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MDM2 amplification and fusion gene ss18-ssx in a poorly differentiated synovial sarcoma: A rare but puzzling conjunction

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Abstract

The detection of specific alterations by genetic analyses has been included in the diagnostic criterions of the World Health Organization's classification of soft tissues tumors since 2013. The presence of a *SS18* rearrangement is pathognomonic of synovial sarcoma (SS). *MDM2* amplification is strongly correlated to well-differentiated or dedifferentiated liposarcoma (DDLPS) in the context of sarcoma. We identified one case of poorly differentiated sarcoma harboring both *SS18-SSX2* fusion and *MDM2* amplification. The review of the literature showed high discrepancies, concerning the incidence of *MDM2* amplification in SS: from 1.4% up to 40%. Our goal was to precisely determine the specific clinico-pathological features of this case and to estimate the frequency and characteristics of the association of *SS18-SSX* fusion/*MDM2* amplification in sarcomas. We performed a retrospective and prospective study in 96 sarcomas, (56 SS and 40 DDLPS), using FISH and/or array-CGH to detect *MDM2* amplification and *SS18* rearrangement. None of the 96 cases presented both genetic alterations. Among the SS, only the index case (1/57: 1.7 %) presented the double anomaly. We concluded that *MDM2* amplification in SS is a very rare event. The final diagnosis of the index case was a SS with *SS18-SSX2* and *MDM2* amplification as a secondary alteration. If the detection of *MDM2* amplification is performed first in a poorly differentiated sarcoma, that may lead to not search other anomalies such as *SS18* rearrangement and therefore to an erroneous diagnosis. This observation emphasizes the strong complementarity between histomorphology, immunohistochemistry and molecular studies in sarcoma diagnosis.

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Introduction

Genetics is of major importance in the recognition and clinical management of Soft Tissue Sarcomas (STS). Over the last decades, it has allowed the creation of a modern classification of STS.[1] Roughly, four groups of STS can be distinguished, according to their genetic alterations: STS with translocations leading to formation of fusion genes, STS with specific amplification, STS with specific mutations and STS with complex genome. However, while the discovery of pathognomonic anomalies

Abbreviations: STS, Soft Tissue Sarcomas, NGS, Next Generation Sequencing, FISH, fluorescence in situ hybridization, RT-PCR, Reverse transcription polymerase chain reaction, SS, synovial sarcoma, ALT, atypical lipomatous tumors, WDLPS, well-differentiated liposarcoma, DDLPS, dedifferentiated liposarcoma, CGH, comparative genomic hybridization

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related to morphological and clinical tumor types has served as novel bases of diagnosis, prognosis and targeted therapy of STS, their increasing number has also raised novel issues. Notably, it appeared more and more frequently that a given fusion gene may be present in several, apparently very distinct, entities.[2–8] Conversely, Next Generation Sequencing (NGS) studies have increased the number of fusion genes related to a same tumor entity.[9,10] This brought up several points about the role of the fusion genes in the initiation and progression of the tumor cells. It also raises the issue of the role and potential prominence of the genomic background of a fusion gene.

So far, the *SS18-SSX* (*SYT-SSX*) chimeric gene robustly remains related to synovial sarcoma (SS).[11–13] This fusion *SS18-SSX* has never been described in any STS other than SS.[14] It results from the t(X;18)(p11;q11) that fuses *SS18* either with *SSX1* or with *SSX2*. Molecular variants involving *SSX4*, *SS18L1* and *NEDD4* are very rare.[11–13,15,16] The detection of *SS18* and *SSX* rearrangements can be done routinely by fluorescence in situ hybridization (FISH) using break-apart probes. Reverse transcription polymerase chain reaction (RT-PCR) and RNA sequencing are also practical methods for detection of *SS18-SSX* fusion gene.[17–19] These molecular analyses are useful for confirmation of histological diagnosis. They are mandatory in challenging cases that can be mistaken for other mesenchymal tumors, such as cellular superficial fibromatosis, solitary fibrous tumor, spindle cell carcinoma, malignant peripheral nerve sheath tumor and Ewing's sarcoma/primitive neuroectodermal tumors. The ubiquitous localization and variable morphologic presentation of SS contributes to these difficulties.[1,17] In a series of 47 SS cases, Oda et al.[20] observed an amplification of the *MDM2* gene at a frequency as high as 40%. The amplification of *MDM2* was described by other authors in several series of SS but such an elevated frequency was not confirmed.[21–23] Though it can be observed occasionally in STS, such as intimal sarcoma or paraosteal osteosarcoma, *MDM2* amplification is strongly related to atypical lipomatous tumors (ALT), well-differentiated liposarcoma (WDLPS) and dedifferentiated liposarcoma (DDLPS).[24–26] Whether the amplification of *MDM2* is a recurrent or an exceptional feature of SS, has to be clearly and definitively established because of such a potential impact in molecular diagnosis. *MDM2* amplification can be detected routinely either by FISH or comparative genomic hybridization on arrays (array-CGH). It is mainly used for distinguishing ALT/WDLPS from lipomas or DDLPS from poorly differentiated sarcomas.

Among the 384 cases of STS or suspicion of STS included in the GENSARC trial (NCT 00847691),[27] one case harbored both *SS18-SSX2* fusion and *MDM2* amplification. We present here the detailed clinical, histological and genetic description of this novel *SS18+/MDM2+* case. In addition, in order to specify the frequency and impact on diagnosis of this double alteration, we have investigated the presence of *MDM2* amplification in 56 molecularly confirmed SS (*SS18+*), as well as the presence of *SS18* rearrangement in a series of 40 *MDM2*-amplified DDLPS (*MDM2+*).

Materials and methods

Index case SS18+/MDM2+

The patient was a 70-year-old man who presented in December 2009 with neuropathy symptoms, pain and alteration of his general condition, notably asthenia and weight loss. Medical examination showed an intramuscular tumor mass of the left thigh that had been noticed one year earlier by the patient. The pathological examination of a biopsy sample led to suspect a poorly differentiated sarcoma. A large surgical excision of a tumor measuring $4 \times 2.5 \times 2.5$ cm^3 was performed. Resection margins were *in sano* (R0). The results of microscopical histopathological analysis indicated a poorly differentiated sarcoma. The patient was informed of the possibility of inclusion in the GENSARC study that focused on molecular diagnosis of main sarcoma types.[27] He was included in the study in agreement with the current French law regarding non-interventional studies. Molecular analyses showed the presence of both *SS18-SSX2* fusion and *MDM2* amplification. The patient was treated by radiotherapy. No recurrence was detected on his last clinical examination in 2013, showing no recurrence.

Cohorts 1 (SS18+ cases) and cohort 2 (MDM2+ cases)

Fifty-six samples of SS showing *SS18* rearrangement from 53 patients (cohort 1; Table 1) and 40 samples of DDLPS showing *MDM2* amplification (cohort 2; Table 2), collected between May 1992 and September 2019, for which sufficient amount of tumor material was available for additional molecular analyses (*MDM2* amplification status for cohort 1 and *SS18* rearrangement status for cohort 2) were retrieved from the files of the Laboratory of Solid Tumor Genetics of Nice University Hospital and of the Pathology Department of Timone Hospital in Marseilles. The design of the study and protection of patient's data were in accordance with the local institutional rules, the current French legislation, and the European Union 2016/679 General Data Protection Regulation. In cohort 1, there were 30 male and 23 female patients whose ages ranged from 13-89 years. Tumor locations were: limbs (29/56 cases), retroperitoneum (3/56 cases), head and neck (5/56 cases) and trunk wall (14/46 cases). For five cases data on tumor localisation were unavailable. Forty-five tumors were primary, three were lung metastases and three tumors was a local recurrence. For five cases this information was not recorded in patient's files. The *SS18* rearrangement involved *SSX1* in 26 cases, *SSX2* in 15 cases and *SSX4* in one case, respectively. In 14 cases the partner gene of *SS18* could not be determined. In cohort 2, there were 24 male and 16 female patients whose ages ranged from 38 to 93 years. Tumors were located in limbs (7/40 cases), retroperitoneum (26/40 cases) and in other locations (7/40 cases). For one case data on tumor localisation was unavailable. Thirty-height tumors were primary, one tumor was a metastasis and for one case this information was not available.

FISH analyses

FISH analysis on FFPE sections was performed using dual-color probe for *MDM2* and centromere 12 (MDM2 Zytolight SPEC MDM2/CEN12 (Clinisciences)) and break-apart probes for *SS18* (3′ green signal, 5′ red signal; Vysis LSI SS18 Break Apart -Abbott Molecular), *SSX1* and *SSX2*. Probes for *SSX1* and *SSX2*: RP11-38O23 (5′ *SSX1*, green signal); CTD-3022H5 (3′ *SSX1*, red signal); CTD-3062G21, CTD-3141N23, RP11-204I15 (3′ *SSX2*, green signal); CTD-2009K1; RP11-258C19 (5′ *SSX2*, red signal), were made of Bacterial Artificial Chromosomes (BAC) from the Roswell Park Cancer Institute library that had been selected according to their location on the University of California Santa Cruz database (http://genome.ucsc.edu/; February, 2009 (GRCh37/hg19); January release) and obtained from Life Technologies (Carlsbad, CA) and prepared as probes for FISH analysis according to standard procedures. *MDM2* amplification was defined as the presence of at least 10 clustered fluorescent signals per cell in ≥1% of cells. *SS18, SSX* and *SSX2* rearrangements were assessed when at least one couple of the two fluorescent signals (red and green) were separated in ≥15% of cells. Results were independently interpreted by two observers. Microscopic analysis was performed using a DM6000B microscope (Leica Microsystems, Wetzlar, Germany). FISH images were processed using the ISIS software (Metasystems, Altlussheim, Germany).

Table 1. Characteristics of cohort 1 (SS with *SS18* rearrangement).

Case Number	ID	Age (years)	Sex	Tumor Localisation	*SS18* partner gene (method of detection)	Histologic type
1#	92.T233	24	M	N.A	*SSX1* (FISH)	N.A.
2#	93.T440	84	M	N.A	N.A.	N.A.
3# °	96.T1205	21	M	Trunk wall	*SSX2* (FISH)	N.A.
4	07.T151	42	M	Pharynx	*SSX1* (FISH and RT-PCR)	Biphasic
5	07.T555	34	M	Limbs	*SSX2* (FISH)	Monophasic
6	08.T124	51	M	Trunk wall	N.A.	N.A.
7	08.T335	42	M	Trunk wall	*SSX1* (FISH and RT-PCR)	Monophasic
8	08.T572	52	M	Limbs	*SSX1* (FISH and RT-PCR)	Monophasic
9	09.T109	51	F	Trunk wall	*SSX1* (FISH and RT-PCR)	N.A.
10	09.T354	87	M	Trunk wall	*SSX1* (FISH and RT-PCR)	N.A.
11	09.T382	53	F	Limbs	*SSX1* (FISH and RT-PCR)	Monophasic
12	09.T437	44	F	Limbs	*SSX2* (FISH and RT-PCR)	Monophasic
13	09.T592	89	F	Limbs	*SSX2* (FISH)	Monophasic
14	09.T658	49	M	Limbs	*SSX1* (FISH)	Monophasic
15	10.T589	47	M	Trunk wall	*SSX4* (FISH)	Monophasic
16	10.T1015	25	M	N.A.	*SSX1* (FISH)	N.A.
17	11.T009	79	M	Trunk wall	N.A.	N.A.
18	11.T161	72	F	Limbs	*SSX2* (FISH)	Monophasic
19	11.T873	66	F	Limbs	*SSX2* (FISH)	Monophasic
20	11.T934	52	M	Limbs	*SSX1* (FISH)	Monophasic
21	12.T014	46	F	Pharynx	*SSX1* (FISH)	Biphasic
22	12.T156	51	M	Limbs	*SSX1* (FISH)	Monophasic
23	12.T666	61	M	Limbs	*SSX1* (FISH and RT-PCR)	Monophasic
24	13.T047	46	F	Limbs	N.A.	Monophasic
25	13.T128	69	F	Limbs	*SSX1* (FISH)	Monophasic
26	13.T1054	68	F	Trunk wall	*SSX2* (FISH)	Monophasic
27*	141109	69	F	Trunk wall	*SSX1* (FISH)	Monophasic
28	150027	72	F	Retroperitoneum	N.A.	Monophasic
29	151017	36	M	N.A.	N.A.	N.A.
30	152131	51	F	Limbs	*SSX1* (FISH and RT-PCR)	Monophasic
31	152368	45	M	Limbs	*SSX1* (FISH)	N.A.
32	152460	70	F	Trunk wall	*SSX1* (FISH)	Monophasic
33	152462	56	F	Trunk wall	*SSX2* (FISH)	Monophasic
34	152463	64	M	Trunk wall	*SSX1* (FISH and RT-PCR)	N.A.
35	152464	58	F	N.A.	*SSX2* (FISH)	Monophasic
36	161408	32	F	Limbs	*SSX1* (FISH)	Monophasic
37	161824	27	M	Limbs	N.A.	Biphasic
38	162736	13	F	Limbs	*SSX2* (FISH)	N.A.
39**	163421	66	F	Trunk wall	*SSX2* (FISH)	Monophasic
40	174658	32	F	Limbs	*SSX1* (FISH)	Monophasic
41	174860	36	M	Limbs	*SSX1* (FISH)	Biphasic
42***	174993	44	F	Limbs	*SSX2* (FISH)	Monophasic
43	174997	47	M	Trunk wall	*SSX1* (FISH)	N.A.
44	174998	34	F	Trunk wall	*SSX2* (FISH)	N.A.
45	181359	27	M	Limbs	*SSX1* (FISH)	Monophasic
46	183946	25	M	Cervical spine	*SSX1* (FISH)	Monophasic
47	192589	81	M	Limbs	*SSX2* (FISH)	Monophasic
48	193044	28	M	Limbs	N.A.	Monophasic
49	193045	23	F	Limbs	*SSX2 (FISH)*	Monophasic
50	193046	40	F	Head	N.A.	Monophasic
51	193048	17	F	Limbs	N.A.	Monophasic
52	193049	32	M	Limbs	N.A.	Biphasic
53	193050	50	M	Limbs	*SSX1 (FISH)*	Monophasic
54	193362	31	M	Retroperitoneum	N.A.	Monophasic
55	193364	58	F	Limbs	N.A.	Monophasic
56	194048	61	M	Thyroid	N.A.	Monophasic

SS: synovial sarcoma; M: male; F: female; N.A: data not available; FISH: fluorescence in situ hybridization; RT-PCR: Reverse transcription polymerase chain reaction; * and **: Cases 27 and 39 were the lung metastases of cases 25 and 19, respectively; ***: case 42 was the local recurrence of case 12. #: these cases were also studied using conventional cytogenetic analysis that showed the presence t(X;18) and absence of large markers or double minute chromosomes. Notably, case 3 showed trisomy 12 consistent with the observation of three *MDM2* signals per cell using interphase FISH. °: case 3 was the pulmonary metastasis of a primary tumor located in the left calf detected two years earlier.

Array-CGH

Index case: DNA extraction from a frozen sample of the surgical excision was done using a standard phenol–chloroform procedure (Phase Lock Gel Light, Eppendorf, Hamburg, Germany). DNA purity and concentration were evaluated using a NanoDrop spectrophotometer (ThermoFisher, Waltham, MA) (absorbance for an optimal labeling yield: A260/A280 $\geq$ 1.8 and A260/A230 $\geq$ 1.9) and Qubit dsDNA BR Assay Kit (Invitrogen, Waltham, MA), respectively. Human Reference DNA was extracted from human blood from healthy control. Labeling of tumor DNA (1000 ng) by Cyanine 5 (Cy5) and of reference DNA by Cyanine 3 (Cy3) was followed by purification, co-hybridization in equal quantity (1ug) to the NimbleGen Arrays (Roche NimbleGen, Madison, WI) and washing according to the manufacturer's recommendations. Arrays were

Table 2. Characteristics of cohort 2 (DDLPS with MDM2 amplification).

Case number	ID	Age (years)	Sex	Tumor Localisation
57	151402	38	M	Retroperitoneum
58	151704	65	M	Retroperitoneum
59	151764	66	M	Para testicular
60	152810	70	M	Retroperitoneum
61	153174	91	F	Limbs
62	153341	80	M	Skin
63	153483	58	M	Limbs
64	153938	92	F	Limbs
65	160319	72	F	Retroperitoneum
66	160786	57	F	N.A.
67	161168	69	F	Retroperitoneum
68	161706	83	F	Retroperitoneum
69	161840	79	M	Limbs
70	161914	90	F	Limbs
71	162021	66	F	Retroperitoneum
72	162267	81	F	Retroperitoneum
73	162552	74	M	Testicular cord
74	162650	75	F	Retroperitoneum
75	162699	66	M	Retroperitoneum
76	163566	66	F	Retroperitoneum
77	163902	93	M	Retroperitoneum
78	164303	89	M	Limbs
79	170217	86	M	Para testicular
80	170357	71	M	Retroperitoneum
81	170705	45	M	Retroperitoneum
82	171883	51	M	Retroperitoneum
83	172526	79	M	Para testicular
84	173207	59	F	Retroperitoneum
85	173208	77	M	Limbs
86	173404	73	M	Retroperitoneum
87	173079	69	M	Retroperitoneum
88	185252	81	M	Retroperitoneum
89	190363	56	M	Retroperitoneum
90	192224	65	M	Para testicular
91	192994	55	F	Retroperitoneum
92	192997	93	F	Retroperitoneum
93	193215	81	F	Retroperitoneum
94	193199	81	F	Retroperitoneum
95	193259	70	M	Retroperitoneum
96	194010	67	M	Retroperitoneum

DDLPS: dedifferentiated liposarcoma; M: male; F: female. N.A: data not available

scanned using GenePix 4000B scanner and analyzed using GenePix V.6.6 Software (Molecular Devices, Sunnyvale, CA). Raw data were normalized and processed using the NimbleScan V.2.5 Software (Roche NimbleGen). Files produced by Nimblescan software were then analyzed on SignalMap V.1. Deletion was defined by a log2 ratio Cy5/Cy3 < - 0.4 and gains by a log2 ratio Cy3/Cy5 > 0.4. Amplifications were defined by a log2 ratio Cy5/Cy3 > 1. Results were provided according to hg18 (NCBI International Human Genome Sequencing Consortium 36; www.genome.ucsc.edu/).

Cohorts 1 and 2: DNA was extracted from FFPE samples using a QiAmp DNA mini kit (Qiagen, Hilden, Germany). The reference non tumor DNA was Human Genomic DNA (Promega, Madison, WI). Labeling of tumor DNA by Cy5 and of reference DNA by Cy3 was followed by purification and co-hybridization in equal quantity on a genome-wide oligonucleotide-based microarray Sureprint G3 Human CGH 180 k (average resolution 13 kb) (Agilent, Santa Clara, CA). Hybridization and washing were performed as specified by the manufacturer (Agilent). Hybridized slides were scanned using SureScan scanner (Agilent) and image analysis was performed using Cytogenomics software (v2.9.2.4, Agilent). Results were provided according to hg19 (GRCh37 Genome Reference Consortium Human Reference 37; www.genome.ucsc.edu/). Gene amplification was defined by a log2 ratio Cy5/Cy3 > 1.1 and gain was defined by a log2 ratio Cy5/Cy3 between 0.2 and 1.1.

Immunohistochemistry

Immunohistochemistry of the index case was performed on paraffin-embedded tissue sections using antibodies against pan-cytokeratin AE1-AE3 (monoclonal mouse antihuman pan-cytokeratin, clone AE1/AE3, M3515; Dako Corp., Carpinteria, CA), Epithelial membrane antigen (EMA; clone E29, M0613; Dako), MDM2 (clone IF2; Zymed Laboratories, San Francisco, CA), HMGA2 (clone AB52039; Abcam, Paris, France), Cyclin Dependent Kinase 4 (CDK4; clone AHZ 0202; Invitrogen, Waltham, MA), TLE1 (clone 1F5; Cell Marque Sigma-Aldrich, Darmstadt, Germany), INI1/BAF47/SMARCB1 (clone 25, Becton Dickinson, San Jose, CA) and H3K27me3 (Diagenode, Seraing, Belgium).

RNA-sequencing (RNA-seq)

Total RNA was extracted from snap frozen sample of the index case using the Trizol/chloroform method (Thermo Fisher Scientific, Carlsbad, CA) and qualified with fragment analysis by TapeStation 4200 (Agilent Technologies, Carlsbad, CA). Libraries were prepared with TruSeq mRNA stranded library kits (Illumina, San Diego, CA). One µg of total RNA was purified, retrotranscripted, fragmented, indexed and amplified for preparation of the RNA libraries. Libraries were sequenced over 2x150pb using a 500 High Output v2 on NextSeq500 (Illumina). Expres-

sion data were generated using Star Aligner (V2.5.3a) and count matrices using FeatureCount (V1.6.0). The count matrices were normalized in Fragments Per Kilobase Million (FPKM). Data were used for clustering analysis (Ward method and correlation Sparman or Pearson with or without Internal Quantil Range) and for boxplot generation. A fusion analysis was performed from FastQ with two approaches: 1) a targeted analysis using a dedicated reference fusion sequences implemented with known fusions of each tumor type; 2) an exploration analysis using 5 fusions finder tools (TopHat fusion v2.0.6, Defuse V0.6.0, StarFusion V2.5.3, Fusion Catcher v1.00 and FusionMap). The fusion interpretation combined results of the targeted fusion and those of the exploration analysis.

Results

Histological and molecular features of index case SS18+/MDM2+

Histologically, the tumor was composed of a monotonous proliferation of monomorphic spindle cells arranged in highly cellular and long fascicles. They had scarce cytoplasm and ovoid hyperchromatic nuclei. Stromal changes were focally abundant with collagen bundles or perivascular myxoid nests containing histiocytes. These stromal changes were devoid from tumor cells, confirmed by the negativity of HMGA2. There was no epithelioid component. Peripheral striated muscle fibres were infiltrated by tumor cells. A component of scattered mature adipocytes was also present mostly at the periphery but also in the centre of the lesion (Fig. 1A–D). Immunostaining of the spindle tumor cells showed classical features of synovial sarcoma: a weak positivity for EMA and AE1-AE3 (Fig. 1E and F). A heterogeneous nuclear positivity for MDM2 was detected in 5% of the spindle cells (Fig. 1H and I) while a diffuse positivity for CDK4, and HMGA2 was observed in approximately 100 % and 70% of cells, respectively (Fig. 1J and K). On the contrary, adipocytes did not express MDM2, CDK4, or HMGA2. Moreover expression of SMARCB1 and H3K27me3 was conserved. Altogether, the complex morphology of the tumor, the presence of a mature adipocytic component and the immunohistochemical features led to a diagnosis of poorly differentiated sarcoma, possibly a SS or a DDLPS. *MDM2* amplification, *SS18* and *SSX2* rearrangements were detected using FISH analysis (Fig. 2A and B). *MDM2* amplification was observed in 78% of tumor cells. Array-CGH showed a high-level amplification (average $\log_2$ ratio Cy5/Cy3 > 1.3) of a large chromosomal segment, from nucleotide positions 50,927,411 up to 74,029,436 at 12q13.13-12q21.1. This amplified region notably included *MDM2, CDK4, HMGA2, DYRK2, FRS2* and *CPM*. A gain of chromosome 21, a loss of 12q21.3-qter and a loss of chromosome 13 were also detected (Fig. 2C). RNA-Seq analysis confirmed both the presence of the *SS18-SSX2* fusion (Fig. 3A) and of the *MDM2* amplification (Fig. 3B). Firstly, five different algorithms were able to identify a fusion of the exon 10 of *SS18* (5′) with the exon 6 of *SSX2* (3′). A targeted analysis using dedicated reference fusion sequences implemented with known fusions of many sarcoma subtypes showed 103 split reads encompassing the fusion point. The exploration analysis using four of five fusion-finder tools detected the same overexpressed fusion (Tools-Split/Span reads: TopHat fusion-292/5; Defuse-385/183; StarFusion-52/5; Fusion Catcher-27/434). Secondly, count matrices using FeatureCount normalized in FPKM allowed to assess relative expression data. *MDM2* expression showed a significant high-level overexpression ($\log_2$FPKM = 6.38) for the index case in comparison to synovial sarcoma ($\log_2$FPKM = 3.60; $n = 7$). These data have also been used for clustering analysis and showed that the transcriptomic profile of the index case clustered perfectly within SS profiles (Fig. 4). In addition, TLE1 immunostaining was performed and showed a diffuse positivity (Fig. 1G), consistent with a SS.

Detection of MDM2 amplification in a series of 56 SS18+ tumors (cohort 1)

Using FISH in 49 cases and array-CGH in 7 cases (cases 26–28, 30–33, 35), none of the 56 *SS18+* cases showed amplification of *MDM2* (Table 1). In addition, results of array-CGH showed a gain of chromosome 16 in case 26; gain of 3q, 8q and a loss of 22q in case 27; gain of 4q13.1-q13.2 and loss of 16q11.2-q24.3, 18q11.1-q23 in case 28; a gain of whole chromosomes 4, 8, and 21, a gain of 15q,16p,17q,19p, and a loss of 11q, 12p, 15q, 16q, 17p, 19q in case 30; a gain of chromosome 17 in case 31. No quantitative anomalies were detected using CGH-array in cases 32, 33 and 35. Polysomy of chromosome 12 (3–4 copies per cell) was observed in cases 3 and 45.

Detection of SS18 rearrangement in a series of 40 MDM2+ DDLPS (cohort 2)

In 32 out the 40 cases, no structural rearrangement of *SS18* was detected using FISH (Table 2). A few extra-copies of the non-rearranged gene were observed in cases 75, 78, 83, 88 and 91 while amplification of *SS18* was detected in rare cells from case 62. In case 87, we detected an unbalanced rearrangement of *SS18* (gain of the 5′ region) while no rearrangement of *SSX1, SSX2* and *SSX4* were observed. Further investigation of case 87 using array-CGH confirmed the *MDM2* amplification previously detected using FISH and showed amplification of a large segment at 12q13-15, notably containing *CDK4* and *FRS2* in addition to *MDM2*. Amplification of 18q11 close to the *SS18* gene at 18q11 was also observed (Fig. 5A–C). We concluded that the alteration surrounding *SS18* was secondary to breakages generated by 18q amplification and was not related to an oncogenic *SS18-SSX* fusion gene.

Discussion

The main driver alteration of SS is the *SS18-SSX* fusion. Although SS presents a low mutational and copy number variations rate,[28] so-called ``secondary'' genetic structural or quantitative anomalies have been reported in addition to the *SS18-SSX* fusion. A few non-recurrent mutations, affecting oncogenes and tumor suppressor genes, such as *TP53*, *HRAS* and *PTEN*, have been described.[20,29,30] The clinical consequence of these mutations in the background of the *SS18-SSX* fusion is not clearly established yet. Losses and gains of chromosomal segments have been reported in the conventional karyotypic and CGH studies of SS.[21,22,31,32] The most frequent described anomaly was a partial or complete gain of chromosome 8. Low-level gains of 12q were also reported.[21,33] It has been noticed long ago that translocations in STS were frequently associated with secondary chromosomal alterations that could vary from a single extra-chromosome up to multiple alterations.[34–41] The clinical significance, notably prognostic value, of such additional chromosomal alterations has been under debate for years.[22,34–44] Recent data showed that tumors harboring both fusion genes and genomic instability undergo an aggressive outcome.[45] However a few secondary alterations do not always relies on genuine genomic instability. Genomic amplification is a remarkable genomic feature that can be a diagnostic marker when recurrent in a tumor type or a marker of instability and aggressiveness. The presence of genomic amplification in addition to fusion genes has been reported in some STS including SS. In SS, amplification of *MDM2* has been so far described in 23 patients.[20–23,33] The frequency of this association of two alterations – i.e *SS18* fusion and *MDM2* amplification- individually known to be representative of a specific entity has to be determined. Indeed, it was as high as 40% in the series of 47 SS studied by Oda et al.[20] while much lower (1.4% up to 11%) in other series: one out 9 cases (11%),[23] one out 13 cases (7%),[22] one out 67 cases

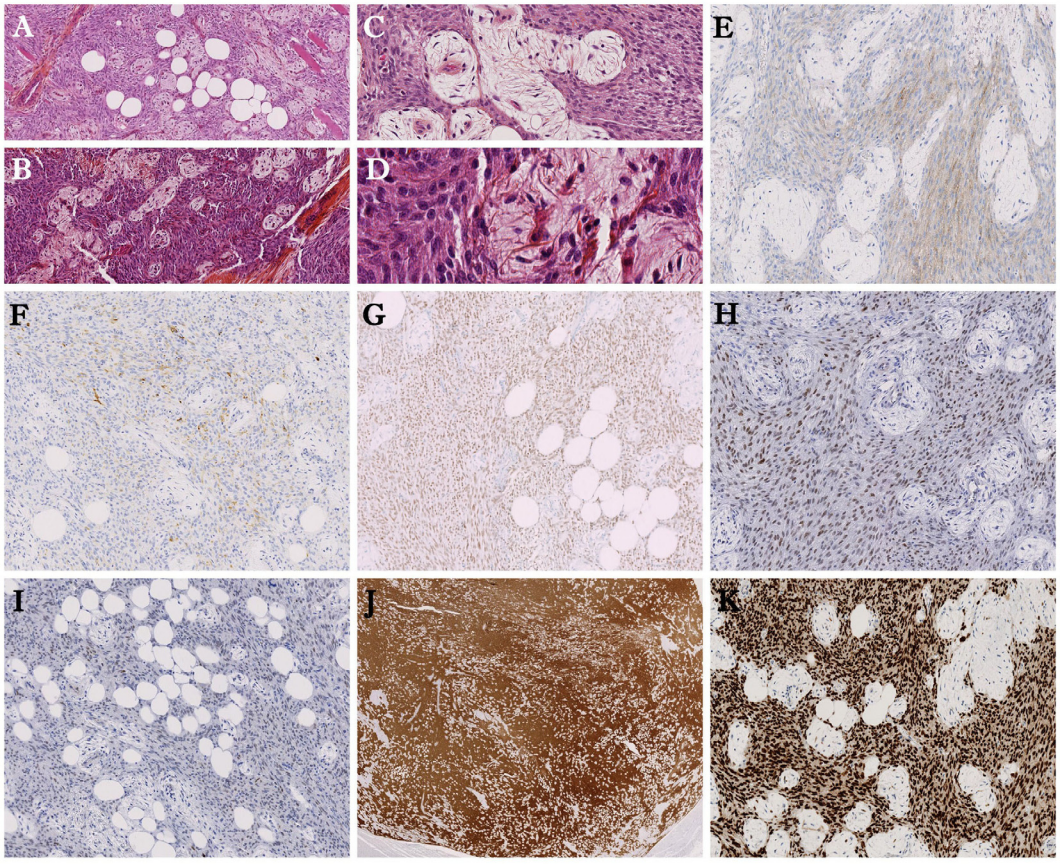

Fig. 1. Histological and immunohistochemical features of index case. A–D: poorly differentiated sarcoma displaying a monomorphic spindle-cell proliferation arranged in long fascicles, surrounding adipocytes mostly at the periphery as well as in the centre of the tumor mass. There are stromal changes with collagen bundles and myxoid nests containing histiocytes. Hematoxylin and eosin (H and E) staining (magnification: A: ×50, B: ×100, C: ×200; D:×400). D-E: weak positivity for EMA and AE1-AE3 in the spindle cells (×100). F: diffuse positivity for TLE1 in spindle cells (×100). G, H: MDM2 heterogeneous nuclear positivity in the spindle cell component and no evidence of positivity in the mature adipocytic component (×100). I: CDK4 positivity in 100% of tumor cells (×6). J: HMGA2 expression is positive in spindle cells and negative in adipocytes (×100).

(1.4%),[33] one out 69 cases (1.4%).[21] In a comprehensive and integrated genomic characterization of six types of sarcomas, only one case of the 10 SS studied showed a low gain of 12q13-15 containing *DDIT3*, *CDK4*, *HMGA2*, *FRS2* and *MDM2* genes.[28] In these previous series, the diagnosis of SS was mostly assessed on morphological bases. Only a part of the cases benefited from a molecular confirmation: 21 out 47 cases in the series from Oda et al.[20] and all the 13 cases in the series from Nakagawa et al.[22] On the basis of the observation of a novel *SS18+/MDM2+* case, we subsequently aimed at exploring more deeply both the clinicopathological characteristics and frequency of such *SS18+/MDM2+* tumors. Indeed, the conjunction of *SS18* rearrangement and *MDM2* amplification raises diagnostic issues with consequences on decision-making and treatment strategies. Notably, the finding of *MDM2* amplification in a soft tissue tumor is never meaningless and deserves peculiar attention. For instance, amplification and expression of *MDM2* has been detected also in malignant peripheral nerve sheath tumor (MPNST), a tumor that can be hard to distinguish from both DDLPS and SS Makise et al.[46] As in the case of SS presented here,[46] had to deal with *MDM2* amplification/overexpression in a case of MPNST. Detection of H3K27me3 expression was a helpful marker in this context. Indeed, PRC2 alteration leading to H3K27me3 deficiency has been reported as the molecular hallmark of MPNST. In our index case, the conservation of H3K27me3 expression was consistent with a tumor other than MPNST. However, Makise et al.[46] showed that some DDLPS showed a complete loss of expression of H3K27me3, suggesting that this marker should not be used alone for an accurate distinction of MPNST from liposarcoma. Altogether, diagnosis has to be made according to the whole clinical, histological and molecular features. In addition, although a reduced expression of SMARCB1 is not specific of SS, it can also contribute to distinguish SS from its histological mimics.[47] Indeed, a disruption of mSWI/SNF (BAF) complex by the SS18-SSX fusion induces a loss of expression of SMARCB1.[48] Immunohistochemical detection of this reduced expression is therefore often observed in SS. However in our index case, SMARCB1 expression was conserved. In the present case, histological features were those of a poorly differentiated sarcoma that exhibited overlapping morphologic features between SS and DDLPS, with an unusual component of scattered intermingled mature adipocytes. In contrast to *SS18-SSX* fusion that has been described as fully specific of SS, *MDM2* amplification has been reported in a variety of STS other than WDLPS/DDLPS and even in tumors other than STS. Therefore, *SS18-SSX* fusion was considered as the relevant alteration of this case, *MDM2* amplification being only a secondary alteration. Moreover, the immunohistochemical diffuse expression of TLE1 as well as the RNA clustering were in favor of a SS. The treatments of primary SS and DDLPS do not sensibly differ: it consists in surgery followed by radiotherapy.[49] In contrast, the recognition of SS is crucial for conducting the treatment in metastatic patients. SS tend to have better survival rate and a higher chemo-sensitivity than other STS.[50,51] Doxorubicin and Ifosfamide are recommended in advanced or

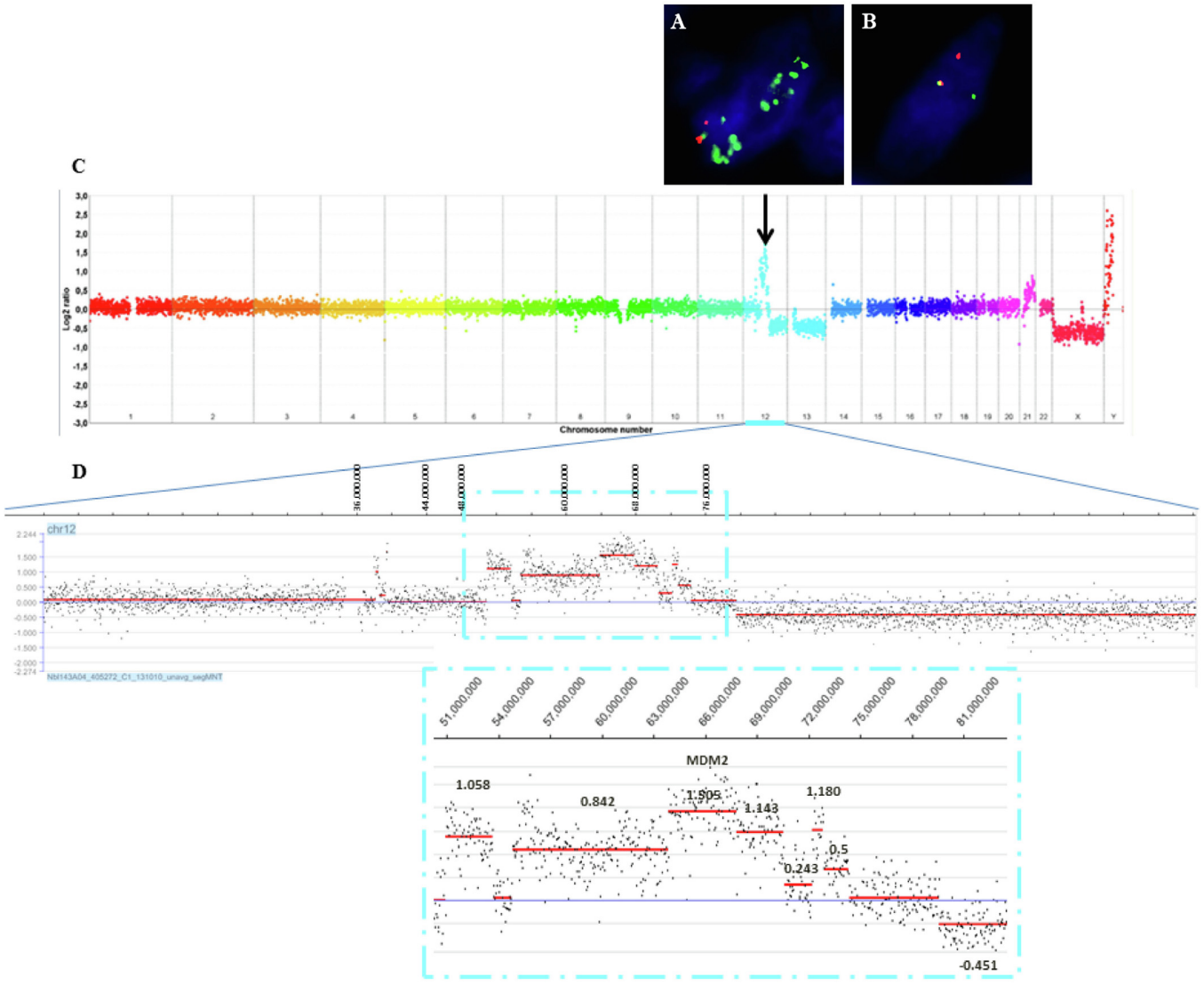

Fig. 2. Fluorescence in situ hybridization (FISH) analyses and comparative genomic hybridization on arrays (array-CGH) of index case. A-B: FISH. A: Dual-color probe for *MDM2* (green signal) and centromere 12 (red signal) on fixed-paraffin embedded sections showing *MDM2* amplification. More than 10 green signals grouped in clusters are observed. B: Break-apart probes for *SS18* (green signal: 3′, red signal: 5′) showing one normal *SS18* allele (juxtaposed red and green signals) and one rearranged *SS18* allele (split red and green signals). C-D: array-CGH. A: Whole genome profile showing the prominent amplification of a large chromosomal segment on chromosome 12q (black arrow). A gain of chromosome 21, a loss of 12q21.3-qter and a loss of chromosome 13 were also detected. D: chromosome 12 profile. Zoom on amplification of 12q13.13-12q21.1: *MDM2* $\log_2$ ratio Cy3/Cy5 > 1.5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metastatic SS.[51–53] Recently, the multikinase inhibitor pazopanib became the first targeted agent to be approved for the treatment of advanced SS after failure of chemotherapy.[54] An accurate molecular diagnosis of SS is also important to allow access to clinical trials. Indeed, several molecules are currently under development for the treatment of SS, such as Wnt inhibitors, Þ-catenin inhibitors and immunotherapy.[55–57] In contrast, chemotherapy regimens are generally ineffective for metastatic DDLPS[58] and targeted treatments have been disappointing. For instance, potent and selective MDM2 inhibitors such as nutlins appeared ineffective because of their high toxicities.[59,60] Several clinical trials evaluating MDM2 inhibitors alone or in combination with anti-CDK4 molecules are ongoing.[61–63] MDM2-Pp53 interaction may also constitute an interesting target.[64] Since in routine practice multiple molecular analyses are not usually performed, the detection of *MDM2* amplification is often done in priority in the context of a poorly differentiated sarcoma. In such a situation observation of a high level amplification of *MDM2* might not be followed by further analyses and lead to overlook a *SS18* rearrangement. This may have an unfavourable impact on metastatic patients. The frequency of the association *SS18+/MDM2+* has therefore to be precisely determined in order to be aware of potential misdiagnoses of SS. In some cases, recognition of a SS might be difficult because the immunoprofile that may show some variations. Moreover, the diagnostic value of some markers has been limited by their lack of sensitivity and/or specificity; in particular TLE1 shows a good sensitivity but a limited specificity in the diagnosis of SS. Recently, Baranov et al.[65] described a novel antibody showing 95% of sensitivity and 100% of specificity for the fusion

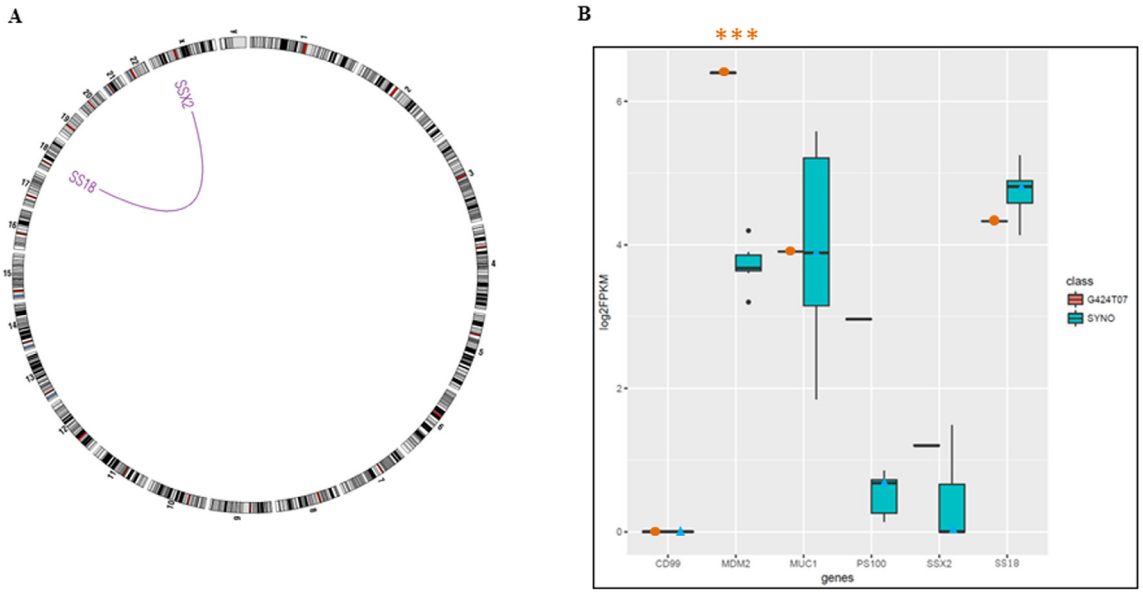

Fig. 3. RNA-sequencing (RNA-Seq) of index case. A: Circos plots illustrating chromosomal translocations *SS18-SSX2*. Chromosomes are drawn into scale around the rim of the circle and data are plotted on these coordinates. Interchromosomal fusion is indicated by an arc. B: Boxplot presentation based on expression data obtained by Star alignment and count matrices, normalized in Fragments Per Kilobase Million (FPKM), generated using FeatureCount. Relative expression in $\log_2$FPKM of *CD99*, *MDM2*, *MUC1*, *PS100*, *SSX2* and *SYT* are plotted for the index case (G424T07) and a SS+ control group (n = 7). Significant overexpression of *MDM2* is highlighted by asterisks.

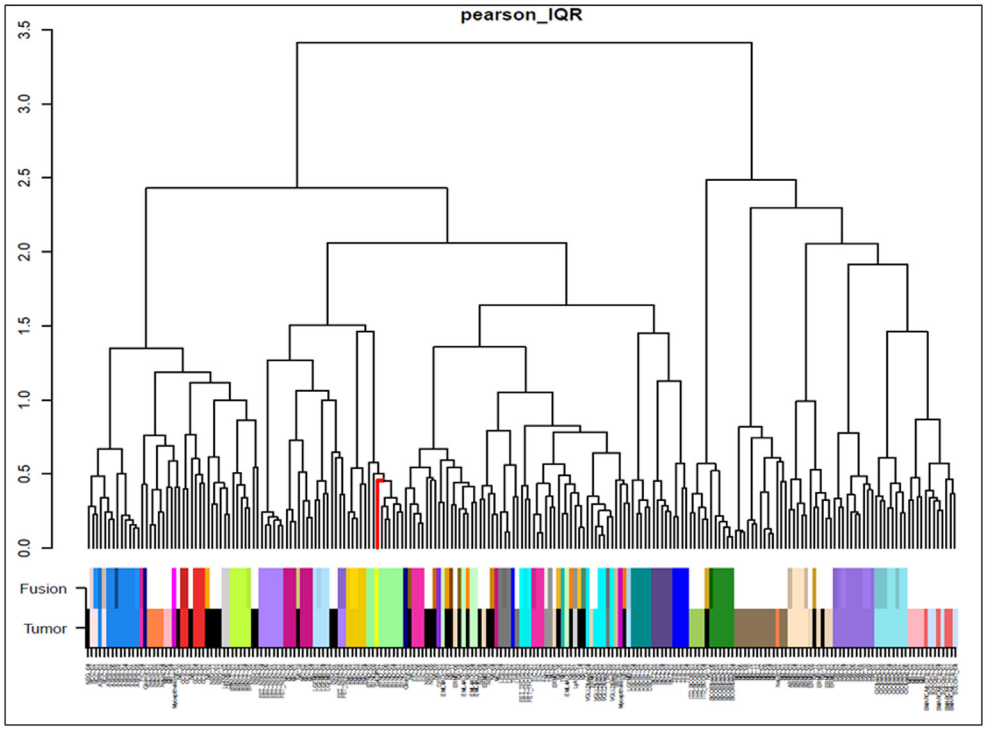

Fig. 4. Clustering analysis of index case. Count matrices, normalized in Fragments Per Kilobase Million (FPKM) data have been used for clustering analysis (Ward method and correlation Pearson with Internal Quantile Range). Well-defined collection of sarcomas was used to assess each specific tumor type or entity (bottom box in x-range), presence of absence of characteristic fusion is represented in the upper box. The index case is figured by a red branch in the dendrogram and yellow box in the light green cluster containing SS samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

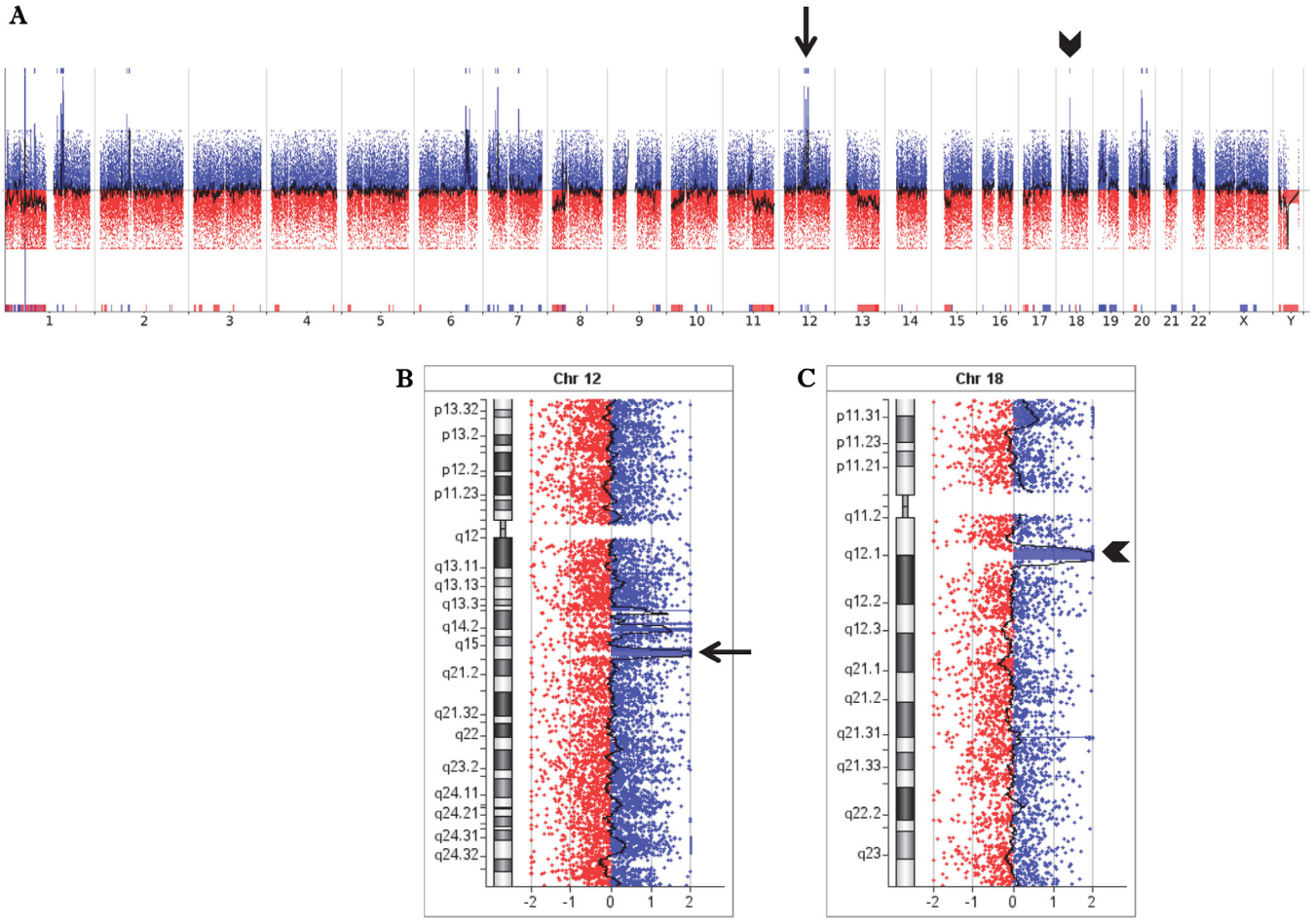

Fig. 5. Comparative genomic hybridization on arrays (array-CGH) of case number 87. A: Whole genomic profile showing complex alterations. *X*-axis coordinates represent chromosomes positions along the genome. Amplifications at 12q13-15, including *MDM2* (black arrow) and at 18q11, close to *SS18* gene (arrowhead) were observed. B and C: details of chromosomes 12 and 18, respectively.

SS18-SSX. This marker is likely to become a useful IHC tool, especially in centers with limited access to molecular biology or as an additional element in challenging cases.

Poorly differentiated histology is more frequent in SS from the elderly and be a source of diagnostic difficulties.[66] The location of the tumor is an element that has to be taken into account: though SS may arise in any anatomical site and that a significant proportion of DDLPS are located in limbs, a retroperitoneal location is more consistent with a DDLPS than a SS.

Our study is, to the best of our knowledge, the only one to investigate specifically both *SS18* rearrangements in DDLPS and *MDM2* amplification in molecularly confirmed SS in a large series of patients. Only the index case presented both alterations. The *SS18+/MDM2+* frequency in our series was 1%, closer to the results of 1.4% from Szymanska et al.[33] than the 40% found by Oda et al.[20]. This discrepancy was probably due to the methods used for detection of *MDM2* amplification (differential PCR[20] *versus* CGH or FISH[35]) as well as to the determination of the threshold for the definition of the amplification of *MDM2*. Possibly, the term ``amplification'' when it is related to the differential PCR method may be confusing when compared to its use in genomic studies where it usually refers to a number of more than 8 copies/per cell for a given gene. The term ``gain'' might be more appropriate for extra-copy number/cell <8.

Conclusions

Therefore, our results and the review of literature indicate that *MDM2* amplification is a very rare event in SS. Whether this rare event has a clinical impact on prognosis remains to be established. *MDM2* amplification might act synergistically with *SS18-SSX* fusion by promoting TP53 ubiquitination and degradation.[67] Moreover, this observation emphasizes the strong complementarity of clinical data, especially tumor location, histomorphology, immunohistochemistry and molecular studies to perform more accurate subtyping of sarcomas and to increase our knowledge of these tumors.

Authors' contributions

IDM and FP designed the research study; LMM, BC, ML, GP, AB and CB performed the research; IDM, FP and BDM analysed the data; IDM and FP wrote the paper; BDM and JFM gave technical support.

Declarations of interest

None

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