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Novel *EWSR1-SMAD3* Gene Fusions in a Group of Acral Fibroblastic Spindle Cell Neoplasms

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Abstract

Benign/low grade fibroblastic tumors encompass a broad spectrum of tumors with different morphologies and molecular genetic abnormalities. However, despite significant progress in recent genomic characterization, there are still tumors in this histologic spectrum that are difficult to classify, lacking known molecular characteristics. Triggered by a challenging congenital spindle cell neoplasm arising in the heel of a 1 year-old boy, we applied RNA sequencing for genetic discovery and identified a novel *EWSR1-SMAD3* gene fusion. Based on the index case superficial acral location and fibroblastic appearance with a non-specific immunophenotype, we searched our files for similar cases and screened them by FISH for these abnormalities. Thus an identical *EWSR1-SMAD3* fusion was identified in 2 additional spindle cell tumors with similar clinicopathological features. Both cases occurred in the feet of adult women (58 and 61 years old) and were characterized by distinctive nodular growth with zonation pattern of peripheral hypercellular areas arranged in short fascicles, transitioning to hypocellular central areas of hyalinization and infarction. Focal stippled calcification in the collagenous area was present in one case. All 3 tumors had similar immunoprofiles, being negative for SMA, CD34, CD31 and S100, but showing consistent ERG positivity of uncertain significance. Follow-up information was available in 2 patients who developed local recurrences after incomplete initial excisions, at 5 and 14 months, respectively. None developed metastatic disease. In summary, we report a group of locally recurrent superficial acral tumors, characterized by bland spindle cell fascicular growth, occasional zonation pattern, ERG positivity, and recurrent *EWSR1-SMAD3* gene fusions.

Keywords

EWSR1; *SMAD3*; ERG; spindle cell tumor; fibroblastic tumor; acral

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INTRODUCTION

Benign/low grade fibroblastic tumors are a diverse group of tumors with overlapping morphologies and clinical presentations that can pose diagnostic challenge due to their rarity and lack of a specific immunoprofile. In recent years, with the advent of next generation sequencing, novel genetic alterations, including mutations or recurrent gene fusions, have been unraveled, increasingly refining the classification of fibroblastic and myofibroblastic neoplasms. Few examples in this morphologic spectrum with newly described genetic abnormalities include: calcifying aponeurotic fibroma showing recurrent *FNI-EGF* fusions,¹ fibrous hamartoma of infancy with *EGFR* internal tandem duplications,² myofibroma/myopericytoma with *PDGFRB* mutations,^{3,4} and lipofibromatosis-like neural tumors with recurrent *NTRK1*-related gene fusions.⁵ Triggered by a challenging congenital fibroblastic lesion, which did not fit in any of the known pathologic entities, we have applied whole transcriptome sequencing for further genomic characterization. Thus a novel *EWSR1-SMAD3* fusion was identified and found to be recurrent in 2 additional cases with similar acral clinical presentation and immunoprofile, suggesting the possibility of a new subtype of fibroblastic lesions with propensity for local recurrence.

MATERIALS AND METHODS

Index case and patient selection

The index case was that of a 1 year-old boy presenting with a skin-colored papule since birth on the left heel. The excisional specimen showed a 1.0 cm dome-shaped tumor involving the dermis and subcutaneous tissues, with flattened overlying epidermis, entrapped skin appendages in the center, and an infiltrative growth toward the subcutaneous fat (Fig. 1A,B). The tumor was composed of intersecting fascicles of uniform plump spindle cells with fine chromatin and occasional small but distinct nucleoli (Fig. 1C,D). Immunohistochemically, the tumor was negative for smooth muscle actin, desmin, caldesmon, HHF35, S100, CD34, panCK, and EMA and was initially diagnosed as lipofibromatosis. RNA extracted from the frozen tissue was submitted for RNA sequencing (RNAseq).

After the identification of the fusion candidate in the index case, we performed fluorescence in situ hybridization (FISH) to validate the gene fusion and screen 2 other cases (cases #2 and #3), selected based on a similar spindle cell tumor morphology, lack of a specific line of differentiation by immunohistochemistry and the presence of an *EWSR1* gene rearrangement. The study was approved by the Institutional IRB.

Whole transcriptome sequencing and analysis

Total RNA was extracted from frozen tissues of case #1 using RNeasy Plus Mini (Qiagen), followed by mRNA isolation with oligo(dT) magnetic beads and fragmentation by incubation at 94°C in fragmentation buffer (Illumina) for 2.5 minutes. After gel size-selection (350–400bp) and adapter ligation, the library was enriched by PCR for 15 cycles and purified. Paired-end RNAseq at read lengths of 50 or 51 bp was performed with the HiSeq 2000 (Illumina). After being independently aligned by STAR (ver 2.3) and BowTie2

against the human reference genome (hg19), the reads were analyzed by STAR-Fusion and TopHat-Fusion algorithms, respectively, for fusion discovery.

Fluorescence in situ hybridization (FISH)

To validate the fusion candidates found by RNA sequencing, we performed FISH for *EWSR1* and *SMAD3* break-apart. Custom probes were made by bacterial artificial chromosomes (BAC) clones flanking the genes of interest according to UCSC genome browser (<http://genome.ucsc.edu>) and obtained from BACPAC sources of Children's Hospital of Oakland Research Institute (Oakland, CA; <http://bacpac.chori.org>) (Supplementary Table 1). DNA from each BAC was isolated according to the manufacturer's instructions. The BAC clones were labeled with fluorochromes (fluorescent-labeled dUTPs, Enzo Life Sciences, NY, USA) by nick translation and validated on normal metaphase chromosomes. The 4 µm-thick FFPE slides were deparaffinized, pretreated, and hybridized with denatured probes. After overnight incubation, the slides were washed, stained with 4', 6-diamidino-2-phenylindole (DAPI), mounted with an antifade solution, and then examined on a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany) controlled by Isis 5 software (Metasystems). The 2 additional cases were also tested by FISH for *EWSR1*, followed by *SMAD3*.

RESULTS

Novel *EWSR1-SMAD3* fusion identified in a congenital acral spindle cell tumor

RNAseq performed in the index case identified an *EWSR1-SMAD3* fusion candidate, with exon 7 of *EWSR1* (22q12.2) fused to exon 5 of *SMAD3* (15q22.33) (Fig. 2A,B). The predicted chimeric amino acid sequence was in-frame and contained the N-terminal transcriptional activation domain of *EWSR1* and part of the linker region and the entire MH2 (MAD homology 2) domain of *SMAD3*. The gene fusion was further confirmed in the index case by break-apart FISH assay of both genes. FISH showed unbalanced rearrangements, with telomeric deletion of *EWSR1* (3') and centromeric deletion of *SMAD3* (5') (Fig. 2C).

Unsupervised clustering and gene expression analysis are in keeping with a fibroblastic lineage

By unsupervised hierarchical clustering of the whole transcriptome RNAseq data of >100 various soft tissue tumors, our index case clustered closely to other pediatric fibroblastic lesions available on the array, such as a calcifying aponeurotic fibroma with *FNI-EGF* fusion, a lipofibromatosis sample (no known genetic abnormalities) and a lipofibromatosis-like neural tumor with *TPR-NTRK1* fusion (Fig. 2D). The expression level of *SMAD3* in the index case was not significantly different from other soft tissue tumors. However, the index case showed up-regulation of *FNI* mRNA expression, at similar levels with the calcifying aponeurotic fibroma harboring an *FNI-EGF* fusion (Fig. 2E, left). Of note, *ERG* mRNA expression was significantly up-regulated in the index case, at even higher levels than 3 vascular neoplasms (2 head and neck and one breast angiosarcomas)(Fig. 2E, right), which may explain the ERG positivity seen immunohistochemically.

Recurrent *EWSR1-SMAD3* fusions were detected in 2 additional acral tumors in adult patients

Following the identification of the index case, we screened 2 previously unclassified spindle cell neoplasms with bland and uniform cytomorphology, non-specific immunoprofile, and known *EWSR1* gene rearrangements but unknown fusion partner. Both cases # 2 and #3 were found to have *EWSR1* gene rearrangements by FISH during a molecular work-up for a possible diagnosis of soft tissue myoepithelial tumor. However, a myoepithelial line of differentiation could not be confirmed by immunohistochemical studies. FISH screening for *SMAD3* gene abnormalities were tested and identified as the *EWSR1* fusion partner. Both cases occurred in the feet of adult female patients (58 and 61 years old, respectively) and involved dermis and/or subcutaneous tissues. The tumors had a nodular growth pattern, showing an infiltrative growth into the adipose tissues in case #2 (Fig. 3A). Microscopically, the tumors were composed of fascicles of uniform ovoid to spindle cells showing a distinctive zonation pattern, with hypercellular areas at the periphery and acellular central areas. Similar to the index case, the lesional cells had an ovoid to short spindled phenotype, with light eosinophilic cytoplasm and fusiform nuclei with open chromatin, lacking significant pleomorphism or increased mitotic activity. The central zone revealed densely collagenized or infarction-like areas with ghost cell shadows (Fig. 3B–D, F–G). Focal stippled calcification in the hyalinized component was present in case #2 (Fig. 3E).

Immunohistochemically, the tumors lacked a specific line of differentiation, being negative for SMA, desmin, caldesmon, S100, and CD34. Intriguingly, both cases showed diffuse and strong ERG (rabbit monoclonal C-terminus Ab EPR3864, Roche) nuclear staining similar to the index case (Fig. 3I). Despite strong ERG immunoreactivity and *ERG* mRNA overexpression in the index case, FISH showed no *ERG* break-apart or other copy number abnormalities in case #3. Keratin and EMA showed equivocal weak staining in case #2, while being negative in case #3 and index case. Additional immunostains performed showed negative SOX10 in 2 cases and NTRK1 in case #3.

EWSR1-SMAD3 fusion positive tumors are prone for local recurrence when incompletely excised

The tumors in all 3 cases were intra-lesionally or marginally excised with positive margins. Case #1 recurred as a 0.5 cm tumor at the junction of dermis and subcutis 14 months after the initial excision. Case #3 also recurred 5 months later, as a subcutaneous tumor with irregular borders, composed of cellular spindle cell fascicles without a hypocellular center (Fig. 3H). No follow-up information was available for case #2. No metastatic disease developed in these 2 patients with available follow-up.

DISCUSSION

In this study, we report novel *EWSR1-SMAD3* gene fusions in 3 superficial acral soft tissue tumors with unusual histologic features, difficult to classify into any existing pathologic entities. The tumors presented as small superficial tumors involving the dermis and subcutis of the feet in an infant and 2 adult patients. Based on their morphologic appearance and non-specific immunoprofile, the tumors appear to have a fibroblastic differentiation, being

composed of short fascicles of bland spindle cells. The 2 tumors occurring in adult patients showed a unique zonation pattern, with acellular centers of hyalinization or infarction-like areas, surrounded by more cellular peripheral areas. All 3 cases showed infiltrative border to the adipose tissues, and local recurrences were observed following incomplete initial excision.

The fibroblastic lineage of these tumors was also suggested by the whole transcriptome sequencing analysis of the index case, which, by unsupervised clustering, grouped together with other fibroblastic neoplasms, such as calcifying aponeurotic fibroma and lipofibromatosis. In addition, both the index case and the calcifying aponeurotic fibroma showed a significant *FNI* mRNA up-regulation. *FNI* gene encodes for fibronectin, a glycoprotein secreted by fibroblasts and other cell types and involved in extracellular matrix formation.⁶ Also similar to calcifying aponeurotic fibroma, focal calcification was present in one case, although both the morphology and genetic hallmark are otherwise distinct from calcifying aponeurotic fibroma.

Fibroblastic tumors include a wide spectrum of different pathologic entities, each with its own distinct clinicopathologic characteristics. However, the 3 cases reported here do not fit well into any of the currently known entities. In our index case, the infantile setting, acral location, and the morphology of bland spindle cell fascicles and focal infiltrative growth in the subcutis suggested a diagnosis of lipofibromatosis. However, the tumor showed limited adipose tissue infiltration compared to the typical long dissecting fascicles of fibroblastic cells seen in lipofibromatosis. Similar patterns have also been recently described in lipofibromatosis-like neural tumors with *NTRK1* fusion,⁵ but the index case was negative for both S100 and CD34 immunostains. The two adult cases displayed a peculiar zonation pattern, with an acellular center and cellular peripheral areas, distinctive from most other tumors considered in the differential diagnosis. Other fibroblastic tumors considered in the differential diagnosis based on the acral anatomic location, included a superficial acral fibromyxoma and an infantile digital fibroma. In contrast to typical findings of superficial acral fibromyxoma, the lesions in our study cohort showed a distinct fascicular growth pattern and lacked myxoid stroma. Despite the similar presentation of our index case (acral, infant) to an infantile digital fibroma, no cytoplasmic inclusions were present. The lack of staining for muscle markers further excluded a myofibroblastic line of differentiation. Quite unexpectedly was the consistent diffuse and strong ERG immunostaining in all 3 cases. ERG is a known marker for vascular and cartilaginous differentiation, and is often positive in Ewing sarcomas with the variant *EWSR1-ERG* gene fusion.^{7–9} Remarkably, the RNAseq data from the index case (Case #1) showed corresponding high levels of *ERG* mRNA levels; however, this upregulation was not due to gene amplifications or gene rearrangements, as FISH was negative in one case tested. Thus, the mechanism of aberrant ERG expression in this subset of *EWSR1-SMAD3* fusion positive fibroblastic tumors remains elusive. Of note, prostate carcinomas with *ERG* related fusions show TGF- β signaling pathway activation and up-regulation of several *SMAD* genes;¹⁰ as it has been shown that ERG co-precipitates and interacts with SMAD3.¹¹ Another interesting finding is that ERG immunoreactivity has also been reported in phosphaturic mesenchymal tumors, with variable positive rates (38–100%).^{12–14} Similar to our cohort, an upregulated *FNI* mRNA expression is also seen in a subset of

phosphaturic mesenchymal tumors with *FNI-FGFR1/FGF1* fusions,¹⁵ suggesting a potential correlation between the *FNI* and *ERG* transcriptional co-activation.

SMAD3 gene is a novel *EWSR1* fusion partner in translocation-associated neoplasia. SMAD3 functions as an important signal transducer in the TGF- β /Smad signaling pathway, which is involved in several biological processes, including extracellular matrix synthesis by fibroblasts.^{16,17} TGF- β receptor activation induces phosphorylation of the downstream SMAD3, which forms a heteromeric complex with SMAD4 and translocates into the nucleus to bind the promoter regions of its targets, such as type I collagen, fibronectin, and connective tissue growth factor (CTGF/CCN2).^{18,19} In addition to inducing extracellular matrix production-related genes, SMAD3 also inhibits their degradation through TIMP-1 activation.¹⁸ Dysfunction of SMAD family is associated with scleroderma, renal fibrosis, and radiation-induced fibrosis.^{17,20,21} Therefore, *SMAD3* gene abnormalities through chromosomal translocation is also in keeping with the presumed fibroblastic differentiation. Although *SMAD3* mRNA expression level is not significantly upregulated in the index case, the truncation of the SMAD3 protein due to the translocation event might lead to abnormal localization and functional alterations of its downstream transcriptional targets. In our index case, the mRNA expression levels of several SMAD3 target collagen genes, including *COL1A1*, *COL1A2*, *COL3A1*, and *COL6A3*, were also up-regulated (data not shown). The breakpoint involves the linker region between MH1 and MH2 domains of SMAD3, and thus the predicted fusion protein retains the MH2 domain, which is associated with oligomerization, phosphorylation, and transactivation. In contrast, the MH1 domain is responsible for nuclear localization and DNA binding of the chimeric protein.¹⁶

In summary we report recurrent *EWSR1-SMAD3* gene fusions in a distinctive group of acral superficial tumors with presumed fibroblastic lineage and local recurrence potential. Despite an otherwise non-specific immunoprofile, diffuse ERG expression was a consistent feature of this unique cohort, which correlated with a significant *ERG* mRNA upregulation in the index case. At transcriptional level, the tumors also show overexpression of *FNI* (fibronectin), at similar levels with other tumors with *FNI*-related gene fusions, such as calcifying aponeurotic fibroma and phosphaturic mesenchymal tumor, the latter also showing ERG immunoreactivity. Dysfunction of SMAD family, previously associated with extracellular matrix abnormalities, such as scleroderma and renal fibrosis, are now implicated for the first time in translocation-associated mesenchymal neoplasia. Their similar morphology, anatomic location, and novel *EWSR1-SMAD3* gene fusion strongly suggest that these tumors represent a novel and distinct entity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Histologic features of the index case, a heel tumor in a one year-old boy

The tumor presented as a bulging nodule involving the dermis and subcutaneous tissues with infiltrative border (A,B). It is composed of intersecting fascicles of uniform plump spindle cells with fibrillary cytoplasm and bland fusiform nuclei (C,D).

Figure 2. (A) A novel *EWSR1-SMAD3* gene fusion, resulting from a t(15;22)(q22.33;q12.2) was identified in the index case by whole transcriptome sequencing. (B) The fusion transcript was composed of exon 7 of *EWSR1* fused in-frame to exon 5 of *SMAD3*. The deduced chimeric protein contains the N-terminal transcriptional activation domain of *EWSR1* and the MH2 domain with part of the linker region of *SMAD3*. (C) The fusion was validated by break-apart FISH assays for *EWSR1* and *SMAD3*, respectively. *SMAD3* shows unbalanced rearrangement, with single telomeric (green) signals and deletion of the centromeric (red) signals (arrows). FISH for *EWSR1* similarly shows unbalanced pattern with deletion of telomeric signals (not shown). (D) Unsupervised clustering of RNA sequencing data shows the index case (red) clusters with a calcifying aponeurotic fibroma with *FNI-EGF* fusion (purple), a lipofibromatosis (light blue), and a lipofibromatosis-like neural tumor with *TPR-NTRK1* fusion (dark blue), among other soft tissue tumors. (E) Left panel: high *FNI* mRNA expression in the index case (red), calcifying aponeurotic fibroma (purple), and lipofibromatosis-like neural tumor (dark blue). Right panel *ERG* mRNA level is

significantly up-regulated in the index case (red), even at higher levels than 3 angiosarcomas in the same dataset (green bars in D, E).

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Figure 3. Histologic features of the additional two adult acral spindle cell neoplasms with *EWSR1-SMAD3* fusions

(A–E) Case #2, a subcutaneous nodule in the foot of a 61 year-old female showing an infiltrative border (A) and a distinctive zonation pattern with hypercellular periphery (B), transitioning to hypocellular collagenous area (C), and an acellular central zone (D). Focal fine calcifications were also present (E). (F–H) Case #3, a toe lesion in a 58 year-old female, displayed a similar zonation pattern in the primary lesion (F,G) and the cellular component only in the local recurrent lesion 5 months later. (I) All 3 cases show diffuse and strong ERG immunoreactivity.

Table 1Clinicopathologic Features of Cases with *EWSR1-SMAD3* Fusions

Case#	Age/Sex	Location	Depth	Size (cm)	Immunohistochemistry				Follow-up
					ERG	CD34	SMA	S-100	
1	1/M	Heel	Dermis & Subcutis	1.0	+	-	-	-	LR (14 mon)
2	61/F	Foot	Subcutis	2.0	+	-	-	-	NA
3	58/F	Toe	Dermis & Subcutis	1.1	+	-	-	-	LR (5 mon)

M, male; F, female; LR, local recurrence; mon, months