J Neuropathol Exp Neurol
Vol. 80, No. 5, May 2021, pp. 415–418
doi: 10.1093/jnen/nlab033

OXFORD

INVITED REVIEW ARTICLE

What Every Neuropathologist Needs to Know: Practical Aspects and Pitfalls in Molecular Diagnosis of Brain Tumors

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Abstract

Molecular testing has become part of the routine diagnostic workup of brain tumors after the implementation of integrated histomolecular diagnoses in the 2016 WHO classification update. It is important for every neuropathologist to be aware of practical preanalytical, analytical, and postanalytical factors that impact the performance and interpretation of molecular tests. Prior to testing, optimizing tumor purity and tumor amount increases the ability of the molecular test to detect the genetic alteration of interest. Recognizing basic molecular testing platform analytical characteristics allows selection of the optimal platform for each clinicopathological scenario. Finally, postanalytical considerations to properly interpret molecular test results include understanding the clinical significance of the detected genetic alteration, recognizing that detected clinically significant genetic alterations are occasionally germline constitutional rather than somatic tumor-specific, and being cognizant that recommended and commonly used genetic nomenclature may differ. Potential pitfalls in brain tumor molecular diagnosis are also discussed.

Key Words: Analytical sensitivity, Genetic nomenclature, Germline, Integrated diagnosis, Molecular testing, Somatic, Tumor purity.

INTRODUCTION

The 2016 update of the fourth edition of the World Health Organization (WHO) classification of CNS tumors incorporated molecular parameters in the diagnostic scheme of brain tumors for the first time. Integrated histomolecular diagnoses replaced preceding morphological diagnoses for a subset of brain tumors, including adult-type diffuse gliomas and medulloblastoma (1). The 2016 WHO classification update was succeeded by multiple updates from the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy-Not Official WHO (cIMPACT-NOW) to clarify/recommend diagnostic terminologies, specify diagnostic criteria, and rapidly integrate emerging clinically relevant molecular parameters until the next edition of the WHO classification (2–9). Technological advances have decreased costs and increased availability of molecular testing, which became part of the routine diagnostic workup of many brain tumor types. Herein, we discuss practical factors that impact molecular testing as well as potential pitfalls in brain tumor molecular diagnosis to improve the practicing neuropathologist's ability to select molecular testing platforms, interpret molecular test results and perform molecular diagnosis of brain tumors.

MOLECULAR PARAMETERS AND MOLECULAR TESTING PLATFORMS

Three types of molecular parameters have been incorporated in the 2016 WHO classification update: mutations (i.e. clinically significant sequence variants), copy number variants (CNVs) and gene fusions. A "variant" is a change in genetic material (i.e. DNA, RNA) that deviates from the "normal" reference and is often referred to as a "genetic alteration." A "sequence variant" is a change in the spelling of the genetic material at the gene level, whereas a "CNV" is a change in the number of copies of the genetic material at a gene, chromosome, or genome level. Molecular testing is typically performed using DNA for detection of mutations and CNVs and using DNA or RNA for detection of gene fusions.

The 4 main molecular testing platforms utilized to evaluate these 3 molecular parameters and their key specifications are compared in Table 1. PCR-based and sequencing (e.g. next-generation sequencing [NGS], pyrosequencing, Sanger sequencing) assays are robust testing platforms to detect mutations and gene fusions. Although PCR-based and sequencing assays can detect CNVs, fluorescence in situ hybridization (FISH) and chromosomal microarray are the primary testing platforms to evaluate CNVs. FISH and chromosomal microarray can also infer the presence of gene fusions but are unable to define the specific breakpoint/junction of the fusion events.

PREANALYTICAL FACTORS IMPACTING MOLECULAR TESTING

Prior to testing, 2 critical preanalytical factors impact molecular testing: tumor purity and tumor amount. Tumor

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No financial support was received for the writing of this article.
The authors have no duality or conflicts of interest to declare.

TABLE 1. Four Main Molecular Testing Platforms

	PCR-based	Sequencing	FISH	Array
Analytical sensitivity	+++	++/+++	+++	++/+++
Target(s)	+/++	++/+++	+/++	++/+++
Specimen requirement	+	++/+++	+	++/+++
Cost/TAT	+	++/+++	++	++/+++
Assay/strategy	Allele-specific PCR ddPCR	Sanger Pyrosequencing NGS	Enumeration Break-apart Dual fusion	Chromosomal microarray Array CGH

FISH, fluorescence in situ hybridization; TAT, turnaround time; ddPCR, droplet digital PCR; NGS, next-generation sequencing; CGH, comparative genomic hybridization.

specimens are composed of variable proportions of tumor cells and non-neoplastic cells (e.g. neurons, inflammatory cells). Tumor purity is the fraction of tumor cells present in a tumor specimen and is usually expressed as a tumor percentage. Nucleated non-neoplastic cells decrease tumor purity and impact DNA and RNA-based molecular testing. Red blood cells are anucleate and lack DNA; thus, hemorrhage does not influence tumor purity for DNA-based molecular testing but may impact tumor purity for RNA-based molecular testing. Tumor purity should be above a minimal level to avoid false-negative test results. For example, NGS testing of an IDH-mutant tumor with low tumor purity generates fewer IDH-mutant sequencing reads than an IDH-mutant tumor with high tumor purity. If the proportion of mutant sequencing reads relative to all sequencing reads, also known as variant allele frequency (VAF), falls below the test predefined lower VAF cut-off (i.e. analytical sensitivity), the test is resulted as negative. In this circumstance, the negative result is a false-negative result caused by the inability of the molecular test to confidently call the IDH mutation due to low tumor purity.

Tumor amount is the total number of viable tumor cells present in a tumor specimen. Tumor necrosis decreases tumor amount. The surgical procedure whereby the specimen was obtained also impacts tumor amount as biopsy specimens yield substantially lower quantities of nucleic acid compared to resection specimens with similar tumor purity. Tumor amount should be above a minimal level to generate a valid (not failed) result. For example, NGS testing of an IDH-mutant tumor with low tumor amount generates an insufficient total number of sequencing reads, also known as depth of coverage, to confidently distinguish the IDH mutation from background sequencing artifacts even if tumor purity is high. NGS testing platforms usually require at least 100 sequencing reads to allow for confident variant calling. Failure to reach the test's predefined minimum depth of coverage means that the evaluation for sequence variants within the low coverage target region may be inaccurate and cannot be trusted. Insufficient depth of coverage is particularly problematic for large NGS panels and whole exome/genome sequencing as they evaluate hundreds to thousands of gene targets simultaneously and may not achieve enough coverage for every target.

Specimens with low tumor purity and/or low tumor amount wherein the fraction and/or quantity of tumor cells may be overestimated represent a potential pitfall for brain tumor molecular diagnosis. Examples include tumor specimens with a prominent inflammatory cell component (e.g. abscess-like tumor necrosis) and biopsies with few scattered infiltrating tumor cells or with atypical cells wherein morphological distinction between definite tumoral and reactive glial atypia is challenging.

Practical strategies to optimize molecular testing when dealing with specimens with low tumor purity and/or low tumor amount are described in Table 2.

ANALYTICAL FACTORS IMPACTING MOLECULAR TESTING

Analytical factors are inherent to the molecular testing platform and are unaffected by the specimen characteristics (preanalytical factors). There are 2 analytical factors to consider when selecting the molecular testing platform for a given clinicopathological scenario: analytical sensitivity and technical limitations.

Analytical sensitivity of a test is the test's lower limit of detection for the tested target(s) and is a crucial factor, especially when testing specimens with low tumor purity and/or low tumor amount. PCR-based assays and FISH are testing platforms with high analytical sensitivity; they evaluate one to a few targets and have low tissue requirements, costs and turnaround time. Sequencing and microarray technologies, on the other hand, evaluate multiple targets at the expense of lower analytical sensitivity, higher tissue requirements and costs and increased turnaround time when compared to PCR-based assays and FISH (Table 1). As an example, droplet digital PCR is an emerging technology with high analytical sensitivity (10). This testing platform is able to detect mutations at levels that would be called negative by other commonly used molecular testing platforms and is particularly suitable for specimens with low tumor purity and/or low tumor amount (11).

All testing platforms have technical limitations. For instance, FISH only detects CNVs located within the regions evaluated by the designed probes, which cover a relatively small portion of the targeted chromosomal regions. Therefore, FISH is unable to distinguish CNVs that involve whole-arm versus partial-arm chromosomal regions, and this technical limitation is specifically relevant when testing for 1p/19q co-deletion. The 1p/19q co-deletion that, along with an IDH mutation, genetically defines the 2016 WHO "oligodendroglioma, IDH-mutant and 1p/19q-codeleted" is a

TABLE 2. Strategies to Optimize Tumor Testing for Specimens with Low Tumor Purity and/or Low Tumor Amount

	Low Tumor Purity	Low Tumor Amount
Strategies to optimize molecular testing	Select tissue block(s) with highest tumor purity by avoiding block(s) with substantial adjacent normal brain and/or inflammatory cells	Increase number of tissue sections submitted for nucleic acid extraction (e.g. 20–30 versus 10–15 unstained slides)
	Tumor enrichment (e.g. macrodissection)	Combine multiple tissue blocks if from the same tumor site
		Prioritize testing based on diagnostic/clinical utility when multiple molecular tests are indicated
	Use high analytical sensitivity testing platforms (e.g. ddPCR, targeted NGS)	

ddPCR, droplet digital PCR; NGS, next-generation sequencing.

whole-arm 1p/19q co-deletion resulting from an unbalanced translocation (1). Combined partial losses of 1p and 19q arms that span the location of commonly used FISH test probes and lead to a positive FISH 1p/19q co-deletion result occur in approximately 3.5%–4.5% of IDH-mutant/IDH-wildtype diffuse astrocytic gliomas (12). The diagnostically false-positive FISH result due to combined partial 1p/19q losses in a diffuse astrocytic glioma is a potential pitfall for brain tumor molecular diagnosis as misdiagnosis may occur if a discrepancy between morphological, immunohistochemical, and molecular findings is unnoticed or unapparent.

POSTANALYTICAL FACTORS IMPACTING MOLECULAR TESTING

After testing, postanalytical factors to consider when interpreting molecular test reports include the categorization system used to evaluate the clinical significance and the verbiage/genetic nomenclature used to describe the detected variant (i.e. genetic alteration).

Two types of variants are detected in tumor testing: somatic and germline. Somatic variants are genetic alterations that occur only in the tumor cells and are absent in non-neoplastic cells, including germ cells; thus, somatic variants are not inheritable. Somatic variants include mutations (e.g. IDH mutations) that we intentionally target in tumor testing. Since somatic variants are present only in the tumor cells, the frequency of somatic variants is proportional to tumor purity. Germline variants are genetic alterations that occur in tumor and non-neoplastic cells, including germ cells, and are inheritable. For the most part, germline variants are not clinically significant, with some exceptions that are discussed below. As germline mutations are present in all cells, the frequency of germline variants is unaffected by tumor purity.

Variants detected in tumor testing are evaluated according to evidence-based guidelines (13, 14). The strength of available clinical and experimental evidence regarding therapeutic, diagnostic and prognostic implications was stratified into 4 levels; based on these levels of evidence, variants were categorized into 4 tiers (I–IV). Tiers I/II variants are variants that show some level of evidence supporting an oncogenic role and these variants should be reported. Sequence variants classified as Tiers I/II variants are often referred to as "mutations." These clinically significant Tiers I/II variants are mostly somatic but may occasionally be germline and associated with hereditary cancer predisposition syndromes (15). Examples of genes associated with brain tumors that may show clinically significant germline variants include *TP53*, *NF1*, and *SMARCB1*. Tier IV variants are variants that do not show evidence of cancer association and have been reported in at least 1% of individuals in germline population databases. Such variants are classified as benign/likely benign, and they do not need to be reported as Tier IV variants are thought to represent normal genetic variation. Lastly, Tier III variants are variants that do not qualify as either clinically significant or benign/likely benign; these variants are classified as a variant of uncertain/unknown significance (VUS). As an example, an *IDH1* sequence variant classified as a Tier III VUS does not carry the same diagnostic/clinical implications as a Tier I *IDH1* mutation like the canonical R132H mutation. Thus, the finding of an *IDH1* VUS in the absence of an *IDH2* mutation in an adult-type diffuse glioma would support an "IDH-wildtype" designation. Misinterpretation of the clinical significance of an *IDH1* VUS would result in a misdiagnosis of an IDH-wildtype tumor as IDH-mutant, illustrating another potential pitfall in brain tumor molecular diagnosis.

Germline mutations in cancer predisposition genes detected in tumor testing have been reported in approximately 7%–8.5% of pediatric and 8% of adult patients (16–18). Tumor testing performed with paired normal non-neoplastic tissue allows for distinction between somatic and germline variants. Variants identified in the non-neoplastic tissue are germline variants that can be filtered out from variants detected in the tumor such that the remaining variants observed only in the tumor are the somatic, tumor-specific variants. Most clinical laboratories, however, perform tumor-only testing. In tumor-only testing, it is not possible to definitively distinguish somatic variants from germline variants although it is often possible to infer a somatic versus germline origin based on the variant frequency, estimated tumor purity and gene copy number status. Whenever a medically relevant germline mutation is suspected in tumor-only testing, follow-up germline testing on a normal sample along with genetic counseling/consultation should be recommended through discussions with referring clinicians and/or specific comments on the molecular test report (19). A brief comment may also be included in the neuropathology report to reinforce the recommendation for follow-up germline testing in this clinico-pathological scenario. In brain tumors, atypical teratoid/rhabdoid tumor is a tumor type with

high frequency of germline mutations: *SMARCB1* mutations are germline in origin and diagnostic of rhabdoid tumor predisposition syndrome in approximately 35%–40% of patients (20–22).

Molecular test results are reported using recommended genetic nomenclature. The nomenclature for human genes and genetic variation follows the recommendations of the Human Genome Organization (23, 24). Commonly used genetic nomenclature and recommended genetic nomenclature are occasionally discrepant, which may cause confusion and lead to misinterpretation and/or misdiagnosis (25). For example, the recommended nomenclature for the gene described as *KIAA1598* in the *KIAA1598-FGFR2* fusion in "polymorphous low-grade neuroepithelial tumor of the young (PLNTY)" (26) has been updated to *SHTN1*; the "*KIAA*" designation is a placeholder symbol until the protein function is identified. Another example is the commonly used nomenclature of the *TERT* promoter C228T and C250T mutations, which was coined in initial melanoma research studies (27, 28). The C228T and C250T mutation nomenclature consists of a combination of the 3 last digits of the coordinates of the recommended nomenclature at the genomic DNA level (i.e. Chr5[GRCh37]: g. 1,295,**228** and Chr5[GRCh37]: g.1,295,**250**) flanked by the nucleotide changes of the recommended nomenclature at the coding DNA level (NM_198253: c.-124**C**>**T** and NM_198253: c.-250**C**>**T**), respectively.

CONCLUSION

We have entered a precision diagnostics era for brain tumors. The brain tumor classification system is rapidly evolving and integrates molecular parameters that convey new advances in our understanding of tumor molecular biology. The practicing neuropathologist plays a central role in brain tumor molecular diagnosis by selecting molecular tests and integrating molecular findings into a final integrated histomolecular diagnosis. To optimally utilize molecular testing and avoid potential diagnostic pitfalls, it is important to be aware of practical preanalytical, analytical, and postanalytical factors that impact molecular testing.

REFERENCES

1. WHO Classification of Tumours of the Central Nervous System. 4 ed. Lyon: International Agency for Research on Cancer, 2016
2. Louis DN, Aldape K, Brat DJ, et al. cIMPACT-NOW (the consortium to inform molecular and practical approaches to CNS tumor taxonomy): A new initiative in advancing nervous system tumor classification. Brain Pathol 2017; 27:851–2
3. Louis DN, Wesseling P, Paulus W, et al. cIMPACT-NOW update 1: Not Otherwise Specified (NOS) and Not Elsewhere Classified (NEC). Acta Neuropathol 2018;135:481–4
4. Louis DN, Giannini C, Capper D, et al. cIMPACT-NOW update 2: Diagnostic clarifications for diffuse midline glioma, H3 K27M-mutant and diffuse astrocytoma/anaplastic astrocytoma, IDH-mutant. Acta Neuropathol 2018;135:639–42
5. Brat DJ, Aldape K, Colman H, et al. cIMPACT-NOW update 3: Recommended diagnostic criteria for "Diffuse astrocytic glioma, IDH-wildtype, with molecular features of glioblastoma, WHO grade IV". Acta Neuropathol 2018; 136:805–10
6. Ellison DW, Hawkins C, Jones DTW, et al. cIMPACT-NOW update 4: Diffuse gliomas characterized by MYB, MYBL1, or FGFR1 alterations or BRAF(V600E) mutation. Acta Neuropathol 2019;137:683–7
7. Brat DJ, Aldape K, Colman H, et al. cIMPACT-NOW update 5: Recommended grading criteria and terminologies for IDH-mutant astrocytomas. Acta Neuropathol 2020;139:603–8
8. Louis DN, Wesseling P, Aldape K, et al. cIMPACT-NOW update 6: New entity and diagnostic principle recommendations of the cIMPACT-Utrecht meeting on future CNS tumor classification and grading. Brain Pathol 2020;30:844–56
9. Ellison DW, Aldape KD, Capper D, et al. cIMPACT-NOW update 7: Advancing the molecular classification of ependymal tumors. Brain Pathol 2020;30: 863–6
10. Olmedillas-Lopez S, Garcia-Arranz M, Garcia-Olmo D. Current and emerging applications of droplet digital PCR in oncology. Mol Diagn Ther 2017;21:493–510
11. Ge J, Liu MY, Li L, et al. Detection of IDH1 and TERT promoter mutations with droplet digital PCR in diffuse gliomas. Int J Clin Exp Pathol 2020;13:230–8
12. Ball MK, Kollmeyer TM, Praska CE, et al. Frequency of false-positive FISH 1p/19q codeletion in adult diffuse astrocytic gliomas. Neurooncol Adv 2020;2:vdaa109
13. Li MM, Datto M, Duncavage EJ, et al. Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer: A Joint Consensus Recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists. J Mol Diagn 2017;19:4–23
14. Mikhail FM, Biegel JA, Cooley LD, et al. Technical laboratory standards for interpretation and reporting of acquired copy-number abnormalities and copy-neutral loss of heterozygosity in neoplastic disorders: A joint consensus recommendation from the American College of Medical Genetics and Genomics (ACMG) and the Cancer Genomics Consortium (CGC). Genet Med 2019;21: 1903–16
15. Futreal PA, Coin L, Marshall M, et al. A census of human cancer genes. Nat Rev Cancer 2004;4:177–83
16. Zhang J, Walsh MF, Wu G, et al. Germline mutations in predisposition genes in pediatric cancer. N Engl J Med 2015;373:2336–46
17. Gröbner SN, Worst BC, Weischenfeldt J, ICGC MMML-Seq Project, et al. The landscape of genomic alterations across childhood cancers. Nature 2018;555:321–7
18. Huang KL, Mashl RJ, Wu Y, Cancer Genome Atlas Research Network, et al. Pathogenic Germline Variants in 10,389 Adult Cancers. Cell 2018;173: 355–70.e14
19. Li MM, Chao E, Esplin ED, ACMG Professional Practice and Guidelines Committee, et al. Points to consider for reporting of germline variation in patients undergoing tumor testing: A statement of the American College of Medical Genetics and Genomics (ACMG). Genet Med 2020;22:1142–8
20. Sevenet N, Sheridan E, Amram D, et al. Constitutional mutations of the hSNF5/INI1 gene predispose to a variety of cancers. Am J Hum Genet 1999; 65:1342–8
21. Eaton KW, Tooke LS, Wainwright LM, et al. Spectrum of SMARCB1/INI1 mutations in familial and sporadic rhabdoid tumors. Pediatr Blood Cancer 2011;56:7–15
22. Bourdeaut F, Lequin D, Brugieres L, et al. Frequent hSNF5/INI1 germline mutations in patients with rhabdoid tumor. Clin Cancer Res 2011;17: 31–8
23. Braschi B, Denny P, Gray K, et al. Genenames.org: The HGNC and VGNC resources in 2019. Nucleic Acids Res 2019;47:D786–92
24. den Dunnen JT, Dalgleish R, Maglott DR, et al. HGVS recommendations for the description of sequence variants: 2016 update. Hum Mutat 2016;37:564–9
25. Ogino S, Gulley ML, den Dunnen JT, Association for Molecular Pathology Training and Education Committtee, et al. Standard mutation nomenclature in molecular diagnostics: Practical and educational challenges. J Mol Diagn 2007;9:1–6
26. Huse JT, Snuderl M, Jones DTW, et al. Polymorphous low-grade neuroepithelial tumor of the young (PLNTY): An epileptogenic neoplasm with oligodendroglioma-like components, aberrant CD34 expression, and genetic alterations involving the MAP kinase pathway. Acta Neuropathol 2017;133: 417–29
27. Horn S, Figl A, Rachakonda PS, et al. TERT promoter mutations in familial and sporadic melanoma. Science 2013;339:959–61
28. Huang FW, Hodis E, Xu MJ, et al. Highly recurrent TERT promoter mutations in human melanoma. Science 2013;339:957–9