Undifferentiated round cell sarcomas with *CIC-DUX4* gene fusion: expanding the clinical spectrum

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Summary

Round cell sarcomas are a heterogeneous group of mesenchymal neoplasms with overlapping morphology and immunohistochemical profile. Ewing sarcoma is the most well-known tumour in this group characterised by *EWSR1/FUS* rearrangements with members of the ETS family of transcription factors. Undifferentiated round cell sarcomas lacking these rearrangements, known as 'Ewing-like' sarcomas, usually show atypical clinical presentation and focal CD99 positivity. This group of tumours can be subdivided into: capicua transcriptional repressor (*CIC*)-rearranged sarcomas, Bcl6 corepressor (*BCOR*)-rearranged sarcomas, sarcomas with *EWSR1* fusion to non-ETS family members and unclassified round cell sarcomas.

We describe seven new cases of *CIC-DUX4* rearranged sarcomas with their clinicopathological features, two of which presented in unusual locations (skin and lymph node). Patient age ranged between 23 and 54 years, three of whom were female. In five cases, aggressive behaviour was observed with rapid disease progression and lethal outcome within 15 months. One patient achieved a complete response after chemotherapy. The last patient whose tumour was located purely in the dermis demonstrated no residual tumour in the re-resection specimen, was not given any further treatment and showed no sign of disease after 24 months. Immunohistochemically, tumour cells in all cases showed focal membranous CD99 positivity, while WT1 N-/C-terminus were positive in all 5/5 cases (nuclear and/or cytoplasmic). NGS analysis revealed a *CIC-DUX4* fusion in all cases.

This study expands the spectrum of anatomical locations of *CIC-DUX4* rearranged sarcomas, highlighting the inclusion of this rare entity in the differential diagnosis of undifferentiated tumours in various anatomical locations outside of soft tissues.

Key words: *CIC-DUX4*; sarcomas; rearrangement.

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INTRODUCTION

Ewing sarcoma (ES) and undifferentiated round cell sarcomas (URCS) are a heterogeneous group of bone/soft tissue neoplasms characterised by small blue round cell morphology and unspecific/overlapping immunohistochemical findings.^{1–4} ES commonly occurs in paediatric and young adult patients and is mainly located in the bones, but in up to 20% of cases ES can be found in an extra-osseous location.⁵ ES typically shows diffuse membranous positivity for CD99 and is characterised genetically by specific fusions between two families of genes, namely TET/FET (*EWSR1* and *FUS*) and E26 transformation-specific (ETS), most commonly *EWSR1-FLI1* or *EWSR1-ERG*.⁶

URCS lacking these rearrangements are known as 'Ewing-like' sarcomas according to the current (2013) World Health Organization (WHO) classification.⁷ They frequently show atypical clinical presentation and focal weak positivity for CD99 and therefore pose a diagnostic challenge for both clinicians and pathologists. Some of these URCS cases have been shown to harbour alternative fusions, including fusions between the TET/FET family and non-ETS family genes (e.g., *ZSG*, *SMARCA5*, *POU5F1* and *NCATc2*) as well as fusions between the non-TET/FET family and other genes (*BCOR-CNB3*, *BCOR-MAML3*, *ZC3H7B-BCOR* and *ETV6-NTRK*).^{8–10}

In the latter group, a distinct subset of URCS harbours a capicua transcriptional repressor (*CIC*) rearrangement, predominantly characterised by gene fusion between *CIC* (19q13) and one of the two *DUX4* retro-genes (4q35 or 10q26).^{4,11–16} Recently, *CIC-FOXO4* and *NUTM2A-CIC* fusions have been described.^{17–20} In the central nervous system, another fusion

partner alternative to *DUX* has been identified, namely NUT midline carcinoma family member 1 (*NUTM1*).²¹ Clinically, *CIC*-rearranged sarcomas usually present at extraskeletal sites, behave aggressively with the development of metastases (mostly lungs and brain), and have poor prognosis.^{13,22}

In this study, we describe seven novel cases of *CIC-DUX4* sarcomas, two of which occurred in unusual locations with clinicopathological features and follow-up. Expression of immunohistochemical markers and genetic findings were compared with previously published data including differential diagnoses.

MATERIAL AND METHODS

Patients and tumour characteristics

Five cases of *CIC*-rearranged sarcomas with small blue round cell morphology were retrieved from surgical pathology files and consultation cases of one of the authors (BL-A). Patients were diagnosed between 2014 and 2018 at the Institute of Pathology, Medical University of Graz. Institutional ethical approval was obtained from the Institutional Review Board. All cases were reviewed by two pathologists (IB and BL-A).

Immunohistochemical and molecular analysis

For immunohistochemical and molecular analyses, we used formalin-fixed, paraffin-embedded (FFPE) material. Immunohistochemistry was performed on 4 µm thick whole tissue sections. The following antibodies with appropriate positive controls were used: CK, EMA, S100, CD34, SMA, desmin, ERG, WT1 (N-terminus and C-terminus), and CD99 (Table 1). WT1 staining was evaluated as nuclear or cytoplasmic if at least 5% of tumour cells showed moderate-to-strong staining.

Molecular analysis (fusion gene detection) was performed using the next-generation sequencing (NGS)-based anchored multiplex polymerase chain reaction (PCR) technique in the Laboratory for Diagnostic Genome Analysis, Institute of Pathology, Medical University of Graz, Austria, and Sanger sequencing was performed at the Department of Clinical Pathology, Medical University Vienna.

RNA workflow

For each sample, 5–8 × 10 µm FFPE sections were cut from a single representative block. Macrodissection was performed with a scalpel to enrich for tumour content. RNA was isolated using the Maxwell RSC RNA FFPE kit (Promega, USA) according to the manufacturer's instructions.

Targeted next generation sequencing RNA was quantified by ribogreen fluorescence and 250 ng total RNA was used for the FusionPlex Sarcoma kit (Archer, USA). NGS libraries were sequenced on the Ion Torrent Proton platform using the Ion PI Hi-Q Sequencing 200 kit (Thermo Fisher, USA). Analysis was performed with the ArcherDX Analysis software version 5.1.3.

Sanger sequencing One microgram of total RNA was reverse-transcribed into complementary DNA (cDNA) by super script VILO master mix (Applied Biosystems, USA) cDNA was subjected to RT-PCR using JumpStart REDTaq DNA Polymerase (Merck, Germany). The following primers were used: *CIC* Exon 19 Fwd: 5'-AGGACGTGCTTGGGGAGCTAGAGT-3'; *DUX* Exon 2 Rev: 5'-CCGCGGAGTGGAGTCTCTCACCG-3' and *DUX42R2* CGCTGTGTGGAGTCTCTCACCG. Amplified PCR products were subjected to Sanger sequencing using BigDye terminator v3.1 Kit (Thermo Fisher) and the Applied Biosystems 3500 Genetic Analyzer. Obtained sequences were submitted to BLAST searches (<http://blast.ncbi.nlm.nih.gov/>) to identify fusion events.

RESULTS

Clinical findings

Patients' clinical data are summarised in Table 2. There were three female and four male patients; the mean age was 35 years (age range 23–54 years). Tumours were located in an axillary lymph node (unknown primary tumour), the wall of

the small bowel, the pelvic bone, the skin (dermis) of the right ear, the soft tissues of the right lower leg, the thorax and the abdomen. The patient whose tumour was located in the axillary lymph node underwent additional imaging and no other 'primary' tumour was detected.

Morphological, immunohistochemical and molecular findings

Cut sections of all specimens showed a circumscribed tan-brown fleshy mass with areas of necrosis and haemorrhage. Histological analysis revealed tumour tissue growing in a diffuse or lobular pattern. The tumour was composed of sheets or nests of small to medium size round to oval blue cells with areas of (geographical) necrosis. Focally, a myxoid matrix was also observed (Fig. 1). At higher magnification, the tumour cells had irregular nuclei with coarse chromatin and conspicuous nucleoli and scant eosinophilic cytoplasm was also seen (Fig. 1H). Mitotic figures were frequent. Immunohistochemistry revealed at least a focal positive membranous staining with CD99 (Fig. 2A,B). Nuclear and/or cytoplasmic staining with WT1 (N-terminus and C-terminus) was seen in 5/5 cases (Fig. 2C). Positive nuclear staining with ERG was observed only in the case located in the dermis (Fig. 2D). Stainings for CK, S100, desmin and SMA were all negative. The detailed findings of the immunohistochemical staining results are presented in Table 2.

In all cases, NGS analysis detected the t(4;19)(q35;q13) translocation. A gene fusion involving a 'G' nucleotide insertion in the breakpoint between exon 20 of the *CIC* gene and exon 1 of the *DUX4* gene was observed in five of the cases tested in Graz. In the other cases, the same fusion was verified by Sanger sequencing. Visualisation of fusion events for the tumour located in the skin (Case 4) is presented in Fig. 3.

Treatment and follow-up

Six patients received chemotherapy (see Table 2). Clinical follow-up data were available for all patients, ranging from 6 to 30 months with a median of 14 months. At the time of the last follow-up, five patients succumbed to disease 6–15 months after diagnosis, two developed distant metastases and three progressed locally under chemotherapy. The last two patients, however, had a different course of the disease. The patient whose tumour was located in the lymph node (Case 3) showed no sign of disease 30 months after receiving chemotherapy. The last patient (Case 2), with a superficially located tumour of

Table 1 Antibodies used for immunohistochemical analysis

Antibody	Source	Clone	Dilution
CK	Dako/Agilent	MNF116	1:100
EMA	Ventana/Roche	E29	RTU
S100	Dako/Agilent	Polyclonal	1:2000
CD34	Dako/Agilent	QBEnd 10	RTU
SMA	Sigma	1A4	1:20,000
Desmin	Dako/Agilent	D33	RTU
ERG	Abcam	EPR3864	1:200
WT1 (N-terminus)	Dako/Agilent	6F-H2	1:20
WT1 (C-terminus)	LifeSpan Biosciences	C-terminus	1:100
CD99	Dako/Agilent	12E7	RTU

CK, cytokeratin; RTU, ready to use; SMA, smooth muscle actin; WT1, Wilms' tumour.

Table 2 Summary of clinical, immunohistochemical and molecular data of all patients

Case	1	2	3	4	5	6	7
Age	54	35	25	36	47	26	23
Sex	F	F	M	M	F	M	M
Location	Small bowel	Skin, right ear	LN/left axilla	Pelvic bone	Right lower leg	Abdomen	Thorax
Metastasis	Local progression	None	None	Lung	Stomach, pancreas, brain, lungs, liver, spleen, mediastinal and parahilar LN, bones	Local progression	Local progression
CD99	Focal +	Focal +	Focal +	Focal +	Focal +	Focal +	Focal +
WT1 (N-terminus)	Cytoplasmic	NA	Cytoplasmic	NA	Cytoplasmic	Nuclear	Nuclear
WT1 (C-terminus)	Cytoplasmic	Nuclear	Cytoplasmic	Cytoplasmic	Cytoplasmic	NA	NA
ERG	Negative	Focal +	Negative	NA	Negative	NA	NA
Archer	CIC-DUX4 t(4;19)(q35;q13)	CIC-DUX4 t(4;19)(q35;q13)	CIC-DUX4 t(4;19)(q35;q13)	CIC-DUX4 t(4;19)(q35;q13)	CIC-DUX4 t(4;19)(q35;q13)	CIC-DUX4 t(4;19)(q35;q13)	CIC-DUX4 t(4;19)(q35;q13)
Therapy	Doxorubicin, ifosfamide	None	VIDE; VAI	VIDE; ITB	VAIA; pazopanib	VIDE	VIDE
Follow-up	10 months	24 months	30 months	15 months	14 months	8 months	6 months
Outcome	DOD	NED	NED	DOD	DOD	DOD	DOD

Archer, Archer FusionPlex Sarcoma panel; AWD, alive with disease; DOD, died of disease; F, female; ITB, irinotecan, temozolomide, bevacizumab; LN, lymph nodes; M, male; NA, not available; NED, no evidence of disease; VAI, vincristine, actinomycin, ifosfamide; VAIA, vincristine, dactinomycin, ifosfamide, doxorubicin; VIDE, vincristine, ifosfamide, doxorubicin, etoposide.

the ear, showed no evidence of disease 24 months after initial diagnosis without receiving chemotherapy.

DISCUSSION

This study describes seven new cases of *CIC-DUX4* sarcomas, two of which presented in unusual anatomical locations, i.e., the dermis and the axillary lymph node. Sarcomas with a *CIC-DUX4* fusion account for up to 68% of *EWSR1/FUS*-negative rearranged USRCSs.^{4,23} Overlapping morphological features with other small blue round cell tumours (SBRCT; see below) make them challenging to diagnose. Clinically, they present more frequently in young adults and arise almost exclusively in the soft tissue, usually showing an aggressive behaviour with rapid progression.¹⁶ Histologically, these sarcomas are composed of small to medium-sized round to oval cells arranged in patternless sheets or nests. The cells have vesicular nuclei with prominent nucleoli and variable amounts of cytoplasm.^{3,4,12,14} Tumour stroma can be desmoplastic, sclerotic, myxoid or oedematous and geographic necrotic areas are frequently found.²⁴ Mitotic count is high (ranging from 11 to 125/10 high power fields).²⁴ Other members of the *CIC*-rearranged sarcomas, i.e., *CIC-FOXO4* and *NUTM2A-CIC*, share similar morphological features.^{17,18,20}

All of our cases showed the described histological features. Five of seven tumours were located at previously reported anatomical sites and demonstrated aggressive clinical behaviour. Interestingly, two cases occurred in unusual anatomical locations, including an axillary lymph node and the dermis, and showed an indolent clinical course without signs of disease (even without therapy, as seen in one of the reported cases). Based on our observations, *CIC-DUX4* sarcomas need to be included in the differential diagnosis of SBRCT in anatomical locations outside of the soft tissues and bone.

Within the heterogeneous group of SBRCTs, the most common differential diagnosis is ES. Histologically, ES is characterised by tumour cells with blue round cell

morphology, and varying degrees of neuroectodermal differentiation.²⁵ Immunohistochemistry shows typical strong, diffuse membranous staining for CD99. Recently, NKX2.2 was found to be positive in up to 93% of ESs tested. Nevertheless, its role in the diagnosis of ES is still questionable, as it was found to be positive in non-Ewing tumours as well.^{26,27}

The diagnosis of ES is confirmed by the detection of a rearrangement of the *EWS* gene on chromosome 22q12. However, in this regard, it should be noted that the fluorescence *in situ* hybridisation (FISH) assay for *EWSR1* is not specific, as detection of a break-apart signal may not exclude other ES mimics with *EWSR1* rearrangement (see below). Additionally, the group of the ES family of tumours also have round cell sarcomas with a *FUS* rearrangement. For this reason, PCR-based techniques and especially NGS techniques should be applied in this setting.

Besides ES, other SBRCTs must be considered, in particular round cell sarcomas with *EWSR1*-non ETS rearrangement, *BCOR*-rearranged sarcomas, desmoplastic small round cell tumours, alveolar rhabdomyosarcoma, extraskeletal mesenchymal chondrosarcoma, poorly differentiated synovial sarcoma, lymphoblastic lymphoma (LBL) and neuroblastoma. High-grade myxoid liposarcoma should also be excluded, as it is known that it can present as solid sheets of back-to-back primitive round cells without intervening myxoid stroma. Due to the resemblance of *CIC-DUX4* sarcomas to ES and other SBRCTs, correlation between clinical data, radiological findings, histology and ancillary immunohistochemical and/or molecular techniques are necessary for diagnosis.

In *CIC-DUX4* sarcomas, immunohistochemistry frequently reveals focal to multifocal weak membranous positivity for CD99 and nuclear staining for WT1 (N-terminus), whereas ERG and NKX2.2 are in most cases negative.^{12,23,24,26} Recently, diffuse nuclear ETV4 positivity in up to 100% of cases has been reported.^{24,26,28} It is important to mention that

Fig. 1 Morphology of *CIC*-rearranged sarcomas showing patternless sheets of medium-sized round blue tumour cells. Tumour cells have round to oval nuclei and contain variably coarse chromatin with discernible to prominent nucleoli, and scant to moderate amounts of eosinophilic cytoplasm. (A,B) Tumour situated in the dermis; (C,D) tumour localised in a lymph node; (E,F) tumour localised in the small bowel; (G) tumour showing area of necrosis; (H) higher power showing morphological features of *CIC-DUX4* sarcoma. The tumour cells show moderate nuclear pleomorphism and a syncytial appearance.

nuclear ETV4 and WT1 immunoreactivity, although usually weak and present in a low percent of tumour cells, can be found in up to 5% and 19% of non-*CIC*-rearranged tumours, respectively.^{24,26} In addition, DUX4 immunohistochemistry has been proposed as a diagnostic marker for *CIC-DUX4* rearranged sarcomas in one study.²⁸ DUX4 immunohistochemistry may help to detect this rare subgroup of sarcomas. However, at least

in our opinion, molecular studies are needed to confirm the diagnosis of a *CIC-DUX4* sarcoma.

In our study, all cases showed focal membranous positivity for CD99 and all tested cases (5/5, 100%) showed WT1 (C-terminus) expression in at least a subset of tumour cells. In all of the cases tested, we observed a nuclear staining pattern and, in some cases, also a cytoplasmic positivity for WT-1

Fig. 2 Immunohistochemical findings. (A) Staining with CD99 with more diffuse membranous positivity and (B) with more focal staining pattern. (C) Staining with WT1 (N-terminus) shows nuclear staining. (D) Staining with ERG shows nuclear positivity in tumour cells (endothelial cells depicted in dermis used as positive internal control).

(N-terminus) was observed (according to various authors, nuclear positivity is regarded as positive reaction). Only the superficially located tumour arising in the skin (dermis) stained positive for ERG. NKX2.2, ETV4 and DUX4 antibodies were not available at our institution, as molecular testing is part of the routine work-up for SBRCT.

A gold standard for the identification of known (and unknown) genetic alterations is still not established. As mentioned earlier, the FISH assay for *EWSR1* is not specific and should be interpreted together with morphological and immunohistochemical findings. Considering CIC break-apart

FISH analysis, a false-negative rate of 14% for *CIC*-rearranged sarcomas was reported.²⁹ An NGS-based PCR approach, although not available at every centre, could be a very helpful ancillary technique. The high sensitivity of this method when compared to FISH and RT-PCR enables the detection of certain fusions in cases with small amounts of tumour tissue, making exclusion of other differential diagnoses possible. In our study, we used NGS-based anchored multiplex PCR (Archer FusionPlex Sarcoma panel) or Sanger sequencing and were able to detect *CIC-DUX4* fusion with t(4;19)(q35;q13) translocation in all of the presented cases.

Fig. 3 Visualisation of the fusion event. (A) In Case 4, a fusion of *CIC* exon 20 to *DUX4* exon 1 was detected. (B,C) The nucleotide sequence of the fusion transcript obtained by NGS was submitted to a NCBI Blast search, yielding 100% mapping to *CIC*, transcript variant 1, mRNA and *DUX4*, transcript variant 1, mRNA.

Based on the findings of different chromosomal alterations in ES, 'ES-like' sarcomas and other URCSs, Antonescu *et al.* suggested that the *CIC*-rearranged sarcomas represent a specific genetically defined group of tumours with distinct biological behaviour.¹⁶ They analysed the clinical outcome of 57 patients with *CIC*-rearranged sarcomas, showing that the 2-year and 5-year overall survival (OS) were 53% and 43%, respectively.¹⁶ Very recently, Kao *et al.* demonstrated that the OS of *CIC*-rearranged sarcomas is less favourable when compared to *BCOR*-rearranged sarcomas, while no significant survival differences were observed between *BCOR*-rearranged sarcomas and ES or SS, respectively.³⁰ In addition, *CIC*-rearranged sarcomas are found to be less sensitive to standard Ewing chemotherapy regimens.^{1,31} In our series, the behaviour of 2/7 patients differed from the cases described in the literature. The lesion in the skin presented as a nodular mass within the dermis. After re-excision, no residual tumour was found and the patient, without undergoing any additional treatment, currently demonstrates no evidence of disease after a 24-month follow-up. This could be compared to superficial Ewing sarcoma, which represents a very rare entity in the ES family, but appears to have a relatively favourable prognosis.³² The same finding was described by Yoshida *et al.* in 2016.²³ The authors reported five patients with superficially located tumours (including one involving the skin and one in the subcutis) who were all alive and disease-free. In contrast, 12 of the 15 patients whose tumours involved deep soft tissues or organs, died of disease, demonstrating an aggressive clinical behaviour. Furthermore, our second patient with the tumour in the lymph node achieved a complete response to induction chemotherapy with VIDE (vincristine, ifosfamide, doxorubicin, etoposide) followed by VAI (vincristine, actinomycin D, ifosfamide), with no sign of disease after 30 months. The remaining patients (5/7, 71.4%) progressed rapidly under chemotherapy with lethal outcome in all cases within 6–15 months. As clear treatment guidelines for patients with *CIC-DUX4* fusion sarcomas are lacking, retrospective case series such as ours should be collected in prospective registries. Thus, important information and data on diagnostics, treatment and follow-up of respective patients could be collected and shared within the international sarcoma community in order to guide future clinical management of this patient cohort. Taking that into consideration together with the distinctive aggressive clinical behaviour in almost every case published, it is mandatory that these tumours are separated from ES, as well as from other URCSs and SBRCTs. Therefore, correct classification has an immense impact on the appropriate management and establishment of new treatment protocols for these patients.

In conclusion, our study confirms previous data that *CIC*-rearranged sarcomas present with distinct morphological, immunophenotypic and molecular features and, in most cases, demonstrate aggressive behaviour and poor outcome. However, this present study demonstrates the occurrence of this rare tumour entity at unusual sites. Interestingly, the tumour located purely in the dermis showed an indolent clinical course with 24 months of follow-up, supporting the prior observation that superficially located tumours may behave differently. In addition, the majority of our cases with molecularly confirmed *CIC-DUX4* rearranged sarcomas did indeed show the classical immunohistochemical profile with nuclear expression of WT1 (N-terminus) in association with CD99. Therefore, pathologists should consider *CIC*-rearranged sarcomas in the

differential diagnosis even in an unusual anatomical location. Molecular studies analysing *CIC* fusions should be performed to confirm the diagnosis. Larger studies on *CIC*-rearranged sarcomas, especially in superficial locations, could help to further evaluate the role of tumour location and/or size as a parameter of biological behaviour.

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