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Review with novel markers facilitates precise categorization of 41 cases of diagnostically challenging, "undifferentiated small round cell tumors". A clinicopathologic, immunophenotypic and molecular analysis

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ABSTRACT

Background: Despite extensive immunohistochemical (IHC) and molecular studies combined with morphologic findings, a group of round/ovoid cell tumors histologically similar to Ewing sarcomas (ES) but lacking *EWSR1*-rearrangements may remain unclassifiable.

Design: We retrospectively analyzed 41 Ewing-like tumors (formalin-fixed, paraffin-embedded) previously determined as negative or non-informative for *EWSR1*-rearrangements by FISH and/or RT-PCR. A new histopathology revision and additional IHC and molecular analyses were carried out in order to investigate whether additional IHC and/or molecular testing in combination with the morphological findings may help in reaching a definitive diagnosis.

Results: Almost all the tumors (n = 40) involved soft tissue and/or bone and half the patients died of disease. In the archival cases all diagnoses were Ewing sarcoma (ES), Ewing-like sarcoma (ELS), myoepithelial tumor and undifferentiated sarcoma (US). In the new review all the tumors were re-classified as, ES (n = 16), Ewing-like tumor with *EWSR1* rearrangement and amplification and possible *EWSR1-NFATC2* gene fusion (n = 1), *CIC*-rearranged sarcomas or undifferentiated sarcoma, most consistent with *CIC*-rearranged sarcoma (n = 7), sarcoma with BCOR-alteration or undifferentiated sarcoma, consistent with BCOR-associated sarcoma (n = 3), neuroblastoma (n = 2), unclassifiable neoplasm with neuroblastic differentiation (n = 1), malignant rhabdoid tumor (n = 2), lymphoblastic lymphoma (n = 1), clear cell sarcoma of the gastrointestinal tract (n = 1), small cell carcinoma (n = 1), sclerosing rhabdomyosarcoma (n = 1), desmoplastic small round cell tumor (n = 1), malignant peripheral sheath nerve tumor (n = 1), poorly-differentiated synovial sarcoma (n = 1), Possible gastrointestinal stromal tumor/GIST with predominant round cells (n = 1) and possible SMARCA4-deficient-sarcoma (n = 1). NKX2.2, ETV4 and BCOR immunoreactivity was observed in all ES, *CIC*-rearranged sarcomas and sarcomas with BCOR alteration, respectively. *CIC*-rearrangement by FISH was observed in many of the *CIC*-rearranged sarcomas.

Conclusion: Our analysis of 41 Ewing-like tumors confirms that there may be a significant pathological and IHC overlap among Ewing-like tumors, with prognostic and therapeutic impacts. Additional IHC (NKX2.2, ETV4 and BCOR) and molecular studies including *FUS*, *CIC* or *BCOR* analysis may support the final diagnosis when FISH or RT-PCR fail to detect *EWSR1*-rearrangements. Any molecular findings should always be interpreted in relation to the specific clinical and pathological context.

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1. Introduction

The histopathological and immunohistochemical (IHC) differential diagnostic findings in small round cell sarcomas (SRCS) of soft tissue and bone overlap significantly [1,2]. Thus, a careful histologic examination combined with additional ancillary testing (IHC and molecular studies) is required to reach a conclusive diagnosis [1-8]. Specific chromosomal translocations frequently allow identification of most SRCS arising in soft tissue or bone [3-9]. Ewing sarcoma (ES) represents the prototypic bone and soft tissue SRCS with strong and membranous CD99 immunoexpression and *EWSR1* rearrangement in about 98% of tumors. ES are almost always characterized by reciprocal translocations between *EWSR1* and genes of the *ETS* family of transcription factors (*FLI-1, ERG, FEV, ETV4*), although they may occasionally reveal unusual gene fusions (*EWSR1-non-ETS*, *FUS* rearrangement instead of *EWSR1* or a very rare non-*TET/ETS* gene fusion) [1,2,8,10-14].

Many of the SRCS arising in bone and soft tissue with variable CD99 immunoreactivity but lacking *EWSR1* rearrangement belong to the new emerging category of Ewing-like sarcomas (ELS) [9,15-29]. Round or oval/spindle tumors with *CIC* or *BCOR* rearrangements represent the vast majority of ELS. *CIC-DUX4* and *BCOR-CCNB3* are the most prevalent gene fusions among ELS, although *CIC-FOXO4*, *BCOR-MAML3*, *ZC3H7B-BCOR* as well as *BCOR* internal tandem duplication (ITD) have also been reported [9,15-29].

At present, despite the extensive IHC and molecular assays (fluorescence in situ hybridization/FISH and/or reverse transcription polymerase chain reaction/RT-PCR) a small proportion of SRCS with Ewing-like morphology remain unclassified due to negative or non-informative molecular results, leading to ambiguity in diagnosis and treatment options [8,9,17,19,20]. In addition, testing for gene fusions is not available in all pathology departments.

In 2016 our working group published a series of 200 SRCS with morphology and immunophenotype of Ewing tumor in which the EWSR1 FISH translocation was non-informative or negative. Although several specific transcripts were tested in this series, some cases remained unclassified [30].

Our previous study had some drawbacks; first, we did not perform *FUS*, *CIC* or *BCOR* break-apart analysis by FISH, and thus, we could not excluded the existence of any of these rearrangements in tumors without the *EWSR1* translocation [30]. In addition, we did not test NKX2.2, ETV4 or BCOR immunoreactivity. It is now well documented that IHC expression of NKX2.2, ETV4 and BCOR may help in the differential diagnosis between ES and ELS, given that NKX2.2 immunoreactivity is highly sensitive and specific for ES, ETV4 immunoexpression is very suggestive of CIC-rearranged sarcomas and BCOR positivity is frequently encountered in BCOR-associated sarcoma [8,9,12-14,19,20,25].

The purpose of the present study was to re-review the morphological and IHC profile of these unclassified/undifferentiated tumors to investigate whether additional IHC and/or molecular testing in combination with the morphological findings may help in reaching a definitive diagnosis.

2. Materials and methods

Archival tumor specimens with morphological and IHC features (CD99 positivity) suggestive of ES or ELS in which *EWSR1* FISH translocation and RT-PCR for *EWSR1-FLI-1*, *EWSR1-ERG*, *EWSR1-FEV*, *EWSR1-ETV4*, *CIC-DUX4*, *CIC-FOXO4*, *BCOR-CCNB3*, *SYT-SSX(1,2,4)*, *PAX3-7-FOX01* and *YWHAE-NUTM2B* were negative or non-informative were retrieved from surgical pathology files from the University of Valencia (Spain), Instituto Valenciano de Oncología, Valencia (Spain), Hospital de Cancer de Barretos, Sao Paulo (Brasil) and the Rizzoli Orthopedic Institute Bologna (Italy). A total of 41 cases were selected for this study, comprising cases obtained through personal consultations with one of the authors (ALLB, $n = 34$), with additional cases ($n = 7$), from the collections of the PROgnosis and THerapeutic Targets in the Ewing's Family of TumorS (PROTHETS/European Union) project and EuroBonet consortium. Approval for data acquisition and analysis was obtained from the ethics committees of all institutions involved in the study.

The tumors reviewed had previously been diagnosed as undifferentiated Ewing-like sarcoma with at least focal round/oval cell morphologies that had challenged definitive classifications during the review processes of the previous study [30]. In addition, tumors that had been originally diagnosed as ES despite the absence of EWSR1 translocation were also reviewed.

The histopathology review comprised two phases. The first review was performed by two coauthors (IM and ALLB) and a presumptive diagnosis was rendered in Valencia, Spain. A second review with a discussion of the histopathology findings and clinical-pathologic correlation was performed by IM and AY in Japan.

Specific immunohistochemical studies and/or FISH analysis were performed at the National Cancer Center Hospital in Tokyo, Japan, based upon the clinical and morphological findings and tumor tissue availability. Further diagnostic interpretations were then made according to the results of IHC and molecular studies.

2.1. Immunohistochemistry

The primary antibodies, source, dilution and staining pattern criteria used are listed in Table 1. Sections (4 μm thick) from formalin-fixed paraffin-embedded tissue (whole tissue sections) were processed for IHC analysis as per conventional protocols. The analysis was performed on a single representative block for each primary tumor. The reactions were detected using the EnVision system (Dako, Glostrup, Denmark). Staining intensity was graded as negative, or weak, moderate or strong positive. The extent of positive IHC reaction was scored as focal (< 10%), patchy (10–50%) or diffuse (> 50%). NKX2.2 and BCOR positivity was defined as weak, moderate or strong nuclear immunoreactivity in at least 5% of tumor cells. ETV4 expression in at least 30% of nuclear tumor cells was defined as moderate or strong nuclear positivity [19]. Overall, NKX2.2 was performed in those tumors with specific morphology and clinical context very suggested of ES, ETV4 in tumors with histological appearance of CIC-rearranged sarcoma and BCOR for those cases resembling a sarcoma with a BCOR alteration. All sections were evaluated independently and read in a blind manner by three pathologists (IM, AY and ALLB). Discordant cases were evaluated at a multi-head microscope to achieve consensus. NKX2.2, ETV4, and BCOR staining reliability had previously been validated at the National Cancer Center Hospital, Tokyo, using a sufficient number of ES, CIC-rearranged sarcomas, and BCOR-associated sarcomas. Additional and pertinent immunostainings were made according to the histopathology review findings. Standard positive and negative controls were used throughout. The scores by all observers were recorded, and in cases of disagreement, the score was determined by consensus.

2.2. Fluorescence in situ hybridization

FISH analysis to detect gene rearrangements was performed on formalin-fixed, paraffin-embedded, 4-mm-thick tumor sections. Break-apart probes were used for the *EWSR1* (Vysis *EWSR1* Break Apart FISH Probe Kit; Abbott Molecular, Abbott Park, IL), *FUS* (Vysis FUS Break apart FISH Probe Kit; Abbott Molecular), *SS18* (Vysis SS18 Break Apart FISH Probe Kit; Abbott Molecular), *CIC* (custom-made probe; Chromosome Science Lab), and *BCOR* (custom-made probe; Chromosome Science Lab) genes. The *CIC* probe design has been documented previously [17]. The *BCOR* break-apart probe hybridizes with the neighboring telomeric (RP11-91I16 and RP11-1082P20, labeled with SpectrumGreen) and centromeric (RP11-77G22 and RP11-665O2, labeled with SpectrumOrange) sequences of the *BCOR* gene. The FISH images were captured using the Metafer Slide Scanning