

## Short report

## Detection of SYT-SSX fusion transcripts in both epithelial and spindle cell areas of biphasic synovial sarcoma using laser capture microdissection

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**Abstract**

To investigate the distribution of tumour cells expressing the SYT-SSX fusion gene in biphasic synovial sarcoma, modified reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using microdissected specimens from haematoxylin and eosin stained sections of archival paraffin wax embedded tissues. This modified RT-PCR included a stage with degenerate oligonucleotide primed (DOP) PCR, which randomly amplified cDNA after reverse transcription. SYT-SSX fusion transcripts were detected in both epithelial and spindle cell areas of all three biphasic synovial sarcomas examined. Subsequent sequence analysis confirmed that the detected messages were derived from the SYT-SSX1 fusion gene in two cases and from SYT-SSX2 in one. These results indicate that SYT-SSX fusion transcripts are found in both epithelial and spindle cell areas of biphasic synovial sarcoma, and RT-DOP-PCR-PCR analysis is a useful method for detection of extremely small amounts of mRNA in microdissected samples from archival formalin fixed, paraffin wax embedded tumour tissues.

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The SYT-SSX chimaeric transcript is generated as a result of the reciprocal translocation t(X;18)(p11;q11), which is the primary and specific cytogenetic abnormality in synovial sarcoma.<sup>1</sup> Molecular analysis of SYT-SSX fusion transcripts by reverse transcription polymerase chain reaction (RT-PCR) has been applied to archival formalin fixed, paraffin wax embedded tumour tissues as a feasible and reliable technique for the diagnosis of synovial sarcoma.<sup>2-5</sup>

Molecular analysis of RNA and DNA extracted from whole tissues or sections does not provide information regarding the topological organisation of the cells concerned. An in situ hybridisation study by Hiraga *et al* to detect SYT-SSX fusion gene expression in synovial sarcoma provided topological information, showing that it was expressed in both the epithelial and fibrous areas<sup>6</sup>; however, the sensitivity of this assay is limited and the different types of SYT-SSX fusion transcript could not be identified. Recent developments in laser technology allow various molecular analyses of microdissected samples from routinely stained sections or immunostained frozen sections to be carried out.<sup>7-9</sup>

In our study, we conducted a modified RT-PCR assay that included degenerate oligonucleotide primed (DOP) PCR after a step of reverse transcription for the detection of the SYT-SSX fusion gene using laser capture microdissected samples from both epithelial and spindle cell areas of haematoxylin and eosin stained sections of formalin fixed, paraffin wax embedded tissues. The specimens were biphasic synovial sarcomas from three patients.

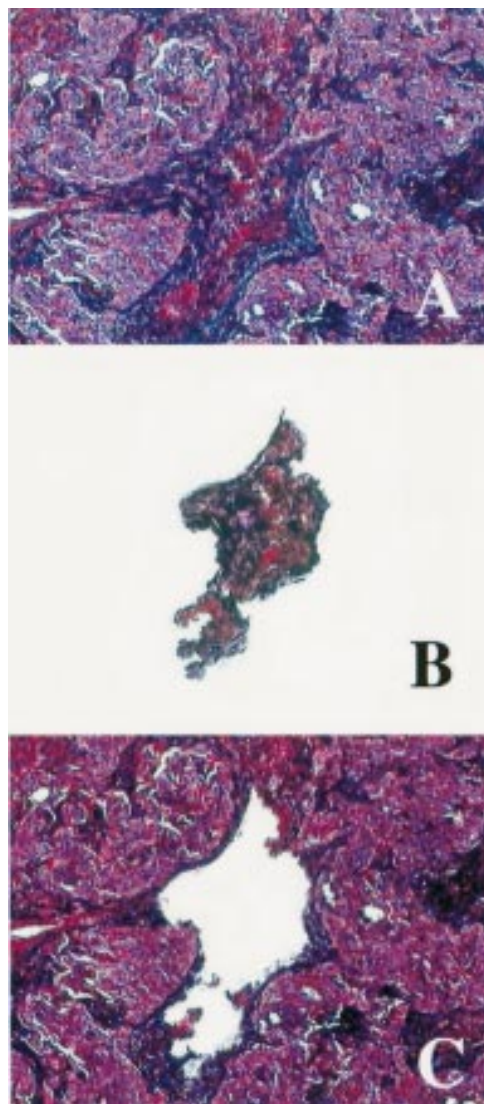
**Materials and methods**

We retrieved three samples of biphasic type synovial sarcoma, in which SYT-SSX fusion transcripts had been detected previously by RT-PCR using archival paraffin wax embedded tissue.<sup>4</sup> Three samples of pulmonary adenocarcinoma were also analysed as negative controls. Table 1 summarises the clinical data. One 5 µm thick section was prepared from each representative paraffin wax embedded

Table 1 Clinicopathological and molecular features of biphasic synovial sarcomas

Case number	Age/Sex	Site	Component	RT-DOP-PCR-PCR		
				PBGD	SYT-SSX	Fusion type
Biphasic synovial sarcoma						
1	15/F	Thigh	E	+	+	1
			S	+	+	1
2	13/F	Knee	E	+	+	1
			S	+	+	1
3	62/F	Knee	E	+	+	2
			S	+	+	2
Pulmonary adenocarcinoma						
1	64/M			+	-	
2	72/F			+	-	
3	56/M			+	-	

F, female; M, male; E, epithelial areas; PBGD, porphobilinogen deaminase gene; S, spindle cell areas.



**Figure 1** (A) Paraffin wax embedded section of biphasic synovial sarcoma (case 1) stained with haematoxylin and eosin. (B) Captured sample from the spindle cell areas containing about 100 spindle tumour cells. (C) The section after capture.

tumour sample. To avoid cross contamination of samples, a new microtome blade was used for each patient. The area of the microtome around the blade was cleaned with 70% ethanol between samples. The sections were stained with haematoxylin and eosin in the usual way, paying attention to the effect of DNAase and RNAase and cross contamination of samples. The stained sections were used for microdissection using a PixCel laser capture microscope (laser capture microdissection system, LM100; Olympus, Tokyo, Japan) with an infrared diode laser (Arcturus Engineering, California, USA).<sup>10 11</sup> In brief, each section was overlaid with a thermoplastic membrane and cells were captured by focal melting of the membrane by laser activation. The parameters of one laser shot were as follows: a spot size was 30 µm in diameter, its power was 30 mW, and its exposure duration was 5 ms. Each of the captured samples, containing 50–100 tumour

cells (fig 1), was immersed in 200 µl of lysis buffer (20 mmol/litre Tris/HCl, pH 8.0, 20 mmol/litre EDTA, and 2% sodium dodecyl sulphate) and then 10 µl of proteinase K solution (100 mg/ml) was added to the sample, which was incubated overnight at 55°C.

RNA extraction and reverse transcription were performed as described previously.<sup>4</sup>

The modified DOP-PCR was performed in two separate phases.<sup>12</sup> First, four cycles (a preamplification step) were carried out in a 5 µl reaction mixture (using ThermoSequenase; Amersham, Cleveland, Ohio, USA) in low stringency conditions, followed by 30 cycles in a 25 µl reaction volume (using AmpliTaq polymerase, LD; Perkin Elmer, Norwalk, Connecticut, USA) under high stringency conditions. UN1 primer (5'-CCG ACT CGA GNN NNN NAT GTG G-3', with N = A, C, G, or T) was used in both reactions. Table 2 gives the reagents, volumes, and reaction conditions. After DOP-PCR, 5 µl of each sample was used in the next PCR step, as described previously.<sup>4</sup> The primer set was FP (SYT): 5'-CCA GCA GAG GCC TTA TGG ATA-3' and RP (SSX): 5'-TTT GTG GGC CAG ATG CTT C-3'.<sup>2</sup> As positive controls for the integrity of mRNA in each sample, PCR for the ubiquitously expressed porphobilinogen deaminase (PBGD) gene transcripts was performed using the following primers: PBGD-S (5'-TGT CTG GTA ACG GCA ATG CGG CTG CAA C-3') and PBGD-A (5'-TCA ATG TTG CCA CCA CAC TGT CCG TCT-3').<sup>13</sup> These primers amplify a 98 bp fragment of SYT-SSX mRNA and a 127 bp fragment of PBGD mRNA, respectively. In each PCR procedure, a control lacking reverse transcription (to exclude cDNA contamination) and a negative control containing all reagents but no cDNA template were included.

To confirm the type of SYT-SSX fusion gene, the PCR products were cloned into a pCR2.1 vector (Invitrogen, San Diego, California, USA) by TA ligation and sequenced using an automated sequencing system, namely the ALF express DNA sequencer (Pharmacia Biotech, Uppsala, Sweden).

## Results

Microscopically, all three tumours consisted of two alternating components; one was made up of fibroblast like spindle cells arranged in fascicles, and the other was composed of epithelioid cells forming glandular structures or arranged in sheets. Immunohistochemically, the epithelial components detected were cytokeratins (detected with CAM5.2 (Becton Dickinson, San Jose, California, USA) and AE1/AE3 (Boehringer Mannheim, Mannheim, Germany)) and epithelial membrane antigen (Dako, Kyoto, Japan).

Both the predicted 98 bp transcript of the SYT-SSX fusion gene and the transcript of the PBGD gene were identified in all six microdissected samples from both epithelial and spindle cell areas of the three specimens (fig 2). The nucleotide sequences of these products were confirmed by sequence analysis. Two of

Table 2 Protocol of DOP-PCR

Reagent	Volume ( $\mu$ l)	Reaction
Preamplification step		
10 $\times$ high salt buffer	0.5	1 minute denaturation at 94 $^{\circ}$ C
2 mM dNTP	0.5	1 minute annealing at 25 $^{\circ}$ C
10 $\mu$ M UN1 primer	0.5	3 minute ramp from 25 $^{\circ}$ C to 74 $^{\circ}$ C
ThermoSequenase (4 U/ $\mu$ l)	0.5	2 minutes extension at 74 $^{\circ}$ C
Sample	3.0	4 cycles in total
	5.0 (total)	
Second amplification step add		
10 $\times$ low salt buffer	2.0	1 minute denaturation at 94 $^{\circ}$ C
2 mM dNTP	5.0	1 minute annealing at 56 $^{\circ}$ C
Water	12.2	2 minutes extension at 72 $^{\circ}$ C
100 $\mu$ M UN1-primer	0.3	30 cycles in total
Taq polymerase LD (5 U/ $\mu$ l)	0.5	
	25.0 (total)	

10 $\times$  high salt buffer: 200 mM Tris/HCl (pH 9.2), 600 mM KCl, and 20 mM MgCl<sub>2</sub>.

10 $\times$  low salt buffer: 100 mM Tris/HCl (pH 8.4), 100 mM KCl, and 15 mM MgCl<sub>2</sub>.

DOP-PCR, degenerate oligonucleotide primed polymerase chain reaction.

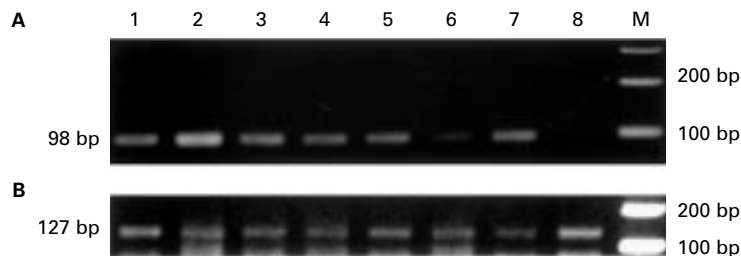


Figure 2 Modified reverse transcription polymerase chain reaction (RT-PCR) amplification of (A) 98 bp SYT-SSX fusion gene transcripts and (B) 127 bp porphobilinogen deaminase (PBGD) gene transcripts from haematoxylin and eosin stained sections of formalin fixed, paraffin wax embedded tissues. Lane 1, positive control of synovial sarcoma; lane 2, epithelial area from case 1; lane 3, spindle cell area from case 1; lane 4, epithelial area from case 2; lane 5, spindle cell area from case 2; lane 6, epithelial area from case 3; lane 7, spindle cell area from case 3; lane 8, negative control (pulmonary adenocarcinoma); M, molecular weight marker. The double bands in (B) were probably formed by DNA conformational changes, because the DNA sequences of the PCR products in both upper and lower bands were confirmed to be identical by sequence analysis.

the three tumours had an SYT-SSX1 fusion transcript, and one had an SYT-SSX2 fusion transcript. No PCR products of the SYT-SSX fusion gene were detected in the three pulmonary adenocarcinomas, although transcripts of the PBGD genes were detected (table 1).

### Discussion

SYT-SSX fusion transcripts have been detected in most synovial sarcomas, of both the monophasic and biphasic type.<sup>14</sup> The distribution of tumour cells expressing the SYT-SSX gene in biphasic synovial sarcoma remains to be elucidated, although Hiraga *et al* detected SYT-SSX fusion probe binding in both epithelial and spindle cell areas of biphasic synovial sarcomas by in situ hybridisation.<sup>6</sup> Our study confirmed that SYT-SSX fusion transcripts are present in microdissected specimens from both areas of biphasic synovial sarcoma using a modified RT-PCR assay. Our results suggest that the products of SYT-SSX fusion transcripts may not be related directly to the morphogenesis of epithelial or spindle cell components in synovial sarcomas, although recently the products of SYT-SSX fusion transcripts have been shown to exist in the tumour cell nuclei of synovial sarcoma and might be transcription coactivators of target genes that regulate both cell proliferation and differentiation.<sup>15 16</sup>

The mean total RNA quantity from the microdissected samples containing 50–100

tumour cells was 0.01–0.5% of that of five 5  $\mu$ m thick sections, as used in previously reported methods using paraffin wax embedded tissue (data not shown). Most of the previous reports of RNA analysis using laser assisted microdissection have used conventional RT-PCR methods.<sup>8 9</sup> We tried increasing the cycle numbers of our previous protocols for PCR or nested PCR,<sup>4 17 18</sup> but still could not detect SYT-SSX fusion transcripts or PBGD gene transcripts in the microdissected samples used in our study. The ability to detect these gene transcripts is likely to be influenced by the quality and size of RNA extracted from routinely stained sections of formalin fixed, paraffin wax embedded tissues, being affected by fixative, fixation time, duration of prefixation, and/or the time that has elapsed since death.<sup>19</sup> Recently, DOP or primer extension preamplification PCR, both of which use whole genome amplification of a small amount of DNA extracted from microdissected or flow sorted samples, have been reported to be useful for the detection of p53 and K-ras point mutations and for microsatellite analysis.<sup>9 19 20</sup> Therefore, we performed DOP-PCR after a reverse transcription step for amplification of a small amount of cDNA, and found that RT-DOP-PCR-PCR analysis could optimise and expand the detection of target RNA from microdissected samples using haematoxylin and eosin stained sections from archival material. This is the first report of a molecular assay using a RT-DOP-PCR-PCR protocol for a small amount of RNA from microdissected samples.

In conclusion, SYT-SSX fusion transcripts are detected in both epithelial and spindle cell areas of biphasic synovial sarcoma using laser assisted microdissection.

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