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Identification of *SYT-SSX* transcripts from synovial sarcomas using RT-multiplex PCR and capillary electrophoresis

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Synovial sarcomas are highly malignant tumors of soft tissue which are characterized by the t(X;18) resulting in *SYT-SSX* fusion transcript production. Diagnosis of these tumors based on histology can be challenging, particularly when minimal biopsy specimens are presented to the pathologist. Demonstration by molecular methods of *SYT-SSX* transcripts is a useful adjunct for diagnosis in these situations. We have developed an assay, which combines one-step RT-multiplex PCR with capillary electrophoresis to detect and genotype the *SYT-SSX* transcripts from synovial sarcomas. Small amplicons from chimeric transcripts as well as *GAPD* transcripts are differentially labeled with fluorophores, allowing detection and size discrimination by capillary electrophoresis. In a study of 32 formalin-fixed soft tissue tumor specimens, the assay detected chimeric transcripts from 17/22 (77%) synovial sarcomas. All five assay negative specimens yielded no intact RNA as evidenced by lack of a *GAPD* amplicon. Chimeric transcripts were not detected in 9/9 malignant peripheral nerve sheath tumors or 1/1 epithelioid sarcoma. Representative amplicons were sequenced and confirmed the genotype results obtained by capillary electrophoresis. One-step RT-multiplex PCR combined with capillary electrophoresis is a rapid and accurate method for the detection and genotypic classification of *SYT-SSX* transcripts from fixed tissue specimens.
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Synovial sarcomas account for approximately 5–10% of all soft tissue sarcomas. They are aggressive, highly malignant tumors, typically developing around the large joints of the extremities, especially the knee and thigh. They occur most frequently in adolescents and young adults (annual incidence approximately 1 per million) and with equal frequency in males and females. The overall prognosis associated with these tumors is poor, with 25% of patients succumbing to their disease within 5 years of diagnosis in spite of aggressive treatment modalities.[1] The name *synovial* sarcoma was applied to the tumors because they were initially believed to originate from synovium; however, the tumors typically display morphological and phenotypic characteristics of epithelial, not synovial, differentiation. The true cell of origin remains unknown, but it is currently believed that these tumors arise from a type of pluripotent stem cell.[2]

Histologically, synovial sarcomas are generally divided into two subtypes: biphasic and monophasic. Biphasic tumors contain both epithelial cells arranged in gland-like structures and spindle shaped cells, whereas monophasic tumors are composed of spindle cells only. Histologic diagnosis of synovial sarcoma can be challenging, particularly for the monophasic type, as they can closely resemble other spindle cell tumors.

The t(X;18) translocation was first recognized as a hallmark of synovial sarcomas in the 1980s.[3,4] This translocation has been shown to be present in over 95% of synovial sarcomas, regardless of histologic subtype.[2] The molecular characterization of the breakpoints involved in the translocation identified novel genes, designated *SSX* (at Xp11) and *SYT* (at 18q11).[5] Protein products of both the *SYT* and *SSX* genes appear to function as transcription regulatory factors, although their target genes are presently unknown.[6] Subsequent work has shown that the breakpoint at Xp11 can alternatively involve one of at least three closely related members of an *SSX* gene family, designated *SSX1, SSX2,* and *SSX4.*[7–9]

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The translocation breakpoints consistently occur in intron 10 of the *SYT* gene and intron 4 of the *SSX* genes.[6,10] Transcription of the resulting chimeric gene typically produces an in-frame transcript composed of exons 1–10 of the *SYT* gene fused to exons 5 and 6 of an *SSX* gene.

As a result of its high prevalence in synovial sarcomas, the t(X;18) translocation and more specifically the resulting *SYT-SSX* fusion transcript, is a highly sensitive diagnostic marker for these tumors. Its specificity for synovial sarcoma has been shown to approach 100% in studies of morphologically similar sarcomas.[11–13] The ratio of tumors harboring *SYT-SSX1* fusions to those with *SYT-SSX2* fusions is approximately 2:1, while tumors harboring the *SYT-SSX4* fusion are extremely rare. Although the clinical significance of the alternate *SYT-SSX* fusion types is unclear,[14–16] there is a strong correlation between biphasic tumor morphology and the presence of an *SYT-SSX1* fusion.[17]

Demonstration by molecular methods of an *SYT-SSX* transcript is a useful adjunct to the morphologic diagnosis of synovial sarcomas, particularly when minimal biopsy specimens are presented to the pathologist. Our goal was to establish a rapid, sensitive and specific assay for the detection of *SYT-SSX* transcripts, from fresh or fixed tissue specimens, that can be routinely performed in a clinical laboratory. We have developed an assay combining one step RT-multiplex PCR with capillary electrophoresis to simultaneously detect *SYT-SSX* fusions involving any *SSX* gene family member and genotype those involving the *SSX1* or *SSX2* genes.

Materials and methods

Specimen Selection

Formalin-fixed, paraffin-embedded tissue blocks from 32 cases of previously diagnosed soft tissue tumors were retrieved from the files of the University of Michigan Department of Pathology (22 synovial sarcomas, nine malignant peripheral nerve sheath tumors, one epithelioid sarcoma). Diagnoses were made based upon histomorphology and available ancillary studies, which were reviewed by two of the authors (DRL and RER).

RNA Preparation

A Leitz 1512 rotary microtome was used to cut two 20 μm sections from each tumor block, with a new blade being used for each block. The sections were deparaffinized with xylene/ethanol, and the tissue digested overnight with proteinase K. RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and precipitated with isopropanol essentially as described.[18] The extracted RNA was resuspended in DEPC-treated deionized water, quantitated spectrophotometrically, and stored at -70°C until use.

Multiplex RT-PCR and Capillary Electrophoresis

Genomic sequences, including intron–exon boundaries, for all genes and gene products analyzed were obtained from the public database of the National Center for Biotechnology Information. Oligonucleotide primers for use in multiplex PCR were designed using the software programs Primer Express v2.0 (Applied Biosystems, Foster City, CA, USA) and Vector NTI Advance (Informax/Invitrogen). Sequences of all primers used in these studies are shown in Table 1. Fluorochrome-labeled and unlabeled primers were purchased from Applied Biosystems or Integrated DNA Technologies (Coralville, IA, USA). All assays were performed in a total volume of 50 μl using the GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems). For each multiplex assay, 0.1–1.0 μg of total RNA was combined with random hexamers and all labeled and unlabeled primers: forward and reverse *GAPD* specific primers were used at 75 nM final concentration, while the *SYT* exon 10 labeled primer was used at 150 nM and unlabeled *SSX* primers at 200 nM. Thermal cycling was carried out in a GeneAmp® PCR System 9700 instrument (Applied Biosystems) as follows: 42°C for 12 min, 95°C for 10 min, then 43 cycles of 95°C for 20 s, 58°C for 30 s, 72°C for 1 min. Following PCR amplification, 1 μl of each sample was analyzed by capillary electrophoresis on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) using POP-4™ polymer and a 36 cm capillary array. The GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems) was used as internal size standards. Fragment analysis was performed using GeneMapper® ID Software v3.1 (Applied Biosystems).

DNA Sequencing

PCR amplicons from representative chimeric transcript-positive tumor samples were purified from low-melting temperature agarose gels and ligated

Table 1 Multiplex PCR and sequencing primers

GAPD assay	
GAPD exon 1	5′-NED-GGAAGGTGAAGGTCGGAGTCAA
GAPD exon 3	5′-GACGGTGCCATGGAATTTGC
SYT-SSX assay	
SYT exon 10	5′-6FAM-AGGTCAGCAGTATGGAGGATATA GACC
SSX common	5′-GCTGGCTTCTTGGGCATGAT
SSX1	5′-GGCCAGATGCTTCTGACACTC
SSX2	5′-CACTTCCTCCGAATCATTTCCT
SSX common primer for transcript sequence verification	5′-TTTCCCATCGTTTTGTGGGCCAGATGC

6FAM, 6 carboxy-fluorescein tag; NED, Applied Biosystems proprietary yellow fluorochrome tag. Bases which are unique to either the *SSX1* or *SSX2* gene and confer genotype specificity are underlined.

into the pCR®4-TOPO® vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen). Ligation products were used to transform chemically competent TOP10 cells (Invitrogen). Colonies were screened for the presence of inserts by PCR, and then grown overnight in broth culture. Plasmid DNA was purified using the Wizard® *Plus* Minipreps DNA Purification System (Promega, Madison, WI, USA), then subjected to cycle sequencing in both the forward and reverse directions using the ABI PRISM® BigDye™ Terminator v3.0 Cycle Sequencing kit (Applied Biosystems). Sequencing reactions were analyzed on an ABI PRISM® 3100 Genetic Analyzer using POP-6™ polymer and a 50 cm capillary array.

Results

For detection of the *SYT-SSX* chimeric transcripts, a one step reverse transcription multiplex PCR assay was designed. Following reverse transcription of RNA using random hexamers, the cDNA is subjected to multiplex PCR amplification using one set of primers specific for *GAPD* transcripts and a second set of primers specific for *SYT-SSX* transcripts. The *SYT-SSX* transcript specific primer set is composed of one sense primer, annealing to a sequence in exon 10 of the *SYT* gene and three antisense primers, the first specific for a sequence common to exon 5 of all members of the *SSX* gene family, the second specific for a sequence unique to exon 5 of the *SSX1* gene, and the third specific to a sequence in exon 5 of the *SSX2* gene (see Figure 1). The sense primer of each set is differentially labeled with a fluorochrome, allowing specific detection and sizing of the resulting amplicons by capillary electrophoresis.

Using these primers, as illustrated in Figure 1, amplification of cDNA generated from either an *SYT-SSX1* or and *SYT-SSX2* transcript results in the production of two amplicons: one amplicon is identical in size (102 bp) when produced from either type of transcript, whereas the second amplicon differs in size between an *SYT-SSX1* transcript (146 bp) or an *SYT-SSX2* transcript (130 bp). In the rare case of an *SYT-SSX4* transcript, only the 102 bp amplicon would be produced. Within the same reaction, a slightly larger (176 bp) amplicon is also produced from *GAPD* transcripts. Representative assay results from both an *SYT-SSX1* and an *SYT-SSX2* positive tumor specimen are shown in Figure 2.

The clinical specificity of the assay was evaluated by analyzing RNA extracted from a panel of formalin-fixed, paraffin-embedded tumor specimens. All cases had been diagnosed prior to this study based upon histology and the use of immuno-

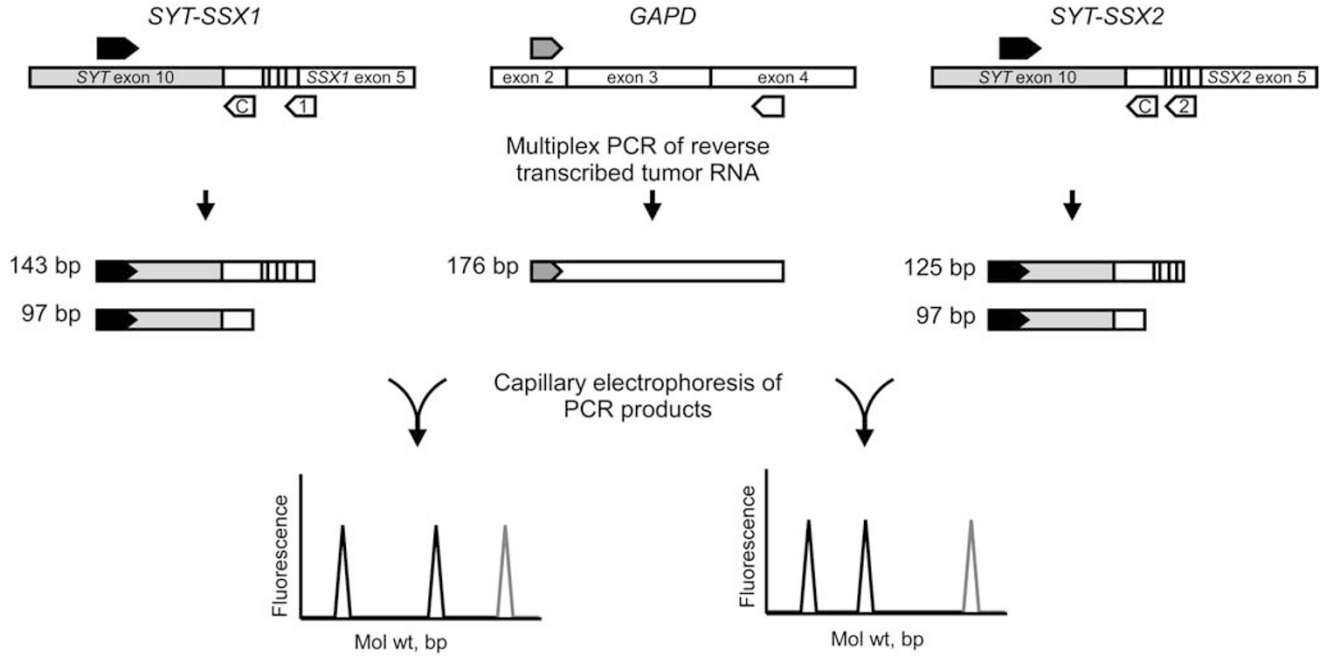

Figure 1 Schematic diagram of RT-multiplex PCR for detection and genotypic characterization of *SYT-SSX* transcripts, depicting relative locations of primers, PCR amplicon sizes, and expected capillary electrophoresis results from tumors harboring either *SYT-SSX1* or *SYT-SSX2* transcripts. RNA extracted from FFPE tissue blocks is combined with all RT-PCR reagents in a single tube. A 176 bp NED-labeled amplicon is produced from *GAPD* transcripts and 6-FAM-labeled amplicons are produced from the *SYT-SSX* fusion transcripts, one (102 bp) common to *SYT* fused to any *SSX* gene, and a second unique to either *SYT-SSX1* (146 bp) or *SYT-SSX2* (130 bp) transcripts. Products are analyzed by capillary electrophoresis and genotype is assigned based on amplicon sizes. Relative primer locations are depicted by chevrons, C indicates a common priming site, 1 and 2 are unique sites. Locations of nucleotides, which differ between exon 5 of *SSX1* and *SSX2* are shown by vertical lines. Labels on the 5′ ends of primers and amplicons are represented as black (6-FAM) or gray (NED).

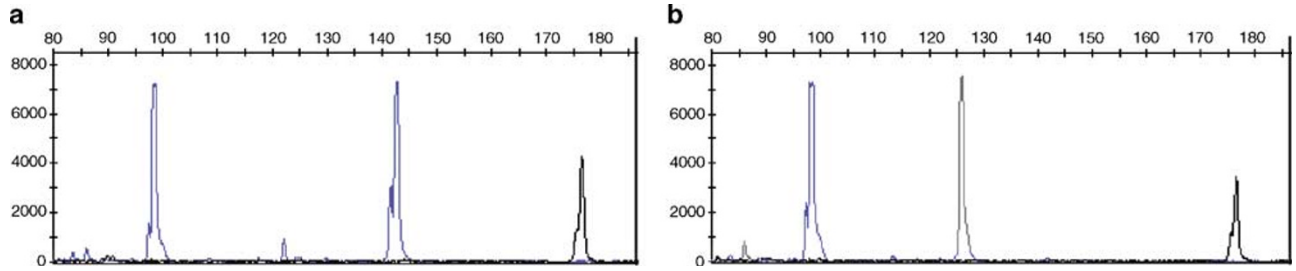

Figure 2 Capillary electrophoresis of RT-multiplex PCR assay products. (**a**) Results from an *SYT-SSX1* positive tumor specimen, while results from an *SYT-SSX2* positive specimen are shown in (**b**). Blue peaks represent *SYT-SSX* amplicons labeled with 6-FAM, black peaks represent *GAPD* amplicons labeled with NED. The scale at the top of each electropherogram indicates the amplicon size, in base pairs. The 6-FAM labeled products routinely appear 4 bp smaller than their actual size, eg, the 102 bp *SYT-SSX* common amplicon runs at 98 bp, while the 130 and 146 bp amplicons run at 126 and 142 bp, respectively.

Table 2 Patient cases, tumor location, diagnosis, transcript findings

Case no.	*Age/Sex*	*Location*	*Diagnosis*	*Assay results*	
				GAPD	*SYT-SSX*
SS1	37M	Lung	SS, Biphasic	Pos	*SYT-SSX1*
SS2	19M	Ankle	SS, Monophasic	Neg	None
SS3	72M	Thigh	SS, Monophasic	Pos	*SYT-SSX1*
SS4	24F	Finger	SS, NOS	Neg	None
SS5	20F	Ankle	SS, Monophasic	Pos	*SYT-SSX2*
SS6	31F	Leg	SS, Monophasic	Pos	*SYT-SSX1*
SS7	7F	Thigh	SS, Monophasic	Neg	None
SS8	47M	Knee	SS, Biphasic	Pos	*SYT-SSX1*
SS9	71M	Thigh	SS, Monophasic	Pos	*SYT-SSX1*
SS10	15M	Thigh	SS, Biphasic	Pos	*SYT-SSX1*
SS11	26F	Hand	SS, Monophasic	Neg	None
SS12	52M	Scrotum	SS, Biphasic	Pos	*SYT-SSX1*
SS13	53M	Thigh	SS, Biphasic	Pos	*SYT-SSX1*
SS14	36F	Lung	SS, Biphasic	Pos	*SYT-SSX2*
SS15	58M	Lymph node	SS, Biphasic	Pos	*SYT-SSX1*
SS16	44M	Thigh	SS, Biphasic	Pos	*SYT-SSX1*
SS17	18M	Foot	SS, Monophasic	Neg	None
SS18	18F	Face	SS, biphasic	Pos	*SYT-SSX1*
SS19	21F	Thigh	SS, Monophasic	Pos	*SYT-SSX2*
SS20	39M	Shoulder	SS, Biphasic	Pos	*SYT-SSX1*
SS21	59M	Lung	SS, Monophasic	Pos	*SYT-SSX2*
SS22	11M	Neck	SS, Monophasic	Pos	*SYT-SSX2*
C1	44M	Parotid gland	MPNST	Pos	None
C2	26F	Calf	MPNST	Pos	None
C3	41F	Chest wall	MPNST	Pos	None
C4	35M	Flank	MPNST	Pos	None
C5	4M	Brachial plexus	MPNST	Pos	None
C6	52F	Neck	MPNST	Pos	None
C7	52F	Thigh	MPNST	Pos	None
C8	9M	Lumbar fascia	MPNST	Pos	None
C9	28M	Buttock	MPNST	Pos	None
C10	12M	Hand	Epithelioid sarcoma	Pos	None

SS, synovial sarcoma; NOS, not otherwise specified; MPNST, malignant peripheral nerve sheath tumor.

histochemical markers, including antibodies to cytokeratin filaments, epithelial membrane antigen, and S100 protein, among others. Of 22 synovial sarcoma specimens, 11 were monophasic, 10 were biphasic, and for one the histomorphology was uncertain due to a small sample size and loss of architectural integrity.

The results of the RT-multiplex PCR assay and the histomorphological characteristics of the corresponding tumor specimens are presented in Table 2. For 17/22 synovial sarcoma specimens, both a *GAPD* amplicon and *SYT-SSX* amplicons were produced. In all, 12 of 17 genotyped as *SYT-SSX1* (nine biphasic, three monophasic), while 5/17 genotyped as *SYT-SSX2* (one biphasic, four monophasic). For the remaining 5/22 synovial sarcoma specimens, neither a *GAPD* amplicon nor any *SYT-SSX* amplicons were produced, suggesting either the lack of intact RNA or

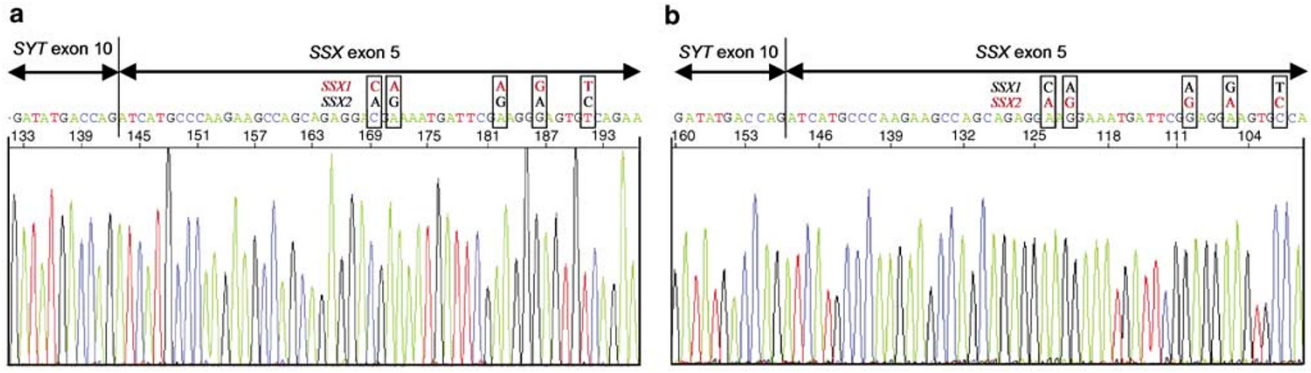

Figure 3 Sequence analysis of cDNA from *SYT-SSX* transcripts. RNA from synovial sarcoma specimens was used to generate RT-PCR amplicons using the *SYT* exon 10 sense primer in combination with an antisense *SSX* exon 5 primer annealing to a region downstream of the genotype specific *SSX* sequences. The amplicons were ligated into a plasmid vector and sequenced in both the forward and reverse directions. (**a**) Results from an *SYT-SSX1* tumor; (**b**) results from an *SYT-SSX2* tumor. The fusion junctions of the *SYT* and *SSX* exons are indicated. Nucleotides which define the *SYT-SSX* genotype are indicated above each sequence and are enclosed in boxes.

the presence of a PCR inhibitor. Re-analysis of a diluted RNA specimen failed to produce a positive result in all five cases (data not shown). No *SYT-SSX* amplicons were produced from RNA extracted from 9/9 malignant nerve sheath tumors or 1/1 epithelioid sarcoma; however, each of these specimens did yield a *GAPD* amplicon.

In order to confirm the genotype specificity of the assay, RNA from an *SYT-SSX1*-positive tumor and an *SYT-SSX2*-positive tumor was reverse transcribed and amplified using an unlabeled version of the *SYT* specific primer in conjunction with an antisense primer located in a sequence common to the SSX genes, downstream of the sequences targeted by the genotype-specific primers. The amplicons were ligated into the pCR4-TOPO plasmid and the resulting constructs sequenced in the forward and reverse directions. As shown in Figure 3, in each case examined the genotype predicted by the RT-multiplex PCR assay was confirmed by sequence analysis.

Discussion

We have developed an assay that utilizes one-step RT-multiplex PCR coupled with capillary electrophoresis to detect and genotype the *SYT-SSX* chimeric transcripts that characterize synovial sarcomas. In our analysis of 32 previously diagnosed soft tissue tumor specimens, a positive result was obtained with this assay from 17/22 formalin-fixed synovial sarcoma specimens (77% sensitivity) while 9/9 malignant peripheral nerve sheath tumors and 1/1 epithelioid sarcoma were negative (100% specificity).

The failure to produce an *SYT-SSX* amplicon from five of the 22 synovial sarcoma cases could result from poor RNA quality, an atypical fusion transcript, or the absence of a translocation in those particular tumor specimens. Significantly, there was a 100% correlation between *SYT-SSX* negativity and the apparent absence of intact RNA in the extracted specimens, as demonstrated by the inability to produce a *GAPD* amplicon from these same specimens. This finding might reflect differences in fixation time or in the age of the specimens at the time of RNA extraction, as both of these factors have been shown to affect the quality of extractable nucleic acids.[19] Although the fixation time used for each of these specimens is not known, we found no definitive correlation between the age of the specimen and the presence of intact RNA (data not shown).

Rare cases of synovial sarcoma in which the *SYT* gene is fused to the *SSX4* gene have been reported;[9,20] however, our assay should detect transcripts from this type of fusion as well, by virtue of its use of a primer site common to all *SSX* gene family members (ie, 102 bp amplicon, Figure 1) in addition to the *SSX1* and *SSX2* gene-specific priming sites. In any event, a 77% sensitivity is not unlike that which has been previously reported for RT-PCR-based studies on fixed tissue specimens.[21]

As increasing emphasis is placed upon minimally invasive biopsy strategies, molecular approaches to the diagnosis of soft tissue tumors are frequently required in routine practice. As a result of this, it is desirable for a clinical laboratory to have available an assay which can be utilized on fresh or fixed tissue specimens, has a rapid turn around time, and provides results which are rapidly and accurately interpretable. A variety of approaches to the detection of chimeric fusion genes or their transcripts can be utilized, including conventional cytogenetics, fluorescence *in situ* hybridization (FISH) and several variations of RT-PCR.[22–27] The RT-multiplex PCR/capillary electrophoresis assay described here was designed to produce small (<150 bp), genotype-specific amplicons from *SYT-SSX* fusion transcripts as well as a slightly larger amplicon from the normal *GAPD* transcript, making it well suited for use on RNA extracted from fixed tissue specimens. Through the use of *SSX* genotype-specific and

non-specific primers and a multiplex design, the assay has the ability to detect fusion transcripts involving any *SSX* gene, and simultaneously genotype the two most common forms. The reliability of this design for correctly genotyping tumor-derived transcripts was confirmed by direct sequencing of PCR products. The single tube format of our assay simplifies interpretation, in that the presence or absence of a *GAPD* amplicon helps to distinguish a true negative result (ie, *GAPD* amplicon present without fusion gene amplicon) from a false negative result due to PCR inhibitors or poor RNA quality (neither *GAPD* amplicon nor fusion gene amplicon present). Although little can be done to circumvent poor RNA quality, the effect of a PCR inhibitor, once recognized, can frequently be overcome by reanalysis of a diluted specimen. Finally, because the *GAPD* gene may be expressed at a level above that of the fusion gene in some cases, the sensitivity of this assay was intentionally biased to favor amplification of the fusion gene transcript by adjusting the ratio of primer concentrations. Sample dilution experiments (data not shown) demonstrate that, in all cases examined, *GAPD* amplicons become undetectable prior to the point at which fusion gene amplicons become undetectable. This reduces the likelihood of a false negative interpretation of the assay (ie, *GAPD* amplicon present but fusion gene amplicon present at a level below the detection limit of the assay).

The detection of specific chimeric fusion gene transcripts as an adjunct to the histological diagnosis of soft tissue tumors, such as Ewing's sarcoma, desmoplastic small round cell tumor, alveolar rhabdomyosarcoma, and synovial sarcoma has become an important tool for the pathologist. Assays for these markers can provide an increased level of confidence in the accurate diagnosis of soft tissue tumors, particularly for those cases where biopsy specimens are small or in which the morphologic features present may be seen in more than one tumor type. The utility of RT-PCR-based assays for this purpose is well recognized.[28,29] The RT-multiplex PCR/capillary electrophoresis assay described here is easily performed in a clinical molecular diagnostics laboratory and provides reliable results from formalin-fixed tissue specimens. The assay results are readily interpreted and, through the use of an internal amplification control, questions concerning RNA integrity or PCR inhibition frequently encountered with fixed tissue specimens are minimized.

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