Intra- and extra-cranial BCOR-ITD tumours are separate entities within the BCOR-rearranged family

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Abstract

BCOR-ITD tumours form an emerging family of aggressive entities with an internal tandem duplication (ITD) in the last exon of the BCOR gene. The family includes cerebral tumours, termed central nervous system BCOR-ITD (CNS BCOR-ITD), and sarcomatous types described in the kidney as clear cell sarcoma of the kidney (CCSK), in the endometrium as high-grade endometrial stromal sarcoma, and in the bone and soft tissue as undifferentiated round cell sarcoma or primitive myxoid mesenchymal tumour of infancy. Based on a series of 33 retrospective cases, including 10 CNS BCOR-ITD and 23 BCOR-ITD sarcomas, we interrogated the homogeneity of the entity regarding clinical, radiological, and histopathological findings, and molecular signatures. Whole-transcriptomic sequencing and DNA methylation profiling were used for unsupervised clustering. BCOR-ITD tumours mostly affected young children with a median age at diagnosis of 2.1 years (range 0–62.4). Median overall survival was 3.9 years and progression-free survival was 1.4 years. This dismal prognosis is shared among tumours in all locations except CCSK. Histopathological review revealed marked differences between CNS BCOR-ITD and BCOR-ITD sarcomas. These two groups were consistently segregated by unsupervised clustering of expression (n = 22) and DNA methylation (n = 21) data. Proximity between the two groups may result from common somatic changes within key pathways directly related to the novel activity of the ITD itself. Conversely, comparison of gene signatures with single-cell RNA-Seq atlases suggests that the distinction between BCOR-ITD sarcomas and CNS BCOR-ITD may result from differences in cells of origin.

Keywords: CNS BCOR-ITD; BCOR-ITD sarcomas; CCSK; ESS; clustering; transcriptome; methylome

Received 29 June 2021; Revised 3 December 2021; Accepted 9 December 2021

No conflicts of interest were declared.
Introduction

BCOR-internal tandem duplication (ITD) tumours are a heterogeneous group of neoplasms comprising sarcomas and neuroepithelial tumours of the brain. Sarcomas are described in the kidney as clear cell sarcomas of the kidney (CCSK) [1–8], in the endometrium as high-grade endometrial stromal sarcomas (HG-ESS) [9–11], and in the bone [12,13] and soft tissue as undifferentiated round cell sarcoma (URCS), but also including primitive myxoid mesenchymal tumour of infancy (PMMTI) [12,14,15]. In the central nervous system (CNS), tumours with BCOR-ITD alteration were first identified by DNA methylation analyses and termed high-grade neuroepithelial tumours BCOR (HGNET-BCOR) [16–24]. They are now referred to as CNS BCOR-ITD in the latest (2021) version of the WHO classification of brain tumours [25].

BCOR-ITD tumours mostly affect infants and young children, except for the endometrial tumours, which occur exclusively in adult patients. The radiological and histopathological features of BCOR-ITD tumours are not sufficiently specific to ascertain the diagnosis. Definitive diagnosis is achieved by molecular analysis through RNA-Seq and DNA methylation studies or direct sequencing of the characteristic in-frame partial duplication of BCOR exon 15 by RT-qPCR.

The diversity of anatomical locations questions whether a single molecular alteration, i.e. BCOR-ITD, drives similar oncogenic pathways throughout the various tumour entities. CNS BCOR-ITD tumours were analysed through their methylation profiles, which proved to be specific [17,22,26]. BCOR-ITD sarcomas were also found to localise within the group of BCOR-rearranged tumours based on their transcriptomic profile [27]. Recently, hierarchical clustering based on the methylome of 1,077 prototypical sarcomas also identified the BCOR-rearranged sarcomas as a coherent group [28].

To our knowledge, the molecular profiles of BCOR-ITD sarcomas and CNS BCOR-ITD have not been compared to date. Here, we report a retrospective study including BCOR-ITD tumours from all described locations, with emphasis on the clinical, therapeutic, radiological, and histopathological aspects. We describe the features shared among all locations as well as the key differences, in particular the prognosis, histopathology, and immunohistochemical profiles. From this diversity emerges two distinct molecular signatures as revealed by unsupervised analysis of expression and methylation profiles: cranial versus extracranial BCOR-ITD tumours.

Materials and methods

Study population

The population under study was defined as all patients with a BCOR-ITD tumour diagnosed in France over the period 2010–2020, including retrospective diagnoses on archival material. Within this population, our cohort comprised all cases of BCOR-ITD identified by RNA-Seq analysis, as performed routinely for diagnostic purposes. This analysis was performed either at Institut Curie (Paris, France) or Centre Léon Bérard (Lyon, France), which were the sole centres to provide physicians with diagnostic RNA-Seq. All BCOR-ITD cases identified either by RT-PCR or droplet-digital PCR in other centres were not included because expression data were not available. Thirty-three cases were identified: 7 CCSK, 4 HG-ESS, 10 CNS BCOR-ITD, 10 URCS, and 2 bone tumours. Five cases of URCS were reported previously in the study by Watson et al, although without clinical, radiological, and histopathological details [27]. Another case of URCS (P16) and one case of CNS BCOR-ITD (P23) were also previously reported (in Refs [12] and [16], respectively).

This study was approved by the Institutional Clinical Research Board of Institut Curie, and complied with the reference methodology MR-004 from the Commission Nationale de l’Informatique et des Libertés (CNIL). All fastq files have been deposited in the European Genome-phenome Archive (URL pending).

Clinical review

The 33 patients were cared for within 17 medical centres in France and 1 centre abroad (Padova Hospital, Italy). Data collection in all 18 centres followed ethical rules approved by the Clinical Research Board of Institut Curie. Medical record could be collected for 31 cases (10 CNS BCOR-ITD, 7 CCSK, 4 HG-ESS, 8 URCS, 2 bone tumours).

Radiological review

Radiological images at diagnosis were available for 23 cases (10 CNS BCOR-ITD, 5 CCSK, 2 HG-ESS, 5 URCS, 1 bone tumour). Depending on the location, radiological review was based on centralised analysis of ultrasound, computed tomography (CT) scans, and/or magnetic resonance imaging (MRI). Images were qualitatively analysed using the viewer of the picture archiving and communicating system (PACS) of Institut Curie (Carestream-Philips v 12.1.6).
Histopathological review and immunostaining
Central pathology review was performed jointly by a neuropathologist (AT-E) and a pathologist expert in paediatric sarcomas (AG) on 21 samples (9 CNS BCOR-ITD, 4 CCKS, 2 HG-ESS, 6 URCS). Of note, two bone tumours could not be evaluated histologically. Unstained 3-μm-thick slides of formalin-fixed paraffin-embedded (FFPE) tissues were obtained and submitted for immunostaining. Conditions of use of all primary antibodies are described in the Supplementary materials and methods. External positive and negative controls were used for all antibodies and stains.

Paired-end RNA sequencing
Paired-end RNA sequencing was performed on 22 frozen samples in Institut Curie (Paris, France) and 11 FFPE samples in Centre Léon Bérard (Lyon, France). Frozen and FFPE samples correspond to distinct cases: samples analysed in Paris and Lyon were obtained from different tumours. Details are given in supplementary material, Figure S1.

Identification of the BCOR-ITD sequence
For frozen samples, gene expression values were extracted after alignment on Hg19 (GRCh37) genome annotation with SALMON (v0.13.1). For fusion gene discovery, sequencing reads were injected into various available tools: Defuse (v0.6.2), FusionCatcher (v1.0), STARFusion (v2.5), Arriba, and FusionMap integrated in Oshell (v10.0.1.50). Only fusion transcripts supported by at least two tools with at least two split reads were considered. Search for expressed mutations was performed after alignment on Hg19 genome (GRCh37) with STAR, using HaplotypeCaller and Mutect2 (GATK4). The BCOR-ITD cases were identified by hierarchical clustering, using the R package Cluster v2.0.3 using the Pearson correlation distance and the Ward clustering method. Each sample, represented by its Salmon expression matrix, was compared with a panel of well-characterised tumours including BCOR-rearranged and BCOR-ITD tumours. A sample was labelled ‘BCOR-ITD’ if it segregates within the BCOR-ITD family and is negative after the fusion search. The result was then confirmed by RT-qPCR: total RNA was transcribed as cDNA (Multiscribe Reverse Transcriptase with random hexamers; Life Technologies, Carlsbad, CA, USA) and BCOR exon 15 was amplified with primers 5'-3' ACCATTGCGAGCGCAAAAT and 3'-5' ATGACACATGACACAGGATTAAC.

A similar approach was applied to FFPE samples, as detailed in the Supplementary materials and methods.

Methylation analysis
DNA was extracted and converted by bisulphite treatment for 21 samples from both frozen (n = 15) and FFPE (n = 6) samples, without overlap between the two sample types. DNA methylation profiling was performed as described [29] at the Integragen Core Facility (Paris, France) with Illumina HumanMethylation450 BeadChip array (450k array) (Illumina, San Diego, CA, USA).

Clustering studies
Two segregation methods were applied separately to the RNA-Seq dataset and the methylome dataset.

The RNA-Seq dataset was compiled from the 22 available frozen samples (8 CNS BCOR-ITD, 6 CCKS, 7 URCS, 1 bone, no HG-ESS). Paraffin samples were excluded as there is no guarantee that their expression matrices are comparable with frozen samples, given the differences in the methods employed to produce libraries. The DNA methylation dataset was compiled from 21 BCOR-ITD samples (9 CNS BCOR-ITD, 6 CCKS, 6 URCS). Control samples were sourced differently depending on the dataset. Tumours in the RNA-Seq dataset were compared with 181 control sarcoma cases extracted by Watson et al [27], and 165 control brain tumours from an in-house collection. Control cases for the methylome dataset were taken from the publicly available database provided by the German Cancer Research Center DKFZ and the Heidelberg University Hospital [28,29]. They included 310 cerebral tumours and 212 sarcomas. Methods for data alignment, scaling, and segregation are detailed in Supplementary materials and methods.

Differential expression analysis on RNA-Seq dataset
Reads were aligned with STAR 2.6.0a [30] to the human genome (gencodeV19). Gene expression values (FPKM = fragments per kilobase per million reads) were computed with Cufflinks v2.2.1 [31] on Ensembl Release 75 and further normalisation between samples was done using quantile normalisation (log2 (FPKM + 2), R/Bioconductor package limma [32]). Differential analysis was performed with the Welch’s t-test and the P value was adjusted using the Benjamini–Hochberg correction. For differential analysis,
significance was given by $p < 0.05$ and $|\text{fold-change}| > 1.5$. All control cases used in this analysis were obtained from in-house clinical cohorts: medulloblastomas (21 samples), ependymomas (10 samples), and Ewing sarcomas (31 samples). The list of significantly different genes was further analysed using the ToppGene suite, in particular ToppFun (Cincinnati Children’s Hospital Medical Center).

**BCOR-ITD signatures from single-cell RNA-Seq atlases**

Differential expression analysis (see previous section) between 8 CNS BCOR-ITD and 15 BCOR-ITD sarcomas using two different fold-change thresholds (1.5 and 2.5) led to 356 and 117 differentially expressed genes (DEGs) enriched in CNS BCOR-ITD and 129 and 54 DEG enriched in BCOR-ITD sarcomas, respectively. These gene sets were compared with two human single-cell/single-nuclei RNA-Seq atlases, namely Cao et al and Han et al [33,34], taken from the https://descartes.brotmanbaty.org website (‘sampled data’) and from the Gene Expression Omnibus website under GEO accession number GSE134355, respectively. They contain over 70 and 100 carefully annotated cell identities, respectively, extracted from adult, foetal, cord, and induced pluripotent stem (iPS) tissue. Counts in both atlases were natural log-normalised with the NormalizeData function in Seurat 4.0.3 with default options and signatures for all gene sets were calculated on the transformed data using Seurat’s in-built AddModuleScore method, which accounts for background expression. Signature intensities can thus be assimilated to an average natural log-fold change with the background expression (i.e. a signature intensity of 0.4 can be considered as a fold-change of about $e^{0.4} = 1.5$). Dot plots were built such that dot colour represents the average signature intensity for a given cell type and dot size the number of cells with a signature intensity strictly higher than 0 (brought to the [0, 1] range for each row).

**GSEA on RNA-Seq dataset**

The GSEA (Gene Set Enrichment Analysis) software was used as described originally in [35] after downloading from https://www.gsea.msigdb.org/gsea. Gene sets representing canonical pathways were selected in the Molecular Signature Database (MSigDB). The list is provided in Supplementary materials and methods.

**Results**

Clinical features, therapeutic data, and survival analyses

Thirty-three cases of BCOR-ITD tumours were identified and distributed as follows: 10 URCS, 10 CNS BCOR-ITD, 4 HG-ESS, 7 CCSK, and 2 bone tumours (supplementary material, Figure S1). Clinical and therapeutic data were collected for 31 cases. The demographic features as well as the disease presentation at diagnosis are shown in Table 1 and, patient by patient, in supplementary material, Table S1. The disease was mainly localised at presentation, with the exception of HG-ESS. The median age at diagnosis was 2.1 years (range 0–62.4). Adult cases were found exclusively in the HG-ESS and bone groups. The overall sex ratio was balanced, 1:1.1 (M:F), without considering the four female cases of HG-ESS. The differences in sex ratio within each group were not significant. Overall, these findings show that demographic features are homogeneous among all locations, except the adult cases of endometrium and the bone sites.

The initial therapeutic strategy was mainly multimodal including most often the first-line surgery and...
Distinctive features of intra- and extra-cranial BCOR-ITD tumours

Figure 1. Legend on next page.

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adjuvant chemotherapy. Eighteen cases underwent complementary radiotherapy. Details are shown in supplementary material, Table S2. The median follow-up was 50.3 months (range 1–200), with a median OS of 47 months (95% CI 38–not applicable [NA]) (Figure 1A,C) and median PFS of 17 months (95% CI 12–NA) (Figure 1B,D). Our data suggest that this overall dismal prognosis did not affect the CCSK group: OS and PFS were significantly higher in this subgroup (Figure 1E,F).

Radiological features

Imaging results are summarised in supplementary material, Table S3 and detailed in supplementary material, Text S1, for 23 reviewed cases. All BCOR-ITD tumours were well-circumscribed, non-infiltrative, heterogeneous masses of variable volumes (median volume 128 ml [range 6–918]). Using CT scan or MRI, the injection of a contrast medium brings out the density/signal moderately and heterogeneously in most cases (supplementary material, Figure S2). BCOR-ITD sarcomas (URCS, CCSK, bone, HG-ESS) differed from CNS BCOR-ITD, the latter displaying more necrosis (9/10 versus 5/13), more calcification (1/10 versus 1/13), and more haemorrhage (8/10 versus 4/13).

Histopathological and immunohistochemical analyses

The histopathological findings were available for 21 cases (9 CNS BCOR-ITD, 4 CCSK, 2 ESS, and 6 URCS) and are summarised in Table 2. Complete description is provided in supplementary material, Text S2. All cases presented as highly cellular tumours, well-delimited from host tissue. The predominant histopathological pattern varied according to the subtype: ependymoma-like or fascicular in CNS BCOR-ITD (Figure 2A,E), fascicular or diffuse in URCS (Figure 2B), diffuse in HG-ESS (Figure 2C), and alveolar in CCSK (Figure 2D). Of note, in our series, a myxoid background was encountered only in CNS BCOR-ITD and not in sarcomas, excluding PMMTI in our series. Additional patterns were frequently identified, such as perivascular pseudorosettes in CNS BCOR-ITD cases, non-constant expression of the glial markers GFAP and Olig2 (Figure 2M), with varying degrees of distribution.

**Table 2. Main histopathological features of the BCOR-ITD tumours**

| Number of cases | Histological pattern | Cytology     | Pseudorosettes | Microcystic background | Haemorrhage | Calcification | Necrosis                          | Mitotic count per 10 hpf |
|-----------------|----------------------|--------------|----------------|-------------------------|-------------|--------------|-----------------------------|--------------------------|
| CNS BCOR-ITD    | 9                    | EPN-like     | Spindle cells  | 9/9                     | 7/9         | 6/9          | 2/9                         | 5–62                     |
|                 |                      | Fascicular   |                |                         |             |              |                             |                          |
|                 |                      | Fascicular   |                |                         |             |              |                             |                          |
|                 |                      | Fascicular   |                |                         |             |              |                             |                          |
| URCS            | 6                    | Fascicular   | Round-to-ovoid | 1/6                     | 0           | 4/6          | 0                           | 1.7–13.7                 |
|                 |                      | Diffuse      | Spindle cells  | 2/2                     | 2/2         | 2/2          | 0                           | 14–21                    |
|                 |                      | Diffuse      |                |                         |             |              |                             |                          |
| HG-ESS          | 2                    | Diffuse      | Spindle cells  | 1/2                     | 0           | 1/4          | 0                           | 0                        |
|                 |                      | Alveolar     | Round-to-ovoid | 0                      | 2/2         | 2/2          | 0                           | 14–21                    |
| CCSK            | 4                    | Diffuse      | Spindle cells  | 0                       | 1/4         | 0            |                            | 0–18                     |

Table 2. Main histopathological features of the BCOR-ITD tumours

EPN, ependymoma; hpf, high-power field.

Figure 1. Prognosis of BCOR-ITD tumours. Kaplan–Meier plots showing OS and PFS of the whole BCOR-ITD cohort (A, B) and the five histological subgroups (C, D). The two metrics are also shown for the CCSK subgroup in comparison with all other groups (E, F). Survival was calculated as the delay between the date of first treatment and the date of decease or last medical visit. Log-rank test P values are shown in C–F. NS, not significant; ρ, P value.
These co-stained with the neuronal markers neurofilament and, almost constantly, with NeuN. In contrast, no staining for glial or neuronal markers was found in BCOR-ITD sarcomas (Figure 2N–P). There was shared staining among all BCOR-ITD tumours for BCOR protein and four additional markers: SATB2, Cyclin D1, BCL2, and TLE1. These markers were tested for specificity by comparing the mean mRNA levels of each marker in BCOR-ITD tumours versus selected families of sarcomas and brain tumours. The resulting boxplots show that BCOR, SATB2, and TLE1 tend to be overexpressed in the family of BCOR-rearranged tumours, which includes BCOR-ITD, BCOR-CCNB3, and YWHAE-NUTM2A/B members (supplementary material, Figure S3).

Among potential therapeutic targets, we identified EGFR as strongly and diffusely expressed in all CNS BCOR-ITD cases and three cases of sarcoma. YAP1, as a surrogate of Shh signalling, was expressed diffusely in most cases. NTRK (pan-Trk) was expressed only in CNS BCOR-ITD. No significant nuclear accumulation of β-catenin was observed in any case.

Transcriptome and methylome profiles identify BCOR-ITD sarcomas and CNS BCOR-ITD tumours as two close coherent subgroups

Unsupervised clustering was performed on RNA-Seq data using 22 tumours from this series compared to 346 reference tumours. Visualisation after dimension reduction by the Uniform Manifold Approximation and Projection (UMAP) method delineated closely related but distinct clusters of BCOR-ITD sarcomas and CNS BCOR-ITD (Figure 3A). CNS BCOR-ITD samples clustered with neither glial nor neuroectodermal tumours. Hierarchical clustering was applied to the same dataset leading to a higher resolution picture:
while CNS BCOR-ITD and BCOR-ITD sarcomas were grouped within a single common clade of BCOR-rearranged tumours (Figure 3B,C), they clearly formed two distinct subclusters. The same methods were applied to the DNA methylation data from 21 BCOR-ITD cases compared with 528 reference tumours. Similar to transcriptomic data, CNS BCOR-ITD and BCOR-ITD sarcomas formed two distinct branches of the same clade (Figure 4).

Overall, these data identify BCOR-ITD as a homogeneous and reproducible molecular signature and define two related entities, intracranial and extracranial.

Differential analysis of expression data identifies biological differences between CNS BCOR-ITD and BCOR-ITD sarcomas and sheds light on their respective cell of origin

We aimed to define the molecular processes that drive the transcriptional traits underlying the differences between BCOR-ITD sarcomas and CNS BCOR-ITD.

The expression levels of 2,306 genes were found to be significantly different between CNS BCOR-ITD and BCOR-ITD sarcomas. In comparison, CNS BCOR-ITD was more distant from medulloblastoma and ependymoma with 5,587 and 4,763 significantly different genes, respectively. Similarly, comparison between BCOR-ITD sarcomas and bone or soft tissue Ewing sarcomas found 4,921 significant genes (supplementary material, Figure S4A–D).

The top 20 Gene Ontology (GO) Biological Processes enriched in CNS BCOR-ITD in comparison with BCOR-ITD sarcomas all refer to CNS development, neurogenesis, and glial cell differentiation (supplementary material, Table S5). Reciprocally, BCOR-ITD sarcomas display an over-representation of GO terms related to embryonic skeletal development and embryonic morphogenesis, in line with the 33 Hox genes found in the top 100 listed DEGs (supplementary material, Table S6).

DEGs were used as marker genes for CNS BCOR-ITD and BCOR-ITD sarcomas. Using publicly available single-cell/single-nuclei RNA-Seq atlases, namely Cao et al. (human foetal gene expression) and Han et al. (adult, foetal, cord, and iPS tissue), we evaluated the enrichment of the BCOR-ITD sarcoma signature and the CNS BCOR-ITD signature in specific cell identities available in these atlases. The signature of CNS BCOR-ITD sarcomas was found to be enriched in neurons, astrocytes, and oligodendrocytes (Figure 5 and supplementary material, Table S7A,C and Figure S5), while the signature of BCOR-ITD sarcoma was enriched in stromal, endothelial, and mesangial cells of various organs (Figure 5 and supplementary material, Table S7B,D and Figure S5).

This clear dichotomy suggests both types of tumours derive from distinct cells of origin.

We then analysed the top listed DEGs to better characterise the molecular pathways governing CNS BCOR-ITD and BCOR-ITD sarcoma oncogenesis. Genes involved in targetable signalling pathways were scrutinised in particular. Compared with reference tumours of medulloblastoma and ependymoma types, CNS BCOR-ITD displayed overexpression of NTRK2, NGFR, PDGFRA, and WNT7B (Table 3). Activation of the corresponding pathways could be confirmed by GSEA only for the PDGFRA pathway (CNS BCOR-ITD versus medulloblastomas, Normalised Enriched Score [NES] 1.42, False Discovery Rate q-value [FDRq] 0.085) (supplementary material, Figure S4E).

In contrast, BCOR-ITD sarcomas were better defined by overexpression of NTRK3, EGFR, and PDGFRA, in comparison with Ewing sarcomas (Table 4), as confirmed by the GSEA approach (NES 1.20, FDRq 0.244) (supplementary material, Figure S4F). Interestingly, two genes remained significantly differentially expressed when CNS BCOR-ITD and BCOR-ITD sarcomas were compared, NTRK2 and WNT7B, suggesting that these two may participate in the molecular subgrouping between the two entities.

Discussion

We present here a comprehensive description of 33 BCOR-ITD tumours, putting together sarcomas of all known locations and CNS BCOR-ITD. Although this study is retrospective, with a relatively small number of cases in each subgroup, treated following disparate protocols, we explored the main similarities and differences, as summarised in Table 4. This analysis showed no clear clinical, radiological, or pathological specificity of BCOR-ITD tumours, but a common transcriptomic signature. We speculate that a common tumorigenic scheme, driven by the ITD itself and applied to distinct cells of origin in different environments, leads to two distinctive lineages: BCOR-ITD sarcomas and CNS BCOR-ITD.

Similarities and differences of the clinical presentations

The family of BCOR-ITD tumours offers a heterogeneous set of presentations, reflecting the variety of locations. The main features in common are the median age at diagnosis as previously reported in
CCSK (2.0 years over 33 cases [1(p),2,3]), URCS (0.8 year over 28 cases [12]), and CNS BCOR-ITD (3.5 years over 49 cases [16–24, 26, 36–38]); the local presentation; and the poor prognosis.

Two entities appear as clinical outliers. First, CCSK with BCOR-ITD alteration display higher survival rates: among the seven cases in our series, six were alive at the end of the study and one was still
on therapy. A remission was obtained in five cases after the first-line treatment. No histological feature could easily explain this better prognosis. Protagonizing the therapeutic strategy reveals that all CCSK cases underwent upfront extracapsular surgery with R0 outcomes. First-line complete resections were also performed on most CNS BCOR-ITD cases, but microscopic mid-range dissemination of the disease remains possible as brain tumours are not encapsulated. In addition, the chemotheraphy plan of CCSK includes platinum derivatives and anthracyclines, both being excluded from the first-line treatment of other localisations.

HG-ESS also appears to be a separate entity as it occurs exclusively in adult uterine tissue, with a more severe presentation at diagnosis, either because the symptoms are detected later or the disease progresses faster. Again, there is no histopathological clue to decipher why this type of tumour presents differently.

We report two cases primarily localised in bones, which is a rare situation as compared with the related BCOR-CCNB3 subgroup. Three previous cases were reported in adolescents [12,13]. Our cases were older, in the fifth decade, and both presented with synchronous pulmonary metastases at diagnosis. As CCSK are known to evolve with very long-term pulmonary and bone metastasis [39], it was tempting to document these bone/pulmonary tumours as metastases from an occult CCSK. We found no argument in the medical records arguing in favour of this hypothesis.

Cranial and extracranial BCOR-ITD tumours form two distinct subgroups and question the cell of origin

Transcriptomic profile is becoming a major tool for the molecular diagnosis of paediatric and adult sarcomas. Classification of cerebral tumours routinely uses DNA methylation data. A recent report shows that methylome profiling also reliably defines clusters of sarcomas, coherently with histopathology and expression data [28]. We showed here that both techniques could be used to define the whole family and its subgroups.

CNS BCOR-ITD and BCOR-ITD sarcomas present close but distinct transcriptomic and DNA methylation signatures. This may result from a common oncogenic programme, acquired somatically and applied to cells of distinct types, within distinct environments. This was corroborated by immunohistopathological data that shows neuro-glial markers in CNS BCOR-ITD (GFAP, Olig2, NeuN, NF70) and not in BCOR-ITD sarcomas. Conversely, the sharp segregation between the two groups may result from a ‘host-tissue effect’. Such bias cannot be formally excluded at this stage but is rather unlikely because the same methods of molecular analysis were applied to compare renal, soft tissue, and cerebral rhabdoid tumours, and did not document a host–tissue effect (compare malignant rhabdoid tumours and atypical teratoid rhabdoid tumours in Figure 4A).

We argue that the divergence between CNS BCOR-ITD and BCOR-ITD sarcomas comes from differences in their cell of origin. Accordingly, the distance

Figure 3. Unsupervised clustering using RNA-Seq data. (A) Unsupervised clustering of gene expression levels of 22 BCOR-ITD samples, with 346 reference tumours of known histology (previously published sarcomas from Watson et al [27] and unpublished brain tumours from in-house clinical cohorts), using UMAP dimensionality reduction. (B) Dendrogram of hierarchical clustering using Euclidian distances and the complete linkage method applied to the same dataset as in (A). Locations of BCOR-ITD sarcomas and CNS BCOR-ITD tumours are depicted in red and blue, respectively. The grey square outlines the position of the zoom-in shown in (C). (C) Zoom-in on the dendrogram. The ‘Source’ bar refers to the control tumours in grey and the cases of the series in yellow, with the corresponding P values. The ‘Tumour type’ bar allocates a tumour type to each case, with the same colour code as in (A). Abbreviations: ALCL, anaplastic lymphoma; aRMS, alveolar rhabdomyosarcoma; ASPS, alveolar soft part sarcoma; ASTRO, astrocytoma; BRAIN, normal brain tissue from GTEX database; CCS, clear cell sarcoma of soft tissue; CCSK, clear cell sarcoma of the kidney; CIC-fused, CIC-fused sarcoma; DFSP, dermatofibrosarcoma protuberans; DSRCT, desmoplastic small round cell tumour; EMCS, extraskeletal myxoid chondrosarcoma; EML4-ALK-S, EML4-ALK sarcoma; EPN, ependymoma; eRMS, embryonal rhabdomyosarcoma; ETMR, embryonal tumour with multi-layered rosettes; EWS, Ewing sarcoma; FET-NFATC2, FET-NFATC2 sarcoma; FET-TCP2, FET-TCP2 sarcoma; GBM, glioblastoma; IFS, infantile fibrosarcoma; IMT, inflammatory myofibroblastic tumour; LGFMS, low-grade fibromyxoid sarcoma; LGG, low-grade astrocytoma and ganglioglioma; MB, medulloblastoma; MCS, chondrosarcoma; MLL, myxoid liposarcoma; MNG, meningioma; MYOE, myoepithelioma; NB, neuroblastoma; NTRK, NTRK fused sarcoma; NUT, NUT midline carcinoma; O, anaplastic oligodendroglioma; OMF, ossifying fibromyxoid tumour; PATZ, EWSR1-PATZ1 sarcoma; PIN, pineoblastoma; PLEX, choroid plexus carcinoma; RCC, renal cell carcinoma; SCCC, small cell carcinoma of the ovary hypercalcemic type; SFT, solitary fibrous tumour; SMARCA4-4T5, SMARCA4-deficient thoric sarcoma; SMARCB1-4T5, SMARCB1-deficient thoracic sarcoma; SYSA, synovial sarcoma; VGLL2, VGLL2-fused sarcoma.
between the two subgroups was higher in the UMAP based on methylation data compared to transcriptome data. Second, differential analyses of expression data revealed a gene signature for both subgroups, based on the DEG. The signature of CNS BCOR-ITD was enriched in genes expressed in neuro-glial cells while BCOR-ITD sarcomas predominantly expressed embryonal/developmental genes. Furthermore, those gene signatures were compared to two single-cell/single-nuclei RNA-Seq human atlases. Analysis of the top listed cell

Figure 4. Legend on next page.
Expression data shed light on the activated pathways

As an attempt to elucidate the tumourigenesis of BCOR-ITD tumours and identify the possible therapeutic targets, we studied the most significant DEGs and identified differential upregulation of NTRK2, WNT7B, and PDGFRa in CNS BCOR-ITD cases (in comparison with medulloblastomas and BCOR-ITD sarcomas) and NTRK3, EGFR, and PDGFRa in BCOR-ITD sarcomas (in comparison with Ewing sarcomas).

Increased expression of NTRK3/TrkC receptors has already been reported in CCSK [40], BCOR-ITD, and more generally in BCOR-rearranged tumours [41]. Our data confirm this result and further argue that the whole NTRK3 pathway is active as GSEA shows its members to be significantly enriched in BCOR-ITD sarcomas. Our study also shows that NTRK signalling is active in CNS BCOR-ITD cases, given positive NTRK immunolabelling and upregulation of NTRK2 mRNA.

Results on Wnt7b in CNS BCOR-ITD were corroborated neither by nuclear translocation of β-catenin (as reported in Ref. [17]) nor by GSEA. EGFR mRNA upregulation in BCOR-ITD sarcomas and, to a lesser extent, in CNS BCOR-ITD contrasts with the strong staining of EGFR protein in CNS BCOR-ITD cases and in only a few BCOR-ITD sarcomas. Despite these discrepancies, most probably related to technical artefacts, EGFR signalling appears to be an active pathway in the whole family, in line with the recent work showing strong positivity in nine cases of BCOR-ITD UCs [42].

Concordant clues point towards NTRK, EGFR, and PDGFRa, but preclinical studies are required to evaluate the corresponding pathways and possibly document the role of the BCOR-ITD protein in their activation.
Acknowledgements

We would like to thank the clinicians who shared medical reports: G Bisogno, R Alaggio, J Grill, P Berlanga, K Beccaria, S Puget, JC Gentet, C Coze, AS Defachelles, J Probert, P Schneider, S Le Gallic, S Ducassou, and M Toulmonde. We also thank the technicians who helped managing imaging transfer from one centre to the other: E Gasparollo, G Lemaire, S Kankonde, C Courio, F Becker, and K Legoupil.

This work was supported by the Institut National de la Santé et de la Recherche medical (INSERM).

Figure 5. Dot plot summarising the distributions of CNS BCOR-ITD and BCOR-ITD sarcoma signatures over cell types independently analysed by single-cell/single-nuclei RNA-Seq. The signatures were computed from the DEGs between CNS BCOR-ITD and the BCOR-ITD sarcoma with a minimal fold-change of 2.5. Dot colour represents the average of each distribution and size represents the count of cells within a cell type with a signature intensity strictly higher than 0 brought to the [0, 1] range: a gene signature is highly represented in a cell type of the atlas when the dot gets bigger (several cells of one type express the signature) and tends towards black colour (several genes of the signature are expressed within a cell type). (A) Dot plot of the CNS BCOR-ITD signature (upper line) and the BCOR-ITD sarcomas signature (lower lines) over selected cell types of the Cao et al’s atlas. Only significant cell types are shown. The complete list of this atlas’ cell types is shown in supplementary material, Figure S5. The number of genes in common between the signatures and the reference database is as follows: CNS BCOR-ITD n = 114; BCOR-ITD sarcomas n = 43. (B) Dot plot of the CNS BCOR-ITD signature (upper line) and the BCOR-ITD sarcoma signature (lower line) over selected cell types of the Han et al’s atlas. Only significant cell types are shown. The complete list of this atlas’ cell types is shown in supplementary material, Figure S5. Number of genes in signatures is also in the reference database: CNS BCOR-ITD n = 103; BCOR-ITD sarcomas n = 38.

Table 3. Key DEGs

| Group A (light grey) versus Group B (dark grey) | NTRK1 | NTRK2 | NTRK3 | NGFR | EGFR | PDGFRA | Wnt7B |
|-----------------------------------------------|-------|-------|-------|------|------|--------|-------|
| CNS BCOR-ITD, n = 8                           | n/s   | n/s   | n/s   | n/s  | n/s  | 15.8   |       |
| CNS BCOR-ITD, n = 8 Ependymomas, n = 10       | n/s   | 5.7   | 7.3   | 24.9 | 4.0  | 22.2   | 14.0  |
| CNS BCOR-ITD, n = 8 Medulloblastomas, n = 21  | n/s   | 33.0  | 1.8   | 26.4 | 9.4  | 13.1   | 39.1  |
| BCOR-ITD sarcomas, n = 30                      | 1.6   | 2.2   | 29.4  | 5.6  | 12.7 | 28.8   | 2.4   |

The fold-change of expression of seven genes between tumours of group A (light grey) and tumours of group B (dark grey) is presented. The number of cases in each group is indicated. Fold-change is shown in favour of the tumours of group A (light grey). Values shown in bold are considered of high significance.
Table 4. Summary of differences and similarities between BCOR-ITD sarcomas of all localisations

| Clinical data | Histology | Radiology | Molecular biology |
|---------------|-----------|-----------|------------------|
|               | Age, median (range) | Prognosis | Mitotic counts per 10 hpf (range) | Immunostaining | (features apply to all tumour types) | DEGs |
| BCOR-ITD sarcomas | CCSK | 2.0 (0.5–18.9) | 0/7 | 0–18 | BCOR, SATB2, Cyclin D1, BCL2, TLE1, WT1-neg, SMARCB1 + | Heterogeneous masses, Necrosis, Haemorrhage, Low-to-moderate contrast enhancement | NTRK3, EGRF, PDGFRa |
| HG-ESS | 51.1 (25.8–62.4) | 2/4 | 14–21 | BCOR, SATB2, Cyclin D1, BCL2, TLE1, Desmin-neg, Caldesmon-neg | |
| URCS | 0.7 (0.1–19.7) | 4/8 | 1.7–13.7 | BCOR, SATB2, Cyclin D1, BCL2, TLE1 | |
| BCOR-ITD CNS tumours | HGNET-BCOR | 1.8 [1.2–7.6] | 6/10 | 5–62 | BCOR, SATB2, Cyclin D1, BCL2, TLE1, NeuN, GFAP+/-, Olig2 +/- | |

hpf, high-power field.

Institut Curie, the Institut National du Cancer (INCa), and the Direction Générale de l’offre de soins INCa-DGOS_13219 (iMAPS project).

We are indebted to the following associations for providing essential support: L’Étoile de Martin, la Course de l’Espoir, M la vie avec Lisa, ADAM, Couleur Jade, Dans les pas du Géant, Courir pour Mathieu, Marabout de Ficelle, Les Bagouzamanon, Enfants et Santé, Les Amis de Claire, and Liv et Lumière.

Author contributions statement

YB, AT-E, AG, LC, GS, FB, OD, GP and FD designed the study. YB, AT-E, AG, LC, OD, GP and FD wrote the manuscript. YB and MC acquired and analysed the clinical data. LC reviewed all imaging data. AT-E and AG reviewed all histopathological data, and performed immunohistochemistry. DG, DB, JV, SG, CQ, SW, FT, JM-P and GP processed and analysed RNA-Seq and methylome data. DO, NC, GS, FB, MS, CD, VM-C, MB, FT, DP, MK, M-CM, OD and FD recruited patients and provided samples and clinical information. All authors reviewed the manuscript.

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References 43 and 44 are cited only in the supplementary material.
SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Text S1. Detailed imaging characteristics of BCOR-ITD tumours
Text S2. Detailed histopathological and immunohistochemical analyses

Figure S1. Structure of the BCOR-ITD cohort. For RNA-Seq, the FFPE and frozen samples correspond to distinct tumours. There is no overlap between the two sample types

Figure S2. Examples of typical imaging findings in BCOR-ITD sarcomas at diagnosis

Figure S3. Boxplots showing the mRNA levels of five markers in the BCOR-ITD family in comparison with other families of sarcomas and brain tumours

Figure S4. Molecular divergence between CNS BCOR-ITD and BCOR-ITD sarcomas

Figure S5. Dot plot summarising loose (FC > 1.5) and stringent (FC > 2.5) CNS BCOR-ITD and BCOR-ITD sarcoma signature distributions over cell types independently analysed by sc/sn RNA-Seq

Table S1. Tumour features at diagnosis, patient by patient.
Table S2. Treatment and prognosis, patient by patient
Table S3. Radiological features of BCOR-ITD tumours

Table S4. Immunohistochemistry results

Table S5. Analysis of the DEGs between CNS BCOR-ITD and BCOR-ITD sarcomas

Table S6. Analysis of the DEGs between BCOR-ITD sarcomas and CNS BCOR-ITD

Table S7. List of the top 10 cell types where the gene signatures of CNS BCOR-ITD and BCOR-ITD sarcomas are enriched, based on two single-cell/single-nuclei RNA-Seq atlases