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The Utility of *ETV1, ETV4*, and *ETV5* RNA In Situ Hybridization in the Diagnosis of CIC-DUX4 Sarcomas

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ABSTRACT

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 protooncogenic transcription factors *ETV1, ETV4*, and *ETV5*. To test the utility of an ancillary diagnostic technique for these tumors, 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 6 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 5/6 cases, while *ETV5* was positive in 6/6. No Ewing sarcoma or other sarcoma tested, showed co-expression of these transcripts, while one *ETV1, ETV4, ETV5* positive previously unclassified round cell sarcoma, was identified as harboring a *CIC* rearrangement by break-apart 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 proven to harbor 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.

INTRODUCTION

There has been an exponential increase in the identification of recurrent and/or pathognomonic gene fusions in a rapidly expanding group of mesenchymal lesions of varying biologic 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 harboring fusions of *CIC* (*Capicua* transcriptional repressor) on chromosome 19 and either, most frequently, the gene *DUX4* (*double homeobox 4*) on chromosome 4 or, less frequently, the highly related gene *DUX4L* (*double homeobox 4-like*) 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 tumors 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 tumors 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 protooncogenic 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 tumors are more aggressive than Ewing sarcoma, an assumption supported by a recent large Japan-based cohort of twenty cases [7].

It is the opinion of the authors that the shared expression of ETS-family protooncogenic 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 co-expression of both FLI1 and ERG in these tumors by immunohistochemistry (IHC)[4], comprehensive transcriptional profiling by another group[6] has confirmed upregulation 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 reliably distinguishing expression of each of these three by IHC, we have recently developed and validated a facile, exquisitely paralog-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, 9 alveolar, 1 adult spindle cell); and a TMA of high grade synovial sarcomas (n=38). Additional whole sections of each of the 6 CIC-DUX4 sarcomas were also used for evaluation of the variability of staining across the sections.

Chromogenic RNA in situ hybridization

Our chromogenic RNA in situ hybridization protocol for detection of *ETV1, ETV4*, and *ETV5* transcripts, including its validation with respects to exquisite specificity to *ETV1*, *ETV4*, or *ETV5* expression (proven by comparison to prostate carcinomas proven to harbor *ETV1*, *ETV4*, or *ETV5* rearrangements) has been detailed previously[10]. We used RNA scope probes, designed by Advanced Cell Diagnostics, Hayward, CA, 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 FFPE tissue sections were baked at 60 °C for 1 hr, deparaffinized in xylene twice for 15min, washed in 100% ethanol twice for 3min, and air-dried for 5min . Slides were treated with pretreatment 1, 2, and 3 buffers as previously described[10], rinsed in deionized water, and then incubated with the ETV1, 4, 5 or control probes for 2 hours 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 minutes, washes and counterstaining in 50% hematoxylin, 0.01% ammonium hydroxide wash, dehydration in ethanol and xylene, and mounting in Cytoseal XYL (Thermo Scientific, #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 previously validated analytically[10] and clinically[11]. Sections were evaluated at using a 20X objective based on the number of punctate dots/cell. As previously reported, staining level was assessed as one of five levels, from 0 to 4. Level 0 was defined as no staining or less than 1 dot/cell; level 1 was defined as 1–3 dots/cell in > 5% of the tumor; level 2 defined as 4–10 dots/cell with minimal clustering apparent in >5% of the tumor; level 3 defined as more than 10 dots/cell with dot clustering apparent in <10% of cells, and level 4 defined 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 5/6 cases, for *ETV4* in 5/6 cases, and for *ETV5* in 6/6 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 extra skeletal Ewing sarcomas, no case demonstrated coincident aberrant positive staining for *ETV1*, *ETV4* or *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, 8 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 increasingly must be recognized 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 only detect the small subset of Ewing sarcomas harboring *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 very reason for our prior 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].

We observed in this study a sensitivity (83%, 83% or 100%, for *ETV1, ETV4* and *ETV5*, respectively) and specificity (100%) for CIC-DUX4 sarcomas, which compares favorably to 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* 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]). Yet, we note that these remain rare tumors with little in the way of diagnostic adjuncts to support their identification from the significant subset of round cell sarcomas that remain unclassified.

We have previously reported promising findings for use of a panel of IHC markers including ETV4[21], and others have recently reported utility for ETV4 and WT1 as IHC markers for CIC-rearranged sarcomas[22, 23]. Notably, we have not tested any BCOR-CCNB3 sarcomas[24], though we do note that ETV4 IHC has been negative in 9 cases tested in two recent reports[22, 23]. Despite the potential utility, the ETV4 IHC findings remain of unproven 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 leading 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 up front (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 tumors[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. 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.

Going forward, while molecular studies including break apart FISH (or 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 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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FIGURE LEGENDS

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 ISH slides performed on consecutive sections to that of (A), even at low power inspection, positive staining for the indicated ETS-family member transcript was readily identified (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.

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 TMA studied by the ISH 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, proven by break apart FISH (F).

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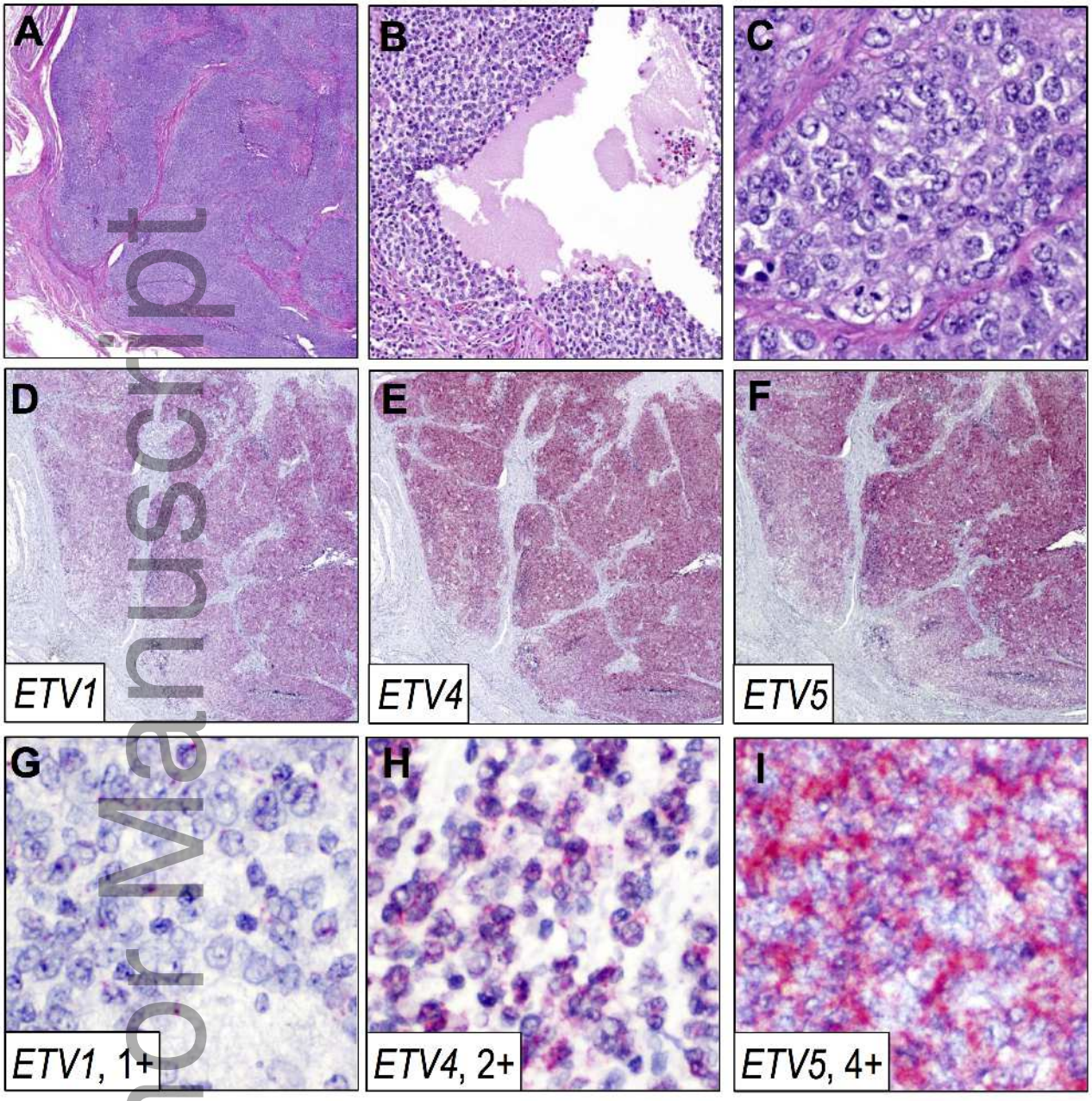

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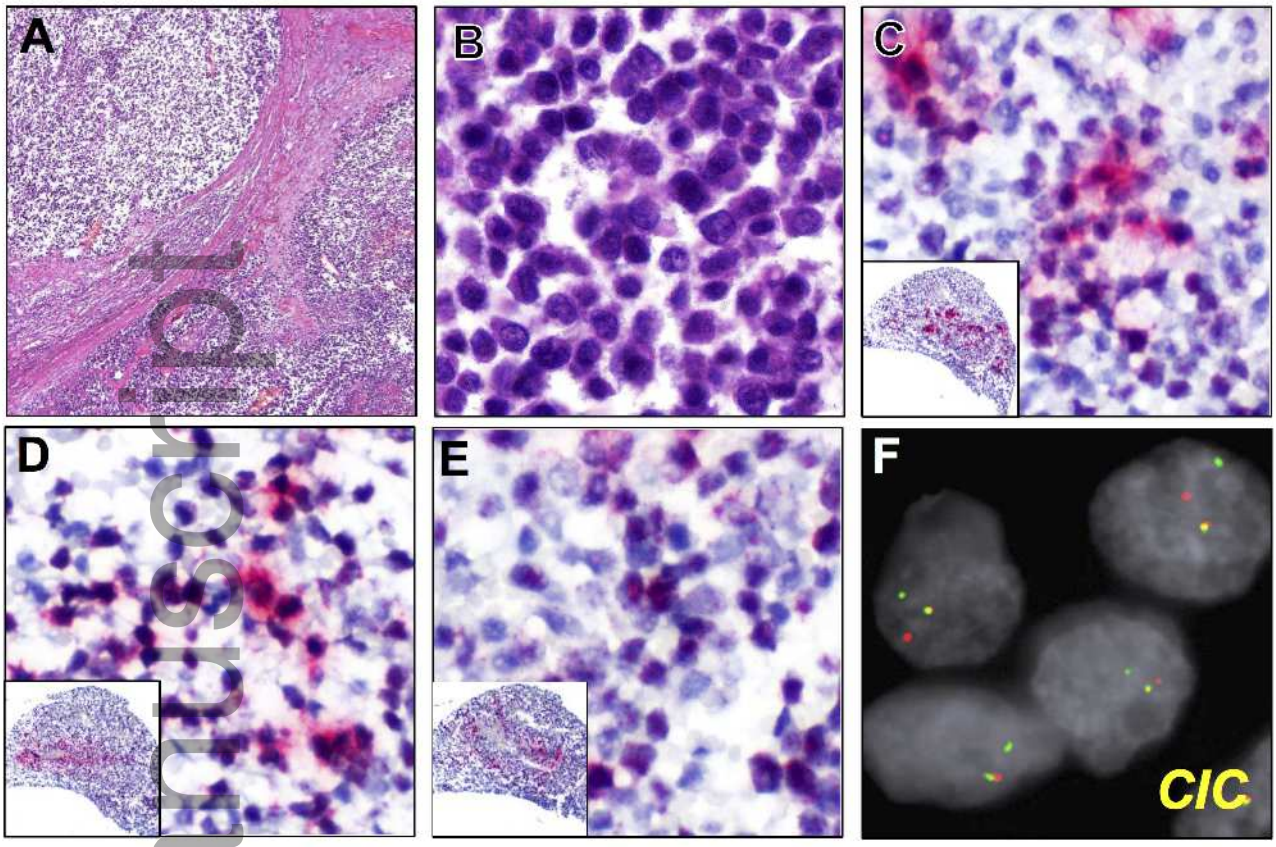

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