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Nuclear DUX4 immunohistochemistry is a highly sensitive and specific marker for the presence of *CIC*::*DUX4* fusion in CIC-rearranged sarcomas: a study of 48 molecularly confirmed cases

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Nuclear DUX4 immunohistochemistry is a highly sensitive and specific marker for the presence of *CIC*::*DUX4* fusion in CIC-rearranged sarcomas: a study of 48 molecularly confirmed cases

Aims: *CIC*-rearranged sarcomas (CRS) are clinically aggressive undifferentiated round cell sarcomas (URCS), commonly driven by *CIC::DUX4*. Due to the repetitive nature of *DUX4* and the variability of the fusion breakpoints, *CIC::DUX4* fusion may be missed by molecular testing. Immunohistochemical (IHC) stains have been studied as surrogates for the *CIC::DUX4* fusion. We aim to assess the performance of DUX4 IHC in the work-up of CRS and its expression in non-CRS round cell or epithelioid neoplasms.

Methods and results: Cases of molecularly confirmed CRS (n = 48) and non-CRS (n = 105) were included. CRS cases consisted of 35 females and 13 males, with ages ranging from less than 1 year to 67 years (median = 41 years). Among the molecularly confirmed non-CRS cases, C-terminal DUX4 expression was investigated in Ewing sarcomas (38 cases), alveolar rhabdomyosarcomas (18 cases), desmoplastic small round cell tumours (12 cases) and synovial sarcomas (n = five), as well as in non-mesenchymal neoplasms such as SMARCA4/SMARCB1-deficient tumours (n = five), carcinomas of unknown primary (n = three) and haematolymphoid neoplasms (four cases). DUX4 IHC was considered positive when strong nuclear expression was detected in more than 50% of neoplastic cells. When used as a surrogate for the diagnosis of CRS, the sensitivity and specificity of DUX4 IHC was 98 and 100%, respectively. Only one CRS case was negative for DUX4 IHC and harboured a *CIC::FOXO4* fusion.

Conclusions: DUX4 IHC is a highly sensitive and specific surrogate marker for the presence of *CIC::DUX4* fusion, demonstrating its utility in establishing a diagnosis of CRS.

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Abbreviations: CRS, CIC-rearranged sarcomas; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NGS, next-generation sequencing; URCS, undifferentiated round cell sarcomas.

Keywords: *CIC::DUX4*, *CIC*-rearranged sarcomas, immunohistochemistry, round cell sarcoma, small round cell tumours

Introduction

Undifferentiated round cell sarcomas (URCS) comprise a category of malignant mesenchymal tumours with histological features that preclude a definitive diagnosis based on histomorphology alone.[1] They are characterised by round-to-ovoid cytomorphology and a high nucleus-to-cytoplasm ratio, arranged in diverse architectural patterns.[1] This group includes more prevalent sarcomas with round-cell morphology such as Ewing sarcoma and desmoplastic small round cell tumour, as well as rare fusion-defined entities such as *CIC*-rearranged sarcoma (CRS), sarcomas with *BCOR* genetic alterations and round cell sarcomas, with fusions involving *EWSR1* and non-*ETS* family genes such as *NFATC2* and *PATZ1*.[1]

CRS are URCS characterised by rearrangements involving the *Capicua* transcriptional repressor gene (*CIC*) located on chromosome 19, which encodes the CIC protein, a highly conserved peptide that can act as a tumour repressor by inhibiting the transcription of *PEA3* subfamily transcription factors, such as *ETV1*, *ETV4* and *ETV5*.[2–4] The most frequently found genetic alteration associated with CRS is a gene fusion between *CIC* located on chromosome 19 and the double homeobox 4 (*DUX4*) located on chromosome 4 or its paralogue double homeobox 4-like (*DUX4L*), located on chromosome 10.[5–7] Fusion occurs most frequently between *CIC* exon 20 and *DUX4* exon 1, located within the D4Z4 repeat, a subtelomeric array of homologous tandem repeat units present on both 4q35 and 10q26.[5,8]

Given the worse prognosis of CRS when compared to Ewing sarcoma[3,9] and other URCS, and the different therapeutic approaches that may be deployed to treat these entities,[10] it is paramount to appropriately distinguish CRS from their histological mimickers.[11,12] The absence of gold-standard morphological or immunophenotypical features poses a diagnostic challenge. In addition, fluorescence *in-situ* hybridisation (FISH) break-apart assays demonstrate a significant number of false-negative results,[13,14] due to possible cryptic insertions beyond the lower limit of detection. The applicability of real-time polymerase chain reactions (RT-PCR) in the diagnostic work-up of CRS can be complicated by the variability of the exon–exon breakpoint locations in each gene, especially for the *DUX4* partner.[13] Finally, next-generation sequencing (NGS) methods that rely on fusion discovery algorithms may filter-out *CIC::DUX4* fusions due to the highly repetitive sequences on the *DUX4* gene.[13] Therefore, a combination of techniques is pivotal for a precise diagnosis of CRS.

The immunohistochemical (IHC) phenotype of CRS has been studied previously, demonstrating variable expression of WT-1, CD99, FLI-1 and, less frequently, ERG.[7,11,12,15,16] Nuclear WT-1 immunohistochemistry is a less sensitive marker that lacks specificity.[11] ETV4 has been evaluated as a potential IHC or RNA chromogenic *in-situ* hybridisation marker for CRS,[11,17] showing promising sensitivity and specificity, although it can occasionally be detected in other URCS such as Ewing sarcomas.[12] The performance of DUX4 IHC as a surrogate for the *CIC::DUX4* gene fusions diagnostic of CRS has also been studied previously, showing perfect sensitivity and specificity, although restricted to a very limited number of cases.[18] The present study aims to further assess the performance of DUX4 IHC in the diagnostic work-up of CRS, as well as to study its expression in non-CRS round cell neoplasms in a large cohort of cases from two different laboratories.

Methodology

The cases were identified from cases reviewed at the Biopticka laborator in Pilsen, Czech Republic (cohort 1) and the Cleveland Clinic in Cleveland, Ohio (cohort 2) (Table 1).

To assemble cohort 1, the institution's database was queried for cases of various round cell sarcomas diagnosed from 1993 to 2024, such as CRS, alveolar rhabdomyosarcoma, *EWSR1::PATZ1*-rearranged sarcoma and others (Table 2). No CRS cases originated from bone were identified. Cases were required to have formalin-fixed paraffin-embedded (FFPE) tissue blocks available for additional immunohistochemical studies. After review, cases were retrospectively stained with DUX4 antibody. To assemble cohort 2, the institution's database was queried for cases dating from 2014 to 2024 in which DUX4 immunohistochemical staining was performed as part of the diagnostic work-up. Inclusion criteria encompassed non-CRS cases with at least

Table 1. Clinical and molecular features of study cohorts (*CIC*-rearranged sarcomas)

Case number	Age (years)	Gender	Location	Confirmatory diagnostic modality	Gene breakpoint (reference genome) between *CIC* (NM_015125) and *DUX4* (NM_033178.4)
Cohort 1: molecularly confirmed *CIC*-rearranged sarcomas					
Case 1	55	Female	Small intestine (jejunum)	FISH	NA
Case 2	57	Female	Soft tissue (peroneus muscle)	FISH	NA
Case 3	47	Female	Soft tissue (back muscles)	NGS and FISH	NA
Case 4	27	Male	Soft tissue (calf)	FISH	NA
Case 5	52	Female	Mesentery	NGS	NA
Case 6	25	Female	Large intestine (rectum)	NGS	NA
Case 7	NA	Male	Soft tissue (gluteal muscle)	NGS	NA
Case 8	56	Female	Scalp	FISH	NA
Case 9	18	Male	Soft tissue (thigh)	NGS	NA
Case 10	36	Male	Not available	NGS	NA
Case 11	52	Female	Uterus	NGS	NA
Case 12	57	Female	Brain	NGS	NA
Case 13	13	Male	Soft tissue (thigh)	NGS and FISH	NA
Case 14	< 1	Male	Soft tissue (hand, palm)	NGS and FISH	*CIC* exon 20, *DUX4* exon 1
Case 15	NA	Female	Soft tissue (pelvis)	NGS, FISH and MP	*CIC* exon 20, *DUX4* exon 1
Case 16	14	Female	Soft tissue (thigh)	NGS and MP	*CIC* exon 20, *DUX4* exon 1
Case 17	18	Female	Soft tissue (retroperitoneum)	NGS, FISH and MP	*CIC* exon 21, *DUX4* exon 1
Case 18	46	Male	Soft tissue (peritoneum)	FISH	NA
Case 19	35	Female	Soft tissue (retroperitoneum)	NGS	*CIC* exon 20, *DUX4* exon 1
Cohort 2: molecularly confirmed *CIC*-rearranged sarcomas					
Case 1	33	Female	Soft tissue (left hip)	FISH*	NA
Case 2	27	Female	Skin (right vulva)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 3	38	Female	Soft tissue (right rectus muscle)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 4	55	Male	Skin (right lower leg)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 5	33	Female	Soft tissue (proximal right leg)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 6	58	Male	Soft tissue (pelvis)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 7	60	Female	Lung (right lower lobe)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 8	67	Female	Soft tissue (right thigh)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 9	34	Female	Soft tissue (left hip extrafascial mass)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 10	42	Female	Skin (chest)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 11	16	Female	Soft tissue (right scapula)	NGS	*CIC* exon 20, *DUX4* exon 1

Continued

Table 1. (*Continued*)

Case number	Age (years)	Gender	Location	Confirmatory diagnostic modality	Gene breakpoint (reference genome) between *CIC* (NM_015125) and *DUX4* (NM_033178.4)
Case 12	26	Female	Soft tissue (right buttock)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 13	10	Male	Soft tissue (right elbow)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 14	12	Female	Skin (right upper back)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 15	27	Female	Small intestine (portion of jejunum)	NGS	NA
Case 16	29	Female	Soft tissue (left foot)	NGS	NA
Case 17	31	Female	Soft tissue (right arm)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 18	24	Female	Soft tissue (right medial thigh)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 19	18	Male	Soft tissue (right knee)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 20	15	Female	Soft tissue (right axilla)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 21	30	Male	Soft tissue (back)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 22	53	Female	Right colon (terminal ileum)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 23	62	Female	Right parotid gland	NGS	*CIC* exon 20, *DUX4* exon 1
Case 24	33	Female	Skin (left buttock)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 25	57	Female	Soft tissue (peritoneum)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 26	39	Male	Soft tissue (right posterior neck)	NGS	*CIC* exon 21 (NM_001304815.1) and *FOXO4* exon 3 (NM_001170931.1)
Case 27	26	Female	Soft tissue (right anterior knee)	NGS	*CIC* exon 20, *DUX4* exon 1
Case 28	24	Female	Skin (left hand)	PCR*	NA
Case 29	29	Female	Soft tissue (right thigh)	NGS	*CIC* exon 15, *DUX4* exon 1

FISH, fluorescence *in-situ* hybridisation; NGS, next-generation sequencing; MP, methylation profiling; NA, not available.
*Diagnostic modality performed at an outside institution.

a DUX4 immunohistochemical stain available for review, molecular evidence of non-CRS defining genomic alterations or convincing histomorphological and immunophenotypical evidence of a non-CRS diagnosis. For both cohorts, additional immunohistochemical markers, ancillary studies, clinical data related to patient's age, gender and tumour location were recorded from surgical pathology reports. Positive DUX4 staining is defined as diffuse nuclear expression in more than 50% of neoplastic cells. Absence of staining or cytoplasmic staining only was considered negative for DUX4 immunoreactivity. For purposes of DUX4 IHC performance evaluation, only cases of CRS that demonstrated evidence of *CIC* rearrangement through at least one confirmatory study, i.e. FISH, NGS or multiplex PCR, were included (cohorts 1 and 2). The study was approved by the institutional review board.

IMMUNOHISTOCHEMICAL STAINING

DUX4 immunohistochemical staining at both institutions was performed following a similar protocol: 4-μm FFPE tissue sections were stained with an identical commercially available monoclonal DUX4 antibody (clone P4H2) from ThermoFisher (Waltham, MA, USA; dilution 1:800 and 1:400 at institutions 1 and 2, respectively) using a Ventana Benchmark Ultra automated immunostainer (Ventana Medical Systems, Oro Valley, AZ, USA). Fully automated deparaffinisation, followed by automated standard CC1 epitope retrieval was performed. The antigen–antibody complex was localised with the Ventana Medical Systems OptiView DAB detection kit with peroxidase and DAB chromogen and displayed brown colour with the UltraView Universal diaminobenzidine tetrahydrochloride (DAB) detection

Table 2. DUX4 expression profile among *CIC*-rearranged sarcomas and non-*CIC* rearranged neoplasms

Diagnosis	Number of cases	DUX4 nuclear expression
Cohort 1		
CIC-rearranged sarcoma	19	19/19
Alveolar rhabdomyosarcoma	12	0/12
BCOR-rearranged sarcoma*	6	0/6
Desmoplastic small round cell tumour	12	0/12
Ewing sarcoma	18	0/18
NFATC2-rearranged sarcoma	4	0/4
PATZ1-rearranged sarcoma	4	0/4
Cohort 2*		
CIC-rearranged sarcoma	21	20/21†
Alveolar rhabdomyosarcoma	6	0/6
Blastic plasmacytoid dendritic cell neoplasm	1	0/1
Carcinoma of unknown primary	3	0/3
Ewing sarcoma	20	0/20
Plasmacytoma/myeloma	3	0/3
SMARCA4/SMARCA2-deficient undifferentiated tumour	5	0/5
Solitary fibrous tumour (including malignant and anaplastic types)	5	0/5
Synovial sarcoma	5	0/5

Only CRS cases with molecular evidence of *CIC* gene rearrangement and DUX4 IHC available for review were included, and non-CRS cases were negative for CRS-defining rearrangements or were clearly defined entities from both histomorphological and immunophenotypical standpoints.

*Among *BCOR*-rearranged sarcomas, four had *ZC3H7B::BCOR* fusions, one had a *BCOR::KMT2D* fusion and one had a *BCOR::CCNB3* fusion.

†The CRS case negative for DUX4 IHC harboured a *CIC::FOXO4* fusion. DUX4, double homeobox 4; *CIC*, *Capicua* transcriptional repressor gene; CRC, *CIC*-rearranged sarcomas; CRS, *CIC*-rearranged sarcoma.

kit. Despite different antibody dilutions, no discernible differences in the staining intensity of DUX4 were observed between both institutions. The positive controls used were normal testis—where DUX4 is normally expressed[19]—and known *CIC::DUX4* sarcomas. Other immunohistochemical stains, either performed at the referring institution and provided with the case or additionally performed at the authors' institutions, included WT-1, CD99, calretinin, ERG, CD31, AE1/AE3, CAM 5.2, CD138, CK7, desmin, epithelial membrane antigen (EMA), smooth muscle actin (SMA), synaptophysin and FLI-1 (only markers tested in at least five cases were included). Staining protocols and antibody sources are available upon request.

TARGETED RNA SEQUENCING (NGS)

RNA sequencing at both institutions were performed using a customised version of FusionPlex assay (ArcherDX Inc, Boulder, CO, USA), as described previously in detail.[20,21] This is a laboratory-developed test based on anchored multiplex polymerase chain reaction (PCR) that identifies fusion transcripts in targeted regions of RNA from total nucleic acid (TNA) isolated from FFPE tissue specimens. Total nucleic acid extracted from the specimen was subjected to nested multiplex PCR enriching 59–88 gene targets. The amplicons were subjected to massive parallel sequencing with 150 × 2 cycle pair-end reads. Specifically, unidirectional gene-specific primers targeting *CIC* (NM_015125) exons 12, 17–20 were included in the panel, enabling detection of known and novel gene partners. To report a gene fusion, a minimum of five unique reads spanning the fusion junction were required alongside its detection in at least three unique start sites. In addition, at least 10% of reads surrounding the breakpoint should support the fusion event.

FLUORESCENCE *IN-SITU* HYBRIDISATION (FISH)

For *CIC* rearrangement detection by FISH, custom-designed probes with chromosomal locations (chrX:39508922-39909216 and chrX:40036437-40435921) were used (SureFish/Agilent Technologies, Santa Clara, CA, USA). Cut-off values were set to more than 10% of the nuclei with break-apart signals. The FISH procedure has been described in more detail previously.[22]

Results

CLINICAL SUMMARY OF *CIC*-REARRANGED SARCOMAS

The clinical features of all cases are listed in Table 1. A total of 19 CRS cases were obtained from cohort 1, consisting of 12 female and seven male patients, with ages ranging from less than 1 to 57 years (median = 41 ± 16.1 years). This sarcoma most

frequently involved the soft tissues of the extremities (n = six) and the truncal/pelvic axis (n = six). Within cohort 2, 29 cases of molecularly confirmed CRS (cohort 2) were identified, encompassing 23 female and six male patients, with ages ranging from 10 to 67 (median = 31 ± 15.6 years). The most common location was the soft tissues of the extremities (n = 13), followed by the trunk (n = seven).

MORPHOLOGICAL FINDINGS

Histologically, CRS was characterised by a spectrum of architectural patterns and cytomorphologies that is more diverse than other URCS. The tumours ranged from round cells with high nuclear-to-cytoplasmic ratio arranged in solid sheets to spindled or epithelioid cells set in variably myxoid to collagenous stroma (Figure 1A,B). One of the cases from cohort 2 also demonstrated a pseudoalveolar pattern (Figure 1C). Geographic-type tumour necrosis was seen frequently (Figure 1D). Cytologically, the cells contained round to ovoid nuclei, fine chromatin and prominent nucleoli, and displayed a higher degree of nuclear pleomorphism than other URCS, such as Ewing sarcoma (Figure 1E,F).

MOLECULAR FINDINGS

In cohort 1, *CIC* gene rearrangements were detected by FISH using *CIC* break-apart probes in four cases; targeted RNA sequencing was performed as a stand-alone method or in association with FISH or methylation profiling in an additional 15 cases, revealing *CIC::DUX4* fusions in all of them. In cohort 2, *CIC* gene rearrangements were detected in 28 cases by targeted RNA sequencing: 27 cases demonstrated *CIC::DUX4* fusions and one exhibited a *CIC::FOXO4* fusion. One case demonstrated *CIC* gene rearrangement through FISH analysis performed at an outside laboratory. Among the cases with *CIC::DUX4* fusion breakpoint data, the most common exonic breakpoints involved *CIC* exon 20 (NM_015125) and *DUX4* exon 1 (NM_033178.4) (27 of 29, 93%). One case each harboured *CIC* exon 21 or exon 15 fused to *DUX4* exon 1, respectively (Table 1).

DUX4 IHC PERFORMANCE

DUX4 nuclear expression was evaluated in CRS and non-CRS cases (Table 2). Cohort 1 comprised an additional 56 non-CRS cases encompassing multiple URCS, such as Ewing sarcoma (18 cases), alveolar rhabdomyosarcoma (12 cases), desmoplastic small round cell tumour (12 cases) and other fusion-defined entities (Table 2), which were retrospectively stained with DUX4 IHC. All CRS (19 of 19) demonstrated strong and diffuse nuclear DUX4 expression (Figure 2A–H), whereas none (none of 56) of the non-CRS cases exhibited nuclear expression.

Of the 29 molecularly confirmed CRS cases (cohort 2), 21 had DUX4 immunohistochemical staining performed and 20 demonstrated strong and diffuse nuclear DUX4 expression. The one CRS case that was negative for DUX4 IHC nuclear staining harboured a *CIC::FOXO4* fusion.

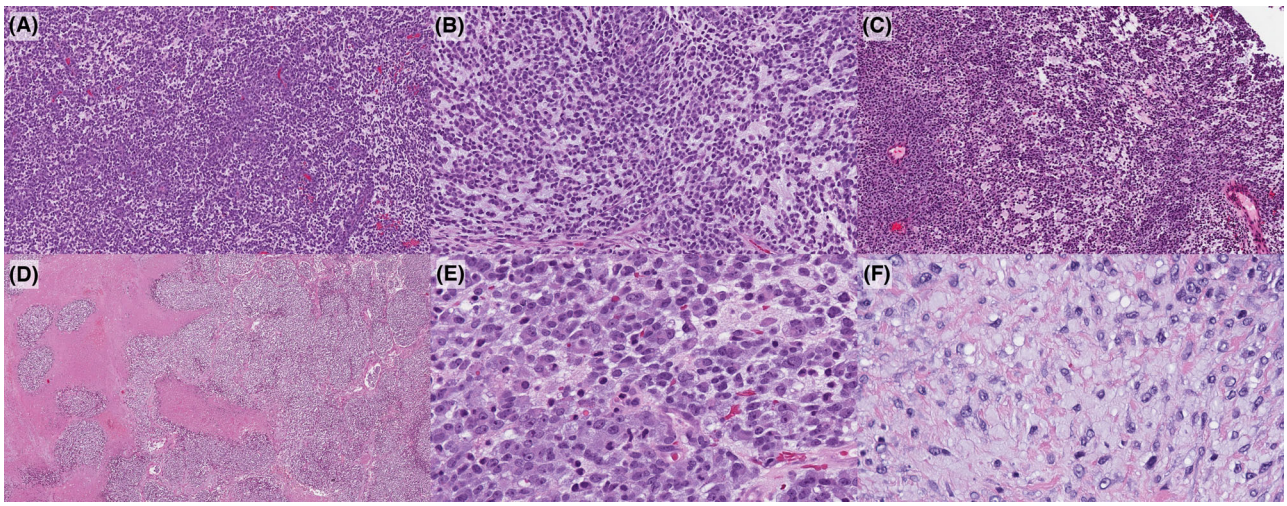

Figure 1. Architectural and cytomorphological spectrum of *CIC*-rearranged sarcomas (CRS): solid sheets of monotonous round cells (**A**), myxoid stroma (**B**), pseudoalveolar pattern (**C**) and geographic type necrosis (**D**). The cytomorphology can range from epithelioid to spindled cells (**E,F**) with more pronounced pleomorphism than other URCS, such as Ewing sarcoma.

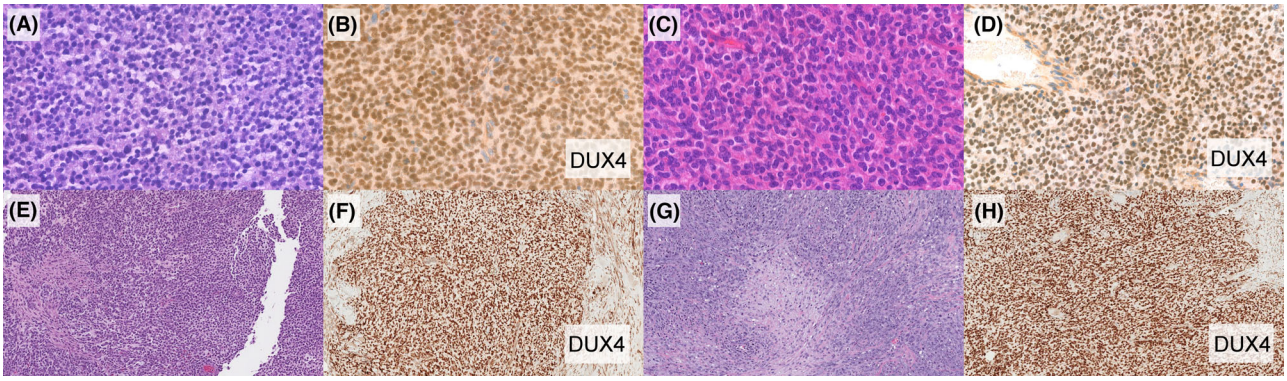

Figure 2. Four different cases from two independent institutions—cohorts 1 (**A–D**) and 2 (**E–H**)—demonstrating nuclear DUX4 expression. When strong and diffuse nuclear expression of this immunohistochemical marker is present, it can be used as a surrogate for *CIC::DUX4* fusion. **A,C** (cohort 1): CRS haematoxylin and eosin; **B,D** (cohort): C-terminal DUX4; **E,G** (cohort 2): haematoxylin and eosin; **F,H** (cohort 2): C-terminal DUX4.

Among the 48 non-CRS cases from cohort 2, there were three epithelial (6%), four haemato-lymphoid (8%) and 41 mesenchymal (85%) neoplasms, including 26 small round blue cell tumours (Table 2). None (of 48) demonstrated nuclear staining of DUX4. Forty-three cases (89.6%) demonstrated non-specific patchy to diffuse cytoplasmic staining), and in five cases (10.4%) DUX4 IHC was completely absent (Figure 3A–J). Cases with true focal nuclear DUX4 staining were not identified. Only cases with confirmed alternate diagnoses (by IHC or molecular testing) or molecularly confirmed absence of *CIC* rearrangements were included in the non-CRS cohort.

Combining both cohorts, the sensitivity and specificity of DUX4 for the diagnosis of CRS with *CIC* rearrangements was 98% (39 of 40) and 100% (104 of 104), respectively.

OTHER IMMUNOMARKERS OF RELEVANCE

Additional immunohistochemical markers were examined in CRS cases of both cohorts (Table 30). In cohort 1, CD99 (12 of 12; mostly patchy), WT-1 (eight of eight) and FLI-1 (four of four) were expressed in all CRS cases in which these markers were performed, while ERG (three of five) and CD31 (two of four) were expressed in 60 and 50% of tested CRS cases, respectively. In cohort 2, among molecularly confirmed CRS cases, CD99 (16 of 19), WT-1 (six of six), FLI-1 (two of two) and CAM 5.2 (three of six) were expressed in 50% or more cases of CRS.

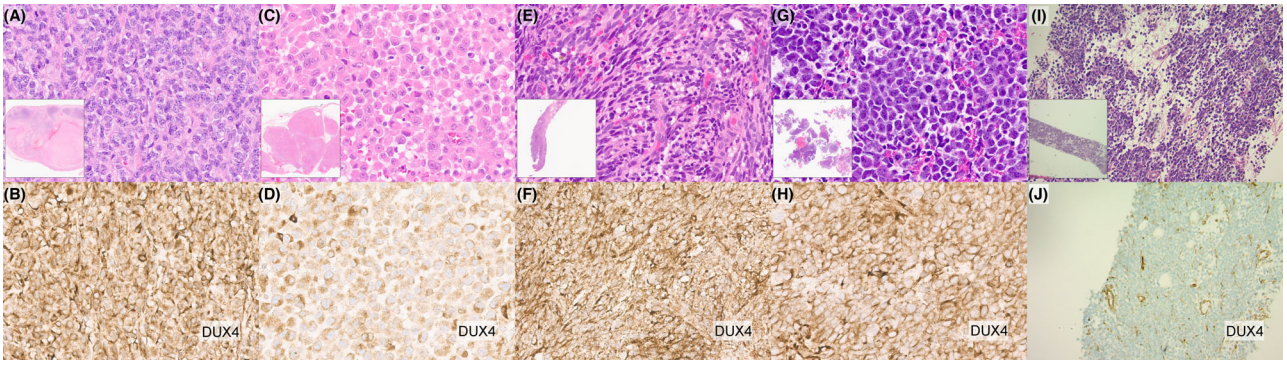

Figure 3. The interpretation of DUX4 can be challenging when strong cytoplasmic staining is present, leading the pathologist to overinterpret areas of limited nuclear sampling as positive nuclear staining. In some cases, DUX4 expression might be absent. **A–J,** DUX4 expression in non-CRS cases; **A,B,** haematoxylin and eosin and cytoplasmic DUX4 expression in a case of endometrial stromal sarcoma; **CD,** haematoxylin and eosin and cytoplasmic DUX4 expression in a case of epithelioid sarcoma; **E,F,** haematoxylin and eosin and cytoplasmic DUX4 expression in a case of melanoma; **G,H,** haematoxylin and eosin and cytoplasmic DUX4 expression in a case of a carcinoma of unknown primary; **I,J,** haematoxylin and eosin and complete absence of DUX4 expression in a case of a diffuse large B cell lymphoma.

Table 3. DUX4 and other immunohistochemical markers expressed in CRS

	Cohort 1	Cohort 2	Total
AE1/AE3	NP	1/9	1/9 (11%)
Calretinin	5/5	2/2	7/7 (100%)
CAM5.2	NP	3/6	3/6 (50%)
CD138	NP	1/5	1/5 (20%)
CD31	2/4	1/5	3/9 (33%)
CD99	12/12	16/19	28/31 (90%)
CK7	NP	0/5	0/5 (0%)
Desmin	NP	3/22	3/22 (14%)
DUX4	19/19	20/21	39/40 (98%)
EMA	NP	1/9	1/9 (11%)
ERG	3/5	2/7	5/12 (42%)
FLI-1	4/4	2/2	6/6 (100%)
SMA	NP	2/14	2/14 (14%)
Synaptophysin	NP	1/5	1/5 (20%)
WT-1	8/8	5/8	13/16 (81%)

Any documented expression or positive expression after slide review was included as positive. The rate of positive per all cases of each cohort, expressed as positive/total is demonstrated. NP, not performed; SMA, smooth muscle actin; DUX4, double homeobox 4.

Discussion

We report the largest study to date, to our knowledge, investigating the diagnostic utility of DUX4 immunohistochemistry in CRS. The widespread utilisation of molecular-based approaches during the diagnostic work-up of soft tissue lesions has led to the discovery of a multitude of fusion-defined entities demonstrating oncoproteins that, most frequently, behave mechanistically as oncogenic drivers.[23] Because these oncoproteins are often expressed at significantly higher levels, immunohistochemical detection can be applied as a surrogate for the diagnosis of fusion-driven sarcomas, representing a much more cost-effective approach with shorter turnaround times than molecular confirmation.[23,24] Characteristic diagnoses that may be rendered by this approach include solitary fibrous tumour, in which IHC expression of STAT6 acts as a surrogate for *NAB2::STAT6* gene fusion, epithelioid haemangioendothelioma and CAMTA1 expression (*WWTR1::CAMTA1*), synovial sarcoma and SS18–SSX expression (*SS18::SSX1/2/4*)[25] and myxoid liposarcoma and DDIT3 expression (*FUS/EWSR1::DDIT3*).[23]

It was hypothesised that nuclear expression of *CIC::DUX4*, detected by the DUX4 antibody targeting its conserved region in the chimeric protein, can be utilised as a surrogate of *CIC*-gene rearrangements. Indeed, a small series of CRS demonstrated a perfect correlation between nuclear DUX4 IHC expression and the presence of *CIC::DUX4* fusions.[18] In addition, downstream targets such as ETV4 and WT-1 have been identified through gene expression profiling,[2,3,11,17,24] and the IHC expression of the former showed 90% sensitivity and 95% specificity for CRS if diffuse nuclear expression was present,[12] a performance similar to DUX4, allowing pathologists to identify CRS cases using multiple tools.

The present study analysed the performance of DUX4 IHC as a surrogate for the detection of *CIC* gene rearrangement in CRS in two different cohorts performed at two different laboratories. Of the 40 cases of CRS, the single case negative for nuclear DUX4 expression harboured a *CIC::FOXO4* rearrangement, pointing to the specificity of this marker for the detection of *CIC::DUX4* fusions. As CRS cases can present with *CIC* rearrangements involving diverse gene partners other than *DUX4*, including *FOXO4*, *NUTM1*, *LEUTX*, etc.,[26–28] the absence of DUX4 expression does not completely exclude a diagnosis of CRS. However, these alternate fusion partners are rare in *CIC* sarcomas (approximately 5%).[3] In the absence of DUX4 nuclear reactivity, if CRS remains in the differential diagnosis we recommend performing molecular testing to detect for the presence of *CIC* gene rearrangements.

Interestingly, a subset of CRS in our study also expressed vascular markers at least partially, such as ERG, CD31 and FLI1. This finding re-ignites the ongoing debate regarding whether CRS cases with vasculogenic differentiation should be classified as a distinct entity or a variant of angiosarcoma. This morphological conundrum was assessed previously, and the authors demonstrated both *CIC*-rearrangements or *CIC* mutations in angiosarcomas[29] or the presence of vascular markers in CRS.[30] Addressing DUX4 IHC expression in angiosarcomas presents a potential opportunity for further assessment of this debate and is beyond the scope of the present study.

Given the idiosyncratic nature of *CIC* and *DUX4* rearrangements, with variable exonic breakpoints,[13] multiple different probes targeting both *CIC* and *DUX4* should be used to account for this gene fusion

heterogeneity. This caveat to CRS molecular identification is also present in our study. Case 29 from cohort 2 exhibited strong and diffuse DUX4 expression, but the targeted gene panel utilised initially did not include probes that cover the *CIC* exon 15 breakpoint. An expanded NGS panel that included this exon was able to ultimately confirm the fusion. This highlights the utility of C-terminal DUX4 IHC in the diagnosis of *CIC::DUX4* sarcomas.

Similarly to the assessment of beta-catenin immunohistochemistry in desmoid fibromatosis, nuclear rather than cytoplasmic expression of DUX4 is required for positive cases. Occasionally, cases with prominent cytoplasmic expression of DUX4 can be misinterpreted as nuclear staining, due to the varying planes of sections that may give a false impression of strong nuclear expression. Therefore, for better reproducibility, only strong and diffuse nuclear expression of DUX4 should be considered positive.

Because of its sensitivity and specificity, especially in limited biopsies, the diagnosis of CRS has been established by DUX4 IHC without molecular confirmation in some centres. In our practice, we have begun to render the diagnosis of CRS based on clinical and histopathological findings, including the expression of nuclear DUX4 through IHC, without the utilisation of molecular testing. One of the limitations of the present study is the selection bias stemming from the inclusion of cases where DUX4 IHC was performed as part of the diagnostic work-up when CRS is strongly suspected. This selection bias may lead to potential inflation of the sensitivity and specificity of this marker. Additional independent validation of the sensitivity and specificity of this marker in future studies would be helpful.

A potential pitfall of DUX4 IHC not examined by this study is the possible expression of this marker in a subset of B cell precursor acute lymphoblastic leukaemias harbouring *IGH::DUX4* or *ERG::DUX4* fusions.[31] However, in these leukaemias the fusion gene loses the 3′ end of *DUX4*, culminating in a truncated C-terminal end of DUX4 in the chimeric protein.[31] As the antibody used to detect DUX4 in this study is against the C-terminal end of this protein, its expression is not expected to be positive in those leukaemias, as demonstrated in a previous series.[31] However, different clones that target the N-terminus of DUX4 can demonstrate positive expression in DUX4-fused leukaemias.[31] Therefore, precautions must be taken when selecting and interpreting the DUX4 antibody clone.

Because CRS are characterised by fusion-driven oncogenesis, both the aberrant oncoprotein or its downstream targets may serve as IHC surrogates of *CIC* gene rearrangements.[11,12,17,18,32,33] The current evidence suggests that the *CIC::DUX4* fusion oncoprotein drives nuclear localisation of DUX4,[2,5,7,12,15] given that the chimeric protein derived from the fusion between *CIC* and *DUX4* preserves the C-terminal domain of DUX4 in addition to most of CIC protein domains.[2,13,34] In addition, the C1 and HMG motifs of CIC, alongside the activation domain of DUX4, which are retained in the chimeric protein, maintain CIC's DNA-binding ability. This culminates in the nuclear localisation of DUX4 and a paradoxical oncogenic activity of the CIC::DUX4 protein,[35,36] where it interacts and activates CIC-binding gene targets such as members of the *PEA* gene family, inducing cell cycle progression via the cyclin E pathway, and cell growth and migration through the overexpression of *IGFR1*.[13,36,37]

In conclusion, the present study demonstrated that DUX4 IHC can be used as a surrogate marker for the presence of *CIC::DUX4* fusion due to its excellent sensitivity and specificity.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

Data availability statement

Data that support the findings of this study are available from the corresponding author on request.

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