Regular Article

Academic Pathology: Volume 6
DOI: 10.1177/2374289519848353
journals.sagepub.com/home/apc

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Molecular Neuropathology in Practice: Clinical Profiling and Integrative Analysis of Molecular Alterations in Glioblastoma

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Abstract

Molecular profiling of glioblastoma has revealed complex cytogenetic, epigenetic, and molecular abnormalities that are necessary for diagnosis, prognosis, and treatment. Our neuro-oncology group has developed a data-driven, institutional consensus guideline for efficient and optimal workup of glioblastomas based on our routine performance of molecular testing. We describe our institution's testing algorithm, assay development, and genetic findings in glioblastoma, to illustrate current practices and challenges in neuropathology related to molecular and genetic testing. We have found that coordination of test requisition, tissue handling, and incorporation of results into the final pathologic diagnosis by the neuropathologist improve patient care. Here, we present analysis of *O[6]-methylguanine-DNA-methyltransferase* promoter methylation and next-generation sequencing results of 189 patients, obtained utilizing our internal processes led by the neuropathology team. Our institutional pathway for neuropathologist-driven molecular testing has streamlined the management of glioblastoma samples for efficient return of results for incorporation of genomic data into the pathological diagnosis and optimal patient care.

Keywords

neuro-oncology pathway, *EGFR* variant III, glioblastoma, *O[6]-methylguanine-DNA-methyltransferase* promoter methylation, molecular profile, next-generation sequencing

Received May 1, 2018. Received revised February 27, 2019. Accepted for publication March 25, 2019.

Introduction

Glioblastoma (GBM) is the most common and aggressive malignant primary brain neoplasm in adults and has a poor prognosis.[1] In recent years, in-depth genomic studies of large cohorts of primary brain tumors have yielded genetic information that has proven to be clinically useful and is now incorporated into the diagnoses of primary brain tumors for optimal patient care.[2] These advances and their impact on the field of neuro-oncology are evident in the 2016 update to the World Health Organization

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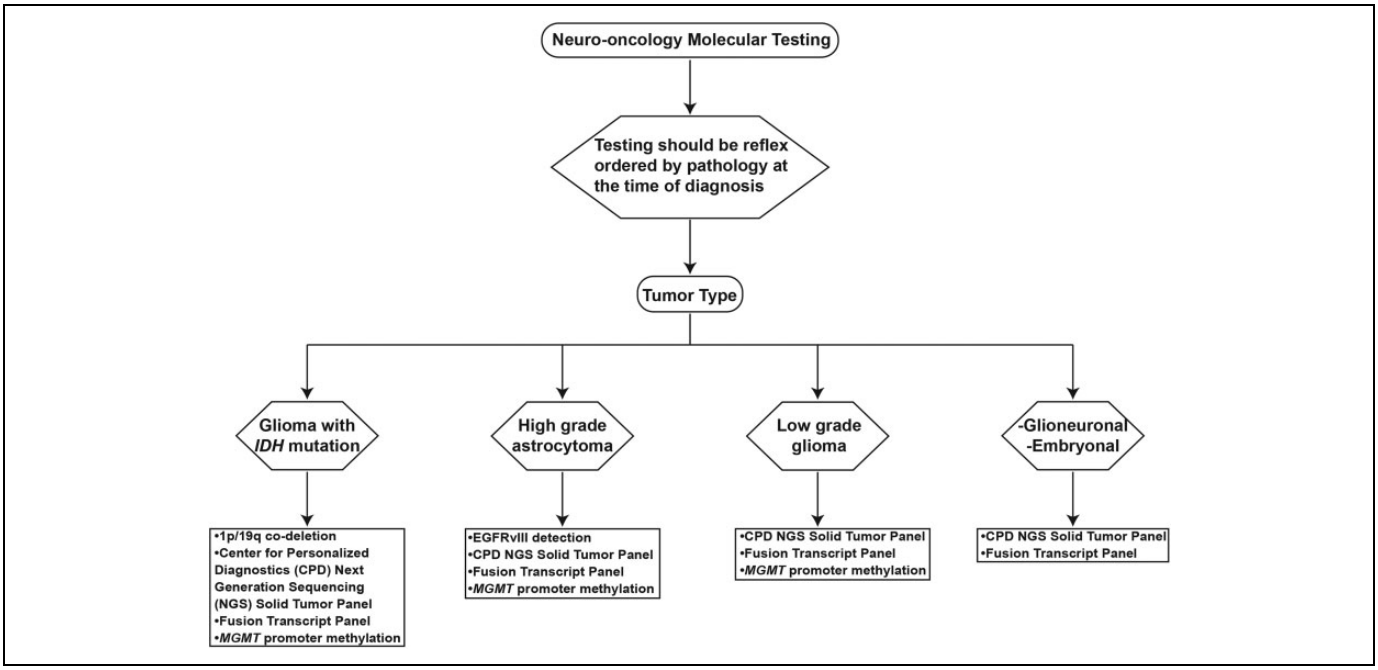

Figure 1. Penn Molecular Neuro-Oncology pathway flowchart. If the surgeon requests molecular testing at the time of resection, the neuropathologist orders NGS, EGFRvIII testing, MGMT promoter methylation testing, and/or 1p/19q codeletion studies as appropriate for the tumor type at the time of histological review. EGFRvIII indicates *EGFR* variant III; NGS, next-generation sequencing.

(WHO) Classification of Tumours of the Central Nervous System,[3] which requires, for the first time, the integration of molecular/genetic information into diagnoses of brain tumors.

At our institution, in parallel with the histological and immunohistochemical workup for diagnosis of GBM, we follow a consensus guideline for molecular testing of these tumors, including recurrences. The guideline was developed through the collaborative effort of neuro-oncology, neurosurgery, neuropathology, radiation oncology, and neuroradiology, based on the molecular assays whose results are deemed essential to patient care and which had been routinely ordered by care providers on primary brain tumors. The tests include *O^6-methylguanine-DNA-methyltransferase* (*MGMT*) promoter methylation analysis, epidermal growth factor receptor (EGFR) variant III (EGFRvIII) analysis, and interrogation of a wider array of recurrently mutated genes in solid tumors by next-generation sequencing (NGS).

O^6-methylguanine-DNA-methyltransferase promoter methylation is present in 40% to 50% of isocitrate dehydrogenase (*IDH*)-wild-type GBMs and is predictive of improved progression-free survival and overall survival in response to the alkylating chemotherapeutic agent, temozolomide.[4,5] Knowledge of tumor *MGMT* promoter methylation status is crucial to neuro-oncologists' treatment decisions throughout these patients' complicated courses, as it weighs into the clinicians' complex and ongoing therapeutic decision-making, not only of how to use temozolomide and other agents in the initial treatment of the patient but also at each recurrence of tumor. For some patients, other agents may be preferred to temozolomide.[6]

EGFR variant III is a deletion of 267 amino acids (exons 2 to 7) in the extracellular domain present in 20% to 30% of GBMs.[7,8] *EGFR* variant III results in the creation of a novel glycine residue at the junction of exons 1 and 8 and leads to ligand-independent constitutive activation of intracellular signaling cascades.[9-11] This novel glycine is, therefore, specific to the surface of tumor cells, and a potential immunogenic epitope, which has been targeted by our group with chimeric antigen receptor T-cell therapy.[12]

This article describes the assays and coordinated detection of gene variants by NGS, the EGFRvIII RNA transcriptional variant by NGS, and *MGMT* promotor methylation, as well as use of immunohistochemistry (IHC). These tests target a spectrum of clinically relevant and potentially actionable biomarkers, with testing coordinated and summarized in the final pathological report by the neuropathology team. Combined molecular results across a cohort of patients are presented. This work culminated in the development of the institutional Molecular Neuro-Oncology Pathway (Figure 1), ensuring that all GBM patient samples receive appropriate and efficient genomic testing to assist in diagnosis and prognosis.

Materials and Methods

Tumor Specimens and Nucleic Acid Preparation

Tumor specimens consisted of 189 GBM (both *IDH*-wild-type and *IDH*-mutant) biopsy or resection specimens at the University of Pennsylvania. The GBMs were diagnosed by a clinical neuropathologist in the course of service. Diagnosis of a GBM required neuropathology assessment showing a high-grade

glial tumor with nuclear atypia, mitotic activity, and either necrosis or microvascular proliferation, or both. Immunohistochemistry was performed on all tumors with GFAP, IDH1-R132 H, p53, EGFR, and Ki-67, with a subset of specimens also stained for ATRX. Control specimens for molecular test development were obtained from temporal lobe nontumor resections and adipose samples. All studies were performed in accordance with the ethical standards of the University of Pennsylvania's institutional review board and the Helsinki Declaration of the World Medical Association.

Assays

Next-Generation Sequencing–Based EGFR *Variant III Detection Assay*

A clinically validated assay for the detection of EGFRvIII was utilized in these patients. In brief, the assay was an amplicon-based NGS assay using total nucleic acids extracted from formalin-fixed paraffin-embedded tissue, requiring a minimum of 10% tumor cellularity (Supplementary Figures 1 and 2). The assay detects the ratio of EGFRvIII (defined by sequence reads joining exons 1 to 8) compared with total EGFR (defined by the total of EGFRvIII sequence reads joining exons 1 to 8 plus sequence reads joining exons 1 to 2, indicating wild type). A positive result was called if EGFRvIII was present in 5% or greater of the total EGFR reads, with at least 100 reads supporting the call of reads joining exon 1 to 8.

Targeted Tumor Panel Next-Generation Sequencing

Targeted gene panel NGS was used to detect single-nucleotide variants (SNVs), insertions, and deletions (indels). Two different assays were used over the course of this study. The initial panel (Penn-47) used for detection of variants in brain tumor specimens included 47 genes, *ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CSF1 R, CTNNB1, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL*. A more recent sequencing panel includes 153 genes (Penn-153), *ABL1, AKT1, AKT2, AKT3, ALK, APC, AR, ARAF, ARID1A, ARID2, ATM, ATRX, AURKA, BAP1, BRAF, BRCA1, BRCA2, BRIP, BTK, CBP, CCND1, CCND2, CCND3, CCNE1, CDH1, CDK4, CDK6, CDKN2A, CHEK2, CIC, CRKL, CSF1 R, CTNNB1, DAXX, DDR2, DNMT3A, EGFR, EP300, EPHA3, ERBB2, ERBB3, ERBB4, ERCC2, ERG, ESR1, ESR2, EZH2, FBXW7, FGF3, FGFR1, FGFR2, FGFR3, FGFR4, FLT3, FUBP1, GATA3, GNA11, GNAQ, GNAS, HRAS, H3F3A, IDH1, IDH2, IGF1 R, JAK1, JAK2, JAK3, KCNG1, KDM5A, KDM5C, KDM6A, KDR, KIT, KMT2C, KRAS, LRRK2, MAP2K1, MAP2K2, MAP2K4, MAPK1, MAPK3, MAX, MCL1, MDM2, MDM4, MED12, MEN1, MET, MITF, MLH1, MRE11A, MSH2, MSH6, MTOR, MYC, MYCN, NBN, NF1, NF2, NTRK1, NTRK2,*

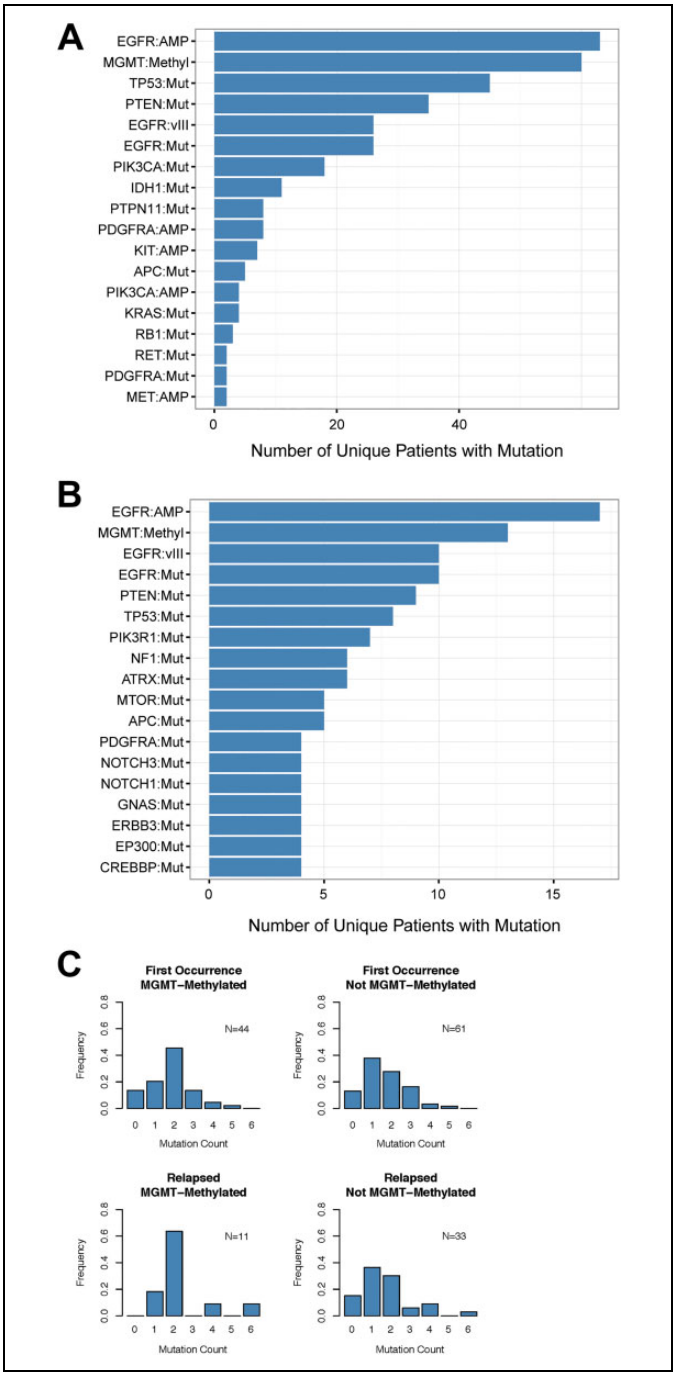

Figure 2. Mutations and copy number gains identified in the sample cohort with a comparison of the first and second versions of the NGS panel. A, Using Penn-47, a total of 272 mutations and copy number gains, excluding 26 EGFRvIII mutations and MGMT promoter methylation, were identified across 164 patients. These mutations were all independent of EGFRvIII status. B, Using Penn-153, a total of 250 mutations and copy number gains were identified across 32 patients. Shown are the most frequently occurring alterations. C, Pairwise Kolmogorov-Smirnov tests showed no statistically significant difference between mutation count distributions between initial glioblastoma occurrences and recurrent tumors when comparing MGMT methylated versus MGMT unmethylated tumors sequenced on Penn-47. AMP indicates copy number gain; EGFRvIII, *EGFR* variant III; Methyl, methylation; mut, mutation; NGS, next-generation sequencing.

NTRK3, NKX2-1, NOTCH1, NOTCH2, NOTCH3, NRAS, PAK1, PALB2, PBRM1, PDGFRA, PIK3CA, PIK3CB, PIK3R1, PTCH1, PTEN, PTPN11, RAB35, RAC1, RAD50, RAD51, RAD51B, RAD51C, RAD51D, RAF1, RB1, RET, RHOA, RNF43, SETD2, SF3B1, SLIT2, SMAD4, SMARCA4, SMO, SPOP, SRC, STAG2, STK11, SUFU, SUZ12, SYK, TET2, TGFBR2, TP53, TRAF7, TSC1, TSC2, TSHR, U2AF1, VHL, WT1, XRCC2. In addition, copy number gains of *EGFR*, *KIT*, *PDGFRA*, *MET*, *FGFR3*, *PIK3CA*, *FLT3,* and *ERBB2* were interrogated on both versions of the panel.

The threshold for sensitivity was determined to require a minimum of 10% estimated tumor cellularity for both panels. The initial panel was a clinically validated panel using the TruSeq Custom Amplicon Cancer Panel kit (Illumina, San Diego, CA), which targeted hotspot variants. Samples were multiplexed and sequenced on a MiSeq to an average depth of coverage of 2500×. All variants were identified using an in-house data processing bioinformatics pipeline capable of detecting SNVs, insertions and/or deletions (indels), and copy number events which include aneuploidy and amplification, referred to in this manuscript as copy number gains.[13,14] Next-generation sequencing often cannot distinguish between aneuploidy in a high number of cells and true amplification occurring in a smaller number of cells; copy number gains refer to both scenarios.

Based on clinical demand, the larger Penn-153 panel was validated and implemented in clinical practice and incorporates additional actionable, prognostic, and diagnostic gene information. The larger panel was designed to have full gene coverage of 153 genes, using the Agilent Haloplex design with unique molecular identifiers. Samples were multiplexed and sequenced on a HiSeq with total deduplicated reads of 6.5 million reads/sample; duplicate reads were removed based on incorporation of unique molecular identifiers. All variants were identified using an in-house data processing bioinformatics pipeline capable of detecting SNVs, insertions and/or deletions (indels), and copy number gains for a subset of genes, based on increased read depth.

Variants identified by sequencing were classified internally as one of the following: disease-associated variants, probably disease-associated variants, variants of uncertain significance (VUS), likely benign, or benign polymorphisms. The category of disease-associated variants included variants associated with disease in any tumor type and was determined using an in-house curated database incorporating data available from the current literature as well as public databases such as COSMIC. Probably disease-associated variants, VUS, and likely benign variants are reported together under the category VUS. Benign variants were those for which there was no functional data and which occur in the population at greater than 0.1%, based on the ExAC database (http://exac.broadinstitute.org/). Synonymous changes were not evaluated or reported unless they were located within the conserved positions of a splice site. Intronic changes at the −1, −2, +1, and +2 positions were evaluated and reported if pathogenic or VUS.

O6-Methylguanine-DNA-Methyltransferase Promoter Methylation Assay

To assess *MGMT* promoter methylation status, formalin-fixed paraffin-embedded tissue samples containing at least 20% tumor cellularity were selected by a neuropathologist. DNA was extracted from paraffin rolls or macrodissected from paraffin sections if necessary to enrich for tumor cellularity above 20% as previously described.[15] The DNA then underwent bisulfite conversion, which converted unmethylated cytosines to uracils (EZ DNA Methylation Kit; Zymo Research, Irvine, CA). Subsequently, bisulfite-converted genomic DNA was amplified by polymerase chain reaction, creating a fragment spanning 4 CpG sites in exon 1 (DMR2) of *MGMT*. This fragment was pyrosequenced to evaluate for the presence of methylation at each of the 4 CpG sites (PyroMark Q24; Qiagen, Hilden, Germany). A result was considered positive when both the mean and median percentage methylation across the 4 interrogated CpG sites was greater than or equal to 10%. *O^6-Methylguanine-DNA-Methyltransferase* promoter methylation testing was performed on a single block per patient, given that this biomarker tends to be consistent throughout an individual GBM.[16]

Statistical methods for molecular alteration correlation analysis. In order to measure and illustrate the relative positive or negative association of different pairwise gene alterations across patients, binary similarity measures were computed using 2 complementary methods: Pearson correlation coefficient (r) and Yule coefficient of association (also known as Yule Q), which both fall within the range of (−1 to 1). By both methods, positive coefficients indicate alterations that tend to occur together, negative coefficients indicate alterations that tend to occur separately, and a zero coefficient indicates no association. However, these 2 similarity measures differ significantly when alterations occur at different population frequencies—for example, when alteration A occurs at 50% frequency ($F_A = 50\%$) and alteration B occurs at 10% frequency ($F_B = 10\%$), their maximum possible Pearson coefficient is only $r = 1/3$, whereas their maximum possible Yule coefficient is $Q = 1$. Therefore, an advantage of Yule coefficient is that it adjusts for mismatches in the relative population frequency of gene alterations, so that $Q = 1$ indicates that one alteration always co-occurs with the other alteration across patients, and $Q = -1$ indicates that the 2 alterations have the minimum possible overlap across patients (ie, $Q = -1$ implies that alterations are mutually exclusive provided that $F_A + F_B \leq 1$). To assess the statistical significance of association between pairwise combinations of variants, we used the Fisher exact test. Plots of pairwise correlation coefficients were visualized using the R programming language.

Results

Detection of Genetic Changes in Glioblastoma

Cohorts. Given the heterogeneity in the design, method, and analysis for *MGMT* promoter methylation across various

Table 1. Co-Occurrence of Genetic Alterations.

Correlations with *MGMT* promoter methylation					
IDH1-mutant		OR = 9.4	95% CI = 1.9-90.9	Fisher exact test $P = .001$	
Positive trends:					
De novo GBM, *IDH1*-wild type	*TP53* variants	OR = 1.5	95% CI = 0.6-3.4	Fisher exact test $P = .41$	
De novo GBM, *IDH1*-wild type	*PTEN* G > A variants	OR = 3.1	95% CI = 0.3-38.3	Fisher exact test $P = .33$	
Recurrent/residual GBM, *IDH1*-wild type	*TP53* variants	OR = 4.3	95% CI = 0.6-31.1	Fisher exact test $P = .08$	
Number of Co-Occurring Variants Per Tumor on Sequencing Panels	Mean	Median	Range	Cases with > 1 Variant	
Penn-47	0.9	1	0-3	26%	Damaging or likely damaging
	0.2	0	0-3	4%	VUS
	1.1	1	0-5	32%	All
Penn-153	2.3	2	0-6 (outlier 15)	56%	Damaging or likely damaging
	8.2	2	0-7 (outliers 71, 122)	69%	VUS
	10.4	4	0-8 (outliers 86, 128)	94%	All
Co-occurrences					
EGFRvIII	*EGFR* amp	OR = 13.5	95% CI = 4.8-47.6	Fisher exact test $P = 2.23 \times 10^{-9}$	
EGFR variant (excluding EGFRvIII)	*EGFR* amp	OR = 12.8	95% CI = 4.5-45.1	Fisher exact test $P = 6.34 \times 10^{-9}$	
PDGFRA amp	*KIT* amp	OR = 400	95% CI = (33-16384)	Fisher exact test $P = 7.87 \times 10^{-9}$	
Mutual exclusions					
IDH1 variants	*EGFR* amp	OR = 0.00	95% CI = 0.00-0.51	Fisher exact test $P = .004$	

Abbreviations: CI, confidence interval; GBM, glioblastoma; MGMT, *O*^{6}*-methylguanine-DNA-methyltransferase;* OR, odds ratio; VUS, variants of uncertain significance.

laboratories, we chose to restrict our analysis to the subset of 189 unique patients with GBM (both *IDH*-wild-type and *IDH*-mutant) whose *MGMT* studies were performed at our institution, who also had results for the solid tumor panel and the EGFRvIII platform. These patients were further subdivided into 2 overlapping cohorts: 164 unique patients with sequencing on the Penn-47 solid tumor panel and 32 unique patients with sequencing on the Penn-153 solid tumor panel. Seven patients were common to both cohorts. When multiple tumor specimens were available for a given patient within each cohort, only the earliest tumor specimen was included. Overall demographics of the 2 cohorts showed a male to female ratio of 1.33:1. The median age at the time of surgery was 60 years.

Within the Penn-47 cohort, further diagnostic subcategories were defined for first occurrence of primary GBMs (N = 127 unique patients), recurrent/residual primary GBMs (N = 44 unique patients), and GBMs arising from lower grade astrocytomas (N = 15 unique patients). To ensure independence for statistical comparisons between the first occurrence primary GBM cohort and the recurrent/residual primary GBM cohort, 22 patients with first occurrence GBM were excluded from analysis because they overlapped with the recurrent/residual primary GBM cohort.

Penn-47 findings. In the cohort of 164 patients who have results for Penn-47, the EGFRvIII assay, and *MGMT* methylation testing, the NGS assay detected copy number gains in *EGFR*, *KIT*, *PDGFRA*, *MET*, and *PIK3CA* and point mutations in *TP53*, *PTEN*, *EGFR*, *PIK3CA*, *IDH1*, *PTPN11*, *APC, RB1*, *KRAS*, and *RET* (Figure 2A). Among gliomas, *EGFR* copy number gain, which often reflects amplification, but in some instances reflects aneuploidy, is found essentially only in GBMs.[17] EGFR copy number gain is the most common alteration found in the cohort (63 patients, 38%), followed by *MGMT* promoter methylation (60 patients, 37%), *TP53* variants (45 patients, 27%), *PTEN* variants (35 patients, 21%), EGFRvIII (26 patients, 16%), and *EGFR* point mutations exclusive of EGFRvIII (26 patients, 16%). Full genetic data are available in Supplementary Table 1.

Penn-153 findings. Thirty-two patients have results for Penn-153, the EGFRvIII assay, and *MGMT* methylation testing, with

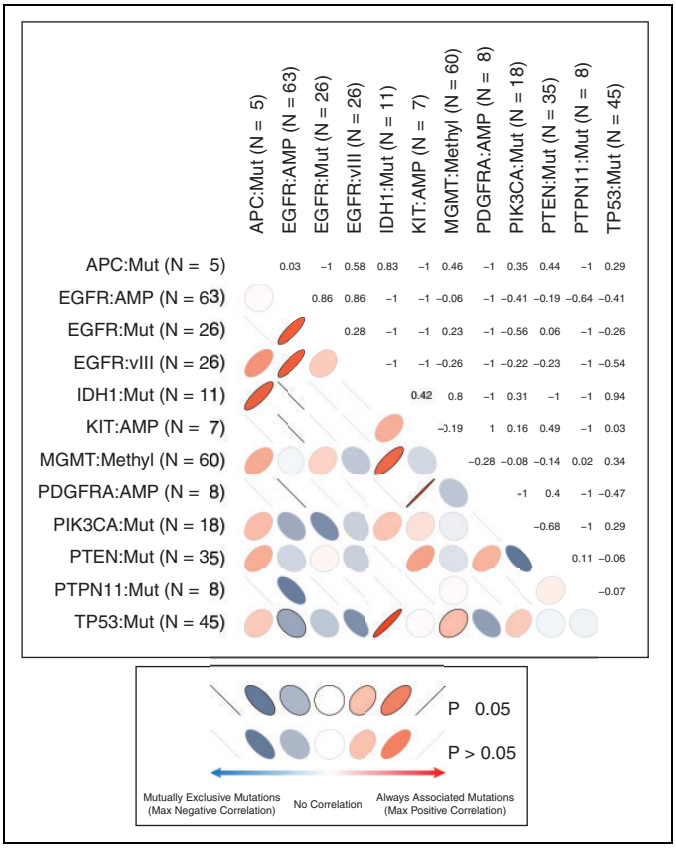

Figure 3. Correlations of mutations and amplifications found in gliomas. Pairwise correlation plot of the 11 most common mutations and copy number gains found in the cohort of glioblastoma samples analyzed on Penn-47. Positive (red) and negative (blue) correlations are illustrated with elliptical eccentricity and color depth proportional to Yule coefficient of association. Pairwise correlations with Fisher exact test $P \leq .05$ are highlighted in bold outline.

7 of these patients' prior resections run on Penn-47. Across these 7 patients, 8 mutations were detected on the Penn-47 panel, of which 5 (62.5%) mutations were also detected on the Penn-153 panel. For all 7 patients, the Penn-153 panel was run on a later resection; detected mutations in genes shared between the 2 panels that were not observed on the Penn-47 panel likely reflect both a difference in assay design and spatial and temporal molecular heterogeneity. The NGS assay detected disease-associated variations in 71 different genes on the 153-gene panel, including copy number gains of *EGFR*, *KIT,* and *PDGFRA*. Again, the most common alteration is *EGFR* copy number gain (17 patients, 53%), followed by *MGMT* methylation (13 patients, 41%), EGFRvIII mutations (10 patients, 31%), *EGFR* point mutations exclusive of EGFRvIII (10 patients, 31%), *PTEN* variants (9 patients, 28%), and *TP53* (8 patients, 25%; Figure 2B and Supplementary Figure 3). Full genetic data are available in Supplementary Table 2. A subset of the additional genes can be important in meeting diagnostic criteria for specific brain tumors. Variants were found in *CIC*, *FUBP1*, *MDM2,* and *ATRX*, among other genes, which would not have been detected by the Penn-47 panel.

O^6-Methylguanine-DNA-methyltransferase promoter methylation results and correlations across cohorts. Analysis of 189 cases of GBM with *MGMT* promoter methylation and genomic testing showed a correlation between tumors containing *IDH1* mutations with hypermethylated *MGMT* (odds ratio [OR] = 8.88, 95% confidence interval [CI] = 1.80-59.18, Fisher exact $P = .002$, False Discovery Rate = 0.028), as expected from the hypermethylated profile seen in *IDH*-mutated tumors.[18-21] The mutation rate was not significantly different between initial GBM occurrences and recurrent tumors when comparing *MGMT* methylated versus *MGMT* unmethylated tumors in either cohort sequenced (Figure 2C, Supplementary Figure 4). Among the subcohort of recurrent/residual GBMs that are negative for *IDH1* mutations, a trend toward increased *TP53* variants when *MGMT* is methylated (4 tumors had *TP53* variants out of 8 *MGMT* methylated recurrent/residual GBMs) compared to unmethylated (6 tumors had TP53 variants out of 33 *MGMT* unmethylated recurrent/residual GBMs) was observed. However, this result did not reach statistical significance ($P = .082$), in contrast to the 2008 TCGA data.[22] *O^6-Methylguanine-DNA-methyltransferase* promoter methylation must be assayed in each case, given the lack of absolute correlation with mutational profile or other tumor characteristics and its importance in predicting response to therapy.

Co-Occurrence of Genetic Alterations

Analysis of co-occurring or mutually exclusive alterations detected in the Penn-47 panel demonstrated both positive and negative correlations for specific patterns (Figure 3). We preferentially illustrated correlations via Yule coefficient (Figure 3) instead of Pearson coefficient (Supplementary Figure 5), as Yule correlation better illustrated instances where variants were maximally correlated or mutually exclusive (see Methods). Additional co-occurrence data are tabulated in Table 1.

Co-Occurrence of EGFR Changes

Disease-associated variants co-occurring with *EGFR* changes include additional variants in *EGFR* and/or variants in other genes.[9] Copy number gains of *EGFR* were found in 39.7% of the GBMs studied (data not shown). *EGFR* disease-associated variants other than EGFRvIII are seen in 28 (37.3%) out of the 75 cases with *EGFR* copy number gains. In agreement with previous studies,[23] *IDH1* mutations and *EGFR* copy number gain are mutually exclusive events in our cohort, demonstrating statistically significant anticorrelation (OR = 0.00, 95% CI = 0.00-0.51, Fisher exact test $P = .004$). Seventeen (9.0%) cases showed co-occurrence of *EGFR* and *PTEN* changes. Identification of these cases may be therapeutically important, as the loss of *PTEN* may lead to resistance to EGFR kinase inhibitors.[24]

Within our combined cohort of 189 total patients with GBM, 29 out of 34 EGFRvIII-mutated tumors have *EGFR* copy number gains (85.3%; Figure 4A and C), demonstrating a high degree of association between EGFRvIII and *EGFR* copy

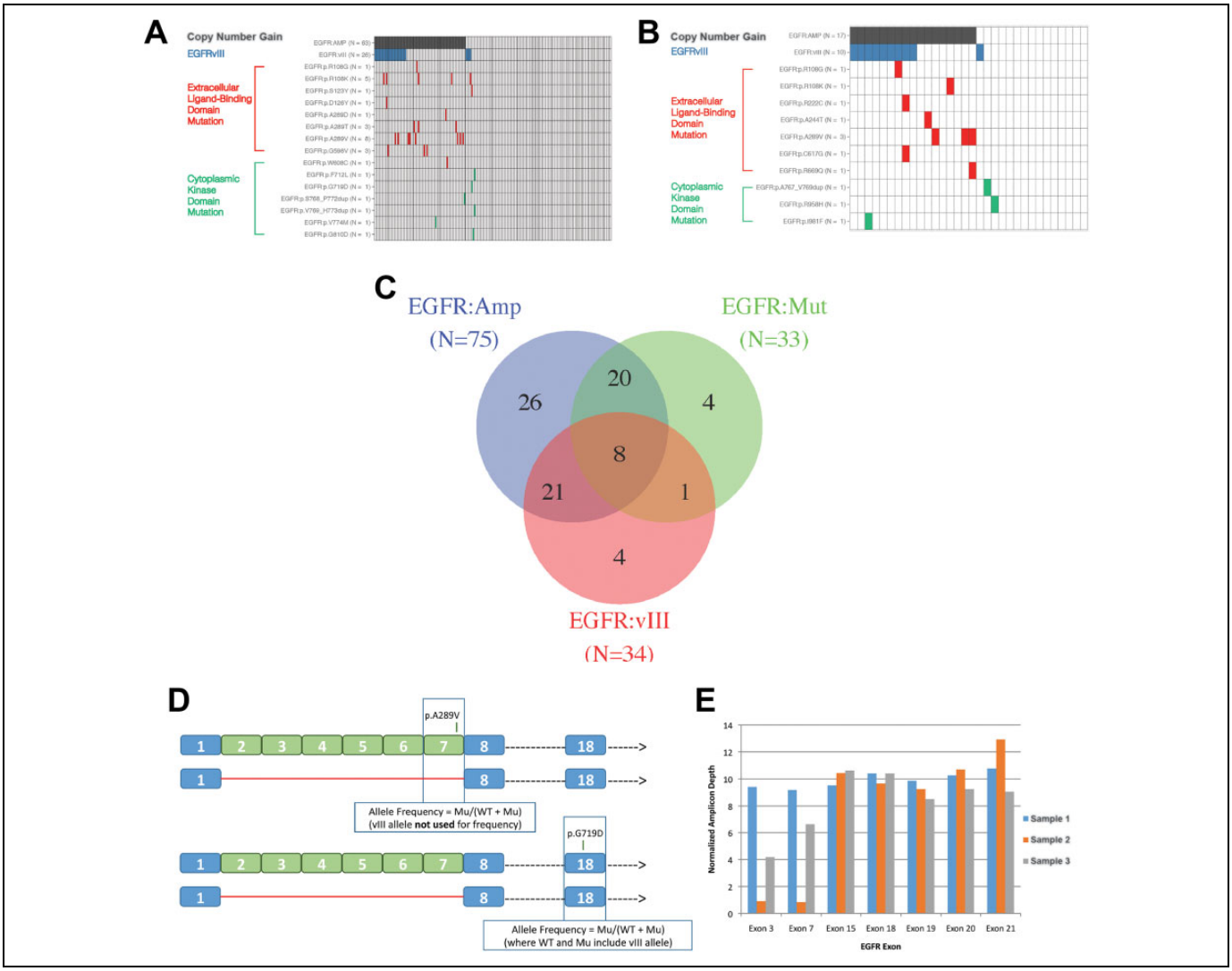

Figure 4. EGFR changes identified in the sample cohort with a comparison of the first and second versions of the NGS panel. There was significant co-occurrence of EGFRvIII mutant isoform with other mutations in the samples with Penn-47 (A) and Penn-153 (B). Venn diagrams demonstrating the significant overlap of alterations in EGFR changes for all patients (C). EGFRvIII mutant isoform, other point mutations, and copy number gains all show positive co-occurrence. (D) The *EGFR* gene is depicted with the EGFRvIII deleted exons in green and the retained exons in blue. Formulas for calculating allele frequencies of mutations depending on their location are shown. With the p.G719D mutation, we cannot determine if it is located in cis or trans with the EGFRvIII allele based on our NGS assay. (E) Raw EGFR amplicon depths for amplicons covering exons 3, 7, 15, 18, 19, and 20 were normalized to the sample mean depth of coverage and then normalized to the average read depth for each amplicon in nontumor brain parenchyma. Comparison of normalized amplicon depths between exons 3 and 7 and those of exons 15, 18, 19, 20, and 21 identified the amplified allele (EGFRvIII and/or wild-type EGFR). EGFRvIII indicates *EGFR* variant III; NGS, next-generation sequencing.

number gains (OR = 13.5, 95% CI = 4.8-47.6, Fisher exact test $P = 2.23 \times 10^{-9}$). Similarly, 28 out of 33 patients with *EGFR* variants excluding EGFRvIII are also likely *EGFR* amplified (84.8%; Fisher exact test $P = 6.34 \times 10^{-9}$). The combination of *EGFR* copy number gain, EGFRvIII splice variant, and *EGFR* SNVs was identified in 8 (4.2%) cases. In GBM, *EGFR* point mutations are more often found in the extracellular domain, as opposed to the cytoplasmic kinase domain (Figure 4A and 4B), as has been seen in previous studies, and may partially underlie the resistance of GBMs to kinase domain inhibition.[25-27]

Notably, interpretation EGFR mutation variant allele frequency is complicated by the presence of concurrent copy number alterations, including EGFRvIII (deletion of exons 2-7) and EGFR copy number gain (Figure 4D). In our cohort, analysis of normalized amplicon depths across *EGFR* in EGFRvIII-positive tumors demonstrated distinct patterns reflecting these allelic differences (Figure 4E). The scenarios encountered include gain of wild-type EGFR (Figure 4E, sample 1), gain of the EGFRvIII allele (sample 2), and gains of both the wild-type and EGFRvIII alleles (sample 3), which could occur in either single or multiple clones. This makes variant

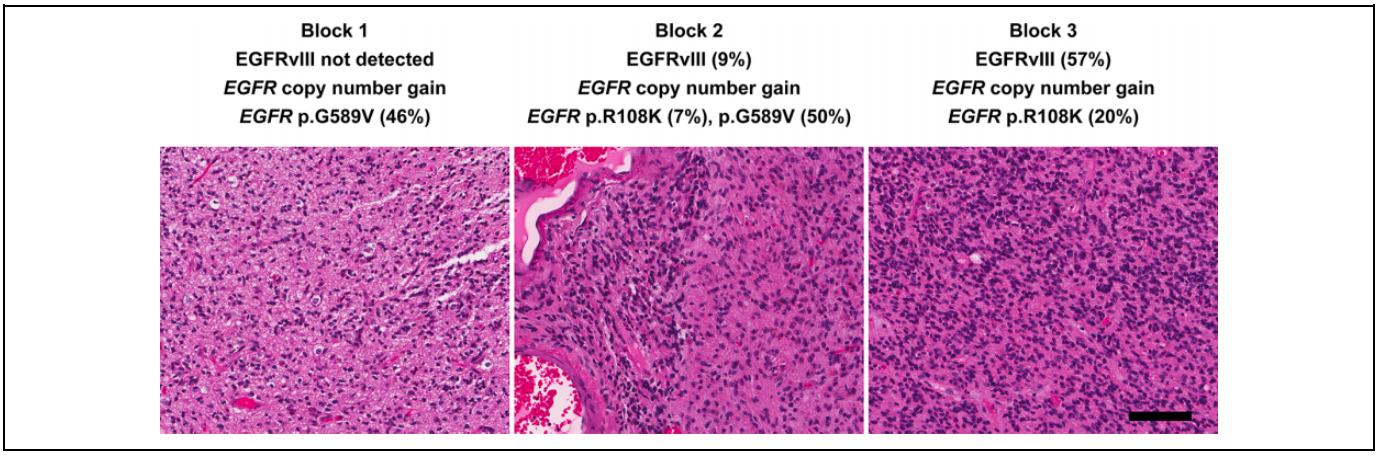

Figure 5. Histological features of GBM with heterogeneous EGFR changes. Different EGFR mutations were observed in distinct blocks of tissue from a single GBM. Focal histological features are demonstrated for each block, with the uniform hypercellular infiltrative tumor shown for block 1 (left), leptomeningeal spread and dense tumor shown for block 2 (middle), and dense tumor shown for block 3 (right). Scale bar 100 microns (lower right). GBM indicates glioblastoma.

interpretation complex and necessary to consider during assay validation, especially when designating thresholds for these relatively common covariants.

IDH-*Mutant Glioblastomas Versus "Secondary Glioblastomas"*

Glioblastoma can arise *de novo*, clinically referred to as "primary GBM," or may progress from a lower grade astrocytoma as what is clinically known as a "secondary GBM." Many, but not all, GBMs that progress from lower grade astrocytomas have an *IDH* mutation. It is now understood that the *IDH* mutational status is more relevant to prognosis than histologic grade at initial tumor presentation.[28-30] The prognostic value of *IDH* mutational status is reflected in the 2016 update of the WHO classification of tumors, in which a diagnosis of any grade astrocytoma requires the mutational status of *IDH*.[3] Since the majority of our cohort predated the change in terminology, we compared the designation of "secondary GBM" with *IDH* mutational status, retrospectively.

Our cohort includes 13 cases of lower grade tumors that progressed to GBM ("secondary GBMs"): 5 tumors with *IDH1* mutations and 8 *IDH1*-wild-type tumors. Of the 8 *IDH1*-wild-type tumors, 5 cases had *EGFR* abnormalities (ie, copy number gain, *EGFR* point mutation, and/or EGFRvIII), findings that would be inconsistent with co-occurrence of an *IDH* variant, including those not covered by the panel. Therefore, of our patients diagnosed clinically with "secondary GBM," more patients had *IDH*-wild-type tumors than *IDH*-mutant tumors, crucial information for the patient and treating physicians. An additional 6 cases presented at first occurrence ("primary GBM") as *IDH*-mutant GBM (grade IV). These findings illustrate the importance of testing *IDH* mutational status, given that *IDH* mutation does not consistently correlate with "secondary GBM," and not all "primary GBM" are *IDH*-wild type.[28,30] Additionally, of the remaining 3 *IDH1*-wild-type cases that lacked *EGFR* changes, one case showed *PTPN11* and *TP53* mutations, another showed an *MLH1* mutation, and the last case showing no changes on Penn-47. These inconclusive results highlight the importance of the broader Penn-153 panel, which includes *IDH2* and *ATRX*.

Complexity of Intratumoral Heterogeneity

Tumor heterogeneity is common in GBM; our studies and others have shown the presence of multiple alterations based on varying allele frequencies suggestive of subclones and complexity.[23] To evaluate tumor heterogeneity, 3 different tumor blocks from a single procedure for a recurrent/residual GBM were analyzed by the EGFRvIII and NGS assays. The tumor percentage was assessed by a neuropathologist as >50% for each of the 3 blocks. In 3 different blocks from resection of the recurrent/residual GBM, EGFRvIII detection varied, with values of 0%, 9%, and 57% (Figure 5). Similarly, the presence or absence of the *EGFR* p.R108 K and p.G589 V variants and their allele frequencies varies among tumor blocks, despite no significant difference in the tumor percentage of each block. *EGFR* copy number gain was detected in all 3 blocks. These results exemplify the known molecular heterogeneity present in primary GBM[31] and the complexity of EGFR alterations in GBM.

In addition to the differences in variants, histologic variation was also present. Block 1 contains much less tissue than blocks 2 and 3, but is essentially all tumor, densely infiltrating cortex (Figure 5, block 1, left). In contrast, block 2 contains much more tissue, with a variegated histological appearance and predominantly dense tumor including leptomeningeal involvement (Figure 5, block 2, middle), but also has areas of less dense cortical infiltration, as well as dramatic treatment-related changes, including numerous macrophages and hemosiderin deposition. Block 3 shows solid and infiltrating tumor,

with areas of incipient pseudopalisading necrosis, and more perivascular and infiltrating lymphocytes, but less treatment-related change than block 2 (Figure 5, block 3, right).

Consensus Institutional Molecular Neuro-Oncology Pathway

Development and Use of the Pathway

The institutional experience combining histopathologic findings with molecular findings in brain tumors has led to the decision by our neuro-oncology group to create a brain tumor molecular testing pathway in which the choice of tests is determined by the neuropathologist. The Molecular Neuro-Oncology Pathway (Figure 1) was formulated and agreed upon by all departments that collaborate in caring for neuro-oncology patients, including the departments of neuropathology, neuro-oncology, neurosurgery, radiation oncology, and neuroradiology. Curated by the institution's Center for Evidence-Based Practice, the Pathway allows the pathologist to coordinate molecular testing, based on histological and immunohistochemical assessment of the specimen. As depicted, a Fusion Transcript Panel was recently incorporated into the Pathway; methods and results from this assay are beyond the scope of this manuscript.

The Pathway begins at the time of resection of any central nervous system (CNS) tumor, glial or other, at our institution. When sending the specimen to the pathology laboratory, the surgeon checks a box on the requisition sheet for molecular testing, which initiates the Pathway. The Pathway scheme indicates the applicable tests for each type of tumor, such that testing is performed to provide the appropriate molecular information to the pathologist when rendering a final diagnosis and to the clinician when treating the patient.

For example, in the case of a tumor histologically and immunohistochemically diagnosed as a GBM, including recurrent/residual GBM, the pathologist would submit the sample for NGS, EGFRvIII testing, and potentially the *MGMT* promoter methylation assay. Testing for 1p/19q codeletion might be performed on specimens demonstrating *IDH* mutation, detected either by IHC for IDH1-R132 H or by sequencing, depending on the patient's age and the immunohistochemical profile of the tumor.[32]

Communicating Results of Molecular Testing

The results of the array of testing (NGS, EGFRvIII, 1p/19q codeletion, and MGMT promoter methylation) are issued in separate reports subsequent to the surgical pathology report. To alleviate the need to identify the multiple test results and to summarize the findings in context with the pathological diagnosis, an addendum to the surgical pathology reports is issued to report a final diagnosis that requires integration of the molecular results or, when necessary for clarity of diagnosis. Results and consequent management of patients are discussed at a weekly Brain Tumor Conference, with neurosurgery, neuro-oncology, neuropathology, radiation oncology, neuroradiology, and clinical trial teams present.

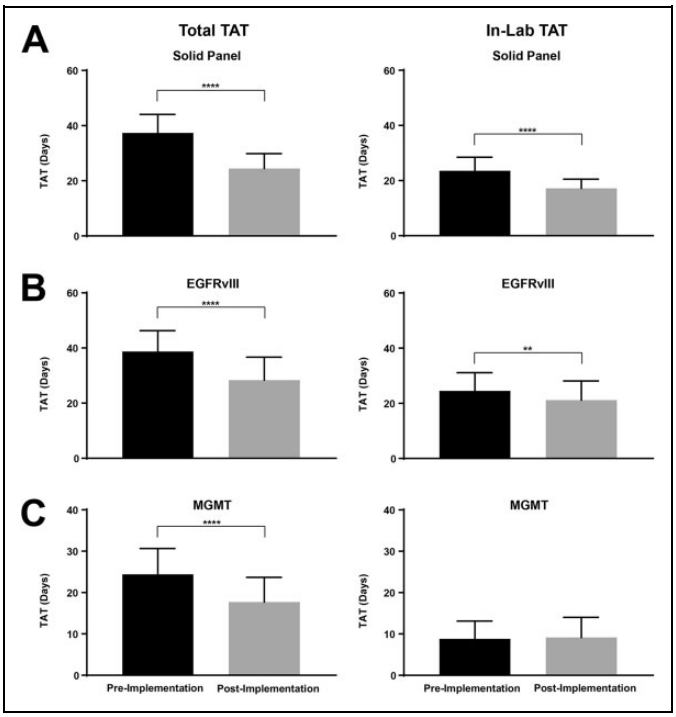

Figure 6. Turnaround time comparisons pre- and postpathway implementation, covering a 5-month span before and after pathway implementation. Total turnaround time (left) and lab-specific turnaround time (right) for the (A) solid panel testing, (B) *EGFR* variant III (EGFRvIII) testing, and (C) MGMT methylation status. Turn-around time (TAT) quantification counts all calendar days and is not limited to working days. Error bars represent standard deviation, significance $^{**}P \leq .01$, $^{****}P \leq .0001$. The turnaround time for reporting molecular results since implementation of the Pathway has reduced by 13 days for the solid tumor panel next-generation sequencing and by 10 days for the EGFRvIII assay. Improvements of in-lab TAT account for approximately 6 and 3 days of the reductions, respectively, with the remaining week of the reduction due to the Pathway workflow (see Supplementary Table 3). EGFRvIII indicates *EGFR* variant III.

Impact of the Pathway

Implementation of this Pathway has resulted in more efficient use of tissue and decreased turnaround time (TAT) for molecular testing. For solid tumor panel sequencing, the TAT measured from the date of surgery to the date of report was reduced by 13 days after implementation of the Pathway; likewise, for EGFRvIII testing, the TAT from the date of surgery to report reduced by 10 days (Figure 6). Improvements of in-laboratory TAT account for approximately 6 and 3 days of the reductions, respectively, with the remaining week of the reduction due to the Pathway workflow (Supplementary Table 3). Similarly, a 7-day reduction in TAT for *MGMT* promoter methylation assay occurred. These reductions in TAT were detected by analyzing the 5-month period prior to implementation of the Pathway compared to the 5-month period subsequent to its implementation. At both time points, all tests were run in batches, which

is cost-effective. For assays such as NGS, batching leads to better TAT given the long instrument time. Despite the many additional laboratory, technical, personnel, and workflow changes occurring during these periods, the decrease in time for a patient to receive sequencing results after surgery is significant.

Case Study Illustrating Clinical Impact

For a number of cases, it has been essential that the neuropathologist direct testing through the institutional pathway. In instances when resected tissue is scant (eg, when a tumor is located near an eloquent area of the brain, in the midline, in the brain stem or in the spinal cord), the tissue is carefully apportioned, the formalin-fixed paraffin-embedded tissue carefully cut, and appropriate molecular testing is prioritized, with the extracted nucleic acids shared among assays whenever possible. The benefits of this approach are illustrated by a case of a minute biopsy of a spinal cord tumor with an histological differential that included pilocytic astrocytoma (grade I) and H3 K27M-mutant diffuse midline glioma (DMG, grade IV).[3] In contrast to DMG, pilocytic astrocytomas often show changes in the mitogen-activated protein kinase (MAPK) pathway, including *KIAA1549/BRAF* fusion.[33] An initial limited panel of immunohistochemical stains for H3 K27M, IDH1-R132H, and Ki-67 was performed for tumor classification and histological grading, and the order to cut for molecular testing was placed simultaneously with the order for these immunohistochemical stains to avoid resurfacing the block. The H3 K27M IHC appeared potentially positive but showed high background, making it difficult to interpret. When placing the order for sequencing, the neuropathologist communicated the specific question to the molecular team, with the request to communicate and reassess for next steps in the case of insufficient DNA for testing. *O^6-Methylguanine-DNA-methyltransferase* promoter methylation testing was held. The initially extracted DNA was insufficient for the full solid tumor panel; therefore, the additional cuts were extracted for DNA, rather than the routine protocol which would direct that total nucleic acids be extracted for EGFRvIII/Fusion Transcript Panel testing. The second extraction yielded sufficient DNA; sequencing was successful and confirmed the H3K27 M variant. The diagnosis of this case depended on the neuropathologist coordinating molecular testing as delineated by the Pathway.

Prior to implementation of the Pathway, the clinician likely would have placed orders for all of the molecular tests at one time, subsequent to the issuance of the initial surgical pathology report with a pending integrated diagnosis. Results of "quantity not sufficient" for both the solid tumor panel and the EGFRvIII test, and possibly *MGMT* promoter methylation would have been the likely outcome. The tissue would have been exhausted and the diagnosis would have remained unconfirmed.

Clinical Implications

In recognition of the diagnostic, prognostic, and therapeutic implications of molecular subcategories of GBM, the publication of the 2016 update to the WHO classification for CNS tumors has standardized incorporation of molecular information into pathologic diagnosis by requiring certain genetic changes as elements of the final diagnosis, underscoring the importance of molecular profiling to providing optimal patient care. In addition to the WHO update, the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy, cIMPACT-NOW, sponsored by the International Society of Neuropathology, issues periodic consensus recommendations on CNS tumor classifications due to the rapid rate of change, primarily in the field of molecular neuropathology.[34]

Allowing the pathologist to direct the molecular testing based upon the neuro-oncology group's consensus ensures that the necessary tests are ordered immediately and that potential additional molecular tests are suitably triaged. Although not every test listed in the guideline must be ordered, the pathologist may order testing as deemed appropriate. The workflow facilitates communication between the pathologist and the downstream laboratories, in contrast to when a clinician places an order. Both the timely ordering and the close communication are likely to lead to the improved TAT that has occurred. An additional benefit to having the pathologist guide the testing is that the pathologist may test multiple histologically distinct regions of a tumor to assess tumor heterogeneity, only apparent by pathological assessment. The pathologist identifies optimal tissue blocks at the time of histological examination and the molecular orders are placed before the report is verified. Recurrent GBMs are handled following the same algorithm, with increased focus on detection and testing morphologically heterogeneous sections for testing when patients have had targeted therapy.

Impact on Therapy

In addition to describing the Pathway, we share our assay development and results. Analysis of the data from our cohort illustrates the spectrum of unique and recurrent molecular changes in GBMs, consistent with previous findings.[9] In addition, the results highlight the value of increasing the panel size for NGS studies, given the increasing number of both ongoing and upcoming clinical treatment trials that target various genetic changes. Co-occurrence is clinically relevant because the constellation of variants found in a tumor can be helpful in clarifying the grade of the tumor, and on occasion the diagnosis. For example, if a tumor that appears radiologically to be a GBM is biopsied, but the sampled tissue shows a low grade glioma, the molecular profile may allow the neuropathologist diagnose the tumor as "with molecular features of GBM, WHO grade IV" as determined by cIMPACT-NOW.[35] Although the histology may not warrant the diagnosis of a grade IV tumor, the molecular changes are the prognostically relevant finding and may make the patient eligible for clinical trials to treat GBM. These include IDH-targeted therapies, off-label use of BRAF-MEK inhibition for tumors with the BRAF V600E variant, and EGFR-targeted therapies including CellDex, Abbvie, CAR T trials, and use

of lapatinib. For example, a patient with an BRAF-mutated GBM recurred with leptomeningeal spread of disease. Given the molecular finding, the patient was treated off-label with trametinib, an MEK inhibitor, and dabrafenib, an BRAF inhibitor. The patient had a striking clinical and radiographic response, although of limited duration. The currently understood importance of a subset of the genes and their variants with respect to diagnostic, prognostic, and therapeutic relevance is depicted in Supplementary Table 4.

Clinical trials evaluating immunotherapy in GBM with a hypermutated genotype, particularly seen after temozolomide therapy, may indicate greater efficacy of checkpoint blockade therapy.[36-39] However, there is evidence that only a limited subset of patients respond, indicating that determination of the appropriate biomarkers in GBM is critical for clinical trial and treatment decisions.[40] Detection of a hypermutated genotype was initially performed using whole-exome data, but subsets of the genome, as low as 0.5 megabase, have been correlated with increased tumor mutational burden.[41] Although we do not measure tumor mutational burden or hypermutated genotypes in our clinical assay, the modest increase in the number of pathogenic variants observed and even more dramatically numerous VUS changes present most often in MGMT promoter-methylated recurrent GBMs on sequencing on Penn-153 are consistent with the known hypermutated genotype observed in many temozolomide-treated tumors. Some post-temozolomide therapy changes in mismatch repair genes, proposed as a mechanism of resistance to alkylating chemotherapy, are detectable with our panel.[39,40,42] However, it should be noted that there were numerous low variant allele frequency changes in some of these specimens. These low-level changes may represent sequencing artifact, treatment effect, low level clones, or passenger mutations. Detection of many low-level variants may be indicative of tumor complexity and demonstrates the real-world detection of variants in these complex tumors and clinical settings.

Testing GBMs for EGFRvIII has become common practice to determine eligibility for clinical trials involving therapies directed against the neoantigen, which is an ideal target as it is present only in tumors, most commonly in GBMs.[12,43] Assessment of multiple regions of a single tumor resulting in varied EGFRvIII levels is suggestive of tumor evolution and heterogeneity. This level of molecular detail may help guide patients toward appropriate targeted therapy, and it has the potential to explain the variety of phenotypic outcomes associated with EGFRvIII.[11,44-46]

EGFR alterations in a single tumor often do not occur in isolation. In our cohort, some tumors had multiple point mutations in *EGFR*, others showed EGFRvIII co-occurring with point mutations, and some had copy number gain of *EGFR* but also EGFRvIII and *EGFR* point mutations. Patients with an extensively altered EGFR pathway may benefit from therapies currently in development that target activated EGFR.[47] Identification of the specific domain altered may result in more effective therapy through tailoring of the EGFR inhibitor to that domain or functional change.[27] A recent case highlights the value of EGFR mutation identification. After first-line therapy, a patient's GBM recurred with multifocal disease. Given the identification through the pathway of an activating EGFR mutation in the tumor, the patient was treated off-label with a third generation tyrosine kinase inhibitor, osimertinib, to which the tumor initially responded. Although the tumor did eventually progress, the patient derived benefit from the mutation profile-based treatment decision.

The additional genes in Penn-153 allow a more complete molecular profiling of tumors related to common therapeutic targets, as well as provide molecular information necessary for diagnosis in some cases. For example, *ATRX*, *MDM2*, *MYCN*, *IDH2*, *H3F3A*, *SETD2*, *CIC*, *FUBP2*, and multiple genes encoding proteins involved in transcriptional control through histone modification are included on the newer panel. Interrogation of *IDH2* for mutations in gliomas of younger patients allows the definitive call of an astrocytoma as "*IDH*-wild type" or "*IDH*-mutant" and, in the case of an *IDH2* mutation, allows the diagnosis of "oligodendroglioma, *IDH*-mutant, and 1p/19q-codeleted." Although in the absence of complete *IDH* mutation analysis, a tumor may be designated "not otherwise specified," the *IDH* mutational status is a major determinant of patient prognosis and course of therapy. Therefore, addition of *IDH2* to the sequencing panel represents a significant improvement in patient care, with interrogation of *ATRX* and potentially *CIC* and *FUBP2* complementing the findings to give the neuropathologist greater confidence in the distinction between astrocytoma and oligodendroglioma, if 1p/19q codeletion studies are pending or unavailable.

Similarly, gliomas with midline location must be tested for the change in histone 3 at amino acid residue 27 from lysine to methionine (K27M), which is most commonly seen in histone variant 3.3, to make a diagnosis of "DMG, H3 K27M-mutant, WHO grade IV."[3] To this end, Penn-153 includes *H3F3A*, which encodes histone variant 3.3. Patients with diffuse gliomas harboring this variant have been shown to have a poor prognosis, regardless of the tumors' histological features, whose assessment is the classic method of grading gliomas. Therefore, the DMG is a WHO grade IV glioma based simply on the presence of the variant, making the inclusion of *H3F3A* on the sequencing panel critical for diagnosis and patient care. In addition, recent studies suggest the H3.3 K27 M variant may be targetable, such as with T cell-based therapy.[48]

In cases of tumors with unique biology, such as rarer *IDH* mutations, *BRAF* variants, *FGFR* variants, and the EGFRvIII variant, having the molecular results raises the possibility of response to targeted therapies and/or allows screening for eligibility to enroll in clinical trials for treatments targeting specific mutations. H3 K27M-mutant DMGs and GBM with extracellular domain variants in EGFR, such as A289 V, R108 K, and changes at residue 598, may be added to this list in the future, given ongoing studies.[48,49] In addition, the sequencing results occasionally highlight the need to consider screening for germline mutations, because our assay cannot determine germline versus somatic status of variants.

Conclusion

The current process of molecular testing of gliomas at our institution serves to inform the clinician at the point-of-care to the benefit of the patient. These tests are helpful in establishing predicted response to treatment, selecting therapy agents, and in both determining the prognosis and counseling regarding the prognosis. In our cohort, 135 (82%) of 164 brain tumors sequenced on Penn-47 and 31 (97%) of 32 tumors sequenced on Penn-153 demonstrate pathogenic variants. Although many of these targets are not currently actionable, incorporation into the diagnosis is increasingly relevant as the field of molecular neuropathology is adapting to the incorporation of these biomarkers. Our practice continues to move with the rapidly changing field, with expanding panels both for gene mutation detection and fusion detection of additional targets seen in brain tumors.

Authors' Note

MacLean P. Nasrallah, Zev A. Binder, Derek A. Oldridge, Donald M. O'Rourke, and Jennifer J. D. Morrissette have contributed equally to this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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