TITLE

An undifferentiated sarcoma of bone with a round to epithelioid cell phenotype harboring a novel EWSR1-SSX2 fusion identified by RNA-based next-generation sequencing

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Abstract:

Due to the increased application of RNA-based next-generation sequencing techniques on bone and soft tissue round cell sarcomas new fusions are frequently found, thereby expanding the molecular landscape of these tumors. In this report, we describe and discuss the finding of an undifferentiated sarcoma of the bone with a round to epithelioid cell phenotype harboring a novel $EWSR1$-$SSX2$ fusion. Treatment of this new bone tumor entity according to the Euro Ewing 2012 protocol led to complete pathologic response.

1. Introduction

In recent years, the molecular landscape of undifferentiated small round cell sarcomas of bone and soft tissue has greatly expanded by the increased application of RNA-based next-generation sequencing technologies in clinical practice. This has led to the introduction of new sarcoma entities, such as $CIC$-rearranged sarcomas, sarcomas with $BCOR$ genetic alterations and round cell sarcomas with $EWSR1$-non-ETS (in particular $NFATC2$ and $PATZ1$) fusions, which are now incorporated in the recent 2020 5th edition World Health Organization (WHO) Classification.\(^1\) Despite the significant advancement in the molecular classification of small round cell sarcomas of bone and soft tissue, pathologists are still facing cases with an undifferentiated phenotype that do not fit within the current WHO classification and remain ‘unclassified’. In this brief communication, we illustrate this further with a case of an undifferentiated round cell sarcoma of the bone with a novel $EWSR1$-$SSX2$ fusion.

2. Methods and results

2.1 Case presentation

A 47-year-old man, with a history of sarcoidosis, showed a flair up when tapering off his corticoid therapy. He suffered from severe pain of the left hip, and sarcoidosis of the bone was clinically suspected. Computed Tomography (CT) showed an osteolytic lesion in the subtrochanteric region. Magnetic Resonance Imaging (MRI) confirmed the osteolytic lesion,
revealing destruction of the cortical bone and reactive changes of the surrounding soft tissues. After determination of the most suspect location for viable tumor tissue retraction, during a multidisciplinary discussion, an open surgical biopsy was taken from the lesion.

2.2 Pathological features and immunohistochemical studies
Microscopy of the biopsy showed a cellular, undifferentiated tumor composed of primitive round to epithelioid cells arranged in solid sheets. The tumor cells had scant cytoplasm with vesicular nuclei and prominent nucleoli. This uniform round to epithelioid cell component was mixed with solid areas of more spindle tumor cells showing more pleomorphism. Brisk mitotic activity, as well as apoptosis and marked tumor necrosis were observed. In the background small ‘ectatic’ vessels could be seen (Figure 1). Immunohistochemically, tumor cells showed strong and diffuse membranous staining for CD99, together with strong and diffuse nuclear expression for TLE1, SATB2, cyclin D1 and BCOR (Figure 1). There was no expression of cytokeratin AE1/AE3, EMA, CD45, TdT, MPO, S100, SOX10, NKX2.2, NKX3.1, aggrecan, ETV4, WT-1, CD34, ERG, desmin, myogenin, MyoD1, SS18-SSX and ALK. Pan-tropomyosin receptor kinase (pan-TRK) (clone EPR17341, Roche Diagnostics) showed a moderate strong cytoplasmatic and membranous staining in 30% of the tumor cells.

2.3 Fluorescence in situ hybridization (FISH)
FISH was performed on formalin-fixed paraffin-embedded tissue using break-apart probes for SS18 (SYT), EWSRI and FUS, which revealed rearrangement of EWSRI (Figure 2), but not of FUS and SS18 (SYT).

2.4 RNA-based next-generation sequencing
RNA extraction from formalin-fixed paraffin-embedded tissue (FFPE) was performed using the Maxwell(R) RSC RNA FFPE kit (protocol on https://be.promega.com/products/nucleic-acid-extraction/rna/maxwell-rsc-rna-ffpe-kit/?catNum=AS1440). RNA-based next-generation sequencing with the Archer FusionPlex Expanded Sarcoma panel (40 genes) was carried out.
and revealed an in-frame fusion transcript between exon 15 of the *EWSR1* gene (NM_005243.3, hg19; ENSE00003684056: chr22: 29,299,234-29,299,331) and exon 8 of the *SSX2* gene (NM_175698.2, hg19; ENSE00003521258: chrX: 52,698,088-52,697,984) (Figure 3) using the Archer Analysis software program. Further, this targeted RNA sequencing approach did not show any *NTRK1, NTRK2* nor *NTRK3* fusions.

### 2.5 Treatment

The patient was treated according to the Euro Ewing 2012 protocol. In this protocol 9 cycles of two combinations of chemotherapy, namely vincristine-doxorubicin-cyclophosphamide (VDC) and ifosfamide-etoposide (IE), are alternately given every two weeks in a neo-adjuvant setting, followed by a resection of the tumor and 5 cycles of chemotherapy in an adjuvant setting. However, after 3 cycles (approximately 2 months after the start of the chemotherapy) there was tumor progression on MRI with an increase in volume of the tumor in the proximal femur, especially at the site of the collum. In a multidisciplinary clinical consultation, the decision was made to resect the tumor. The resected bone tumor showed a complete histological tumor response with tumor necrosis. There was evidence of cortex invasion with local breakthrough in the surrounding soft tissue but histologically there were no vital tumor cells detectable. The volume increase, as seen on MRI, was due to the reaction to the chemotherapy and gave a false-positive image of tumor progression. Chemotherapy was continued after the resection based on the original Euro Ewing 2012 protocol. The interval between the diagnosis and this report was 1 year, in which there were no signs of recurrence or metastasis.

### 3. Discussion

In this brief communication, we describe an undifferentiated sarcoma of bone with a round to epithelioid cell phenotype harboring an *EWSR1* gene rearrangement, detected by FISH and
additionally confirmed by RNA-based next-generation sequencing, showing a novel EWSR1-SSX2 fusion transcript.

With the advent of widely used modern molecular techniques during the last decades, it became obvious that EWSR1 is the most common rearranged gene in soft tissue tumors and can show a variety of breakpoints. EWSR1 gene rearrangements are seen in a wide spectrum of mesenchymal tumors including Ewing sarcoma (ES), small round cell sarcoma with EWSR1-non-ETS fusions, desmoplastic small round cell tumour, myxoid liposarcoma, extra skeletal myxoid chondrosarcoma, angiomatoid fibrous histiocytoma, clear cell sarcoma of soft tissue and clear cell sarcoma-like tumours of the gastrointestinal tract, primary pulmonary myxoid sarcoma, extrasalivary myoepithelial tumours, low-grade fibromyxoid sarcoma, sclerosing epithelioid fibrosarcoma, epithelioid/spindle cell rhabdomyosarcoma and myxoid neoplasms harboring EWSR1-CREM fusions.¹-²,⁵

EWSR1 (being the 5’ partner) can fuse with many partner genes and was first noted to be rearranged in ES.²-⁴,⁶ In ES, translocations occur between EWSR1 and genes encoding members of the ETS (E-Twenty-Six) family of transcription factors (frequently ERG, FLI1, ETV1/4 and FEV). Rarely, EWSR1 fuses with non-ETS genes. These gene fusions include EWSR1-NFATC2, EWSR1-PATZ, EWSR1-VEZF1, EWSR1-POU5F1, EWSR1-SMARCA5 and EWSR1-SP3.²-⁴,⁶-¹³ This last group was recently incorporated as a new emerging entity (‘round cell sarcoma with EWSR1-non-ETS fusions’) in the recent 5th WHO Classification of soft tissue and bone tumors.¹ However, in this chapter, only the EWSR1-NFATC2 and EWSR1-PATZ1 sarcomas are mentioned (for now). This new group of ‘round cell sarcoma with EWSR1-non-ETS fusions’ differs from Ewing sarcoma, CIC-rearranged sarcomas and sarcomas with BCOR genetic alterations based on their genetic/molecular profile.¹-³,⁶-⁷

The morphology (monotonic round to epithelioid cell tumor population mixed with a spindle cell morphology) and immunohistochemical profile (strong staining for CD99, BCOR, TLE1,
SATB2 and cyclin D1) of the current case strongly mimicked that of a sarcoma with BCOR genetic alteration. The clinical presentation (primary osseous involvement) and the immunohistochemical profile (no expression of ETV4, ERG and WT-1) were not compatible with the diagnosis of a CIC-rearranged sarcoma. Further, the molecularly proven EWSR1 gene rearrangement ruled out the diagnosis of a BCOR-rearranged sarcoma and CIC-rearranged sarcoma. The diagnosis of Ewing sarcoma could be considered in this case given the monotonic round cell tumor morphology, the strong immunohistochemical expression of CD99 and the EWSR1 gene rearrangement. Moreover, a subset of Ewing sarcoma can show nuclear variations in size and shape, prominent nucleoli and spindle cell morphology (‘atypical Ewing sarcoma’). However, the relatively old age of the patient and negative immunohistochemical staining for NNX2.2 (which is a highly sensitive immunohistochemical marker for Ewing sarcoma) argued against this differential diagnosis. In the group of round cell sarcomas with EWSR1-non-ETS fusions, EWSR1-NFATC2 sarcoma was considered because these sarcomas occur predominantly in long bones. Though, NNX3.1 (a recently described useful marker helping in distinguishing EWSR1-NFATC2 sarcomas from other round cell sarcomas) and aggrecan (a novel diagnostic marker for NFATC2-rearranged sarcomas) were both negative in this case. Further, small cell osteosarcoma (given the strong SATB2 positivity) and small cell synovial sarcoma (given the strong TLE1 positivity) were taken into account. The absence of osteoid cancelled out the diagnosis of a small cell osteosarcoma in this case. However, we had to bear in mind that the given material was only a biopsy and sampling error could have occurred. A primary osseous location is very unusual for a synovial sarcoma. Moreover, additional immunohistochemical and molecular features (no immunohistochemical expression for SS18-SSX; no SS18 gene rearrangement by FISH) were argumenting against the diagnosis of synovial sarcoma or an unusual undifferentiated round cell sarcoma with SS18 fusion. Other line of differentiations (e.g. epithelial,
melanocytic, hematolymphoid, vascular, myogenic, rhabdomyoblastic and neurogenic) were excluded by immunohistochemistry. Based on the morphology, immunohistochemical profile and the molecular FISH findings, a preliminary diagnosis of an ‘undifferentiated round cell sarcoma with EWSR1 rearrangement’ was proposed.

In an attempt to further classify this lesion RNA-based next-generation sequencing was performed, which showed a novel EWSR1-SSX2 fusion. SSX (synovial sarcoma X-breakpoint) is a transcription factor expressed in different epithelial and mesenchymal tumors, which was first discovered as part of the SS18-SSX fusion in synovial sarcoma. In synovial sarcoma the SS18-SSX fusion transcript most often contains exons 6 and 8 of the SSX2 gene. This differs from our case, where the EWSR1-SSX2 fusion transcript only contains exon 8 of the SSX2 gene, coding for the last 33 amino acids of SSX2 (Figure 3). This, however short, SSX2 domain still contains the functional SSX repression domain (SSX RD) and in turn could explain the immunohistochemical expression for BCOR and TLE seen in this case. Still, little is known about the cellular functions of the SSX family of proteins and their specific role in oncogenesis. Studies have shown that SSX2 induces senescence and can activate several important mitogenic pathways, such as MAPK and Wnt, suggesting an important role in cancer cell proliferation. The breakpoint of EWSR1 in our case lies at 3’ of exon 15, which is not a common breakpoint in EWSR1 (most common being exon 7). Interestingly, the transcriptional activation domain (TAD) and the RNA recognition motif (RRM) of EWSR1 are maintained in the fusion product (Figure 3). This is also described in the EWSR1-NR4A3 fusions in which the break point occurs after exon 12 or 13 of EWSR1.

To our best knowledge, an EWSR1-SSX2 fusion has not yet been described in the literature. This newly found gene rearrangement was detected by FISH and confirmed by RNA-based NGS. It included known oncogenes but with breakpoints not occurring in hotspot locations.
However, the gene fusion resulted in an in-frame fusion, containing functionally important exons of both oncogenes, and therefore could be considered to play a role in tumorigenesis.

This could be a novel gene fusion defining a new member belonging to the heterogeneous group of round cell sarcomas with EWSR1-non-ETS fusion. Only very recently, Antonescu et al. described 2 unusual cases of poorly differentiated sarcomas with round cell to epithelioid morphology, both harboring a novel EWSR1-SSX1 fusion.\textsuperscript{29} There are some overlapping morphologic (round to epithelioid cell phenotype) and immunohistochemical features (expression of TLE1, BCOR and CD99) with our case but the clinical presentation (primary soft tissue tumors) and immunohistochemical profile (immunoreactivity for pankeratin and EMA) of these sarcomas with EWSR1-SSX1 fusion are different from our case. The possible relationship between the cases of Antonescu et al. and our case is unclear, based on the limited information that is available.

In conclusion, we described the first case of a high-grade sarcoma with a round to epithelioid cell phenotype harboring an EWSR1-SSX2 fusion, illustrating the role of RNA-based next-generation sequencing techniques for further classification of the heterogeneous group of undifferentiated round cell sarcomas. Confirmation by additional cases of undifferentiated sarcomas with EWSR1-SSX2 fusions are necessary to unravel the underlying molecular mechanisms guiding this novel EWSR1-SSX2 fusion and to better characterize their place in the evolving classification of fusion-positive undifferentiated round cell sarcomas.
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Informed Consent: Informed consent was obtained from the patient.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figures legend:

**Figure 1** A, B Cellular, undifferentiated tumor with solid growth and marked tumor necrosis. Presence of small ‘ectatic’ vessels in the background (H&E, original magnification 100x). C Round and epithelioid tumor component with brisk mitotic activity (H&E, original magnification 200x). D Solid tumor areas of more spindle tumor cells (H&E, original magnification 200x). Strong diffuse expression of the tumor cells for BCOR (E), CD99 (F), Cyclin D1 (G), SATB2 (H) and TLE1 (I) (original magnification 200x).

**Figure 2** Photomicrograph of the dual-color fluorescence in situ hybridization (FISH) used for the detection of *EWSR1* translocation: tumor cell with one fusion (yellow, yellow arrow), one green and one red split (arrowhead) signal pattern, indicating the presence of rearrangement.

**Figure 3** *EWSR1*-SSX2 fusion product: the predicted fusion protein contains functionally important domains of both the *EWSR1* and SSX2 oncogene.
References


**EWSR1** (NP_005234, 656aa)

- TAD = transcriptional activation domain
- RG = arginine-glycine-rich domain
- RRM = RNA recognition motif
- ZF = zinc finger domain
- PY = proline-tyrosine nuclear localisation signal

**SSX2** (NP_783629, 188aa)

- KRAB = Kruppel-associated box domain
- DD = divergent domain
- RD = repression domain

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**EWSR1-SSX2**

- TAD
- RG
- RRM
- ZF
- RD