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A novel FISH assay for *SS18–SSX* fusion type in synovial sarcoma

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Synovial sarcoma is a morphologically, clinically and genetically distinct entity that accounts for 5–10% of all soft tissue sarcomas. The t(X;18)(p11.2;q11.2) is the cytogenetic hallmark of synovial sarcoma and is present in more than 90% of the cases. It produces three types of fusion gene formed in part by *SS18* from chromosome 18 and by *SSX1*, *SSX2* or, rarely, *SSX4* from the X chromosome. The *SS18–SSX* fusions do not seem to occur in other tumor types, and it has been shown that in synovial sarcoma a clear correlation exists between the type of fusion gene and histologic subtype and, more importantly, clinical outcome. Previous analyses regarding the type of fusion genes have been based on PCR amplification of the fusion transcript, requiring access to good-quality RNA. In order to obtain an alternative tool to diagnose and follow this malignancy, we developed a fluorescence *in situ* hybridization (FISH) assay that could distinguish between the two most common fusion genes, that is, *SS18–SSX1* and *SS18–SSX2*. The specificity of the selected bacterial artificial chromosome clones used in the detection of these fusion genes, as well as the sensitivity of the analysis in metaphase and interphase cells, was examined in a series of 28 synovial sarcoma samples with known fusion gene status. In all samples, the type of fusion was correctly identified by FISH. Thus, the assay described here should be useful for clarifying unresolved chromosome markers and for identifying fusion gene status in samples from which RNA of sufficient quality for PCR could not be extracted.
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Synovial sarcoma accounts for 5–10% of all soft tissue sarcomas.[1] It is most prevalent in adolescents and young adults between 15 and 40 years of age, occurs predominantly in males, and is often found in juxta-articular locations, particularly in the area of the knee. However, synovial sarcoma occasionally occurs in areas with no apparent relationship to synovial structures, such as the head and neck region and the trunk.

Histologically, synovial sarcoma may be subdivided into four variants, the biphasic and the monophasic subtypes being the most common. Biphasic synovial sarcoma is characterized by epithelial and spindle cell components in varying proportions, whereas the monophasic type has a spindle cell component alone. Monophasic epithelial and poorly differentiated subtypes have also been discerned, but are much less common. Poorly differentiated synovial sarcoma, in particular, poses a diagnostic problem, as it is difficult to distinguish from other round cell sarcomas. Thus, molecular genetic techniques may be useful in reaching a correct diagnosis.[2]

The t(X;18)(p11.2;q11.2) is the cytogenetic hallmark of synovial sarcoma, being present in more than 90% of all the cases reviewed in the literature.[3–5] It produces three types of fusion gene formed in part by *SS18* (also known as *SYT*) from chromosome 18 and by *SSX1*, *SSX2* or *SSX4* from the X chromosome.[6] The *SS18–SSX* fusion gene is always retained during tumor progression and is transcribed from the der(X) chromosome. In the oncogenic fusion protein, the eight carboxy (C)-terminal amino acids of SS18 are replaced with the last 78 amino acids of SSX.[7]

It has previously been shown that a correlation exists between histologic subtype and type of fusion gene, with biphasic and monophasic histologies being more characteristic of the *SS18–SSX1* and *SS18–SSX2* fusions, respectively.[8] Furthermore, patients with *SS18–SSX1* fusion genes have a worse clinical outcome than patients with

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SS18–SSX2-positive tumors.[9] The *SS18–SSX4* fusion gene is much less frequent than the other two types, making up less than 1% of the cases, and nothing is known about its clinical impact.[9,10]

Although the different *SS18–SSX* fusion genes, as well as the variant reported in a single case,[11] are readily detected by reverse transcriptase-polymerase chain reaction (RT-PCR), sufficient amounts of good-quality RNA may not always be available. Thus, we wanted to develop a fluorescence *in situ* hybridization (FISH) assay that could distinguish between the two most common fusion genes, that is, *SS18–SSX1* and *SS18–SSX2*. For this purpose, we identified bacterial artificial chromosome (BAC) clones, specific for the *SSX1, SSX2* and *SS18* genes, which should allow the detection of synovial sarcoma fusion genes by FISH not only in metaphase cells, but also in interphase cells. The specificity of these probes, as well as the sensitivity of the analysis, was examined in a series of 28 synovial sarcoma samples with known fusion gene status.

Materials and methods

A total of 28 samples from 21 patients with synovial sarcoma were processed for FISH investigations. From three patients, multiple (2–5) samples were investigated. Culturing, harvesting of tumor cells and chromosome banding were performed as previously described.[12] The karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[13]

All the samples were analyzed beforehand by RT-PCR, as reported elsewhere,[14] in order to detect the type of fusion gene (*SS18–SSX1* or *SS18–SSX2*). The clinical, cytogenetic and molecular features are listed in Table 1.

FISH was carried out as described.[15] The following BAC clones were used as probes in the FISH experiments: RP11-38O23 (Accession No. AL356464; map position 47022510–47096260), RP11-552E4 (AL683817; 47096261–47173328) and RP11-344N17 (AL606490; 47173329–47311004) as a pool for the *SSX1* gene; RP11-552J9 (AL450023; 51569622–51753153) and RP13-77O11 (AL445236; 51753154–51900350) as a pool for the *SSX2* gene; and RP11-737G21 (AC027229; 21729417–21788220), RP11-786F14 (AC091021; 21788221–21981898) and RP11-399L5 (AC016839; 21981899–22097735) as a pool for the *SS18* gene. The clones belong to the RPCI library (http://www.chori.org/bacpac/) and were obtained from Resources for Molecular Cytogenetics (http://www.biologia.uniba.it/rmc/). The UCSC database (University of California Santa Cruz, http://genome.ucsc.edu/index.html, April and July 2003 releases) was queried for the location of the clones. The BAC clones RP11-552E4 and RP11-344N17 were selected and used previously by Storlazzi *et al*[11] as a pool for the whole cluster of *SSX* genes, on the basis of the June 2002 release of the UCSC database. Probes for the *SSX1* gene were directly labeled with Cy3-dUTP (Amersham Biosciences, UK); probes for the *SSX2* gene were indirectly labeled with biotin–dUTP (Enzo, Roche, Germany) and detected with streptavidin–diethylaminocoumarin (Roche, Germany and Molecular Probes, The Netherlands); probes for the *SS18* gene were directly labeled with Fluor-X-dCTP (Amersham Biosciences, UK). The labeling of all the clones was performed by use of Amersham's Mega Prime kit (Amersham Biosciences, UK). The hybridizations were performed on metaphase and interphase cells from short-term cultures, and were analyzed with the aid of the Chromofluor System (Applied Imaging, Newcastle, UK).

In the present FISH analysis, a pool of BAC probes mentioned before spanning the *SSX1*and *SSX4* genes was cohybridized with a pool of BACs covering the entire *SSX2* gene and with a pool of BAC probes specific for the *SS18* gene in order to recognize, in a single hybridization experiment, if an *SS18–SSX1* or an *SS18–SSX2* fusion gene was formed. These clones were selected with the aim of covering more than the entire length of the involved genes. Hence, wherever the breakpoints are located in the *SSX1, SSX2* and *SS18* genes, the two parts originating from the splitting of the pools of BAC probes would always be large enough to be detected by FISH. This is particularly important for revealing fusion genes in cases with unusual breakpoints or with more complex translocations, for example, three-way translocations.

All the BAC clones were tested one by one on metaphase and interphase cells of healthy donors in order to analyze the strength of the signals, the lack of crosshybridization or chimeric signals and the absence of background. Then, the pools of clones were also cohybridized on normal male cells with the purpose of evaluating the pattern of the FISH signals and the efficiency of the probes when used together. The results of the latter experiment are shown in Figure 1a.

Then, 28 samples of synovial sarcoma were analyzed. These samples had been selected so as to include both *SSX1*- and *SSX2*-positive cases, as well as one of the very rare cases with the *SS18–SSX4* fusion gene, and to represent both cytogenetically simple and complex variants of the t(X;18). All signal observations were performed in a blinded manner, without knowing the results of the karyotypic or RT-PCR analyses. A total of 10 metaphases and 50 interphase nuclei for each case were counted. Signals in overlapping and incomplete nuclei were not recorded. A schematic illustration of the expected FISH signals in normal male and female nuclei and in nuclei carrying the translocation t(X;18)(p11.2;q11.2), together with the ideograms of the rearranged chromosomes, is shown in Figure 2.

Table 1 Cytogenetic, PCR and FISH findings in 28 synovial sarcomas

Sample[a]	*Sex*	*PCR*[b]	*Karyotype*[c]	*FISH*[d]					
				SSX1 %		*SSX2 %*		*Other*[e] *%*	
				M	*I*	*M*	*I*	*M*	*I*
1a P	F	*SSX2*	44,−X,t(X;18)(p11;q11),add(5)(p11),−15/44,idem,t(3;12)(p23;q12)	0	0	100	76	0	24
1b R	F	*SSX2*	44,−X,t(X;18)(p11;q11),t(3;12)(p23;q12),add(5)(p11),−15/44,idem,−t(3;12),+r	0	0	70	32	30	68
1c M	F	*SSX2*	40–49,−X,t(X;18)(p11;q11),t(3;12)(p23;q12),add(5)(p11),+1–3r,inc	0	0	100	76	0	24
1d R	F	*SSX2*	44,−X,t(X;18)(p11;q11),t(3;12)(p23;q12),add(5)(p11),−15	0	0	60	92	40	8
1e M	F	*SSX2*	44,−X,t(X;18)(p11;q11),add(5)(p11),−15	0	0	100	88	0	12
2a M	M	*SSX1*	44,Y,t(X;18)(p11;q11),del(1)(p12p22),del(3)(p12p25),inv(9)(p11q12)c,−11,−22	100	88	0	0	0	12
2b M	M	*SSX1*	41–44,same as above,−4,add(6)(q21),−8,−14,add(16)(p11),+mar	100	96	0	0	0	4
2c M	M	*SSX1*	42–44,Y,t(X;18),del(1)(p12p22),del(1)(p22),+-der(1;?)(p10;?)t(?;3)(?;q21),−2,−3,der(4)t(4;5)(q24;q13),add(5)(p14),−8,inv(9)(p11q12)c,−11,add(11)(p15),−12,?del(13)(q22),−14,−15,+der(?)t(?;12)(?;--15, +*der*(?)*t*(?; 12)(?; *q*15), +4*mar*	100	90	0	0	0	10
3a R	M	*SSX1*	43,Y,t(X;18)(p11;q11),del(2)(p23),dic(3;?)(p11;?),der(5)del(5)(q11q13)ins(5;?)(q11;?)add(5)(p15),t(7;22)(q11;q13),−11,add(15)(q26),−18,−19,−21,+mar	100	90	0	0	0	10
3b M	M	*SSX1*	Same as above/43,idem,t(4;13)(p14;q13)/85–86,idemx2	70	90	0	0	30	10
4 P	M	*SSX1*	44–46,XY,add(3)(q13),der(5)t(5;8)(p15;q13)ins(5;?)(p15;?),der(5)t(5;8)(q35;q13)ins(5;?)(q35;?),−8,−9,add(9)(p24),der(10;13)(q10;q10),+del(10)(q22),+add(15)(q22),−17,−17,+der(?)t(?;8)(?;q13)ins(?;5)(?;?)t(?;8)(?;q13)	40	0	0	0	60	100
5 P	M	*SSX2*	46,Y,t(X;18)(p11;q11)/45,Y,der(X)t(X;18),del(5)(q13),der(7)t(7;8)(q11;p21),der(8)t(X;8)(p11;p21),−10,ins(14;?)(q24;?) or der(14)t(5;14)(q31;q24),-der(18)t(X;18)(p11;q11)t(X;7)(p11;q11)	0	0	100	86	0	14
6 M	M	*SSX1*	45,Y,t(X;18)(p11;q11),der(2)t(2;8)(q13;q21)t(2;2)(p13;q37)add(2)(q13),der(8)t(2;8)(p13;q21),add(9)(q32),der(10;14)(q10;q10)/45,idem,der(6)t(1;6)(q21;q27)	100	84	0	0	0	16
7 P	M	*SSX2*	58–59,Y,der(X)t(X;18)(p11;q11)x2,+Y,−1,der(2)t(2;3)(p25;q21),−3,−3,−4,−5,−6,−10,−11,+12,−13,−13,−14,−15,add(15)(q22),−16,−17,−18,−19,−20,−21,+22,add(22)(q13)x2,+der(?)t(?;1)(?;q21),+3mar	0	0	100	30	0	70
8 P	M	*SSX2*	46,Y,t(X;18)(p11;q11),der(9)t(9;9)(p21;q21)	0	0	100	84	0	16
9 P	M	*SSX2*	46,Y,t(X;18)(p11;q11)	0	0	100	74	0	26
10 P	M	*SSX2*	86–87,Y,der(X)t(X;18)(p11;q11)x2,−Y,add(1)(p36),add(1)(q11),del(1)(q11),+der(1)t(1;5)(p13;q13)x2,−2,−3,del(3)(q12),−4,−5,add(6)(q15),−7,−9,−10,−10,−11,?add(12)(q13),−13,?−14,−19,−19,−20,−21,−21,+2−3r,inc	0	0	100	82	0	18
11 P	M	*SSX1*	43,Y,t(X;18)(p11;q11),−6,der(12)t(12;?13)(p12;q12),−13,−14,−15,add(16)(q24),add(22)(q13),+mar	100	70	0	0	0	30
12 P	F	*SSX1*	94–96,XX,t(X;18)(p11;q11)x2,+4,+6,+der(8;18)(q10;q10)x2,+9,add(11)(q25)x2,+12,+13,−14,−15,+17,add(17)(q23)x2,+22,+22/95–101,idem,+13,+17/95–99,idem,+22/98,idem,−2,−4,+22	90	74	0	0	10	26
13 P	F	*SSX4*	46,X,der(X)t(X;18)(p11;q11)del(X)(q13),+der(X)t(X;18)del(X),der(5)t(5;6)(q31;q21),add(6)(q13),del(12)(q22),−18	NA	8	NA	0	NA	92
14 P	M	*SSX2*	46,Y,t(X;16;18)(p11;q22;q11),t(1;15)(q21;q24)	NA	0	NA	72	NA	28
15 P	M	*SSX1*	46,Y,t(X;20;8;15;18)(p11;p13;p11;q23;q11)	100	84	0	0	0	16
16 P	F	*SSX2*	46,X,t(X;18)(p11;q11)	NA	0	NA	88	NA	12
17 P	F	*SSX1*	51,X,t(X;18)(p11;q11),+2,+12,+15,+16,+17	NA	88	NA	0	NA	12
18 P	F	*SSX2*	49–52,X,t(X;18)(p11;q11),del(3)(p21p22),t(6;7)(q11;p21),+8,+add(12)(p12)x1–2,del(13)(q22q32),+16,+21	NA	0	NA	96	NA	4
19 P	F	*SSX1*	47,X,t(X;18)(p11;q11),+2	NA	90	NA	0	NA	10

Table 1 Continued

Sample[a]	Sex	PCR[b]	Karyotype[c]	FISH[d]					
				SSX1 %		SSX2 %		Other[e] %	
				M	I	M	I	M	I
20 P	F	SSX1	45,X,t(X;18)(p11;q11),add(1)(p34),del(3)(p13), der(10)t(10;?14)(p12?2;q2?4),−13,−14,der(16)t(?13;16)(q12–14;q24),+mar	NA	90	NA	0	NA	10
21 P	F	SSX2	45,X,der(X)t(X;18)(p11;q11),−3,−11,t(11;12)(q13;q24),+12, −13,add(14)(p11),der(16)t(3;16)(q12–21;q12–21),−18,−20,+21, +2mar/41–44,X,der(X)t(X;18),del(1)(p22),−3,−4,add(4)(p1?), der(5)t(5;14)(p11;q11),−6,−11,t(11;12),+12,−13,−14,der(16)t(3;16),?inv(16) (p11q12),−18,+2mar	NA	0	NA	84	NA	16

[a]Numbers refer to different patients; lower case letters refer to different samples from the same patient; capital letters: P = primary lesion; R = local recurrence; M = metastasis.
[b]*SSX1* = *SS18–SSX1* fusion transcript; *SSX2* = *SS18–SSX2* fusion transcript; *SSX4* = *SS18–SSX4* fusion transcript.
[c]The complete karyotypes of all samples except 1b, 1d, 1e and 16–21 have been reported before.[14]
[d]NA = not analyzed; M = metaphase; I = interphase.
[e]Includes cells with normal hybridization signals.

Results

In the present study, metaphase and interphase nuclei from 28 synovial sarcoma samples with known fusion gene status were analyzed with FISH probes specific for the *SSX1*, *SSX2* and *SS18* loci. In all samples, the type of fusion gene was correctly identified by FISH (Table 1). At FISH, 19 samples showed a simple translocation t(X;18)(p11.2;q11.2), with the formation of *SS18–SSX1* (10 samples) or *SS18–SSX2* (nine samples) fusion genes. In the samples displaying the *SS18–SSX1* fusion, the distinctive features were the splitting of the *SSX1* (red) and *SS18* (green) signals into two parts and the colocalization of *SSX1* and *SS18* (red + green) signals, as observed both in metaphase and interphase cells (Figure 1b–d). In the samples with a breakpoint in *SSX2*, the splitting of the *SSX2* (violet) and *SS18* (green) signals and the colocalization of *SSX2* and *SS18* (violet + green) signals were always detected (Figure 1e and f). Each case showed only one type of fusion gene and was always consistent with the results of the cytogenetic and RT-PCR studies.

Nine samples displayed variant hybridization patterns. Sample 4 was t(X;18)-negative and showed a highly complex karyotype with many unresolved aberrations at cytogenetic analysis, but it was positive for the *SS18–SSX1* fusion gene by RT-PCR. FISH revealed the *SS18–SSX1* fusion gene in 40% of the metaphase cells, but not in interphase cells due to the high number of normal cells. Thus, a cryptic rearrangement involving the *SS18* and *SSX1* genes had occurred, but the chromosomal location of the fusion gene could not be determined.

Sample 5 exhibited the expected pattern of *SS18–SSX2* FISH signals, but in 80% of the metaphase cells the der(18) was longer than usual, suggesting a more complex translocation involving also chromosome 7. The remaining 20% of the metaphase cells displayed an ordinary t(X;18).

Sample 7 showed an atypical pattern of FISH signals because, in addition to the expected *SS18–SSX2* fusion gene on the der(X) and the *SS18* green signal on the normal chromosome 18, it displayed two *SS18* signals on two marker chromosomes and two *SSX1* and *SSX2* signals on two other markers. No colocalization of green, violet and red signals was seen, indicating the absence of a der(18).

Sample 10 was at G-banding analysis interpreted to have two copies of a der(X)t(X;18), but no der(18)t(X;18). However, FISH experiments using centromeric and whole chromosome painting probes specific for chromosomes X and 18 revealed also the der(18)t(X;18) and a third chromosome carrying a part of the X chromosome. The colocalization of the three signals specific for the *SS18, SSX1* and *SSX2* genes, consistent with an *SSX2–SS18* fusion gene, was seen on the der(18).

Sample 11 displayed a karyotype that at G-banding was interpreted to contain a balanced

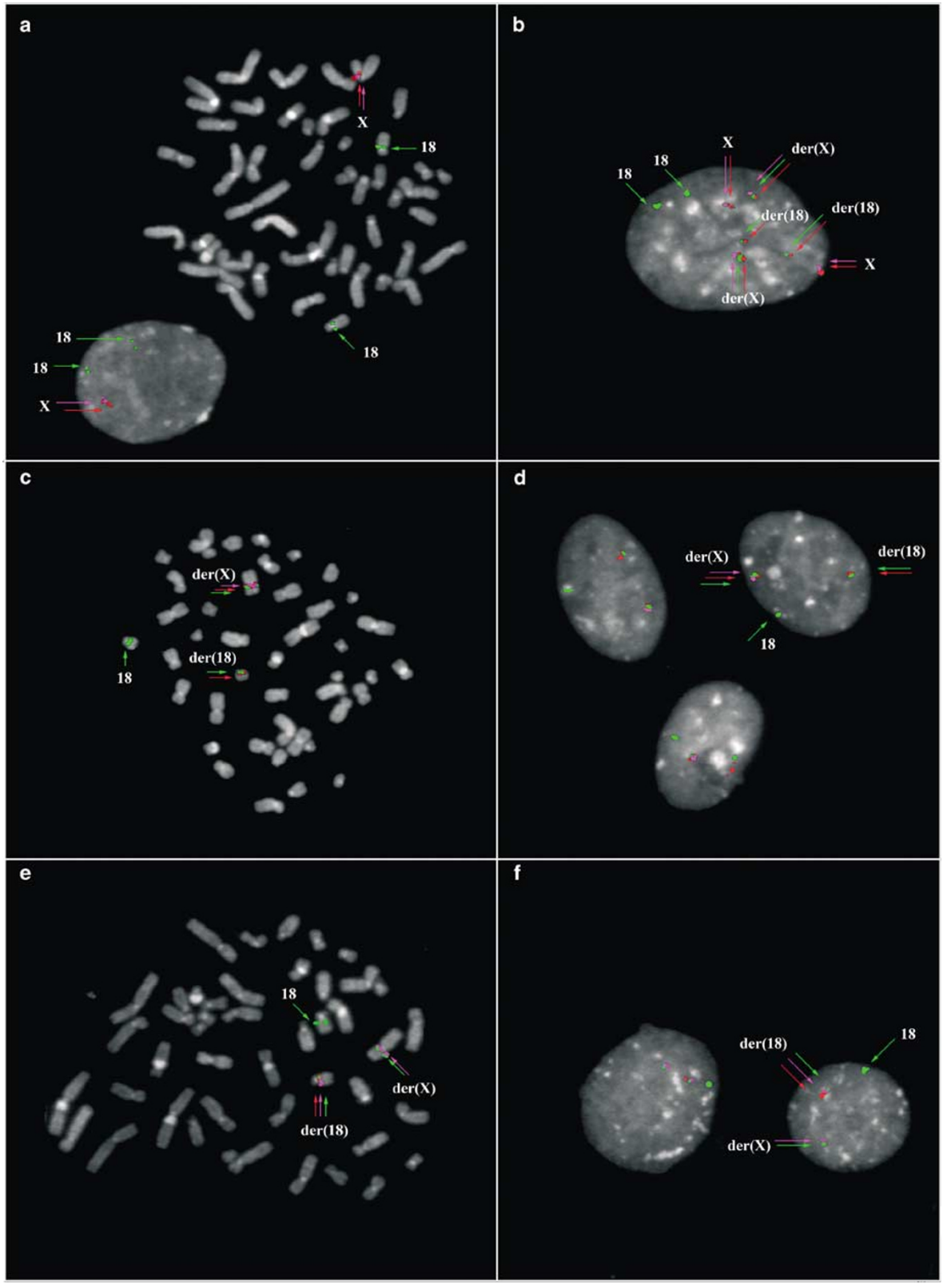

Figure 1 Results of some FISH experiments: *SSX1* in red, *SSX2* in violet, *SS18* in green. (**a**) Pattern of FISH signals in interphase nucleus and metaphase of a normal male. (**b**) Distribution of signals in a tetraploid nucleus of a female (Sample 12) carrying the t(X;18) and an *SS18–SSX1* fusion gene. (**c** and **d**) Signals in metaphase and interphase nuclei of two male patients (Samples 6 and 3b) with t(X;18) and the *SS18–SSX1* fusion. (**e** and **f**) Signals in a metaphase cell and interphase nuclei of two different male patients (Samples 9 and 8) carrying the t(X;18) and the *SS18–SSX2* fusion.

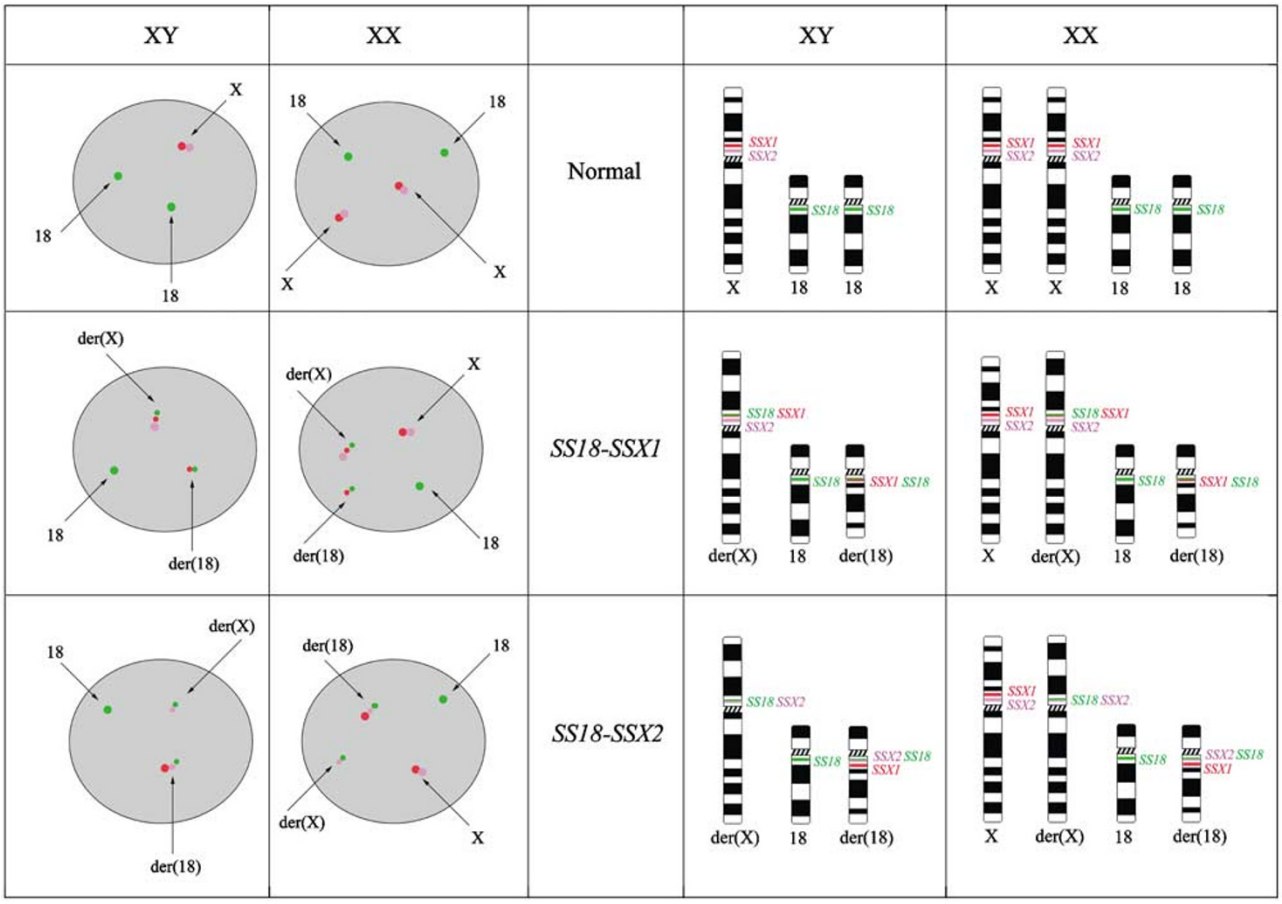

Figure 2 Schematic representation of the patterns of the expected FISH signals in normal male and female nuclei and in nuclei carrying the translocation t(X;18)(p11.2;q11.2) (left side). Ideograms of male and female chromosomes X and 18, showing the localizations of the genes *SSX1*, *SSX2* and *SS18* in the normal chromosomes and in rearranged ones (right side).

t(X;18), and RT-PCR identified an *SS18–SSX1* fusion gene. However, apart from displaying a typical der(X), FISH studies revealed a more complex rearrangement of the der(18), which exhibited a split and/or duplicated *SS18* signal, but no *SSX1* signal, and which was rearranged with a third chromosome that displayed the *SSX1* signal. What was originally interpreted as the der(18)t(X;18) turned out to be a der(?) consisting in most part of chromosome X material.

Sample 15, in addition to the colocalization of three signals on the der(X) and the *SS18* green signal on chromosome 18, displayed the green and red signals specific for the *SS18* and *SSX1* genes on two different chromosomes. The karyotype showed a complex translocation t(X;20;8;15;18), which was in agreement with this variant pattern of FISH signals.

In samples 13, 14 and 16–21 only interphase nuclei were analyzed. In sample 13, the nuclei showed colocalization of the *SS18* and *SSX1* signals, but the RT-PCR results had revealed an *SS18–SSX4* fusion gene. The FISH data fit with the RT-PCR results because *SSX4* is located close to *SSX1* and was included in BAC clone RP11-344N17, the most proximal clone among the three clones of the pool used for *SSX1*.

In sample 14, almost 3/4 of the nuclei (72%) showed the colocalization of signals specific for the *SS18* and *SSX2* genes, but not the colocalization of green, violet and red signals. This is consistent with the complex translocation between the chromosomes X, 16 and 18, as shown by G-banding.

In sample 21, the signal for *SSX2* was split and colocalized with the signal for *SS18*. No signal pattern corresponding to a der(18)t(X;18) was seen, in agreement with the cytogenetic findings.

Discussion

In the present study, we used eight BAC clones covering the genes *SSX1*, *SSX2* and *SS18* to identify the type of fusion gene (*SS18–SSX1* or *SS18–SSX2*) in 28 synovial sarcoma samples. Since the identification of the *SSX* and *SS18* genes,[6,16] this is, to our knowledge, the largest series of synovial sarcoma samples screened for the type of fusion gene by FISH. Previously, FISH assays have been performed

with whole chromosome painting, alphoid, YAC, PAC and cosmid probes, mainly with the purpose of detecting the t(X;18), irrespective of the type of *SSX* gene involved.[17–30]

The BAC clones that we selected allowed us not only to detect a simple t(X;18), but also more complex variant translocations. In fact, at FISH analysis, only 19/28 samples displayed a simple t(X;18), whereas the remaining nine samples showed further aberrations. The former consisted of 10 samples having the *SS18–SSX1* fusion gene and nine samples with *SS18–SSX2*. Among the samples with more complex rearrangements, three showed the *SS18–SSX1* fusion gene, five had *SS18–SSX2* and one displayed *SS18–SSX4*. We never observed the presence of both *SS18–SSX1* and *SS18–SSX2* fusion genes in the same sample, which is in contrast to a recent publication in which RT-PCR and FISH findings were interpreted to suggest the simultaneous occurrence of the two fusion genes in a subset of synovial sarcoma.[30] This discrepancy could be due to chance; as only 10% of the cases reported by Yang *et al*[30] were positive for both *SS18–SSX1* and *SS18–SSX2*, such cases might not have been included in our series. On the other hand, synovial sarcomas expressing more than one fusion gene have not been reported by others, why artefacts from nested RT-PCR must be considered a possible explanation for this phenomenon.

In the present study, we had included one of the very rare cases of synovial sarcoma with the *SS18–SSX4* gene. Obviously, this type of fusion could not be distinguished from the *SS18–SSX1* fusion gene because the *SSX4* gene is included in the clone RP11-344N17, the most proximal clone in the BAC pool used for the detection of *SSX1* rearrangement. Since *SSX1* is contained in the clone RP11-552E4, the separate use of RP11-552E4 or RP11-344N17 could, theoretically, distinguish between *SSX1* and *SSX4* rearrangements. However, bearing in mind the low frequency of *SS18–SSX4* fusions, we preferred to add the RP11-344N17 clone in order to obtain strong and unequivocal split signals. Furthermore, there are so far no data indicating that patients with *SS18–SSX4*-positive synovial sarcoma should have a better prognosis than those with *SS18–SSX1*-positive tumors.

In almost all the cases, the percentage of interphase cells showing *SS18–SSX1* or *SS18–SSX2* fusion gene by FISH was lower than in the corresponding metaphase cells. Most probably, there are several explanations for this discrepancy. First, a higher mitotic activity of the t(X;18)-carrying cells *in vitro* would lead to a relative enrichment of sarcoma cells among the dividing cells. Second, hybridization patterns are in general more difficult to interpret at the interphase level than at the metaphase level, especially in cases with complex rearrangements. Third, interphase nuclei often yield signals that are located in different planes, making them difficult to capture simultaneously in the microscope. Hence, the interpretation of FISH signals was sometimes not straightforward, especially in the samples displaying complex chromosome rearrangements. However, because the FISH assay that we set up is based on the simultaneous detection of split signals for the involved genes and their colocalization, we were able to minimize the problems in interpreting unusual hybridization patterns that may arise from, for example, polyploidization or three-way translocations. Furthermore, it should be noted that the cases that were included in our study were selected not only on the basis of their *SS18–SSX* fusion status, but also so that as many complex karyotypes and varieties of chromosome X and 18 rearrangements as possible could be analyzed. In an unselected series of synovial sarcoma, the frequency of cases with an ordinary t(X;18) would be expected to be much higher.

Thus, apart from being a valuable tool, complementary to RT-PCR and independent on RNA degradation, in characterizing the status of the *SSX* and *SS18* genes in metaphase spreads, the results from interphase nuclei also suggest that the BAC probes can be employed in FISH studies on archived samples.

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