

The SYT-SSX1 Variant of Synovial Sarcoma Is Associated with a High Rate of Tumor Cell Proliferation and Poor Clinical Outcome¹

Gunnar Nilsson,² Björn Skytting,² Yuntao Xie,² Bertha Brodin,² Roland Perfekt, Nils Mandahl, Joakim Lundeberg, Mathias Uhlén, and Olle Larsson³

Cellular and Molecular Tumor Pathology, CCK, R8: 04 [G. N., Y. X., B. B., O. L.] and Department of Orthopedics [G. N.], Karolinska Hospital, SE-171 76 Stockholm, Sweden; Department of Orthopedics, Stockholm Söder Hospital, SE-100 64 Stockholm, Sweden [B. S.]; Department of Biotechnology, Royal Institute of Technology (KTH), SE-100 44 Stockholm, Sweden [B. B., J. L., M. U.]; and Southern Swedish Regional Tumor Registry [R. P.] and Department of Clinical Genetics [N. M.], University Hospital, SE-221 85 Lund, Sweden

ABSTRACT

Cytogenetically, synovial sarcoma (SS) is characterized by the translocation t(X;18)(p11.2;q11.2), resulting in a fusion between the *SYT* gene on chromosome 18 and *SSX1* or *SSX2* on the X chromosome and the formation of new chimeric genes, *SYT-SSX1* or *SYT-SSX2*. We examined the potential clinical relevance of *SYT-SSX1* and *SYT-SSX2* fusion transcripts together with tumor proliferation. In a series of 33 patients with primary SS, the type of fusion transcript was assessed by reverse transcription-PCR and sequence analysis. The proliferation rate was analyzed using anti-Ki-67 antibodies. One case carrying an atypical transcript with a 57-bp insert was excluded, leaving 13 *SYT-SSX1* and 19 *SYT-SSX2* cases for analysis. The hazard ratio (with respect to metastasis-free survival for patients with *SYT-SSX1* versus patients with *SYT-SSX2* fusion transcripts was 7.4 (95% confidence interval, 1.5–36; log-rank $P = 0.004$). There was also an association with reduced overall survival for patients with *SYT-SSX1* compared to patients with *SYT-SSX2* (hazard ratio, 8.5; 95% confidence interval, 1.0–73; log-rank $P = 0.02$). The 5-year metastasis-free survival for patients with *SYT-SSX1* was 42% versus 89% for patients with *SYT-SSX2*. There was a significant association between *SYT-SSX1* and a high tumor proliferation rate ($P = 0.02$). We conclude that the findings suggest that the type of *SYT-SSX* fusion transcript determines the proliferation rate and is an important predictor of clinical outcome in patients with SS.

INTRODUCTION

SS⁴ accounts for 5–10% of soft tissue sarcomas and is mainly located in the extremities. SS can occur at any age, including childhood, but is most commonly seen in young adults (1). Histologically, a biphasic variant composed of varying proportions of epithelial and spindle cells and a monophasic variant predominantly containing spindle cells are recognized (2).

Cytogenetically, SS is characterized by the translocation t(X;18)(p11.2;q11.2) (3). Cloning of the breakpoints of this translocation revealed the fusion of two novel genes, *SYT* and *SSX* (4). The *SYT* gene, located on chromosome 18, is fused with one of three closely related genes, *SSX1*, *SSX2*, or *SSX4*,⁵ located on the X chromosome (5, 6). The frequency of *SYT-SSX4* is still unknown. The fused genes form a chimeric protein in which 8 amino acids of the COOH-terminal of *SYT* are replaced by 78 amino acids from the COOH-terminal of

either of the *SSX* proteins. Five highly homologous *SSX* genes (*SSX1–5*) have been described, all of which are located in chromosome band Xp11.2 (5, 7, 8). In contrast to the *SYT* gene, which is widely expressed in human tissues, the *SSX* genes seem to be expressed only in testis and thyroid (5). The biological properties of normal *SYT* and *SSX* proteins are largely unknown. However, recent studies indicate that wild-type *SYT* and *SSX* play an active role in transcription, although they lack direct DNA-binding domains. The *SYT* protein is rich in proline, glutamine, and glycine, which is characteristic of transcriptional activators (9). The *SSX* proteins have two well-preserved areas: (a) one resembling Krüppel-associated box-A; and (b) the other located in the COOH-terminal, both of which have repression activity (10). However, the Krüppel-associated box-related domain is not retained at the fusion with *SYT*. The resulting chimeric gene most probably shows an altered transcriptional pattern, possibly through *SSX*-mediated binding sites.

Besides large tumor size, which is a well-known factor associated with poor clinical outcome in SS (11, 12), there are few objective markers predicting prognosis. In a recent study, however, it was demonstrated that Ki-67, a proliferation marker, is an independent prognostic factor in SS (13).

A recent study comparing clinical data and the type of *SYT-SSX* fusion suggests that *SYT-SSX1* is less favorable in terms of metastasis-free survival (14). Kawai *et al.* (14) investigated the metastasis-free survival rate in material from 39 SS cases with RT-PCR analysis using *SSX1*- and *SSX2*-specific primers. However, a substantial number of their samples were from metastases or local recurrences, and only a limited amount of their material was sequenced. Although there seems to be a low rate of polymorphism in the *SYT-SSX* genes, aberrant cases with insertions in exon 5 of the *SSX* genes have been reported previously (5). Here we report on material from 33 primary SSs. No patients had metastases at diagnosis, and all fusion transcripts were sequenced.

MATERIALS AND METHODS

Patients. From 1988 to 1998, 33 patients with histologically verified SS and material available for cytogenetical analysis were referred to a Scandinavian Sarcoma Group center (Table 1). There were 16 males and 17 females, with a mean age at diagnosis of 40 years (range, 10–80 years). Eighteen tumors were located in the lower extremities, eight tumors were located in the upper extremities, and seven tumors were located in the central axis. The mean tumor size was 7 cm (range, 3–23 cm), based on the largest tumor diameter as assessed by preoperative magnetic resonance imaging or computer tomography. The tumor specimen used for analysis came from primary lesions in all 33 patients. All histological material was reviewed by a single pathologist at the Karolinska Hospital (O. L.) and classified according to histological type, subtype, and Ki-67 index. Histologically and immunohistochemically, all tumors were found to be SSs. Four tumors were considered biphasic, and 28 tumors were considered monophasic (case 28 is missing due to preoperative treatment). All tumors were high-grade lesions (grade III and grade IV) on a four-grade scale (15, 16), and all but one (case 7) was deep-seated. All patients were treated surgically with a curative intent. The final surgical margins were

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² These authors contributed equally to this work.

³ To whom requests for reprints should be addressed, at Cellular and Molecular Tumor Pathology, CCK, R8-04, Karolinska Hospital, SE-171 76 Stockholm, Sweden. E-mail: olle.larsson@onkpat.ki.se.

⁴ The abbreviations used are: SS, synovial sarcoma; RT-PCR, reverse transcriptase-PCR; CI, confidence interval; HR, hazard ratio.

⁵ B. T. Skytting, G. Nilsson, B. Brodin, Y. Xie, J. Lundeberg, M. Uhlén, and O. Larsson. A novel fusion gene, *SYT-SSX4*, in synovial sarcoma. *J. Natl. Cancer Inst.*, in press, 1999.

Table 1 Characteristics of 33 primary SS patients^a

Patient no.	Age (yr)/ Sex	Primary site	Tumor size (cm)	Sample source	Histological subtype	SYT-SSX fusion type	Ki-67 ^b (%)	Adjuvant therapy	Time to recurrence (mo)	Metastasis		Follow-up (mo)	Status
										Site	Time after diagnosis (mo)		
1	50/F	Thigh	6	P	Monophasic	2	1	R	50			68	NED
2	18/F	Foot	5	P	Monophasic	2	0	R				29	NED
3	37/F	Low trunk	7	P	Monophasic	2	1	R		Lung	16	29	NED
4	31/F	Gluteal	8	P	Monophasic	2	1	R		Lung	15	16	AWD
5	80/F	Foot	3	P	Monophasic	2	0	R				25	NED
6	31/F	Low arm	5	P	Monophasic	2	0					74	NED
7	72/M	Thigh	5	P	Monophasic	2	0					21	DWD
8	74/M	Retro	23	P	Monophasic	2		R	15			30	NED
9	72/F	Low arm	3	P	Monophasic	2	0					111	NED
10	59/M	Chest wall	5	P	Monophasic	2	0	R	23			40	NED
11	12/M	Shoulder	4	P	Monophasic	2	0	C				17	NED
12	67/F	Knee	13	P	Monophasic	2		R				7	NED
13	44/F	Foot	5	P	Monophasic	2	1					74	NED
14	56/M	Low leg	11	P	Monophasic	2	0					84	NED
15	10/M	Neck	3	P	Monophasic	2	1	C				47	NED
16	38/F	Thigh	10	P	Monophasic	2	0					60	NED
17	26/F	Low arm	3	P	Monophasic	2	1					31	NED
18	18/M	Thigh	12	P	Monophasic	2	0	C+R				22	NED
19	20/F	Thigh	5	P	Monophasic	2	0					2	NED
20	31/F	Shoulder	10	P	Monophasic	2 ^c	1	R				42	NED
21	46/F	Foot	7	P	Monophasic	1	0			Lung	7	36	DOD
22	42/M	Low arm	4	P	Monophasic	1	1		25			59	NED
23	42/F	Low trunk	5	P	Biphasic	1	1			Soft tissue	37	56	DOD
24	18/M	Chest wall		P	Biphasic	1	1		64	Lung	77	96	DOD
25	58/F	Foot	4	P	Biphasic	1	1			Lung	15	25	DOD
26	50/F	thigh	13	P	Monophasic	1	1	R		Lung	5	22	DOD
27	57/F	Low arm	6	p	Biphasic	1	0					50	NED
28	20/M	Thigh	4	P		1		C+R				9	NED
29	45/M	Groin	9	P	Monophasic	1	1	R		Lung	10	16	AWD
30	35/M	Thigh	4	P	Monophasic	1	1	R		Lung	23	69	NED
31	36/M	Knee	10	P	Monophasic	1	1	R				18	NED
32	17/M	Elbow	9	P	Monophasic	1	1	C+R				26	NED
33	14/M	Neck	6	P	Monophasic	1	1	C+R				22	NED

^a P, primary tumor; C, chemotherapy; R, radiotherapy; AWD, alive with disease; DOD, dead of disease; NED, no evidence of disease; DWD, Dead without evidence of disease.

^b 0, Ki-67 index <10%; 1, Ki-67 index ≥10%.

^c Sequencing revealed an insertion of 57 bp between SYT and SSX2 genes.

as follows: 4 intralesional; 16 marginal; 9 wide; and 4 compartmental. Radiotherapy was given to 17 patients. Six patients received adjuvant chemotherapy, all according to an Ifosfamide-based protocol. The mean follow-up period was 46 months (range, 2–111 months).

RT-PCR. Total RNA was isolated using Qiaquick Rneasy (Qiagen, Hilden, Germany). A reverse-transcription using the primer SSX-A 5'-CACT-TGCTATGCACCTGATG-3' (17) was then performed at 42°C for 1 h. The resulting cDNA was amplified by PCR with SYT primer 5'-AGACCAACA-CAGCCTGGACCA-3' (17) and SSX primer 5'-TGCTATGCACCTGAT-GACGA-3' (5). The SSX primer is a consensus primer for both SYT-SSX1 and SYT-SSX2. Amplification was performed at 94°C for 30 s, 64°C for 30 s, and 72°C for 30 s for 35 cycles, and a final elongation was performed for 10 min. SSX1-specific primer 5'-GGTGCAGTTGTTTCCCATCG-3' (17) and SSX2-specific primer 5'-TCTCGTGAATCTTCTCAGAGG-3' (5) were used in a subsequent PCR reaction to discriminate between the two fusion types. A control without reverse transcriptase was included in every case to ensure that the generated PCR products did not represent DNA contamination. In addition, negative controls were included in every step. The actin gene was used as a positive control. In all RT-PCR experiments, strict precautions were taken to avoid cross-contamination or product carry-over. The pre- and postamplification steps were separated from each other. The PCR products were detected by ethidium bromide staining on a 2% agarose gel.

Sequence Analysis. To analyze the breakpoint sequences, PCR products were directly sequenced by cycle sequencing with dye-labeled terminators (BigDye Terminators; Perkin-Elmer, Norwalk, CT) and analyzed on the DNA sequencer ABI PRISM 377XL (PE Applied Biosystems, Foster City, CA). Primers used in the PCR amplification were used as sequencing primers.

Immunohistochemistry. Immunostaining was performed according to the standard Avidin-Biotin Complex technique (Elite Standard Kit catalogue number PK-6100; Vector Laboratories, Burlingame, CA). Paraffin sections were deparaffinized, rehydrated, and pretreated. Antigen retrieval was performed by immersing the specimens for 10 min in citrate buffer (pH 6) and heating in a microwave oven (700 W) for 10 min. After rinsing, the endogenous peroxidase activity was blocked

by hydrogen peroxide dissolved in methanol (3% hydrogen peroxide: methanol, 1:5 v/v) for 30 min. The sections were rinsed and incubated with blocking serum (normal horse serum) for 20 min and then incubated with the primary antibody, anti-Ki-67 (MIB-1; Immunotech, Marseilles, France), diluted 1:50. Incubations were performed overnight at 8°C. After the ABC complex, a biotinylated anti-mouse IgG was used as a secondary antibody. The peroxidase reaction was developed using 3,3-diaminobenzidine for 6 min. Nuclear counterstaining was performed with hematoxylin. Tris-PBS (pH 7.6) was used for rinsing between the steps. The staining was checked with negative and positive controls.

A semiquantitative score was used to assess the percentage of cells that were positively stained, regardless of staining intensity. The percentage of Ki-67 per 10 high-power fields (×250) was graded as follows: 0–1%; 2–9%; 10–24%; 25–49%; 50–74%; and 75–100%. Specimens with a Ki-67 index of <10% were considered to have a low proliferation rate, and specimens with a Ki-67 index of ≥10% were regarded as highly proliferative (13, 18, 19). All of the immunohistochemically stained slides (which were coded) were analyzed

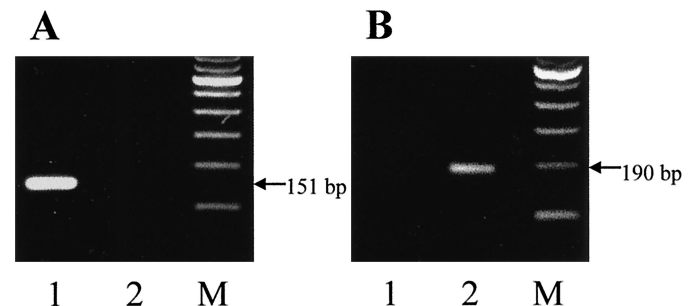


Fig. 1. Detection of fusion transcripts by seminested PCR using SSX1-specific (Lane 1) and SSX2-specific primers (Lane 2) in SS. A, SYT-SSX1 (case 24). B, SYT-SSX2 (case 9). M denotes a 100-bp ladder.



Fig. 2. Alignment of sequenced SYT-SSX1 and SYT-SSX2 fusion transcripts. Cases 23–33 exactly matched the sequence of SYT-SSX1 (GenBank S79325). Cases 1–19 exactly matched with the sequence of SYT-SSX2 (GenBank S79332). In cases 21 and 22, a variant representing a SYT-SSX1 polymorphism (C→T) at position 452 was found. Top, the 57-bp insertion of the variant SYT-SSX2 (case 20). Arrow, the fusion point between SYT and SSX. Exon 5 of the SSX gene is shown between vertical dashed lines.

microscopically by O. L., without knowledge of the clinical characteristics. In each case, more than 1000 cells were analyzed.

Statistical Analyses. Metastasis-free survival and overall survival were analyzed univariately using Kaplan-Meier survival curves and HR estimates from Cox’s proportional hazards model. Patients were followed from time of diagnosis, and the log-rank test was used to evaluate differences between survival curves. One patient who died from non-tumor-related reasons was censored at the time of death in the analysis of metastasis-free survival. Two-sided *P*s from Fisher’s exact test were used to assess associations between categorical variables. In view of the relatively small number of patients, we did not further extend the analyses with multivariate Cox modeling.

RESULTS

RT-PCR Analysis and DNA Sequencing. RT-PCR analysis of 33 SSs using outer primers revealed a transcript of the predicted length (401 bp) in 32 cases. In case 20, the product was 57 bp larger. Seminested PCR was able to discriminate SYT-SSX1 from SYT-SSX2 (Fig. 1). Sequence analysis, which was performed in all 33 cases, showed transcripts that were identical or nearly identical to SYT-SSX1 in 13 cases and identical or nearly identical to SYT-SSX2 in 19 cases. In two of the SYT-SSX1 products, a sense mutation (C→T at position 452; Fig. 2) was observed. All SYT-SSX2 transcripts except for a 57-bp longer variant (case 20) corresponded exactly to those described previously (5). The extra 57 bp represent an insertion between the ordinary SYT and SSX2 fusion (Fig. 2). The origin of this insertion is unknown. Due to the heterogeneity of the fusion product in case 20, this case was excluded from the statistical analysis.

Statistical Analysis using Data from 32 Patients. The characteristics of the SS cases, including SYT-SSX subtype and Ki-67 score, are summarized in Table 1. For patients with SYT-SSX1 transcripts, the mean tumor size was 7 cm, the mean age was 37 years, three patients received Ifosfamide-based adjuvant treatment, and the mean follow-up period was 39 months. For patients with SYT-SSX2 transcripts, the mean tumor size was 7 cm, the mean age was 43 years, three patients received Ifosfamide-based adjuvant treatment, and the mean follow-up period was 41 months. The HR with respect to metastasis-free survival for patients with SYT-SSX1 versus SYT-SSX2 fusion transcripts was 7.4 (95% CI, 1.5–36; log-rank, *P* = 0.004;

Table 2). There was also an association with reduced overall survival for patients with SYT-SSX1 compared to patients with SYT-SSX2 (HR, 8.5; 95% CI, 1.0–73; log-rank *P* = 0.02). The 5-year metastasis-free survival for patients with SYT-SSX1 was 42% compared to 89% for patients with SYT-SSX2 (Fig. 3). The metastasis-free survival for patients with a Ki-67 index of <10% was significantly better than that for patients with a Ki-67 index of ≥10 (HR, 8.3; 95% CI, 1.0–69; log-rank *P* = 0.02) (Table 2; Fig. 4), but no significant difference was found with respect to overall survival (log-rank *P* = 0.53). Neither tumor size (≤5 cm versus >5 cm) nor patient age (≤20 years versus >20 years) was a significant factor for metastasis-free survival (log-rank *P* = 0.34 and 0.37, respectively; Table 2). There was a significant association between the SYT-SSX1 fusion type and Ki-67 index of ≥10% (*P* = 0.02; Fig. 5). An association was also seen between fusion type and histological subtype, because all 19 tumors with SYT-SSX2 fusion transcripts were considered to be of the monophasic subtype, and 4 of 12 tumors with SYT-SSX1 were biphasic (*P* = 0.02).

DISCUSSION

Since the discovery of the tumor-specific translocation t(X; 18)(p11.2;q11.2) in SS, many studies have been performed utilizing the translocation for diagnosis. In well-defined materials, this aberration, including variant translocations, is seen in almost all cases of SS. The finding that cytogenetically indistinguishable translocation results in fusion of the SYT gene with either SSX1 or SSX2 has led to investigations of the clinical implications of the fusion variants. It is possible that the type of fusion transcript is a better indicator of clinical outcome than other genetic changes. Secondary genetic aberrations, as assessed by comparative genomic hybridization, were found in 55% of all SSs (20), but no difference was seen regarding metastasis-free survival or overall survival among patients with or without secondary aberrations.⁶ Over the last few years, several

⁶ B. T. Skytting, J. Szymanska, Y. Aalto, T. Lushnikova, C. Blomqvist, I. Elomaa, O. Larsson, and S. Knuutila. Clinical importance of secondary aberrations in synovial sarcoma evaluated by comparative genomic hybridization, Cancer Genet. Cytogenet., in press, 1999.

Table 2 Univariate analysis of metastasis-free survival in SS^a

Variable	No. of patients (n=32)	5-Year MFS (%) ^b	Log-rank P	HR	95% CI
Age			0.37	2.5	0.3–20
≤20 yr	9	100			
>20 yr	23	63			
Tumor size			0.34	2.0	0.5–8.3
≤5 cm	16	72			
>5 cm	15	67			
Fusion product			0.004	7.4	1.5–36
SYT-SSX1	13	42			
SYT-SSX2	19	89			
Ki-67 index			0.02	8.3	1.0–69
<10%	13	92			
≥10%	16	51			
Histological subtype			0.13	0.4	0.1–1.5
Biphasic	4	50			
Monophasic	27	76			

^a Five-year survival estimates by Kaplan-Meier method; HRs from Cox regression analyses.

^b MFS, metastasis-free survival.

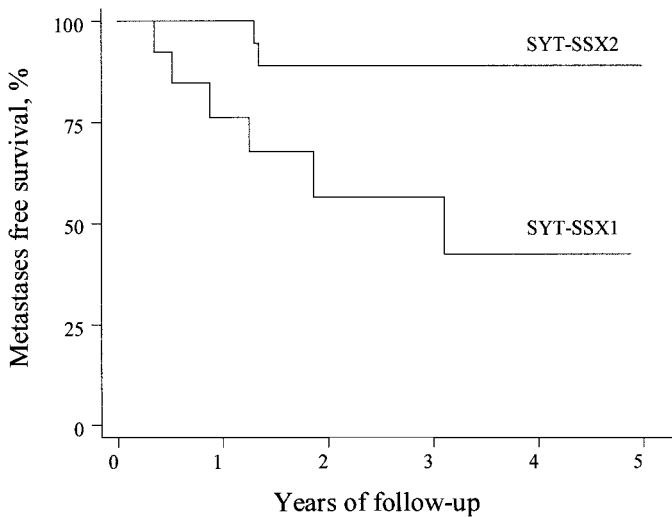


Fig. 3. Metastasis-free survival in 19 patients with SYT-SSX2 and in 13 patients with SYT-SSX1. Metastasis-free survival was significantly shorter among patients with SYT-SSX1 fusion transcripts (log-rank $P = 0.004$).

groups have reported associations between histopathological features and the two different fusion types, but none except Kawai *et al.* (14) have systematically compared the type of fusion transcript and clinical outcome. Kawai *et al.* (14) found that patients with tumors positive for SYT-SSX1 had a high risk of early metastases compared to patients with SYT-SSX2, who had a higher risk for late metastases, resulting in similar metastasis-free survival curves after 4–5 years. However, their material of 39 samples included 12 metastases and 4 local recurrences, representing more than 40% of the material. This subset of cases also had the longest follow-up. Including patients solely on the basis of the availability of frozen tumor material, without respect to whether the samples come from primary tumor, local recurrence, or metastasis, may lead to selection bias.

Several studies have shown aberrant variants of SYT-SSX1 and SYT-SSX2 (5, 6, 17). In the present study, we demonstrate a 57-bp insertion at the SYT-SSX2 fusion point (case 20), and we recently described a new SYT-SSX fusion gene involving SSX4.⁵ The use of SSX1- and SSX2-specific PCR primers failed to separate SYT-SSX4 from SYT-SSX1 and SYT-SSX2. Therefore, in studies of the impact of the type of fusion transcript on clinical outcome, we believe that it may be of great importance to sequence all transcripts; for this reason, we excluded case 20 to analyze a homogeneous cohort. We were

unable to find out how many cases from the study of Kawai *et al.* (14) actually were sequenced, but as far as we can understand, one case with an alternative fusion point (17), considered to represent a SYT-SSX2 transcript, was included in their prognostic analysis. In this study, we present material exclusively from primary tumors in which all PCR products were sequenced. Our findings show that patients with SYT-SSX1 have a significantly reduced metastasis-free survival and overall survival, which corroborates the findings of Kawai *et al.* (14). However, in contrast to the previous study, we did not find any increase in late-occurring metastases among patients with SYT-SSX2.

Tumor size, which is a well-accepted prognostic factor for SS (21, 22), was not significant in our series (log-rank $P = 0.34$). It is possible that this could be due to the small number of cases investigated.

Because only six of our patients had received chemotherapy (three patients with SYT-SSX1 and three patients with SYT-SSX2), the difference in metastasis-free survival could not be attributed to adjuvant chemotherapy.

Tumor proliferation assessed by the Ki-67 index (<10% versus ≥10%) was significant (log-rank $P = 0.02$) for metastasis-free survival, which is in conformity with recent results regarding SS presented by us (13). Ki-67 is a well-established proliferation marker and is only expressed during the proliferation (late of G₁, S phase, G₂, and

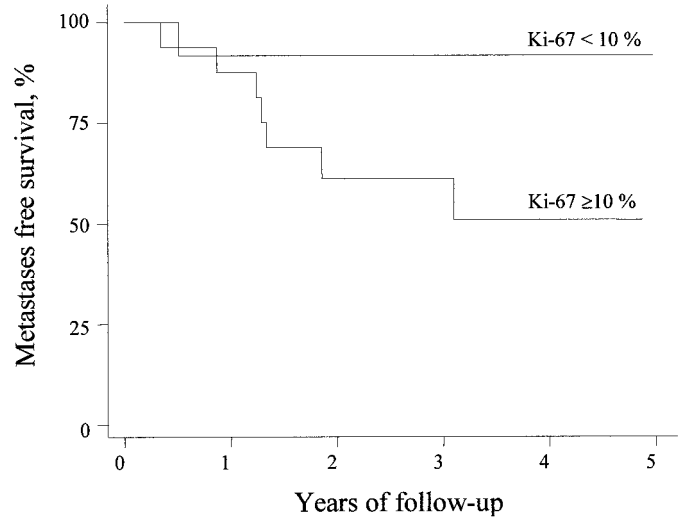


Fig. 4. Metastasis-free survival for patients with a Ki-67 index of ≥10% was significantly shorter than for patients with a Ki-67 index of <10% (log-rank $P = 0.02$).

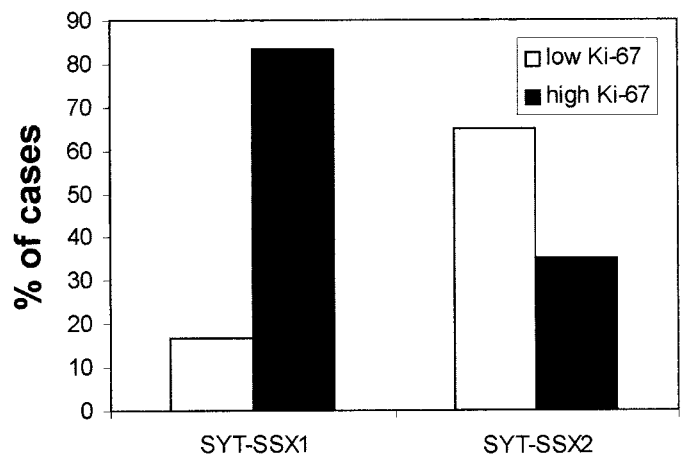


Fig. 5. SYT-SSX1 was significantly associated with high Ki-67 expression ($P = 0.02$). Ten of 12 tumors with SYT-SSX1 had a high Ki-67 expression.

M phase). In two large studies of SS, a cut-off level of the Ki-67 index at 10% was considered appropriate for separating high-proliferating from low-proliferating tumors (13, 19). Interestingly, we found a significant association between a high rate of tumor cell proliferation and *SYT-SSX1* fusion transcript, indicating different biological properties for the two fusion proteins of *SYT-SSX1* and *SYT-SSX2*.

An additional support for a biological difference between *SYT-SSX1* and *SYT-SSX2* is that all biphasic tumors had a *SYT-SSX1* transcript. This observation has also been reported in a number of other studies (14, 23–25), but a substantial number of the monophasic tumors also have a *SYT-SSX1* transcript. Although one case of a biphasic tumor with the *SYT-SSX2* transcript has been reported (5), it seems reasonable to suggest that *SYT-SSX1* is important for epithelial differentiation.

The breakpoints in *SYT* and *SSX* are identical in both *SYT-SSX1* and *SYT-SSX2*, implying that for the *SSX* gene, the break always occurs between exons 4 and 5, leaving exons 5 and 6 to fuse with the 3' of the *SYT* gene (8). Previous studies (5, 8) have shown that the COOH-terminal regions of both *SSX1* and *SSX2* are highly conserved, and that the major bp heterologies in *SYT-SSX1* and *SYT-SSX2* are found in exon 5 of the *SSX* gene (Fig. 2). In the predicted amino acid sequence, there is a 73% homology between *SSX1* and *SSX2*. Exon 5 in both *SSX1* and *SSX2* contains a comparable number of residues for phosphorylation. There are five such residues in *SSX1* (five serines), and six such residues in *SSX2* (four serines and two threonines). Four of these potential sites are common for the two fusion variants. Moreover, *SSX1* contains two *N*-linked glycosylation sites, and *SSX2* contains one *N*-linked glycosylation site, one of which is in common. Because there are differences in both potential phosphorylation and *N*-linked glycosylation sites, it is tempting to speculate that this might explain the biological and clinical difference between *SYT-SSX1* and *SYT-SSX2*. Both *SSX1* and *SSX2* belong to a family called cancer/testis antigens because they share a distinct feature of expressing mRNA in normal testis and in certain types of human cancers (8).

In conclusion, our findings suggest that besides having an influence on morphology and clinical outcome, the *SYT-SSX* fusion transcript is also associated with tumor cell proliferation in SS. However, larger studies suitable for multivariate analysis are preferable to conclude the definitive impact of the different *SYT-SSX* fusion types in SS.

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