Undifferentiated Small Round Cell Sarcomas with Rare *EWS* Gene Fusions

Identification of a Novel EWS-SP3 Fusion and of Additional Cases with the EWS-ETV1 and EWS-FEV Fusions

Lu Wang,* Rohit Bhargava,* Tao Zheng,*
Leonard Wexler,* Margaret H. Collins,[†]
Diane Roulston,[‡] and Marc Ladanyi*[§]

From the Department of Pathology* and Human Oncology and Pathogenesis Program,[§] Memorial Sloan-Kettering Cancer Center, New York, New York; the Department of Pathology,[†] Cincinnati Children's Hospital and Medical Center, Cincinnati, Ohio; and the Department of Pathology,[‡] University of Michigan Medical School, Ann Arbor, Michigan

Ewing family tumors (EFTs) are prototypical primitive small round blue cell sarcomas arising in bone or extraskelatal soft tissues in children or adolescents. EFTs show fusions of *EWS* with a gene of the ETS family of transcription factors, either *EWS-FLI1* (90 to 95%) or *EWS-ERG* (5 to 10%). Rare cases with fusions of *EWS* to other ETS family genes, such as *ETV1*, *E1AF*, and *FEV*, have been identified, but their clinicopathological similarity to classic EFTs remains unclear. We report four new cases of EFT-like tumors with rare *EWS* fusions, including two with *EWS-ETV1*, one with *EWS-FEV*, and a fourth case in which we cloned a novel *EWS-SP3* fusion, the first known cancer gene fusion involving a gene of the Sp zinc finger family. Analysis of these three new cases along with data on nine previously reported cases with fusions of *EWS* to *ETV1*, *E1AF*, or *FEV* suggest a strong predilection for extraskelatal primary sites. EFT-like cases with fusions of *EWS* to non-ETS translocation partners are also uncommon but involve the same amino-terminal portion of *EWS*, which in our novel *EWS-SP3* fusion is joined to the SP3 zinc-finger DNA-binding domain. As these data further support, these types of *EWS* fusions are associated with primitive extraskelatal small round cell sarcomas of uncertain lineage arising mainly in the pediatric population. (*J Mol Diagn* 2007, 9:498–509; DOI: 10.2353/jmoldx.2007.070053)

mors in the soft tissues, chest wall, and visceral sites, as well as tumors previously termed peripheral neuroectodermal tumor, which show more neural features. EFTs are also defined by translocations, resulting in the fusion of the *EWS* gene, located at 22q12, and a gene of the ETS family of transcription factors.^{1–3} The ETS family of transcription factors is defined by the ETS domain, which interacts with DNA at sequences containing a common sequence motif.⁴ In 90 to 95% of cases, the gene fusion is *EWS-FLI1*, consisting of the N-terminal portion of *EWS* and the C-terminal portion of *FLI1* including the ETS DNA-binding domain. An *EWS-ERG* fusion, where the *ERG* gene from 21q22 substitutes for *FLI1*, is found in 5 to 10% of cases.^{2,5} *EWS* is a ubiquitously expressed protein containing an RNA-binding domain in its C-terminal portion, whereas its N-terminal domain can function as a strong transactivation domain when juxtaposed to a DNA-binding domain.⁶ The *EWS* promoter is strongly and broadly activated, leading to relatively unrestricted high-level expression of the resulting fusion genes.^{7,8} In contrast, expression of native *FLI1* is tightly regulated and lineage restricted.⁹ Details of the biology of native *EWS* and *FLI1*, as well as functional aspects of *EWS-FLI1*, are reviewed in detail elsewhere.^{10,11} In less than 1% of cases, variant translocations namely t(7;22)(p22;q12), t(17;22)(q12;q12), and t(2;22)(q33;q12) involving fusion of *EWS* gene with *ETV1*, *E1AF* (also known as *ETV4*), and *FEV* genes, respectively, have been described.^{12–15} *ETV1*, *E1AF*, and *FEV* are all three members of the ETS family of transcription factors that are highly related to *FLI1* and *ERG*.

Although most other well-known *EWS* fusions have involved other transcription factor gene families and have been described in tumors that are pathologically clearly distinct from EFTs, such as clear cell sarcoma, extraskelatal myxoid chondrosarcoma, myxoid liposarcoma, and angiomatoid fibrous histiocytoma,^{11,16,17} there have

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L.W. and R.B. contributed equally to this study.

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Address reprint requests to Marc Ladanyi, M.D., Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021. E-mail: ladanyim@mskcc.org.

Classic Ewing's sarcoma is a primitive bone sarcoma of children or adolescents composed of small round cells showing limited neural differentiation. The Ewing family of tumors (EFTs) also includes identical or very similar tu-

Table 1. Primer Sequences for *EWS-ETS* Fusion Transcripts

Primers	Sequences
Forward primers	
EWS exon 7 (22.3)	5'-TCCTACAGCCAAGCTCCAAGTC-3'
EWS exon 7 (RACE1)	5'-AGCAGCCTCCCACCTAGTTAC-3'
EWS exon 7 (22.14)	5'-GCACCTCCATCCTACCCTCC-3'
Reverse primers	
FLI1 exon 6	5'-GTTGAGGCCAGAATTCATGTTA-3'
FLI1 exon 9	5'-ACTCCCCGTTGGTCCCTCC-3'
ERG exon 9	5'-AAAGCTGGATCTGGCCACTG-3'
ETV1	5'-CCGATACATTCTGGCTCTTG-3'
ETS1 (consensus for FLI1, ERG, and ETV1)	5'-AGGAA-T/C-TGCCA-A/C-AGCTGGATCTG-3'
ETS2 (consensus for ETV1 and E1AF)	5'-CC-A/C-GTCCAGGCAAT-A/G-AAATG-3'

been rare reports of unique *EWS* fusions in EFT-like cases that are more difficult to fit in existing categories. For instance, a single case has been reported of a small round cell tumor of the chest wall with a fusion of *EWS* to a zinc finger family gene, *ZNF278* (also known as *ZSG*).¹⁸ The only other translocation fusing *EWS* to a zinc finger family gene is the *EWS-WT1* fusion of the desmoplastic small round cell tumor (DSRCT).^{19–21} Indeed, the case bearing the *EWS-ZNF278* fusion displayed some features more typical of DSRCT, such as positivity for desmin and negativity for MIC2, but lacked desmoplasia. Finally, another unique *EWS* fusion has recently been reported in a bone tumor, involving a POU homeodomain family gene, *POU5F1*.²²

Studies evaluating the clinicopathological correlates of the different *EWS* gene fusions in EFTs have focused on cases with the common *EWS-FLI1* and *EWS-ERG* fusion transcripts. Overall, no significant clinical or pathological differences have been found between cases with *EWS-FLI1* and those with *EWS-ERG*.²³ Among *EWS-FLI1* cases, the most common type of *EWS-FLI1* fusion transcript (type 1) may be associated with a somewhat more favorable prognosis compared with type 2 *EWS-FLI1* and other types.^{24–26} In contrast, little or nothing has been published concerning the clinicopathological features of patients with tumors harboring rare variant *EWS* fusions (*EWS-ETV1*, *EWS-E1AF*, and *EWS-FEV*) after their initial discovery.

We report four new cases of EFT-like tumors with rare *EWS* fusions, including two with *EWS-ETV1*, one with *EWS-FEV*, and a fourth case in which we have cloned a novel *EWS-SP3* fusion, the first known cancer gene fusion involving a gene of the Sp zinc finger family. We have also reviewed the clinical and pathological data on these cases along with other reported cases in the literature and highlight the clinicopathological features of these primitive round cell sarcomas with rare variant *EWS* fusions.

Materials and Methods

RNA Extraction

Total RNA was isolated from snap-frozen tissue using acid guanidium-isothiocyanate followed by phenol-chloroform extraction and isopropanol precipitation. RNA was

resuspended in diethylpyrocarbonate-treated water and stored at -70°C .

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using the Qiagen (Valencia, CA) one-step RT-PCR kit. The reaction was performed in one tube in a sequential manner, in a final volume of 50 μL . Each reaction tube contained 1 μg of total RNA diluted in 26.5 μL of RNase-free water, 10 μL of 5 $\times$ buffer containing 2.5 mmol of MgCl_2 , 2 μL of dNTP mix (final concentration: 400 $\mu\text{mol/L}$ of each dNTP), 0.5 μL of RNase inhibitor (5 U), 8 μL of primers (each primer concentration: 0.5 to 1 $\mu\text{mol/L}$), and 2 μL of one-step enzyme mix. The thermal cycling conditions were as follows: reverse transcriptase at 50°C for 30 minutes, initial PCR activation step at 95°C for 15 minutes, 35 cycles of denaturation (94°C for 30 seconds), annealing (60°C for 1 minute), and extension (72°C for 30 seconds). The final extension was performed at 72°C for 7 minutes. The primers are listed in Table 1. PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide in 1 $\times$ Tris acetic acid ethylenediamine tetraacetic acid (TAE) buffer.

Sequencing

The RT-PCR product was purified with a Qiagen purification kit and sequenced on an Applied Biosystems (Foster City, CA) automated sequencer using the dideoxy dye terminator method.

Rapid Amplification of cDNA Ends (RACE)

RACE was performed using the 3' RACE system from Invitrogen (Carlsbad, CA). In brief, first-strand cDNA synthesis was initiated at the poly(A) tail of the mRNA using the adapter primer (5'-GGCCACGCGTCGACTAG-TACTTTTTTTTTTTTTTTT-3'). After the first-strand cDNA synthesis, the original mRNA template was destroyed with RNase H. The first-round amplification was performed using a universal amplification primer that binds the adapter sequence in the adapter primer (AUAP; 5'-GGCCACGCGTCGACTAGTAC-3') and a forward primer that anneals to *EWS* exon 6 (*EWS*22.14; Table 1). To

Table 2. EFTs with Rare Variant Fusions of *EWS* to *ETS* Family Transcription Factor Genes

Case no. (reference)	Age/sex	Presentation	Histology
<i>EWS-ETV1</i> cases			
No. 1 (12)	2/F	Primary in gluteal region; lung metastases	SRCT
No. 2, present series, patient A	8/M	Primary adjacent to fibula; no metastases	SRCT
No. 3, present series, patient B	2/F	Primary in pelvis and thigh; lung metastases	Undifferentiated tumor with spindling (post-CT)
No. 4 (42,43)	0/M	Skin and paraspinal; primary and metastatic sites unclear	Round, polygonal and spindle cells with scant cytoplasm
No. 5* (44)	12/F	Primary in femur; no data on metastatic disease	NA
<i>EWS-FEV</i> cases			
No. 6 (15)	2/F	Paraspinal primary; no data on metastatic disease	SRCT
No. 7 (15)	15/M	Extraosseous maxillary primary; no data on metastatic disease	SRCT
No. 8 (45)	15/M	Paratesticular primary; distant metastases	Large round polygonal cells with clear cell features
No. 9 (46)	24/M	Retroperitoneal primary; no metastases	SRCT with foci of pleomorphic large cells
No. 10, present series, patient C	40/M	Primary in retroperitoneum and pelvis; no metastases	Poorly differentiated neoplasm
<i>EWS-ETAF</i> cases			
No. 11 (13)	0/F	Primary in cheek; no metastases	SRCT
No. 12 (14;47)	10/M	Extraosseous chest wall primary; no data on metastatic disease	SRCT

F, Female; M, male; NA, not available; SRCT, small round cell tumor; IHC, immunohistochemistry; EM, electron microscopy; CG, cytogenetics; CT, chemotherapy; RT, radiotherapy; PBSCT, peripheral blood stem cell transplant; NED, no evidence of disease; DOD, dead of disease, AWD, alive with disease.

*No molecular confirmation of fusion transcript on this case.

(table continues)

increase the specificity of the process, a seminested PCR was performed after the first round PCR, using the same AUAP reverse primer and a nested forward primer, EWS22.3 (Table 1), which primes from exon 7 of *EWS*. One μ l of a 1:100 dilution of the first-round PCR product was used as the template for the second-round PCR. The products of the seminested PCR were cloned using the pGEM-T easy AT cloning system (Promega, Madison, WI), and multiple clones were sequenced as described above.

Results

We identified four new cases (Tables 2 and 3, patients A to D) of variant *EWS* fusions. Two of these cases showed the *EWS-ETV1* fusion transcript, one showed an *EWS-FEV* fusion, and the fourth involved a novel *EWS* translocation partner.

Patients A, B, and C: New *EWS-ETV1* and *EWS-FEV* Cases

Patient A was an 8-year-old male child who had a history of trauma to his leg 1.5 years before presenting with a parosteal soft tissue tumor. The nature of the trauma was unknown, but plain films at that time failed to show any lesion. He presented with swelling in the region of trauma, and an magnetic resonance imaging showed a mass near the dorsal aspect of the left fibula. There were no other sites of disease. A resection was performed, showing a primitive tumor with small round cell morphology and diffuse positivity for CD99 by immunohistochemistry (Figure 1, A and B). The tumor karyotype was interpreted as 46,XY,t(7;22)(p15;q11) (Figure 1C). This suggested the presence of the *EWS-ETV1* fusion, and this was confirmed by RT-PCR followed by direct sequencing. The fusion structure was identical to that originally reported, ie, a fusion of *EWS* exon 7 to the same *ETV1* exon (num-

Table 2. Continued

IHC	Cytogenetic translocation	Treatment	Outcome
+ Vimentin, NSE, desmin, MSA, myoglobin	t(7;22)(p21;q12)	NA	NA
+ Vimentin, CD99, Ki 67 >10%, S100, SMA, chromogranin, CD79A, CD34, desmin, EMA, cytokeratin	t(7;22)(p15;q11)	Resection followed by CT (see text for details)	NED at 60 months
+ Leu-7, CD99 (focal), vimentin (weak), SMA, S100, CG, SYN, MSA, CK, EMA, NSE, GFAP, desmin, myogenin, CD34, CD31, factor VIII, CD3, CD45, CD79a	t(7;22)	Neoadjuvant CT, resection (see text for details)	DOD at 60 months
+ S100, CD99, focal CK, CG, SYN	t(7;22)(p21;q11.2)	Surgery, CT, RT, and PBSCT	DOD at 96 months
NA	t(7;22)(p22;q12)	Neoadjuvant CT	DOD at 3 months
+ CD99, desmin, NSE, LCA	t(2;21;22)(q33;q22;q12)	Resection. CT information NA	NA
+ Vimentin, desmin, LCA, S100, NSE, CD99, chromogranin	NA	Resection. CT information NA	NA
+ Vimentin, NSE, PGP9.5, CD99, CD57, S100, NF, CK, EMA, muscle markers	t(2;22)(q13;q22)	Excision followed by CT (vincristine, adriamycin, cisplatin) and local RT	DOD at 28 months
+ CD99, NSE, and weakly for vimentin. Large cells also positive for CEA, CAM 5.2, EMA, actin, desmin, CD45	der(17)t(17;2;22;13)(q21::q11-q33::q12-q13::q14), del(22)(q12)	Partial resection. CT for 6 months	DOD at 12 months
+ CD57, SYN, CAM5.2, CD99, CK7, CK20, EMA, PSA, PSAP, CG	NA	CT, RT (see text for details)	AWD at 42 months
+ Vimentin, NSE, CD99, desmin, MSA, SMA, LCA, S100, NF	t(17;22)(q12;q12)	Partial resection and CT (carboplatin, etoposide, cyclophosphamide, pirarubicin, cisplatin)	DOD at 2 years
+ CD99	NA	Resection	NA

bered 10 or 11 based on two alternative splice forms of *ETV1*).¹² He was treated on the CCG 7942 protocol. At 3.5 years after diagnosis, he developed pain in his right upper extremity and on plain film had a lytic lesion in the proximal humerus. On biopsy, this was found to be an osteosarcoma histologically unrelated to the previous small round cell tumor. By immunohistochemistry, the osteosarcoma showed a different staining pattern for CD99, with only scattered round and spindled cells expressing this marker. This osteosarcoma was not near the radiation field for the first tumor. He then received chemotherapy for osteosarcoma and had a limb salvage procedure with insertion of an allograft. Imaging studies of his extremities and chest do not show recurrent or metastatic disease at 5 years after initial EFT diagnosis.

Patient B was a 2-year-old female child who developed fever and acute right groin and right lower quadrant abdominal pain. Her mother discovered groin lumps that were mildly mobile and hard. A magnetic resonance imaging revealed a large soft tissue mass in the proximal thigh measuring 6 cm in greatest dimension and an intrapelvic mass measuring 8 cm in greatest dimension. Her initial extent of disease evaluation also showed several pulmonary metastases. A biopsy of the pelvic mass

revealed a high-grade round cell sarcoma that was focally reactive for CD99 and negative for lineage markers (Table 2), and the patient was treated with five-drug chemotherapy consisting of alternating cycles of vincristine/doxorubicin/cyclophosphamide and ifosfamide/etoposide. She received 3600 cGy of radiation therapy followed by complete surgical resection of her primary tumor in the upper thigh and pelvis. The excised tumor showed spindle cell morphology (Figure 2), which was deemed to be a therapy-related change. The karyotype reportedly showed t(7;22) but further cytogenetic details were not available. RT-PCR showed the *EWS-ETV1* fusion transcript. The fusion structure was identical to that originally reported,¹² as described for patient A above. One year after completion of her therapy, she presented with local-regional recurrence of the tumor in her right pelvis. At that time, she underwent resection of the tumor. Several months later she began having significant progression of her pulmonary disease. Multiple types of salvage chemotherapy were given with partial responses. She died of progressive disease 5 years after diagnosis.

Patient C was a 40-year-old man who presented with lower abdominal pain radiating into the extremity. Imaging studies revealed a retroperitoneal and pelvic mass

Table 3. EFT-Like Tumors with Noncanonical *EWS* Fusions

Case no. (reference)	Age/sex	Presentation	Histology
No. 1 (18)	16/M	Extraosseous chest wall primary	SRCT
No. 2 (22)	39/F	Primary in pubic bone; pulmonary metastases	SRCT
No. 3, present series, patient D	16/M	Disseminated disease: forehead, bones, lungs, right kidney	SRCT

For abbreviations, see Table 2.

(table continues)

without skeletal involvement. A biopsy of the mass showed a poorly differentiated malignant tumor diffusely positive for CD99 and with dot-like positivity for CAM5.2 (Figure 3, A–C). The differential diagnosis was between an EFT and a high-grade neuroendocrine carcinoma. RT-PCR analysis of the fresh-frozen tumor tissue using the *EWS* exon 7 forward primer and the *ETS1* reverse primer revealed the *EWS-FEV* fusion, confirming a diagnosis of an EFT. The fusion joined *EWS* exon 7 to *FEV* exon 2, as described in the original report of this fusion.¹⁵ No tissue was submitted for cytogenetics. Radiation to the primary site was given in 25 fractions to a dose of 45 Gy, along with chemotherapy (vincristine, doxorubicin, dexamethasone, cyclophosphamide). Surgical exploration of the primary site was then performed and showed no viable tumor in extensive sampling of the residual unresectable masses. At 3.5 years after original diagnosis, he relapsed with metastatic disease in his left lung and left hilar lymph nodes and is now on second line therapy. The morphology and immunophenotype of the metastatic tumor in the lung was similar to that of the primary. In addition to strong positivity for CD99, some reactivity with AE1/AE3 antibody was also noted.

Patient D: Identification of a Novel EWS-SP3 Fusion in an Extraskeletal SRCT

Patient D was a 16-year-old Caucasian male who presented with progressive enlargement of a right forehead mass. The initial magnetic resonance imaging and a computerized automated tomography (CT) scan revealed a heterogeneous extra-axial, extradural mass in the right frontal region measuring 5 × 3.8 × 5 cm³. In addition to the right frontotemporal mass, abnormal radiotracer accumulation was also identified in the right femoral, left clavicular, and left proximal ulnar regions by bone scan. CT scan revealed seven pulmonary nodules bilaterally in the lower lobes and a large mass in the right kidney. An excisional biopsy of the extradural mass was performed. The histopathology showed an undifferentiated small round cell tumor. Immunohistochemistry studies did not clarify the diagnosis, only showing strong positivity for BCL2 and weak and focal expression of CD99, NSE, and neurofilament (Figure 4, A–C; and see Table 3 for details). Cytogenetic analysis revealed a t(2;

22)(q31;q12) (Figure 5A). Fluorescence *in situ* hybridization (FISH) performed with dual-color, break-apart probes flanking the *EWS* gene (Abbott-Vysis, Downer's Grove, IL) showed a split in the *EWS* gene locus and a simple translocation of *EWS* gene material to 2q31 (Figure 5, B and C).

The patient was initially treated with alternating cycles of cyclophosphamide/doxorubicin/vincristine, and ifosfamide/etoposide. Because of progressive disease, he was placed on a more intensive high-dose ifosfamide/etoposide regimen with partial response. A radical nephroureterectomy was performed. He then underwent an autologous peripheral stem cells transplant as consolidation therapy. Approximately 3 months after the transplant, he relapsed with multiple bony metastases. Despite several cycles of irinotecan, the bone lesions progressed. The patient was then treated with palliative radiation therapy and palliative chemotherapy (oral etoposide). He died ~20 months from initial diagnosis.

To address the hypothesis that a novel gene might be fused to *EWS*, 3' RACE was performed using 3' RACE kit (Invitrogen) with nested *EWS* forward primers (EWS22.14 for the first PCR step, EWS22.3 for the nested PCR step). Cloning and sequencing of the 3' RACE products revealed two aberrant transcripts, both of which represented chimeric mRNAs encoding an in-frame transcript encoding a fusion of the *EWS* amino-terminal domain (NTD) to the zinc finger DNA-binding domain of *SP3*. Because the *SP3* gene is located at 2q31, this was consistent with the cytogenetic and FISH data in this case. Of the two cloned chimeric *EWS-SP3* transcripts, the shorter one (designated type I fusion product) represented an in-frame fusion of *EWS* exon 7 to nucleotide 48 of *SP3* exon 6 (Figure 6A). This exon encodes the first zinc finger of *SP3*. The second, longer chimeric *EWS-SP3* transcript (designated type II fusion product) showed an insertion of a 31-bp sequence between *EWS* exon 8 and nucleotide 26 of *SP3* exon 6; this insertion resulted in restoration of the reading frame that would otherwise have been lost in a direct fusion between *EWS* exon 8 (end phase = 2) and *SP3* exon 6 (start phase = 1) (Figure 6B). Sequence homology searches revealed that this additional 31-bp sequence, except the last T, is located within *EWS* intron 8, suggesting that it was functioning as a cryptic exon in this tumor. The extra T at the fusion point of this chimeric

Table 3. *Continued*

IHC	Fusion type and cytogenetic translocation	Treatment	Outcome
+ SYN, desmin, CAM5.2, CD99, neurofilament	<i>EWS-ZNF278</i> t(1;22)(p36.1;q12)	NA	AWD (pulmonary metastases) at 2 years
+ vimentin, S100, NSE, focal CK, MIC2, MSA,	<i>EWS-POU5F1</i> t(6;22)(p21;q12)		DOD at 6 months
+ BCL2, CD99 (focal), NF (focal), CK cocktail (focal), CG, SYN, S100, desmin, ALK1, EMA, CD45, CD20, CD30, CD43, CD3, CD79a, EBER	<i>EWS-SP3</i>	Neoadjuvant CT, resection (kidney) irinotecan, palliative RT (see text for details)	DOD at 20 months

transcript may be attributable to error-prone nonhomologous end joining. Interestingly, the two alternative junction points within *SP3* exon 6 were both at cryptic splice acceptor sites (Figure 6C). Both *EWS-SP3* fusion transcripts were then confirmed by RT-PCR using *EWS* forward and *SP3* reverse primers (Figure 7). RT-PCR assays consistently showed that the shorter fusion transcript was more prevalent. The predicted protein structure of the *EWS-SP3* fusion product is schematized in Figure 8.

Although we did not clone the genomic junctions of the *EWS-SP3* fusion, the structure of the type II fusion product indicates that the genomic *EWS* breakpoint in this tumor is most likely in intron 8. Presumably, most chimeric *EWS-SP3* transcripts underwent splicing that removed *EWS* exon 8 to produce the in-frame type I fusion product that predominated in our case. Splicing out of *EWS* exon 8 is also observed in *EWS-FLI1* rearrangements when the genomic break is in *EWS* intron 8.²⁷ As for the *SP3* gene, the genomic break is most likely in intron 5, or is at least upstream of nucleotide 26 of *SP3* exon 6 (based on the type I fusion product).

Discussion

Based on the patterns of associations between different translocation partners in sarcomas, the notion emerged that variant gene fusions were linked by strong similarities in the genes involved and that this in turn could be a defining feature of these specific entities. Thus, EFTs show fusions of *EWS* to related members of the *ETS* family of transcription factors (*FLI1*, *ERG*, *ETV1*, *E1AF*, and *FEV*). Likewise, alveolar rhabdomyosarcomas contain fusions of *FKHR* to members of the *PAX* gene family (*PAX3*, *PAX7*), clear cell sarcomas contain fusions of *EWS* to the *CREB* transcription factor family (*ATF1*, *CREB1*), in extraskeletal myxoid chondrosarcoma the *TEC* transcription factor is fused to a member of the *TET* family of RNA-binding proteins (*EWS*, *FUS*, and *TAFI168*), and so on.¹¹ This has been a useful conceptual framework because tumors with related variant fusions are invariably more similar to each other than to tumors with completely different fusions. Some of the more common variant fusions have been frequent enough to allow comparisons of clinical features, including survival, within a given sarcoma entity according to fusion type. Our recent

experience with three additional cases with rare variant fusions of *EWS* to *ETS* family transcription factor genes reported here prompted us to review the cumulated experience with these cases (summarized in Table 2) to begin to define their clinical and pathological phenotype.

EFTs are considered to affect primarily children and young adults, and this also holds true for EFTs with the rare variant translocations (*EWS-ETV1*, *EWS-E1AF*, *EWS-FEV*). Based on the data summarized in Table 2, the *EWS-ETV1* and *EWS-FEV* fusions may be more common than the *EWS-E1AF* fusion. In the current study, analyzing all of the reported cases of rare variant translocations confirmed at the molecular level, the age group ranges from newborn to 40 years of age. However, 83% (10 of 12) of patients were <18 years of age at diagnosis. The two older patients had the *EWS-FEV* fusion. One recurrent feature of tumors with these rare variant translocations appears to be their extraskeletal location. The location in our patient A was described as adjacent to the left fibula; however, gross and microscopic examination revealed a predominantly soft tissue mass around the bone with focal periosteal involvement but no evidence of cortical bone involvement. Overall, the proportion of extraskeletal primaries is 11 of 12 cases (92%). Most cases showed a small round cell tumor on histological examination. The tumor from our patient B (*EWS-ETV1* fusion transcript) showed spindle cell areas, which is unusual for EFT, but this specimen was obtained after the patient received high-dose chemotherapy. Therefore, this may reflect a therapy-related change. One case with the *EWS-FEV* fusion transcript (case 8 in Table 2) showed large polygonal cells with clear cell features; however, this phenotype is not uncommonly seen in EFT containing abundant glycogen. Another case of *EWS-FEV* fusion (case 9 in Table 2) showed a predominantly small round cell tumor with foci of large pleomorphic cells. Like most EFTs, 9 of 10 cases with immunohistochemical data for CD99/MIC2 showed positive staining, which was only focal in one case. The majority of the tumors were also positive for vimentin and variably positive for neuroendocrine markers. Other immunohistochemical data are summarized in Table 2. Clinical outcomes were quite variable, with some patients presenting with metastatic disease and progressing rapidly, whereas others have shown extended disease-free survival. Other genetic factors may account

Figure 1. Patient A. **A:** H&E-stained section showing characteristic small round blue cell tumor morphology. **B:** Diffuse and strong positivity for CD99 (O13 antibody). **C:** Partial karyotype showing a t(7;22) translocation. **Arrows** indicate the derivative chromosomes.

for occasional aggressive behavior. For instance, a patient whose tumor contained the *EWS-FEV* fusion (case 9 in Table 2) died 16 months after diagnosis, 12 months after surgery despite two courses of adjuvant chemotherapy. This case showed a p53 point mutation, which is known to have a strong negative impact on chemotherapeutic response and survival in EFT.²⁸

Studies in NIH3T3 cells have demonstrated some functional differences between the five known EWS-ETS fu-

Figure 2. Patient B. Undifferentiated tumor showing areas of spindling in a specimen obtained after neoadjuvant chemotherapy.

sions. Introduction of *EWS-FLI*, *EWS-ERG*, or *EWS-FEV* into NIH3T3 leads to anchorage-independent growth, whereas *EWS-ETV1* and *EWS-E1AF* do not.²⁹ Intriguingly, these associations parallel the structural similarities in these ETS family members; *FLI*, *ERG*, and *FEV* are more closely related to each other than to *ETV1* and *E1AF*.

We also report for the first time a sarcoma with an *EWS-SP3* fusion, making this at least the 12th *EWS* fusion observed in this group of cancers and the first involvement of a gene encoding a SP family zinc finger protein in a cancer-associated gene fusion. Similar to all previously described fusions involving *EWS*, the fusion transcript encodes a predicted chimeric protein consisting of the *EWS* amino-terminal domain known to function as a transactivation domain in the context of *EWS* fusion proteins¹¹ fused to a DNA-binding domain, in this instance the zinc finger domain from the carboxy-terminal portion of *SP3* (Figure 8).

SP3 is a member of the mammalian SP transcription factor family,³⁰ which is ubiquitously expressed along with *SP1* and is indistinguishable from *SP1* in its DNA binding properties.³¹ Functionally, *SP3* seems to be a bifunctional transcription factor, ie, it can either inhibit or activate target gene expression.³² SP family members have similar domain structures, consisting of three zinc fingers close to the C terminus and glutamine-rich activation domains in their N-terminal region.³⁰ The last two zinc fingers are conserved classical zinc fingers, whereas the first zinc finger is less conserved. *SP3* also possesses an inhibitory domain (ID) between the second glutamine-rich domain and the first zinc finger (Figure 8).³⁰ The inhibitory domain of *SP3* acts as an independent module in *cis*-. It can be joined to other activation domains causing them to lose their activation properties.³² The amino acid triplet KEE within its inhibitory domain has been demonstrated to be essential for repressor function of *SP3*.³² Splicing and alternate use of open reading frames has been postulated to affect *SP3* function.³³ Thus, one can speculate that a transcript that excludes the exon coding for the inhibitory domain would deprive *SP3* of repressor function. In the case presented

Figure 3. Patient C. **A:** H&E-stained section showing small round blue cell tumor with crush artifact. **B:** Immunostain for CD99 showing diffuse and strong staining. **C:** CAM5.2 immunostain showing dot-like positivity for cytokeratin in the tumor cells.

Figure 4. Patient D. **A:** H&E-stained section showing small round blue cell tumor with high mitotic rate. **B:** Immunostain for CD99 showing focal weak staining. **C:** BCL2 immunostain showing strong diffuse positivity in the tumor cells.

here, the chimeric protein included the EWS N-terminal domain and the SP3 zinc finger DNA-binding domain but not the inhibitory domain of SP3 (Figure 8).

It is notable that this feature of exclusion of an inhibitory domain is also observed in the two other sarcoma fusions involving *EWS* and a zinc finger gene, *EWS-WT1* and *EWS-ZNF278*. WT1, the first zinc finger protein involved in a fusion with *EWS*,¹⁹ also acts as a transcriptional acti-

vator or repressor depending on the cellular or chromosomal context.³⁴ WT1 protein contains a domain N-terminal to the zinc finger region, which functions as a strong transcriptional repressor.³⁵ ZNF278 contains a POZ transcriptional repressor domain at the N terminus.¹⁸ Neither WT1's repressor domain nor ZNF278's POZ domain are included in their respective *EWS* fusion proteins. This suggests that part of the biology of these *EWS*-zinc finger

Figure 5. Patient D. **A:** Partial karyotype showing t(2;22)(q31;q12). **Arrows** indicate the derivative chromosomes. **B:** FISH using dual-color, break-apart *EWS* probe on a metaphase preparation. The **small arrow** shows the normal fusion signal (red and green signals together) on the intact chromosome 22; the **large arrow** indicates the der(22)t(2;22) with only red signal (*EWS* centromeric probe); the **midsize arrow** indicates the der(22)t(2;22) with the green signal (*EWS* telomeric probe). **C:** Same FISH on interphase nuclei showing one normal fusion signal and separation of the other red and green signals.

gene fusions may involve disruption of gene expression patterns normally regulated by the alternative activator and repressor functions of these transcription factors. The clinicopathological features of these EFT-like tumors with noncanonical *EWS* fusions, including *EWS-ZNF278*, *EWS-SP3*, and *EWS-POU5F1*, are summarized in Table 3.

The existence of a growing number of rare *EWS* variant translocations complicates the molecular diagnosis of this group of undifferentiated small round cell sarcomas, whether by RT-PCR or FISH. To achieve a nearly definitive molecular diagnosis, the RT-PCR-based approach requires the use of multiple specific or consensus reverse primers, usually in a stepwise manner proceeding from

Figure 6. Patient D. **A:** Partial sequence of the type I *EWS-SP3* fusion transcript is an in-frame junction of *EWS* exon 7 with *SP3* exon 6 (see Results for details). The translated protein sequence is shown below, with the codon at the junction in red. **B:** Partial sequence of the type II *EWS-SP3* transcript. The transcript has a full open reading frame and is a fusion of *EWS* exon 8 to *SP3* exon 6 with an intervening cryptic exon. The **arrowhead** indicates the extra T (see Results for details). The translated protein sequence is shown below, with the codons at the junctions in red. **C:** Partial genomic sequence of *SP3*. The intron sequence is in lower case and the exon sequence is in upper case. The portion of *SP3* exon 6 coding for the zinc finger is underlined. The two cryptic splice acceptor sites in *SP3* exon 6 are shown in green (used in type I fusion transcript) and red (used type II fusion transcript), respectively.

common fusions to rare ones. As the numbers of different possible fusions rise, the RT-PCR approach is increasingly labor-intensive, making it more pressing to develop

Figure 7. Patient D: RT-PCR detection of *EWS-SP3* fusion. The presence of both type I and type II fusion transcripts was confirmed by RT-PCR using an *EWS* primer (sequence: 5'-CTGGATCCTACAGCCAAGCTCCAAGTC-3') and an *SP3* reverse primer (sequence: 5'-CAGAATGCCAAGCAGATGA-3'). **Lane ptD** is the RT-PCR product obtained from tumor RNA. Band A is 120 bp, and band B is 375 bp. Sequencing of A and B confirmed that A represents the type I *EWS-SP3* fusion transcript, whereas B represents the type II fusion, both of which are in-frame (see Figure 6). Sequencing of band C revealed an out-of-frame *EWS-SP3* fusion transcript (sequence data not shown). **Lane neg** is the no RNA negative control. Size marker (**lane M**) is ϕ X174/*Hae*III (Invitrogen).

Table 4. EFT-Like Tumors with Non-EWS Fusions

Case no. (reference)	Age/ sex	Primary site	Histology
No. 1 (39)	9/M	Chest wall; no data on metastatic disease	SRCT, high mitotic rate
No. 2 (39)	7/F	Chest wall; no data on metastatic disease	SRCT, high mitotic rate
No. 3 (39)	15/F	Chest wall; no data on metastatic disease	SRCT, high mitotic rate, pleomorphism
No. 4 (39)	21/M	Femur; no data on metastatic disease	SRCT
No. 5 (40)	62/F	Pelvic soft tissue mass; no metastatic disease	SRCT, glycogen granules
No. 6 (40)	31/M	Shoulder soft tissue; no metastatic disease	SRCT, glycogen granules
No. 7 (41)	10/M	Iliac bone; no metastatic disease	SRCT, high mitotic rate

For abbreviations, see Table 2.

(table continues)

comprehensive diagnostic strategies, possibly chip-based. This may well be a continuing process because there also likely remain additional rare uncloned variant *EWS* fusions in these tumors, based on cytogenetic reports of cases with unusual translocations involving 22q12.^{36–38} The FISH approach based on *EWS* break-apart probes is comprehensive in terms of the detection of *EWS* rearrangements but does not identify the translocation partner and, therefore, may not provide clarification of cases with ambiguous pathological features.

Finally, to complicate further molecular diagnosis and our biological concepts of this tumor group, there are also isolated reports of EFT-like small round cell sarcomas that do not contain *EWS* fusions (Table 4). The recent report of a recurrent *FUS-ERG* gene fusion in four cases of EFT, three of which involved the chest wall,³⁹ suggests that even a negative *EWS* FISH may not be sufficient to definitively exclude EFT. The existence of these *FUS-ERG* fusions suggests that *FUS* fusions with other *EWS* partners in EFTs may also be possible. Beyond *EWS* and *FUS* fusions, there are rare EFT-like cases with seemingly completely biologically unrelated fusions. Specifically, there are two reported cases of soft tissue sarcoma diagnosed as Ewing-like sarcoma, both with a

t(4;19)(q35;q13), encoding a novel *CIC-DUX4* fusion joining the *CIC* high-mobility group box transcription factor gene to the double-homeodomain gene *DUX4*.⁴⁰ In addition, there has been a report of an EFT-like sarcoma harboring the *BRD4-NUT* fusion originally described in aggressive midline carcinomas of the chest.⁴¹ These rare non-*EWS* EFT-like cases are summarized in Table 4. Overall, these cases, together with the present data, highlight the growing complexity of sarcoma molecular diagnostics. Understanding the diverse or shared mechanisms underlying transformation by these distinct but often related fusions should help to clarify the biology of these aggressive cancers.

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Figure 8. Schematic diagrams of EWS, SP3, and the predicted EWS-SP3 protein. NTD, amino-terminal domain; AD, transactivation domain; ID, inhibitory domain; zinc finger, zinc finger DNA-binding domain.

Table 4. Continued

IHC	Fusion type and cytogenetic translocation	Treatment	Outcome
+ CD99, vimentin, neural markers, myogenic, lymphoid, myeloid markers	<i>FUS-ERG</i> t(16;21;22)(p11;q22;p11.2)	NA	DOD at 2 years
+ CD99 (focal weak), neural, myogenic, lymphoid, myeloid markers	<i>FUS-ERG</i> t(16;21)(p11.2;q22.3)	NA	NA
+ CD99, neural, myogenic, lymphoid, myeloid markers	<i>FUS-ERG</i> der(21)t(16;21)(p11;q22)	NA	NA
+ CD99, other myogenic, neural, lymphoid, myeloid markers	<i>FUS-ERG</i> t(16;21)(p11;q22)	NA	NA
+ CD99 (weak), vimentin	<i>CIC-DUX4</i> t(4;19)(q35;q13)	No curative surgery. Palliative RT and CT.	DOD at 10 months
+ CD99 (weak), vimentin	<i>CIC-DUX4</i> t(4;19)(q35;q13)	Neoadjuvant RT and CT (adriamycin, cisplatin). Resection.	NED at 30 months
+ CD99 (weak), CG, SYN, CD45, NSE, S100, CAM5.2, AE1/AE3, PLAP, AFP, OCT3/4, CD34	<i>BRD4-NUT</i> t(15;19)(q13;p13)	No resection. RT and CT (vincristine, doxorubicin, ifosfamide, cisplatin)	NED at 13 years

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