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Ewing Sarcoma with Novel Translocation t(2;16) Producing an In-Frame Fusion of *FUS* and *FEV*

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Ewing family tumors are molecularly characterized by expression of chimeric transcripts generated by specific chromosomal translocations, most commonly involving fusion of the *EWS* gene to a member of the ETS family of transcription factors (including *FLI1*, *ERG*, *ETV1*, *E1AF*, and *FEV*). Approximately 85% of reported cases of Ewing sarcoma bear an *EWS-FLI1* fusion. In rare cases, *FUS* can substitute for *EWS*, with translocation t(16;21)(p11;q24) producing a *FUS-ERG* fusion with no *EWS* rearrangement. We report a case of Ewing sarcoma, presenting as a pathological fracture of the distal clavicle in a 33-year-old male, in which cytogenetic analysis revealed a single t(2;16)(q35;p11) balanced translocation. Fluorescence *in situ* hybridization using a commercially available diagnostic probe was negative for an *EWS* gene rearrangement; instead, break-apart fluorescence *in situ* hybridization probes for *FUS* and *FEV* were positive for a translocation involving these genes. Cloning and sequencing of the breakpoint region demonstrated an in-frame fusion of *FUS* to *FEV*. In conclusion, this represents the first reported case of Ewing family tumors demonstrating a variant translocation involving *FUS* and *FEV* and highlights the need to consider alternative permutations of fusion partners for molecular diagnosis of sarcomas. *(J Mol Diagn 2007, 9:459–463; DOI: 10.2353/jmoldx.2007.070009)*

Ewing sarcoma is a highly malignant small round cell tumor of bone. It is the second most common bone malignancy of childhood, with approximately 50% of cases occurring between 10 and 20 years of age, although it may occur in adults, including the elderly.[1,2] Males are affected more frequently than females, with a ratio of approximately 1.5:1. Extraosseous soft tissue and visceral locations are also well described.[3,4] Based on their shared immunophenotypes and molecular signatures, several diagnostic entities, previously considered distinct, are now amalgamated as the Ewing family of tumors (EFT). These include classic Ewing sarcoma of bone as well as extra-osseous Ewing sarcoma, peripheral primitive neuroectodermal tumor, and Askin tumor (peripheral primitive neuroectodermal tumor of chest wall).[5]

In light of the effectiveness of chemotherapeutics in its treatment, establishing the correct diagnosis of EFT as opposed to other small round cell sarcomas is of particular clinical relevance.[1] The diagnostic pathological criteria for EFT include a spectrum of histological, immunophenotypic, and molecular features. The malignant cells display intense cytoplasmic membrane-associated immunoreactivity with antibodies to CD99. In approximately 85% of cases, the chromosomal translocation t(11;22)(q24;q12) can be detected by cytogenetic or molecular analysis of the tumor cells. This tumor-specific translocation results in an in-frame fusion of *EWS*, at chromosome band 22q12, with *FLI1*, a member of the ETS transcription factor family,[6] at 11q24. The precise breakpoint sites within the *EWS* gene may have prognostic significance.[7] Less commonly, *EWS* becomes fused with another ETS member, including *ERG* in approximately 5 to 10% of cases,[8,9] and even less frequently with *ETV1*,[10] *E1AF* (*ETV4*),[11] or *FEV*.[12] In a recent report, four cases of EFT showed a fusion of *FUS* to *ERG* through a t(16;21)(p11;q24) instead of the more typical *EWS* gene rearrangement.[13] FUS, belonging to the TET family of RNA-binding proteins, shows considerable homology with EWS.[14] Such a finding highlights the possibility of variant gene participation in both the 5′ and 3′ portions of

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EFT fusion transcripts. This has particular diagnostic relevance, since the current methods of reverse transcription-polymerase chain reaction (RT-PCR) and commonly used fluorescence *in situ* hybridization (FISH) probes may overlook the involvement of alternative translocation partners when such permutations are not considered. To illustrate further this important issue, we report here a case of EFT showing a novel t(2;16)(q35;p11) translocation that results in an in-frame fusion of *FUS* and *FEV*.

Materials and Methods

Clinical History

A 33-year-old Caucasian male fell while snowboarding, injuring his left, nondominant shoulder. X-rays revealed a distal clavicle fracture through a lytic lesion with permeative borders. Magnetic resonance imaging showed a 3.2-cm expansile lesion of the distal clavicle. The initial core needle biopsy showed reactive bone and a tiny 100-μm fragment of small round blue cells. After sections were taken for routine histology, immunohistochemistry was performed, with the tumor cells staining strongly positive for CD99 and negative for CD20; however, the specimen was cut through, and paraffin-based FISH analysis could not be performed. Because there was insufficient material for a definitive diagnosis, an open biopsy, placed directly over the distal clavicle, was performed and further tissue obtained. This allowed definitive diagnosis, and treatment was instituted for EFT.

Neoadjuvant chemotherapy and external beam radiation therapy were administered and well tolerated. Surgical resection of the distal clavicle was performed, dividing the clavicle on the medial side of the coracoclavicular ligaments and dividing the acromion sagittally. Examination of the specimen revealed that the lesion had been removed with a wide margin, and the tumor exhibited 100% pathological response. The patient made an excellent early recovery and was free of disease at last follow-up, 10 months following initial diagnosis.

Tissue Handling for Morphological and Genetic Studies

The needle core biopsy was formalin-fixed and paraffin-embedded *in toto*. The subsequent open biopsy additionally had a representative sample of fresh tumor tissue submitted for cytogenetic evaluation and a portion snap-frozen in liquid nitrogen and stored at −70°C for molecular studies. The remainder of the tissue was fixed in formalin for routine histology.

Cytogenetic Studies and Fluorescence in Situ *Hybridization*

Chromosomal analysis was performed on tissue from the open biopsy using standard tissue culture and harvesting procedures. Metaphases were stained by the GTG method. The karyotype alterations were described according to International System for Human Cytogenetic Nomenclature 1995.[15] FISH was performed using commercially available dual-color break-apart probes for *EWS* and *FUS* (Vysis, Des Plaines, IL). An in-house probe was prepared that consisted of the bacterial artificial chromosomes (BACs) RP11-96D18 and FP11-42612 that flank the *FEV* gene. These BACs were labeled with SpectrumRed and SpectrumGreen (Vysis), respectively, to create a dual-color break-apart probe to detect possible rearrangements within the *FEV* gene. A second BAC probe combination was generated with BAC RP11-270E5 from band 2p12 (labeled with SpectrumGreen) and RP11-207M4 that spanned the *FEV* locus (labeled with SpectrumRed).

Sequencing Analysis of Fusion Transcript

Total RNA was extracted from a frozen portion of tumor tissue using a standard protocol with TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA was reverse transcribed into cDNA with Superscript II (Invitrogen) and then used as template for PCR amplification of the fusion breakpoint. Primers were designed to flank the probable breakpoints within the *FUS* and *FEV* genes. The primer sequences used were as follows: FUS-IF, 5′-gtgcgcggacatggcctcaaacg-3′, derived from exon 1 of *FUS*; and FEV-IR, 5′-tgttgggcttgctcttgcgctc-3′, derived from exon 3 of *FEV*. The reaction was subjected to 35 cycles of PCR using the following conditions: denaturation at 94°C for 1 minute, annealing at 62°C for 30 seconds, and extension at 72°C for 45 seconds. The amplification product was resolved on 1% agarose gel electrophoresis, and a band of the expected size of ~1.4 kb was excised and gel-purified. This was cloned into a TOPO TA vector, cultured overnight following transformation into TOP10 *Escherichia coli* cells. A minipreparation of the plasmid DNA was performed, and the insert was recovered using *Eco*RI digestion. Clones containing the appropriate-sized insert were submitted for sequencing.

Tissue Microarray

A tissue microarray (TMA) consisting of 168 cases of pediatric tumors was constructed consisting of 1.0-mm tissue cores, using standard methods.[16] It included 22 cases of EFT, two of which were negative for both *EWS-FLI1* and *EWS-ERG* fusions by RT-PCR on molecular diagnostic testing and three of which with unavailable molecular diagnostic results. Other tumor types included neuroblastoma (30 cases), ganglioneuroma (14 cases), medulloblastoma (14 cases), embryonal rhabdomyosarcoma (25 cases), alveolar rhabdomyosarcoma (21 cases), Wilms' tumor (24 cases), fibromatosis (10 cases), congenital fibrosarcoma (five cases), and one case each of alveolar soft part sarcoma, clear cell sarcoma of kidney, and neurofibroma. All cases were diagnosed at the British Columbia Children's Hospital pathology service and reviewed by a pediatric pathologist during the construction of the TMA. Sections from this TMA were analyzed by FISH for disruption of *FUS* using the commer-