**Clinical
Cancer
Research**

Prospective Feasibility Trial for Genomics-Informed Treatment in Recurrent and Progressive Glioblastoma

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Abstract

Purpose: Glioblastoma is an aggressive and molecularly heterogeneous cancer with few effective treatment options. We hypothesized that next-generation sequencing can be used to guide treatment recommendations within a clinically acceptable time frame following surgery for patients with recurrent glioblastoma.

Experimental Design: We conducted a prospective genomics-informed feasibility trial in adults with recurrent and progressive glioblastoma. Following surgical resection, genome-wide tumor/normal exome sequencing and tumor RNA sequencing were performed to identify molecular targets for potential matched therapy. A multidisciplinary molecular tumor board issued treatment recommendations based on the genomic results, blood–brain barrier penetration of the indicated therapies, drug–drug interactions, and drug safety profiles. Feasibility of generating genomics-informed treatment recommendations within 35 days of surgery was assessed.

Results: Of the 20 patients enrolled in the study, 16 patients had sufficient tumor tissue for analysis. Exome sequencing was

completed for all patients, and RNA sequencing was completed for 14 patients. Treatment recommendations were provided within the study's feasibility time frame for 15 of 16 (94%) patients. Seven patients received treatment based on the tumor board recommendations. Two patients reached 12-month progression-free survival, both adhering to treatments based on the molecular profiling results. One patient remained on treatment and progression free 21 months after surgery, 3 times longer than the patient's previous time to progression. Analysis of matched nonenhancing tissue from 12 patients revealed overlapping as well as novel putatively actionable genomic alterations.

Conclusions: Use of genome-wide molecular profiling is feasible and can be informative for guiding real-time, central nervous system–penetrant, genomics-informed treatment recommendations for patients with recurrent glioblastoma. *Clin Cancer Res; 24(2); 295–305. ©2017 AACR.*

See related commentary by Wick and Kessler, p. 256

Introduction

Glioblastoma is a rapidly progressing disease with poor outcome, with a median overall survival (OS) of less than 15 months for patients with newly diagnosed glioblastoma (1). Though glioblastoma is a genetically diverse tumor type with multiple molecular subgroups, the current standard-of-care treatment of maximally safe surgical resection followed by temozolomide (TMZ) chemotherapy both during and after radiotherapy is

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Translational Relevance

Glioblastoma is a clinically challenging brain tumor associated with rapid recurrence, limited therapeutic options, and poor patient outcome. Application of molecularly guided treatment strategies in recurrent glioblastoma has been impeded by concerns regarding intratumor heterogeneity, minimal efficacy of single-agent strategies, and limited brain penetration of potential therapies. This study provides one of the first prospective demonstrations of using genome-wide molecular profiling to guide treatment recommendations for patients with recurrent glioblastoma within a clinically actionable time frame, and points to the role of considering central nervous system penetration and combination therapy approaches for these tumors. These findings provide a rationale and framework for larger prospective studies to further assess the efficacy of employing genomics-guided treatment for patients with recurrent glioblastoma.

broadly applied across patients with glioblastoma. Alternating electric fields used in combination with TMZ in the adjuvant setting were recently shown in an open label, phase III trial to improve median survival and OS in newly diagnosed disease (2); currently, no patient-specific predictive biomarkers are associated with use of this device.

Nearly all glioblastomas progress or recur. Although several treatment strategies have been explored, there is no consensus standard of care to improve outcomes for patients with recurrent glioblastoma, and participation in clinical trials is encouraged (3). Median progression-free survival (PFS) for patients with recurrent glioblastoma who enroll in clinical trials remains less than 4 months (4).

Retrospective studies suggest that the majority of primary glioblastoma tumors possess potentially clinically actionable genomic alterations (5, 6). A recent prospective study using panel-based, tumor-only sequencing for patients with newly diagnosed or recurrent high-grade glioma reported detection of therapeutically actionable alterations for nearly all patients (7). However, despite an encouraging high impact of profiling on treatment decisions, with 30% of patients receiving targeted treatment based on the profiling results, none of the patients responded to the predominantly single-agent, genomics-based treatment, with an average OS for patients treated with targeted therapy of less than 6 months (7).

Results from clinical trials with molecularly targeted agents in glioblastoma have likewise been disappointing (8). Lack of efficacy of these agents has been attributed to evaluation predominantly as single agents and in biomarker unselected patient populations. Most agents being tested lack validated predictive biomarkers, aside from O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation and TMZ response. Glioblastoma treatment carries additional concerns of drug distribution to the brain and insufficient central nervous system (CNS) penetration of the therapeutic agents, as well as spatial heterogeneity of the tumor that may limit efficacy of single-agent strategies (9–11). Clonal and subclonal evolution over time and as a consequence of treatment is an additional concern in the setting of progressive disease (11–13).

Although genomic profiling analysis has shown promise in patients with advanced cancers (14–17), the role for molecular profiling in patients with recurrent or progressive glioblastoma is unclear, and clinical benefit from these precision medicine approaches has yet to be demonstrated in this patient population. Here, we report our experience using genome-wide exome sequencing and RNA sequencing to guide treatment recommendations for adult patients with recurrent, progressive glioblastoma within a single-arm feasibility study.

Materials and Methods

Patients

Adult patients with recurrent glioblastoma were enrolled in a single-arm feasibility study conducted at the University of California, San Francisco (UCSF; NCT02060890). Patients who were candidates for surgery for their clinical management were eligible for the study. Enrollment was independent of the number of prior therapies, but patients must have received prior radiotherapy and have progressive disease based on imaging despite standard-of-care treatment. The study was approved by the UCSF Institutional Review Board and by the Western Institutional Review Board (TGen). All study participants provided written informed consent prior to study entry.

Sample processing and analysis

Fresh-frozen tumor tissue and whole blood (for constitutional DNA analysis) samples were collected. A board-certified neuropathologist (J.J. Phillips) reviewed histologic sections for tumor content estimations. Median tumor content was estimated at 70% (range, 20%–95%). Genome-wide exome sequencing and RNA sequencing were performed by Ashion (http://www.ashion.com), a Clinical Laboratory Improvement Amendments–certified laboratory. Additional samples were collected for correlative research studies, including tissue from the infiltrating tumor margin (nonenhancing tissue), tumor tissue for establishment of patient-derived tumor models, and longitudinal collection of plasma samples for circulating tumor DNA analysis.

Genome sequencing and analysis

Tumor/normal genome-wide exome sequencing (GEM GW) was performed to identify somatic coding point mutations, small insertions and deletions, copy-number changes, and structural events. Tumor RNA sequencing was performed for differential expression and gene fusion analysis. The GEM GW assay provides clinical whole-exome analysis for identification of mutations within exons and regional whole-genome analysis for detection of copy-number variants and translocation breakpoints. The mean target coverage for exome sequencing was 377X (range, 248X–438X) for tumor samples and 178X (range, 114X–261X) for peripheral blood samples. On average, more than 90% of target bases had at least 100X coverage (average across samples, 92.4%; range, 81.7%–95.0%) in the tumor samples. RNA sequencing averaged >242 million aligned reads (range, 173 million–365 million). Sequence alignment and variant calling were performed as previously described (18–20). Data were aligned to build 37 of the human reference genome. Somatic single-nucleotide variants (SNV) and small indels were identified with Seurat (21), with a minimum tumor allele ratio of 0.05 and a minimum quality score of 20. Copy-number variants were detected using a read depth–based comparative method (https://github.com/tgen/tCoNuT), and structural variants were

detected as previously described (22). Focal copy-number events with a length less than 25 Mb and an absolute $\log_2$ fold change greater than 1 were reported. Fusions were called using TopHat (v2.0.8b), with a quality score cutoff of 100 (23). Differential expression was determined using Cuffdiff (version 2.2.1) comparison against a brain homogenate control, with a P value cutoff of 0.005 (24). EGFRvIII was detected by *de novo*–guided assembly of the reads that map to EGFR. In this approach, reads are assembled into contigs using a De Brujin graph that connects across the exons for EGFRvIII. Hypermutation was defined as tumors with more than 500 nonsynonymous coding mutations per exome, similar to previous reports in glioblastoma (25). This study has been deposited in the database of Genotypes and Phenotypes (dbGaP) under accession number phs001460.v1.p1 (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001460.v1.p1).

Study pharmacopeia

The study pharmacopeia consisted of more than 180 FDA-approved agents, including all FDA-approved oncology agents and selected FDA-approved nononcology (repositioned) drugs. There is growing interest in neuro-oncology toward repositioned agents, as these drugs are well tolerated, and several are known to penetrate the brain and have preclinical evidence suggesting potential activity in cancer (26). Matching of specific alterations to potential therapeutics in the study pharmacopeia was performed using a custom set of expert-annotated drug rules (19, 20). When available, glioma-specific data were included in the supporting evidence for drug–gene associations, though data from other tumor types were also leveraged in the drug rule base. Report generation was performed as previously described (20). The molecular profiling results were presented to the molecular tumor board in the form of an interpretive genomic report listing the somatic events identified with a focus on potential targets amenable to treatment. In addition to variant-specific content, this report included drug-specific content, from an in-house custom blood–brain barrier database, that described pharmacokinetic features of the indicated therapies, including experimental evidence (based on expert curation from published literature) or predictive model (27) information on whether the drug may cross the blood–brain barrier and/or have CNS activity.

Molecular tumor board

Interpretive genomic reports were reviewed by a multidisciplinary, expert molecular tumor board. At least two clinical neuro-oncologists, one neuropathologist or neurogenomics specialist, one neuropharmacologist, the tumor board chair, and two genomics experts were required to reach a quorum. The median number of tumor board participants was 16 (range, 11–20). Following presentation of the clinical history and genomics report, the results were reviewed and discussed by a neuro-oncologist from an outside institution, neuropharmacologist, and the treating physician, followed by open discussion among all tumor board members to reach a consensus treatment recommendation. Combination of up to four FDA-approved drugs was allowed. The tumor board considered evidence supporting the drug–gene association, blood–brain barrier penetration for recommended therapies, drug–drug interactions, and drug safety profiles of the potential therapeutic options. The treating oncologist reviewed the recommendation with the patient. Treatment based on the tumor board recommendation was optional.

Patients treated based on the tumor board recommendation were followed for toxicity and efficacy, including progression and survival. Patients who decided not to use the tumor board recommendation were followed for progression and survival.

Immunohistochemistry

A subset of altered genes and downstream pathways were selected for validation at the protein level. Immunohistochemistry (IHC) was performed at UCSF using a Ventana BenchMark autostainer. Sections were immunostained with commercially available antibodies, including anti-ATRX (Sigma-Aldrich; HPA001906), anti-IDH1 R132H (Dianova; H09), anti-EGFR (Dako; M3563, H11), anti-TP53 (Dako; M7001), anti-RB1 (RB1; BD Biosciences; 554136), anti–phospho-RPS6 (Ser240/244; Cell Signaling Technology; 2215), anti–phospho-AKT1S1 (PRAS40; Thr246; Cell Signaling Technology; 2997, C77D7), and anti–phospho-p44/42 MAPK1/MAPK3 (ERK1/2; Thr202/Tyr204; Cell Signaling Technology; 4370, D13.14.4E). All slides, including positive and negative controls, were reviewed and scored by a neuropathologist (J.J. Phillips).

Nonenhancing adjacent tissue analysis

Nonenhancing tissue biopsies were collected at the time of tumor resection of the contrast-enhancing tumor region. Locations of the acquired enhancing and nonenhancing tissue samples were estimated by the surgical team and recorded as screenshots and image coordinate values of the associated MRI images using Brainlab. Estimated distance between enhancing and nonenhancing samples was calculated using the three-dimensional Cartesian coordinate annotations. The median estimated distance between nonenhancing and enhancing tissue samples was 18 mm (estimated range, 8–34 mm). The median tumor estimate from nonenhancing regions was 10%, (range, <5%–60%.) Nonenhancing tissue samples were flash frozen and shipped to Ashion for DNA extraction, and exome sequencing was performed in the research setting at the Collaborative Sequencing Center at TGen. The mean target coverage for exome sequencing of the nonenhancing tissue samples averaged 268X (range, 179X–482X).

Statistical methods

The primary endpoint of the study was time from tumor resection to reporting of genomics-guided treatment recommendations to the treating physician. Feasibility was assessed based on the number of treatment recommendations that were completed within 35 calendar days of tissue collection. Demonstration of feasibility required that 85% of evaluable patients (with sufficient DNA and RNA for analysis) receive treatment recommendations within this specified time frame. A sample size of 15 evaluable patients was selected prior to initiating the study. The study would terminate if the specialized tumor board could not issue a treatment recommendation in a total of 5 patients with sufficient DNA and RNA for molecular analysis. A safety-stopping rule was also included such that if 3 or more patients experience dose-limiting toxicity as a result of following the recommended treatment regimen, the study would be closed for enrollment. The secondary objective was to assess whether tumor tissue taken from the nonenhancing tumor edge presented distinct therapeutic targets compared with tissue from the enhancing region of the tumor from the same patient. Estimating efficacy of genomics-guided treatment was included as an exploratory objective. April 1, 2017, was used as the cutoff date for analysis; all patients who were

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Table 1. Clinical data summary for patients profiled on the trial ($N = 16$)

Characteristic	Number of patients (%)
Gender	
Male	12 (75%)
Female	4 (25%)
Age at diagnosis (years)	
Median (range)	51 (29–66)
Year of diagnosis	
Median (range)	2013 (2006–2014)
Tumor recurrence	
First	8 (50%)
Second	5 (31.25%)
Third	3 (18.75%)
Tumor location	
Temporal lobe	5 (31.25%)
Frontal lobe	5 (31.25%)
Parietal lobe	2 (12.5%)
More than one lobe	3 (18.75%)
Other	1 (6.25%)
Extent of resection	
Gross total	11 (68.75%)
Subtotal	5 (31.25%)
Molecular markers	
MGMT methylated	9 (60%)[a]
TERT promoter mutation	12 (80%)[a]
IDH1 R132H	3 (18.75%)
Previous treatment	
Chemoradiation (concurrent TMZ + RT)	14 (87.5%)
Adjuvant TMZ	15 (93.75%)
Bevacizumab, at any time	7 (43.75%)
Non-TMZ chemotherapy	4 (25%)
Previous investigational agent trial	4 (25%)

Abbreviation: RT, radiotherapy.
[a]15 evaluable patients.

progression free (PFS) or alive (OS) on this date were censored on their date of last follow-up.

Results

Patient overview and feasibility assessment

This prospective trial aimed to assess the feasibility of using genome-wide exome and RNA sequencing to generate real-time tumor board treatment recommendations for patients with recurrent glioblastoma [World Health Organization (WHO) grade IV]. Supplementary Fig. S1 outlines the study workflow. Twenty adult patients with recurrent glioblastoma were enrolled in this study between September 2014 and August 2015. Sixteen patients were eligible for genomic profiling, and 4 patients were ineligible due to low tumor cellularity (<10% estimated tumor content). Table 1 provides a description of patient demographics. All patients had been treated with radiotherapy at the time of initial diagnosis, and the majority also received concurrent and adjuvant TMZ chemotherapy. Seven patients had previously been treated with bevacizumab and were characterized as bevacizumab failures, and 4 patients had previously been enrolled on a clinical trial and progressed on treatment with an investigational agent.

Feasibility was assessed based on (i) completion of both genome-wide exome sequencing and RNA sequencing and (ii) delivery of a tumor board treatment recommendation within 35 calendar days following surgery. Exome sequencing was completed for 16 of 16 eligible patients; RNA sequencing was completed for 14 of 16 patients. Tumor board treatment recommendations were provided within 35 days of surgery for 15 of 16 (94%) patients. The median time from surgery to molecular results and tumor board

treatment recommendations was 27 calendar days (range, 23–34). Thirteen of 16 (81%) patients met the predefined feasibility requirements of the trial. In one case, the molecular profiling results were not available within the required timeline due to initial sequencing of a region of the tissue sample representing extensive gliosis. A second tissue sample with confirmed tumor was sequenced and a genomics report generated, but delivery of these results exceeded the feasibility time frame. Upfront neuropathology review was added to the study workflow after this sample. The other 2 patients were classified as feasibility failures because the RNA failed to meet quality control metrics for sequencing. For both patients, a genomics report was generated and tumor board treatment recommendations were made based on DNA-level alterations alone. Of patients with sufficient DNA and RNA for analysis, 13 of 14 (93%) received treatment recommendations within 35 calendar days, demonstrating the feasibility of performing comprehensive sequencing analysis to guide treatment selection for patients with recurrent, progressive glioblastoma.

Genomic alterations and therapeutic recommendations

Therapeutically informative alterations were identified for all 16 patients (Fig. 1). The most common genes altered included *EGFR* ($n = 10/16$, 63%), *PTEN* ($n = 9/16$, 56%), *CDKN2A* (7/16, 44%), *NF1* (7/16, 44%), *RB1* (5/16, 31%), and *TP53* (5/16, 31%). These somatic alterations include missense, nonsense, frameshift, and splice-site mutations; focal copy-number gains and losses; structural variants; and gene fusions. RNA sequencing revealed expression of the mutated allele for 80% of the therapeutically informative somatic SNVs detected in the 14 patients with tumor exome and RNA sequencing.

The tumor board treatment recommendations are listed in Table 2. The recommended therapies included options for targeted cancer therapies, chemotherapies, immunotherapies, and repositioned agents. Treatment recommendations consisted of an average of 3.4 therapies per patient (range, 1–4 therapies per patient), reflecting the tumor board's view that blocking multiple pathways with combination therapy may be more effective than single-agent therapy in treating recurrent, progressive glioblastoma.

Treatment based on the tumor board recommendation was optional. Seven of the 15 (47%) patients decided to pursue treatment based on the tumor board's genomics-informed treatment recommendations (Table 2). Of the 8 patients that elected to not pursue these treatment recommendations, 3 patients participated in other ongoing clinical trials (2 of which were supported by an alteration detected by molecular profiling results), and 3 patients pursued treatment with lomustine (CCNU) and bevacizumab. The decision to pursue these other treatments was based on physician and patient preference and, in some cases, concern around timely access to the recommended therapies. Two patients experienced clinical decline and elected not to pursue any further treatment.

Of the 7 patients that were treated based on the tumor board treatment recommendation, 2 remained on treatment >365 days after surgery without evidence of disease progression, 1 of whom was still on study and progression free >665 days after surgery (Fig. 2). These 2 patients are discussed in detail below.

GBM-011. GBM-011 is a 58-year-old woman with left frontal lobe glioblastoma that progressed on standard-of-care treatment (focal radiotherapy with TMZ chemotherapy, followed by TMZ). She
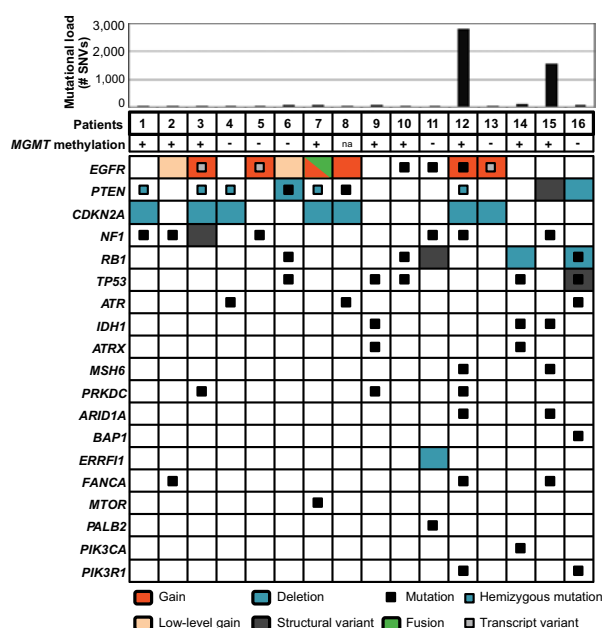
Figure 1.

Summary of genomic alterations. Potentially therapeutically informative genomic alterations detected in patients with recurrent glioblastoma enrolled in the prospective genomics-enabled medicine feasibility trial. Patients are represented in columns, with the number of nonsynonymous coding SNVs for each sample shown in the top plot, followed by a summary of genomic alterations, with genes presented in rows. Transcript variants include EGFRvIII (GBM-003 and GBM-013) and an EGFR c-terminal deletion variant (GBM-005). *MGMT* status is based on the recurrent tumor profiled in this study, when available, or from the primary tumor or previous recurrent tumor tissue. * *MGMT* methylation status was not available for GBM-008.

enrolled in this trial and underwent subtotal resection of the progressive disease in 2015, with a portion of the enhancing tumor region provided for molecular profiling. The pathology report was consistent with recurrent glioblastoma and noted the tumor was negative for *EGFR* amplification and *PTEN* deletion by FISH, and was *MGMT* promoter methylation negative. Exome

sequencing was performed, but RNA did not pass preset quality-control metrics. From the exome-sequencing data, several alterations of potential therapeutic relevance were identified: *EGFR* missense mutation (V292L), *NF1* frameshift (T956fs), *PALB2* frameshift (S700fs), *ERRFI1* deletion, and *RB1* breakpoint. The profiling results were presented to the molecular tumor board 29 days after surgery.

The tumor board discussion centered on the alterations in *NF1* and *PALB2*. The *EGFR* mutation was discussed but was not prioritized in the treatment recommendation due to a lower tumor DNA allele fraction for this mutation. Preclinical studies in glioblastoma cell lines suggest that *NF1* alterations may be associated with sensitivity to MEK inhibition, particularly in cell lines without PI3K pathway activation (28). A recent case report described clinical and radiologic benefit for a patient with neurofibromatosis-associated glioblastoma treated with the MEK inhibitor trametinib (29), supporting potential activity for MEK inhibition in glioblastoma. Though mutations in *PALB2*, a binding partner for BRCA2, are rarely seen in glioblastoma, loss of *PALB2* has been associated with sensitivity to PARP inhibitors and platinum agents in a variety of other tumor types (30, 31). Although germline *PALB2* mutations have largely been the focus (32, 33), somatic *PALB2* mutations have also been identified and associated with sensitivity and clinical response to PARP inhibitors and platinum agents (34, 35). The *PALB2* mutation reported in this recurrent glioblastoma tumor is a somatic alteration. Recent studies suggest the FDA-approved PARP inhibitor olaparib may reach therapeutic concentrations in the brain (36).

Based on the *NF1* and *PALB2* frameshift mutations, the molecular tumor board recommended treatment with trametinib, olaparib, and carboplatin. Concerns around potential toxicity of this combination were discussed by the treating oncologist, neuropharmacologist, and other neuro-oncologists on the tumor board. The consensus was to use low-dose olaparib (200 mg twice a day) and carboplatin (AUC 4 once every 4 weeks), along with trametinib (2 mg daily), monitoring for hematologic and liver toxicity and increasing the doses if tolerated. The patient and treating oncologist agreed to pursue the tumor board treatment recommendation. This patient continued on treatment without disease progression >665 days after surgery. This corresponds to a longer time to progression (TTP) than the patient experienced on

Table 2. Treatment summary

Patient number	Tumor board treatment recommendation	Treatment received
1	Chlorpromazine, metformin, trametinib	Chlorpromazine, metformin, trametinib
2	Minocycline, temozolomide, trametinib	Minocycline, temozolomide
3	Chlorpromazine, erlotinib, propranolol	Bevacizumab, CCNU
4	Disulfiram, metformin, mebendazole, palbociclib	Metformin, palbociclib
5	No tumor board recommendation (feasibility failure >35 days)	Nivolumab
6	Everolimus, metformin, minocycline, propranolol	Clinical trial
7	Erlotinib, everolimus, minocycline, palbociclib (propranolol if palbociclib not available)	Erlotinib, everolimus, minocycline, propranolol
8	Erlotinib, everolimus, palbociclib, propranolol	Clinical trial
9	Carboplatin, CCNU, metformin	CCNU, metformin
10	Erlotinib, metformin, propranolol	Bevacizumab, CCNU
11	Carboplatin, olaparib, trametinib	Carboplatin, olaparib, trametinib
12	Palbociclib, pembrolizumab, propranolol, vismodegib	Bevacizumab, CCNU, radiotherapy
13	Erlotinib, minocycline, palbociclib, propranolol	Clinical trial
14	Carboplatin, everolimus, metformin, propranolol	No further treatment
15	Nivolumab or pembrolizumab	Nivolumab
16	Everolimus, metformin, propranolol, vorinostat	No further treatment

NOTE: Therapeutic options pursued following molecular profiling and tumor board recommendations.
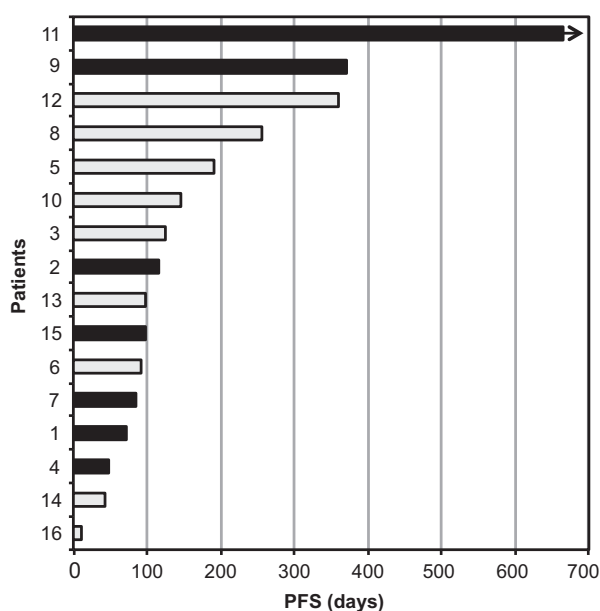
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Figure 2.
PFS data for patients with progressive glioblastoma profiled on this study. PFS is displayed as days from surgery for progressive disease and molecular profiling until radiographic or clinical evidence of disease progression. Black bars indicate patients treated based on genomics-guided tumor board recommendations. Gray bars indicate patients that did not pursue treatment with a tumor board–recommended therapy.

prior therapy, with a TTP ratio of 3.7 for the genomics-guided treatment, surpassing the general TTP ratio cutoff of >1.3 used to indicate clinical benefit (37). Although the prolonged TTP seen in this patient provides an initial signal of potential efficacy for MEK inhibitors in *NF1*-mutated glioblastomas and/or PARP inhibitors/platinum agents in *PALB2*-mutant glioblastomas, additional preclinical and clinical studies will be needed to determine the role of genomic context and combination therapy in the response observed for this patient.

GBM-009. GBM-009 is a 35-year-old man originally diagnosed in 2009 with right frontal-parietal glioblastoma. Following gross total resection, the patient participated in a phase II trial of TMZ, bevacizumab, and erlotinib during and following radiotherapy. He completed treatment and was followed without evidence of tumor progression for 6 years. Disease progression was noted, and the patient enrolled in this trial and underwent surgery in 2015. Clinical pathology evaluation demonstrated recurrent glioblastoma, methylation of the *MGMT* promoter, *IDH1* mutation (p. R132H), and IHC evidence for lack of ATRX expression and strong nuclear staining for TP53 in the majority of tumor nuclei (suggestive of mutations in *ATRX* and *TP53*). The clinical history and profile were consistent with secondary glioblastoma. Although *IDH1*-mutant secondary glioblastomas have been associated with longer OS compared with *IDH1*–wild-type glioblastoma (38, 39), a recent retrospective analysis evaluating the impact of *IDH1* mutation status on clinical outcomes in recurrent glioblastoma clinical trials reported similar median PFS for patients with *IDH1*-mutant recurrent glioblastoma compared with patients with *IDH1*–wild-type recurrent glioblastoma (4).

Genome-wide exome and RNA sequencing were performed, and an interpretive molecular report was presented to the tumor board 23 days after surgery. The genomic report outlined alterations of potential relevance that included *ATRX* frameshift mutation (G1368fs), *IDH1* mutation (R132H), *PRKDC* frameshift mutation (I166fs), and *TP53* mutation (R273C). The tumor board discussion focused on the *IDH1* and *ATRX* mutations. These mutations were detected in both the DNA and RNA at mutant allele ratios greater than 30%. The *TP53* mutation also occurred at high DNA and RNA allele ratios (81% and 89%, respectively) but had limited therapeutic options within the study pharmacopeia for targeting alterations in this gene. Point mutations in IDH1/2 have been shown to alter cell metabolism and induce epigenetic changes (reviewed in ref. 40). Although investigational agents targeting mutant IDH are currently in clinical trials, preclinical evidence suggests that IDH mutation may confer increased sensitivity to various FDA-approved agents, including nitrosoureas (carmustine and lomustine), DNA methyltransferase inhibitors/DNA-demethylating agents (5-azacytidine and decitabine), and metabolic agents (metformin; refs. 41–44). Disruption of *ATRX* can result in genetic instability and has been associated with increased sensitivity to DNA-damaging agents (i.e., platinum agents and topoisomerase inhibitors) in preclinical studies involving multiple cell types, including glioma (45, 46). The tumor board discussed the options and recommended treatment with metformin, CCNU, and carboplatin. Concerns for combined myelosuppression from CCNU and carboplatin were discussed, with a consensus recommendation to start with low doses of both agents (CCNU: 75 mg/m^2 once every 6 weeks; carboplatin: AUC 5 once every 4 weeks) and monitor for hematologic toxicity. The patient and treating oncologist decided to pursue treatment with CCNU and metformin. This patient remained on treatment and progression free for just over 1 year, at which time progression was noted.

Hypermutated genotype. Hypermutation has been reported in approximately 17% of recurrent glioblastomas, post-TMZ exposure, and associated with TMZ-induced mutations in mismatch repair genes such as *MSH6, MSH2, MSH4, MSH5, PMS1, PMS2, MLH1,* and *MLH2* (13, 25, 47–49). Two patients, GBM-012 and GBM-015, showed a hypermutated tumor genotype, with >1,500 nonsynonymous coding variants detected in each sample—more than 20 times the median number of mutations seen in non-hypermutated tumors (median = 64 SNVs; range, 40–135; Fig. 1). This mutational load is similar to previous reports of TMZ-induced hypermutation in glioblastoma (25).

Both of the hypermutated tumors in this feasibility trial had previous TMZ exposure, somatic *MSH6* mutations detected in the recurrent tumor, and a mutational signature consistent with TMZ-associated hypermutation (Fig. 1, data not shown). GBM-012 was diagnosed with glioblastoma in 2006, received radiotherapy with concurrent and adjuvant TMZ, and then received additional TMZ treatment following tumor progression in 2014. Progression was again noted in 2015, at which time, the patient enrolled on this trial. Clinical pathology reported the 2014 progressive disease as IDH wild type with *EGFR* gain and *PTEN* loss by FISH. GBM-015 was diagnosed with IDH1 R132H-mutant, WHO grade III anaplastic oligoastrocytoma in 2013 and treated with TMZ alone. Progression was noted in 2014, at which time, the patient received radiotherapy followed by CCNU. The patient progressed on CCNU, underwent surgical resection and bevacizumab treatment, and enrolled on this study after disease progression in 2015.

Though the *MGMT* promoter methylation status of the primary tumors was not available for either patient, both patients had previous or current progression samples documented as *MGMT* promoter methylation positive, consistent with the reported association between *MGMT* promoter methylation and hyper-mutation in patients treated with TMZ (25).

There are several emerging reports in other tumor types that a high number of overall mutations or mutations in specific DNA repair genes may be associated with increased sensitivity to immune checkpoint inhibitors (50–53). In both of these hyper-mutated recurrent glioblastoma tumors, the tumor board recommended treatment with an immune checkpoint inhibitor. One patient was treated with nivolumab but showed disease progression and discontinued treatment after 2 months. Several ongoing clinical trials are evaluating immune checkpoint inhibitors in glioblastoma, including trials in recurrent glioblastoma. Recent results from CheckMate-143, a phase III study evaluating nivolumab compared with bevacizumab in patients with recurrent glioblastoma, failed to show improved OS with the immune checkpoint inhibitor (54) despite promising phase II data. Molecular biomarkers may prove beneficial for application of immune checkpoint inhibitors to this disease. Indeed, initial case reports of clinical responses in recurrent pediatric glioblastoma patients with germline biallelic mismatch repair deficiency and in adult glioblastoma patients (including a patient with a *POLE* germline alteration) are now emerging (55–57). The efficacy of immune checkpoint inhibitors in adult glioblastoma tumors with TMZ-associated hypermutation remains to be determined in ongoing clinical trials.

IHC validation of selected targets

We evaluated concordance between the genomic alterations identified and protein-level events by performing IHC for five of the most frequently altered, potentially clinically informative genes observed in this cohort: EGFR, IDH1, ATRX, TP53, and RB1. Representative IHC images are shown in Fig. 3A–F. Five of the eight samples with focal *EGFR* copy-number gain showed positive EGFR staining, with one additional sample with low level (and potentially subclonal) *EGFR* gain showing robust expression in a subset of cells. Two samples with *EGFR* mutation in the absence of *EGFR* amplification did not show EGFR overexpression at the protein level. The three *IDH1* R132H mutations and five *TP53* genomic alterations were all validated by IHC. Likewise, genomic events predicted to result in loss of *ATRX* (2 patients) or *RB1* (5 patients) showed loss of the proteins by IHC. Together, a majority (>85%) of the staining patterns were concordant with the genomic results.

Downstream pathway activation was also evaluated by IHC, using phosphorylated MAPK1/3 (pERK1/2) as a readout for MAPK pathway activation and phosphorylated AKT1S1 (pPRAS40) and phosphorylated S6-ribosomal protein (pRPS6) as readouts for activation of the PI3K/AKT/mTOR pathway. Representative IHC images are shown in Fig. 3G–L. Seven of the nine samples with genomic alterations in *PTEN* showed activation of the PI3K/AKT/mTOR pathway. The other two *PTEN* altered samples (GBM-012 and GBM-015) were hypermutated and showed weak activation of this pathway by IHC. GBM-014 had a canonical *PIK3CA* E545K mutation but showed weak PI3K/AKT/mTOR pathway staining by IHC. Four of the five samples with *NF1* alterations stained positive for pMAPK1/3 (pERK1/2).

GBM-005 was the exception, with lack of pMAPK1/3 (pERK1/2) staining despite the detection of a frameshift mutation in *NF1* by exome sequencing. In this feasibility study, treatment recommendations were based on results from exome and RNA sequencing. These IHC results demonstrate that protein measures can provide complementary insight into the functional consequences of genomic alterations, both related to the target protein and to downstream signaling pathway activation, and may help facilitate prioritization of targets for therapeutic intervention.

Genomics of the nonenhancing region

Although the genomic profiling and target selection for each patient in the clinical trial were performed from tissues obtained from the enhancing tumor core region, glioblastoma intratumoral heterogeneity creates significant challenges. It is well appreciated that different regions in the same tumor comprise multiple genetically distinct subpopulations that can express different therapeutic targets. This may lead to differences in therapeutic options and recommendations, because the genetic profiles from the region removed during surgery may not accurately reflect another subregion that remains following surgery, contributing to poor or incomplete treatment response. To address whether tumor taken from the "edge" of the enhancing disease presents distinct therapeutic targets compared with the tumor "core" from the same patients, we performed exome sequencing on the matched nonenhancing tissue samples that represent the tumor typically left behind after surgery. Nonenhancing tissue samples were collected at the time of surgery for 12 of the 16 patients enrolled in this trial.

As shown in Fig. 4, the majority of the informative alterations identified in the enhancing region of the tumor were also identified in the matched nonenhancing tissue samples. This was particularly true for genes recurrently altered in glioblastoma and considered drug targets or pathway modifiers, such as *EGFR*, *PTEN*, *CDKN2A*, and *NF1*. In 6 of 9 patients, focal copy-number changes of therapeutic interest were concordant between the enhancing and nonenhancing tissue samples. Most patients had at least one genomic alteration detected in the enhancing tumor that was not detected in the nonenhancing tissue sample. Tumor heterogeneity may account for some of these differences, such as in GBM-016 where copy-number events, such as *PTEN* deletion, were detected only in the enhancing tissue sample despite adequate tumor content (30%–40%) in the nonenhancing tissue samples. Lower tumor content of the nonenhancing tissue samples can also influence variant detection. For example, the *IDH1* mutation reported in the enhancing tumor sample for GBM-009 was not called in the nonenhancing tissue sample. However, the nonenhancing tissue sample, which had a tumor tissue estimate of <5%, showed *IDH1* mutation upon visual inspection of the data in Integrative Genomics Viewer (58). This discrepancy is likely not due to tumor heterogeneity but rather reflects the differences in tumor content and read depth between these matched samples. Nonenhancing tissue samples with low tumor content (e.g., GBM-008 and GBM-009) showed the greatest discordance between variants detected in enhancing and matched nonenhancing tissue.

For 2 patients, the same gene was altered in both the enhancing and nonenhancing tissues, and the same therapeutic indication reported, but different alterations in the gene were identified in the two tissue regions. For GBM-001, *NF1* alterations were
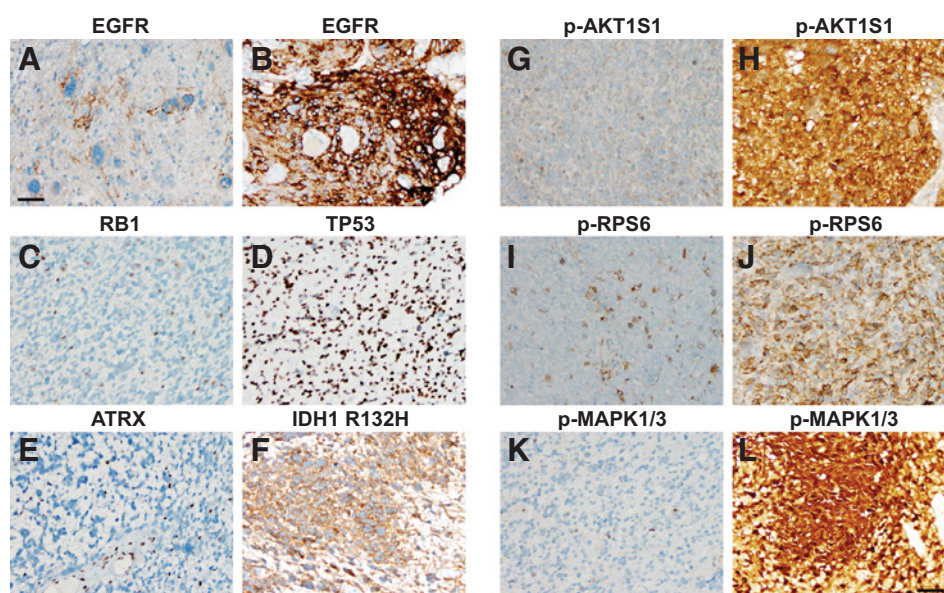
Figure 3.
Validation of selected mutations and copy-number alterations as well as signaling pathway activity by immunohistochemical staining. **A,** GBM-006 has scattered cells with robust EGFR protein expression, consistent with low-level focal copy-number gain. **B,** In contrast, GBM-008 has diffuse, robust expression of EGFR, consistent with multiple alterations in *EGFR*, including high-level focal copy-number gain and *EGFR* mRNA overexpression. **C–F,** In GBM-014, there is (**C**) loss of RB1 immunostaining in the majority of tumor cells, consistent with copy-number loss of *RB1*; (**D**) robust TP53 immunostaining, suggestive of *TP53* mutation; (**E**) loss of immunostaining for ATRX, consistent with a loss-of-function mutation in *ATRX* (K425fs); and (**F**) positive immunostaining for the R132H-mutated IDH1. For both RB1 and ATRX, immunostaining is retained in non-neoplastic cells, including microglia/macrophages and endothelial cells. **G–J,** Activation of the PI3K/AKT/mTOR signaling pathway, as demonstrated by robust positive staining for phosphorylated-AKT1S1/PRAS40 (Thr246) and phosphorylated-ribosomal S6 protein (RPS6; Ser240/244) in GBM-007 (**H, J**), as compared with only weak activation in GBM-015 (<25% of tumor cells are immunopositive; **G, I**). **K** and **L,** Activation of the MAPK pathway, as demonstrated by robust positive staining for phosphorylated-p44/42 MAPK1/3 (ERK1/2) protein (Thr202/Tyr204) in GBM-001 (**L**), as compared with minimal staining in GBM-014 (**K**). Representative images: magnification, x200; bar, 20 μm.

detected in both the enhancing and nonenhancing tumor samples. However, the enhancing region showed an *NF1* frameshift mutation (F1247fs), whereas the nonenhancing region showed two nonsense *NF1* mutations (R1534X and R2517X). For GBM-007, both enhancing and nonenhancing tissue samples showed *EGFR* copy-number gain, though an *EGFR–SEPT14* fusion was detected in the enhancing tumor sample and an *EGFR* mutation (A289V) was identified only in the nonenhancing tissue samples. Although the same therapeutic recommendations were reported for the alterations in both of these patients, intratumoral genomic heterogeneity, even affecting the same driver gene, has the potential to influence pathway activation and therapeutic sensitivity.

Two patients showed new alterations of potential therapeutic interest in the nonenhancing tumor samples that were not observed in the enhancing region. These alterations were typically at a low DNA allele fraction (≤10%) and included an *FANCC* mutation (E101Q) in GBM-001 and a *RET* mutation (T492I) in GBM-016. The functional consequences and therapeutic implications for these mutations are not clear, as neither mutation has been previously identified in cancer or functionally characterized. The hypermutated tumor, GBM-012, showed several common alterations across enhancing and nonenhancing samples, including *MSH6* mutations, *EGFR* gain and mutation, and *PTEN* mutation. Distinct mutations were also detected in the nonenhancing samples from GBM-012, including mutations in *ATR, ATRX, BAP1*, and *MTOR*.

Compared with the actionable therapeutic targets initially identified in the enhancing tumor sample, profiling the matched nonenhancing tissue samples did not alter the treatment recommendation for these 12 patients (Table 2).
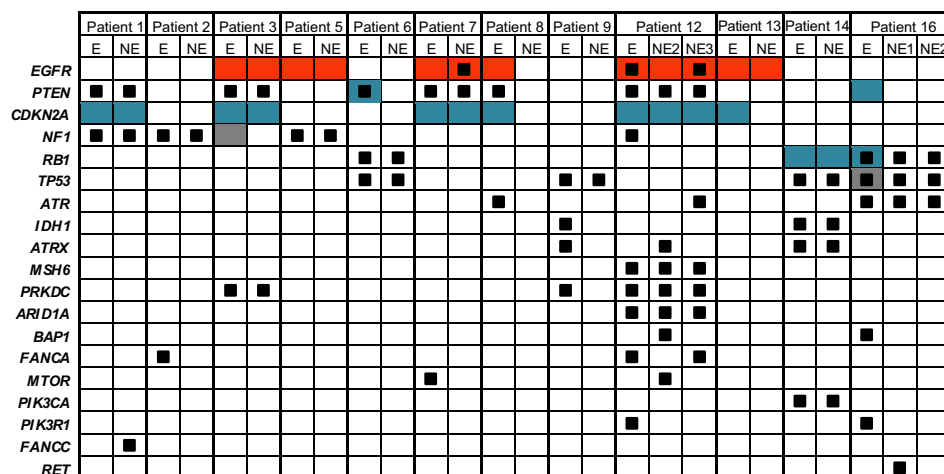
Discussion

This study demonstrates the feasibility of using genome-wide molecular tests to guide treatment in recurrent glioblastoma, with the majority (15/16, 94%) of patients receiving genomics-informed treatment recommendations by a molecular tumor board within the study's preset feasibility time frame of 35 calendar days. Despite the late stage in disease course, with nearly half of the profiled patients failing bevacizumab treatment prior to enrollment, 7 patients were treated based on the tumor board recommendations. Notably, 2 patients experienced PFS greater than a year, with 1 of these patients progression free at 21 months—more than 3 times longer than the TTP on their previous therapy. To our knowledge, this is the first report of a prospective profiling study in recurrent glioblastoma to show a patient with extended TTP following treatment with genomics-informed therapy (7).

This integrated multidimensional data approach allowed RNA-sequencing data to add additional insight into the exome-sequencing data, such as confirming coding mutations detected in the DNA were expressed in the RNA, detection of transcript

Figure 4.

Therapeutically informative alterations in nonenhancing (NE) tumor rim samples compared with enhancing (E) tumor core samples. Comparison of the genomic alterations of potential therapeutic interest that were detected in E and/or NE regions of the tissue collected at surgery. Two patients had two distinct NE tissue samples collected and profiled (GBM-012 and GBM-016). Somatic, nonsynonymous coding mutations are indicated by black boxes; focal copy-number gains are indicated in orange; focal copy-number deletions are indicated in blue; and structural variants are indicated in gray.

Gene	P1 E	P1 NE	P2 E	P2 NE	P3 E	P3 NE	P5 E	P5 NE	P6 E	P6 NE	P7 E	P7 NE	P8 E	P8 NE	P9 E	P9 NE	P12 E	P12 NE2	P12 NE3	P13 E	P13 NE	P14 E	P14 NE	P16 E	P16 NE1	P16 NE2
EGFR					orange	orange	orange	orange			orange	orange■					orange	orange	orange■							
PTEN	■	■			■	■			blue■		■	■	■				■	■	■						blue	
CDKN2A	blue	blue			blue	blue					blue	blue	blue				blue	blue	blue							
NF1	■	■	■	■	gray		■	■									■									
RB1											■	■										blue		■	■	■
TP53											■	■			■	■						■	■	gray	■	■
ATR												■							■			■	■	■	■	■
IDH1															■							■	■			
ATRX															■			■				■	■			
MSH6																	■	■	■							
PRKDC					■	■									■		■	■	■							
ARID1A																	■	■	■							
BAP1																		■					■			
FANCA				■													■		■							
MTOR										■								■								
PIK3CA																						■	■			
PIK3R1																	■						■			
FANCC		■																								
RET																									■	

variants (EGFRvIII and EGFR c-terminal deletion variants), RNA evidence for gene fusions (*EGFR-SEPT14*), and co-incident gene expression and copy-number changes. Selected IHC validation showed strong overall concordance between DNA and protein or pathway level changes. However, there were also examples where the DNA alteration did not lead to the expected change at the protein level. In addition to helping guide prioritization of genomics-informed treatment recommendations, protein measures and knowledge of pathway alterations may reveal additional tumor vulnerabilities and therapeutic options to consider in this patient population.

A small number of patients were needed to evaluate feasibility and to optimize the workflow necessary for a larger efficacy trial. The sample size, extent of intra- and interpatient heterogeneity, and various treatment recommendations limit conclusions about the benefit of this strategy. Larger numbers of patients will be needed to either validate or reject this approach. Validation of tissue and blood biomarkers will also require larger patient groups and, eventually, will necessitate the use of a control group. An adaptive approach, within a multicenter clinical trial network, will likely be needed in terms of clinical design given the lack of any validated predictive biomarker in recurrent glioblastoma. This trial was ambitious from a number of standpoints, including use of multiple drug recommendations, sampling of enhancing and nonenhancing tumor regions, collecting sequential blood biomarkers, and creating tissue resources for additional preclinical testing. Caveats include the need for additional knowledge concerning drug–gene relationships and contexts of vulnerability to improve therapeutic selection based on genomics, how to leverage combination therapies to improve efficacy, and the need to better understand the full extent of spatial heterogeneity within each patient.

From a research perspective, validating pharmacologic treatment recommendations in preclinical, patient-derived *in vitro* cell sources and *in vivo* xenograft models is valuable, allowing comparison of those models with patient outcomes, as well as testing of single agents, combination treatments, and novel therapeutic strategies in glioblastoma. Characterization of patient-derived xenograft models established in this study is underway.

Investigating spatial intratumoral heterogeneity was felt to be an important step toward optimizing a prospective efficacy trial.

The enhancing component of disease likely underrepresents the spectrum of genomic alterations associated with individual patient tumors, and we wished to gain further experience as to the potential changes within adjacent tumor regions that might inform the molecular tumor board recommendations. Exome sequencing of adjacent nonenhancing tissue showed overall concordance in therapeutically actionable alterations with those identified from the enhancing tumor, supporting use of profiling the enhancing tumor tissue to inform treatment of adjacent tissue left behind following surgery. However, only one nonenhancing region was collected and profiled for most patients. As glioblastoma is highly heterogeneous, evaluation of additional, distinct nonenhancing tissue regions may provide deeper appreciation for the spectrum of actionable alterations present in the tumor remaining after surgery. In addition, sequential imaging using MR-based anatomic features in this patient population remains problematic as to specificity/sensitivity of response and/or progression, and the possibility of using an early tumor biomarker in blood is worthy of further investigation. Sequential plasma samples were collected under this protocol for use in follow-on circulating tumor DNA research studies.

Although the trial was small and conducted in a single institution, there was enthusiasm for the approach from patients and families. The idea of "personalized"- or "precision"-based therapeutic recommendations was well received and even encouraged by patients. Many patients are currently receiving similar recommendations using various genomic platforms outside of a clinical trial setting. Expanding this strategy toward a larger prospective clinical trial would likely accrue well given the lack of any effective current therapies and the large unmet need. A coordinated approach beginning with a treating physician interacting with patients and family members, and including excellent surgical and pathology support and high-quality tissue acquisition and deep molecular sequencing are critical requirements. Based upon the current trial, we feel these steps are in place at many academic settings.

Although glioblastoma is a challenging disease, there is renewed optimism for continued, prospective efforts toward patient-specific approaches. A large, international adaptive, genomics-based clinical trial is now being developed in newly diagnosed glioblastoma. This and other precision-based, prospective

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studies in newly diagnosed and progressive/recurrent glioblastoma will be very helpful going forward in order to address the significant unmet need of this disease.

Disclosure of Potential Conflicts of Interest

Authors' Contributions

Acknowledgments

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Prospective Feasibility Trial for Genomics-Informed Treatment in Recurrent and Progressive Glioblastoma

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