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A new subtype of high-grade mandibular osteosarcoma with RASAL1/MDM2 amplification

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RUNNING HEAD : MANDIBULAR OSTEOSARCOMA WITH MDM2/RASAL1 AMPLIFICATION

NO CONFLICT OF INTEREST AND FUNDING DISCLOSURES

ABSTRACT

In contrast to long bone osteosarcoma, mandibular osteosarcoma are highly heterogeneous and morphologically overlap with benign tumors, obscuring diagnosis and treatment selection. Molecular characterization is difficult due to the paucity of available specimens of this rare disease. We aimed to characterize the spectrum of mandibular osteosarcoma using immunohistochemistry and molecular techniques (quantitative polymerase chain reaction, sequencing) and compare them with benign fibro-osseous lesions. Forty-nine paraffin-embedded mandible osteosarcoma tissue samples were collected retrospectively and compared with 10 fibrous dysplasia and 15 ossifying fibroma cases. These were analyzed for molecular markers thought to differ between the different diseases and subtypes: MDM2 (murine double-minute type-2) overexpression; *GNAS* (guanine nucleotide-binding protein/alpha subunit) mutations; and amplification of *MDM2* and/or *RASAL1* (RAS protein activator like-1). Five fibroblastic high-grade osteosarcoma subtypes showed *MDM2* amplification, including two with a microscopic appearance of high-grade osteosarcoma with part low-grade osteosarcoma (differentiated/dedifferentiated osteosarcoma) and MDM2 overexpression. The other three contained a co-amplification of *MDM2* and *RASAL1*, a signature also described for juvenile ossifying fibroma, with no overexpression of MDM2. These were of the giant cell-rich high-grade osteosarcoma, with areas mimicking juvenile ossifying fibroma (ossifying-fibroma-like osteosarcoma). Our results show that some diagnosed high-grade osteosarcoma are differentiated/dedifferentiated osteosarcomas and harbor an overexpression and amplification of *MDM2*. In addition, juvenile ossifying fibromas can potentially evolve into giant cell-rich high-grade osteosarcomas and are characterized by a *RASAL1* amplification (osteosarcoma with juvenile ossifying fibroma-like genotype). Thus, the presence of a *RASAL1* amplification in ossifying fibroma may indicate a requirement for closer follow-up and more aggressive management.

Key words: osteosarcoma, bone tumor, ossifying fibroma, molecular analysis, RASAL1, MDM2

NON-STANDARD ABBREVIATIONS

GFPO: Groupe Français de Pathologistes Osseux,
RESOS: Réseau de référence en sarcomes osseux
GSF-GETO: Groupe Sarcome Français – Groupe d'Etude des Tumeurs Osseuses
REFCOR: Réseau d'Expertise Français des Cancers ORL Rares
RCN: Rare Cancer Network

1.1 INTRODUCTION

Mandibular osteosarcoma (MOS) account for less than 10% of all osteosarcoma (OS) [1] and, in contrast to their non-head and neck counterparts, they are characterized by a later onset and a lower propensity for distant metastases. Most series of MOS cases are of limited statistical value for the molecular characterization of different mandibular subtypes due to their rarity and histological heterogeneity. Some maxillary OS have been linked with the overexpression and amplification of MDM2 (murine double-minute type 2) and CDK4 (cyclin-dependent kinase 4), and the overexpression of Ezrin (a membrane-cytoskeleton linker protein involved in metastases), but these are the only molecular characterization studies reported thus far and have provided no prognostic value [2–4].

According to the WHO 2013 classification [5], low-grade OS (LGOS) represent 5-7% of all OS and are characterized by a paucicellular stroma with fusiform cells exhibiting minor cytonuclear atypia and well-differentiated bone trabeculae. Their genomic profile includes the presence of episomal ring neochromosomes containing a high-level amplification of the 12q13-15 region harboring the *MDM2* and *CDK4* genes. In 10-36% of LGOS cases some dedifferentiation into high-grade OS (HGOS) can occur, such that areas of low- and high-grade tumor can coexist microscopically (defined here as HGOS with part LGOS, or differentiated/dedifferentiated OS). Compared to the LGOS part, the HGOS contingent is composed of cells with larger atypia and a more immature neoplastic osteoid production [5]. Tumour necrosis may also be present. Amplification of the *MDM2* and *CDK4* genes is also preserved in the dedifferentiated form of OS and is not observed in benign fibro-osseous lesions, providing an important diagnostic tool and prognostic factor [6]. When the LGOS in biopsy or surgical resection specimens, the diagnosis of dedifferentiated OS may instead be made from its molecular signature [5,6, 7].

In terms of osteosarcoma subtypes, HGOS include primary and secondary conventional sub-types, telangiectatic and small cell OS [5]. Conventional HGOS represent 90% of cases and are divided into osteoblastic/sclerosing (76-96%), chondroblastic (10-13%) and fibroblastic (10%) OS, based on the type of matrix produced by the tumor cells. Other subtypes include osteoblastoma-like (which are rich in giant cells), epithelioid, clear cell, and chondroblastoma-like OS. Secondary OS often develop in Paget's disease or in irradiated areas and occasionally after bone infarction or in the presence of benign tumors such as fibrous dysplasia. They have never been reported in cases of ossifying fibroma. Conventional HGOS have a complex genomic profile, with different molecular alterations of *MDM2* and *CDK4* reported than those observed in LGOS or differentiated/dedifferentiated OS [8].

MOS may morphologically overlap with benign tumors such as fibrous dysplasia and ossifying fibromas [1]. Fibrous dysplasia tumors exhibit immature irregularly-shaped curvilinear trabeculae of woven bone associated with bland fibroblastic cells exhibiting rare mitoses. The juvenile type of ossifying fibroma (JOF) may resemble osteoblastoma [9]. JOF contain fusiform and giant plurinucleated cells surrounded by immature peripheral osseous trabecular structures that are then surrounded by osteoblastic cells. Activating mutations in the *GNAS* (guanine nucleotide-binding protein/alpha subunit) gene occur in up to 50% of fibrous dysplasias [10;11] and occasionally in LGOS [12] but have not been identified in ossifying fibromas [11]. The amplification of chromosome 12 which harbors the *MDM2* and *RASAL1* (RAS protein activator like-1; telomeric to *MDM2* on chromosome 12) genes has also been recently identified as a molecular diagnostic marker for ossifying fibromas (mostly of juvenile-type) and is associated with aggressive tumors [13]. Alterations to the *RASAL1* and *MDM2* genes have not been reported in OS, however in some fibrous dysplasias and ossifying fibromas *MDM2* amplification has been reported without an accompanying overexpression of the MDM2 protein [6].

Due to the significant overlap in the morphological and molecular presentation of benign and malignant mandibular tumors, a more comprehensive molecular characterization is required and should have a significant prognostic and therapeutic impact. In this study we have characterized the spectrum of MOS using immunohistochemistry and molecular techniques (quantitative polymerase chain reaction: qPCR, sequencing) and compared these with benign fibro-osseous lesions to investigate whether MOS that are histologically different exhibit distinct molecular features.

1.2 MATERIALS AND METHODS

1.2.1 Patient and tumor characteristics

Institutional and national ethics committees approved this retrospective study. Patient samples were obtained after authorization by the French Ministry of Higher Education and Research (declaration DC 2009-989; DC-2011-1388; transfer agreement AC-2008-820; AC-2011-130). The institution that granted permission was the "Comité de Protection des Personnes Sud Ouest et Outre Mer I", Agence Régionale de Santé Midi-Pyrénées, Toulouse. Clinical and biological annotations were consistent with the CNIL (Comité National Informatique et Libertés) guidelines. All patient records and information were anonymized and de-identified prior to analysis. Samples were stored in the “CRB cancer des Hôpitaux de Toulouse; BB-0033-00014” collection.

Forty-nine MOS and 25 benign fibro-osseous tumor samples were collected from patients treated between 1997 and 2013. Clinical data (age, gender) and histological characteristics (grade, histological subtype) are reported in Table 1 (see Results section 1.3.1).

Paraffin-embedded tissue samples were collected from different institutions belonging to the Groupe Sarcome Français - Groupe d’Etude des Tumeurs Osseuses (French Sarcoma Group - Bone Tumor Study Group; GSF-GETO), the Rare Cancer Network (RCN) and the

Reseau d'Expertise Français des Cancers ORL Rares (French Network of Expertise on Rare Ear, Nose and Throat Cancers; REFCOR). Hematoxylin-eosin-stained histology slices were blindly examined by two pathologists (AGB, MG) from the French Group of Bone Tumor Pathologists (GFPO) who further selected blocks to be used for tissue microarrays (TMAs). TMAs (triplicate sampling) were performed as recommended by Kononen et al. [14]. Since fixation and decalcification protocols have altered over time (some samples were fixed in formol and some in picric acid), wherever possible tumor samples from different tumor blocks for a given patient were used to compensate for any potential differences due to fixation and decalcification methods.

The 49 tumors were reviewed by the GFPO and reclassified into three subtypes (chondroblastic/osteoblastic/fibroblastic) based on the prominent component and according to the WHO 2013 classification. Fibroblastic (FB) osteosarcomas were subdivided based on the presence or absence of giant cells, with "FB*" denoting suspected HGOS with part LGOS (differentiated/dedifferentiated OS) and "FB giant cells" denoting suspected ossifying-fibroma-like OS (see Tables 1 and 2, Results sections 1.3.1 and 1.3.2 respectively). The 25 maxillary benign fibro-osseous tumors were collected from the University Hospital of Toulouse and included 10 fibrous dysplasias and 15 ossifying fibromas.

1.2.2 Immunohistochemistry

The Ki67 proliferation index and MDM2 immunostainings were performed. Vessels were stained to detect the presence of CD34. Preparations were dried for 1 hour at 58°C, then overnight at 37°C. Sections were deparaffinized with toluene and rehydrated in ethanol. They were then pretreated with the high pH target retrieval solution (DAKO, EnVision Flex, Denmark), and a heat-based antigen retrieval method was used before incubation. Endogenous peroxidase was blocked using a 3% H_2O_2 incubation for 5 minutes. The primary

CD34 antibody was used at a 1:200 dilution (Beckman Coulter, CA, USA; clone QBEnd 10) and the primary Ki67 antibody was used at a 1:150 dilution (DAKO, Denmark; clone MIB-1). Sections were incubated for 20 minutes at room temperature. For MDM2 immunostaining, 4 µm-thick paraffin sections were mounted onto glass slides (Superfrost, DAKO, Trappes, France). Preparations were dried for 1 hour at 58°C, and overnight at 37°C. Sections were then deparaffinized with toluene and rehydrated in ethanol. They were pretreated with EDTA (MS-Unmasker-Diapath, Martinengo, Italy) and then a heat-based antigen retrieval method was used before incubation. Endogenous peroxidase was blocked using a 3% H_2O_2 incubation for 5 minutes. The primary MDM2 antibody was used at a 1:25 dilution (Zymed Laboratories, CA, USA; clone IF2) and sections were incubated for 1 hour at 22°C. Staining was performed with the Envision kit (DAKO, Carpinteria, CA, USA); sections were revealed by incubation in a diaminobenzidine solution for 7 minutes then staining with hematoxylin for 16 seconds. MDM2 immunostaining was performed for each case of ossifying fibroma, fibrous dysplasia and for all subtypes of MOS. Only well-defined nuclear reactivity was considered positive. Immunoreactivity was considered positive when 1-100% of cells were stained, irrespective of staining intensity.

1.2.3 Molecular studies

1.2.3.1. DNA Extraction

Genomic DNA was extracted using the QIAamp DNA FFPE Extraction kit (Qiagen, Courtaboeuf, France) from 10 mm sections cut from tissue blocks of each of the 49 cases. Thick sections of FFPE tissue were dewaxed by extraction in 100% xylene and washed with 100% ethanol. Samples were then air-dried before DNA extraction. Extracted DNA was quantified using a spectro-photometer (Cary100Scan Varian, Agilent, Massy, France).

1.2.3.2 qPCR

qPCR was performed using a Light Cycler 480 (Roche, Boulogne, France) with 50 ng DNA using the Sybr Green I Master kit (Roche). The *ALB* gene was used as a reference. Primer sequences are given in Table 1. PCR was carried out as follows: following 5 minutes at 95°C, 45 amplification cycles were run, each consisting of 10 seconds at 95°C, 10 seconds at 60°C and 10 seconds at 72°C. The relative amounts of *MDM2* and *RASAL1* were established as a ratio of the reference gene (*ALB*) using Light Cycler Relative Quantification software

1.2.3.3 GNAS mutations

GNAS mutations in patients were determined as previously described [11]. All PCR and sequencing reactions were run in duplicate. Briefly, exon 8 of *GNAS* was screened by high resolution melt (HRM) using High Melting Master Kit (Roche). The *GNAS* HRM primers were: forward 5'-TCCATTGACCTCAATTTTGTTTCAG-3' and reverse 5'-AAGTTGACTTTGTCCACCTGGAACT-3' (Invitrogen, ThermoFisher Scientific, USA). Alternatively, *GNAS* mutations (exon 8: p.R201C and p.R201H, exon 9: p.Q227L) were genotyped by allele-specific PCR using a Light Cycler 480 SYBR Green I Master Kit (Roche), as previously described [11]. HRM and allele-specific PCR positive findings were further sequenced using bidirectional Sanger sequencing.

1.3 RESULTS

1.3.1 Clinical and pathological findings

Of the 49 patients treated for MOS in this series, 28 were male and 21 were female (Table 1). Eight occurred in irradiated areas. All were HGOS, with 18 chondroblastic, 16 osteoblastic and 15 fibroblastic OS. Eight of the fibroblastic cases included a high- and a low-grade component suggestive of a possible dedifferentiated osteosarcoma (shown by FB* in

Table 1). In general, the low-grade component resembled either a desmoid tumor, with areas of moderately atypical fibroblastic cells in a fibrous stroma, or areas of well-differentiated osteogenesis associated with moderately atypical fibroblastic cells (Figure 1). Seven other fibroblastic cases that were rich in giant cells (see Table 1) had fibroblastic areas resembling JOF (areas of immature osteogenesis interspersed with fibroblastic areas and giant cells) (Figure 2).

Due to the low number of patients in each category, no significant prognostic correlations could be made.

1.3.2 MDM2 immunohistochemistry

Of the 49 cases, 36 were suitable for immunohistochemistry analysis. Thirteen cases were excluded since control CD34 and Ki67 immunostainings were negative and these samples were assumed to have inadequate tissue quality due to decalcification. All of the 25 benign fibro-osseous tumor samples had positive CD34 and Ki67 staining and all were negative for MDM2 overexpression. Three MOS cases showed nuclear MDM2 immunostaining (Figure 3 and Table 3); these were all of fibroblastic subtype and differentiated/dedifferentiated OS was suspected. All other subtypes, including the seven giant cell fibroblastic OS cases, were also negative for MDM2 staining.

1.3.3 Molecular findings

Fifteen cases were suitable for molecular analysis based on the quantity and/or quality of their extracted DNA (Table 2).

1.3.3.1 Amplification of *MDM2* and *RASAL1*

Five cases showed *MDM2* amplification by qPCR. These were fibroblastic osteosarcomas, and included two cases in which differentiated/dedifferentiated osteosarcoma

was observed microscopically. These two cases had also shown MDM2 overexpression by IHC analysis. Unfortunately qPCR could not be used in the third case exhibiting overexpressed MDM2 due to insufficient DNA quality. The other three cases showing *MDM2* amplification by qPCR also contained an amplification of *RASAL1*, but did not have MDM2 overexpression. These three cases were of the giant cell-rich fibroblastic subtype, with areas mimicking JOF. There were four other giant cell-rich fibroblastic OS, all of which had no MDM2 overexpression. One was negative for *MDM2* and *RASAL1* amplification but molecular analyses could not be performed on the other three due to poor DNA quality.

1.3.3.2 *GNAS* mutations

Due to insufficient quality or quantity of the DNA extracted, 35 samples had inconclusive results regardless of the techniques used. However, *GNAS* mutations were not detected in the remaining 14 cases.

1.4 DISCUSSION

This study is the largest to date to compare high-grade MOS samples. We have shown that different subtypes of high-grade MOS (graded according to the WHO 2013 classification) can be characterized based on their morphology, immunohistochemistry and molecular biology. This has important implications in terms of the prognosis and understanding of the pathobiology of the MOS spectrum. MOS are usually treated based on their grade [15, 16], with recent data suggesting that complete resection with neoadjuvant chemotherapy gives the best outcome for HGOS while LGOS are better treated with surgery alone [2]. However, our results suggest that this strategy overlooks potentially different behaviors within a given HGOS subtype.

According to the molecular criteria of the WHO 2013 classification, LGOS and its dedifferentiated contingent (HGOS with part LGOS) are characterized by the amplification of *MDM2*, whereas conventional HGOS are thought to harbor a complex genomic profile with no distinct molecular signature.

Recent studies have suggested that benign fibro-osseous tumors of the jaw exhibit specific molecular characteristics. JOF can harbor a *RASAL1* and *MDM2* amplification that is associated with a greater clinical aggressiveness [13]. In addition, in both its monostotic or polyostotic presentations, or when associated with McCune Albright syndrome, fibrous dysplasia is characterized by somatic mutations of *GNAS* located on chromosome 20q13.3. [11,17]. However, *GNAS* mutations have not been reported in other benign fibro-osseous lesions. In LGOS, the presence of *GNAS* mutations is controversial [11,12,18], but a recent study of 31 cases of LGOS (confirmed through molecular analysis), led by the French Group of Bone Tumor pathologists (GFPO), did not find any *GNAS* mutations in either LGOS or its dedifferentiated contingent, supporting the specificity of *GNAS* mutations to fibrous dysplasia [18]. In accordance with this [11, 18], none of the 14 OS cases in our cohort with adequate DNA quality contained *GNAS* mutations.

Despite the heterogeneous fixation and decalcification methods used and the limitations of using tissue microarrays rather than slides, we were able to assess the diagnostic value of MDM2 overexpression, *MDM2* gene amplification, *GNAS* mutations and *RASAL1/MDM2* amplifications. MDM2 overexpression can also be confirmed by FISH or CGH array analysis [10,11], however qPCR is usually used for decalcified tissues since FISH is not usually interpretable.

Our results show that molecular biology, alongside immunochemistry and morphological examination, can distinguish between the three subtypes of HGOS.

Two HGOS exhibited MDM2 overexpression by immunohistochemistry analysis and *MDM2* amplification by qPCR. These two OS exhibited morphologically-distinct areas of low-grade and high-grade fibroblastic OS. The third case, which was morphologically similar to the other two, overexpressed MDM2 but qPCR could not be performed because the tissues were damaged due to fixation and/or decalcification. Thus, based on the molecular pathology guidelines of the WHO classification, these three OS cases were reclassified as differentiated/dedifferentiated OS (HGOS with part LGOS), which have a better prognosis than conventional HGOS with its complex genomic profile [14].

Three other OS in our series contained a co-amplification of *RASAL1/MDM2*, without an accompanying MDM2 overexpression. Microscopic examination showed areas of giant cells or areas resembling JOF. Chromosome 12 amplifications of both the *RASAL1* and *MDM2* genes were recently identified as being potentially useful for the molecular diagnosis of ossifying fibromas and as a marker of clinical aggressiveness [13]. The observation of such amplification in high-grade giant cell (fibroblastic) OS harboring morphological resemblance with JOF questions the possibility that there is a continuum between the two entities. Malignant transformation of ossifying fibromas into OS has never been reported. Our results show that the two entities share molecular abnormalities and support the hypothesis of a *RASAL1/MDM2* co-amplification as well as a poor prognostic value / transformation risk when such molecular abnormality is found in ossifying fibromas. A *RASAL1/MDM2* co-amplification was absent in one giant cell-rich case, and three other giant cell-rich OS could not be analyzed because of tissue damage due to fixation and/or decalcification. Such technical issues were unavoidable owing to the rarity of disease, bone involvement and retrospective multicenter nature of the study.

This large study shows that high-grade MOS can be divided into subtypes of distinct pathogenesis (Table 4). This could potentially improve prognosis and guide therapies. In

future, the use of non-decalcified tissues or other extraction procedures should improve DNA integrity and permit the use of techniques such as CGH arrays or NGS. This will further develop these unique results and will ultimately improve our understanding of the etiological molecular pathways involved in MOS.

1.5 CONCLUSION

This large study is the first description of a comprehensive overview of MOS, assessed by MDM2, RASAL1 and GNAS status. Two unique conclusions can be made from our study: 1) Some high-grade MOS may result from the transformation of LGOS (defined by an overexpression and amplification of *MDM2*), as has been reported for long bone OS; 2) It is possible that JOF could evolve into HGOS characterized by *MDM2* and *RASAL1* co-amplification. Thus, a new entity, OS with "JOF-like genotype", could be established. In addition, the presence of a *RASAL1* amplification in ossifying fibromas may constitute an early molecular signature that requires closer follow-up and more aggressive management.

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FIGURE LEGENDS

Figure 1. **A.** Histological appearance of dedifferentiated osteosarcoma with fibrous dysplasia-like area (right) and atypical fibroblastic cells (left) (X 2.5). HE, hematoxylin eosin. **B**. High-grade fibroblastic osteosarcoma area (X 200) (HE).

Figure 2: Histological appearance of fibroblastic osteosarcoma rich in giant cells.

A. Osteoblastoma-like pattern with immature osteogenesis, fibroblastic cells and osteoclastic cells (X 2.5). **B**. Abnormal mitosis and atypical fibroblastic or osteoblastic cells producing tumoral osteoid matrix (X 200). **C.** Histological appearance of juvenile trabecular variant of ossifying fibroma with immature osteogenesis and giant cells.

Figure 3. **A.** MDM2 nuclear immunostaining in a dedifferentiated osteosarcoma. **B**. Fibroblastic high-grade osteosarcoma.

Patient	Histology	Grade	Sex	Age	Response to treatment
1	CB	HG	M	61	GR
2	CB	HG	M	34	PR
3	CB	HG	F	29	PR
4	CB	HG	M	36	None
5	CB	HG	F	56	PR
6	CB	HG	M	64	PR
7	CB	HG	M	15	PR
8	CB	HG	F	25	PR
9	CB	HG	M	32	-
10	CB	HG	M	29	None
11	CB	HG	M	65	-
12	CB	HG	F	44	PR
13	CB	HG	M	19	PR
14	CB	HG	M	19	PR
15	CB	HG	M	77	None
16	CB	HG	F	33	None
17	CB	HG	F	35	-
18	CB	HG	M	31	GR
19	CB	HG	M	35	PR
20	FB giant cells	HG	F	80	None
21	FB giant cells	HG	-	-	-
22	FB giant cells	HG	M	55	PR
23	FB giant cells	HG	-	-	-
24	FB giant cells	HG	F	80	-
25	FB giant cells	HG	M	31	None
26	FB giant cells	HG	F	68	PR
27	FB IIr	HG	F	18	PR
28	FB*	HG	F	21	None
29	FB*	HG	M	21	PR
30	FB*	HG	F	31	PR
31	FB*	HG	F	61	None
32	FB*	HG	M	49	PR
33	FB*	HG	M	26	PR
34	FB*	HG	F	13	PR
35	FB*	HG	M	77	None

36	OB	HG	F	32	PR
37	OB	HG	F	45	-
38	OB	HG	M	46	GR
39	OB	HG	F	49	None
40	OB	HG	M	25	GR
41	OB	HG	M	50	PR
42	OB	HG	F	68	PR
43	OB IIr	HG	F	64	-
44	OB IIr	HG	M	25	-
45	OB IIr	HG	M	47	-
46	OB IIr	HG	M	34	-
47	OB IIr	HG	M	60	GR
48	OB IIr	HG	F	65	PR
49	OB IIr	HG	F	36	-

Table 1. Clinical and pathological findings in patients with mandibular osteosarcoma.

Abbreviations: CB, chondroblastic; FB, fibroblastic; OB, osteoblastic; IIr, secondary osteosarcoma (post-radiation); FB* (suspected differentiated/dedifferentiated osteosarcoma); FB giant cells (suspected ossifying-fibroma-like OS); HG, high-grade; M, male; F, female; GR, good responder; PR, poor responder; -, no information.

Patient	Histology	IHC MDM2	qPCR *MDM2*	qPCR *RASAL1*	*GNAS*	CD34	KI67
1	CB	-	-	-	-	-	+
4	CB	-	NA	NA	NA	+	-
6	CB	-	NA	NA	NA	+	-
7	CB	-	NA	NA	NA	+	-
9	CB	-	-	-	-	+	-
14	CB	-	NA	NA	NA	-	+
15	CB	-	NA	NA	NA	-	+
17	CB	-	NA	NA	NA	-	+
18	CB	-	NA	NA	NA	+	+
20	FB giant cells	-	+	+	-	+	+
21	FB giant cells	-	NA	NA	NA	+	+
22	FB giant cells	-	+	+	NA	-	+
23	FB giant cells	-	-	-	-	-	+
24	FB giant cells	-	+	+	-	+	+
25	FB giant cells	-	NA	NA	NA	+	-
27	FB IIr	-	NA	NA	NA	+	+
28	FB*	-	-	-	-	+	+
29	FB*	-	NA	NA	NA	+	-
30	FB*	-	-	-	-	+	+
31	FB*	+	+	-	-	-	+
32	FB*	+	+	-	-	-	+
33	FB*	-	-	-	-	-	+
34	FB*	+	NA	NA	NA	-	+
35	FB*	-	NA	NA	NA	+	-
36	OB	-	NA	NA	NA	+	-
37	OB	-	NA	NA	NA	-	+
38	OB	-	-	-	-	+	+
39	OB	-	NA	NA	NA	+	+
40	OB	-	NA	NA	NA	+	+

41	OB	-	NA	NA	NA	-	+
42	OB	-	-	-	-	+	+
43	OB IIr	-	NA	NA	NA	-	+
44	OB IIr	-	NA	NA	NA	+	+
45	OB IIr	-	NA	NA	NA	+	+
47	OB IIr	-	-	-	-	+	-
48	OB IIr	-	NA	NA	NA	+	+

Table 2. Immunohistochemistry and molecular results in mandibular osteosarcomas

Abbreviations: CB, chondroblastic; FB, fibroblastic; OB, osteoblastic; IIr, secondary osteosarcoma (post-radiation); FB* (suspected differentiated/dedifferentiated osteosarcoma); FB giant cells (suspected ossifying-fibroma-like OS); IHC, immunohistochemistry; NA, not assessable; -, negative; +, positive.

Gene (locus)	Sequence
MDM2-F (12q13-15)	5'-CCGGAT GATCGCAGGTG-3'
MDM2-R	5'-AAAAGCTG AGTCAACCTGCCC-3'
RASAL1-F (12q23-24)	5'-TGGATTTCTCTTCTTGCGATTCT-3'
RASAL1-R	5'-TGTTGGTCCCGAAGGTCAAA-3'
ALBUMIN-F (4q13.3)	5'-TGAAACATACGTTCCCAAAGAGTTT-3'
ALBUMIN-R	5'-CTCTCCTTCTCAGAAAGTGTGCATAT-3'

Table 3. Sequences of primers used for quantitative polymerase chain reaction.

IHC MDM2	**qPCR *MDM2* amplification**	**qPCR *RSAL1* amplification**	***GNAS* mutation**	**Diagnosis**
+	+	-	-	Differentiated/ dedifferentiated osteosarcoma
-	+	+	-	Fibroblastic high-grade osteosarcoma with giant cells
-	+	+	-	Ossifying fibroma
-	+/-	+/-	+	Fibrous dysplasia

Table 4. Summary of IHC and molecular data.

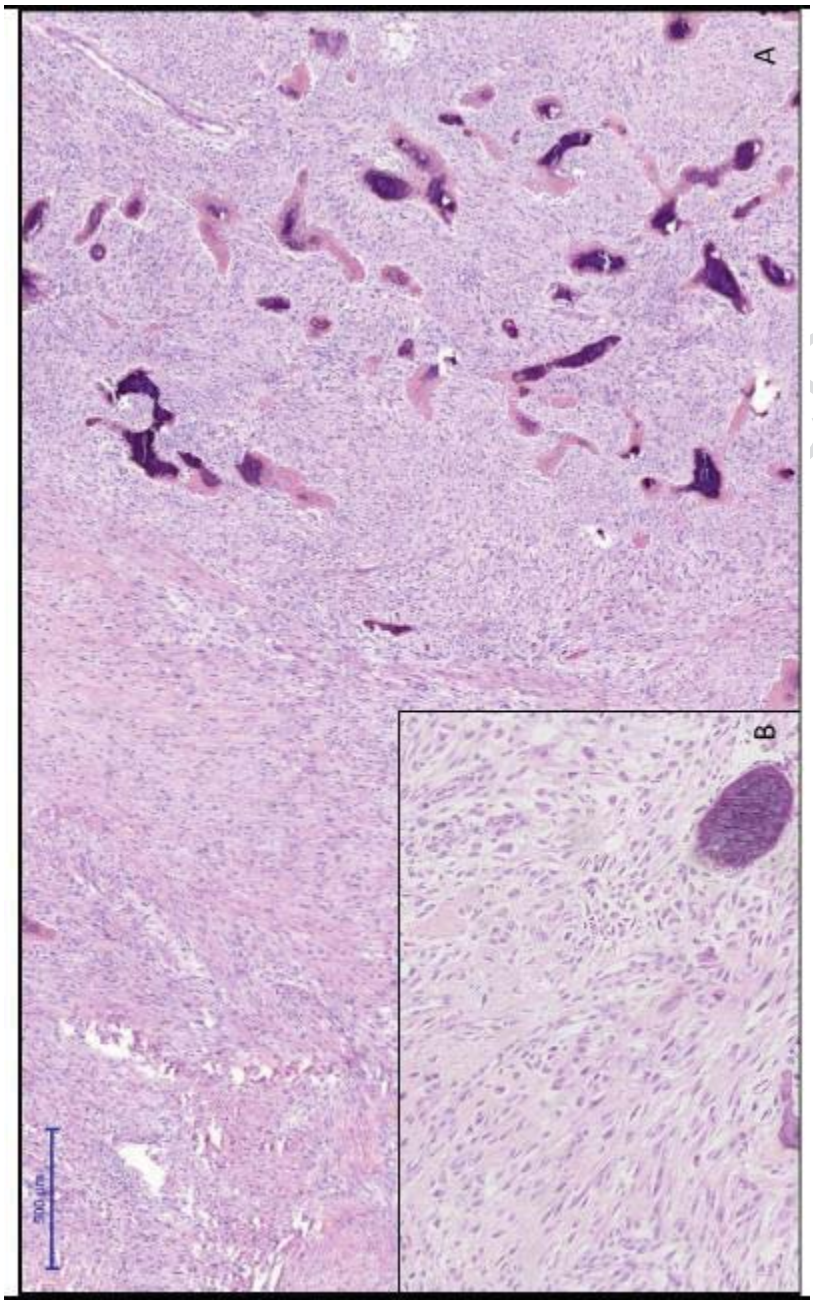

Figure 1

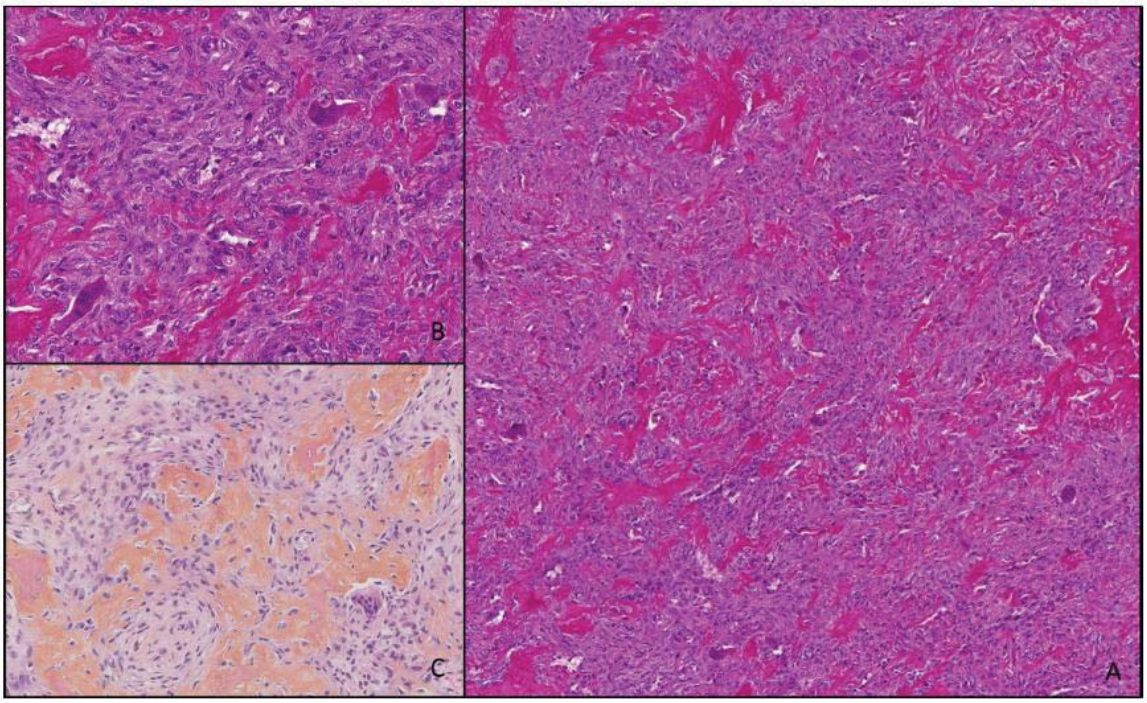

Figure 2

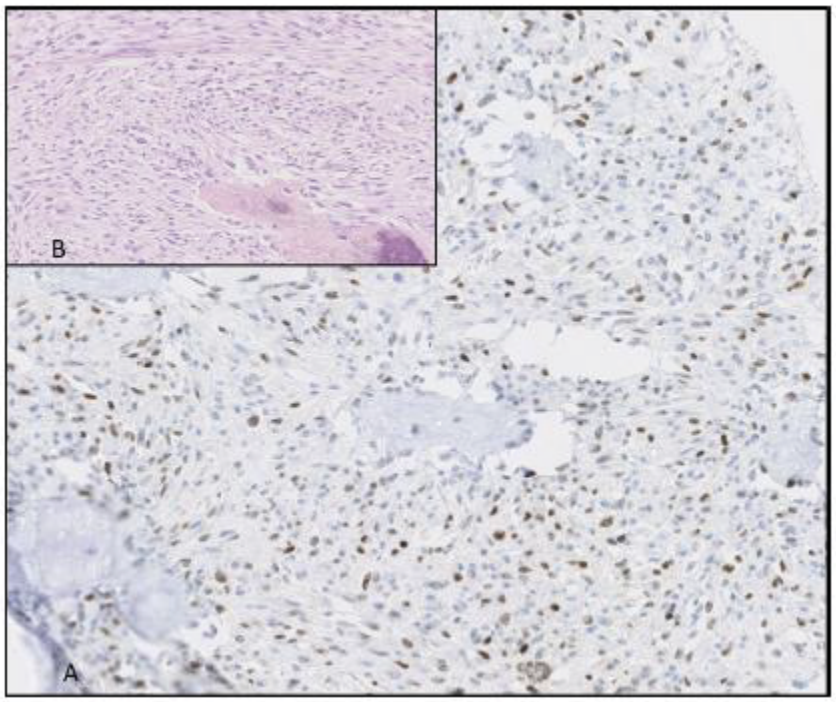

Figure 3