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ORIGINAL PAPER

Genomic Profiling of the Craniofacial Ossifying Fibroma by Next-Generation Sequencing

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Abstract

Background Ossifying fibroma (OF) of the craniofacial skeleton is a fibro-osseous lesion characterized by various patterns of bone formation in a cellular fibroblastic stroma. The molecular landscape of OF remains mostly unknown. There are a few known pathogenic abnormalities in OF, including *HRPT2* mutations in conventional OF and *SATB2* translocations in juvenile psammomatoid OF. On the other hand, conflicting reports exist regarding *MDM2* gene amplification and chromosomal copy number alterations (CNA) in OF.

Methods Surgically removed biopsies and curettage specimens from OF patients were obtained. Clinical, radiographic, and pathologic features of tumors were reviewed. Genomic DNA was extracted from formalin-fixed, paraffin-embedded blocks of tumor tissue. Capture-based DNA next-generation sequencing targeting the coding regions 529 cancer genes and select introns was performed.

Results We identified 17 OF cases from 8 male and 8 female patients with mean age of 22 years (range 1–58 years). Nine case occurred in the gnathic bones and 8 in the extragnathic craniofacial bones. These cases included 3 juvenile psammomatoid OF, 6 conventional OF and 8 juvenile trabecular OF. Large-scale CNAs were present in 6 of 17 cases. Seven cases (41%) had focal amplifications including *FOSB* ($n=2$, 11%), *FOS* ($n=4$, 23%), *COL1A1* ($n=4$, 23%) and *TBX3* ($n=5$, 29%). Three cases (17%) had pathogenic *CDC73* mutations. No cases showed focal *MDM2* amplification.

Conclusions Here, we provided a comprehensive molecular characterization of OF that reveals a heterogeneous genetic profile with occasional large-scale CNAs ($n=6$, 35%). *FOS*, *FOSB*, and *TBX3* genes that regulate AP-1 transcriptional complex are frequently altered in OF ($n=7$, 41%), chiefly in juvenile trabecular OF. These genes encode transcription factors that act as downstream effectors of the MAP kinase signaling pathway. *MDM2* amplification is an exceedingly rare event in OF, if present at all, so identification of this event should continue to raise concern for low-grade gnathic osteosarcoma. In summary, our findings suggest that OF represents a heterogeneous group of tumors at the genetic level but dysregulation of the AP-1 pathway may play a role in pathogenesis of juvenile trabecular OF.

Keywords Ossifying fibroma · Next-generation sequencing · AP-1 · MDM2

Introduction

Fibro-osseous lesions of the craniofacial bones are a heterogeneous collection of tumors characterized by fibrous tissue containing varying proportions of immature osteoid and/or calcified bone [1]. This diagnostic category includes benign (e.g., ossifying fibroma) and malignant (e.g., low-grade osteosarcoma) neoplasms, developmental dysplastic processes (e.g., cemento-osseous and fibrous dysplasia), and reactive/inflammatory diseases (e.g., chronic sclerosing osteomyelitis). Among these, ossifying fibroma (OF) of the craniofacial bones is distinguished by diverse patterns of bone development within a cellular fibroblastic stroma consisting of a conventional subtype (cemento-OF) and two juvenile subtypes (juvenile psammomatoid [JPOF] and juvenile trabecular OF [JTOF]) [2].

The genetics of some fibro-osseous lesions have been elucidated allowing more accurate classification and

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an improved understanding of pathogenesis. For example, fibrous dysplasia (FD) frequently harbors activating mutation of the *GNAS* gene while low-grade gnathic osteosarcoma demonstrates amplification of the chromosome 12q13-15 region resulting in overexpression of *MDM2* and *CDK4* genes [3–7].

On the other hand, the molecular pathogenesis of OF remains poorly understood. Inactivating mutations in *CDC73*, a tumor suppressor gene formerly known as *HRPT2*, can be seen in syndromic cases of OF in the setting of hyperparathyroidism-jaw tumor syndrome (HPT-JT syndrome) and occasionally in sporadic cases of OF [8]. Dysregulation of Wnt/Beta-catenin pathway is implicated in the pathogenesis of OF [9] but *CTNNB1* and *APC* gene mutations are infrequent [10]. The evidence for the role of *MDM2* gene amplification in OF is conflicting. One study reported *MDM2* gene amplification in a subset of OF by quantitative polymerase chain reaction (qPCR) but these results were not corroborated by immunohistochemistry or by cytogenetics [11]. Copy number detection with low-coverage whole-genome sequencing revealed copy number alterations (CNAs) in about half of OFs. CNA-associated genes with amplified expression included *MDM2* and *CDK4* by qPCR [12]. Conversely, we recently demonstrated that *MDM2* gene amplification and MDM2 and CDK4 protein overexpression are exceptionally rare in OF by fluorescent in situ hybridization (FISH) and by immunohistochemistry, respectively [13]. Little is known about gene rearrangements in OF. Cytogenetic findings in three cases of JPOF revealed the presence of chromosomal breakpoints occurring at bands 2q33 and Xq26 corresponding to a recurrent gene fusion between *SATB2* located on chromosome 2q33.1 and *AL513487.1* located on chromosome Xq26 [14, 15]. A separate study of 20 non-odontogenic OF detected no fusion transcripts using ArcherPlex [16]. Pathogenic mutations appear to be rare in OF. Next-generation sequencing (NGS) of a panel of 50 genes revealed no pathogenic mutations in 7 cases of COF [9].

Given the limited and sometimes conflicting results described above, a need exists for a comprehensive analysis of the molecular-genetic profile of a large number of OF. A clearer picture of the widespread genomic landscape of OF may provide insight to the mechanisms of tumorigenesis and may uncover potential diagnostic or therapeutic targets. Furthermore, qPCR and FISH data of 12q13-15 and *MDM2* gene amplification in OF conflict and thus deserve further investigation using an independent method. To address the above points, we employed a comprehensive genomic profiling assay of targeted hybrid capture DNA sequencing of 529 cancer-related genes as well as genomic copy number analysis in a group of well-characterized OF.

Material and Methods

Patient Cohort and Tumor Samples

We identified 17 cases of OF from the pathology archives at our institution. The clinical and radiographic data were obtained and reviewed. The diagnoses were based on light microscopic, radiographic, and clinical features. Except for case 15, all cases were previously reported [13].

Targeted Next-Generation Sequencing

Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) blocks of tumor tissue from the initial biopsy, or curettage specimen using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germantown, MD). Undecalcified FFPE blocks were used whenever possible. Furthermore, if decalcification was noted in the gross description, it was recorded. In each case, DNA was extracted from two 2 mm cores punched from the FFPE block. Capture-based DNA NGS was performed using an assay that targets a total footprint of ~3.5 Mb that includes all coding exons of 529 cancer-related genes, select introns and upstream regulatory regions of 47 genes to enable detection of TERT promoter variants and some structural variants including gene fusions (Supplemental Table 1). The assay also includes baits for ~2000 unique DNA segments containing common single-nucleotide polymorphisms within regions devoid of constitutional copy number variants to enable genome-wide copy number and zygosity analysis. Multiplex library preparation was performed using the KAPA Hyper Prep Kit (Roche, Santa Clara, CA) according to the manufacturer's specifications. Hybrid capture of pooled libraries was performed using our UCSF500 custom oligonucleotide library (Nimblegen SeqCap EZ Choice). Captured libraries were sequenced as paired-end 100 bp reads on a NextSeq 500 instrument (Illumina, San Diego, CA). Sequence reads were mapped to the reference human genome build GRCh37 (hg19) using the Burrows-Wheeler aligner. Recalibration and deduplication of reads was performed using the Genome Analysis Toolkit (Broad Institute, Cambridge, Massachusetts). Coverage and sequencing statistics were determined using Picard CalculateHsMetrics and Picard CollectInsertSizeMetrics (Broad Institute). Single-nucleotide variant, insertion/deletion and structural variant calling was performed using Freebayes (Erik Garrison), Unified Genotyper (Broad Institute), Pindel (EMBL, Heidelberg, Germany), and DELLY (EMBL). Variant annotation was performed with Annovar (Kai Wang). Single-nucleotide variants, insertions/deletions, and structural variants were visualized

and verified using the Integrated Genome Viewer (Broad Institute). Genome-wide copy number analysis based on on-target and off-target reads was performed by CNVkit and visualized using NxClinical (Biodiscovery, El Segundo, CA). All molecular data were evaluated by an expert molecular pathologist.

Results

Clinicopathologic Features

The clinicopathologic features are summarized in the Table 1 and representative histologic and radiographic findings are shown in Fig. 1. Seventeen cases of craniofacial OF from 16 patients (8 males, 8 females) were included in this study. The ages ranged from 1 to 58 years (mean = 22 years). Nine cases arose in the gnathic bones and 8 in the extragnathic craniofacial bones. The cohort consisted of 6 conventional/cemento-OF, 3 JPOF and 8 JTOF. MDM2 and CDK4 expression and *MDM2* amplification status reported in our prior study [13] are included in Table 1 to allow direct comparison to the current data. We were unable to perform NGS on the remaining 27 cases from the prior study due to insufficient tissue or DNA yield.

Next-Generation Sequencing Results

The NGS data are summarized in Fig. 2 and representative copy number changes are demonstrated in Fig. 3. Full details of mean target coverage, SNVs and copy number alterations are reported in Supplemental Tables 1, 2 and 3, respectively. Large-scale copy number changes were identified in 6/17 (35%) cases, ranging from 1 to 10 events (mean = 2.8). One

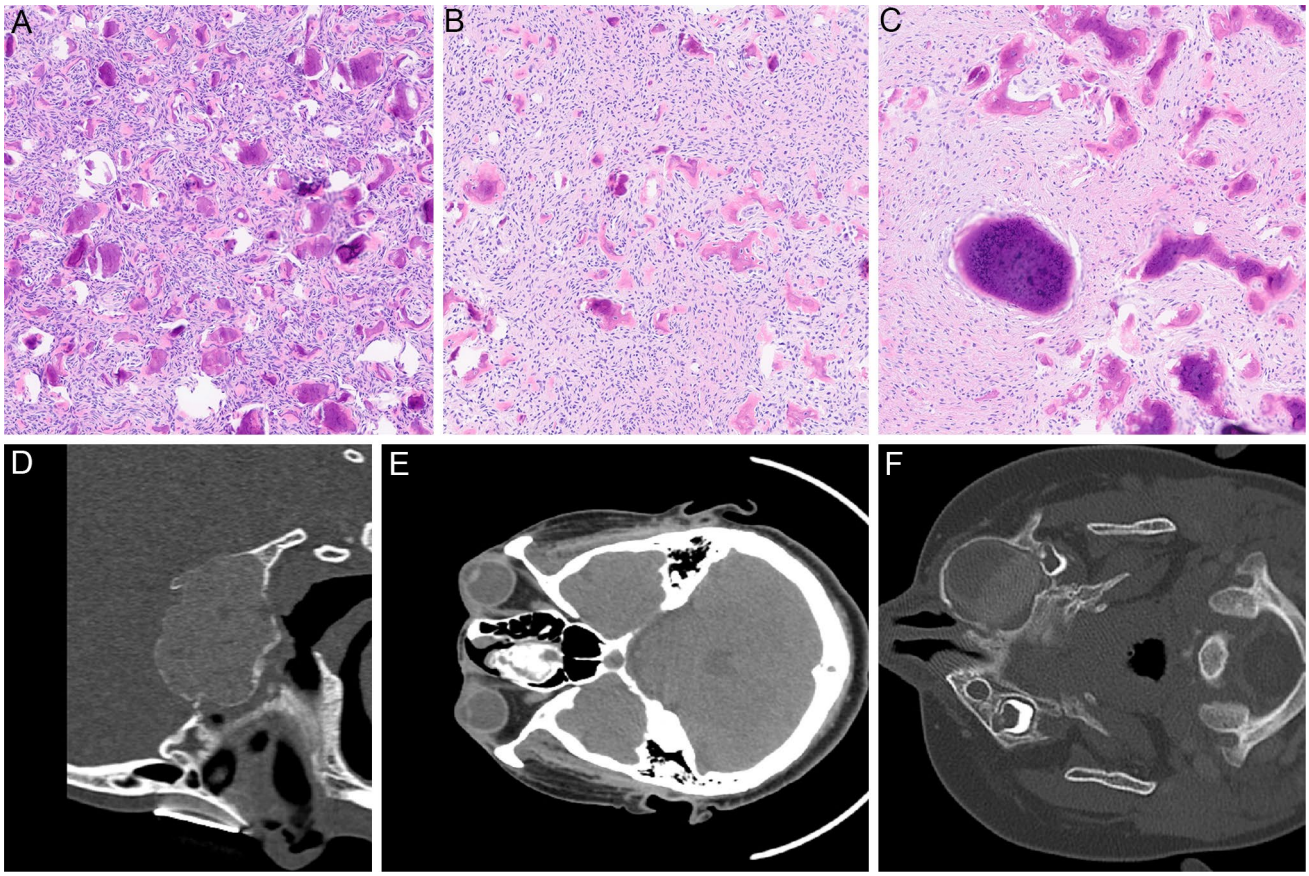

Fig. 1 Representative morphologic and radiographic findings of ossifying fibroma. **A**, **C** Juvenile psammomatoid ossifying fibroma (case 3) with a uniform distribution of psammomatoid bony metaplasia and intervening stroma of bland spindle cells and giant cells; central skull base mass centered within the sphenoid sinuses with gross expansion of all local structures and erosion of surrounding bones without invasion. **B**, **D** Conventional/cemento-ossifying fibroma (case 6) with low-grade dense cellular fibroblastic stroma that forms new bone; well-defined soft tissue mass in the right a mass centered in the right ethmoid bone with high density material which may be appears to invade the nasal septum, with extension into the left ethmoid sinus. **C**, **E** Juvenile trabecular ossifying fibroma (case 12) with cellular osteoid and woven bone in a trabecular pattern set in a variably cellular storiform to fascicular monotonous bland fibrous stroma; well-circumscribed mixed expansile bony lesions in the left maxilla causing bony remodeling and expansion of neighboring structures without aggressive radiologic characteristics

Table 1 Summary of clinical, pathologic, and genetic results of ossifying fibromas

Case#	Age	Gender	Location	Diagnosis	Immunohistochemistry**		FISH**	
					MDM2	CDK4	*MDM2*	Decalcified
1	11	F	Maxilla	JPOF	+	–	–	No
2	13	F	Maxilla	JPOF	+	–	–	No
3	17	M	Sphenoid	JPOF	–	–	ND	No
4	9	F	Mandible	COF	+	–	–	No
5	29	M	Mandible	COF	–	–	–	Yes
6	58	F	Ethmoid	COF	–	–	+	No
7	47	F	Maxilla	COF	+	–	–	No
8	26	M	Mandible	COF	–	–	–	No
9	26	M	Mandible	COF	+	+	–	No
10*	12	M	Mandible	JTOF	–	–	–	No
11*	12	M	Mandible	JTOF	+	–	–	No
12	1	M	Maxilla	JTOF	+	–	–	No
13	12	F	Mandible	JTOF	–	–	–	No
14	28	F	Mandible	JTOF	–	–	–	No
15	29	M	Mandible	JTOF	–	–	NP	No
16	12	M	Maxilla	JTOF	–	–	–	No
17	31	F	Mandible	JTOF	+	–	–	Yes

FISH Fluorescence in situ hybridization, *ND* Non-diagnostic, *NP* Not performed, *JPOF* Juvenile Psammomatoid Ossifying Fibroma, *COF* Conventional/Cemento Ossifying Fibroma, *JTOF* Juvenile Trabecular Ossifying Fibroma

*Cases 10 and 11 are from the same patient

**Immunohistochemistry and FISH results from our prior study [13]

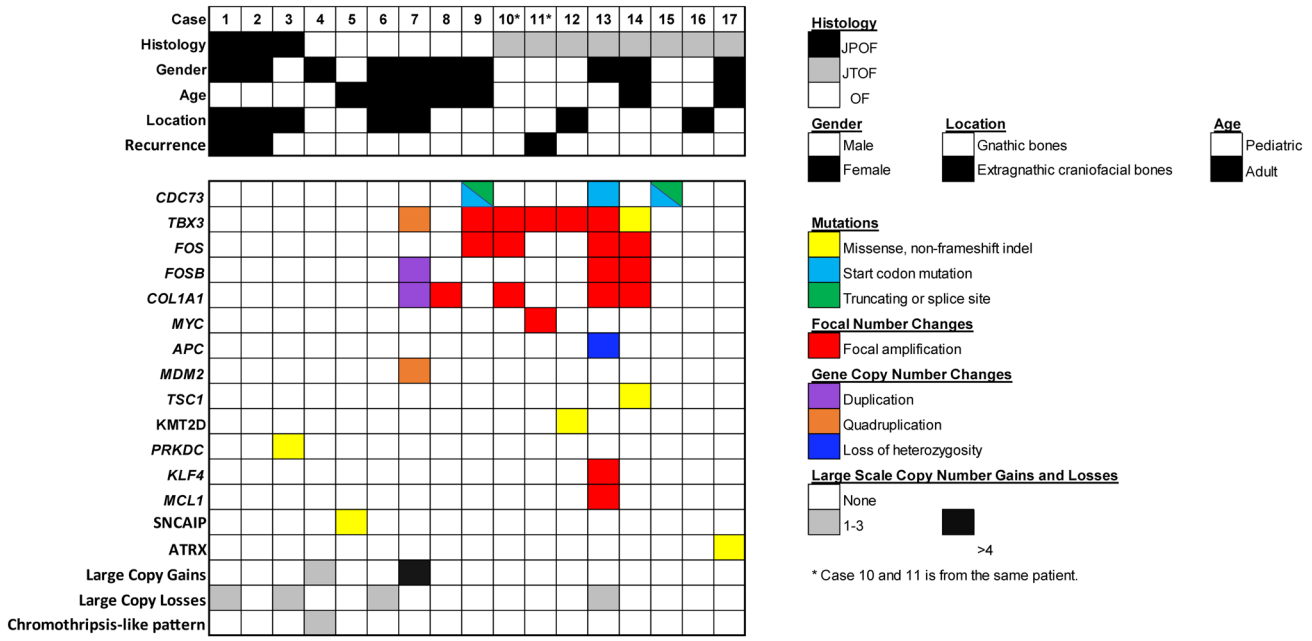

Fig. 2 Genomic profiles of 17 ossifying fibromas highlighting recurrent alterations. Each row represents a gene and columns represent individual tumors

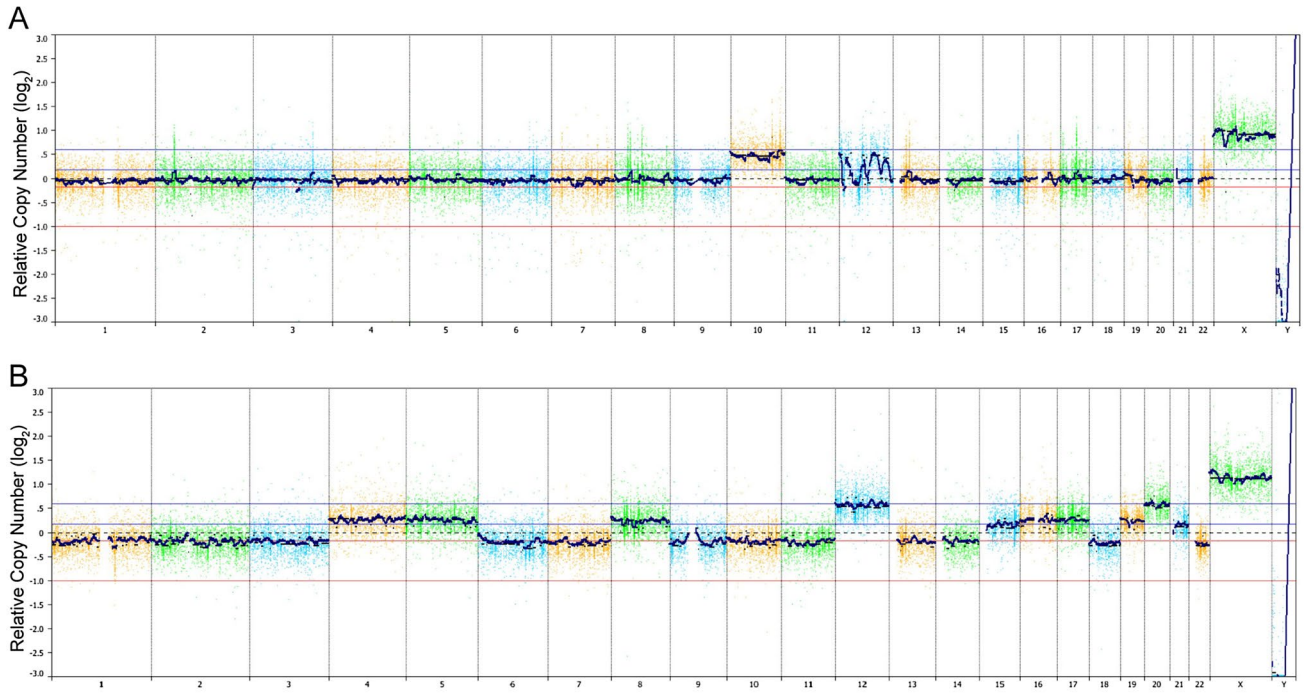

Fig. 3 Representative copy number alterations of case 4 (**A**) and 7 (**B**)

case (6%) showed a chromothripsis-like pattern with gain of many short segments in chromosome 14. Three cases (17%) had pathogenic mutations in *CDC73*. Additional findings included focal amplification in *FOSB* ($n=2$, 11%), *FOS* ($n=4$, 23%), *COL1A1* ($n=4$, 23%), and *TBX3* ($n=5$, 29%). A single case (case 14, 7%) showed a missense mutation in *TBX3*. In case number 7, there was tetrasomy of chromosome 12 that includes the genes for *MDM2* and *TBX3* as well as a trisomy of chromosomes 17 and 19 that includes the genes for *COL1A1* and *FOSB*, respectively. *MYC* amplification was seen in one case (5%), which was a recurrence. No case demonstrated focal *MDM2* gene amplification or GNAS mutation by NGS. While *FOS* family genes (i.e., FOS and FOSB) and *TBX3* amplifications were more frequent in JTOF (respectively, $n=3$, 37% and $n=4$, 50%), copy number alterations were more common in JPOF ($n=2$, 66%) and OF ($n=3$, 60%). We did not identify any other correlation between genomic alteration identified in this study and histologic findings. No gene fusions were identified, but analysis is limited to the targeted genes on the panel, which do not include *SATB2*.

Discussion

OF is a benign fibro-osseous neoplasm of craniofacial skeleton showing various histomorphologic patterns with the diagnosis typically relying on the synthesis of radiographic and clinicopathologic findings [2]. The genomic landscape of OF is poorly understood and existing studies have reported conflicting results regarding *MDM2* amplification and CNA. A recent study reported *MDM2* amplification in a majority of JTOF and JPOF [11]. In another study, copy number changes were observed in OF, including a case with the gain of chromosome 12 spanning *MDM2* and *CDK4* [12]. However, neither of the above studies correlated the genetic findings with MDM2 and CDK4 RNA or protein expression. The above findings raise a significant diagnostic problem. Previously, amplification and/or overexpression of *MDM2* (24–56%) and *CDK4* (84–89%) were reported in craniofacial osteosarcomas supporting the diagnostic utility of these genetic events to support osteosarcoma over benign fibro-osseous lesions. [7]. A separate study using FISH demonstrated *MDM2* amplification in only 2% of OF without concordant protein overexpression [13]. Similar conflicting results were also reported in the copy number profiles of OF; whereas some studies showed large-scale CNAs, others showed none [12, 16]. Few other pathogenic molecular alterations have been reported in OF. These include *CDC73* mutation in patients with HPT-JT syndrome [8] and *SATB2* rearrangement in JPOF [14, 15].

To our knowledge, this is the first study to perform comprehensive genomic profiling of a group of well-characterized OF. This analysis suggests a heterogeneous genetic landscape with occasional large-scale CNAs in approximately one third of the cases (6/17). Recurrent focal copy number gains were seen in *FOS*, *FOSB*, *TBX3* and *COL1A1*

genes. Additionally, the sequencing data demonstrated *CDC73* pathogenic mutations in 17% of cases (3/17). Focal *MDM2* amplification was not identified in any case though case 7 showed gain of *MDM2* secondary to tetrasomy of chromosome 12. The only case (case 6) that showed *MDM2* amplification by FISH in our prior study [13] did not display *MDM2* amplification by NGS in the current analysis. This discrepancy is difficult to explain. The detection of copy loss in case 6 argues against insufficient tumor fraction in the NGS sample. Based on the NGS data, *MDM2* amplification in case 6 may represent a technical false-positive in the prior FISH result. The imaging studies of case 6 revealed a well-circumscribed, expansile homogeneous mass with a thin bony capsule that lacked infiltration into surrounding soft tissue or bone or other aggressive radiographic features (Fig. 1D), consistent with a benign fibro-osseous lesion. The resection specimen contained no nuclear atypia, trabeculae of woven bone or permeation of the fibrous component into native lamellar bone (Fig. 1B). Furthermore, no recurrence, progression or metastasis has been observed in 16 years following the surgical treatment. Although low-grade osteosarcoma was considered, the constellation of radiographic, clinicopathologic and molecular findings are most consistent with ossifying fibroma. In any event, the lack of amplification by NGS, even in the single case with amplification detected by FISH, underscores the rarity of *MDM2* amplification in OF. Consequently, identification of *MDM2* gene amplification in a craniofacial fibro-osseous lesion should continue to raise suspicion of low-grade osteosarcoma over OF.

In the current study, focal amplification and gene copy number gains were observed in members of the activator protein 1 (AP-1) transcription factor family including *FOSB* ($n=2$, 11%), *FOS* ($n=4$, 23%), or its downstream target *TBX3* ($n=5$, 29%). Overall, nearly 40% of the cases (7/17) showed gain or focal amplification in at least one of these genes with *TBX3* being the most common. AP-1 is a transcription factor formed by dimerization of a member of the FOS family of proteins (FOS, FOSB, FOSL1, and FOSL2) with a member of the JUN family (JUN, MAF and ATF1). The AP-1 complex participates in various cellular processes including cell proliferation, differentiation, apoptosis, and transformation [17]. AP-1 activity is regulated by a complex network of signaling pathways involving growth factors and cytokines that increase the activity of mitogen-activated protein kinases (MAPKs), including the ERK, p38, and JNK [18]. These MAPKs regulate the activity of AP-1 including dimer composition, transcriptional, and post-translational events and interaction with coactivators and corepressors [19]. The cell type and differentiation state, tumor stage, and genetic background of the tumor also determine whether AP-1 is oncogenic or tumor suppressive [19].

Several members of AP-1 complex have been implicated in bone development, homeostasis, and neoplasia [20]. The members of FOS family are differentially expressed during normal osteoblast maturation [21] and tissue-specific gene knock out studies in mice showed that deletion of *FOS* results in osteopetrosis and impaired osteoblast proliferation and absence of tooth eruption [22, 23]. The FOS proteins play a significant role in osteoclast differentiation through induction interferon β gene (*IFNB1*) by RANKL in *FOS*-dependent manner [24]. Furthermore, FOS and Jun, AP-1 complex transcription factors are also highly expressed in fibroblast-like stromal cells in bone following mechanical injury, suggesting a potential role in bone fibroblasts [25]. FOS transcription factor has been linked to the genesis of bone tumors since its discovery as a viral oncogene (*VFOS*) from mouse osteosarcomas, the overexpression of which resulted in malignant transformation of cultured fibroblasts [26]. *FOS* and *FOSB* rearrangements have previously been described in vascular tumors such as epithelioid hemangioma and pseudomyogenic hemangioendothelioma [27–30] and bone tumors such as cementoblastoma, osteoid osteoma and osteoblastoma [31–33]. These alterations drive *FOS* overexpression, rendering the protein resistant to degradation [30, 34], or upregulate gene transcription [27, 29, 35]. *FOS* overexpression is also observed in fibrous dysplasia by RNA in situ hybridization, but without a structural rearrangement in the *FOS* gene [36]. Due to various functions of FOS/AP-1 in different cells types taking part in bone development and homeostasis, it is perhaps not surprising that FOS and FOSB oncogenic alterations may be seen in both osteogenic and fibro-osseous lesions (e.g., ossifying fibroma). Amplification of *FOS* or *FOSB* may contribute to the pathogenesis of OF by promoting AP-1 dependent cell proliferation.

Our data suggest a pathogenic role for alteration in TBX3, chiefly in JTOF (63% of JTOF cases). TBX3, a T-box transcription factor gene family member, primarily acts as a transcription repressor by binding to other transcription factors and cofactors [37]. TBX3 does, however, have activation and repression domains that function in different cellular environments [38, 39]. TBX3 plays a role in regulation of several structures in embryonic development, including the limbs, mammary glands, lungs, and heart [40]. Ulnar-mammary syndrome is a developmental disorder characterized by limb, mammary gland, tooth, and genital abnormalities. It is caused by *TBX3* gene mutations that diminish TBX3 protein function [41]. TBX3 is upregulated in many cancers, including breast, liver and melanoma where it plays a role in the oncogenic process, including tumor formation, metastasis, and invasion [38, 42]. In vitro and in vivo studies indicate that AP-1

mediates the activation of the *TBX3* gene by binding to the *TBX3* promoter [43].

There are contradictory data reported on CNAs in OF. In the present study, we demonstrated that CNAs in OF are not uncommon, occurring in 35% of cases. In a single case (case 7), CNAs resulted in gene copy number gain in *MDM2* and *CDK4* due to tetrasomy 12, consistent with the findings of CNA-associated *MDM2* and *CDK4* overexpression in OF by Ma et al. [12]. In alignment with the CNAs, case 7 also previously demonstrated nuclear MDM2 immunoreactivity [13]. Tabareau-Delalande et al. reported chromosome 12 long arm rearrangement covering MDM2 and RASAL1 in a subset of OF (33%), particularly in JPOF and JTOF, which correlated with *MDM2* amplification by qPCR [11]. Although *MDM2* amplification is exceedingly rare if not non-existent, we identified recurrent amplifications in *TBX3* in about nearly one third of the total cases (35%, 6/17) [13]. *MDM2* and *RASAL1* are located in the chromosomal regions 12q15 and 12q24, respectively. *TBX3* is localized to the chromosomal region 12q23-24, approximately 1.5 Mb from *RASAL1*, suggesting that a subset of OF carries a modification in this region of chromosome 12, leading to focal amplification of the neighboring genes (e.g., *RASAL1*, *TBX3*). *RASAL1* is not on our gene panel, thus, unfortunately we cannot confirm if *RASAL1* is simultaneously amplified with *TBX3* in our cohort.

Pathogenic mutations in the tumor suppressor gene *CDC73* (also known as *HRPT2*), which encodes parafibromin, are identified in HPT-JT syndrome and sporadic parathyroid tumors [8]. HPT-JT is an autosomal-dominant syndrome characterized by parathyroid adenoma and carcinoma, craniofacial OFs, and kidney tumors [44]. Thirty percent of individuals with HPT–JT may develop OFs, primarily of the mandible and maxilla [45], and *CDC73* mutations are seen 5–22% of sporadic OFs. [46, 47]. In the current series, three patients (17%) had *CDC73* pathogenic point mutation with mean allele frequency ranging between 43 and 86%, which raises the possibility of germline variants associated with HPT-JT syndrome [48]. These cases were from authors' consultation files but none had a reported history of HPT-JT syndrome and all were solitary lesions clinicopathologically. However, germline *CDC73* mutations could not be excluded in these patients.

In summary, genomic profiling of OF by high-throughput next-generation DNA sequencing showed large-scale CNAs in approximately one third of craniofacial OF. Alterations in transcription factors downstream of the MAP kinase signaling pathway, including *FOS*, *FOSB*, and *TBX3*, appear to be common events in juvenile trabecular OF. Along with *RASAL1* amplification reported in OF [11], *TBX3* amplification seen in this study also suggests recurrent copy gains involving chromosome 12q23-24. Conversely, *MDM2* and *CDK4* amplification appears to be an extremely rare event in OF.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12105-022-01523-9.

Author Contributions All authors confirm they have meaningfully contributed to the research and read and approved the final manuscript. Data from this manuscript was presented, in part, at the annual meeting of United States and Canadian Academy of Pathology, with an embargo on publication until its completion, March 16, 2023.The full research will be published in Head and Neck Pathology.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code Availability Not applicable.

Disclosures Data from this manuscript was presented, in part, at the annual meeting of United States and Canadian Academy of Pathology, with an embargo on publication until its completion, March 16, 2023. The full research will be published in Head and Neck Pathology.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This study has obtained IRB approval the Institutional Review Board at the University of California, San Francisco (IRB#11-05361). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to Participate The need for informed consent was waived by the Institutional Review Board at the University of California, San Francisco (IRB#11-05361).

Consent for Publication This type of study consent for publication is not required.

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