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ORIGINAL ARTICLE

Histological and immunohistochemical characteristics of undifferentiated small round cell sarcomas associated with *CIC-DUX4* and *BCOR-CCNB3* fusion genes

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Abstract *CIC-DUX4* and *BCOR-CCNB3* fusion-gene-associated small round cell sarcomas account for a proportion of pediatric small round cell sarcomas, but their pathological features have not been sufficiently clarified. We reviewed a large number of soft tissue tumors registered at our institution, retrieved the cases of unclassified tumors with a small round cell component, and subjected them to histopathological, immunohistochemical, and gene profile analysis. We reviewed 164 cases of unclassified tumors with a small round cell component and analyzed them by RT-PCR and FISH. Tumors positive for a specific fusion-gene were also subjected to histopathological and immunohistochemical examinations. We identified 16 cases of *BCOR-CCNB3*/*CIC*-associated (*CIC-DUX4* or *CIC* gene rearrangement-positive) sarcomas. These included seven *BCOR-CCNB3* sarcomas and nine *CIC*-associated sarcomas. Heterogeneous elements included a myxoid spindle cell component in three *BCOR-CCNB3* sarcomas and an epithelioid cell component in two *CIC*-associated sarcomas (one *CIC-DUX4*-positive and one *CIC-DUX4*-negative sarcomas). Mitotic activity was low in both heterogeneous components. By immunohistochemistry, in seven *BCOR-CCNB3* sarcomas expression of EMA was positive in two cases, of p63 in three, of CD56 in six, of TLE1 in seven, of NKX2.2 in two, of CCNB3 in seven, and of BCOR in six cases (one case could not be tested for BCOR). In nine cases of *CIC*-associated sarcoma, CD56 was expressed

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in five, alpha-smooth muscle actin in one, ERG in three, and CD99, WT1 and TLE1 each in eight cases. Both sarcoma types showed not only a small round cell component, but also a myxoid/epithelioid component with low mitotic activity.

Keywords Small round cell sarcoma · CIC-DUX4 · BCOR-CCNB3 · Fusion gene · Heterogeneous component

Introduction

Small round cell sarcoma (SRCS), which includes rhabdomyosarcoma and Ewing sarcoma (EWS), is one of the most important tumor groups in pediatric oncology. SRCS is characterized by small-sized round-shaped tumor cells with scant cytoplasm, known as "blue cells." The latest WHO classification categorizes undifferentiated/unclassified SRCS (USRCS) into undifferentiated/unclassified sarcoma with round cell morphology [1]. For a diagnosis of USRCS, a wide variety of neoplastic lesions, such as carcinoma, malignant melanoma, hematopoietic malignancy, and epithelioid-type soft tissue tumors, need to be ruled out.

Recently, recognition of *CIC-DUX4*, *CIC-FOXO4*, and *BCOR-CCNB3* fusion-gene-associated SRCSs has improved because of their aggressive biological behavior and novel gene aberrations [2–20]. However, it can be difficult to differentiate these sarcomas from other small round cell tumors, especially EWS family tumors, by means of morphology and immunohistochemistry. In addition, due to the rarity of these tumors' documentation of characteristics useful for diagnosis still needs to be improved.

We reviewed a large number of cases of soft tissue tumor registered at our institution, identified the specific-fusion gene-positive SRCSs among them, and examined histopathological, immunohistochemical, and gene-aberration profiles of the specific-fusion gene-positive SRCSs.

Materials and methods

Materials

Histopathology reports on 20,000 cases of soft tissue tumor, registered from 1975 to 2016 in the files of the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, were reviewed. The authors initiated this investigation by reviewing histologically all unclassified tumors with a small round cell component registered in our institution. Ewing sarcoma, rhabdomyosarcoma, and synovial sarcomas were excluded. In this way, we retrieved 164 cases of an unclassified tumor with a small round cell component, including USRCS and round cell tumor, not otherwise specified. Available tumors were prepared for further examination as described below.

We used three different techniques to identify *CIC-DUX4* and *BCOR-CCNB3* fusion-gene-associated small round cell sarcomas. As a first step, CCNB3 immunohistochemical staining (IHC) was performed, followed by fluorescent in situ hybridization (FISH) with a split *CIC* gene probe and finally by reverse transcription-polymerase chain reaction (RT-PCR) (Supplementary Fig. 1). The CCNB3-positive tumors were directly analyzed by RT-PCR and direct sequencing instead of *CIC* gene FISH.

Methods

Clinicopathological and histopathological review

We reviewed clinicopathological and histopathological data of *BCOR-CCNB3*/*CIC-DUX4* fusion-gene-positive or *CIC* gene-rearrangement-positive cases retrieved by the above processes, with a focus on age, sex, site, tumor cell shape, nucleoli, mitotic figures, growth pattern and capsule, as well as heterogeneous components other than round tumor cells.

Immunohistochemistry

Immunohistochemical staining was performed on all sarcomas with *BCOR-CCNB3*, *CIC-DUX4*, or *CIC* gene rearrangement. Formalin-fixed, paraffin-embedded tissue was sectioned at 3 μm thickness. The primary antibodies, their dilution, and the applied antigen retrieval procedure are summarized in Supplementary Table 1. Immunoreactivity was detected with the EnVision Detection System (DAKO, Glostrup, Denmark).

Fluorescent in situ hybridization

To assess rearrangement of the *CIC* gene, split dual-color FISH was performed on all available cases using ready-made FISH probes (GSP Laboratories, Kawasaki, Japan) on 3 μm thick sections. Fifty nuclei were counted to estimate the proportion of tumor cells having a *CIC* gene rearrangement. A split signal was defined by a distance between the red and green signals of at least twice the estimated signal diameter. *CIC* gene-rearrangement-positive tumors are called "*CIC*-associated sarcomas" in this report.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from frozen or paraffin samples using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) to prepare the first-strand complementary DNA. *CIC-DUX4* and *BCOR-CCNB3* fusion assays were

performed using newly designed primers (Supplementary Table 2) that specifically amplify the fusion gene transcripts. Each PCR product (5 μL) was loaded onto 2% agarose gel with ethidium bromide and visualized under UV illumination. The PCR products were also evaluated by direct sequence analysis using the BigDye Terminator method (version 1.1; Applied Biosystems, Foster City, CA, USA) to confirm the breakpoints of the fusion transcripts. All non-specific PCR bands were confirmed by direct sequence analysis.

Results

Clinicopathological and histopathological review

The clinicopathological data of all tumors are listed in Table 1 and Supplementary Table 3. *BCOR-CCNB3* sarcomas occurred in five females and two males with an age range of 12 to 32 years (mean 16.5 years); two were located in an extremity, four in the trunk, and one in the cauda equina. *CIC*-associated sarcomas occurred in five females and four males with an age range of 5 to 81 years (mean 37.1 years); four were located in an extremity, two in the trunk, one in the omentum, one in the iliac bone, and one in the tongue.

Histopathological findings and representative figures are summarized in Table 2 and Fig. 1. All seven *BCOR-CCNB3* sarcomas had similar histological features: short, spindle-shaped to round cells, scant cytoplasm, and irregular but not bizarre nuclei (Fig. 1a, b). Similarly, all nine *CIC*-associated sarcomas had similar histological features such as oval to round cells, scant cytoplasm, irregular but not bizarre nuclei, and conspicuous nucleoli (Fig. 1c, d). Three evaluable sarcomas of each type showed a multinodular and focally infiltrative growth pattern. As heterogeneous elements, a myxoid spindle cell component was observed in three *BCOR-CCNB3* sarcomas (Fig. 1e, f), and an epithelioid cell component was observed in two *CIC*-associated sarcomas (1 *CIC-DUX4*-positive sarcoma and 1 *CIC-DUX4*-negative sarcoma) (Fig. 1g, h). Myxoid areas showed proliferation of short spindle-shaped cells with bland or mildly atypical nuclei arranged in loose fascicular or haphazard pattern and embedded in a myxoid matrix. Epithelioid areas were composed of epithelioid tumor cells with mildly atypical nuclei arranged in a sheet-like pattern. Mitotic activity was low in the heterogeneous components: 5–76/10 HPF (mean 31.4) in the small round cell component vs 1–6/10 HPF (mean 3) in the myxoid spindle cell component in *BCOR-CCNB3* sarcomas. In *CIC*-associated sarcomas this was 4–58/10 HPF (mean 23.7) in the small

Table 1 Clinicopathological data

Case no.	Age	Sex	Site	Initial diagnosis	CIC-DUX4[a]	CIC-FOXO4[a]	CIC rearrangement[b]	BCOR-CCNB3[a]	CCNB3 IHC
BCOR-CCNB3-positive cases									
1	16	F	Rt. back	Monophasic synovial sarcoma	−			+	Nuclear
2	12	F	Cauda equina	MPNST	−			+	Nuclear
3	32	M	Rt. upper arm	Poorly differentiated synovial sarcoma	−			+	Nuclear
4	12	F	Lt. sacral bone	Undifferentiated small round cell sarcoma	−			+	Nuclear
5	14	F	Lt. lower leg	Undifferentiated small round cell sarcoma	−			+	Nuclear
6	15	F	Sacral region	Poorly differentiated synovial sarcoma or MPNST	−	−		+	Nuclear
7	15	M	Sacral region	Undifferentiated small round cell sarcoma	−	−		+	Nuclear
CIC-DUX4/CIC gene rearrangement-positive cases									
8	5	M	Hand	Undifferentiated small round cell sarcoma	−	−	+	−	−
9	11	F	Rt. lower leg	Undifferentiated small round cell sarcoma	+		N.A.	−	−
10	19	F	Omentum	Undifferentiated small round cell sarcoma	+		+	−	−
11	81	F	Lt. hand	Undifferentiated small round cell sarcoma	+		+	−	−
12	60	F	Tongue	Undifferentiated small round cell sarcoma	+		+	−	−
13	71	F	Rt. iliac region	Undifferentiated small round cell sarcoma	−	−	+	−	−
14	40	M	Rt. Buttock	Undifferentiated small round cell sarcoma	−	−	+	−	−
15	12	M	Lt. Pelvis	Undifferentiated small round cell sarcoma	−	−	+	−	−
16	35	M	Rt. Thigh	Undifferentiated small round cell sarcoma	−	−	+	−	−

IHC immunohistochemical stain, *N.A.* not available, *MPNST* malignant peripheral nerve sheath tumor

[a] RT-PCR and direct sequence analysis

[b] FISH analysis

Table 2 Histopathological findings

	BCOR-CCNB3	CIC-DUX4
Tumor cell shape	Short spindle to round shaped cells, scant cytoplasm, and irregular but not bizarre nuclei. Pseudolipoblasts (case 2).	Oval to round shaped cells, scant cytoplasm, irregular but not bizarre nuclei, and conspicuous nucleoli.
Heterogeneous component	Myxoid spindle cell component (cases 2, 4, and 5)	Epithelioid cell component (Cases 8 and 9)
Fibrous capsule	Incomplete	Incomplete
Growth pattern	Multinodular/focally infiltrative	Multinodular/focally infiltrative
Mitotic figure	Myxoid area 1–6/10 HPFs	Epithelioid area 3–6/10 HPFs
	SRC area 5–76/10 HPFs	SRC area 4–58/10 HPFs

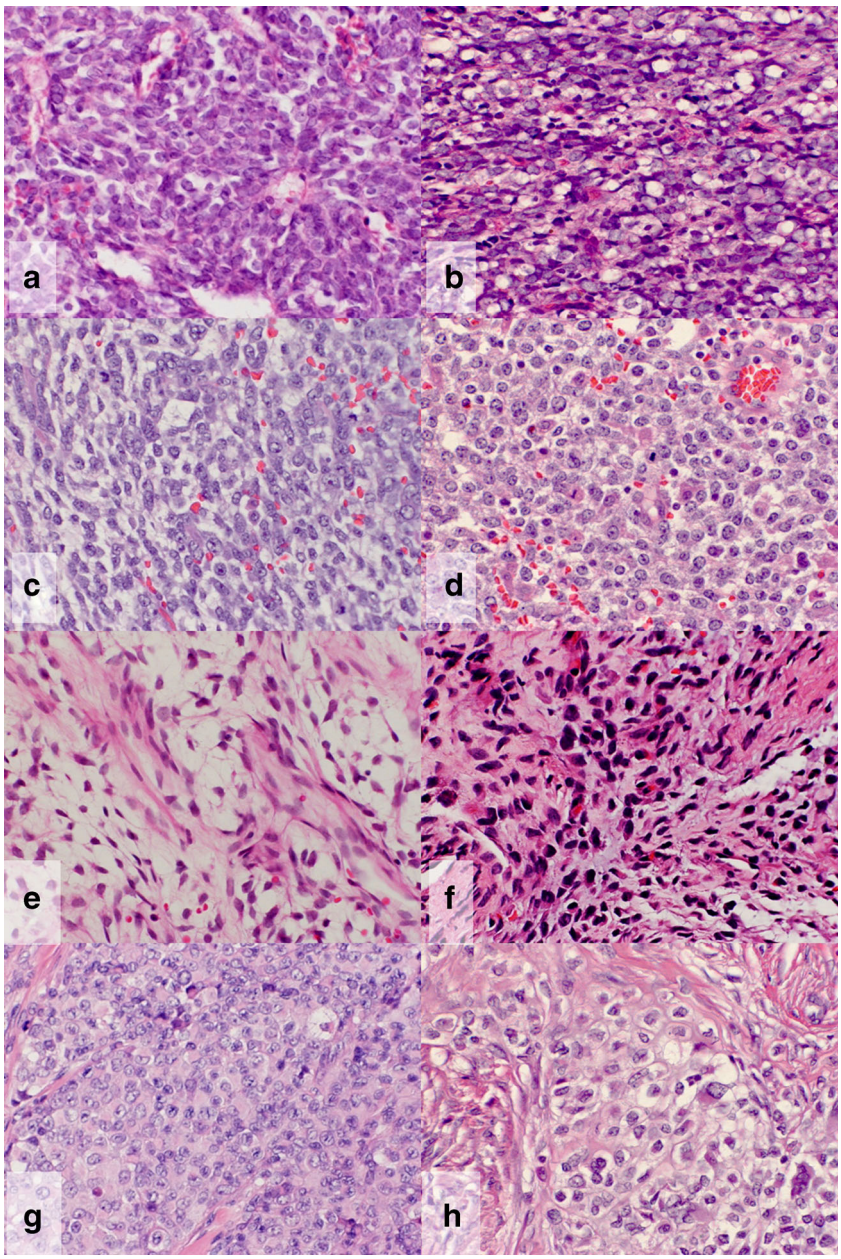

Fig. 1 Histopathological features of the small round cell tumors. **a**,**b** Small round cell component of BCOR-CCNB3 sarcoma, **c**,**d** small round cell component of CIC-DUX4 sarcoma, **e**,**f** myxoid component of BCOR-CCNB3 sarcoma, and **g**,**h** epithelioid cell component of CIC-DUX4 sarcoma

round cell component vs 3–6/10 HPF (mean 4.5) in the epithelioid cell component.

One *BCOR-CCNB3* sarcoma case initially presented as a tumor as a myxoid predominantly tumor and later recurred with an increased small cell component. Systemic metastases of the small round cell component and tumor death were confirmed in one case (case 2) by autopsy. One *CIC*-associated sarcoma (case 9) was resected under a clinical diagnosis of a benign cutaneous tumor, but subsequently diagnosed as undifferentiated small round cell sarcoma and additionally treated by chemotherapy. This tumor initially appeared to consist entirely of small round cell sarcoma, although the resected tumor after chemotherapy presented with a focal epithelioid cell component.

Immunohistochemistry

Representative images of immunohistochemical staining are presented in Fig. 2 and the results can be summarized as follows. All seven *BCOR-CCNB3* sarcomas were positive for CCNB3 (diffuse and nuclear in both myxoid and round cell areas) (Fig. 2a, b) and for TLE1 (diffuse and nuclear) (Fig. 2c), while six cases were positive for CD56 (Fig. 2d), two for EMA (weakly positive), three for p63, two for NKX2.2 (nuclear staining), and six for BCOR (nuclear staining) (Fig. 2e). All were negative for AE1/AE3, S-100 protein, alpha-smooth muscle actin, desmin, myogenin, NY-ESO-1, ERG, WT1, and CD99. Of nine *CIC*-associated sarcomas eight were positive for CD99 (Fig. 2f), five for CD56,

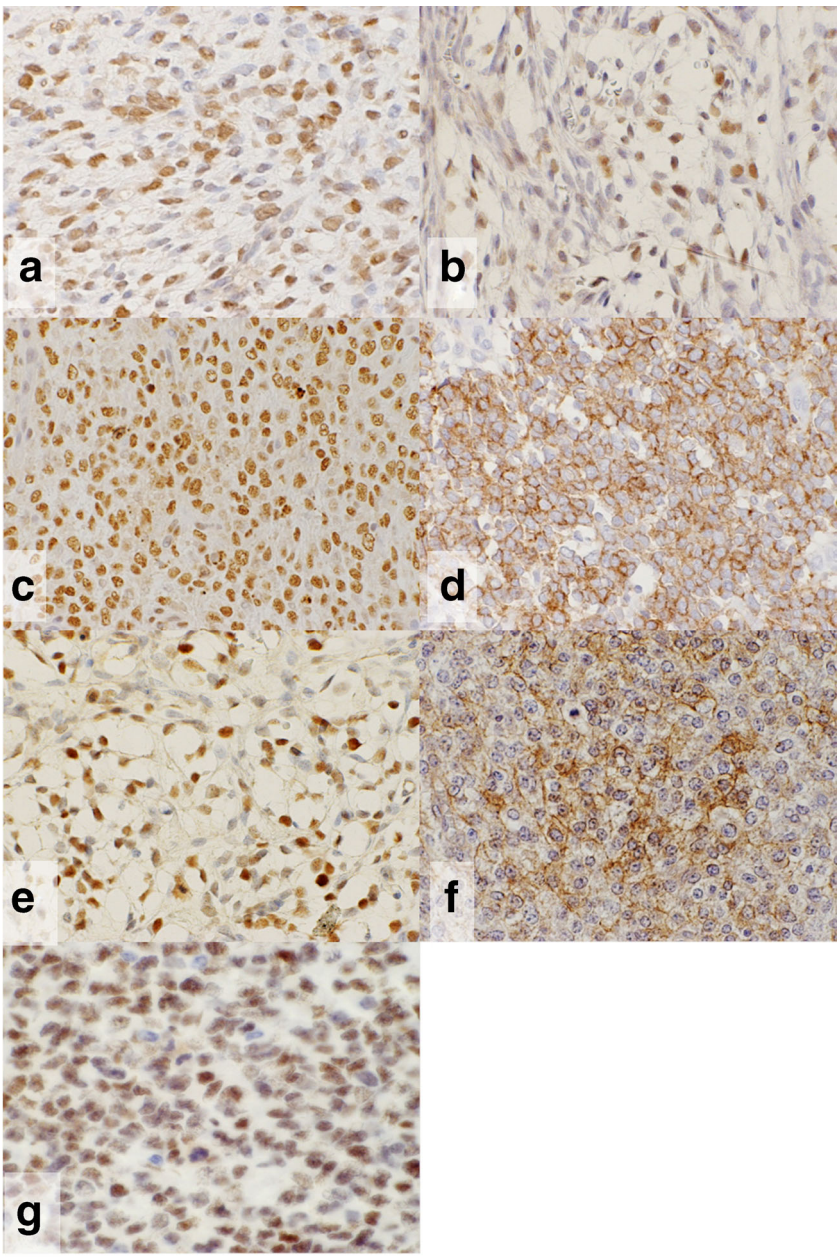

Fig. 2 Immunohistochemical features of the small round cell tumors. **a**,**b** BCOR-CCNB3 sarcoma with CCNB3 IHC (**a** round cell area, **b** myxoid area), **c** BCOR-CCNB3 sarcoma with TLE1 IHC, **d** BCOR-CCNB3 sarcoma with CD56 IHC, **e** BCOR-CCNB3 sarcoma with BCOR IHC, **f** CIC-DUX4 sarcoma with CD99 IHC, **g** CIC-DUX4 sarcoma with WT1 IHC

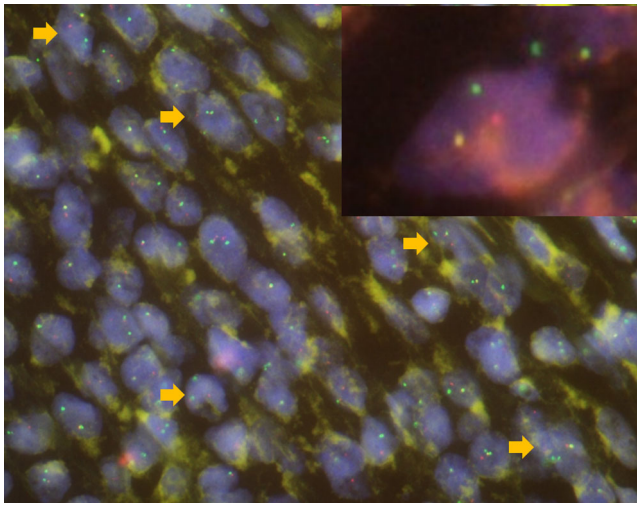

Fig. 3 *CIC* split FISH in case 9. Clear split signals of Texas red and fluorescein were observed

eight for WT1 (Fig. 2g), eight for TLE1 (weakly), one for alpha-smooth muscle actin (focally) and three for ERG. All were negative for CCNB3, AE1/AE3, EMA, p63, S-100 protein, desmin, myogenin, NY-ESO-1, NKX2.2 and BCOR. INI-1 was expressed all 11 tumors.

Fluorescent in situ hybridization

Red and green fluorescent signals were obtained. Eight cases of *CIC* gene-rearrangement-positive tumors were identified (Fig. 3). *CIC* gene rearrangement was detected in both round cell and epithelioid areas.

RT-PCR

BCOR-CCNB3 and *CIC-DUX4* fusion genes were detected in seven and four tumors, respectively. This was in cases 1–5 *BCOR* exon 15-*CCNB3* exon 5 (Fig. 4a), in cases 9, 10, and 12 *CIC* exon 20-*DUX4* exon 1 and in case 11 *CIC* exon 20-*DUX4* exon 2 (Fig. 4b). *BCOR-CCNB3*/*CIC-DUX4* was not detected in cases 8 and 13, and *CIC-FOXO4* was not detected in any case.

Discussion

CIC-DUX4 sarcomas were first identified in 2009 by Kawamura and Saito using chromosomal analysis, and were initially regarded as Ewing-family tumors [2]. In 2014, Specht et al.

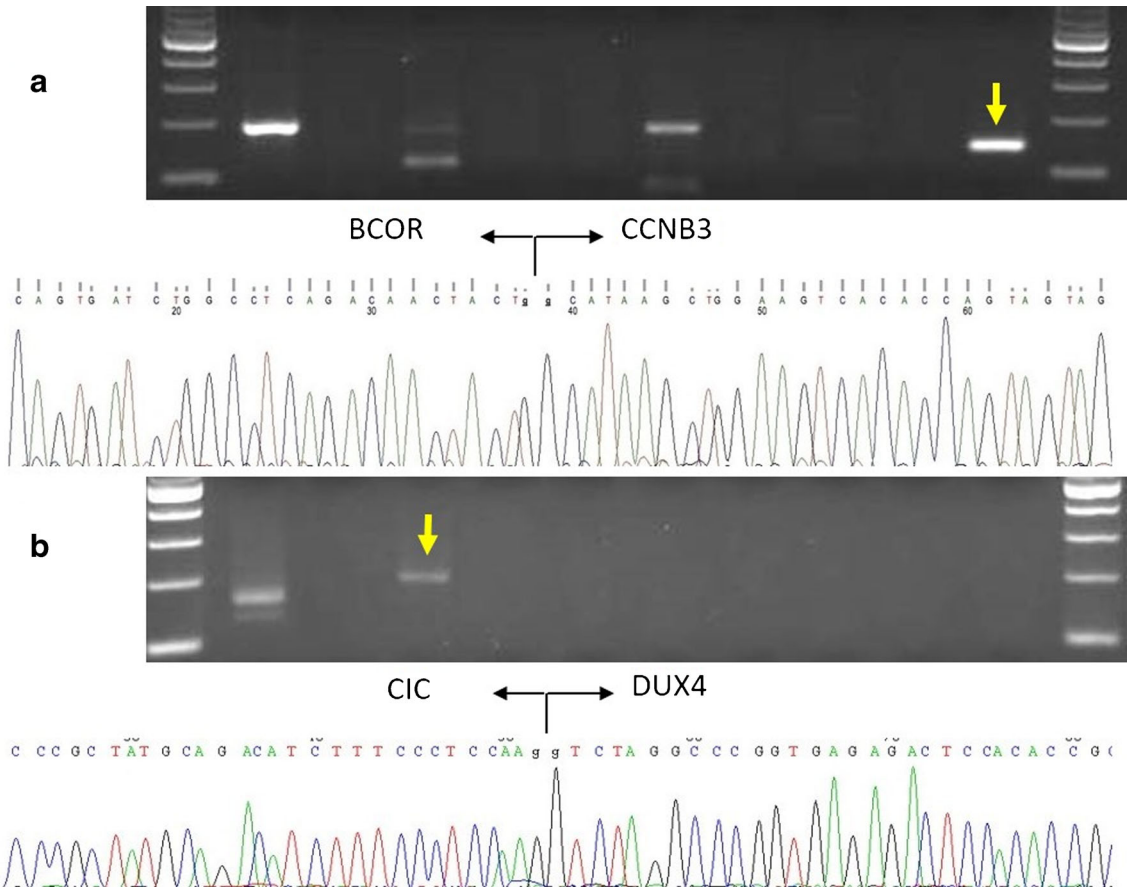

Fig. 4 *BCOR-CCNB2* and *CIC-DUX4* fusion gene analysis in **a** case 2 and **b** case 8. RT-PCR and direct sequencing were performed. The *arrows* mean true PCR bands of fusion gene products confirmed by direct sequencing

analyzed a larger number of these tumors and showed that their histopathological features and immunohistochemical profile are distinct from those of Ewing sarcomas [3]. *BCOR-CCNB3* sarcomas were first reported in 2012 by Pierron et al. and also considered as part of the Ewing family. Although these specific fusion gene-positive SRCTs were mostly classified as so-called atypical Ewing sarcoma or Ewing-like sarcomas in the past, recent research suggests that these two tumors are entities distinct from Ewing sarcoma. We reviewed the 164 unclassified tumors that had round tumor cells at least focally, and identified seven *BCOR-CCNB3* and nine CIC gene-rearrangement-positive sarcomas. Although both tumors types tended to occur in young patients, our study shows that the tumors, especially those *CIC* gene-rearrangement-positive, occur not only in pediatric patients but also regularly in adult or senior patients (mean age 37.1 years). Moreover, unlike in a previous investigation, our *BCOR-CCNB3* sarcomas series does not show male predominance. [19] In our series, the location of both *BCOR-CCNB3* and *CIC* gene-rearrangement-positive sarcomas varied and showed no predilection for bone. We contend that unclassified small round cell tumors include a small proportion of the above sarcomas.

BCOR-CCNB3- and *CIC*-associated sarcomas are histological mimics of Ewing sarcomas, although heterogeneous elements unlike EWS have also been reported as minor component [4]. Both tumor types can have myxoid areas as well as a prominent spindle component. In our study, both tumors were composed not only of small round cells but also of short, spindle- or epithelioid-shaped cells, proliferating in multinodular and sheet-like patterns with incomplete fibrous capsules. Interestingly, some of the tumors contained heterogeneous components, i.e. myxoid spindle cells and epithelioid cells in addition to small round cells. These features make for diagnostic pitfalls in the differentiation between *BCOR-CCNB3* sarcomas and malignant peripheral nerve sheath tumors, synovial sarcomas, and myxofibrosarcomas. They also make it difficult to differentiate between *CIC-DUX4*/*CIC* gene-rearrangement-positive sarcomas and carcinomas, malignant melanomas and epithelioid sarcomas. One of our *BCOR-CCNB3* cases was predominantly composed of myxoid spindle cells with small foci of small round cells. Moreover, one *CIC*-associated sarcoma presented as a wholly epithelioid cell tumor.

These heterogeneous components showed a lower proliferative activity than the small round cell components. One patient with a *BCOR-CCNB3* sarcoma (case 2) died due to systemic metastasis of the small round cell component, confirmed by autopsy. These observations underline the heterogeneous nature of both tumor types: a relatively low-grade component composed of myxoid spindle cell/epithelioid cells and a high-grade component composed of small round cells.

CCNB3 is a useful immunohistochemical marker for *BCOR-CCNB3* sarcoma [4]. For *CIC*-associated sarcomas, there is currently no validated immunohistochemical marker. We found in both tumor types focal to diffuse immunostaining for CD56 and TLE1, but no consistent staining for the other antigens. In *BCOR-CCNB3* sarcomas, we observed focal or diffuse immunoreactivity for CCNB3, TLE1, and NKX2.2. Most CIC-associated sarcoma cases were WT1-positive. We conclude that even though *BCOR-CCNB3* and *CIC*-associated sarcomas are histological and immunohistochemical mimics of Ewing sarcomas, immunostaining for CCNB3 and WT1 can be used to differentiate them from Ewing sarcoma.

TLE1 is a diagnostic marker of synovial sarcoma, one of the differential diagnoses of small round cell sarcoma, but its specificity has been contested. [21] We observed diffuse TLE1 expression in all cases, but not associated with the *SS18-SSX* fusion gene. As a consequence, poorly differentiated synovial sarcoma should be carefully ruled out by FISH or RT-PCR.

The differential diagnosis of *BCOR-CCNB3*/*CIC*-associated sarcoma covers a broad spectrum. In particular, translocation-associated small round cell tumors need to be excluded. FISH and CCNB3 immunohistochemical staining allowed us to differentiate *CIC-DUX4* and *BCOR-CCNB3* sarcomas from other SRCSs. In two tumors, we failed to confirm a *CIC* gene rearrangement by RT-PCR, which suggests that these tumors may have carried other *CIC*-associated gene aberrations. Genome analysis by FISH or RT-PCR would be necessary for a definitive diagnose of both tumor types. For screening, we recommend a *CIC* split FISH probe and CCNB3 immunohistochemical staining.

In our institution, a diagnosis of typical Ewing sarcomas was based in the past only on histological findings and extensive surveys of IHC and FISH did not include *BCOR-CCNB3*− or *CIC*–associated sarcomas (data not shown). Typical cases of Ewing sarcoma can be distinguished from these sarcomas by histology.

We conclude that *BCOR-CCNB3*− and *CIC*–associated sarcomas show a myxoid/epithelioid component in addition to a small round cell component. Moreover, these heterogeneous components showed low mitotic activity. The morphological heterogeneity of these sarcomas may be responsible for misdiagnoses and misguided therapy decisions.

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Compliance with ethical standards This study was approved by the Ethics Committee of Kyushu University (Nos. 25-111, 25-143) and conducted according to the principles embodied in the Declaration of Helsinki. Informed consent was obtained from the subjects or guardians.

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Conflict of interest The authors declare that they have no conflict of interest.

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