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Absence of Microsatellite Instability In Soft Tissue Sarcomas

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Key Words
Soft tissue sarcomas · Microsatellite instability · DNA repair proteins

Abstract
Objective: Here, we analyze a series of soft tissue sarcomas (STS), which are a heterogeneous group of mesenchymal neoplasms, for the presence and frequency of microsatellite instability (MSI). MSI has been proposed to be clinically relevant for colorectal cancer, yet on STS its role is not consensual, partly due to the limited number of cases analyzed and methodology issues. ***Methods:*** The detailed evaluation of MSI in tumor samples from 71 STS patients was performed by pentaplex PCR of the MSI markers NR-27, NR-21, NR-24, BAT-25, and BAT-26, followed by capillary electrophoresis. The expression of DNA mismatch repair (MMR) proteins (MLH1, MSH2, MSH6, and PMS2) was also evaluated in suspected MSI-positive cases by immunohistochemistry. ***Results:*** The MSI analysis showed instability of one MSI marker in a total of 3 cases (4.2%). However, MMR protein expression was not affected, demonstrating that all cases were microsatellite stable. ***Conclusion:*** Our results suggest that MSI does not play a role in STS tumorigenesis.

Introduction

Soft tissue sarcomas (STS) are a heterogeneous group of mesenchymal neoplasms with distinct histological characteristics that are classified according to the adult tissue they resemble [1–4]. Although STS are relatively rare, accounting for only 0.7% of adult malignancies and approximately 6.5% of childhood cancers, effective treatment options remain limited, particularly for advanced disease [1, 5]. In 2014, 12,020 new STS are expected to be diagnosed in the USA, with 4,740 predicted deaths [6].

STS can occur anywhere in the body but they are most common in the extremities (75%), followed by the trunk (10%) and the retroperitoneum (10%) [4]. Histologically, around 75% of STS are classified as undifferentiated pleo-

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morphic sarcoma (previously denominated by malignant fibrous histiocytoma), liposarcoma, leiomyosarcoma, myxofibrosarcoma, synovial sarcoma, or malignant peripheral nerve sheath tumors [4]. The most common STS subtype in childrens in rhabdomyosarcoma [4]. Despite the different types, they share many clinical and pathological features. The current American Joint Committee on Cancer (AJCC) has established a grading system for STS based on the histological grade, tumor size, and the presence of nodal or distant metastases [4, 7].

STSs are generally locally aggressive and have a high risk of recurrence and invasive behavior leading to metastasis [1]. Most STS metastasize hematogenously, most commonly to the lung parenchyma both at presentation and at recurrence [4]. The principal treatment for STS is surgery, with or without radiotherapy [3]. This can cure localized disease in approximately 50% of patients, but within 2–3 years following diagnosis the remaining 50% of patients develop metastases [8]. Although several chemotherapy regimens are used for the treatment of metastatic disease, there remains a poor median survival rate of 8–12 months, which has shown little improvement in the last 20 years [8]. Therefore, uncovering the molecular causes of STS could lead to better disease management and/or the development of much needed new therapies.

Genetically, some events have recently gained attention in sarcomas; one of these is the microsatellite instability (MSI) phenotype. MSI was first described in tumors associated with hereditary nonpolyposis colorectal cancer (or Lynch syndrome) [9]. It arises in short repetitive DNA sequences (or microsatellites) due to defects in the DNA mismatch repair (MMR) system, which normally recognizes and repairs errors that occur during DNA replication, as well as repairing some forms of DNA damage [10, 11]. MSI is defined as altered lengths of repetitive sequences in tumor DNA compared to corresponding normal DNA [10, 11]. The presence of MSI in some tumor types, particularly in colorectal cancer, appears to be related to specific clinical and histopathological features, including location, tumor lymphocyte infiltration, tumor differentiation, frequency of distant metastasis, and prognosis [12]. Moreover, MSI-positive tumors are prone to accumulate mutations in other genes that harbor microsatellite regions in their coding or regulatory regions, known as MSI target genes [13, 14].

Currently, the determination of MSI phenotype in colorectal cancer tumors can help to predict the therapy response and the prognosis of the patients [12, 15]. On the other hand, in STS, there are no reliable molecular markers that can predict the prognosis of the STS patients [16]. Because of this and considering the poor characterization of the MSI phenotype, due in part to insufficient and controversial results [17–20], in this work we assessed this phenomenon using accurate MSI methodology, correlating it with loss of MMR protein expression in a series of Brazilian STS.

Materials and Methods

Subjects

We analyzed 71 patients with the diagnosis of STS submitted for surgery at the Barretos Cancer Hospital. Relevant clinical-pathological data available included patient's age, race and gender, tumor localization, diagnostic and grade (according to the French Federation of Cancer Centers Sarcoma Group (FNCLCC)) [7], disease progression, presence of pleomorphic cells, presence of metastasis, and disease recurrence, as specified in table 1. This study was approved by the local ethics committee (CEP-331/2010).

DNA Isolation

Paraffin-embedded tumor samples from 71 patients were retrieved from the Pathology Department of Barretos Cancer Hospital. The tissues were deparaffinized by a serial extraction with xylene and ethanol, and selected areas of tumor were macrodissected using a sterile needle. DNA was isolated using the QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions and as previously described by our group [21]. The quality and concentration of DNA were measured using a NanoDrop 2000 U-Vis Spectrophotometer, followed by storage at –20°C until molecular analysis.

The DNA from the HCT-15 cancer cell line was extracted using Trizol reagent (Life Technologies, Gaithersburg, Md., USA), following the manufacturer's protocol. HCT-15 was the positive MSI-high (MSI-H) control in all MSI analysis.

MSI Analysis

The MSI evaluation was performed using multiplex PCR for five quasimonomorphic mononucleotide repeat markers (NR-27, NR-21, NR-24, BAT-25, and BAT-26) previously described [22–25]. Briefly, each antisense primer was end-labeled with a fluorescent dye, as follows: FAM (6-carboxyfluorescein) for BAT-26 and NR-21, VIC (2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein) for BAT-25 and NR-27 and NED (2,7,8-benzo-5-fluoro-2,4,7-trichloro-5-carboxyfluorescein) for NR-24. PCR was performed using the Qiagen Multiplex PCR Kit (Qiagen) with 0.5 μl of DNA at 50 ng/μl and the five markers were coamplified in a standard multiplex PCR (denaturation at 95°C for 15 min and 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 90 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 40 min). PCR products were then submitted to capillary electrophoresis on an ABI 3500 XL Genetic Analyzer (Applied Biosystems, Carlsbad, Calif., USA), according to the manufacturer's instructions, and the results were analyzed using GeneMapper v4.1 software (Applied Biosystems).

Table 1. Clinical-pathological features of STS patients

Variable	n	%
Age, years		
>51	41	57.7
≤51	30	42.3
Gender		
Female	27	38.6
Male	43	61.4
Race		
Caucasian	52	74.3
Not Caucasian	18	25.7
Location		
Inferior limb	51	71.8
Superior limb	20	28.2
Diagnostic		
Leiomyosarcoma	12	16.9
Pleomorphic leiomyosarcoma	3	4.3
Round cells/myxoid liposarcoma	4	5.6
Myxoid liposarcoma	5	7.1
Round-cell liposarcoma	2	2.8
High-grade undifferentiated pleomorphic sarcoma	11	15.5
Monophasic fibrous synovial sarcoma	6	8.5
High-grade myxofibrosarcoma	5	7.0
Malignant peripheral nerve sheath tumor	7	9.9
Low-grade fibromyxoid sarcoma	1	1.4
Poorly differentiated synovial sarcoma	2	2.8
High-grade myofibroblastic sarcoma	2	2.8
Clear cell sarcoma	1	1.4
Biphasic synovial sarcoma	1	1.4
Fibrosarcoma	1	1.4
Angiomatoid histiocytoma	1	1.4
Well-differentiated liposarcoma	1	1.4
Undifferentiated liposarcoma	1	1.4
Pleomorphic liposarcoma	1	1.4
Low-grade myofibroblastic sarcoma	1	1.4
Giant-cell-rich high-grade undifferentiated pleomorphic sarcoma	1	1.4
Malignant solitary fibrous tumor	1	1.4
Alveolar sarcoma	1	1.4
Cellular type		
Absence of pleomorphic cells	39	56.5
Presence of pleomorphic cells	30	43.5
Grade		
Low grade (I)	10	14.3
High grade (II or III)	60	85.7
Disease progression		
No	21	31.8
Yes	45	68.2
Disease recurrence		
Absent	39	56.5
Present	30	43.5
Metastasis		
Absent	34	49.3
Present	35	50.7

DNA from the cell line HCT-15 (MSI-H) was used as a positive control for MSI. In a recent study, our group determined the quasimonomorphic variation range of each marker for the Brazilian population [25]. Accordingly, samples were considered MSI-H when two or more markers were altered and MSI-low (MSI-L) when only one marker was altered, with further validation by MSI analysis of normal tissue or immunohistochemistry of the MMR enzymes [25].

MMR Immunohistochemistry

Formalin-fixed paraffin-embedded tissue specimens were cut into 4-μm sections for immunohistochemistry using the avidin-biotin peroxidase complex method with the Dako EnVision™ FLEX detection system Kit (Dako) and the Autostainer Link 48 equipment (Dako), in accordance with the manufacturer's instructions. The antigen retrieval process was performed at 97°C for 20 min (pH = 9.0). Endogenous peroxidases were blocked with EnVision FLEX peroxidase-blocking reagent. The primary antibodies (rabbit polyclonal) used in this study were the following: anti-MLH1 (dilution 1:100, clone G168-728, Cellmark); anti-MSH2 (dilution 1:100, clone G219-1129, Cellmark); anti-PMS2 (dilution 1:25, clone MRH-28, Cellmark), and anti-MSH6 (dilution 1:600, clone 44, Cellmark). The DAB solution was used for visualization. Slides were counterstained with hematoxylin. Nuclear immunostaining of normal epithelial cells, lymphocytes and stromal cells served as internal positive controls in each case, and colorectal cancer cases known to exhibit an MSI-H phenotype and known to lack MLH1 and MSH2 served as negative controls [26]. All cases were quantitatively scored as positive (defined as >10% of stained nuclei of tumor cells) or negative (<10% of stained nuclei of tumor cells), as previously described [27, 28]. Tumors negative for any of the MMR proteins were classified as MMR-deficient, whereas tumors positive for all MMR proteins were considered MMR-proficient [26, 27].

Results

In 3 out of 71 patients (4.2%), we observed one altered MSI marker (BAT-26) (fig. 1; table 2). Of these 3 patients, 2 were male and 1 was female, with the diagnoses of leiomyosarcoma, pleomorphic leiomyosarcoma and high-grade undifferentiated pleomorphic sarcoma, and all exhibited a high grade histologically (table 2). Since we previously reported that the presence of instability in one marker in the Brazilian population can be due to polymorphic variants associated with African ethnic background [25], we further analyzed these subjects by MMR immunohistochemistry to accurately determine their MSI status. In the 3 cases that exhibited instability in one marker, we detected positivity, with strong immunostaining, for all MMR proteins analyzed (MLH1, MSH2, MSH6, and PMS2; fig. 2), suggesting that the MMR pathway was active. Thus, these findings indicate that all 3 cases were microsatellite stable.

Table 2. Clinical-pathological and molecular features of the 3 cases with instability in one marker

Case	Clinical-pathological data					MSI					MMR			
	age, years	gender	location	diagnostic	grade	NR-27	NR-21	NR-24	BAT-25	BAT-26	MLH1	MSH2	MSH6	PMS2
E093102D2	46	M	SL	HUPS	III	86/87	106/106	123/123	145/146	166*/178	+	+	+	+
E1022014	41	F	IL	PL	III	86/86	106/106	122/122	145/147	166*/176	+	+	+	+
E077056	75	M	SL	L	II	85/86	105/105	123/123	145/145	166*/177	+	+	+	+

* Marker altered. SL = Superior limb; IL = inferior limb; HUPS = high-grade undifferentiated pleomorphic sarcoma; PL = pleomorphic leiomyosarcoma; L = leiomyosarcoma.

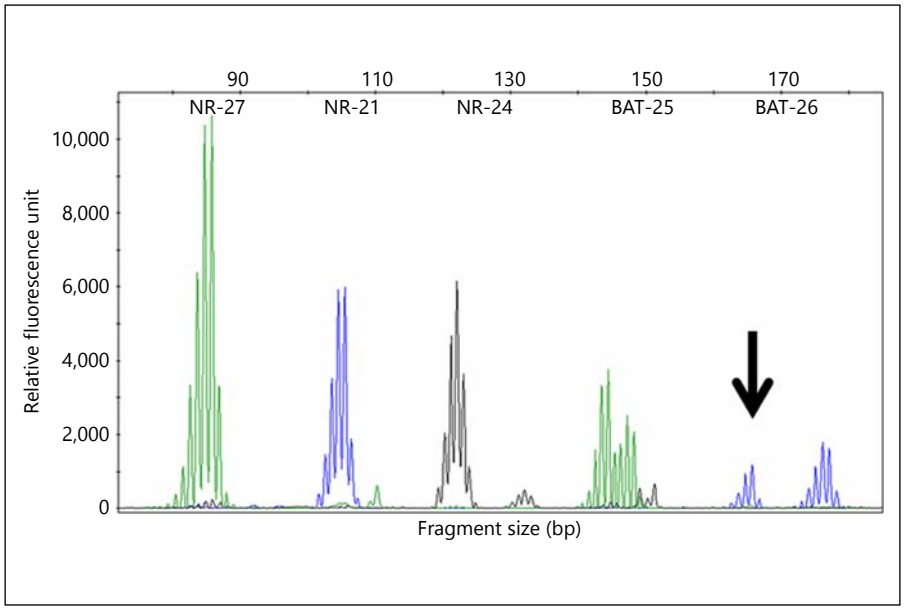

Fig. 1. Fragment analysis of 1 case with instability (deletion of 8 base pairs) in BAT-26 marker, as indicated by the arrow.

Discussion

Genetically, several genes have been associated with STS tumorigenesis. The gene amplification of the oncogenes *MDM2*, *N-MYC*, *ERBB2*, and mutations of members of the *RAS* family are implicated both in the development of STS and with adverse outcomes in STS patients [3, 29]. Additionally, mutations in *TP53* were observed in 25.6% of STS [30]. Recently, mutations in the *TERT* gene promoter have also been reported [31]. Despite these findings, there are no effective molecular markers that can predict the prognosis of STS patients.

MSI testing has been used in the discrimination of prognosis in colorectal cancer patients, predicting the therapy response and specific clinical and histopathological features such as tumor location, lymphocyte infiltration, tumor differentiation, and frequency of distant metastasis [12]. In STS the presence and clinical implication of MSI is largely unknown.

In the present study we analyzed 71 STS cases, using both molecular and immunohistochemistry approaches – the largest study so far to evaluate MSI status using molecular techniques. Sophisticated PCR and capillary electrophoresis techniques with appropriated controls were used and showed no evidence of MSI in these tumors.

The Bethesda panel for MSI evaluation includes five microsatellite loci: two mononucleotides (BAT-25 and BAT-26) and three dinucleotides (D5S346, D2S123 and D17S250) [22]. A following updated international con-

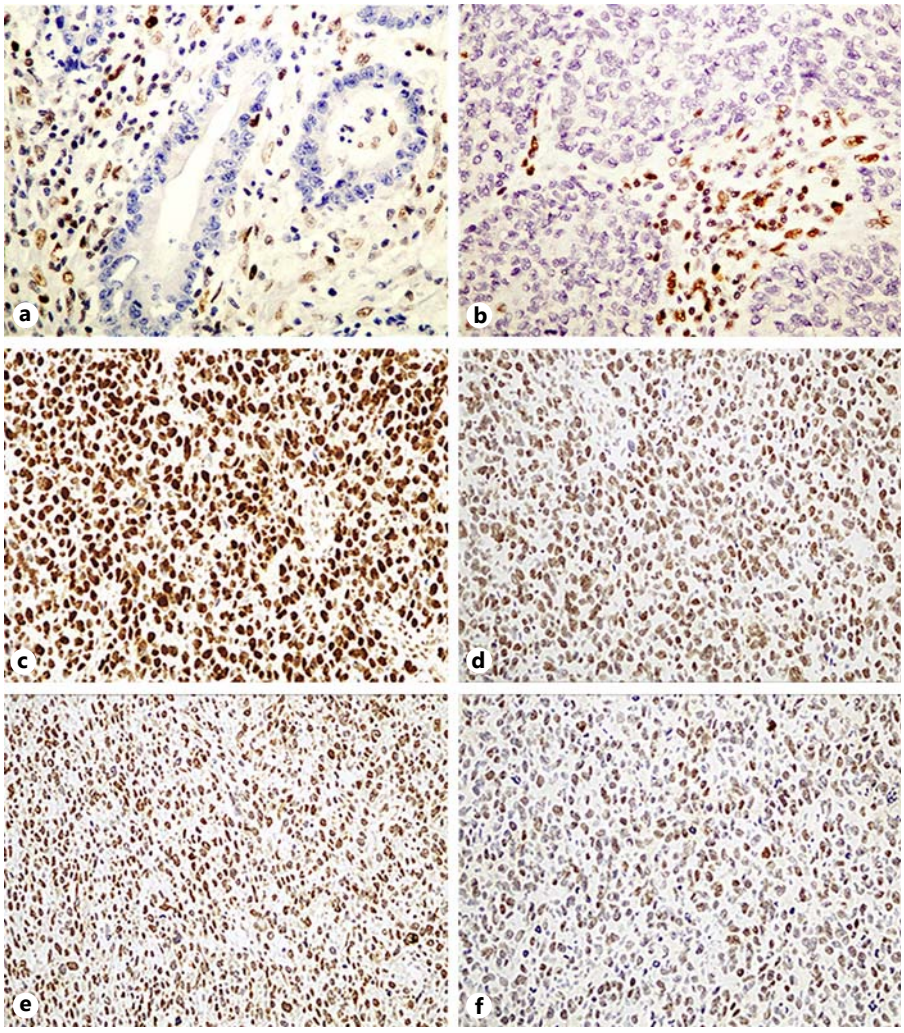

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Fig. 2. Immunohistochemistry of MMR proteins in 1 case with instability in one marker. **a** Negative control to MLH1. **b** Negative control to MSH2. **c** Expression of MLH1. **d** MSH2. **e** MSH6. **f** PMS2. Amplification ×200.

sensus meeting held in December 2002 reevaluated this panel [23] and concluded that there were caveats in its use, and it was recommended that dinucleotide repeats be substituted by mononucleotide repeats [23, 32]. In the present study we used the newly recommended mononucleotide repeats, which were recently validated by our group for the Brazilian population [25].

The MSI phenotype in STS is poorly characterized, and published results are contradictory (table 3). The first study analyzing MSI in STS was reported in 1994, and the authors observed MSI in 11% of cases (2/18) [17]. Using similar methodologies, Rucinska et al. [18] reported the absence of MSI in 8 cases of low-grade STS and the presence of MSI in 8 high-grade STS cases. The authors found 4 cases with one altered MSI marker, 3 cases with two and 1 case with three altered markers. Furthermore, Kawaguchi et al. [19] studied 40 STS cases and described 25% of the tumors with the MSI phenotype – 2 exhibiting MSI-H and 8 MSI-L. In addition, they reported loss of MLH1 expression in 5 MSI-positive cases (1 MSI-H and 4 MSI-L) and loss of MSH2 expression in 1 MSI-H case, as well as in 5 microsatellite stable cases (17%) [19]. In accordance with these results, using immunohistochemistry of MMR proteins, other studies found loss of expression of MSH2 and MSH6 [33], and MLH1 and MSH6 [34] in MSI-positive STS. However, loss of expression of MMR proteins was also observed in microsatellite stable cases, hampering interpretation [33, 34].

The discrepancy of our results with those previously published may be due to multiple factors. Firstly, there are many subtypes of STS that may represent different entities and, therefore, have distinct genetic features. Secondly, the number of cases analyzed in previous studies

Table 3. Summary of MSI evaluation in STS

Author	Country	STS analyzed, n	Methodology	Criteria	Conclusion
Wooster et al. [17], 1994	Several European countries	18	Molecular (di-, tri- and tetranucleotide markers)	1 or more markers with instability	11% (2/18) MSI-positive cases
Saito et al. [34], 2003	Japan	8	Immunohistochemistry (MSH2 and MLH1)	Loss of expression	25% (2/8) MSI-positive cases
Ericson et al. [33], 2004	Sweden	209	Immunohistochemistry (MLH1, MSH2 and MSH6)	Loss of expression	0.9% (2/209) MSI-positive cases (loss of MSH2 and MSH6)
Rucinska et al. [18], 2005	Poland	16	Molecular (di- and tetranucleotide markers)	MSI-L (1 marker with instability) and MSI-H (2 or more markers with instability)	25% (4/16) MSI-L and 18.7 (3/16) MSI-H cases
Kawaguchi et al. [19], 2005	Japan	40	Molecular (dinucleotide markers) and immunohistochemistry (MLH1 and MSH2)	MSI-L (<40% of markers with instability) MSI-H (>40% of markers with instability)	20% (8/40) MSI-L and 5% (2/40) MSI-H cases MSI-L (4 loss of MLH1) and MSI-H (1 loss of MLH1)
Present article	Brazil	71	Molecular (mono- and dinucleotide markers) and immunohistochemistry (MLH1, MSH2, MSH6, and PMS2)	MSI-L (1 marker altered) and MSI-H (2 or more markers altered)	MSS

was small. Here, we analyzed 71 STS cases – the largest study so far to evaluate MSI status using molecular techniques. Thirdly, distinct methodologies were used for MSI assessment, and it is well known that the accuracy of MSI detection is highly dependent on the techniques used. In our study we used the gold standard panel of MSI markers widely used in colorectal cancer MSI testing [9, 22, 25].

In conclusion, our study suggests that MSI does not play a role in STS tumorigenesis.

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Disclosure Statement

The authors declare no competing financial interests.

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