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Malignant Round Cell Tumor of Bone with *EWSR1-NFATC2* Gene Fusion

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Abstract

Gene rearrangements involving the Ewing sarcoma breakpoint region 1 (*EWSR1*) gene are seen in a broad range of sarcomas and some non-mesenchymal neoplasms. Ewing sarcoma is molecularly defined by a fusion of the *EWSR1* gene (or rarely the related *FUS* gene) to a member of the ETS family of transcription factors, frequently the *EWSR1-FLI1* fusion. More recently, *EWSR1* gene fusion to non-ETS family members, including the nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 (*NFATC2*) gene has been reported in a histological variant of Ewing sarcoma. Here we report a malignant round cell tumor of bone with an *EWSR1-NFATC2* fusion gene. This report builds upon the unusual morphological and clinical presentation of bone neoplasms containing an *EWSR1-NFATC2* fusion gene.

Keywords

EWSR1-NFATC2 fusion gene; *EWSR1*; Ewing's sarcoma; osteosarcoma

Introduction

Ewing sarcoma is small round cell, high grade tumor that primarily affects the bone and soft tissue of young adults and children. Ewing sarcoma is molecularly defined by translocations resulting in the fusion of the *EWSR1* gene, located at 22q12 (or rarely the *FUS* gene, located at 16p11), and a gene of the ETS family of transcription factors [1]. In 85 to 90% of cases the translocation is t(11;22)(q24;q12) resulting in *EWSR1-FLI1* fusion gene [2, 3]. The *ERG* gene, located at 21q22, is the fusion partner to *EWSR1* in about 5 to 10% of cases of Ewing sarcoma [4]. Other fusion partners described in the ETS family members include *ETV1* (7p22) [5], *ETV4* (17q12) [6], and *FEV* (2q33) genes [7]. These translocations result in the fusion of the N-terminal transactivation domain of *EWSR1* with the C-terminal DNA-

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binding domain of the ETS family member generating a potent and oncogenic transcription factor [8]. *EWSR1* gene fusions are presumed to be the important oncogenic event in Ewing sarcoma [9]. The *EWSR1-FLI1* fusion promotes numerous oncogenic properties, including cell proliferation, transformation, *in vivo* tumor growth, and chemoresistance in experimental models and is considered a therapeutic target [9–13].

More recently, rare cases of Ewing sarcoma-like tumors with fusions between *EWSR1* and non-ETS family members have been described. These cases showed fusion partner genes, *PATZ1* [14] and *SP3* [15], encoding proteins in the zinc-finger family; *SMARCA5* [16], encoding a chromatin-remodeling protein; and *NFATC2* [17], encoding a member of the NFAT-transcription family. The rearrangement of the *EWSR1* gene is not specific to the Ewing sarcoma family of tumors. Distinct translocations involving the *EWSR1* gene and non-ETS transcription family members are seen in several other mesenchymal neoplasms, including desmoplastic small round cell tumor [18], clear cell sarcoma of soft tissue [19], angiomatoid fibrous histiocytoma [20], extraskeletal myxoid chondrosarcoma [21], myxoid liposarcoma [22], primary pulmonary myxoid sarcoma [23], myoepithelial tumors [24–26], and hemangioma of bone [27]. In addition to mesenchymal tumors, *EWSR1* gene fusions have also been described in hyalinizing clear cell carcinoma of salivary gland [28], and in rare cases of mesothelioma [29] and mucoepidermoid carcinoma of salivary gland [30].

Here we report an aggressive tumor of bone with focal small round cell features in a young male adult characterized by the amplification of the *EWSR1-NFATC2* fusion gene. Bone tumors characterized as Ewing sarcoma variants [17, 31] and myoepithelioma-like sarcoma [32] have been recently described to contain the amplified *EWSR1-NFATC2* gene fusion. The case described in this report builds upon prior cases and expands the morphological spectrum of tumors described to contain amplification of this gene fusion.

Case Report

A previously healthy 30 year-old man presented with constant burning and stabbing pain of the left thigh over a 6-week period. He reported no history of trauma or infection. A mixed lytic and blastic lesion involving the mid-left femoral diaphysis was identified on plain radiograph. The lesion had a benign-appearing surrounding sclerosis and periosteal reaction. No soft tissue extension was seen on CT. Curettage with bone graft was performed, and after correlation with the radiographic findings, a diagnosis of aggressive osteoblastoma was rendered on histologic examination.

Two and half years later, the patient returned with complaint of persistent left leg pain and no other constitutional symptoms. There was a worsening appearance of the mid-left femur lesion. An aggressive, ill-defined, lytic-type process, measuring 8.3 cm in length, and involving the cortex with thick periosteal reaction and a calcified matrix was seen on plain radiographs (Figure 1). Curettage biopsy and subsequent surgical resection were performed. The tumor extended into the surrounding connective tissue and skeletal muscle. In conjunction with outside expert consultation, the recurrent lesion was initially diagnoses as an osteosarcoma with small cell features arising from an aggressive osteoblastoma. Due to positive surgical margins, the patient underwent three months of etoposide and ifosfamide

chemotherapy, with complications of renal failure and sepsis. Chemotherapy was discontinued and he was followed by close surveillance. Four months after his resection, his radiological scans revealed intact hardware with no evidence of tumor recurrence or metastasis. Two more months later, the patient was lost on follow up.

Methods

The biopsy and curettage specimens were received fresh, fixed in 10% buffered formalin and processed for routine histological analyses. Histological and immunohistochemical analyses were done on 5 µm thick sections and using a Leica processor (Leica Microsystems, Buffalo Grove, IL). Antibodies used were against AE1/3 (1:400; Leica Microsystems), CAM 5.2 (1:10; BD Bioscience, San Jose, CA), CD3 (1:175; Leica Microsystems), CD15 (1:50; BD Bioscience), CD20 (1:250; Dako, Carpinteria, CA), CD30 (1:60; Dako), CD31 (1:25; Dako), CD34 (1:80; BD Bioscience), CD56 (1:25; Invitrogen, Grand Island, NY), CD57 (1:20; ThermoFisher Scientific, Waltham, MA), CD68 (1:2000; Dako), CD99 (1:150; Dako), CD117 (1:75; Dako), CK7 (1:200; Dako), CK19 (1:25; Leica Microsystems), CK20 (1:30; Dako), desmin (1:100; Dako), EMA (1:100; Dako), FLI-1 (1:25; Cell Marque, Rocklin, California), SMA (1:600; Dako), LCA (1:200; Dako), melanin A (1:50; Dako), MSA (1:75; Dako), myogenin (1:200; Dako), CD99 (1:100; Signet, Dedham, MA), OCT4 (1:700; Dako), neurofilament (1:20; Dr. Trojanowski Laboratory), p63 (1:100; Biocare, Concord, CA), synaptophysin (1:50; Zymed, San Francisco, CA), S100 (1:3750; Dako), vimentin (1:60; Dako), WT1 (1:100; Dako).

Fluorescence *in situ* hybridization (FISH) for rearrangement of the EWSR1 locus was performed using an EWSR1 dual-color break-apart probe (22q12) (Vysis/Abbot Laboratories, Downers Grove, IL). FISH analysis of the *EWSR1-FLI1* fusion was performed using a *FLI1 EWSR1* translocation dual fusion probe (Cytocell, Cambridge, MA). These two FISH assays were performed according to manufacturers' instructions. The *EWSR1-NFATC2* fusion was investigated by a dual-color FISH assay with Spectrum Green-labeled BAC clones RP11-367E7 and RP11-480L23 corresponding to the *EWSR1*-5' region and Spectrum Red-labeled BAC clones RP11-2L23 and RP11-73P15 corresponding to the *NFATC2*-3' region (Empire Genomics, Buffalo, NY). The FISH assay was performed on tumor sections as described previously [33].

An RT-PCR assay for the EWSR1-NFATC2 fusion transcript was performed with an EWSR1 exon 8 forward primer (TGGGTGTTTATGGGCAGGAGTC) and a *NFATC2* exon 5 reverse primer (GCTCAATGTCGGCGTTTCTAAG) to amplify a 573 base pair product. As a control assay, the wild-type FOXO1 transcript was assayed by RT-PCR with a *FOXO1* exon 1 forward primer (GCAGATCTACGAGTGGATGG) and a *FOXO1* exon 2 reverse primer (AACTGTGATCCAGGGCTGTC) to amplify a 323 base pair product. The RT-PCR was performed with the Superscript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Life Technologies, Carlsbad, CA).

Results

The original curettage revealed osteoblast-rimmed irregular bony trabecula with loose fibrovascular intertrabecular spaces (Figure 2A-B). There were foci of atypical cells with pale to clear cytoplasm, oval nuclei, and nucleoli (Figure 2C). These cells were vimentin positive and had unusual 'dot-like' AE1-3 staining (Figure 2D); all other cytokeratins (Cam5.2, CK7, CK19, CK20) were negative. Other negative stains included p63, CD34, SMA, myogenin, desmin, EMA, MSA, S100, Melan A, HMB45, synaptophysin, CD117, CD99, Oct3/4, LCA, CD3, CD15, CD20, CD30, and CD68. Rare larger hyperchromatic nuclei were identified. No infiltration into adjacent tissue was identified. After consideration of morphologic, immunohistochemical, and benign radiologic findings, the diagnosis of an aggressive osteoblastoma was rendered.

Two and half years later, the subsequent biopsy and resection showed areas resembling the prior biopsy with similar vascularity, bony trabecula and osteoid rimmed osteoblasts (Figure 3A-B). There were also diffuse sheets and clusters of atypical cells with eosinophilic cytoplasm, irregular nuclear contours and prominent nucleoli (Figure 3C-3D). Within the loose fibrovascular stroma were foci of smaller cells with scant cytoplasm, oval nuclei and occasional small nucleoli (Figure 3E-3F). There were large areas of necrosis with variable mitotic activity (up to 8 per high power field), consistent with a high grade malignancy. Ki-67 revealed a proliferation index of approximately 60%. The neoplastic cells were positive for vimentin and CD57, focally positive for CD99 and FLI-1. The tumor cells were negative for muscle differentiation markers (desmin, SMA, MSA, myogenin), vascular markers (CD31, CD34), keratins (CAM 5.2, AE1-3), neuronal markers (S100, neurofilament), LCA, CD56, CD68, WT1, synaptophysin, and EMA. In conjunction with outside expert consultation, the recurrent lesion was initially diagnosed as an osteosarcoma with small cell features arising from an aggressive osteoblastoma.

Given the foci of small blue cells with CD99 immunoreactivity in the recurrent specimen, an interphase FISH assay using an *EWSR1* dual-color break-apart probe was performed on paraffin sections of non-decalcified tissue. The FISH assay showed numerous copies (>15/nucleus) of the proximal region flanking the *EWSR1* gene while the distal region remained normal in copy number but separate from centromeric region (Figure 4A). These results suggested an amplification of the proximal region flanking *EWSR1* and potential break apart in *EWSR1*. To look for rearrangement partner, interphase FISH using a dual-fusion *EWSR1-FLI1* probe set was performed and did not reveal an *EWSR1-FLI1* fusion. Based on the previous finding of bone tumors with *EWSR1-NFATC2* amplification, FISH using an *EWSR1-NFATC2* fusion probe set was performed and revealed numerous copies of the *EWSR1-NFATC2* fusion (>15/nucleus; Figure 4B-C). The amplification pattern was in concordance with the the proximal *EWSR1-5'* probe hybridization in the *EWSR1* break apart probe assay. To further characterize the fusion, RT-PCR was performed for the *EWSR1-NFATC2* fusion transcript, and detected the predicted 573 base pair fragment (Figure 4D). Sequence analysis of the PCR product showed a junction between the 3' end of *EWSR1* exon 8 of and the 5' end of *NFATC2* exon 3, forming an in-frame transcript (Figure 4E). Based on these findings, the break apart and fusion FISH assays were retrospectively performed on the original curettage material and revealed similar results.

Discussion

The discovery of the *EWSR1-NFATC2* gene fusion in both the initial and follow-up specimens best supports the characterization of this tumor as an Ewing sarcoma variant. In retrospect, the lack of CD99 and neuroendocrine marker immunoreactivity along with the misinterpretation of the florid osteoblastic proliferation as part of the tumor, rather than a reaction to the tumor, led to a misclassification of the original lesion as an aggressive osteoblastoma.

Two years after conservative treatment, the patient developed a local malignant recurrence. The *EWSR1-NFATC2* amplification was detected in both the initial presentation and malignant recurrence and the copy number did not increase with disease progression. The presence of *EWSR1-NFATC2* fusion, focal small round cell morphology and CD99 immunopositivity in the reoccurrence favor an Ewing-like sarcoma. Two different neoplastic cell populations were seen in the recurrent tumor. There were foci of small cells with scant clear cytoplasm, regular oval-shaped nuclei and small nucleoli (Figure 3E-F) that resemble typical Ewing sarcoma. There were also diffuse sheets of larger cells with eosinophilic cytoplasm, irregular nuclear contours and prominent nucleoli (Figure 3C-D) that are more consistent with 'atypical' Ewing sarcoma [34]. The clinical presentation of only local recurrence without metastasis in more than two years after conservative curettage treatment is less common in typical Ewing sarcoma [35].

Small cell osteosarcoma is a rare morphological variant of osteosarcoma that morphologically resembles Ewing sarcoma. Although malignant osteoid formation remains the hallmark of osteosarcoma, Ewing sarcoma can have osteoid deposits, most likely reactive osteoid formation secondary to bone destruction [35]. Like Ewing sarcoma, small cell osteosarcoma can also show positive CD99 membrane staining [36], although additional FLI1 immunoreactivity favors Ewing sarcoma [37]. Moreover, *EWSR1* gene fusions, such as *EWSR1-CREB3L1* [38], have been described in small cell osteosarcomas [39]. These findings have been challenged by some that believe these reported tumors should be classified in the Ewing sarcoma family regardless of whether they produce osteoid; implying that it is possible that some of the tumors classified as small cell osteosarcomas actually are Ewing sarcoma with divergent differentiation [40]. Distinguishing between these two entities is not a trivial matter given the differences in therapeutic approach and response to chemoradiation therapy [41, 42].

Molecular detection of tumor-specific translocations has been widely used as a reliable diagnostic tool in soft tissue sarcomas and is beginning to be more commonly applied to bone tumors [43]. However, the diagnostic implication of any molecular finding needs to be interpreted with morphologic, radiological, and clinical correlation as translocations with the same gene fusion are increasingly being discovered in different tumors with distinct morphology and clinical presentation. For example, *EWSR1-ATF1* is present in clear cell sarcoma of soft part and angiomatoid histiocytoma, two morphologically and clinically unrelated soft tissue tumors [20]. The amplification of the *EWSR1-NFATC2* fusion gene present in our case has been described in a series of Ewing sarcoma-like tumors [17] as well as a myoepithelioma-like sarcoma of bone [32]. Our case shares similarities with Ewing

sarcoma-like tumors previously described [17, 31], being present in a patient older than 18 years of age, showing CD99 positivity (only on the reoccurrence) and unusual morphology with atypical cells and occasional prominent nucleoli (primarily in the reoccurrence). The diffuse dot-like cytokeratin immunoreactivity has previously been described in Ewing sarcoma [34].

In summary, we reported an additional case of malignant bone sarcoma in a young male adult containing the recently reported *EWSR1-NFATc2* fusion gene. Similar to the few prior reported cases, the *EWSR1-NFATc2* fusion gene was amplified in the current case [17, 31]. Although the previously reported cases were mostly regarded as Ewing sarcoma variant based on the morphologic features and finding of the *EWSR1* translocation, little is known about the biology of these rare tumors. Before being lost to follow-up, the current case was followed for almost three years. The unusual adjacent reactive osteoblastic proliferation, atypical morphology, and only local recurrence two and a half years following conservative curettage treatment suggest a very unusual variant of Ewing sarcoma family that has not been well described.

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Figure 1. Conventional radiograph showing a lytic lesion involving the proximal mid-diaphysis of the left femur with a thick periosteal reaction.

Figure 2.

A-B, The tumor consisted primarily of bony trabecula rimmed by osteoblasts with loose fibrovascular stroma. C, Foci of cells with clear to eosinophilic cytoplasm, oval nuclei, and nucleoli were seen. C, These cells showed unusual 'dot-like' AE1-3 positivity.

Figure 3.

A, The tumor consisted of areas resembling the prior biopsy with irregular bony trabecula lined by osteoblasts. B, Atypical cells were seen within the loose fibrovascular stroma. C-D, Diffuse sheets and clusters of atypical cells were also seen. These atypical cells had eosinophilic cytoplasm, displayed nuclear pleomorphism, and prominent nucleoli. E-F, In addition, there are scattered foci of smaller cells with oval nuclei and scant cytoplasm, arranged in cohesive clusters or nests. These smaller cells focally showed moderate cytological atypia.

Figure 4.

A, Dual color FISH using commercial probes flanking the centromeric (red) and telomeric (green) regions of the *EWSR1* gene. Break apart and amplification of centromeric region of *EWSR1* gene in tumor cells (yellow arrow). Non-tumor cells in analysis show colocalization of two probes (white arrows). B-C, Dual color FISH using probes from the 3' NFATC2 region on chromosome 20 and the 5' *EWSR1* region on chromosome 22. B, Normal human metaphase cells from peripheral blood lymphocytes from a healthy donor. C, Tumor cells showing colocalization and amplification of the *EWSR1* and NFATC2 regions. D, RT-PCR to detect expression of wild-type FOXO1 and EWSR1-NFATC2 mRNA sequences. The lower panel shows a specific amplicon of 573 bp from RT-PCR using primers for the *EWSR1* and *NFATC2* genes. No amplicon was seen in control cell lines (SU-CCC-S1 and A673) and no template control. E, Partial sequence chromatogram showing the junction of the end of exon 8 of *EWSR1* and beginning of exon 3 of *NFATC2* genes (dashed lines). The fusion results in an inframe transcript, as shown by the resulting amino acid sequence below the sequence readout.