A novel Krüppel-associated box containing the SSX gene (SSX3) on the human X chromosome is not implicated in t(X;18)-positive synovial sarcomas

B. de Leeuw, M. Balemans, and A. Geurts van Kessel
Department of Human Genetics, University Hospital, Nijmegen (The Netherlands)

Abstract. The human synovial sarcoma-specific translocation t(X;18) results in the fusion of the SYT gene on chromosome 18 with either one of the Krüppel-associated box (KRAB) containing SSX1 or SSX2 genes on the X chromosome, depending on the exact location of the breakpoint within band Xp11.2. Screening of a testis cDNA library yielded several SSX-positive clones. Subsequent sequence analysis revealed that one third of these clones represent an SSX gene that differs from both SSX1 and SSX2. This novel member of the family of KRAB containing SSX genes, which we designated SSX3, is 90% homologous to SSX1 and 95% homologous to SSX2 at the cDNA level. Somatic cell hybrid analysis indicated that SSX3 maps within Xp11.2→p11.1, the region that also harbors the SSX1 and SSX2 genes. However, we conclude from our RT-PCR data and from results reported in the literature that SSX3 does not act as a fusion partner to SYT in any of the 44 independent synovial sarcomas thus far tested.

Synovial sarcomas are soft-tissue tumors that occur mainly in adolescents and young adults. The chromosomal translocation t(X;18)(p11.2;q11.2) is found in the majority of these sarcomas and, as such, is thought to play a causative role in tumor formation (Turc-Carel et al., 1987). Two distinct chimeric products have been identified in different t(X;18)-positive synovial sarcomas, resulting from the fusion of the SYT gene on chromosome 18 to either the SSX1 or the SSX2 gene on the X chromosome (Clark et al., 1994; Leeuw et al., 1994a, 1995; Crew et al., 1995). These alternative fusion products have been correlated with different X-chromosomal breakpoints in fluorescence in situ hybridization experiments using Xp11.2-specific YACs as probes (Leeuw et al., 1993a, b, 1994b; Olde Weghuis et al., 1994; Shipley et al., 1994; Janz et al., 1995). Interestingly, there appears to be a positive relationship between the occurrence of these alternative X-chromosomal breakpoints and the most predominant histologic characteristics of the tumors, namely, whether they are monophasic or biphasic (Leeuw et al., 1994b; Janz et al., 1995). Here, we report the identification and chromosomal localization of a third member of the family of Krüppel-associated box containing SSX genes. This gene, however, is not implicated in t(X;18)-positive synovial sarcomas.

Materials and methods

Library screening and sequence analysis
The human fibrosarcoma HT1080 (Clonetech) and the human testis 5' stretch (Clonetech) cDNA libraries were used for screening, using essentially the same standard procedures described as before (Leeuw et al., 1993b, 1994a). DNA sequences were analyzed on an automated DNA sequencing kit (ABI 373A) using a Taq dye deoxy terminator cycle sequencing kit (Applied Biosystems).

Patient material and RT-PCR analysis
The patient material used in this study included nine synovial sarcomas that were extensively analyzed before (Leeuw et al., 1995) and six novel tumors. Diagnosis of these latter six tumors as synovial sarcomas was confirmed via a positive SYT-SSX RT-PCR score. RT-PCR and subsequent restriction enzyme analyses were performed as described previously (Leeuw et al., 1995).

Southern blot analysis
DNAs of hybrid and parental cell lines were isolated as described before (Leeuw et al., 1994a); digested to completion with a variety of restriction endonucleases (Life Technologies); and, after agarose gel electrophoresis,
SSX1 ATGAACGGAG AGCACACCT TGCAAAAGAGA CCCAGGGATG
SSX2 ATGAACGGAG AGCACGCCT TGCAAAGGAGA CCCACGGTTG
SSX3 ATGAACGGAG AGCAACCTT TGCAAAGGAGA CCCACGGTTG
SSX1 ATGCTAAAGC ATCAGAGAAG AGAAGCAAGG CCTTTGATGA
SSX2 GTGCTCAAAT ACCAGAGAAG ATCCAAAAGG CCTTCGATGA
SSX3 GTGCTCAAAT ACCAGAGAAG ATACAAAAGG CCTTCGATGA
SSX1 TATTGCCACA TACTTCTCTA AGAAAGAGTG GAAAAAGATG
SSX2 TATTGCCAAA TACTTCTCTA AGGAAGAGTG GGAAAAGATG
SSX3 TATTGCCAAA TACTTCTCTA AGGAAGAGTG GGAAAAGATG
SSX1 AAATACTCAG AGAAAATCAG CTATGTGTAT ATGAAGAGAA
SSX2 AAAGCCTCAG AGAAAATCTT CTATGTGTAT ATGAAGAGAA
SSX3 AAAGTCTCGG AGAAAATCGT CTATGTGTAT ATGAAGAGAA
SSX1 ACTATAAGGC CATGACTAAA CTAGGTTTCA AAGTCACCCT
SSX2 AGTATGAGGC TATGACTAAA CTAGGTTTCA AGGCCACCCT
SSX3 AGTATGAGGC CATGACTAAA CTAGGTTTCA AGGCCATCCT
SSX1 CCCACCTTTC ATGTGTAATA AACAGGCCAC AGACTTCCAG
SSX2 CCCACCTTTC ATGTGTAATA TACGGGCCGA AGACTTCCAG
SSX3 CCCATCTTTC ATGCGTAATA AACGGGTCAC AGACTTCCAG
SSX1 GGGAATGATT TTGATAATGA CCATAACCGC AGGATTCAGG
SSX2 GGGAATGATT TGGATAATGA CCCTAACCGT GGGAATCAGG
SSX3 GGGAATGATT TTGATAATGA CCCTAACCGT GGGAATCAGG
SSX1 TTGAACATCC TCAGATGACT TTCGGCAGGC TCCACAGAAT
SSX2 TTGAACGTCC TCAGATGACT TTCGGCAGGC TCCAGGGAAT
SSX3 TTCTACGTCC TCAGATGACT TTCGGCAGGC TCCAGGGAAT
SSX1 CATCCCGAAG ATCATGCCCA AGAAGCCAGC AGAGGACGAA
SSX2 CTCCCCGAAG ATCATGCCCA AGAAGCCAGC AGAGGAAGGA
SSX3 CTTCCCGAAG ATCATGCCCA AGAAGCCAGC AGAGGAAGGA

Fig. 1. The SSX3 cDNA sequence compared to those of SSX1 and SSX2. Differences are printed in boldface type. The locations of the BglII, EcoRV, Smal, and Lspl restriction sites are indicated.

SSX1 AATGATTCGA AGGGAGTGTC AGAAGCATCT GGCCCACAAA
SSX2 AATGATTCGG AGGAAGTGCC AGAAGCATCT GGCCCACAAA
SSX3 AATGTTTCGA AGGAAGTGCC AGAAGCATCT GGCCCACAAA

Lspl
SSX1 ACGATGGGAA ACAACTGCAC CCCCCAGGAA AAGCAAATAT
SSX2 ATGATGGGAA AGAGCTGTGC CCCCCGGGAA AACCAACTAC
SSX3 ACGATGGGAA ACAGCTGTGC CCCCCGGGAA AACCAACTAC

SmaI
SSX1 TTCTGAGAAG ATTAATAAGA GATCTGGACC CAAAAGGGGG
SSX2 CTCTGAGAAG ATTCACGAGA GATCTGGACC CAAAAGGGGG
SSX3 CTCTGAGAAG ATTAACATGA TATCTGGACC CAAAAGGGGG

EcoRV/BglII
SSX1 AAACATGCCT GGACCCACAG ACTGCGTGAG AGAAAGCAGC
SSX2 GAACATGCCT GGACCCACAG ATTGCGTGAG AGAAAACAGC
SSX3 GAACATGCCT GGACCCACAG ACTGCGTGAG AGAAAACAGC

SSX1 TGGTGATTTA TGAAGAGATC AGCGACCCTG AGGAAGATGA
SSX2 TGGTGATTTA TGAAGAGATC AGCGACCCTG AGGAAGATGA
SSX3 TGGTGATTTA TGAAGAGATC AGCGATCCTG AGGAAGATGA

SSX1 CGAGTAACTC CCCTGGGGGA TACGACACAT GCCCTTGATG
SSX2 CGAGTAACTC CCCTGGGGGA TACGACACAT GCCCATGATG
SSX3 TGAGTAACTC CCCTGGGGGA TATGACACAT GCCCATGATG

SSX1 AGAAGCAGAA CGTGGTGACC TTTCACGAAC ATGGGCATGG
SSX2 AGAAGCAGAA CGTGGTGACC TTTCACGAAC ATGGGCATGG
SSX3 AGAAGCAGAA CGTGGTGACC TTTCACGAAC ATGGGCATGG

SSX1 CTGCGGCTCC CTCGTCATCA GGTGCATAGC AAGTG
SSX2 CTGCGGCTCC CTCGTCATCA GGTGCATAGC AAGTG
SSX3 CTGCTGGACCC CTCGTCATCA GGTGCATAGC AAGTG

Results and discussion

Screening of a human fibrosarcoma cDNA library with a synovial sarcoma-derived SYT-SSX RT-PCR product (Leeuw et al., 1995) yielded several partial SSX2 fragments. One of these fragments was used as a probe to screen a human testis cDNA library, which resulted in 15 positive clones. Five of these full-length clones (pDR2-30, pDR2-33, pDR2-34, pDR2-36, and pDR2-42) were found to lack a BglII site that is normally present in both the SSX1 and SSX2 cDNAs (Leeuw et al., 1995). Sequencing of these five clones yielded identical results and revealed a novel SSX transcript that was significantly different from SSX1 and SSX2 (for comparison, see Fig. 1). Overall, this novel sequence, which we designated SSX3, is 90% homologous to SSX1 and 95% homologous to SSX2 at the cDNA level. At the position of the BglII site in SSX1 and SSX2, blotted onto Genescreen Plus membranes (Dupont) using standard protocols. Blots were hybridized overnight in 0.5 mM PBS, 0.1 mM Na2EDTA, and 7% (v/v) SDS at 65° C; washed once in 40 mM phosphate, 0.1 % SDS and then in 10 mM phosphate, 0.1 % SDS at 65° C; and, lastly, exposed to X-ray film (Kodak) at -80° C for 1-3 d, using intensifying screens.

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Screening of a human fibrosarcoma cDNA library with a synovial sarcoma-derived SYT-SSX RT-PCR product (Leeuw et al., 1995) yielded several partial SSX2 fragments. One of
A single SYT-SSX fusion transcript was detected in the synovial sarcomas revealed a BglII site and either an SmaI or LspI site, whereas none showed an EcoRV site or both SmaI and LspI sites (Fig. 3). From these results we conclude that the SSX3 gene is not fused to SYT in any of our 15 independent synovial sarcomas.

Crew et al. (1995) published a series of 29 synovial sarcomas which showed either a SmaI site or LspI site within the SYT-SSX RT-PCR products, but never both sites. They also mentioned that only SSX1 or SSX2 sequences were encountered. Therefore, we conclude that SSX3 is not included in the fusion with SYT in any of the 44 independent synovial sarcomas thus far tested. In addition, three synovial sarcomas without any evidence for the presence of SYT-SSX1 or SYT-SSX2 fusion products were reported (Crew et al., 1995). Whether in these cases SSX3 is involved cannot, at present, be excluded with certainty, but it should be expected that aberrant SSX transcripts would have been detected by the investigators using the highly homologous (95%) SSX2 as a probe.

Figure 4 compares the amino acid sequences of SSX1, SSX2, and SSX3. It can be deduced from this comparison that, at this level, the identity between SSX2 and SSX3 is relatively high (90%), except for the Krüppel-associated box B (KRAB B) region, where it is only 73%. The overall identity between SSX1 and SSX2 is only 76%. Whereas the KRAB A domain appears to be involved in the repression of transcription, the KRAB B domain seems to be dispensable for this particular activity under in vitro conditions (Witzgall et al., 1994). It has been suggested that KRAB-containing proteins may exert their effects through interactions with transcriptional activators containing a glutamine-rich activation domain (Licht et al., 1993).
have arisen by reversed transcription. In this respect, they are different from, for example, the interspersed OAT-like sequences, some of which have been reported to be processed pseudogenes (Lafreniere et al., 1991; Geraghty et al., 1993). It has been hypothesized before that the OATL1 and OATL2 clusters may have evolved via a duplication event of the entire region (Shipley et al., 1994). Our present results indicate that during the course of evolution, several of these duplication events must have occurred within this particular genomic segment.

In addition to the SSX3 gene reported here, we have evidence for the existence of at least two other SSX-like genes. The first one, designated SSX4, was found after RT-PCR with two SSX internal primers on RNA extracted from a primary human fibrosarcoma. Preliminary sequence data indicate that this gene may give rise to a protein truncated just after the KRAB A box. The second one, SSX5, was detected via the presence of an exon in one of our OATL1 YAC-derived cosmids (Leeuw et al., 1993b). This exon shows between 80% and 90% base-pair homology to the corresponding exons in SSX1, SSX2, SSX3, and SSX4. Whether SSX4 and SSX5 actually represent functional genes still remains to be established. The possible involvement of any of these novel SSX sequences in the development of other neoplastic disorders carrying X-autosome translocations, such as renal cell carcinomas (Sinke et al., 1993; Dijkhuizen et al., 1995), is currently under investigation.

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As such, the observed differences at the amino acid level between the KRAB B domains of SSX2 and SSX3 may give rise to functionally different interactions. The exact nature of these putative interactions, however, remains to be established.

The chromosome location of the SSX3 gene was established through the analysis of a panel of human × rodent somatic cell hybrids including the X-only line 578 (Sinke et al., 1993); its radiation-reduced derivative 578K17, which contains the Xp11.4→p11.1 segment as the only human constituent (Berger et al., 1992); and the synovial sarcoma-derived line H1synsarc containing Xp11.2→qter (Leeuw et al., 1993a). Since 578, 578K17, and H1synsarc exhibit restriction fragments similar to those present in control total human genomic DNA that hybridizes to our SSX3 cDNA probe (absent in the control hamster DNA under the stringency conditions applied [Fig. 5]), we conclude that the SSX3 gene must reside within Xp11.2→p11.1. This proximal Xp segment contains several low-copy repeats, among which are the OAT-like sequences (Sinke et al., 1993; Leeuw et al., 1994b). Interestingly, SSX1 is positioned precisely within the OATL1 cluster (Leeuw et al., 1993a). The identification of yet another SSX gene (SSX3) in Xp11.2→p11.1 is in full agreement with the repeated nature of this particular chromosomal segment (Lafreniere et al., 1991). Since SSX1 and SSX2 are functional, expressed genes containing intronic sequences (unpublished data), they do not seem to...
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