A Case Series of BCOR Sarcomas With a New Splice Variant of *BCOR/CCNB3* Fusion Gene

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Abstract. *Background/Aim:* Undifferentiated round cell sarcomas are a heterogeneous group of sarcomas. Identification of *BCOR* alterations, such as *BCOR/CCNB3* and *BCOR/MAML3* fusion genes and *BCOR* ITD has recently contributed in the precise diagnosis of these neoplasms, defining a new entity of the current classification of soft tissue and bone sarcomas. *BCOR* sarcomas share both morphological and genetic characteristics distinct from Ewing sarcomas. The scope of our study was to retrospectively identify *BCOR* sarcomas and find the correlations with the clinical outcome of these patients. *Patients and Methods:* Histopathology and immunohistochemistry of pediatric tumor samples were combined with molecular testing (PCR) and fluorescent in situ hybridization to find *BCOR* sarcomas. *Results:* We, herein, present our experience with *BCOR* sarcomas in a referral center of Greece. Moreover, we report in one case the detection of a variant *BCOR/CCNB3* fusion not previously described. *Conclusion:* We are the first to report a splice variant of *BCOR/CCNB3* which reveals the central position of *BCOR* in the oncogenesis of these tumors, furthermore we highlight the importance of molecular

diagnostics in Ewing-like sarcomas and discuss the current treatment options for this rare entity.

Undifferentiated round cell sarcomas are a heterogeneous group of mesenchymal malignant tumors which until recently were classified as Ewing sarcomas (1). Genetic characterization of these tumors revealed *BCOR* alterations to be a crucial element of their molecular pathology. Identification either of *BCOR* internal tandem duplication (ITD) or *BCOR/MAML3* and *BCOR/CCNB3* fusion genes; is important for the diagnosis of this group of tumors (2-7). Interestingly, these tumors not only share genetic similarities and overlap in gene expression level, but also share morphological features (8). These genetic and morphological similarities are seen both in soft tissue and bone undifferentiated sarcomas, thus making this subgroup an important new entity in the current classification of sarcomas.

BCOR genetic alterations have been also described in other sarcomas (9-13). The identification of *YWHAE/FAM22* fusion gene in endometrial stromal sarcomas (ESS) is mutually exclusive for *BCOR* internal tandem duplication (14-17). Similarly to ESSs, clear cell sarcomas of the kidney with *YWHAE/FAM22* fusion gene, do not present with *BCOR* genetic alterations neither *BCOR* (ITD) nor *BCOR/CCNB3* fusion gene, which appears to be the genetic alternative leading to cyclin D1 up-regulation and high mitotic activity (17). A recent publication raises the possibility of a new category of high-grade uterine sarcomas with *BCOR* ITD (15).

BCOR genetic alterations have been described in hematological malignancies including Non-Hodgkin's B and T cell lymphoma, acute myeloid leukemia, pediatric acute myeloid leukemia and promyelocytic leukemia (18-22).

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Table I. Clinicopathological characteristics of the 5 patients with BCOR sarcomas.

Lab number	Gender	Age at diagnosis	Primary tumor site	Operation	Perioperative/adjuvant chemotherapy	Relapse	First-line chemotherapy	RT	Status	OS (months)
1094-13	M	14	Tibia	Excision	VIDE, VAI	N	N/A	N	Alive	61.7
527-13	M	2	Spine	Biopsy	VIDE, VAI	Y	N/A	N	Dead	21.6
1318-18	M	10	Paraspinal	Excision	VIDE, VAI	N	N/A	Y	Alive	14.3
2756-09	M	3	Lung	Excision	IRS	Diaphragm, ribs	ICE	Y	Alive	118.3
1561-13	M	13	Spine	Excision	VIDE, VAI	N	N/A	Y	Alive	74.1

M: Male, N: no, Y: yes, N/A: non applicable, VIDE: vincristine, etoposide, ifosfamide, doxorubicin, VAI: vincristine, actinomycin-d, ifosfamide, ICE: ifosfamide, etoposide, carboplatin, IRS: vincristine, actinomycin-d, cyclophosphamide, OS: overall survival.

Herein we present a case series of patients with soft tissue undifferentiated sarcomas sharing a BCOR translocation. Moreover we are the first to report a new splice variant of BCOR/CCNB3 fusion gene, which was detected in one of the tumors.

Patients and Methods

Patients. Our series comprised 10 pediatric patients with the probable histologic diagnosis of a Ewing-like sarcoma. Five of these patients were diagnosed with a BCOR sarcoma. The clinicopathological data of those patients are summarized in Table I. Interestingly, all 5 patients were males indicating the importance of BCOR localization to X chromosome. Median age was 8.4 years (range=2-14 years). Localization of the primary tumor was the spine and paraspinal tissues in 3 cases, tibia and lung to each one of the other patients. Staging before surgery revealed localized disease for all the patients without signs of metastases. Four out of them are still alive after the administration of perioperative/adjuvant chemotherapy. One patient received first line chemotherapy for relapse. One patient died 21 months after the diagnosis. Overall survival (OS) for each patient ranged from 13 months to 118 months. The treatment schemes that were offered are Euro-Ewing (vincristine, cyclophosphamide, doxorubicin alternating with ifosfamide, etoposide) with or without RT to 4 patients and one patient received IRS (vincristine, doxorubicin, cyclophosphamide) in the perioperative/adjuvant setting. ICE protocol (ifosfamide, cyclophosphamide, etoposide) was given as first-line treatment option. Written consent for publication was given from all 5 cases presented. This study was approved from the ethics committee of Aghia Sofia Children's Hospital and Panagiotis and Aglaia Kyriakou Children's Hospital.

Histopathology. Formalin-fixed, 4-micrometer-thick, paraffin embedded tissue sections of all tumors were immunostained using the Bond polymer refine kit in the Bond max, autostainer, Leica Biosystems as described, for the detection of: CD99 (clone 12E7, DAKO, Santa Clara, CA, USA; clone O-13, Invitrogen 1:100, Carlsbad, CA, USA), Fli-1 (polyclonal, Santa Cruz, TX, USA, rabbit antimouse), CD56/NCAM (clone 123C3, Monosan/Sanbio, 1:50, Uden, the Netherlands). Neural and neuroendocrine markers were: Synaptophysin (clone SY38, Monosan, Sanbio), neurofilaments [clone 2F11(70+200 kda) DAKO], S-100 (clone, Biogenex, 1/100, Fremont, CA, USA), SOX-10 (Rabbit monoclonal 1/20, Cell Marque,

Darmstadt, Germany), GFAP (clone GA-5, Novocastra, Buffalo Grove, IL, USA). Epithelial markers were: cytokeratin AE1/AE3 (1/100, DAKO, Santa Clara, CA, USA), cytokeratin 8.18 (1/100, Novocastra), cytokeratin 7 (1/50, Monosan), cytokeratin19 (1/50, Novocastra), EMA (clone E29, DAKO), smooth muscle actin (clone 1A5, 1/200, DAKO), desmin (clone D33, DAKO), myogenin/Myf-4 (clone 1/20, Novocastra), CD34 (clone QBend 10, DAKO 1/50), bcl-2 (clone 100/D5, Leica, Buffalo Grove, IL, USA), c-kit (polyclonal, DAKO), and INI-1/BAF47 (Becton Dickinson, Franklin Lakes, NJ, USA), ERG (prediluted, DAKO), TLE-1 (monoclonal 1/50, BIOSB, Santa Barbara, CA, USA), SATB2 (rabbit monoclonal 1/50, BIOSB). The immunohistochemical expression was evaluated by two independent pathologists (K.S., A.P.), as the percentage of positive tumor cells in the most stained areas out of the total number of counted tumor cells.

Genetics. Polymerase chain reaction (PCR) and Sanger sequencing. Total RNA extraction was performed according to the manufacturer's instructions, by using FFPE sections with NucleoSpin total RNA FFPE Mini Kit (Macherey-Nagel, GmbH, Duren, Germany). SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers were used to reverse transcribe approximately 500 ng of total RNA. cDNA was subjected to PCR analysis, for the detection of the BCOR/CCNB3 fusion gene using Platinum TAQ Polymerase (Invitrogen), with specific primers, as listed:

BCOR3.1F: 5'-GGCAGGTTTCTGCAAGTCTC-3'
CCNB3.1R: 5'-AGATGCCTCCTCAGTGTGG-3'
CCNB3.2R: 5'-GCTTCAGACGGGACCTCTTC-3'
GAPDH-F: 5'-AACAGCCTCAAGATCATCAGC-3'
GAPDH-R: 5'-GGATGATGTTCTGGAGAGCC-3'

PCR conditions were: 40 cycles of 94°C for 50 sec, 60°C for 40 s and 72°C for 1 min. PCR products were analyzed in agarose gel electrophoresis. GAPDH was also amplified as housekeeping gene to monitor the integrity of the isolated RNA material. The PCR products were excised from the gel and subjected to Sanger sequencing using the same primers.

Fluorescence in situ hybridization (FISH). The pathologist selected representative 4-µm-thick formalin-fixed, paraffin-embedded tissue sections applied to silinized slides to be analyzed for the detection of translocations involving the BCOR gene at Xp11.4. A section from normal tissue was used as negative control. Pretreatment of the slides included, baking at 60°C for 4 h, deparaffinization in 2

Figure 1. A. HE sections (i-vi). (i) Spindle and clear cell morphology. (ii) Round and spindle cells, with myxoid stroma and presence of giant cells. (iii) Hyalinization. (iv) Clear cell nuclei. (v) Pseudoadenoid morphology. (vi) Hyalinization and giant cells. B. Immunohistochemistry (vii-x). (vii) CD56. (viii) CD99. (ix) FLI-1. (x) Ki67/MIB-1 >40% of the nuclei.

changes of fresh xylene for 10 min at room temperature, dehydration for 5 minutes in 100% (twice), 90% and 70% ethanol solutions and air-drying before application of the pretreatment kit (Zytolight FISH-Tissue kit, by Zytovision, Bremerhaven, Germany) according to the manufacturer's instructions. For hybridization procedures, the FISH probe "BCOR split FISH probe by Abnova" was used, according to the manufacturer's instructions. Two post-hybridization washes were performed in $2\times$ SSC/0.3%NP40. Slides were air-dried and counterstained using 10 μ l DAPI. Hybridization signals were analyzed on a Zeiss Axioplan, Oberkochen, Germany, fluorescence microscope equipped with the appropriate filter combination and the ISIS digital imaging system and software (Metasystems, Altlusheim, Germany). Microscopic evaluation of FISH signals met the criteria: a) no overlapping cells are counted, b) split (break-a-part) signal is counted when the separation of

orange and green signals is at least twice the size of one hybridization signal, c) when the number of nuclei found to carry the break apart signals exceeds the cutoff of the control sample set at 20%, then this sample is considered to be positive for BCOR gene translocation.

Results

Round cell primitive morphology was the predominant feature of the 5 neoplasms with the BCOR alteration. However, cell spindling, clear cell cytology and myxoid stroma, which were common in all tumors, were suggestive features of undifferentiated sarcomas with BCOR alterations. It is interesting that the tumor of the tibia and the paraspinal

Table II. FISH and PCR results for the detection of BCOR fusion.

Lab number	PCR	FISH
248/09	+	–
107/2013	+	+
188/2013	+	+
261/2013	+	+
454/19	+	
Alternative splicing	+	

tumor showed a characteristic considerable pericellular wire-like hyalinization. In all five tumors, the heterogeneous CD99 immunohistochemical expression, mainly cytoplasmic, the diffuse membranous expression of CD56 and absence of Fli-1 detection supported the morphological suspicion of a Ewing like sarcoma. The paraspinal tumor with the new BCOR fusion gene variant co-expressed TLE-1 and SATB2, as described in few sarcomas with a BCOR alteration (23) (Figure 1A and B).

The results of FISH and PCR are presented in Table II. FISH analysis was positive to four of the 10 tested samples (Figure 2). Additional confirmation of BCOR fusion was performed with PCR analysis detecting BCOR/CCNB3 chimeric gene. The fusion gene, as expected, was between BCOR exon 15 and CCNB3 exon 5. One case was positive with PCR, but not with FISH analysis.

However, in one of these 5 samples (paraspinal tumor), an alternative PCR product was detected. Sequencing of the latter revealed a not previously reported fusion between BCOR exon 15 and a part of the CCNB3 exon5. The break point in CCNB3 exon 15 was between nucleotides 898-899(NM_033031). The new fusion gene was found to have a 4-nucleotide deletion with a synchronous 2 nucleotide insertion in the BCOR exon15 - CCNB3 exon5 junction leading to an in-frame fusion product (Figure 3). In order to detect this alternatively spliced variant we designed a new reverse primer (CCNB3.2R). The predicted fusion protein product contains the usually involved BCOR exon 15 amino acid except the deletion of the last amino acid (aa186, AAH63536.1). The CCNB3 part of the fusion protein predicted to be 88 amino acids (aa) shorter. The deleted part was from aa113 to aa200 (NP_149020).

Discussion

BCOR sarcomas define a new category of sarcomas characterized by BCOR genetic alterations. Internal tandem duplication of the BCOR gene and formation of the fusion genes BCOR/CCNB3 or BCOR/MAML3 are the genetic alterations identified with diagnostic meaning (2, 3, 5, 6, 9, 11, 14, 24).

Figure 2. Interphase FISH using BCOR brake-apart probe labeled in Spectrum-Green and Spectrum-Red show one bicolor signal in normal cells, whereas in BCOR-CCNB3 sarcoma cells, two separated unicolor signals (white arrowheads) are seen.

During the last 5 years a number of solid tumors were sent to our laboratory for genetic analysis to diagnose a Ewing-like sarcoma. All specimens were subjected to qPCR or PCR analysis, for the detection of any of the defined chimeric genes whose expression characterizes Ewing sarcomas. When all other tests were negative, tumors were analyzed for the presence of the rare BCOR/CCNB3 chimeric gene. The case series reported above includes undifferentiated round cell sarcomas, where pathologist's reports raised the possibility of a non-Ewing sarcoma. From the 10 cases analyzed only 5 were positive for BCOR fusion with FISH and PCR. The discrepancy between PCR and FISH that was found in one of the samples tested is possibly related to differences in sensitivity and/or sample heterogeneity, which is described repeatedly in molecular diagnostics of sarcomas (25).

BCOR is implicated in chromatin remodeling and histone modification being part of the PRC1.1 complex (26, 27). The C-terminal BCOR PUF domain binds to the RAW domain of PCGF1 (26, 27). The predicted BCOR-CCNB3 fusion oncoprotein includes the CCNB3 cyclin box. It must be highlighted that all BCOR genetic abnormalities related to sarcomagenesis, including BCOR-CCNB3, BCOR-MAML3 and BCOR ITD, involve the last exon of BCOR, which encodes the PUF domain. The variant reported here, includes the N terminal of BCOR encoding PUF domain.

On the other hand, CCNB3's product was predicted to be 88-aa shorter. The deleted part does not directly affect any of the functionally important CCNB3 domains namely, the Nuclear Localization Signals (located in aa 99-109, aa251-

Figure 3. Electrophoresis gel showing the 700 bp fusion variant. On the right sequencing of the product.

Table III. BCOR sarcomas treated with non-Ewing backbone chemotherapy, reported in the recent literature.

Author	RT	Chemotherapy regimen	Survival*	Outcome
Peters <i>et al.</i>	Y	Ifosfamide/doxorubicin	11	NED
Peters <i>et al.</i>	Y	Ifosfamide/doxorubicin	94	NED
Peters <i>et al.</i>	Y	Vincristine/actinomycin-d/ cyclophosphamide	93	NED
Puls <i>et al.</i>	Y	Ifosfamide/doxorubicin/ methotrexate	78	NED
Kao <i>et al.</i>	N	OS regimen	60	AWD
Kao <i>et al.</i>	Y	Ifosfamide/doxorubicin	26	NED
Kao <i>et al.</i>	N	Ifosfamide/doxorubicin	91	NED
Kao <i>et al.</i>	Y	Ifosfamide/cisplatin	22	DOD
Kao <i>et al.</i>	Y	Epirubicin-Cyclophosphamide- Nedaplastin	16	AWD
Kao <i>et al.</i>	N	Cisplatin/doxorubicin	N/A+	N/A+
Kao <i>et al.</i>	Y	Cisplatin/methotrexate	47	DOD

Y: Yes, N: no, RT: radiotherapy, NED: no evidence of disease, AWD: alive with disease, DOD: dead of disease. *Survival is measured from the date of diagnosis since the last follow up or date of death. +Recent case, outcome and survival were not available.

aa320, aa471-aa511), coiled-coil domain, cyclin box and leucine zipper. The deleted region resides in a non-evolutionary conserved region of the CCNB3 gene (aa119-aa366).

The detection of BCOR alterations in several sarcomas has shed light to a new category of neoplasms with distinct molecular and morphological characteristics (3). Together with CIC, YFAM and NUTM1 fusion genes, BCOR alterations seem to change the classification of sarcomas, revealing new categories and depicting the importance of genetic alterations and genetic background in the development of many sarcomas (8). It is clear that BCOR's implication in oncogenesis and signal networks of sarcoma

cells is highly important to better understand the biology of these tumors. Given the paucity of therapeutic options when treating these sarcoma patients (28-31), it is intriguing to speculate that BCOR might be an important candidate as a therapeutic target.

BCOR sarcomas are the so-called "3Bs tumors" (Boys, Bones and Better prognosis), which is confirmed to our small cohort. All our patients were males indicating the importance of BCOR localization to X chromosome. The localization of the primary tumor was bone in 4 of our patients. It should be highlighted that 4 out of the 5 cases are alive with OS ranging from 14 to 118 months.

Furthermore, it should be noted that the patient who was offered the IRS protocol (vincristine, doxorubicin, cyclophosphamide) is a long survivor. Interestingly, the literature includes 11 cases of BCOR sarcomas that received other than Ewing sarcoma backbone chemotherapy regimens (2, 4, 14) (Table III). Peters *et al.*, treated 3 patients with rhabdomyosarcoma (RMS) (vincristine/dactinomycin/cyclophosphamide) and non-rhabdomyosarcoma soft tissue tumors (NRSTS), (doxorubicin/ifosfamide) chemotherapy regimens with NED after 11, 93 and 94 months since the last follow-up (2). Puls *et al.*, included one patient who received ifosfamide, methotrexate and doxorubicin being alive and with no evidence of disease (NED) after 78 months of follow-up (4). Kao *et al.*, in their publication reported 7 cases of patients who were offered osteosarcoma regimen (1 case), ifosfamide-adriamycin (2 cases), ifosfamide-Cisplatin (2 cases), epirubicin-cyclophosphamide-nedaplastin (1 case) and doxorubicin-cisplatin (1 case) (14). Available survival data range from 16 to 91 months of OS (14). Additionally, expression data from BCOR sarcomas analyses show a different profile from Ewing sarcomas (3, 14). Taken together the differences in the morphology, the genetics and the expression profile and combining them with the generally better outcome of BCOR sarcomas, it is reasonable to raise

the question whether BCOR sarcomas should be treated as Ewing sarcomas, or a different approach might be adequate. Targeting Ewing sarcomas with tyrosine kinase inhibitor Cabozantinib has been beneficial for patients (32, 33); however BCOR sarcomas due to their rarity are not yet studied to any clinical trials. BCOR sarcomas ideal therapeutic strategy is yet to be discovered.

To conclude, we herein present our experience from a reference Center in Greece in the detection of BCOR sarcomas. We are the first to report a splice variant of BCOR/CCNB3 which reveals the central position of BCOR in the oncogenesis of these tumors. Further studies to fully understand the functional aspects of BCOR alterations are needed.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

ED, AK were the writers of the article. Data acquisition was performed by AK, NT, AP, KS, VT, ES, MB, SP and AKa. Molecular tests were performed by AP, DM, MaL. Histology was performed by AP and KS. ED and AK contributed to conception and design of the study. Manuscript editing and the revision were performed by ML, KS, MB, AKa and FZ. All Authors have read and approved the final manuscript.

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