Molecular pathology in bone and soft tissue tumors: a multifunctional key for diagnosis and prediction

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Chapter 7

Mismatch repair deficiency is rare in bone and soft tissue tumors

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Abstract

Introduction

There has been an increased demand for mismatch repair (MMR) status testing in sarcoma patients after the success of immune checkpoint inhibition (ICI) in MMR deficient tumors. However, data on MMR deficiency in bone and soft tissue tumors is sparse, rendering it unclear if routine screening should be applied. Hence, we aimed to study the frequency of MMR deficiency in bone and soft tissue tumors after we were prompted by two (potential) Lynch syndrome patients developing sarcomas.

Materials and methods

Immunohistochemical expression of MLH1, PMS2, MSH2 and MSH6 was assessed on tissue microarrays (TMAs) and included 353 bone and 539 soft tissue tumors. Molecular data was either retrieved from reports or microsatellite instability (MSI) analysis was performed. In MLH1 negative cases, additional *MLH1* promoter hypermethylation analysis followed. Furthermore, a systematic literature review on MMR deficiency in bone and soft tissue tumors was conducted.

Results

Eight MMR deficient tumors were identified (1%), which included four leiomyosarcoma, two rhabdomyosarcoma, one malignant peripheral nerve sheath tumor and one radiation-associated sarcoma. Three patients were suspected for Lynch syndrome. Literature review revealed 30 MMR deficient sarcomas, of which 33% were undifferentiated/unclassifiable sarcomas. 57% of the patients were genetically predisposed.

Conclusion

MMR deficiency is rare in bone and soft tissue tumors. Screening focusing on tumors with myogenic differentiation, undifferentiated/unclassifiable sarcomas and in patients with a genetic predisposition / co-occurrence of other malignancies can be helpful in identifying patients potentially eligible for ICI.

Introduction

Immune checkpoint inhibitors have proven their utility in the past several years across many cancer subtypes. Particularly, antibodies blocking the programmed death (PD-1) pathway have been approved as second-line or first-line therapies for melanomas and an ever-growing list of mostly epithelial malignancies [1]. Activation of the PD-1 pathway in T cells represses Th1 and cytotoxic responses in the presence of its ligands (PD-L1 or PD-L2). The former can be abundantly expressed in tumor microenvironments by both cancer and immune cells. The blocking of this pathway with therapeutic antibodies reinvigorates antitumor immune responses and elimination of cancer cells [1, 2]. Since the success of checkpoint blockade immunotherapy, the identification of predictive biomarkers for immune checkpoint inhibition (ICI) response has been the subject of investigation. Although no single biomarker can predict which patients will likely benefit from immunotherapy, PD-L1, with its limitations, has been identified as a predictive biomarker. However, in contrast to other solid cancers the predictive value of PD-L1 for ICI response is limited in sarcomas [3-5]. Another strong association of ICI response is the tumor mutation burden (TMB), where a high TMB leads to production of more neo-antigens that might be recognized by the immune system, thereby eliciting an anti-tumor response. This partially explains why therapy response is more often seen in tumors with a high TMB, e.g., lung carcinomas and melanomas, than in tumors with a low mutational burden, such as sarcomas, which are mostly refractory to ICI [6]. This theory is further supported by the finding that mismatch repair (MMR) deficiency is associated with a high sensitivity to ICI, as the defect in the MMR machinery leads to a high mutational load [7, 8]. The tumor agnostic approach of some clinical trials, the so-called basket trials, has led to an increased demand for MMR status testing in advanced cancer patients, irrespective of the tumor type, and thus also including advanced sarcoma patients[9]. However, in contrast to other cancer types, such as colon- and endometrial carcinoma, MMR deficiency does not seem to play a major role in sarcomagenesis and only anecdotal cases of sarcomas have been reported in Lynch syndrome patients [10-12]. Yet, we were prompted by two (potential) Lynch syndrome patients with leiomyosarcoma and pleomorphic rhabdomyosarcoma, respectively. Since reliable data on MMR deficiency in soft tissue sarcomas is sparse, and almost absent for bone sarcomas, we aimed to study the frequency of MMR deficiency in sarcomas by immunohistochemical testing of MMR proteins in a large cohort of different bone and soft tissue tumors and by systematically reviewing the literature in order to determine if there is a rationale for routine MMR testing in advanced sarcoma patients.

Material and methods

Sample collection

Two index cases displaying MMR deficiency were identified. In addition, tissue

microarrays (TMAs) of two institutions (Leiden University Medical Center (LUMC) and UZ Leuven) were used to assess MMR deficiency. For most of the LUMC TMAs, clinicopathological data were previously published, and the series included conventional chondrosarcoma (n=137), dedifferentiated chondrosarcoma (n=28), mesenchymal chondrosarcoma (n=21), clear cell chondrosarcoma (n=20), leiomyosarcoma (n=87), angiosarcoma (n=60), different subtypes of liposarcoma (n=42), undifferentiated pleomorphic sarcoma (n=22), vestibular schwannoma (n=22), Ewing sarcoma (n=19), malignant peripheral nerve sheath tumor (MPNST) (n=19), myxofibrosarcoma (n=17), enchondroma (n=11), neurofibroma (n=10), osteochondroma (n=9), undifferentiated spindle cell sarcoma (n=7), radiation-associated sarcoma (n=4), rhabdomyosarcoma (n=2) and osteosarcoma (n=2) [13-20]. In addition, TMA's of synovial sarcoma (n=69), osteosarcoma (n=65), MPNST (n=20), rhabdomyosarcoma (n=13), dedifferentiated liposarcoma (n=20), radiation-associated tumors (n=11) were constructed as previously described [21]. Samples were handled according to the ethical guidelines described in "code for Proper Secondary Use of Human Tissue in the Netherlands" in a coded (pseudonymized) manner, as approved by the Leiden University Medical Center ethical board (B17.020, B17.036, B17.030, and B20.064). Furthermore, previously constructed TMAs from the UZ Leuven institute included alveolar soft part sarcoma (n=59), different subtypes of liposarcoma (n=42), inflammatory myofibroblastic tumor (n=33) and alveolar rhabdomyosarcoma (n=21) [22]. The analysis of anonymized data and use of archival FFPE tumor samples were approved by the Medical Ethics Committee, UZ Leuven (S51495, S59181). All tumors were classified according to the WHO classification of bone and soft tissue tumors, fifth edition.

Immunohistochemistry

Immunohistochemistry was performed with commercially available antibodies using a standard lab protocol, as described previously [21]. In short, microwave antigen retrieval in either TRIS-EDTA (pH 9.0) or Citrate (pH 6.0) was performed using deparaffinized sections, followed by overnight incubation with the primary antibody. Details of antibodies are summarized in **Supplementary Table 1**. The following day, detection using power vision poly-HRP (ImmunoLogic, the Netherlands) and visualization with a DAB+ substrate chromogen system (Dako, Glostrup, Denmark) followed. Finally, slides were counterstained with hematoxylin, dehydrated and mounted.

Nuclear expression of the MMR proteins was scored as positive, heterogeneous or negative. If heterogenous or negative, the expression of the internal control was evaluated and staining was repeated using a whole slide section of the same tumor. Subsequently, immunohistochemistry for PD-1, PD-L1 and CD3 was performed on MMR deficient tumors. The scoring system was adapted from previous studies: PD-L1: negative: <1%, +: 1–49% and ++: ≥50% [23]. The degree of T cell infiltration was graded

as low if ≤5 T cells/HPF or high if >5 T cells/HPF. PD-1 expression was assessed on T cells and was considered positive if membranous staining was present.

MLH1 promoter methylation assay

Since the loss of MLH1 and PMS2 expression is commonly caused by somatic promoter hypermethylation of *MLH1*, *MLH1* promoter status was analyzed in MLH1/PMS2 negative cases using methylation specific PCR. Briefly, using the EZ DNA methylation Gold kit (Zymo Research, Orange, US) bisulfite conversion of tumor DNA was performed. Bisulfite-converted DNA was amplified using specific methylated and unmethylated primers in a PCR reaction, as described previously [24, 25].

Microsatellite instability (MSI) analysis

MSI analysis was performed using MSI analysis system, version 1.2 (Promega), according to the manufacturer's instructions. In short, PCR using five MSI Markers (BAT26-, BAT-25, NR-24, NR21, MONO-27) was performed and PCR products were analyzed using the SeqStudio genetic analyzer (ThermoFisher, Waltham, Massachusetts, U.S.). Samples were classified as microsatellite stable (MSS) if none of the markers were altered, MSI-Low if 1 out of 5 markers was unstable and MSI-High if ≥2 out of 5 markers were unstable.

Literature search

A Pubmed search matching the terms of HNPCC, Lynch syndrome, mismatch repair deficiency, microsatellite instability and sarcoma(s), soft tissue tumor(s), bone tumor(s) was conducted. Studies were included if the full text was available and if reference to an internal control was made in case no expression of MMR proteins detected in tumor cells.

Results

Index cases

The first index patient was a 55-year-old male presenting with a pleomorphic rhabdomyosarcoma in the lower extremity (**Figure 1**). Subsequently, he developed a pancreatic adenocarcinoma at the age of 60 and two years later an urothelial carcinoma of the ureter. He was referred to the clinical geneticist, where a germline mutation in *MSH2* (p.Cys697Tyr) was found. The second index patient involved a male of 42 years presenting with a leiomyosarcoma of the psoas (**Figure 2**). Seven years later, the patient developed acute myeloid leukemia, a sebaceous gland carcinoma and adenocarcinoma of the coecum. Although no mutation analysis was performed, the leiomyosarcoma showed a MSI-high phenotype (instability of three out of five microsatellite markers) and the coecum tumor a MSI-low phenotype

(instability of one maker). Combined with the loss of MSH2/MSH6 expression, it is highly suspicious that this patient developed diverse tumors in the context of Muir-Torre syndrome, a variant of Lynch syndrome.

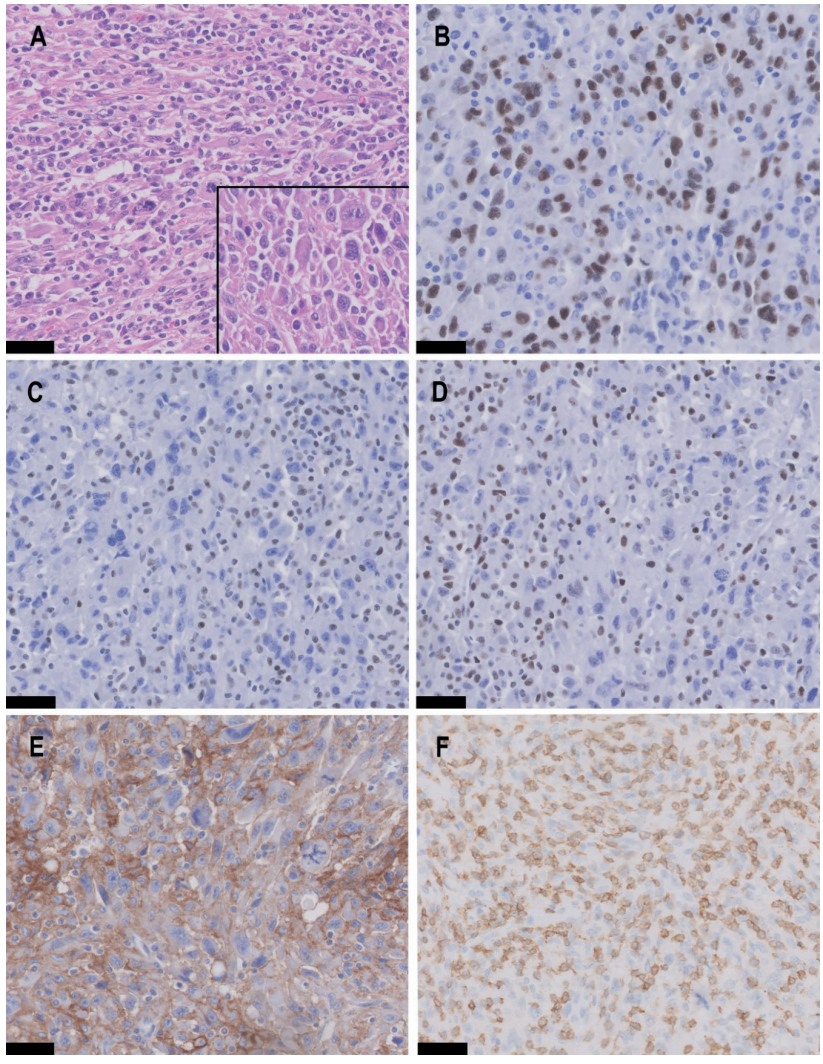

Figure 1. Pleomorphic rhabdomyosarcoma of first index patient with a *MSH2* germline mutation. H&E staining showing numerous lymphocytes intermingled between tumor cells. Cells are pleomorphic with enlarged nuclei, prominent nucleoli and surrounded by abundant eosinophilic cytoplasm, resembling rhabdomyoblasts (insert)(**A**). Immunohistochemistry for MyoD1 confirms skeletal muscle differentiation (**B**). Loss of expression of MSH2 (**C**) and MSH6 (**D**) is seen in tumor cells, while expression in immune and stromal cells is retained. Expression of PD-L1 is seen on tumors cells (**E**). Note the abundance of T cells in the CD3 immunohistochemical detection (**F**). Scale bar: 50µm.

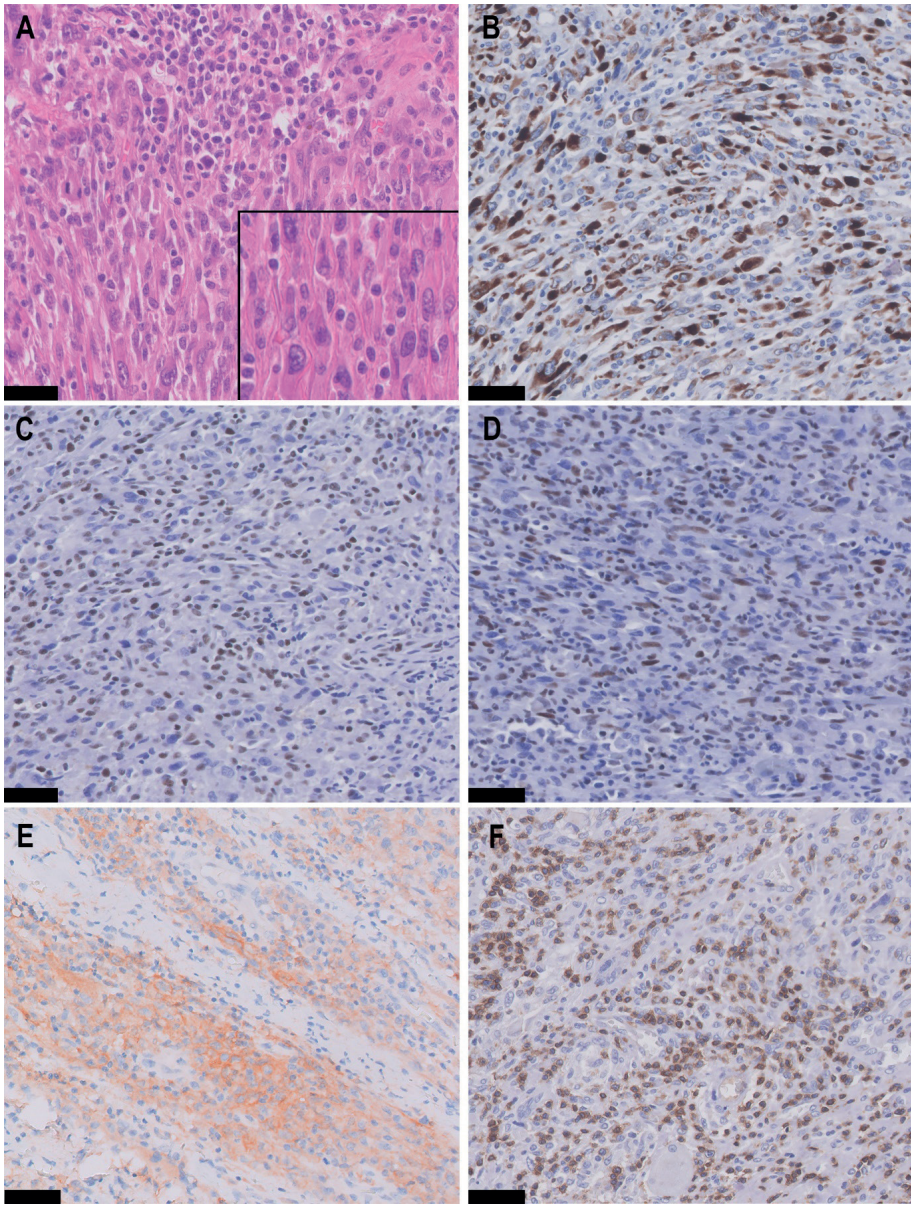

Figure 2. Leiomyosarcoma of second index patient. H&E staining showing a prominent lymphocytic infiltrate between tumor cells. The tumor is arranged in long bundles of spindle cells. Nuclei are enlarged, ovoid to spindled and surrounded by bipolar eosinophilic cytoplasm (insert) (**A**). Smooth muscle differentiation is confirmed by positivity for desmin (**B**). Loss of expression of MSH2 (**C**) and MSH6 (**D**) is seen, while expression of MLH1 and PMS2 is retained (not shown). Positivity for PD-L1 is seen on tumor cells (**E**). Numerous T cells are scattered throughout the tumor (CD3 staining) (**F**). Scale bar: 50µm.

Protein expression

The index cases showed loss of expression of both MSH2 and MSH6, while MLH1 and PMS2 were retained (**Figure 1** and **2**). In addition, a total of six other tumors (three leiomyosarcomas, one embryonal rhabdomyosarcoma, one MPNST and one radiation-associated soft tissue sarcoma) showed loss of expression of one or more MMR proteins, leading to a total of eight cases with potential MMR defects (1%) (**Table 1**). Loss of MLH1 and PMS2 was seen in three cases (two leiomyosarcomas and one radiation-associated sarcoma), loss of MSH2 and MSH6 was present in one embryonal rhabdomyosarcoma and one MPNST. Isolated loss of PMS2 was seen in one leiomyosarcoma (**Table 2**).

In the remaining 786 bone and soft tissue tumors, no loss of expression was observed (**Table 1**). Five out of eight tumors with loss of MMR protein expression displayed expression of PD-L1 and a high influx of T cells. In two of these cases expression of PD-1 was observed. Among the three PD-L1 negative tumors, the majority showed a low amount of tumor-infiltrating T cells (**Table 2**).

Clinical and genotypic analysis

In addition to the index cases, molecular information was available for one other leiomyosarcoma, which showed a *MLH1* mutation (p.Val7Argfs*18) in the tumor sample. Clinical data of this patient revealed a breast tumor and a rectal carcinoma. The patient was referred to the clinical genetics, though additional information could not be retrieved. Among the other MMR deficient sarcoma patients, one was known with neurofibromatosis type 1 and one developed adenocarcinoma of the prostate, while the remaining patients had no other tumors. The MMR deficient radiation-associated sarcoma occurred 10 years after radiation therapy of a liposarcoma. None of the examined MLH1 negative tumors (n=3) showed *MLH1* promoter hypermethylation. MSI analysis revealed one microsatellite stable tumor, while analysis failed on the remaining tumors due to insufficient quality of the DNA (**Table 2**).

Mismatch repair deficient bone and soft tissue sarcomas in literature

A total of 30 MMR deficient bone and soft tissue sarcomas were encountered in literature (details are summarized in **Table 3**). Histologically classifiable tumors included liposarcoma (n=5), osteosarcoma (n=5), rhabdomyosarcoma (n=4), alveolar soft part sarcoma (n=3), clear cell sarcoma (n=2), leiomyosarcoma (n=1), PEComa (n=1). Undifferentiated pleomorphic/unclassifiable sarcoma accounted for eight cases and in one cases the subtype was not specified [4, 26-42]. While most studies referred to case reports or case series, Doyle and colleagues investigated the frequency of MMR deficiency in a cohort of 279 cases and identified 6 MMR deficient cases (2%) [26]. In all these studies, seventeen patients had a germline mutation in one of the mismatch repair genes (Lynch syndrome n=13; Muir-Torre syndrome

n=2; Constitutional Mismatch Repair Deficiency n=2). A predominance of *MSH2* mutations, either germline of somatic, was found in MMR deficient sarcomas.

Table 1. Mismatch repair deficiency in bone and soft tissue tumors.

Tumor type	n	MMRd	%
Enchondroma	11	0/8	0
Osteochondroma	9	0/5	0
Chondrosarcoma	206	0/181	0
Subtypes: Conventional	137	0/118	0
Dedifferentiated	28	0/26	0
Clear cell	20	0/18	0
Mesenchymal	21	0/19	0
Osteosarcoma	67	0/65	0
Angiosarcoma of bone	37	0/24	0
Ewing sarcoma	19	0/18	0
Radiation-associated bone sarcoma	4	0/3	0
Schwannoma	22	0/22	0
Neurofibroma	10	0/10	0
Inflammatory myofibroblastic tumor	33	0/29	0
Liposarcoma	104	0/101	0
Subtypes: Myxoid	49	0/48	0
Dedifferentiated	31	0/30	0
Well differentiated	13	0/12	0
Pleomorphic	11	0/11	0
Leiomyosarcoma	88	4/88	5
Synovial sarcoma	69	0/65	0
Alveolar soft part sarcoma	59	0/31	0
Malignant peripheral nerve sheath tumor	35	1/32	3
Rhabdomyosarcoma	37	2/33	6
Subtypes: Alveolar	22	0/18	0
Pleomorphic	3	1/3	33
Embryonal	4	1/4	25
Spindle cell	6	0/6	0
NOS	2	0/2	0
Undifferentiated soft tissue sarcoma	29	0/29	0
Angiosarcoma of soft tissue	23	0/19	0
Myxofibrosarcoma	17	0/17	0
Radiation-associated soft tissue sarcoma	15	1/14	7

MMRd, mismatch repair deficient; NOS, not otherwise specified

Table 2. Summary of immunohistochemical and molecular analysis of MMRd cases.

Histology	Grade*	MSH2	MSH6	MLH1	PMS2	Molecular data	PD-1 (%)	PD-L1 (%)	T cells/ HPF
Pleomorphic rhabdomyosarcoma	N/A	-	-	+	+	*MSH2* p.Cys697Tyr	64	80	140
Embryonal rhabdomyosarcoma	N/A	-	-	+	+	NA	-	40	50
Leiomyosarcoma	1	-	-	+	+	MSI-High	23	40	47
Leiomyosarcoma	1	+	+	-	-	*MLH1* p.Val7Argfs*18	-	90	60
Leiomyosarcoma	1	+	+	-	-	failed	-	-	<5
Leiomyosarcoma	1	+	+	+	-	MSS	-	-	<5
Radiation-associated sarcoma	N/A	+	weak	-	-	failed	-	-	<5
MPNST	N/A	-	-	+	+	NA	-	5	18

*, grading according to FNCLCC; HPF, high-power field; het, heterogenous; +, positive; -, negative; N/A, not applicable; NA, not assessed; MPNST, malignant peripheral nerve sheath tumor

Table 3. Overview of mismatch repair deficient bone and soft tissue sarcoma published in the literature

Authors	Year	Sarcoma	Associated tumors/syndrome	MMR loss IHC on sarcoma	genotypic analysis
De Angelis de Carvalho, et al	2020	Liposaroma Osteosarcoma Osteosarcoma	Lynch syndrome Lynch syndrome	MSH2 and MSH6 MSH2 and MSH6	*MSH2* c.2152C>T *MSH2* c.1661+1G>A
Doyle L, et al	2019	PEComa	NA	MSH2 and MSH6	*MSH2* copy deletion
		Rhabdomyosarcoma	Lynch syndrome	MSH2 and MSH6	*MSH2* Y678*
		UPS	NA	MSH2 and MSH6	*MSH2* R389*
		Undifferentiated sarcoma	NA	PMS2	*PMS2* R315*
		Undifferentiated sarcoma	NA	MSH6	*MSH6* F1088Sfs*2
Kim S, et al	2017	Sarcoma NOS	NA	MSH2 or MLH1	NA
Daou B, et al	2015	Osteosarcoma	CMMRD		*PMS2* c.400C>T
			colorectal adenocarcinoma		*PMS2* c.1579del
			anaplastic ganglioglioma		
			acute myeloid leukemia		
Cranmer L, et al	2013	Pleomorphic rhabdomyosarcoma	Lynch syndrome	MLH1 and PMS2	*PMS2* G857A
			colorectal adenocarcinoma		
Lee N, et al	2013	UPS	cutaneous sebaceous tumor	MSH2	NA
			Muir-Torre syndrome		
Yozu M, et al	2013	Pleomorphic liposarcoma	colorectal cancer	MSH2 and MSH6	*MSH2* mutation
			sebaceous neoplasm		
			Muir-Torre syndrome		

Authors	Year	Sarcoma	Associated tumors/syndrome	MMR loss IHC on sarcoma	genotypic analysis
Urso E, et al	2012	Leiomyosarcoma	Lynch syndrome	MSH2 and MSH6	*MSH2*; deletion of exon 1-16
			mucinous adenocarcinoma colon		
			kidney cancer		
Ahmed H, et al	2012	Osteosarcoma	Invasive duct carcinoma	NA	*MSH2* mutation and *MLH1* mutation
Brieger A, et al	2011	UPS	Lynch syndrome	MSH2	*MSH2* c.2038C>T
			prostate cancer		
			gliosarcoma		
		UPS	Lynch syndrome	MSH2	*MSH2* c.942 + 3A>T
			breast cancer		
			cervix carcinoma		
Kratz CP, et al	2009	Embryonal Rhabdomyosarcoma	CMMRD	NA	*PMS2* p.Cys73*
			Adenocarcinoma colon		
		Undifferentiated sarcoma	anaplastic astrocytoma	PMS2	NA
Nilbert M, et al	2009	Liposarcoma	Lynch syndrome	MSH2 and MSH6	*MSH2* c.942 + 3A>T
		Liposarcoma	Lynch syndrome	MSH2 and MSH6	*MSH2* c.1-?_366 + ?del
Hirata K, et al	2006	Liposarcoma	Lynch syndrome	MSH2	AT deletion at codon 677 in exon 13 of *MSH2*
Garcia J, et al	2006	Clear cell sarcoma		MSH6	NA
		Clear cell sarcoma		MSH6	NA

Authors	Year	Sarcoma	Associated tumors/syndrome	MMR loss IHC on sarcoma	genotypic analysis
Lynch HT, et al	2003	Osteosarcoma	Lynch syndrome rectal carcinoma	NA	*MSH2* mutation in exon 4
den Bakker MA, et al	2003	Pleomorphic Rhabdomyosarcoma	Lynch syndrome	MSH2	*MSH2* mutation
Saito T, et al	2003	ASPS (n=3)		MSH2 and MLH1 in 2 cases MLH1 in one case	NA
Sijmons R, et al	2000	UPS	Lynch syndrome	MSH6	*MSH6* mutation

ASPS; alveolar soft part sarcoma, CMMRD, constitutional mismatch repair deficiency; UPS, undifferentiated pleomorphic sarcoma; NOS, not otherwise specified; LMS, leiomyosarcoma; NA, not available

Discussion

This study provides a comprehensive immunohistochemical evaluation of MMR protein expression in a large series of bone and soft tissue tumors. We show that MMR deficiency is a rare phenomenon in bone and soft tissue tumors but can be relatively more frequent in soft tissue sarcomas with myogenic differentiation and in patients with a genetic predisposition / co-occurrence of other malignancies.

MMR deficiency was detected in 1% of the total bone and soft tissue tumor cohort and was enriched to up to 5% in tumors with myogenic differentiation. The only non-myogenic MMR deficient tumors were a radiation-associated bone sarcoma and a MPNST. Notably, MMR deficiency was completely absent in a relatively large series of osteosarcomas and chondrosarcomas. Among the MMR deficient tumors, three patients were suspected to have or had an established diagnosis of Lynch syndrome / Muir-Torre syndrome. Our findings are in keeping with the study of Doyle *et al*., who also reported an overall frequency of 2% but a marked enrichment (10%) among undifferentiated/unclassifiable sarcomas using parallel sequencing followed by immunohistochemical evaluation of MMR protein expression. The fact that the frequency of MMR deficiency is comparable between their study, starting with an NGS approach, and the present study, starting with immunohistochemistry, suggests that immunohistochemistry could serve as a cost-effective surrogate marker for MMR deficiency.

The current study includes a relatively large cohort of bone sarcomas, including osteogenic, chondrogenic tumors and Ewing sarcoma, thereby representing the three most common bone sarcomas. Among the soft tissue sarcomas, also the most common subtypes are included (liposarcoma, leiomyosarcoma and undifferentiated soft tissue sarcoma). However, given the high amount of sarcoma subtypes it is not possible to evaluate a completely representative cohort. In addition, some tumor types are overrepresented, including those with myogenic differentiation (leiomyosarcoma, rhabdomyosarcoma and inflammatory myofibroblastic tumor), which was based on the myogenic differentiation in the tumors of our two index patients. In addition, we included a series of alveolar soft part sarcomas which was based on data from literature. In contrast to our findings and those from Doyle *et al*., two other groups reported a higher frequency of MMR deficiency varying between 23% and 85% in soft tissue sarcoma and osteosarcoma, respectively [43, 44]. In our series, none of the 65 osteosarcomas investigated demonstrated loss of MMR protein expression. Since MMR deficient sarcomas often show a significantly elevated TMB relative to MMR proficient sarcomas [45], and the TMB in osteosarcoma is reportedly low [46], with low to moderate response to ICI [46-48], it seems very unlikely that the majority of osteosarcomas would be MMR deficient. Given the lack of reporting on a positive internal control and the lack of molecular validation in these publications, these cases were not taken along in Table 3.

This is the first systematic analysis of MMR deficiency in cartilaginous tumors, which showed complete absence of MMR deficiency in 181 patients. Based on this specific biomarker, these patients would not be eligible to ICI therapy. We previously also showed the absence of PD-L1 expression in conventional, clear cell and mesenchymal chondrosarcoma. However, PD-L1 expression and the presence of an immune infiltrate were found in 52% of the dedifferentiated chondrosarcomas, which were also included in the current study, and PD-L1 expression was restricted to the dedifferentiated component [23]. Response to immunotherapy in clinical trials was observed in few (dedifferentiated) chondrosarcoma patients [47, 49]. This again illustrates that in the current era of immunotherapy, with the lack of definitive biomarkers, evaluation of tumors based on both their immune phenotype and genomic mutation profile is needed to determine which patients would likely be responsive to ICI treatment.

For alveolar soft part sarcoma, loss of expression of MSH2 and MLH1 was previously reported in two (18.2%) and three (27.3%) of eleven cases, respectively [40]. Hypermethylation of *MSH2* and *MLH1* promoter region was absent, but three of eight (37.5%) cases were found to be MSI-low. Moreover, alveolar soft part sarcoma, despite a low mutational load and lack of inflammatory infiltrate, was observed to be able to respond to immune checkpoint inhibitors [50]. Two patients with sustained partial response showed a MMR mutational signature after sequencing, however, staining for MMR protein expression was intact [51]. This led us to include a relatively large series of this very rare sarcoma subtype in our studies, as TMAs were previously constructed and available from the EORTC-CREATE study [22, 52]. We did not find loss of MMR protein expression in 31 evaluable cases. Thus, we cannot confirm previous results of MMR deficiency in alveolar soft part sarcoma, and other mechanisms underlying sensitivity to immune checkpoint inhibitors in these tumors seem more likely.

Despite the selection bias in our cohort, both tumors of the index patients demonstrated myogenic differentiation, most of the other MMR deficient sarcomas also displayed myogenic differentiation. Notably, all MMR deficient leiomyosarcoma were low-grade (grade 1). Since leiomyosarcomas often show a poor response to chemotherapy, it would be worthwhile to examine MMR status in this selected tumor group, ultimately providing these patients novel treatment options. Moreover, we previously showed PD-L1 expression together with high T cell infiltrate and HLA class I expression in around 30% of high grade leiomyosarcoma, reflecting an active immune microenvironment [53]. Thus far, results of clinical trials of PD-1 blockade therapy in leiomyosarcoma patients are diverse. Single reports with successful treatment or a mixed partial response or stable disease are described, while others report no effect to treatment [5, 49, 54, 55].

Of note, one of the leiomyosarcomas with loss of PMS2 expression showed a microsatellite stable phenotype. Although the MSI analysis kit is commonly used in

colorectal cancer, it is not widely applicable in other tumors. In addition, concordance between MMR protein expression and MSI is variable between tumor types with percentages varying between 68% in epithelial ovarian tumors to 97% in colorectal carcinomas [56]. However, no data is available for sarcoma. It would be highly interesting to see whether this patient is carrying a germline variant in one of the mismatch repair genes. However, germline analysis was not covered by the IRB approval.

Most of the MMR deficient bone and soft tissue sarcomas in the current study showed presence of infiltrating immune cells and five cases also showed expression of PD-L1 on the tumor cells. This may indicate that these patients could benefit from ICI. Thus far, effectiveness of ICI in sarcoma patients has only been studied in limited trials with variable results. In the SARC028 study, Pembrolizumab showed promising results in patients with undifferentiated pleomorphic sarcoma and dedifferentiated liposarcoma, while in the PEMBROSARC and Alliance *A091401* trial no response was observed. Also, PD-L1 expression alone was not a predictive biomarker [47, 48, 57]. Clearly, there is an urgent need for predictive biomarkers, and it remains to be answered if the MMR status contributes to the selection of patients who will respond to ICI.

To conclude, MMR deficiency is rare in bone and soft tissue tumors. Screening focusing on tumors with myogenic differentiation, undifferentiated/unclassifiable sarcomas and in patients with a genetic predisposition / co-occurrence of other malignancies can be helpful identifying patients potentially eligible for ICI, while for other bone and soft tissue tumors reflex testing remains debatable.

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Author contributions

The study was designed, written and reviewed by S.W. Lam, M. Kostine and J.V.M.G. Bovée. All authors contributed to the data collection, data analysis and interpretation. The manuscript was approved by all authors.

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Supplementary Table

Supplementary Table 1. Details of antibody

Antibody	Clone	Company	AR	Dilution
MSH6	EPR3945	Gene tech	Tris-EDTA	1/3200
PMS2	EP51	DAKO	Tris-EDTA	1/50
MLH1	ES05	DAKO	Tris-EDTA	1/100
MSH2	FE11	DAKO	Tris-EDTA	1/100
PD-L1	E1L3N	Cell Signaling	Tris-EDTA	1/400
PD-1	polyclonal	R&D systems	citrate	1/40
CD3	A0452	DAKO	Tris-EDTA	1/400

AR, antigen retrieval;