Neuro-Oncology
23(S5), S16–29, 2021 | https://doi.org/10.1093/neuonc/noab143

S16

DNA methylation profiling as a model for discovery and precision diagnostics in neuro-oncology

Drew Pratt, Felix Sahm, and Kenneth Aldape

Department of Pathology, University of Michigan, Ann Arbor, Michigan, USA (D.W.); Department of Neuropathology, Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany (F.S.); Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland, USA (K.A.)

Corresponding Authors: Kenneth Aldape, MD, Laboratory of Pathology, National Cancer Institute, Bethesda, MD 20814, USA (kenneth.aldape@nih.gov); Felix Sahm, MD, PhD, Department of Neuropathology, University Hospital Heidelberg and Clinical Cooperation Unit Neuropathology (B300), German Cancer Research Center (DKFZ), Im Neuenheimer Feld 224, 69120 Heidelberg, Germany (felix.sahm@med.uni-heidelberg.de).

Abstract

Recent years have witnessed a shift to more objective and biologically-driven methods for central nervous system (CNS) tumor classification. The 2016 world health organization (WHO) classification update ("blue book") introduced molecular diagnostic criteria into the definitions of specific entities as a response to the plethora of evidence that key molecular alterations define distinct tumor types and are clinically meaningful. While in the past such diagnostic alterations included specific mutations, copy number changes, or gene fusions, the emergence of DNA methylation arrays in recent years has similarly resulted in improved diagnostic precision, increased reliability, and has provided an effective framework for the discovery of new tumor types. In many instances, there is an intimate relationship between these mutations/fusions and DNA methylation signatures. The adoption of methylation data into neuro-oncology nosology has been greatly aided by the availability of technology compatible with clinical diagnostics, along with the development of a freely accessible machine learning-based classifier. In this review, we highlight the utility of DNA methylation profiling in CNS tumor classification with a focus on recently described novel and rare tumor types, as well as its contribution to refining existing types.

Keywords

brain tumor | DNA methylation | neuro-oncology

Current paradigms of cancer revolve around the shaping and fitness of neoplastic cells through an interrelated series of genetic alterations and epigenetic modifications.[1] Cancer epigenetics involves a disruption in gene expression that is not mediated through primary DNA sequence alterations and often results in dysregulation of cell signaling pathways and changes in cellular phenotype. Cancer cells primarily achieve this epigenetic aberrancy through DNA methylation, histone modifications, remodeling and repositioning of nucleosomes, and non-coding RNA (e.g. microRNA).[1] Within this epigenetic landscape, DNA methylation is among the most characterized.

In mammals, DNA methylation primarily occurs at the carbon-5 position of CpG cytosines (termed 5-methylcytosine, or 5-mC). This covalent transfer is achieved via DNA methyltransferases (DNMT) and is largely conserved through cell division.[2] The location and methylation state of CpG-rich sites, called CpG islands or CGI, influences gene expression: transcriptional repression is frequently associated with promoter and enhancer CGI methylation, whereas CGI methylation of gene bodies often correlates with increased expression.[3] In cancer, a global reduction of CpG methylation is observed, often coupled with CGI hypermethylation and subsequent transcriptional silencing of tumor suppressor genes. Beyond facilitating oncogenesis, there is also a cell type-specific pattern of CpG methylation that may persist in tumor development and progression.[4] This combination of aberrant

methylation and cell-of-origin patterns lends itself to being particularly useful for tumor classification and potentially identifying clinically relevant subtypes.

In this review, we discuss the increasing role and impact of DNA methylation profiling in the classification of central nervous system (CNS) tumors with a focus on the discovery of novel and rare tumor types, as well as its contribution to refining established types from the revised 4th edition of the WHO Classification of Central Nervous System Tumors.[5] We briefly introduce the most commonly used array-based platform for measuring CpG methylation and the bioinformatic methods that are often employed for its interpretation. It is anticipated that >50% of the diagnostic entries in the upcoming 5th edition of the WHO Classification of CNS Tumors will include DNA methylation profiles as an essential or recommended diagnostic feature. Consequently, the following discussion will highlight *possible* changes in nomenclature based on novel molecular findings, with an emphasis on types where methylation profiling has demonstrated a significant clinical and/or biologic impact on classification. Finally, we have structured the review around the traditional histogenic classification of CNS tumors in an effort to bridge current and future directions of nosology in neuro-oncology.

Measuring CpG Methylation

A variety of methods exist to quantify DNA methylation, each with various applications in clinical oncology. Restriction endonuclease digestion, affinity enrichment methods such as methylated DNA immunoprecipitation (MeDIP), and electrochemical assays are examples used to interrogate genome-wide methylation (see Laird, 2010[6] for a comprehensive review). Bisulfite sequencing of genomic DNA, the most commonly used method, converts unmethylated cytosine to uracil after exposure to sodium bisulfite.[7] Methylated cytosine (5-mC), however, is resistant to this reaction and can be detected in subsequent sequencing steps. This method provides single base resolution of CpG methylation and, when applied at a genome-wide scale (whole-genome bisulfite sequencing, or WGBS), can quantify the methylation state of the approximately 29 million CpGs in the human genome.[8] While WGBS provides an unprecedented scale and resolution of the human methylome, it is currently prohibitively expensive and computationally intensive. Of the approximately 11,000 cancer samples catalogued in The Cancer Genome Atlas (TCGA), only 39 have been profiled with WGBS.[9]

A comprehensive and cost-effective alternative is the complementary use of bisulfite-converted DNA and microarray technology. Illumina microarrays for CpG methylation are based on the hybridization of fragmented whole-genome amplification products to oligonucleotide bead arrays which contain oligomers linked to specific CpGs.[10,11] This approach is based on their Infinium genotyping assay and, when first introduced in 2008, provided coverage of over 27,000 CpG sites (HumanMethylation27 DNA Analysis BeadChip, or HM27). This has since been followed by the HumanMethylation450[12] (HM450, >450,000 CpGs, "450k") and HumanMethylationEPIC (EPIC, >850,000 CpGs, "850k") arrays released in 2011 and 2016, respectively. The HM450 array, of which EPIC covers >90%, contains probes pre-selected for biologically meaningful regions of the genome including RefSeq genes (98.9%), CpG islands (96%) and adjacent regions (e.g. shores, shelves), FANTOM4 promoters, predicted enhancers, MHC regions, and DNase hypersensitivity sites.[12] Thus, while the Infinium BeadChip arrays (HM450, EPIC) cover only ~1–3% of the human DNA methylome, it provides comprehensive coverage of cancer-relevant CpG sites at a comparatively lower cost and higher throughput than WGBS, as well as being compatible with formalin-fixed tissue and demonstrating robustness to pre-analytic factors. Figure 1 provides an overview of the current diagnostic applications of DNA methylation profiling and its role in surgical neuropathology.

Recently, a novel adaption of long-read DNA sequencing using the Oxford Nanopore MinION sequencer has enabled accurate profiling of DNA modifications such as CpG methylation.[13] The technique infers the methylation status using a modified hidden Markov model (HMM) to learn ionic current distributions and can achieve an accuracy up to 95% in identifying 5-mC.[13,14] The method does not require bisulfite treatment and can be performed in a significantly shorter amount of time (~hours) compared to methylation-based assays (~days). A recent study[15] successfully extended this technology to classifying CNS tumors using the Heidelberg brain tumor classifier (classifier details discussed later). Notably, the relatively quick turnaround time may also allow for intraoperative tumor classification.

Bioinformatic Methods

To translate methylation array data into practical use, a variety of analytic and computational steps need to be performed. The proprietary Illumina file format (*.IDAT) contains the raw signal intensities which can be processed using the R programming language[16] (with or without R Studio, an easy-to-use graphical user interface for R); a popular R package to process methylation array data is *Minfi*.[17] After parsing the.IDAT files, intensities are typically normalized to correct for unwanted signal variation, and probes that are unreliable or map to polymorphic regions of the genome[18] are removed. The most informative probes are then selected for further analysis (feature selection) based on variability in the dataset (e.g., choosing the top 10,000 most variable probes based on standard deviation).

A frequently used and practical approach for delineating tumor types with methylation data is dimensionality reduction. Similar to principal component analysis (PCA), these unsupervised algorithms reduce high-dimensional data (e.g. thousands of brain tumor samples, each with ~20–30k of data points) to a lower dimension (2 or 3) for visualization. The most widely used techniques are t-Distributed Stochastic Neighbor Embedding (t-SNE)[19] and Uniform Manifold Approximation and Projection (UMAP).[20] The underlying mathematics is beyond the scope of this review, suffice it to say both techniques produce similar visual information when clustering bulk CNS tumor samples (Figure 2). However, a few details are worth mentioning. The scalability of t-SNE is limited and, thus, PCA is performed before embedding. It is also thought that UMAP preserves the global structure of the data better than t-SNE. Finally, as a general rule, the distances between the observed clusters

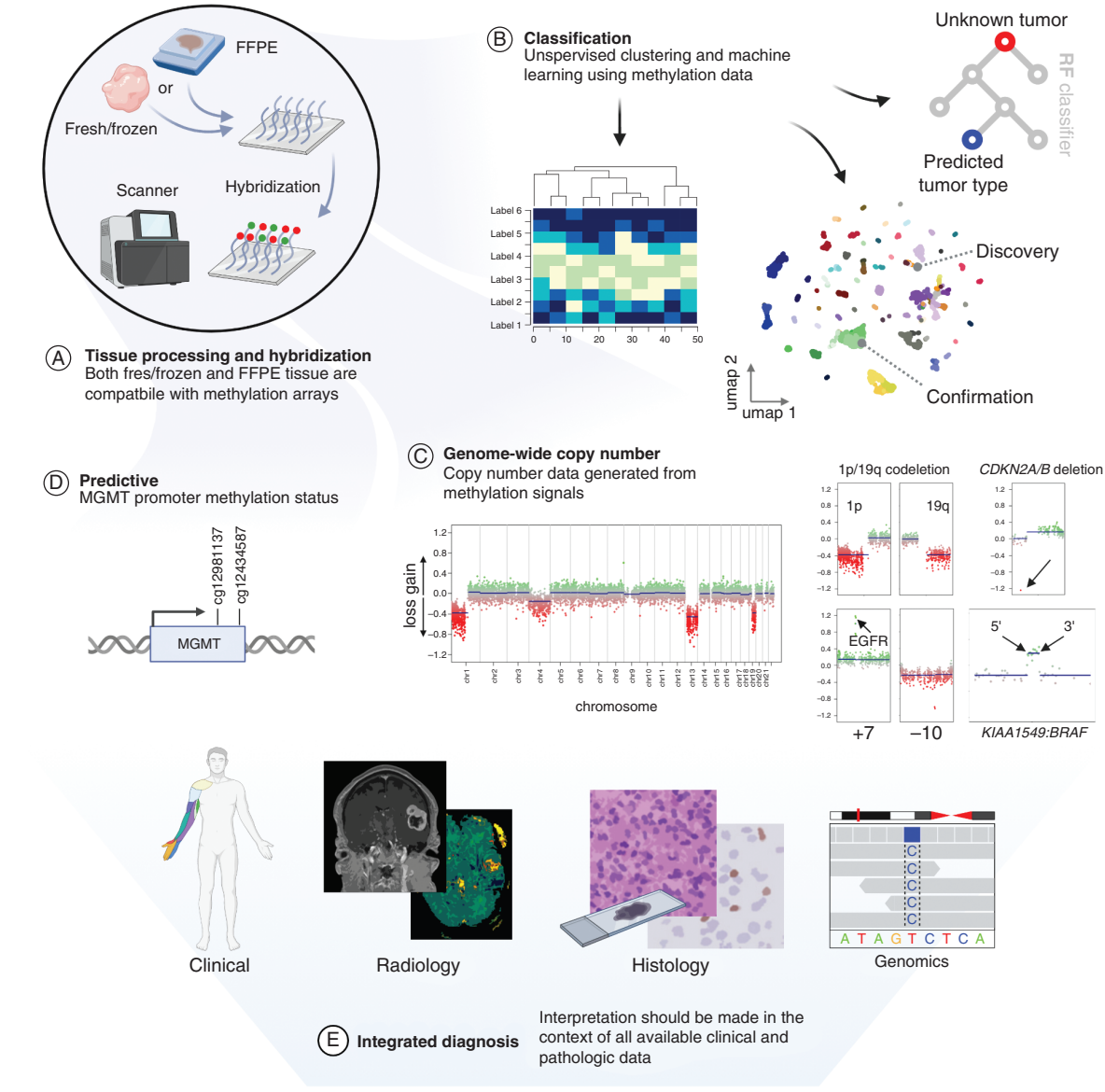

Fig. 1 Workflow and applications of DNA methylation profiling in surgical neuropathology. The Illumina 450k and HumanMethylationEPIC (EPIC) arrays are compatible with both fresh/frozen tissue and Formalin-Fixed Paraffin-Embedded (FFPE) material (A). Following extraction, DNA is bisulfite-converted and hybridized to the BeadChip. The fluorescent signals are then processed with the iScan or NextSeq 550 readers and two separate data files (*.idat) are produced, one for each color channel (i.e. red and green). Following various preprocessing steps, methylation data (often represented as beta values) can then be used as input for clustering or data visualization (B). In addition to hierarchical clustering, dimensionality reduction with t-Distributed Stochastic Neighbor Embedding (t-SNE) or Uniform Manifold Approximation and Projection (UMAP) provides a novel technique for confirming and identifying new tumor types. The basis for this method of tumor diagnostics relies on epigenetically (and often genetically) similar tumor types grouping together in the two- or three-dimensional space; subtypes can also be identified within these macro-clusters. Machine learning algorithms, such as random forest, can be used to train a classifier based on pre-defined cluster labels using these dimensionality reduction techniques. The combined intensities of the signals from the methylated and unmethylated channels may be used to infer focal and broad copy number changes (C). Diagnostic markers in central nervous system (CNS) tumors, such as whole-arm 1p/19q codeletion and the +7/–10 signature (with or without *EGFR* amplification) can be reliably detected with this method; furthermore, copy number breakpoints are useful to infer fusion events in the correct diagnostic context. A two-CpG methylation signature can be used to assess O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status and has been independently associated with response to alkylating therapy in isocitrate dehydrogenase (IDH)-wildtype glioblastoma (GBM) (D). Finally, as with any diagnostic assay in surgical pathology, methylation profiling should be interpreted in conjunction with all available clinical data, including history, imaging, histology, and other ancillary molecular techniques such as sequencing (E).

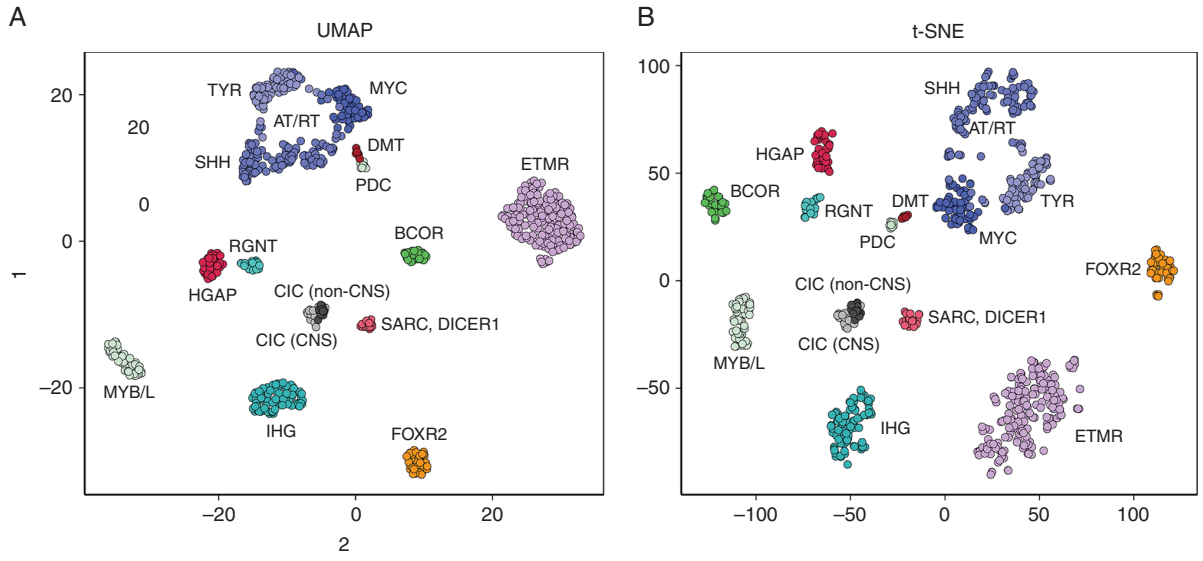

Fig. 2 Dimensionality reduction techniques for the visual assessment of central nervous system (CNS) tumor clustering. Two frequently used methods for visualizing tumor types are Uniform Manifold Approximation and Projection (UMAP) (A) and t-distributed stochastic neighbor embedding (t-SNE) (B). Illustrated are selected novel and rare CNS tumor types that are discussed in this review (not included: diffuse glioneuronal tumor with oligodendroglioma-like features and nuclear clusters (DGONC) and cribriform neuroepithelial tumor (CRINET), and those presented in "epigenetic subtyping"). As a rule of thumb, the distance between the groups is less meaningful than the relationship within the groups. UMAP is thought to preserve global structure better than t-SNE, but both produce comparable results for classifying CNS tumor types. Abbreviation: AT/RT, atypical teratoid/rhabdoid tumor (TYR, SHH, MYC subtypes); DMT, desmoplastic myxoid tumor; PDC, poorly differentiated chordoma; ETMR, embryonal tumor with multilayered rosettes; BCOR, CNS tumor with *BCOR* with internal tandem duplication; SARC, *DICER1*, primary intracranial sarcoma, *DICER1*-mutant; CIC, *CIC*-rearranged sarcoma; RGNT, rosette-forming glioneuronal tumor; HGAP, high-grade astrocytoma with piloid features; MYB/L, diffuse astrocytoma, *MYB* or *MYBL1*-altered; IHG, infant-type hemispheric glioma; FOXR2, CNS neuroblastoma, *FOXR2*-activated.

are not as meaningful as the distances within the clusters. For example, points that cluster together are more similar than points in other clusters; however, the distances between clusters are not as informative.

It is also important to mention other diagnostically useful information that can be derived from methylation array data. It was recognized early on that the sum of methylated and unmethylated signal intensities can be used to infer genome-wide copy number.[21] The segmented copy number data can then be used to detect diagnostically useful alterations such as whole chromosome (+7/–10 signature) and arm-level (1p/19q codeletion) changes, as well as gene-level alterations (e.g. *EGFR* amplification, *CDKN2A/B* homozygous deletion) (Figure 1C). Furthermore, analysis of copy number breakpoints can also be used to infer *possible* fusion events resulting from unbalanced translocations (e.g. *BRAF*, *FGFR* fusions). The diagnostic impact of integrating methylation and copy number data in neuropathology practice was recently reviewed.[22] Finally, both O-6-methylguanine-DNA methyltransferase (MGMT)[23] and mutL homologue 1 (MLH1)[24] promoter methylation can be quantified with the array-based platform (Figure 1D). While discrepancies with pyrosequencing-based methods have been noted, DNA methylation-based MGMT promoter methylation status has been shown to provide clinically relevant prognostic information.[25,26] Both copy number and MGMT promoter methylation status are included in the report generated from the "Classifier" (see below).

Diagnostic Accuracy and Impact on Clinical Outcome

Since its adaptation as an investigative diagnostic tool in 2018,[27] Infinium methylation arrays have led to the identification of new CNS tumor types and subtypes, many of which harbor meaningful associations with clinical course and outcome. One of the most important contributions from this study is the implementation of a machine learning-based classifier to prospectively evaluate new samples (the Heidelberg "Classifier," https://www.molecularneuropathology.org/mnp). Benchmarking of the Classifier with a routine diagnostic work-up (e.g. histology, IHC) revealed high concordance (838/1104, 76%) with a correspondingly low number of cases with profiles that were discordant with clinical, pathologic, and molecular features ("misleading" profile). Notably, methylation results led to diagnostic revisions in a significant proportion (129/139 or 12% of the entire cohort) of cases. This finding was confirmed in five external centers (50/401, 12%), with reclassification rates in 6%-25% of cases.

A recent study independently applied the Classifier to a large cohort of samples in the work-up of routine and challenging cases.[28] The authors included 502 samples from 480 pediatric and adult primary and recurrent tumors. To assess its diagnostic impact, they adopted similar outcome categories for the Classifier results, including confirmation/

refinement of diagnosis, establishment of a new diagnosis, misleading profile, discrepant but non-contributory, and "no match." The results extended those reported by Capper et al.[27]: 54.4% of cases were concordant with histopathology, with a precise match achieved in 41% of samples and 13.3% receiving a refined diagnosis. Classifier performance was comparable between primary and recurrent tumors (66.1% vs. 65.5% matching a class, respectively). A new diagnosis was established in favor of the methylation class in 9.8% of cases (vs. 12%[27]). The majority of cases in which a new diagnosis was rendered (49/502) also resulted in a change in WHO grade: 22.5% (11/49, vs. 30%[27]) were downgraded and 48.9% (24/49, vs. 41%[27]) were upgraded. A similar proportion of cases had a misleading profile (5/502 vs. 10/1104[27]). Interestingly, there was a demonstrated benefit in cases not meeting the cutoff for a class match (calibrated score <0.9). In unmatched cases with a calibrated score between 0.3 and 0.9 (130/502), the histopathologic diagnosis could be confirmed or refined in 50.7%, and a new diagnosis rendered in 7.7% of cases. These results provide independent confirmation of the diagnostic and prognostic impact of DNA methylation-based classification.

Finally, differences in clinical outcome based on methylation data were recently demonstrated in tumors previously classified as primitive neuroectodermal tumors, or CNS-PNET, which are now known to resolve into biologically distinct tumor types with specific alterations.[29] These include embryonal tumor with multilayered rosettes (ETMR) with a high frequency of C19MC alterations, CNS neuroblastoma with *FOXR2*-activation (CNS-NB-FOXR2), CNS tumors with *BCOR* internal tandem duplication (CNS-BCOR-ITD), *CIC*-rearranged sarcomas (EFT-CIC), CNS tumors with *MN1* alteration, and a subset of high-grade gliomas (HGG). While detection of these alterations can be done through targeted sequencing or cytogenetic techniques, methylation array profiling can resolve CNS-PNET types independent of the underlying alteration,[27,29] some of which have disparate clinical outcomes. A retrospective survival analysis of CNS-PNET types showed prolonged 5-year progression-free (5y-PFS) and overall survival (5y-OS) in CNS-NB-FOXR2 (52%, 96%, respectively) compared with HGG (12%, 12%) and ETMR (12%, 18%).[30] Although *MN1*-altered tumors typically contain an astroblastoma-like histology,[29,31] a subset may present with PNET-like features. Distinction from other embryonal tumors is thus important as *MN1*-altered tumors have a comparatively favorable overall survival.[29,31–33] Collectively, distinctions based on unsupervised analysis of DNA methylation data among CNS-PNET subtypes have important clinical implications.

Novel and Rare Tumor Types

In addition to its utility in recognizing common CNS tumor types, an emerging advantage of methylation profiling is its ability to discover novel types and provide confirmation/refinement of existing types. For example, *IDH1/2* mutations define a broad subtype of diffuse gliomas that are clinically and molecularly distinct from isocitrate dehydrogenase (IDH)-wildtype diffuse gliomas.[34] This genetic distinction was *confirmed* with DNA methylation profiling and has recently been *refined* to identify a clinically-distinct subtype (e.g. mismatch repair-deficient[35]). For many of the newly-described tumor types, however, methylation profiling has largely served as a tool for discovery. Below, we outline selected new and anticipated tumor types where methylation profiling has significantly contributed to its discovery or refinement. In the majority of cases, this method represents a highly sensitive assay that captures a wide range of histology and genetic alterations (e.g. mutations, CNVs, fusions) that converge on a common epigenetic signature.

Gliomas

High-grade astrocytoma with piloid features (HGAP) is a recently described tumor type that comprises a distinct methylation group and harbors a variable combination of alterations in telomere maintenance (*ATRX*, *TERT* promoter mutations), the cell cycle pathway (*CDKN2A/B* deletion/mutation, *CDK4* amplification), and the MAPK pathway[36]; the latter can include *NF1* deletion/mutations (somatic or germline), *BRAF* fusions (e.g. *KIAA1549*:*BRAF*), *FGFR1* fusion/mutations, or, rarely, *KRAS* or *BRAF* p.V600E mutations. The histologic findings largely overlap with other gliomas and can include features of pilocytic astrocytoma (PA) and pleomorphic xanthoastrocytoma (PXA), but can also mimic prototypic glioblastoma (GBM) with increased mitotic activity, microvascular proliferation, and necrosis.[36,37] HGAP are most commonly seen in young adults (median 40 years) and are found predominantly in the posterior fossa but can be seen in the supratentorial region. Notably, HGAP is thought to be rare in children (age 0–16), with the majority of morphologically-diagnosed "pilocytic astrocytoma with anaplasia" in this age group clustering with other methylation classes, including pilocytic astrocytoma and IDH-wildtype GBM.[38]

HGAP forms a distinct group on t-SNE or UMAP embedding of DNA methylation array data (Figure 2). The methylation class "anaplastic pilocytic astrocytoma" (ANA PA, not to be confused with the aforementioned "pilocytic astrocytoma with anaplasia") is included in the current version of the Classifier (v11b4),[27] and is anticipated to be included in an upcoming expanded iteration as HGAP (v12). This change in designation is intended to underline the aggressiveness and wider morphological spectrum compared to pilocytic astrocytoma. Presently, methylation profiling is the only method to diagnose this tumor type. The genetic alterations found in HGAP (e.g., *CDKN2A/B* homozygous deletion, *ATRX* mutation, *BRAF* fusion) are, in isolation, not specific and can be seen in other CNS tumor types. However, the combination of these genetic alterations, or even the presence of ATRX loss by immunohistochemistry in a pilocytic-like IDH- and H3-wildtype astrocytoma, should raise the suspicion of HGAP. Clinically, distinction from other high-grade gliomas is important as HGAP has a more aggressive outcome than PA and PXA, but significantly better overall survival than IDH-wildtype GBM.[36] The importance of an accurate diagnosis is underscored by the fact that HGAP is not infrequently misdiagnosed as GBM.[37] As highlighted in our review, resolving histologically-similar tumor types into biologically and clinically relevant types is among the significant advantages of DNA methylation-based classification (Figure 3).

Infant-type (infantile) hemispheric glioma (IHG) is a genetically heterogeneous group of high-grade gliomas that are primarily seen in children less than one year of age (median age 2.8 months).[39] The histopathology of IHG is not specific and may include gemistocytic, gangliocytic, ependymal, and primitive features, in addition to the typical appearance of a high-grade glioma.[40,41] IHG tend to involve the leptomeninges and can disseminate within the neuraxis.[40] A common genetic feature in this group is the presence of receptor tyrosine kinase (RTK) alterations consisting of fusions in the *ALK*, *ROS1*, *NTRK1/2/3*, and *MET* genes present in 61–83% of reported cases.[39,41]

Despite the range of histopathology and the variety of fusion genes, a common DNA methylation signature helps define IHG. However, identifying the specific fusion can provide valuable options for therapeutic targeting. In the US, two drugs are currently FDA-approved for use in any solid tumor harboring an NTRK fusion or TRK oncoprotein ("tumor-agnostic"): entrectinib and larotrectinib.[42,43] Importantly, entrectinib recently showed *in vitro* efficacy and sustained CNS exposure in an intracranial tumor model.[44] While *ALK* and *ROS1* fusions are relatively specific to IHG among gliomas, NTRK fusions have also been observed in adult-type glioblastoma subtypes,[41] pilocytic astrocytoma,[45] pleomorphic xanthoastrocytoma,[46] and H3K27M-mutant diffuse midline glioma.[47] Methylation profiling thus serves as a sensitive diagnostic assay for IHG and can direct therapeutically-relevant sequencing.

There are currently two CNS tumor types harboring an *MYB* or *MYBL* alteration: *diffuse astrocytoma, MYB or MYBL-altered* (DA-MYB/L), and *angiocentric glioma* (AG). Collectively, these are IDH/H3-wildtype diffuse gliomas with low-grade histologic features that harbor structural alterations in the *MYB* or *MYBL* genes. These tumors commonly arise in the cerebral hemispheres but can rarely present in the infratentorial region.[48,49] DA-MYB/L are frequently associated with seizures due to their cortical location in many cases and belong to a group of developmental CNS neoplasms called long-term epilepsy-associated tumors (LEAT).[50] There are often overlapping histologic features between DA-MYB/L and AG. The monomorphic appearance of DA-MYB/L, fine fibrillary matrix, and diffuse infiltration led to its initial description as "isomorphic diffuse astrocytoma" in 2004.[51] AG frequently consists of bipolar spindle tumor cells oriented radially in a rosette-like pattern around vessels. In 22 sequenced cases, eight DA-MYB/L contained an *MYBL1* fusion and three contained an *MYB* fusion. In contrast, the majority of AG harbor an *MYB-QKI* fusion (found in up to 99% of cases).

The methylation class in the current version of the Classifier, "LGG, *MYB*/*MYBL*-altered," contains both isomorphic diffuse glioma and angiocentric glioma. However, unsupervised analysis of these tumors with other high- and low-grade gliomas has demonstrated a clear distinction between the two on both dimensionality reduction and hierarchical clustering.[49] While the *MYB-QKI* fusion is present in the vast majority of AG, non-*QKI* partners have also been detected.[52] Interestingly, t-SNE embedding also suggests a separation between these tumors and the previously reported "pediatric-type *MYB*/*MYBL* diffuse astrocytoma".[49,53] It is likely that diffuse gliomas harboring an *MYB* or *MYBL* alteration (fusion or CNV) comprise multiple methylation classes with distinct clinicopathologic features.

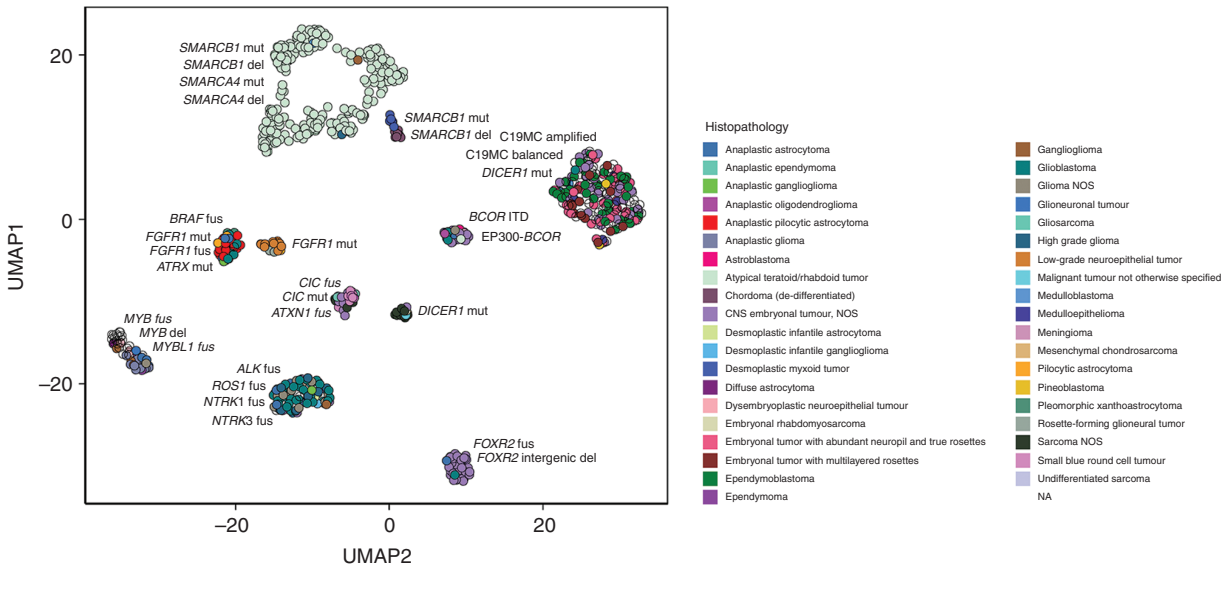

Fig. 3 Heterogeneity in histologic diagnoses and genetic alterations among central nervous system (CNS) tumor types from Figure 2. Clustering of CNS tumors by methylation profiling frequently reveals remarkable variability in histopathology within tumor types, often with discrepancies in the world health organization (WHO) grade. Additionally, large-scale studies and case reports have also revealed previously unrecognized heterogeneity in the genetic landscape of some tumors. Abbreviation: mut, mutation; fus, fusion.

Glioneuronal Tumors

Diffuse glioneuronal tumor with oligodendroglioma-like features and nuclear clusters (DGONC) is a recently proposed, methylation-defined tumor type discovered through unsupervised clustering of >25,000 CNS tumors.[54] This represents true class discovery as a result of the increasing use of the Classifier. The existence of this class was further supported through iterative resampling and cluster stability analysis, as well as the identification of copy number changes involving chromosome 14 in almost all cases. This was independently confirmed in a separate series that also noted chromosome 1p and 3p loss.[55] The histologic appearance of DGONC may have significant overlap with other tumor types, including oligodendroglioma and CNS-PNET.[54] Genetic alterations typically seen in low-grade glial or glioneuronal tumors, such as *FGFR1* and *BRAF*, have not been identified in sequenced cases.[54,55] As genetic drivers have yet to be elucidated in this tumor type, DNA methylation profiling remains the only method for its detection.

Rosette-forming glioneuronal tumor (RGNT) is a low-grade (WHO grade 1) glioneuronal tumor with hybrid histologic features including neurocytic rosettes/pseudorosettes and a glial component most frequently resembling pilocytic astrocytoma. A subset of RGNT may lack this biphasic appearance and contain a predominantly oligodendroglioma-like or PA-like histology.[56,57] Indeed, 3 of 10 methylation-defined RGNT in a recent report were initially characterized as "low-grade oligodendroglial tumor NOS (not otherwise specified)" and lacked the characteristic neurocytic rosettes.[57] Genetically, RGNT are among the *FGFR1*-altrered spectrum of LGG/LGNT and harbor either an *FGFR1* p.N546 or p.K656 mutation, identified in 40 of 40 methylation-defined cases to date.[56,57] There is also a high frequency of co-occurring *PIK3CA*/*PIK3R1* and *NF1* mutations detected in 28/40 and 14/40 cases, respectively.[56,57] The combination of hotspot *FGFR1* and *PIK3CA*/*PIK3R1* or *NF1* mutations is reasonably specific for RGNT with compatible histology.

RGNT forms a distinct cluster on both dimensionality reduction (t-SNE, UMAP) and hierarchical clustering.[27,56,57] When faced with incomplete histologic features, RGNT may closely resemble PA. Furthermore, *FGFR1* p.N546K or p.K656 hotspot mutations have been detected in methylation-defined PA.[57] Conversely, tumors epigenetically aligned with PA may contain RGNT-like histology with neurocytic rosettes.[57] Therefore, a subset of tumors clustering with RGNT may contain overlapping genetic and histologic features of PA, and *vice versa*. Further studies are required to determine the clinical and/or biologic significance of these discrepancies. Nevertheless, methylation profiling serves as a sensitive method for the diagnosis of RGNT without the need for hotspot detection of *FGFR1* mutations. Confirmation of *FGFR1* mutations as a ubiquitous finding in RGNT may also support methylation profiling as a useful surrogate for this alteration and for directing targeted therapy (e.g. *FGFR1* inhibition[58]).

Embryonal Tumors

Embryonal tumor with multilayered rosettes (ETMR) is a high-grade (WHO grade 4) primitive CNS tumor with distinct histologic features consisting of foci of pseudostratified primitive neuroepithelial cells arranged circumferentially around a lumen (true "ependymoblastic" rosettes) and containing variable amounts of interspersed neuropil.[59] ETMR is now the term given for three morphologic variants: embryonal tumor with abundant neuropil and true rosettes (ETANTR), ependymoblastoma, and medulloepithelioma.[60] The common molecular alteration is a structural alteration in the microRNA cluster on chromosome 19q13.42 (C19MC); this mostly frequently manifests as a *TTYH1-C19MC* fusion with concurrent C19MC amplification, the latter present in 87.9% (167/190) of primary ETMR in a recent large series,[61] but can also include *DICER1* mutations (germline or somatic) in 11.4% (8/70), or amplification of the *miR-17–92* miRNA cluster on chromosome 13 in 4.2% (3/70). A subset of ETMR do not show miRNA cluster amplification or *DICER1* mutations and can harbor alternative C19MC partners including *MYO9B*.[61] This genomic heterogeneity is anticipated to refine the "embryonal tumor with multilayered rosettes, C19MC-altered" nomenclature in the 5th edition of the WHO blue book to encompass non-amplified and *DICER1*-mutant cases.

ETMR methylation profiles show clear separation from other CNS tumor types and form a relative homogenous group regardless of C19MC amplification or *DICER1* mutation status.[61] The characteristic histology of multilayered rosettes with admixed regions of neuropil is present in the vast majority of cases. In combination with LIN28A immunohistochemistry, the diagnosis can be readily made at the microscope. However, reports of a glioneuronal-like appearance[62] and divergent differentiation (osteoid, myeloid, epithelial)[63,64] may complicate its recognition, particularly in relapse specimens.[65] Despite its high sensitivity, LIN28A expression is not specific to ETMR and has been reported in AT/RT,[66,67] germ cell tumors,[68] and HGG.[66] DNA methylation profiling thus serves as a sensitive and specific method for the diagnosis of ETMR, regardless of the underlying histopathology or genetic alteration. While outcome differences between amplified and non-amplified ETMR have yet to be established, methylation-based copy number assessment may also serve as a useful feature to identify potential subtypes.

CNS tumor with BCOR internal tandem duplication (CNS-BCOR-ITD) is a recently described high-grade tumor of uncertain differentiation (often termed "neuroepithelial"). These rare tumors can occur on either side of the tentorium cerebelli and are primarily seen in children and young adults (median age 3.5 years).[69] High-grade histology is typically encountered (increased proliferation, necrosis) with a conspicuous paucity of microvascular proliferation in one series.[69] A myxoid background is usually seen, and ependymoma-like (perivascular pseudorosettes) or embryonal-like (Homer Wright-like rosettes) features may be encountered.[69,70] Currently, the defining genetic alteration for this tumor type is an internal tandem duplication

(ITD) in exon 15 of the *BCOR* gene on Xp11.4. Clinical follow-up is limited, but 4/10 patients in one series had evidence of recurrence (range 4–49 months) and combined survival analysis of 24 patients showed a poor prognosis (median OS: 1.7 years).[69]

While specific among CNS tumors, the exon 15 ITD in *BCOR* that defines this tumor type is also present in clear cell sarcoma of the kidney[71] and undifferentiated round cell sarcoma/primitive myxoid mesenchymal tumor of infancy.[72,73] The methylation profile of CNS-BCOR-ITD is distinct[29] and the diagnosis can be reliably made with methylation array profiling.[27] Interestingly, genetic alterations other than *BCOR* ITD may also occur in tumors related to this methylation class. A recent study reported two high-grade CNS tumors harboring an *EP300:BCOR* fusion that clustered with the "CNS high grade neuroepithelial tumor with *BCOR* alteration" methylation class[74]; we independently found a sample from the TCGA harboring an *EP300:BCOR* fusion also clustered near tumors of this methylation class (Figure 3). While the further characterization is needed, *BCOR*-altered CNS tumors may extend beyond the exon 15 ITD.

CNS neuroblastoma, FOXR2-activated (CNS-NB-FOXR2) is a rare embryonal tumor with typical primitive histologic features (Homer Wright rosettes) accompanied by occasional neurocytic and/or gangliocytic differentiation. The spectrum of genetic alterations in CNS-NB-FOXR2 is limited to a single study. In 6/8 sequenced samples, structural alterations involving the *FOXR2* gene on Xp11.21 were identified, including three samples harboring *FOXR2* fusion transcripts mediated through gene translocation or tandem duplication. A novel mitochondrial-nuclear fusion was also reported. As discussed previously, the distinction of CNS-NB-FOXR2 from other tumors manifesting as CNS-PNET may have clinical relevance, with a reported better outcome compared to other CNS embryonal tumors. The utility of methylation profiling for CNS-NB-FOXR2 may encompass both diagnosis and surrogate identification of the underlying alteration with copy number changes. In ~28% of cases (13/46), 450k-derived copy number demonstrated *FOXR2* alterations including deletion and breakpoints, of which three cases that were sequenced harbored a *FOXR2* fusion trancript.[29]

There are currently four *SMARCB1*-inactivated (INI-1-deficient) primary CNS tumor types that are either established or provisionally recognized: *atypical teratoid/rhabdoid tumor* (AT/RT), *desmoplastic myxoid tumor* (DMT), *cribriform neuroepithelial tumor* (CRINET), and *poorly differentiated chordoma* (PDC). Comprehensive analyses of AT/RT (WHO grade 4) have revealed distinct methylation subtypes (SHH, TYR, MYC) and their clinicopathologic significance is discussed in detail elsewhere.[75,76] As shown in Figure 2, these tumor types often cluster in close proximity to each other (CRINET data not available). Loss of the INI-1 protein by immunohistochemistry has classically been associated with AT/RT and is due to inactivation of the *SMARCB1* gene (22q11.2) through structural alterations or mutations[77,78]; however, a small subset can harbor an inactivating alteration in the *SMARCA4* gene with corresponding loss of the BRG1 protein.[79] DMT are, in contrast, low-grade appearing tumors restricted to the pineal region and similarly harbor inactivating alterations in *SMARCB1*.[80] CRINET are low-grade neuroepithelial tumors that show INI-1 loss primarily through 22q hemizygous deletion with a "second hit" inactivating mutation/deletion in *SMARCB1*.[81] PDC are primarily sacrococcygeal tumors with focal rhabdoid histology and INI-1 loss.[82] *SMARCB1* inactivation in PDC has only been demonstrated through gene deletion, with no *SMARCB1* mutations reported to date.[83–85]

The distinction between these tumor types is clinically important and can be frequently resolved with histopathology and imaging. However, atypical cases may pose diagnostic difficulty. AT/RT and PDC (spinal) have a mean OS of 14.4[86] and 51[82] months, respectively; however, survival estimates vary with AT/RT subtype. Survival data for CRINET and DMT are limited but have been reported with a mean OS of 125 (vs. 37 for AT/RT-TYR)[81] and 36[80] months, respectively. Specific DNA methylation profiles for AT/RT, PDC, and DMT are evident despite sharing a common genetic alteration (Figure 2). Limited data on CRINET has shown that it clusters with the AT/RT-TYR subtype on methylation profiling.[81] The data so far has shown that DNA methylation profiling can resolve most *SMARCB1*-inactivated CNS tumors that may pose diagnostic challenges.

Mesenchymal Tumors

CIC-rearranged sarcoma is a rare high-grade mesenchymal neoplasm that occurs in both CNS and extra-CNS sites. The extra-CNS counterpart was recently introduced in the 5th edition of the WHO Classification of Soft Tissue and Bone Tumors in 2020.[87] Histologically, *CIC*-rearranged sarcomas are characterized by spindled and/or round tumor cell morphology with high-grade features (increased proliferation, necrosis) and variable amounts of myxoid matrix.[88] The characteristic genetic alteration is a translocation involving *CIC* with various partner genes resulting in a fusion that acts as a dominant oncogene.[89] In the CNS, this most frequently occurs as a *CIC-NUTM1* fusion resulting from a t(15;19) translocation.[88] False negatives with *CIC* break-apart FISH were been reported in 14% of *CIC* fusion-positive cases in one series.[90] Additionally, a *CIC* frameshift mutation had previously been identified in a fusion-negative case.[29] A recent report also noted the occurrence of a non-*CIC* fusion gene involving *ATXN1* and *DUX4*.[91] Originally termed a "Ewing-like" sarcoma, survival outcomes of *CIC*-rearranged sarcomas are significantly worse than Ewing sarcoma.[92] Furthermore, treatment with Ewing sarcoma-based therapy showed an inferior pathologic response in 70% of *CIC*-rearranged sarcomas.[92] Thus, distinction from EWS is clinically and prognostically important. *CIC*-rearranged sarcomas cluster in a distinct group on methylation profiling independent of the underlying fusion gene/mutation or anatomic site (Figure 2).

DICER1-mutant primary intracranial sarcoma is a newly-recognized high-grade sarcoma that is defined by either somatic and/or germline mutation(s) in the *DICER1* gene on chromosome 14q32.13.[93] Tumors that occur in the context of a germline *DICER1* mutation may represent a hereditary or syndromic association (i.e. *DICER1*

syndrome).[93] Histologic features have been frequently reported with myogenic differentiation and morphologic and immunophenotypic overlap with embryonal rhabdomyosarcoma.[93] Consequently, a tentative name "spindle cell sarcoma with rhabdomyosarcoma-like features, *DICER1* mutant" was given. Similar to *DICER1*-mutant pineoblastoma,[94] a subset of these tumors do not harbor a germline mutation and may have multiple somatic *DICER1* mutations or copy number loss that results in biallelic inactivation.[93]

DICER1 mutations are not specific to intracranial sarcomas in this tumor class. As previously discussed, a subset of methylation-defined ETMR also harbors somatic *DICER1* mutations in the absence of C19MC alterations. A recent report also noted a loss of the H3K27me3 epigenetic mark in *DICER1*-mutant tumors including intracranial sarcoma, pineoblastoma, and ETMR, thus expanding the spectrum of H3K27me3-negative CNS tumors.[95] In this context, morphologic and immunohistochemical distinction from H3K27me3-negative MPNST may be difficult. Additionally, the histologic findings of *DICER1*-mutant intracranial sarcoma are not specific and may overlap those of other sarcomas such as synovial sarcoma, fibrosarcoma, and gliosarcoma. DNA methylation profiling readily distinguishes *DICER1*-mutant primary intracranial sarcoma from its histologic mimics. However, further studies are needed to assess whether it can be differentiated from metastatic *DICER1*-mutant tumors.

Epigenetic Subtyping of Established CNS Tumors

Here we present examples of the contribution of DNA methylation profiling to the subtyping of established WHO CNS tumors. The 5th edition of the WHO classification for CNS tumors is anticipated to include an increased number of molecularly-defined subtypes. Methylation array profiling has been shown to effectively identify and, in some cases, define these subtypes, as outlined below.

Diffuse leptomeningeal glioneuronal tumor (DLGNT) was recently introduced in the revised 4th edition of the WHO Classification of CNS Tumors in 2016.[5] DLGNT are rare, predominantly pediatric tumors characterized by glial and neuronal differentiation, leptomeningeal involvement, and a monomorphous oligodendroglial-like cellular morphology.[96]The histologic features, however, can be variable and may show substantial overlap with other tumor types including pilocytic astrocytoma, anaplastic astrocytoma, primitive neuroectodermal tumor, ganglioglioma, and atypical neurocytoma.[97] While they generally contain low-grade histologic features, a subset of DLGNT demonstrate features of anaplasia (mitoses, microvascular proliferation, necrosis) and have been associated with shorter overall survival in this context.[96] Genetically, there is a high frequency (75%) of the *KIAA1549-BRAF* fusion,[98] chromosome 1p deletion (59–100%),[97,98] and, less commonly, 1p/19q codeletion (18–30%).[97,98] *IDH1* or *IDH2* mutations were not detected in sequenced cases (n=10 and n=5, respectively).[97] Importantly, alternative mechanisms of MAPK pathway activation may be seen in DLGNT including *BRAF* p.V600E mutations,[99,100] *NTRK1/2/3*, and *RAF1* fusions,[97] and *FGFR1* mutations.[101] MAPK alterations are not specific and can be seen in other glial or glioneuronal tumors. The presence of the *KIAA1549-BRAF* fusion, in conjunction with an oligodendroglial-like low-grade histology, may be difficult to distinguish from pilocytic astrocytoma; this is further confounded by the fact that Rosenthal fibers and eosinophilic granular bodies (EGBs) may also be seen in DLGNT.[97] Thus, there appears to be significant molecular and histologic heterogeneity in DLGNT which can be diagnostically challenging at both the microscopic and genetic level.

In DLGNT, methylation profiling is useful both as a diagnostic and prognostic assay. A recent study analyzing 30 methylation-confirmed cases of DLGNT identified two subclasses: MC-1 and MC-2.[97] In this series, methylation array-based copy number profiling demonstrated chromosome 1p loss as a frequent finding in DLGNT. A focal gain of 7q34 was observed in 20/30 DLGNT cases, indicating the likely presence of the *KIAA1549-BRAF* gene fusion. Age at presentation was also significantly different between the two methylation subclasses, with a median age of 5 for MC-1 and 14 years for MC-2. Most significant, however, was that the DLGNT MC-2 subclass showed shorter OS and PFS in this cohort.[97] While this finding needs to be confirmed, it is likely DNA methylation-based DLGNT subtypes will provide valuable prognostic information going forward.

Posterior fossa ependymomas are a clinically and molecularly heterogeneous group of ependymal tumors. They are the prototypic epigenetically-driven CNS tumor and have been shown to (largely) lack recurrent genetic alterations.[102] There are two broad types of posterior fossa ependymomas supported by clinical, gene expression, and DNA methylation data: group A (PFA) and group B (PFB). Morphologic features alone are insufficient to distinguish the two. At the microscope, loss of the H3K27me3 mark is currently diagnostic of PFA in the correct histologic and anatomic context,[103] and results from PRC2 inhibition via EZHIP overexpression.[104] PFA ependymomas have a significantly worse overall survival compared to PFB,[103] highlighting the importance of this distinction. Histologic grading is likely to have little impact on prognosis within molecular subgroups of ependymomas, including PFA and PFB.[104–106] A subset of subependymomas within the fourth ventricle and posterior fossa (methylation class "subependymoma, posterior fossa") may contain mixed histologic features of ependymoma and subependymoma.[107,108] In our experience, features of classic ependymoma may be encountered in this class even in the total absence of characteristic subependymoma histology. The clinical significance of these histologically-confirmed ependymomas that cluster with posterior fossa subependymoma is unclear.

Methylation array profiling is a powerful diagnostic tool for posterior fossa ependymomas and may also provide tumor type-specific prognostic information. In a study of 675 PFA ependymomas, two subgroups and nine subtypes were identified.[104] Within the PFA-1 subgroup, poorly prognostic subtypes were identified by methylation clustering. Currently, distinguishing PFA from PFB is the most clinically relevant use of methylation profiling in posterior fossa ependymomas; however, additional analyses may identify or confirm these clinically aggressive PFA subtypes. PFB ependymomas have

similarly been shown to harbor molecular heterogeneity. Five distinct methylation-based subtypes were identified in a cohort of 212 PFB ependymomas.[109] Differences in patient demographics and copy number (13q loss as a prognostic marker) were the predominant findings from subtype analysis. Thus, in addition to the reliable separation of PF ependymoma types by methylation profiling, copy number analysis may provide type-specific prognostic information.

Medulloblastomas (MB) are among the most histologically, molecularly, and clinically characterized CNS tumors. The revised 4th edition of the WHO blue book[5] introduced genetically-defined entries for MB subtypes that reflect their significant biologic and clinical heterogeneity. These subtypes include WNT-activated (wingless signal transduction pathway), SHH (sonic hedgehog signaling pathway), and non-WNT/non-SHH (groups 3 and 4). It is clinically important to make this molecular distinction: WNT-activated MB have a significantly better prognosis (>95% OS at 5 years in pediatric patients), while groups 3 and 4 have poor OS and often have metastatic disease at presentation. The clinical and genetic features that comprise these subtypes has been reviewed elsewhere.[110] A recent large-scale combined analysis of 1,501 group 3 and 4 MB revealed eight subtypes using both methylation and gene expression data.[111] In addition to genetic differences among these subtypes, three risk groups were identified with significantly different survival outcomes: group 1 ("high-risk") had a 5-year OS of 50%, compared to the "standard risk" group with a 5-year OS of 82%. This refinement of group 3/4 MB by methylation profiling was also translated into an ancillary random forest classifier that can be used in conjunction with the original Heidelberg Classifier. The additional refinement of medulloblastoma subtypes by methylation profiling is likely to be translated into the clinic in the future.

Conclusions, Limitations, and Future Directions

The integration of DNA methylation profiling into the routine work-up of CNS tumors has demonstrated improved diagnostic precision and clinically meaningful stratification of tumor types. The ability to capture the immense morphologic and genetic heterogeneity in primary CNS tumors remains the most robust feature of DNA methylation profiling. This is particularly valuable in histologically-ambiguous tumor types that may harbor targetable alterations (e.g., IHG). The technique has been demonstrated to be robust in confirming histologic diagnoses and may soon be adapted to providing useful diagnostic information even prior to receiving fixed tissue (H&E slides) for evaluation (e.g. intraoperative long-read sequencing).

A notable limitation of the Infinium BeadChip assay is the relatively higher amount of DNA that is required for hybridization (~250 ng). While this is significantly less than WGBS and RRBS requirements, brain tumor biopsies are often small and frequently yield a suboptimal amount of DNA. However, in our practice, we have been able to achieve meaningful classification results down to 20 ng of DNA (unpublished data). An inherent limitation to the use of bulk tissue for analysis is the presence of admixed non-neoplastic cells that may attenuate or obscure the signal of interest. These contaminants are particularly prevalent in brain tumor specimens where infiltration of normal brain tissue is a defining feature of many CNS tumor types. While other variables exist (DNA quality, batch effects), DNA yield and tumor purity are notable challenges in the translation of DNA methylation arrays to routine clinical practice.

Not infrequently, classification results may be non-contributory (suboptimal classifier score) or not congruent with the clinical, histopathologic, or molecular features of the case ("misleading profile"). While this can be due to the aforementioned pre-analytic factors, interpretation can often be improved with visual inspection of unsupervised UMAP or t-SNE embedding(s). In our experience, the power of this approach is largely dependent on the sample size and distribution of tumor types. In the setting of a "suggestive" classifier score (e.g. below 0.85), our experience has shown that concordance of orthogonal methods to assess methylation class (t-SNE, UMAP) serves to add confidence in suggesting a specific diagnosis for a tumor. Alternatively, in the instance where these complementary methods are discordant, confidence for a specific tumor class is lower, and often in these cases a purely descriptive diagnosis is rendered. Additionally, appropriate bioinformatics techniques and quality control should be carefully employed. As we move forward with machine learning-based classifier development, real-time unsupervised clustering of tumor samples and employment of complementary methods of classification will undoubtedly form a useful adjunct for difficult-to-classify cases.

Finally, the recent implementation of DNA methylation array-based classification in bone and soft tissue pathology[112] may herald a similar paradigm shift for other subspecialties. Of the 33 cancer types profiled through the TCGA, comprehensive molecular profiling has revealed biologically and/or prognostically-meaningful subgroups in *all* types based on unsupervised clustering of DNA methylation data (see references in the Supplementary Material), further underscoring its potential clinical utility in other cancers. Given the increasing reliance on methylation profiling in CNS tumor diagnostics and its adoption in clinical classification criteria, further study and utilization of this clinically useful platform are warranted in the practice of diagnostic neuropathology, in particular for uncommon and rare CNS neoplasms.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

Funding

The NCI Comprehensive Oncology Network for Evaluating Rare CNS Tumors (NCI-CONNECT) is a program within the Rare Tumor Patient Engagement Network (RTPEN), an initiative supported

by Cancer Moonshot℠ funds and managed at the National Institutes of Health, National Cancer Institute, Center for Cancer Research, Neuro-Oncology Branch. This research was funded in part through the National Institutes of Health/National Cancer Institute Cancer Center Support Grant P30 CA008748.

Acknowledgments

The authors wish to acknowledge Brittany Cordeiro, the NCICONNECT staff, and the Society for Neuro-Oncology staff for their assistance in developing the Rare CNS Cancer symposium and Kendal Morgan for her editorial assistance.

Supplement sponsorship. This supplement was sponsored by the National Institutes of Health.

Conflict of interest statement. None declared.

References

1. You JS, Jones PA. Cancer genetics and epigenetics: two sides of the same coin? *Cancer Cell*. 2012;22(1):9–20.
2. Tost J. DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. *Methods Mol Biol*. 2009;507:3–20.
3. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet*. 2012;13(7):484–492.
4. Klughammer J, Kiesel B, Roetzer T, et al. The DNA methylation landscape of glioblastoma disease progression shows extensive heterogeneity in time and space. *Nat Med*. 2018;24(10):1611–1624.
5. Louis DN, Perry A, Reifenberger G, et al. The 2016 world health organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol*. 2016;131(6):803–820.
6. Laird PW. Principles and challenges of genomewide DNA methylation analysis. *Nat Rev Genet*. 2010;11(3):191–203.
7. Frommer M, McDonald LE, Millar DS, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A*. 1992;89(5):1827–1831.
8. Lister R, Pelizzola M, Dowen RH, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*. 2009;462(7271):315–322.
9. Zhou W, Dinh HQ, Ramjan Z, et al. DNA methylation loss in late-replicating domains is linked to mitotic cell division. *Nat Genet*. 2018;50(4):591–602.
10. Bibikova M, Le J, Barnes B, et al. Genome-wide DNA methylation profiling using Infinium® assay. *Epigenomics*. 2009;1(1):177–200.
11. Steemers FJ, Gunderson KL. Whole genome genotyping technologies on the BeadArray platform. *Biotechnol J*. 2007;2(1):41–49.
12. Bibikova M, Barnes B, Tsan C, et al. High density DNA methylation array with single CpG site resolution. *Genomics*. 2011;98(4):288–295.
13. Rand AC, Jain M, Eizenga JM, et al. Mapping DNA methylation with high-throughput nanopore sequencing. *Nat Methods*. 2017;14(4):411–413.
14. Simpson JT, Workman RE, Zuzarte PC, David M, Dursi LJ, Timp W. Detecting DNA cytosine methylation using nanopore sequencing. *Nat Methods*. 2017;14(4):407–410.
15. Kuschel LP, Hench J, Frank S, et al. Robust methylation-based classification of brain tumors using nanopore sequencing. *medRxiv*. 2021:2021.2003.2006.21252627.
16. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: *R Foundation for Statistical Computing [Computer Program]*; 2020.
17. Aryee MJ, Jaffe AE, Corrada-Bravo H, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics*. 2014;30(10):1363–1369.
18. Zhou W, Laird PW, Shen H. Comprehensive characterization, annotation and innovative use of Infinium DNA methylation BeadChip probes. *Nucleic Acids Res*. 2017;45(4):e22.
19. van der Maaten L, Hinton G. Visualizing Data using t-SNE. *J Mach Learn Res*. 2008;9:2579–2605.
20. McInnes L, Healy J, Melville J. UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction. 2018:arXiv:1802.03426. https://ui.adsabs.harvard.edu/abs/2018arXiv180203426M. Accessed February 01, 2018.
21. Feber A, Guilhamon P, Lechner M, et al. Using high-density DNA methylation arrays to profile copy number alterations. *Genome Biol*. 2014;15(2):R30.
22. Capper D, Stichel D, Sahm F, et al. Practical implementation of DNA methylation and copy-number-based CNS tumor diagnostics: the Heidelberg experience. *Acta Neuropathol*. 2018;136(2):181–210.
23. Bady P, Delorenzi M, Hegi ME. Sensitivity analysis of the MGMT-STP27 model and impact of genetic and epigenetic context to predict the MGMT methylation status in gliomas and other tumors. *J Mol Diagn*. 2016;18(3):350–361.
24. Benhamida JK, Hechtman JF, Nafa K, et al. Reliable clinical MLH1 promoter hypermethylation assessment using a high-throughput genome-wide methylation array platform. *J Mol Diagn*. 2020;22(3):368–375.
25. Bady P, Sciuscio D, Diserens AC, et al. MGMT methylation analysis of glioblastoma on the Infinium methylation BeadChip identifies two distinct CpG regions associated with gene silencing and outcome, yielding a prediction model for comparisons across datasets, tumor grades, and CIMP-status. *Acta Neuropathol*. 2012;124(4):547–560.
26. van den Bent MJ, Erdem-Eraslan L, Idbaih A, et al. MGMT-STP27 methylation status as predictive marker for response to PCV in anaplastic oligodendrogliomas and oligoastrocytomas. A report from EORTC study 26951. *Clin Cancer Res*. 2013;19(19):5513–5522.
27. Capper D, Jones DTW, Sill M, et al. DNA methylation-based classification of central nervous system tumours. *Nature*. 2018;555(7697):469–474.
28. Priesterbach-Ackley LP, Boldt HB, Petersen JK, et al. Brain tumour diagnostics using a DNA methylation-based classifier as a diagnostic support tool. *Neuropathol Appl Neurobiol*. 2020;46(5):478–492.
29. Sturm D, Orr BA, Toprak UH, et al. New brain tumor entities emerge from molecular classification of CNS-PNETs. *Cell*. 2016;164(5):1060–1072.
30. von Hoff K, Haberler C, Robinson G, et al. Diagnostic re-evaluation and pooled clinical data analysis of patients with previous diagnosis of Cns-Pnet. *Neuro-Oncology*. 2018;20:72.
31. Łastowska M, Trubicka J, Sobocińska A, et al. Molecular identification of CNS NB-FOXR2, CNS EFT-CIC, CNS HGNET-MN1 and CNS

HGNET-BCOR pediatric brain tumors using tumor-specific signature genes. *Acta Neuropathol Commun.* 2020;8(1):105.
32. Wood MD, Tihan T, Perry A, et al. Multimodal molecular analysis of astroblastoma enables reclassification of most cases into more specific molecular entities. *Brain Pathol.* 2018;28(2):192–202.
33. Lehman NL, Usubalieva A, Lin T, et al. Genomic analysis demonstrates that histologically-defined astroblastomas are molecularly heterogeneous and that tumors with MN1 rearrangement exhibit the most favorable prognosis. *Acta Neuropathol Commun.* 2019;7(1):42.
34. Eckel-Passow JE, Lachance DH, Molinaro AM, et al. Glioma groups based on 1p/19q, IDH, and TERT promoter mutations in tumors. *N Engl J Med.* 2015;372(26):2499–2508.
35. Suwala AK, Stichel D, Schrimpf D, et al. Primary mismatch repair deficient IDH-mutant astrocytoma (PMMRDIA) is a distinct type with a poor prognosis. *Acta Neuropathol.* 2021;141(1):85–100.
36. Reinhardt A, Stichel D, Schrimpf D, et al. Anaplastic astrocytoma with piloid features, a novel molecular class of IDH wildtype glioma with recurrent MAPK pathway, CDKN2A/B and ATRX alterations. *Acta Neuropathol.* 2018;136(2):273–291.
37. Reinhardt A, Stichel D, Schrimpf D, et al. Tumors diagnosed as cerebellar glioblastoma comprise distinct molecular entities. *Acta Neuropathol Commun.* 2019;7(1):163.
38. Gareton A, Tauziède-Espariat A, Dangouloff-Ros V, et al. The histomolecular criteria established for adult anaplastic pilocytic astrocytoma are not applicable to the pediatric population. *Acta Neuropathol.* 2020;139(2):287–303.
39. Guerreiro Stucklin AS, Ryall S, Fukuoka K, et al. Alterations in ALK/ROS1/NTRK/MET drive a group of infantile hemispheric gliomas. *Nat Commun.* 2019;10(1):4343.
40. Olsen TK, Panagopoulos I, Meling TR, et al. Fusion genes with ALK as recurrent partner in ependymoma-like gliomas: a new brain tumor entity? *Neuro Oncol.* 2015;17(10):1365–1373.
41. Clarke M, Mackay A, Ismer B, et al. Infant high-grade gliomas comprise multiple subgroups characterized by novel targetable gene fusions and favorable outcomes. *Cancer Discov.* 2020;10(7):942–963.
42. Mullard A. FDA notches up third tissue-agnostic cancer approval. *Nat Rev Drug Discov.* 2019;18(10):737.
43. Drilon A, Laetsch TW, Kummar S, et al. Efficacy of larotrectinib in TRK fusion-positive cancers in adults and children. *N Engl J Med.* 2018;378(8):731–739.
44. Fischer H, Ullah M, de la Cruz CC, et al. Entrectinib, a TRK/ROS1 inhibitor with anti-CNS tumor activity: differentiation from other inhibitors in its class due to weak interaction with P-glycoprotein. *Neuro Oncol.* 2020;22(6):819–829.
45. Jones DT, Hutter B, Jäger N, et al.; International Cancer Genome Consortium PedBrain Tumor Project. Recurrent somatic alterations of FGFR1 and NTRK2 in pilocytic astrocytoma. *Nat Genet.* 2013;45(8):927–932.
46. Torre M, Vasudevaraja V, Serrano J, et al. Molecular and clinicopathologic features of gliomas harboring NTRK fusions. *Acta Neuropathol Commun.* 2020;8(1):107.
47. Wu G, Diaz AK, Paugh BS, et al. The genomic landscape of diffuse intrinsic pontine glioma and pediatric non-brainstem high-grade glioma. *Nat Genet.* 2014;46(5):444–450.
48. Ryall S, Zapotocky M, Fukuoka K, et al. Integrated molecular and clinical analysis of 1,000 pediatric low-grade gliomas. *Cancer Cell.* 2020;37(4):569–583 e565.
49. Wefers AK, Stichel D, Schrimpf D, et al. Isomorphic diffuse glioma is a morphologically and molecularly distinct tumour entity with recurrent gene fusions of MYBL1 or MYB and a benign disease course. *Acta Neuropathol.* 2020;139(1):193–209.
50. Slegers RJ, Blumcke I. Low-grade developmental and epilepsy associated brain tumors: a critical update 2020. *Acta Neuropathol Commun.* 2020;8(1):27.
51. Blümcke I, Luyken C, Urbach H, Schramm J, Wiestler OD. An isomorphic subtype of long-term epilepsy-associated astrocytomas associated with benign prognosis. *Acta Neuropathol.* 2004;107(5):381–388.
52. Chiang J, Harreld JH, Tinkle CL, et al. A single-center study of the clinicopathologic correlates of gliomas with a MYB or MYBL1 alteration. *Acta Neuropathol.* 2019;138(6):1091–1092.
53. Qaddoumi I, Orisme W, Wen J, et al. Genetic alterations in uncommon low-grade neuroepithelial tumors: BRAF, FGFR1, and MYB mutations occur at high frequency and align with morphology. *Acta Neuropathol.* 2016;131(6):833–845.
54. Deng MY, Sill M, Sturm D, et al. Diffuse glioneuronal tumour with oligodendroglioma-like features and nuclear clusters (DGONC) - a molecularly defined glioneuronal CNS tumour class displaying recurrent monosomy 14. *Neuropathol Appl Neurobiol.* 2020;46(5):422–430.
55. Pickles JC, Mankad K, Aizpurua M, et al. A case series of diffuse glioneuronal tumours with oligodendroglioma-like features and nuclear clusters (DGONC). *Neuropathol Appl Neurobiol.* 2021;47(3):464.
56. Sievers P, Appay R, Schrimpf D, et al. Rosette-forming glioneuronal tumors share a distinct DNA methylation profile and mutations in FGFR1, with recurrent co-mutation of PIK3CA and NF1. *Acta Neuropathol.* 2019;138(3):497–504.
57. Lucas CG, Gupta R, Doo P, et al. Comprehensive analysis of diverse low-grade neuroepithelial tumors with FGFR1 alterations reveals a distinct molecular signature of rosette-forming glioneuronal tumor. *Acta Neuropathol Commun.* 2020;8(1):151.
58. Dieci MV, Arnedos M, Andre F, Soria JC. Fibroblast growth factor receptor inhibitors as a cancer treatment: from a biologic rationale to medical perspectives. *Cancer Discov.* 2013;3(3):264–279.
59. Korshunov A, Remke M, Gessi M, et al. Focal genomic amplification at 19q13.42 comprises a powerful diagnostic marker for embryonal tumors with ependymoblastic rosettes. *Acta Neuropathol.* 2010;120(2):253–260.
60. Korshunov A, Sturm D, Ryzhova M, et al. Embryonal tumor with abundant neuropil and true rosettes (ETANTR), ependymoblastoma, and medulloepithelioma share molecular similarity and comprise a single clinicopathological entity. *Acta Neuropathol.* 2014;128(2):279–289.
61. Lambo S, Gröbner SN, Rausch T, et al. The molecular landscape of ETMR at diagnosis and relapse. *Nature.* 2019;576(7786):274–280.
62. Antonelli M, Korshunov A, Mastronuzzi A, et al. Long-term survival in a case of ETANTR with histological features of neuronal maturation after therapy. *Virchows Arch.* 2015;466(5):603–607.
63. Al-Hussaini M, Abuirmeileh N, Swaidan M, et al. Embryonal tumor with abundant neuropil and true rosettes: a report of three cases of a rare tumor, with an unusual case showing rhabdomyoblastic and melanocytic differentiation. *Neuropathology.* 2011;31(6):620–625.
64. Buccoliero AM, Castiglione F, Rossi Degl'Innocenti D, et al. Embryonal tumor with abundant neuropil and true rosettes: morphological, immunohistochemical, ultrastructural and molecular study of a case showing features of medulloepithelioma and areas of mesenchymal and epithelial differentiation. *Neuropathology.* 2010;30(1):84–91.
65. Woehrer A, Slavc I, Peyrl A, et al. Embryonal tumor with abundant neuropil and true rosettes (ETANTR) with loss of morphological but retained genetic key features during progression. *Acta Neuropathol.* 2011;122(6):787–790.
66. Spence T, Sin-Chan P, Picard D, et al. CNS-PNETs with C19MC amplification and/or LIN28 expression comprise a distinct histogenetic diagnostic and therapeutic entity. *Acta Neuropathol.* 2014;128(2):291–303.
67. Weingart MF, Roth JJ, Hutt-Cabezas M, et al. Disrupting LIN28 in atypical teratoid rhabdoid tumors reveals the importance of the mitogen

activated protein kinase pathway as a therapeutic target. *Oncotarget.* 2015;6(5):3165–3177.

68. Cao D, Liu A, Wang F, et al. RNA-binding protein LIN28 is a marker for primary extragonadal germ cell tumors: an immunohistochemical study of 131 cases. *Mod Pathol.* 2011;24(2):288–296.
69. Ferris SP, Velazquez Vega J, Aboian M, et al. High-grade neuroepithelial tumor with BCOR exon 15 internal tandem duplication-a comprehensive clinical, radiographic, pathologic, and genomic analysis. *Brain Pathol.* 2020;30(1):46–62.
70. Yoshida Y, Nobusawa S, Nakata S, et al. CNS high-grade neuroepithelial tumor with BCOR internal tandem duplication: a comparison with its counterparts in the kidney and soft tissue. *Brain Pathol.* 2018;28(5):710–720.
71. Roy A, Kumar V, Zorman B, et al. Recurrent internal tandem duplications of BCOR in clear cell sarcoma of the kidney. *Nat Commun.* 2015;6:8891.
72. Santiago T, Clay MR, Allen SJ, Orr BA. Recurrent BCOR internal tandem duplication and BCOR or BCL6 expression distinguish primitive myxoid mesenchymal tumor of infancy from congenital infantile fibrosarcoma. *Mod Pathol.* 2017;30(6):884–891.
73. Antonescu CR, Kao YC, Xu B, et al. Undifferentiated round cell sarcoma with BCOR internal tandem duplications (ITD) or YWHAE fusions: a clinicopathologic and molecular study. *Mod Pathol.* 2020;33(9):1669–1677.
74. Tauziède-Espariat A, Pierron G, Siegfried A, et al. The EP300:BCOR fusion extends the genetic alteration spectrum defining the new tumoral entity of "CNS tumors with BCOR internal tandem duplication". *Acta Neuropathol Commun.* 2020;8(1):178.
75. Johann PD, Erkek S, Zapatka M, et al. Atypical Teratoid/Rhabdoid tumors are comprised of three epigenetic subgroups with distinct enhancer landscapes. *Cancer Cell.* 2016;29(3):379–393.
76. Torchia J, Golbourn B, Feng S, et al. Integrated (epi)-genomic analyses identify subgroup-specific therapeutic targets in CNS rhabdoid tumors. *Cancer Cell.* 2016;30(6):891–908.
77. Biegel JA, Zhou JY, Rorke LB, Stenstrom C, Wainwright LM, Fogelgren B. Germ-line and acquired mutations of INI1 in atypical teratoid and rhabdoid tumors. *Cancer Res.* 1999;59(1):74–79.
78. Versteege I, Sévenet N, Lange J, et al. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature.* 1998;394(6689):203–206.
79. Hasselblatt M, Nagel I, Oyen F, et al. SMARCA4-mutated atypical teratoid/rhabdoid tumors are associated with inherited germline alterations and poor prognosis. *Acta Neuropathol.* 2014;128(3):453–456.
80. Thomas C, Wefers A, Bens S, et al. Desmoplastic myxoid tumor, SMARCB1-mutant: clinical, histopathological and molecular characterization of a pineal region tumor encountered in adolescents and adults. *Acta Neuropathol.* 2020;139(2):277–286.
81. Johann PD, Hovestadt V, Thomas C, et al. Cribriform neuroepithelial tumor: molecular characterization of a SMARCB1-deficient non-rhabdoid tumor with favorable long-term outcome. *Brain Pathol.* 2017;27(4):411–418.
82. Shih AR, Cote GM, Chebib I, et al. Clinicopathologic characteristics of poorly differentiated chordoma. *Mod Pathol.* 2018;31(8):1237–1245.
83. Mobley BC, McKenney JK, Bangs CD, et al. Loss of SMARCB1/INI1 expression in poorly differentiated chordomas. *Acta Neuropathol.* 2010;120(6):745–753.
84. Hasselblatt M, Thomas C, Hovestadt V, et al. Poorly differentiated chordoma with SMARCB1/INI1 loss: a distinct molecular entity with dismal prognosis. *Acta Neuropathol.* 2016;132(1):149–151.
85. Shih AR, Chebib I, Deshpande V, Dickson BC, Iafrate AJ, Nielsen GP. Molecular characteristics of poorly differentiated chordoma. *Genes Chromosomes Cancer.* 2019;58(11):804–808.
86. von Hoff K, Hinkes B, Dannenmann-Stern E, et al. Frequency, risk-factors and survival of children with atypical teratoid rhabdoid tumors (AT/RT) of the CNS diagnosed between 1988 and 2004, and registered to the German HIT database. *Pediatr Blood Cancer.* 2011;57(6):978–985.
87. Kallen ME, Hornick JL. The 2020 WHO Classification: what's new in soft tissue tumor pathology? *Am J Surg Pathol.* 2021;45(1):e1–e23.
88. Le Loarer F, Pissaloux D, Watson S, et al. Clinicopathologic features of CIC-NUTM1 sarcomas, a new molecular variant of the family of CIC-fused sarcomas. *Am J Surg Pathol.* 2019;43(2):268–276.
89. Kawamura-Saito M, Yamazaki Y, Kaneko K, et al. Fusion between CIC and DUX4 up-regulates PEA3 family genes in Ewing-like sarcomas with t(4;19)(q35;q13) translocation. *Hum Mol Genet.* 2006;15(13):2125–2137.
90. Yoshida A, Arai Y, Kobayashi E, et al. CIC break-apart fluorescence in-situ hybridization misses a subset of CIC-DUX4 sarcomas: a clinicopathological and molecular study. *Histopathology.* 2017;71(3):461–469.
91. Pratt D, Kumar-Sinha C, Cieślik M, et al. A novel ATXN1-DUX4 fusion expands the spectrum of 'CIC-rearranged sarcoma' of the CNS to include non-CIC alterations. *Acta Neuropathol.* 2021;141(4):619–622.
92. Antonescu CR, Owosho AA, Zhang L, et al. Sarcomas With CIC-rearrangements are a distinct pathologic entity with aggressive outcome: a clinicopathologic and molecular study of 115 cases. *Am J Surg Pathol.* 2017;41(7):941–949.
93. Koelsche C, Mynarek M, Schrimpf D, et al. Primary intracranial spindle cell sarcoma with rhabdomyosarcoma-like features share a highly distinct methylation profile and DICER1 mutations. *Acta Neuropathol.* 2018;136(2):327–337.
94. de Kock L, Sabbaghian N, Druker H, et al. Germ-line and somatic DICER1 mutations in pineoblastoma. *Acta Neuropathol.* 2014;128(4):583–595.
95. Alexandrescu S, Meredith DM, Lidov HG, et al. Loss of histone H3 trimethylation on lysine 27 and nuclear expression of transducin-like enhancer 1 in primary intracranial sarcoma, DICER1-mutant. *Histopathology.* 2021;78(2):265–275.
96. Rodriguez FJ, Perry A, Rosenblum MK, et al. Disseminated oligodendroglial-like leptomeningeal tumor of childhood: a distinctive clinicopathologic entity. *Acta Neuropathol.* 2012;124(5):627–641.
97. Deng MY, Sill M, Chiang J, et al. Molecularly defined diffuse leptomeningeal glioneuronal tumor (DLGNT) comprises two subgroups with distinct clinical and genetic features. *Acta Neuropathol.* 2018;136(2):239–253.
98. Rodriguez FJ, Schniederjan MJ, Nicolaides T, Tihan T, Burger PC, Perry A. High rate of concurrent BRAF-KIAA1549 gene fusion and 1p deletion in disseminated oligodendroglioma-like leptomeningeal neoplasms (DOLN). *Acta Neuropathol.* 2015;129(4):609–610.
99. Dodgshun AJ, SantaCruz N, Hwang J, et al. Disseminated glioneuronal tumors occurring in childhood: treatment outcomes and BRAF alterations including V600E mutation. *J Neurooncol.* 2016;128(2):293–302.
100. Appay R, Pages M, Colin C, Jones DTW, Varlet P, Figarella-Branger D. Diffuse leptomeningeal glioneuronal tumor: a double misnomer? A report of two cases. *Acta Neuropathol Commun.* 2020;8(1):95.
101. Dyson K, Rivera-Zengotita M, Kresak J, et al. FGFR1 N546K and H3F3A K27M mutations in a diffuse leptomeningeal tumour with glial and neuronal markers. *Histopathology.* 2016;69(4):704–707.
102. Mack SC, Witt H, Piro RM, et al. Epigenomic alterations define lethal CIMP-positive ependymomas of infancy. *Nature.* 2014;506(7489):445–450.
103. Panwalkar P, Clark J, Ramaswamy V, et al. Immunohistochemical analysis of H3K27me3 demonstrates global reduction in group-A childhood posterior fossa ependymoma and is a powerful predictor of outcome. *Acta Neuropathol.* 2017;134(5):705–714.

104. Pajtler KW, Wen J, Sill M, et al. Molecular heterogeneity and CXorf67 alterations in posterior fossa group A (PFA) ependymomas. *Acta Neuropathol*. 2018;136(2):211–226.

105. Pajtler KW, Witt H, Sill M, et al. Molecular classification of ependymal tumors across all CNS compartments, histopathological grades, and age groups. *Cancer Cell*. 2015;27(5):728–743.

106. Upadhyaya SA, Robinson GW, Onar-Thomas A, et al. Molecular grouping and outcomes of young children with newly diagnosed ependymoma treated on the multi-institutional SJYC07 trial. *Neuro Oncol*. 2019;21(10):1319–1330.

107. Kim Y, Lee SY, Yi KS, et al. Infratentorial and intraparenchymal subependymoma in the cerebellum: case report. *Korean J Radiol*. 2014;15(1):151–155.

108. Bi Z, Ren X, Zhang J, Jia W. Clinical, radiological, and pathological features in 43 cases of intracranial subependymoma. *J Neurosurg*. 2015;122(1):49–60.

109. Cavalli FMG, Hübner JM, Sharma T, et al. Heterogeneity within the PF-EPN-B ependymoma subgroup. *Acta Neuropathol*. 2018;136(2):227–237.

110. Kumar R, Liu APY, Northcott PA. Medulloblastoma genomics in the modern molecular era. *Brain Pathol*. 2020;30(3):679–690.

111. Sharma T, Schwalbe EC, Williamson D, et al. Second-generation molecular subgrouping of medulloblastoma: an international meta-analysis of Group 3 and Group 4 subtypes. *Acta Neuropathol*. 2019;138(2):309–326.

112. Koelsche C, Schrimpf D, Stichel D, et al. Sarcoma classification by DNA methylation profiling. *Nat Commun*. 2021;12(1):498.