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BCOR expression in paediatric pineoblastoma

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Abstract

BCOR is expressed in a new brain tumour entity, i.e. 'CNS tumour with BCOR internal tandem duplication' (HGNET BCOR) but not in several other high grade paediatric brain tumours investigated. Immunohistochemical detection of BCOR expression may therefore serve as a potential diagnostic marker. Nevertheless, in rare paediatric glioma cases recurrent EP300-BCOR fusions were detected, which resulted in strong BCOR immunopositivity. We have therefore examined other, not analysed so far, types of central nervous system (CNS) tumours, pineoblastoma and germinoma, to assess a potential involvement of BCOR in these tumours. Levels of BCOR RNA expression were investigated by NanoString nCounter system analysis in a series of altogether 66 high grade paediatric tumours, including four pineoblastoma cases. Immunohistological detection of BCOR was performed in eight pineoblastoma, five germinoma and four atypical teratoid rhabdoid tumours (ATRTs), all located in the pineal region. We detected BCOR expression in all pineoblastomas, at the RNA and protein levels, but not in germinomas and ATRTs. Further analysis of pineoblastoma samples did not reveal the presence of either BCOR internal tandem duplication or BCOR fusion involvement. Positive immunohistological BCOR nuclear reaction in pineoblastoma may therefore differentiate this type of tumour from other high grade tumours located in the pineal region.

Key words: *pineoblastoma, BCOR, gene expression.*

Introduction

The fifth edition of the 2021 WHO Classification of Central Nervous System (CNS) Tumours recognizes a new entity, namely 'CNS tumour with *BCOR* internal tandem duplication' [13]. This type of tumour was discovered by Sturm *et al*. in 2016 using genes methylation profiling and was originally named as 'CNS high-grade neuroepithelial tumour with *BCOR* alteration' (HGNET-BCOR) due to the presence of in-frame internal tandem duplications in exon 15 of the BCL6 co-repressor (*BCOR*) gene. This resulted in high expression of this gene in tumour cells [20]. In the same study, an elevated expression of BCOR at the RNA level was also detected in a CNS high grade neuroepithelial tumour with *MN1* alteration (HGNET-MN1), but not in 8 other types of CNS tumours investigated. Overall, low expression of BCOR was observed so far in high grade gliomas (HGGs) of different molecular types, medulloblastomas, ependymomas, CNS neuroblastoma with *FOXR2* activation (NBFOXR2), CNS Ewing sarcoma family tumour with *CIC* alteration (EFTCIC), atypical teratoid rhabdoid tumours (ATRTs), embryonal tumour with multilayered rosettes and choroid plexus carcinomas (CPCs) [16,17,20]. The evidence of BCOR nuclear expression detected by immunohistochemistry in HGNET-BCOR

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tumours was presented in several recent studies [2,6,7,16], therefore indicating a potential diagnostic usefulness of this marker.

Nevertheless, different than BCOR internal tandem duplication (ITD) abnormalities were found in 3 cases of paediatric gliomas, where recurrent *EP300-BCOR* fusions were detected and resulted in strong BCOR nuclear immunopositivity, similar to that observed in cases with *BCOR* ITD [22]. This implies that a further investigation is necessary to establish the frequency and nature of *BCOR* involvement in CNS paediatric tumours.

We have, therefore, examined other types of CNS tumours, pineoblastomas and germinomas, which have not been analysed so far, to assess a potential involvement of *BCOR* in these tumours. We showed evidence of BCOR expression in pineoblastomas, but without presence of neither *BCOR* ITD nor *BCOR* fusion involvement. Moreover, positive immunohistological BCOR nuclear reaction in pineoblastoma could differentiate this type of tumour from other types of high grade tumours located in the pineal region.

Material and methods

Patients and tumour material

Paediatric patients diagnosed between 1998 and 2019 with high grade brain tumours in the Children's Memorial Health Institute in Warsaw, Poland, were included in the analysis. The analysis was performed on archive tumour material obtained at diagnosis. Hematoxylin-eosin-stained preparations (H&E) were used for pathological re-analysis to confirm the original diagnosis and to determine the tumour tissue content by three experienced neuropathologists. Whole preparations were scanned in Hamamatsu NanoZoomer 2.0 RS scanner at the original magnification 40×.

Seventeen tumours located in the pineal region were analysed, including eight pineoblastomas, five germinomas and four ATRT tumours. For comparative analysis, additional 62 supratentorial tumours were re-analysed from the previously published series: 20 HGGs, 13 CPCs, 10 ATRTs, eight ependymoma *RELA* fusion positive tumours (EPN-RELA), four HGNET-BCOR, three NB-FOXR2 and four HGNET-MN1 tumours [15,16].

Informed consent was obtained to use the tumour material, in accordance with the procedures outlined by the Bioethics Committee at the Children's Memorial Health Institute, Warsaw, Poland, approval No. 1/KBE/2017 and No. 67/KBE/2017.

Detection of genes expression at the RNA level

NanoString nCounter system analysis (NanoString Technologies, Seattle, USA) was applied in a series of altogether 66 tumours. Total RNA was extracted from frozen or FFPE tumour samples using RNeasy kits (Qiagen). RNA integrity and quantity were assessed using an Agilent 2100 Bioanalyzer.

For BCOR expression level analysis and group assignment, a custom NanoString CodeSet was applied, which consisted of marker genes and three housekeeping genes (*ACTB*, *GAPDH* and *LDHA*).

Probes were designed to target the regions of analysed genes. The sequences for *BCOR* and marker genes for HGNET-BCOR and HGNET-MN1 tumours are presented in our previous paper [16]. Due to the lack of sufficient expression microarray data for pineoblastoma, we chose four genes previously described as highly expressed in the pineal tissue, as the markers for pineoblastoma tumours. These include *TPH1* involved in melatonin synthesis, *PDC* and *IMPG2* involved in phototransduction and *OTX2* transcription factor, all four genes highly expressed in pinealocytes [1,4,5,18,21]. The sequences for pineoblastoma marker genes are presented in Table I.

Hybridization of the probes to the tumour RNA samples was performed in the Clinical Research Centre, Medical University of Białystok, Poland, following NanoString Technologies procedures for hybridization, detection and scanning. The raw counts for each gene were subjected to technical and biological normalization using nSolver 4.0 software (NanoString Technolo-

Table I. The target regions of the marker genes for pineoblastoma

Gene	Accession	Target sequence
TPH1	NM_004179.1	TTCTGACCTGGACCATTGTGCCAACAGAGTTCTGATGTATGGATCTGAACTAGATGCAGACCATCCTGGCT TCAAAGACAATGTCTACCGTAAACGTCGA
OTX2	NM_021728.3	ATACTCAGGCTTCAGGTTATAGTCAAGGATATGCTGGCTCAACTTCCTACTTTGGGGGCATGGACTGTGGA TCATATTTGACCCCTATGCATCACCAGCT
IMPG2	NM_016247.2	CTTGGAGTATAAAGCCCTGGAGCAAAGATTCTTAGAATTGCTGGTTCCCTATCTCCAGTCAAATCTCACGG GGTTCCAGAACTTAGAAATCCTCAACTTC
PDC	NM_002597.4	ACTGCCTTCGTAAATACCGTAGACAGTGTATGCAGGATATGCACCAGAAGCTGAGTTTTGGGCCTAGATAT GGGTTTGTGTATGAGCTGGAAACTGGAAA

gies, Seattle, USA). Clustering of the samples was performed using Euclidean distance metrics and average settings.

Detection of internal tandem duplication in the BCOR gene

Genomic DNA was extracted from eight pineoblastoma tumour samples using the QIAamp DNA FFPE Tissue Kit (Qiagen). The duplicated region in exon 15 of *BCOR* was detected by targeted PCR using the following primers: BCOR_15F: TCCTCCCGCATATTTCGCTG and BCOR_15R: ACACACTGTACATGGTGGGTCC (35 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 120 s). Bidirectional sequencing was performed using a 3500 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The sequences were determined on both DNA strands from at least two independent PCR products. The analysed sequence fragments were compared with the *BCOR* cDNA (Gen-Bank RefSeq: NM_001123385.1) sequence using Mutation Surveyor software version 3.30 (Soft Genetics, LLC, State College, PA, USA). The presence of internal tandem duplication was also validated using Archer FusionPlex® Pan Solid Tumor v2, next-generation sequencing (NGS) panel.

Immunohistochemistry

Expression of BCOR protein was detected using commercially available antibody clone C10 (sc-514576; Santa Cruz, Dallas, TX), at a dilution of 1 : 400. Antigen retrieval was performed using Target Retrieval Solution, High pH, (DAKO, Glostrup, Denmark) for 30 min in 99.5°C. The specificity of BCOR immunoexpression was tested in the classic medulloblastoma sample and normal testis.

Detection of INI1 protein was performed using mouse monoclonal anti-INI-1 antibody (clone MRQ-27, concentration 0.31 µG/ml) on the Ventana BenchMark ULTRA IHC/ISH auto-staining system. After antigen retrieval in CC1 buffer, detection of the signal was followed with the Ultra View HRP system (Roche/Ventana). Whole preparations were scanned in the Hamamatsu NanoZoomer 2.0 RS scanner (Hamamatsu Photonics, Hamamatsu, Japan) at the original magnification of 40×.

Detection of BCOR fusion by targeted next-generation sequencing

Targeted cancer panel sequencing – Archer FusionPlex® Pan Solid Tumor v2 (for Illumina) was used to detect *BCOR* gene fusions in pineoblastoma tumour samples. This panel uses Archer's Anchor multiplex PCR chemistry to target regions of interest. This technology enables detection of both known and unknown analysed gene fusion partners. Prior to the library preparation, total RNA was extracted from fresh-frozen or FFPE tumour samples using RNasy Mini Kit (Qiagen) and quantified with QuantiFluor RNA system (Promega). Libraries were performed using Archer FusionPlex reagent kit for Illumina with 200 ng input of RNA, according to the manufacturer's assay protocol and quantified using the KAPA Universal Library Quantification Kit (KAPA Biosystems, Wilmington, MA). Then libraries were normalized, pooled and sequenced on Illumina MiniSeq instrument (Illumina) using the MiniSeq High Output Kit (2 × 150 cycles). At the end of sequencing, FASTQ files were generated using Illumina's bcl2fastq software version 2.17.1 and analysed with the Archer data analysis pipeline (Archer™ analysis software version 6.0). Analysed samples passed quality control criteria: pre-sequencing RNA Ct ≤ 28, unique RNA starting site reads per GSP control ≥ 10 and library quantity > 2 nmol/l. The criteria for calling a positive fusion were 5 or more unique supporting RNA reads and 3 or more unique starting sites among the reads.

The BCOR expression analysis using NGS-based targeted DNA sequencing panel

For evaluation of BCOR expression, the Archer analysis software was used. Gene expression was calculated on the basis of the ratio of unique reads between the *BCOR* gene and genes used as internal controls in Archer FusionPlex panels. Relative gene expression was visualized in a heat map and displayed as numerical values.

Statistical analysis

Statistical analyses were performed using pairwise Mann-Whitney test using the SPSS software (version 26; SPSS Inc., Chicago, IL, US).

Results

In the first step, eight tumours with morphologically diagnosed pineoblastomas were investigated. The age of patients ranged from 3 to 16 years, five patients were males and three patients were females. Four tumours were analysed by the NanoString method and all eight tumours were analysed by immunohistochemistry.

The expression level of BCOR analysed by NanoString method

Comparative analysis included altogether 66 tumours – four pineoblastomas, four HGNETBCOR tumours, four HGNETMN1 tumours, 20 HGGs, eight EPN-RELA

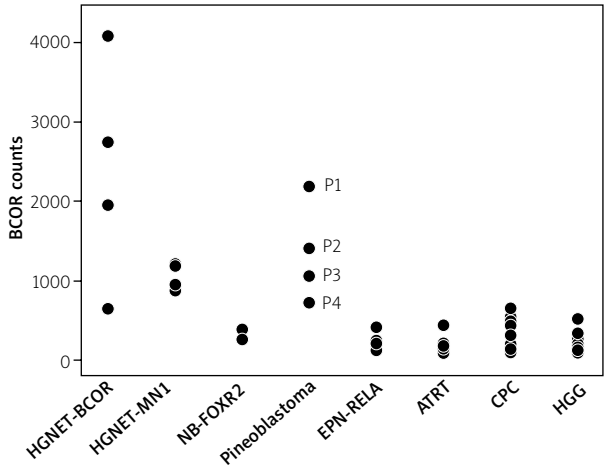

Fig. 1. BCOR mRNA levels in 66 central nervous system (CNS) tumours. The level of expression was measured as 'counts' after normalization using NanoString method in 8 types of CNS tumours.

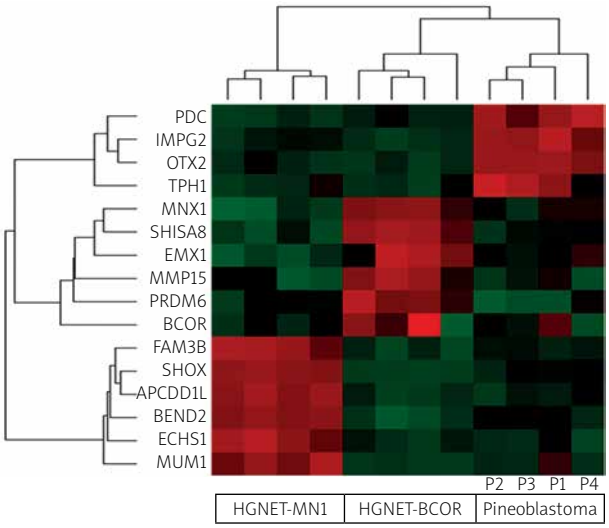

Fig. 2. Clustering of brain tumours according to the expression level of marker genes using NanoString method. Clustering of 12 supratentorial tumours using 16 marker genes reveals a cluster of 4 tumours with high expression of 4 pineoblastoma marker genes, separated from HGNET-MN1 and HGNET-BCOR clusters. Colours represent log2 gene expression differences.

tumours, 13 CPCs, 10 ATRTs, and three NBFOXR2 tumours.

Levels of BCOR expression were the highest in HGNET-BCOR tumours (mean = 2323 counts), followed by pineoblastomas (mean = 1317 counts) and HGNET-MN1 tumours (mean = 1022 counts) (Fig. 1). Pairwise analysis indicates a significant increase in BCOR expression levels in pineoblastomas vs. HGGs (p = 0.002), EPN-RELA tumours (p = 0.004), ATRTs (p = 0.004), CPCs (p = 0.003) and NB-FOXR2 tumours (p = 0.034) but, as expected, not significant against HGNET-BCOR and HGNET-MN1 tumours.

Clustering of tumours with an increased BCOR expression

In order to confirm that diagnosed pineoblastomas with a high *BCOR* expression are distinct from HGNET-BCOR and HGNET-MN1 tumours, NanoString analysis was applied using a panel of altogether 16 marker genes. These included six marker genes for HGNET-BCOR and six marker genes for HGNETMN1 tumours, which were presented in our previous paper [16] and four marker genes for pineoblastoma, as described in the material and method section.

Unsupervised hierarchical clustering analysis of 12 tumours revealed three distinct clusters: four samples with an expression of HGNET-MN1 signature, four samples with HGNET-BCOR signature and four samples expressing pineoblastoma marker genes, but no other signature genes (Fig. 2). Therefore, morphologically diagnosed pineoblastoma tumours, which expressed high BCOR levels are distinct from other BCOR-expressing tumours.

Histological findings and detection of BCOR expression by immunohistochemistry in pineoblastoma

All eight analysed pineoblastomas appeared as small round blue cell tumours, composed of poorly defined cells with a high nuclear-cytoplasmic ratio (Fig. 3A). The tumour cells exhibited round, oval or slightly irregular, hyperchromatic nuclei and scant cytoplasm. Occasionally rosettes of Flexner-Wintersteiner type with a central lumen or true Homer-Wright rosettes with tumour cells arranged around central eosinophilic areas could be seen. The tumours showed mitotic activity and high proliferation index. Apoptotic bodies and foci of necrosis were commonly observed. Neoplastic cells exhibited neuronal differentiation with diffuse synaptophysin immunopositivity (Fig. 3B). All tumours showed retained SMARCB1 (INI1) nuclear immunostaining (Fig. 3C).

BCOR immunohistochemistry was performed altogether on eight pineoblastomas, including four tumours analysed by NanoString method. Seven tumours

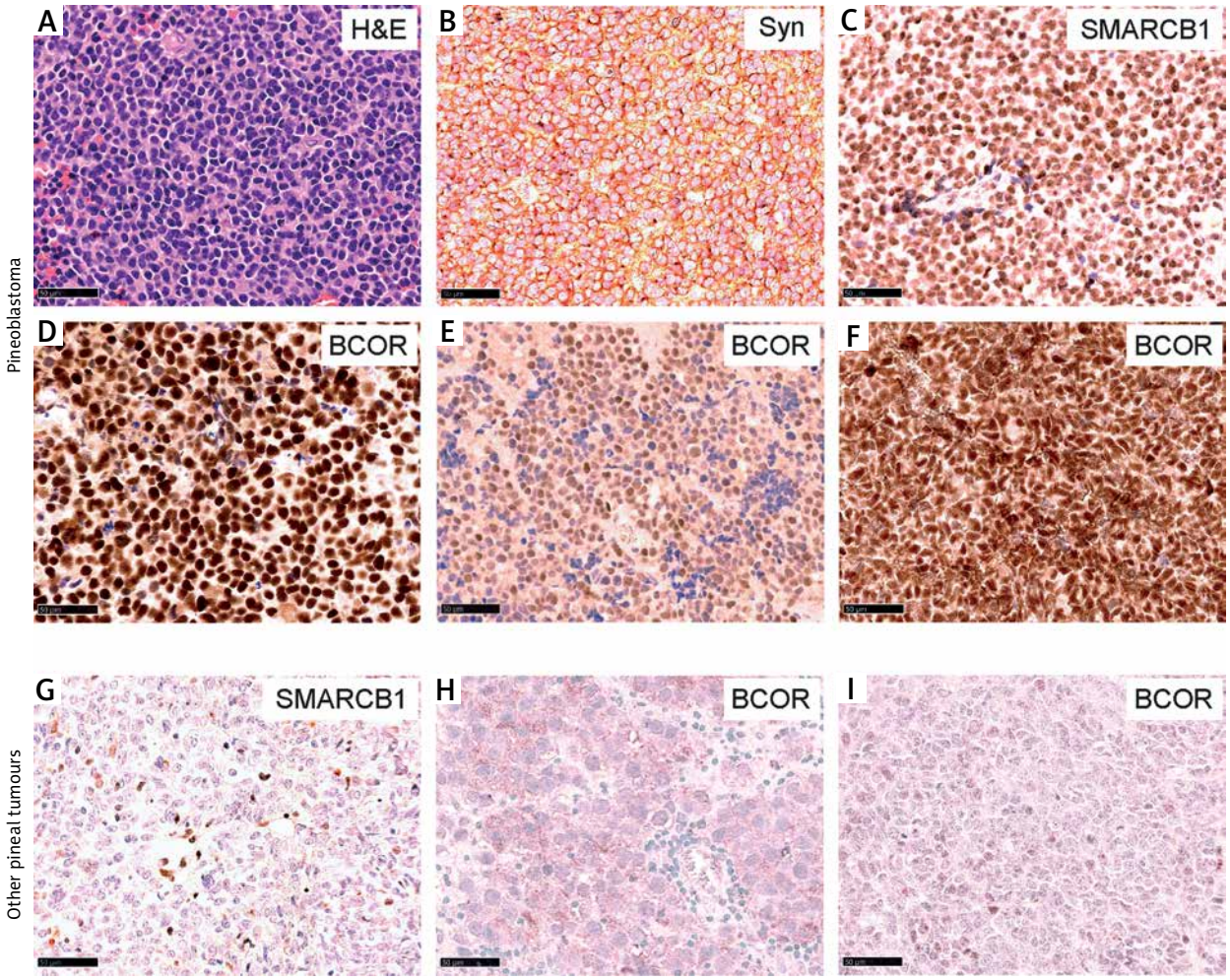

Fig. 3. Histopathological and immunohistochemical features of pineoblastoma and other tumours in the pineal region. **A**) Pineoblastoma composed of densely packed, small cells with a high nuclear-cytoplasmic ratio. H&E. **B**) Diffuse expression of synaptophysin in pineoblastoma cells. **C**) Retained SMARCB1 (INI1) immunoreactivity in pineoblastoma cell nuclei. **D**) Pineoblastoma with strong, widespread nuclear BCOR immunoreactivity. **E**) Nuclear BCOR staining in a large percentage of neoplastic pineoblastoma cells. **F**) Extensive nuclear expression of the BCOR protein in almost all neoplastic cells of pineoblastoma. **G**) ATRT with a loss of SMARB1 nuclear immunoreactivity in tumour cells and retained expression in non-neoplastic cells. **H**) ATRT with negative BCOR immunostaining. **I**) Germinoma with negative BCOR immunostaining. The scale bars: A-I) 50 µm.

showed diffuse and strong BCOR nuclear immunopositivity in > 90% of neoplastic cells (Fig. 3D). One tumour showed heterogeneous intensity of BCOR expression with focal strong nuclear staining in a large percentage of tumour cells (Fig. 3E).

All four pineoblastomas, which were not analysed at the RNA level by NanoString method, demonstrated diffuse BCOR nuclear immunoreactivity. In two of them, almost all neoplastic cells nuclei showed strong immunostaining (Fig. 3F).

Therefore, all eight pineoblastomas expressed BCOR at the protein level (Table II), including seven tumours with nuclear reactivity similar to CNS HGNET-BCOR tumours.

Detection of BCOR expression by immunohistochemistry in other tumours located in the pineal region

In order to differentiate pineoblastoma from other tumours located in the pineal region we analysed five germinomas and four ATRTs, the latter tumours showing loss of nuclear SMARCB1 (INI1) staining (Fig. 3G).

Table II. BCOR analysis in pineoblastoma patients

ID	Sex	Age (years)	BCOR NanoString counts	BCOR mRNA NGS	BCOR IHC	BCOR ITD	BCOR Fusion
P1	F	16	2156	high	+	no	no
P2	M	10	1381	nd	++	no	nd
P3	M	5.5	1036	high	+++	no	no
P4	M	3	693	high	+++	no	no
P5	F	6	nd	moderate	++	no	no
P6	M	11	nd	moderate	++	no	no
P7	M	12	nd	high	+++	no	no
P8	F	5	nd	high	+++	no	no

Counts – levels of mRNA expression after normalization using NanoString method, NGS – next generation sequencing, IHC – immunohistochemistry, BCOR IHC: + 10-50%, ++ 50-90%, +++ > 90% of positive cells, ITD – internal tandem duplication, nd – not done

In contrast to pineoblastoma, all analysed tumours showed negative BCOR immunoreactivity (Fig. 3H, I).

Analysis of internal tandem duplication in the BCOR gene (BCOR ITD)

Tandem duplication in exon 15 of the *BCOR* gene was assessed in DNA from eight pineoblastoma samples. None of analysed tumours showed the presence of BCOR ITD, despite the fact that 7 out of 8 tumours showed nuclear BCOR immunoreaction present in > 80% of tumour cells.

Analysis of BCOR fusions *via* targeted NGS

Archer FusionPlex® Pan Solid Tumor v2 (for Illumina) was used to detect *BCOR* gene fusions in seven pineoblastoma tumour samples. None of the analysed tumours showed the presence of *BCOR* fusion event.

The BCOR expression analysis using NGS-based targeted DNA sequencing panel

Seven pineoblastomas and one control sample were also evaluated for BCOR expression using Archer FusionPlex panel and Archer analysis software. Five pineoblastoma tumour samples (P1, P3, P4, P7 and P8) displayed a relatively high expression ratio of unique reads for the *BCOR* gene compared to two remaining pineoblastoma samples (P5 and P6) and the control ependymoma sample (Fig. 4).

Discussion

Rare CNS tumours with *BCOR* ITD and an expression of the BCOR gene are now recognized as a distinct CNS tumour type by both the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT) and the 2021 WHO Classification [13,14]. In addition to this new category, we showed that high BCOR expression is also present in pineoblastoma, both at the RNA and protein levels. Recently, transcriptomic analysis by RNA-sequencing (RNA-seq) was performed in 16 pineoblastomas, but the investigation was focused on presentation of subgroup-specific expression signatures in relation to gene methylation profiles [11]. Therefore, potentially elevated *BCOR* expression frequently present across pineoblastoma samples could be omitted by such a statistical approach.

We found a robust BCOR expression in 7 out of 8 analysed pineoblastoma tumours, what suggests that *BCOR* may play an important role in pineoblastoma, similarly to other malignancies with presence of high BCOR expression. In addition to HGNET-BCOR tumours, upregulated expression and *BCOR* ITD was described in clear cell sarcoma of the kidney, suggesting that *BCOR* alteration is the critical oncogenic driver in some tumours [19,23]. High BCOR expression also resulted from rearrangements other than ITD. For example, in BCOR-expressing sarcomas, several types of *BCOR* fusions were detected, including *BCOR-CCNB3*, *KMT2D-BCOR*, *ZC3H7B-BCOR* or *BCOR-MAML3* [3,8].

However, we did not find either *BCOR* ITD or *BCOR* fusions in analysed pineoblastoma samples. Recently published results based on the next generation sequencing of pineoblastoma tumours did not also mention any abnormalities in the *BCOR* gene [10-12].

Up-regulation of BCOR in pineoblastoma does not seem to reflect pineal gland tissue specificity, since recent analysis by single cell sequencing did not reveal BCOR expression either in pinealocytes or in other cell types present in the pineal gland [5]. Therefore, BCOR up-regulation may have an oncogenic impact on pineoblastoma but this is neither a consequence of *BCOR* ITD nor a fusion event, suggesting involvement of epi-

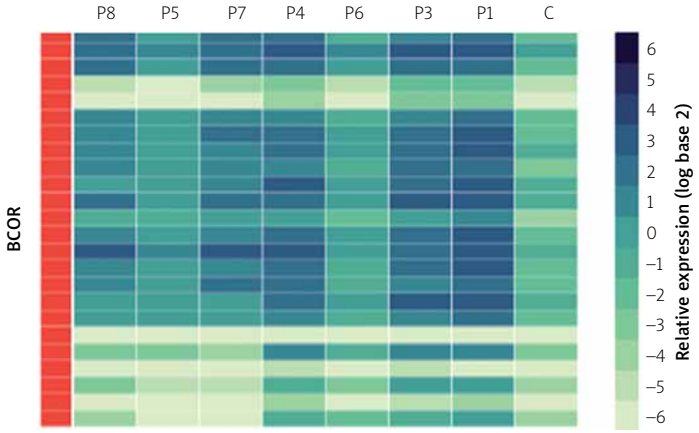

Fig. 4. BCOR expression levels in pineoblastoma tumours using NGS-based targeted DNA sequencing. Relative BCOR expression using Archer FusionPlex panel in 7 pineoblastoma samples (P1-P7) and one control sample other than pineoblastoma (C) is visualized in a heat map. Colours represent log2 gene expression differences. Five pineoblastoma tumours (P1, P3, P4, P7 and P8) showed a higher expression ratio of unique reads for the BCOR gene compared to other pineoblastoma samples and the control sample.

genetic regulations. Similarly, in rare cases of undifferentiated round cell sarcomas with diffuse and strong BCOR positivity, no *BCOR* rearrangements have been found [9]. Following these results, the exact nature of *BCOR* involvement in pineoblastoma requires further clarification.

Strong BCOR immunopositivity present in pineoblastoma, but not in other tumours located in the pineal region, namely ATRTs and germinomas, provides a diagnostic opportunity for the differentiation of those tumours. Although BCOR immunohistochemical reaction is present in both pineoblastoma and 'CNS tumour with *BCOR* internal tandem duplication', the distinct tumour location may discriminate those tumours from each other.

In conclusion, we detected the second type of CNS tumour, pineoblastoma, with a strong expression of BCOR. Although our analysis did not reveal a presence of *BCOR* rearrangements, it is still possible that this gene plays an important role in pineoblastoma oncogenesis. From the diagnostic point of view, the positive immunohistochemical BCOR nuclear reaction should be taken into account in the classification of other CNS tumours.

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Disclosure

The authors report no conflict of interest.

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