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An undifferentiated sarcoma with BCOR-CCNB3 fusion transcript - pathological and clinical retrospective study

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The *BCOR-CCNB3* positive sarcoma is a recently identified sarcoma morphologically and clinically similar to Ewing sarcoma in adolescents and young adults. The *BCOR-CCNB3* fusion transcript originates from a paracentric inversion on the X chromosome with an in-frame fusion between the last codon of *BCOR* and the exon 5 of *CCNB3* gene. We report morphological and molecular genetic analysis of 8 undifferentiated sarcomas positive for the *BCOR-CCNB3* fusion. Six of the eight *BCOR-CCNB3* positive sarcoma patients were male. Five of the eight patients were in their second decade of life (median of all patients 14 years at diagnosis). The bone marrow involvement was demonstrated in 2 of 4 patients tested. Detection of the fusion transcripts *BCOR-CCNB3* in the bone marrow suggests that patients with positive findings are at high risk of the tumor progression.

Key words: undifferentiated sarcoma, BCOR-CCNB3, CIC-DUX4, Ewing-like sarcoma, bone marrow

Undifferentiated sarcomas are a group of rare mesenchymal tumors lacking any evident line of differentiation detected by standard histopathological, immunohistochemical and molecular-genetic examinations [1, 2, 3].

Recently, Pierron et al. identified a new recurrent gene fusion of *BCOR* (encoding Bcl 6 interacting corepressor) and *CCNB3* (encoding the testis specific cyclin B3) in primitive and unclassifiable small cell sarcomas in adolescents and young adults [4]. This alteration originates from a paracentric inversion on the X chromosome with an in-frame fusion between the last codon of *BCOR* and the exon 5 of *CCNB3* gene. In vitro studies suggest that the fusion protein BCOR-CCNB3 has an oncogenic function and drives proliferation in this type of tumors [4, 5]. *BCOR-CCNB3* positive sarcomas showed a distinct gene expression profile when compared with classic Ewing sarcoma [4]. Tumors with the *BCOR-CCNB3* fusion gene appear to share some clinical and morphological overlap with the Ewing tumor family, including the frequent occurrence in long bones of adolescents and young adults, but also appear to have differences, including a strong male predilection and a potentially less aggressive clinical outcome [1, 4]. This is in contrast with the second "new" recurrent genetic alteration in a subset of "Ewing-like" undifferentiated sarcomas with the translocation t(4;19)(q35;q13) or t(10;19)(q26.3;q13) with fusion of the *CIC* gene (capicua homolog) and the *DUX4* gene (double homeobox 4) [1]. These types of sarcomas represent a clinically aggressive subset of undifferentiated sarcomas [6, 7].

In this study, we aimed to further characterize the morphological and clinical characteristics as well as the relative incidence of sarcomas with *BCOR-CCNB3* fusion in the group of undifferentiated sarcomas.

Patients and methods

Patient samples. Archival samples from 2000–2017 of undifferentiated sarcomas without a specific line of differentiation and without a detected specific translocation were screened for a *BCOR-CCNB3* fusion transcript using the RT-PCR method. In total, 71 cases of bone or soft-tissue sarcomas with final diagnosis: undifferentiated sarcoma, spindle cell undifferentiated sarcoma, Ewing sarcoma without *EWSR1* rearrangement, hemangiopericytoma and sarcoma not otherwise specified were tested for *BCOR-CCNB3* fusion

transcript by RT-PCR. Eight patients (6 males and 2 females) were positive for BCOR-CCNB3. They were 6 children and 2 adults. Because of morphological similarities of one of the *BCOR-CCNB3* positive cases with clear cell sarcoma of the kidney (CCS), six further CCSs of the kidney were added to the study. Ten sarcomas with other specific genetic mutations were analyzed as controls. They included 2 rhabdomyosarcomas with *PAX3-FOXO1* fusion, 1 sarcoma with *CIC-DUX* fusion transcript, 4 synovial sarcomas with the *SS18-SSX* fusion gene, 2 solitary fibrous tumors with *NAB2-STAT6* fusion and an alveolar soft part sarcoma with *ASPSCR1-TFE3* fusion. Table 1 summarizes the data of *BCOR/CCNB3* positive patients with regards to clinical presentation at diagnosis, primary histopathological diagnosis, treatment and the outcome. In two cases, post-chemotherapy samples were also available for analyses. In three cases, biopsies of distant metastases (kidney, sternum, retroperitoneum and ethmoidal sinus; lung and bone metastases; lung and lymph nodes in retroperitoneum) were available and included for evaluation beside the primary tumors. Also bone marrow aspirates obtained from 4 patients at the time of diagnosis were available for the testing.

Detection of BCOR-CCNB3 fusion transcript. Reverse transcription polymerase chain reaction (RT-PCR) was performed on formalin-fixed, paraffin-embedded tissue samples (49) except for 22 cases in which fresh frozen tumor tissue was available. Total RNA was extracted from fresh frozen material using Trizol (Invitrogen, Ltd., Carlsbad, CA, USA) and from formalin-fixed, paraffin-embedded sections using High Pure RNA Paraffin Kit (Roche Diagnostic, Mannheim, Germany) following the manufacturer's instructions for total mRNA isolation.

The complementary DNA (cDNA) was synthesized using MMLV Reverse Transcriptase (Invitrogen) from 10 μl total mRNA in a volume of 20 μl. RT-PCR was performed using 2x PCRBIO HS Taq Mix Red (PCR Biosystems Ltd., London, UK) with primers which generate a 140 bp product [5]. The primers used were as follows: BCOR-FFPE: 5′ GGC TCC ACC CCA GTG ATC T and CCNB3-FFPE: 5′ GGG TGT TTT GGA GGT GGT GGA T. For the first round of nested RT-PCR used for the detection of *BCOR/CCNB3* fusion gene in bone marrow aspirates, we used primers generating a 290 bp PCR product [4] with primers BCOR: 5′ GGC AGG TTT CTG CAA GTC TC and CCNB3: 5′ AGA TGC CTC CTC AGT GTT GG and for the second round we used primers BCOR-FFPE and CCNB3-FFPE. Amplification of a 208 bp amplicon of an abl house-keeping gene was used to confirm the presence of intact and amplifiable cDNA.

Direct Sanger sequencing was performed using Big Dye Terminator v 3.1 chemistry (LifeTechnologies) on positive cases.

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed paraffin-embedded biopsy specimens. Representative 3 μm thick sections of the diagnostic biopsy and recurrent samples were used for immunohistochemistry. The primary antibodies, clones and dilutions were: CD99 (O13, 1:500, NeoMarkers) and Ki-67 (MIB-1, 1:150, Dako), Vimentin (V9, 1:100, Dako), SM Actin (1A4, 1:50, Dako), Sarcomeric Actin (α-Sr-1, 1:75), EMA (E29, 1:100, Dako), Cytokeratin AE (AE1/AE3, 1:50, Dako), BCL2 (124, 1:100, Dako), Myogenin (F5D, 1:30, Dako), S100 (polyclonal, 1:5000, Dako), CD34 (QB/10, 1:30, BioGenex).

Results

Out of 71 undifferentiated sarcoma cases, RT-PCR on either frozen or formalin-fixed paraffin-embedded specimens followed by direct sequencing identified eight cases (6 children and 2 adults) that were positive for *BCOR-CCNB3* fusion transcript (11% of undifferentiated sarcoma cases). In all patients the fusion of the last codon of *BCOR* with the exon 5 of *CCNB3* gene was detected (Figure 1A, B, C). All other sarcomas with other specific chromosomal rearrangements (including renal CCSs) were negative for *BCOR-CCNB3* fusion transcript. Table 1 summarizes the data regarding clinical presentation at the time of diagnosis, primary pathological diagnosis, metastatic involvement and outcome of the patients.

Six out of eight *BCOR-CCNB3* positive sarcoma patients were males. Five out of eight patients were in their second decade of life (age ranged from 9 to 23 years, median 14 years). Six out of eight patients had localized tumors. Five out of the eight tumors arose in soft tissues, but in three of them there was an extensive local bone involvement. Tumors were distributed between axial (1 paraspinal, 2 chest wall) and

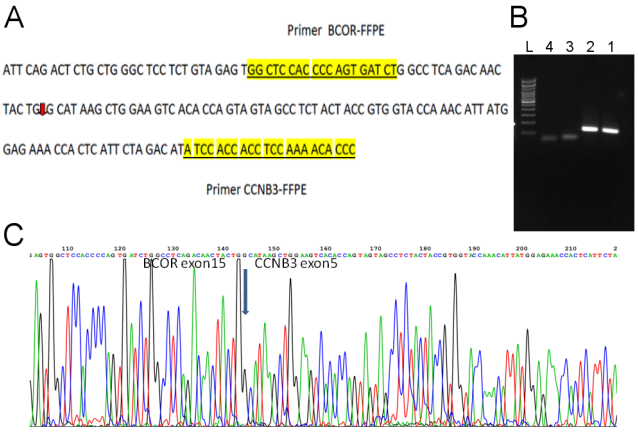

Figure 1. A) BCOR/CCNB3 partial cDNA sequence. The breakpoint between the exon 15 of BCOR gene and the exon 5 of CCNB3 is indicated with an arrow. B) RT-PCR detection of a specific 140-bp fragment of BCOR-CCNB3 fusion transcript. Total RNA was extracted from FFPE and subjected to RT-PCR using BCOR-FFPE and CCNB3-FFPE primers: Lane 1: *BCOR-CCNB3* positive sarcoma. Lane 2: *BCOR-CCNB3* fusion transcript positive control. Lane 3: negative control of reverse transcription (no template). Lane 4: negative control of PCR (water). L: DNA molecular weight ladder (100 bp ladder). C) Sequencing analysis of BCOR-CCNB3 positive sarcoma.

Table 1. Patients' characteristics.

Case	Age/Sex	Site	Primary diagnosis	Mets at diagnosis	FISH and/or PCR	Outcome at months
1	13/M	Sacral area with obliteration of the sacral canal (ST)	BCOR/CCNB3+ sarcoma	None	EWSR1- ETV6- ETV6/NTRK3- EWSR1/ATF1- EWSR1/CREB- CIC/DUX4-	NED (28)
2	14/M	Chest wall with the destruction of skeleton and promotion into the spinal canal (ST)	UDSa	Lung	SS18/SSX- EWSR1-	DOD (23)
3	14/M	Gastrocnemius (ST)	UDSa	None	PAX3-7/FOXO1-	NED (187)
4	20/F	Head of the femur (B, ST)	Low grade fibromyxoid sarcoma	None	SS18/SSX-	DOD (91)
5	15/M	Proximal fibula (B)	Mesenchymal chondrosarcoma	None		DOD (73)
6	23/M	Neck, compression of the trachea and esophagus (ST)	UDSa	None	EWSR1- SS18/SSX-	NED (137)
7	13/F	Chest wall (ST)	UDSa	None	SS18/SSX-	NED (139)
8	9/M	Lung	BCOR/CCNB3+ sarcoma	diaphragm	EWSR1-CIC/DUX4-	Therapy (1)

Age – age at diagnosis; M – men; F – female; B – bone; ST – soft tissue; UDSa – undifferentiated sarcoma; NED – alive, no evidence of disease; DOD – died of disease

extra-axial (1 neck, 3 lower limb, 1 lung) locations. Adjuvant and/or neoadjuvant therapy was administered to all patients, and it included chemotherapy and radiation therapy, one patient started with therapy at the time of this study. Three out of seven patients experienced a relapse at median time of 13.5 months (range 13–54 months) after the initial diagnosis. Four out of seven patients are alive in complete remission at 9; 168; 108 and 119.5 months, one patient is undergoing therapy. Three patients died, all from progressive disease 23.5; 91 and 73 months after the diagnosis.

Histopathologic features of *BCOR-CCNB3* positive sarcomas. Biopsies of primary tumors without previous chemotherapy were available in all eight patients. Generally, the tumors were hypercellular, although in one case (case 6) the cellularity assessment was complicated by an extensive intralesional hemorrhage. The tumors were composed of two cell types – uniform small ovoid to round cells and/or short spindle cells. Four cases were exclusively composed of discohesive ovoid/round cells (arranged in sheets similar to that in Ewing´s sarcoma), 2 cases were exclusively composed of short spindle cells arranged in short fascicles comparable to spindle cell sarcomas and the remaining two were formed by a both round and spindle cells. In four cases a perivascular condensation of the tumor cells was observed. A whirling pattern was seen in one case. Both, the ovoid and spindle cells possessed scant to moderate amounts of pale eosinophilic cytoplasm. The tumor cell nuclei were vesicular, oval or angulated in shape with finely dispersed chromatin. The mitotic activity varied significantly, being brisk in 5 cases (more than 10 mitoses per 10 high-power fields ×40) and relatively low in 3 tumors (with less than 5 mitoses per 10 high-power fields). None of the tumors displayed nuclear pleomorphism that would be compatible with the diagnosis of undifferentiated pleomorphic sarcoma. In all tumors, the cells were embedded within an edematous to myxoid matrix with focal fibrous component or even chondroid-like changes (in one case). The stroma of all tumors was richly vascularized with thin-walled, angulated and sometimes gaping vessels (Figure 2). The areas of tumor necrosis were observed in 3 out of 8 cases, both of them with a high mitotic rate.

Table 2. The results of the immunohistochemical study.

IHC marker	Number of positive cases
CD99	7 weak/8
Vimentin	4 positive/4
SM actin	0/4
Sarcomeric actin	0/2
EMA	1 focal positive/6
cytokeratins	0/7
BCL2	2/3
Myogenin	0/5
S100	2 focal positive/7
CD34	0/6

Immunohistochemically, all but one tumor expressed CD99, but the reaction was weak and/or focal unlike that seen in typical Ewing sarcoma. The proliferative activity as assessed immunohistochemically by Ki67 (MIB1) varied from 10% to 40%. Table 2 summarizes immunohistochemical staining of *BCOR/CCNB3* positive patients (Figure 4). Post-treatment samples were available for evaluation from two patients including metastatic tumors. In addition, the

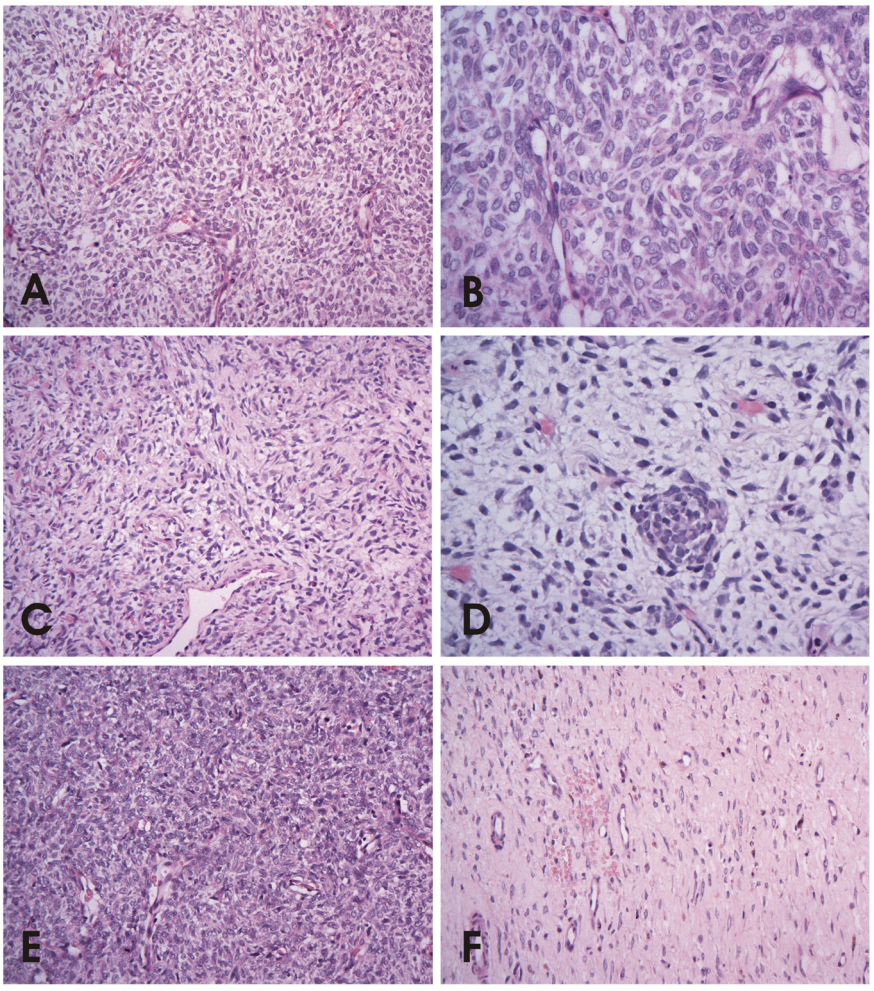

Figure 2. Histological features of BCOR-CCNB3 positive sarcomas. A, B) The most common morphological pattern consisted of discohesive uniform small ovoid to round cells with oval or angulated nuclei embedded within an edematous to myxoid matrix with thin-walled angulated vessels. C) In some tumors, short spindle cells were arranged in short fascicles within a stroma with focal fibrosis. D) a focal whirling pattern was seen in one case. E) pulmonary metastasis revealing ovoid to round cell morphology with significantly increased cellularity. F) in posttreatment samples, the tumors were hypocellular with bland spindle cell morphology. Hematoxylin and eosin stain, original magnification ×200 (A, C, E, F) and ×400 (B, D).

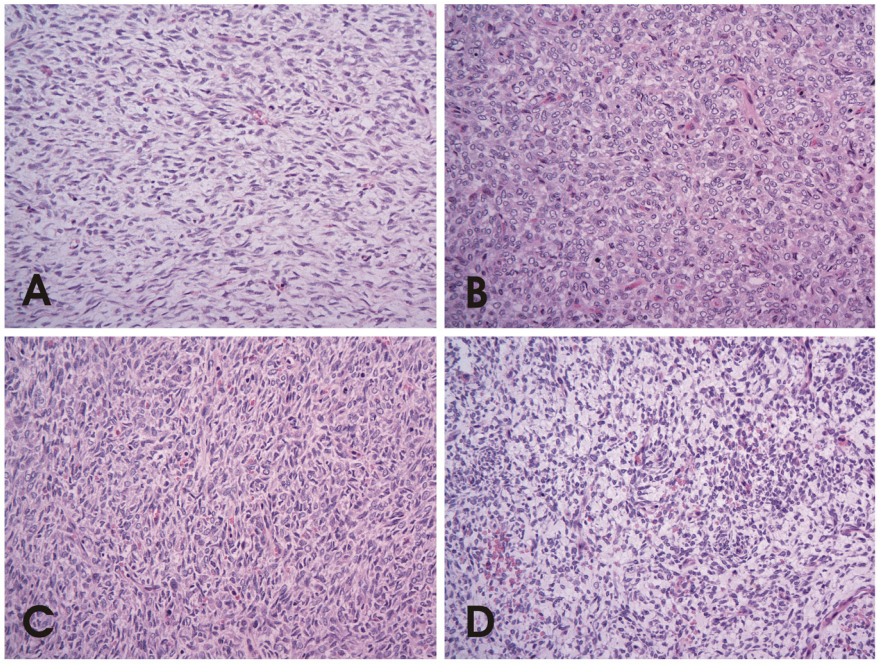

Figure 3. Case No. 4 – multiple tumors. Although all were shown to be BCOR-CCNB3 positive, the morphology of the tumors differed from spindle cell proliferation in primarily resected soft tissue tumor (A) to more epithelioid pattern with oval to round cell morphology similar to renal clear cell sarcoma in the right kidney (B) and also later in the left one (C). Myxoid changes with perivascular condensation of oval/round tumor cells with focal whirling pattern were evident in retroperitoneal metastasis (D). Hematoxylin and eosin stain, original magnification ×200 (A–D).

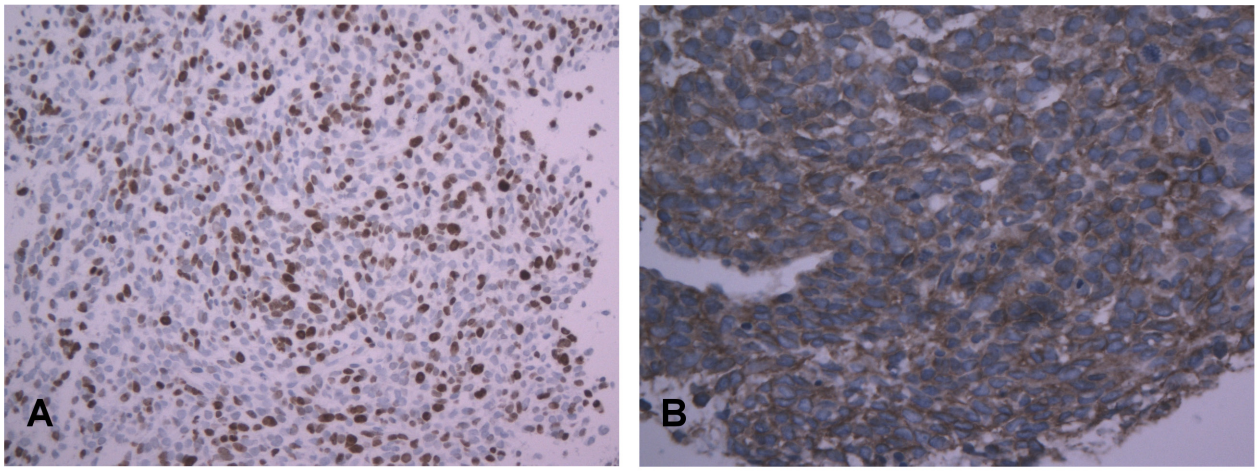

Figure 4. Immunohistochemical staining. Representative positive staining for A) Ki-67 and B) CD99 antigen expression.

case 4 presented with multiple tumors, and it is discussed in detail below (Figure 3).

Resection of the primary tumor shortly after the first-line chemotherapy was performed in two patients allowing repeated histopathological examination (cases No. 2 and 5). In both samples, the tumors revealed a decreased cellularity and a bland spindle cell morphology when compared to the primary examination of the tumors. The tumor cells were without atypia and mitotic activity. In both samples, the RT-PCR for *BCOR-CCNB3* fusion transcript was positive as in the primary lesions.

Resection of pulmonary metastatic tumors was available for the same two patients. Pulmonary metastases showed ovoid to round cell morphology similar to the primary tumor, but with a significantly increased cellularity and areas of necrosis. Overall survival of our *BCOR-CCNB3* positive patient is shown in Figure 5.

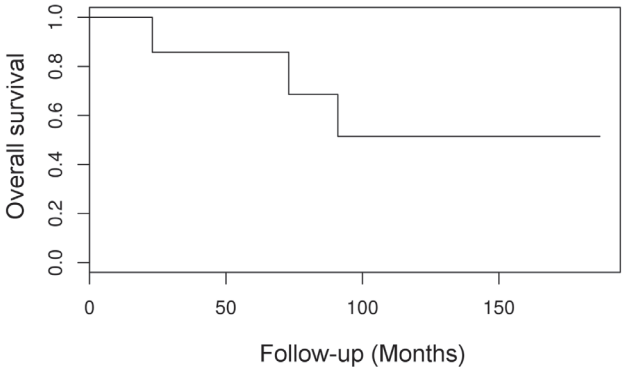

Figure 5. Overall survival estimated according to Kaplan-Meier method.

Case report – case No. 4. This patient was in her 20 years of age diagnosed with a tumor of the left femur with soft tissue involvement. The primary tumor consisted mostly of spindle cells arranged in fascicles with intercellular deposits of collagen with focal myxoid change. The diagnosis of a low grade fibromyxoid sarcoma was established at the time of diagnostic surgery. No metastases were revealed by routine staging examinations at that time.

Four years later (3 years after cessation of the therapy) the patient presented with a second tumor localized in the right kidney with a different morphology: the tumor consisted of a mixed population of mitotically active ovoid to round cells with pale to clear cytoplasm and of spindle cells which were focally arranged in long fascicles. The tumor was diagnosed as renal clear cell sarcoma. The patient underwent nephrectomy of the right kidney and combined chemoradiotherapy. However, one year later (two months after the therapy ending), the patient had a clear cell sarcoma also in the left kidney. Nine months later (in the 26th week of pregnancy), numerous bone metastases (sternum, sphenoids, ethmoids and the scull of the base) were revealed by MRI; the radiotherapy of the cranium was performed and the metastasis in sternum was resected due to the tumor progression. One month later, during the cesarean section, metastases were found in retroperitoneum and omentum and these lesions were resected. All metastases showed ovoid to round cell morphology similar to the kidney tumors, but with a significantly increased cellularity. The last investigated metastasis was obtained from the previously irradiated ethmoidal sinus. This sample showed, similar to other post-treatment tumors, rather a hypocellular pattern with elongated spindle cells in myxoid stroma. Four months later, numerous metastatic lesions in the skeleton were revealed by PET/CT examination and the patient died of the disease progression 91 months after the initial diagnosis.

All available tumor samples including patient metastases were tested for *BCOR-CCNB3* fusion transcript by RT-PCR. And despite the atypical localization of tumors in kidneys and varying morphology of the tumors, all were confirmed as *BCOR-CCNB3* positive sarcomas.

Bone marrow involvement. Bone marrow aspirates obtained from 4 patients at the time of diagnosis were tested. The samples of bone marrow aspirates (right and left iliac trepan biopsies) in case 2 were positive for *BCOR-CCNB3* fusion transcript. The sample from the right sided aspirate was positive in the second round PCR and that from the left in the first round PCR. The bone marrow aspirate from the left iliac trepan biopsy obtained at the time of diagnosis from patient case 5 was positive in the second round RT-PCR. Both patients developed metastatic disease and died of the tumor progression. Bone marrow samples of patients (cases 3 and 7) were negative in both rounds of RT-PCR. Both patients are in a complete clinical remission 14.5 and 10.5 years from the initial diagnosis.

Discussion

Our study disclosed eight cases (11%) of *BCOR-CCNB3* positive tumors in the group of 71 patients diagnosed with "undifferentiated sarcoma". The higher prevalence of BCOR-CCNB3 positive cases when compared to other studies (10 of 134 in the study of Puls et al, 26 of 631 by Cohen-Gogo et al) is probably caused by focusing on pediatric tumors with *EWSR1* and *SS18* negative rearrangement [5, 8]. When compared with typical Ewing sarcoma, a finding of an "atypical morphology" is crucial for identifying *BCOR-CCNB3* positive sarcoma. In correlation with previous studies, *BCOR-CCNB3* positive sarcomas exhibited a higher degree of heterogeneity in nuclear size and morphology in comparison with the typical uniform cellular pattern in Ewing sarcoma. In agreement with previous published data and with our observations as well, it is clear that *BCOR-CCNB3* positive tumors can be morphologically characterized by ovoid plump cells intermixed with areas of spindle cellularity and myxoid stroma.

In contrast to the published series that reported the majority of cases to be primary bone tumors, five out of eight cases in our study were found in soft tissues, one patient had primary pulmonary localization of the tumor. The *BCOR-CCNB3* positive sarcomas in our study were seen predominantly in older children (median age 14 years). These findings are in concordance with other reports [1] showing that this sarcoma is more common in teenage males. Six out of eight *BCOR-CCNB3* positive patients in the series were males and together with the cases published to date we observed a clear male predominance of BCOR-CCNB3 positive sarcomas.

The patient described as case 4 shows a similarity between *BCOR-CCNB3* positive sarcomas and renal clear cell sarcomas (CCSs) which are characterized by internal tandem duplication of *BCOR* (ITD BCOR CCSs) or *YWHAE-NUTM2* gene fusion similarly as a primitive myxoid mesenchymal tumor of infancy and an undifferentiated round cell sarcoma of infancy [9]. All of our investigated renal CCSs, similar to sarcomas with other specific fusion transcripts were negative for the *BCOR-CCNB3* fusion transcript.

The similarities of our case 4 and renal CCSs were in clear cell morphology of the tumors investigated microscopically, and also in the clinical behavior with bone metastatic disease. The patient developed metastases in bones, as it is commonly observed in CCSs. The appearance of a tumor in the femur four years before the kidney lesion was diagnosed is difficult to explain. As all the tumors had the same *BCOR-CCNB3* fusion we consider them as a part of one disease. Therefore, we can speculate in two different aspects. The femur lesion could be considered a metastatic disease from undiscovered kidney tumor and the treatment of the femur lesion might have influenced a further progression of the kidney tumor which remained hidden until the time of diagnosis. In addition to that, in the recent publication by Wong et al., authors identified the first renal clear cell sarcoma carrying the *BCOR-CCNB3* fusion, which is typical for *BCOR-CCNB3* positive undifferentiated sarcomas [10]. On the other hand, it cannot be ruled out that the primary tumor of the femur metastasized first to the right kidney and then to the left kidney, and to other sites as mentioned above. Discussing this patient, we wanted to call the attention to this rather unusual neoplasm with clear cell morphology and *BCOR-CCNB3* genetic hallmark.

Fusion transcripts generated through chromosomal translocations have been demonstrated to represent excellent targets for monitoring minimal residual disease in hematological malignancies [11, 12, 13]. Many studies suggest a direct correlation between the number of residual tumor cells and the prognosis of the patients. Dissemination of tumor cells in the bone marrow (BM) and peripheral blood (PB) has also been observed in patients with solid malignancies [14, 15]. Assessment of bone marrow dissemination by light microscopy has a limited sensitivity. Using RT-PCR for detection of the tumor specific aberrations we can reveal minimal amounts of tumor cells contaminating BM. We examined BM samples from 4 patients with undifferentiated *BCOR-CCNB3* sarcomas at the time of diagnosis. Three patients were presented with a localized disease, one had distant metastases at presentation. *BCOR-CCNB3* positive cells were detected in the BM of one patient with localized disease and in one with clinically detectable metastases at diagnosis. The former patient with localized sarcoma and BM involvement at the molecular detection level developed metastatic disease and died of the tumor progression, the latter patient also died of the disease dissemination. The prognostic value of the identification of fusion transcripts in bone marrow aspirates or in peripheral blood samples has to be proven by further prospective studies. Detection of the fusion transcripts *BCOR-CCNB3* in the bone marrow suggests that patients with positive findings are at high risk of the tumor progression. The frequency of BM RT-PCR positive cases in clinically non-metastatic patients was similar to that described in patients with Ewing sarcomas [16, 17, 18], in rhabdomyosarcomas [19, 20, 21], or in neuroblastoma [22].

Routine molecular analysis of *BCOR-CCNB3* fusion using RT-PCR is the most reproducible diagnostic approach for this type of undifferentiated sarcoma and would provide information about bone marrow involvement and help to determine risk stratification outcome.

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