Small blue round cell tumor of the interosseous membrane bearing a t(2;22)(q34;q12)/EWS-CREB1 translocation: a case report

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Abstract

Background: The group of small blue round cell tumors encompasses a heterogeneous group of neoplasms characterized by primitive appearing round cells with few distinguishing histologic features.

Results: We report the case of a small blue round cell tumor with an EWS gene rearrangement detected by fluorescent in situ hybridization (FISH) analysis that mimicked Ewing sarcoma, but with unusual histology and immunohistochemical features. Multi-color karyotyping identified the presence of a t(2;22)(q34;q12) that was initially expected to represent a variant EWSR1-FEV translocation. After an extensive workup, the lesion is considered to represent a clear cell sarcoma harboring an EWSR1-CREB1 fusion transcript.

Conclusions: This case appears to represent a rare variant of clear cell sarcoma arising in peripheral soft tissues with unusual histology and unique immunophenotype. In this circumstance, FISH for all EWSR1 translocation partners or RT-PCR for a spectrum of possible transcript variants is critically important for diagnosis, since cytogenetic analysis or clinical FISH assay using only commercial EWSR1 probes will be misleading.

Background

The accurate diagnosis of small blue round cell neoplasms can be difficult. The differential diagnosis includes sarcomas (such as the Ewing family of tumors (EFT), alveolar rhabdomyosarcoma, poorly differentiated synovial sarcoma, myxoid/round cell liposarcoma, desmoplastic small round cell tumor, and cellular variants of extraskeletal myxoid chondrosarcoma), small cell and lymphoid lymphomas, neuroblastoma, melanoma and small cell carcinoma among others.

Although certain histologic features may be useful in differentiating these entities, their general morphology is generic by light microscopy and a large battery of ancillary studies is required. Immunohistochemistry is the first line supplemental methodology and is sufficient for diagnosis in many cases of small round cell tumors. For example, myogenin and myoD1 are specific and sensitive for the diagnosis of rhabdomyosarcoma [1] and lymphoid markers such as CD20, CD3, CD30 and CD45 are very useful in the diagnosis of lymphoma. However, many other markers, although helpful, are not so specific and require interpretation in the context of an immunohistochemical panel. For example, epithelial markers are essential for the diagnosis of carcinoma, but they can also be positive in poorly differentiated synovial sarcoma, Merkel cell carcinoma and in rare Ewing family tumors [2]. S-100 is positive in melanoma but also in clear cell sarcoma, myxoid liposarcoma, extraskeletal myxoid chondrosarcoma and some Ewing family tumors; desmin is strongly positive in rhabdomyosarcoma but is also positive in desmoplastic small round cell tumor; and CD99 immunoreactivity is seen in EFT, but also in mesenchymal chondrosarcoma and lymphoblastic lymphoma [3].

FISH analyses for chromosomal translocations can be extremely helpful in this setting, but even these findings may not be specific. EWSR1 gene rearrangement is characteristic of EFT, but is also present in extraskeletal myxoid chondrosarcoma, desmoplastic small round cell tumor, and a subset of myxoid/round cell liposarcomas...
Karyotype analysis is a global genome scan that has the ability to detect gross chromosomal alterations such as translocations, but the precise chromosomal bands and breakpoints involved in the identified translocations may be inaccurate due to the very low resolution of this technique.

Here we report on a small blue round cell tumor with an unusual combination of histological and immunohistochemical findings. Results from standard first line molecular cytogenetic studies turned out to be misleading for both diagnosis and therapy. A complete workup including karyotype analysis, multicolor FISH and construction of new FISH probes was required for the definitive diagnosis of what we consider to represent a variant of clear cell sarcoma bearing an EWSR1-CREB1 fusion transcript and expressing an aberrant immunophenotype.

Case presentation
Clinical details
54-year-old female presented with pain and swelling of one year duration in her left leg. An MRI scan revealed a 7 cm enhancing mass lying in the posterior calf, at the level of popliteus muscle and extending through the interosseous membrane. The tibial nerve and popliteal vessels were encased in the tumor. Systemic imaging revealed no metastases.

A core needle biopsy was taken, but proved insufficient for a definitive diagnosis. An open biopsy was performed and a working diagnosis of soft tissue Ewing sarcoma rendered. The patient received pre-operative systemic chemotherapy as for Ewing sarcoma, but failed to respond with any tumor shrinkage.

For local treatment of her tumor she was advised to undergo above knee amputation due to the anticipated poor functional results of limb salvage in this situation. Despite extensive counseling and corroborating second opinions she refused amputation. As limb salvage was technically feasible, she underwent pre-operative radiation therapy and a complex wide resection of the tumor was performed, with tibial nerve resection, vascular reconstruction with saphenous vein grafts, allograft reconstruction and internal fixation of the tibial defect, as well as reconstruction of the soft tissue defect with a free tissue transfer from the scapular region. Wide margins were achieved. The patient developed a wound infection with methicillin-resistant S. aureus 14 days postoperatively. At day 22, she suffered an anastomotic leak of the vessel reconstruction. Although vascularity was restored and her limb at this stage was still viable, she was very disappointed with the functional results of her procedure and requested an above knee amputation. She was clear of disease at last follow up.

Results
Core needle biopsy
Sections showed a small blue round cell tumor growing in poorly cohesive sheets. The tumor cells had uniform plasmacytoid cytology (Fig. 1). A morphologically suspected diagnosis of plasma cell myeloma was ruled out based on the lack of immunoreactivity for kappa or lambda light chains. Instead, the tumor cells showed striking CD99, synaptophysin and desmin immunoreactivity. In contrast, other immunohistochemical markers were all negative, including melanocytic markers such as S-100, HMB-45 and Melan-A. The results of immunohistochemistry are shown in Table 1 with some relevant immunohistochemical staining patterns illustrated in Figure 1.

The main differential diagnoses were considered to be Ewing family tumor and alveolar rhabdomyosarcoma. Alveolar rhabdomyosarcoma was ruled out by negative myogenin and myoD1 and by absence of PAX3/PAX7-FKHR translocations by FISH. EWSR1 FISH, in contrast, showed a break-apart signal pattern in most of the interphase nuclei. A provisional diagnosis of Ewing sarcoma was rendered. However, the histology, the absence of Fli1 staining and the strong desmin immunoreactivity were all considered unusual by several local and consultant pathologists who reviewed the case.

Additional FISH assays for WT1, CHN and DDIT3 (3’ partners of EWSR1 in desmoplastic round cell tumor, extraskeletal myxoid chordrosarcoma and round cell liposarcoma, respectively) were negative. All core needle biopsy tissue had been consumed after these assays.

In view of the major therapeutic implications engendered by a diagnosis of Ewing sarcoma, a decision was made to perform an open biopsy to obtain more tissue including fresh tissue for cytogenetics.

Open biopsy
An open biopsy was performed 6 weeks after the original core needle procedure. Histological examination of this specimen revealed plasmacytoid cells with small, eccentric, uniform, moderately vesicular nuclei, most with a single central nucleolus. The tumor cells were arranged in poorly cohesive sheets with no associated necrosis and occasional mitotic figures were evident. Results of immunohistochemical studies were similar to those found on the core biopsy. Other immunohistochemical markers were ordered and found to be negative, including EMA, CD68, PNL2 and WT1.

The cultured tumor specimen did not yield metaphases of sufficient quality to be analyzed by G-banding. Multicolor karyotyping identified a few metaphases that contained a reciprocal translocation between 2 q and 22 q in the context of additional numerical and structural

[4]. FUS rearrangements are seen in a majority of myxoid/round cell liposarcomas but also in EFT [5,6].
changes. The M-FISH stem line karyotype was established as 46, XX, t(2;22)(q36;q12), +5, t(14;21)(q32;q22), -21 [5] (Fig. 2). On this basis the diagnosis of Ewing sarcoma was considered confirmed as these findings were consistent with the variant t(2;22) EWS-FEV fusion. At this point neoadjuvant chemotherapy was initiated for Ewing sarcoma.

Characterization of the translocation t(2;22)

To confirm the suspected EWS-FEV translocation, a dual-color break-apart probe for FEV on chromosome band 2q36 [6] was applied to the cultured specimen. The scoring with this probe revealed two intact signals in the interphase nuclei, with one metaphase showing an intact copy of FEV abnormally located on der(22)t(2;22). FISH for EWSR1 was performed on the same slide and showed rearrangement in most of the interphase nuclei with the 3' probe signal located on der(22)t(2;22) (Fig. 2b). These findings indicated that FEV was not rearranged as expected, and that the breakpoint on the der(2) t(2;22) was in fact centromeric to the FEV locus.

A candidate gene approach was initiated to establish the variant EWSR1 fusion partner. CREB1 is the only other gene in this region previously identified in sarcoma translocations, and a break-apart CREB1 probe was created and applied to the cultured specimen. With this FISH experiment, break-apart signals in ~80% of the interphase nuclei were observed (Fig. 2c). The reciprocal t(2;22)(q34;q12) was confirmed using a EWSR1-CREB1 dual-fusion probe that revealed the expected fusion signals.

Agarose gel electrophoresis of the RT-PCR product showed a ~120 bp band (Fig. 3a). The size of the product corresponded to the predicted product size of the fragment spanning the fusion transcript breakpoint based on the primer design from published cDNA sequences for EWSR1 (NM_013986.2) and CREB1 (NM_004379.3). Cloning and sequencing of this product showed an in-frame fusion between EWSR1 exon 7 and CREB1 exon 7 (Fig. 3b).

Following this workup and considering also the lack of response to neoadjuvant therapy, a diagnosis of clear

Figure 1 Microscopic appearance and immunohistochemical features of the tumor. A) Representative area of the core needle biopsy specimen showing a homogeneous plasmacytoid appearance of the tumor cells (H&E, ×100 magnification). B) Desmin and C) CD99 strong immunoreactivity (×200 magnification). D) S100 and E) HMB-45 immunohistochemical staining (×200 magnification). F) Representative area of the resection specimen showing nests of tumor cells with clear cytoplasm divided by thin fibrous septa (×100 magnification).
cell sarcoma with unusual histology and variant immunophenotype was considered.

**Surgical specimen**

The resection specimen revealed a high-grade sarcoma with areas similar to those found in the biopsies, but also other areas with neoplastic cells with clear cytoplasm arranged in a nested pattern (Fig. 1f). Extensive infiltration of malignant cells through soft tissues and the dense fascia of the periosteum and interosseous membrane was evident as was extensive lymphovascular invasion. Immunohistochemistry was repeated and while HMB45 remained negative and CD99 strongly positive, S-100 and Melan-A now revealed patchy positive staining. These new findings led us to favor a final diagnosis of clear cell sarcoma.

**Discussion**

Clear cell sarcoma (CCS) is an aggressive neoplasm of uncertain histogenesis, accounting for 1% of soft tissue sarcomas. The deep soft tissues of the distal extremities are most frequently involved, often in association with tendons and aponeuroses [7]. Unlike most sarcomas, CCS has a high propensity for lymph nodes metastasis. The tumor cells show immunoreactivity for melanocytic markers [8,9] as they contain melanosomes in different stages of development [10], and display melanocytic gene expression signatures [11]. Despite its similarity with melanoma, CCS is a distinct entity genetically characterized by the presence of a chromosomal translocation involving EWSR1 most frequently partnered with ATF1 [10,12-14]. More recently, the alternative chimeric transcript EWSR1-CREB1 has been described in three cases of clear cell sarcoma of the gastrointestinal tract that, interestingly, did not show overt melanocytic differentiation [15]. The same chimeric transcript, resulting from a presumed t(2;22)(q34;q12) has been described in three cases of CCS of soft tissue to date [13,14]. Hisaoka et al [13] found this chimeric transcript in 2 of 33 cases of CCS (6%). One presented as a 1.5 cm mass in the finger of a 67-year-old male, and the other as a 15 cm mass in the pelvis of a 31-year-old female. Wang et al

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**Table 1 Methods and results of immunohistochemistry**

| Antibody   | Source               | Dilution/antigen retrieval | Detection method | Reactivity |
|------------|----------------------|-----------------------------|------------------|------------|
| CK AE1/AE3 | Dako                 | 1:200/protease digestion    | SA/Bi            | -          |
| CK7        | Dako                 | 1:200/protease digestion    | SA/Bi            | -          |
| CK20       | Dako                 | 1:500/protease digestion    | SA/Bi            | -          |
| TTF1       | Dako                 | 1:100/CC1/95°C/30 min       | SA/Bi            | -          |
| EMA        | Dako                 | 1:200/CC1/95°C/30 min       | SA/Bi            | -          |
| Vimentin   | Biogenex             | 1:10000/CC1/95°C/30 min     | SA/Bi            | -          |
| CD20       | Dako                 | 1:250/CC1/95°C/30 min       | SA/Bi            | -          |
| CD30       | Dako                 | 1:50/CC1/95°C/30 min        | SA/Bi            | -          |
| CD34       | Cell Marque          | 1:50/CC1/95°C/30 min        | SA/Bi            | -          |
| Kappa light chain | Dako            | 1:5000/protease digestion  | SA/Bi            | -          |
| Lambda light chain | Dako         | 1:10000/protease digestion | SA/Bi            | -          |
| S-100      | Dako                 | 1:800/CC1/95°C/8 min        | SA/Bi            | -          |
| HMB-45     | Dako                 | 1:100/untreated             | SA/Bi            | -          |
| Melan-A    | Cell Marque          | 1:50/CC1/95°C/30 min        | SA/Bi            | -          |
| PNL2       | Private source       | RTU/untreated               | Polymer based    | -          |
| SMA        | Dako                 | 1:200/untreated             | SA/Bi            | -          |
| Desmin     | Dako                 | 1:200/CC1/95°C/30 min       | SA/Bi            | ++         |
| H-Caldesmon| Dako                 | 1:200/CC1/95°C/30 min       | SA/Bi            | -          |
| Myogenin   | Dako                 | 1:50/CC1/95°C/60 min        | Polymer based    | -          |
| Myo-D1     | Dako                 | 1:60/citrate buffer (pH6.0) | Polymer based    | -          |
| CD99       | Signet               | 1:200/CC1/95°C/30 min       | SA/Bi            | +++        |
| Synaptophysin | Cell Marque       | 1:250/CC1/95°C/30 min       | SA/Bi            | +++        |
| E11        | BD Pharmingen        | 1:200/CC1/95°C/60 min       | Polymer based    | -          |
| WT1        | Dako                 | 1:50/CC1/95°C/60 min        | Polymer based    | -          |
| Ki 67      | Lab Vision           | 1:200/CC1/95°C/30 min       | SA/Bi            | >10%       |

Dako, Carpinteria, CA, USA; Biogenex, San Ramon, CA, USA; Cell Marque, Rocklin, CA, USA; Signet, Dedham, MA, USA; BD Pharmingen, Franklin Lakes, NJ, USA. SA/Bi: Streptavidin/biotin method; RTU: ready to use; CC1: Cell conditioning 1 (pH8.0) (Ventana medical systems, Tucson, AZ, USA). *Negative on the core and open biopsies, but patchy positive staining was identified in the resection specimen. Pacheco et al. Molecular Cytogenetics 2010, 3:12 http://www.molecularcytogenetics.org/content/3/1/12 Page 4 of 8
Figure 2 Characterization of the t(2;22) by molecular cytogenetic techniques. A) Partial multicolor karyotype showing normal chromosome 2, normal chromosome 22 and both derivative chromosomes resulting from t(2;22)(q36;q12). B) Metaphase FISH with EWSR1 break-apart probe. An intact dual-color EWSR1 signal can be seen on normal chromosome 22, a green signal on the der(2) and a red signal on the der(22). C) Interphase FISH with the CREB1 break-apart probe showing break-apart signals (1 fused, 1 green and 1 red signals).

Figure 3 Agarose gel of EWSR1-CREB1 RT-PCR product and sequence of the junction point. A) RT-PCR product of ~120 bp corresponding to the predicted size of the fragment spanning the break point based on the pair of primers used. M, 100 bp molecular marker; NC, negative control (molecularly confirmed clear cell sarcoma with an EWSR1-ATF1 translocation); NTC, no template control. B) Sequence electropherogram showing an EWSR1 exon7/CREB1 exon 7 in-frame fusion.
fibrous pseudocapsule with a pericapsular lymphoplas-
tic cells, pseudoangiomatoid blood-filled spaces,
superficial mass with a distinctive histology (nodules of
young adults [16,17] that typically presents as a small
ympathic neoplasm of borderline malignancy of children and
young adults [16,17] that typically presents as a small

EWSR1-ATF1 and EWSR1-CREB1 are not exclusively
found in CCS. They also are the most common gene
fusions in angiomatoid fibrous histiocytoma, a mesench-

Areas with rhabdoid tumor cells were
histology has been seen in some cases of CCS
[10,13,18]. Areas with rhabdoid tumor cells were
detected in 24% and 16% of the molecularly confirmed
cases reported by Antonescu et al [10] and Hisaoka
et al [13]. However, to the best of our knowledge, des-
min expression by CCS cells has not been described
previously [13,19] and CD99 immunoreactivity has been
found only in one molecularly confirmed case of CCS of
the stomach [20]. Nevertheless, these are markers that
are not consistently tested in the clinical workup for
clear cell sarcoma, as small blue round cell tumors such
as Ewing sarcoma and rhabdomyosarcoma are not part
of the usual histological differential diagnosis of CCS.

CCS of soft tissue is typically characterized by the
expression of S-100 and melanosome-associated markers
[10,13,14,18,21]. In this regard too, the present tumor
had a unique immunoprofile, as S-100, HMB45 and
Melan-A were all negative in both the core needle and
incisional biopsies. It was only in the resection specimen
that some patchy staining for S-100 and Melan-A was
seen. Lack of vimentin expression was also an unex-
pected finding.

In a setting of supportive histomorphology and
immunohistochemistry, detection of EWSR1 rearrange-
ments by FISH is a very useful diagnostic tool that can
support the diagnosis for the known set of EWSR1-
translocation bearing tumors (Ewing family tumors, clear
cell sarcoma, extraskeletal myxoid chondrosar-
coma, desmoplastic round cell tumor, and variant myx-
oid/round cell liposarcomas) [4]. Nevertheless, in light
of the lack of specificity of EWSR1 break apart probes in
the differential diagnosis of small round cell sarcomas,
the search for a 3′ partner is very important in the
follow-up analysis when the histology is not entirely

fusions in angiomatoid fibrous histiocytoma, a mesench-
ymal neoplasm of borderline malignancy of children and
young adults [16,17] that typically presents as a small

EWSR1 rearrangement in CCS by FISH has been variously reported to be 70%, 88% or
100% [18,22,23], raising the possibility of EWSR1 being
substituted by other genes as a 5′partner in a small
subset of CCS.

Our case would represent the fourth soft tissue CCS
reported harboring this fusion transcript variant, and
the first in which the cytogenetic features of this reci-
procal translocation t(2;22)(q34;q12) are detailed. The
predicted structure of EWSR1-CREB1 in this case is
similar to that described previously [14-16] in which
the oncogenic chimeric transcript retains its CREB1
carboxyl-terminal basic leucine zipper DNA binding
and dimerization domain, fused to the amino-terminal
transcriptional activation domain of EWS which
confers oncogenic properties by transcriptional
dysregulation.

Conclusions
This case supports the fact that EWSR1-CREB1 is not a
translocation variant exclusive to clear cell sarcomas
arising in the gastrointestinal tract.

The described variation in histology and immunohis-
tochemical features displayed by CCS must be taken
into account when considering the differential diagnosis
for an unusual small blue round cell tumor. In this set-
ing, immunohistochemistry and even karyotype can be
misleading, and FISH for both of the translocation part-
ers or PCR primers accounting for all of the fusion
transcript variants is important for accurate diagnosis.

Methods
Tissue handling
The core needle biopsy was fixed in neutral-buffered
formalin and processed for routine histology. Unstained
6 μm paraffin sections were submitted for interphase
FISH. Representative tissue from the incisional biopsy
and from the excision specimen were submitted
fresh for cytogenetic analysis and a portion was snap
frozen for molecular studies. The remaining tissue
was fixed and submitted for routine histology and immunohistochemistry.

**Immunohistochemistry**

Standard immunohistochemical studies were done using a Ventana Benchmark XT Instrument (Ventana medical systems, Tucson, AZ, USA). The source and dilution of the antibodies, antigen retrieval and the detection methods are presented in Table 1.

**Cytogenetic studies, FISH and multicolor FISH**

Chromosome analysis was performed by standard methods after 6 days culture in RPMI 1640 medium supplemented with 20% fetal calf serum and L-glutamine. Metaphase chromosomes were banded by the GTG method and the karyotypes were described according to the International System for Human Cytogenetic Nomenclature 2005 [24].

FISH was performed on sections from the core needle biopsy and on cell preparations from the cultured incisional biopsy specimen. Commercial probes for **EWSR1, FUS** and **DDIT3** (Abbott Molecular, Des Plaines, IL, USA) and “in-house” dual-color break-apart (**CHN, WT1, FEV**) and dual-color dual-fusion (**PAX3-FKHR** and **PAX7-FKHR**) bacterial artificial chromosome (BAC) probes were used. An “in house” dual-color break-apart probe was prepared for the detection of **CREB1** rearrangements using BACs RP11-354H1 and RP11-135B21. An “in-house” dual-color, dual-fusion **EWSR1-CREB1** probe was prepared to confirm the reciprocal t(2;22) (q34;q12) using BACs RP11-135B21/RP11-354H1 (chromosome 2) and RP11-945M21/RP11-1126O13 (chromosome 22).

The BAC probes were directly labeled by nick translation using either Spectrum Green or Spectrum Orange (Abbott Laboratories, Abbott Park, IL, USA). The chromosomal locations of the BACs were initially confirmed by hybridization to normal metaphases from a peripheral blood culture. Each probe was scored by counting 200 interphase nuclei under fluorescent microscopy. For confirmation of true breakapart, >10% of cells showing a clear pattern of one fused, one red and one green signal was required. For confirmation of dual fusions, >5% of nuclei with a clear two fused, one red, one green pattern was required.

Multicolor FISH was performed using the 24 XCyte color kit (Metasystems, Altlussheim, Germany) following the manufacturer’s protocol.

**Reverse transcription-PCR of the fusion transcript**

Total RNA from the frozen open biopsy tumor tissue was isolated using the RNeasy mini kit (Qiagen, Maryland, USA). Two micrograms of total RNA were reverse transcribed using the qScript cDNA SuperMix system (Quanta Biosciences, Maryland, USA) and used as template for PCR amplification of the **EWS-CREB1** fusion breakpoints using the following primers [14] **EWSx7-F1** primer (‘-TCCTACAGCACAATCCTCAAAGTCC3′) and **CREBe7-REVC** primer specific for **CREB1** (‘-GTACCCCATCGGTACCATGGT3′). The PCR amplification started with 5 minutes at 95°C; followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 58°C, and 45 seconds at 72°C; and a final extension of 10 minutes at 72°C. PCR product was detected by 1.5% agarose gel electrophoresis.

**cDNA cloning and sequencing analysis of the fusion transcript**

The cDNA of the break-point-crossing fragment of the **EWS-CREB1** chimeric transcript was first amplified with HindIII-EWSx7-FW (5′TATCTacgttTcCTAcaGC-CAAAGCTCAAAGTCC3′) and XhoI-CREBe7-RV (5′-TTTCTcagagGTACCCCATCGGTACCATGGT3′), then subcloned into pcDNA3.1 (+) vector. Purified plasmid DNA was verified using the same restriction enzymes. The clones with an insert of the appropriate size were then submitted for cDNA sequencing using a T7-FW primer.

**Consent**

Written informed consent was obtained from the patient for publication of this case report. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

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**Authors’ contributions**

MP carried out the candidate gene approach and the preparation of the “in-house” FISH probes, led the molecular studies, performed the cloning of the fusion transcript and drafted the manuscript. DEH led the cytogenetic and molecular cytogenetic studies, their analysis and interpretation and reviewed the manuscript. MMH was involved in the histopathologic diagnosis of the patient’s sample and the review of the manuscript. PWC provided the clinical data of the patient and helped drafting the manuscript. HHI organized the original histopathologic diagnosis of the patient. TON initiated and led the pathologic investigations and co-wrote the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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