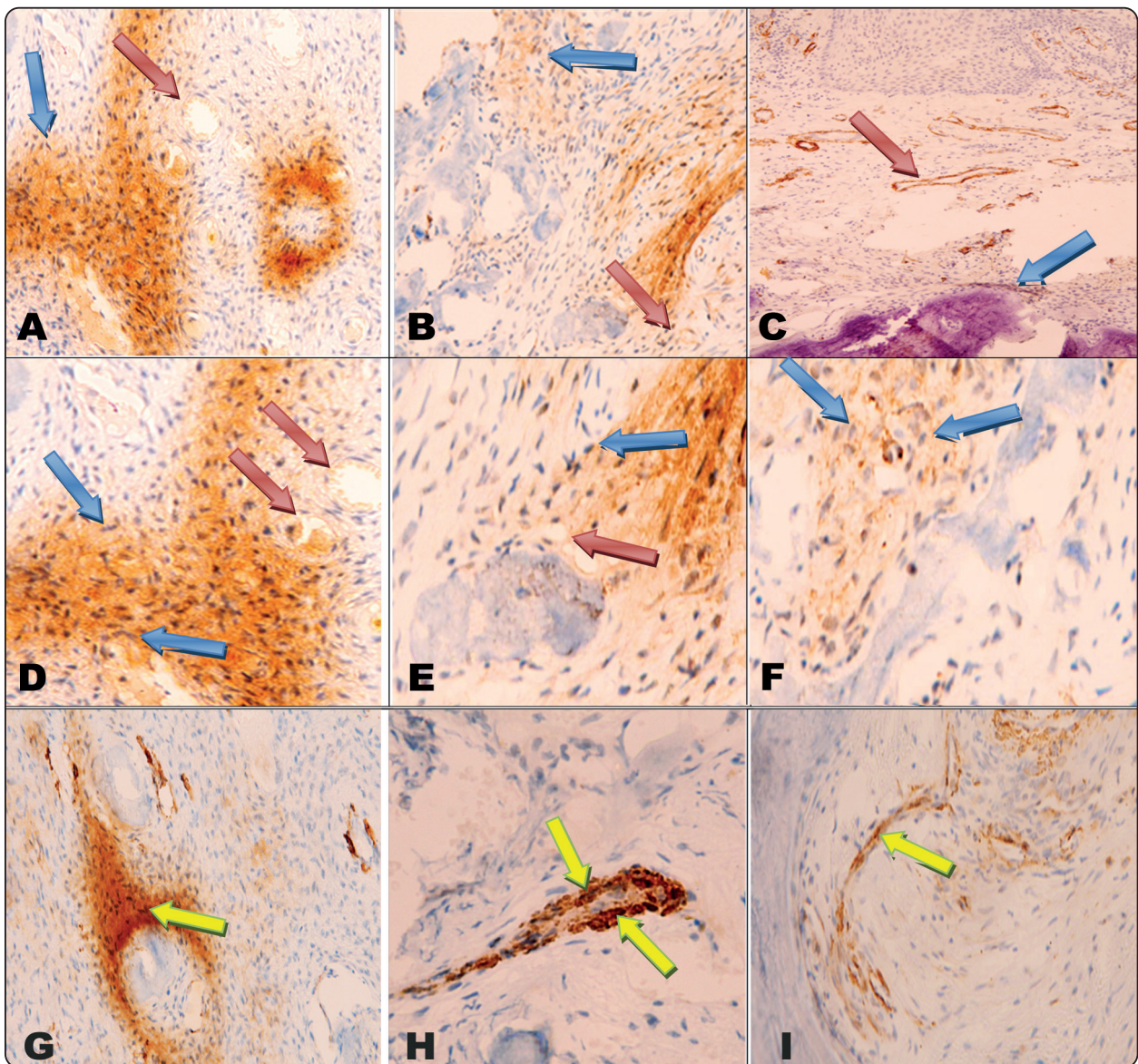
#### A. Smooth Muscle Actin:

All 10 cases of POF, COF and JOF showed  $\alpha$ -SMA immunopositivity. The immunoreactivity was seen in the cytoplasm of stromal spindle cells in the lesions studied. The endothelial cells lining the blood vessels in the stroma of the three categories

also demonstrated immunoreactivity of  $\alpha$ -SMA in their cytoplasm (Fig.1. A, B, C, D, E, F).

#### B. Ki 67:

All 10 cases of POF, COF and JOF demonstrated positive Ki 67 immunoreactivity. The stromal spindle cells in the three lesions showed nuclear and cytoplasmic Ki 67 expression. In some cases of POF the hyperplastic covering epithelial cells showed nuclear immunopositivity (Fig.1. G, H, I, J)



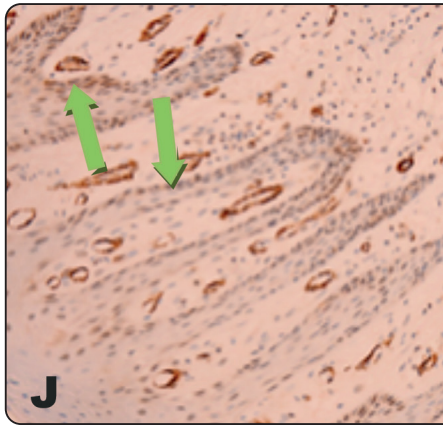


Fig. (1): Photomicrographs of immunohistochemical results of  $\alpha$ -SMA (A, B, C, D, E, F) showing the cytoplasmic expression of  $\alpha$ -SMA in stromal spindle cells (blue arrows) and vascular endothelial cells (red arrows) of JOFs (A,D), COFs (B,E) and POFs (C,F) (Orig. Mag. X20, Orig. Mag. X40, respectively). and Ki 67 (G,H,I,J) showing the cytoplasmic and nuclear expression of Ki 67 in stromal spindle cells (yellow arrows) of JOFs (G), COFs (H) and POFs (I) and nuclear immunopositivity in the hyperplastic covering epithelial cells of POFs (J) (green arrows) (Orig. Mag. X40)

**2- Statistical results:**

**A- Comparisons**

The statistical tests used showed that the difference in expression of  $\alpha$ -SMA & Ki 67 in the three categories studied (POFs, COFs and JOFs) was of statistical significance (P-value  $\leq 0.05$ ).

JOFs demonstrated the highest mean value for  $\alpha$ -SMA & Ki 67 expression (16.69 $\pm$ 5.25)

(17.04 $\pm$ 2.75) followed by COFs (5.93 $\pm$ 1.94) (8.33 $\pm$ 1.23) then POFs (1.54 $\pm$ 0.64) (3.14 $\pm$ 0.95), respectively (Table 1, Fig.2, 3)

**B- Correlations:**

The relation between  $\alpha$ -SMA and Ki 67 expression was positive with statistical significance (P-value  $\leq 0.05$ ) in the lesions studied (Table 2, Fig.4).

TABLE (1): Comparison of  $\alpha$ -SMA & Ki 67 between POFs, COFs and JOFs

	Peripheral ossifying fibromas (POFs)	Central ossifying fibromas (COFs)	Juvenile ossifying fibromas (JOFs)	P value
$\alpha$ -SMA (%Area fraction)	1.54 $\pm$ 0.64	5.93 $\pm$ 1.94 <sup>a</sup>	16.69 $\pm$ 5.25 <sup>bc</sup>	<0.001*
Ki67 (%Area fraction)	3.14 $\pm$ 0.95	8.33 $\pm$ 1.23 <sup>a</sup>	17.04 $\pm$ 2.75 <sup>bc</sup>	<0.001*

Data expressed as mean $\pm$ SD

SD: standard deviation

P: Probability \*:significance <0.05

Test used: One way ANOVA followed by post-hoc tukey

a: significance between POFs & COFs, b: significance between POFs & JOFs, c:significance between COFs & JOFs

TABLE (2): Correlation between  $\alpha$ -SMA & Ki 67 in POFs, COFs and JOFs groups.

		r	P
$\alpha$ -SMA & Ki67	All cases	0.83	<0.001*

r: Pearson's correlation coefficient P: Probability \*:significance <0.05

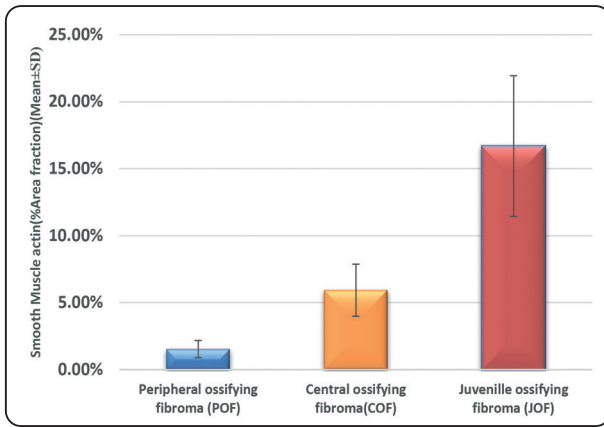


Fig. (2): A bar chart showing the mean values of  $\alpha$ -SMA expression in POFs, COFs & JOFs groups.

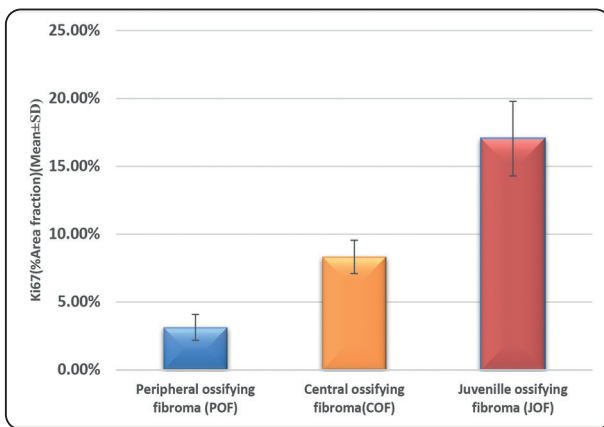


Fig. (3): A bar chart showing the mean values of Ki 67 expression in POFs, COFs & JOFs groups.

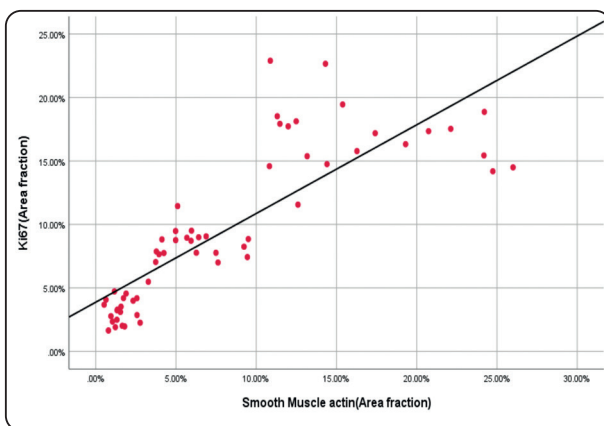


Fig. (4): Scatter diagram representing the correlation between  $\alpha$ -SMA & Ki 67 in POFs, COFs & JOFs groups together.

## DISCUSSION

Benign fibro-osseous lesions are composed of variable categories of lesions which involve developmental lesions, reactive or dysplastic disease, and tumors. They represent a group of lesions histopathologically known by presence of by fibrous stroma with variable quantities of mineralized material that looks like bone or cementum (Rai and Shetty, 2011).

In several cases of FOLs of the jaws, particularly in FD and OFs, it is very difficult to state final diagnosis in spite of all the clinical, radiographic and histological assessments. Therefore, there were many trials to find a solution for this diagnostic difficulty (Pimenta et al., 2006, Toyosawa et al., 2007, Prabhu et al., 2013).

OFs are benign, solitary, progressively growing, fibro-osseous neoplastic lesions occurring mainly in jaws (Whitten et al., 2006). These neoplasms can be classified into POFs, which occurs in the gingiva superficially, and COFs, which are present inside jaw bones (Kumar et al., 2006), while JOFs are diseases of debate, and have been recognized from the adult type of OFs based on patient's age, most common site of occurrence, and how they behave clinically. Most of them usually occur in extra-gnathic bones; some could be localized in maxillary and craniofacial bones but rarely found in mandibular bones. Their clinical symptoms are variable such as swelling of face, expanding hard mass, inflammation of sinuses, obstruction of nasal cavity, teeth displacement, eye proptosis, and pain (Aggarwal et al., 2012).

In the current study, the difference in the immunopositivity of  $\alpha$ -SMA in POFs, COFs and JOFs was statistically significant ( $P$ -value  $\leq 0.05$ ), JOFs showed the highest mean value of  $\alpha$ -SMA immunoreactivity ( $16.69 \pm 5.25$ ) followed by COFs ( $5.93 \pm 1.94$ ) then POFs ( $1.54 \pm 0.64$ ). The cytoplasm of stromal spindle cells and vascular endothelial cells showed immunopositivity. These

findings were similar to the results of Goel et al., who reported that COFs displayed high expression of  $\alpha$ -SMA followed by FD and cement-osseous dysplasia and explained that by the nondestructive nature and mild clinical behavior of FD compared to COFs (2019). Al-attas et al., in their study also demonstrated intense  $\alpha$ -SMA immunoreactivity in POFs more than FD and normal mucosa (2012).

The highest expression of  $\alpha$ -SMA in JOFs in the present study could be explained by the argument stating that MFs function in the breakdown of adjacent matrix enhancing tumor island growth, increase in lesion size and finally invasion of surrounding structures as well as transdifferentiation of MFs was reported to be involved in the local invasion of ameloblastoma, as the MFs provide a "pre-invasive niche" in the proximity of invading neoplastic cells and islands which could be in relation with intrabony growth (Goel et al., 2019), this may explain the aggressive behavior of JOFs more than COFs and POFs.

Our results were also in accordance with the results of another study by De Marcos et al, who showed intense cytoplasmic immunopositivity of  $\alpha$ -SMA in most of cells of POFs (2010). This supported that  $\alpha$ -SMA positive proliferating spindle cells are of myofibroblastic nature and indicated that POFs are myofibroblastic proliferation in agreement with the possible origin of these diseases in the peridontium (Marcos et al, 2010).

Another proof for the myofibroblastic nature of  $\alpha$ -SMA positive cells was the study conducted by Fernandez et al., demonstrating  $\alpha$ -SMA and HHF35 positive cells in POFs confirming myofibroblastic properties of the lesion (2017). The  $\alpha$ -SMA immunopositivity in the vascular endothelial cells could be explained by the fact that in normal compact and cancellous bone,  $\alpha$ -SMA is limited to the wall of blood vessels (Hemingway et al., 2012).

To conclude, MFs function in the progression of the lesion by stromal modulation which includes the

growth of the disease and breakdown of ECM (Goel et al., 2019)

In our study, the difference in Ki67 immunoreactivity in POFs, COFs and JOFs was statistically significant ( $P$ -value  $\leq 0.05$ ), JOFs showed the highest mean value of Ki67 immunopositivity ( $17.04 \pm 2.75$ ) followed by COFs ( $8.33 \pm 1.23$ ) then POFs ( $3.14 \pm 0.95$ ). The stromal spindle cells showed nuclear and cytoplasmic immunoreactivity. In some cases of POFs the hyperplastic covering epithelial cells showed nuclear immunopositivity. The present study results of high Ki 67 immunoexpression in COFs more than POFs are in accordance with the results of Garcia et al., who showed high expression of PCNA proliferation marker in COFs more than POFs which assures more the non-neoplastic reactive nature of POFs (2013). The statistically significant differences in Ki67 expression in stromal cells of COFs and POFs could be attributed to the possible differences in how they behave because of the neoplastic nature of COFs in comparison with the reactive inflammatory nature of POFs. This finding is similar to the results of Ono et al., (2007).

Regarding JOFs results, the high proliferation index reported here could be attributed to the aggressive behavior of the lesion as proved by other studies such as that of Aggarwal et al., who demonstrated Ki 67 immunoreactivity in JOFs indicating their aggressiveness (2012) and the findings of Bohn et al. (2011). Ki 67 is a nuclear protein which is present in proliferating cells and used for identification of proliferation index (Omer et al., 2008), it could be also an indicator for the aggressiveness of JOFs (Ariyasathitman et al., 2012). In accordance to the results of our study, another study by Tabareau-Delalande et al. reported MDM2 proliferation marker amplification by qPCR in JOFs (2015).

The pathogenesis of JOFs was not fully clarified, but there were studies reporting that JOFs were related to non-random break points at Xq26

and 2q33. Some cases of trauma to facial bones were reported as the possible cause for the JOFs occurrence. Due to its progressive behavior and as it recurred frequently, early diagnosis and complete surgical removal is mandatory (Aggarwal et al., 2012).

In the present study, some cases of POFs showed nuclear Ki 67 immunopositivity in hyperplastic covering epithelium; this could be attributed to the reactive inflammatory nature of POFs which causes the epithelial and the mesenchymal cells have a similar proliferation potentiality. It could be concluded that the mesenchymal component has a pivotal role causing differences in the biological behavior when comparing central lesions with peripheral ones (Garcia et al., 2013).

In the present study, a statistically significant positive relation between  $\alpha$ -SMA and Ki 67 expression was observed between all lesions studied. As there are few studies working on  $\alpha$ -SMA and Ki 67 in types of OFs, this correlation is considered to be similar to the results of Schmelting et al., who demonstrated  $\alpha$ -SMA and Ki 67 immunoeexpression in stromal spindle cells of POFs in monkeys (2011). This correlation could be explained by the statement that proliferating MFs influence matrix formation and enhance cellular migration, angiogenesis, and extracellular proteolytic activity (Goel et al., 2019) resulting in aggressive behavior of different lesions (Ariyasathitman et al., 2012). This explanation supports the aggressive behavior of JOFs when compared with COFs and POFs.

### Conclusion:

High expression of  $\alpha$ -smooth muscle actin (myofibroblasts marker) and Ki67 in JOF when compared with COF and POF indicates that the constituents of the mesenchyme could have a role in the differences in how central lesions behave, in comparison with peripheral lesions. Also, as we found that the relation between  $\alpha$ -SMA and Ki 67 expression in JOFs, COFs, and POFs was positive

with statistical significance, this supports the belief that MFs have a pivotal role in the aggressiveness of JOFs when compared with COFs and POFs by facilitating an increase in cellular proliferation, cellular movement, angiogenesis, and breakdown of extracellular matrix and affect matrix formation.

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