Differentiating Ewing's sarcoma from other round blue cell tumors using a RT-PCR translocation panel on formalin-fixed paraffin-embedded tissues

Tracey B Lewis¹, Cheryl M Coffin^{1,2} and Philip S Bernard^{1,2,3}

¹The ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT, USA; ²Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT, USA and ³Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA

Ewing's sarcoma is a common malignancy of bone and soft tissue that occurs most often in children and young adults. Differentiating Ewing's sarcoma from other round blue cell tumors can be a diagnostic challenge because of their similarity in histology and clinical presentation. Thus, ancillary molecular tests for detecting disease-defining translocations are important for confirming the diagnosis. We analyzed 65 round blue cell tumors, including 53 Ewing's sarcoma samples from 50 unique cases. Samples were processed for RNA from archived formalin-fixed paraffin-embedded tissue blocks. Real-time RT-PCR assays specific for Ewing's sarcoma (*EWS-FLI1*, *EWS-ERG*, *EWS-ETV1*, *EWS-ETV4*, and *EWS-FEV*), synovial sarcoma (*SYT-SSX1* and *SYT-SSX2*), and rhabdomyosarcoma (*PAX3-FKHR* and *PAX7-FKHR*) were tested across the samples. The translocation panel had a sensitivity of 81% (43 out of 53 samples) for diagnosing Ewing's sarcoma when using the histological criteria as the 'gold' standard. None of the Ewing's specific translocations were found in the non-Ewing's samples (100% specificity). Of the 43 samples with translocations detected, 26 (60%) had an *EWS-FLI1* type 1 translocation, 13 (30%) had an *EWS-FLI1* type 2 translocation, 3 (7%) had an *EWS-ERG* translocation, 1 had an *EWS-ETV1* translocation, and 1 sample had both an *EWS-FLI1* type 1 and type 2 translocation. Our real-time RT-PCR assay for detecting sarcoma translocations has high sensitivity and specificity for Ewing's sarcoma and has clinical utility in differentiating small round blue cell tumors in the clinical lab.

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Round blue cell tumors are a diverse group of cancers that include the Ewing's sarcoma family, rhabdomyosarcoma, poorly differentiated synovial sarcoma, desmoplastic sarcoma, neuroblastoma, and malignant lymphoma.¹ Although these tumors share similar histology, the prognosis and treatment is vastly different. There are now a variety of molecular techniques that can help differentiate these tumor types. For instance, the histological diagnosis of Ewing's sarcoma can be corroborated with immunohistochemistry (CD99-positive, Flt1-positive)^{2,3} or identification of specific chromosomal

translocations using either fluorescence *in situ* hybridization or RT-PCR.^{4–6}

Ewing's sarcoma commonly presents in adolescence, either at skeletal or extraskkeletal sites, and there are often metastases at the time of diagnosis. Balanced translocations between the *EWS* gene and members of the *ETS* family are characteristic for Ewing's sarcoma. Approximately 85% of cases have an *EWS-FLI1* gene fusion in which the DNA-binding domain of the *ETS* transcript fuses to the trans-activation domain of *FLI1* (t(11;22)(q24;q12)).^{7,8} In ~60% of cases with *EWS-FLI1* translocations, exons 1–7 of *EWS* are fused to exons 6–9 of *FLI1* (type 1 fusion). The remaining cases involving *FLI1* result from *EWS* exons 1–7 joining to exons 5–9 of *FLI1* (type 2 fusion).⁹ *EWS* translocations involving *ERG* as a fusion partner (t(22;21)(q22;q12)) are the second most common translocation in Ewing's, accounting for ~10% of cases.^{7,10,11} Fusions between *EWS* and

Correspondence: Dr PS Bernard, Huntsman Cancer Institute, University of Utah, 2000 Circle of Hope, Suite 3345, Salt Lake City, UT 84105-5550, USA.

E-mail: phil.bernard@hci.utah.edu

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other ETS family members occur less frequently but are also specific for Ewing's: *EWS-ETV1* t(7;22), *EWS-ETV4* t(17;22), *EWS-FEV* t(2;22).^{12–15} Other mesenchymal tumors with specific translocations that should be considered in the differential diagnosis of Ewing's include alveolar rhabdomyosarcoma and synovial sarcoma.

Rhabdomyosarcoma is predominantly a disease of children and adolescents accounting for 5–8% of cancers in the pediatric population. Alveolar rhabdomyosarcoma is the most aggressive type of rhabdomyosarcoma and can be distinguished from embryonal rhabdomyosarcoma and other mesenchymal tumors by the detection of a *PAX-FKHR* gene translocation. In ~55% of alveolar rhabdomyosarcoma cases, the *PAX3* gene on chromosome 2q35 is juxtaposed to the *FKHR* gene on chromosome 13q14. Fusions between *PAX7* on chromosome 1p36 and the *FKHR* gene account for ~22% of cases. The specific translocation type, as well as the tumor type, has prognostic significance—alveolar rhabdomyosarcomas containing a *PAX7-FKHR* translocation are usually less invasive and have a better prognosis than those with the *PAX3-FKHR* gene fusion.¹⁶

Synovial sarcomas frequently present during the second decade of life and should be considered in the differential diagnosis along with other mesenchymal tumors that occur during adolescence. In synovial sarcoma, the *SYT* gene on chromosome 18 is fused to a member of the *SSX* gene family (t(X;18)(p11;q11)). The *SSX* gene family contains nine members, all clustered on the X chromosome, with *SSX1* and *SSX2* being most often involved in synovial sarcoma. Fusion to the *SSX1* gene accounts for ~64% of synovial sarcomas, whereas the *SSX2* gene is involved in the vast remainder of cases. Ladanyi *et al*¹⁷ have reported that cases involving the *SYT-SSX1* gene fusion have a worse prognosis although a more recent study by Guillou and co-workers^{18,19} found no significant correlation.

In this current study, 65 round blue cell tumors were analyzed using a real-time RT-PCR translocation panel for detecting gene fusion transcripts specific for Ewing's sarcoma (*EWS-FLI1*, *EWS-ERG*, *EWS-ETV1*, *EWS-ETV4*, and *EWS-FEV*), synovial sarcoma (*SYT-SSX1* and *SYT-SSX2*), and rhabdomyosarcoma (*PAX3-FKHR* and *PAX7-FKHR*). Alternate splice sites were detected using either dual-labeled probes or the dsDNA dye SYBR Green and post-amplification fluorescent melting curve analysis. Our translocation panel is accurate in detecting Ewing's sarcoma and has general utility in the clinical laboratory for the differentiating round blue cell tumors.

Materials and methods

Cohorts

Institutional Review Board approval was obtained from the University of Utah to study patients

diagnosed with sarcoma at Primary Children's Medical Center (Salt Lake City, UT, USA) or the University of Utah Health Sciences Center. Diagnosis was established based on WHO defined histological criteria.^{20,21}

RNA Extraction and Reverse Transcription

Full-face 10 μ m sections were cut from each formalin-fixed paraffin-embedded tissue block with a microtome and sections were stored in a microfuge tube at -80°C . A single unstained section from each block was deparaffinized with Hemo-De (Scientific Safety Products, Keller, TX, USA) and total cellular RNA was extracted using the High Pure RNA Paraffin Kit (Roche Applied Science, Indianapolis, IN, USA) according to the supplier's instructions. RNA was quantified on a NanoDrop ND-1000 Spectrophotometer (Wilmington, DE, USA) and concentrations greater than 30 ng/ μ l were considered acceptable for reverse transcription. First-strand cDNA synthesis was performed from 500 ng purified total RNA using Superscript III reverse transcriptase (1st Strand Kit, Invitrogen, Carlsbad, CA, USA) and gene specific primers (each anti-sense primer at 1 pmol/ μ l) in a final volume of 25 μ l. Each cDNA was diluted in TE (10 mM Tris-HCl, 0.1 mM EDTA) to a concentration of 5 ng/ μ l.

Primers and Probes

Two different real-time RT-PCR systems were employed for detecting transcripts. Dual-labeled probes were used in the assays for the housekeeper gene (*MRPL19*), the common gene fusions of Ewing's sarcoma (*EWS-FLI1*, type 1 and *EWS-FLI1*, type 2), and alveolar rhabdomyosarcoma (*PAX3-FKHR* and *PAX7-FKHR*). The dsDNA dye SYBR Green was used for the more rare Ewing's translocations (*EWS-ERG*, *EWS-ETV1*, *EWS-ETV4*, and *EWS-FEV*) and synovial sarcoma (*SYT-SSX1* and *SYT-SSX2*). Table 1 provides the primer and probe sequences, the amplicon length, and amplicon T_m (SYBR Green based assays only) for each system. A schematic showing primer and probe placement for each translocation assay is shown in Figure 1.

Real-Time PCR

The dual-labeled probe assays were performed in a final reaction volume of 25 μ l containing 12.5 μ l TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA), 10 ng cDNA, 0.25 μ M gene specific fluorescent probe (Operon, Huntsville, AL, USA), 0.5 μ M each forward and reverse primers (Operon, Huntsville, AL, USA), 0.5 mg/ μ l of bovine serum albumin (Sigma-Aldrich, catalogue # B6917), and 0.1 U UNG (Applied Biosystems, Foster City, CA, USA). Uracil-N-glycosylase and dUTPs were

Table 1 Primers and probes sequences

Assay	Translocation	Forward primer	Reverse primer	Probe	Amplicon (bp)	T _m ^a (°C)
Housekeeper	MRPL19	ggaagaggacttggagctact	tcctggacccgaggattat	caaattctcgacacctgttccttga	95	—
Ewing's sarcoma	EWS/FLI1, type 1	ccaagtcaatatagccaacag	ggccagaattcatgttattgc	acgggcagcaga/acccttcttat	100	—
	EWS/FLI1, type 2	ccaagtcaatatagccaacag	ggccagaattcatgttattgc	acgggcagcaga/gttcactgct	166	—
	EWS/ERG	ccaagtcaatatagccaacag	tccaggaggaactgccaaag	SYBR Green	154	88.49 ± 0.17
	EWS/ETV1	acagccaagctccaagtc	tgtgggtccttcccatac	SYBR Green	98	83.32 ± 0.18
	EWS/ETV4	tctacagccaagctcc	gagaagccctctgtgtgg	SYBR Green	104	85.00 ± 0.16
	EWS/FEV	tggtggacccatggatgaag	tcgggttcttcccgctcttg	SYBR Green	80	82.87 ± 0.11
Rhabdomyosarcoma	PAX3/FKHR	ccgacagcagctctgcctac	tgaacttgctgtgtaggacag	cctctcacctcag/aattcaattcgtca	171	—
	PAX7/FKHR	ccgacagcagctctgcctac	tgaacttgctgtgtaggacag	acggcctgtctcctcag/aattcaatt	159	—
Synovial sarcoma	SYT/SSX1	ccagcagaggccttatggata	acactcccttcgaatcatttctg	SYBR Green	77	82.67 ± 0.26
	SYT/SSX2	ccagcagaggccttatggata	gcacttccctcgaatcatttct	SYBR Green	77	80.98 ± 0.34

^aT_m range based on 2 s.d. from the mean across three separate runs.

Figure 1 A schematic showing primer and probe placement for different sarcoma translocations. (a) Representation of the Ewing's EWS-FLI1 type 1, EWS-FLI1 type 2, and EWS-ERG assays. The type 1 and type 2 EWS-FLI1 translocations are identified during real-time PCR using fusion-specific dual-labeled probes, whereas the EWS-ERG translocation is identified by a characteristic T_m using fluorescent melting curve analysis. (b) The SYT-SSX translocations are identified using a common forward SYT primer and a reverse primer specific for either SSX1 or SSX2, which are placed in separate reactions. (c) The PAX-FKHR translocations are amplified using the same forward and reverse primers and fusion-specific dual-labeled probes differentiate the PAX3-FKHR from the PAX7-FKHR fusion.

included in all PCR master mixes to prevent the possibility of subsequent re-amplification of PCR products. Each sample was amplified in duplicate in the LightCycler (Roche). Prior to amplification, each run was held at 50°C for 5 min (UNG step) in order to remove any contaminating amplicon. A two-step amplification was performed using an initial denaturation step (95°C for 5 min) followed by 50 cycles of denaturation (95°C for 15 s) and annealing/extension (60°C for 1 min). Fluorescence (494 nm) was acquired each cycle during the annealing/extension phase.

The SYBR Green assays (*EWS-ERG*, *EWS-ETV1*, *EWS-ETV4*, *EWS-FEV*, *SYT-SSX1*, and *SYT-SSX2*) were performed in a final reaction volume of 20 µl containing 1 × PCR buffer 3 (Idaho Technology, Salt Lake City, UT, USA), 10 ng cDNA, 0.5 µM each forward and reverse primers, 0.2 mM each of dATP, dCTP, and dGTP, 0.1 mM dTTP, 0.3 mM dUTP (Applied Biosystems, Foster City, CA, USA), 0.1 U UNG (Applied Biosystems, Foster City, CA, USA), 0.2 U Taq (Invitrogen, Carlsbad, CA, USA), and SYBR Green (Molecular Probes/Invitrogen, Carlsbad, CA, USA) at a 1/3000 dilution. PCR reactions were carried out in the LightCycler using an initial UNG step (50°C for 5 min) followed by a denaturation step (95°C for 5 min) and 50 cycles of denaturation (94°C for 3 s), annealing (58°C for 6 s), and extension (72°C for 6 s). A post-amplification melting curve analysis was carried out immediately following PCR by heating to 94°C for 15 s, rapidly cooling to 45°C for 15 s, and slowly heating to 97°C at 0.1°C/s while continuously monitoring fluorescence (494 nm).

Controls

As a control for cDNA synthesis and sample quality, each sample was reverse transcribed and amplified for the housekeeper gene *MRPL19*.²² All samples

were assayed in duplicate, and positive and negative controls were included for each translocation. Samples with a RT-PCR crossing threshold greater than 35 for the housekeeper gene were excluded from further analysis. Positive controls consisted of cell lines, plasmid constructs, and sequence-verified patient samples (Table 2).

The A673 cell line was used as a positive control for the *EWS-FLI1*, type 1 translocation. Interestingly, A673 was originally thought to be derived from a rhabdomyosarcoma until it was found to have a characteristic Ewing's sarcoma translocation.²³ The cell line RDES was used as a control for the *EWS-FLI1*, type 2 translocation (<http://www.biotech.ist.unige.it/cldb/cl4129.html>). Plasmids containing cDNA sequences surrounding the breakpoints for *EWS-ERG*, *EWS-ETV1* and *EWS-ETV4* were used as controls for the remaining Ewing's translocations.

The cell line FU-SY-1 (*SYT-SSX1*) and a sequence confirmed sample (*SYT-SSX2*) were used as positive controls for synovial sarcoma. Controls for human alveolar rhabdomyosarcoma included the cell lines SJRH30 and CW9019, known to contain the *PAX3-FKHR* and *PAX7-FKHR* translocations respectively.²⁴

Results

There were initially 69 Ewing's sarcoma samples extracted for RNA from formalin-fixed paraffin-embedded tissue. Twelve (17%) failed RNA extraction and 4 (6%) were excluded due to poor sample quality as determined by a late Cp (>35) for the housekeeper control gene. Failure in extraction or PCR was not associated with sample age. Forty-three out of 53 (81%) Ewing's samples had a detectable *EWS* translocation by RT-PCR (Table 2). Of the 43 samples with translocations detected, 26 (60%) had an *EWS-FLI1* type 1 translocation, 13 (30%) had an *EWS-FLI1* type 2 translocation, 3 (7%) had an *EWS-ERG* translocation, 1 had an *EWS-ETV1* translocation, and 1 sample had both an *EWS-FLI1* type 1 and type 2 translocation. Figure 2 shows typical results for detecting *EWS-FLI1* variants with fusion-specific dual-labeled probes, and detecting the *EWS-ERG* and *SYT-SSX* gene fusions by fluorescent melting curve analysis.

There were 50 unique cases of Ewing's, since two patients had multiple samples. Out of 50 patients with a diagnosis of Ewing's, 41 (82%) had an *EWS* translocation detected in at least one of their samples. One of the subjects in which replicate samples were obtained had an *EWS-FLI1* type 2 translocation in the primary (femur) and an *EWS-FLI1* type 1 translocation in the metastasis (chest wall). Another subject had three samples with two of the blocks positive for the *EWS-FLI1* type 2 translocation and the other block with no detectable transcript.

Sensitivity (TP/(TP + FN)) and specificity (TN/(TN + FP)) of the RT-PCR translocation panel was

determined for Ewing's sarcoma using the histological diagnosis as the gold standard. Ewing's samples that had no detectable *EWS* translocation were tested on the extended translocation panel and none had a translocation of another type. In addition, the translocation panel was tested on other round blue cell samples (two neuroblastomas, one leiomyosarcoma, one desmoplastic sarcoma, three synovial sarcomas, and four rhabdomyosarcomas samples) and there were no false positives. Overall, the translocation panel had 81% sensitivity and 100% specificity for Ewing's.

Discussion

Round blue cell tumors can have considerable overlap in epidemiology, histology, and immunophenotype. For instance, rhabdomyosarcomas that lack myoblastic differentiation can be difficult to distinguish from other round cell neoplasms, such as Ewing's. In addition, synovial sarcomas may have a spectrum of morphologies from spindle shaped to rounder epithelial-like cells, which can resemble other mesenchymal and non-mesenchymal tumors. Immunohistochemical markers can assist in the differential diagnosis, but the current panels have limitations in specificity.²⁵ For this reason, fluorescence *in situ* hybridization has been useful in difficult cases. For instance, there have been several reported cases of sarcomas suspected to be desmoplastic small round cell tumors or osteosarcomas that upon translocation analyses were shown to have characteristic Ewing's *EWS-FLI1* translocations.^{26–29} Although fluorescence *in situ* hybridization break-apart probes are commonly employed clinically, most assays do not discern the specific fusion type (eg *EWS-FLI1* type 1 and type 2).^{5,30}

Using our RT-PCR translocation panel, we found that 81% of the Ewing's samples had an *EWS* translocation, consistent with previous reports estimating the frequency at 85%.^{7,8} There were an additional 16 Ewing's samples that were rejected due to low RNA quality or quantity. These samples are removed from the analyses upfront so that they do not affect the sensitivity of the assay. While most of the Ewing's samples had an *EWS-FLI1* translocation, we also detected the *EWS-ERG* gene fusion in three samples and the *EWS-ETV1* gene fusion in one sample. No samples had translocations involving the *EWS-FEV* or *EWS-ETV4* translocations, which others have only rarely detected.^{12–15,31} Finally, none of our 11 non-Ewing's samples had an *EWS* fusion.

In a single case, we found *EWS-FLI1* type 1 and type 2 fusion transcripts coexisting in the same sample. The coexistence of defining translocations in Ewing's sarcoma and synovial sarcoma patients has been previously reported.^{1,32} Different fusion transcripts identified in the same patient could be explained by independent chromosomal fusion events or alternate splicing events that occur either

Table 2 Clinico-pathological information

Case	Patient age (in years)	Gender	Site	Histological diagnosis	Translocation	Age of block (in years)
EWS1	18	M	Pelvis	Ewing's Sarcoma	EWS-FLI1, type 1	3
EWS2	15	M	Presacral soft tissue	Ewing's Sarcoma	EWS-FLI1, type 1	1
EWS3	20	M	Calf	Ewing's Sarcoma	EWS-FLI1, type 1	8
EWS4	9	M	Abdomen	Ewing's Sarcoma	EWS-FLI1, type 1	6
EWS5	7	F	Left buttock	Ewing's Sarcoma	EWS-FLI1, type 1	2
EWS6	11	M	Temporal lobe	Ewing's Sarcoma	EWS-FLI1, type 1	6
EWS7	16	M	Left femur	Ewing's Sarcoma	EWS-FLI1, type 1	12
EWS8	21	F	Right 10th rib	Ewing's Sarcoma	EWS-FLI1, type 1	12
EWS9	15	M	Cranium	Ewing's Sarcoma	EWS-FLI1, type 1	7
EWS10	13	F	Spine	Ewing's Sarcoma	EWS-FLI1, type 1	1
EWS11	8	F	Spinal canal T8-L3	Ewing's Sarcoma	EWS-FLI1, type 1	15
EWS12	6	F	Left femur	Ewing's Sarcoma	EWS-FLI1, type 1	8
EWS13	12	F	Right kidney	Ewing's Sarcoma	EWS-FLI1, type 1	2
EWS14	14	M	Right fibula	Ewing's Sarcoma	EWS-FLI1, type 1	4
EWS15	18	M	Right chest and rib	Ewing's Sarcoma	EWS-FLI1, type 1	1
EWS16	17	M	Ilium	Ewing's Sarcoma	EWS-FLI1, type 1	8
EWS17	1	M	Right scapula	Ewing's Sarcoma	EWS-FLI1, type 1	4
EWS18	11	M	Brain metastasis from left 3rd rib	Ewing's Sarcoma	EWS-FLI1, type 1	1
EWS19	22	M	Right 2nd rib	Ewing's Sarcoma	EWS-FLI1, type 1	8
EWS20	18	M	Right thigh	Ewing's Sarcoma	EWS-FLI1, type 1	1
EWS21	17	M	Right lung lower lobe	Ewing's Sarcoma	EWS-FLI1, type 1	3
EWS22	20	M	Right pelvis	Ewing's Sarcoma	EWS-FLI1, type 1	3
EWS23	20	M	Sternum	Ewing's Sarcoma	EWS-FLI1, type 1	2
EWS24-m ^a	20	F	Metastasis right chest wall	Ewing's Sarcoma	EWS-FLI1, type 1	10
EWS25	16	F	Left thigh	Ewing's Sarcoma	EWS-FLI1, type 1	12
EWS26	13	M	Right femur	Ewing's Sarcoma	EWS-FLI1, type 1	6
EWS27	14	M	Right knee	Ewing's Sarcoma	EWS-FLI1, type 2	4
EWS35-p ^b	16	M	Right scapula	Ewing's Sarcoma	EWS-FLI1, type 2	4
EWS28	23	M	Left groin	Ewing's Sarcoma	EWS-FLI1, type 2	11
EWS29	16	M	Right ilium	Ewing's Sarcoma	EWS-FLI1, type 2	2
EWS30	14	F	Right ilium	Ewing's Sarcoma	EWS-FLI1, type 2	6
EWS31	23	M	Right ilium	Ewing's Sarcoma	EWS-FLI1, type 2	6
EWS32	23	F	Left flank	Ewing's Sarcoma	EWS-FLI1, type 2	12
EWS33	10	F	Right humerus	Ewing's Sarcoma	EWS-FLI1, type 2	7
EWS34	15	M	Left neck	Ewing's Sarcoma	EWS-FLI1, type 2	3
EWS35-lr ^b	18	M	Right scapula	Ewing's Sarcoma	EWS-FLI1, type 2	2
EWS36	9	M	Left scapula	Ewing's Sarcoma	EWS-FLI1, type 2	2
EWS37	20	F	Pelvis	Ewing's Sarcoma	EWS-FLI1, type 2	11
EWS24-p ^a	16	F	Left femur	Ewing's Sarcoma	EWS-FLI1, type 2	14
EWS38	26	F	Third and 4th ribs	Ewing's Sarcoma	EWS-ERG	15
EWS39	15	F	Left fibula	Ewing's Sarcoma	EWS-ERG	2
EWS40	12	F	Left 3rd rib	Ewing's Sarcoma	EWS-ERG	10
EWS41	2	M	Back	Ewing's Sarcoma	EWS-ETV1	2
EWS42	10	M	Left calcaneus	Ewing's Sarcoma	Negative	3
EWS43	7	M	Paraspinal	Ewing's Sarcoma	Negative	4
EWS44	18	M	Left chest wall	Ewing's Sarcoma	Negative	13
EWS35-p ^b	16	M	Right scapula	Ewing's Sarcoma	Negative	4
EWS45	1	M	Calf	Ewing's Sarcoma	Negative	2
EWS46	12	M	Right buttock	Ewing's Sarcoma	Negative	6
EWS47	17	M	Right pelvis	Ewing's Sarcoma	Negative	5
EWS48	21	M	Right pelvis	Ewing's Sarcoma	Negative	8
EWS49	25	M	Right pelvis	Ewing's Sarcoma	Negative	13
EWS50	11	M	Right temporal	Ewing's Sarcoma	Negative	14
A673	NA	NA	Control cell line	Ewing's Sarcoma	EWS-FLI1, type 1	NA
03-344	NA	NA	Control sample	Ewing's Sarcoma	EWS-FLI1, type 1	NA
RDES	NA	NA	Control cell line	Ewing's Sarcoma	EWS-FLI1, type 2	NA
02-060	NA	NA	Control sample	Ewing's Sarcoma	EWS-FLI1, type 2	NA
03-1434	NA	NA	Control sample	Neuroblastoma	Negative	NA
03-1359	NA	NA	control sample	Neuroblastoma	Negative	NA
02-415	NA	NA	Control sample	Leiomyosarcoma	Negative	NA
03-558	NA	NA	Control sample	Desmoplastic sarcoma	Negative	NA
03-556	NA	NA	Control sample	Synovial sarcoma	SYT-SSX2	NA
03-563	NA	NA	Control sample	Synovial sarcoma	SYT-SSX1	NA
FU-SY-1	NA	NA	Control cell line	Synovial sarcoma	SYT-SSX1	NA
SJRH30	NA	NA	Control cell line	Rhabdomyosarcoma	PAX3-FKHR	NA
PCO32	NA	NA	Control cell line	Rhabdomyosarcoma	PAX3-FKHR	NA
CW9019	NA	NA	Control cell line	Rhabdomyosarcoma	PAX7-FKHR	NA
PCO33	NA	NA	Control cell line	Rhabdomyosarcoma	PAX7-FKHR	NA

m = metastatic; p = primary; lr = local reoccurrence; NA = not applicable.

^aEWS 24.

^bEWS35.

Figure 2 Detection and differentiation of Ewing's and synovial sarcoma translocations by amplification curves and T_m . Each sample is run in duplicate. (a) A plot of fluorescence vs cycle number shows amplification curves specific for EWS-FLI1 type 1 in the cell line (A673) and the patient sample (03-344), while no amplification is seen in the RDES cell line containing the type 2 translocation. (b) The cell line RDES and the patient sample 02-060 show amplification curves specific for EWS-FLI1 type 2 translocation, while no amplification is seen in the A673 cell line containing the type 1 translocation. (c) Samples are PCR amplified in the presence of the dsDNA dye SYBR Green and post-amplification melting curve analysis is performed. A plot of dF/dT vs fluorescence shows a characteristic T_m for the control plasmid (pERG) and the patient sample (EWS39) containing an EWS-ERG translocation. Non-Ewing's sarcoma samples are negative. (d) The SYT-SSX genes are reverse transcribed using common primers and amplified using primers specific for SSX1 and SSX2. Fluorescent melting curve analysis distinguishes SYT-SSX1 (FUSY1) from SYT-SSX2 (03-556) by a reproducible T_m shift of 0.5°C .

in the same or different cells. Bielack *et al*³³ have reported a case of two primary Ewing's sarcomas in the same patient defined by different translocations, suggesting that the translocations were independent events arising from different clones. Here, we report a case with a type 2 translocation in the primary and a type 1 in the metastasis. Assuming that the metastasis arose from the primary, it suggests that either there was another chromosomal fusion event and the first translocation was lost from the cell or there was a switch in the splicing. It is uncertain whether the loss of exon 5 contributed to the metastatic potential or whether other alterations were responsible.

Studies have suggested that the EWS fusion type may have prognostic importance, with type 1 being associated with lower proliferation and an improved outcome in patients with localized disease.³⁴⁻³⁶ However, outcome differences in Ewing's patients based on fusion type have been statistically difficult to show due to the rarity of the disease and previous

technical challenges in resolving the different fusion types. Large-scale retrospective studies will need to be carried out to determine if our assay has prognostic, as well as diagnostic, value.

A variety of real-time PCR techniques have been used for identifying translocations in hematological malignancies and soft tissue tumors. For instance, dual-labeled probes have been used to detect translocations in chronic myeloid leukemia (*BCR-ABL* transcript) and synovial sarcoma (*SYT-SSX* transcripts),³⁷⁻³⁹ and fluorescent melting curve analysis has been used to detect the *MBR-JH* translocation in follicular lymphoma.⁴⁰ The decision whether to use sequence specific probes or a dsDNA dye as a probe depends on the sequences of the fusion partners and the resolution needed to distinguish different fusion types. In general, dsDNA probes are less expensive and technically easier, making them an attractive alternative to sequence-specific probes. We found that we could distinguish different SYT-SSX fusions using fluor-

escent melting curve analysis and the dsDNA dye SYBR Green. This was possible since the *SSX1* and *SSX2* sequences varied enough to create a characteristic T_m for each fusion type. In contrast, the *EWS-FLI1* fusions were best distinguished by sequence-specific probes.

The advantages of using real-time PCR over conventional PCR with gel electrophoresis are that there is increased sensitivity, less sample manipulation, faster results, and the potential for quantification. For instance, monitoring the amount of *BCR-ABL* transcript in the blood and bone marrow has been useful for detecting relapse in chronic myeloid leukemia.^{37,38} Thus, our translocation assays could also be used quantitatively for monitoring minimal residual disease in sarcomas.

In conclusion, we have developed a translocation panel accurate in detecting Ewing's sarcoma, synovial sarcoma, and rhabdomyosarcoma. Our real-time RT-PCR assays allow rapid detection of translocations specific for these sarcomas. The identification of the different gene fusion partners may add prognostic value to existing fluorescence *in situ* hybridization assays. The RT-PCR panel has utility in the clinical laboratory for diagnosing of sarcomas from formalin-fixed paraffin-embedded tissue and could potentially be used for monitoring minimal residual disease.

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