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The Essentials of Molecular Testing in CNS Tumors: What to Order and How to Integrate Results

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Abstract

Purpose of Review Molecular testing has become essential for the optimal workup of central nervous system (CNS) tumors. There is a vast array of testing from which to choose, and it can sometimes be challenging to appropriately incorporate findings into an integrated report. This article reviews various molecular tests and provides a concise overview of the most important molecular findings in the most commonly encountered CNS tumors.

Recent Findings Many molecular alterations in CNS tumors have been identified over recent years, some of which are incorporated into the 2016 World Health Organization (WHO) classification and the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy-Not Official WHO (cIMPACT-NOW) updates. Array-based methylation profiling has emerged over the past couple of years and will likely replace much of currently used ancillary testing for diagnostic purposes.

Summary A combination of next-generation sequencing (NGS) panel and copy number array is ideal for diffuse gliomas and embryonal tumors, with a low threshold to employ in other tumor types. With the recent advances in molecular diagnostics, it will be ever more important for the pathologist to recognize the molecular testing available, which tests to perform, and to appropriately integrate results in light of clinical, radiologic, and histologic findings.

Keywords Molecular testing · Next generation sequencing · Methylation profiling · Glioma · Embryonal · Ependymoma

Introduction

In today's world of surgical neuropathology, providing accurate, efficient, and useful results requires an up-to-date molecular diagnostics laboratory capable of identifying a wide array of important single nucleotide variants (SNVs), insertions and/or deletions (indels), copy number variants (CNVs), fusions, and DNA methylation changes. We will first briefly review the uses, strengths, and weaknesses of various molecular and ancillary tests, which will be followed by a concise overview of the most important diagnostic, prognostic, and predictive molecular findings in the most commonly encountered CNS tumors (summarized in Table 1). For more detailed information, the reader is referred to additional articles and books on the subject [1•, 2•].

This article is part of the Topical Collection on *Neuro-Oncology*

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Overview of Main Testing Methods

Detection of Single Nucleotide Variations and Small Insertions/Deletions

IHC

In specific situations, immunohistochemistry (IHC) can screen for clinically relevant molecular aberrations (such as the canonical p.R132H SNV in *isocitrate dehydrogenase 1* [*IDH1*]), mutations resulting in loss/truncation of tumor suppressor proteins (e.g., Atrx loss in *IDH*-mutant astrocytomas), mutations resulting in altered subcellular localization (e.g., β-catenin nuclear staining in *WNT*-activated medulloblastomas) and can serve as a surrogate of underlying molecular alterations (e.g., L1cam for

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Table 1 Overview of major molecular findings in CNS tumors

Tumor type [references]	Molecular IHC	CNVs	SNVs, indels, fusions
Diffuse astrocytic and oligodendroglial tumors			
GBM, *IDH*-wildtype/diffuse astrocytic glioma, *IDH*-wildtype, with molecular features of GBM [18••, 33]	- Negative for Idh1 R132H - Retention of Atrx	Combined whole chromosome gain of 7/loss of 10 and/or amplification of *EGFR*	- *TERT*-p mut. - Alterations of RTK, p53 and/or Rb pathways
Astrocytoma, *IDH*-mutant, WHO grades 2–4 [4, 38, 40, 41•]	- Positive for Idh1 R132H (~90%) - Loss of Atrx	Hz loss of *CDKN2/B* (WHO grade 4)	- *IDH1* p.R132 or *IDH2* p.R172 SNVs - Alterations of *ATRX* and *TP53*
"Anaplastic astrocytoma with piloid features," NEC* [30•]	- Negative for Idh1 R132H- Loss of Atrx (~45%)	Loss of *CDKN2A/B* (~80%)	- MAPK pathway alterations (~75%) - Alterations of *ATRX* (~45%)
Diffuse midline glioma, H3 K27M-mutant, WHO grade 4 [43–45]	- Positive for H3 K27M - Loss of H3K27me3		p.K27M mut. in *H3F3A/B* or *HIST1H3A/B/C*
H3 G34-mutant glioma, *IDH*-wildtype, NEC [43, 46, 47]	- Negative for Idh1 R132H and Olig2 - Loss of Atrx, strong p53		p.G34R/V mutation in *H3F3A*
Pediatric diffuse gliomas with *BRAF*, *FGFR1*, *MYB*, or *MYBL1* alterations [48, 49]	- Negative for Idh1 R132H and H3 K27M - Positive Braf V600E (in subset)	Single alterations of *MYB*, *MYBL1*, *BRAF*, *FGFR1*, or other MAPK pathway genes	Single alterations of *MYB*, *MYBL1*, *BRAF*, *FGFR1*, or other MAPK pathway genes
Oligodendroglioma, *IDH*-mutant and 1p/19q-codeleted [17••, 40]	- Positive for Idh1 R132H (~90%) - Atrx retained	Whole arm 1p/19q codeletion	- *IDH1* p.R132 or *IDH2* p.R172 SNVs - *TERT*-p mut.
Non-infiltrative astrocytoma variants and glioneuronal tumors			
Pilocytic astrocytoma [17••, 54–56]	Positive Braf V600E (~5–10%)	Tandem duplication of 7q34 (causing a *BRAF* fusion, ~70%)	*KIAA1549-BRAF* fusion (~70%), *BRAF* p.V600E (~5–10%) or other MAPK pathway alterations
Pleomorphic xanthroastrocytoma [56, 57]	Positive Braf V600E (~60–80%)	Hz loss of *CDKN2A/B* (50–70%)	*BRAF* p.V600E (60–80%)
Ganglioglioma [56]	Positive Braf V600E (~20–50%)		*BRAF* p.V600E (~20–50%)
Dysembryoplastic neuroepithelial tumor [48, 59–61]	Positive Braf V600E in subset (~4–30%)	*BRAF* gain (~30%)	*FGFR1* alterations (~60%) *BRAF* p.V600E (~4–30%)
Rosette-forming glioneuronal tumor [62]			*FGFR1* (majority, if not all) and *PIK3CA* (~63%)/*NF1* (~33%) mut.
Papillary glioneuronal tumor [63]			*SLC44A1-PRKCA* fusion
Ependymomas			
Ependymoma, *RELA*-fusion [27, 64]	Positive for L1cam	Chromothripsis or CNVs of 11q	*C11orf95-RELA* fusion most common
Ependymoma, *YAP1*-fusion [27]		Segmental CNVs of 11q (*YAP1*)	*YAP1-MAMLD1* fusion most common
PFA ependymoma* [27, 65]	Loss of H3K27me3	Balanced genome in majority	
PFB ependymoma* [27, 65]	Retention of H3K27me3	Multiple CNVs	
Spinal ependymoma [27, 58]		Many CNVs, including loss of 22q (*NF2*)	*NF2* mut.
Myxopapillary ependymoma [27, 58]		Many CNVs, including gains of 5, 7, 9, 16 and 18	
Embryonal tumors			
Medulloblastoma, *WNT*-activated [66–68]	- Nuclear β-catenin - Yap1 positive, Gab1 negative	Monosomy 6	*CTNNB1* (most common) or *APC* mut.
Medulloblastoma, *SHH*-activated [66–69]	Yap1 and Gab1 positive	- Loss of 9q and 10q - Chromothripsis (*TP53*-mut.)	Alterations in *PTCH1*, *SMO*, *SUFU*, *GLI1*, *GLI2*, *MYCN*, *TP53* (in subset)
Medulloblastoma, group 3* [66–68]	Negative for Gab1 and Yap1	- i(17q) (~60%) - *MYC* amp. (~20%)	
Medulloblastoma, group 4* [66–68]	Negative for Gab1 and Yap1	i(17q) (~80%)	
Atypical teratoid/rhabdoid tumor [70–72]	Ini1 (or Brg1) loss	*SMARCB1* (or *SMARCA4*) CNVs	*SMARCB1* (or *SMARCA4*) alterations
ETMR, C19MC-altered [73]	Positive for Lin28a	*C19MC* amp.	*TTYH1-C19MC* fusion
CNS neuroblastoma with *FOXR2* alteration* [29•]		*FOXR2* CNVs	*FOXR2* alterations
	Nutm1 positive (in subset)		*CIC* alterations

Table 1 (continued)

Tumor type [references]	Molecular IHC	CNVs	SNVs, indels, fusions
CNS Ewing sarcoma family tumor with *CIC* alteration* [29•]			
CNS high-grade neuroepithelial tumor with *MN1* alteration* [29•]			*MN1* alterations
CNS high-grade neuroepithelial tumor with *BCOR* alteration* [29•]	Nuclear β-catenin (in subset)	*BCOR* CNVs	*BCOR* alterations
Other tumors			
Meningioma [74–81, 83, 84]		- Loss of 22q (*NF2*, ~40–70%) - Loss of 1p, 10, 14q (higher grade) - Loss of 9p21 (*CDKN2A*) (poorer prognosis)	- *NF2, SMO*, *AKT1*, *PIK3CA* mut. - *KLF4* and *TRAF7* (secretory), *SMARCE1* (clear cell), *BAP1* mut. (rhabdoid) - *TERT*-p mut. (poorer prognosis)

*Genome-wide methylation profiling may be needed for accurate diagnosis

amp. amplification, *CNS* central nervous system, *CNV* copy number variant, *ETMR* embryonal tumor with multilayered rosettes, *GBM* glioblastoma, *Hz* homozygous, *IDH* isocitrate dehydrogenase, *IHC* immunohistochemistry, *indel* insertion/deletion, *MAPK* mitogen-activated protein kinase, *mut.* mutation, *NEC* not elsewhere classified, *Rb* retinoblastoma, *RTK* receptor tyrosine kinase, *SNV* single nucleotide variant, *TERT-p* telomerase reverse transcriptase promoter

RELA-fusion positive supratentorial ependymomas) [3]. The most widely used IHC marker in neuropathology is the antibody specific for Idh1 R132H, which detects ~90% of *IDH*-mutant gliomas [4].

Advantages of IHC include quick turnaround time (only one tissue section needed per IHC marker), the ability to pick out sparse tumor cells amongst more numerous nonneoplastic cells, and the relative ease of deployment (including at institutions without molecular labs). Weaknesses include the difficulty of creating reliable IHC antibodies, as only a small number of molecular IHC markers are robust enough for routine use. Also, some molecular alterations do not cause protein conformational changes large enough to facilitate mutation-specific antibodies [3]. Additionally, IHC against specific hotspot mutations will not detect other less common mutations (e.g., non-canonical *IDH1* or *IDH2* mutations in *IDH*-mutant gliomas) [4]. Further, some stains, like those directed against Braf V600E, H3 K27M, and H3K27 trimethylation (H3K27me3) are challenging to optimize. Finally, no IHC marker is 100% sensitive or specific, and their reliability can be compromised by necrosis and thermal cautery artifact. Best outcomes are when molecular IHC markers are interpreted by experienced neuropathologists, with a low threshold for reflex confirmation by sequencing in the event of ambiguous results.

Targeted Mutation Detection

Recurrent hotspot mutations can be targeted for detection using any one of a number of techniques. For the detection of multiple mutations within one or more exons, sequencing by termination (e.g., Sanger dideoxy sequencing) [5] or sequencing by synthesis (e.g., pyrosequencing) [6] are common. These methods use polymerase chain reaction (PCR) amplification to target exons of interest followed by sequencing and analysis. DNA is the usual template, although RNA can also be used after reverse transcription of the RNA to complementary DNA (i.e., RT-PCR). These techniques are optimal for detection of SNVs and small indels in single exons (e.g., *IDH1* p.R132, *BRAF* p.V600, *H3F3A* exon 1). However, these methods require at least 20% tumor cellularity. Droplet digital PCR (ddPCR) is highly sensitive even with low DNA quality, enabling the detection of mutations well below 1% variant allele frequencies [7, 8]. However, each mutation must be specifically targeted in ddPCR, so the number of targetable mutations is more limited. SNaPshot® incorporates multiple probes for multiple targets to detect a panel of known SNVs and indels simultaneously across many genes [9]. However, this can only be used to detect recurrent hotspot mutations.

Next-Generation Sequencing

Next-generation sequencing (NGS) refers to high-throughput technologies that perform massive, rapid sequencing of DNA and/or RNA (RNA-Seq) [10, 11]. Common platforms include Illumina® and Ion Torrent™. NGS is efficient, cost-effective, feasible on formalin-fixed, paraffin-embedded (FFPE) tissue and allows simultaneous evaluation of the majority of the most common alterations (SNVs, small indels, small CNVs, and fusions) in CNS tumors. Relevant genes are targeted using hybrid capture methods or custom sets of multiplexed PCR primers. Panels can target genes known to be most relevant for brain tumors (e.g., GlioSeq) [12], all exons of hundreds of cancer-relevant genes (e.g., Foundation One® CDx) or whole exome or whole genome analysis. Larger panels more

accurately estimate tumor mutational burden (TMB), which is relevant when considering anti-tumor immunotherapies [13]. Because NGS can screen for known, as well as novel, mutations in thousands of exons (with better sensitivity than either Sanger sequencing or pyrosequencing) NGS is our primary assay, with targeted methods as necessary for confirmation. For example, we confirm *TERT* promoter (*TERT*-p) mutations by pyrosequencing or ddPCR and confirm fusions via RT-PCR followed by Sanger sequencing.

When developing an NGS assay, care should be taken to select a panel of appropriate breadth, including all mutations (SNVs and small indels), small CNVs and fusions relevant to brain tumors. Additionally, there must be sufficient depth of coverage (i.e., sensitivity) for regions of interest (e.g., exons, introns including regulatory regions). This can be of particular importance in biopsies containing relatively sparse tumor or when treatment-resistant subclones may be present. The technology is not optimal for large CNVs, such as whole chromosome or whole arm gain/deletion. Patient-matched nonneoplastic tissue may be needed to distinguish germline versus somatic mutations [14]. Our NGS panel screens for the most relevant SNVs, small indels, small CNVs, and fusions in CNS tumors, including those found in gliomas, glioneuronal tumors, and embryonal tumors.

One drawback to NGS is the requirement for high-quality bioinformatics analysis of the raw data. While many commercial bioinformatics pipelines exist, each algorithm has variable strength of accuracy in variant calling across the genome. As a result, more detailed, manual post-processing analysis or additional coverage via direct sequencing methods are sometimes required for certain regions (e.g., *TERT*-p). Additionally, tissues that are several years old, mostly necrotic, sparsely cellular, or suboptimally processed may be unsuitable for NGS (especially for RNA-Seq, given the lability of RNA compared to DNA) [3].

Detection of Copy Number Variations

FISH

Fluorescence in situ hybridization (FISH) is still the most widely used tool for assessing single copy number variants and fusions. It is particularly well-suited when the biopsy material is very small, when only a portion of tissue contains tumor, when there is intratumoral heterogeneity, when searching for specific copy number gains (e.g., *EGFR* amplification) and when searching for specific fusions. Deletions are a little more challenging, as tissue sectioning can make some truncated nuclei falsely appear to have copy number loss [1•]. FISH only assesses a single locus (or if multiplexed, a handful of loci). It cannot fully cover whole-arm losses or gains (occasionally resulting in false positives) [15], and it cannot detect SNVs or small indels. Break-apart probes will not discern a specific fusion partner gene, and fusion probes will require knowledge of both fusion partners. Fusion detection by FISH may thus be complicated when targeted genes have multiple fusion partners and/or multiple breakpoints (e.g., *BRAF*) [16]. Furthermore, FISH is relatively labor-intensive and requires considerable technical staff training. We use FISH only when 1p/19q status needs to be determined quickly and for molecular aberrations not well-covered in our NGS panel or copy number array (e.g., C19MC alterations).

Copy Number Arrays

Assessment of genome-wide CNVs by copy number array is an important, though frequently overlooked, molecular tool in the assessment of brain tumors. Unlike FISH, the technology can simultaneously assess multiple loci throughout the entire genome. Different platforms include single nucleotide polymorphism (SNP) arrays, array comparative genomic hybridization (aCGH), and methylation arrays (see below), all of which can be performed on FFPE tissue [3]. The most well-known CNV in CNS tumors is 1p/19q-codeletion, which is now required for the diagnosis of oligodendroglioma in the most recent WHO classification [17••]. Additionally, combined whole chromosome 7 gain/10 loss can be used to diagnose a histologic grade 2–3 diffusely infiltrating *IDH*-wildtype astrocytoma as "diffuse astrocytic glioma, *IDH*-wildtype, with molecular features of glioblastoma, WHO grade 4" [18••].

Hybridization-based copy-number arrays require more tumor tissue than either NGS or FISH and cannot determine the fraction of cells with a particular CNV [1•]. We routinely employ copy number arrays in the workup of diffusely infiltrating gliomas and embryonal tumors and frequently use it in the workup of other CNS tumors.

Detection of Fusions

Gene fusions can be helpful for the diagnosis of some brain tumors, such as *RELA* fusion in supratentorial ependymomas or *BRAF* fusion in pilocytic astrocytomas. They can also provide predictive information like *NTRK* and *FGFR* fusions in diffuse gliomas. Fusions can be detected by PCR-based copy arrays, FISH, DNA-based NGS panels, and RNA-Seq. False negatives can occur, mainly because it is difficult to create a panel that captures all breakpoint variants and fusion partners [1•]. Because of potential false positives, fusions should be screened by one method and confirmed via another. We routinely screen for selected fusions using RNA-Seq as part of our NGS panel and confirm all positives with RT-PCR followed by Sanger sequencing.

DNA Methylation

MGMT Promoter Methylation

DNA methylation, an epigenetic mechanism of gene regulation, occurs on cytosine residues of cytosine/guanine (CG) sequences. Methylation on CG clusters usually induces transcriptional silencing. The *MGMT* gene encodes O-6-methylguanine-DNA methyltransferase, an enzyme that repairs DNA damage induced by alkylating chemotherapeutic agents such as temozolomide (TMZ). *MGMT* transcription is regulated by its promoter methylation status, whereby increased methylation leads to decreased protein. *MGMT* promoter methylation is thus associated with a better response to TMZ chemotherapy [19]. There are many ways to detect *MGMT* promoter methylation, including quantitative methylation-specific PCR (qMS-PCR), methylation-specific high-resolution melting, MethyLight™ and pyrosequencing. While *MGMT* pyrosequencing correlated best with overall survival in one study [20], qMS-PCR is the most widely used method in clinical trials and provides a three-tiered classification of methylation extent [21]. We analyze *MGMT* promoter methylation by pyrosequencing on any tumor in which TMZ therapy is being considered (e.g., diffuse gliomas).

Genome-Wide Methylation Profiling

Genomic array-based DNA methylation profiling is revolutionizing how primary CNS and metastatic tumors are being diagnosed. This technology assesses methylation at hundreds of thousands of CpG loci across the entire genome. The results are then paired with a classifier algorithm, originally derived via unsupervised clustering (machine learning). Brain tumor types with different molecular underpinnings possess different methylation "fingerprints," allowing accurate discrimination even amongst tumors with similar histologic appearances [22••]. Metastatic tumors retain the methylation "fingerprint" of their tissue of origin [23]. In recent large series, methylation profiling substantially revised CNS tumor diagnoses in approximately 12–14% of cases originally diagnosed by expert neuropathologists [22••, 24]. This assay also provides large CNV and *MGMT* promoter methylation data [25], can be performed on FFPE tissue, and is ideal for difficult-to-classify tumors, including those with indistinct histology or crush/cautery artifact.

Methylation profiling can reliably subtype medulloblastomas (e.g., group 3 vs group 4) [26] and ependymomas (e.g., posterior fossa group A vs B) [27]. It is superior to histology-based WHO grading for the prognostic stratification of meningiomas [28••]. It has also proven essential for the discovery of new entities, such as CNS embryonal tumors with alterations of *FOXR2*, *BCOR*, *CIC*, or *MN1* [29•] and the "anaplastic astrocytoma with piloid features [30•]." However, samples need to be > 50% tumor tissue, ideally > 70%, and this assay requires deep bioinformatics expertise. Not all CNS tumors cluster with other known methylation classes and, thus, a tumor that is difficult to classify by more conventional methods may remain unique by methylation profiling [1•]. As with all molecular testing, results must be interpreted within a patient-specific clinical and pathologic context. We currently employ array-based methylation profiling for tumors that are challenging to classify by our other aforementioned methods and will soon be expanding testing to all CNS tumors.

Overview of Molecular Findings in CNS Tumors

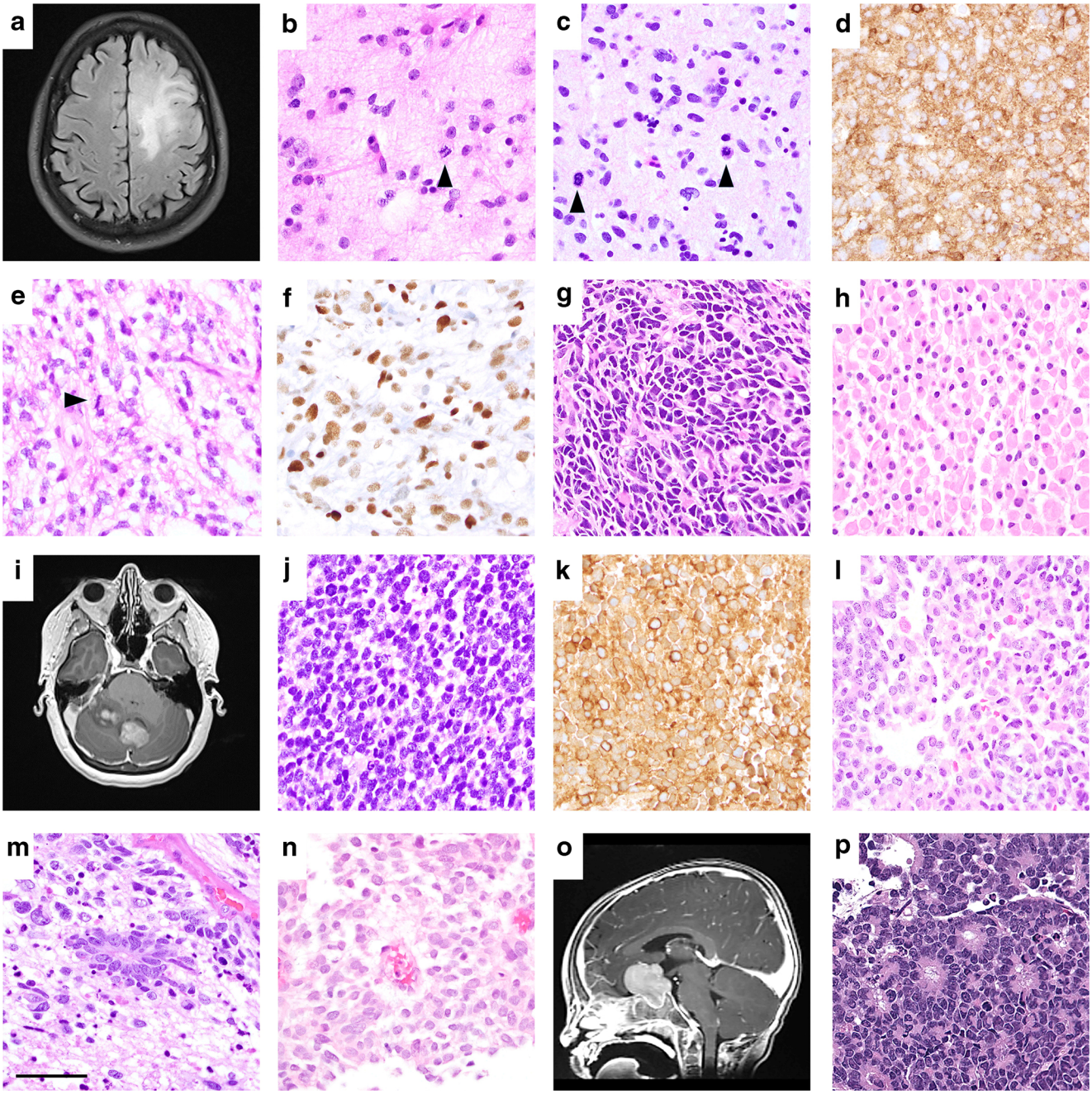
Diffuse Gliomas

Glioblastoma, IDH-Wildtype

Diffusely infiltrating *IDH*-wildtype astrocytomas are the most commonly encountered glioma [31•]. Most of these tumors show classic WHO grade 4 glioblastoma histology, including mitotic activity with tumor necrosis and/or microvascular proliferation. A minority (10–15%) have only WHO grade 2 or 3 features [32]. However, with the third update from cIMPACT-NOW, if such a tumor also harbors a *TERT*-p mutation, combined whole chromosome gain of 7/loss of 10 and/or amplification of *EGFR*, it can be diagnosed as "diffuse astrocytic glioma, *IDH*-wildtype, with molecular features of glioblastoma, WHO grade 4 [18••] (Fig. 1a, b)." *IDH*-wildtype glioblastomas will typically harbor alterations in receptor tyrosine kinase (RTK) (e.g., *EGFR*, *PTEN*, *NF1*, *PIK3CA*, *PDGFRA*), retinoblastoma (Rb) (e.g., *RB1*, *CDKN2A/B*, *CDK4*, *CDK6*) or p53 (e.g., *TP53*, *MDM2*, *MDM4*) pathways [33]. Certain molecular aberrations are emerging as therapeutic targets, such as *FGFR* and *NRTK* fusions, *MET* and *EGFR* alterations, *BRAF* p.V600E and immunotherapies for high tumor mutational burden [34–37].

IDH-Mutant Astrocytomas

Greater than 80% of WHO grade 2–3 astrocytomas, as well as approximately 10% of histologic WHO grade 4 astrocytomas, harbor SNVs in *IDH1* or *IDH2*, with ~ 90% positive for IHC against the Idh1 R132H mutation [38] (Fig. 1c, d). Compared to *IDH*-wildtype infiltrative gliomas, *IDH*-mutant astrocytomas tend to be less aggressive, respond better to adjuvant therapy, are more often *MGMT* promoter methylated, and harbor mutations in *ATRX* and *TP53* [4, 39, 40]. Functional loss of *ATRX* (which often results in IHC loss of Atrx staining) results in alternative lengthening of telomeres, a process that is mutually exclusive with *TERT*-p mutations, the method of telomere maintenance employed by oligodendrogliomas and *IDH*-wildtype glioblastomas [40]. In addition to high-grade



histologic features of tumor necrosis and/or microvascular proliferation, the fifth cIMPACT-NOW update recommends homozygous *CDKN2A/B* loss as a diagnostic feature of WHO grade 4 *IDH*-mutant astrocytomas [41•]. Alterations of *CDK4*, *MYCN*, *PDGFRA*, *PIK3CA*, *PIK3R1*, and *RB1* may also affect the prognosis of patients with *IDH*-mutant astrocytomas [41•].

Anaplastic Astrocytoma with Piloid Features

Methylation profiling of previously diagnosed, histologically defined anaplastic pilocytic astrocytomas and cerebellar glioblastomas has shown that subsets of these tumors represent a unique methylation class designated "anaplastic astrocytoma with piloid features," not elsewhere classified (NEC) [30•, 42]. Such tumors often possess a unique combination of molecular findings: (i) *IDH*-wildtype, (ii) *CDKN2A/B* deletion, (iii) mutations in *ATRX* or loss of Atrx IHC, and (iv) activating mitogen-activated protein kinase (MAPK) pathway alterations (e.g., *NF1*, *BRAF*, or *FGFR1* alterations), though methylation profiling may be the only way to provide a definitive diagnosis when the full combination of molecular alterations is not present [30•]. The MAPK alterations may be targetable and the *MGMT* promoter is often methylated

◀ **Fig. 1** Examples integrating molecular findings. Case 1 is a 67-year-old male with an expansile, infiltrative, T2-hyperintense, non-enhancing left frontal lobe lesion (**a**, T2-weighted magnetic resonance imaging (MRI), pre-contrast). Sections showed an infiltrating glioma with scattered mitoses (**b**, arrowhead) without tumor cell necrosis or microvascular proliferation. IHC for Idh1 R132H was negative and Atrx nuclear staining was retained (not shown). NGS panel and copy number array showed no *IDH1*/*IDH2* mutations but did reveal a *TERT*-p mutation, *EGFR* amplification, and whole chromosome gain of 7/loss of 10. The final integrated diagnosis was diffuse astrocytic glioma, *IDH*-wildtype, with molecular features of glioblastoma, WHO grade 4. Case 2 is a 45-year-old female with an enhancing temporal lobe lesion (not shown). Sections showed an infiltrating glioma with mitoses (**c**, arrowhead) without tumor cell necrosis or microvascular proliferation. IHC was positive for Idh1 R132H (**d**) with loss of Atrx (not shown). NGS panel and copy number array showed *IDH1* R132H, *ATRX* and *TP53* mutations as well as homozygous loss of 9p21.3 (*CDKNA/B*). The final integrated diagnosis was astrocytoma, *IDH*-mutant, WHO grade 4. Case 3 is a 7-year-old male with an enhancing pontine mass (not shown). Sections showed an infiltrating glioma with mitoses (**e**, arrowhead) without tumor cell necrosis or microvascular proliferation. IHC was positive for H3 K27M (**f**). NGS panel confirmed an *H3F3A* p.K27M mutation. The final integrated diagnosis was diffuse midline glioma, H3 K27M-mutant, WHO grade 4. Case 4 is a 16-year-old female with an enhancing right frontal lobe lesion (not shown). Sections showed an infiltrating tumor with embryonal-like histology (**g**). By IHC, the tumor was positive for GFAP and p53, negative for Olig2 and Idh1 R132H and showed loss of Atrx (not shown). NGS revealed an *H3F3A* p.G34R mutation. The final integrated diagnosis was H3 G34-mutant glioma, *IDH*-wildtype. Case 5 is a 41-year-old female with a non-enhancing left frontal lobe mass (not shown). Sections showed an infiltrating glioma with diffuse gemistocytic features (**h**) without mitotic activity, tumor cell necrosis or microvascular proliferation. IHC was positive for Idh1 R132H with retention of Atrx nuclear staining and no p53 staining (not shown). NGS panel and copy number array showed *IDH1* R132H and *TERT*-p mutations as well as whole arm 1p/19q-codeletion. The final integrated diagnosis was oligodendroglioma, *IDH*-mutant and 1p/19q-codeleted, WHO grade 2. Case 6 is a 36-year-old female with enhancing lesions of the cerebellar vermis and right cerebellar hemisphere (**i**, magnetization prepared rapid acquisition gradient echo (MP-RAGE) sequence MRI, post-contrast). Sections demonstrated sheets of infiltrating tumor cells with embryonal features (**j**). IHC was positive for Gab1 (**k**) and Yap1 (not shown) and negative for nuclear β-catenin and p53 (both not shown). NGS panel and copy number array showed *TERT*-p and *SUFU* mutations, no *TP53* alterations, amplification of 2q (including *GLI2*) and losses of 9q and 10q. The final integrated diagnosis was classic medulloblastoma, *SHH*-activated and *TP53*-wildtype, WHO grade 4. Case 7 is a 2-year-old male with an enhancing right parietal lobe tumor (not shown). Sections showed sheets of tumor cells with embryonal and rhabdoid features (**l**). IHC for Ini1 showed loss of normal staining (not shown). Copy number array detected homozygous loss of 22q11.2 (*SMARCB1*). The final integrated diagnosis was atypical teratoid/rhabdoid tumor, WHO grade 4. Case 8 is a 3-year-old female with an enhancing lesion of the left parietal lobe (not shown). Sections showed tumor cells with embryonal features, true rosettes and neuropil-like areas (**m**). Tumor cells were positive for Lin28a (not shown). FISH and copy number array demonstrated amplification of 19q13.42 (C19MC). The final integrated diagnosis was embryonal tumor with multilayered rosettes, C19MC-altered. Case 9 is a 2 year-old male with an enhancing lesion of the left frontal lobe (not shown). Sections showed mildly pleomorphic tumor cells with round to oval profiles and questionable perivascular pseudorosettes (**n**). In areas, there was abundant tumor cell necrosis (not shown). IHC was positive for NeuN and Olig2, negative for synaptophysin and EMA, and showed retention of both Ini1 and Brg1 staining; β-catenin showed nuclear reactivity (not shown). NGS panel and copy number array were positive for tandem duplication of Xp11.4 (*BCOR*). Array-based methylation profiling subsequently confirmed the final integrated diagnosis of CNS high-grade neuroepithelial tumor with *BCOR* alteration. Case 10 is a 1-year-old male with an enhancing sellar/suprasellar mass (**o**, T1-weighted MRI, post-contrast). Sections showed sheets of tumor cells with embryonal features and focal Flexner-Wintersteiner-like rosettes (**p**). IHC panel was positive for synaptophysin and negative for Olig2, GFAP, NeuN, Lin28a, desmin, myogenin, Nkx2.2, pancytokeratin, and TdT; Ini1 and Brg1 were retained (not shown). FISH was negative for alterations of *BCOR*, *CIC*, *EWSR1*, or C19MC. Copy number array revealed chromothripsis of chromosome 13, including 13q14.2 (*RB1*). Array-based methylation profiling classified the tumor as pineoblastoma group A/intracranial retinoblastoma. The final integrated diagnosis was intracranial retinoblastoma with *RB1* alteration. Scale bar = 50 μM in **m** and also applies to **b**, **c**, **d**, **e**, **f**, **g**, **h**, **j**, **k**, **l**, **n**, and **p**

[30•]. Outcomes in these tumors are more favorable than *IDH*-wildtype glioblastomas [30•, 42].

Histone Mutations in Gliomas

High-grade gliomas in children and young adults are usually genetically distinct from their older adult counterparts, often including mutations in histone H3 variants H3.3, and to a lesser extent H3.1, encoded by genes *H3F3A/B* and *HIST1H3A/B/C* [43]. These histone mutations are mutually exclusive with *IDH* mutations. H3 K27M is seen in midline gliomas (e.g., pons, thalamus, spinal cord, cerebellum) [44] (Fig. 1e). These tumors will show IHC staining for H3 K27M (Fig. 1f) and loss of H3K27me3, though the latter finding can be seen in other entities that do not have histone mutations (e.g., posterior fossa group A ependymomas). Furthermore, H3 K27M SNVs have also been found in ependymomas, pilocytic astrocytomas, and gangliogliomas. Thus, the diagnosis of diffuse midline glioma, H3 K27M-mutant, WHO grade 4, must be reserved for gliomas harboring this mutation which are both infiltrating and midline [45]. Subsets of these tumors may exhibit additional alterations of *TP53*, *ATRX*, *PDGFRA*, *CDK4*, *CDK6*, and/or *ACVR1* [46].

In contrast to H3 K27M, H3 G34R/V mutations are seen in pediatric and young adult hemispheric high-grade gliomas [43], including some tumors that show embryonal-like histologies (Fig. 1g). H3 G34R/V should be suspected in tumors negative for Idh1 R132H and Olig2 that also show strong p53 expression and loss of Atrx [46]. These tumors often show alterations in *TP53* and *ATRX* (as predicted by IHC), and subsets have additional alterations in *PDGRFA* or *CCND2* [46, 47].

Pediatric Diffuse Low-Grade Gliomas

Special subsets of diffuse low-grade gliomas of children are *IDH*–wildtype/H3-wildtype, show astrocytoma- or oligodendroglioma-like histologies, rarely show anaplastic progression, do not exhibit 1p/19q-codeletion and are associated with favorable outcomes (despite being *IDH*-wildtype) [48, 49]. The majority of these tumors show single driver alterations of *MYB*, *MYBL1*, *FGFR1* or *BRAF*, or other activating alterations of the MAPK pathway [48], warranting

integrated classifications as outlined by cIMPACT-NOW update 4 [49].

Oligodendroglioma, IDH-Mutant, and 1p/19q-Codeleted

Oligodendrogliomas comprise the other major subset of adult diffuse gliomas (Fig. 1h). These grade 2–3 tumors, while still lethal, are even less aggressive than *IDH*-mutant astrocytomas. Diagnosis requires both an *IDH* mutation and whole arm 1p/19q-codeletion [17••]. By IHC, these tumors typically show reactivity for Idh1 R132H, Atrx retention and low or no p53 staining. Nearly all of these tumors contain a *TERT*-p mutation, which is mutually exclusive with *ATRX* mutations, further distinguishing them from *IDH*-mutant astrocytomas [40]. *IDH*-mutant gliomas with mixed astrocytoma and oligodendroglioma histologic features can be separated based on alterations in *ATRX* and *TP53* (*IDH*-mutant astrocytoma) or 1p/19q-codeletion and *TERT*-p mutations (*IDH*-mutant oligodendroglioma), thus making the "oligoastrocytoma" term obsolete [40, 50]. Some oligodendrogliomas also show inactivating mutations of *FUBP1* (1p31.1) and *CIC* (19q13.2). Alterations of *NOTCH1* and polysomy of 1p and 19q are associated with poorer outcomes [51, 52].

Non-diffuse Astrocytoma Variants and Glioneuronal Neoplasms

Pilocytic astrocytomas (PAs), WHO grade 1, generally occur in the cerebellum, optic nerve, cerebral hemispheres, and spinal cord and arise mostly in children [53]. Because PAs are non-infiltrative, many can be cured by surgical excision if they are in a safely accessible location. Most PAs harbor an activating alteration of the MAPK pathway, most commonly the *KIAA1549-BRAF* fusion, though a much wider range of *BRAF* fusion partners and breakpoints exist [54, 55]. Approximately 5–10% of PAs harbor *BRAF* p.V600E SNVs, especially supratentorial tumors [17••, 56]. Other MAPK pathway alterations involve *NF1*, *RAF1*, the *NTRK* family, *FGFR1* and *KRAS* [17••, 55]. PAs which harbor *CDKN2A/B* deletions and *ATRX* mutations may be better classified as "anaplastic astrocytomas with piloid features;" such tumors are more likely to recur and progress than grade 1 PAs [30•].

Sixty to 80% of grade 2–3 pleomorphic xanthroastrocytomas (PXAs) possess *BRAF* p.V600E SNVs, often with concomitant homozygous *CDKN2A/B* deletions (50–70%) [56–58]. The distinction between anaplastic PXAs and glioblastomas with *BRAF* p.V600E mutations can be challenging; however, array-based methylation profiling can resolve such cases into prognostically distinct groups [37].

Among glioneuronal tumors, 20–50% of gangliogliomas harbor *BRAF* p.V600E mutations [56]. *FGFR1* alterations are present in the majority of dysembryoplastic neuroepithelial tumors [59] with *BRAF* p.V600E SNVs and *BRAF* copy number gains being described in a minority of cases [48, 60, 61]. The majority of, if not all, rosette forming glioneuronal tumors harbor *FGFR1* mutations with subsets showing concomitant *PIK3CA* and *NF1* mutations, this in contrast to other low grade gliomas/glioneuronal tumors which usually exhibit only single driver alterations [62]. Finally, the majority of, if not all, papillary glioneuronal tumors contain *SLC44A1-PRKCA* fusions [63].

Ependymomas

Ependymomas can be subdivided into distinct molecular subgroups across different CNS compartments [27]. *RELA*-fusion positive ependymomas are the most common supratentorial subtype with *C11orf95-RELA* the most common alteration [17, 27]. This fusion activates the NF-κB pathway and increases L1cam expression, allowing the latter to be used as a surrogate IHC marker [64]. *YAP1*-fusion ependymomas comprise the other supratentorial subclass of ependymomas [27].

Group A posterior fossa (PFA) ependymomas are seen predominantly in infants and younger children, are more aggressive, tend to have balanced genomes (few, if any, CNVs) and lose H3K27me3 staining [27, 65]. By contrast, group B posterior fossa (PFB) ependymomas are found in older children and adults, exhibit multiple CNVs, show retention of H3K27me3 and have more favorable outcomes [27, 65]. Array-based methylation profiling can reliably distinguish between PFA and PFB ependymomas [27].

In the spine, WHO grade 2 ependymomas, tend to harbor multiple CNVs including 22q deletions (involving *NF2*), as well as mutations of the remaining *NF2* allele [27, 58]. Grade 1 myxopapillary ependymomas also tend to show multiple CNVs, most commonly gains of 5, 7, 9, 16 and 18 [27, 58].

Embryonal Tumors

Medulloblastoma, the prototypical embryonal WHO grade 4 malignancy, arises in the cerebellum of pediatric patients and younger adults. It consists of four molecular subgroups, each with different clinical and biologic features [17••, 66, 67]. *WNT*-activated tumors are the least common and least aggressive type, exhibiting monosomy 6 and *CTNNB1* mutations (less commonly *APC* mutations) [66, 67]. This subtype shows IHC nuclear localization of β-catenin (though it can be very focal) as well as positivity for Yap1 [67]. Common alterations in *SHH*-activated medulloblastomas (Fig. 1i, j) involve *PTCH1*, *SUFU*, *SMO*, *GLI1*, *GLI2*, and *MYCN* [68]. These tumors are positive for both Gab1 (Fig. 1k) and Yap1 and, classically, show losses of both chromosomes 9q and 10q [67, 68]. *TERT*-p mutations are more common in *SHH*-activated medulloblastomas from adults [68] and *SHH* tumors with *TP53* mutations tend to behave aggressively [68, 69]. Non-*WNT*/non-*SHH* (group 3/4) tumors comprise approximately

60% of medulloblastomas [66]. The majorities of both groups exhibit i(17q) [66]. *MYC* amplifications are seen in approximately 20% of group 3 tumors and impart a poorer prognosis [68]. Array-based methylation profiling discriminates amongst all four subtypes [22••, 58].

Atypical teratoid/rhabdoid tumors (ATRTs) are highly aggressive neoplasms, most commonly occurring in the cerebral hemispheres of infants (Fig. 1l). ATRTs harbor biallelic alterations of Ini1 (*SMARCB1*) or, rarely, Brg1 (*SMARCA4*) [70, 71]. Loss of Ini1 or Brg1 staining by IHC provides a good surrogate of these underlying alterations [71, 72].

"Embryonal tumors with multilayered rosettes, C19MC altered" are rare, aggressive malignancies of young children characterized by primitive multilayered rosettes in a neuropil-rich stroma, along with amplifications and fusions involving the microRNA cluster in chromosome 19q13.42 (C19MC) [17••] (Fig. 1m). C19MC alterations can be detected by FISH or copy number array [73].

Array-based methylation profiling of previously diagnosed CNS primitive neuroectodermal tumors (PNETs) has shown that most actually represent other well-defined CNS tumor entities [29•]. Among the remaining tumors, some clustered within four new entities: "CNS neuroblastoma with *FOXR2* activation," "CNS Ewing sarcoma family tumor with *CIC* alteration," "CNS high-grade neuroepithelial tumor with *MN1* alteration" and "CNS high-grade neuroepithelial tumor with *BCOR* alteration" [29] (Fig. 1n). NUTM1 and nuclear β-catenin staining can be seen in subsets of *CIC*- and *BCOR*-altered tumors, respectively [29•]. The variety of alterations in these four genes is still being elucidated and may not be well-covered by current NGS, FISH and targeted sequencing assays [29•]. For many of these tumors, and also with other rare tumors in the pediatric population, diagnosis relies on array-based methylation profiling (Fig. 1o, p).

Meningiomas

Mutations in meningiomas tend to vary according to tumor site and clinical context: *NF2* in cerebral convexities and radiotherapy-induced tumors and *SMO*, *AKT1*, *TRAF7*, and *PIK3CA* mutations in skull base tumors [74–77]. Other mutations correlate with specific histologic subtypes: *KLF4* and *TRAF7* (secretory), *SMARCE1* (clear cell), and *BAP1* (rhabdoid) [78–80]. *TERT*-p mutations, *DMD* deletions and *CDKN2A* alterations impart more frequent and rapid recurrences [81–83]. The most common CNV is whole or partial chromosome 22 loss; more aggressive tumors accrue additional CNVs, such as losses of 1p, 10, and 14q [84]. Though histology-based grading is the current standard, array-based methylation profiling is better at predicting which tumors will behave more aggressively [28••].

Conclusions

Molecular diagnostics are essential for the optimal workup of CNS tumors. A combination of NGS panel and copy number array is ideal for all diffuse gliomas and embryonal tumors, with a low threshold to employ in any tumor type where it is expected to yield useful data. In certain settings, immunostains and FISH can be used to screen for molecular alterations, with subsequent confirmation by more definitive methods. In the coming years, genome-wide methylation profiling will likely become more widely used. It is therefore critical for pathologists and clinicians to recognize the diversity of molecular testing available, know which tests to order, and know how to incorporate molecular data to provide the best patient care possible.

Compliance with Ethical Standards

Conflict of Interest Alexander Z. Feldman, Lawrence J. Jennings, Nitin R. Wadhwani, Daniel J. Brat, and Craig M. Horbinski declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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