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Original Paper

Detection of *SYT–SSX1/2* Fusion Transcripts by Reverse Transcriptase–Polymerase Chain Reaction (RT–PCR) is a Valuable Diagnostic Tool in Synovial Sarcoma

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Cytogenetically, most synovial sarcomas are characterised by a specific chromosomal translocation [(X;18)(p11.2;q11.2)], which results in the generation of fusion transcripts comprising *SYT* (18q11) and either *SSX1* or *SSX2* (Xp11) sequences. By using a sensitive reverse transcriptase–polymerase chain reaction (RT–PCR) protocol, specific *SYT-SSX1/2* fusion transcripts were detected in 10 histopathologically confirmed synovial sarcomas. Control tumours with morphological spindle cell patterns mimicking monophasic synovial sarcoma tested negative (18/19) in the RT–PCR protocol, with the exception of one spindle cell sarcoma originally classified as a fibrosarcoma. Furthermore, the established RT–PCR protocol was used to evaluate the feasibility of *SYT–SSX1/2* fusion transcript detection for minimal residual disease analysis. Analyses of surgical margins revealed a fusion transcript in two of four operations for synovial sarcoma analysed, one of which was diagnosed with tumour free margins by conventional histopathology. These data suggest that the RT–PCR amplification of *SYT–SSX1/2* fusion transcripts is a valuable tool in the differentiation of synovial sarcomas, especially in cases of equivocal morphology. Additionally, the RT–PCR approach may be used for the detection of residual tumour cells in synovial sarcoma patients. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: sarcoma, translocation, RT–PCR, minimal residual disease, *SYT–SSX1/2*
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INTRODUCTION

SPECIFIC TRANSLOCATIONS resulting in gene fusions make soft tissue sarcomas exceptional among solid tumours [1]. In synovial sarcomas, a stable translocation involving chromosomes X and 18 has been detected cytogenetically [2–5]. Cloning of the affected chimeric fusion genes led to the identification of the *SYT* gene (18q11) positioned at the 5′ end and either the *SSX1* gene or the *SSX2* gene at the 3′ end [6]. The *SSX1* and *SSX2* genes are located approximately 2 Mb apart on Xp11 and are characterised by a high sequence homology [7–9]. Little is known about the function of the genes involved in this translocation. The *SYT* gene is ubiquitously expressed in early embryogenesis [10]. However, in mature mouse tissues, only in cartilage tissue, some neuronal and epithelial cells, as well as in primary spermatocytes, has *SYT* gene expression been demonstrated [10]. Expression of the *SSX2* gene has been identified through serological screening of cDNA expression libraries from malignant melanoma with sera from melanoma patients [11]. Furthermore, hepatocellular, breast and colon cancer have been shown to express *SSX2* in 25–30% of cases analysed. Recently, the *SYT-SSX* fusion protein has been localised in the nucleus, and the *SYT* domain of the *SYT-SSX* fusion transcript has been found to serve as a transcriptional activator in reporter assay experiments [12, 13].

Clinically, synovial sarcomas represent malignant soft tissue tumours observed mostly in association with tendon

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sheaths, bursae and joint capsules [14, 15]. Morphologically, biphasic and monophasic fibrous tumours can be distinguished. While biphasic tumours exhibit an unambiguous characteristic pattern of epithelial and mesenchymal components, monophasic fibrous tumours consist predominantly of mesenchymal spindle cells rendering a diagnostic classification based on morphology difficult [16, 17].

In adult patients with synovial sarcoma, metastatic dissemination and local recurrence are frequently observed, even after radical surgery. This suggests tumour cell dissemination either before or during surgical removal of the primary tumour. The detection of disseminated tumour cells may be relevant in terms of prognosis and treatment.

In this study, we determined the frequency of *SYT-SSX1/2* fusion transcripts in tumour specimens from patients with morphologically and immunohistochemically proven synovial sarcoma. To evaluate the potential role of a reverse transcriptase–polymerase chain reaction (RT–PCR) based detection of fusion transcripts as a diagnostic tool, various spindle cell sarcomas with different lines of differentiation were also analysed for fusion transcripts. We also analysed the feasibility of a RT–PCR protocol using *SYT–SSX1/2* fusion transcripts as a marker for minimal residual disease in peripheral blood, bone marrow samples and resection margins.

MATERIALS AND METHODS

Patients and investigated samples

Thirty-two tumour samples were studied from 29 patients surgically treated for soft tissue sarcoma at the Department of Surgery, University of Heidelberg, Heidelberg, Germany between 1990 and 1997. These included 10 synovial sarcomas (seven with a monophasic spindle cell and three with a biphasic morphology) and 19 further sarcomas with spindle cell morphology (i.e. leiomyosarcomas, malignant peripheral nerve sheath tumours, gastrointestinal stromal sarcomas, fibrosarcomas). Diagnosis was based on standard histopathological criteria according to WHO classification [14] with additional immunohistochemistry. Tumour stage and grading were defined according to the 1987 UICC classification [18]. Immediately after surgical removal, tumour specimens and normal tissues were snap frozen in liquid nitrogen. Total RNA was extracted from 20 µm thick cryosections. Haematoxylin–eosin staining of 4 µm cryocuts confirmed the selection of tumour samples carrying at least 70% tumour cells, or of normal samples without microscopically visible tumour cells. For analysis of minimal residual disease, pre-, intra- and postoperative blood samples, bone marrow aspirates and macroscopically tumour free tissue from the resection margins were collected. Ten blood samples from healthy donors and bone marrow aspirates from 10 patients with colon cancer served as negative controls. The study protocol was approved by the local ethics committee.

Blood and bone marrow samples

Peripheral blood (10 ml) was obtained through a central venous catheter placed in the superior vena cava either pre-, intra- and 24 h postoperatively and diluted with 10 ml phosphate buffered saline (PBS). After density centrifugation (30 min, 400 ***g***) through Ficoll-Paque (Pharmacia Biotech, Freiburg, Germany), mononuclear peripheral blood cells were harvested from the interphase and washed twice in PBS. The cell pellet was subsequently snap frozen in liquid nitrogen and stored until further analysis at − 70°C. Bone marrow aspirate (10 ml) was collected from both sides of the superior iliac crest under general anaesthesia prior to tumour resection. One sample was used for cytological analysis. The second sample was diluted with 10 ml PBS and nucleated cells were harvested through Ficoll-Paque density centrifugation as described above.

RNA extraction

Total RNA of tumour and normal tissue samples, peripheral mononuclear blood cells and bone marrow aspirates was isolated using a commercially available RNA isolation kit (Glassmax®; Life Technologies Gibco BRL, Eggenstein, Germany) according to the recommendations of the supplier. To eliminate contaminating DNA within RNA preparations, samples were digested with RNase free DNaseI as recommended by the supplier (Life Technologies Gibco BRL).

RT–PCR and cDNA sequencing of SYT-SSX1/2 *transcripts*

Total RNA (1 µg) was reverse transcribed (Superscript, Life Technologies Gibco BRL) in 20 µl of a reaction mixture utilising hexanucleotide random priming (analysis of tumour tissue) or oligonucleotide primer psx2 (minimal residual disease analysis). To control for RNA integrity and reverse transcription efficacy, PCR reactions employing glyceraldehyde phosphate dehydrogenase (GAPDH) specific primers served as an internal control [19]. *SYT-SSX1/2* specific oligonucleotide primers were designed based on GenBank database entries HSSYT, X86174 and X86175: psx1, 5′-ATGGAAATTCACAGTATGGC-3′, psx2, 5′-CTTGCTATGCACCTGATGAC-3′; psx3, 5′-CCAACAGCAAGATGCATACCAGGGA-3′; psx4, 5′-CAGCCATGCCCATGTTCGTGAAAG-3′. The locations of the reverse oligonucleotide primers are in areas of complete sequence identity between the *SSX1* and *SSX2* genes. First strand cDNA (2.5 µl; 7.5 µl for minimal residual disease analysis) was subjected to PCR amplification (25 pmoles of primers psx1 and psx2, 200 µM of each dNTP, 2.5 units of *Taq* DNA polymerase (Life Technologies Gibco BRL) and 1.25 mM $MgCl_2$) in a final volume of 50 µl using an Omnigene ThermoCycler (Hybaid, Teddington, U.K.). After initial template denaturation (94°C/3 min), the amplification of transcripts was performed in 20 cycles of 94°C/30 sec, 54°C/45 sec, 72°C/1 min and a final extension step at 72°C/5 min. The PCR products (1 µl; 15 µl for minimal residual disease analysis) were further subjected to a second, nested PCR reaction applying identical PCR conditions, but using primers psx3 and psx4 and increasing the annealing temperature to 69°C. All PCR reactions were performed with negative (devoid of RNA or containing RNA from non-sarcoma cell lines or tumours) and positive controls (known samples with *SYT–SSX1/2* fusion transcripts). All reactions were repeated at least twice from independent RNA samples. The specificity of the PCR products was confirmed by Southern blot hybridisation experiments using a cDNA probe encompassing nucleotides 956–1152 of the *SYT* gene and nucleotides 422–554 of the *SSX1/2* cDNA sequence. After agarose gel electrophoresis (0.9%), the PCR products were blotted on to a Hybond N^+ membrane (Amersham Life Science, Buckinghamshire, U.K.) and cross-linked by ultraviolet light exposure. The *SYT-SSX1/2* specific cDNA probe was radioactively labelled with [α-^{32}P] dATP using a random priming DNA labelling kit (MBI Fermentas, Vilnius, Lithuania). Membrane filters were hybridised for 16 h at 65°C in 0.5 M

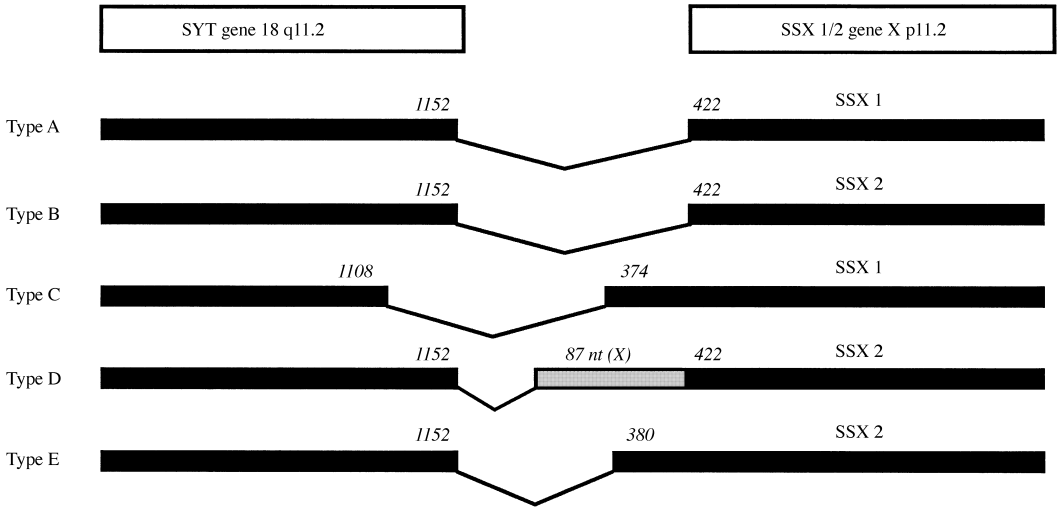

Figure 1. Structure of variant chimeric fusion transcripts of *SYT-SSX1/2* [7, 9]. Enumeration of the nucleotides is given for the *SYT* gene (GenBank #HSSYT) and the *SSX1/2* genes (GenBank # X86174 and X86175). In the type D transcript, the grey box indicates 87 nucleotides which were mapped to the X chromosome and are located between the *SYT* and the *SSX2* part of the fusion gene [7].

sodium phosphate (pH 7.2), 7% sodium dodecyl sulphate (SDS), subsequently washed (3×30 min at 65°C in 0.5×SSC, 0.1% SDS), and exposed to XAR5 autoradiographic films (Eastman Kodak Co., Rochester, New York, U.S.A.) All *SYT–SSX1/2* fusion transcripts were ligated into vector pCR2.1 (Invitrogen, San Diego, California, U.S.A.), and subsequently sequenced using a Cy5′-AutoRead sequencing kit (Pharmacia Biotech). Sequencing reactions were analysed on denaturing 6.6% polyacrylamide/7 M urea gels using an ALFexpress DNA sequencing device (Pharmacia Biotech).

RESULTS

Specificity of SYT-SSX1/2 *RT–PCR*

We developed a nested RT–PCR protocol to selectively amplify the *SYT-SSX1/2* fusion transcripts generated by the specific translocation [(X;18)(p11.2;q1.2)], which is observed in most synovial sarcomas. Oligonucleotide primers were chosen to permit the amplification of all five previously reported chimeric transcripts (Figure 1) [7, 9, 20, 21]. In order to determine the sensitivity of the RT–PCR protocol, we performed a simulation experiment. Decreasing amounts of RNA extracted from a *SYT–SSX2* positive tumour were diluted in 1 μg RNA extracted from peripheral blood monocytes of healthy volunteers. Using this approach, we repeatedly generated a fusion transcript in a 10^{-6} dilution of tumour RNA (Figure 2). To test the specificity of the RT–PCR protocol, peripheral blood from 10 healthy volunteers and bone marrow from 10 patients with colon cancer were analysed. All these samples tested negative in the RT–PCR protocol established for minimal residual disease analysis.

RT–PCR results in synovial sarcomas and in spindle cell sarcomas with different lines of differentiation

In contrast to biphasic synovial sarcomas, differential diagnosis of monophasic fibrous subtypes may be challenging, due to various soft tissue malignancies also demonstrating a spindle cell pattern (Figure 3). Therefore, we employed the nested RT–PCR protocol for the analysis of *SYT–SSX1/2* fusion transcript expression. Amplicons of the expected length of approximately 550 nucleotides were generated in 12 tumour samples of all 10 patients with synovial sarcomas. 18 of 19 control spindle cell sarcomas proved negative in the established RT–PCR protocol. One case of recurrent spindle cell sarcoma of the abdominal wall, originally classified as a fibrosarcoma, revealed a specific *SYT-SSX2* fusion transcript. In this case, the recurrent tumour tissue available for analysis consisted only of two small intramuscular nodules. This material was not sufficient to exclude or confirm the diagnosis of fibrosarcoma established by another institution involved in the treatment and diagnosis of the primary tumour. Clinical characteristics of patients harbouring *SYT–SSX1/2* fusion transcripts in the respective tumours are summarised in Table 1. DNA sequence analysis of all individual fusion transcripts obtained from tumour tissue revealed *SYT–SSX1* fusion transcripts (Figure 1, type A) in 6 of 11 tumours and *SYT–SSX2* transcripts (Figure 1, type B) in another five cases (Figure 4). All three recurrent tumours of 1 patient with a monophasic fibrous synovial sarcoma carried the identical fusion transcript. Three tumours demonstrated a biphasic histomorphological pattern which all harboured a *SYT-SSX1* fusion. Analysis of monophasic fibrous subtypes revealed *SYT-SSX1* in three and *SYT-SSX2* fusion transcripts in another four cases.

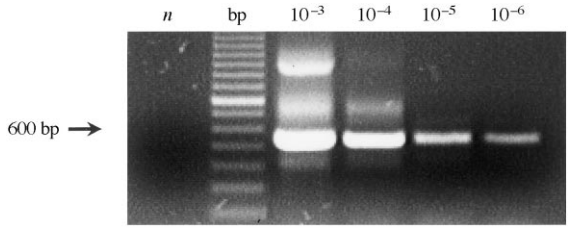

Figure 2. Simulation experiment to define the sensitivity of the reverse transcriptase–polymerase chain reaction (RT–PCR) protocol in analysing minimal residual disease. Total RNA extracted from tumour tissue demonstrating a fusion transcript was diluted in RNA derived from peripheral blood monocytes from healthy volunteers. *SYT/SSX* fusion transcripts were detectable up to a 10^{-6} dilution of tumour RNA. *n*, negative control; bp, 100 bp ladder.

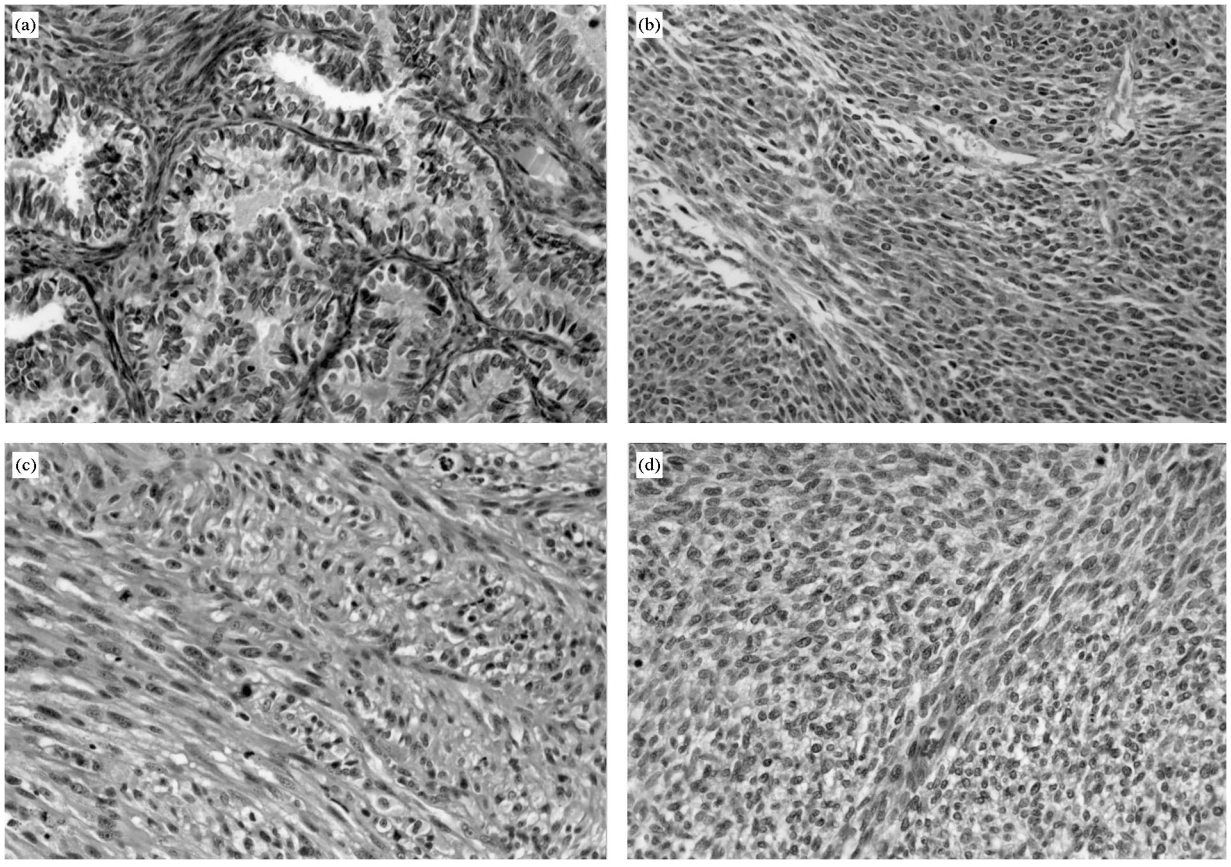

Figure 3. Haematoxylin–eosin stained sections of a biphasic synovial sarcoma with a dominating epithelial component (a) and a monophasic fibrous synovial sarcoma with focal myxoid change (b). Examples for sarcomas with a spindle cell morphology possibly mimicking monophasic fibrous synovial sarcoma are shown in (c) (gastrointestinal stromal sarcoma) and in (d) (malignant peripheral nerve sheath tumour). Magnification ×280.

Minimal residual disease—patient study

2 very recent patients undergoing resection for histologically confirmed synovial sarcoma tested positive for a fusion transcript generated in the analysed tumour and were eligible for examination of minimal residual disease. In 1 patient with a synovial sarcoma of the distal thigh, pre-, intra- and postoperative blood samples and tissue samples from the resection margins tested negative for the *SYT–SSX1* fusion transcripts generated by the tumour. In the other patient, pre-, intra- and postoperative blood samples, as well as a bone marrow aspirate, also tested negative for a fusion transcript in the nested PCR. However, two of three resection margins which were shown to be microscopically free of tumour revealed a fusion transcript. The RT–PCR results of this case including tumour tissue, bone marrow aspirate, peripheral blood and soft tissue from the resection margins are shown in Figure 5. All generated PCR products were subsequently shown to be specific for *SYT-SSX1/2* by Southern blot hybridisation (data not shown). Furthermore, DNA sequencing demonstrated the authenticity of the *SYT–SSX1* fusion transcript previously detected in the tumour of the patient.

Table 1. *Clinical characteristics of all patients whose tumours revealed* SYT-SSX1/2 *fusion transcripts*

Patient	Age (years)	Source	Localisation	Histology	Fusion partner of *SYT*
1	53	LR	Shoulder	Monophasic SS	*SSX1*
2	13	PT	Thigh	Monophasic SS	*SSX2*
3	22	PT	Chest wall	Monophasic SS	*SSX2*
4	62	LR	Knee	Monophasic SS	*SSX1*
5	24	PT	Thigh	Monophasic SS	*SSX2*
6	67	PT	Knee	Biphasic SS	*SSX1*
7	57	DM	Lung	Biphasic SS	*SSX1*
8	73	DM	Lung	Monophasic SS	*SSX2*
9	25	PT	Thigh	Biphasic SS	*SSX1*
10	44	PT	Thigh	Monophasic SS	*SSX1*
11	38	LR	Abdominal wall	Fibrosarcoma?*	*SSX2*

*Thorough re-evaluation by morphology and immunohistochemistry impossible due to lack of tumour material. LR, local recurrence; PT, primary tumour; DM, distant metastasis; SS, synovial sarcoma.

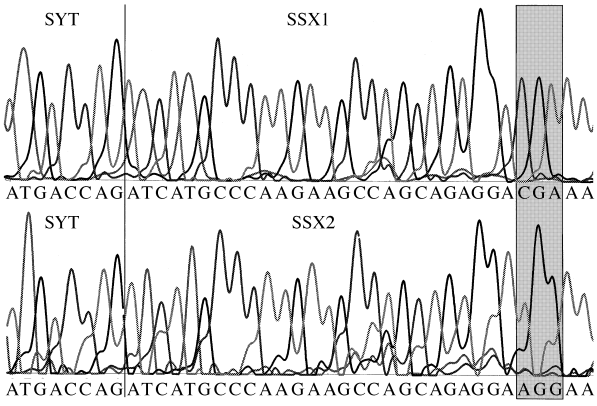

Figure 4. Junction sites of *SYT-SSX1* and *SYT–SSX2* fusion transcripts, demonstrated by sequence analyses of polymerase chain reaction (PCR) fragments from two different tumours. On the left, the 3′ part of the *SYT* gene is shown. The adjacent 26 nucleotides are shared by *SSX1* and *SSX2*, respectively. The grey box highlights nucleotides allowing a distinction between *SSX1* and *SSX2*.

Retrospectively, we analysed two further precedent operations for local recurrences in this patient. While resection margins in one operation proved negative, in the other operation a positive RT–PCR result was detected in one resection margin. However, in this procedure, pathohistological examination also revealed microscopic tumour infiltration of the resection margins. The patient developed one further local recurrence 12 months later, despite postoperative radiotherapy.

DISCUSSION

Since stable chromosomal translocations have been described in tumour cells, tremendous efforts have been undertaken to characterise and analyse these molecular events. Cytogenetic detection of chromosomal translocation was evaluated for classifying soft tissue tumours long before cloning of the affected genes allowed more detailed examinations [22–25]. In synovial sarcoma, one challenge is the microscopic differentiation of monophasic fibrous types from other types of sarcomas with spindle cell morphology. As a recurrent translocation [(X;18)(p11.2;q11.2)] has been detected in 90% of synovial sarcomas [2–5], karyotyping has been evaluated as a tool to support histological diagnosis of synovial sarcoma. However, since short term culture of tumour cells has only been successful in less than half of the cases [26], this technique is not suitable for routine application. Fluorescence *in situ* hybridisation (FISH) has been applied for the identification of synovial sarcomas carrying chromosomal (X;18) translocations [20, 23, 27, 28]. This approach has the advantage that archival paraffin-embedded tissues can be used for the analysis.

The recent cloning of the affected fusion genes [6–9] allowed us to select a RT–PCR approach in evaluating various soft tissue sarcomas with spindle cell morphology, including a panel of 10 synovial sarcomas with exclusive or partial fibrous differentiation for the expression of *SYT-SSX1/2* genes. This approach shares two advantages with FISH analysis, one being the small quantity of specimen necessary for the investigation. Furthermore, once the analytical process is established, results are obtained after 24–48 h [26]. The RT–PCR approach additionally leaves the option of sequencing the RT–PCR products in order to characterise exactly the structure of the chimeric transcripts and the involved fusion genes. Finally, this examination should yield objective and reproducible results and, therefore, would be independent of personal experience necessary for a profound classification based on histomorphology and histochemical examination [16, 29].

The results of our study reinforce these findings. We were able to extract sufficient RNA in all cases and analyse all selected tumours with this approach. All synovial sarcomas were found to generate a *SYT-SSX1/2* fusion transcript. In 19 cases of spindle cell sarcomas with divergent lines of differentiation (i.e. leiomyosarcomas, malignant peripheral nerve sheath tumours, gastrointestinal stromal sarcomas, fibrosarcomas), only one recurrent tumour showed a *SYT–SSX2* fusion transcript. Since material from the primary tumour excised elsewhere was not available for thorough histomorphological and immunohistochemical re-evaluation, its exact classification remains questionable. However, since synovial sarcomas of the abdominal wall, although being rare, have been described previously [30], the patient analysed herein may well have had a synovial sarcoma. If further studies will support the high specificity of the *SYT–SSX1/2* fusion in synovial sarcomas, reclassification as a synovial sarcoma on the basis of RT–PCR analysis might be justified. Therefore, the RT–PCR based detection of *SYT-SSX1/2* fusion transcripts might be considered as a valuable addition in the diagnosis of monophasic fibrous synovial sarcomas.

DNA sequence analysis of all affected tumours in our series demonstrated the two most frequently detected fusion transcripts (type A and type B) of *SYT-SSX1/2* in this panel of synovial sarcomas (Figure 1). All biphasic synovial sarcomas ($n = 3$) demonstrated a *SYT–SSX1* fusion transcript. When the identification of the affected fusion genes revealed two different fusion partners of the *SYT* gene, a relationship between the respective fusion partner and the phenotypic

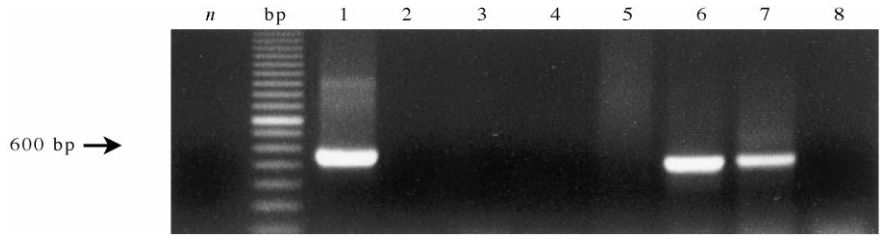

Figure 5. Minimal residual disease analysis in a patient undergoing resection of a recurrence of a synovial sarcoma of the shoulder girdle. A *SYT-SSX1* transcript was detected in the primary tumour and two resection margins after radical resection of the tumour. Analysis of *SYT-SSX1* fusion transcript occurrence in the tumour (1); bone marrow (2); pre- (3), intra- (4) and postoperative (5) blood samples; proximal (6), medial (7) and lateral (8) resection margins. *n*, negative control; bp, 100 bp ladder.

characteristics of the tumour seemed an attractive explanation for the variant morphology in synovial sarcomas. Early results indeed suggested a positive relationship [8], but other groups were unable to confirm these findings [7]. In a very recent report, Kawai and colleagues analysed 45 patients with synovial sarcoma regarding the influence of the *SSX* partner gene and the prognosis of the respective patients [31]. In this study, analogous to the 3 patients analysed in our series, all 12 biphasic synovial sarcomas demonstrated a *SYT–SSX1* gene fusion. Furthermore, patients with a *SYT–SSX2* fusion transcript and localised disease demonstrated a significantly better prognosis than the respective patients harbouring *SYT–SSX1* gene fusions in a multivariate analysis. These findings suggest a biological difference between both fusion genes resulting in variant clinical courses. The underlying mechanism remains unclear at the moment.

Although preliminary, the data provided in this study suggest that the RT–PCR based detection of fusion transcripts may provide a sensitive tool in the diagnosis of disseminated tumour cells in synovial sarcoma patients. Fusion transcripts generated through chromosomal translocation have been demonstrated to represent excellent targets for determining minimal residual disease in haematopoietic malignancies [32]. Here, various studies suggest a direct correlation between the amount of residual tumour cells and the prognosis of the affected patients. Dissemination of tumour cells in the peripheral blood or bone marrow has also been observed in patients with solid cancers, even in the absence of metastatic disease by conventional staging techniques [19, 33, 34]. However, in some cases of selected target genes, illegitimate transcription may significantly influence results and interpretation of the gained data [35]. The *SYT–SSX1/2* fusion transcripts are selectively present in synovial sarcomas and have not been demonstrated in any normal, non-neoplastic tissues or in other tumours. The potential clinical value of determining minimal residual disease by *SYT-SSX1/2* fusion transcript RT–PCR is underlined by our finding that fusion transcripts can be detected in microscopically tumour free resection margins. The tendency of local tumour recurrence observed in synovial sarcoma may well find its explanation in disseminated tumour cell clusters located distant from the tumour apparent on diagnostic imaging and macroscopic as well as microscopic evaluation. This assumption needs further support by a larger series of samples.

In conclusion, we have demonstrated that RT–PCR is a valuable tool in confirming the diagnosis of synovial sarcoma based on the detection of fusion transcripts resulting from the translocation [(X;18)(p11.2;q11.2)]. Furthermore, our results point to the potential of minimal residual disease diagnostics as a more sophisticated tool for staging patients with synovial sarcoma. The prognostic impact of these findings has to be proven in further prospective studies. The value of fusion genes and fusion proteins as targets for therapeutical intervention remains to be established.

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