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Odontogenic Myxomas Harbor Recurrent Copy Number Alterations and a Distinct Methylation Signature

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Abstract: Odontogenic myxoma is a rare, benign, and locally aggressive tumor that develops in the tooth-bearing areas of the jaw. The molecular mechanisms underlying odontogenic myxomas are unknown and no diagnostic markers are available to date. The aim of this study was to analyze DNA methylation and copy number variations in odontogenic myxomas to identify new molecular signatures for diagnostic decision-making. We collected a cohort of 16 odontogenic myxomas from 2006 to 2021 located in the mandible (n = 10) and maxilla (n = 6) with available formalin-fixed paraffin-embedded or fresh frozen tumor tissue from a biopsy or resection material. Genome-wide DNA methylation and copy number variation data were generated from 12 odontogenic myxomas using the Illumina Infinium Methylation EPIC array, interrogating >850,000 CpG sites. Unsupervised clustering and dimensionality reduction (Uniform Manifold Approximation and Projection) revealed that odontogenic myxomas formed a distinct DNA methylation class. Copy number profiling showed recurrent whole-chromosome gains (trisomies) of chromosomes 5, 8, and 20 in all cases, and of chromosomes 10, 12, and 17 in all except one case. In conclusion, odontogenic myxomas harbor recurrent copy number patterns and a distinct DNA methylation profile, which can be used as an additional diagnostic tool in the appropriate clinical and radiologic context. Further research is needed to explain the genetic mechanisms caused by these alterations that drive these locally aggressive neoplasms.

Key Words: odontogenic myxoma, DNA methylation, copy number profiling, genome-wide profiling, recurrent alterations

(*Am J Surg Pathol* 2024;48:1224–1232)

Odontogenic myxoma is a rare, benign, and locally aggressive tumor of ectomesenchymal origin characterized by invasive growth and a high local recurrence rate.[1] It is the third most frequent odontogenic tumor after odontoma and ameloblastoma,[1] with an incidence rate of ~0.07 per million individuals per year.[2] There is a slight

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Funding was provided by the Basel Research Centre for Child Health to Baptiste Ameline and Daniel Baumhoer.

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The methylation data sets generated in this study are deposited in the European Genome–Phenome Archive (EGA) under accession number EGAS00001007876.

Conflicts of Interest and Source of Funding: T.G.K. was supported by a travel grant from the Melanoma and Sarcoma Foundation, Groningen. For the remaining authors none were declared.

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Supplemental Digital Content is available for this article. Direct URL citations are provided in the HTML and PDF versions of this article on the journal's website, www.ajsp.com.

DOI: 10.1097/PAS.0000000000002293

female predominance, and most odontogenic myxomas occur in the second to fourth decades of life.[3] Odontogenic myxomas exclusively develop in the tooth-bearing areas of the jawbones, with the majority occurring in the posterior mandible.[3] Surgery is the main therapeutic modality, ranging from less invasive procedures, such as enucleation or curettage, to marginal or extended resections.[4] More conservative treatment strategies tend to result in high recurrence rates of up to 31%.[3]

The standard diagnostic approach for odontogenic myxoma involves the integration of clinicoradiologic features with histopathologic findings. Clinically, odontogenic myxoma commonly manifests as a painless, slowly enlarging, and expansile tumor of the jawbones, often accompanied by tooth loosening or displacement. As the tumor expands, it frequently invades the adjacent structures. Odontogenic myxomas of the maxilla tend to extend into the sinuses, whereas those located in the mandibular body frequently invade the ramus.[5] Radiologically, odontogenic myxoma appears most commonly as a unilocular or multilocular radiolucency with ill-defined or less commonly well-defined borders with retention of fine, bony septae, resulting in a "honeycomb" or "soap-bubble" appearance.[6,7] The main clinical and radiographic differential diagnoses include ameloblastoma, central giant cell granuloma, dentigerous cyst, odontogenic keratocyst, and solitary bone cyst.[2]

Microscopically, odontogenic myxoma resembles the mesenchymal tissue of the dental follicle and papilla of the developing tooth. It is an unencapsulated tumor composed of spindle to stellate-shaped cells arranged in a loose myxoid background of delicate collagen fibers and abundant ground substance. The proportion of collagen varies among tumors. Immunohistochemistry in odontogenic myxomas is nonspecific and, therefore, generally not helpful other than to rule out myxoid neurofibroma.

The molecular pathogenesis of odontogenic myxoma remains poorly understood.[8] A comparative analysis of methylation patterns demonstrated hypomethylation of the tumor suppressor genes *CDKN1A*, *CDKN1B*, *CDKN2A*, *TP53*, and *RB1* in odontogenic myxomas compared with dental pulp tissue.[9] Perdigão et al[10] identified *PRKAR1A* mutations in 2 of 17 odontogenic myxomas, with reduced immunostaining for the corresponding protein. However, odontogenic myxoma is not associated with Carney syndrome.[8] *GNAS* mutations, often associated with intramuscular myxomas, have not been detected in odontogenic myxomas.[11] Furthermore, next-generation sequencing (NGS) analyses targeting 50 genes commonly mutated in human cancers, including *KRAS*, *HRAS*, *NRAS*, and *BRAF*, did not reveal any pathogenic mutations in 9 odontogenetic myxomas.[12] Odintsov et al[13] sequenced 7 odontogenic myxomas using a broader sequencing panel covering 447 genes, and this approach likewise did not identify recurrent pathogenic mutations. However, they identified copy number variations (CNVs), including heterogeneous nonrecurrent gains and losses, in 4 of these tumors.[13] Pahl et al[14] performed chromosome analysis on a maxillary odontogenic myxoma with an aggressive clinical course, revealing an abnormal hypertetraploid chromosome composition with structural abnormalities affecting chromosomes 1, 3, 4, 6, and 12.

The diagnosis of odontogenic myxomas can be challenging due to a nonspecific clinical and radiologic presentation, overlapping histology with normal structures of developing teeth, and lack of specific immunohistochemical and molecular markers. In this study, we analyzed DNA methylation and CNVs in a cohort of odontogenic myxomas to identify new molecular signatures to support diagnostic decision-making.

PATIENTS AND METHODS

Patient Samples

The archives of the pathology departments at University Medical Center Groningen, Erasmus University Medical Center, Leiden University Medical Center, Maastricht University Medical Center, and Radboud University Medical Center in the Netherlands were searched for odontogenic myxomas with sufficient amounts of well-preserved tumor tissue for molecular studies. In total, 16 odontogenic myxomas from 2006 to 2021 were retrieved. The histology of all tumors was reviewed by expert bone tumor pathologists (A.H.G.C. and D.B.) to confirm the diagnosis based on standard criteria according to the current WHO classification of head and neck tumors,[1] exclude mix-up of samples and guide DNA extraction. Sinonasal tract myxomas were excluded.

Samples were retrieved from the bone and soft tissue tumor archives as approved by the ethical board (UMCG RR202200287; LUMC: B21.022). Samples were coded (pseudonymized) according to the Dutch Code Proper Secondary Use of Human Tissue according to the Dutch Society of Pathology (Federa).

DNA Extraction

Genomic DNA was extracted from 14 formalin-fixed paraffin-embedded (FFPE) and 2 fresh frozen odontogenic myxomas, using only representative tissue with a tumor content of at least 60%. DNA extraction from FFPE tissue was performed using the QIAamp DNA FFPE Tissue Kit (QIAGEN) according to the manufacturer's instructions and from fresh frozen tissue using a salt/chloroform-based protocol. The DNA was quantified using a Qubit Fluorometer. Tumors with a total of more than 100 ng DNA were selected for array-based DNA methylation analysis. Four FFPE samples were excluded due to limited genomic DNA availability, leaving 12 odontogenic myxoma samples for analysis.

DNA Methylation Data Sets

Genome-wide methylation data were generated from 12 odontogenic myxomas using the Illumina Infinium Human MethylationEPIC v1.0 BeadChip or its successor v2.0 BeadChip (EPICv2), which covered 850,000 and 935,000 CpG sites across the genome, respectively. FFPE DNA was restored using the Illumina FFPE DNA Restoration Kit according to the manufacturer's instructions.

Raw methylation data from odontogenic myxomas were processed together with published external data sets[15–20] to compare DNA methylation profiles in odontogenic myxomas with other benign and malignant bone tumors. Taken together, the DNA methylation data set comprised 12 odontogenic myxomas, 28 aneurysmal bone cysts, 5 ameloblastomas, 15 chondroblastomas, 24 chordomas, 7 chondromyxoid fibromas, 16 chondrosarcomas (head and neck region only), 18 fibrous dysplasias, 18 giant cell tumors of bone, 39 high-grade osteosarcomas, 13 low-grade osteosarcomas, 2 intramuscular myxomas, and 29 osteoblastomas. Sample details are provided in Supplemental Material (Supplemental Table S1, Supplemental Digital Content 1, http://links.lww.com/PAS/B918).

Methylation Array Processing

Raw intensity data files (IDATs) from the MethylationEpic v1.0 and v2.0 BeadChips were processed with the R-package "minfi" (https://bioconductor.org/packages/minfi/). Thanks to a handmade edit of the "convertArray" function from the R-package "minfi," EPICv2 arrays were converted into a virtual EPICv1 array for joint normalization and processing of data from both platforms. Probes associated with known single-nucleotide polymorphisms, non-CpG islands, and sex chromosomes were not taken into account for the evaluation. Moreover, samples with a mean detection P value of >0.03 were discarded. The "preprocessQuantile" function was used before generating dimension reduction visualization, whereas the "preprocessIllumina" function was preferred before deriving copy number profiles. Finally, batch effect corrections were applied to the beta values to remove any bias related to the sample type (FFPE/fresh frozen) and the array type (EPICv1/EPICv2) using the R package "ChAMP" (https://bioconductor.org/packages/ChAMP/).

Unsupervised Clustering

The set of probes was then restricted to the top 40,000 most differentially methylated probes. The unsupervised nonlinear dimension reduction method "Uniform Manifold Approximation and Projection" was performed on the results of a principal component analysis calculated through the singular value decomposition of the beta methylation matrix. The R-package "uwot" (https://github.com/jlmelville/uwot) was used for generating the graph. The settings used to generate the nonlinear dimensional reduction model were as follows: principal component analysis = 50; neighbors = 7; remaining parameters were left unchanged. The selection of the number of principal component analysis fulfilled 2 criteria: (1) explained variance >70% and (2) the reference samples used as positive controls were displayed in their expected clusters.

TABLE 1. Clinicopathologic Characteristics of Study Cases

#	Age (y)	Sex	Tumor location	Size (mm)	Treatment	Recurrence (free period, y)
1	37	F	Mandible	NA	Resection	No[3]
2	30	M	Mandible	45	Resection	No[4]
3	22	M	Maxilla	58	Resection	No[12]
4	49	M	Mandible	70	Resection	No[8]
5	25	M	Mandible	10	Enucleation	No[3]
6	25	F	Maxilla	40	Resection	No[3]
7	37	F	Mandible	35	Resection	No[3]
8	42	M	Mandible	30	Enucleation	No[17]
9	21	F	Maxilla	42	Resection	NA
10	25	M	Maxilla	50	Resection	No[11]
11	37	F	Mandible	18	Enucleation	No[10]
12	17	F	Mandible	29	Resection	No[7]
13	29	F	Maxilla	18	Resection	NA
14	3	M	Mandible	46	Resection	No[6]
15	10	F	Mandible	60	Enucleation	NA
16	24	F	Maxilla	45	Resection	NA

NA indicates not available.

CNV Analysis

Copy number profiles were derived from the methylation array data using the R-package "conumee" (http://bioconductor.org/packages/conumee/), after the preprocessing of data described previously. The settings for copy number segmentation were as follows: (1) minimum number of probes per bin equal to 25 and (2) minimum bin size equal to 50,000 bp. CNVs were called based on a minimum of 5 bins. All copy number profiles were reviewed individually. To identify statistically significant recurrent CNVs, we used GISTIC (https://broadinstitute.github.io/gistic2/) and the R package "CNsummaryplots" (https://github.com/dstichel/CNsummaryplots). The segmentation generated by the "conumee" R package served as the input for GISTIC2 and "CNsummaryplots." In GISTIC2, we considered events with False Discovery Rate q values <0.05 as significant at a 90% confidence level.

Copy number profiles of 3 published dental follicle cases[16] were created in the same manner as described previously for comparison with odontogenic myxomas. Sample details are provided in Supplemental Material (Supplemental Table S1, Supplemental Digital Content 1, http://links.lww.com/PAS/B918).

RESULTS

Clinicopathologic Characteristics of Odontogenic Myxomas

The median age at presentation in our study cohort was 25 years (range: 3 to 49 y; Table 1). Seven patients (44%) were males and 9 were females. Preoperative imaging showed tumors located in the mandible (10 patients) and maxilla (6 patients; Fig. 1A, B). The median tumor size was 42 mm (range: 10–70 mm). In 4 patients, the tumor was enucleated, and in 12 patients, gross total resection was achieved with free margins. On macroscopic examination, the odontogenic myxomas had a whitish appearance and a soft gelatinous consistency (Fig. 1C). Histologically, they were characterized by spindle-to-stellate-shaped cells lacking cellular atypia within a loose myxoid to fibromyxoid background (Fig. 1D). The proportion of collagen varied among tumors with 15/16 cases having predominantly loose myxoid background of

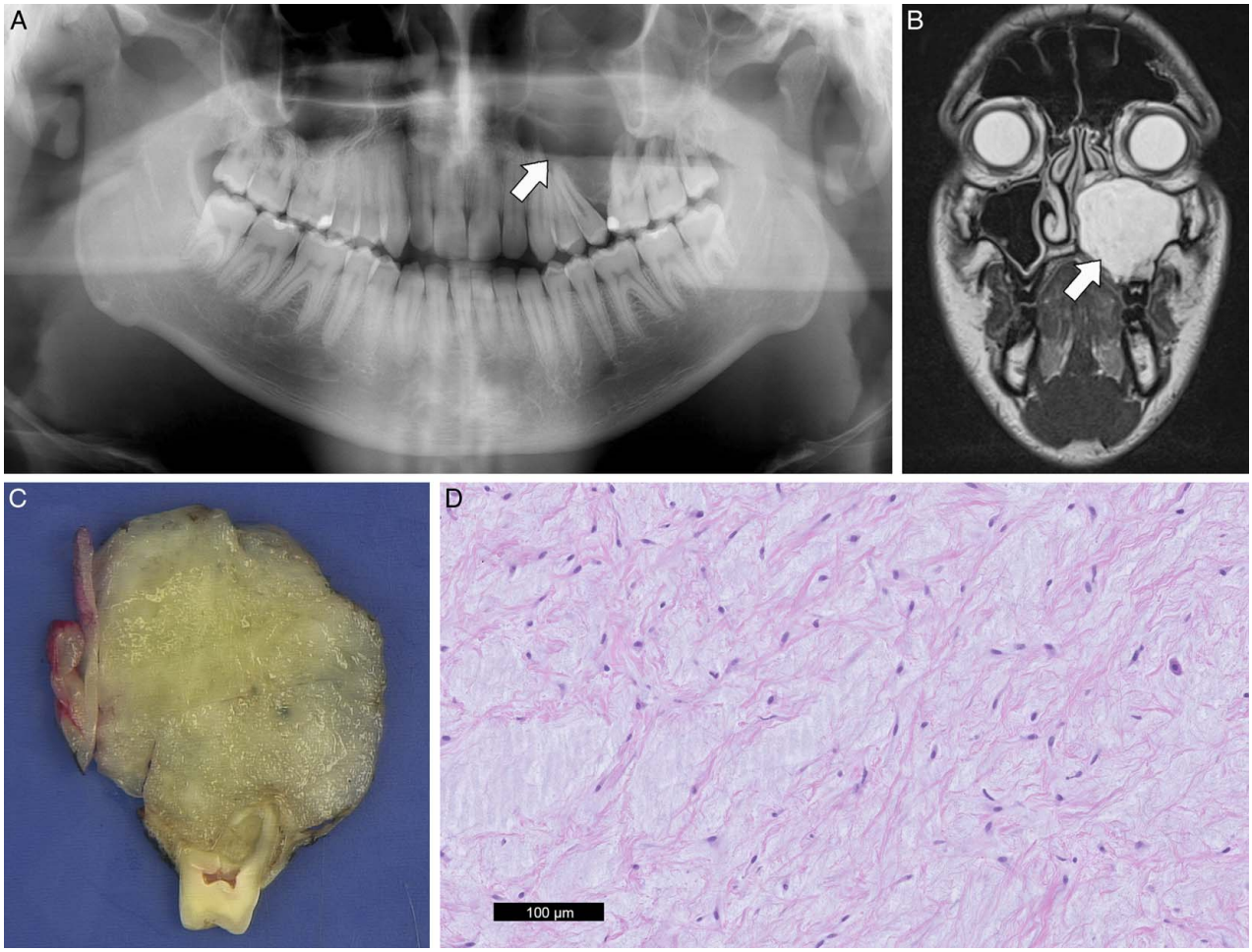

FIGURE 1. Radiologic and histopathologic characteristics of odontogenic myxoma case #3. A, Orthopantomogram and B, coronal view of T2-weighted T2-weighted turbo spin echo magnetic resonance imaging (MRI) showing typical features of odontogenic myxoma, as a well-defined mass with a maximal tumor diameter of 58 mm in the left hemimaxilla (indicated by white arrows) with extension into the left maxillary sinus and nasal cavity. C, Macroscopic examination revealed a whitish gelatinous mass. D, Histology showed typical stellate, spindle-shaped cells within a myxoid background of delicate collagen fibers (hematoxylin and eosin staining). The scale bar represents 100 µm. MRI indicates magnetic resonance imaging.

delicate collagen fibers and abundant ground substance. Case #2 had a more prominent collagenous stroma. Follow-up data were available for 12 patients, with duration ranging from 3 to 17 years. All patients remained in good health with no signs of recurrence.

Methylome Analysis and Uniform Manifold Approximation and Projection–based Classification

Unsupervised methylation-based clustering revealed that 11/12 odontogenic myxomas clustered closely together and formed a distinct methylation group separated from other bone tumors available in the reference cohort (Fig. 2). Surprisingly, one odontogenic myxoma (case #2) clustered within the group of conventional high-grade osteosarcomas. Reevaluation of this case confirmed the diagnosis of odontogenic myxoma based on the radiologic and morphologic findings (Fig. 3).

Copy Number Analysis

Copy number analysis of our 12 odontogenic myxomas revealed whole-chromosome gains (trisomies) of chromosomes 5, 8, and 20 in all cases (Fig. 4A, B), and of chromosomes 10, 12, and 17 in all except one case (case #2). Trisomies of other chromosomes were observed in a subset of our cohort (chr19 in 10 cases, chr16 in 9 cases, and chr4 in 8 cases). No recurrent hemizygous or homozygous deletion (copy number loss) was observed. The individual copy number profiles are summarized in Supplemental Figure S1 (Supplemental Digital Content 2, http://links.lww.com/PAS/B919).

The majority of benign tumors in our DNA methylation reference cohort, including intramuscular myxoma, ameloblastoma, aneurysmal bone cyst, chondroblastoma, fibrous dysplasia, giant cell tumor of bone, and osteoblastoma, showed no chromosomal alterations and exhibited a flat copy number profile. In con-

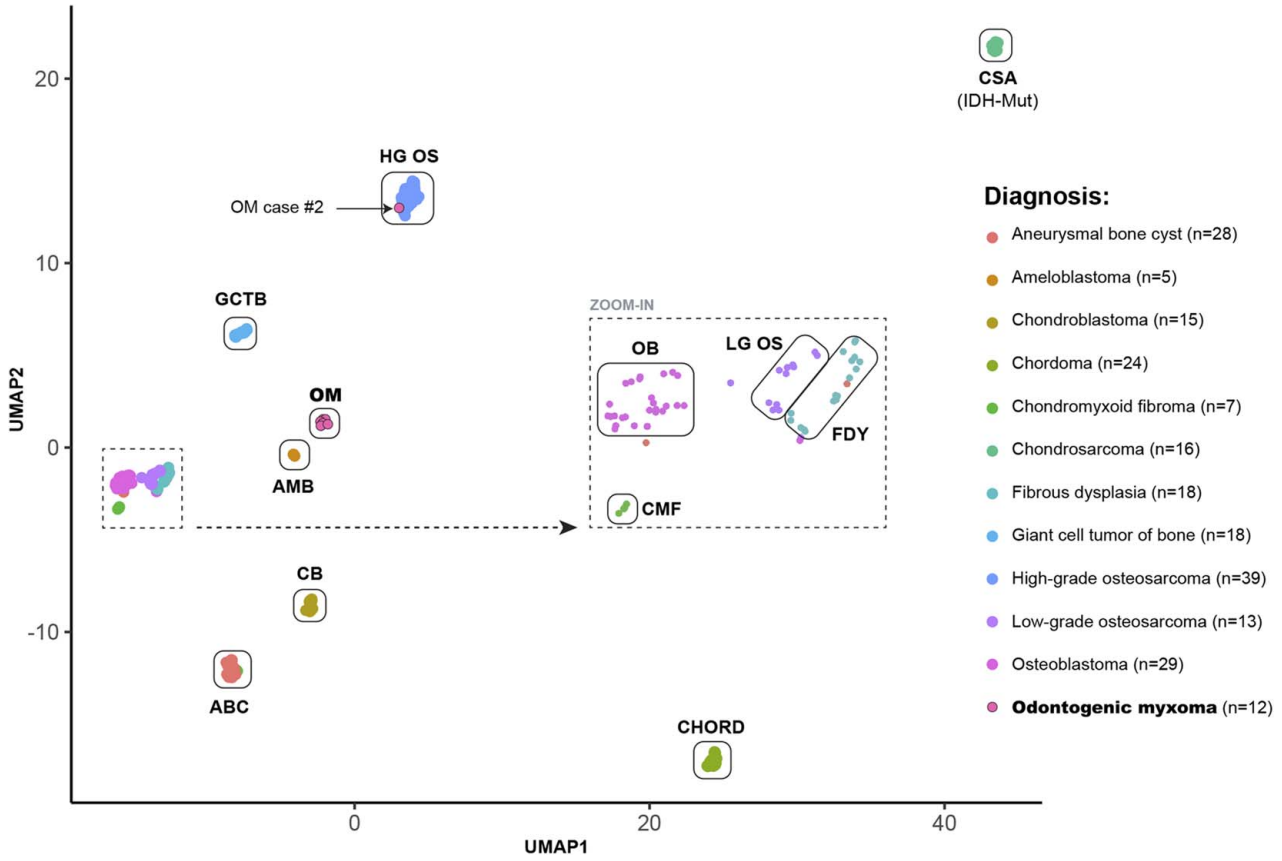

FIGURE 2. Methylation-based clustering of odontogenic myxomas. Using unsupervised UMAP analysis, 11/12 OM clustered together and formed a distinct DNA methylation group separated from other tumor entities, including ABC, AMB, CB, CHORD, CMF, CSA, FD, GCTB, HG OS, LG OS, and OB. One odontogenic myxoma (case #2) clustered with the group of high-grade osteosarcomas. ABC indicates aneurysmal bone cyst; AMB, ameloblastoma; CB, chondroblastoma; CHORD, chordoma; CMF, chondromyxoid fibroma; CSA, chondrosarcoma; FD, fibrous dysplasia; GCTB, giant cell tumor of bone; HG OS, high-grade osteosarcoma; LG OS, low-grade osteosarcoma; OB, osteoblastoma; OM, odontogenic myxomas; UMAP, Uniform Manifold Approximation and Projection.

trast, malignant tumors, such as conventional high-grade osteosarcomas and central chondrosarcomas (grades 2 and 3), displayed multiple but generally non-recurrent copy number alterations.

Searching the Mitelman database,[21] no other tumors within the differential diagnostic spectrum of odontogenic myxoma had similar patterns of copy number alterations as in our study cohort.

OM case #2, which clustered in the group of conventional high-grade osteosarcomas based on DNA methylation, had in addition to whole-chromosome gains (trisomies) of chromosomes 5, 8, and 20, similar to the other odontogenic myxomas, additional small copy number losses (chr17p, chr18) and gains (chr12p and chr13; Fig. 4C). Similar copy number profiles were not observed in our reference group of high-grade osteosarcomas.

DISCUSSION

Odontogenic myxoma is a benign neoplasm that can display a destructive growth pattern, of which the underlying molecular mechanisms are still largely unknown. An accurate diagnosis is mandatory to develop optimal treatment plans that might severely affect facial esthetics and function. Here, we report that odontogenic myxomas harbor a recurrent copy number signature and form a DNA methylation cluster distinct from various other bone tumors. These features seem useful to support the diagnosis of odontogenic myxoma also from a molecular point of view.

Our study revealed recurrent whole-chromosome gains (trisomies) of chromosomes 5, 8, and 20 in all odontogenic myxomas, and of chromosomes 4, 10, 12, 16, 17, and 19 in most cases. In contrast, the majority of other benign tumors in our DNA methylation reference cohort showed no chromosomal alterations and exhibited a flat copy number profile, whereas malignant tumors harbored multiple but usually nonrecurrent CNVs. To the best of our knowledge, no other tumor type entering the differential diagnosis of odontogenic myxoma has been reported to show patterns of CNVs identical to our set of odontogenic myxomas. Thus, these recurrent large-scale aberrations appear to be a specific and reliable copy

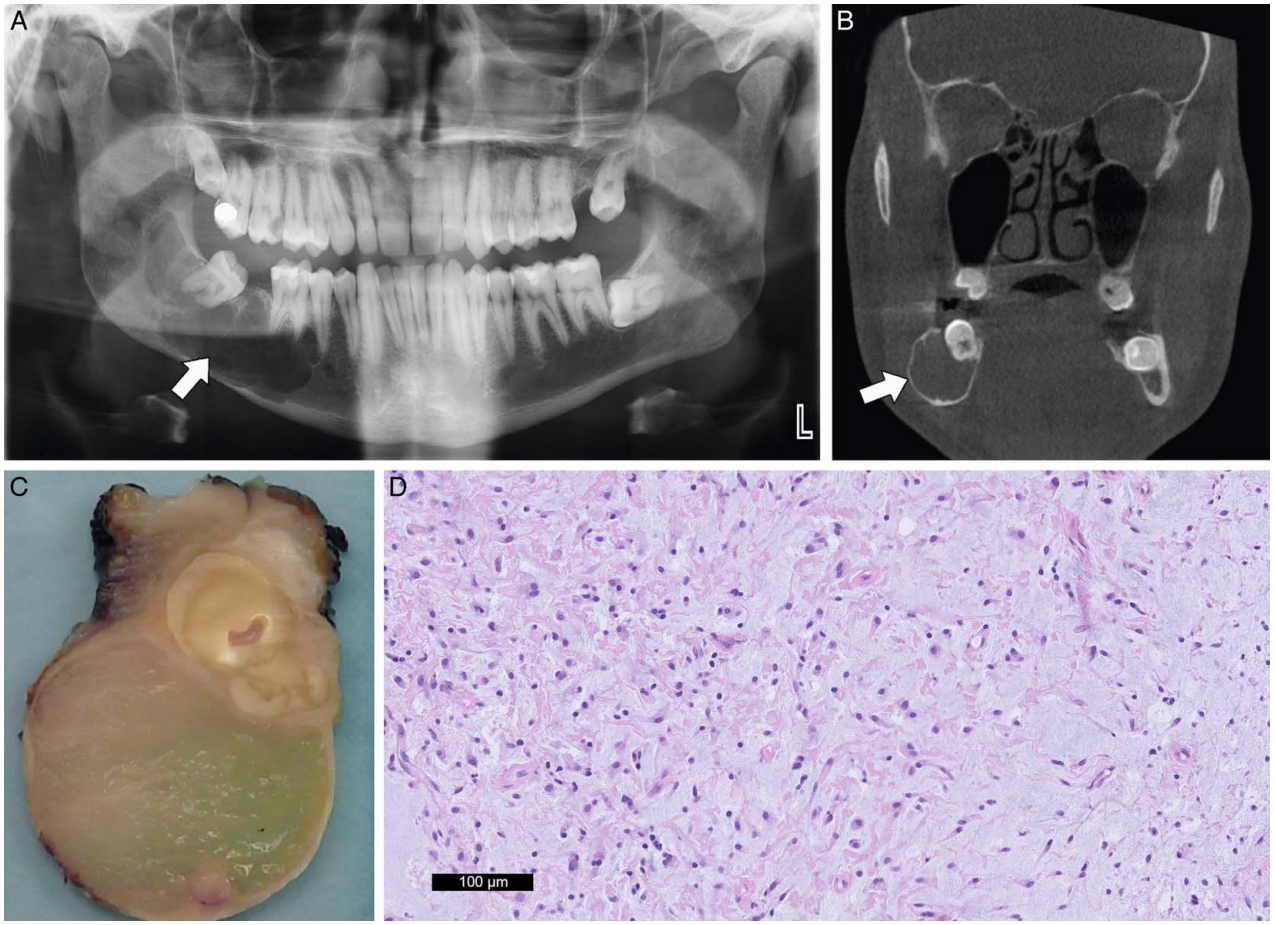

FIGURE 3. Radiologic and histopathologic features of odontogenic myxoma case #2, which clustered with the high-grade osteosarcomas. A, Orthopantomogram and B, coronal CT image showing an odontogenic myxoma with a maximal tumor diameter of 45 mm in the right mandibular body (indicated by white arrows) extending into the ramus, characterized by a well-defined mass with bone expansion and thinning of the cortical borders. C, Macroscopic examination revealed a well-defined whitish gelatinous mass. D, Histology showed stellate, spindle-shaped cells in loose myxoid to more fibromyxoid background (hematoxylin and eosin staining). The scale bar represents 100 µm. CT indicates computed tomography.

number signature that can be used as a molecular diagnostic marker for odontogenic myxoma.

Clinical genetic studies on chromosomal abnormalities in odontogenic myxomas are limited. Pahl et al[14] analyzed the karyogram of an aggressive maxillary case, revealing an aberrant hypertetraploid chromosome composition and structural abnormalities involving chromosomes 1, 3, 4, 6, and 12. Recently, Odintsov et al[13] employed targeted NGS to analyze 7 odontogenic myxomas revealing heterogeneous nonrecurrent gains and losses frequently at the arm or whole-chromosome level, with one case showing a gain of chromosome 12 and another displaying gains of chromosomes 4, 5p, and 10. Although these findings partially overlap with our results, consistent recurrent copy number gains, as observed in our study, have not been reported, likely reflecting the targeted NGS approach used by Odintsov and colleagues compared with our whole genome-wide approach. A limitation of an NGS-based approach is the difficulty to detect low-level copy number gains as seen in our cohort.[22] Alternative approaches, such as single nucleotide polymorphism-array analysis or multiplex ligation-dependent probe amplification, are more sensitive than an NGS-based approach to detect these chromosomal alterations.[22]

Trisomies involving single or multiple chromosomes are frequently reported in benign and malignant neoplasms.[21] In particular, trisomy 8 has been identified as a nonrandom chromosomal abnormality in desmoid tumors, salivary gland pleomorphic adenomas, and a subgroup of benign fibrous lesions, either as the sole karyotypic event or in conjunction with other chromosomal changes.[23,24] In addition, trisomy 8 is a common secondary genetic alteration in various mesenchymal neoplasms, such as dermatofibrosarcoma protuberans, myxoid liposarcoma, clear cell sarcoma, and Ewing sarcoma.[24–26] Approximately 16% of genes located on chromosome 8 are implicated in cancer development. Trisomies of chromosomes 5 and 20 have also been reported frequently in various tumors.[21] The presence of an

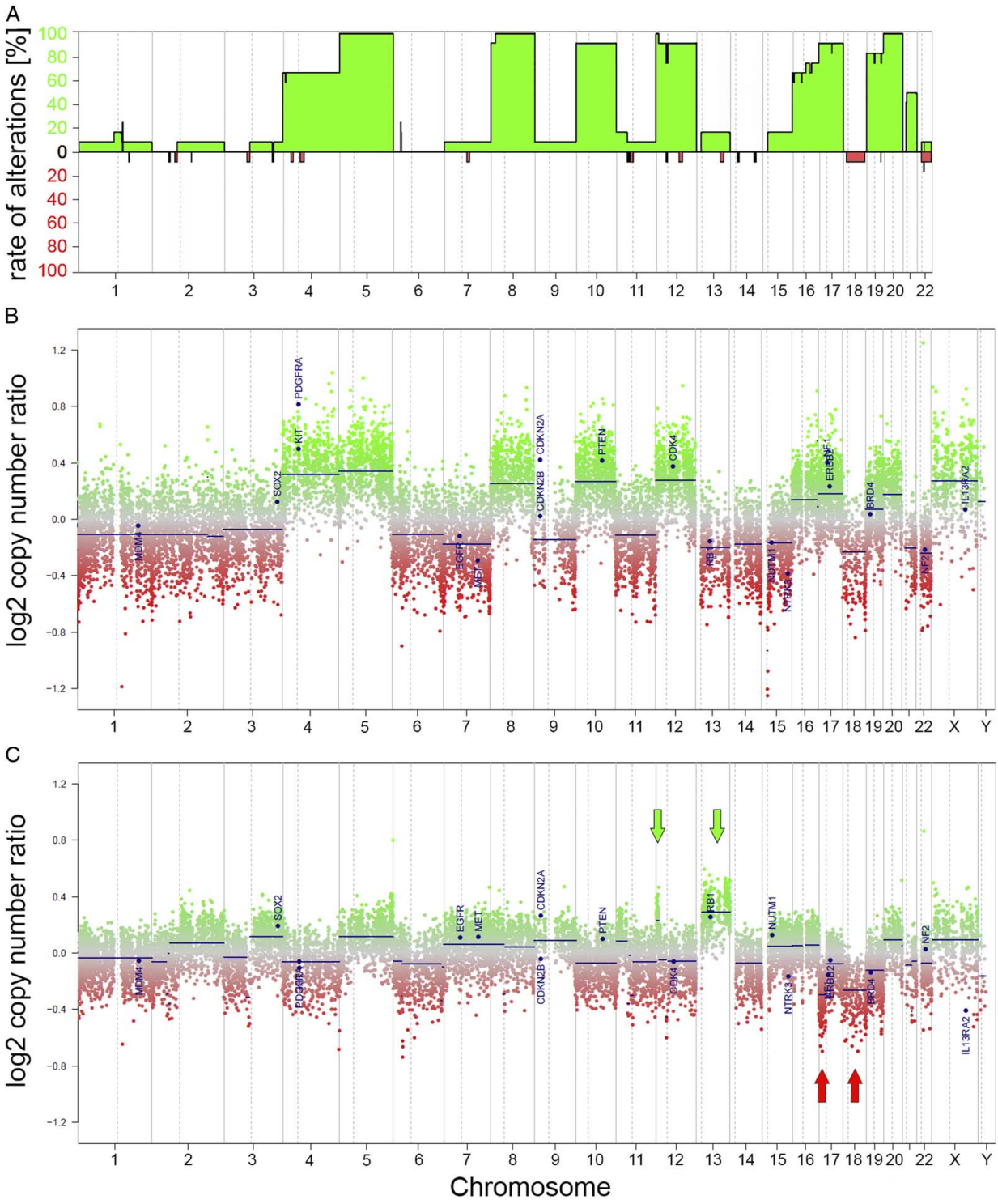

FIGURE 4. CNV plots of odontogenic myxomas calculated from the DNA methylation array data. A, The frequency of CNVs in 12 odontogenic myxomas showed whole-chromosome gains (trisomies) of chromosomes 5, 8, and 20 in all cases, and of chromosomes 10, 12, and 17 in all except one case (case #2). Trisomies of other chromosomes were observed in a subset of our cohort (chr19 in 10 cases, chr16 in 9 cases, and chr4 in 8 cases). B, CNV plot of case #5. C, Case #2 showed additional small gains in chromosomes 12p and 13, as well as losses in chromosomes 17p and 18 (indicated with arrows).

extra copy of chromosome 12 has been observed in more than 70% of mesenchymal chondrosarcomas.[27] Most likely, these chromosomal alterations play a role in the tumorigenesis of odontogenic myxomas. Further research is required to identify the exact genetic events that drive tumorigenesis and to determine whether these chromosomal alterations are responsible for initiating tumor development or rather represent secondary pathogenic events. As an additional step for future research, we will conduct gene expression profiling and pathway analysis in correlation with DNA methylation and copy number profiles to gain a better understanding of the underlying mechanisms involved in odontogenic myxomas.

One odontogenic myxoma (case #2) in our study shows typical morphology and imaging features, and trisomies of chromosomes 5, 8, and 20, similar to the other odontogenic myxomas, surprisingly clustered with conventional high-grade osteosarcomas based on DNA methylation. The only differences we observed between case #2 and the other odontogenic myxomas were that case #2 had more collagenous stroma, and its copy number profile showed additional small gains and losses. Due to the more collagenous stroma, this tumor could be referred to as an odontogenic fibromyxoma; however, there are currently no clear histologic criteria to differentiate myxoma from fibromyxoma, and both entities are considered part of the same disease spectrum,[1,28] which is in line with the recurrent copy number gains we found in our series of odontogenic myxomas including case #2 with more prominent collagenous stroma. The different clustering cannot be explained unequivocally but might indicate that methylome data of more cases of odontogenic myxomas and their mimics are needed to capture the full spectrum of epigenetic changes of this rare neoplasm and emphasize the need for an integrated approach for accurate diagnosis.

Recently, Miettinen et al[29] evaluated the sarcoma classifier from the German Cancer Research Center (Deutsches Krebsforschungszentrum) using an independent set of 62 soft tissue and bone tumor types and found that the classifier had a high sensitivity and specificity for fusion-driven sarcomas, which are well represented in the reference cohort. In contrast, a rather lower sensitivity was found for diagnosing tumors that are underrepresented in this cohort, including desmoid fibromatosis, neurofibroma, and schwannoma. Probably, adding more and well-defined sets of tumor subtypes with high-quality DNA content will improve the diagnostic accuracy of this sarcoma classifier[15] and other open-source platforms (eg, epidip.org), as has been shown for brain tumors.[30]

The differential diagnosis of odontogenic myxoma includes normal structures of the developing teeth, such as hyperplastic dental follicles and developing dental papilla, which are histologically identical but clinically and radiographically distinct. Unfortunately, in many practice settings radiologic correlation is frequently not available, particularly in consultation cases. A limitation of this study is the lack of DNA methylation data from these normal structures and other important differential diagnoses, such as odontogenic fibroma, for comparison. Published DNA methylation data from 3 dental follicle cases were available,[16] which is insufficient for unsupervised DNA methylation profiling;[31] interestingly, their copy number profiles were flat, in contrast to the specific recurrent gains observed in odontogenic myxomas, suggesting that based on copy number patterns odontogenic myxomas can be distinguished from normal dental follicles.

CONCLUSION

Herein, we first report that copy number and methylome profiling can be a valuable diagnostic add-on in the diagnosis of odontogenic myxomas, strengthening the confidence of clinicians when making impactful decisions regarding potentially mutilating surgery.

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