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Atypical Ewing sarcoma breakpoint region 1 fluorescence *in-situ* hybridization signal patterns in bone and soft tissue tumours: diagnostic experience with 135 cases

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Aims: Recurrent Ewing sarcoma breakpoint region 1 (*EWSR1*) gene rearrangements characterize a select group of bone and soft tissue tumours. In our routine diagnostic practice with fluorescence *in-situ* hybridization (FISH), we have occasionally observed *EWSR1* gene rearrangements in tumours not associated classically with *EWSR1* translocations. This study aimed to review our institutional experience of this phenomenon and also to highlight the occurrence of unusual *EWSR1* FISH signals (i.e. 5′ centromeric region or 3′ telomeric region signals) that do not fulfil the published diagnostic criteria for rearrangements.

Methods and results: Using an *EWSR1* break-apart probe, we performed FISH assays on formalin-fixed paraffin-embedded tissue sections from 135 bone and soft tissue specimens as part of their routine diagnostic work-up. *EWSR1* gene rearrangements were identified in 51% of cases, 56% of which also showed an abnormal FISH signal pattern (in addition to classically rearranged signals). However, atypical FISH signals were present in 45% of the non-rearranged cases. In addition, we observed tumours unrelated to those described classically as *EWSR1*-associated that were technically *EWSR1*-rearranged in 6% of cases. Borderline levels of rearrangement (affecting 10–30% of lesional cells) were present in an additional 17% of these cases.

Conclusions: While our study confirmed that FISH is a sensitive and specific tool in the diagnosis of *EWSR1*-associated tumours, atypical FISH signals and classical rearrangement in entities other than EWSR1-associated tumours can occur. Therefore, it is

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essential that the FISH result not be used as an isolated test, but must be evaluated in the context of clinical features, imaging, pathological and immunohistochemical findings.

Keywords: *EWSR1*-associated tumours, fluorescence *in-situ* hybridization, rearrangement

Introduction

The Ewing sarcoma breakpoint region 1 (*EWSR1*) gene, mapping to chromosome 22q12, is one of the most commonly translocated genes identified by routine diagnostic fluorescence *in-situ* hybridization (FISH) in a wide range of entities.[1,2] In the diagnostic setting, *EWSR1* FISH is used mainly in the differential diagnosis of Ewing sarcoma (ES), clear cell sarcoma (CCS), extraskeletal myxoid chondrosarcoma (ESMCS), desmoplastic small round cell tumour (DSRCT), angiomatoid fibrous histiocytoma (AFH), low-grade fibromyxoid sarcoma (LGFMS) and sclerosing epithelioid fibrosarcoma (SEF).[1,2,4,8,13] Less commonly encountered entities associated with *EWSR1* gene fusions include pulmonary myxoid sarcoma,[6] clear cell sarcoma-like tumour of the gastrointestinal tract,[7] myoepithelial tumours of the soft tissue,[8–10] a proportion of salivary gland and odontogenic carcinomas[9–13] and even skin adnexal tumours.[14]

Unusual signal patterns detected by FISH for the *EWSR1* break-apart probe are described poorly in the bone and soft tissue tumour (BST) literature to date, and the contribution of true translocations to such abnormal FISH signals (i.e. gains or losses of signals) have only been alluded to in brief descriptions[3,5,15] but have not, to the best of our knowledge, been evaluated systematically in the literature to date. In addition, performance characteristics for normal reference ranges for the interpretation of *EWSR1* FISH signals are not well established and different cut-offs for specific probes might result in false positive or negative results when interpreted in isolation of the clinical and pathological findings.

Royal Prince Alfred Hospital (RPAH) is a tertiary referral centre and one of the largest BST treatment centres in the southern hemisphere. Since commencing paraffin-based FISH for the diagnosis of BST tumours in 2010, *EWSR1* has been performed in 135 cases, with classical rearrangements identified in 51% of cases ($n = 69$). During routine clinical practice, we have noted that this assay can occasionally pose challenges in diagnostic interpretation and subsequently also for patient management. Only very limited data exist on the frequency, appearance and significance of 'atypical' FISH patterns in BST tumours. Although the significance of these unusual FISH patterns cannot be elucidated, based on the findings of this study, we present our results to emphasize the importance of interpreting FISH in the appropriate clinical, imaging, histopathological and immunhistochemical (IHC) context.

Methods

All FISH tests performed in the Department of Tissue Pathology and Diagnostic Oncology are stored in a database. We have analysed all *EWSR1* FISH tests performed on BST specimens at the Royal Prince Alfred Hospital, Sydney, Australia since the use of FISH was implemented (in January 2010). This study was approved by the Human Research and Ethics Committee (HREC) at Royal Prince Alfred Hospital (Sydney Local Health District), approval numbers X15-0103 and LNR/15/RPAH/143. The diagnosis of BST tumours in our unit is performed exclusively by experienced bone and soft tissue pathologists (S.W.M., A.M., R.Z.K., R.A.S, S.F.B. and F.M.). All cases are discussed in a weekly multidisciplinary, multi-institutional team meeting (MDTM), where a final consensus diagnosis is reached by pathologists, radiologists and orthopaedic surgeons with specialized expertise in the diagnosis and management of BSTs. The FISH results are integrated to reach a final diagnosis, which was modified if appropriate, taking the consensus clinicopathological diagnosis of the MDTM as the gold standard.

FISH studies were performed on interphase nuclei on 3 μm formalin-fixed paraffin-embedded (FFPE) tissue sections using the Vysis *EWSR1* break-apart FISH probe kit (Abbott Molecular, Abbott Park, IL, USA). The FISH protocol was performed following the manufacturers' instructions, except that Invitrogen pretreatment solution (Life Technologies, Carlsbad, CA, USA) was used at 98–102°C for 20 min. FISH interphase signals were counted in at least 50 nuclei by two independent observers [a senior FISH scientist (C.S.) and a molecular pathologist (S.A.O.T., W.A.C. or R.G.)]. *EWSR1* gene rearrangement was considered positive if the nuclei analysed showed a split 5′ centromeric and 3′ telomeric signals of at least one signal distance apart in at least 15% of the cells analysed, as reported previously,[17] and following in-house

validation of this break-apart probe. Validation involved analysis of the *EWSR1* rearrangement present in 15 normal tissue samples. The percentage of cells with the *EWSR1* rearrangement was used to calculate the normal reference range using the binomial expansion formula (Beta Inverse calculation[46]) and confidence intervals. Using this method, we calculated a threshold of positivity of 15%. The mean percentage of balanced split signals observed in non-tumour cases was 2%, with a range of 0–12% observed. In addition, our cut-off has been validated independently and externally on a different cohort of positive and negative cases at external clinical reference laboratories (SydPath, St Vincent's Hospital, Sydney and Peter MaCallum Cancer Centre, Melbourne) with expertise in FISH testing. FISH signal patterns were classified as atypical (non-classical) when gain or loss of the red, 5′ centromeric or green, 3′ telomeric was identified or an increased copy number of fused signals was observed. Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) for the *EWSR1* probe were calculated using the MedCalc statistical software tool.

A literature review of the cut-offs and methodology for *EWSR1* FISH interpretation was performed in PubMed using the following terms: '*EWSR1*', 'FISH', 'Fluorescent *in situ* hybridization' and 'Soft tissue'.

Results

EWSR1 FISH was performed in 135 cases with rearrangement identified in 69 (51% of cases, with a mean of classically split signals within those cases of 61% of cells; range of rearranged cells: 16–99%). In 93% of cases ($n = 64$), the FISH result supported the MDTM diagnosis of a tumour from the classically described the *EWSR1*-rearranged group of tumours, which included ES (40 of 48 rearranged cases), CCS (seven of nine rearranged cases), ESMCS (seven of eight rearranged cases) and other entities associated with *EWSR1* rearrangement (Figure 1). The remaining five *EWSR1*-rearranged assays (7%) were identified in entities which do not characteristically harbour an *EWSR1* translocation, and therefore the FISH results did not change the original MDTM diagnosis. For instance, a *CIC-DUX4* translocated sarcoma diagnosed in a 30-year-old male with a cervical mass showed an unexpected but consistent *EWSR1* rearrangement pattern (30% split signals) in two separate FISH tests (Figure 2A) at our institution and confirmed independently at the Memorial Sloan Kettering Cancer Center, New York (where the diagnosis of a *CIC-DUX4* translocated sarcoma was also made on review of the pathology including detection of *CIC-DUX4* translocation). The other unexpectedly *EWSR1* 'rearranged' cases were a synovial sarcoma (SS; with proven *SS18* gene fusion and with clinical, imaging, histopathological and immunohistochemical results supporting the diagnosis), an ossifying fibromyxoid tumour, a high-grade neuroendocrine carcinoma and a gastrointestinal stromal tumour (GIST). Interestingly, the *EWSR1* 'rearranged' SS showed loss of one copy of the red (5′ centromeric) signal in 95% of the cells (Figure 2B,C).

For the non-rearranged *EWSR1* cases (49% of diagnostic cases; $n = 66$ with a mean of 5% of cells with classically split signals, range: 0–15%), the majority (47 of 66, 71%) were instances where the diagnosis of an *EWSR1*-rearranged tumour was not favoured based upon review of all clinical, radiological, pathological and immunohistochemical findings (i.e. poorly differentiated carcinomas, metastatic melanomas and undifferentiated tumours) but difficult to rule out entirely on the basis of the clinicopathological features (e.g. for a tumour favoured to represent a poorly differentiated carcinoma in a young patient it is difficult to entirely rule out a myoepithelial carcinoma). In 23% (15 of 66) of the non-rearranged tumours, a member of the *EWSR1*-associated tumour group was the preferred clinicopathological diagnosis (eight ES, two CCS, two myoepithelial carcinomas, one ESMCS and one DSRCT), but FISH did not confirm an *EWSR1* gene translocation (an additional case of LGFMS with absence of *FUS* rearrangement was also included in this group). In these cases, however, the final diagnosis remained unchanged despite the absence of a positive FISH result because other typical clinical, imaging, histological and immunohistochemical features provided sufficient evidence to support the diagnosis in each case, and also recognizing that the *EWSR1* gene rearrangement is reported in fewer than 100% of cases of these entities.[2] Finally, in 6% of the cases ($n = 4$), the diagnosis was modified from ES to that of undifferentiated small round cell sarcoma as a result of the negative FISH analysis. Overall, FISH for *EWSR1* in our department showed a sensitivity and specificity of 81% and 91%, respectively (PPV: 93%; NPV: 77%).

In addition to the fused and split paired signals, we noted atypical signal patterns characterized by the presence of unusual red (5′ centromeric region) or green (3′ telomeric region) signals in a significant proportion of both rearranged (56%, $n = 39$) and non-rearranged (45%, $n = 30$) cases. For instance, numerous 5′ centromeric signals suggestive of

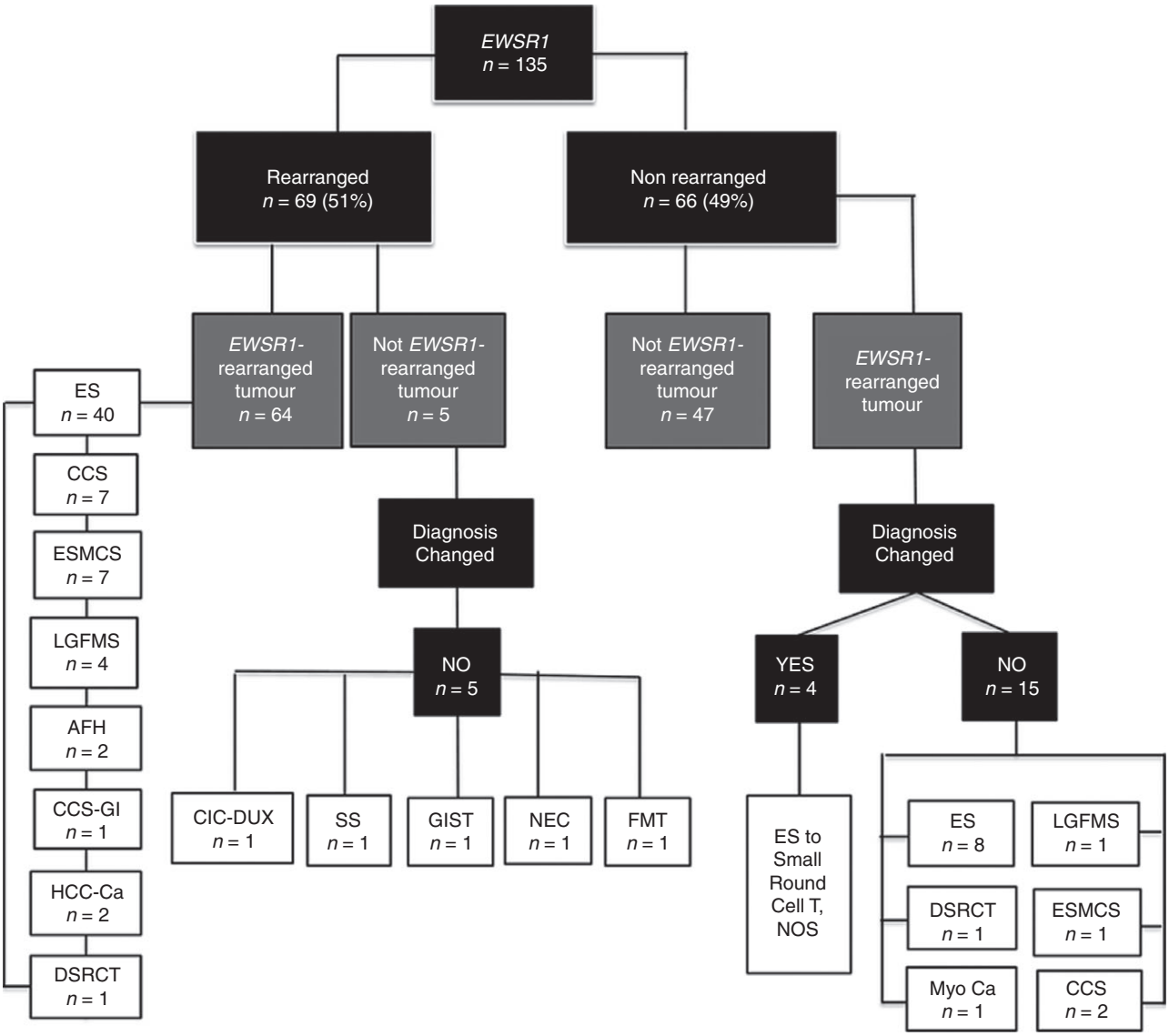

Figure 1. Diagnostic flow diagram of cases tested for Ewing sarcoma breakpoint region 1 (*EWSR1*) fluorescence *in-situ* hybridization (FISH). The final clinicopathological diagnosis after integration of the FISH results is shown. ES, Ewing sarcoma; CCS, clear cell sarcoma; ESMCS, extraskeletal myxoid chondrosarcoma; LGFMS, low-grade fibromyxoid sarcoma/sclerosing epithelioid fibrosarcoma; AFH, angiomatoid fibrous histiocytoma; CCS-GI, clear cell sarcoma-like of the gastrointestinal tract; HCC-CA, hyalinizing clear cell carcinoma of the salivary gland; DSRCT, desmoplastic small round cell tumour; CIC-DUX, *CIC-DUX*-associated sarcoma; SS, synovial sarcoma; GIST, gastrointestinal stromal tumour; NEC, neuroendocrine carcinoma; OFT, ossifying fibromyxoid tumour of the soft tissue; Myo CA, myoepithelial carcinoma.

amplification of the *EWSR1* gene (sclerosing epithelioid fibrosarcoma, Figure 2D) increased (metastatic melanoma) or decreased (sarcomatoid mesothelioma) gene copy number and loss or gain of green/red isolated signals (Figure 2E,F) were identified. Atypical signals were observed in a mean of 16% (range: 2–76%) of tumour cells in non-rearranged cases and a mean of 24% (range: 4–95%) of tumour cells in rearranged cases. The interpretive challenge of atypical signals is illustrated by a case with the differential diagnosis between CCS and metastatic melanoma, unresolvable by morphology, IHC and molecular findings, which showed only 12% of classical split *EWSR1* signals (diagnostic cut-off 15%), with a further 16% of tumour cells with additional atypical signals. However, as atypical signals are of uncertain clinical significance, and are largely disregarded in almost all series published to date in the literature (Table 1), the case was interpreted as technically non-rearranged. A further issue that must be

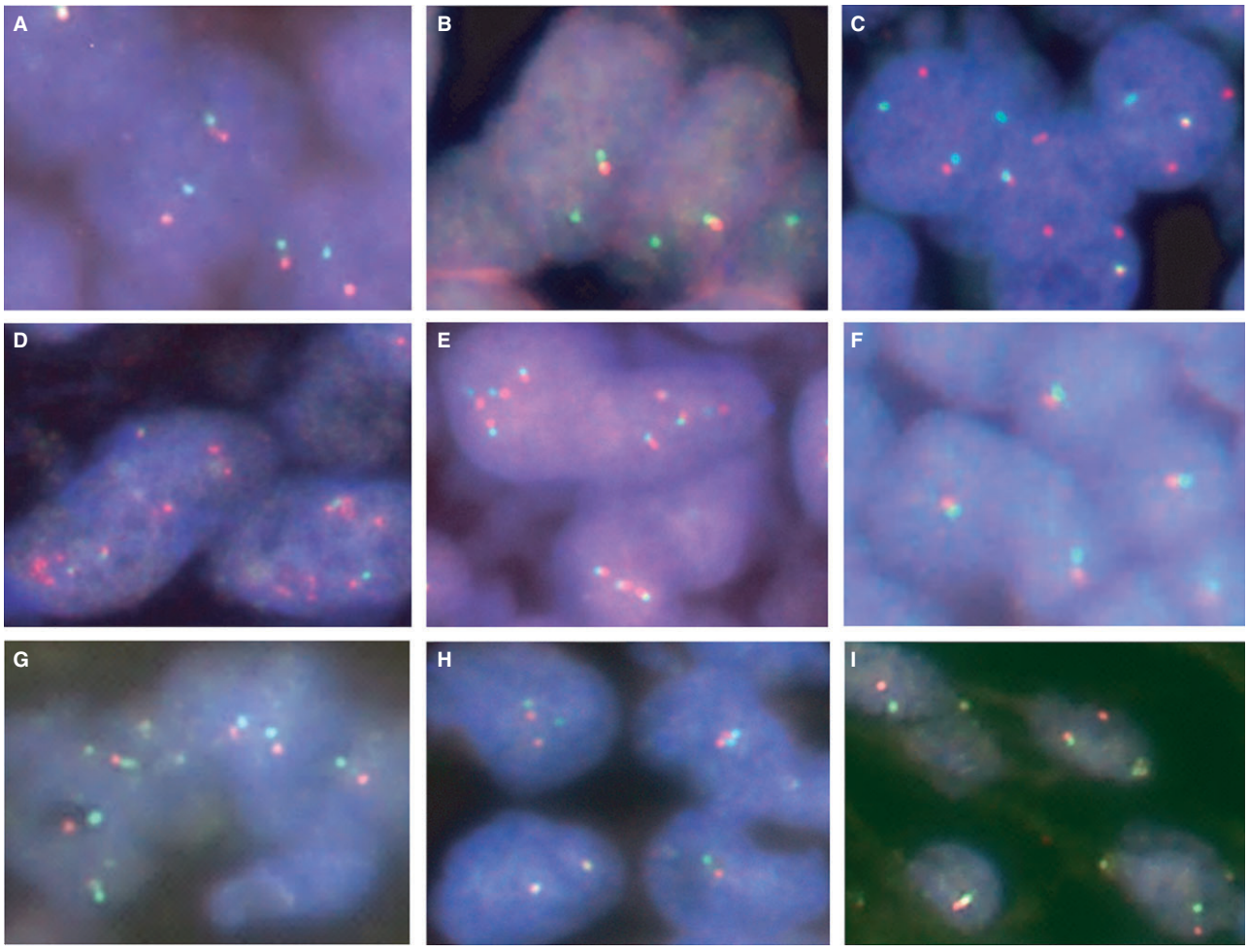

Figure 2. Cases showing atypical signal patterns by fluorescence *in-situ* hybridization (FISH). (**A**) *CIC-DUX4* rearranged sarcoma showing typical Ewing sarcoma breakpoint region 1 (*EWSR1*) rearrangement. (**B**) *EWSR1*-rearranged synovial sarcoma, which demonstrated loss of the red 5′ centromeric signals in the majority of the cells. (**C**) This case showed classical *SS18* rearrangement in 92% of the nuclei. (**D**) Sclerosing epithelioid fibrosarcoma showing an increase in the *EWSR1* isolated red (5′ centromeric) signals consistent with gene amplification. (**E**) Melanoma with increased *EWSR1* copy number (>4 fused signals per nucleus. (**F**) Sarcomatoid mesothelioma with monosomy for chromosome 22 showing loss of one pair of signals. (**G**) Poorly differentiated primary pancreatic adenocarcinoma showing classical rearrangement for *EWSR1*. (**H**) Case of epithelioid malignant peripheral nerve sheath tumour in a 30-year-old male showing classical plus atypical *EWSR1* FISH signals. (**I**) Mesenteric mass with the preferred histological diagnosis of myofibroblastic sarcoma showing classical split signals. Dual-colour break-apart probes.

considered in the interpretation of cases with borderline signal count and equivocal morphology is that by using a lower cut-off (10%), as in other series (Table 1), the case would have been interpreted as *EWSR1*-rearranged. This diagnostic dilemma has major clinical implications, because the treatment and prognosis is quite different for primary CCS and metastatic melanoma. Another case with a high proportion of atypical signals (76% of cells with loss of the green, 3′ telomeric signals and a further 45% of cells with up to three red signals) was a sclerosing epithelioid fibrosarcoma (SEF) primary from the bone with associated positive mucin 4 (MUC4) immunostaining.

We were prompted by these experiences to investigate the presence of atypical signals in high-grade malignant neoplasms completely unrelated to the *EWSR1*-associated tumours (mainly undifferentiated carcinomas) in a separate cohort included in a tissue microarray (TMA; $n = 24$, Supporting information, Table 1). In this set enriched for high-grade tumours, atypical signals were observed in up to 26% of the tumour cells in 12 of 24 cases (where atypical signals were seen in >10% of lesional cells). In addition, even

Table 1. Literature review (PUBMED) search for series of cases for Ewing sarcoma breakpoint region 1 (*EWSR1*) fluorescence *in-situ* hybridization (FISH)

Reference	No. of cases (*n*)	No. of cells counted	Cut-off	Entities included	Criteria to establish *EWSR1* break-apart probe cut-off	Additional scoring criterion
Tanas *et al.*[15]	230	–	10%	Round cell sarcomas	Not discussed	Atypical signals described
Arbajian *et al.*[3]	15	–	30%	LGFMS/SEF	Not discussed, confirmation with RT–PCR, mRNA and sequencing	Atypical signals described
Antonescu *et al.*[9]	23	200	20%	Hyalinizing CCC	Not discussed, RT–PCR confirmation	
Flucke *et al.*[10]	18	50	20%	Cutaneous myoepithelial tumours	Not discussed	
Skalova *et al.*[11]	94	100	10%	Salivary gland tumours	Reference to Ventura *et al.*[42]	
Bilodeau *et al.*[12]	22	–	20%	Clear cell odontogenic Carcinoma	Not discussed	
Shah *et al.*[13]	15	100	10%	Hyalinizing CCC	Not discussed	Only tumour nuclei with all 4 signals present were evaluated
Antonescu *et al.*[14]	66	200	20%	Soft tissue myoepithelial tumours	Not discussed: RT–PCR confirmation	Only tumour nuclei with all 4 signals present were evaluated
Bridge *et al.*[17]	67	100	15%	Round cell tumours	Calculated using a probe–specific normal range: 3 SD from the mean + PCR correlation	
Warren *et al.*[18]	32	–	–	ES	Not discussed; confirmation with RT–PCR, sequencing and conventional cytogenetics	
Miura *et al.*[19]	280	50	10%	Range of sarcomas	Reference to Yamaguchi *et al.*[23]	Distance between the green and the red signals: 2–3 signal diameter
Horn *et al.*[20]	64	100	10%	ES/CCS	Calculated using a probe–specific normal range: 3 SD from the mean + PCR correlation	
Neuville *et al.*[21]	286	–	–	PNET/DSRCT,ML	Not discussed: RT–PCR confirmation	
Italiano *et al.*[22]	22	200	20%	Small round cell tumours	Not discussed: RT–PCR confirmation	
Downs-Kelly *et al.*[16]	61	100	10%	ESMCS,ML,LGFMS	Calculated using in–house validation and PCR–based confirmation	Only tumour nuclei with all 4 signals present were evaluated
Yamaguchi *et al.*[23]	28	100	–	ES/DSRCT/CCS	Not discussed: RT–PCR confirmation	
Wang *et al.*[24]	20	200	–	CCS	Not discussed; confirmation with RT–PCR, sanger sequencing and cytogenetics	Distance between the green and the red signals > than 1 signal diameter

Table 1. *(Continued)*

Reference	No. of cases (*n*)	No. of cells counted	Cut-off	Entities included	Criteria to establish *EWSR1* break-apart probe cut-off	Additional scoring criterion
Wang *et al.*[25]	16	–	20%	ESMCS	Calculated using a cut–off of 5% and negative controls from bone marrow aspirates	Only tumour nuclei with all 4 signals present were evaluated
Song *et al.*[26]	18	100	10%	CCS and melanoma	Calculated using in–house validation and PCR–based confirmation	Distance between the green and the red signals > than 1 signal diameter
Patel *et al.*[27]	42	100	10%	CCS and melanoma	Calculated using probe–specific normal range ≤4%; 2 SD from the mean	Only tumour nuclei with all 4 signals present were evaluated
Hantschke *et al.*[28]	12	50	–	Cutaneous CCS	Calculated using a probe–specific range: 2 SD from the mean + PCR correlation	Only tumour nuclei with all 4 signals present were evaluated
Machado *et al.*[29]	9	200	15%	Atypical ES	Not discussed: RT–PCR confirmation	
Shi *et al.*[30]	21	–	15%	AFH	Not discussed	
Thway *et al.*[31]	17	–	–	AFH	Not discussed: RT–PCR confirmation	
Milione *et al.*[32]	7	100	–	ES from small bowel	Not discussed: RT–PCR confirmation	Only tumour nuclei with all 4 signals present were evaluated
Kao *et al.*[33]	11	100	20%	AFH	Not discussed: RT–PCR confirmation	Only tumour nuclei with all 4 signals present were evaluated (>2 signal diameter)
Tanas *et al.*[34]	18	–	–	AFH	Confirmation with RT–PCR cytogenetics; reference to Downs–Kelly *et al.*[16]	
Shingde *et al.*[35]	7	100	10%	Cutaneous ES	Not discussed	Distance between the green and the red signals > than 1 signal diameter
Noguchi *et al.*[36]	18	100–120	–	ESMCS	Not discussed: RT–PCR confirmation	Distance between the green and the red signals: 2–3 signal diameter

LGFMS/SEF, low-grade fibromyxoid sarcoma/sclerosing epithelioid fibrosarcoma; CCC, clear cell carcinoma; ES, Ewing sarcoma; CCS, clear cell sarcoma; PNET, primitive neuroectodermal tumour; DSRCT, desmoplastic small round cell tumour; ML, myxoid liposarcoma; ESMCS, extraskeletal myxoid chondrosarcoma; AFH, angiomatoid fibrous histiocytoma; RT–PCR, reverse transcription–polymerase chain reaction; SD, standard deviation.

by using a higher diagnostic cut-off of 15% (adopted in some institutions including our own), the *EWSR1* FISH assay again proved problematic, as a case of a poorly differentiated pancreatic adenocarcinoma would have been classified technically as 'positive' (16% split signals) based exclusively on the FISH result (Figure 2G). Moreover, five further cases [a myofibroblastic sarcoma (Figure 2I), an undifferentiated endometrial carcinoma, an epithelioid malignant peripheral nerve sheath tumour (MPNST, Figure 2H), a neuroendocrine carcinoma and a radiotherapy-associated sarcoma] would have been interpreted as *EWSR1*-rearranged if a 10% cut-off had been used (Supporting information, Table S1).

Our literature search (Table 1) revealed 30 papers (series with at least six cases) where *EWSR1* break-apart probes have been used in the classification and differential diagnosis of *EWSR1* translocated tumours. In fact, we identified that a third of these published series do not describe the cut-off selected to establish gene rearrangement ($n = 10$, 33% of the papers). In the remainder of the studies, 10% is the most common cut-off used ($n = 9$, 30% of the studies) followed by 20% ($n = 7$, 23% of the studies), 15% ($n = 3$; 10%) and 30% (3%; $n = 1$). In our institution, we have selected a 15% cut-off following the study by Bridge *et al.*,[17] who published the first large series demonstrating the utility of *EWSR1* FISH in the differential diagnosis of round cell tumours (and still today the most frequently cited paper in the differential diagnosis of round cell tumours), and which is in agreement with our internal in-house validation.

Discussion

The use of *EWSR1* FISH to detect characteristic gene rearrangement has been an invaluable tool for the more accurate diagnosis and treatment of patients with BSTs at our institution. In our cohort, FISH was supportive of the preferred clinicopathological diagnosis of an *EWSR1*-rearranged tumour group member in 93% of the rearranged cases but did not support the preferred diagnosis in 23% of the non-rearranged cases, producing an overall sensitivity and specificity of 81% and 91%, respectively (PPV: 93%; NPV: 77%). Although our sensitivity and specificity are similar to that described in prior reports in the literature,[17–20] it should be noted that accurate calculation of these parameters for a specific FISH probe is problematic. For instance, it has been shown that in cases with classical histopathological features, FISH demonstrates the specific rearrangement in more than 95% of the cases, but the level of specificity and sensitivity decreases for cases with equivocal histopathological features in which greater discrepancy between histopathology and FISH tends to be observed.[15,21] It must also be emphasized that other tumours, apart from *EWSR1*-associated tumours (pleomorphic sarcomas, carcinomas, lymphomas, melanomas, rhabdomyosarcomas mesotheliomas and small cell osteosarcoma), can show *EWSR1* rearrangement.[19–21,37–39] Our assessment of *EWSR1* FISH on a TMA containing mainly high-grade tumours (none of which are recognized as characteristically containing an *EWSR1* translocation) confirms these findings (Supporting information, Table 1). In addition, secondary complex rearrangements involving the *EWSR1* gene may occur. For instance, it can be hypothesized that the *CIC-DUX4*-associated round cell tumour and SS identified in our series (Figure 2A–C) harbour a secondary *EWSR1* rearrangement. This has been described for SS[40] and was supported by the conventional cytogenetics result in our case, but has not yet been documented in the recently described entity *CIC-DUX4*-associated round cell tumour.[22,41]

In our routine diagnostic practice using the *EWSR1* FISH assay, atypical signals (gain or loss of the red, 5′ centromeric or green, 3′ telomeric) and borderline levels of rearrangement around the cut-off posed significant interpretative problems. We noted atypical signal patterns in 56% and 45% of rearranged and non-rearranged cases, respectively. Such abnormal signal patterns are reported very infrequently in the literature; the majority of the BST series reported to date only counted cells containing four intact signals, assuming that any other signal pattern was indicative of a sectioning-related truncation artefact (Table 1). However, the limited reports of this phenomenon in the literature suggest that atypical signal patterns identified by FISH might instead be associated with true translocations and/or other gene abnormalities. Ventura *et al.* and Wolff *et al.*, in the context of haematological malignancies, have described that 'unusual' patterns (i.e. extra fusion and/or gain or loss of loci signals) should not be ignored when they are present in the vast majority of the cells, as they might be predictive of a gene rearrangement or other gene alterations (e.g. a concurrent deletion).[42,47] However, other studies have shown that, although these patterns can account for a significant proportion of the cases, a subset is unrelated to a concurrent translocation, requiring thorough investigation with alternative methods.[48] Arbajian *et al.*, using break-apart FISH for *EWSR1*, *FUS* and *CREB3L1-2* in a series of SEFs, interpreted

that loss of the *EWSR1* 5′ part in combination with loss of the 3′ part of *CREB3L1* or *CREB3L2* was equivalent to a split signal, and therefore indicative of a gene fusion [confirmed by reverse transcription–polymerase chain reaction (RT–PCR).[3] Similarly, Tanas *et al.* and Antonescu *et al.* have indicated that loss of the telomeric (3′ part) of the *EWSR1* probe is suggestive of unbalanced translocations.[9,15] In addition, *EWSR1* gene amplification can produce an atypical signal pattern characterized by increased 5′ centromeric signals,[5] which was identified in a case of SEF in our series (Figure 2D). Moreover, in a single case report it was suggested that *EWSR1* cryptic translocations can potentially give rise to additional signals by FISH.[43] Adding to the complexity, pseudogenes have been shown to contribute to gene amplification detected by FISH and PCR-based techniques,[1,37] but this requires further study for confirmation. Finally, poor hybridization due to poor fixation or suboptimal performance of the assay might also be a contributing factor in such cases. From the findings of our study, the significance of these atypical signals is unclear. The fact that such atypical signals were identified more commonly in tumour cells from rearranged cases than those in non-rearranged (25% versus 16%) cases supports the possibility that these atypical signal patterns represent gene rearrangements. It would appear appropriate, in selected cases, to consider adding the atypical signal count to the final split signal estimation if this is a repetitive pattern in most of the nuclei analysed and in the context of appropriate clinicopathological correlation, as discussed by Ventura *et al.*[42] Some of the cases identified in our study with a high percentage of atypical signals were consistent with an *EWSR1*-associated tumour based on clinicopathological features, but showed no definite gene rearrangement when the established cut-off (>15%) was utilized. Examples of this include two myoepithelial soft tissue tumours (26% and 68% of atypical signals, respectively), a CCS and an SEF (16% and 18%, respectively).

We also encountered problems assessing cases with levels or rearrangement near the cut-off. In our search of the literature for FISH interpretation of these BST assays, we identified significant inconsistency in the cut-offs reported by authors from different institutions for *EWSR1* (e.g. 10%, 15%, 20% versus 30%, Table 1). Although this would not be a problem for the majority of the cases in which a clear rearrangement is present or absent, this would not be the case for samples with borderline levels of rearrangement. Therefore, cases with a borderline level or rearrangement (e.g. between 10% and 30% of rearranged cells) testing at different institutions may result in either positive or negative FISH results, probably with very different prognosis and therapeutic approaches, depending on the cut-off utilized. When assessing such cases, technical aspects of the FISH performance, such as the degree of separation of the break-apart signals expected by an interchromosomal rearrangement, the hybridization efficiency of the test and the degree of signal visualization should be assessed carefully to ensure the reliability of the result. Although stringent performance characteristics might be followed by different laboratories, as can be observed in Table 1, unified standardization of in-house validation for FISH probes has yet to be published. Conversely, selection of scoring criteria based on specific publications instead of establishing internal properly validated cut-offs can also lead potentially to incorrect results.

In our opinion, it seems sensible to adopt the approach recommended by Ventura *et al.* and others[42,47] that atypical cell patterns in a high proportion of tumour nuclei probably represent a rearrangement event, although it is essential to always interpret the FISH result in the appropriate clinicopathological context. Ideally, such cases with equivocal or borderline FISH results should be tested with another molecular assay, where available, to avoid potential misclassification and instigation of inappropriate management.[44,45] Unfortunately, this may be extremely challenging if there is only FFPE material available. Many tissue pathology departments will not have access to a second molecular assay, where FISH may be the only modality available to identify a translocation on FFPE.

Our study has a number of limitations. By using break-apart FISH as the single modality to detect *EWSR1* gene rearrangement, it is not possible to elucidate the true nature of the atypical signal patterns identified in our cases and a definite rearrangement or lack thereof cannot be confirmed. Similarly, we cannot distinguish false negative results, which can occur as a result of rearrangements below the sensitivity of the test such as cryptic translocations or even true negative results associated with alternative genetic mechanisms (e.g. non-ETS genes). Further molecular techniques, such as RT–PCR, conventional cytogenetics and alternative FISH strategies (i.e. dual fusion probes), would provide insight into these challenging cases, increasing our understanding of FISH interpretation and tumour biology. However, we believe that there is value in presenting our findings, as this phenomenon is essentially unrecognized outside the research setting and can lead potentially to misdiagnosis in centres with no routine access to

other molecular diagnostic tools such as ours. Another significant limitation of our study, which is addressed only briefly in the current World Health Organization (WHO) classification,[2] is regarding the classification of ES in the absence of the expected genetic abnormality. According to the WHO classification,[2] the absence of a molecular confirmation does not rule out the diagnosis of ES, but it should prompt a clinicopathological review.[2] Nonetheless, there are no universal guidelines with regards to the minimum clinicopathological diagnostic criteria for a small round cell tumour to be regarded as ES or, alternatively, when the diagnosis should be modified strictly to that of small round cell tumour. With the current molecular characterization and biological diversification of small round cell primitive sarcomas, soft tissue pathologists are relying more upon specific genetic abnormalities, which are becoming the defining features of emerging specific subgroups. However, this field is evolving rapidly, and until robust data are available specific classification of these tumours is likely to be subjected to variability in the application of clinicopathological criteria based on individual cases. This might account for a change in the diagnosis in a subset of our cases, but not for all. Moreover, we cannot entirely exclude that the discussion of the FISH findings at MDTM can potentially introduce bias into the final tumour classification, but this is also likely to occur in any other tertiary centres where discussion of the pathology and the molecular findings is an essential aspect of patient care and clinical management. Importantly, ES and 'Ewing-like' small round cell tumours are currently subjected to the same therapeutic strategies regardless of the underlying genetic abnormality. It is possible that this will change in the near future with the advent of personalized targeted therapy and, hence, the need to recognize the molecular driving event.

In conclusion, there is poor formal recognition of atypical FISH patterns in the published literature to date, and further investigation of their significance will be important for better classification and understanding of *EWSR1*-associated tumours. The increasing availability of high-throughput technologies may assist in characterizing the underlying changes associated with such atypical signals, although their use in FFPE material remains challenging.

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Conflicts of interest

There are no conflicts of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Results of *EWSR1* FISH performed in a tissue microarray containing a range of entities non related to *EWSR1* associated tumours.