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SHORT REPORT

The utility of *ETV1*, *ETV4* and *ETV5* RNA *in-situ* hybridization in the diagnosis of *CIC–DUX* sarcomas

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The utility of *ETV1*, *ETV4* and *ETV5* RNA *in-situ* hybridization in the diagnosis of *CIC–DUX* sarcomas

Aims: A recently characterized group of undifferentiated small round cell sarcomas harbours fusions of the genes *CIC* and *DUX4*. Studies report a distinctive gene expression profile for these sarcomas, including expression of E26 transformation-specific (ETS) family proto-oncogenic transcription factors *ETV1*, *ETV4* and *ETV5*. To test the utility of an ancillary diagnostic technique for these tumours, we evaluated chromogenic RNA *in-situ* hybridization assays for *ETV1*, *ETV4* and *ETV5* as diagnostic adjuncts for this emerging group of highly malignant sarcomas.

Methods and results: We tested six confirmed CIC–DUX4 sarcomas and 105 lesions in the differential, including 48 Ewing sarcomas for expression of *ETV1*, *ETV4* and *ETV5*, scoring expression utilizing a previously validated scale. *ETV1* and *ETV4* were positive in five of six cases, while *ETV5* was positive in six of six. No Ewing sarcoma or other sarcoma tested showed coexpression of these transcripts, while one ETV1/ETV4/ETV5 triple positive previously unclassified round cell sarcoma was identified as harbouring a *CIC* rearrangement by break-apart fluorescence *in-situ* hybridization (FISH).

Conclusion: We identified overexpression of *ETV1*, *ETV4* and *ETV5* transcripts *in situ* in CIC–DUX4 sarcomas using a robust assay in routine archival sections. One previously unclassified round cell sarcoma showed *ETV1/4/5* positivity, and was proved to harbour a *CIC* rearrangement by break-apart FISH. The sensitivity and specificity observed with our *in-situ* hybridization assay implies potential utility as an ancillary diagnostic technique, particularly when faced with limited biopsy samples.

Keywords: biomarkers, CIC–DUX sarcomas, ETS-family transcription factors, RNA *in-situ* hybridization

Introduction

There has been an exponential increase in the identification of recurrent and/or pathognomonic gene fusions in a rapidly expanding group of mesenchymal

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lesions of varying biological potential.[1] This is particularly true with regard to small round cell sarcomas, where an increasing spectrum of *EWSR1* rearrangement-negative small round cell sarcomas have been recognized,[2] including an intriguing group of sarcomas harbouring fusions of *Capicua* transcriptional repressor (*CIC*) on chromosome 19 and either, most frequently, the gene *double homeobox 4* (*DUX4*) on chromosome 4 or, less frequently, the highly related gene *double homeobox 4-like* (*DUX4L*) on chromosome 10. Our experience[3,4] and that of others[5,6] with this group of sarcomas suggests that they are most commonly soft tissue-based tumours arising across a wide age range, but predominantly among young adults. They demonstrate a degree of atypia and pleomorphism that is beyond that of classic Ewing sarcoma, with higher-grade nuclear features, vesicular chromatin and more prominent nucleoli. These tumours also show patchy, clear cell foci and distinctive areas of myxoid change. Complicating their recognition, especially in limited core biopsies, these sarcomas show highly prevalent expression of the ETS-family proto-oncogenic transcription factors, FLI1 and ERG, similar to Ewing sarcoma, but with more variable and less distinctive membranous CD99 expression.[4,6] Underscoring the importance of their recognition, cumulative evidence suggests that these tumours are more aggressive than Ewing sarcoma, an assumption supported by a recent large Japanese-based cohort of 20 cases.[7]

It is the opinion of the authors that the shared expression of ETS-family proto-oncogenic transcription factors seen in Ewing sarcoma and CIC–DUX sarcomas represents not only a diagnostic challenge, but also a potential avenue for diagnosis. While, in Ewing sarcomas, expression of FLI1 and ERG is related to translocation and oncogenic fusion of *EWSR1* to *FLI1* or (less frequently) *ERG*, in experimental systems, expression of the CIC–DUX4 fusion oncoprotein appears sufficient to induce[8] expression of multiple other members of the PEA3 subfamily of the ETS-family of transcription factors. While we have seen coexpression of both FLI1 and ERG in these tumours by immunohistochemistry (IHC),[4] comprehensive transcriptional profiling by another group[6] has confirmed up-regulation of ETS-family transcription factors, including *ETV1*, *ETV4* and *ETV5*, as part of a core gene signature of CIC–DUX4 sarcomas that is distinctive from Ewing sarcomas. Based on experience assaying for ETS-family gene fusions[9] in archival sections of prostatic adenocarcinoma, where the high degree of homology between ETV1, ETV4 and ETV5 prevented our distinguishing expression of each of these three reliably by IHC, we have recently developed and validated a facile, exquisitely paralogue-specific chromogenic RNA *in-situ* hybridization strategy for their detection from standard archival sections.[10] This technique results in reliable, quantitative detection of the specific RNA species as a punctate cytoplasmic dot of red chromogen. Herein, we evaluated the potential utility of this technology for distinguishing CIC–DUX4 sarcomas from Ewing sarcoma and a subset of other round cell sarcomas in the differential.

Materials and methods

COHORTS TESTED

A retrospective cohort of archival tissues, consisting of whole sections and tissue microarrays, was assembled for testing. This included a tissue microarray (TMA) of Ewing sarcomas (n = 37) and CIC–DUX4 sarcomas (n = 6, cases 1–4 and 7–8, as reported previously[4]); a TMA of assorted small round cell sarcomas including additional Ewing sarcomas, n = 11; high-grade myxoid liposarcomas with round cell morphology (n = 8); rhabdomyosarcomas (n = 10, nine alveolar, one adult spindle cell); and a TMA of high-grade synovial sarcomas (n = 38). Additional whole sections of each of the six CIC–DUX4 sarcomas were also used for evaluation of the variability of staining across the sections.

CHROMOGENIC RNA *IN-SITU* HYBRIDIZATION (ISH)

Our chromogenic RNA *in-situ* hybridization protocol for detection of *ETV1*, *ETV4* and *ETV5* transcripts, including its validation with respect to exquisite specificity to *ETV1*, *ETV4* or *ETV5* expression (proved by comparison to prostate carcinomas proved to harbour *ETV1*, *ETV4* or *ETV5* rearrangements) has been detailed previously.[10] We used RNA scope probes, designed by Advanced Cell Diagnostics (Hayward, CA, USA) for *ETV1* (transcript region 998–2031, #311411), *ETV4* (transcript region 431–1891, #491521) and *ETV5* (region 2638–3839, #590371) and *POLR2A* (positive control). In brief, archival sections of TMAs and whole formalin-fixed paraffin-embedded (FFPE) tissue sections were baked at 60°C for 1 h, deparaffinized in xylene twice for 15 min, washed in 100% ethanol twice for 3 min and air-dried for 5 min. Slides were treated with pretreatment 1, 2 and 3 buffers as described previously,[10] rinsed in deionized water and then incubated with the ETV1, 4, 5 or control probes for 2 h at 40°C in a humidity chamber. Slides were then treated with Amp

solutions 1–6, before chromogen was developed by adding a solution of 1:60 fast red B:fast red A for 10 min, washes and counterstaining in 50% haematoxylin, 0.01% ammonium hydroxide wash, dehydration in ethanol and xylene and mounting in Cytoseal XYL (ThermoScientific, Waltham, MA, USA; #8312-4) for routine microscopy.

EVALUATION OF ISH STAINING

ETV1, *ETV4* and *ETV5* expression, present as distinct, punctate cytoplasmic dots, was evaluated using our scoring system for quantitative expression differences, which has been validated previously analytically[10] and clinically.[11] Sections were evaluated using a ×20 objective based on the number of punctate dots/cell. As reported previously, staining level was assessed as one of five levels, from 0 to 4. Level 0 was defined as no staining or fewer than 1 dot/cell; level 1 was defined as 1–3 dots/cell in >5% of the tumour; level 2 was defined as 4–10 dots/cell with minimal clustering apparent in >5% of the tumour; level 3 was defined as more than 10 dots/cell with dot clustering apparent in <10% of cells and level 4 was defined as more than 10 dots/cell with >10% of the cells with clusters of dots. As validated previously, scores of 0 or 1+ were considered negative, while scores of 2–4 were considered positive. Each sample was scored based on the highest intensity observed.

Results

Overall, CIC–DUX4 sarcomas demonstrated positive staining for *ETV1* in five of six cases, for *ETV4* in five of six cases and for *ETV5* in six of six cases (one case was only positive for ETV5). In many cases the staining was strong and evident at low power (Figure 1A–F). Overall, *ETV4* and *ETV5* expression scores tended to be greater than that of *ETV1* ($P = 0.0091$, Friedman's test). Of 48 well-characterized, classic and extraskeletal Ewing sarcomas, no case demonstrated coincident aberrant positive staining for *ETV1*, *ETV4* and *ETV5*; two Ewing cases showed positive staining for *ETV4* only (2+) or *ETV5* only (2+). No staining in the positive range was seen in 36 synovial sarcomas, eight myxoid liposarcomas or 11 rhabdomyosarcomas. One EWSR1 rearrangement-negative unclassified round cell sarcoma included in the TMA evaluable in the ISH slides demonstrated *ETV1*/*ETV4*/*ETV5* triple positivity; based on this suggestive finding, we performed *CIC* break-apart FISH,[3] confirming a rearrangement at the *CIC* locus (Figure 2A–F).

Discussion

The differential diagnosis of malignancies with small round cell morphology, especially small round cell sarcomas, is complicated by several factors in tandem. These include a rapid proliferation of newly appreciated, clinically relevant entities and variants[2] that must be recognized increasingly in smaller samples from minimally invasive diagnostic image-guided sampling,[12] performed to guide increasing use of neoadjuvant therapeutic regimens.[13] Such factors have driven increasing use of immunohistochemical biomarkers for sarcoma diagnosis[14] and triage for definitive molecular testing based on tiny tissue samples. In Ewing sarcoma, for instance, several markers with varying sensitivity and specificity have been proposed with varying success, including most recently NKX2-2,[15] as well as FLI1,[16] a marker also detected at higher concentrations by monoclonal antibodies to the highly homologous ETS-family member, ERG.[17] At lower concentrations, ERG immunostain may detect only the small subset of Ewing sarcomas harbouring *ERG* rearrangements,[18] emphasizing the challenges in this area. In any case, both markers seem positive in CIC–DUX4 sarcomas in this differential.[4,6] Indeed, the challenges of IHC validation of transcription factors in the closely related ETS family of transcription factors were the reason for our previous efforts to develop and validate this RNA *in-situ* hybridization platform for use in the detection of overexpression of *ETV1*, *ETV4* and *ETV5* driven by oncogenic gene fusions of these genes with androgen-responsive genes in subsets of prostatic adenocarcinomas.[10]

In this study we observed a sensitivity (83%, 83% and 100% for *ETV1*, *ETV4* and *ETV5*, respectively) and specificity (100%) for CIC–DUX4 sarcomas, which compares favourably with markers currently in use for this and other differential diagnoses in soft tissue pathology. One *EWSR1* rearrangement-negative undifferentiated soft tissue sarcoma in the TMA, arising in the groin of a 31-year-old patient of African descent who developed pulmonary and epidural metastases and died of disease, showed triple positivity (3+ each for *ETV1/4/5*). Rearrangement was detected at the *CIC* locus by break-apart FISH, supporting the potential utility for this assay to identify cases prospectively, including in tiny samples of the size of a TMA core.

Certainly, these findings suffer from limitations of sample size, retrospective design and limitation to the CIC–DUX4 sarcomas tested (we were unable to test any CIC-rearranged sarcomas with the *CIC–DUX4L* fusion[4,6] or recently reported *CIC–FOXO4*

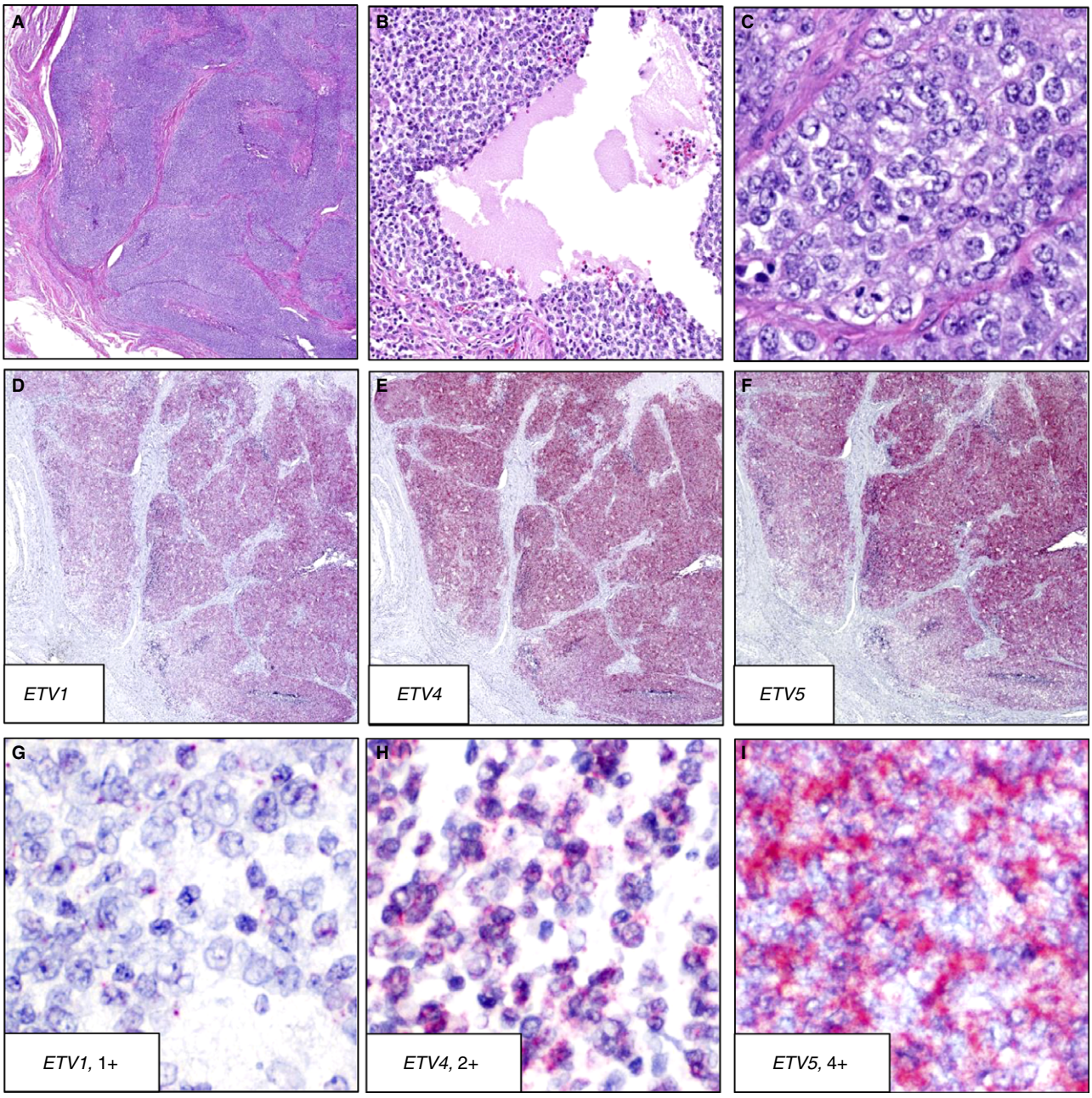

Figure 1. CIC–DUX4 sarcomas show prominent nodular growth and a primitive round cell morphology (**A**) with frequently observed myxoid change (**B**) and high-grade nuclear cytology (**C**). As seen in *in-situ* hybridization slides performed on consecutive sections to that of (**A**), even at low power inspection, positive staining for the indicated ETS-family member transcript was identified readily (**D–F**). A staining pattern ranging from only punctate dots (**G**; 1+, considered negative) to positive staining at 2+ (**H**) up to 4+ (**I**) was observed.

fusions,[19,20] while the presumptive *CIC* fusion partner in the positive case we discovered remains unknown, as do many cases tested by FISH alone[7]). However, we note that these remain rare tumours, with little in the way of diagnostic adjuncts to support their identification from the significant subset of round cell sarcomas that remain unclassified.

Previously, we have reported promising findings for use of a panel of IHC markers including ETV4,[21] and others have reported recently utility for ETV4 and WT1 as IHC markers for CIC-rearranged sarcomas.[22,23] Notably, we have not tested any BCOR–CCNB3 sarcomas,[24] although we note that ETV4 IHC has been negative in nine cases tested in two recent

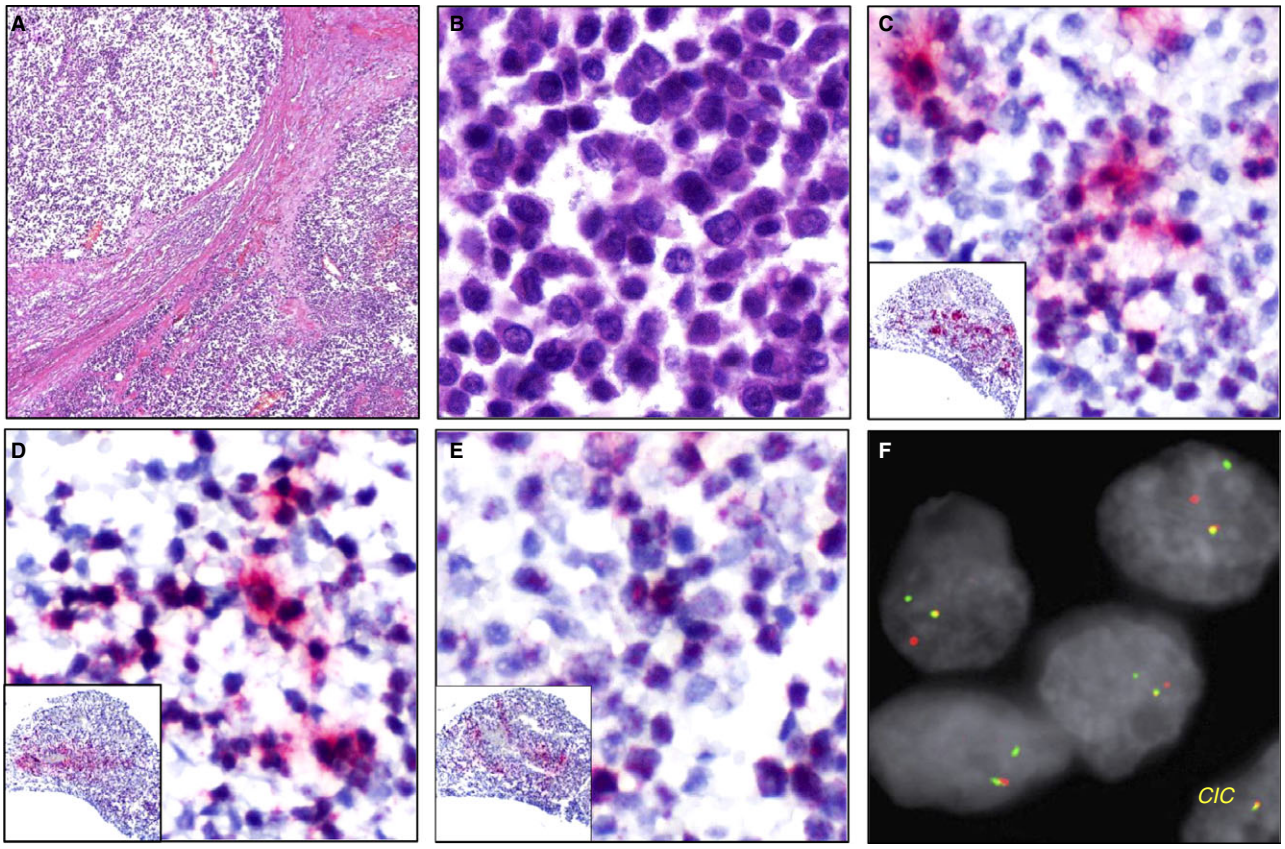

Figure 2. A previously unclassified (*EWSR1* rearrangement negative) round cell sarcoma arising in the soft tissues of the groin of a 31-year-old male patient was included in the tissue microarray (TMA) studied by the *in-situ* hybridization assays. Morphologically, it demonstrated septate, nodular growth of high-grade, undifferentiated primitive round cells with prominent necrosis and discohesion (**A**). At higher power, the nuclear pleomorphism and atypia were somewhat more than expected for a classic Ewing sarcoma (**B**). TMA cores demonstrated positive (3+) staining for *ETV1* (**B**), *ETV4* (**C**) and *ETV5* (**D**; each inset low power showing proportion positivity), a suspicious finding that we found to be associated with rearrangement at the *CIC* locus, proved by break-apart fluorescence *in-situ* hybridization (**F**).

reports.[22,23] Despite the potential utility, the ETV4 IHC findings remain of unproved technical specificity to ETV4, as opposed to the other highly homologous ETS-family members (FLI1, ERG, ETV1, ETV5, etc.) overexpressed in CIC–DUX sarcomas,[6] the initial concern of which led to our use of RNA-based specific detection. Overall, at present WT1 (N-terminal) remains the IHC marker that we have used most frequently as a marker for triage of *EWSR1* rearrangement-negative undifferentiated soft tissue sarcomas in the differential with CIC-rearranged sarcoma, which the published data suggest is quite sensitive but not necessarily specific.[6,22]

In fact, our experience with the technical advantages of this RNA ISH system suggest that this technology might have broad potential for assay development not only where *in-vitro* diagnostic-quality antibodies are unavailable, but where discrimination among highly homologous proteins based on specific RNA species is of utility. In this RNA ISH approach, specificity is conferred not only by the sequence specificity of probes hybridizing to the target RNA species, but by use of paired, adjacent 'ZZ' probes, hybridization of both of which is required for amplification and detection.[25] While requiring implementation of a new workflow in advance (compared to introducing a new IHC marker into existing processes), RNA ISH allows rapid, specific implementation of any transcript as a diagnostic marker, independent of availability of a robust, specific antibody for detection. This particular advantage was illustrated recently for detection of *FGF23* RNA species in phosphaturic mesenchymal tumours,[26] a similar scenario without available specific antibodies. Moreover, the platform is quite amenable for use in cases where IHC-based detection remains suboptimal, such as for detection of viral RNA species [e.g. human papilloma virus (HPV) in head and neck

cancer specimens[27]], such that addition and validation of markers such as these ETS family members might be even more facile.

In future, while molecular studies including break-apart FISH [or reverse transcription–polymerase chain reaction (RT–PCR)] are currently deemed the gold diagnostic standard for CIC-rearranged sarcomas, and which we continue to use for confirmation in any case of uncertainty, we note that such studies often require significant samples of tissue. While, as used herein, our ETV studies would require three routine sections, we note that recently reported duplex or multiplex RNA *in-situ* hybridizations[28] might be used for specific, simultaneous detection of multiple ETS-family species in the same tissue section, further sparing tissue for downstream studies. In the context of the rapid proliferation of characteristic and even pathognomonic molecular lesions in mesenchymal tumours, our observations lend even further support for the development of RNA *in-situ* hybridization as a means for rapid translation of promising markers from profiling studies into diagnostic use.

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Conflicts of interest

The authors declare there are no conflicts of interest.

References

1. Fletcher CDM; World Health Organization, International Agency for Research on Cancer. *WHO classification of tumours of soft tissue and bone*. 4th ed. Lyon: IARC Press, 2013.
2. Antonescu C. Round cell sarcomas beyond ewing: emerging entities. *Histopathology* 2014; **64**; 26–37.
3. Choi EY, Thomas DG, McHugh JB *et al.* Undifferentiated small round cell sarcoma with t(4;19)(q35;q13.1) cic-dux4 fusion: a novel highly aggressive soft tissue tumor with distinctive histopathology. *Am. J. Surg. Pathol.* 2013; **37**; 1379–1386.
4. Smith SC, Buehler D, Choi EY *et al.* CIC–DUX sarcomas demonstrate frequent myc amplification and ETS-family transcription factor expression. *Mod. Pathol.* 2015; **28**; 57–68.
5. Italiano A, Sung YS, Zhang L *et al.* High prevalence of CIC fusion with double-homeobox (DUX4) transcription factors in EWSR1-negative undifferentiated small blue round cell sarcomas. *Genes Chromosom. Cancer* 2012; **51**; 207–218.
6. Specht K, Sung YS, Zhang L, Richter GH, Fletcher CD, Antonescu CR. Distinct transcriptional signature and immunoprofile of CIC–DUX4 fusion-positive round cell tumors compared to EWSR1-rearranged Ewing sarcomas: further evidence toward distinct pathologic entities. *Genes Chromosom. Cancer* 2014; **53**; 622–633.
7. Yoshida A, Goto K, Kodaira M *et al.* CIC-rearranged sarcomas: a study of 20 cases and comparisons with ewing sarcomas. *Am. J. Surg. Pathol.* 2016; **40**; 313–323.
8. Kawamura-Saito M, Yamazaki Y, Kaneko K *et al.* Fusion between CIC and DUX4 up-regulates PEA3 family genes in Ewing-like sarcomas with t(4;19)(q35;q13) translocation. *Hum. Mol. Genet.* 2006; **15**; 2125–2137.
9. Tomlins SA, Rhodes DR, Perner S *et al.* Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 2005; **310**; 644–648.
10. Kunju LP, Carskadon S, Siddiqui J, Tomlins SA, Chinnaiyan AM, Palanisamy N. Novel RNA hybridization method for the *in situ* detection of ETV1, ETV4, and ETV5 gene fusions in prostate cancer. *Appl. Immunohistochem. Mol. Morphol.* 2014; **22**; e32–e40.
11. Warrick JI, Tomlins SA, Carskadon SL *et al.* Evaluation of tissue PCA3 expression in prostate cancer by RNA *in situ* hybridization – a correlative study with urine PCA3 and TMPRSS2-ERG. *Mod. Pathol.* 2014; **27**; 609–620.
12. Noebauer-Huhmann IM, Weber MA, Lalam RK *et al.* Soft tissue tumors in adults: ESSR-approved guidelines for diagnostic imaging. *Semin. Musculoskelet. Radiol.* 2015; **19**; 475–482.
13. Gaspar N, Hawkins DS, Dirksen U *et al.* Ewing sarcoma: current management and future approaches through collaboration. *J. Clin. Oncol.* 2015; **33**; 3036–3046.
14. Doyle LA. Soft tissue tumor pathology: new diagnostic immunohistochemical markers. *Semin. Diagn. Pathol.* 2015; **32**; 370–380.
15. Hung YP, Fletcher CD, Hornick JL. Evaluation of NKX2-2 expression in round cell sarcomas and other tumors with EWSR1 rearrangement: imperfect specificity for Ewing sarcoma. *Mod. Pathol.* 2016; **29**; 370–380.
16. Folpe AL, Hill CE, Parham DM, O'Shea PA, Weiss SW. Immunohistochemical detection of FLI-1 protein expression: a study of 132 round cell tumors with emphasis on CD99-positive mimics of Ewing's sarcoma/primitive neuroectodermal tumor. *Am. J. Surg. Pathol.* 2000; **24**; 1657–1662.
17. Tomlins SA, Palanisamy N, Brenner JC *et al.* Usefulness of a monoclonal ERG/FLI1 antibody for immunohistochemical discrimination of Ewing family tumors. *Am. J. Clin. Pathol.* 2013; **139**; 771–779.
18. Wang WL, Patel NR, Caragea M *et al.* Expression of ERG, an ETS family transcription factor, identifies ERG-rearranged Ewing sarcoma. *Mod. Pathol.* 2012; **25**; 1378–1383.
19. Solomon DA, Brohl AS, Khan J, Miettinen M. Clinicopathologic features of a second patient with Ewing-like sarcoma harboring CIC–FOXO4 gene fusion. *Am. J. Surg. Pathol.* 2014; **38**; 1724–1725.
20. Sugita S, Arai Y, Tonooka A *et al.* A novel CIC–FOXO4 gene fusion in undifferentiated small round cell sarcoma: a genetically distinct variant of Ewing-like sarcoma. *Am. J. Surg. Pathol.* 2014; **38**; 1571–1576.
21. Thomas DG, Smith SC, Choi EK, McHugh JB, Lucas DR, Patel RM. Unsupervised hierarchical clustering of immunohistochemical stains suggests that CIC–DUX4 sarcomas can be differentiated from Ewings family of tumors. *Connective Tissue Oncology Society Annual Meeting*, Berlin, Germany, 2014.
22. Hung YP, Fletcher CD, Hornick JL. Evaluation of ETV4 and WT1 expression in CIC-rearranged sarcomas and histologic mimics. *Mod. Pathol.* 2016; **29**; 1324–1334.
23. Le Guellec S, Velasco V, Perot G, Watson S, Tirode F, Coindre JM. ETV4 is a useful marker for the diagnosis of CIC-rearranged

undifferentiated round-cell sarcomas: a study of 127 cases including mimicking lesions. *Mod. Pathol.* 2016; 29: 1523–1531.
24. Pierron G, Tirode F, Lucchesi C *et al.* A new subtype of bone sarcoma defined by BCOR–CCNB3 gene fusion. *Nat. Genet.* 2012; **44**; 461–466.
25. Wang F, Flanagan J, Su N *et al.* RNAscope: a novel *in situ* RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J. Mol. Diagn.* 2012; **14**; 22–29.
26. Carter JM, Caron BL, Dogan A, Folpe AL. A novel chromogenic *in situ* hybridization assay for FGF23 MRNA in phosphaturic mesenchymal tumors. *Am. J. Surg. Pathol.* 2015; **39**; 75–83.
27. Bishop JA, Ma XJ, Wang H *et al.* Detection of transcriptionally active high-risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel e6/e7 MRNA *in situ* hybridization method. *Am. J. Surg. Pathol.* 2012; **36**; 1874–1882.
28. Anderson CM, Zhang B, Miller M *et al.* Fully automated RNAscope *in situ* hybridization assays for formalin-fixed paraffin-embedded cells and tissues. *J. Cell. Biochem.* 2016; **117**; 2201–2208.