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

Gunvar Nilsson, Björn Skyetting, Yuntiao Xie, Bertha Brodin, Roland Perfekt, Nils Mandahl, Joakim Lundeberg, Mathias Uhlen, and Olle Larsson

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

SS 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, located on the X chromosome (5, 6). The frequency of SYT-SSX4 is still unknown. The fusion 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.

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 Qiagen RNeasy (Qiagen, Hilden, Germany). A reverse-transcription using the primer SSX-25'-CAGCCCAGCTGGACCA-3' (5) was used in a 9-Minutes (range, 2–111 months). The resulting cDNA was amplified by PCR with SYT-5'-TGCTATGCACCTGATG-3' (17) and SSX2-specific primer 5'-GTGTCGACTGTGTTCTCCATCG-3' (17) and SSX2-specific primer 5'-TCTCGTGAATCTTCTCAGAGG-3' (9). The SYT-SSX1 Amplification was performed at 94°C for 30 s, 64°C for 30 s, and 95°C for 2 min. The cDNA was amplified by PCR with SYT-SSX1-5'-AGACCAACA-CAGCCTGGACCA-3' (17) and SSX primer 5'-TGCTATGCACCTGATG-3' (9). The resulting cDNA was amplified by PCR with SYT-5'-TGCTATGCACCTGATG-3' (17) and SSX2-specific primer 5'-GTGTCGACTGTGTTCTCCATCG-3' (17) and SSX2-specific primer 5'-TCTCGTGAATCTTCTCAGAGG-3' (9).

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 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 deparaf finized, 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 using the hematoxylin. The slides were dehydrated, cleared, and mounted. The sections were examined under a microscope. 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 1000 cells 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).

The Ki-67 index of 10% was regarded as highly proliferative (13, 18, 19). All of the immunohistochemically stained slides (which were coded) were analyzed 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).
Seminested PCR was able to discriminate (401 bp) in 32 cases. In case 20, the product was 57 bp larger. SSs using outer primers revealed a transcript of the predicted length.

RESULTS

each case, more than 1000 cells were analyzed. microscopically by O. L., without knowledge of the clinical characteristics. In Two-sided censored at the time of death in the analysis of metastasis-free survival. diagnosis, and the log-rank test was used to evaluate differences between Cox’s proportional hazards model. Patients were followed from time of analyzed univariately using Kaplan-Meier survival curves and HR estimates

we did not further extend the analyses with multivariate Cox modeling. In view of the relatively small number of patients, 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 Ps 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 32 cases, showed transcripts that were identical or nearly identical to SYT-SSX1 fusion transcripts. Cases 23–33 exactly matched the sequence of SYT-SSX1 (GenBank S79332). 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.

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

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 SSX4.3 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 $\geq 10\%$) 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 M)
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 terminal regions of both SSX1 and SSX2 genes, 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/phosphorylation. There are five such residues in SSX1 (five serines), and more over, 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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