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Abstract: Mesenchymal chondrosarcoma (MCS) is a rare and highly aggressive tumour of soft tissue and bone that is defined by an underlying and highly specific fusion transcript involving HEY1 and NCOA2. Histologically, the tumours show a biphasic appearance consisting of an undifferentiated blue and round cell component as well as islands of highly differentiated cartilage. Particularly in core needle biopsies, the chondromatous component can be missed and the non-specific morphology and immunophenotype of the round cell component can cause diagnostic challenges. We applied NKX3.1 immunohistochemistry which was recently reported as a highly specific marker as well as methylome and copy number profiling to a set of 45 well characterised MCS cases to evaluate their potential diagnostic value. Methylome profiling revealed a highly distinct cluster for MCS. Notably, the findings were reproducible also when analysing the round cell and cartilaginous component separately. Furthermore, four outliers were identified by methylome profiling for which the diagnosis had to be revised. NKX3.1 immunohistochemistry showed positivity in 36% of tumours, the majority of which was rather focal and weak. Taken together, NKX3.1 expression showed a low sensitivity but a high specificity in our analysis. Methylome profiling on the other hand represents a sensitive, specific and reliable tool to support the diagnosis of MCS, particularly if only the round cell component is obtained in a biopsy and the diagnosis is not suspected. Furthermore, it can aid in confirming the diagnosis in case RNA sequencing for the HEY1::NCOA2 fusion transcript is not available.

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ANATOMICAL PATHOLOGY

NKX3.1 immunohistochemistry and methylome profiling in mesenchymal chondrosarcoma: additional diagnostic value for a well-defined disease?

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Summary

Mesenchymal chondrosarcoma (MCS) is a rare and highly aggressive tumour of soft tissue and bone that is defined by an underlying and highly specific fusion transcript involving *HEY1* and *NCOA2*. Histologically, the tumours show a biphasic appearance consisting of an undifferentiated blue and round cell component as well as islands of highly differentiated cartilage. Particularly in core needle biopsies, the chondromatous component can be missed and the non-specific morphology and immunophenotype of the round cell component can cause diagnostic challenges. We applied NKX3.1 immunohistochemistry which was recently reported as a highly specific marker as well as methylome and copy number profiling to a set of 45 well characterised MCS cases to evaluate their potential diagnostic value.

Methylome profiling revealed a highly distinct cluster for MCS. Notably, the findings were reproducible also when analysing the round cell and cartilaginous component separately. Furthermore, four outliers were identified by methylome profiling for which the diagnosis had to be revised. NKX3.1 immunohistochemistry showed positivity in 36% of tumours, the majority of which was rather focal and weak.

Taken together, NKX3.1 expression showed a low sensitivity but a high specificity in our analysis. Methylome profiling on the other hand represents a sensitive, specific and reliable tool to support the diagnosis of MCS, particularly if only the round cell component is obtained in a biopsy and the diagnosis is not suspected. Furthermore, it can aid in confirming the diagnosis in case RNA sequencing for the *HEY1::NCOA2* fusion transcript is not available.

Key words: Mesenchymal chondrosarcoma; Bone tumour; NKX3.1; Immunohistochemistry; Methylome profiling; Copy number analysis.

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INTRODUCTION

Mesenchymal chondrosarcoma (MCS) is a rare high-grade sarcoma, which was first described by Lichtenstein and Bernstein in 1959.[1] It affects predominantly young individuals with a peak incidence in the third decade of life with no sex predilection and shows a broad anatomical distribution with most cases arising in the bone and soft tissues.[2] Visceral location can also rarely occur.[3] The prognosis is unfavourable with 5- and 10-year overall survival rates of 51% and 43%, respectively.[2] Morphologically, MCS has a characteristic biphasic pattern, showing sheets of undifferentiated small and round cells admixed with islands of mature cartilage. Given the typical morphological appearance, the histological diagnosis is usually straight forward, provided that both components are present in the tissue obtained for biopsy. However, the amount of cartilage can differ significantly and therefore can be easily missed in a core needle biopsy. Since the small cell component is morphologically non-specific, the differential diagnoses are broad and include other undifferentiated round cell sarcomas, small cell carcinoma, lymphoma, and melanoma. In 2012, Wang *et al.* identified a highly specific gene fusion between *HEY1* (located 8q21.1) and *NCOA2* (located 8q13.3) that has emerged as a reliable and specific molecular marker for MCS.[4] Recently, it has been demonstrated that the fusion enhances cell proliferation and directly upregulates *PDGFB* and *PDGFRA*, which stimulates PI3K-AKT signalling.[5] This might pave the way for exploring new therapeutic targets, making an accurate diagnosis even more relevant. Although the *HEY1::NCOA2* gene fusion is present in virtually all MCS, some studies have shown alternative alterations with one case arising in the soft tissue harbouring an *IRF2BP2::CDX1* gene fusion and one arising in the kidney with no detectable fusion transcript, suggesting genomic heterogeneity in at least a small subset of MCS.[3,6] Immunophenotypically, the small cell component can express various markers with low sensitivity and specificity, although

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SOX9 might be useful in distinguishing MCS from other small cell tumours.[7,8]

Recently, studies using array-based DNA methylation profiling showed that MCS forms a distinct and highly specific methylation cluster.[9,10] Moreover, NKX3.1, a marker protein for prostatic adenocarcinoma, has been proposed to be highly specific also for MCS with partly contradictory results.[11,12] The aim of this study was to validate these two new and innovative diagnostic tools in a set of 45 well characterised MCS.

MATERIALS AND METHODS

Patient samples

All cases were retrieved from the archives of the institutes of pathology in Basel and Zürich, Switzerland. Representative haematoxylin and eosin (H&E) stained slides were re-evaluated by experienced bone and soft tissue pathologists (DB, CP). In total, 45 samples from 42 patients, including both initial biopsies and resection specimens, were analysed (from one patient four different manifestations were included). The study was approved by the Ethikkommission beider Basel (reference 274/12) and the the Cantonal Ethical Committee in Zurich (BASEC-2021-00417).

Next-generation sequencing (NGS)

Due to limited tissue availability and preservation, only samples from 26 of 42 patients were eligible for *HEY1::NCOA2* fusion gene testing. Semi-automated purification of RNA from formalin-fixed, paraffin-embedded (FFPE) tissue sections was performed using the miRNeasy FFPE kit (Qiagen, USA) on the QIAcube instrument (Qiagen). The TruSight RNA Fusion Panel (covering 507 fusion-associated genes) was used for hybrid capture-based library preparation as described by Illumina. Sequencing was conducted on the MiniSeq system (High Output Reagent Kit, 150 cycles; Illumina, USA). Following the manufacturer's instructions, abundance of 28S rRNA was used as quality control to exclude samples with low RNA content.

Genome-wide array-based methylome profiling

The slides with the highest available tumour content were chosen for DNA extraction. All biopsies were subjected to DNA methylation analysis based on the Infinium Human Epic Array (850K) platform (Illumina) according to the manufacturer's instructions. For comparison, pre-existing datasets of 50 conventional osteosarcomas, 26 epithelioid sarcomas, 75 Ewing sarcomas, 24 giant cell tumours of bone, 21 cutaneous melanomas, 23 malignant rhabdoid tumours, 81 rhabdomyosarcomas and RMS-like sarcomas, 60 synovial sarcomas, and 18 undifferentiated pleomorphic sarcomas were used ('comparison set').[10,13] All methylation data were deposited in the European Nucleotide Archive under the study accession number: EGAS00001007042.

Raw data were processed as described earlier.[14] Briefly, raw intensity data files (IDATs) from the Methylation Epic (850K) BeadChips (Illumina) were processed with the R package minfi (https://www.rdocumentation.org/packages/minfi). Epic arrays were converted to a virtual 450K array for joint normalisation and processing of data from both platforms. Probes associated with known single nucleotide polymorphisms, non-CpGs and sex chromosomes were not taken into account for the evaluation. Moreover, samples with a mean detection p value >0.02 were discarded. The 'preprocessIllimuna' function was used before generating the dimension reduction visualisation whereas the 'preprocessQuantile' was preferred before deriving copy number profiles.

Unsupervised methylation-based clustering

Batch effects related to the different array types (450K/850K) as well as the different tissue conditions (FFPE and fresh frozen) were corrected using the R package ChAMP (https://bioconductor.org/packages/release/bioc/html/ChAMP.html) in order to correct the beta values. The set of probes was then restricted to the top 25,000 most differentially methylated (based on the standard deviation) determined on 15,500 reference datasets mostly derived from the cancer genome atlas (TCGA) and gene expression omnibus (GEO) as previously described.[14] Uniform manifold approximation and projection (UMAP) was performed on the results of a principal component analysis (30 PCs) calculated via the singular value decomposition of the beta methylation matrix. The R package used for generating the graph can be found at https://github.com/jlmelville/uwot.

Copy number analysis derived from methylation array

Copy number variations were inferred from the Infinium Human Epic Array platform using the R package conumee (https://bioconductor.org/packages/release/bioc/html/conumee.html), after pre-processing the data as described above. A set of 14 control samples was used as a reference (tissue with reactive changes n=10 and blood n=4 obtained from the study of Koelsche *et al.*).[15] The settings for copy number variation inference were as follows: a minimum number of probes per bin equal to 25; minimum bin size equal to 100,000 bp. Any sample with a background noise superior to 0.9 was excluded. Copy number events were called against their background noise. Any copy number variation inferior to a third of the individual background noise was considered non-significant and was thus filtered out. Finally, all copy number profiles were reviewed individually. Subsequently, a score representing the percentage of the whole genome involved by copy number variations was calculated (sum of the length of each CNV divided by the sum of the length of the 22 autosomes, after exclusion of the centromeric and telomeric regions).

Immunohistochemistry

In total, slides from 43 MCS samples were stained with a NKX3.1 rabbit monoclonal antibody (EP356; Cell Marque, Roche, Switzerland). Immunohistochemistry was performed on 4 µm tissue sections according to routine protocols on a Ventana BenchMark Ultra stainer (Roche Tissue Diagnostics, Switzerland). We used stains against INI-1 to test the general preservation of antigens. We extended the analysis of NKX3.1 to a sarcoma tissue microarray containing samples from 21 leiomyosarcomas, 21 liposarcomas, nine angiosarcomas, 13 plexiform neurofibromas, five epithelioid sarcomas, 13 myxofibrosarcomas, 16 dermatofibrosarcoma protuberans, 22 malignant mixed mesodermal tumours, 24 gastrointestinal stromal tumours, 12 rhabdomyosarcomas, three rhabdoid tumours, two alveolar soft part sarcomas, 16 osteosarcomas, six Ewing sarcomas, nine synovial sarcomas, and 18 malignant peripheral nerve sheath tumours.

Immunohistochemistry for NKX3.1 was evaluated only for nuclear staining and semiquantitatively, as described previously:[11] lack of positive cells (0), <5% positive tumour cells (1+), 5–25% positive tumour cells (2+), 26–50% positive tumour cells (3+), and >50% of positive tumour cells (4+).

Radiology

The corresponding imaging of all patients with MCS was re-evaluated by an experienced musculoskeletal radiologist (DH).

RESULTS

Clinicopathological characteristics

Our study included 45 tumour samples from 42 patients. There were 17 men and 25 females (ratio 1:1.47), the average age was 29.2 years (range 10–68 years). Sixty-eight point nine percent (31 of 45) of the tumours were located inside bone, 26.7% (12 of 45) in the soft tissue and 4.4% (two of 45) had visceral tumours. Detailed clinicopathological data are provided in Table 1.

Radiological aspects of MCS

For most of the patients, the corresponding imaging was available. All cases inside bone presented primarily lytic and showed varying amounts of chondroid-like matrix mineralisation. Soft tissue tumours presented as mass lesions with heterogeneous signal intensity in magnetic resonance imaging and were partially calcified on computed tomography scans.

HEY1::NCOA2 fusion transcript

Among the 26 tumour samples tested for the *HEY1::NCOA2* chimeric transcript, seven of 26 (26.9%) samples did not pass

Table 1 Clinicopathological data of patients including NKX3.1 immunostaining, fusion panel sequencing, methylome profiling, and final diagnosis

Case no.	Gender	Age at diagnosis	Site	NKX3.1 scoring	Fusion panel	Methylome profiling	Final diagnosis
1	F	24	Spine, C3/C4	3	Positive	MCS	MCS
2	F	44	Femur	4	Positive	MCS	MCS
3	F	55	Nuchal soft tissue	4	Positive	MCS	MCS
4	F	19	Kidney	2	Positive	MCS	MCS
5	M	42	Femur	4	Positive	MCS	MCS
6	F	40	Intramuscular NOS	4	Positive	MCS	MCS
7	F	23	Mandible	0	QC failed	QC failed	MCS
8	F	27	Fibula	0	–	QC failed	MCS
9	M	23	Mediastinum	0	–	MCS	MCS
10	F	48	Spine NOS	0	–	DICER1 Sarcoma	Uncertain
11	F	40	Femur	0	–	MCS	MCS
12	M	26	Femur	2	Positive	QC failed	MCS
13	F	19	Spine, TH9	2	Positive	MCS	MCS
14	F	19	Orbita	0	QC failed	MCS	MCS
15	F	32	Spine, C7	0	Negative	QC failed	MCS
16	F	43	Scapula	0	Negative	MCS	MCS
17	F	18	Spine, TH2	0	QC failed	MCS	MCS
18	M	35	Zygomatic bone	0	QC failed	QC failed	MCS
19	M	15	Maxillary sinus	0	–	MCS	MCS
20	M	24	Spine NOS	0	–	OS	OS
21	M	32	Pelvic	0	QC failed	OS	OS
22	M	23	Femur	0	–	QC failed	MCS
23	F	12	5th metacarpal	0	–	QC failed	MCS
24	F	31	Thigh	0	–	–	MCS
25	M	48	Alveolar bone	0	–	–	MCS
26	M	17	Mandible	0	–	MCS	MCS
27	F	13	Maxillary sinus	0	–	QC failed	MCS
28	M	25	Pelvic bone	0	–	MCS	MCS
29	F	10	Orbital and temporal bone	0	–	–	MCS
30	M	14	Pubic bone	0	–	MCS	MCS
31	F	15	Alveolar bone	2	Positive	MCS	MCS
32	F	57	Sacrum	0	–	–	MCS
33	F	39	Alveolar bone	0	–	QC failed	MCS
34	M	25	Spine, TH NOS	0	Negative	QC failed	MCS
35	M	24	Proximal phalanx Dig II	0	Negative	QC failed	MCS
36	F	68	Maxillary sinus	0	Negative	MELA	MELA
37	F	22	Maxillary sinus	1	QC failed	QC failed	MCS
38	1	NA	Left maxilla	0	Negative	QC failed	MCS
39	F	39	Radius	0	QC failed	QC failed	MCS
40	M	27	Gluteus maximus muscle	1	Positive	–	MCS
41	M	22	Iliac bone	1	Positive	QC failed	MCS
42	F	49	Primary (tibia)[a]	1	Positive	MCS	MCS
43	F	51	Metastasis I (vertebra)[a]	1	–	MCS	MCS
44	F	51	Metastasis II (femur)[a]	–	–	MCS	MCS
45	F	51	Metastasis III (vertebra)[a]	0	Positive	MCS	MCS

MCS, Mesenchymal chondrosarcoma; MELA, malignant melanoma; NOS, not otherwise specified; OS, conventional high-grade osteosarcoma.
[a] Tumour samples from the same patient.

the quality control. The gene fusion was confirmed in 13 of 26 (50.0%) cases whereas six of 26 (23.1%) cases had no detectable fusion transcript. Five of six fusion negative cases showed borderline quality controls due to low content of interpretable sequencing reads which might explain the relatively high proportion of fusion-negative cases.[4] One of six fusion-negative cases was found to represent a malignant melanoma by subsequent methylome profiling and immunophenotyping. Another fusion-negative case was confirmed to represent MCS by morphology and methylome profiling, and all other cases were confirmed by morphology only. The presence of *HEY1::NCOA2* fusion independent MCS cannot be excluded in those cases[3,6] (Table 1).

NKX3.1 expression of MCS

NKX3.1 immunostaining was positive in 14 of 39 (35.9%) cases, whereas 25 of 39 (64.1%) cases were negative. Of the positive tumours, four of 14 (28.6%) cases showed moderate to strong staining in >50% of tumour cells (4+), one case (7.1%) showed strong staining in 26–50% of tumour cells (3+), and nine cases (64.3%) showed positivity in less than 26% of tumour cells (1+ and 2+). The intensity of the staining of the latter cases varied from weak to moderate, with only two of them showing moderate to strong positivity (Fig. 1). The results were restricted to the undifferentiated round cell component; the cartilaginous lobules remained negative in all cases studied. Detailed evaluation of NKX3.1 immunohistochemistry is provided in Table 1. All samples included in the sarcoma tissue microarray (210/210) were completely negative for NKX3.1 immunostaining, underlining a high specificity of the antibody despite a rather low sensitivity (35.9%) compared to fusion testing (72.2%) and methylome profiling (100%) as outlined in Fig. 2. Ten cases of prostate cancer were used as positive controls and consistently showed strong staining in >50% of tumour cells (4+) (data not shown).

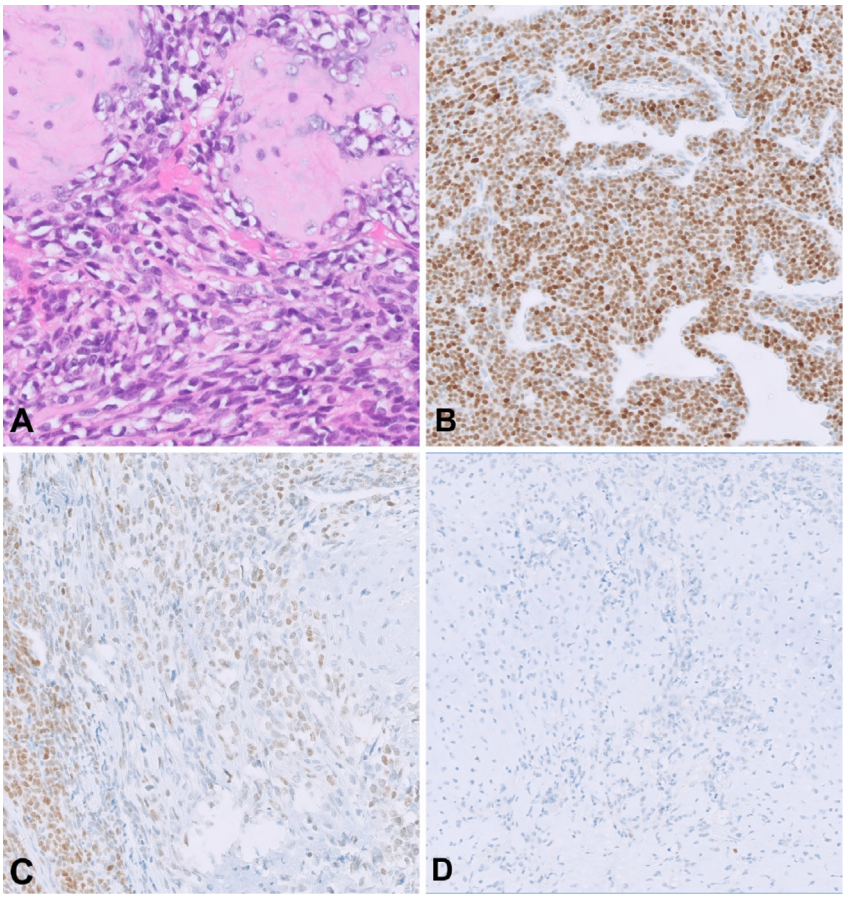

Fig. 1 NKX3.1 immunohistochemistry tested on MCS. (A) Representative histological features of a mesenchymal chondrosarcoma, showing both the cellular and the cartilaginous component. (B) More than 50% of tumour cells were positive for NKX3.1 (Score 4+). (C) Less than 50% of the tumour cells positive for NKX3.1 (Score 3+). Note the cartilaginous component which is negative. (D) Scattered tumour cells positive for the antibody. The amount of positivity shown here is similar to that found in a different study in one conventional osteoblastic osteosarcoma.

		MCS +	MCS –
Methylation-based prediction MCS+		21	0
Methylation-based prediction MCS–		0	4
	Sensitivity	100% (21/21)	
	Specificity	100% (4/4)	

	MCS+	MCS –
Fusion POS	13	0
Fusion NEG	5	1
Sensitivity	72.2% (13/18)	
Specificity	100% (1/1)	

	Methylome profiling			
Fusion	**Correct prediction**	**Wrong prediction**	**QC failed**	**Not tested**
POS	10	0	2	1
NEG	1	0	4	0
QC failed	2	0	4	0
Not tested	8	0	5	4

	MCS+	MCS –
NKX3.1 POS	14	0
NKX3.1 NEG	25	4 + 210*
Sensitivity	35.9% (14/39)	
Specificity	100% (214/214*)	

Fig. 2 Comparison between immunostaining, fusion panel sequencing and methylome profiling including statistics (only MCS cases, cases with revised diagnoses excluded). *Sarcoma tissue microarray samples.

Genome-wide array-based methylation profiling of MCS

Methylome analysis yielded evaluable results in 25 samples which were investigated by two different approaches. Firstly, the DNA methylation arrays were submitted to the 'Sarcoma Heidelberg Classifier'. Four of these cases were originally used to establish the reference class of the classifier and, as a consequence, were not re-evaluated.[15] Among the 21 remaining cases, 16 (76%) were properly classified as MCS (Table 1; Supplementary Table 1, Appendix A), whereas one case (P21) clustered in another tumour class (high-grade osteosarcoma). The remaining four samples did not yield confidence scores above the internal threshold and were regarded as non-classifiable.

Secondly, we evaluated our MCS data together with the 'comparison set' of other tumours using an unsupervised clustering approach[16] (UMAP, uniform manifold approximation and projection). All methylome profiles were put on a dimension reduction graph (Fig. 3) with each individual tumour positioned according to its similarity to the methylome profiles of the other tumour samples included. Twenty-one of 25 MCS (84%) clustered closely together and apart from the samples of the 'comparison set'. The remaining four cases clustered together with samples from other established methylation classes including RMS-like sarcoma (P10), osteosarcoma (P20, P21) and melanoma (P36). Notably, methylome profiling provided diagnostic aid in three of seven (42.9%) samples that did not pass quality control for RNA fusion testing (Table 1 and Fig. 2).

In three tumours (P1, P3, P6), the undifferentiated round cell and the cartilaginous component were microdissected and investigated for their methylome profiles independently. Three other fusion-positive tumour samples composed exclusively of round cells (P2, P4, P5) served as additional positive controls. Notably, using the same clustering analysis, all nine samples clustered in close proximity to each other within the cluster of MCS without any ambiguity. The 'Heidelberg Sarcoma Classifier' also correctly predicted the tumour class of all these specimens with the maximum confidence score. Despite being morphologically distinct, both tumour components therefore share the same methylome profiles.

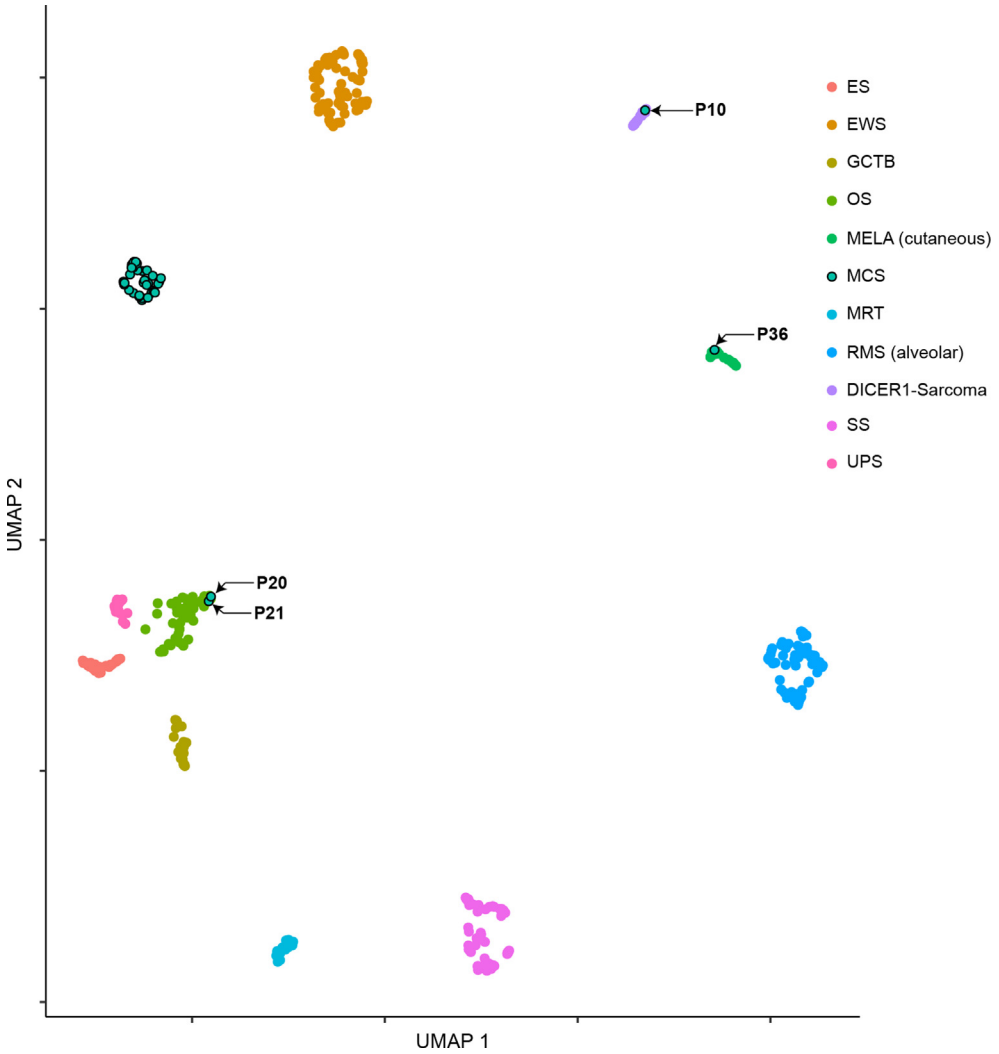

Fig. 3 DNA methylation-based clustering of MCS and potential differential diagnoses after non-linear dimension reduction (UMAP). Each tumour type is associated with a dedicated colour. Mislocalised samples are highlighted by an arrow and labelled with its case number. ES, epithelioid sarcoma; EWS, Ewing sarcoma; GCTB, giant cell tumour of bone; MCS, mesenchymal chondrosarcoma; MELA, melanoma; MRT, malignant rhabdoid tumour; OS, conventional osteosarcoma (high-grade); RMS, rhabdomyosarcoma; SS, synovial sarcoma; UPS, undifferentiated pleomorphic sarcoma.

Copy number variations derived from methylation array

Together with recently published data on MCS,[10,15] we evaluated the copy number variations of 29 tumours. On average, 10.9% (range 0.03–33.1%) of MCS genomes were affected by CNV (Fig. 4). As the sole recurrent variation identified in our study, a gain of the whole chromosome 12 was observed in 69.7% of cases (23/33). The four samples not clustering together with the remaining MCS cases (P10, P20, P21, P36) showed highly rearranged genomes and were not excluded from the above described analysis (Fig. 4).

Re-evaluation of the outliers

The four samples with highly rearranged genomes and distinct methylome profiles were histologically re-evaluated (P10, P20, P21, P36). Both cases falling into the methylation class of conventional osteosarcoma (P20 and P21) showed evidence of focal immature bone formation (Fig. 5A–D) and therefore were re-classified as osteosarcomas (Supplementary Table 1, Appendix A). Case P36 clustered with the methylation class melanoma and showed focal expression of HMB45 and S100 on immunohistochemistry (Fig. 5E,F). As a consequence, the tumour was re-classified as melanoma. Finally, case P10 was assigned to the methylation class of RMS-like sarcoma carrying *DICER1*

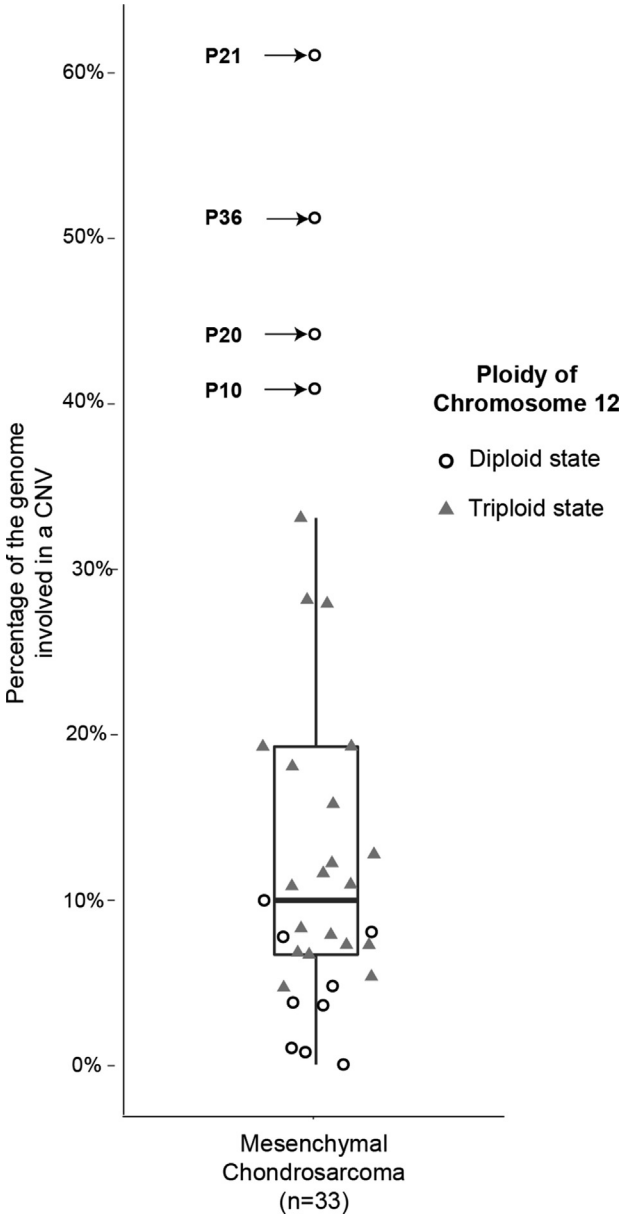

Fig. 4 Distribution of the percentage of genomes recombined in our cohort of MCS. All the outliers are designated by an arrow and marked with their case number. The dot shape indicates the ploidy of chromosome 12.

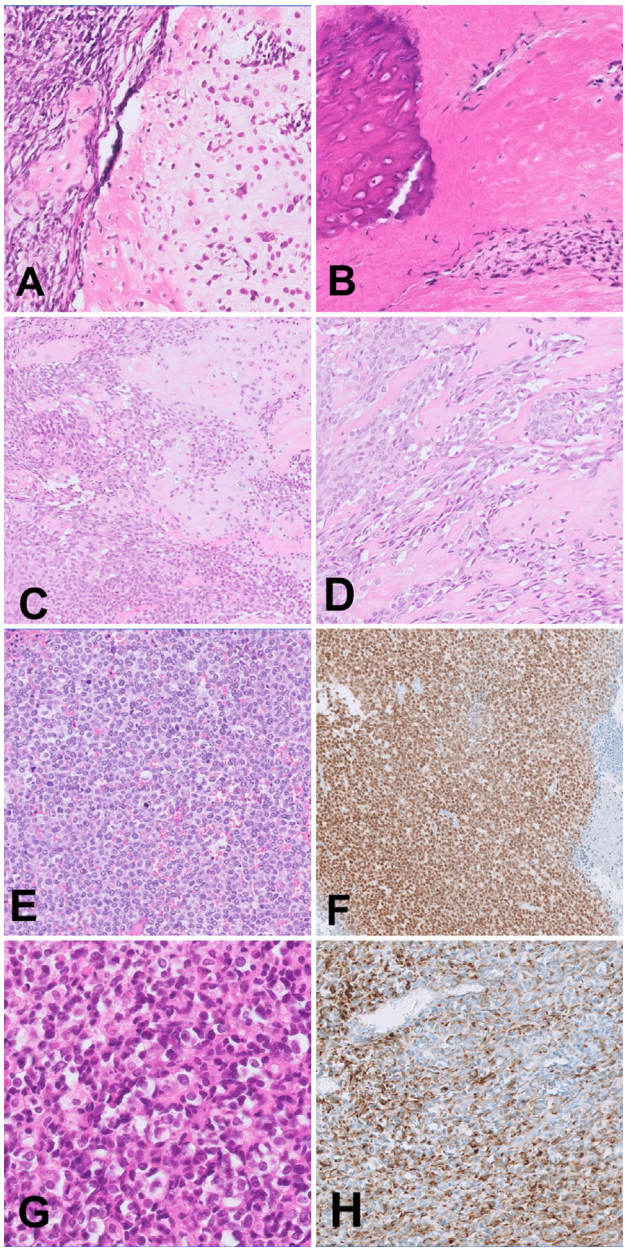

Fig. 5 Revised cases. (A,B) Case P20: conventional osteoblastic osteosarcoma with (A) focal chondromatous differentiation resembling mesenchymal chondrosarcoma whereas (B) focal areas of bone formation can be observed. (C,D) Case P21: conventional osteosarcoma showing (C) chondromatous differentiation and (D) focal osteoid deposition. (E,F) Case P36: the histology shows atypical cells with vesicular nuclei, conspicuous nucleoli and scattered mitotic figures (E). The tumour cells are diffusely positive for SOX 10 (F), focal positive for HMB45, as well as for a melanoma cocktail (not shown). (G,H) Case P10: the histology shows a tumour composed of medium-sized atypical cells with pleomorphic nuclei and a eosinophilic to clear cytoplasm (G). The cells are focally positive for desmin (H), but negative for myogenin and myoD1 (not shown).

mutation.[9] However, NGS gene panel sequencing (Oncomine Comprehensive Assay Plus; ThermoFisher Scientific, USA) failed to detect a *DICER1* mutation. Immunohistochemistry showed focal desmin positivity whereas Myogenin and MyoD1 were negative. The case was considered unclassifiable (Fig. 5G,H). Taken together, methylome profiling showed the highest sensitivity in diagnosing MSC (100%) compared to fusion testing (72.2%) and NKX3.1 immunohistochemistry (35.9%) (Fig. 2).

DISCUSSION

Mesenchymal chondrosarcoma has a unique histological appearance and generally shows a defining fusion transcript involving *HEY1* and *NCOA2*. Particularly in larger biopsies in which both the undifferentiated round cell and the cartilaginous component are present, the diagnosis is usually straight forward and can be molecularly confirmed by RNA sequencing. However, in smaller biopsies the highly differentiated chondromatous islands can be missed and the morphology as well as the immunophenotype of the undifferentiated component is usually non-specific. SOX9 expression can suggest MCS but is generally not sufficient to make the diagnosis with certainty and not many pathology departments in the world have the possibility to detect the fusion transcript by RNA sequencing or fluorescence *in situ* hybridisation.

NKX3.1 is a well-established diagnostic marker in adenocarcinoma of the prostate. The corresponding gene is located on chromosome 8p21, a region affected by loss of heterozygosity (LOH) in 40% of prostatic adenocarcinoma.[17,18] Interestingly, this haplo-insufficiency does not affect the NKX3.1 protein expression but appears to be correlated with disease recurrence in prostate cancer.[19] The same location is also affected by the *HEY1::NCOA2* gene fusion in MCS which might enhance the expression of the protein although the exact mechanism is still unclear. In a recent study published by Yoshida *et al.*, nuclear NKX3.1 expression has indeed been shown as a consistent finding in the undifferentiated round cell component of MCS (12/12 cases, 20–90% of cells).[12] Similar findings have been reported by Wang *et al.* (expression in 12/12 cases) and Syed *et al.* (expression in 21/32 cases, 66.7%).[20,21] A validation study from Chen and colleagues that analysed a series of 25 MCS with two different antibodies, however, did not find NKX3.1 expression in any of their tumours.[11] Therefore, we assembled the largest series so far of 45 MCS samples, and used the same rabbit polyclonal antibody as in the first study, but identified NKX3.1 nuclear positivity in only 14 of 39 evaluable samples (36%). Since we could not detect NKX3.1 expression in any of the other sarcomas analysed in our study, the marker seems highly specific and thus could still be useful in selected cases, although positivity has also been described in the majority of *EWSR1::NFACT2* sarcomas and rarely in osteosarcomas.[12]

DNA methylation is a stable and the most intensely studied epigenetic modification. Its fingerprint can provide clues to both cellular differentiation and cell of origin, as well as to acquired aberrations during tumourigenesis.[15] Recently, DNA methylation patterns were used to develop tumour maps that proved helpful and reliable in classifying brain tumours but also soft tissue and bone tumours, including MCS.[9,10,15,22] The analysis presented here confirms a highly reproducible methylation cluster for MCS that remained stable even when microdissecting only the cartilaginous component; this is diagnostically irrelevant but in keeping with the theory that methylation patterns correlate with the common cell of origin of MCS despite the morphological (and immunophenotypic) differences of both components. Four tumour samples included in our series were erroneously diagnosed as MCS and were all correctly identified by methylome profiling. This should have been noticed before and we do not claim that methylome profiling is required to correctly classify these tumours but it underlines the potential of the method.

The copy number profile represents another interesting and independent tool worth mentioning. Indeed, we demonstrated that mesenchymal chondrosarcoma usually harbours comparably few chromosomal rearrangements, involving 10.8% of the genome on average, with a recurrent aneuploidy of chromosome 12 in 70% of cases which is in keeping with the literature.[23] In contrast, conventional osteosarcomas generally show a much higher degree of copy number changes due to chromosomal instability.[24] These features can help to distinguish MCS from conventional (or small cell) osteosarcoma as illustrated in this study, particularly in cases that show only focal neoplastic bone formation.

According to the WHO classification of soft tissue and bone tumours, the histological appearance together with the identification of the *HEY1::NCOA2* gene fusion are the gold standard for diagnosing MCS.[2] We fully agree and would not recommend NKX3.1 immunohistochemistry or methylome profiling in unequivocal cases. However, whereas NKX3.1 staining is cheap, highly specific and probably easily available in many departments due to its specificity for prostate cancer, methylome profiling is by far more sensitive to reach the correct diagnosis. Particularly in unusual cases and when the diagnosis is not suspected, this new and innovative technique can help to correctly classify tumours with an undifferentiated small and round cell morphology.

Data availability: All raw intensity data files (IDATs) from the Methylation Epic (850K) generated during this study have been deposited in the European Nucleotide Archive under the study accession number: EGAS00001007042.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2023.03.003.

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